# Papel de la cascada del ácido araquidónico en la función epitelial de barrera en un modelo de células intestinales Caco-2

M. José Rodríguez Lagunas

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FACULTAD DE FARMACIA

DEPARTAMENTO DE FISIOLOGÍA

# Papel de la cascada del ácido araquidónico en la función epitelial de barrera en un modelo de células intestinales Caco-2

M. José Rodríguez Lagunas

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FACULTAD DE FARMACIA

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# Papel de la cascada del ácido araquidónico en la función epitelial de barrera en un modelo de células intestinales Caco-2

Memoria presentada por M. José Rodríguez Lagunas para optar al Título de Doctor por la Universidad de Barcelona

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INFORMAN:

Que la memoria titulada "Papel de la cascada del ácido araquidónico en la función epitelial de barrera en un modelo de células intestinales Caco-2" presentada por M. JOSÉ RODRÍGUEZ LAGUNAS para optar al título de Doctor por la Universidad de Barcelona, ha sido realizada bajo nuestra dirección en el Departamento de Fisiología, y considerándola finalizada, autorizamos su presentación para ser juzgada por el tribunal correspondiente.

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Dra. Ruth Ferrer Roig                Dr. Juan José Moreno Aznárez

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"Las cosas importantes de la vida no son cosas"

A mis padres,
A Martina y Francisco

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| | |
|---|---|
| AAS | Ácido acetilsalicílico |
| AA | Ácido araquidónico |
| AGPI | Ácido graso poliinsaturado |
| AINE | Antiinflamatorio no esteroideo |
| AJ | *Adherens junction*, unión adherente |
| ALA | Ácido α-linolénico |
| AMPc | Adenosin monofosfato cícliclo |
| ATP | Adenosin trifosfato |
| BLT | Receptor para LTB |
| CD | *Chron's Disease*, enfermedad de Chron |
| COX | Ciclooxigenasa |
| CYP | Citocromo P450 |
| CysLT | Cisteinil leucotrieno |
| $CysLT_1R$ | Receptor para CysLT |
| DHA | Ácido docosahexaenoico |
| DHETE | Ácido dihidroxieicosatetraenoico |
| DP | Receptor para PGD |
| EET | Ácido epoxieicosatrienoico |
| EGF | *Epidermal Growth Factor*, factor de crecimiento epidérmico |
| EP | Receptor para PGE |
| EPA | Ácido eicosapentaenoico |
| ERK | *Extracellular regulated protein kinase* |
| FABP | *Fatty acid binding protein,* proteína transportadora de ácidos grasos |
| FLAP | *5-lipoxygenase-activating protein,* proteína activadora de la 5-LOX |
| FP | Receptor para $PGF_{2\alpha}$ |
| GJ | *Gap junction*, unión de comunicación |
| HEPE | Ácido hidroxieicosapentaneoico |
| HETE | Ácido hidroxieicosatetraenoico |
| HODE | Ácido hidroxioctadecadienoico |
| HPETE | Ácidos hidroperoxieicosatetraenoicos |
| IBD | *Inflammatory Bowel Disease*, enfermedad inflamatoria intestinal |
| IL-1 | Interleuquina 1 |
| INF-γ | Interferón γ |
| IP | Receptor para $PGI_2$ |
| JAM | *Junctional Adhesión Molecules*, moléculas de adhesión de la unión |
| LA | Ácido linoleico |
| LOX | Lipoxigenasa |
| LT | Leucotrieno |

| | |
|---|---|
| MLC | *Myosin Light Chain,* cadena ligera de miosina |
| MLCK | *Myosin Light Chain Kinase*, quinasa de la cadena ligera de miosina |
| NFkB | Factor nuclear kB |
| PDZ | Dominio denominado por las siglas de: proteína de densidad postsináptica (**P**SD95), proteína supresora de tumores en Drosophila (**D**lgA) y proteína Zonula Occludens-1 (**Z**O-1) |
| PG | Prostaglandina |
| PGI | Prostaciclina |
| PKA | Proteína quinasa A |
| PKC | Proteína quinasa C |
| $PLA_2$ | Fosfolipasa $A_2$ |
| PLC | Fosfolipasa C |
| PPAR | *Peroxisome Proliferator Activated Receptor,* receptor activador de la proliferación de peroxisomas |
| sEH | Enzima epóxido hidrolasa soluble |
| TER | Resistencia eléctrica transepitelial |
| TJ | *Tight junction*, unión estrecha |
| TNF-$\alpha$ | Factor de necrosis tumoral $\alpha$ |
| TP | Receptor para $TXA_2$ |
| TX | Tromboxano |
| UC | *Ulcerative colitis*, colitis ulcerosa |
| ZO | *Zonula Occludens* |

