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No. 05-074973-00016-0000

Fecha: 2011-11-03 11:49:12 Dep. 2020 DIR.NUEVASOR
Tra. 11 PATENTEIYII Eve: 379 FASENACIONALI
Act. 523 RESPUESTA Folios: 63

Trámite : 011
Evento : 379
Actuación : 444

Referencia : Expediente No. 05074973
Asunto : Solicitud de patente para: "Medicamento para la profilaxis y el tratamiento de arteriosclerosis e hipertensión".
Solicitante : DAIICHI SANKYO COMPANY, LIMITED
Fecha de solicitud: 29 de Julio de 2005.

1. Yo, Luz Clemencia DE PÁEZ, en mi condición de apoderada de la sociedad solicitante de la patente de la referencia, me refiero al oficio No. 12484 notificado por fijación en lista del 11 de Agosto de 2011.

2. Mediante oficio No. 12484 se puso en conocimiento del solicitante el informe que contiene las observaciones hechas por su Despacho sobre la presente solicitud de patente. Las observaciones realizadas en el oficio son las siguientes:

3. Reivindicaciones.

El Examinador sugiere al solicitante eliminar la expresión "o ii) una sal farmacológicamente aceptable del mismo" de la reivindicación 1 del pliego reivindicatorio.

Con el fin de atender la anterior observación y de acuerdo con el artículo 34 de la Decisión 486, se presenta un nuevo capítulo reivindicatorio conformado por 5 reivindicaciones como sustituto del anteriormente presentado, en el cual no ha sido incluida la expresión "o ii) una sal farmacológicamente aceptable del mismo", de conformidad con las sugerencias del Sr. Examinador. De tal manera que, la objeción formulada a este respecto se debe tener por superada.

Sin embargo, en el evento que el Despacho considere que una modificación adicional sea necesaria, respetuosamente le solicito hacer uso de la facultad que le otorga el artículo 45 inciso 2. de la Decisión 486 de la Comisión de la Comunidad Andina a efecto de requerir nuevamente de nuestra parte cualquier complementación o aclaración.

4. Análisis de patentabilidad

El Examinador concluye que el nivel inventivo de la presente solicitud se ve afectado por el documento US221049384 (D1) y el documento "norwood Daryl, et al. "Olmesartan Medoxomil for Hypertension: A Clinical Review", Drug Forecast, Vol. 27, No.12, december 2002" (D3). Su Despacho enuncia:

- "La solicitud se diferencia de D1 en que el antagonista de angiotensina II es olmesartan medoxomilo. El efecto técnico que genera tal diferencia es que la composición genera un efecto antihipertensivo mayor al de la combinación de valsartán y amlodipino besilato, siendo así, útil para el tratamiento de la hipertensión".
- "No obstante, D3 enseña que el olmesartán medoxomilo presenta una mayor eficacia antihipertensiva según resultados reportados en estudios comparativos (estudio Ball y estudio de Oparil), al ser olmesartán medoxomilo más efectivo en disminuir la presión en pacientes con hipertensión que valsartán, losartán e irbesartán".
- "De manera tal, que la persona del oficio normalmente versada en la materia, de acuerdo con las divulgaciones de D1 y D3, cambiaría el valsartán de las composiciones de D1 por olmesartán medoxomilo, ya que éste presenta un mejor efecto antihipertensivo que el valsartán como se divulga en D3, con la expectativa razonable de obtener una composición que tenga mejor efecto antihipertensivo que las de D1, dadas las enseñanzas de D3; por tanto, no son inesperados los resultados presentados para la composición reclamada, relacionados con un efecto antihipertensivo mayor al de la combinación de valsartán y amlodipino besilato".

Con respecto a lo señalado por su Despacho, mi representada desea poner de presente lo siguiente:

De acuerdo con el artículo 18 de la Decisión 486:

"Artículo 18.- Se considerará que una invención tiene nivel inventivo, si para una persona del oficio normalmente versada en la materia técnica correspondiente, esa invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica".

De lo anterior, es claro que para determinar si una invención es inventiva o no, el examinador debe determinar si la misma se deriva de manera obvia a partir del estado de la técnica.

El estado de la técnica es el conjunto de conocimientos técnicos dentro del campo de la invención que hayan sido publicados por cualquier medio, antes de la fecha de solicitud de la invención o de la prioridad reivindicada. A pesar de que esta búsqueda de anterioridades se realiza a posteriori, es decir, tomando como punto de partida la invención y la forma como efectivamente se solucionó el problema técnico, al realizar la evaluación de nivel inventivo el

examinador debe ponerse en el lugar del inventor al momento de realizar la invención junto con los conocimientos técnicos existentes al momento de la solicitud con el fin de entender el esfuerzo y análisis que dicho inventor requirió para resolver el problema técnico planteado. Lo anterior, por cuanto si la invención es analizada con los avances tecnológicos hoy conocidos y adicionalmente se toma como punto de partida la forma como efectivamente se solucionó el problema técnico, la invención en todos los casos le resultará obvia y evidente.

De otro lado, se debe tener en cuenta el alcance de las palabras obvio y evidente, y cómo estas deben ser tenidas en cuenta al momento que el técnico medio hace el examen de nivel inventivo.

Según la jurisprudencia del Tribunal, es obvio "aquello que salta a la vista de manera clara y directa; lo "que se encuentra o pone delante de los ojos, muy claro o que no tiene dificultad"". ¹

Por otro lado, el Tribunal define aquello que es evidente como "la certeza clara y manifiesta de lo que no se puede dudar; cierto, claro, patente y sin la menor duda". ²

Así entonces, es posible concluir que para ser pertinentes en la evaluación de nivel inventivo de la presente invención, los documentos citados deberían revelar sugerencias y enseñanzas claras para el técnico en la materia que lo lleven a obtenerla. Para este fin, el Señor Examinador debe examinar si, de conformidad con dichos documentos (el estado de la técnica más próximo), una persona versada en la materia habría o no propuesto las características técnicas que distinguen la invención reivindicada y que permiten alcanzar los resultados obtenidos por dicha invención.

En tal sentido consideramos oportuna la cita del tratadista GOMEZ SEGADE, mencionada por el Tribunal de Justicia del Acuerdo de Cartagena en la sentencia proferida, dentro del Proceso No. 26-IP-99, el 23 de julio de 1999:

"Tal y como lo establece GOMEZ SEGADE (obra citada) el inventor debe reunir los méritos que le permitan atribuirse una patente, sólo si la invención fruto de su investigación y desarrollo creativo constituyen "un salto cualitativo en la elaboración de la regla técnica", actividad intelectual mínima que le permitirá que su invención no sea evidente (no obvia) del estado de la técnica.".

Teniendo en cuenta el anterior, podemos concluir que una invención no se considera obvia u evidente y por tanto cumple con el requisito de nivel inventivo cuando la misma haya resultado a través de estudio y experimentación (investigación) y constituya un adelanto tecnológico, materializado en un resultado inesperado (mejorado) y además no evidente para alguien del oficio.

¹ Proceso No. 192-IP-2005. Tribunal de Justicia de la Comunidad Andina.

² Ibídem.

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Con el fin de demostrar que los argumentos esgrimidos por el Señor Examinador no resultan de recibo en el presente caso, toda vez que sus conclusiones no son adecuadas frente a la metodología que debe adelantarse para determinar el nivel inventivo de una solicitud de patente, mi representada desea poner de presente lo siguiente:

4.1 El efecto de la composición farmacéutica reivindicada es inesperado y no se puede derivar de manera obvia a partir del estado de la técnica.

En primer lugar, deseamos poner de presente que tal como se evidencia en el documento D3, la interacción de una droga con un bloqueador de los receptores de angiotensina II (ARB) no necesariamente se observa con un ARB estructuralmente diferente, tal como el olmesartan medoxomilo. En efecto, D3 divulga que las diferencias estructurales entre compuestos ARBs resulta en diferencias farmacológicas y farmacocinéticas (Ver páginas 611, 613). En efecto, D3 muestra que los compuestos ARB no proporcionan resultados similares cuando son empleados en combinación con otras drogas. Por ejemplo, a partir de la tabla 1 de D3 se observa que el losartan tiene interacciones farmacológicas significativas con la rifampina y el fluconazol, más sin embargo dichas interacciones no se observan con otros ARBs listados en la tabla 1.

Adicionalmente, a partir de la tabla 1 de D3 se observa que el telmisartan tiene interacciones importantes con la digoxina. Dichas interacciones tampoco se observan con otros ARBs listados en la tabla 1.

Así entonces, las interacciones del telmisartan en combinación con el digoxin, las cuales no son observadas con otros ARBs listados en la tabla 1 incluyendo el olmesartan medoxomilo, demuestran que se presentan distintas interacciones con diferentes ARBs en combinación con compuestos que afectan los niveles de calcio en el sistema cardiovascular.

Las diferencias que se presentan entre distintos compuestos ARBs son asimismo divulgadas en la publicación: Eklind-Cervenka et al., "Association of Candesartan vs Losartan With All-Cause Mortality in Patients With Heart Failure", JAMA, Vol. 305, No.2, 175-182. January 12, 2011, radicado ante su Despacho el 10 de Marzo de 2011, en donde se establece que diferentes ARBs actúan a través de diferentes mecanismo que resultan en diferentes tasas de mortalidad en pacientes que dependen del ARB específico administrado. En particular, en dicho documento se revela que los antagonistas de la angiotensina II: candesartan y losartan, tienen efectos diferentes en pruebas para la mortalidad de pacientes con insuficiencia cardíaca a partir de un estudio realizado a 30,254 pacientes que se les administraba candesartan o losartan con el cual se demostró que el candesartan se asocia con menores causas de mortalidad en comparación con el losartan.

Entre las razones que explican la mayor efectividad del candesartan en comparación con el losartan, se encuentran las siguientes:

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- (1) El Candesartan tiene 4 sitios de unión en el receptor AT1 mientras que el losartan tiene 2 sitios de unión.
- (2) La unión del losartan es débil mientras que las uniones del candesartan son fuertes.
- (3) Una mutación de la Lisina¹⁹⁹ disminuye 45 veces la afinidad del candesartan y solo 5 veces la del losartan.
- (4) Estudios en ratas han demostrado que el candesartan es entre 10 hasta 30 veces más potente que el losartan.
- (5) Estudios clínicos relacionados con la hipertensión también sugieren que el candesartan puede ser más efectivo que el losartan por cuanto únicamente el candesartan puede disminuir los niveles de plasma del activador-inhibidor del plasminógeno 1 y la proteína quimiatrayente de monocitos.
- (6) El candesartan inhibe en mayor grado que el losartan el incremento de la angiotensina II-inducida en filtraciones renales y la secreción de aldosterona.
- (7) El candesartan (16mg/d) en comparación con el losartan (100mg/d) es más efectivo al bajar la presión arterial.
- (8) En un estudio de hipertensión, el candesartan se asoció con menos fallas del corazón (no presentadas anteriormente) en comparación con el losartan y adicionalmente, en estudios realizados en pacientes de edad avanzada con fallas del corazón, el losartan se asoció con menos tasas de supervivencia, en comparación con el irbesartan, valsartan y candesartan.

En vista de las anteriores diferencias entre dos compuestos ARBs y su interacción con diferentes drogas, es claro que una persona versada en la materia no podría predecir de forma razonable los efectos observados a partir de la administración de un ARB en combinación con un bloqueador de los canales de calcio (CCB) o predecir los efectos de diferentes SRBM en combinación con el mismo CCB. Por lo tanto, ninguno de los documentos D3-D1 proporcionan enseñanza o sugerencia alguna que dirija a una persona versada en la materia a prever de forma absoluta y certera que al combinar el olmesartan medoxomilo y la amlodipina se obtendrían resultados satisfactorios.

4.2. La anterioridad D1 no enseña un efecto sinérgico de una composición que consiste de un ARB y una dihidropiridina CCB.

La anterioridad D1 (US2001049384) revela un método de tratamiento y una composición farmacéutica que comprende un antagonista de la angiotensina II, valsartan, en combinación con un bloqueador de los canales de calcio, el cual se encuentra ejemplificado mediante 18 compuestos, los cuales incluyen los tres bloqueadores de canales de calcio revelados en la reivindicación 1 de la presente invención.

Así en el párrafo [0017] dicha anterioridad afirma que la combinación de valsartan y un bloqueador de los canales de calcio "...resulta no solo en un efecto sinérgico sino también en beneficios adicionales...". Lo anterior es la única mención en D1 con respecto a un efecto sinérgico; sin embargo, D1 no incluye prueba alguna en donde se establezca la efectividad terapéutica de las composiciones allí reveladas, o en donde dicha efectividad corresponda a un efecto sinérgico. En efecto, D1 reconoce que la larga lista de indicaciones

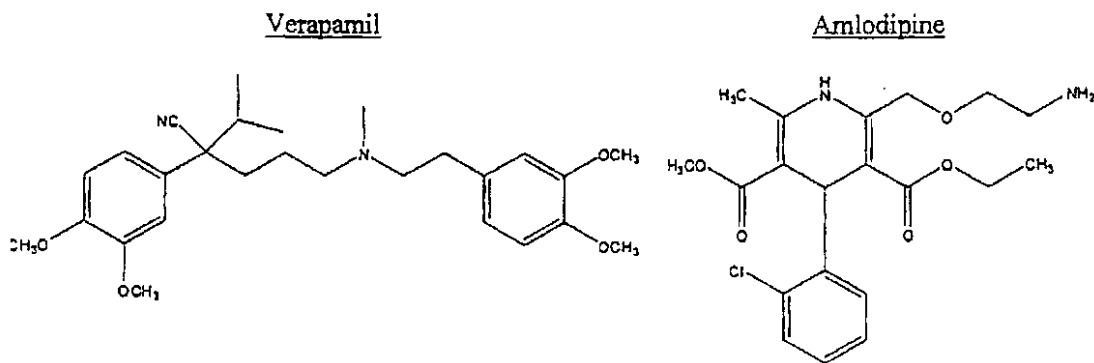
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terapéuticas proporcionadas carecen de base científica alguna y establece explícitamente en el párrafo [0021] que una persona versada en la materia necesitaría seleccionar "un modelo relevante para probar las indicaciones terapéuticas mencionadas...".

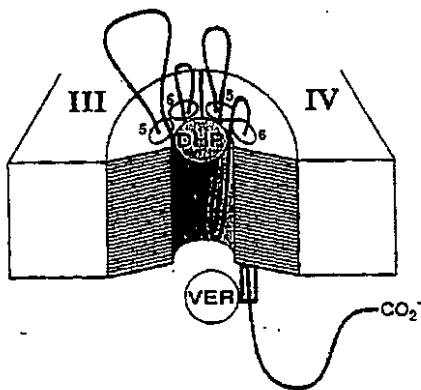
Los únicos datos experimentales divulgados en D1 se refieren a la combinación de valsartan y verapamil. El verapamil es un CCB que es estructuralmente diferente a las dihidropiridinas CCBs y exhibe propiedades muy distintas. Las diferencias estructurales y farmacológicas significativas entre el verapamil y una dihidropiridina no motivarían a una persona versada en la materia a predecir, a partir de la actividad de una combinación de un ARB y verpamil, las propiedades de diferentes combinaciones de distintos ARBs y dihidropiridinas CCB, tal como la amlodipina.

Sin embargo, tal como se explica en la publicación: Nayler et al., "Calcium antagonists and their mode of action: an historical overview", Br. J.clinic. Pharmac. 21: 97s-107s (1986), la cual se encuentra anexa al presente memorial, los compuestos bloqueadores de los canales de calcio son considerados como un grupo heterogéneo toda vez que estos compuestos presentan entre sí diferentes sitios de unión, farmacocinética y formas de interacción. Por ejemplo, como se muestra a continuación, la estructura química del verapamil no comparte un núcleo en común con la estructura de la amlodipina:



Adicionalmente, a partir del estado del arte se sabe que las CCB del tipo fenilalquilamina, tal como el verapamil, se unen a sitios de unión diferentes a los de las CCB del tipo dihidropiridina, tal como la amlodipina. Por ejemplo, la publicación : Nakayama et al. "Identification of 1,4-dihydropyridine binding regions with the $\alpha 1$ subunit of skeletal muscle Ca^{2+} channels by photoaffinity labeling with diazipine", Proc. Natl. Acad.Sci, Vol 88, pp 9203-9207 (October 1991), la cual se encuentra anexa al presente memorial, divulga un modelo para los diferentes sitios de unión, el cual se reproduce a continuación:

Fig. 6



A partir de dicha publicación es claro que los sitios de unión de CCBs del tipo dihidropiridina se localizan en la superficie exterior, mientras que el verapamil interactúa con un sitio receptor intracelular. De modo que, en vista de la heterogeneidad de los compuestos CCB, las propiedades y actividades observadas para un tipo de CCB, tal como los CCB del tipo fenilalquilamina (i.e. verapamil) no sería tenido en cuenta por una persona versada en la materia para predecir el comportamiento de una CCB del tipo dihidropiridina, tal como la amlodipina, la cual se une a sitios de unión completamente distintos a los de las fenilalquilaminas. Así entonces, de lo anterior es claro que una persona versada en la materia no podría predecir de manera certera y evidente la actividad de una combinación de olmesartan medoxomilo y una amlodipina a partir de las enseñanzas de D1.

4.3 Resultados inesperados contra la hipertensión.

La combinación de olmesartan medoxomilo y una amlodipina proporciona un efecto ventajoso en comparación con las combinaciones reveladas en los documentos D1 y D3. Lo anterior, por cuanto la combinación reivindicada no solo proporciona una mayor reducción de la presión en comparación a cuando cada uno de los compuestos por separado es empleado sino que adicionalmente, dicha combinación permite a un mayor porcentaje de pacientes tratados, alcanzar los niveles de presión arterial deseados. Con el fin de demostrar el efecto inesperado obtenido con la combinación acá reivindicada, nuevamente se dirige la atención del Señor Examinador a la publicación: Chrysant, "Amlodipine/ARB Fixed Dose Combination" *Drugs Today*, vol. 44, issue 6, pages 443-453, 2008, presentada anexa al memorial de respuesta de la anterior acción oficial No.14387 y radicado ante su Despacho en Marzo del presente año, en donde se presentan datos comparativos entre la combinación de olmesartan medoxomil y amlodipina frente a cualquiera de los dos componentes tomados individualmente (Ver Tabla II). En dichos datos presentados en la tabla II se tiene lo siguiente:

Monotherapy						
Treatment	PL	AB	AB	OM	OM	OM
Dose (mg/day)	0	5	10	10	20	40
SDBP (mmHg)	-3.1	9.4	12.7	8.3	9.2	10.2
SSBP (mmHg)	-4.8	14.9	19.7	11.5	13.8	16.1
Combination Therapy						
Treatment	AB/OM	AB/OM	AB/OM	AB/OM	AB/OM	AB/OM
Dose (mg/day)	5/10	5/20	5/40	10/10	10/20	10/40
SDBP (mmHg)	-13.8	14.0	15.5	16.0	17.0	19.0
SSBP (mmHg)	-24.2	23.6	25.4	25.3	29.2	30.1

Adicionalmente, en dicha publicación se afirma que "todos los grupos de tratamiento muestran una reducción mas grande en la presión arterial en comparación con el placebo ($P<0.001$) y los efectos de disminución en la presión arterial son mas grandes que los de las monoterapias ($P<0.001$)" (Ver p. 446, columna izquierda, líneas 36 a 40). Es decir, la combinación de olmesartan medoxomil y besilato de amlodipina proporciona un beneficio relacionado con la disminución en la presión arterial en comparación con la monoterapia de los componentes tomados individualmente. Adicionalmente, los datos proporcionado en la Figura 2 de la publicación muestra que un mayor numero de pacientes tratados con la terapia de combinación alcanzaron las metas de presión arterial ($<140/90$ mmHg) que los que recibieron solo la monoterapia. Específicamente, los datos de dicha Figura 2 del muestran que más del 50% de los pacientes que recibieron 5/40, 10/10, o 10/20 mg/día de amlodipina/olmesartan alcanzaron el control de la presión arterial (definido como $<140/90$ mmHg), mientras a un porcentaje mucho más bajo de pacientes suministrados con el máximo de la dosis de la monoterapia de amlodipina (10 mg/día, 36.1% de pacientes que alcanzaron el objetivo) o de olmesartan (40 mg/día, 38.5% de pacientes que alcanzaron el objetivo), alcanzó el control de presión arterial. Se encontraron diferencias aún más grandes cuando se comparan dosis de monoterapia menores a las máximas con las dosis de las terapias de combinación (ver página 449, Figura 2).

En consecuencia, el elevado número de pacientes que alcanzaron los controles de presión arterial corresponde a un resultado ventajoso y sorprendente. De hecho, no existe evidencia alguna para predecir que en donde solo el 36% o el 38% de pacientes que recibían las dosis máximas de amlodipina (es decir 10mg/día) o de olmesartan (es decir 40mg/día), pudieran controlar su presión arterial de, un porcentaje mayor de pacientes (es decir el 46.6%) que recibían dosis más bajas de cada ingrediente en combinación (es decir 5mg/día de amlodipina y 20mg/día de olmesartan), pudieran controlar su presión arterial. Es así como dicho incremento en el porcentaje de pacientes que son capaces de controlar su presión arterial, no es predecible a partir de las enseñanzas del estado del arte (D1 o D2) y por lo tanto, corresponde a un resultado ventajoso.

Aún más, los datos mencionados anteriormente resuelven una necesidad insatisfecha, ya que la hipertensión sin control representa una necesidad latente en el campo médico y los datos arriba mencionados proporcionan evidencia de que la composición farmacéutica de la

presente invención se comporta de manera diferente frente a las composiciones enseñadas en el estado del arte. Específicamente, deseamos aclarar que la composición de la presente invención permite que un número mas elevado de pacientes alcancen el control de la presión arterial, que lo que alcanzarían con las dosis máximas de la monoterapia con amlodipina (es decir 10mg/día) o con olmesartan (40mg/día), respectivamente. Así, dicho incremento es altamente significativo ya que hace que ocurran disminuciones sustanciales en eventos cardiovasculares aún con pequeñas disminuciones en la presión arterial.

4.4 Resultados inesperados en comparación con combinaciones de otros ARBs con amlodipina.

De otro lado, se tiene que la combinación de olmesartan medoxomilo y el bloqueador de los canales de calcio 1, 4 dihidropiridina, i.e. amlodipina, proporciona un resultado inesperado en comparación con la combinación de valsartan y amlodipina, teniendo en cuenta las enseñanzas de la publicación: C.V.S. Ram, "Antihypertensive efficacy of olmesartan medoxomil or valsartan in combination with amlodipine: A review of factorial-design studies", Current Medical Research and Opinion, vol. 25, no. 1, p. 177-185, 2009, la cual se presento ante su Despacho el 10 de Marzo de 2011.

En dicho documento, se proporciona evidencia relacionada con el hecho de que la composición de la presente invención produce un efecto material más alto en comparación con la combinación de valsartan y amlodipina. Específicamente, allí se menciona que la combinación "amlodipina 10mg/olmesartan medoxomil 40mg proporcionó las reducciones corregidas por placebo mas altas en tanto el promedio de mínimos cuadrados para presión arterial sistólica en posición sedente (SeSBP) como para la presión arterial diastólica en posición sedente (SeDBP) (15.9 y 25.7 mmHg, respectivamente, por el contrario de 9.8 y 15.5 mmHg, con dosis máximas de amlodipina (10mg) y de valsartan (320mg), respectivamente)" (Ver p. 184, columna izquierda, líneas 37 a 52). Con relación a la reducción de la presión arterial diastólica en posición sedente (SeDBP), los datos reportados demuestran que el promedio de mínimos cuadrados para la SeDBP para la reducción máxima corregida por placebo de amlodipina y olmesartan medoxomil, es de 6.1 mmHg o acerca de 62% (6.1mmHg / 9.8 mmHg) más alta que la máxima reducción corregida por placebo de amlodipina y valsartan. De igual manera, los datos revelados para el promedio de mínimos cuadrados de la SeSBP para la reducción más alta corregida por placebo para una combinación de amlodipina y olmesartan medoxomil es de 10.2mmHg o cerca del 66% (10.2mmHg/15.5mmHg) más alta que el la reducción máxima corregida por placebo de una combinación de amlodipina y valsartan. Aún más, en dicho documento se afirma que "se ha demostrado que la morbilidad por riesgo cardiovascular se duplica con cada incremento de 20/10 mmHg en la presión arterial arriba de 115/75 mmHg" (Ver columna derecha, página 177, líneas 2 a 5).

En particular, en los datos mostrados en la tabla 4 de la publicación arriba mencionada, se observa que una dosis de 10mg de olmesartan no es en promedio, más efectiva en reducir la presión diastolica de la

sangre (SeDBP) en comparación con 320 mg de valsartan administrados solos. Sin embargo, la tabla 4 reporta que 10 mg de olmesartan proporcionaron una reducción del placebo en SeDBP de 5.3.

En contraste, la tabla 4 reporta que 320 mg de valsartan proporcionan una reducción del placebo en SeDBP de 6.6 y 4.5. En todo caso, en dicha tabla también se observa que cuando se compara la dosis de 10mg de olmesartan, con la dosis de 320 mg de valsartan, la combinación de olmesartan y amlodipina de manera sorprendente proporciona una reducción mayor en SeDBP en comparación con la combinación de valsartan y amlodipina.

Amlodipine	Olmesartan	Valsartan	Placebo Adjusted Reduction in SeDBP
-----	10 mg	-----	5.3
-----	-----	320 mg	6.6
-----	-----	320 mg	4.5
5 mg	10 mg	-----	10.8
5 mg	-----	320 mg	9.1
10 mg	10 mg	-----	13.2
10 mg	-----	320 mg	9.8

Adicionalmente, los datos reportados en la tabla 4 muestran que una dosis de 10mg de olmesartan no es en promedio, más efectiva en reducir la presión sistólica de la sangre (SeSBP) en comparación con 320 mg de valsartan administrados solos. Sin embargo, la tabla 4 reporta que 10 mg de olmesartan proporcionaron una reducción del placebo en SeSBP de 8.1.

En contraste, la tabla 4 reporta que 320 mg de valsartan proporcionan una reducción del placebo en SeSBP de 9 y 6.9. En todo caso, en dicha tabla también se observa que cuando se compara la dosis de 10mg de olmesartan, con la dosis de 320 mg de valsartan, la combinación de olmesartan y amlodipina de manera sorprendente proporciona una reducción mayor en SeSBP en comparación con la combinación de valsartan y amlodipina.

Amlodipine	Olmesartan	Valsartan	Placebo Adjusted Reduction in SeDBP
-----	10 mg	-----	8.1
-----	-----	320 mg	9
-----	-----	320 mg	6.9
5 mg	10 mg	-----	19.8
5 mg	-----	320 mg	16
10 mg	10 mg	-----	22
10 mg	-----	320 mg	15.5

En consecuencia, teniendo en cuenta la información revelada en dicho documento, una persona versada en la materia apreciaría que la magnitud de la mejora en la reducción del promedio de mínimos

cuadrados tanto de la SeDBP como de la SeSBP mediante la composición de la presente invención en comparación con la combinación de amlodipina con valsartan, proporciona cerca del doble de la reducción en el riesgo de muerte cardiovascular, el cual es claramente un resultado inesperado y ventajoso.

