

Clark

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 09-008201- -00004-0000

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P-2089

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIVISION DE NUEVAS CREACIONES
E. S. D.

Re: Expediente No. **09.008.201**
Solicitud de patente a nombre de **KYORIN PHARMACEUTICAL CO., LTD**

Yo, DILIA MARIA RODRIGUEZ D'ALEMAN, actuando como apoderada e identificada como aparece al pie de mi firma, domiciliada en la carrera 11 A No. 86-53, piso 6, de la sociedad **KYORIN PHARMACEUTICAL CO., LTD** domiciliada en Chiyoda-ku, Tpkyo Japón, solicitante de la patente de la referencia, en respuesta al oficio No. 12525 notificado por fijación en lista el 18 de julio de 2012, me permito manifestar:

1. Con fundamento en la facultad que confiere el artículo 34 de la Decisión 486 al solicitante para modificar la solicitud de patente en cualquier momento del trámite, presento junto con este memorial nuevo capítulo reivindicatorio que consta de 7 cláusulas dirigidas al derivado amino fosfato representado por la fórmula general (Ia).

En este sentido, el señor examinador podrá notar que en el capítulo reivindicatorio se realizaron las siguientes modificaciones:

La reivindicación original 1 se canceló.

La reivindicación original 2 se modifica en forma independiente como la reivindicación de la nueva redacción.

Las reivindicaciones originales 3 y 4 se modifican según la nueva redacción a las reivindicaciones 2 y 3.

En cuando a la reivindicación 3 de nueva redacción, el término "ácido fosfónico" en la reivindicación original 4 se modifica al término "ácido fosfórico".

En este sentido, respetuosamente manifestamos que un experto en la material puede dar cuenta que los compuestos de la reivindicación 3 de la nueva redacción son ácido **fosfórico** y no ácido fosfónico, con base en la estructura y los datos de NMR, MS y el análisis elemental, mostrado en los Ejemplos 40, 41, 42, 43 y 50 consignados en la memoria descriptiva.

Así las cosas, es claro que el término “ácido fosfónico” incluido en los nombres de los compuestos de la reivindicación original 4 son un error de digitación, y estas modificaciones no introducen nueva materia a la solicitud como fue originalmente presentada.

Además, se suprimió una duplicación del monoéster del ácido ((R)-2-amino-5-[2-cloro-4-(3-trifluorometilfeniltio) fenil]-2-metilpentilfosfónico) en la reivindicación original 4.

La reivindicación original 5 se modifica en forma independiente según la reivindicación 4 de la nueva redacción, así como también se corrigió el error de digitación.

Además, en la reivindicación 4 de la nueva redacción, los grupos funcionales de los compuestos como materias primas se limitan según se describe en el nuevo capítulo reivindicatorio.

La nueva reivindicación 5 cubre la materia objeto consignada en los Ejemplos 40 y 41 de la memoria descriptiva. Una persona versada en el arte entenderá fácilmente que los compuestos de los Ejemplos 40 y 41 son isómeros (+) por sus valores medidos de rotación óptica.

Las reivindicaciones originales 6 y 7 corresponden a las reivindicaciones 6 y 7 de la nueva redacción del pliego reivindicatorio presentado con este escrito.

Adicionalmente, el señor examinador podrá notar que en el capítulo descriptivo se realizaron las siguientes modificaciones:

Se corrigieron las páginas 11 y 79 a 89 donde en los nombres de los compuestos de los Ejemplos 40 a 51, se reemplazó "ácido fosfónico" por "ácido fosfórico". Los que expertos en la técnica pueden darse cuenta de que los compuestos de los Ejemplos 40 a 51 son ácido fosfórico y no ácido fosfónico, basándose en los datos de RMN, MS y análisis elemental.

Sobre este particular, nos permitimos manifestar que las modificaciones anteriormente mencionadas no amplían el alcance original de la invención y se dan con el ánimo de subsanar las objeciones de novedad, establecidas por el señor examinador en su concepto técnico del oficio No. 12525.

Teniendo en cuenta el concepto técnico emitido por el Señor Superintendente Delegado para la propiedad intelectual GianCarlo Marcenaro Jiménez a la doctora Ana Maria Castro Wey, que da alcance a las comunicaciones No. 09-001524 y 09-012566, no se presenta tasa adicional por reivindicaciones adicionales, toda vez que la solicitud solo tiene ahora 7 cláusulas.

2. Claridad

En relación con las objeciones planteadas por el señor examinador, atentamente nos permitimos decir que con el nuevo capítulo reivindicatorio se superan dichas objeciones.

En cuanto a las objeciones relacionadas con el alcance de la solicitud representado por la fórmula general, amablemente nos permitimos decir que con el nuevo pliego reivindicatorio, el compuesto de la presente invención es limitado. Prácticamente, los compuestos representados por la fórmula general (1a) se describen en los ejemplos de la especificación y un experto en la materia se dará cuenta de la presente invención a partir de la especificación.

En consecuencia, el alcance representado por la fórmula general (1a) es claro y sustentado por debidamente por la descripción y solicitamos amablemente que las objeciones sean retiradas.

Aunado a lo anterior, el compuesto representado por la fórmula general (1a) de la nueva reivindicación 1, en donde R^5 es un grupo etilo no se describe en los ejemplos de la descripción. Sin embargo, los ejemplos de la memoria descriptiva divulgan el compuesto representado por la fórmula general (1a) en la que R^5 es un grupo metilo y el compuesto representado por la fórmula general (1a) en la que R^5 es un grupo propilo. Por lo tanto, una persona experta fácilmente comprenderá que el compuesto representado por la fórmula general (1a) en donde R^5 es un grupo etilo también se incluye en el alcance de la presente invención.

3. Novedad

El señor examinador argumenta que la solicitud en estudio carecería de novedad en vista de lo reportado en el documento D1 (US20060160771).

Sobre este particular, es deseo del solicitante dirigir la atención del señor examinador al artículo 16 de la Decisión 486 donde se establece que: “Una invención se considera nueva cuando no está comprendida en el estado de la técnica. El estado de la técnica comprenderá todo lo que haya sido accesible al público por una descripción escrita u oral, utilización, comercialización o cualquier otro medio antes de la fecha de presentación de la solicitud de patente o, en su caso, de la prioridad reconocida...” (El resaltado es nuestro).

Si de acuerdo con el espíritu de la norma legal indicada, la invención no se considera como nueva si está “comprendida” en el estado de la técnica, dicha norma se está refiriendo, naturalmente, a la totalidad del invento y no a alguno o a algunos de los componentes que hacen parte de la nueva solicitud.

En efecto, para que la invención pierda su calidad de novedosa, es claro que ella, TODA ELLA, ha de estar contenida en el “conjunto de conocimientos existentes que son aplicables al proceso industrial” como define el estado de la técnica el

Tribunal de justicia del Acuerdo de Cartagena (Proceso No. 6-IP-89, sentencia de 31 de octubre de 1989, publicada en Jurisprudencia del Tribunal de Justicia del Acuerdo de Cartagena, Tomo 11, BID-INTAL, Buenos Aires, 1994, página 60).

Esto se conoce como NOVEDAD ABSOLUTA, de conformidad con lo cual para desvirtuar el requisito de novedad de una invención será necesario que lo reivindicado por la invención bajo estudio corresponda de forma total con lo revelado en un documento o anterioridad señalada, conclusión a la que se llega después de un análisis exhaustivo mediante una comparación entre el capítulo reivindicatorio de la invención en estudio y la divulgación citada por ese despacho como anterioridad.

Ahora bien, si un determinado elemento, condición o circunstancia de la nueva creación ya hace parte del estado de la técnica no quiere ello decir, desde luego, que la invención deba ser rechazada por carecer del requisito de novedad. No sin razón ha dicho el mencionado Tribunal: “hay que advertir que toda creación es en cierta forma, producto del estado de la técnica existente, en el sentido de que el inventor ha de tener en cuenta un conjunto de conocimientos técnicos que le permitan crear nuevos procedimientos aplicables a la industria” (Ibidem, páginas 53 y 54) (El subrayado es nuestro).

Por lo tanto y teniendo en cuenta lo anteriormente expuesto, como mínimo la novedad debe contemplar los siguientes aspectos:

- Que la invención en estudio esté contenida de forma TOTAL y ABSOLUTA en el documento que se menciona como anterioridad; y

- Que la invención en cuestión puede partir de conocimientos conocidos, caso en el cual habrá que hacer la comparación no de lo conocido sino de lo inédito y novedoso, teniendo que analizarse el valor agregado que aporta la invención con sus nuevas características al campo técnico al cual pertenece la solicitud en estudio.

Aplicando lo anterior al caso concreto, para que las razones que aduce ese despacho para desvirtuar la novedad fuesen ciertas, sería forzoso que se demostrara que la invención de mi mandante está comprendida en el estado de la técnica de forma TOTAL, lo cual no sucede toda vez que las características mostradas en el nuevo capítulo reivindicatorio modificado, especialmente en la reivindicación 1 no son enseñadas ni sugeridas por la anterioridad D1.

En efecto, el documento reportado por el señor examinador no divulga una un patrón específico de sustitución como el ahora cubierto por la nueva reivindicación 1. Por consiguiente, solicitamos amablemente sean retiradas las objeciones de novedad.

4. Nivel inventivo

En relación con este requisito de patentabilidad, atentamente nos permitimos decir que el documento US20060160771 (D1) citado por el examinador colombiano pertenece a la misma familia de patentes del documento EP1602660 (D1) citado por el examinador de la EPO.

En este sentido, atentamente nos permitimos decir que los receptores S1P tienen varios tipos subsidiarios que incluyen receptores S1P₁ y S1P₃. Los receptores S1P₁ son importantes para expresar actividad de inmuno-regulación. En contraste a esto, los receptores S1P₃ pueden conducir a reacciones indeseables tal como hiperpermeabilidad vascular e hiper actividad respiratoria (véase por favor el anexo 1 o el anexo 2).

En ese orden de ideas, es importante hacer énfasis a que es esperado que, el compuesto que tenga una actividad agonista más alta contra los receptores S1P₁ y una selectividad más alta pudiera tener actividad farmacéutica mejorada sin que se presenten efectos colaterales.

Aunque la reivindicación 1 de D1 muestra una fórmula de Markush amplia, todos los ejemplos de acuerdo con las enseñanzas de esta anterioridad tienen un R¹ igual a BnO y un R³ igual a un alquilo. Los ejemplos 239 y 240 de D1 se compararon con el compuesto del Ejemplo 41 en la siguiente tabla:

	Actividad Agonista SIP ₁	Actividad Agonista SIP ₂
Compuesto de la presente invención Ejemplo 41 	+++	-
EP1602660 Ejemplo 239  (+)	+++	+++
EP1602660 Ejemplo 240  (-)	++	+

Evaluación de actividad : $EC_{50} > 10 \mu M = "-"$
 $1 \mu M > EC_{50} > 0.1 \mu M = "+"$
 $0.1 \mu M > EC_{50} > 0.01 \mu M = "++"$
 $0.01 \mu M > EC_{50} = "+++"$

Como se puede observar en la tabla anterior, el compuesto de la presente invención tiene una actividad agonista alta de espingosina 1-fosfato (S1P) y una

alta selectividad de subtipo. Por lo tanto, se espera que muestre una actividad farmacéutica superior sin que conlleve a efectos colaterales.

Aunado a lo anterior, es importante señalar que debido a que D1 no enseña ni sugiere un patrón de sustitución específico como el mostrado en la presente invención en la nueva reivindicación 1 o que las anteriores ventajas pudieran conseguirse con dichos compuestos. Por tal razón nos permitimos manifestar que la presente invención cumple también con el requisito de Nivel Inventivo.

Habiendo atendido cabalmente las objeciones presentadas por ese despacho, atentamente solicitamos se conceda el privilegio de patente de invención a la solicitud en estudio o si el señor examinador considera que subsisten razones para objetar la presente invención, rogamos expedir otro examen de fondo con los debidos motivos y razones que sustenten su objeción con el fin de que nuestro mandante tenga la posibilidad de exponer sus argumentos con base en la interpretación de los artículos 45 de la Decisión 486 -el cual otorga la facultad al examinador para emitir los exámenes de fondo-, 35 y 59 del Código Contencioso Administrativo que señala que: “habiéndose dado oportunidad a los interesados para expresar sus opiniones, y con base en las pruebas e informes disponibles, se tomará la decisión que será motivada al menos en forma sumaria si afecta a particulares” y “(..) deberá proferirse la decisión definitiva. Esta se motivará en sus aspectos de hecho y de derecho, y en los de conveniencia si es el caso”, respectivamente (El subrayado es nuestro).

En consecuencia, solicitamos a ese despacho pronunciarse en relación con todos los argumentos presentados en este memorial dando estricta aplicación al principio del debido proceso consagrado en el artículo 29 de la Constitución Nacional

Señor Superintendente,



DILIA MARIA RODRIGUEZ D'ALEMAN

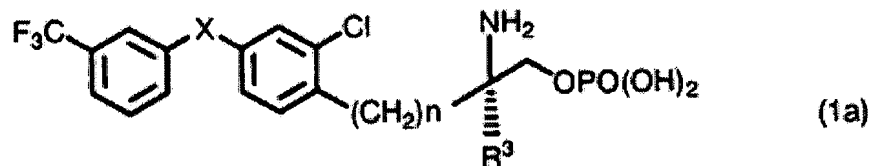
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REIVINDICACIONES

1. Un derivado amino fosfato representado por la formula general (1a),



5

caracterizado porque R^3 representa un grupo alquilo de cadena lineal que tiene uno a tres átomos de carbono, X representa un átomo de oxígeno o azufre y n indica 2 o 3, o una sal aceptable farmacéuticamente o hidrato del mismo.

- 10 2. El derivado aminofosfato de acuerdo con la reivindicación 1, caracterizado porque en la fórmula general, R^3 es un grupo metilo, o una sal aceptable farmacéuticamente o un hidrato del mismo.

- 15 3. El derivado aminofosfato de acuerdo con la reivindicación 1, caracterizado porque el compuesto representado por la fórmula general (1a) es,

1)

Monoester del ácido (R)-2-amino-5-[2-cloro-4-(3-trifluorometilfenóxi)fenil]-2-metilpentil fosfórico,

2)

- 20 Monoester del ácido (R)-2-amino-5-[2-cloro-4-(3-trifluorometilfeniltio)fenil]-2-metilpentil fosfórico,

3)

Monoester del ácido (R)-2-amino-4-[2-cloro-4-(3-trifluorometilfenóxi)fenil]-2-metilbutil fosfórico

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4)

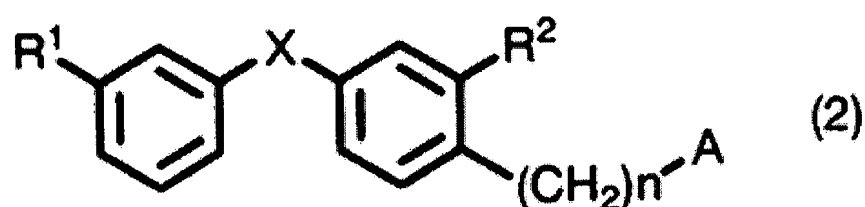
Monoester del ácido (R)-2-amino-4-[2-cloro-4-(3-trifluorometilfeniltio)fenil]-2-metilbutil fosfórico

o

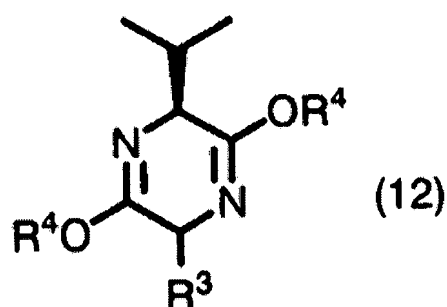
5 5)

Monoester del ácido (R)-2-amino-5-[2-cloro-4-(3-trifluorometilfeniltio)fenil]-2-propilpentil fosfórico, o una sal aceptable farmacéuticamente o hidrato del mismo.

- 10 4. Un derivado aminofosfato que se puede obtener por la etapa que permite que un compuesto representado por la fórmula general (2)



- en la que R¹ representa un grupo trifluorometilo, R² representa un átomo de cloro; A representa un átomo de halógeno, X representa un átomo de oxígeno o un átomo de azufre, y n indica uno 2 o 3], y un compuesto representado por la fórmula general (12)

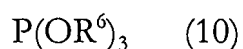


- en el que R³ representa un grupo alquilo de cadena lineal que tiene 1 a 3 átomos de carbono y R⁴ representa un grupo alquilo que tiene 1 a 6 átomos de carbono
20 reaccione en presencia de una base,

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una etapa que somete el producto resultante a descomposición ácida, protegiendo un átomo de nitrógeno con un grupo terbutóxicarbonilo, y luego reduciendo el producto,

una etapa en la que se hace reaccionar el producto de reducción con un
5 compuesto representado por la fórmula general (10),



en la que R^6 representa un grupo alquilo que tiene 1 a 6 átomos de carbono o un grupo bencilo, y

una etapa en la que el producto resultante obtenido por las etapas previas
10 se somete a descomposición ácida o a tratamiento con halogenosilano,
o una sal aceptable farmacéuticamente o hidrato del mismos.

5. Un derivado aminofosfato representado por

Monoester del ácido (+)-2-amino-5-[2-cloro-4-(3-trifluorometilfenóxi)fenil]

15 -2-metilpentil fosfórico,

Monoester del ácido (+)-2-amino-5-[2-cloro-4-(3-trifluorometilfeniltio)fenil]-2-metilpentil fosfórico, o una sal aceptable farmacéuticamente o hidrato del mismo.

20 6. Un modulador del receptor S1P que contiene el derivado aminofosfato, o una sal aceptable farmacéuticamente o hidrato del mismo, de acuerdo con cualquiera de las reivindicaciones 1 a 5 como un ingrediente activo.

7. Un fármaco que contiene el derivado aminofosfato, o una sal
25 aceptable farmacéuticamente o hidrato del mismo, de acuerdo con cualquiera de las reivindicaciones 1 a 5 como un ingrediente activo.