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El epitelio intestinal forma una barrera que permite el paso de nutrientes pero restringe el de substancias potencialmente nocivas. La alteración de dicha función se relaciona con enfermedades gastrointestinales como la enfermedad inflamatoria intestinal (IBD) en la que, además, la producción de eicosanoides en la mucosa intestinal se encuentra incrementada. Por ese motivo, el objetivo del presente trabajo ha sido investigar el papel de los eicosanoides producidos en la cascada del ácido araquidónico en la regulación de la función epitelial de barrera en un modelo intestinal de células Caco-2. La permeabilidad paracelular (PP) se ha estudiado en cultivos mantenidos sobre filtros, a partir de la determinación de la resistencia eléctrica transepitelial (TER) y de los flujos de dextrano. La concentración intracelular de $Ca^{2+}$ ($[Ca^{2+}]_i$) se ha determinado por espectrofluorimetría, y la de AMPc así como la activación de NFκB por enzima inmuno ensayo. La fosforilación de la cadena ligera de miosina (MLC) se ha realizado por western blot y la localización de las proteínas de la unión estrecha (TJ) por inmunofluorescencia. Los resultados revelan que la $PGE_2$, la $PGE_3$, el $LTD_4$ y los 5-, 12-(R)-, 12-(S)- y 15-HETE son capaces de romper la función barrera. En cambio, la $PGD_2$, el $LTB_4$, el 13-HODE, el 20-HETE, los 11,12- y 14,15-EET, los 11,12 y 14,15-DHETE y el 12-HEPE no tienen este efecto. La $PGE_2$ y $PGE_3$ incrementan la PP al interaccionar con los receptores $EP_1$ y $EP_4$ que a su vez activan la vía de la PLC-$IP_3$-$Ca^{2+}$ y la vía del AMPc-PKA, respectivamente. En el caso de $LTD_4$, se ha observado que el receptor implicado en la disrupción de la función barrera es el $CysLT_1R$. En el caso del 5-HETE se ha descartado la participación de $BLT_1$, $BLT_2$, $CysLT_1R$ y $CysLT_2R$. En el caso del $LTD_4$ y 5-HETE, además de inducir un incremento de la $[Ca^{2+}]_i$, se ha demostrado la activación de la vía de la PLC-$Ca^{2+}$/PKC y de la vía de la PKA independiente de AMPc y de NFκB. En el caso de 12-(R)-, 12-(S)- y 15-HETE también se ha observado un incremento de la $[Ca^{2+}]_i$ pero no de AMPc, a excepción de 12-(S)-HETE que sí que incrementa la concentración de AMPc. En todos los casos en los que un eicosanoide ha alterado la función epitelial de barrera se ha observado una relación entre el incremento de la PP y la redistribución hacia el citosol de las proteínas de la TJ ocludina y claudina-4, sin alteración de la localización de ZO-1, claudina-1 o claudina-2. Dichos eicosanoides, a excepción del 5-HETE, también incrementan la actividad de la MLCK provocando la desorganización del anillo de actina. A pesar de que la disrupción de la función epitelial de barrera observada en pacientes con IBD se ha atribuido tradicionalmente al efecto de citocinas proinflamatorias como TNF-α e IFN-γ, en la presente memoria se aportan evidencias del papel de los eicosanoides en la regulación de la PP. Además se aporta una posible explicación para los efectos negativos de la inhibición de la COX en pacientes con IBD y la posibilidad de nuevas estrategias terapéuticas. Si bien se ha descrito un efecto beneficioso del consumo de aceite de pescado o ácidos grasos n-3 en pacientes con IBD, éste no puede ser atribuido a la reducción de la relación $PGE_2/PGE_3$ ya que ambas PG tienen un efecto negativo sobre la función epitelial de barrera. Sin embargo, dicho efecto sí que podría atribuirse a la generación de 12-HEPE que al contrario que 12-HETE no altera la PP.