Por lo tanto, no puede ser afirmado que la composición de la presente invención podría ser derivada de manera obvia, ya que por medio de los resultados mencionados, se ha demostrado el efecto mayor al esperado alcanzado por la composición de la presente invención en comparación con el resultado obtenido en la anterioridad D1, lo que es demuestra a su vez que la presente invención no pudo haber sido derivada de manera obvia a partir de D1.

Adicionalmente, además de las reducciones más bajas en el presión arterial, en comparación con la composición de la presente invención, la combinación de amlodipina y valsartan tiene ciertas deficiencias, por cuanto "la terapia de combinación con valsartan 160mg con 10mg de amlodipina ha sido reportada como incapaz de atenuar la activación simpática inducida por la amlodipina" (Ver Pág. 184, columna derecha, líneas 1 a 4) y no se proporciona explicación para dicha diferencia entre la combinación de amlodipina/valsartan en comparación con la combinación de amlodipina/olmesartan. Por lo tanto, es claro que la actividad obtenida con la composición acá reivindicada es inesperada y lo impredecible que es la combinación de dos ingredientes activos.

Adicionalmente, se presenta anexo al presente memorial una pruebas comparativas que demuestran la efectividad en la disminución de la presión en la sangre al emplear la combinación de olmesartan medoxomilo y besilato amlodipina en comparación con la combinación de losartan y besilato de amlodipina, a partir de los cuales se observa lo siguiente:

Table 1

Test Substance	n	24-hour average of mean blood pressure (mmHg)				
		1 st Day	2 nd Day	3 rd Day	4 th Day	5 th Day
Vehicle	6	184±2	186±4	181±3	182±3	180±3
Amlodipine besylate	6	163±3	164±3	164±3	166±3	166±3
Amlodipine besylate + olmesartan medoxomil	6	149±2	147±1	149±1	151±1	152±2
Amlodipine besylate + losartan potassium	6	165±3	158±2	159±2	160±2	160±1

Values represent mean ± S.E.M.

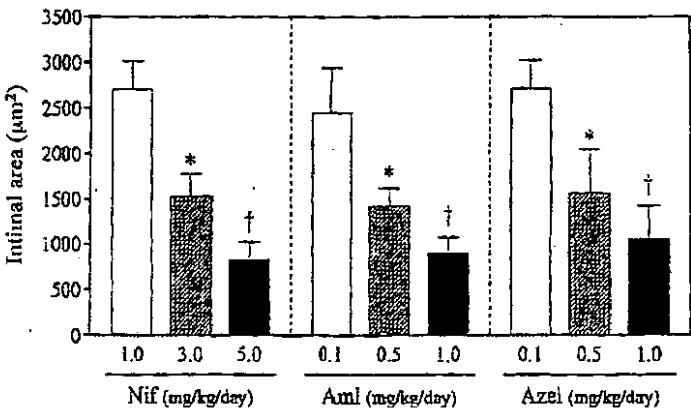
Una combinación de 2mg/kg de besilato de amlodipina con 0.3 mg/kg de olmesartan medoxomilo proporcionan una reducción aproximada de 10mmHg en la presión sistólica en comparación con una combinación de 2 mg/kg

de besilato de amlodipina con 0.3 mg/kg de losartan. De modo que, teniendo en cuenta que una disminución de 5 a 6 mmHg puede reducir el riesgo de un derrame en un 40%, una enfermedad coronaria del corazón en un 15 a 20%, fallas del corazón y mortalidad por una enfermedad vascular, es claro que el resultado obtenido con la composición reivindicada representa una mejora significativa toda vez que reduce el riesgo de que el paciente sufra de eventos coronarios fatales.

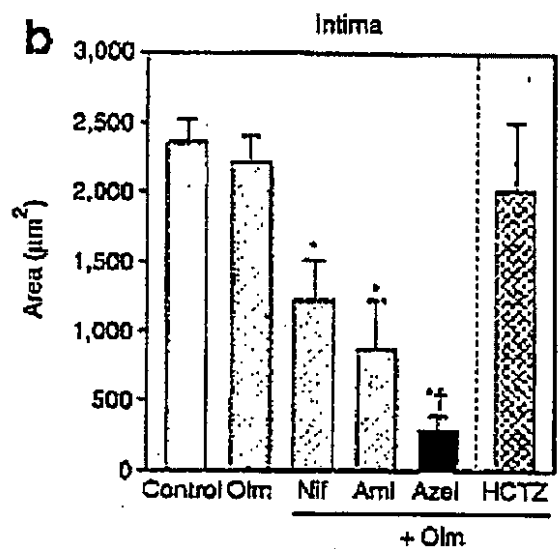
4.5 Resultados inesperados frente a otras enfermedades cardiovasculares.

La composición acá reivindicada también presenta un efecto inesperado frente a otras enfermedades cardiovasculares, incluyendo la arteriosclerosis. A este respecto, se presenta anexo al presente memorial la publicación: Inaba et al., "Prevention of Vascular Injury by Combination of an AT1 Receptor Blocker, Olmesartan, With Various Calcium Antagonists", *American Journal of Hypertension*, 22, 145-150 (2009), la cual proporciona evidencia de que la composición reivindicada produce un efecto inesperado frente otras enfermedades cardiovasculares, tal como inflamación vascular y arteriosclerosis.

En dicha publicación, se reportan un experimento en el cual cada droga se uso a una dosis subefectiva, en la cual no se pudiera inhibir de manera significativa una formación neointimal. Las drogas fueron administradas a ratones con lesiones vasculares y los datos se muestran a continuación:

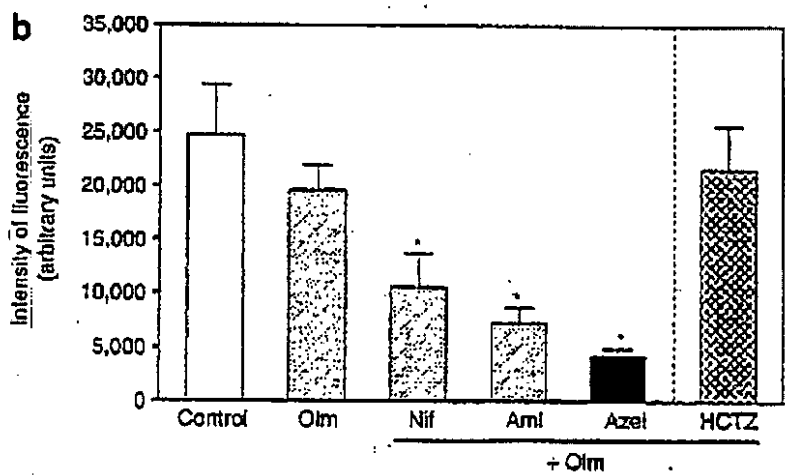


De lo anterior, es claro que una dosis de 1 mg/kg/día de nifedipina, 0.1mg/kg/día de amlodipina, 0.1mg/kg/día de azelnidipina no proporcionan una reducción significativa en el area transversal de la neointima. Similarmente, la dosis subefectiva de olmesartan tampoco proporcionó una reducción significativa. De modo que, no se esperaría que la combinación de dos dosis sub-efectivas proporcionara una reducción significativa, tal como se muestra en la Fig. 1b que se muestra a continuación:



En consecuencia, la combinación de olmesartan y amlodipina o azelnidipina proporciona una reducción inesperada de la respuesta inflamatoria en arterias dañadas y por lo tanto, el riesgo de arteriosclerosis, en comparación cuando se emplea cada una de estas drogas por separado. Teniendo en cuenta lo anterior, es claro que los efectos de la composición acá reivindicada son inesperados.

La combinación de olmesartan y amlodipina o azelnidipina también proporciona un resultado inesperado en la inhibición de la producción de aniones super-óxidos. Tal como se señala Inaba et al, la producción excesiva de especies reactivas de oxígeno, juega un papel importante en la inflamación vascular y se encontró que la combinación de olmesartan con amlodipina o azelnidipina, proporcionaban reducciones significativas en la producción de aniones super-óxidos (alrededor del 70%) a pesar de que cada uno de estos componentes por separado no afectaban de ninguna manera la producción de los super-óxidos:



La descripción de la presente solicitud, también proporciona evidencias en relación con que la composición reivindicada presenta efectos inesperados contra la arteriosclerosis. La descripción presenta datos experimentales in vitro en la Figura 8 (descrita en el ejemplo de ensayo 1), que demuestran que cuando dosis bajas de azelnidipina y olmesartan son insuficientes por si solas para producir cualquier efecto ventajoso, al ser administrados conjuntamente se observa que la síntesis de DNA en músculos blandos de rata se suprime considerablemente.

En la descripción de la presente solicitud también se divulga que los datos del ejemplo de ensayo 1, demuestran de forma clara que la coadministración in vivo de azelnidipina y olmesartan presenta un efecto sinérgico, inhibe la proliferación de células de músculo blando. Como se puede observar a partir de las figuras 5A y 5B, los ratones a los cuales se les administró una combinación de 0.1mg/kg/día del bloqueador de los canales de calcio (dihidropiridina), azelnidipina, y 0.5 mg/kg/día de olmesartan presentaron una reducción mayor en la cantidad de síntesis de DNA de células de músculo blando en comparación a cuando cada uno de estos compuestos era administrado por separado.

Las figuras 6A y 6B también demuestran que la combinación de azelnidipina y olmesartan proporciona una reducción mayor en el área transversal de la neointima en comparación a cuando cada uno de estos compuestos es administrado por separado.

El efecto sinérgico de la composición reivindicada se encuentra soportado en el documento: Jinno et al., "Calcium Channel Blocker Azelnidipine Enhances Vascular Protective Effects of AT1 Receptor Blocker Olmesartan", *Hypertension*, 43, 263-269 (2004), en el cual se divulga que el olmesartan o la azelnidipina por separado en dosis de 0.5 o 0.1 mg/kg por día, no afectan la formación de neointima, el índice Brd U, la activación de ERK, STAT1 y STAT3, la expresión de p22^{phox} y la producción de aniones super-óxidos. Sin embargo, revela que la coadministración de olmesartan y azelnidipina con estas dosis disminuyen de forma significativa estos parámetros (Ver figuras 4 a 6 y II).

De lo anterior, es claro que la composición reivindicada presenta un efecto sinérgico en el tratamiento de enfermedades vasculares y la hipertensión.

Adicionalmente, la información presentada en la descripción de la presente solicitud, se encuentra soportada por el documento: Suzuki et al., "Effect of combination of calcium antagonist, azelnidipine and AT1 receptor blocker, olmesartan, on atherosclerosis in apolipoprotein E-deficient mice", *J. Hypertens*, 23: 1383-1389 (2005), la cual proporciona información que evidencia que la combinación de una dihidropiridona, azelnidipina, y olmesartan proporciona un efecto mejorado en la inhibición de la aterosclerosis. Tal como se puede observar en la Figura 1 de Suzuki, et al., la lesión de aterosclerosis en la aorta de los ratones se veía reducida en mayor proporción cuando se coadministraban el olmesartan y la azelnidipina en comparación a cuando cada uno de estos compuestos era administrado por separado.

Asimismo, en dicho documento se presentan resultados sorprendentes en la figura 2, en la cual se demuestra que la combinación de 0.1 mg/kg/día de azelnidipina y 1 mg/kg/día de olmesartan reducen de forma significativa las lesiones de aterosclerosis en la aorta.

4.5 Conclusiones.

Teniendo en cuenta los anteriores argumentos, respetuosamente ponemos de presente que no compartimos la opinión del Señor Examinador en relación con el hecho que el documento D1 afecta la actividad inventiva de la presente solicitud por cuanto no revela la combinación específica acá reivindicada o la actividad inesperada de la misma. De hecho, sin experimentación alguna, no es posible predecir con certeza que la combinación de olmesartan medoxomilo junto con una amlodipina o una azelnidipina, daría a lugar a un efecto sinérgico en el tratamiento de la hipertensión y enfermedades cardíacas a partir de las enseñanzas de dicho documento.

Por lo tanto, teniendo en cuenta el carácter general de las enseñanzas de D1 toda vez que no incluye prueba alguna en donde se establezca la efectividad terapéutica de las composiciones allí reveladas, o en donde dicha efectividad corresponda a un efecto sinérgico, respetuosamente deseamos poner de presente que considerar este tipo de enseñanzas para las invenciones relacionadas con esta área de la ciencia, impediría el desarrollo de este campo de la ciencia, porque nadie estaría interesado en el desarrollo de nuevas investigaciones sobre este asunto, ya que no sería posible obtener el privilegio de una patente para un nuevo desarrollo o el progreso técnico en este campo.

En este caso particular, es evidente que la predicción de la actividad de una combinación de un antagonista de los receptores de angiotensina II y un bloqueador de los canales de calcio es un tema muy complejo y difícil de realizar toda vez que debido a la heterogeneidad de los CCB, las posibles combinaciones responden de una manera muy distinta. Asimismo, el examinador debe tener en cuenta que no es una tarea sencilla y fácil para una persona experta en el arte la de generalizar y prever la actividad de composiciones comprendiendo un CCB y un antagonista de los receptores de angiotensina II a pesar de que ya se conocen varias combinaciones en el estado del arte que comprenden ambos compuestos. En efecto, vale la pena resaltar que las enseñanzas generales como las reveladas en D1 no pueden ser objeto de patente de invención porque las mismas carecen de total validez desde el punto de vista técnico, pues no solamente son vagas sino que carecen de sustentación científica alguna. De esta manera, el régimen de patentes evita no solo que se otorgue patente de invención a simples enunciados que pueden llegar a constituir ciencia ficción sino que se tenga en cuenta las mismas para impedir la patentabilidad de creaciones que tengan la entidad de una invención, en la medida que no anticipan científicamente la solución a un problema técnico.

Adicionalmente, se reitera nuevamente a su Despacho, que en la práctica no existe previsibilidad con respecto a que si alguna combinación específica de medicamentos del estado del arte pueda o no proporcionar un tratamiento beneficioso para pacientes sin que se

presenten efectos secundarios. Los documentos discutidos anteriormente establecen que dentro de los medicamentos revelados en el estado del arte útiles en el tratamiento de la hipertensión, no existe previsibilidad acerca de que una combinación específica pueda proporcionar un efecto beneficioso sin que se presenten efectos secundarios. Dichos documentos establecen también que la composición farmacéutica tiene un efecto inesperado y ventajoso cuando se usa para tratar la hipertensión, a saber, una disminución sustancial en la presión arterial lo que resulta en los beneficios discutidos anteriormente.

Deseamos aclarar que en la presente invención se han seleccionado dos monoterapias del voluminoso número de monoterapias del estado del arte para proporcionar la composición farmacéutica de la invención, la cual presenta un efecto inesperado y significativo en el tratamiento de pacientes, la cual no podría ser predicho de manera obvia por una persona versada en la materia.

De modo que, de lo anterior es claro que no hay enseñanza o sugerencia alguna en los documentos citados que dirija a una persona versada en la materia a resolver el problema específico planteado en esta solicitud tal como lo hace la presente solicitud, es decir, resolver la necesidad de proporcionar nuevas composiciones farmacéuticas que combinen un antagonista de receptores de angiotensina II y un bloqueador de canales de calcio (derivados de dihidropiridinas) que sean útiles en el tratamiento de la hipertensión y enfermedades cardíacas y al mismo tiempo presenten un efecto mejorado inesperado en comparación con composiciones de este tipo ya conocidas en el estado del arte. Por lo tanto, la materia reivindicada debería considerarse inventiva frente al estado de la técnica.

5. Puesto que se han atendido a cabalidad las observaciones hechas por su Despacho sobre la presente solicitud de patente, respetuosamente solicito se continúe con su tramitación y proceda con la concesión de la patente.

6. ANEXOS

- Nuevo pliego reivindicatorio conformado por 5 reivindicaciones
- Formulario de modificaciones.
- Pruebas comparativas.
- *Nayler et al., "Calcium antagonists and their mode of action: an historical overview", Br. J.clinic. Pharmac. 21: 97s-107s (1986).*
- *Nakayama et al. "Identification of 1,4-dihydropyridine binding regions with the $\alpha 1$ subunit of skeletal muscle Ca^{2+} channels by photoaffinity labeling with diazipine", Proc. Natl. Acad.Sci, Vol 88, pp 9203-9207 (October 1991).*
- *Inaba et al., "Prevention of Vascular Injury by Combination of an AT1 Receptor Blocker, Olmesartan, With Various Calcium Antagonists", American Journal of Hypertension, 22, 145-150 (2009).*

CAVELIER

ABOGADOS

- Jinno et al., "Calcium Channel Blocker Azelnidipine Enhances Vascular Protective Effects of AT1 Receptor Blocker Olmesartan", Hypertension, 43, 263-269 (2004).
- Suzuki et al., "Effect of combination of calcium antagonist, azelnidipine and AT1 receptor blocker, olmesartan, on atherosclerosis in apolipoprotein E-deficient mice", J. Hypertens, 23: 1383-1389 (2005)

Señor Director,

Luz Clemencia de Páez
LUZ CLEMENCIA DE PÁEZ

C.C. 35.456.344 de Usaquén
T.P.A. No. 23.555

COLO-15869-845-002-6

206 MGM\MGM\ID-203822\WPC15869
No. M-2011-203822\MGM



Espacio reservado para el adhesivo de radicación

FORMULARIO UNICO DE CORRECCIONES Y MODIFICACIONES

1

SOLICITUD DE:
☐ Corrección de Error Material ☒ Modificación a una solicitud

2

SOLICITANTE

Nombre: **DAIICHI SANKYO COMPANY, LIMITED**
Sociedad constituida y existente bajo las leyes de Japón.

Dirección: 3-5-1, Nihonbashi Honcho,
Chuo-ku,
Tokio 103-8426,
Japón

Domicilio: Tokio,
Japón

Teléfono: 546 09 70 Fax: 249 40 70
E-mail: clientes@cavelier.com

IDENTIFICACION

C.C. ☐ NIT ☐
C.E. ☐ Otro ☐

Número: _____

3

REPRESENTANTE
O APODERADO

Nombre: Luz Clemencia de PAEZ

Dirección: Carrera 4ª No. 72-35

Teléfono: 347 36 11 Fax: 217 92 11
E-mail: cavelier@cavelier.com

IDENTIFICACION

C.C. ☒ NIT ☐
C.E. ☐ Otro ☐

Número: 35.456.344 TP 23.555

4

Número de expediente: 05.074.973

5

Descripción de la corrección o modificación solicitada:

Presento nuevo capitulo reivindicatorio compuesto por 5 reivindicaciones con el fin de superar las objeciones hechas por Su Despacho a la presente solicitud de patente y pido respetuosamente que el examen de patentabilidad sea realizado sobre estas nuevas reivindicaciones presentadas.

6

Comprobante de pago No. _____ Fecha: _____

7

Anexos

☐ Comprobante de pago de la tasa por \$118.000, por concepto de modificaciones.
☐ Comprobante de pago por reivindicaciones adicionales después de la décima reivindicación por.
☐ Poderes, si fuere el caso
☐ Documento que acredita la existencia y representación legal de las partes, cuando sean personas jurídicas.
☐ Copia auténtica de la sustitución que del poder otorgado por la Sociedad Solicitante hizo el Doctor EMILIO FERRERO a mi favor, y para lo cual solicito me sea reconocida personería para continuar con el trámite del asunto de la referencia.



NOMBRE:

LUZ CLEMENCIA DE PAEZ

FIRMA:

Luz Clemencia De Paez

C.C. 85.456.344 de Usaquén T.P.A. 23.555

MGM

REIVINDICACIONES

1. Una composición farmacéutica que comprende:

(A) el antagonista de los receptores de angiotensina II (5-metil-2-oxo-1,3-dioxolen-4-il)metilo-4-(1-hidroxi-1-metiletil)-2-propil-1-[[2'-(1H-tetrazol-5-il)bifenil-4-il]metil]imidazol-5-carboxilato; y

(B) un bloqueador de los canales de calcio seleccionado de (i) azelnidipina, (ii) amlodipina ó (iii) besilato de amlodipina.

2. Una composición farmacéutica de conformidad con la reivindicación 1, caracterizada porque el antagonista de los receptores de angiotensina II es (5-metil-2-oxo-1,3-dioxolen-4-il)metilo-4-(1-hidroxi-1-metiletil)-2-propil-1-[[2'-(1H-tetrazol-5-il)bifenil-4-il]metil]imidazol-5-carboxilato.

3. Una composición farmacéutica de conformidad con las reivindicaciones 1 a 2, caracterizada porque el bloqueador de los canales de calcio es azelnidipina.

4. Una composición farmacéutica de conformidad con las reivindicaciones 1 a 2, caracterizada porque el bloqueador de los canales de calcio es besilato de amlodipina.

5. Una composición farmacéutica de conformidad con las reivindicaciones 1 a 2, caracterizada porque el bloqueador de los canales de calcio es amlodipina.

COMPARATIVE TEST

OLM + amlodipine besylate Losartan + amlodipine besylate

Test Example: Antihypertensive activity

40, 49-week old male spontaneously hypertensive rats (SHR) were orally administered amlodipine besylate (2 mg/kg), olmesartan medoxomil (0.3 mg/kg) + amlodipine besylate (2 mg/kg), losartan potassium (0.3 mg/kg) + amlodipine besylate (2 mg/kg), or the vehicle (0.5 % carboxymethyl cellulose Na solution, 2 mL/kg) once a day, for 5 days, in groups of 6 animals. Mean blood pressure was measured by a telemetry method during the 3-day observation period and the 5-day treatment period. The 24-hour average value of mean blood pressure was calculated for analysis. Analysis of variance (ANOVA) for repeated measures with the baseline value as a covariate was applied to compare between antihypertensive effects of amlodipine besylate alone and combined with olmesartan medoxomil or losartan potassium. Changes in blood pressure are shown in Table 1. Excellent antihypertensive effects were observed in animals in the group co-administered olmesartan medoxomil + amlodipine besylate. ANOVA for repeated measures revealed that although treatment with losartan potassium did not potentiate the antihypertensive effect of amlodipine besylate ($P=0.2429$), treatment with olmesartan medoxomil potentiated the antihypertensive effect of amlodipine besylate ($P<0.0001$). It was also demonstrated that the antihypertensive effect of combined administration of amlodipine besylate and olmesartan medoxomil was greater than that of combined administration of amlodipine besylate and losartan potassium ($P=0.0004$).

Table 1

Test Substance	n	24-hour average of mean blood pressure (mmHg)				
		1 st Day	2 nd Day	3 rd Day	4 th Day	5 th Day
Vehicle	6	184±2	186±4	181±3	182±3	180±3
Amlodipine besylate	6	163±3	164±3	164±3	166±3	166±3
Amlodipine besylate + olmesartan medoxomil	6	149±2	147±1	149±1	151±1	152±2
Amlodipine besylate + losartan potassium	6	165±3	158±2	159±2	160±2	160±1

Values represent mean ± S.E.M.

Prevention of Vascular Injury by Combination of an AT₁ Receptor Blocker, Olmesartan, With Various Calcium Antagonists

Shinji Inaba^{1,2}, Masaru Iwai¹, Yumiko Tomono¹, Izumi Senba¹, Megumi Furuno¹, Harumi Kanno¹, Hideki Okayama², Masaki Mogi¹, Jitsuo Higaki² and Masatsugu Horiuchi¹

BACKGROUND

A combination of different types of antihypertensive drugs is widely used for the treatment of hypertension. We examined the inhibitory effects of a combination of an AT₁ receptor blocker (ARB), olmesartan, with various calcium channel blockers (CCBs) on inflammatory vascular remodeling.

METHODS

Inflammatory vascular remodeling was induced by polyethylene-cuff placement around the femoral artery of C57BL/6J mice at 10 weeks of age. Olmesartan (0.5 mg/kg/day) was administered intraperitoneally using an osmotic minipump. CCBs (nifedipine 1.0 mg/kg/day, amlodipine 0.1 mg/kg/day, azelnidipine 0.1 mg/kg/day), and hydrochlorothiazide (HCTZ 0.5 mg/kg/day) were administered orally.

RESULTS

In the injured artery, superoxide anion production and expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunits p47^{phox} and Rac-1 were markedly increased, together with

expression of monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor (TNF)- α . Administration of a single drug alone at each concentration did not significantly inhibit these changes in the injured artery. However, a combination of olmesartan with various CCBs inhibited neointimal formation as well as oxidative stress and inflammatory markers in the injured artery. Moreover, among these CCBs, inhibition of these markers by olmesartan with azelnidipine was stronger than that caused by a combination with other CCBs. On the other hand, a combination of subeffective doses of olmesartan and HCTZ did not significantly affect vascular changes after cuff placement.

CONCLUSIONS

These results suggest that the combination of ARB with CCB synergistically inhibits vascular remodeling and that the inhibitory actions of ARB on vascular remodeling may vary depending on the combined CCB.

Am J Hypertens 2009; **22**:145–150 © 2009 American Journal of Hypertension, Ltd.

AT₁ receptor blockers (ARBs) and calcium channel blockers (CCBs) are well established as the most popular and useful drugs for the treatment of hypertension and cardiovascular disease. Furthermore, combination therapy with various types of antihypertensive drugs, e.g., ARB and CCB, is frequently used to enhance their antihypertensive and cardioprotective effects compared with monotherapy.^{1,2} Recent clinical trials demonstrated that strict control of blood pressure was essential to prevent organ damage and reduce mortality of hypertensive patients.^{3,4} In addition, recent studies also suggest that the combination of ARB and CCB has not only an antihypertensive effect, but also a protective action against cardiovascular disease.^{5–9} Accumulating evidence suggests that oxidative stress, an excessive production of reactive oxygen species, plays

an important role in vascular inflammation and consequent remodeling.^{10–12} We previously reported that coadministration of an ARB and a CCB synergistically enhances the beneficial effects of these drugs on vascular remodeling and atherosclerosis by blunting oxidative stress, inflammation and vascular smooth muscle cell proliferation compared with monotherapy.^{5,6} These results suggest a possible synergistic interaction between ARB and CCB in their effects on cardiovascular injury. However, since each drug has a specific structure and characteristics, the combined effects might differ depending on the pair of drugs. In this study, we compared the effects of a combination of an ARB, olmesartan, and a CCB on inflammatory vascular injury using three different CCBs with a subeffective dose of each drug, focusing on their effects on oxidative stress.

METHODS

Animals and treatment. Adult male C57BL/6J mice (aged 10–12 weeks; purchased from Clea Japan, Osaka, Japan) were housed in a room where lighting was controlled (12h on, 12h off) and room temperature was kept at 24°C. They were given free access to standard laboratory chow (MF; Oriental Yeast, Osaka,

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Received 22 July 2008; first decision 30 August 2008; accepted 25 October 2008; advance online publication 4 December 2008. doi:10.1039/ajh.2008.344

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Japan) and water. To induce vascular injury, a polyethylene tube (2-mm-long; PE 90; Becton Dickinson, Franklin Lakes, NJ) was placed loosely around the left femoral artery, as described previously.¹³ The experimental protocol was approved by the Animal Studies Committee of Ehime University. First, we examined the dose-response effects of each CCB alone on neointimal formation. A selective ARB, olmesartan (provided by Sankyo, Tokyo, Japan), was administered continuously at a dose of 0.5 mg/kg/day with an osmotic minipump (Alzet model 1002; Durect, Cupertino, CA) implanted intraperitoneally at the same time as cuff placement. CCBs, nifedipine (1.0, 3.0, and 5.0 mg/kg/day), amlodipine (0.1, 0.5, and 1.0 mg/kg/day) and azelnidipine (0.1, 0.5, and 1.0 mg/kg/day) (provided by Sankyo, Tokyo, Japan), and hydrochlorothiazide (HCTZ; 0.5 mg/kg/day, Sigma, St. Louis, MO) were administered orally from the same time as cuff placement.⁶ We administered ~0.1 ml of CCBs and HCTZ as a suspension with 0.5% carboxymethyl cellulose sodium salt. Control animals had the same volume of the vehicle orally. Blood pressure was measured by the indirect tail-cuff method (MK-1031; Muromachi Kikai, Tokyo, Japan).