[0017]

4) El derivado amino fosfato de acuerdo con 1), donde el compuesto representado por la fórmula general (1) es,

5 Mono-éster del ácido (R)-2-amino-5-[2-cloro-4-(3-trifluorometilfenóxi)fenilo]-2-metilpentil fosfórico,

Mono-éster del ácido (R)-2-amino-5-[2-cloro-4-(3-trifluorometilfeniltio)fenilo]-2-metilpentil fosfórico,

Mono-éster del ácido (R)-2-amino-4-[2-cloro-4-(3-trifluorometilfenóxi)fenilo]-2-metilbutil fosfórico,

10 Mono-éster del ácido (R)-2-amino-4-[2-cloro-4-(3-trifluorometilfeniltio)fenilo]-2-metilbutil fosfórico,

Mono-éster del ácido (R)-2-amino-5-[2-cloro-4-(3-etilfeniltio)fenilo]-2-metilpentil fosfórico,

15 Mono-éster del ácido (R)-2-amino-5-[2-cloro-4-(3-trifluorometilfeniltio)fenilo]-2-metilpentil fosfórico, o

Mono-éster del ácido (R)-2-amino-5-[2-cloro-4-(3-trifluorometilfeniltio)fenilo]-2-propilpentil fosfórico, o una sal aceptable farmacéuticamente o un hidrato de éste.

[0018]

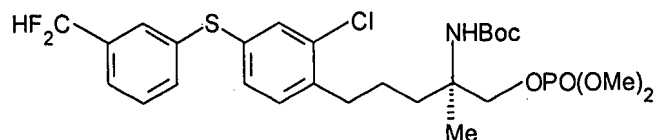
20 5) El derivado amino fosfato de acuerdo con 1) que se produce mediante una etapa que permite la formación de un compuesto representado por la fórmula general (2),

(R)-2-t-butóxicarbonilamino-5-[2-cloro-4-(3-difluorometilfeniltio)fenil]-1-dimetóxfosforiloxi-2-metilpentano.

[0274]

[Fórmula química 80]

5



[0275]

El compuesto del Ejemplo 27 se hizo reaccionar de la misma manera que en el Ejemplo 28 para obtener el producto deseado como un aceite incoloro.

10 $^1\text{H-NMR}$ (CDCl_3 , 400 MHz): δ 1.24 (3H, s), 1.42 (9H, s), 1.51 – 1.67 Hz (3H, m), 1.83 – 1.99 (1H, m), 2.73 (2H, t, $J = 7.6$ Hz), 3.77 (6H, d, $J = 11$ Hz), 4.00 (1H, dd, $J = 9.8, 4.9$ Hz), 4.17 (1H, dd, $J = 9.8, 4.9$ Hz), 4.54 (1H, br s), 6.61 (1H, t, $J = 56$ Hz), 7.19 (2H, d, $J = 1.2$ Hz), 7.34 – 7.42 (4H, m), 7.45 (1H, s).

ESIMS (+): 594 $[\text{M} + \text{H}]^+$.

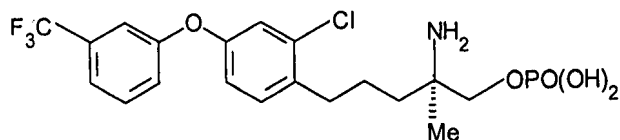
15 [0276]

<Ejemplo 40>

Mono-éster del ácido (R)-2-amino-5-[2-cloro-4-(3-trifluorometilfenóxi)fenil]-2-metilpentil fosfórico.

[0277]

20 [Fórmula química 81]



MA

[0278]

Se agregó yodotrimetilsilano (478 µL) gota a gota bajo enfriamiento con hielo y en atmósfera de argón a una solución del compuesto del Ejemplo 28 (533 mg) en acetonitrilo (8 mL), la solución resultante se agitó bajo enfriamiento con hielo durante 30 minutos. La solución se cargó con agua (100 mL), y luego se agitó bajo enfriamiento con hielo durante otros 30 minutos. Los cristales precipitados luego se filtraron, y se lavaron completamente con agua y acetato de etilo, y luego se secaron para obtener el producto deseado (310 mg) como un polvo blanco.

¹H-NMR (DMSO-d₆-dTFA, 400 MHz): δ 1.16 (3H, s), 1.51- 1.71 (4H, m), 2.66 (2H, t, J = 7.9 Hz), 3.78 (1H, dd, J = 11.0, 4.9 Hz), 3.83 (1H, dd, J = 11.0, 4.9 Hz), 6.98 (1H, d, J = 7.9 Hz), 7.13 (1H, br s), 7.26 (1H, d, J = 7.9 Hz), 7.27 (1H, br s), 7.35 (1H, d, J = 7.9 Hz), 7.44 (1H, d, J = 7.9 Hz), 7.57 (1H, t, J = 7.9 Hz).

FABMS (+): 468 [M + H]⁺.

Análisis elemental: Medido: C 48.28 %, H 4.62%, N 2.86%, calculado para C₁₉H₂₂ClF₃NO₅P.1/4 H₂O : C 48.32%, H 4.80%, N 2.97%

Rotación óptica: [α]_D²⁵ + 6.62 (c 0.55, DMSO-1%TFA)

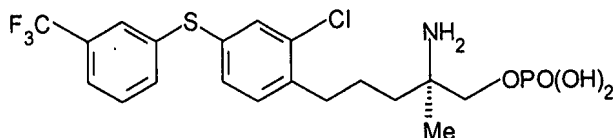
[0279]

<Ejemplo 41>

Mono-éster del ácido (R)-2-amino-5-[2-cloro-4-(3-trifluorometilfeniltio)fenil]-2-metilpentil fosfórico.

[0280]

[Fórmula química 82]



[0281]

El compuesto del Ejemplo 29 se hizo reaccionar de la misma manera que en el Ejemplo 40 para obtener el producto deseado como un polvo blanco.

¹H-NMR (DMSO-d₆-dTFA, 400 MHz): δ 1.15 (3H, s), 1.49- 1.68 (4H, m), 2.67 (2H, t, J = 7.4 Hz), 3.76 (1H, dd, J = 11.0, 4.9 Hz), 3.81 (1H, dd, J = 11.0, 4.9 Hz), 7.32 (1H, dd, J = 7.9, 2.4 Hz) 7.38 (1H, d, J = 7.9 Hz), 7.46 (1H, d, J= 2.4 Hz), 7.53 (1H, d, J= 7.9 Hz), 7.56 (1H, d, J = 7.9 Hz), 7.60 (1H, br s), 7.62 (1H, t, J =7.9 Hz)

FABMS (+): 484 [M + H]⁺.

Análisis elemental: Medido: C 46.85%, H 4.35%, N 2.66%, calculado para C₁₉H₂₂ClF₃NO₄PS : C 47.16%, H 4.58%, N 2.89%

Rotación óptica: [α]_D²⁵ + 7.27 (c 0.55, DMSO-1%TFA)

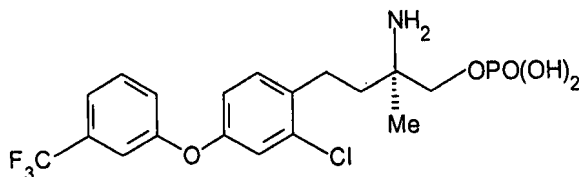
[0282]

<Ejemplo 42>

Mono-éster del ácido (R)-2-amino-4-[2-cloro-4-(3-trifluorometilfenóxi)fenil]-2-metilbutil fosfórico.

[0283]

[Fórmula química 83]



[0284]

El compuesto del Ejemplo 30 se hizo reaccionar de la misma manera que en el Ejemplo 40 para obtener el producto deseado como un polvo blanco.

¹H-NMR (DMSO-d₆-dTFA, 400 MHz): δ 1.30 (3H, s), 1.70- 1.90 (2H, m),
5 2.72 (2H, t, J = 8.6 Hz), 3.88 (1H, dd, J = 11.0, 5.5 Hz), 3.94 (1H, dd, J = 11.0, 5.5 Hz), 7.04 (1H, dd, J = 7.9, 2.4 Hz), 7.20 (1H, d, J = 2.4 Hz), 7.30 (1H, dd, J = 7.9, 2.4 Hz), 7.34 (1H, br s), 7.39 (1H, d, J = 7.9 Hz), 7.51 (1H, d, J = 7.9 Hz), 7.62 (1H, d, J = 7.9 Hz).
FABMS (+): 454 [M + H]⁺.

Análisis elemental: Medido: C 47.83 %, H 4.33%, N 3.02%, calculado para
10 C₁₈H₂₀ClF₃NO₅P.1/2 H₂O : C 46.72%, H 4.57%, N 3.03%

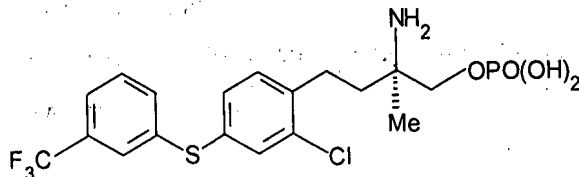
[0285]

<Ejemplo 43>

Mono-éster del ácido (R)-2-amino-4-[2-cloro-4-(3-trifluorometilfeniltio)fenil]-
2-metilbutil fosfórico.

15 **[0286]**

[Fórmula química 84]



20 **[0287]**

El compuesto del Ejemplo 31 se hizo reaccionar de la misma manera que en el Ejemplo 40 para obtener el producto deseado como un polvo blanco.

$^1\text{H-NMR}$ (DMSO- d_6 -dTFA, 400 MHz): δ 1.28 (3H, s), 1.70- 1.90 (2H, m), 2.73 (2H, t, J = 8.6 Hz), 3.87 (1H, dd, J = 11.0, 4.9 Hz), 3.94 (1H, dd, J = 11.0, 4.9 Hz), 7.33(1H, d, J = 7.9 Hz), 7.39 (1H, d, J = 7.9 Hz), 7.46 (1H, br s), 7.50-7.58 (3H, m).

FABMS (+): 470 $[\text{M} + \text{H}]^+$.

- 5 Análisis elemental: Medido: C 45.32 %, H 4.09%, N 2.90%, calculado para $\text{C}_{18}\text{H}_{20}\text{ClF}_3\text{NO}_4\text{PS} \cdot 1/4 \text{H}_2\text{O}$: C 45.57%, H 4.36%, N 2.95%

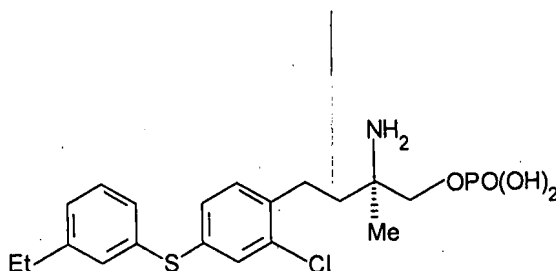
[0288]

<Ejemplo 44>

- 10 Mono-éster del ácido (R)-2-amino-4-[2-cloro-4-(3-etilfeniltio)fenil]-2-metilbutil fosfórico.

[0289]

[Fórmula química 85]



15

[0290]

El compuesto del Ejemplo 32 se hizo reaccionar de la misma manera que en el Ejemplo 40 para obtener el producto deseado como un polvo blanco.

- 20 $^1\text{H-NMR}$ (DMSO- d_6 -dTFA, 400 MHz): δ 1.37 (3H, t, J = 7.3 Hz), 1.28 (3H, s), 1.72-1.85 (2H, m), 2.58 (2H, t, J = 7.3 Hz), 2.67-2.72 (2H, m), 3.86 (1H, dd, J = 11.0, 4.9 Hz), 3.93 (1H, dd, J = 11.0, 4.9 Hz), 7.17-7.21 (3H, m), 7.25-7.26 (2H, m), 7.31 (2H, t, J = 7.3 Hz).

ESIMS(+): 430 $[\text{M} + \text{H}]^+$.

Análisis Elemental: Medido: C 52.56%, H 5.79%, N 3.21%, Calculado para $C_{19}H_{25}ClNO_4PS$.

$1/4H_2O$: C 52.53%, H 5.79%, N 3.21%.

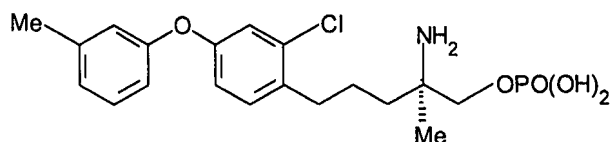
[0291]

<Ejemplo 45>

- 5 Mono-éster del ácido (R)-2-amino-5-[2-cloro-4-(3-metilfenóxi)fenil]-2-metilpentil fosfórico.

[0292]

[Fórmula química 86]



10

[0293]

El compuesto del Ejemplo 33 se hizo reaccionar de la misma manera que en el Ejemplo 40 para obtener el producto deseado como un polvo blanco.

- 15 ^1H -NMR (DMSO- d_6 -dTFA, 400 MHz): δ 1.17 (3H, s), 1.53 – 1.71 (4H, m), 2.28 (3H, s), 2.59 – 2.69 (2H, m), 3.78 – 3.83 (2H, m), 6.80 (1H, dd, J = 7.9, 2.4 Hz), 6.84 (1H, s), 6.92 (1H, dd, J = 7.9, 2.4 Hz), 6.98 (1H, d, J = 7.9 Hz), 7.02 (1H, d, J = 2.4 Hz), 7.27 (1H, t, J = 7.9 Hz), 7.33 (1H, d, J = 7.9 Hz).

HRESIMS(+): 414.12313 (calculado para $C_{19}H_{26}ClNO_5P$ 414.12371).

- 20 Análisis Elemental: Medido: C 54.87%, H 5.89, N 3.27%, Calculado para $C_{19}H_{25}ClNO_5P$: C 55.14%, H 6.09 %, N 3.38%.

Rotación óptica: $[\alpha]_D^{25} + 7.93$ (c 1.20, DMSO-1%TFA)

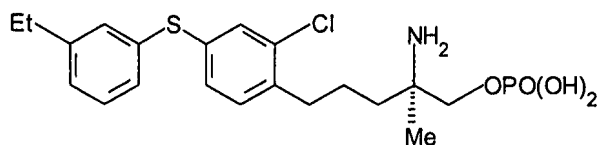
[0294]

<Ejemplo 46>

Mono-éster del ácido (R)-2-amino-5-[2-cloro-4-(3-etilfeniltio)fenil]-2-metilpentil fosfórico.

5 [0295]

[Fórmula química 87]



[0296]

- 10 El compuesto del Ejemplo 34 se hizo reaccionar de la misma manera que en el Ejemplo 40 para obtener el producto deseado como un polvo blanco.

$^1\text{H-NMR}$ (DMSO- d_6 -dTFA, 400 MHz): δ 1.41 (3H, t, $J = 7.3$ Hz), 1.16 (3H, s), 1.51-1.69 (4H, m), 2.58 (2H, t, $J = 7.3$ Hz), 2.63-2.80 (2H, m), 3.78 (1H, dd, $J = 11.0, 4.9$ Hz), 3.81 (1H, dd, $J = 11.0, 4.9$ Hz), 7.16-7.33 (7H, m).

- 15 ESIMS(+): 444 $[\text{M}+\text{H}]^+$.

Análisis Elemental: Medido: C 53.87%, H 6.04%, N 3.11%, Calculado para $\text{C}_{20}\text{H}_{27}\text{ClN}_2\text{O}_4\text{PS}$: C 54.11%, H 6.13%, N 3.16%.

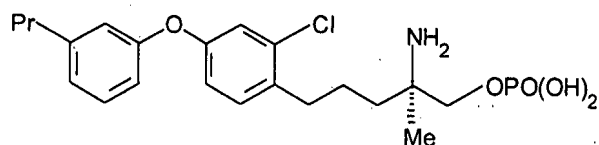
[0297]

<Ejemplo 47>

- 20 Mono-éster del ácido (R)-2-amino-5-[2-cloro-4-(3-propilfenóxi)fenil]-2-metilpentil fosfórico.

[0298]

[Fórmula química 88]



5 [0299]

El compuesto del Ejemplo 35 se hizo reaccionar de la misma manera que en el Ejemplo 40 para obtener el producto deseado como un polvo amarillo pálido.

$^1\text{H-NMR}$ (DMSO- d_6 -dTFA, 400 MHz) : δ 0.86 (3H, t, J = 7.3 Hz), 1.17 (3H, s), 1.51 – 1.67 (6H, m), 2.53 (2H, t, J = 7.3 Hz), 2.64 (2H, t, J = 7.3 Hz), 3.78 (1H, dd, J = 11.0, 4.9 Hz), 3.83 (1H, dd, J = 11.0, 4.9 Hz), 6.81 (1H, dd, J = 7.9, 1.8 Hz), 6.86 (1H, t, J = 1.8 Hz), 6.91 (1H, dd, J = 8.6, 2.4 Hz), 7.00 (1H, d, J = 7.9 Hz), 7.02 (1H, d, J = 2.4 Hz), 7.29 (1H, t, J = 7.9 Hz), 7.33 (1H, t, J = 8.6 Hz).

ESIMS(+): 442 [M + H] $^+$

Análisis Elemental: Medido: C 56.80%, H 6.40%, N 3.04%, Calculado para $\text{C}_{21}\text{H}_{29}\text{ClNO}_5\text{P}$:
15 C 57.08%, H 6.61 %, N 3.17%.

Rotación óptica: $[\alpha]_D^{25} + 8.33$ (c 0.90, DMSO-1%TFA)

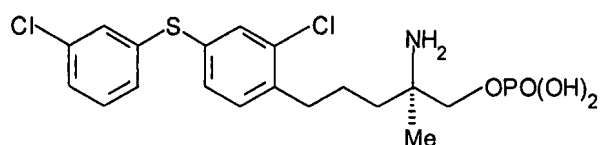
[0300]

<Ejemplo 48>

Mono-éster del ácido (R)-2-amino-5-[2-cloro-4-(3-clorofeniltio)fenil]-2-metilpentil
20 fosfórico.

[0301]

[Fórmula química 89]



[0302]

- 5 El compuesto del Ejemplo 36 se hizo reaccionar de la misma manera que en el Ejemplo 40 para obtener el producto deseado como un polvo blanco.

$^1\text{H-NMR}$ (DMSO- d_6 -TFA, 400 MHz): δ 1.17 (3H, s), 1.53 – 1.71 (4H, m), 2.68 (2H, t, J = 6.7Hz), 3.78 (1H, dd, J = 11, 4.9 Hz), 3.83 (1H, dd, J = 11, 4.9 Hz), 7.24 (1H, dt, J = 7.3, 1.8 Hz), 7.28 -7.40 (5H, m), 7.43 (1H, d, J = 1.8 Hz).