The intestinal epithelium forms a barrier that allows the passage of nutrients but restricts the passage of potentially harmful substances. Alteration of epithelial barrier function is the mechanism responsible for the persistence of inflammation that occurs in gastrointestinal diseases such as inflammatory bowel disease (IBD) in which various eicosanoids are increased in the mucosa. For this reason, the objective of this study was to investigate the role of eicosanoids produced by different pathways of AA metabolism in the regulation of epithelial barrier function in a Caco-2 cell model. The paracellular permeability (PP) was estimated in Caco-2 cell cultures from the transepithelial electrical resistance (TER) and dextran fluxes. Intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) was quantified by spectrofluorimetry, cAMP and NFκB levels by enzyme immunoassay, MLC phosphorylation by Western blot and the state of the tight junction (TJ) proteins by immunofluorescence. The results indicate that $PGE_2$, $PGE_3$, 5-, 12-(R)-, 12-(S)-, 15-HETE and $LTD_4$ are able to break the barrier function, whereas no alteration is induced by 13-HODE, 20-HETE, 11,12- and 14,15-EET, 11,12- and 14,15-DHETE or 12-HEPE. $PGE_2$ and $PGE_3$ alter the barrier function through the interaction with $EP_1$ and $EP_4$ receptors that activate the PLC-$IP_3$-$Ca^{2+}$ pathway and the cAMP-PKA pathway, respectively. Neither $BLT_1$, $BLT_2$, $CysLT_1R$ nor $CysLT_2R$ contribute to the 5-HETE-induced increase in PP. In the case of $LTD_4$ and 5-HETE, along with an increase in $[Ca^{2+}]_i$ there is an activation of PLC-$Ca^{2+}$/PKC and PKA independent of cAMP and NFκB pathways. For 12- and 15-HETE we have also observed an increase in $[Ca^{2+}]_i$ but not in cAMP, except for 12-(S)-HETE which did increase cAMP levels. From the study of the TJ protein immunolocalization, in all cases in which an eicosanoid has altered the epithelial barrier function a relationship can be observed between the increase in PP and the redistribution of occludin and claudin-4 without altering ZO-1, claudin-1 or -2 cellular distribution. Furthermore, the alteration of the actin ring and the increase in MLCK activity has been observed in all the eicosanoids that increased PP except for 5-HETE. Although the disruption of epithelial barrier function observed in IBD patients has been traditionally attributed to the effect of proinflammatory cytokines such as TNF-α and IFN-γ, this thesis evidences a role for the eicosanoids in the regulation of PP, opening up possibilities for new therapeutic strategies. In this regard, the increase in PP induced by LOX metabolites is a possible explanation for the negative effects of the inhibition of COX in IBD patients. Furthermore, the beneficial effect described for fish oil or n-3 fatty acids in IBD patients can not be attributed to the reduction of $PGE_2/PGE_3$ ratio since both PG have a deleterious effect on epithelial barrier function. However, it could be attributed to the generation of 12-HEPE which, unlike 12-HETE, does not increase PP.

# Introducción

# 1. Uniones intercelulares

Las células epiteliales forman barreras selectivas entre los tejidos y los compartimentos del organismo. Dichas células se encuentran polarizadas, presentando un dominio apical y otro basolateral, y se unen unas a otras a través de complejos proteicos que forman uniones intercelulares (Figura 1). Estas uniones no sólo presentan la función de adhesión intercelular, sino que además desarrollan funciones de señalización capaces de regular numerosas funciones fisiológicas.

La integridad estructural del epitelio intestinal se mantiene gracias a cuatro sistemas de adhesión: la unión estrecha (*Tight Junction*, TJ), la unión adherente (*Adherens Junction*, AJ), la unión de comunicación (*Gap Junction*, GJ) y el desmosoma. De entre ellos, la TJ es el componente más apical de la membrana basolateral responsable de formar una barrera a la difusión paracelular de moléculas de elevado peso molecular. Además, forma una barrera que impide el intercambio de componentes de la membrana del dominio apical a la del basolateral y viceversa, manteniendo así la polaridad de la célula. Además, participan en los procesos de proliferación y diferenciación celular. La TJ y la AJ están asociadas al citoesqueleto de actina y el desmosoma se asocia a los filamentos intermedios. La GJ forma poros intercelulares que permiten el intercambio entre células de moléculas de bajo peso molecular hidrofílicas y están distribuidas, al igual que el desmosoma, a lo largo de la membrana basolateral (Matter y Balda, 2003).