Morphometric analysis. The femoral arteries were taken at 14 days after cuff placement and fixed by perfusion with 10% neutral-buffered formalin. Paraffin-embedded cross-sections were prepared as previously described.^{6,14} Intimal area was measured using computer-imaging software (Densitograph; ATTO, Tokyo, Japan) after Elastica van Gieson staining.

Immunohistochemistry. Macrophages were stained as described previously.¹³ The femoral arteries were taken at 7 days after cuff placement and fixed by perfusion with 10% neutral-buffered formalin. The number of macrophage per high power field ($\times 200$) was measured after staining with anti-F4/80 monoclonal antibody (CI:A3-I; BMA Biomedicals, Augst, Switzerland).

Quantitative reverse transcription-PCR. Total RNA was extracted from the femoral arteries at 7 days after cuff placement. Quantitative real-time reverse transcription-PCR was performed using Premix Ex Taq (Takara Bio, Shiga, Japan). The PCR primers for p47^{phox} were 5'-GTCCCTGCATCCTATCTGGA-3' (forward) and 5'-GGGACATCTCGTCC TCTTCA-3' (reverse); for Rac-1, 5'-CCAGTGAATCTGGGCCTATG-3' (forward) and 5'-ACAGTGGTGTCGCACTTCAAG-3' (reverse); for monocyte chemoattractant protein-1 (MCP-1), 5'-TTAACGCCCCACTCACCTGCTG-3' (forward) and 5'-GCTTCTTTGGGACACCTGCTGC-3' (reverse); for tumor necrosis factor (TNF)- α , 5'-CGAGTGACAAGCCTGTAGCC-3' (forward) and 5'-GGTGAGGAGCACGTAGTCG-3' (reverse) and for glyceraldehyde-3-phosphate dehydrogenase, 5'-TGCGACTTCAACAGCAACTC-3' (forward) and 5'-ATGTAGGCCATGAGGTCCAC-3' (reverse).

In situ detection of superoxide anion production in injured arteries. Histological detection of superoxide anion was performed as described previously.¹⁵ In brief, frozen, enzymatically intact, 10- μ m-thick sections of injured femoral

arteries at 7 days after operation were incubated immediately with dihydroethidium (10 μ mol/l) in PBS for 30 min at 37°C in a humidified chamber protected from light. For detection of ethidium with red fluorescence, samples were examined with an Axioskop microscope (Axioskop 2 Plus with AxioCam; Carl Zeiss, Oberkochen, Germany) equipped with a computer-based imaging system. Intensity of the fluorescence was analyzed and quantified using computer-imaging software (Densitograph; ATTO, Tokyo, Japan).

Statistical analysis. Values are expressed as mean $\pm$ s.e.m. in the text and figures. The data were analyzed by one-way ANOVA. If a statistically significant effect was found, post hoc analysis was performed by Fisher's test to detect the difference between the groups. A value of $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Effect of combination of ARB with various CCBs on neointimal formation after cuff placement

Cuff placement around the femoral artery induced neointimal formation (Figure 1). In our previous studies, an ARB or CCB alone was shown to inhibit neointimal formation.^{6,16} Middle and higher doses of each CCB alone inhibited the neointimal formation (see **Supplementary Figure S1** online). On the other hand, a lower dose of nifedipine (1.0 mg/kg/day), amlodipine (0.1 mg/kg/day), and azelnidipine (0.1 mg/kg/day) did not affect neointimal formation (see **Supplementary Figure S1** online). In this study, we used each drug at a subeffective dose, by which neointimal formation could not be significantly inhibited. Each combination of olmesartan with a CCB, nifedipine, amlodipine, or azelnidipine, at subeffective doses significantly inhibited neointimal formation (Figure 1). However, this inhibitory action of the combination was greatest for olmesartan with azelnidipine. On the other hand, a combination of olmesartan and a subeffective dose of HCTZ did not significantly change neointimal formation (Figure 1). These combinations did not change the systolic blood pressure (Table 1).

Effect of combination of olmesartan and CCB on *in situ* superoxide anion production in injured arteries

Figure 2 shows superoxide anion production detected by dihydroethidium. Cuff placement increased superoxide anion production in the injured arteries. Olmesartan and each CCB alone did not change superoxide anion production (data not shown). However, a combination of olmesartan and CCB at these doses decreased superoxide anion production. Among these combinations, the inhibition by olmesartan with azelnidipine was the greatest. On the other hand, a combination of olmesartan and HCTZ did not change superoxide anion production (Figure 2).

Effect of combination of olmesartan and CCB on expression of NADPH oxidase subunits, p47^{phox} and Rac-1, in injured arteries

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase consists of several subunits and produces superoxide

anion.¹² mRNA expression of p47^{phox} and Rac-1 was significantly decreased by olmesartan with any CCB in the injured arteries at 7 days after cuff placement. In addition, among these combinations, olmesartan with azelnidipine inhibited expression of p47^{phox} mRNA more greatly than did olmesartan with amlodipine (Figure 3).

Effect of combination of olmesartan and CCB on inflammatory response in injured artery
Cuff placement induces an inflammatory response in the injured arteries. Expression of MCP-1 and TNF-α was

increased in the injured arteries. As shown in Figure 4, a combination of olmesartan and CCB inhibited expression of MCP-1 and TNF-α in the injured arteries. However, the combination of olmesartan with azelnidipine inhibited these markers more markedly than other combinations. Each combination of olmesartan with a CCB significantly inhibited migration of macrophages around injured vessels (control vs. olmesartan with nifedipine, olmesartan + amlodipine, and olmesartan + azelnidipine; 22.0 ± 1.7 vs. 13.8 ± 3.0, 13.3 ± 2.3, and 11.2 ± 1.8

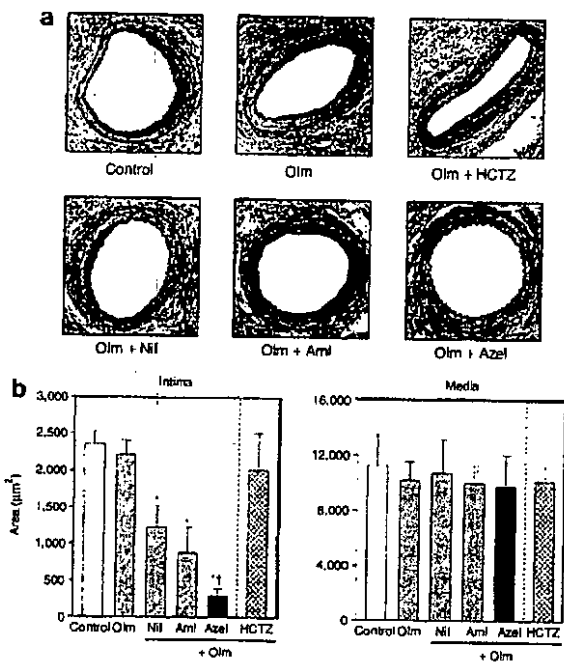


Figure 1 | Effect of Olm with calcium channel blocker on neointimal formation in injured arteries 14 days after polyethylene-cuff placement. Arterial samples were taken 14 days after cuff placement and paraffin-embedded cross-sections were prepared. (a) Representative Elastic van Gieson's staining of injured arteries. (b) Cross-sectional areas of the intima and media as described in Methods. *n* = 5–7 for each group. Values are mean ± s.e.m. Aml, amlodipine (0.1 mg/kg/day); Azel, azelnidipine (0.1 mg/kg/day); HCTZ, hydrochlorothiazide (0.5 mg/kg/day); Nif, nifedipine (1.0 mg/kg/day); Olm, olmesartan (0.5 mg/kg/day). **P* < 0.05 vs. Control or Olm + HCTZ. †*P* < 0.05 vs. Olm + Nif or Olm + Aml.

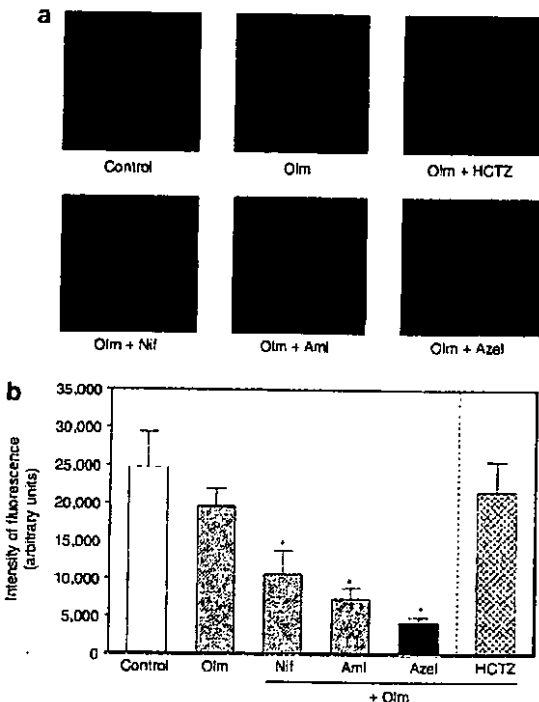


Figure 2 | Detection of superoxide anion production in injured arteries 7 days after cuff placement and effect of Olm with calcium channel blockers. Vascular samples were taken 7 days after operation. *In situ* production of superoxide was detected by dihydroethidium (DHE), as described in Methods. (a) Representative staining of injured arteries with DHE. (b) Intensity of fluorescence after DHE staining in injured arteries. Intensity of fluorescence was determined using computer-imaging software as described in Methods. *n* = 5–7 for each group. Values are mean ± s.e.m. Aml, amlodipine; Azel, azelnidipine; HCTZ, hydrochlorothiazide; Nif, nifedipine; Olm, olmesartan. **P* < 0.05 vs. Control or Olm + HCTZ.

Table 1 | Systolic blood pressure, heart rate and body weight after administration of Olm, CCBs, and HCTZ for 14 days

Control	Olm	Olm + Nif	Olm + Aml	Olm + Azel	Olm + HCTZ
Systolic blood pressure (mm Hg)					
90.5 ± 0.7	90.8 ± 0.6	89.3 ± 1.4	89.2 ± 0.6	89.6 ± 1.2	90.1 ± 1.7
Heart rate (beat/min)					
690 ± 12	710 ± 16	720 ± 13	700 ± 19	694 ± 19	684 ± 19
Body weight (g)					
24.4 ± 0.5	25.6 ± 1.0	24.8 ± 0.6	25.1 ± 1.0	24.7 ± 0.4	25.5 ± 0.8

Olm, HCTZ and/or CCBs were administered and systolic blood pressure was measured after 14 days by the indirect tail-cuff method as described in Methods. *n* = 5–6 for each group. Aml, amlodipine (0.1 mg/kg/day); Azel, azelnidipine (0.1 mg/kg/day); CCBs, calcium channel blockers; HCTZ, hydrochlorothiazide (0.5 mg/kg/day); Nif, nifedipine (1.0 mg/kg/day); Olm, olmesartan (0.5 mg/kg/day).

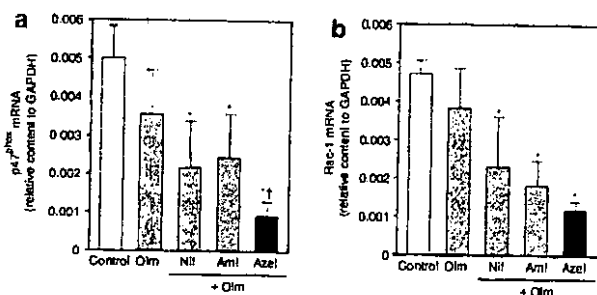


Figure 3 | Effect of Olm with calcium channel blocker on expression of (a) p47^{phox} and (b) Rac-1 in injured arteries 7 days after cuff placement. Arterial samples were taken 7 days after cuff placement and total RNA was purified as described in Methods. Level of mRNA was measured by real-time reverse transcription-PCR as the content relative to that of GAPDH from pooled arteries ($n = 6-7$ for each group). Values are mean $\pm$ s.e.m. Aml, amlodipine; Azel, azelnidipine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Nif, nifedipine; Olm, olmesartan. * $P < 0.05$ vs. Control. [†] $P < 0.05$ vs. Olm + Aml.

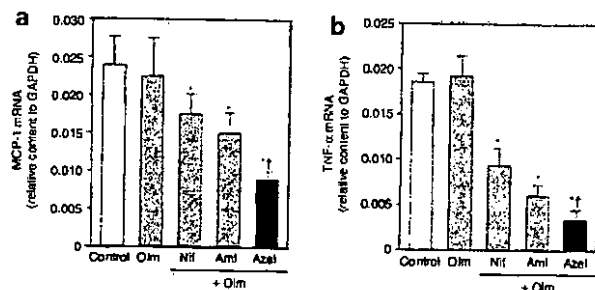


Figure 4 | Effect of Olm with calcium channel blocker on expression of (a) MCP-1 and (b) TNF- α in injured arteries 7 days after cuff placement. Arterial samples were taken 7 days after cuff placement and total RNA was purified as described in Methods. Level of mRNA was measured by real-time reverse transcription-PCR as the content relative to that of GAPDH from pooled arteries ($n = 6-7$ for each group). Values are mean $\pm$ s.e.m. Aml, amlodipine; Azel, azelnidipine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MCP-1, monocyte chemoattractant protein-1; Nif, nifedipine; Olm, olmesartan; TNF, tumor necrosis factor. * $P < 0.05$ vs. Control. [†] $P < 0.05$ vs. Olm + Nif.

count/field, respectively). However, there were no significant differences among any combination.

DISCUSSION

In this study, we demonstrated that the combination of an ARB, olmesartan, with a CCB synergistically inhibited vascular remodeling and the inflammatory response without affecting systolic blood pressure, using a mouse model of vascular injury. We further demonstrated that the combination of sub-effective doses of an ARB and a CCB inhibited oxidative stress, such as superoxide anion production and expression of p47^{phox} and Rac-1, in injured arteries, and that the inhibitory action of olmesartan and azelnidipine was greater than that of other combinations. On the other hand, the combination of olmesartan and a sub-effective dose of HCTZ did not significantly affect vascular changes after cuff placement. These results suggest that the combination of an ARB and a CCB is useful to inhibit vascular remodeling, and that the inhibitory effect of the combination may vary depending on the combined drugs.

We previously reported that CCB as well as ARB inhibited vascular remodeling.^{6,16,17} We also reported that a CCB, azelnidipine, enhanced the inhibitory action of an ARB, olmesartan, on vascular remodeling after cuff placement.⁶ In previous reports, signaling molecules such as Pyk2, c-Src, ERK, and STAT were shown to be involved in AT₁ receptor-mediated actions.^{18,19} CCB may inhibit the activation of these signaling molecules. The mechanism of synergistic action of ARB and CCB is not yet clear. However, some possibilities can be considered to explain this issue. One possibility might be the modulation of intracellular calcium level by ARB and CCB. It has been reported that intracellular Ca²⁺ plays a role in angiotensin II-induced stimulation of MAP kinase and consequently vascular smooth muscle cell proliferation.²⁰ Since angiotensin II increases intracellular calcium level, ARB inhibits the calcium-dependent pathway of angiotensin II signaling by reducing intracellular calcium level. The calcium-dependent pathway may have a threshold. Therefore, sub-effective dose of ARB or CCB decreases intracellular calcium slightly but not under threshold. The combination of sub-effective dose of ARB and CCB lowers intracellular calcium under threshold. Another possibility might be that ARB has Ca²⁺-independent pathway. We have previously reported that a CCB, azelnidipine (0.5 or 1.0 mg/kg/day), significantly decreased neointimal formation even in AT₁AKO mice, whereas olmesartan did not show additional inhibition.⁶

Cuff placement-induced neointimal formation was associated with an increase in oxidative stress determined by superoxide production, NADPH oxidase activity, and NADPH oxidase subunit expression.^{6,14} In this study, superoxide anion production and expression of NADPH subunits, p47^{phox} and Rac-1, were decreased by olmesartan with various CCBs in the injured arteries (Figures 2 and 3). NADPH oxidase consists of subunits of membrane components (Nox1, Nox4, and p22^{phox}) and cytosolic components (p47^{phox} and Rac-1, etc.). We also measured the expression of Nox1 and Nox4, membrane components of NADPH oxidase subunits. Expression of Nox4 was under detectable level in the injured artery. However, expression of Nox1 was significantly lowered only by the combination of olmesartan and azelnidipine (data not shown). These results suggest that the antiatherosclerotic effects of a combination of olmesartan with CCBs are mediated, at least partly, through modulation of oxidative stress. Dihydropyridine-type CCBs are among the most commonly used drugs for the treatment of hypertension. However, the molecular mechanisms by which CCBs reduce atherosclerosis are not fully understood. One of the mechanisms that have been proposed for the beneficial effect of CCBs is antioxidative activity independent of calcium entry blockade.^{6,16,17,21} In fact, the antioxidant action of CCB seemed to be stronger than that of ARB.²²

The cuff injury model stimulates an inflammatory reaction that appears to play a role in increase of neointimal formation. We have also reported that the inflammatory responses, such as infiltration of macrophage or leukocytes and increased expression of chemokines and cytokines, can be observed in the injured artery.¹³ In this study, the expression of MCP-1 and TNF- α was

synergistically inhibited by the combination of olmesartan and CCB, suggesting the synergistic inhibition of inflammatory response by its combination. We also demonstrated that combination of olmesartan with various CCBs significantly decreased macrophage infiltration around injured vessels. In this study, we also measured circulating levels of MCP-1 and TNF- α at 7 days after cuff placement. Circulating level of MCP-1 and TNF- α detected by ELISA (Invitrogen, Camarillo, CA) was not significantly changed by treatment with drugs (data not shown). This result suggests that inhibitory effect by combination of olmesartan and CCB on decrease in neointimal formation might be at least in part due to suppression of local inflammatory response in the injured vascular wall.

In this study, among dihydropyridine-type CCBs, the inhibition of inflammatory change by olmesartan with azelnidipine was stronger than that by combinations with other CCBs. Azelnidipine was developed as a third-generation Ca^{2+} channel blocker. Shinomiya *et al.* reported that azelnidipine more effectively inhibited the production of isoprostane from cultured human arterial endothelial cells stimulated with hydrogen peroxide compared to other CCBs (nifedipine and amlodipine).²³ Therefore, we could expect that azelnidipine has a stronger antioxidative effect than other agents in the same class. Inducible nitric oxide induced-nitric oxide production contributes to the progression of cardiovascular disease. Moreover, it is known that dihydropyridine-type CCBs such as nifedipine and amlodipine inhibit the activation of inducible nitric oxide and contributes to vasculoprotective effects. Ma *et al.* previously reported that azelnidipine exhibited more powerful inhibition of nitric oxide production than other CCBs including nifedipine and amlodipine.²⁴ Although the exact molecular mechanism of the inhibition of inflammatory response by azelnidipine has not been fully elucidated, azelnidipine differs from other dihydropyridine-type CCBs with respect to its pharmacokinetic profile. In azelnidipine, one methyl group is substituted by an amino group in the dihydropyridine ring, whereas other dihydropyridine-type CCBs have two methyl groups, located at positions 2 and 6 of the dihydropyridine ring.²⁵ Moreover, it has been reported that azelnidipine is highly lipophilic, and is thus retained in the vascular wall.^{25,26} These characteristics of azelnidipine may induce strong inhibitory actions on vascular remodeling and vascular oxidative stress, compared with other CCBs.

A recent study showed that the combination of losartan/HCTZ is useful for the management of hypertension.² However, in this study, the combination of subeffective doses of olmesartan and HCTZ did not significantly affect neointimal formation and superoxide anion production after cuff placement, probably because we used these drugs at non-hypotensive and subeffective doses in this study, in order to exclude hemodynamic changes by these various combinations. These data suggested that the combination of an ARB with a CCB might be better to the combination of an ARB with HCTZ with respect to their pleiotropic effects on cuff-induced vascular remodeling. In this study, we did not examine the combined effects with various ARBs. However, it is suggested that

the beneficial effects of drug combinations may vary depending on combined drugs.

Our present findings suggest the possibility that the combination of an ARB with a CCB is better than the combination of an ARB with HCTZ for vascular remodeling, at least when the drugs are used at subeffective doses. The inhibitory effects of the combination of ARB with CCB are, at least partly, dependent on a decrease in oxidative stress. Therefore, we conclude that combination treatment of an ARB with a CCB could be clinically beneficial. Moreover, we also demonstrated that the inhibitory actions of the combination might vary depending on different CCBs. Which kind of CCB is superior for preventing vascular remodeling must be further investigated in the future.

Supplementary material is linked to the online version of the paper at <http://www.nature.com/ajh>

Acknowledgments: This work was supported in part by grants from the Ministry of Education, Science, Sports, and Culture of Japan.

Disclosure: The authors declared no conflict of interest.

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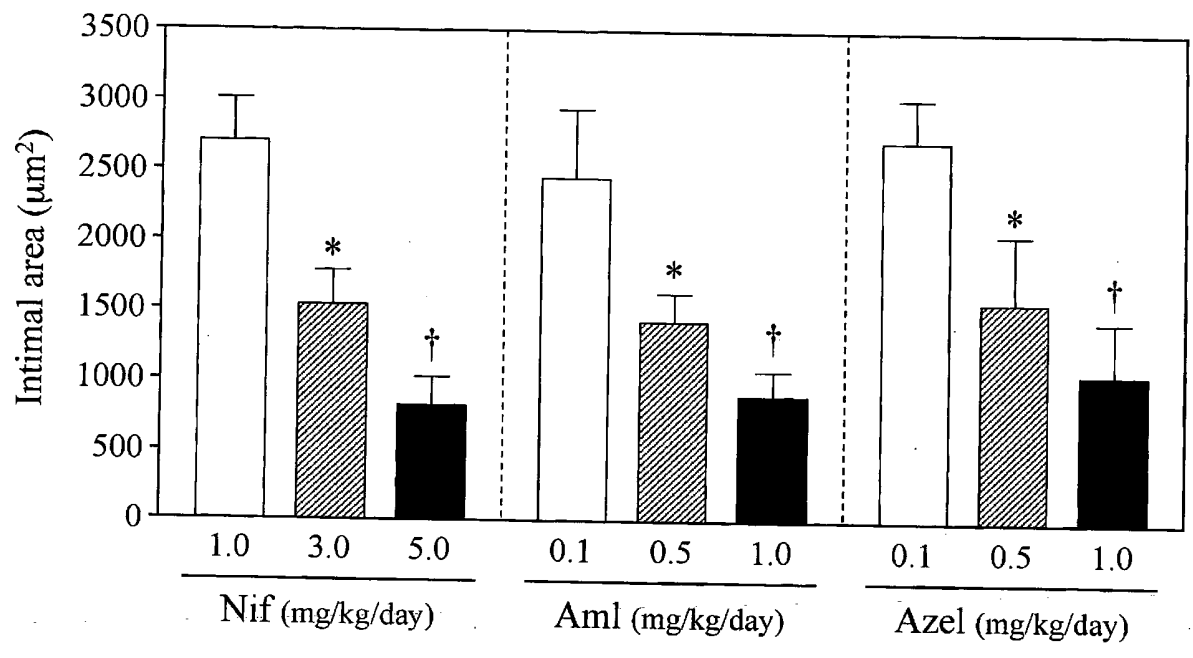


Figure I

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Calcium Channel Blocker Azelnidipine Enhances Vascular Protective Effects of AT 1 Receptor Blocker Olmesartan

Toyohisa Jinno, Masaru Iwai, Zhen Li, Jian-Mei Li, Hong-Wei Liu, Tai-Xing Cui,
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Hypertension 2004;43:263-269; originally published online Jan 5, 2004;

DOI: 10.1161/01.HYP.0000113627.08110.6f

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX
72514

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ISSN: 1524-4563

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Calcium Channel Blocker Azelnidipine Enhances Vascular Protective Effects of AT₁ Receptor Blocker Olmesartan

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Abstract—The present studies were undertaken to investigate the potential effect of a calcium channel blocker (CCB) to enhance the inhibitory effect of an angiotensin (Ang) II type 1 (AT₁) receptor blocker (ARB) on vascular injury and the cellular mechanism of the effect of CCB on vascular remodeling. In polyethylene cuff-induced vascular injury of the mouse femoral artery, proliferation of vascular smooth muscle cells (VSMCs) and neointimal formation associated with activation of extracellular signal-regulated kinase (ERK), and tyrosine-phosphorylation of signal transducer and activator of transcription (STAT)1 and STAT3, inflammatory response assessed by monocyte chemoattractant protein-1 and tumor necrosis factor- α expression, as well as oxidative stress such as expression of NADH/NADPH oxidase p22^{phox} subunit and superoxide production, were less in AT_{1a} receptor null mice. Administration of nonhypotensive doses of a CCB, azelnidipine (0.5 or 1 mg/kg per day) attenuated these parameters in wild-type and AT_{1a} receptor null mice. Coadministration of lower doses of an ARB, olmesartan (0.5 mg/kg per day), and azelnidipine (0.1 mg/kg per day), which did not affect vascular remodeling, significantly inhibited these parameters in wild-type mice. Moreover, the effective dose of azelnidipine (1 mg/kg per day) exaggerated the inhibitory action of olmesartan at effective doses of 1 or 3 mg/kg per day on VSMC proliferation in the injured arteries. These results suggest that azelnidipine could inhibit vascular injury at least partly independent of the inhibition of AT₁ receptor activation and that azelnidipine could exaggerate the vascular protective effects of olmesartan, suggesting clinical possibility that the combination of CCB and ARB could be more effective in the treatment of vascular diseases. (*Hypertension*. 2004;43:263-269.)

Key Words: angiotensin ■ calcium channel ■ oxidative stress ■ vascular remodeling ■ inflammation

Evidence indicates that angiotensin (Ang) II is more than a hormone with hemodynamic and renal actions. It is also a local, biologically active mediator that has direct effects on endothelial and vascular smooth muscle cells (VSMCs) and plays a key role in the initiation and amplification of pathobiological events that lead to vascular disease.¹ Ang II is a major mediator of oxidative stress and reduced nitric oxide (NO) activity by activating NADH/NADPH oxidase, which results in the production of the superoxide anion.² Other mechanisms whereby Ang II may promote vascular remodeling and the formation of vascular lesions are the modulation of VSMC migration and decreased VSMC apoptosis,^{3,4} suggesting that Ang II is a pleiotropic local mediator of vascular remodeling and lesion formation. These major cardiovascular actions of Ang II have been reported to be mediated by the type 1 Ang II (AT₁) receptor, and AT₁ receptor blockers (ARBs) have been widely used as antihypertensive drugs with the expectation of a vascular protective effect.⁵

Calcium channel blockers (CCBs) have been shown to retard atherogenesis in animal models and to prevent the development of early lesions in human coronary arteries. They are used in the treatment of angina pectoris and hypertension. However, the

mechanism by which CCB reverse endothelial dysfunction and prevent vascular damage is largely unknown. One of the mechanisms proposed to explain the beneficial effect of CCBs on endothelial function is antioxidant activity.^{6,7} Recently, Fukuo et al reported that the CCB nifedipine indirectly upregulates endothelial superoxide dismutase expression by stimulating vascular endothelial growth factor production by adjacent VSMC.⁸ It has been reported that DNA synthesis induced by Ang II and growth factors such as platelet-derived growth factor (PDGF) in VSMCs is significantly blunted by CCBs, and that CCBs inhibit neointimal formation in the injured artery.⁹⁻¹¹ Accumulating evidence has suggested that the intracellular signaling mechanisms, by which AT₁ receptors exert hypertrophic and/or hyperplastic effects on their targets such as VSMC, are closely associated with receptor and non-receptor tyrosine kinases, and that some AT₁ receptor-mediated signaling requires Ca²⁺-sensitive tyrosine kinases.¹² These results suggest the possibility that combination therapy of ARB and CCB could more effectively prevent vascular damage than monotherapy.