- 10 ESIMS(+): 450 $[\text{M}+\text{H}]^+$.

Análisis Elemental: Medido: C 47.64%, H 4.72%, N 3.07%, Calculado para $\text{C}_{18}\text{H}_{22}\text{Cl}_2\text{N}_0\text{P}_1\text{S}_1$: C 48.01%, H 4.92%, N 3.11%.

Rotación óptica: $[\alpha]_{\text{D}}^{25} + 8.12$ (c 0.55, DMSO-1%TFA)

[0303]

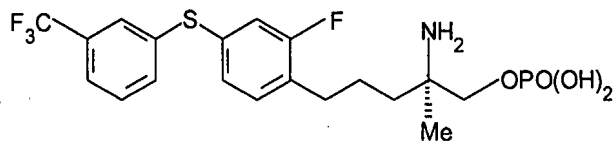
- 15 <Ejemplo 49>

Mono-éster del ácido (R)-2-amino-5-[2-fluoro-4-(3-trifluorometilfeniltio)fenil]-2-metilpentil fosfórico.

[0304]

[Fórmula química 90]

20



[0305]

El compuesto del Ejemplo 37 se hizo reaccionar de la misma manera que en el Ejemplo 40 para obtener el producto deseado como un polvo blanco.

¹H-NMR (DMSO-d₆-dTFA, 400 MHz) : δ 1.16 (3H, s), 1.54 – 1.66 (4H, m) , 2.60 (2H, br s),
5 3.77 (1H, dd, J = 11.0, 4.9 Hz), 3.82 (1H, dd, J = 11, 4.9 Hz), 7.19 (1H, dd, J = 7.9, 1.8 Hz) ,
7.23 (1H, dd, J = 9.8, 1.8 Hz), 7.36 (1H, t, J = 7.9 Hz), 7.52 – 7.66 (4H, m).

ESIMS(+) :468 [M + H]⁺

Análisis Elemental: Medido: C 48.00%, H 4.59, N 2.88%, Calculado para
C₁₉H₂₂F₄N_{0.4}PS.1/2 H₂O: C 47.90%, H 4.87 %, N 2.94%.

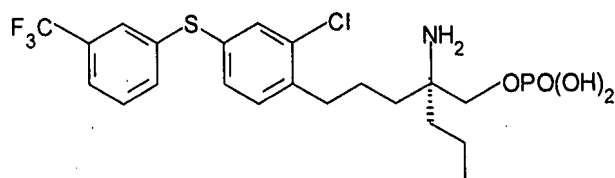
10 **[0306]**

<Ejemplo 50>

Mono-éster del ácido (R)-2-amino-5-[2-cloro-4-(3-trifluorometilfeniltio)fenil]-2-propilpentil fosfórico.

[0307]

15 [Fórmula química 91]



[0308]

20 El compuesto del Ejemplo 38 se hizo reaccionar de la misma manera que en el Ejemplo 40 para obtener el producto deseado como un polvo blanco.

¹H-NMR (DMSO-d₆-dTFA, 400 MHz) : 0.84 (3H, t, J = 7.3 Hz) , 1.21 (2H, q, J = 7.4 Hz),
1.42 – 1.62 (6H, m), 2.64 -2.71 (2H, m), 3.81 (2H, d, J = 4.9 Hz), 7.34 (1H, dd, J = 7.9, 1.8

Hz), 7.40 (1H, d, J = 7.9 Hz), 7.48 (1H, d, J = 1.8 Hz), 7.53 (1H, d, J = 7.9 Hz), 7.54 – 7.66 (3H, m).

FABMS(+): 512 [M+H]⁺.

Análisis Elemental: Medido: C 47.89%, H 4.94%, N 2.65%, Calculado para

5 C₂₁H₂₆ClF₃N₄PS · H₂O: C 47.65%, H 5.33%, N 2.65%.

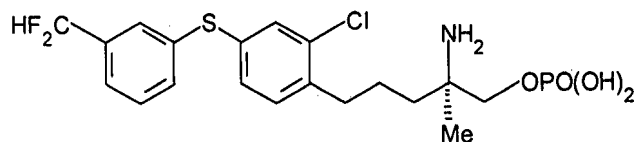
[0309]

<Ejemplo 51>

Mono-éster del ácido (R)-2-amino-5-[2-cloro-4-(3-difluorometilfeniltio)fenil]-2-metilpentil fosfórico.

10 **[0310]**

[Fórmula química 92]



[0311]

15 El compuesto del Ejemplo 39 se hizo reaccionar de la misma manera que en el Ejemplo 40 para obtener el producto deseado como un polvo blanco.

¹H-NMR (DMSO-d₆-dTFA, 400 MHz): δ 1.16 (3H, s), 1.50 – 1.71 (4H, m), 2.62 -2.73 (2H, m), 3.78 (1H, dd, J = 11.0, 4.9 Hz), 3.83 (1H, dd, J = 11.0, 4.9 Hz), 6.98 (1H, dd, J = 56, 1.8 Hz), 7.27 (1H, dd, J = 7.9, 1.8 Hz), 7.37 (1H, d, J = 7.9 Hz), 7.39 (1H, d, J = 1.8 Hz), 7.43 – 7.56 (4H, m).

ESIMS(+): 466 [M + H]⁺

ANEXO 1

Sphingosine-1-Phosphate/Sphingosine Kinase Pathway Is Involved in Mouse Airway Hyperresponsiveness

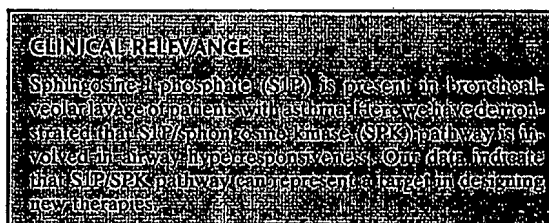
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Dipartimento di Farmacologia Sperimentale, Università di Napoli Federico II; Dipartimento di Medicina Sperimentale, Sezione di Farmacologia L. Donatelli, Seconda Università degli Studi di Napoli; and Dipartimento di Internistica Clinica e Sperimentale, Seconda Università di Napoli, Napoli, Italy

Sphingosine-1-phosphate (S1P) has been shown to regulate numerous and diverse cell functions, including smooth muscle contraction. Here we assessed the role of S1P/Sphingosine kinase (SPK) pathway in the regulation of bronchial tone. Our objective was to determine, using an integrated pharmacologic and molecular approach, (1) the role of S1P as endogenous modulator of the bronchial tone, and (2) the linkage between S1P pathway and bronchial hyperresponsiveness. We evaluated S1P effects on isolated bronchi and whole lungs, harvested from Balb/c mice sensitized to ovalbumin (OVA) versus vehicle-treated mice, by measuring bronchial reactivity and lung resistance. We found that S1P administration on non-sensitized mouse bronchi does not cause any direct effect on bronchial tone, while a significant increase in Ach-induced contraction occurs after S1P challenge. Conversely, in OVA-sensitized mice S1P/SPK pathway triggers airway hyperresponsiveness. Indeed, S1P causes a dose-dependent contraction of isolated bronchi. Similarly, in the whole lung system S1P increased airway resistance only in OVA-sensitized mice. The action on bronchi of S1P is coupled to an enhanced expression of SPK₁ and SPK₂, as well as of S1P₁ and S1P₂ receptors. In these experiments the key role for S1P/SPK in hyperreactivity has been confirmed by pharmacologic modulation of SPKs. S1P/SPK pathway does not seem to play a major role in physiologic conditions, while it may become critical in pathologic conditions. These results open new windows for therapeutic strategies in diseases like asthma.

Keywords: S1P; sphingosine kinase; bronchus; hyperresponsiveness

Sphingosine-1-phosphate (S1P) can act as an extracellular ligand, activating specific G protein-coupled sphingolipid receptors in the plasma membrane (1, 2). Alternatively, S1P produced after activation of specific receptors can act as intracellular messenger and stimulate Ca²⁺ channel on the endoplasmic reticulum (3). Dissection of the relative contribute of intra- or extra-cellular S1P is difficult (4). S1P intracellular levels are regulated via the activation of the enzyme sphingosine kinase (SPK) (5–7). However, the intracellular targets have not been definitively identified. The picture is made even more complicate as S1P, by binding to its receptors, can stimulate sphingosine kinase and thus increase intracellular levels by itself (8, 9). The function of S1P has been intensely investigated over the past years, and it has become clear that S1P is involved in biological functions



including cell growth and survival, differentiation, calcium homeostasis, and in many of the pathways involved in smooth muscle contraction (10–12). The emerging role of S1P in regulating smooth muscle contraction has led to exploration of the possibility that this sphingolipid metabolite could represent a potential therapeutic target. Recent studies have suggested that S1P signaling is involved in hypertension and asthma (13–16). The finding that S1P is an autocrine mediator of activated mast cells further suggests an involvement of S1P in pathophysiology of asthma and/or airway hyperresponsiveness (17, 18). An involvement of S1P in human asthma has been hypothesized on the basis that S1P levels are elevated in the airways of individuals with asthma after segmental allergen challenge (15). In addition, we have recently showed that S1P acts as a chemotactic agent for human eosinophils *in vitro* and *in vivo* (19).

Pathologic changes underlying airway hyperreactivity are characterized, at least in part, by an influx of cells such as lymphocytes and eosinophils that can sustain the ongoing inflammation and provoking airway smooth muscle contraction (20–23). It has been suggested that airway smooth muscle plays a prominent role in orchestrating both the acute inflammatory reaction and the chronic processes supporting airway remodeling (24–26).

The majority of studies published so far has focused on the involvement of S1P in smooth muscle hyperplasia and cytokine production (15). However, it is likely that S1P could contribute to the asthma directly through its effects on smooth muscle contractility. At the present stage the functional role of this signaling pathway in airway smooth muscle has been only partially investigated. In this study we used an integrated pharmacologic and molecular approach to determine (1) the role of S1P as endogenous modulator of the bronchial tone, and (2) the linkage between S1P pathway and bronchial hyperresponsiveness in sensitized mice.

MATERIALS AND METHODS

Drugs

Acetylcholine (Ach), dimethyl sulfoxide (DMSO), pertussis toxin (PTX; from *Bordetella pertussis*), suramine, S1P, and DL-threo-Dihydrosphingosine (DTD) were purchased from Sigma Chemical Co.

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(Milano, Italy). BML-241 was purchased from BIOMOL (Plymouth Meeting, PA).

Tissue Preparation

All studies were performed in accordance with European Union regulations for the handling and use of laboratory animals and were approved by the local committee. Male BALB/c mice (18–20 g; Harlan, Milan, Italy) were housed with a 12-h light/dark cycle and were allowed food and water *ad libitum*. Mice were killed and bronchial tissue was rapidly dissected and cleaned from fat and connective tissue. Rings of 1–2 mm length were cut and placed in organ baths (2.5 ml) filled with oxygenated (95% O₂–5% CO₂) Krebs solution at 37°C and mounted to isometric force transducers (type 7006; Ugo Basile, Comerio, Italy) and connected to a Powerlab 800 (ADInstruments, Chalgrove, UK). The composition of the Krebs solution was as follows (mol/liter): NaCl 0.118, KCl 0.0047, MgCl₂ 0.0012, KH₂PO₄ 0.0012, CaCl₂ 0.0025, NaHCO₃ 0.025, and glucose 0.01. Rings were initially stretched until a resting tension of 0.5 g was reached and allowed to equilibrate for at least 30 min during which tension was adjusted, when necessary, to 0.5 g and bathing solution was periodically changed. In a preliminary study a resting tension of 0.5 g was found to develop the optimal tension to stimulation with contracting agents. In each experiment bronchial rings were previously challenged with acetylcholine (10^{−6} mol/liter) until the responses were reproducible.

Antigen Exposure and Drug Treatment of Mice

Balb/c mice (*n* = 10) were sensitized to ovalbumin (OVA) by subcutaneous injection of 0.4 ml of 10 µg OVA absorbed to 3.3 mg of aluminium hydroxide gel in sterile saline on Days 1 and 8. On Day 21, mice were killed and bronchial tissue was rapidly dissected and cleaned from fat and connective tissue. Isolated bronchi were then used for functional and molecular studies. Vehicle-sensitized mice (*n* = 10), which received OVA-free solutions, acted as a control. Another group (*n* = 10) of OVA-sensitized mice received, 1 h before each OVA challenge, 30 µg of DTD (a sphingosine kinase inhibitor).

Role of S1P in Regulation of Bronchial Tone

To assess the effect of S1P on bronchial tissue, we performed a cumulative concentration–response curve to S1P (10^{−8}–3 × 10^{−3} mol/liter). Next we examined the capacity of S1P to modulate Ach-induced contraction. Briefly we incubated isolated bronchi with S1P at a single concentration of 3 × 10^{−7} mol/liter, inactive by itself. The time course study showed that after 30 min of incubation there was the maximal increase in the concentration–response curve to Ach. To understand the contribute of extracellular and intracellular pathway, we used receptor antagonists such as PTX (1 µg/ml, 2 h), suramine (100 µmol/liter, 1 h), BML-241 (30 µmol/liter, 20 min), and the sphingosine kinase inhibitor, DTD (100 µmol/liter, 1 h). These protocols were used for each group of animals and the optimal dose and incubation time of each inhibitor was previously determined (data not shown).

Isolated Perfused Mouse Lung Preparation

Briefly, lungs were perfused in a nonrecirculating fashion through the pulmonary artery at a constant flow of 1 ml/min, resulting in a pulmonary artery pressure of 2–3 cm H₂O. The perfusion medium used was RPMI 1640 lacking phenol red (37°C). The lungs were ventilated by negative pressure (−3 and −9 cm H₂O) with 90 breaths/min and a tidal volume of ~200 µl. Every 5 min a hyperinflation (−20 cm H₂O) was performed. Artificial thorax chamber pressure was measured with a differential pressure transducer (Validyne DP 45–24; Validyne Engineering, Northridge, CA) and airflow velocity with pneumotachograph tube connected to a differential pressure transducer (Validyne DP 45–15). The lungs respired humidified air. The arterial pressure was continuously monitored by means of a pressure transducer (Isotec Healthdyne Cardiovascular Inc., Philadelphia, PA), which was connected with the cannula ending in the pulmonary artery. All data were transmitted to a computer and analyzed with the Pulmodyn software (Hugo Sachs Elektronik, March Hugstetten, Germany). The data were analyzed through the following formula: $P = V \cdot C^{-1} + R_L \cdot dV \cdot dt^{-1}$, where *P* is chamber pressure, *C* pulmonary compliance, *V* tidal volume, *R_L* airway resistance. The airway resistance value registered was corrected for the resistance of the pneumotachometer and the tracheal cannula of

0.6 cm H₂O · s · ml^{−1}. Lungs harvested from five animals of each group (saline, OVA) were perfused and ventilated for 45 min without any treatment to obtain a baseline state. Subsequently, lungs were challenged with either Ach or S1P. Repetitive dose–response curves of Ach (from 10^{−8} mol/liter to 10^{−5} mol/liter) or S1P (from 10^{−7} mol/liter to 10^{−3} mol/liter) were administered as 50 µl bolus, followed by intervals of 15 min, in which lungs were perfused with buffer only.

Western Blotting

Bronchial tissues were prepared as above reported, then homogenated in Lysis buffer (β-glycerophosphate 50 mmol/liter, orthovanadate sodium 0.1 mmol/liter, Mg Cl₂ 2 mmol/L, EGTA 1 mmol/liter, DTT 1 mmol/liter, PMSF 1 mmol/liter, aprotinin 10 µg/ml, leupeptin 20 µmol/liter, 50 mmol/liter NaF) using a Polytron homogenizer (2 cycles of 10 s at maximum speed). After centrifugation of homogenates at 10,000 rpm for 10 min, equal amounts of the denatured proteins were separated on 10% sodium dodecyl sulfate polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were blocked by incubation in PBS containing 0.1% vol/vol Tween 20 and 5% nonfat dry milk for 2 h, followed by an overnight incubation at 4°C with anti-S1P₁, anti-S1P₂, and anti-SPK₁ or anti-SPK₂.

The filters were washed extensively in PBS containing 0.1% vol/vol Tween 20, before incubation for 2 h with anti-horseradish peroxidase-conjugate secondary antibody. Membranes were then washed and developed using enhanced chemiluminescence substrate (ECL; Amersham Pharmacia Biotech, Piscataway, NJ).

Statistical Analysis

All results are reported as mean ± SEM. To analyze the curve we used a two-way ANOVA followed by Bonferroni post test. A value of *P* < 0.05 was taken as significant.

RESULTS

Effect of S1P on Bronchial Smooth Muscle Tone

Cumulative administration of S1P (1 × 10^{−8}–3 × 10^{−5} mol/liter) on mouse bronchi does not cause any significant change in tone up to 10^{−5} mol/liter, as shown in Figure 1A. Only at the concentration of 3 × 10^{−5} mol/liter S1P produces a weak but not significant constrictor effect. Incubation of bronchial rings with S1P (3 × 10^{−7} mol/liter) before addition of Ach causes a significant increase in Ach-induced contraction (Figure 1B).

Since specific pharmacologic antagonists for S1P receptor subtypes are not commercially available for all receptor subtypes, we incubated tissues with (1) activated PTX (1 µg/ml 2 h), which blocks Gi coupling and S1P₁ receptor activation; (2) suramine (100 µmol/liter, 1 h), which blocks Gs coupling and in turn S1P₂ receptor activation; (3) BML-241, a specific S1P₂ antagonist; and (4) Y-27632, an inhibitor of Rho kinase, since S1P₂ signals through a Rho-dependent pathway (1). Rings were then incubated with S1P (3 × 10^{−7} mol/liter) and a cumulative concentration–response curve to Ach (10^{−8}–3 × 10^{−5} mol/liter) performed. PTX, suramine, and BML-241 do not affect S1P-induced increase in Ach-induced contraction (data not shown). Y-27632 at 10^{−7} mol/liter, a concentration that does not affect Ach-induced contraction, reverses the potentiating effect of S1P on Ach-induced contraction (Figure 1C).