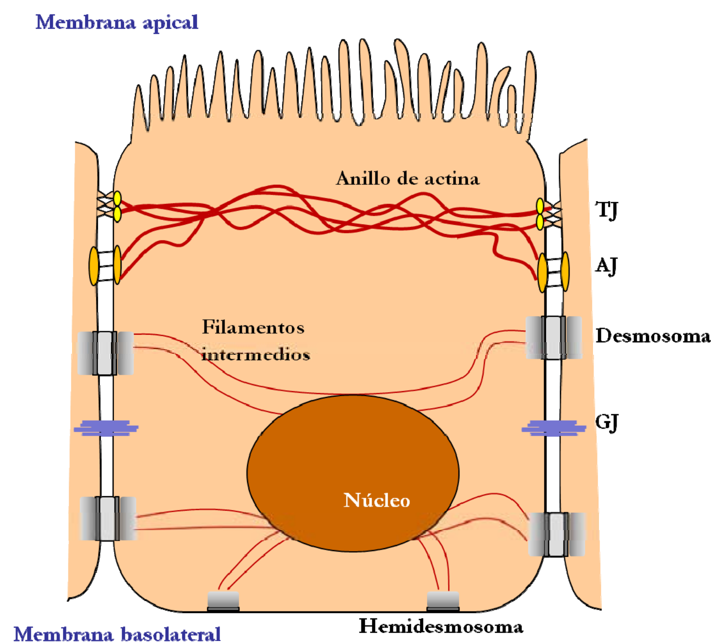


**Figura 1. Uniones intercelulares.** La imagen muestra las uniones intercelulares existentes en células epiteliales: unión estrecha (TJ), unión adherente (AJ), desmosoma y unión de comunicación (GJ).

## *1.1. Unión estrecha o Tight Junction (TJ)*

La TJ está formada por un conjunto amplio de más de 40 proteínas diferentes, que ejercen funciones de señalización, de regulación de la transcripción y del ciclo celular, de mantenimiento de la polaridad, de tráfico intracelular de vesículas, así como de función barrera (Anderson y Van Itallie, 2009).

Las proteínas de la TJ se pueden clasificar en proteínas transmembrana y proteínas citoplasmáticas (que forman la placa citoplasmática). Las proteínas transmembrana, a su vez, se clasifican en función del número de pasos de membrana (*tetraspan* o *singlespan*) (Figura 2). Así, las claudinas, la ocludina y la tricelulina presentan cuatro pasos de membrana y pueden determinar la selectividad de la difusión paracelular. Por otra parte, las moléculas de adhesión de la unión (*Junctional adhesión molecules,* JAM), entre otras, presentan un único paso de membrana (Balda y Matter, 2008).

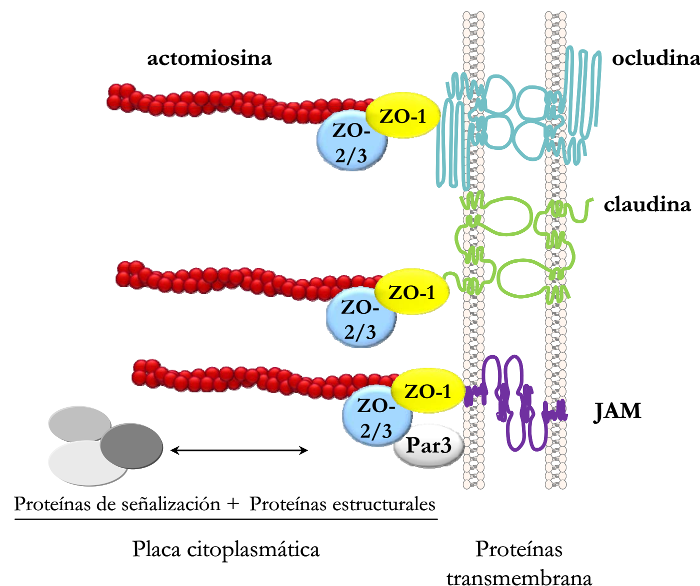


**Figura 2. Esquema de la TJ.** La imagen muestra las principales proteínas transmembrana (claudinas, ocludinas y JAM) de la TJ que interaccionan en la vía paraceular con las proteínas de la célula adyacente. Los dominios intracelulares de las proteínas transmembrana interaccionan con proteínas estructurales que unen las TJ al anillo de actina y con proteínas de señalización. Adaptado de Groschwitz y Hogan (2009).