Arterial neointimal thickening is an important process in the development of atherosclerosis, bypass graft failure, and

Received October 7, 2003; first decision October 27, 2003; revision accepted December 9, 2003.

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Hypertension is available at <http://www.hypertensionaha.org>

DOI: 10.1161/01.HYP.0000113627.08110.6f

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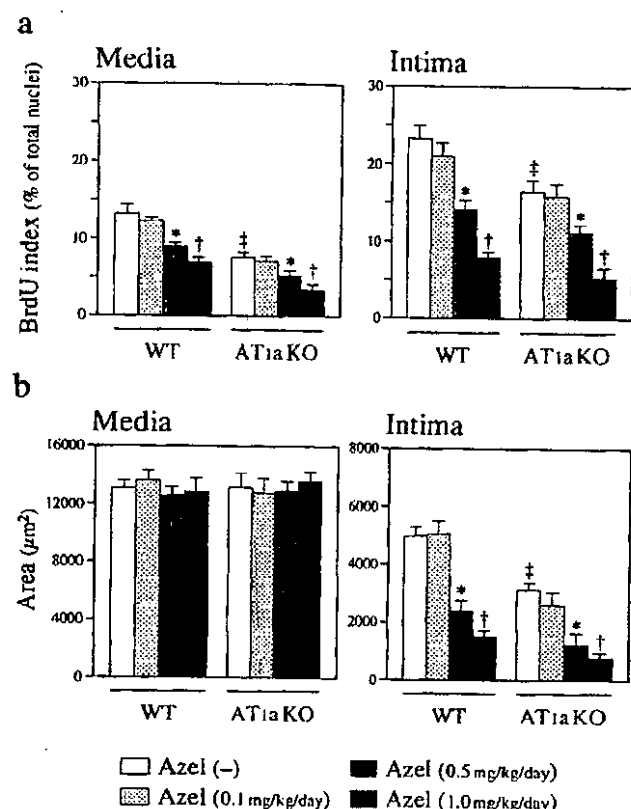


Figure 1. Effects of azelnidipine on DNA synthesis and morphometric changes after cuff placement. Samples were prepared from cuffed femoral arteries of WT and AT₁aKO mice as described in Methods. A, BrdU uptake in VSMC in media and neointima was assayed 7 days after operation. B, Morphometric analysis of media and neointima was performed 14 days after operation. $n=8$ to 10 for each group. * $P<0.05$, † $P<0.01$ versus without azelnidipine. ‡ $P<0.05$ versus WT without azelnidipine.

restenosis after angioplasty. We have developed a mouse model of vascular disease induced by polyethylene cuff placement around the femoral artery, in which the AT₁ receptor is upregulated, followed by VSMC proliferation, vascular inflammation, and neointimal thickening.^{13–16} In the present study, we used this mouse model of cuff-induced vascular injury and explored the following possibilities: (1) dihydropyridine CCB, azelnidipine, can prevent vascular injury, independently of the AT₁ receptor; and (2) possible signaling mechanism of CCB-mediated prevents vascular remodeling, especially when focusing on growth-promoting signals and oxidative stress. Moreover, we investigated that

Systolic Blood Pressure After Administration of Azelnidipine and Olmesartan in WT and AT₁aKO Mice

Groups	(-)	Azelnidipine (mm Hg)	Olmesartan (mm Hg)	Azelnidipine + Olmesartan (mm Hg)
WT	96.4±1.7	97.2±1.3	96.9±2.8	97.2±1.7
AT ₁ aKO	82.4±0.9*	82.9±0.8*	82.8±2.5*	n.d.

Azelnidipine and olmesartan were administered and systolic blood pressure was measured 14 days later by the indirect tail-cuff method as described in Methods. n.d. indicates not determined. Azelnidipine dose was 1.0 mg/kg per day. Olmesartan dose was 3.0 mg/kg per day.

* $P<0.05$ vs WT.

azelnidipine can exaggerate the inhibitory effect of an ARB, olmesartan, on vascular remodeling.

Methods

Animals and Operation

Adult male AT₁a receptor knockout mice (AT₁aKO)¹⁷ (donated by Tanabe Seiyaku Co. Ltd., Osaka, Japan) and wild-type (WT) mice (based on C57BL/6J strain; Clea Japan Inc., Osaka, Japan) aged 10 to 12 weeks were used in this study. The Animal Studies Committee of Ehime University approved the experimental protocol. The surgical procedure for cuff-induced vascular injury in the femoral artery was performed according to the method previously described.^{13–16}

Administration of CCB and ARB

Azelnidipine (CS-905; donated by Sankyo Pharmaceutical Co., Tokyo, Japan) was administered orally after cuff placement for 7 or 14 days. Olmesartan (RNH-6270; donated by Sankyo Pharmaceutical Co., Tokyo, Japan) was administered using an osmotic minipump (model 1002; Alza Corp.) implanted intraperitoneally at the same time as cuff placement, as previously described.¹⁵ Blood pressure was measured by the indirect tail-cuff method with blood pressure monitor (MK-1030; Muromachi Kikai Co. Ltd, Tokyo, Japan).

Morphometric Analysis, Measurement of DNA Synthesis, and Immunohistochemical Study

Morphometric analysis, measurement of DNA synthesis determined by the incorporation of bromodeoxyuridine (BrdU), and immunohistochemical study were performed according to the methods previously described.^{13–16}

Detection of Inflammatory Factors, NADH/NADPH Oxidase, and Signaling Molecules by Western Blot

Total proteins were prepared from pooled arteries (6 to 8 arteries for each group), and Western blot was performed using specific antibodies as previously described.^{13–16} Anti-p22^{phox} antibodies were purchased from Santa Cruz Biotechnology Inc. Densitometric analysis was performed using an image scanner (EPSON GT-8000) and NIH imaging software.

Immunofluorescent Study

Paraffin-embedded sections were incubated with anti-p22^{phox} antibody, washed, and incubated with biotin-labeled secondary antibodies, then incubated with Cy3-labeled streptavidin. Serial sections treated with secondary antibodies alone did not show specific staining. Samples were examined with a Zeiss Axioskop microscope equipped with a computer-based imaging system.¹⁵

Superoxide Detection

Frozen, enzymatically intact, 10-μm-thick sections of sham-operated and injured femoral arteries were incubated at the same time with dihydroethidium (DHE; 10 μmol/L) in PBS for 30 minutes at 37°C in a humidified chamber protected from light.¹⁸ DHE is oxidized on reaction with superoxide to ethidium, which binds to DNA in the nucleus and fluoresces red. For ethidium detection, a 543-nm argon laser combined with a 500- to 550-nm band-pass filter was used.

Statistical Analysis

Values are expressed as mean±SEM in the text and figure. Data were analyzed by 2-way ANOVA, followed by post-hoc analysis. A value of $P<0.05$ was considered to be statistically significant.

Results

Effect of Azelnidipine on Neointimal Formation and VSMC Proliferation After Cuff Placement in WT and AT₁aKO Mice

To examine the possibility of whether CCB could exert a vascular protective effect, in part independently of antagoniz-

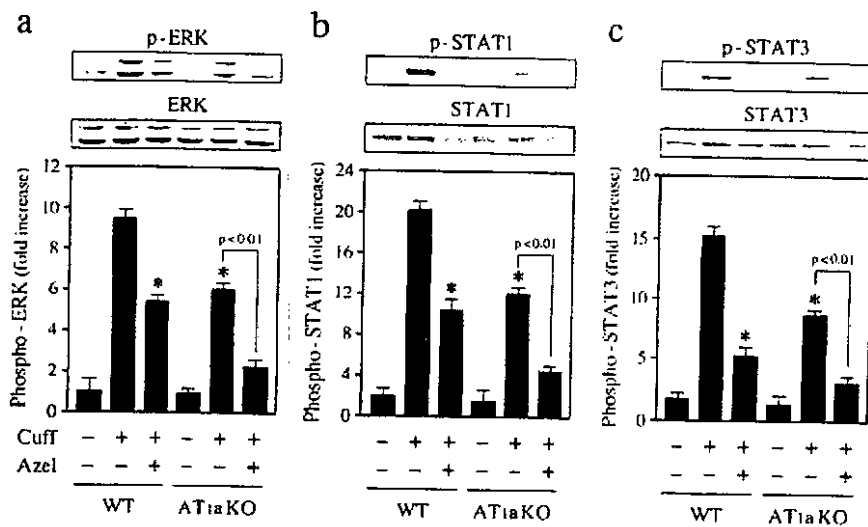


Figure 2. Effects of azelnidipine (1.0 mg/kg per day) on ERK, STAT1, and STAT3. Tissue samples were prepared from cuffed arteries 7 days after operation. A, One representative result of Western blots using 4 different pooled samples is shown. Upper lanes show activated ERK, STAT1, and STAT3 and lower lanes show total proteins. B, Densitometric analysis of Western blots. Values are mean $\pm$ SEM of four different experiments. * P <0.01 versus cuff alone in WT; p indicates phospho.

ing AT₁ receptor stimulation, we used AT₁aKO mice and examined the effect of azelnidipine on vascular injury. As previously reported,¹⁴ BrdU labeling index in VSMCs in the media and neointima measured 7 days after cuff placement was significantly lower in AT₁aKO mice, and neointimal formation 14 days after operation was also significantly less than that in WT mice (Figure 1). These results support the notion that AT₁ receptor stimulation plays a role in vascular remodeling via enhancing VSMC proliferation. Azelnidipine at a dose of 0.5 or 1 mg/kg per day, which did not influence blood pressure and heart rate, significantly inhibited DNA synthesis in VSMC and neointimal formation in WT and AT₁aKO mice (Table, Figure 1).

Signaling Mechanism of Inhibitory Effect of Azelnidipine on Neointimal Formation

To examine the signaling mechanism, we focused on extracellular signal-regulated kinase (ERK) activity, because the ERK pathway, which is activated by the AT₁ receptor and growth factors, is critical for cell proliferation.¹⁹ We also examined the

tyrosine-phosphorylation of signal transducer and activator of transcription (STAT)1 and STAT3 in the injured artery, because STAT seem to be involved in AT₁ receptor-mediated *c-fos* expression in VSMCs.²⁰ Consistent with previous reports,^{15,16} cuff-induced vascular injury increased phosphorylation of ERK, STAT1, and STAT3 in the injured artery 7 days after cuff placement without changes in total protein levels of these proteins, whereas these increases were smaller in AT₁aKO mice (Figure 2). Azelnidipine at a dose of 1 mg/kg per day significantly inhibited activation of ERK and STAT in the injured artery in WT and AT₁aKO mice.

Effect of Azelnidipine on Inflammatory Response and Oxidative Stress Induced by Cuff Placement

Western blot using pooled artery samples showed that the expression of monocyte chemoattractant protein (MCP)-1 and tumor necrosis factor (TNF)- α was increased 7 days after cuff placement in WT and AT₁aKO mice; however, this increase was less in AT₁aKO mice (Figure 1a, available at <http://www.hypertensionaha.org>). Azelnidipine (1 mg/kg per day)

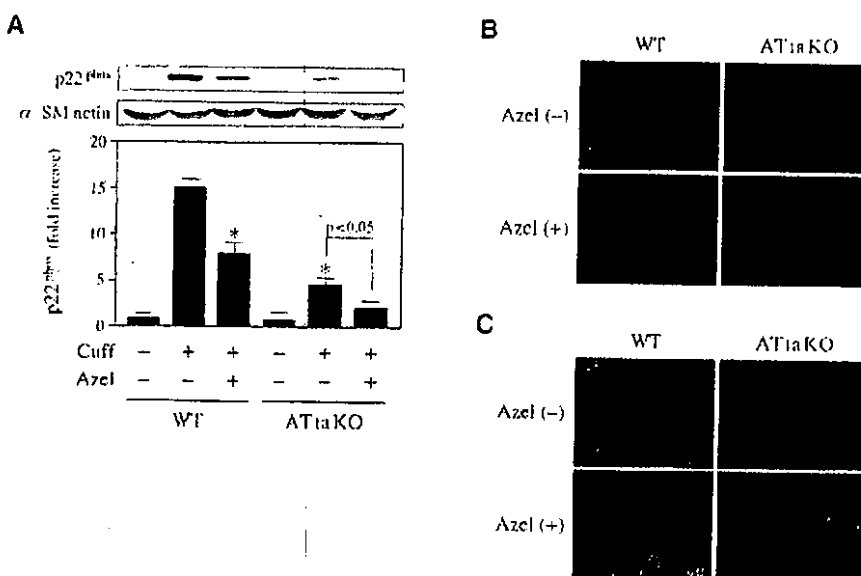


Figure 3. Effect of azelnidipine (1.0 mg/kg per day) on oxidative stress in injured artery. Tissue samples were prepared from injured arteries 7 days after operation. A, One representative result of Western blots (upper) and densitometric analysis (lower) of p22^{phox} using three different pooled samples is shown. B, One representative result of four independent immunohistochemical staining of p22^{phox} in cuffed artery. C, One representative result of four independent fluorescence of superoxide in cuffed artery. * P <0.01 versus cuff alone in WT.

inhibited the expression of MCP-1 and TNF- α not only in WT mice but also in AT₁aKO mice (Figure 1a). Consistent with these results, immunohistochemical staining showed similar results (Figure 1b and 1c, available online at <http://www.hypertensionaha.org>). Next, we examined the effect of azelnidipine on expression of p22^{phox}, a membrane-associated NADH/NADPH oxidase subunit, and superoxide anion production in the injured artery. Expression of p22^{phox} and superoxide anion production was increased after cuff placement in WT and AT₁aKO mice; however, these increases were less in AT₁aKO mice (Figure 3). Azelnidipine inhibited the expression of p22^{phox} and superoxide anion production in both WT and AT₁aKO mice (Figure 3).

Effects of Olmesartan and Azelnidipine on Vascular Remodeling

Olmesartan at 1 or 3 mg/kg per day significantly decreased neointimal formation and decreased BrdU-positive VSMC in the media and neointima in WT mice (Figure 4) without a change in blood pressure (Table), as previously reported.¹⁵ Zhu et al reported that cultured aortic VSMCs prepared from AT₁aKO mice display functional AT₁b receptors, and activation of these receptors leads to calcium entry and metabolism. To examine the role of AT₁b receptor in this cuff-induced vascular injury model, we examined the effect of effective dose of olmesartan (3 mg/kg per day) on DNA synthesis in VSMC in AT₁aKO mice and demonstrated that olmesartan did not significantly inhibit BrdU incorporation in VSMCs (Figure 4). Moreover, we demonstrated that the expression of AT₁b receptor was very low in femoral artery and did not change after cuff placement.²¹ These results suggest that the involvement of AT₁b receptor is less possible in this cuff-induced vascular injury model in femoral artery. Administration of olmesartan (3 mg/kg per day) significantly decreased the activation of ERK, STAT1, and STAT3 without any change in their total protein levels (Figure 11, available online at <http://www.hypertensionaha.org>) and inhibited the expression of MCP-1, TNF- α , and p22^{phox} (Figure 5 and Figure 6). Next, we examined the possibility of whether azelnidipine together with olmesartan could exert a synergistic effect to prevent vascular injury. Olmesartan or azelnidipine alone at lower doses (0.5 or 0.1 mg/kg per day, respectively) did not affect neointimal formation, BrdU index, activation of ERK, STAT1, and STAT3, expression of p22^{phox} and superoxide anion production. However, coadministration of olmesartan and azelnidipine at these doses significantly decreased these parameters (Figure 4 to 6 and 11). We also observed that a lower dose of azelnidipine (0.1 mg/kg per day) significantly exaggerated the inhibitory effects of olmesartan at effective doses (1 or 3 mg/kg per day) on BrdU index (Figure 4). Moreover, we observed that effective dose of azelnidipine (1 mg/kg per day) exaggerated the inhibitory effect of olmesartan at effective doses of 1 or 3 mg/kg per day on VSMC proliferation in the injured arteries (Figure 4).

Discussion

In a mouse model of vascular injury, we have documented that cuff placement around the femoral artery induces neointimal thickening with an increase in VSMC proliferation and inflammation, as well as upregulation of components of the renin-angiotensin system such as the AT₁ receptor in injured

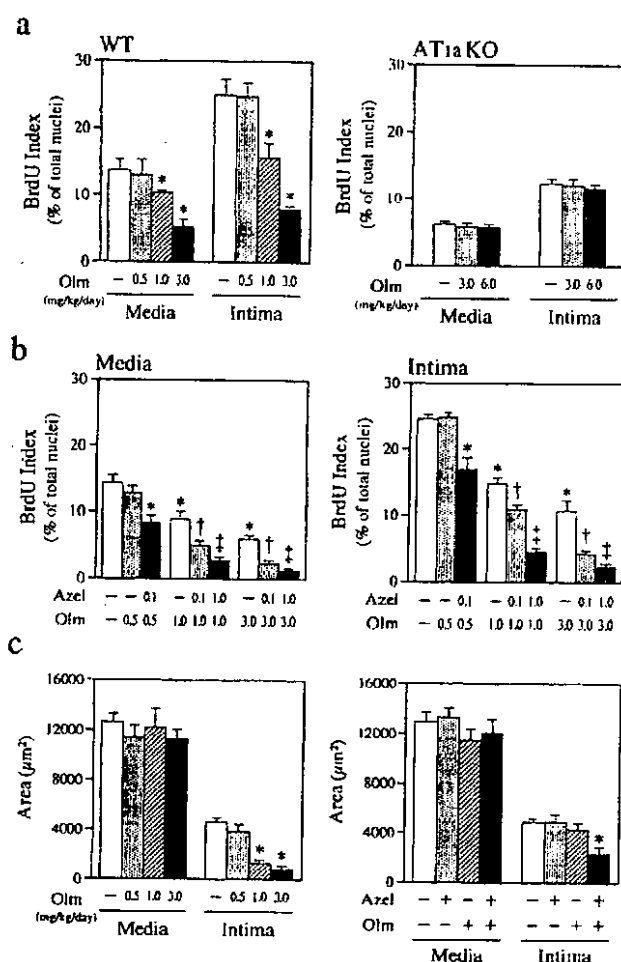


Figure 4. Effect of azelnidipine/olmesartan combination on DNA synthesis and morphometric changes after cuff placement. Samples were prepared from cuffed femoral arteries as in Figure 1. A, Dose-dependent effect of olmesartan on BrdU uptake in media and neointima in cuffed artery of WT and AT₁aKO mice. B, BrdU uptake in media and neointima in cuffed artery of WT mice treated with combination of azelnidipine and olmesartan. C, Morphometric analysis of media and neointima in injured artery with olmesartan alone (left) and the combination of lower doses of azelnidipine at 0.1 mg/kg per day and olmesartan at 0.5 mg/kg per day (right), $n=8$ to 10 for each group. * $P<0.05$ versus without azelnidipine and/or olmesartan. † $P<0.05$ versus without azelnidipine. ‡ $P<0.05$ versus azelnidipine (0.1 mg/kg per day).

arteries.¹⁴ In the present study, we further demonstrated that superoxide anion production and expression of p22^{phox} were increased in injured arteries induced by cuff placement (Figure 3). Because it has been shown that NADH/NADPH oxidases are a major enzymatic source of superoxide generation in vascular cells,²² the upregulation of NADH/NADPH oxidases might cause a burst of superoxides, thereby contributing to the pathophysiology of cuff-induced vascular injury. We demonstrated that proliferation of VSMC, neointimal formation, inflammation assessed by MCP-1 and TNF- α expression, expression of p22^{phox}, and superoxide anion production were attenuated in AT₁aKO mice (Figure 3 and 1). Administration of a nonhypotensive dose of an ARB, olmesartan, significantly attenuated these vascular responses, supporting the notion that Ang II stimulation via the AT₁ receptor exerts pleiotropic effects on vascular remodeling in WT mice (Figure 5 and 6).

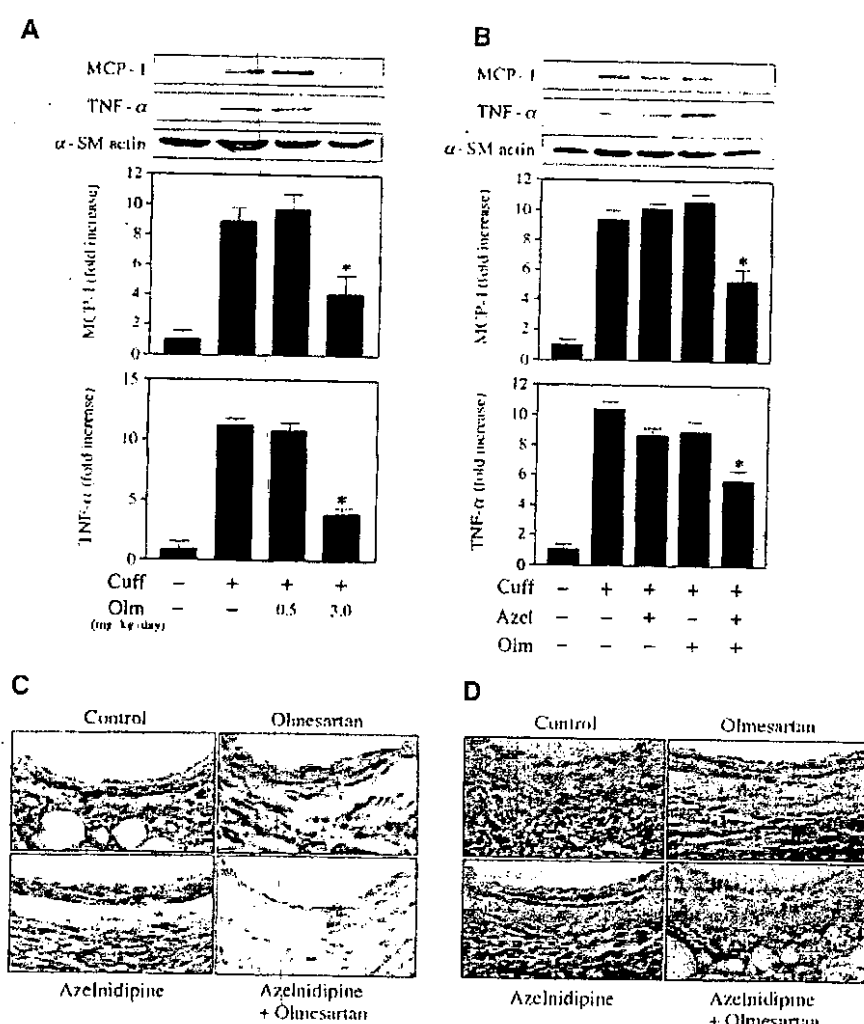


Figure 5. Effects of azelnidipine/olmesartan combination on MCP-1 and TNF- α expression in injured artery. Tissue samples were prepared from cuffed arteries 7 days after operation. A, Dose-dependent effect of olmesartan on MCP-1 and TNF- α expression. B, Effects of azelnidipine/olmesartan combination on MCP-1 and TNF- α expression. One representative result of Western blots (upper) and densitometric analysis (lower) using three or four different pooled samples are shown. One representative result of four different immunohistochemical staining of MCP-1 (C) and TNF- α (D) in cuffed artery. * $P < 0.01$ versus cuff alone.

CCB are also widely used for the treatment of hypertension with the expectation of their potential vascular protective effect. It seems to be possible that combination therapy of an ARB with a CCB could exert more beneficial effects on vascular remodeling than monotherapy, although the effects of CCB on vascular diseases are not well established. To explore this possibility and examine the possible cellular and signaling mechanisms involved in the potential vasoprotective effect of CCB and ARB, we used a cuff-induced vascular injury model, in which Ang II plays a role in the pathogenesis of vascular remodeling as mentioned above. We demonstrated that azelnidipine even at a nonhypotensive dose effectively inhibited DNA synthesis of VSMC and neointimal formation in WT and AT₁AKO mice (Figure 1). These results suggest that azelnidipine could inhibit the VSMC proliferation in vascular injury and, further, that this effect of azelnidipine is at least partly independent of the inhibition of AT₁ receptor activation. Moreover, we demonstrated that olmesartan or azelnidipine alone at lower doses did not affect neointimal formation; however, coadministration of both olmesartan and azelnidipine at these doses (0.5 and 0.1 mg/kg per day, respectively), significantly inhibited VSMC proliferation and neointimal formation (Figure 4).