It is well known that S1P can promote, via S1P₂ activation, its own intracellular synthesis through SPK pathway (8). To assess the role of this intracellular pathway on bronchial tone, bronchial preparations were incubated with DTD, a selective inhibitor of sphingosine kinase and an Ach-induced concentration curve performed in presence of either vehicle or S1P (3 × 10^{−6} mol/liter). Although DTD does not affect by itself Ach-induced contraction (data not shown), incubation of bronchi with DTD prevents S1P-induced potentiation of Ach contraction (Figure 1D).

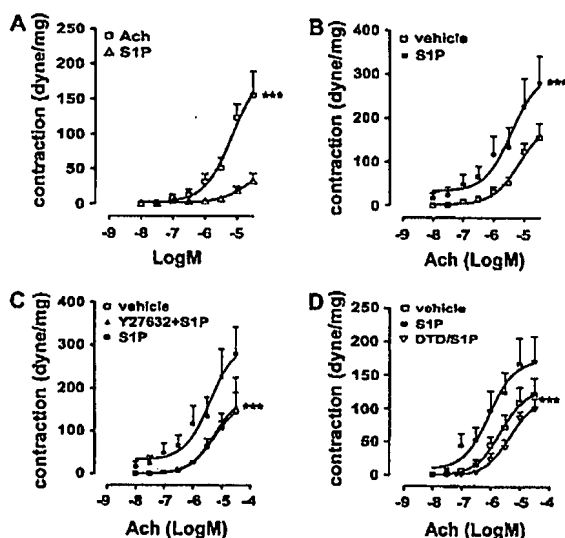


Figure 7. (A) Cumulative administration of S1P (1×10^{-8} – 3×10^{-5} mol/liter) to bronchi isolated from sham-sensitized mice does not cause any significant change in bronchial tone ($***P < 0.001$ versus S1P). (B) Incubation of bronchial preparations with a concentration of S1P (3×10^{-7} mol/liter), inactive by itself, significantly increases ACh-induced contraction ($***P < 0.001$ versus vehicle). (C, D) Pretreatment with the selective Rho kinase inhibitor Y-27632 or the sphingosine kinase inhibitor (DTD) abrogates the enhanced ACh-induced contraction in bronchi previously challenged with S1P ($***P < 0.001$ versus S1P). Results are expressed as the mean $\pm$ SEM.

Role of SPK/S1P in Mouse Airway Hyperresponsiveness

It is well known that OVA sensitization causes a significant increase in airway responsiveness. Indeed bronchi harvested by OVA-sensitized mice show a significant increased response to ACh. Similarly, S1P, inactive on bronchial tone in physiologic conditions, induces a significant and concentration-dependent contraction on bronchi harvested by OVA-sensitized animals (Figure 2A). Next, to verify whether the constriction observed was specific and receptor mediated, we incubated the tissue with PTX, which specifically interferes with S1P₁, or suramine or BML-241, which specifically interfere with S1P₂ signaling. Suramine and BML-241, but not PTX, abrogate the S1P-induced contraction, indicating a major role for S1P₂ in S1P-induced contraction in OVA-sensitized mice (Figure 2B).

To assess the role of intracellular pathway in bronchial hyperresponsiveness, we incubated bronchial preparation with the specific sphingosine kinase inhibitor (DTD). Although DTD does not affect ACh-induced contraction in vehicle-treated mice, it significantly inhibits the hyperresponsiveness to ACh in OVA-sensitized animals (Figure 2D). Furthermore, pretreatment with DTD reverted the enhanced ACh-induced contraction in sensitized bronchi to the contractile value (expressed as dyne/mg) displayed by bronchi harvested by control mice (Figure 2C).

Similarly to what we observed in control mice, preincubation of bronchi with S1P (3×10^{-7} mol/liter) causes a significant and marked increase of ACh-induced contraction (Figure 3A). This finding further implies that S1P signaling becomes critical in pathologic conditions. To gain insight into the molecular mechanisms determining this phenomenon, we performed the same experiments operated on nonsensitized bronchi. While suramine

or BML-241 weakly inhibits the modulation operated by S1P on ACh-induced contraction (Figure 3B), pretreatment of bronchi with DTD reverts this effect (Figure 3C).

To confirm that the changes seen in our experimental conditions in bronchial responsiveness to ACh were coupled to changes in the lung, RL changes to ACh were measured in anesthetized, tracheostomized, and ventilated mice using a whole-body plethysmography. RL measurements in OVA-sensitized animals show a significant airway hyperresponsiveness to ACh (Figure 4A). Similarly, while normal lung did not respond to S1P challenge, OVA-sensitized lung displayed a significant and concentration-dependent response to S1P (Figure 4B) that matched the ACh response (Figure 4A).

Modulation of S1P Receptors and Sphingosine Kinase in Sensitized Animals

Functional study suggests an important role for sphingosine kinase in controlling S1P levels and thus the development of airway hyperresponsiveness. Western blot analysis confirms the up-regulation of sphingolipid pathway, showing an increased expression of S1P₂ and S1P₁ in bronchial tissue harvested from OVA-sensitized animals when compared with sham mice (Figure 5). Conversely, S1P₁ expression was unchanged. SPK1 and SPK2 protein expression is also increased (Figure 5).

The role of SPK in the development of airway hyperresponsiveness was also investigated by administering DTD (30 μ g) to mice before each administration of OVA (Day 1 and Day 7). DTD treatment *in vivo* significantly reduced the increased responsiveness of bronchi *in vitro* to S1P (Figure 6A). Moreover, pretreatment of mice with DTD reduced both the increased responsiveness to ACh (Figure 6B) and the S1P-induced potentiation of ACh-induced contraction (Figure 6C).

DISCUSSION

S1P is normally present in plasma at concentrations ranging from 0.2–0.5 μ mol/liter and its level is under a tight control operated by the balance between sphingosine kinase and sphingosine phosphatases. In addition, S1P can be metabolized by sphingosine lyase to phosphoethanolamine and hexadecenal (11). Growth factors, cytokines, and G protein-coupled receptor agonists can increase rapidly S1P levels upon activation of SPK (27–29). We have recently shown that there is a consistent contribution of this sphingolipid pathway through S1P in cholinergic control of vessel tone (9). It is known that cholinergic system plays an important role in asthma (30–32) and that the levels of S1P are elevated in the airways of individuals with asthma (13). Following this evidence, we have addressed the role of S1P pathway in modulation of bronchial smooth muscle tone in physiologic and pathologic conditions.

Administration of S1P to mouse bronchi did not modify basal bronchial tone. At the highest concentration tested S1P caused a weak, but not significant, contractile effect, suggesting a marginal role, if any, for this lipid mediator in modulation of the bronchial tone in physiologic conditions. Thus, these data fit the concept that S1P pathway is tightly controlled in physiologic conditions. However, exposure of bronchi to S1P caused a marked increase in ACh-induced contraction.

The finding that S1P *per se* was inactive but significantly potentiated the effect of ACh led us to hypothesize that in pathologic conditions a local increase of S1P could be an important trigger of the airway hyperresponsiveness. This hypothesis was assessed by sensitizing mice *in vivo* using a well-known model of allergen sensitization. When bronchi harvested from OVA-sensitized mice were stimulated with S1P, there was a dose-dependent contractile effect. In addition, we confirmed that also in OVA-sensitized bronchi incubation with an inactive dose of

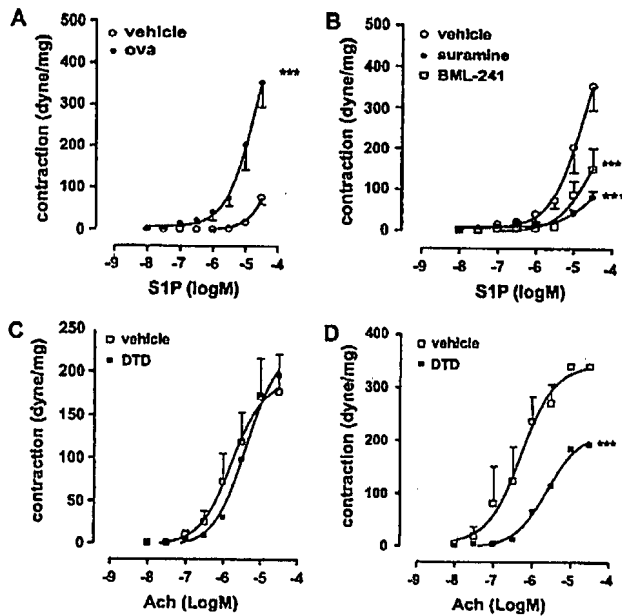


Figure 2. (A) S1P induces a significant and concentration-dependent contraction on bronchi harvested by OVA-sensitized mice (***) $P < 0.001$ versus vehicle). (B) Both suramine or BML-241 inhibit S1P-induced contraction in OVA-sensitized mice (***) $P < 0.001$ versus vehicle). (C, D) DTD does not affect Ach-induced contraction in vehicle (Al(OH)₃)-treated mice, while it inhibits the hyperresponsiveness to Ach in OVA-sensitized animals (D) (***) $P < 0.001$ versus vehicle). Results are expressed as the mean $\pm$ SEM.

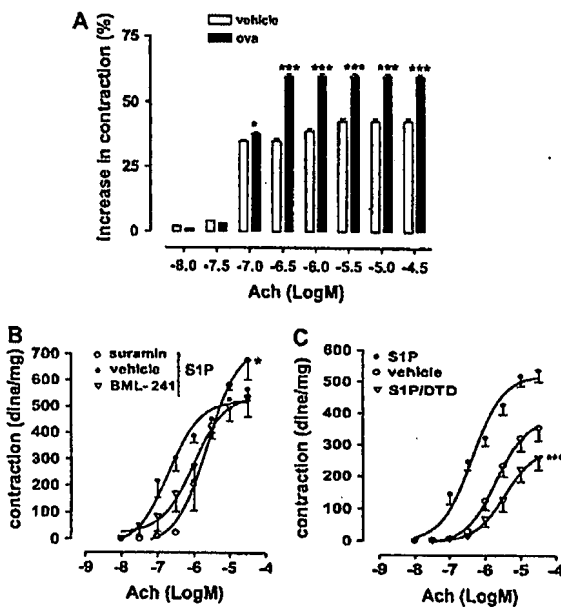


Figure 3. (A) S1P (3×10^{-7} mol/liter) significantly enhances Ach-induced contraction in bronchi harvested either by control (open bars) or by OVA (solid bars)-sensitized mice. Results are expressed as a percentage of increase of Ach-induced contraction (* $P < 0.05$, *** $P < 0.001$ versus vehicle). (B) Suramine, but not BML-241, causes a weak inhibition of S1P-induced enhancement of Ach contraction (* $P < 0.05$ versus vehicle). (C) DTD abrogates S1P-induced potentiation of Ach contraction (***) $P < 0.001$ versus S1P). Results are expressed as the mean $\pm$ SEM of paired data.

S1P caused an exacerbation of Ach-induced response. This result suggests that a local transient increase in S1P levels, after SPK activation, could be involved in triggering bronchial hyperreactivity. The use of SPK inhibitor *in vitro* confirmed this hypothesis. In fact, when either normal bronchi or OVA-sensitized bronchi were pretreated with DTD before the incubation with S1P, S1P-induced Ach potentiation was reverted. Conversely, DDT by itself was ineffective on Ach-induced contraction on normal bronchi while in OVA-sensitized bronchi caused a significant inhibition. These data strongly support our working hypothesis that SPK/S1P pathway is functionally triggered in pathologic conditions. To verify whether the *in vitro* hypothesis could be translated to the complex lung physiology, we performed an *in vivo* experiment on mice. To pursue this aim, we used a whole lung system that replicates the thorax environment in which either S1P or Ach had to travel through the lung to reach the bronchi. In these experimental conditions, S1P by itself was inactive, whereas in sensitized animals it caused an increase in airway resistance comparable to that of Ach. These data

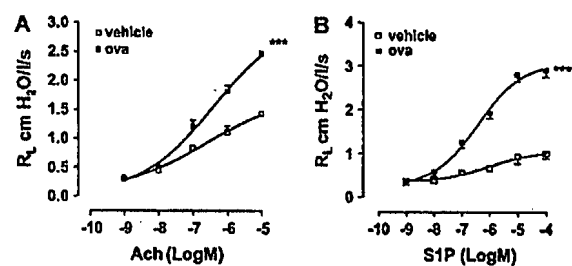


Figure 4. (A) R_L induced by Ach is significantly increased in OVA-sensitized animals. (B) Similarly, while normal lung did not respond to S1P challenge, OVA-sensitized lung displayed a significant and concentration-dependent response to S1P that matched the Ach-response. (***) $P < 0.001$ versus vehicle). Results are expressed as the mean $\pm$ SEM.

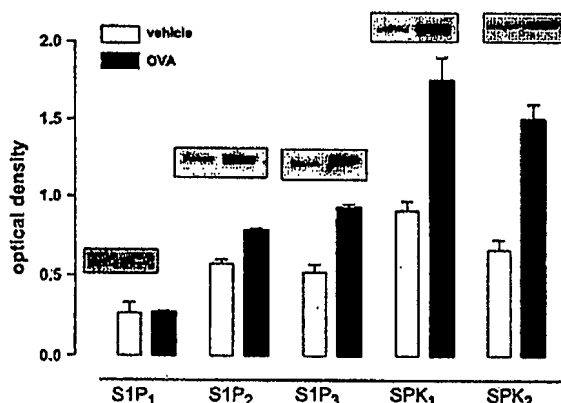


Figure 5. Main bronchi harvested from OVA-sensitized animals show an increased expression of S1P₂ and S1P₃ when compared with vehicle-treated mice. Conversely, S1P₁ expression was unchanged. Western blot analysis showed an increased expression of both of SPK₁ and SPK₂. The blots are representative of three different experiments.

confirmed again that S1P plays a role when the airways are already activated by an inflammatory trigger. By assessing through Western blot analysis the relative protein expression of the S1P receptors we found that S1P₂ and S1P₃ were the most abundantly expressed. To extend to the functional study to these results, we operated a pharmacologic modulation of the response. Since specific pharmacologic antagonists to each single

S1P receptor subtypes are not yet commercially available, we have incubated tissues with (1) activated PTX, which blocks Gi coupling S1P₁ receptor activation; (2) suramine, which blocks Gs coupling and S1P₂ receptor activation; (3) BML-241, a specific S1P₃ antagonist; and (4) Y27163, a selective Rho kinase, since S1P₂ signals through a Rho-dependent pathway. PTX did not affect either S1P-induced contraction or S1P-induced increase in Ach-induced contraction, implying that Gi-sensitive extracellular receptor pathway is not involved or at least does not play a major role. Pretreatment with suramine or BML-241 abrogated S1P-induced contraction, while it did not affect S1P-induced increase in Ach-contraction. Pretreatment with the selective Rho kinase inhibitor Y-27632 abrogates S1P potentiation of Ach-induced contraction. Taken together, these data suggest that S1P is involved in bronchial function at different levels through different specific receptors.

Since S1P levels are tightly regulated by SPK, we next evaluated how SPK is modulated in our experimental conditions. When we measured the levels of sphingosine kinase enzyme subtypes, we found that both isoforms SPK₁ and SPK₂ were up-regulated in bronchi harvested from OVA-sensitized animals. This finding well fits with the data obtained with the SPK inhibitor DTD. Indeed, DTD inhibited Ach-induced contraction only in bronchi harvested by sensitized animals, while it was ineffective in control mice.

Following the above findings we asked the question whether pretreatment *in vivo* of mice with DTD would inhibit bronchial hyperresponsiveness in sensitized animals. Pretreatment of mice with DTD before OVA administration significantly inhibited the enhanced Ach-induced contraction *in vitro*. Similarly, bronchi isolated by the above-described mice showed a reduced S1P-induced contraction *in vitro* and did not present the phenomenon of the potentiating effect induced by S1P on Ach-induced contraction.

In conclusion, our data suggest that S1P/SPK pathway seems not play a major role in physiologic conditions, since exogenous (1) S1P was ineffective in our experimental settings and (2) sphingosine kinase inhibition does not affect bronchial tone. Conversely, in pathologic conditions, such as those resembling an allergen-induced inflammation, the S1P/SPK pathway appears to play a detrimental role. It is worthy of note that S1P at concentrations well below the concentration circulating in the plasma induces a bronchial response. This indicates that a local increase of S1P in the bronchial tissue, most likely mediated through an increase in SPK expression, may mediate bronchial constriction. These findings, taken together with the fact that elevated levels of S1P have been found in BAL of patients with asthma (15), and considering the effects of S1P on cell-contributing allergen-induced lung inflammation (18, 19), suggest that this pathway may represent a novel target in designing new therapies in airway hyperresponsiveness.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

References

- Pyne S, Pyne N. Sphingosine 1-phosphate signalling via the endothelial differentiation gene family of G-protein-coupled receptors. *Pharmacol Ther* 2000;88:115-131.
- Chun J, Goetzl EJ, Hla T, Igarashi Y, Lynch KR, Moolenaar W, Pyne S, Tigyi G. International Union of pharmacology XXXIV. Lysophospholipid receptor nomenclature. *Pharmacol Rev* 2002;54:265-269.
- Ghosh TK, Bian J, Gill DL. Sphingosine 1-phosphate generated in the endoplasmic reticulum membrane activates release of stored calcium. *J Biol Chem* 1994;269:22628-22635.
- Meyer zu Heringdorf D, Niederdraing N, Neumann E, Frode R, Lass H, Van Koppen CJ, Jakobs KH. Discrimination between plasma

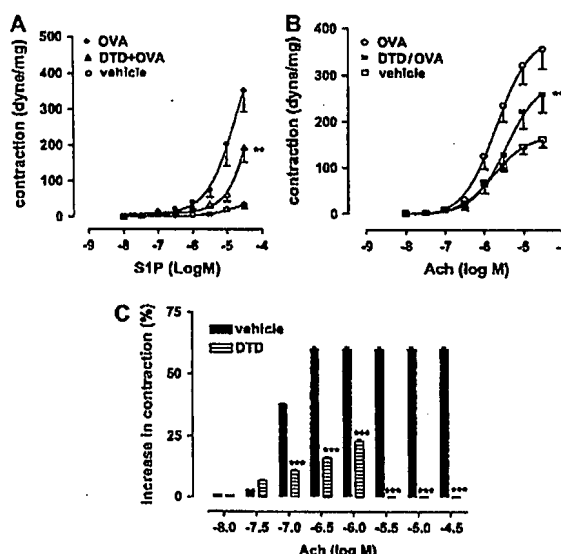


Figure 6. (A) Mice DTD administration (30 μ g intraperitoneally) *in vivo* before OVA sensitization significantly reduced the increased responsiveness of bronchi *in vitro* to S1P. (** P < 0.01 versus OVA). (B, C) DTD reverts the increased responsiveness to Ach and the S1P-induced potentiation of Ach contraction. (C) Results are expressed as a percentage of increase in Ach-contraction after S1P challenge. Results are expressed as the mean $\pm$ SEM.