### 1.1.1. Proteínas transmembrana

La familia de las claudinas está constituida por al menos 24 proteínas expresadas de manera específica en función del tejido. El tipo de claudinas de una TJ determina la selectividad de la vía paracelular a los iones. Los bucles extracelulares de las claudinas crean interacciones homofílicas y heterofílicas con las claudinas de las células adyacentes dando lugar a poros acuosos con cargas selectivas que permiten o restringen la difusión pasiva de iones entre células (Van Itallie y Anderson, 2006).

Las claudinas también interaccionan con proteínas citoplasmáticas con dominios denominados PDZ -por las siglas de tres proteínas: proteína de densidad postsináptica (PSD95), proteína supresora de tumores en *Drosophila* (DlgA) y proteína *Zonula Occludens-1* (ZO-1)- como la ZO-1 que además constituye el nexo de unión con la actina (Balda y Matter, 2008). Las claudinas tienen diferentes funciones y se pueden clasificar en dos grupos: las relacionadas con la formación de la barrera (disminuyen la permeabilidad paracelular, PP) y las implicadas en la formación de poros (incrementan la PP) (Van Itallie y Anderson, 2006). En el epitelio intestinal, las claudinas 1, 3, 4, 5, 8, 9, 11 y 14 están implicadas en el establecimiento de la función barrera, mientras que las claudinas 2, 7, 12 y 15 participan en la formación de poros (Suzuki, 2013).

La ocludina establece interacciones homofílicas a través de bucles extracelulares entre células adyacentes y también contribuye a la creación de una barrera selectiva (Aijaz y col., 2006; Al-Sadi y col., 2011). La ocludina interacciona con diversas proteínas intracelulares de la TJ como las proteínas ZO, que son necesarias para unir la ocludina al citoesqueleto de actina (Furuse y col., 1994). Las funciones de la ocludina no son del todo conocidas, aunque se sabe que es necesaria para mantener la estructura de la TJ y la permeabilidad del epitelio intestinal. El estado de fosforilación de la ocludina regula la localización de dicha proteína y la PP (Suzuki, 2013). Algunas quinasas como la proteína quinasa C (PKC) η y ζ, entre otras, se han identificado como responsables de su fosforilación, además, la inhibición de la expresión y la actividad de estas quinasas inhibe la fosforilación de la ocludina dando lugar a la disrupción de la TJ (Suzuki y col., 2009; Jain y col., 2011).

La tricelulina se encuentra principalmente en las uniones en las que convergen tres células y es esencial para la formación de la función barrera. Al igual que la ocludina, se une a ZO-1 (Riazuddin y col., 2006). En cuanto a las JAM se han descrito cuatro isoformas (A, B, C y D) que interaccionan con proteínas citoplasmáticas como ZO-1 y PAR3. Cuando se unen de manera homofílica participan en la formación de la TJ (Suzuki 2013).

### 1.1.2. Proteínas de la placa citoplasmática

Las proteínas de la placa citoplasmática forman una interfaz entre las proteínas transmembrana y las proteínas del citoesqueleto. Intervienen en la regulación de la adhesión y de la PP así como en procesos relacionados con la expresión génica. Las proteínas de la placa citoplasmática se pueden agrupar en proteínas estructurales periféricas como ZO-1, ZO-2, ZO-3, AF6 y cingulina -que parecen organizar las proteínas transmembrana uniéndolas a otras proteínas citoplasmáticas y a los microfilamentos de actina- y en proteínas de señalización como ZONAB, RhoA, RalA y Raf 1 -que además de participar en la formación de la TJ regulan la transcripción génica- (Forster, 2008).