It has been reported that AT₁ receptor stimulation increases intracellular Ca²⁺ via an influx of extracellular Ca²⁺ by

opening cell membrane calcium channels or release from the intracellular Ca²⁺ pool, which leads to activation of Ca²⁺-sensitive kinases, such as Pyk2 and Src and transactivation of epidermal growth factor (EGF) receptor, and consequent activation of downstream mitogen-activated protein (MAP) kinases such as ERK, thereby contributing to VSMC growth.¹² Seewald et al²³ reported that the Ang II-induced stimulation of ERK is a Ca²⁺-dependent process mainly through the influx of Ca²⁺ from outside of the VSMC and is important for Ang II-induced DNA synthesis in VSMCs. It has also been reported that nifedipine decreased DNA synthesis and total cellular protein content as well as the levels of phosphorylated ERK and Pyk-2 and even the phosphorylation of Pyk-2 in cultured VSMC, and that nifedipine suppressed VSMC proliferation in balloon-injured rat thoracic aorta.¹¹ Consistent with these results, we demonstrated that administration of a nonhypotensive dose of azelnidipine effectively inhibited activation of ERK in the cuff-induced injured artery (Figure 2). It is established that sequentially activated protein kinases such as ERK mediate further transmission of growth signals to the nucleus through expression of the immediate early growth response gene *c-fos*, which is regulated by the net interaction of different transcription factors. STAT are known to be activated by many different extracellular signal-

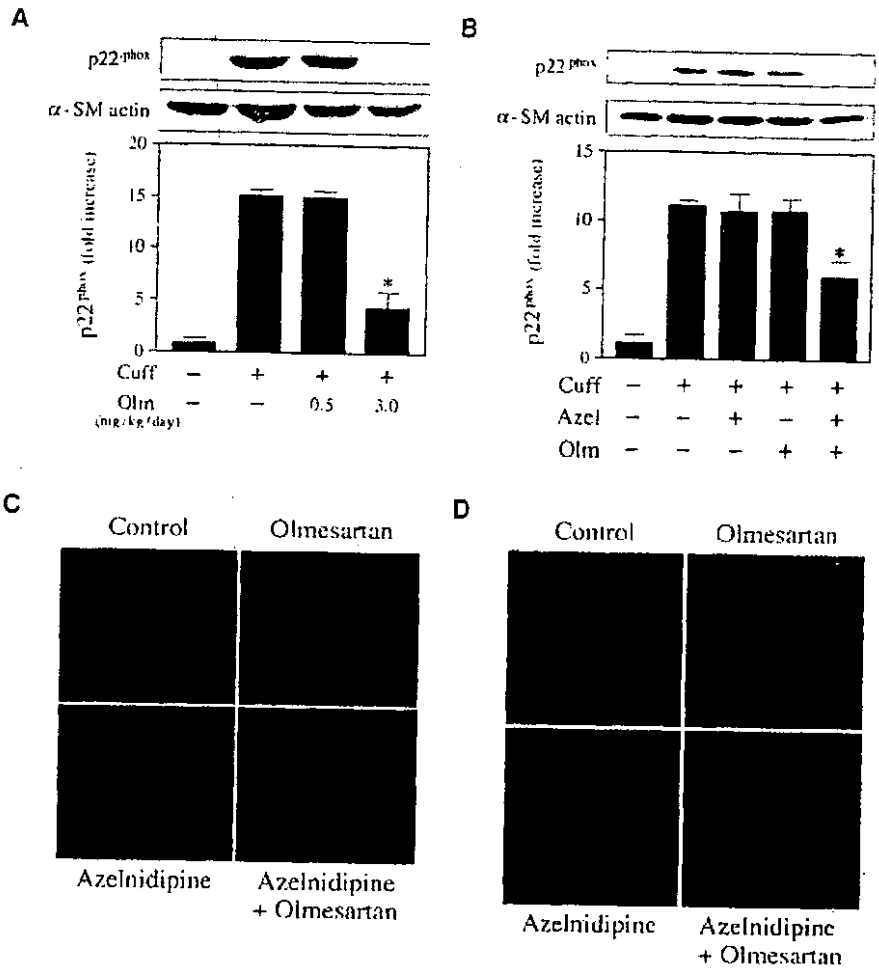


Figure 6. Effect of azelnidipine/olmesartan combination on oxidative stress in injured artery. Tissue samples were prepared from cuffed arteries 7 days after operation. **A**, Dose-dependent effect of olmesartan on p22^{phox} expression. **B**, Effect of azelnidipine/olmesartan combination on p22^{phox} expression. One representative result of Western blots (upper) and densitometric analysis (lower) of p22^{phox} using three or four different pooled samples are shown. **C**, One representative result of four different immunohistochemical staining of p22^{phox} in cuffed artery. **D**, One representative result of four different immunofluorescence of superoxide in cuffed artery. **P*<0.01 versus cuff alone.

ing proteins including cytokines, growth factors, and Ang II via the AT₁ receptor. We observed that azelnidipine decreased the phosphorylation of STAT1 and STAT3 in WT and AT₁aKO mice (Figure 2). Moreover, we observed that coadministration of a lower dose of azelnidipine and olmesartan attenuated the activation of ERK and STAT, although azelnidipine or olmesartan alone at these doses (0.5 or 0.1 mg/kg per day, respectively) did not affect activation of ERK and STAT (Figure II). These results suggest that azelnidipine-mediated inactivation of ERK and STAT may contribute to the exaggeration of the inhibitory effect of olmesartan on vascular injury.

Oxidative stress induces the expression of redox-sensitive genes for chemoattractant proteins, such as MCP-1, and leukocyte adhesion molecules and intracellular adhesion molecules. The superoxide anion may function as a signaling molecule, mediating increased activity of NF- κ B that coordinates the upregulation of these proinflammatory genes.^{24,25} Ang II activates membrane oxidase NADH/NADPH oxidase, which results in production of the superoxide anion, and this proinflammatory action of Ang II on the vessel wall interacts synergistically with those of other cardiovascular risk factors, such as dyslipidemia and diabetes.² We examined the possibility that CCB could exert an antioxidative stress effect in the injured artery, and observed that a nonhypotensive dose of azelnidipine inhibited the expression of p22^{phox}, superoxide anion production, and MCP-1 and

TNF- α expression in WT and AT₁aKO mice (Figure 3 and I). We also observed that coadministration of a lower non-effective dose of azelnidipine and olmesartan (0.5 and 0.1 mg/kg per day, respectively) inhibited oxidative stress in the injured artery. These results suggest that azelnidipine could inhibit AT₁ receptor-mediated as well as AT₁ receptor-independent oxidative stress in vascular injury, at least partly because of inhibition of the expression of p22^{phox}, thereby decreasing superoxide anion production. Once produced, reactive oxygen species can activate several receptor and nonreceptor protein tyrosine kinases possibly via inhibition of a protein tyrosine phosphatase. These tyrosine kinases include the JAK and Src families, Pyk2/CAK, and the receptors for EGF and PDGF.^{7,26} Therefore, it is conceivable that azelnidipine-mediated inhibition of oxidative stress could contribute to the attenuation of ERK and STAT activation in the injured artery, thereby decreasing VSMC proliferation.

Taken together, our results provide the evidence of a synergistic vascular protective effect of CCBs and ARBs, and further extend the clinical notion that the combination of CCBs and ARBs could be more effective in the treatment of vascular diseases and hypertension than monotherapy.

Perspectives

We demonstrated that azelnidipine could exaggerate the vascular protective effects of olmesartan, suggesting that the combination

of CCB amlodipine and ARB olmesartan could be more effective in the treatment of vascular diseases. In addition, a low dosage of combined drugs may be able to reduce the side effects in clinical applications. Moreover, we reported that combination of ARB, valsartan with fluvastatin, or estrogen exaggerate their vascular protective effect in cuff-induced vascular injury model.^{15,16} In contrast, evidences demonstrated that the combination of an ACE inhibitor, perindopril, and an ARB, candesartan cilexetil, may be more effective in the treatment of cardiac and vascular diseases in stroke-prone spontaneously hypertensive rats than the combination of a CCB, amlodipine, with an ACE inhibitor or an ARB or monotherapy with each agent²⁷ and that despite a similar reduction in blood pressure, the addition of the CCB, amlodipine, to the ARB, valsartan, failed to provide similar renoprotection to that observed with an equihypotensive regimen of ARB as monotherapy in diabetic nephropathy.²⁸ In addition, Lassila et al²⁹ reported that CCB, amlodipine, despite similar effects on blood pressure, confers less antitrophic effects in the diabetic heart and blood vessels in diabetic spontaneously hypertensive rats compared with valsartan. These apparent discrepancies would be because of different animal models and different dosages. More detailed analysis of the cellular and signaling mechanism of CCB will provide further insights into the pathogenesis of hypertension and atherosclerosis and may initiate new rational and therapeutic concepts.

Acknowledgments

This work was supported by grants from the Ministry of Education, Science, Sports, and Culture of Japan, the Japan Research Foundation for Clinical Pharmacology, the Tokyo Biochemical Research Foundation, and a grant from the Smoking Research Foundation.

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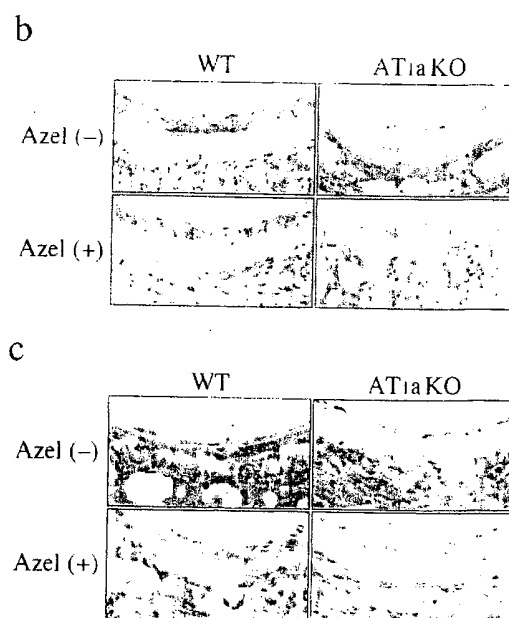
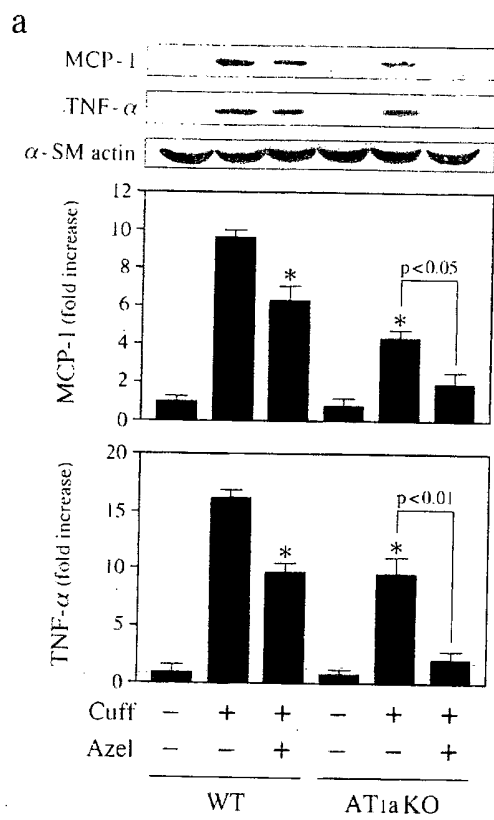


Figure 1

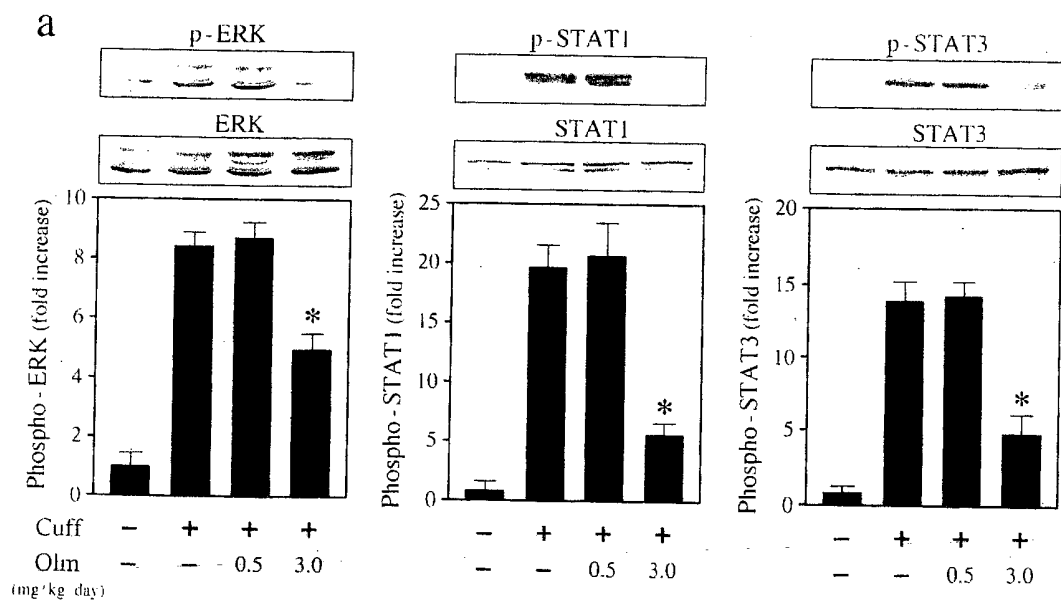


Figure **II**

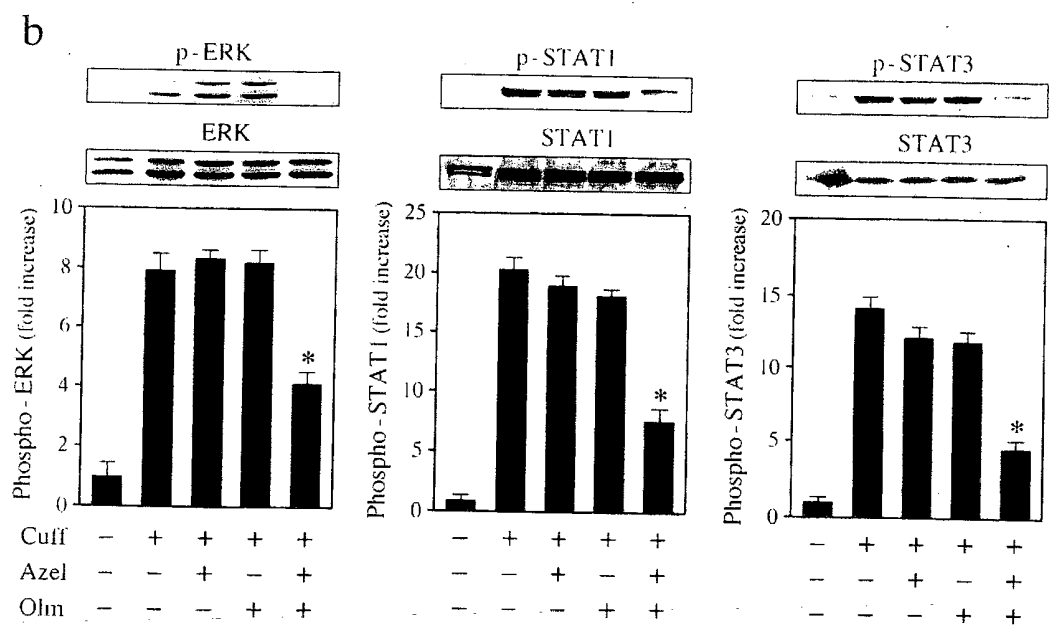


Figure **II**

Calcium antagonists and their mode of action: an historical overview

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1 The Ca^{2+} antagonists are a novel group of drugs useful in management of a variety of cardiac disorders. They differ from one another in terms of their chemistry, tissue specificity and selectivity. As a group, however, they share the common property of slowing Ca^{2+} entry through voltage-activated, ion-selective channels. Some of them exhibit other properties, including that of interfering with Na^+ transport. At least one of them, diltiazem, has an intracellular action.

2 Specific high and low affinity binding sites have been identified for two of the major groups of Ca^{2+} -antagonists, with the binding sites for verapamil and its derivatives being distinct from those which can be occupied by the dihydropyridines. The number (B_{max}) and affinity (K_D) of these binding sites changes under certain pathological conditions—including a reduction in ischaemia and in spontaneous hypertension, an increase in the latter, at present, only demonstrated for the dihydropyridine binding sites.

3 The sensitivity of a particular tissue to these drugs will depend upon a number of factors including the number of binding sites that are present, the contribution made by the Ca^{2+} entering through the voltage-activated channels to the functioning of the tissue, and properties which are peculiar to a particular type of Ca^{2+} antagonist, for example, whether, as in the case of verapamil, they exhibit use-dependence.

Keywords calcium antagonists

Introduction

Although cardioactive substances are known to have been present in many of the medicines administered in antiquity by the Egyptians, and later by the Greeks, there is, as yet, no evidence to suggest that either civilization made use of any of the naturally occurring Ca^{2+} antagonists. By contrast at least one naturally occurring Ca^{2+} antagonist—'Tanshinone', has featured predominantly in Chinese cardiovascular medicine for the past three thousand years (Patmore & Whiting, 1982). Our current interest in these drugs does not stem from ancient Chinese medicine, however, but rather from experiments carried out in Fleckenstein's laboratory in the

mid 1960s. When investigating the coronary vasodilator properties of two, then newly synthesized, compounds, prenylamine and verapamil, Fleckenstein and his colleagues (Fleckenstein *et al.*, 1969) correctly deduced that the peculiar properties these two compounds exhibited warranted the creation of a new class of drugs, to be known as 'the calcium antagonists' (Fleckenstein, 1971). This conclusion was based on the observation that the ability of these drugs, and particularly of verapamil (Figure 1), to uncouple excitation-contraction coupling in heart muscle, and hence to exert a negative inotropic effect, could be reversed by adding Ca^{2+} .

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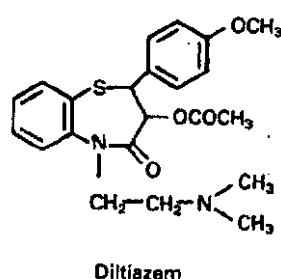
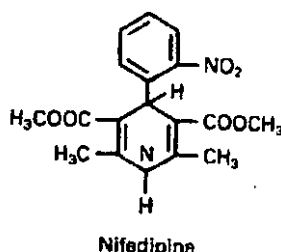
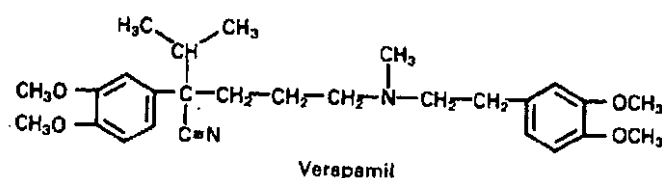


Figure 1 Chemical formulae of Ca^{2+} antagonists.

By the late 1960s, and therefore at a time when verapamil and prenylamine were the only Ca^{2+} antagonists which were available, three important properties of the Ca^{2+} antagonists had already been established, because by then it was known that these drugs are powerful coronary dilators, that they are negatively inotropic and that their negative inotropism can be overcome by raising extracellular Ca^{2+} . Although it took very little time to establish that these drugs lacked β -adrenoceptor blocking activity (Naylor *et al.*, 1968) their vasodilator action on the peripheral circulation was largely ignored until the mid 1970s, when Lewis *et al.* (1978) first described a sustained decrease in the mean arterial blood pressure in a group of patients receiving verapamil. At about the same time, and possibly in association with the introduction of other Ca^{2+} antagonists, their potential use in the management of other cardiovascular disorders, including supraventricular tachyarrhythmias (Schamroth *et al.*, 1972; Krikler & Spurrell, 1974), angina pectoris (Livesley *et al.*, 1973; Parodi *et al.*, 1979), began to attract widespread attention.

Twenty years have elapsed since these initial but critical experiments were performed with

the Ca^{2+} antagonists and in that time not only has the original premise that verapamil and prenylamine were the forerunners of a new class of drugs been amply confirmed: in addition new drugs have been developed, their clinical efficacy (Table 1) established and expanded and, more

Table 1 Therapeutic uses of Ca^{2+} antagonists

Vascular
Angina (effort, vasospastic and rest)
Arterial hypertension
Pulmonary hypertension
Cerebral vasospasm
Raynaud's phenomenon
Migraine
Intestinal ischaemia
Experimental atherosclerosis
Cardiac failure
Myocardium
Hypoxia
Ischaemia
Cardiomyopathies
Cardiopulmonary bypass
Arrhythmias
Skeletal muscle
Malignant hyperthermia

recently, their specific binding sites identified (Glossmann *et al.*, 1984).

Chemistry and classification

One of the most puzzling characteristics of the Ca^{2+} antagonists is their chemical heterogeneity (Nayler & Horowitz, 1983). As a general rule, the currently available drugs of this class can be subdivided into four main groups, on the basis of their chemistry (Table 2). These are the phenylalkylamines, of which verapamil (Figure 1) is the prototype; the dihydropyridines, including nifedipine (Figure 1), nitrendipine and nicardipine; the benzothiazepines, including diltiazem; and the piperazines. Lidoflazine and cinnarizine belong to this latter group. There are, of course, other ways of subdividing these drugs. For example (Nayler, 1983) they can be grouped on the basis of their tissue selectivity. If this method of subdivision is to be used then drugs which exert their greatest potency in the vasculature, lidoflazine for example, can be grouped separately from those which exhibit their greatest potency at other sites, the sino-atrial node for example. Irrespective of how these drugs are subclassified, however, two fundamental questions must be answered. Firstly, there must be some rational explanation as to why such chemically diverse drugs can share the common property of ' Ca^{2+} antagonism', and secondly the basis of their tissue selectivity must be established. The answer to the first of these problems probably lies in the

Table 2 Chemistry of the Ca^{2+} antagonists

Phenylalkylamine-derivatives
verapamil
gallopamil
tiapamil
anipamil
ronipamil
Dihydropyridine-derivatives
nifedipine
nimodipine
niludipine
nitrendipine
nisoldipine
nicardipine
felodipine
Benzothiazepine-derivatives
diltiazem
Piperazine-derivatives
lidoflazine
cinnarizine
flunarizine

recent identification of different receptor or binding sites for the chemically distinct groups of Ca^{2+} antagonists. The answer to the second question is almost certainly multifactorial, involving tissue-dependent differences in receptor distribution, fundamental differences in the Ca^{2+} requirements of the various target tissues, other properties of these drugs not necessarily related to Ca^{2+} antagonism (Table 3), possible tissue-dependent differences in the functioning of the slow channels, and the regional presence of other

Table 3 Other properties of phenylalkylamine type calcium antagonists

Property	Reference
Interaction with other receptors	
Muscarinic	Karliner <i>et al.</i> (1982) Gengo <i>et al.</i> (1984)
5-HT	Angus <i>et al.</i> (1982)
Opiate	Fairhurst <i>et al.</i> (1980)
α -adrenoceptors	Nayler <i>et al.</i> (1982) Karliner <i>et al.</i> (1982) Glossmann & Hornung (1980)
Inhibition of platelet aggregation	Ribeiro <i>et al.</i> (1982)
Inhibition of thromboxane release	Mehta <i>et al.</i> (1983)
Local anaesthetic activity	
Inhibition of fast Na^+ channels	Kass & Tsien (1975)
Inhibition of slow Na^+ channels	Kojima & Sperelakis (1983)
Inhibition of ACTH, GH and TSH release	Eto <i>et al.</i> (1974)
Inhibition of glucose-induced insulin release	Conn <i>et al.</i> (1980)

inotropically or vasoactive substances, for example angiotensin (Hof, 1984).

Receptors for the Ca^{2+} antagonists

The recent availability of radioactively labelled Ca^{2+} antagonists has greatly facilitated the search for the specific binding sites for these drugs. Using classical saturation and displacement binding techniques at least two and probably three chemically-distinct types of saturable, stereospecific binding sites have now been identified. Some of these binding sites are highly selective for the dihydropyridine-based Ca^{2+} antagonists (Triggle & Janis, 1984) whilst others preferentially bind the diphenylalkylamines, including verapamil and gallopamil (Hulthen *et al.*, 1982; Reynolds *et al.*, 1983). Within each group, however, both low and high affinity binding sites have been identified and characterized. In cardiac myocytes these binding sites are predominantly concentrated in the cell membranes, a factor which may explain why the primary action of these drugs is at the cell surface. A similar situation exists in vascular smooth muscle cells (Janis *et al.*, 1982). However, receptor binding sites for the Ca^{2+} antagonists are not limited to the cardiovascular system. For example, they occur in the brain (Janis *et al.*, 1984) and are present in large numbers in skeletal muscle cells (Borsoatto *et al.*, 1984). It follows, therefore, that the mere presence of binding sites or 'receptors' for these compounds alone does not dictate tissue sensitivity, because skeletal muscle, for example, is relatively insensitive to the Ca^{2+} antagonists despite the presence of both high and low affinity binding sites. This lack of sensitivity of skeletal muscle can be most easily explained by recalling that whereas cardiac muscle depends upon the presence of extracellular Ca^{2+} for excitation-contraction coupling, skeletal muscle derives Ca^{2+} for this purpose from its own intracellular stores. In this respect smooth muscle cells, including those of the vasculature, resemble cardiac muscle cells in relying to a large extent upon an influx of extracellular Ca^{2+} for excitation-contraction coupling. In all three muscle types, however, tension development is directly dependent upon the availability of ionized calcium in excess of 10^{-7} M.

Slow Ca^{2+} channels and Ca^{2+} antagonists in cardiac muscle

In cardiac muscle excitation involves the activation of two distinct inward currents (Reuter,

1979). The first of these currents is carried by Na^{+} whilst (Figure 2) the second is carried mainly by Ca^{2+} (Beeler & Reuter, 1970). The Ca^{2+} that acts as the main charge carrier for this second and relatively slowly activated current (hence the term 'the slow Ca^{2+} current') traverses the membrane through channels which are voltage-sensitive, gated, and ion-selective. Each slow channel allows approximately 30000 calcium ions to enter per second and there may be as many as 30 channels μm^{-2} of cell surface. This means that there are as many as 10000 functional Ca^{2+} channels per cardiac muscle cell (Reuter, 1983). As well as being ion-selective and voltage-sensitive some of the slow Ca^{2+} channels are 'receptor' controlled by a mechanism which involves the cyclic-AMP dependent phosphorylation of the channel or of a protein closely associated with the channel. This phosphorylation apparently causes a conformational change in the channel, thereby increasing the probability of its opening on membrane depolarization (Reuter, 1983). Although the precise structure of these slow Ca^{2+} channels has yet to be defined, recent studies have shown them to contain a 278000 dalton protein structure, with a 45000 dalton sub unit capable of binding the dihydropyridine-based Ca^{2+} antagonists (Venter *et al.*, 1983). It is reasonable to surmise (Figure 3) therefore, that there must be a selectivity filter at the orifice of the channel, because certain divalent cations, including Co^{2+} and Mn^{2+} cannot penetrate through it. In addition, the kinetics of slow channel transport are such that it can be assumed that not only must the channel contain voltage sensors, it must also be gated, with the gates opening to allow Ca^{2+} influx (Figure 3).

During each depolarization the slow Ca^{2+} current accounts for the entry of at least 20 $\mu\text{mol Ca}^{2+} \text{ kg}^{-1}$ heart weight (Langer, 1980) and although this is insufficient Ca^{2+} to activate contraction it probably triggers the release of additional Ca^{2+} from intracellular storage sites (Nayler, 1966; Fabiato & Fabiato, 1978). It is these channels, which facilitate the influx of Ca^{2+} during excitation, which are the predominant sites of action of the Ca^{2+} antagonists in cardiac and in the smooth muscle cells of the vasculature. In the case of the dihydropyridines their specific binding sites are thought to be closely associated with, or an integral part of, the outer neck of the slow channel.

Evidence for this comes from two sources:

(a) the number of dihydropyridine binding sites in both cardiac and smooth muscle cells ($1\text{--}10 \mu\text{M}^{-2}$ membrane) closely resembles the density of Ca^{2+} channels as estimated electro-

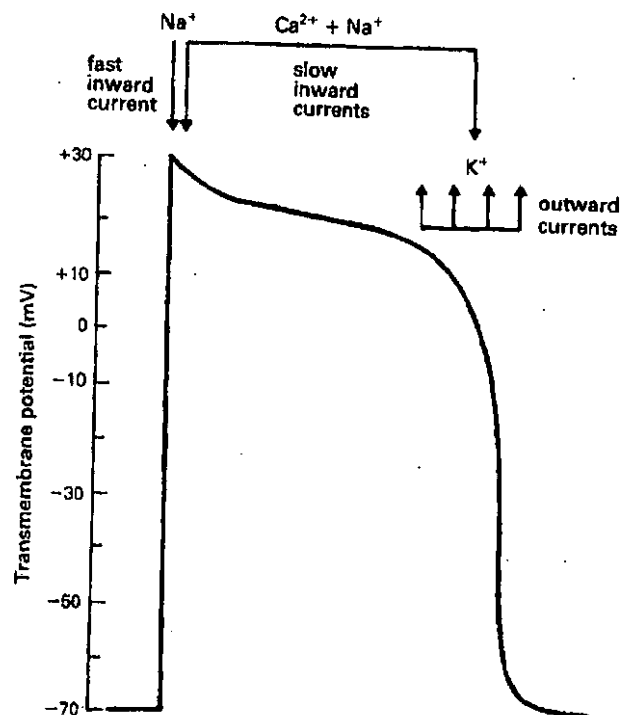


Figure 2 Ion movements associated with a ventricular action potential.