- membrane and intracellular target sites of sphingosylphosphorylcholine. *Eur J Pharmacol* 1998;354:113-122.
5. Young KW, Nahorski SR. Sphingosine 1-phosphate: a Ca^{2+} release mediator in the balance. *Cell Calcium* 2002;32:335-341.
 6. Liu H, Chakravarty D, Maceyka M, Milstien S, Spiegel S. Sphingosine kinases: a novel family of lipid kinases. *Prog Nucleic Acid Res Mol Biol* 2002;71:493-511.
 7. Buehrer BM, Bell RM. Sphingosine kinase: properties and cellular functions. *Adv Lipid Res* 1993;26:59-67.
 8. Meyer zu Heringdorf D, Lass H, Kuchar I, Lipinski M, Alemany R, Rumenapp U, Jakobs KH. Stimulation of intracellular sphingosine-1-phosphate production by G-protein-coupled sphingosine-1-phosphate receptors. *Eur J Pharmacol* 2001;414:145-154.
 9. Roviezzo F, Bucci M, Delisle C, Brancialeone V, Di Lorenzo A, Mayo IP, Fiorucci S, Fontana A, Gratton JP, Cirino G. Essential requirement for sphingosine kinase activity in eNOS-dependent NO release and vasorelaxation. *FASEB J* 2006;20:340-342.
 10. Watterson K, Sankala H, Milstien S, Spiegel S. Pleiotropic actions of sphingosine-1-phosphate. *Prog Lipid Res* 2003;42:344-357.
 11. Spiegel S, Milstien S. Sphingosine-1-phosphate: an enigmatic signalling lipid. *Nat Rev Mol Cell Biol* 2003;4:397-407.
 12. Watterson KR, Ratz PH, Spiegel S. The role of sphingosine-1-phosphate in smooth muscle contraction. *Cell Signal* 2005;17:289-298.
 13. Jolly PS, Rosenfeldt HM, Milstien S, Spiegel S. The roles of sphingosine-1-phosphate in asthma. *Mol Immunol* 2002;38:1239-1245.
 14. Black JL, Johnson PR. Factors controlling smooth muscle proliferation and airway remodelling. *Curr Opin Allergy Clin Immunol* 2002;2:47-51.
 15. Ammit AJ, Hastie AT, Edsall LC, Hoffman RK, Amrani Y, Krymskaya VP, Kane SA, Peters SP, Penn RB, Spiegel S, et al. Sphingosine 1-phosphate modulates human airway smooth muscle cell functions that promote inflammation and airway remodeling in asthma. *FASEB J* 2001;15:1212-1214.
 16. Hemmings DG. Signal transduction underlying the vascular effects of sphingosine 1-phosphate and sphingosylphosphorylcholine. *Naunyn-Schmiedeberg's Arch Pharmacol* 2006;373:18-29.
 17. Baumruker T, Prieschl EE. The role of sphingosine kinase in the signaling initiated at the high-affinity receptor for IgE (FcεRI) in mast cells. *Int Arch Allergy Immunol* 2000;122:85-90.
 18. Prieschl EE, Csonga R, Novotny V, Kikuchi GE, Baumruker T. The balance between sphingosine and sphingosine-1-phosphate is decisive for mast cell activation after FcεRI triggering. *J Exp Med* 1999;190:1-8.
 19. Roviezzo F, Del Galdo F, Abbate G, Bucci M, D'Agostino B, Antunes E, De Dominicis G, Parente L, Rossi F, Cirino G, et al. Human eosinophil chemotaxis and selective in vivo recruitment by sphingosine-1-phosphate. *Proc Natl Acad Sci USA* 2004;101:11170-11175.
 20. James A. Airway remodeling in asthma. *Curr Opin Pulm Med* 2005;11:1.
 21. Amrani Y, Tliba O, Deshpande DA, Walseth TF, Kannan MS, Panettieri RA Jr. Bronchial hyperresponsiveness: insights into new signalling molecules. *Curr Opin Pharmacol* 2004;4:230-234.
 22. Jeffery PK. Remodeling and inflammation of bronchi in asthma and chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 2004;1:176-183.
 23. Bochner BS, Busse WW. Allergy and asthma. *J Allergy Clin Immunol* 2005;115:953-959.
 24. Panettieri RA Jr. Airway smooth muscle: immunomodulatory cells? *Allergy Asthma Proc* 2004;25:381-386.
 25. Chung KF. The role of airway smooth muscle in the pathogenesis of airway wall remodeling in chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 2005;2:347-354. [discussion 371-372.]
 26. Joubert P, Hamid Q. Role of airway smooth muscle in airway remodeling. *J Allergy Clin Immunol* 2005;116:713-716.
 27. Olivera A, Spiegel S. Sphingosine-1-phosphate as second messenger in cell proliferation induced by PDGF and FCS mitogens. *Nature* 1993;365:557-560.
 28. Choi OH, Kim JH, Kinet JP. Calcium mobilization via sphingosine kinase in signalling by the FcεRI antigen receptor. *Nature* 1996;380:634-636.
 29. English D, Welch Z, Kovala AT, Harvey K, Volpert OV, Brindley DN, Garcia JG. Sphingosine 1-phosphate released from platelets during clotting accounts for the potent endothelial cell chemotactic activity of blood serum and provides a novel link between hemostasis and angiogenesis. *FASEB J* 2000;14:2255-2265.
 30. Gosens R, Zaagsma J, Meurs H, Halayko AJ. Muscarinic receptor signalling in the pathophysiology of asthma and COPD. *Respir Res* 2006;7:73.
 31. Belmonte KE. Cholinergic pathways in the lungs and anticholinergic therapy for chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 2005;2:297-304.
 32. Doherty DE. The pathophysiology of airway dysfunction. *Am J Med* 2004;117:11S-23S.

ANEXO 2

Attenuation of Vascular Permeability by Methylnaltrexone

Role of mOP-R and S1P3 Transactivation

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Endothelial cell (EC) barrier dysfunction (i.e., increased vascular permeability) is observed in inflammatory states, tumor angiogenesis, atherosclerosis, and both sepsis and acute lung injury. Therefore, agents that preserve vascular integrity have important clinical therapeutic implications. We examined the effects of methylnaltrexone (MNTX), a mu opioid receptor (mOP-R) antagonist, on human pulmonary EC barrier disruption produced by edemagenic agents including morphine, the endogenous mOP-R agonist DAMGO, thrombin, and LPS. Pretreatment of EC with MNTX (0.1 μ M, 1 h) or the uncharged mOP-R antagonist naloxone attenuated morphine- and DAMGO-induced barrier disruption *in vitro*. However, MNTX, but not naloxone, pretreatment of EC inhibited thrombin- and LPS-induced barrier disruption, indicating potential mOP-R-independent effects of MNTX. In addition, intravenously delivered MNTX attenuated LPS-induced vascular hyperpermeability in the murine lung. We next examined the mechanistic basis for this MNTX barrier protection and observed that silencing of mOP-R attenuated the morphine- and DAMGO-induced EC barrier disruption, but not the permeability response to either thrombin or LPS. Because activation of the sphingosine 1-phosphate receptor, S1P₃, is key to a number of barrier-disruptive responses, we examined the role of this receptor in the permeability response to mOP-R ligation. Morphine, DAMGO, thrombin, and LPS induced RhoA/ROCK-mediated threonine phosphorylation of S1P₃, which was blocked by MNTX, suggesting S1P₃ transactivation. In addition, silencing of S1P₃ receptor expression (siRNA) abolished the permeability response to each edemagenic agonist. These results indicate that MNTX provides barrier protection against edemagenic agonists via inhibition of S1P₃ receptor activation and represents a potentially useful therapeutic agent for syndromes of increased vascular permeability.

Keywords: methylnaltrexone; mu opioid receptor; S1P3 receptor; EC barrier regulation; vascular permeability

Endothelial cells (EC) provide a semi-selective cellular barrier between the blood and underlying vasculature (1), with disruption of this barrier resulting in increased vascular permeability and organ dysfunction (2–4). Agents that enhance EC barrier function are a desirable therapeutic strategy for a variety of inflammatory diseases, tumor angiogenesis, atherosclerosis, and acute lung injury (5). Methylnaltrexone (MNTX) is a charged, peripherally restricted mu opioid receptor (mOP-R) antagonist currently in development for opiate-induced constipation in advanced illness and in Phase III clinical trials for postoperative ileus (6–9). The mOP-R is expressed in a variety of cell types,

including EC (10–12). However, the effects of mOP-R agonists and MNTX on EC barrier regulation are unknown but were examined in this study.

Opioids such as morphine and DAMGO exert their action by binding to the mOP-R present in the central nervous system (CNS) and peripheral tissues (13–15). The mOP-R is a G protein-coupled receptor with multiple isoforms resulting from alternative splicing of mRNA encoded from a single gene (13–17). Most mOP-R antagonists, including naloxone, exist in an uncharged state and readily pass through the blood-brain barrier (BBB) to exert CNS-dependent analgesic effects (18, 19). MNTX, however, is a charged molecule that cannot penetrate the BBB (7, 8), making MNTX a desirable tool by which to examine the effect of opiates and the mOP-R on EC function in the non-CNS peripheral circulations.

Several receptors have been implicated in EC barrier regulation (2–4). One important receptor family is the sphingosine-1-phosphate (S1P) receptors (also called endothelial differentiation gene [Edg] receptors). S1P binds to the plasma membrane G protein-coupled S1P receptors 1 (Edg1), 2 (Edg5), 3 (Edg3), 4 (Edg6), and 5 (Edg8) expressed in a variety of cell types including endothelium (5, 20–24). Human EC exhibit high expression of S1P₁ and S1P₃, with S1P₁ signaling coupled to the G_i pathway and Rac1 activation, whereas S1P₃ signaling couples to the G_q, G_{12/13}, and G_{12/13} pathways and activates RhoA to a much greater extent than Rac1 (5, 20–26). Activated RhoA can bind to and activate the serine/threonine kinase, ROCK (27, 28), which is involved in EC barrier disruption (29, 30). We have previously shown that S1P₁ receptor-dependent activation of Rac1 promotes vascular integrity (2, 3, 22, 29). In contrast, we have shown that the S1P₃ receptor can potentially regulate EC barrier disruption (29, 31).

In this study, we demonstrate that methylnaltrexone (MNTX) inhibits opiate-, thrombin-, and LPS-induced EC barrier disruption via transactivation (RhoA/ROCK-mediated threonine phosphorylation) of the S1P₃ receptor. Further, MNTX inhibited LPS-induced pulmonary vascular hyperpermeability *in vivo*, results suggesting that MNTX may be useful as a potential EC barrier-protective agent.

MATERIALS AND METHODS

Cell Culture and Reagents

Human pulmonary microvascular EC were obtained from Cambrex (Walkersville, MD) and cultured as previously described (2) in EBM-2 complete medium (Cambrex) at 37°C in a humidified atmosphere of 5% CO₂, 95% air, with passages 6–10 used for experimentation. Unless otherwise specified, reagents were obtained from Sigma (St. Louis, MO). Morphine sulfate was purchased from Baxter (Deerfield, IL). Reagents for SDS-PAGE electrophoresis were purchased from Bio-Rad (Richmond, CA), Immobilon-P transfer membrane from Millipore (Millipore Corp., Bedford, MA), and gold microelectrodes from Applied Biophysics (Troy, NY). Rabbit anti-mOP-R antibody was purchased from Abcam (Cambridge, MA). Rabbit anti-S1P₃ receptor antibody was purchased from Affinity Bioreagents (Golden, CO). Mouse

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anti-S1P₁ receptor antibody was purchased from Exalpha Biologicals (Watertown, MA). Rabbit anti-phospho-serine and rabbit anti-phospho-threonine antibodies were purchased from Zymed Laboratories, Inc. (South San Francisco, CA). Rabbit anti-ROCK1, rabbit anti-ROCK2, and rabbit anti-p115 rhoGEF were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-RhoA antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit anti-vWF (Factor VIII) antibody was purchased from Chemicon (Temecula, CA). Mouse anti- β -actin antibody, naloxone, DAMGO, thrombin, LPS, and ionomycin were purchased from Sigma. Secondary horseradish peroxidase (HRP)-labeled antibodies were purchased from Amersham Biosciences (Piscataway, NJ).

Immunoprecipitation and Immunoblotting

Cellular materials from treated or untreated human pulmonary microvascular EC (HPMVEC) were incubated with IP buffer (50 mM HEPES

[pH 7.5], 150 mM NaCl, 20 mM MgCl₂, 1% Nonidet P-40 [NP-40], 0.4 mM Na₂VO₄, 40 mM NaF, 50 μ M okadaic acid, 0.2 mM phenylmethylsulfonyl fluoride, 1:250 dilution of Calbiochem protease inhibitor mixture 3). The samples were then immunoprecipitated with anti-S1P₁ receptor IgG followed by SDS-PAGE in 4–15% polyacrylamide gels, transfer onto Immobilon membranes, and developed with specific primary and secondary antibodies. Visualization of immunoreactive bands was achieved using enhanced chemiluminescence (Amersham Biosciences).

Construction and Transfection of siRNA against mOP-R, S1P₁ receptor, S1P₂ receptor, RhoA, ROCK1, and ROCK2

The siRNA sequence(s) targeting human mOP-R, S1P₁ receptor, and S1P₂ receptor were generated using mRNA sequences from Gen-Bank (gi:56549104, gi:87196352, gi:38788192, gi:33876092, gi:4885582, and

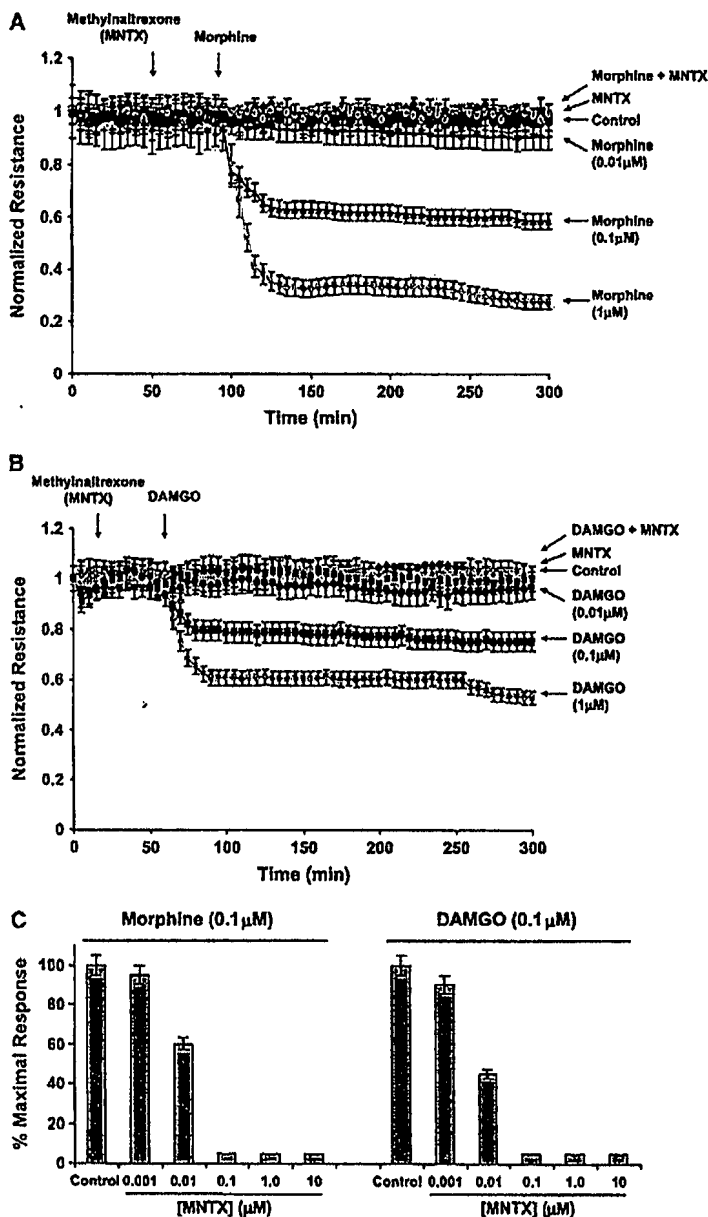


Figure 1. Characterization of MNTX and opiate effects on human EC barrier regulation. (A) HPMVEC were seeded on gold microelectrodes, serum starved for 1 h and either left untreated (control) or incubated with 0.1 μ M methylnaltrexone (MNTX) for 1 h before the addition of morphine sulfate (0.01, 0.1, and 1 μ M). The TER tracing represents pooled data $\pm$ SE from three independent experiments as described in MATERIALS AND METHODS. (B) HPMVEC were plated on gold microelectrodes, serum starved for 1 h, and either untreated (control) or treated with 0.1 μ M MNTX for 1 h before the addition of DAMGO (0.01, 0.1, and 1 μ M). The TER tracing represents pooled data $\pm$ SE from three independent experiments as described in MATERIALS AND METHODS. (C) Bar graph demonstrates the effects of increasing concentrations of MNTX on morphine sulfate- and DAMGO-mediated (0.1 μ M) EC barrier regulation. The bar graph represents the percent maximal TER response (y axis) with morphine sulfate and DAMGO challenge (at least $n = 3$ for each condition).