La ZO-1 tiene funciones estructurales y contiene diferentes dominios de interacción con proteínas, de los cuales tres son dominios PDZ. A través del primer dominio PDZ interacciona con las claudinas, a través del segundo dominio con la ZO-2 o ZO-3 y a través del tercer dominio con la ocludina, la actina, las proteínas de la AJ y otras proteínas de señalización (Suzuki 2013).

### *1.2. Unión adherente o Adherent Junction (AJ)*

Las principales funciones de la AJ son conectar las células entre si para regular la morfogénesis durante el desarrollo embrionario y mantener la estructura de los tejidos sólidos en el adulto (Gumbiner, 1996).

La AJ está formada por dos proteínas transmembrana, las cadherinas y las lectinas, que se unen al citoesqueleto de actina a través de otras proteínas como las cateninas (β-catenina, γ-catenina y p120-catenina) y la afadina, respectivamente (Figura 3). Estas interacciones generan una red transcelular de filamentos de actina (Ebnet, 2008).

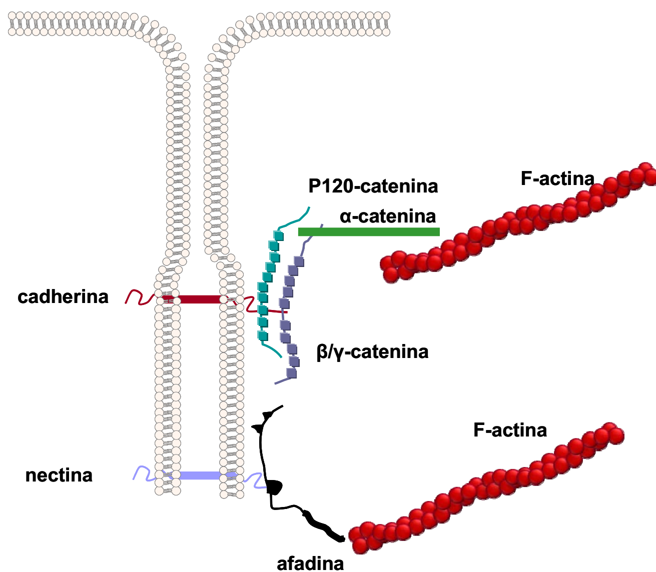


**Figura 3. Esquema de la AJ.** La imagen muestra los principales complejos de proteínas de la AJ en células epiteliales. Un complejo está formado por cadherinas (E-cadherina) unida a catenina (p120-catenina y β/γ-catenina) y el otro por nectina unida a afadina. Ambos complejos interaccionan a través de diferentes proteínas con el anillo subapical de actina. Adaptado de Ebnet (2008)

### *1.3. Desmosomas*

Los desmososmas forman uniones cruciales entre células en tejidos sometidos a estrés físico como el corazón, la vejiga, la piel o el epitelio intestinal. En los desmosomas, se pueden diferenciar tres zonas (Figura 4): el desmoglea, la región más interna, la placa densa citoplasmática exterior y la placa densa interior. En esta

estructura participan glicoproteínas transmembrana de la familia de las cadherinas (desmogleinas y desmocolinas) cuyos extremos extracelulares median la adhesión entre células y forman el desmoglea. Los dominios intracelulares de las cadherinas se asocian con las proteínas de la placa densa exterior. En esta placa citoplasmática las cadherinas se unen a miembros de la familia de las proteínas del armadillo y a miembros de la familia de las proteínas plaquina que a su vez interaccionan con filamentos intermedios del citoesqueleto formados principalmente por queratina y constituyen la placa densa exterior (Delva y col., 2009).