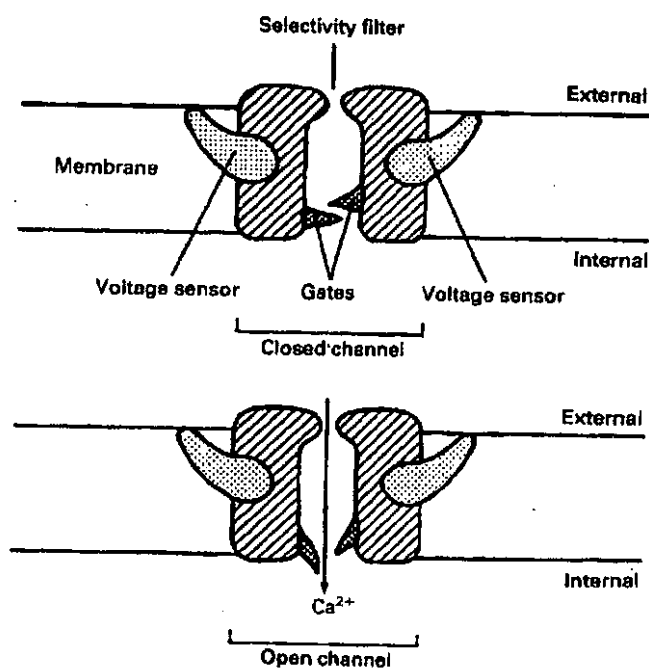


Figure 3 Schematic representation of a slow Ca^{2+} channel.

physiologically; and

(b) the synthesis of channels induced by PGE in certain neuronal cells is accompanied by the appearance of additional dihydropyridine binding sites (Nirenberg *et al.*, 1983).

The situation is less clear for verapamil and its derivatives. Ligand displacement studies have shown that these receptor sites are quite distinct from those occupied by either diltiazem or the dihydropyridines but it is probable that they are allosterically linked to these other binding sites (Figure 4). There is some evidence which indicates that they are located on the cytosolic surface of the membrane, adjacent to the slow channels.

By inhibiting the inward movement of Ca^{2+} through the slow channels, the Ca^{2+} antagonists exert a direct negative inotropic—and hence an energy sparing (Nayler *et al.*, 1980)—effect on the myocardium. In the intact circulation, however, this negative inotropism can be concealed because of reflex changes in the circulation as a whole, unless these reflex changes are suppressed because of disease or because of the presence of other pharmacological agents. The Ca^{2+} antagonists are also powerful vasodilators in the coronary as well as in the peripheral vasculature and again their efficacy depends upon their ability to impede the inward movement of Ca^{2+} through voltage-sensitive ion-selective channels (Figure 5). In addition verapamil and diltiazem, but not nifedipine, slows atrio-ventricular nodal conduction and again this is due to their ability to impede slow channel function. This brings us to the important question as to why the Ca^{2+} antagonists exhibit such a broad spectrum of tissue selectivity within the

circulation. For example, why are the dihydropyridine-based Ca^{2+} antagonists (for example, nifedipine) more potent than the phenylalkylamines (verapamil) in dilating the peripheral vasculature, but less potent in other areas, including the atrio-ventricular nodal tissue when this, as with excitation-contraction coupling in heart muscle, is known to depend upon the inward movement of Ca^{2+} through voltage-activated slow channels? There must also be some explanation as to why some smooth muscle cells, including those of the bronchial tree, are relatively unaffected by the Ca^{2+} antagonists, despite their dependence on the entry of Ca^{2+} through voltage-activated slow channels for the activation of their contractile response.

Tissue selectivity

Many factors seem to be involved in determining the sensitivity of a particular tissue to the Ca^{2+} antagonists. They include:

- (a) the number of binding sites that are present;
- (b) the relative importance of Ca^{2+} entry through the slow channel as far as the functioning of that tissue is concerned;
- (c) the duration of time for which each channel is open;
- (d) the effect of voltage on receptor binding activity;
- (e) other properties of the drugs (Table 3) not necessarily related to Ca^{2+} antagonism;
- (f) the pathology of the tissue, and
- (g) whether or not the drug displays use-dependence.

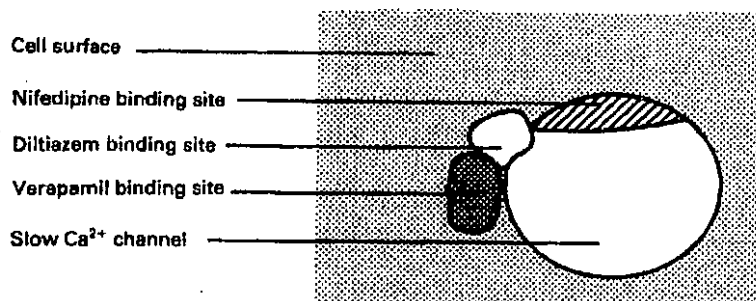


Figure 4 Schematic representation of location of Ca^{2+} antagonist binding sites relative to the orifice of a slow Ca^{2+} channel.

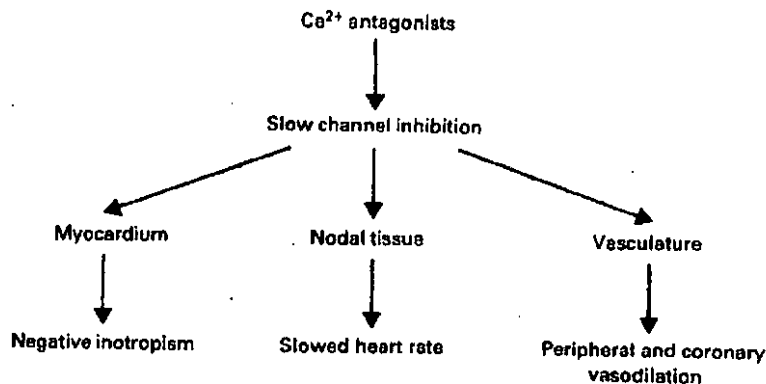


Figure 5 Schematic representation of the effect of Ca^{2+} antagonists on the cardiovascular system.

Using this list as a guide it is relatively easy to account for the relative lack of sensitivity of bronchiolar smooth muscle because that tissue has the capacity to derive a large proportion of the Ca^{2+} it requires for contraction from its own internal stores. We can also explain, in part at least, why verapamil, but not nifedipine, acts preferentially at the atrioventricular node (Rowland *et al.*, 1979). Verapamil exhibits the phenomenon of use-dependence. That is, its potency increases as the frequency of membrane depolarization and hence of channel opening is enhanced, presumably because it can only influence the functioning of the channel when the channel is in its active or open state. Nifedipine exhibits this property to a far lesser degree, and only within a non-physiological range. Hence, at the rapidly firing tissues of the atrio-ventricular node the potency of verapamil is relatively enhanced, relative to that of nifedipine, a factor which underlies its usefulness as an antiarrhythmic agent. Nifedipine, on the other hand, is far more potent in the vasculature, possibly because of tissue-dependent differences in the distribution of the diphenylalkylamine and dihydropyridine binding sites.

Before returning to the overall effects of the Ca^{2+} antagonists on the circulation as a whole two relatively recent findings should be mentioned. These relate to the discovery of Ca^{2+} agonists (Schramm *et al.*, 1983), as opposed to Ca^{2+} antagonists, and to the observation that certain pathological conditions, including myocardial ischaemia and hypertension, influence both the number and affinity of the Ca^{2+} -antagonist receptors (or binding sites).

Ca^{2+} agonists

A newly discovered nifedipine derivative, Bay K 8644 (Schramm *et al.*, 1983) interacts with the same binding sites as the other dihydropyridines, but instead of slowing Ca^{2+} entry through the voltage-sensitive slow channels it enhances Ca^{2+} entry, prompting a positive inotropic response. This is of great interest because it may be the forerunner of a new group of positive inotropes. As yet, however, there is no indication as to whether minor chemical substitutions in any of the other chemically-distinguishable subgroups of the Ca^{2+} antagonists (the phenylalkylamines, benzothiazepines or the piperazines) will produce other Ca^{2+} agonists.

Calcium antagonist binding sites in pathological conditions

Recent results obtained in our laboratories indicate that both the affinity and number (or density) of the specific binding sites for nitrendipine (a nifedipine-derivative) and verapamil in cardiac cell membrane preparations is reduced during an ischaemic episode. Thus, using classical saturation binding techniques on enzymatically characterized sarcolemmal fragments prepared from either aerobically perfused or ischaemic (without reperfusion) hearts, we have found that 60 min normothermic ischaemia reduces both the number (B_{max} , Table 4) and, except in the case of the nitrendipine binding high affinity sites, the affinity of these receptors for the appropriate ligand. Nevertheless Table 4 does show

Table 4 Effect of ischaemia on sarcolemmal binding of [³H]-verapamil and [³H]-nitrendipine*

	High affinity		Low affinity	
[³ H]-verapamil	K _D (μM)	B _{max} (pmol mg ⁻¹)	K _D (μM)	B _{max} (nmol mg ⁻¹)
aerobic perfusion	0.37 ± 0.01	23.4 ± 7.2	74.9 ± 17.9	2.8 ± 0.8
60 min ischaemia	0.17 ± 0.04	3.9 ± 1.5	34.5 ± 0.2	0.8 ± 0.4
	P < 0.05	P < 0.02	P < 0.03	P < 0.02
[³ H]-nifedipine	K _D (nM)	B _{max} (fmol mg ⁻¹)	K _D (nM)	B _{max} (fmol mg ⁻¹)
aerobic perfusion	0.24 ± 0.44	313 ± 110	47.6 ± 8.7	12.4 ± 0.2
60 min ischaemia	0.22 ± 0.55	82.4 ± 14.5	15.8 ± 2.9	6.1 ± 0.2
	NS	P < 0.03	P < 0.03	P < 0.02

Each set of results is mean ± s.e. mean of six experiments. K_D is the concentration of drug needed to saturate half the receptors, and B_{max} is the density of receptors. The sarcolemmal fragments were isolated from rat hearts perfused at 37° C or made ischaemic for 60 min at the same temperature. The sarcolemmal fragments were enzymatically characterized.

that [³H]-nitrendipine and [³H]-verapamil binding receptors survive such a prolonged period of ischaemia. In the case of the surviving [³H]-nitrendipine binding sites we have been able to show that they are still functional because when the nifedipine-based Ca²⁺ agonist is added after 60 min of ischaemia it promotes an increased rate of Ca²⁺ influx.

Hence the reported failure of calcium antagonists that are added only upon reperfusion to influence the sequence of events that occur during post-ischaemic reperfusion (Bourdillon & Poole-Wilson, 1982) cannot be attributed to a failure of the receptors to survive in a functional state. Further evidence of the survival of these receptors is provided by the data shown in Table 5 which indicates that verapamil, when added only upon reperfusion after 30 min normothermic ischaemia, does slow the rate of Ca²⁺ gain. The failure of verapamil (or nifedipine) to abolish the excessive gain in Ca²⁺ that occurs under these conditions can best be explained by recalling that the slow channels are only one of the routes available for excessive Ca²⁺ entry.

Ischaemia is not the only condition which alters the density of the Ca²⁺ antagonist binding

sites. Thus, whereas ischaemia reduces, hypertension increases the number of sites available for dihydropyridine binding sites (Chatelain *et al.*, 1984) a factor which may explain why the cardiovascular system in hypertensives is hypersensitive to these drugs. Comparable data is not yet available for the verapamil-binding sites.

Abnormal tissue pathology is not the only factor which influences the activity of the various Ca²⁺ antagonist binding sites. Binding is known to be pH and temperature sensitive; it is also influenced by the cationic composition of the extracellular fluid, with a raised Ca²⁺ content either decreasing or increasing the ligand binding capacity of the membranes, depending upon which particular ligand is being used.

Is there an intracellular site of action?

Since most of the Ca²⁺ antagonists, including verapamil are lipophilic and accumulate intracellularly (Lullmann *et al.*, 1979; Pang & Sperelakis, 1983) it is not surprising to find that the question as to whether these drugs have an intracellular action, in addition to that of the cell

Table 5 Effect of verapamil when added only upon reperfusion, on reperfusion-induced Ca²⁺ gain*

	Cell Ca ²⁺ (μmol g ⁻¹ dry weight)			
	Time of reperfusion after 30 min ischaemia (min)			
	2	5	10	15
Control reperfusion	4.21 ± 0.79	10.35 ± 2.18	13.65 ± 0.59	17.79 ± 1.83
Reperfusion with 10 ⁻⁶ M verapamil	2.70 ± 0.14	5.00 ± 0.12	5.41 ± 0.76	11.27 ± 1.00

*Each result is mean ± s.e. mean of six experiments. The results relate to cell Ca²⁺ during reperfusion after 30 min of ischaemia.

membrane, has re-emerged. Only in one case has unequivocal evidence of an intracellular site of action been obtained. Thus diltiazem has been shown to slow $\text{Na}^+:\text{Ca}^{2+}$ exchange across the mitochondrial membrane (Matlib *et al.*, 1983) but the significance of this is unknown. There is other evidence, however, which suggests that when relatively high concentrations are achieved many of the Ca^{2+} antagonists interfere with the Ca^{2+} -accumulating and releasing activity of the sarcoplasmic reticulum, in smooth as well as in cardiac muscle cells (Saida & van Breemen, 1983; Wang *et al.*, 1984). There is also some evidence which suggests that at high, but not therapeutically relevant concentrations, they reduce the Ca^{2+} sensitivity of the myofilaments (Morgan *et al.*, 1983).

Problems of definition

Returning then, to the proposition originally outlined by Fleckenstein, that for a substance to be classed as a ' Ca^{2+} antagonist' it must satisfy two requirements:

(a) its predominant characteristic must be that of inhibiting the voltage-activated inward displacement of Ca^{2+} ; and

(b) this inhibitory effect must be overcome by Ca^{2+} .

Presumably this second requirement can be accounted for in terms of the ability of Ca^{2+} to displace Ca^{2+} antagonists that have been previously bound to their respective binding sites (Galizzi *et al.*, 1984).

Whether the term ' Ca^{2+} antagonist' is the most appropriate name for these drugs is still being debated (Lee & Tsien, 1983), particularly in view of the new data which shows that the movement of Ca^{2+} through the slow channels is bidirectional, and that the Ca^{2+} antagonists impede both Ca^{2+} influx and Ca^{2+} efflux through this route.

Ca^{2+} antagonists and the circulation

From the data presented here it is difficult to escape the conclusion that the Ca^{2+} antagonists are a chemically heterogeneous group of drugs (Naylor, 1983). This heterogeneity extends beyond their chemistry to include their binding sites, their pharmacokinetics (Table 6), and even perhaps, their precise mode of interaction with the slow channels themselves. Nevertheless, their one common and outstanding property is that of impeding slow channel function. Since the inotropic state of the myocardium, vasomotor tone and atrio-ventricular conduction is largely dependent upon Ca^{2+} influx through the voltage-sensitive, ion-selective slow channels the efficacy of these drugs in the management of angina, hypertension and when used prophylactically, ischaemic heart disease, is not surprising.

These investigations were supported by a grant from the National Health and Medical Research Council of Australia.

Table 6 Pharmacokinetics of Ca^{2+} antagonists

	<i>Nifedipine</i>	<i>Verapamil</i>
Dose		
Oral	10–40 mg 8 h ⁻¹	80–160 mg 8 h ⁻¹
Intravenous	5–15 µg kg ⁻¹	100–200 µg kg ⁻¹
Plasma concentration	10–75 ng ml ⁻¹	50–250 ng ml ⁻¹
% protein bound	95	90
First pass extraction by liver	40–60%	75–85%
Half-life (<i>t</i> _{1/2})	3–5 h	5–10 h

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Identification of 1,4-dihydropyridine binding regions within the $\alpha 1$ subunit of skeletal muscle Ca^{2+} channels by photoaffinity labeling with diazepam

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Contributed by William A. Catterall, July 30, 1991

ABSTRACT To identify regions that are involved in the formation of the dihydropyridine receptor site of skeletal muscle L-type Ca^{2+} channels, the $\alpha 1$ subunit of the channel complex was specifically labeled with the 1,4-dihydropyridine-receptor-selective photoaffinity probe [^3H]diazepam. Photoaffinity-labeled regions were identified by probing labeled proteolytic fragments with several anti-peptide antibodies recognizing different segments of the $\alpha 1$ sequence. Forty to 50% of the $\alpha 1$ -associated [^3H]diazepam label was contained in the tryptic fragment between Arg-988 and Ala-1023 derived from the loop between segments S5 and S6 in domain III. This region corresponds to a portion of the channel that is believed to contribute to formation of the transmembrane pore. Twenty to 30% of the labeling occurred in a V8 protease fragment between Glu-1349 and Trp-1391. This fragment contains transmembrane segment S6 of domain IV and has previously been shown to form part of the drug receptor for phenylalkylamine Ca^{2+} antagonists. Our data suggest that the dihydropyridine receptor is formed by close apposition of two discontinuous regions of the $\alpha 1$ subunit sequence in domains III and IV. In light of previous work localizing this receptor site to the extracellular surface of the lipid bilayer, it is proposed that amino acid residues at the extracellular surface in the loop connecting segments IIIS5 and IIIS6 and at the extracellular end of segment IVS6 contribute to formation of the dihydropyridine receptor site.

Voltage-gated L-type Ca^{2+} channels mediate Ca^{2+} influx into many excitable cells and serve as voltage sensors for excitation-contraction coupling in skeletal muscle (for review, see refs. 1 and 2). In contrast to other types of Ca^{2+} channels (N, T, and P type; for review, see ref. 3), L-type Ca^{2+} channels are modulated by distinct chemical classes of Ca^{2+} antagonists, such as 1,4-dihydropyridines (DHPs), phenylalkylamines, and benzothiazepines (for review, see ref. 4). These drugs specifically bind with high affinity to distinct allosterically coupled receptor sites on the $\alpha 1$ subunit of the calcium channel complex as revealed by photoaffinity labeling (4-7). Neutron-diffraction and kinetic studies (8) suggest that DHPs approach their receptor site by lateral diffusion after partition into the lipid bilayer of the membrane. This binding domain is thought to be localized close to the outer surface of the $\alpha 1$ subunit as DHPs in a membrane-impermeable charged form only have access to their receptor site if applied from the extracellular side (9, 10). This is in contrast to phenylalkylamine Ca^{2+} antagonists, such as verapamil, that interact with an intracellular receptor site formed in part by the intracellular end of transmembrane helix IVS6 and/or the adjacent intracellular amino acid

residues (11). Knowledge of the regions of the $\alpha 1$ subunit involved in the formation of the DHP receptor site would help to elucidate the molecular basis of channel modulation by DHPs and allosteric interaction between the DHP and phenylalkylamine binding domains. In this report, we define the location of the DHP binding domain within the $\alpha 1$ subunit of skeletal muscle Ca^{2+} channels by photoaffinity labeling with the DHP-receptor-selective ligand trifluoroethyl diazepam [^3H]diazepam (12, 13) and mapping of labeled proteolytic fragments using sequence-directed antibodies.

EXPERIMENTAL PROCEDURES

Materials. [^3H]Diazepam, 2-[4-(1-azi-2,2,2-trifluoroethyl)-benzoylamino]ethyl 2,6-dimethyl-4-(2-trifluoromethyl)phenyl-1,4-dihydropyridine-3,5-dicarboxylate (21.2 Ci/mmol; 1 Ci = 37 GBq), was synthesized as described (13). Unlabeled ($\pm$)-PN200-110 was a gift from Sandoz Pharmaceutical. Unlabeled (-)-desmethoxyverapamil and [N -methyl- ^3H]-LU49888 ((-)-5-[(3-azidophenethyl)(N -methyl- ^3H)methylamino]-2-(3,4,5-trimethoxyphenyl)-2-isopropylvaleronitrile) were gifts from Knoll (Ludwigshafen, F.R.G.). Enzymes and chemicals were obtained from the following sources: digitonin, from Wako Pure Chemical (Osaka) or Sigma; diisopropyl phosphorfluoridate, N -hydroxysuccinimide, m -maleimidobenzoate, bovine serum albumin (essentially globulin free), and porcine thyroglobulin; from Sigma; tosyl L-phenylalanine chloromethyl ketone trypsin (from bovine pancreas), from Worthington; V8 protease, from Boehringer Mannheim; protein A-Sepharose 4B, from Pharmacia; prestained low molecular weight markers, from BRL; unstained high molecular weight standards, from Bio-Rad; Amplify and ACS-II liquid-scintillation fluid, from Amersham; Econofluor and Protosol, from New England Nuclear.

Peptide Synthesis and Antibody Production. Synthetic calcium channel peptides (CPs) corresponding to particular regions of skeletal muscle $\alpha 1$ subunit were synthesized by the solid-phase method (14). Polyclonal antisera were raised in rabbits against peptides corresponding to the following amino acid residues of the $\alpha 1$ sequence (15): 1219-1232 [CP-(1219-1232)], 1338-1351 [CP-(1338-1351)], 964-976 plus C-terminal cysteine [CP-(964-976C)], and 1011-1026 plus C-terminal cysteine [CP-(1011-1026C)]. Peptides were purified by reverse-phase HPLC and their structure was confirmed by amino acid and sequence analysis. For immunization, peptides were coupled through cysteine residues to porcine thyroglobulin using N -hydroxysuccinimide m -maleimidobenzoate (16). Preparation of the site-directed antisera against CP-(743-759), CP-(1339-1354), and CP-(1382-1400) was as described (11).

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Abbreviations: CP, Ca^{2+} -channel peptide; DHP, dihydropyridine.

To obtain tryptic fragments of CP-(1011-1026C), the peptide was digested with trypsin (substrate/enzyme weight ratio = 20) in 50 mM Tris Cl, pH 8.0/2 mM CaCl₂ for 30 min at 37°C. The two fragments, CP-(1011-1022) and CP-(1023-1026C), were isolated by reverse-phase HPLC and their amino acid compositions were determined.

Photoaffinity Labeling of Partially Purified Ca²⁺ Channels. Calcium channels in digitonin extracts of purified T-tubule fractions from rabbit skeletal muscles were purified by wheat germ agglutinin-Sepharose chromatography as described (17, 18). For [³H]diazepine photolabeling, the pooled purified fractions (0.1–0.3 mg of protein per ml, 10–30 nM DHP sites) supplemented with 2 mM CaCl₂ were reversibly labeled with 10 nM [³H]diazepine for 1 h at 25°C in the dark in the absence (total labeling) or presence (nonspecific labeling) of 10 μM (±)-PN200-110. The incubation mixtures were transferred into plastic Petri dishes on ice and irradiated with a Panasonic 20-W black light/blue lamp (at 5 cm) for 30 min. After photolysis, the samples were dialyzed against deionized water, adjusted to 0.1% SDS, lyophilized, and stored at –30°C. Photolabeling of α1 with the phenylalkylamine [*N*-methyl-³H]LU49888 was carried out as described (11).

Purification of [³H]Diazepine-Labeled α1 Subunits. Photolabeled and lyophilized protein (2.9 mg) was dissolved in 0.9 ml of 0.4 M Tris-HCl (pH 8.0) and reduced with 1% 2-mercaptoethanol for 15 min at room temperature before carboxymethylation with 70 mM sodium iodoacetate for 1 h. Photolabeled α1 subunits were then further purified by gel permeation chromatography with two columns of TSK G4000SW as described (19). Fractions corresponding to the α1 subunits were pooled, lyophilized, and stored at –30°C in small samples until use.

Proteolysis of Purified [³H]Diazepine-Labeled α1 Subunits. *Trypsin digestion.* Purified [³H]diazepine-labeled α1 subunits (8 × 10⁵ dpm/20 μg) were dissolved in deionized water (0.5 ml) by brief ultrasonication and dialyzed against 6 M urea followed by distilled water (15) and concentrated to 0.1 ml by lyophilization. The concentrate was digested with trypsin (0.5 μg/ml or 100 μg/ml) in 0.1 M Tris Cl, pH 8.0/2 mM CaCl₂ at 37°C. The reaction was stopped by the addition of a final concentration of 5 mM diisopropyl phosphofluoridate after the indicated incubation times.

V8-protease digestion. Purified [³H]diazepine-labeled α1 was digested with V8 protease (0.2–1.0 mg/ml) in 20–40 mM sodium phosphate in the presence of 0.3% digitonin (pH 7.8) at 37°C as indicated. For immunoprecipitation, the sample was boiled for 5 min and adjusted to final concentrations of 1% Triton X-100, bovine serum albumin (1 mg/ml), and 150 mM NaCl.

Immunoprecipitation. Antibodies were bound to protein A-Sepharose 4B by incubating 1 vol of serum with 1 vol of swollen gel in RIA buffer containing 10 mM Tris Cl (pH 7.2), 150 mM NaCl, and 0.1% or 1.0% Triton X-100 for 2 h at 4°C. The gel was washed with ice-cold RIA buffer before the addition of digested or nondigested [³H]diazepine-labeled α1. After incubation for 2 h at room temperature, the resin was washed with RIA buffer. Immunoprecipitated radioactivity was either directly determined by liquid scintillation counting after resuspension of the resin in 0.1 M sodium citrate (pH 3.0) or analyzed by SDS/PAGE after denaturation of the resin in SDS sample buffer (5 min at 95°C or 15 min at 55°C).

SDS/PAGE, Gel Slicing, and Fluorography. Standard SDS/PAGE was performed as described (ref. 11; gel system 1, 8% polyacrylamide; gel system 2, 8–20% polyacrylamide gradient). For separation of smaller fragments the gel system described by Schagger and von Jagow (20) was used [gel system 3: stacking gel, 4% T/3% C; spacer gel, 10% T/3% C; separating gel, 16.5% T/6% C (where T is total monomer and C is crosslinker)]. To visualize immunoprecipitated photolabeled peptides, gels were fixed or stained with Coomassie

blue R-250, impregnated with Amplify, dried, and exposed to Kodak XAR5 film. Alternatively, individual gel lanes were manually cut into 3-mm slices and radioactivity was measured in ACS-II with 3% (vol/vol) H₂O₂ or in Econofluor with 3% (vol/vol) Protosol.

RESULTS AND DISCUSSION

Photolabeling with [³H]Diazepine. We have described (12, 13) the synthesis and pharmacological characterization of the DHP-receptor-selective photoaffinity ligand [³H]diazepine. [³H]Diazepine is structurally closely related to the phenylazide [³H]azidopine (4) but contains a trifluoroethyl diazirine as the photoreactive group. Both ligands possess similar reversible binding properties for skeletal muscle Ca²⁺-channel-associated DHP receptors (12). In contrast to [³H]azidopine, the covalent bond formed between [³H]diazepine and the receptor site is chemically stable against acid treatment and reducing agents (12, 13). Fig. 1 (lanes 1–3) shows an SDS/PAGE analysis of partially purified skeletal muscle Ca²⁺ channels photolabeled in the absence and presence of 10 μM PN200-110. [³H]Diazepine labeling is specific and occurs exclusively in the α1 subunit of the channel complex. Reductive denaturation did not remove specific α1 labeling (see Fig. 1), which has been reported for [³H]azidopine (21). Photoaffinity labeling of α1 subunits with diazopine was reduced by 60% by the phenylalkylamine (–)-D888 (data not shown), in agreement with the allosteric interactions between the DHP and phenylalkylamine receptor sites observed in reversible binding experiments (4).