gi:41872582, respectively). For each mRNA (or scramble), two targets were identified. Specifically, mOP-R target sequence 1 (5'-AACGC CAGCAATTGCTACTGAT-3'), mOP-R target sequence 2 (5'-AATG TCAGATGCTCAGCTCGG-3'), S1P₁ target sequence 1 (5'-AAGC TACACAAAAGCCTGGA-3'), S1P₁ target sequence 2 (5'-AAAAAGCCTGGATCACTCATC-3'), S1P₂ target sequence 1 (5'-AACAGGGACTCAGGACCAGA-3'), S1P₂ target sequence 2 (5'-AAATGAATGTTCTGGGGCGC-3'), RhoA1 target sequence 1 (5'-AAAACCTTGCTACTGATCAGT-3'), RhoA1 target sequence 2 (5'-AACTTGCTACTGATCAGTTA-3'), ROCK1 target sequence 1

(5'-AAAAAATGGACAACCTGCTGC-3'), ROCK1 target sequence 2 (5'-AAGTGAATTCGGATTGTTTGC-3'), ROCK2 target sequence 1 (5'-AATCTGACTGAGGGGCGGGGA-3'), ROCK2 target sequence 2 (5'-AAGCCGGAGCTAGAGGCAGGC-3'), scrambled sequence 1 (5'-AAGAGAAATCGAAACCGAAAA-3'), and scrambled sequence 2 (5'-AAGAACCAATTAAGCGCAAG-3') were used. Sense and antisense oligonucleotides were provided by the Johns Hopkins University DNA Analysis Facility or purchased from Integrated DNA Technologies (Coralville, IA). For construction of the siRNA, a transcription-based kit from Ambion (Austin, TX) was used (Silencer siRNA

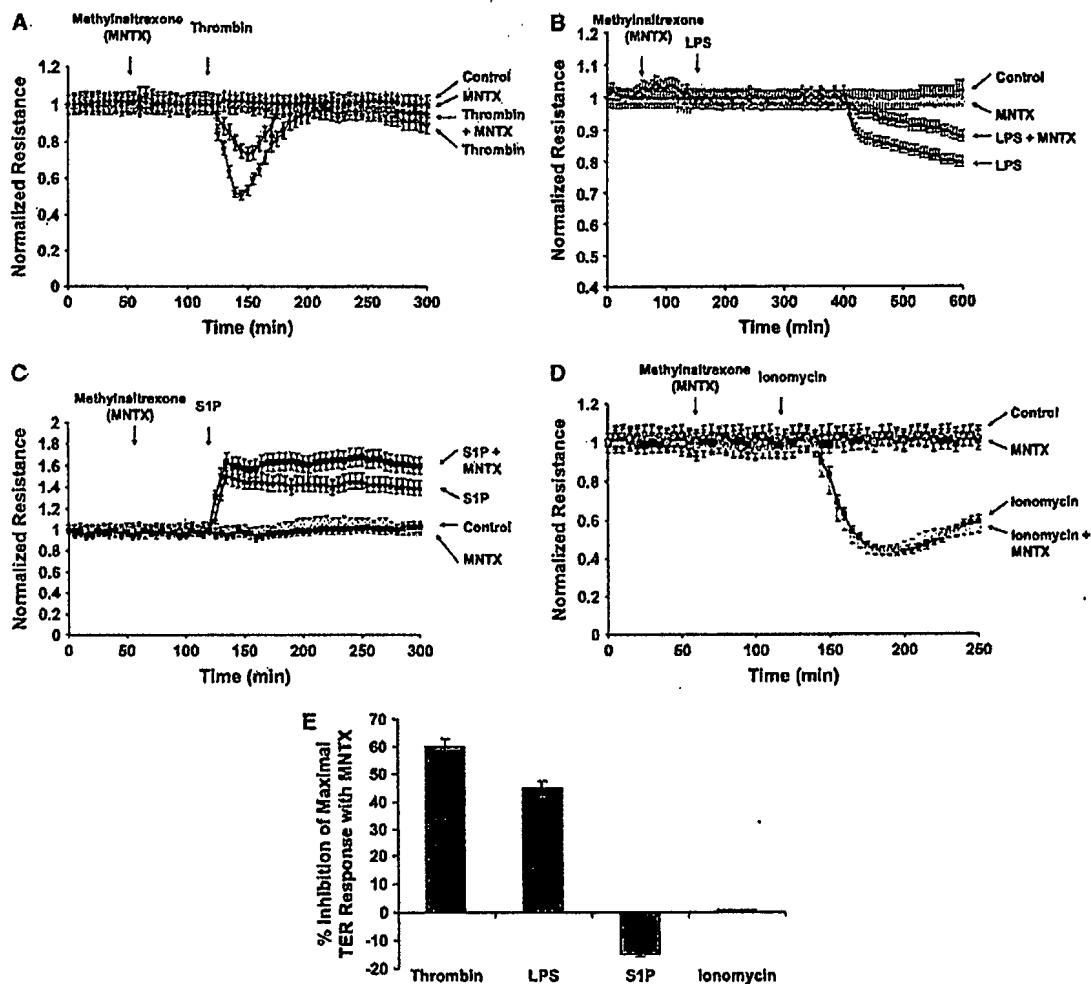


Figure 2. Characterization of MNTX effects on non-opiate agonist-induced human EC barrier regulation. (A) HPMVEC were seeded on gold microelectrodes, serum starved for 1 h, and either left untreated (control) or incubated with 0.1 μ M MNTX for 1 h before the addition of thrombin (1 U/ml). The TER tracing represents pooled data $\pm$ SE from three independent experiments as described in MATERIALS AND METHODS. (B) HPMVEC were seeded on gold microelectrodes, serum starved for 1 h, and either left untreated (control) or incubated with 0.1 μ M MNTX for 1 h before the addition of LPS (1 μ g/ml). The TER tracing represents pooled data $\pm$ SE from three independent experiments as described in MATERIALS AND METHODS. (C) HPMVEC were seeded on gold microelectrodes, serum starved for 1 h, and either left untreated (control) or incubated with 0.1 μ M MNTX for 1 h before the addition of S1P (1 μ M). The TER tracing represents pooled data $\pm$ SE from three independent experiments as described in MATERIALS AND METHODS. (D) HPMVEC were seeded on gold microelectrodes, serum starved for 1 h, and either left untreated (control) or incubated with 0.1 μ M MNTX for 1 h before the addition of ionomycin (10 μ M). The TER tracing represents pooled data $\pm$ SE from three independent experiments as described in MATERIALS AND METHODS. (E) Graphical representation of the data presented in A-D. Bar graph demonstrates the effects of MNTX (1 h pretreatment, 0.1 μ M) on thrombin (1 U/ml)-, LPS (1 μ g/ml)-, S1P (1 μ M)-, and ionomycin (10 μ M)-induced EC barrier regulation. The bar graph represents the percent inhibition of maximal TER response by MNTX (y axis) with agonist challenge (at least $n = 3$ for each condition).

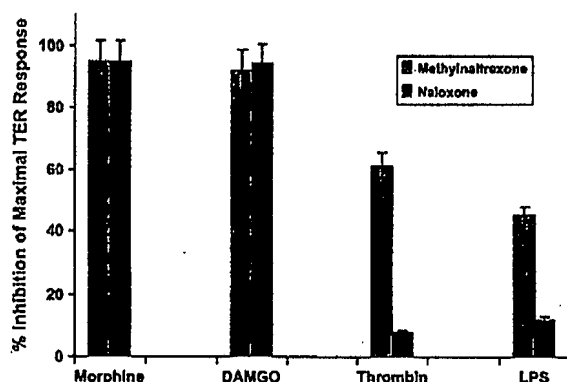


Figure 3. Differential effects of MNTX and naloxone on agonist-induced human EC barrier disruption. Bar graph demonstrates the differential effects of MNTX and naloxone (1 h pretreatment, 0.1 μ M) on morphine sulfate (0.1 μ M)-, DAMGO (0.1 μ M)-, thrombin (1 U/ml)-, and LPS-mediated (1 μ g/ml) EC barrier regulation. The bar graph represents the percent inhibition of maximal TER response by MNTX or naloxone (y axis) with agonist challenge (at least $n = 3$ for each condition).

construction kit). Human lung EC were then transfected with siRNA using siPORTamine as the transfection reagent (Ambion) according to the protocol provided by Ambion. Cells (~40% confluent) were serum-starved for 1 h followed by incubation with 3 μ M (1.5 μ M of each siRNA) of target siRNA (or scramble siRNA or no siRNA) for 6 h in serum-free media. The serum-containing medium was then added (10% serum final concentration) for 42 h before biochemical experiments and/or functional assays were conducted.

Determination of Serine/Threonine Phosphorylation of the S1P₁ Receptor

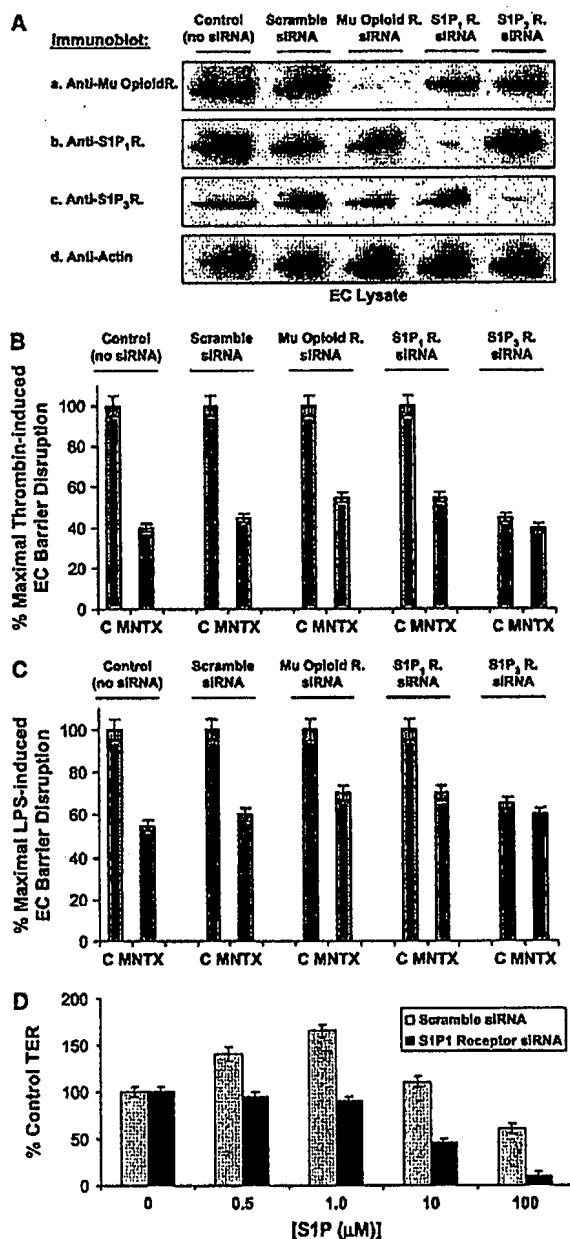
Solubilized proteins in IP buffer (see above) were immunoprecipitated with mouse anti-S1P₁ receptor antibody followed by SDS-PAGE in 4–15% polyacrylamide gels and transfer onto Immobilon membranes

Figure 4. Determination of the effects of silencing mOP-R, S1P₁ receptor, or S1P₃ receptor on MNTX-induced protection from human EC barrier disruption. (A) Immunoblot analysis of siRNA-treated or untreated human EC. Cellular lysates from untransfected (control, no siRNA), scramble siRNA (siRNA that does not target any known human mRNA), mOP-R siRNA, S1P₁ siRNA or S1P₃ siRNA-transfection were analyzed using immunoblotting with anti-mu opioid receptor antibody (a), anti-S1P₁ antibody (b), anti-S1P₃ antibody (c), or anti-actin antibody (d) as described in MATERIALS AND METHODS. Experiments were performed in triplicate each with similar results. Representative data is shown. (B) Bar graph demonstrates the effects of silencing mOP-R, S1P₁ receptor, or S1P₃ receptor on MNTX-induced (1 h pretreatment, 0.1 μ M) protection from thrombin-mediated (1 U/ml) EC barrier disruption. The bar graph represents the percent maximal thrombin-induced TER response (y axis, at least $n = 3$ for each condition). (C) Bar graph demonstrates the effects of silencing mOP-R, S1P₁ receptor, or S1P₃ receptor on MNTX-induced (1 h pretreatment, 0.1 μ M) protection from LPS-mediated (1 μ g/ml) EC barrier disruption. The bar graph represents the percent maximal LPS-induced TER response (y axis, at least $n = 3$ for each condition). (D) Bar graph demonstrates the effects of silencing the S1P₁ receptor on S1P-induced EC barrier regulation. The bar graph represents the percent control (0 μ M S1P) TER response (y axis, at least $n = 3$ for each condition).

(Millipore). After blocking nonspecific sites with 5% bovine serum albumin, the blots were incubated with either mouse anti-S1P₁ antibody, rabbit anti-phospho-serine, or rabbit anti-phospho-threonine antibody, followed by incubation with HRP-labeled goat anti-rabbit or goat anti-mouse IgG. Visualization of immunoreactive bands was achieved using enhanced chemiluminescence (Amersham Biosciences).

Measurement of EC Electrical Resistance

EC were grown to confluence in polycarbonate wells containing evaporated gold microelectrodes, and transendothelial electrical resistance (TER) measurements were performed using an electrical cell-substrate impedance sensing system obtained from Applied Biophysics as previously described in detail (2). TER values from each microelectrode



were pooled at discrete time points and plotted versus time as the mean $\pm$ SE.

Animal Preparation and Treatment

Male C57BL/6J mice (8–10 wk; Jackson Laboratories, Bar Harbor, ME) were anesthetized with intraperitoneal ketamine (150 mg/kg) and acetylpromazine (15 mg/kg). LPS (2.5 mg/kg) or water (control) were instilled intratracheally. Four hours later, mice received methylnaltrexone (MNTX, 10 mg/kg) or saline control intravenously via neck incision to expose the internal jugular vein. The animals were allowed to recover for 24 h after treatment before bronchoalveolar lavage (BAL) protein analysis and/or lung immunohistochemistry.

Murine Lung Immunohistochemistry

To characterize the expression of proteins in mouse lung vascular EC, lungs from control (untreated) mice were formalin fixed, 5- μ m paraffin sections were obtained and hydrated, and epitope retrieval was performed (DakoCytomation Target Retrieval Solution, pH 6.0; DakoCytomation, Carpinteria, CA). The sections were then histologically evaluated by either anti-mOP-R, anti-S1P₃ receptor, or anti-Factor VIII (vWF) antibody and secondary HRP-labeled polymer with DAB staining (Dako EnVision + System, HRP [DAB]; DakoCytomation) followed by hematoxylin QS counterstaining (Vector Laboratories, Burlingame, CA). Negative controls for immunohistochemical analysis were done by the same methods as above but without primary antibody. Immunostained sections were photographed ($\times 100$) using a Leica Axioscope (Bannockburn, IL).

Determination of BAL Protein and Cytokine Concentrations

BAL was performed by an intratracheal injection of 1 cc of Hanks' balanced salt solution followed by gentle aspiration. The recovered fluid was either processed for protein concentration (BCA Protein Assay Kit; Pierce Chemical Co., Rockford, IL) or used to determine TNF- α and TGF- β 1 concentrations using quantitative sandwich enzyme immunoassays (R&D Systems, Minneapolis, MN) as previously described (32).

Statistical Analysis

Student's *t* test was used to compare the means of data from two or more different experimental groups. Results are expressed as means $\pm$ SE.

RESULTS

The Role of MNTX in Agonist-Induced EC Barrier Disruption

EC barrier disruption is an essential feature of a variety of pathologies including atherosclerosis, tumor angiogenesis, and acute lung injury (2–4, 20). We examined the effects of MNTX, a charged peripheral mOP-R antagonist, on pulmonary microvascular EC integrity using measurements of TER. Figures 1A and 1B indicate that ligands for the mOP-R (i.e., morphine sulfate [MS] and DAMGO) induce EC barrier disruption and reductions in TER values in a dose-dependent manner, which is consistent with previous reports (15, 33, 34). These barrier-disruptive effects are blocked by pretreatment with a physiologically relevant dose of MNTX (0.1 μ M) (35). Decreasing the dose of MNTX below 0.1 μ M attenuates its barrier-protective effects, while increasing the dose of MNTX beyond 0.1 μ M did not significantly alter these responses (Figure 1C).