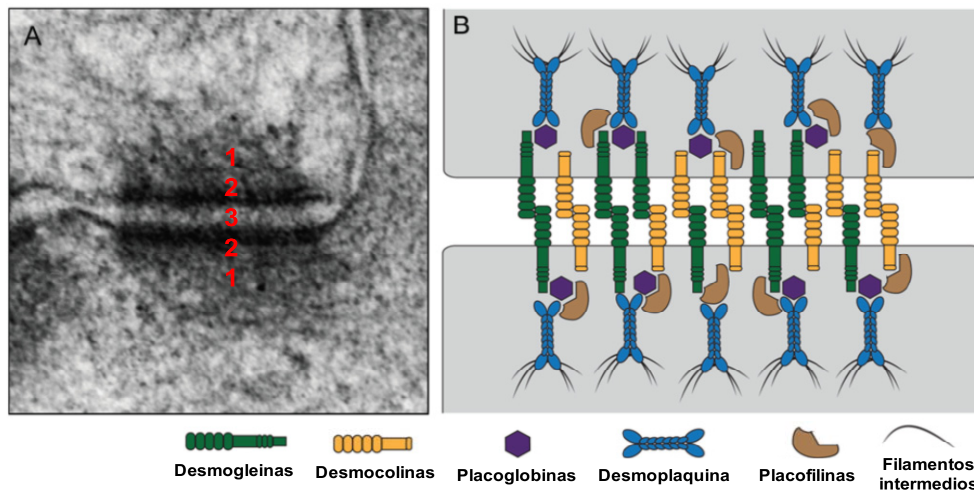


**Figura 4**. **Estrucutra de los desmosomas.** A) Imagen al microscopio electrónico de un desmosoma en formación conectando dos células adyacentes, los números en rojo corresponden a 1) placa densa interna, 2) placa densa externa, 3) desmoglea. B) Representación esquemática de la estructura de un desmosoma. Las desmogleinas y desmocolinas constituyen una unión extracelular entre células y forman homo y heterodímeros con las placoglobinas y las placofilinas. Las placoglobinas se unen a desmoplaquinas y éstas a los filamentos internos de actina. Adaptado de Brooke y col. (2012)

## *1.4. Unión de comunicación o Gap Junction (GJ)*

Las proteínas de la GJ forman canales intercelulares que permiten la difusión directa de iones y moléculas de bajo peso molecular entre células adyacentes. Los canales están formados por seis conexinas unidas formando un conexón (Figura 5).

Se han descrito 20 subtipos diferentes de conexinas, en humanos, la combinación de las cuales da lugar a diferencias en el tamaño del poro y en la expresión tisular. Por ejemplo, el canal Cx32 permite el paso de nucleótidos y glucosa y el canal Cx43 permite el paso de adenosin trifosfato (ATP). A través de los canales se pueden producir procesos como la transmisión de potenciales de acción (sinapsis eléctricas) y la difusión de autacoides y nutrientes (Giepmans, 2004).

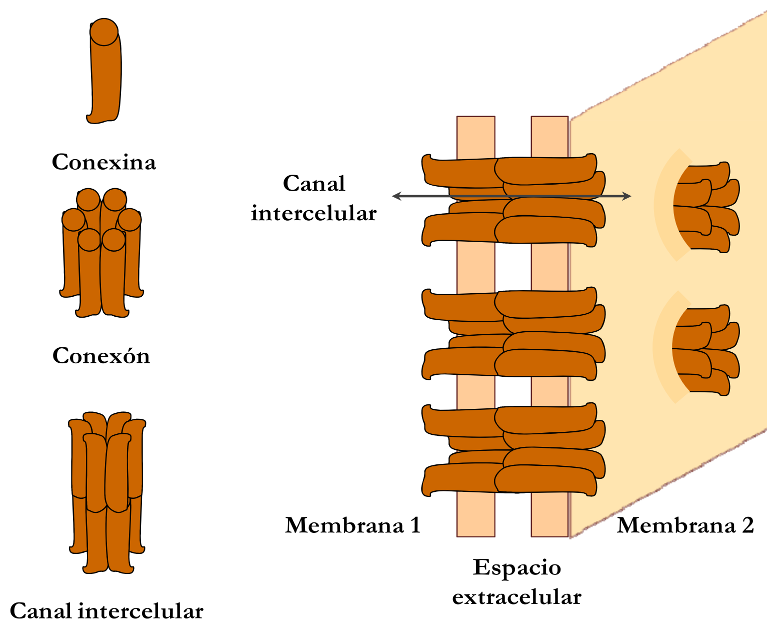


**Figura 5. Estructura de la unión de comunicación.** Las conexinas se unen en hexámeros llamados conexones y se anclan a la superfície de la membrana. Una vez allí, se unen a los conexones de las células adyacentes formando un canal intercelular que comunica las dos membranas plasmáticas y estrecha el espacio extracelular. Adaptado de Goodenough y Paul (2009).