Isolation of Three Fragments Specifically Photolabeled with [³H]Diazepine. Trypsin (0.5 μg/ml) digestion of the purified sample (substrate/enzyme ratio = 200) led to the appearance of labeled 50- to 95-kDa polypeptides, which were further cleaved to labeled fragments of 3.0, 6.2, and 7.4 kDa upon prolonged digestion (Fig. 1, lanes 4–7). These fragments were resistant to further cleavage with very high protease concentrations [trypsin (100 μg/ml); substrate/enzyme ratio = 1] as shown in Fig. 1 (lane 8) after separation in the higher-resolving gel system 3. Quantitative determination of the radioactivity from gel slices revealed that these fragments contained 40–50%, 15–20%, and 5–10% (range, *n* = 4) of the total α1-associated radioactivity, respectively.

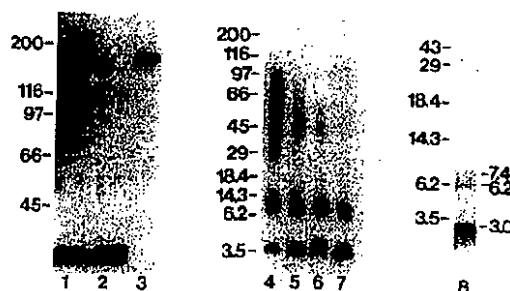


FIG. 1. Tryptic digestion of [³H]diazepine-photolabeled α1 subunit. Partially purified Ca²⁺ channels (10 μg) were photolabeled with [³H]diazepine in the absence (lane 1) or presence (lane 2) of 10 μM (±)-PN200-110 and purified. The purified photolabeled α1 subunit (2 μg, lane 3) was analyzed in gel system 1 (8% polyacrylamide) followed by fluorography of the dried gel. Eighty to 85% (range of three experiments) of labeled α1 was recovered after gel filtration. Purified photolabeled α1 subunit (2 μg, 80,000 dpm) was digested with trypsin (0.5 μg/ml) at 37°C for 0.5 h (lane 4), 1 h (lane 5), 2 h (lane 6), or 4 h (lane 7). The reaction was stopped by addition of 5 mM diisopropyl phosphofluoridate and the photolabeled peptides were separated in gel system 2. A more complete tryptic digest (100 μg/ml for 6 h) separated in gel system 3 is shown in lane 8. Fluorograms of dried gels are shown with molecular masses indicated as kDa.

Identification of Two Specifically Photolabeled Segments of the $\alpha 1$ Subunit by Immunoprecipitation with Sequence-Directed Antibodies. To determine their localization within the primary structure, we probed these tryptic fragments with several sequence-directed antibodies recognizing distinct proposed extracellular portions of the $\alpha 1$ subunit (15). The photolabeled $\alpha 1$ subunits were subjected to immunoprecipitation by incubation with the sequence-directed antibodies anti-CP-(946-976C), anti-CP-(1011-1026C), anti-CP-(1219-1232), anti-CP-(1338-1351), and anti-CP-(1339-1354) bound to protein A-Sepharose before and after tryptic digestion (Fig. 2). All antibodies immunoprecipitated the intact photolabeled $\alpha 1$ subunit. The immunoprecipitation was specific because it was not observed with preimmune sera or after preincubation of the anti-peptide antibodies with excess peptide. After digestion of labeled $\alpha 1$ with trypsin (100 μ g/ml), only anti-CP-(1011-1026C) (Fig. 2), anti-CP-(1338-1351) (Fig. 2), and anti-CP-(1339-1354) (data not shown) were still able to bind radioactivity (40-45% and 30-40% of the specifically bound radioactivity associated with $\alpha 1$, respectively). The results indicated that $\approx 75\%$ of specific [3 H]diazepine labeling occurs in close proximity to the CP-(1011-1026C) and CP-(1338-1351) sequences.

SDS/PAGE analysis of the immunoprecipitated photolabeled peptides revealed the three major photolabeled fragments of 7.4, 6.2, and 3.0 kDa (Fig. 3A). Anti-CP-(1011-1026C) recognized only the 3.0-kDa fragment (Fig. 3A, lane 2), whereas anti-CP-(1338-1351) and anti-CP-(1339-1354) (data not shown) bound only the labeled 6.2-kDa and 7.4-kDa fragments (Fig. 3A, lane 3). Thus, the 3.0-kDa and 6.2/7.4-kDa fragments represent distinct sites of photolabeling in domain III and domain IV, respectively.

Localization of the Site of Photolabeling in Domain III. Inspection of the potential tryptic cleavage sites within ≈ 3.0 kDa of the CP-(1011-1026C) sequence revealed that two tryptic fragments of ≈ 3 kDa can potentially be immunoprecipitated by this antibody (Fig. 3B). These fragments would result from cleavage at Arg-988 (N terminus) and Arg-1022 (C terminus; calculated molecular mass, 4.1 kDa), or Arg-1022 (N terminus) and Arg-1038 (C terminus; calculated molecular mass, 1.7 kDa), respectively. These two fragments contain 12 or 4 amino acid residues of peptide CP-(1011-1026C), respectively. To determine which of these two peptides can be bound by anti-CP-(1011-1026C), immunoprecipitation of a tryptic (100 μ g/ml) $\alpha 1$ digest was carried out in the absence

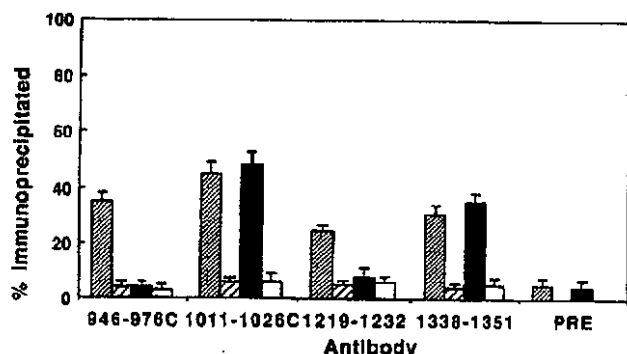


FIG. 2. Immunoprecipitation of [3 H]diazepine-labeled $\alpha 1$ subunit by various sequence-directed antibodies before and after trypsin digestion. Purified photolabeled $\alpha 1$ subunit (5000 dpm; bold-hatched bars and light-hatched bars) and its digests with trypsin (100 μ g/ml; solid bars and open bars) were immunoprecipitated with antisera (200 μ l) directed against the peptides indicated. Radioactivity bound to the protein A-Sepharose-adsorbed antibody was determined directly by scintillation counting. Sera preincubated with the corresponding peptides (lightly hatched bars and open bars) at 10 μ M and preimmune serum (Pre) were measured as control. Data are given as mean $\pm$ SD for $n > 2$.

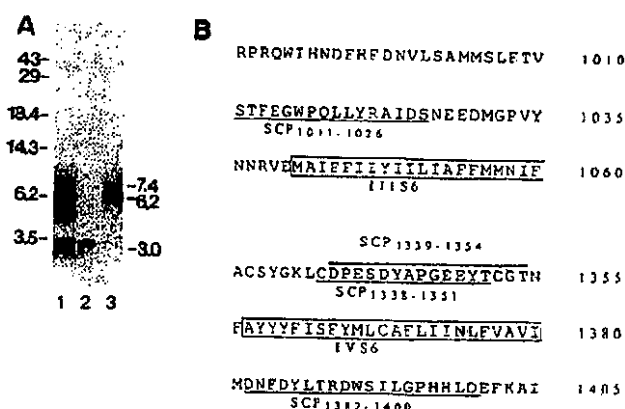


FIG. 3. Analysis of immunoprecipitated labeled tryptic $\alpha 1$ fragments. (A) Purified photolabeled $\alpha 1$ subunit (3×10^5 dpm) was digested with trypsin (100 μ g/ml) for 6 h at 37°C. A sample was analyzed directly by SDS/PAGE in gel system 3 and fluorography (lane 1). One-third of the digested sample was immunoprecipitated with protein A-Sepharose-bound antibodies anti-CP-(1011-1026C) (lane 2) and anti-CP-(1338-1351) (lane 3) and analyzed by SDS/PAGE in gel system 3. A fluorogram of the dried gel is shown. (B) Primary structure of $\alpha 1$ near the CP-(1011-1026), CP-(1338-1351), CP-(1339-1354), and CP-(1382-1400) sequences. Boxed sequences correspond to the proposed transmembrane segments IIIS6 and IVS6.

and presence of either CP-(1011-1022) or CP-(1023-1026C), the two purified tryptic fragments of CP-(1011-1026C). As shown in Fig. 4, only CP-(1011-1022), but not CP-(1023-1026C), was able to compete with anti-CP-(1011-1026C) binding to the ≈ 3 -kDa fragment. We therefore conclude that 40-50% of [3 H]diazepine photolabeling must occur between residues Arg-988 and Ala-1023 of the $\alpha 1$ subunit in the loop between transmembrane segments S5 and S6 of domain III.

Localization of the Site of Photolabeling in Domain IV. Inspection of the potential tryptic cleavage sites near the CP-(1338-1351) and CP-(1339-1354) sequences revealed that labeled tryptic fragments with the experimentally observed molecular masses (6.2 and 7.4 kDa) must be derived from cleavage at Lys-1336 (N terminus) and Arg-1388 (C terminus; calculated molecular mass, 6.3 kDa) or Lys-1403 (C-terminus; calculated molecular mass, 7.9 kDa), respectively.

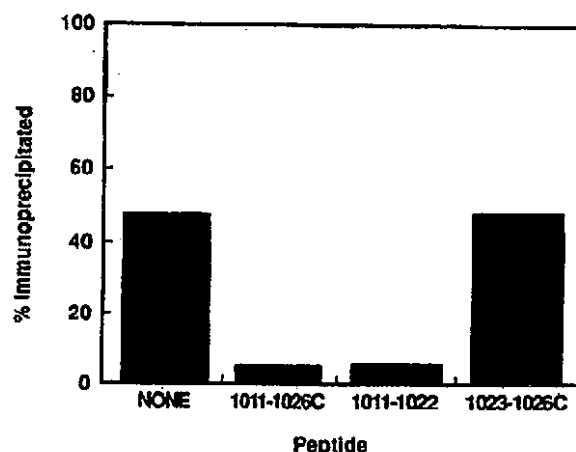


FIG. 4. Anti-CP-(1011-1026C) immunoprecipitation of [3 H]diazepine-labeled $\alpha 1$ fragments in the presence of CP-(1011-1026C) and its tryptic fragments. A tryptic (100 μ g/ml) digest of the purified photolabeled $\alpha 1$ subunit (5000 dpm) was immunoprecipitated with protein A-Sepharose-bound antibody anti-CP-(1011-1026C) preincubated with or without 10 μ M CP-(1011-1026C), 10 μ M CP-(1011-1022), or 10 μ M CP-(1023-1026C). Immunoprecipitated radioactivity was determined as in Fig. 2.

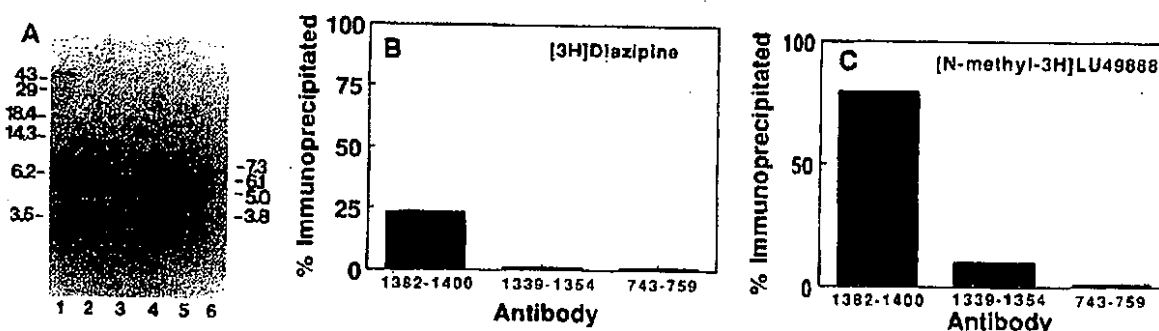


FIG. 5. Immunoprecipitation of [3 H]diazepine-labeled $\alpha 1$ fragments with various sequence-directed antibodies after digestion with V8 protease. (A) Purified [3 H]diazepine-labeled $\alpha 1$ (4000 cpm) was digested with V8 protease (1 mg/ml) for 0.5 h (lanes 1–3) or 2 h (lanes 4–6). Half of the digested sample was immunoprecipitated with protein A-Sepharose-bound antibodies anti-CP-(1382–1400) (50 μ l of serum, lanes 2 and 5) or anti-CP-(743–759) (50 μ l of serum, lanes 3 and 6). These samples and unprecipitated controls (lanes 1 and 4) were analyzed by SDS/PAGE and fluorography. (B) Purified [3 H]diazepine-labeled $\alpha 1$ was digested with V8 protease (0.2 mg/ml) in 40 mM sodium phosphate, pH 7.8/0.1% SDS for 24 h at 37°C. The digested sample was immunoprecipitated with protein A-Sepharose-bound antibodies (50–75 μ l of serum). After incubation, the protein A-Sepharose was collected by centrifugation, and the antibody-bound radioactivity was calculated from the decrease in unbound radioactivity recovered in the supernatant. Immunoprecipitated radioactivity was normalized with respect to the total [3 H]diazepine-labeled $\alpha 1$ added. (C) Reductively carboxyamidomethylated [N -methyl- 3 H]LU49888-labeled $\alpha 1$ was digested with (0.5 mg/ml) or without (control) V8 protease followed by immunoprecipitation as in B. Antibody-bound radioactivity was determined by liquid scintillation counting of a sample of the washed protein A-Sepharose pellet and by SDS/PAGE. Data obtained with the digested sample were normalized with respect to the control samples. SDS/PAGE revealed the presence of a single 6.2-kDa labeled fragment (data not shown).

These fragments make up the transmembrane segment S6 of domain IV and some residues on its proposed extracellular and intracellular sides. To further restrict the site of labeling, we used V8 protease to generate smaller proteolytic fragments in this region. After 120 min of digestion with V8 protease (1.0 mg/ml), three labeled peptides were generated with apparent molecular masses of 6.1, 5.0, and 3.8 kDa, as illustrated for two experiments in Fig. 5A (lanes 1 and 4). The 6.1- and 5.0-kDa peptides were immunoprecipitated by anti-CP-(1382–1400), an antibody against the intracellular side of segment IVS6 (Fig. 5A, lanes 2 and 5), but not by a control antibody directed against a remote region of the calcium channel (Fig. 5A, lanes 3 and 6). Quantitation of the immunoprecipitated radioactivity in a similar experiment with anti-CP-(1339–1354) (Fig. 5B) shows that 23% of the total $\alpha 1$ labeling is recognized by anti-CP-(1382–1400), whereas none is recognized by anti-CP-(1339–1354) or by the control antibody. These results indicate that treatment with V8 protease removes the CP-(1339–1354) peptide from the photolabeled fragment and place its N-terminal end toward the C-terminal end of CP-(1339–1354).

Previous results (11) showed that [N -methyl- 3 H]LU49888, a photoreactive derivative of the phenylalkylamine verapamil, labels V8 fragments of almost identical size that are recognized by anti-CP-(1382–1400). As shown in Fig. 5C, anti-CP-(1382–1400), but not anti-CP-(1339–1354) or the control antibody against a remote region of $\alpha 1$, also immunoprecipitated most of the [N -methyl- 3 H]LU49888 in the photolabeled V8 fragments.

Thus we propose the following localization of the [3 H]diazepine-labeled V8 fragments. The 3.8-kDa fragment is not recognized by anti-CP-(1339–1354) or anti-CP-(1382–1400) and, therefore, must represent a proteolytic fragment of the labeled region in domain III. The 5.0-kDa fragment must have been generated by cleavage at Glu-1349 (N terminus) and Asp-1390 (C terminus; calculated molecular mass, 4.9 kDa) as shown previously for the smallest [N -methyl- 3 H]LU49888-labeled fragment (11). The 6.1-kDa fragment must contain the 5.0-kDa fragment plus additional sequences at its N and/or C termini. We therefore conclude that 25%–30% of specific [3 H]diazepine photolabeling occurs between Glu-1349 and Trp-1391 in domain IV of the $\alpha 1$ subunit.

The Dihydropyridine Receptor Site of L-Type Ca^{2+} Channels. Our results lead directly to the conclusion that diazepam is covalently attached to the calcium channel $\alpha 1$ subunit at

sites in domain III between residues 988 and 1023 and in domain IV between residues 1349 and 1391. These regions are located in the loop connecting transmembrane segments IIIS5 and IIIS6 and in transmembrane segment IVS6 and the adjacent several intracellular and extracellular residues. As only a single high-affinity binding site for DHPs is associated with purified or partially purified skeletal muscle Ca^{2+} -channel preparations, it is very likely that these two specifically photolabeled regions are in close proximity to the high-affinity DHP receptor site in the folded structure of the $\alpha 1$ subunit, as predicted by current folding models (22–24).

Because both of the specifically photolabeled regions contain transmembrane or probable membrane-associated segments, our results do not provide conclusive evidence on whether the DHP receptor site is located within the membrane or on its extracellular or intracellular surfaces. Previous studies with charged DHPs indicated that the DHP binding domain of L-type Ca^{2+} channels is accessible from the outer surface of the calcium channel to both DHPs and protons (9, 10). DHPs are lipophilic molecules with high membrane partition coefficients and are believed to reach their receptor site on the $\alpha 1$ subunit after partition into the lipid bilayer (8). We suggest a model (Fig. 6) for formation of the DHP receptor site that is supported by our present results

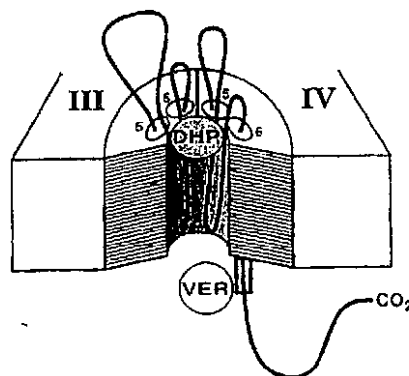


FIG. 6. Tentative model for DHP and verapamil receptor sites. A model of calcium channel structure, adapted from ref. 23, illustrates domains III and IV and the proposed folding of the segment between transmembrane helices S5 and S6 back into the membrane to form the lining of the transmembrane pore. Proposed regions of DHP and verapamil (VER) binding are indicated.

and agrees with these previous studies indicating DHP binding and action at a site accessible from the extracellular surface of the lipid bilayer.

The photolabeled region corresponding to residues 988–1023 in the long connecting loop between the proposed transmembrane segments S5 and S6 of domain III has been proposed to fold back into the membrane in a nonhelical conformation to form two short segments, SS1 and SS2, that may line the transmembrane pores of sodium and potassium channels (22, 24). By analogy with these studies, it is likely that residues 1001–1018 of the calcium channel $\alpha 1$ subunit fold into the membrane and contribute to formation of the transmembrane pore (23). Because DHPs can reach their receptor sites from the extracellular side of the channel and can activate and inhibit channel activity, it seems most likely that their receptor site is formed in a position that is accessible from the extracellular surface of the $\alpha 1$ subunit and does not impede ion access to the transmembrane pore. We suggest that residues in domain III on either side of those that are proposed to form the transmembrane pore (e.g., approximately residues 988–1001 and residues 1018 through the extracellular end of transmembrane segment IIIS6) contribute to formation of the receptor site for DHPs and are photoaffinity labeled in our studies. The amino acid residues in transmembrane segment IVS6 may also contribute to formation of the DHP site since they are thought to be located nearby in the folded structure of the $\alpha 1$ subunit (Fig. 6).

The peptide fragments we have characterized account for the majority of the [3 H]diazepam specifically incorporated into the $\alpha 1$ subunit (65–85%). It is possible that additional minor sites are also photolabeled and contribute to the 15–35% not characterized in this work. For example, hydrophilic intracellular segments in the N- and C-terminal domains of the $\alpha 1$ subunit are also photolabeled by DHPs as determined by amino acid sequencing methods (25). The evidence that the DHP receptor site is located within the lipid bilayer accessible from the extracellular surface suggests that these sequences may not contribute directly to the physiologically active DHP receptor site.

Previous work (11) and the results of Fig. 5 show that the peptide containing residues 1349–1391 is also photolabeled by a verapamil derivative and, therefore, likely contributes to formation of the intracellular receptor site for phenylalkylamines. There are strong allosteric interactions between the binding of DHPs and phenylalkylamines (4). Since the receptor sites for these two classes of drugs may be formed in part by peptide segments at opposite ends of transmembrane segment IVS6, their allosteric interaction may involve conformational changes that are transmitted through the membrane by movements of segment IVS6. Further studies of the molecular properties of these receptor sites may reveal information concerning the conformational changes that are

involved in calcium channel gating and its modulation by DHPs and other calcium channel modulators.

We thank Mr. M. Kanazawa for help in preparing some antibodies. This work was supported by Grants-in-Aid for Scientific Research on Priority Areas (01641501 to Y.K. and 02250102 and 02257201 to H.N.) from the Ministry of Education, Science and Culture of Japan; by grants from the Mitsubishi Foundation, the Toray Foundation, and the Uehara Memorial Foundation to Y.K.; by National Institutes of Health Grant NS22625, a Research Grant from the Muscular Dystrophy Association, and a grant from Miles Laboratories to W.A.C.; by a postdoctoral research fellowship from the Max Kade Foundation to J.S.; and by the Fonds zur Förderung der Wissenschaftlichen Forschung and Österreichische Nationalbank to H.G.

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Effect of combination of calcium antagonist, amlodipine, and AT₁ receptor blocker, olmesartan, on atherosclerosis in apolipoprotein E-deficient mice

Jun Suzuki, Masaru Iwai, Zhen Li, Jian-Mei Li, Li-Juan Min, Ayumi Ide, Toyofumi Yoshii, Akira Oshita, Masaki Mogi and Masatsugu Horiuchi

Objective Angiotensin II type 1 receptor blockers (ARB) are widely recognized to have a vasculoprotective effect. Accumulating data have revealed that calcium antagonists also retard atherosclerosis. We examined the possibility that combination therapy of ARB and calcium antagonists could more effectively prevent atherosclerosis than monotherapy.

Methods and results We observed a marked increase in the atherosclerotic area, associated with the exaggerated expression of nicotinamide adenine dinucleotide (phosphate), reduced form [NAD(P)H] oxidase subunits (p22^{phox} and p47^{phox}) and superoxide anion production, in the aorta of apolipoprotein E-deficient mice maintained on a 1.25% high-cholesterol diet for 10 weeks. A calcium antagonist, amlodipine, at a dose of 1 mg/kg a day or an ARB, olmesartan, at a dose of 3 mg/kg a day, significantly inhibited these parameters, with no change in systolic blood pressure and the blood cholesterol level. Moreover, the co-administration of lower doses of amlodipine (0.1 mg/kg a day) and olmesartan (1 mg/kg a day) significantly inhibited the atherosclerotic area and oxidative stress, whereas amlodipine or olmesartan alone at these doses did not affect these parameters. Furthermore, we observed similar inhibitory effects of amlodipine or olmesartan on angiotensin II-induced NADPH oxidase activity

and Akt activity in cultured vascular smooth muscle cells.

Conclusion These results suggest that the co-administration of calcium antagonists and ARB synergistically blunts oxidative stress at least partly through the inhibition of Akt activity and enhances the beneficial effects of these drugs on atherosclerosis compared with monotherapy. *J Hypertens* 23:1383–1389 © 2005 Lippincott Williams & Wilkins.

Journal of Hypertension 2005, 23:1383–1389

Keywords: AT₁ receptor blocker, atherosclerosis, calcium antagonist, oxidative stress

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Sponsorship: This work was supported by grants from the Ministry of Education, Science, Sports, and Culture of Japan, the Cardiovascular Research Foundation, the Smoking Research Foundation, Takeda Science Foundation and the Novartis Foundation of Gerontological Research.

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Received 7 September 2004 Revised 17 March 2005
Accepted 17 March 2005

Introduction

Increased production of reactive oxygen species (ROS) in the atherosclerotic lesion has been documented in both animal [1] and human [2] studies. Oxidative stress, an excessive production of ROS, plays a central role in the development and progression of atherosclerosis, because it mediates a wide range of pathological processes such as lipid peroxidation [3] and the reduction of nitric oxide bioactivity [4]. Angiotensin II is a principal vasoactive substance of the renin–angiotensin system, which has a variety of physiological actions including vasoconstriction, aldosterone release, and cell growth. Angiotensin II is a potent mediator of oxidative stress through activating nicotinamide adenine dinucleotide (phosphate), reduced form [NAD(P)H] oxidase [5], which contributes to the development of atherosclerosis. Most of the known cardiovascular actions of angiotensin II have been

reported to be mediated by the angiotensin II type 1 (AT₁) receptor, and AT₁ receptor blockers (ARB) have been widely used as antihypertensive drugs with the expectation of vascular protective effects [6].

As a number of molecular and cellular changes in atherogenesis are mediated by the disruption of calcium homeostasis, it has been proposed that calcium antagonists may exert anti-atherosclerotic effects. Calcium antagonists have been shown to limit the development of atherosclerosis [7,8]. Accumulating evidence has suggested that the intracellular signaling mechanisms, by which the AT₁ receptor exerts hypertrophic or proliferative effects on their targets such as vascular smooth muscle cells (VSMC), are closely associated with receptor and non-receptor tyrosine kinases, and that some AT₁ receptor-mediated signaling requires calcium ion-sensitive

tyrosine kinases [9]. These results suggest the possibility that an ARB and calcium antagonist combination therapy could more effectively prevent the development of atherosclerosis than monotherapy. We have recently demonstrated that a calcium antagonist, azelnidipine, enhances the vascular protective effects of an ARB, olmesartan, on an injured artery, with the attenuation of VSMC proliferation, inflammation, and oxidative stress [10]. However, the effects of calcium antagonists combined with ARB on the spontaneous development of atherosclerotic lesions have not been fully elucidated.

In the present study, we examined the possibility that a combination of azelnidipine and olmesartan could be more effective in the treatment of atherosclerosis than monotherapy, using a mouse atherosclerosis model, apolipoprotein E-deficient mice, which exhibit atherosclerotic lesion formation similar to that observed in humans.

Methods

This study was performed in accordance with the National Institutes of Health guidelines for the use of experimental animals. All animal studies were reviewed and approved by the Animal Studies Committee of Ehime University.

Animals and treatment

Adult male apolipoprotein E-deficient mice (The Jackson Laboratory, Bar Harbor, Maine, USA) aged 6 weeks with a body weight ranging from 25 to 30 g were used in this study. The animals were housed in a room where lighting was controlled (12 h on, 12 h off) and the temperature was kept at 25°C. They were given a standard diet (mouse food, Oriental Yeast) or a high-cholesterol diet (1.25% cholesterol, 10% coconut oil in mouse food) for 10 weeks from 6 weeks of age and water was freely available. Azelnidipine (CS-905; donated by Sankyo Pharmaceutical Co., Tokyo, Japan; 0.1 and 1 mg/kg a day) was administered orally from 6 weeks of age. Olmesartan (RNH-6270; donated by Sankyo Pharmaceutical Co.; 1 and 3 mg/kg a day) was administered using an osmotic minipump (Alzet model 1002; DURECT Corporation, Cupertino, California, USA) implanted intraperitoneally from 6 weeks of age. Systolic blood pressure was measured by a non-invasive tail cuff system in conscious mice. The plasma cholesterol level was measured by the cholesterol oxidase method (Cholesterol E-test; WAKO Chemical Industries, Ltd., Tokyo, Japan).