We next examined the potential barrier protective effects of MNTX on mOP-R-independent agonist-induced EC barrier disruption. Consistent with our prior reports, thrombin induces a rapid transient decrease in EC barrier function (36–38) (reflected by TER reductions) (Figure 2A) and LPS induces a delayed (~ 4 h) EC barrier-disruptive response (Figure 2B) (39–41). MNTX (0.1 μ M) attenuated the EC barrier disruption elicited by both thrombin and LPS (Figures 2A and 2B), but not that induced by the Ca²⁺ ionophore, ionomycin (Figure 2D) (42), indicating receptor selectivity in MNTX-mediated EC barrier protection. The protective effects of MNTX are not limited

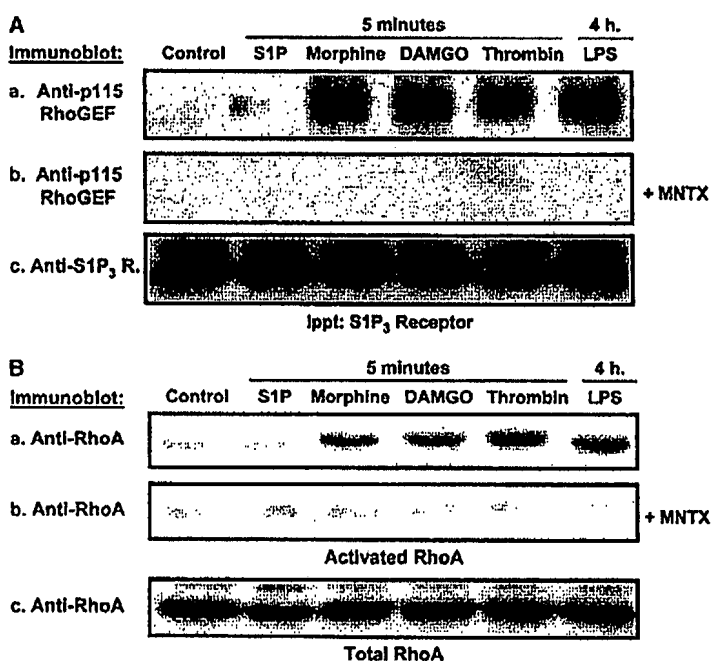


Figure 5. Determination of the role of MNTX on agonist-induced association of p115 RhoGEF with the S1P₃ receptor and RhoA activation in human EC. (A) Immunoblot analysis of the effects of MNTX on p115RhoGEF association with the S1P₃ receptor using various agonists in human pulmonary microvascular EC. Human EC were serum starved for 1 h and were either untreated (control) or pretreated with MNTX (0.1 μ M, 1 h) before the addition of S1P (1 μ M, 5 min), morphine sulfate (0.1 μ M, 5 min), DAMGO (0.1 μ M, 5 min), thrombin (1 U/ml, 5 min), or LPS (1 μ g/ml, 4 h). Cellular lysates were then obtained and immunoprecipitated with anti-S1P₃ receptor antibody as described in MATERIALS AND METHODS. The immunoprecipitated material was immunoblotted with anti-p115RhoGEF (a, b) or anti-S1P₃ receptor (c) antibody. Experiments were performed in triplicate each with similar results. Representative data is shown. (B) Analysis of agonist-induced RhoA activation in human EC. Human EC were serum starved for 1 h and were either untreated (control) or pretreated with MNTX (0.1 μ M, 1 h) before the addition of S1P (1 μ M, 5 min), morphine sulfate (0.1 μ M, 5 min), DAMGO (0.1 μ M, 5 min), thrombin (1 U/ml, 5 min), or LPS (1 μ g/ml, 4 h). EC were then solubilized in IP buffer and incubated with rho-binding domain (RBD)-conjugated beads to bind activated (GTP-bound form) RhoA. The RBD bead-associated material was run on SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-RhoA antibody. Experiments were performed in triplicate with highly reproducible findings (representative data shown).

to barrier-disrupting agents, as MNTX amplified the sustained EC barrier-enhancing effect of S1P (Figure 2C). MNTX is a charged molecule that cannot cross the BBB (35), allowing MNTX to selectively block peripheral mOP-R activity (35). Consistent with this notion, naloxone, which is uncharged and promotes both peripheral and CNS mOP-R antagonist (19, 43), also blocked MS and DAMGO-induced EC barrier disruption (0.1 μ M), but did not display EC barrier protection against either thrombin or LPS challenge (Figure 3). That naloxone is 10 to 30 times as potent as MNTX clinically and in isolated

tissues suggests that the differences we observed are not due to a concentration effect (44). **The Role of S1P₃ Receptor Transactivation in Agonist-Induced EC Barrier Disruption** Considering the actions of MNTX on opiate and S1P-induced EC barrier regulation, we next examined the effects of silencing (siRNA) the expression of the mOP-R or S1P receptor subtypes involved in MNTX-regulated EC integrity (Figure 4). Reductions in mOP-R expression had little effect on MNTX protection

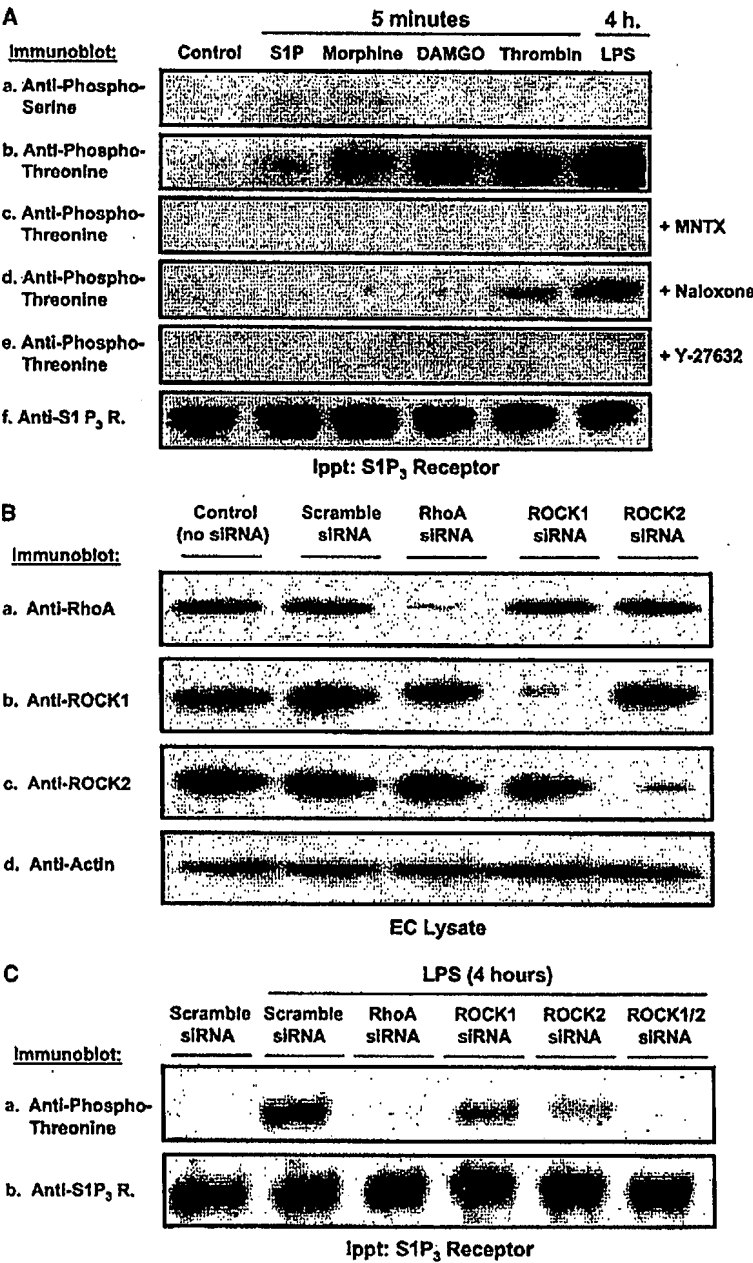


Figure 6. Determination of the role of RhoA and ROCK on agonist-induced S1P₃ receptor transactivation. (A) Immunoblot analysis of the effects of MNTX, naloxone and the ROCK inhibitor, Y-27632, on S1P₃ receptor phosphorylation using various agonists in human pulmonary microvascular EC. Human EC were serum starved for 1 h and were either untreated (control) or pretreated with MNTX (0.1 μ M, 1 h), naloxone (0.1 mM), or Y-27632 (500 nM) before the addition of S1P (1 μ M, 5 min), morphine sulfate (0.1 μ M, 5 min), DAMGO (0.1 μ M, 5 min), thrombin (1 U/ml, 5 min), or LPS (1 μ g/ml, 4 h). Cellular lysates were then obtained and immunoprecipitated with anti-S1P₃ receptor antibody as described in MATERIALS AND METHODS. The immunoprecipitated material was immunoblotted with anti-phospho-serine (a), anti-phospho-threonine (b-e) or anti-S1P₃ receptor (f) antibody. Experiments were performed in triplicate each with similar results. Representative data are shown. (B) Immunoblot analysis of siRNA-treated or untreated human EC. Cellular lysates from untransfected (control, no siRNA), scramble siRNA (siRNA that does not target any known human mRNA), RhoA siRNA, ROCK1 siRNA, or ROCK2 siRNA transfection were analyzed using immunoblotting with anti-RhoA antibody (a), anti-ROCK1 antibody (b), anti-ROCK2 antibody (c), or anti-actin antibody (d) as described in MATERIALS AND METHODS. Experiments were performed in triplicate each with similar results. Representative data is shown. (C) Immunoblot analysis of the effects of RhoA, ROCK1, and ROCK2 siRNA on S1P₃ receptor phosphorylation with LPS treatment of human pulmonary microvascular EC. EC were either untransfected (control, no siRNA) or transfected with scramble siRNA (siRNA that does not target any known human mRNA), RhoA siRNA, ROCK1 siRNA, ROCK2 siRNA, or combined ROCK1/2 siRNA, serum starved for 1 h and were either untreated (control) or treated with LPS (1 μ g/ml, 4 h). Cellular lysates were then obtained and immunoprecipitated with anti-S1P₃ receptor antibody as described in MATERIALS AND METHODS. The immunoprecipitated material was immunoblotted with anti-phospho-threonine (a) or anti-S1P₃ receptor (b) antibody. Experiments were performed in triplicate each with similar results. Representative data are shown.

against thrombin- or LPS-induced EC barrier disruption, indicating mOP-R-independent effects of MNTX on specific models of EC barrier dysfunction (Figures 4B and 4C). We and others have previously determined that EC express both S1P₁ and S1P₃ receptors with the S1P₁ receptor coupled to Rac1-mediated signaling, while the S1P₃ receptor activates RhoA-mediated signaling (3, 5, 21–23, 26). Our prior studies and Figure 4D reveal that silencing of S1P₁ receptor expression completely eliminates the barrier-protective effects of S1P (0.5 and 1 μ M) (22), whereas higher S1P concentrations (10 μ M and 100 μ M) induce barrier disruption due to S1P₃ receptor activation (45, 46). Silencing S1P₃ expression increases basal EC barrier function by $\sim$ 20% (data not shown). Figures 4B and 4C demonstrates that, in contrast to the silencing of the S1P₁ receptor, reductions in S1P₃ receptor expression inhibits both thrombin- and LPS-induced barrier disruption and loss of MNTX protection against these edemagenic agonists.

We have previously shown that S1P₃ receptor transactivation is critical to diverse agonist-induced EC barrier enhancement (3, 22, 47). We and others have shown that the S1P₃ receptor is linked to the barrier-disruptive RhoA signaling pathway (26, 29, 48). Given the previous published reports that the S1P₃ receptor regulates barrier function (29, 49), we hypothesized that S1P₃ receptor transactivation and RhoA signaling may participate as a potentially central mechanism involved in loss of EC barrier integrity. Figure 5 indicates that barrier-disrupting agonists (unlike the barrier-protective 1 μ M S1P) promote association of the RhoA-activating GEF, p115RhoGEF, with the S1P₃ receptor (Figure 5A) and consequent RhoA activation (Figure 5B), which are inhibited by pretreatment with MNTX. Further, barrier-disrupting agonists (unlike the barrier-protective 1 μ M S1P) induce S1P₃ receptor transactivation (threonine phosphorylation) of the S1P₃ receptor (Figure 6A). Both MNTX and naloxone block morphine and DAMGO-induced S1P₃ receptor transactivation. However, MNTX (but not naloxone) also blocks thrombin and LPS-induced S1P₃ receptor transactivation, suggesting mOP-R-independent effects of MNTX. Consistent with the inhibitory effects of MNTX on agonist-induced RhoA activation, treatment of EC with an inhibitor (Y-27632) of the activated RhoA target, the serine/threonine kinase, ROCK, blocked agonist-induced S1P₃ receptor transactivation (Figure 6A). Silencing RhoA and/or ROCK isoforms 1 and 2, like the Y-27632 compound, blocked threonine phosphorylation of the S1P₃ receptor (Figure 6C) as well as attenuated thrombin and LPS-induced EC barrier disruption (Figure 7), indicating the importance of RhoA/ROCK in these events.

Role of MNTX in LPS-Induced Pulmonary Vascular Hyperpermeability *In Vivo*

Similar to our results in human pulmonary microvascular EC, immunohistochemical studies reveal that murine lungs express both mOP-R and S1P₃ receptor (Figure 8A). We next examined whether MNTX was an effective barrier-protective agent in an *in vivo* model of LPS-induced murine lung vascular permeability. LPS administered via an intratracheal route induces murine inflammation and increased vascular leakiness as measured by the protein concentration in BAL fluid (Figure 8B), as we have previously noted (50). Intravenous injection of MNTX (10 mg/kg) alone did not affect pulmonary vascular permeability; however, intravenous injection of MNTX 4 h after LPS delivery attenuated mouse pulmonary hyperpermeability (Figure 8B).

As another measure of pulmonary vascular barrier disruption, we measured the BAL concentrations of two well-characterized EC barrier-disrupting cytokines, TNF- α and TGF- β 1 (Figure 9) (51, 52). Intravenous injection of MNTX (10 mg/kg) alone did not affect the concentrations of these cytokines in murine BAL

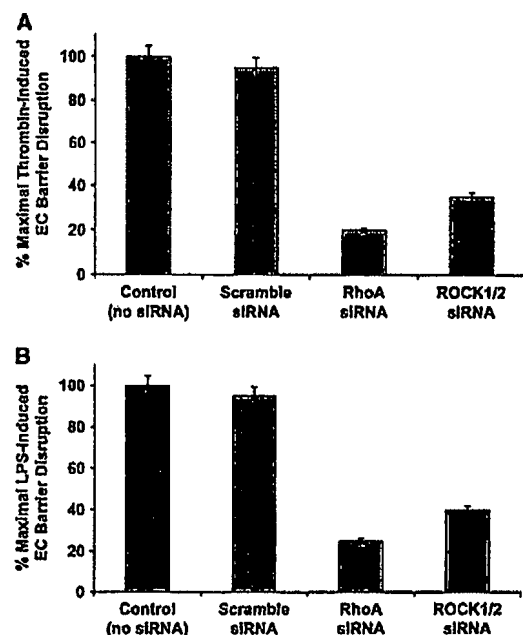


Figure 7. Determination of the role of RhoA and ROCK on agonist-induced human EC barrier disruption. (A) Bar graph demonstrates the effects of RhoA or ROCK1/2 siRNA on thrombin-induced EC barrier disruption. EC were either untransfected (control, no siRNA) or transfected with scramble siRNA (siRNA that does not target any known human mRNA), RhoA siRNA or combined ROCK1/2 siRNA, serum starved for 1 h, and treated with thrombin (1 U/ml). The bar graph represents the percent maximal TER response by thrombin (y axis) with agonist challenge (at least $n = 3$ for each condition). (B) Bar graph demonstrates the effects of RhoA or ROCK1/2 siRNA on LPS-induced EC barrier disruption. EC were either untransfected (control, no siRNA) or transfected with scramble siRNA (siRNA that does not target any known human mRNA), RhoA siRNA or combined ROCK1/2 siRNA, serum starved for one hour and treated with LPS (1 μ g/ml). The bar graph represents the percent maximal TER response by LPS (y axis) with agonist challenge (at least $n = 3$ for each condition).

fluid. However, intravenous injection of MNTX 4 h after LPS delivery attenuated the LPS-induced increase in TNF- α and TGF- β 1 BAL concentrations (Figure 9).

DISCUSSION

The results contained in this report include several novel observations, including the finding that MNTX, a selective peripherally restricted mOP-R antagonist, provides protection against agonist-induced EC barrier disruption via both mOP-R-dependent and -independent mechanisms. In addition, our results highlight a critical role for S1P₃ receptor transactivation (evidenced by RhoA/ROCK-mediated threonine phosphorylation) as an essential regulator of agonist-induced EC barrier disruption. Interestingly, MNTX inhibits not only morphine- and DAMGO-induced S1P₃ receptor transactivation and increased vascular permeability (Figure 10), but also inhibits responses to thrombin and LPS. This indicates that MNTX may be a potential clinically useful therapy for high permeability states such as atherosclerosis, tumor angiogenesis, and acute lung injury. Despite numerous attempts at optimizing doses of mOP-R antagonists,

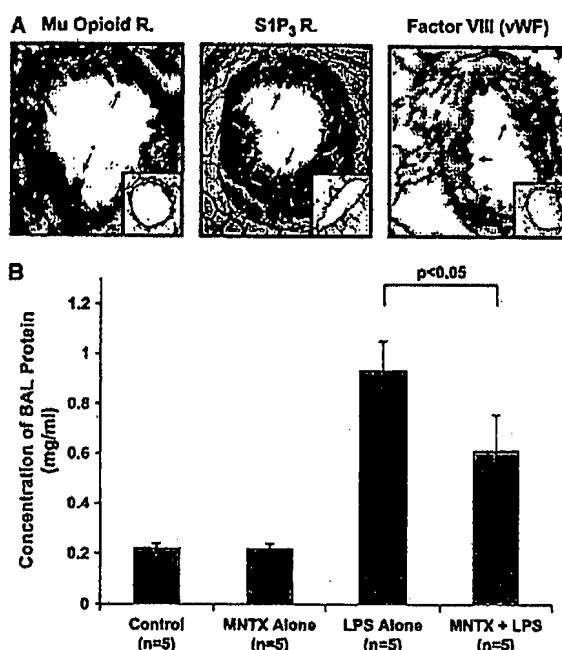


Figure 8. The effect of MNTX on LPS-induced pulmonary vascular hyper-permeability *in vivo*. (A) Immunohistochemical staining of control (untreated) mouse lung using either anti-mOP-R, anti-S1P₃ receptor antibody, or anti-Factor VIII (vWF) antibody and secondary HRP-labeled polymer with DAB staining (Dako EnVision + System, HRP [DAB]; Dako-Cytomation) followed by hematoxylin QS counterstaining (Vector Laboratories, Burlingame, CA) as described in MATERIALS AND METHODS. Images are shown at $\times 100$ magnification. Arrows indicate immunostaining of endothelial cells. Insets in A: Negative controls for immunohistochemical analysis, which were done by the same method as above but without primary antibody. (B) C57BL/6 mice were anesthetized and were either given water (control) or LPS (2.5 mg/kg) intratracheally. After 4 h, mice were given intravenous injections (internal jugular vein) with saline (control) or MNTX (10 mg/kg). The treated mice were allowed to recover for 24 h, BAL fluids were obtained, and protein concentrations were determined (see MATERIALS AND METHODS).

lipid-soluble mOP antagonists (such as naloxone) easily cross the BBB (18, 19, 35) and have proven unsuitable for patients receiving opiates for pain management due to analgesia reversal and breakthrough pain. In contrast, MNTX is a quaternary derivative of the pure narcotic antagonist naltrexone (6), with the addition of the methyl group to naltrexone at the amine in the ring forming the compound N-methylnaltrexone with greater polarity and lower lipid solubility. MNTX does not cross the BBB and thus has significant potential as a therapeutic intervention in reversing the peripheral effects of opiates in palliative care, especially for patients taking elevated doses of opiates for analgesia (7). Two recently completed double-blind clinical trials of subcutaneous MNTX (0.15 mg or 0.3 mg) in patients with advanced medical illness suffering from opiate-induced constipation demonstrated significant reversal of constipation without affecting analgesia (9).