Atherosclerotic lesion area

The mice were killed at the age of 16 weeks, and the atherosclerotic lesions were analysed as described previously [11]. Analysis of the lesion area was performed either within the aortic arch or throughout the aorta. The lesion area of the proximal portion of the thoracic aorta

up to the aortic origin (aortic arch) was determined by means of serial cross-sections, followed by oil red O staining and counterstaining with haematoxylin. A quantitative analysis was performed using Densitograph Imaging Software (ATTO Corporation, Tokyo, Japan). The mean value of five sections was taken as the value for each animal. The lesion area in the whole aorta was also analysed. The excised aorta (from aortic arch to iliac bifurcation) was opened longitudinally. Colour images were taken with a digital camera (Nikon, PIX4500) connected to a computer. The atherosclerotic lesion area was quantified by analysing the open luminal surface of oil red O-stained aorta with Densitograph Imaging Software. The amount of lesion formation in the entire aorta in each animal was measured as the percentage of lesion area per total area of the aortic endothelial surface.

Western blot

Total proteins were prepared from pooled arteries (six to eight arteries for each group) or cultured VSMC, and Western blot was performed as previously described [12]. Anti-p22^{phox}, anti-p47^{phox} and anti-Akt1 antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA). Anti- α -smooth muscle actin antibody (clone 1A4) was purchased from Sigma Chemical Co. (St Louis, Missouri, USA). Antiphosphorylated Akt (Ser 473) was purchased from Cell Signaling Technology, Inc. (Beverly, Massachusetts, USA).

Superoxide production

Superoxide production was quantitatively measured using a cytochrome *c* reduction assay as previously described [13,14]. The aortas were rapidly removed and placed into chilled Krebs/HEPES buffer, and excessive adventitial tissue was cleaned without injuring the endothelium. Three aortic ring sections (2 mm) were placed in buffer containing 145 mmol/l sodium chloride, 4.86 mmol/l potassium chloride, 5.7 mmol/l sodium phosphate, 0.54 mmol/l calcium chloride, 1.22 mmol/l magnesium sulphate, 5.5 mmol/l glucose, 0.1 mmol/l deferoxamine mesylate, and 1 U/ μ l catalase. Cytochrome *c* (50 μ M; Sigma-Aldrich, St Louis, Missouri, USA) was then added, and the samples were incubated at 37°C for 60 min with and without superoxide dismutase (SOD; 125 U/ml). Cytochrome *c* reduction was calculated using absorbance at 550 nm. Superoxide production was quantified in picomoles per milligram of aorta from the difference between absorbance with and without SOD.

Cell culture and treatment

Adult rat aortic VSMC were prepared and cultured as described previously [12]. Subconfluent VSMC from passages 5–7 were used in the following experiments. Before the experiments, VSMC were kept for 24 h in quiescent medium without serum. The cells were then treated with Dulbecco's modified essential medium

containing angiotensin II (Sigma) (10^{-7} mol/l) with or without azelnidipine (CS-905; donated by Sankyo Pharmaceutical Co.; 0.5×10^{-6} and 5×10^{-6} mol/l) or olmesartan (RNH-6270; donated by Sankyo Pharmaceutical Co.; 10^{-10} and 10^{-6} mol/l) as indicated in each experimental condition. As an antioxidant, tempol (10 mmol/l), a SOD mimetic, was purchased from Wako Pure Chemical Industries (Osaka, Japan).

Nicotinamide adenine dinucleotide phosphate, reduced form, oxidase activity

NADPH oxidase activity was measured as previously described, with slight modifications using $5 \mu\text{mol/l}$ lucigenin [15]. Chemiluminescence was monitored by a luminometer (AB-2200; ATTO Corporation) for 5 min after the addition of cell extract.

Statistical analysis

Values are expressed as mean $\pm$ SE in the text and figures. The data were analysed by two-way analysis of variance. If a statistically significant effect was found, post-hoc analysis was performed to detect the difference between the groups. A value of $P < 0.05$ was considered to be statistically significant.

Results

Systolic blood pressure and plasma cholesterol level

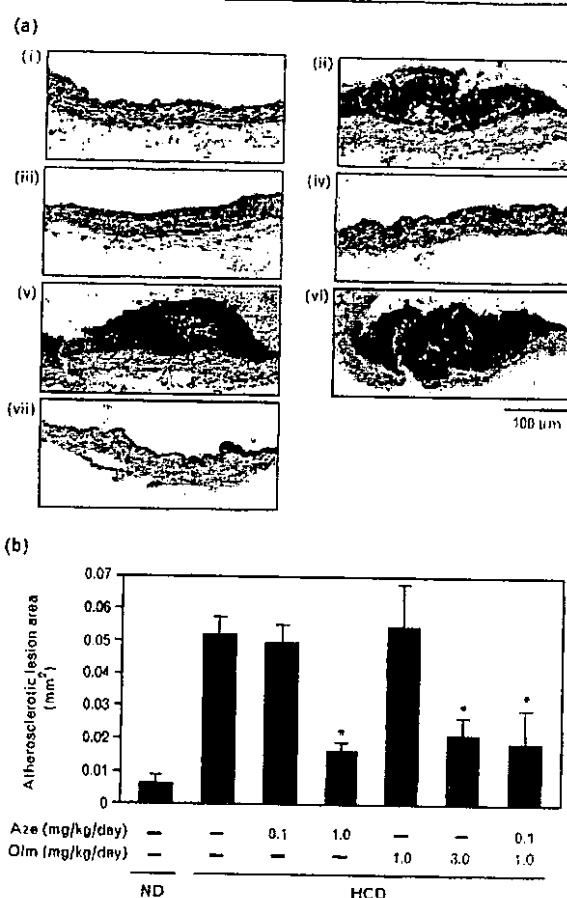
We first examined the effects of a high-cholesterol diet, azelnidipine or olmesartan treatment on systolic blood pressure and the plasma cholesterol level. As shown in Table 1, the plasma cholesterol level was significantly elevated in apolipoprotein E-deficient mice after 10 weeks' feeding of a high-cholesterol diet compared with a normal diet. Azelnidipine (0.1 and 1 mg/kg a day) or olmesartan (1 and 3 mg/kg a day) did not influence either systolic blood pressure or the plasma cholesterol level (Table 1).

Effect of azelnidipine and olmesartan on atherosclerotic lesion area

We next assessed the atherosclerotic lesion area in the proximal and whole aorta. After 10 weeks of high-cholesterol diet feeding, apolipoprotein E-deficient mice

displayed marked atherosclerotic lesion formation in the proximal aorta (Fig. 1). The lesion area was significantly reduced by treatment with azelnidipine at 1 mg/kg a day (24% of high-cholesterol diet only) or olmesartan at 3 mg/kg a day (41% of high-cholesterol diet only; Fig. 1). To examine whether azelnidipine and olmesartan could synergistically inhibit atherosclerotic lesion formation, apolipoprotein E-deficient mice were treated with a lower dose of azelnidipine, olmesartan, or their combination. Azelnidipine or olmesartan at lower doses (0.1 or

Fig. 1



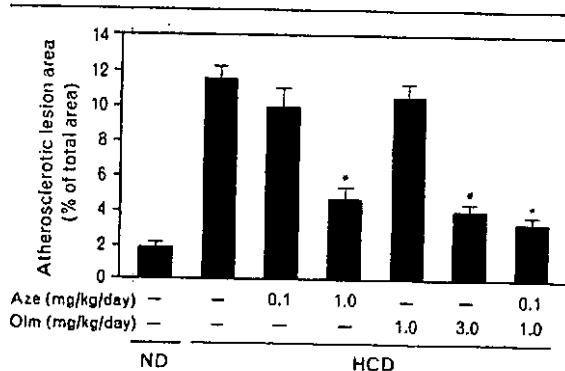
Effects of azelnidipine or olmesartan on atherosclerotic lesion area in proximal aorta. (a) Representative cross-sections of proximal aorta with oil red O staining in apolipoprotein E-deficient mice treated with (i) normal diet (ND), (ii) high-cholesterol diet (HCD), (iii) high-cholesterol diet with 1 mg/kg a day azelnidipine, (iv) high-cholesterol diet with 3 mg/kg a day olmesartan (Olm), (v) high-cholesterol diet with 0.1 mg/kg a day Aze, (vi) high-cholesterol diet with 1 mg/kg a day Olm, and (vii) high-cholesterol diet with 0.1 mg/kg a day Aze and 1 mg/kg a day Olm. (b) Atherosclerotic lesion area in cross-sections of proximal aorta. The mean value of five sections was taken as the value for each animal. Each bar represents mean $\pm$ SE ($n = 8-10$ per group). *Significant difference ($P < 0.05$) compared with high-cholesterol diet only.

Table 1 Systolic blood pressure and plasma cholesterol level in apolipoprotein E-deficient mice

	SBP (mmHg)	Cholesterol (mg/dl)
Normal diet	95.6 $\pm$ 1.0	413.0 $\pm$ 45.0
High-cholesterol diet	98.5 $\pm$ 0.8	2138.0 $\pm$ 254.0*
Azelnidipine (0.1)	98.8 $\pm$ 1.8	2036.0 $\pm$ 121.3*
Azelnidipine (1)	99.8 $\pm$ 1.2	1958.5 $\pm$ 135.0*
Olmesartan (1)	96.7 $\pm$ 1.2	1959.3 $\pm$ 130.7*
Olmesartan (3)	96.8 $\pm$ 0.9	2279.8 $\pm$ 168.1*
Azelnidipine (0.1) + olmesartan (1)	99.3 $\pm$ 1.7	1981.5 $\pm$ 223.2*

Values are mean $\pm$ SE; $n = 11-12$ for each group. Values of azelnidipine and olmesartan are given as mg/kg per day. *Significant difference ($P < 0.05$) compared with normal diet.

Fig. 2



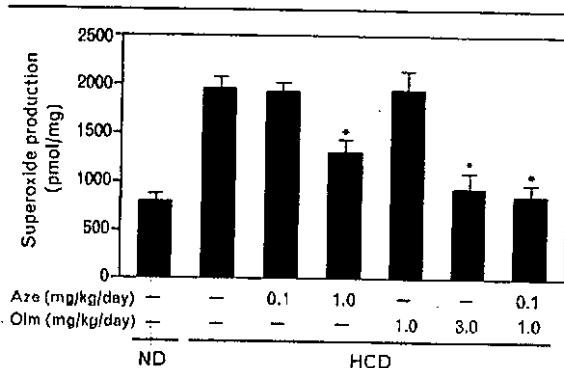
Effects of azelnidipine or olmesartan on atherosclerotic lesion area in the entire aorta. The amount of atherosclerotic lesion formation in the entire aorta was measured as the percentage of the lesion area per total area of the aortic endothelial surface. Each bar represents mean $\pm$ SE ($n = 8-10$ per group). Aze, azelnidipine; HCD, high-cholesterol diet; ND, normal diet; Olm, olmesartan. *Significant difference ($P < 0.05$) compared with high-cholesterol diet only.

1 mg/kg a day, respectively) did not affect the lesion area, whereas a combination of both azelnidipine and olmesartan at these doses significantly reduced the lesion area (37% of high-cholesterol diet only; Fig. 1). Similar effects of azelnidipine or olmesartan on the lesion area were also observed in the whole aorta (Fig. 2).

Effect of azelnidipine and olmesartan on vascular oxidative stress

Oxidative stress is thought to play a critical role in the process of atherosclerotic lesion formation. Therefore, we examined superoxide production and the expression of the NAD(P)H oxidase subunits in the aorta. After 10

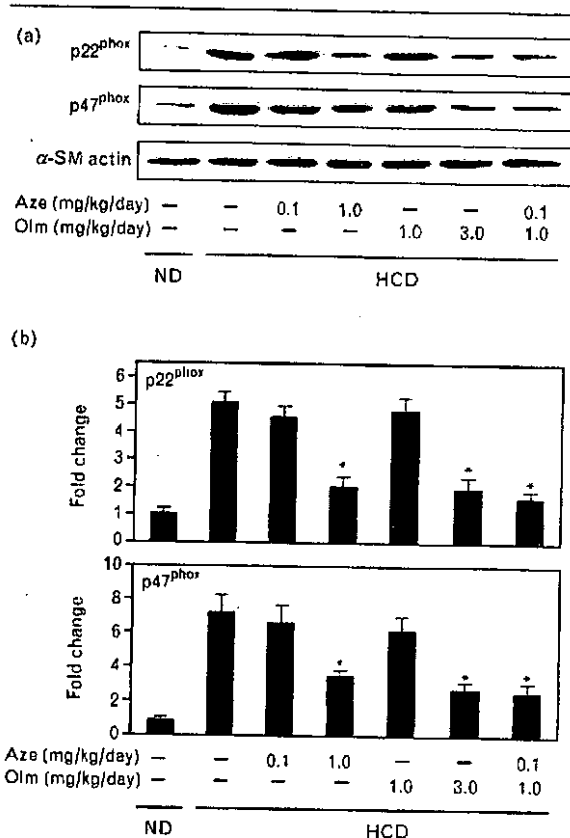
Fig. 3



Effects of azelnidipine or olmesartan on superoxide production in the aorta. The superoxide content was measured by the cytochrome c reduction assay as described in the Methods section. Each bar represents mean $\pm$ SE ($n = 6-8$ per group). Aze, azelnidipine; HCD, high-cholesterol diet; ND, normal diet; Olm, olmesartan. *Significant difference ($P < 0.05$) compared with high-cholesterol diet only.

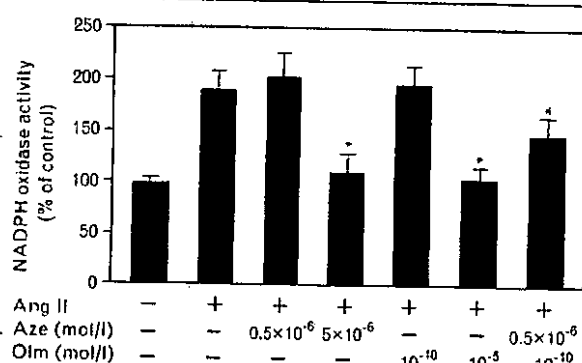
weeks' high-cholesterol diet feeding, superoxide production in the aorta was significantly enhanced compared with normal diet (1970 ± 151 versus 801 ± 83 pmol/mg; Fig. 3). Azelnidipine at 1 mg/kg a day or olmesartan at 3 mg/kg a day attenuated superoxide production (1321 ± 118 and 947 ± 146 pmol/mg, respectively; Fig. 3). Azelnidipine or olmesartan at lower doses (0.1 and 1 mg/kg a day, respectively) did not affect superoxide production (1942 ± 69 and 1954 ± 163 pmol/mg, respectively), whereas a combination of both azelnidipine and olmesartan at these doses significantly attenuated superoxide production (873 ± 112 pmol/mg; Fig. 3). Western blot using pooled aortic samples revealed similar inhibitory effects of azelnidipine or olmesartan on expression of the NAD(P)H oxidase subunits, p22^{phox} and p47^{phox}, in the aorta (Fig. 4).

Fig. 4



Effects of azelnidipine or olmesartan on expression of nicotinamide adenine dinucleotide (phosphate), reduced form [NAD(P)H] oxidase subunits in the aorta. (a) One representative result of Western blots of p22^{phox} and p47^{phox} using three different pooled samples is shown. (b) Densitometric measurements of p22^{phox} and p47^{phox} from Western blots. Values are mean $\pm$ SE ($n = 3$). α -SM actin, α -smooth muscle actin; Aze, azelnidipine; HCD, high-cholesterol diet; ND, normal diet; Olm, olmesartan; p22^{phox} and p47^{phox}, NADH/NADPH oxidase subunit. *Significant difference ($P < 0.05$) compared with high-cholesterol diet only.

Fig. 5



Effects of azelnidipine or olmesartan on angiotensin II (Ang II)-induced nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) oxidase activation in adult rat vascular smooth muscle cells (VSMC). Adult rat VSMC were prepared, cultured and treated with Ang II (10⁻⁷ mol/l) with or without azelnidipine or olmesartan as described in the Methods section. Each bar represents mean ± SE of four independent experiments. Aze, Azelnidipine; Olm, olmesartan. *Significant difference ($P < 0.05$) compared with control.

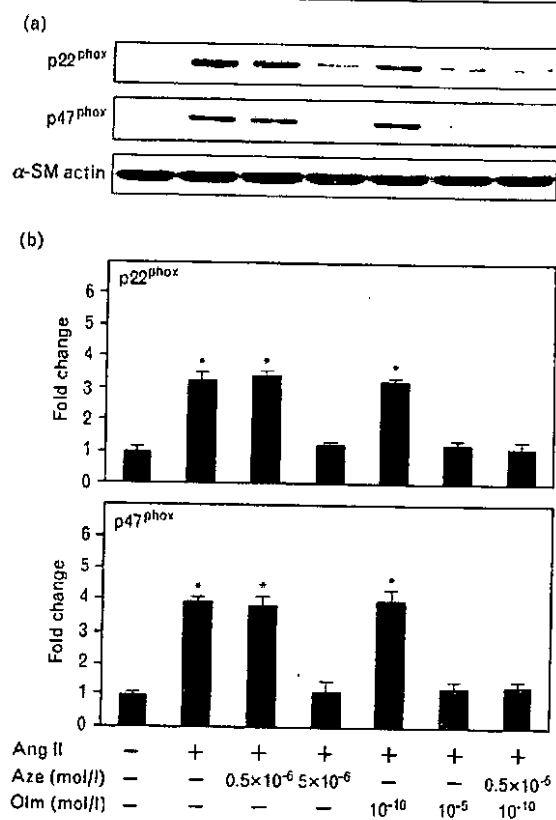
Effect of azelnidipine and olmesartan on angiotensin II-induced NADPH oxidase activation in cultured vascular smooth muscle cells

We next examined the possibility that azelnidipine and olmesartan may attenuate angiotensin II-induced NADPH oxidase activation in VSMC. As shown in Figure 5, angiotensin II (0.1 μmol/l) enhanced NADPH oxidase activity in VSMC. Azelnidipine at 5 μmol/l or olmesartan at 10 μmol/l significantly attenuated angiotensin II-induced NADPH oxidase activation (Fig. 5). Azelnidipine or olmesartan at lower doses (0.5 μmol/l or 0.1 nmol/l, respectively) did not affect NADPH oxidase activity, whereas a combination of both azelnidipine and olmesartan at these doses significantly attenuated angiotensin II-induced NADPH oxidase activation (Fig. 5). Moreover, the combination of lower doses of azelnidipine and olmesartan (0.5 μmol/l or 0.1 nmol/l, respectively) also inhibited the expression of p22^{phox} and p47^{phox} in cultured VSMC, whereas each drug alone did not affect these expressions at these doses (Fig. 6).

Effect of azelnidipine and olmesartan on angiotensin II-induced Akt activation in cultured vascular smooth muscle cells

To assess the signaling pathway involved in the effects of azelnidipine and olmesartan, we measured Akt activity induced by angiotensin II in cultured VSMC. As shown in Figure 7a, the activation of Akt was determined as its phosphorylation. The combination of lower doses of azelnidipine and olmesartan significantly attenuated Akt phosphorylation induced by angiotensin II, whereas each drug did not affect Akt phosphorylation at these doses. Moreover, tempol (10 mmol/l), an antioxidant,

Fig. 6



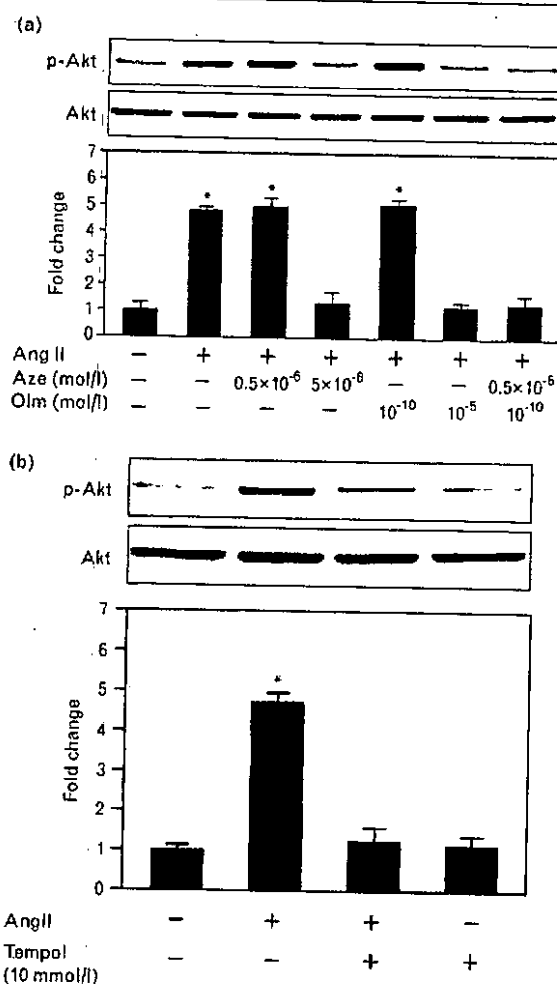
Effects of azelnidipine or olmesartan on the expression of nicotinamide adenine dinucleotide (phosphate), reduced form (NAD(P)H) oxidase subunits in adult rat vascular smooth muscle cells (VSMC). (a) One representative result of Western blots of p22^{phox} and p47^{phox} is shown. (b) Densitometric measurements of p22^{phox} and p47^{phox} from Western blots. Values are mean ± SE ($n = 3$). α-SM actin, α-smooth muscle actin; Aze, azelnidipine; Olm, olmesartan; p22^{phox} and p47^{phox}, NADH/NADPH oxidase subunit. *Significant difference ($P < 0.05$) compared with control.

remarkably inhibited Akt phosphorylation induced by angiotensin II (Fig. 7b).

Discussion

The lack of apolipoprotein E causes a marked increase in plasma cholesterol-rich remnants and subsequent atherosclerotic lesion formation [16]. Apolipoprotein E-deficient mice, therefore, provide a unique model to analyse the pathogenesis of spontaneous atherosclerosis. Consistent with previous observations, we demonstrated in the present study that the atherosclerotic lesion area in this model was significantly reduced by treatment with azelnidipine at 1 mg/kg a day or olmesartan at 3 mg/kg a day [10]. In contrast, Candido *et al.* [17] showed that an ARB, irbesartan, but not a calcium antagonist, amlodipine, reduced plaque size in diabetic apolipoprotein E-deficient mice. Moreover, the authors showed that

Fig. 7



(a) Effects of azelnidipine or olmesartan on angiotensin II (Ang II)-induced Akt activation in adult rat vascular smooth muscle cells (VSMC). Adult rat VSMC were prepared, cultured and treated with Ang II (10⁻⁷ mol/l) with or without azelnidipine or olmesartan as described in the Methods section. (b) Effects of tempol (10 mmol/l) on Ang II-induced Akt activation in adult rat VSMC. Tempol (10 mmol/l) was added simultaneously with Ang II. Each bar represents mean ± SE of three independent experiments. Aze, Azelnidipine; Olm, olmesartan; p-Akt, phosphorylated Akt. *Significant difference ($P < 0.05$) compared with control.

irbesartan but not amlodipine treatment attenuated the collagen content, cellular proliferation, and macrophage infiltration as well as platelet-derived growth factor B, monocyte chemoattractant protein 1 and vascular cellular adhesion molecule 1 overexpression in the aorta [17]. However, we have reported that azelnidipine was able to reduce neointimal formation in a cuff-induced vascular injury model, associated with the attenuation of TNF- α , monocyte chemoattractant protein 1 expression and VSMC proliferation [10]. These apparently conflicting

results could be partly caused by the model (diabetic or non-diabetic apolipoprotein E-deficient mice) or the drug (amlodipine or azelnidipine).

We examined the possibility that calcium antagonists and ARB may synergistically suppress atherosclerotic lesion formation, and demonstrated that a combination of both azelnidipine and olmesartan at low doses, which did not affect blood pressure and the plasma cholesterol level, effectively reduced atherosclerotic lesion formation in apolipoprotein E-deficient mice placed on a high-cholesterol diet for 10 weeks, whereas this inhibitory effect was not observed with these doses of azelnidipine and olmesartan alone. These results suggest that a combination of azelnidipine and olmesartan could be more effective in the treatment of atherosclerosis than monotherapy.

An evaluation of oxidative stress in this model may provide a further understanding of the inhibitory effect of calcium antagonists and ARB on atherosclerosis. We observed that superoxide production in the aorta was significantly enhanced in apolipoprotein E-deficient mice placed on a high-cholesterol diet for 10 weeks. The mechanism by which oxidative stress is increased by hyperlipidemia could involve the exacerbation of the effects of angiotensin II, because LDL has been shown to upregulate AT₁ receptor expression [18]. It has been reported that p22^{phox}, a membrane component of NAD(P)H oxidase, is a critical component of angiotensin II-induced NAD(P)H oxidase activation and vascular hypertrophy [19]. It has also been reported that p47^{phox}, a cytoplasmic component of NAD(P)H oxidase, is required for atherosclerotic lesion progression in apolipoprotein E-deficient mice [20]. These results suggest that ROS production through NAD(P)H oxidase activation plays an important role in the development and progression of atherosclerosis. We, therefore, examined the expression of p22^{phox} and p47^{phox} in the aorta. Consistent with the results of the lesion area, a combination of both azelnidipine and olmesartan synergistically attenuated the expression of p22^{phox} and p47^{phox}. Such an inhibitory effect of the combination on p22^{phox} and p47^{phox} was also observed in VSMC (Fig. 6). Taken together, our observations indicate that a combination of azelnidipine and olmesartan could more effectively reduce the atherosclerosis lesion area associated with the attenuation of oxidative stress than monotherapy.

Akt regulates cell growth and apolipoprotein ptosis in various cell types [21]. It has also been reported that Akt is involved in atherosclerotic lesion formation through the control of VSMC [22]. In our study, the combination of lower doses of azelnidipine and olmesartan attenuated Akt activation induced by angiotensin II in cultured VSMC (Fig. 7a). Moreover, Akt activation was also inhibited by an antioxidant, tempol (Fig. 7b). These

results suggest that the inhibitory action of azelnidipine and olmesartan is mediated by the regulation of Akt activity through the inhibition of oxidative stress. The activation of mitogen-activated protein (MAP) kinase appears to be a major step in the growth signal transduction of several growth-promoting factors. It has been reported that an increase in intracellular free calcium concentration is an important process for angiotensin II-induced activation of MAP kinase [23]. Moreover, a calcium antagonist, nifedipine, has been shown to inhibit intimal thickening by attenuating intimal VSMC proliferation via a MAP kinase pathway [24], suggesting that intracellular calcium kinetics play a critical role in the pathogenesis of atherosclerosis. We demonstrated that NAD(P)H oxidase, a major source of ROS production in the vessel wall, is activated in cultured VSMC in response to angiotensin II, and that the combination of both azelnidipine and olmesartan synergistically attenuates angiotensin II-induced NAD(P)H oxidase activation. The detailed mechanism of the azelnidipine-mediated attenuation of oxidative stress remains to be further investigated. However, the blood pressure and cholesterol-independent attenuation of oxidative stress by azelnidipine, at least partly caused by the inhibition of AT₁ receptor-mediated effects, demonstrates a novel anti-atherosclerotic mechanism that may contribute to the beneficial effects of these agents beyond the lowering of blood pressure and the plasma cholesterol level, especially together with an ARB. The combination of lower doses of two drugs is expected to reduce the side-effects and expand the beneficial effects by interaction between both drugs. Our findings provide further insights into the pathogenesis of hypertension and atherosclerosis and may initiate rational and new therapeutic concepts.

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