The observation that MNTX, but not naloxone, protects against both thrombin- and LPS-induced EC barrier disruption indicates a highly novel finding. Although several mOP-R antagonists can inhibit opioid-induced angiogenesis (10), the effect of

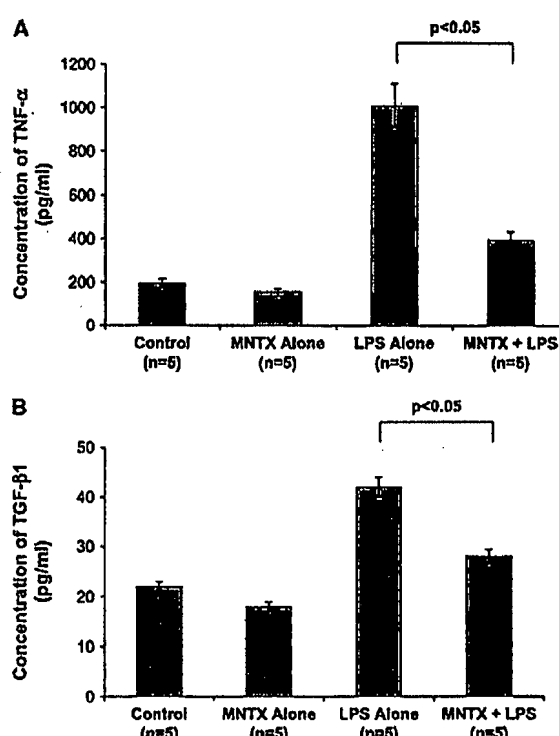


Figure 9. The effect of MNTX on LPS-induced pulmonary EC barrier disruptive cytokine production *in vivo*. C57BL/6 mice were anesthetized and were either given water (control) or LPS (2.5 mg/kg) intratracheally. After 4 h, mice were given intravenous injections (internal jugular vein) with saline (control) or MNTX (10 mg/kg). The treated mice were allowed to recover for 24 h, BAL fluids were obtained, and the concentrations of the EC barrier disruptive cytokines TNF- α (A) and TGF- β 1 (B) were determined using quantitative sandwich enzyme immunoassays (R&D Systems) (see MATERIALS AND METHODS).

ameliorating EC barrier dysfunction with diverse EC barrier-disruptive agents such as thrombin and LPS appears selective for MNTX. Thrombin induces rapid, transient EC barrier disruption through activation of protease-activated receptors with consequent Ca²⁺, RhoA, and Ras/MAP kinase signaling (36–38, 53, 54). In contrast, LPS induces a delayed EC barrier-disruptive response by activating a receptor complex of TLR4, CD14, and MD2 with consequent NF- κ B activation and cytokine production (39–41, 55, 56). We have previously shown that S1P₃ receptor transactivation (AKT-mediated threonine phosphorylation) is a key component in agonist-induced EC barrier enhancement (29, 47). In the current study, we have extended these findings to show that RhoA/ROCK-mediated transactivation (threonine phosphorylation) of the S1P₃ receptor plays an important role in agonist-induced EC barrier disruption. ROCK inhibitors have been used to treat cardiovascular disease and acute lung injury (30, 57). Considering our novel observation that MNTX can inhibit agonist-induced RhoA/ROCK activation, MNTX could potentially be used to treat ROCK-related diseases. Previous reports have indicated that the S1P₃ receptor is also involved in epithelial barrier disruption (49). We are currently investigating the effects of MNTX on S1P₃ receptor-mediated lung epithelial barrier disruption. Further, we are examining the mechanisms

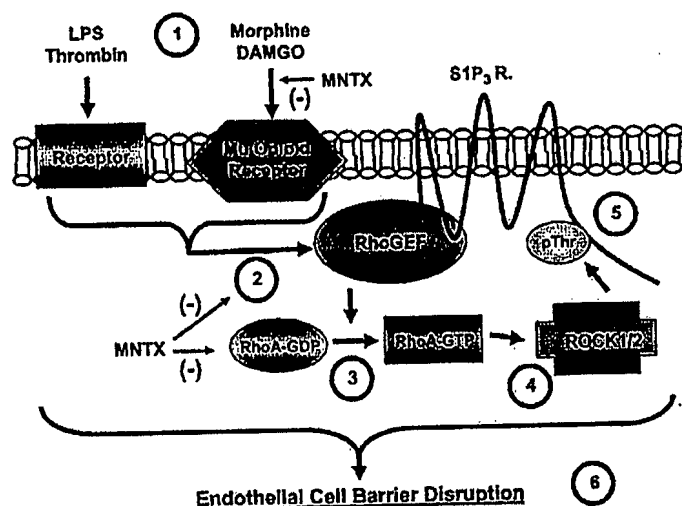


Figure 10. Proposed model for the protective effects of MNTX on agonist-induced EC barrier disruption. We propose that agonists such as LPS, thrombin, morphine, and DAMGO bind to their cell surface receptors (1), which promotes p115 RhoGEF association with the S1P₃ receptor (2), RhoA (3) and ROCK (4) activation, and ROCK-mediated transactivation of the S1P₃ receptor (threonine phosphorylation) (5). Activation of S1P₃ receptor induces EC barrier disruption (6). MNTX inhibits morphine and DAMGO binding to the MOR and inhibits agonist-induced, RhoA and ROCK-dependent, S1P₃ receptor transactivation. These events contribute to the protection from EC barrier disruption by MNTX.

by which MNTX exerts mOP-R-independent protective effects on EC barrier disruption.

In summary, using both *in vitro* and *in vivo* models of pulmonary vascular permeability, we have demonstrated that MNTX alone does not affect basal vascular integrity but significantly attenuates receptor edemagenic agonist-induced vascular barrier disruption. These results occur through the inhibition of a highly novel RhoA/ROCK-mediated transactivation of the S1P₃ receptor and indicate that MNTX may be a potentially useful therapeutic treatment for diseases characterized by high permeability states.

Conflict of Interest Statement: J.M. serves as a consultant to Progenetics Pharmaceuticals, has a financial interest in methylnaltrexone as a patent holder through the University of Chicago, and receives stock options from Progenics. None of the other authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

References

- Luscher TF, Barton M. Biology of the endothelium. *Clin Cardiol* 1997;20:11-3-10.
- Garcia JG, Liu F, Verin AD, Birukova A, Dechert MA, Gerthoffer WT, Bamberg JR, English D. Sphingosine 1-phosphate promotes endothelial cell barrier integrity by Edg-dependent cytoskeletal rearrangement. *J Clin Invest* 2001;108:689-701.
- Dudek SM, Jacobson JR, Chiang ET, Birukov KG, Wang P, Zhan X, Garcia JG. Pulmonary endothelial cell barrier enhancement by sphingosine 1-phosphate: roles for cortactin and myosin light chain kinase. *J Biol Chem* 2004;279:24692-24700.
- Dudek SM, Garcia JG. Cytoskeletal regulation of pulmonary vascular permeability. *J Appl Physiol* 2001;91:1487-1500.
- Spiegel S, Kolesnick R. Sphingosine 1-phosphate as a therapeutic agent. *Leukemia* 2002;16:1596-1602.
- Yuan CS, Foss JF, O'Connor M, Osinski J, Karrison T, Moss J, Roizen MF. Methylnaltrexone for reversal of constipation due to chronic methadone use: a randomized controlled trial. *JAMA* 2000;283:367-372.
- Yuan CS. Clinical status of methylnaltrexone, a new agent to prevent and manage opioid-induced side effects. *J Support Oncol* 2004;2:111-117. (discussion 119-122).
- Yuan CS, Doshan H, Charney MR, O'Connor M, Karrison T, Maleckar SA, Israel RJ, Moss J. Tolerability, gut effects, and pharmacokinetics of methylnaltrexone following repeated intravenous administration in humans. *J Clin Pharmacol* 2005;45:538-546.
- Yuan CS, Israel RJ. Methylnaltrexone, a novel peripheral opioid receptor antagonist for the treatment of opioid side effects. *Expert Opin Investig Drugs* 2006;15:541-552.
- Singleton PA, Lingen MW, Fekete MJ, Garcia JG, Moss J. Methylnaltrexone inhibits opiate and VEGF-induced angiogenesis: Role of receptor transactivation. *Microvasc Res* 2006;72:3-11.
- Cadet P, Mantione KJ, Bilfinger TV, Stefano GB. Differential expression of the human mu opiate receptor from different primary vascular endothelial cells. *Med Sci Monit* 2004;10:BR351-BR355.
- Cadet P. Mu opiate receptor subtypes. *Med Sci Monit* 2004;10:MS28-MS32.
- Law PY, Loh HH. Regulation of opioid receptor activities. *J Pharmacol Exp Ther* 1999;289:607-624.
- Pasternak GW. Multiple opiate receptors: deja vu all over again. *Neuropharmacology* 2004;47:312-323.
- Tegeder I, Geisslinger G. Opioids as modulators of cell death and survival-unraveling mechanisms and revealing new indications. *Pharmacol Rev* 2004;56:351-369.
- Law PY, Loh HH, Wei LN. Insights into the receptor transcription and signaling: implications in opioid tolerance and dependence. *Neuropharmacology* 2004;47:300-311.
- Bailey CP, Connor M. Opioids: cellular mechanisms of tolerance and physical dependence. *Curr Opin Pharmacol* 2005;5:60-68.
- Hoskin PJ, Hanks GW. Opioid agonist-antagonist drugs in acute and chronic pain states. *Drugs* 1991;41:326-344.
- Greenwood-Van Meerveld B, Gardner CJ, Little PJ, Hicks GA, Dehaven-Hudkins DL. Preclinical studies of opioids and opioid antagonists on gastrointestinal function. *Neurogastroenterol Motil* 2004;16:46-53.
- McVerry BJ, Garcia JG. Endothelial cell barrier regulation by sphingosine 1-phosphate. *J Cell Biochem* 2004;92:1075-1085.
- Pyne S, Pyne N. Sphingosine 1-phosphate signalling via the endothelial differentiation gene family of G-protein-coupled receptors. *Pharmacol Ther* 2000;88:115-131.
- Singleton PA, Dudek SM, Chiang ET, Garcia JG. Regulation of sphingosine 1-phosphate-induced endothelial cytoskeletal rearrangement and barrier enhancement by S1P1 receptor, PI3 kinase, Tiam1/Rac1, and alpha-actinin. *FASEB J* 2005;19:1646-1656.
- Spiegel S, Milstien S. Sphingosine-1-phosphate: an enigmatic signalling lipid. *Nat Rev Mol Cell Biol* 2003;4:397-407.
- Krump-Konvalinkova V, Yasuda S, Rubic T, Makarova N, Mages J, Erl W, Vosseler C, Kirkpatrick CJ, Tigyi G, Siess W. Stable knock-down of the sphingosine 1-phosphate receptor S1P1 influences multiple functions of human endothelial cells. *Arterioscler Thromb Vasc Biol* 2005;25:546-552.
- Schaphorst KL, Chiang E, Jacobs KN, Zaiman A, Natarajan V, Wigley F, Garcia JG. Role of sphingosine-1 phosphate in the enhancement of endothelial barrier integrity by platelet-released products. *Am J Physiol Lung Cell Mol Physiol* 2003;285:L258-L267.
- Waeber C, Blondeau N, Salomone S. Vascular sphingosine-1-phosphate S1P1 and S1P3 receptors. *Drug News Perspect* 2004;17:365-382.

27. Sahai E, Alberts AS, Treisman R. RhoA effector mutants reveal distinct effector pathways for cytoskeletal reorganization, SRF activation and transformation. *EMBO J* 1998;17:1350-1361.
28. Fujisawa K, Madaule P, Ishizaki T, Watanabe G, Bito H, Saito Y, Hall A, Narumiya S. Different regions of Rho determine Rho-selective binding of different classes of Rho target molecules. *J Biol Chem* 1998;273:18943-18949.
29. Singleton PA, Dudek SM, Ma SF, Garcia JG. Transactivation of sphingosine 1-phosphate receptors is essential for vascular barrier regulation: novel role for hyaluronan and CD44 receptor family. *J Biol Chem* 2006;281:34381-34393.
30. Tasaka S, Koh H, Yamada W, Shimizu M, Ogawa Y, Hasegawa N, Yamaguchi K, Ishii Y, Richer SE, Doerschuk CM, et al. Attenuation of endotoxin-induced acute lung injury by the Rho-associated kinase inhibitor, Y-27632. *Am J Respir Cell Mol Biol* 2005;32:504-510.
31. Okamoto H, Takuwa N, Yatomi Y, Gonda K, Shigematsu H, Takuwa Y. EDG3 is a functional receptor specific for sphingosine 1-phosphate and sphingosylphosphorylcholine with signaling characteristics distinct from EDG1 and AGR16. *Biochem Biophys Res Commun* 1999;260:203-208.
32. Su X, Wang L, Song Y, Bai C. Inhibition of inflammatory responses by ambroxol, a mucolytic agent, in a murine model of acute lung injury induced by lipopolysaccharide. *Intensive Care Med* 2004;30:133-140.
33. Liu HC, Anday JK, House SD, Chang SL. Dual effects of morphine on permeability and apoptosis of vascular endothelial cells: morphine potentiates lipopolysaccharide-induced permeability and apoptosis of vascular endothelial cells. *J Neuroimmunol* 2004;146:13-21.
34. Ocasio FM, Jiang Y, House SD, Chang SL. Chronic morphine accelerates the progression of lipopolysaccharide-induced sepsis to septic shock. *J Neuroimmunol* 2004;149:90-100.
35. Moss J. Pain relief without side effects: peripheral opiate antagonists, Vol. 33. Philadelphia: Lippincott Williams & Wilkins; 2005.
36. Garcia JG, Patterson C, Bahler C, Aschner J, Hart CM, English D. Thrombin receptor activating peptides induce Ca²⁺ mobilization, barrier dysfunction, prostaglandin synthesis, and platelet-derived growth factor mRNA expression in cultured endothelium. *J Cell Physiol* 1993;156:541-549.
37. Garcia JG, Verin AD, Schaphorst KL. Regulation of thrombin-mediated endothelial cell contraction and permeability. *Semin Thromb Hemost* 1996;22:309-315.
38. Minami T, Sugiyama A, Wu SQ, Abid R, Kodama T, Aird WC. Thrombin and phenotypic modulation of the endothelium. *Arterioscler Thromb Vasc Biol* 2004;24:41-53.
39. Bannerman DD, Goldblum SE. Endotoxin induces endothelial barrier dysfunction through protein tyrosine phosphorylation. *Am J Physiol* 1997;273:L217-L226.
40. Bannerman DD, Goldblum SE. Direct effects of endotoxin on the endothelium: barrier function and injury. *Lab Invest* 1999;79:1181-1199.
41. Dauphinee SM, Karsan A. Lipopolysaccharide signaling in endothelial cells. *Lab Invest* 2006;86:9-22.
42. Garcia JG, Schaphorst KL, Shi S, Verin AD, Hart CM, Callahan KS, Patterson CE. Mechanisms of ionomycin-induced endothelial cell barrier dysfunction. *Am J Physiol* 1997;273:L172-L184.
43. Sykes NP. Using Oral Naloxone in Management of Opioid Bowel Dysfunction. New York: Haworth Medical Press; 2005.
44. Yuan CS, Foss JF, Moss J. Effects of methylnaltrexone on morphine-induced inhibition of contraction in isolated guinea-pig ileum and human intestine. *Eur J Pharmacol* 1995;276:107-111.
45. Shikata Y, Birukov KG, Birukova AA, Verin A, Garcia JG. Involvement of site-specific FAK phosphorylation in sphingosine-1 phosphate- and thrombin-induced focal adhesion remodeling: role of Src and GIT. *FASEB J* 2003;17:2240-2249.
46. Shikata Y, Birukov KG, Garcia JG. S1P induces FA remodeling in human pulmonary endothelial cells: role of Rac, GIT1, FAK, and paxillin. *J Appl Physiol* 2003;94:1193-1203.
47. Finigan JH, Dudek SM, Singleton PA, Chiang ET, Jacobson JR, Camp SM, Ye SQ, Garcia JG. Activated protein C mediates novel lung endothelial barrier enhancement: role of sphingosine 1-phosphate receptor transactivation. *J Biol Chem* 2005;280:17286-17293.
48. Carbajal JM, Gratrix ML, Yu CH, Schaeffer RC Jr. ROCK mediates thrombin's endothelial barrier dysfunction. *Am J Physiol Cell Physiol* 2000;279:C195-C204.
49. Gon Y, Wood MR, Kioussis WB, Jo E, Sanna MG, Chun J, Rosen H. S1P3 receptor-induced reorganization of epithelial tight junctions compromises lung barrier integrity and is potentiated by TNF. *Proc Natl Acad Sci USA* 2005;102:9270-9275.
50. Peng X, Hassoun PM, Sammani S, McVerry BJ, Burne MJ, Rabb H, Pearce D, Tudor RM, Garcia JG. Protective effects of sphingosine 1-phosphate in murine endotoxin-induced inflammatory lung injury. *Am J Respir Crit Care Med* 2004;169:1245-1251.
51. Ferrero E. Assessment of TNFalpha-induced endothelial damage through the loss of its barrier function. *Methods Mol Med* 2004;98:127-136.
52. Lu Q, Harrington EO, Jackson H, Morin N, Shannon C, Rounds S. Transforming growth factor-beta1-induced endothelial barrier dysfunction involves Smad2-dependent p38 activation and subsequent RhoA activation. *J Appl Physiol* 2006;101:375-384.
53. Barnes JA, Singh S, Gomes AV. Protease activated receptors in cardiovascular function and disease. *Mol Cell Biochem* 2004;263:227-239.
54. Hollenberg MD. Physiology and pathophysiology of proteinase-activated receptors (PARs): proteinases as hormone-like signal messengers: PARs and more. *J Pharmacol Sci* 2005;97:8-13.
55. Goldblum SE, Brann TW, Ding X, Pugin J, Tobias PS. Lipopolysaccharide (LPS)-binding protein and soluble CD14 function as accessory molecules for LPS-induced changes in endothelial barrier function, in vitro. *J Clin Invest* 1994;93:692-702.
56. Miller SI, Ernst RK, Bader MW. LPS, TLR4 and infectious disease diversity. *Nat Rev Microbiol* 2005;3:36-46.
57. Noma K, Oyama N, Liao JK. Physiological role of ROCKs in the cardiovascular system. *Am J Physiol Cell Physiol* 2006;290:C661-C668.