## *1.5. Función epitelial de barrera*

El epitelio intestinal forma la mayor y más importante barrera orgánica entre el medio externo y el interno. Está constituido por una monocapa de células que recubre la pared intestinal y cuya función principal es formar una barrera que evita el paso de substancias potencialmente nocivas como antígenos o microorganismos pero que, a su vez, permite el paso de nutrientes, electrolitos y agua desde la luz intestinal a la circulación sistémica. El epitelio intestinal permite el paso de substancias a través de dos vías: la vía paracelular y la vía transcelular. La permeabilidad transcelular consiste en la difusión simple o el transporte de solutos primero a través de la membrana apical y en segundo lugar a través de la membrana basolateral. La vía paracelular está asociada a la difusión simple de substancias a través del espacio intercelular y está determinada por la permeabilidad de las uniones intercelulares. La función barrera se mantiene gracias a la expresión de las AJ y TJ que permiten la unión entre células adyacentes y que proporcionan un anclaje al citoesqueleto (Groschwitz, 2009). Dicha barrera permite el paso de moléculas hidrofílicas de bajo peso molecular. El paso de iones a través de dicha barrera genera una diferencia de potencial que experimentalmente se evalúa a través de la resistencia eléctrica transepitelial (TER) (Anderson y Van Itallie, 2009).

La expresión de las proteínas de la TJ en el intestino está regulada y es específica para cada región del tracto intestinal. La internalización de las proteínas de la TJ hacia el citoplasma, así como su degradación induce un incremento en la permeabilidad intestinal. Cambios en la fosforilación de proteínas como la ZO-1 o la ocludina pueden causar su internalización de manera que se desestabiliza la función barrera. La TJ también puede ver incrementada su permeabilidad por la

contracción del anillo de actina que se asocia a los filamentos de miosina de tal forma que la tensión generada en el anillo subapical de actomiosina incrementa el espacio intercelular. Así, en las células intestinales, la unión del factor de necrosis tumoral (TNF) α a su receptor de membrana induce una cascada de señales que incrementa la expresión y la actividad de la quinasa de la cadena ligera de miosina (*Myosin Light chain Kinase*, MLCK) que a su vez fosforila la miosina y da lugar a la contracción del anillo subapical de actina. Éste es un mecanismo por el cual, la vía paracelular se hace más permeable a patógenos dando lugar a un círculo vicioso en el que la integridad de la barrera se ve comprometida (Barrett, 2008).

### 1.5.1. Alteraciones de la función epitelial de barrera

La alteración de la TJ está implicada en gran variedad de procesos patológicos como las infecciones virales y bacterianas, la inflamación y el cáncer, entre otros. Por ejemplo, la alteración de la ocludina se ha asociado con la colitis colagenosa, la de la claudina-4 con infecciones con *Clostridium perfringens* y la alteración de la ZO-1 con la enfermedad inflamatoria intestinal (IBD) (Forster, 2008).

Muchas citocinas como la interleuquina (IL)-1β, IL-3, IL-4, TNF-α e interferón (IFN) γ alteran la función epitelial de barrera al modificar el estado de la TJ o del citoesqueleto (Forster, 2008). La secreción de TNF-α e IFN-γ en el epitelio intestinal inflamado induce un incremento de la PP que se caracteriza por un aumento de los flujos de dextrano y una disminución de la TER. El mecanismo por el cual TNF-α induce la disrupción de la función barrera se basa en la activación de NFκB y la fosforilación de la MLC por la MLCK (Chen y col., 2012). En el caso del IFN-γ también se produce un incremento de la forforilación de la MLC, pero a diferencia de TNF-α, éste está mediado por la Rho quinasa (Utech y col., 2005). Ambas citocinas actúan de forma sinérgica ya que el IFN-γ induce en las células epiteliales la expresión del receptor para TNF-α de tipo $TNFR_2$ potenciando el efecto del TNF-α (Wang y col., 2006). Además, tanto el TNF-α como el IFN-γ reducen la expresión de la claudina-2 mientras que la citocina proinflamatoria IL-13 incrementa la expresión de dicha proteína (Forster, 2008). A pesar de estas discrepancias y del desconocimiento de los mecanismos exactos involucrados en la disfunción de la función epitelial de barrera inducida por citocinas proinflamatorias, la recuperación de la función barrera es clave para restaurar la homeóstasis del epitelio intestinal. En este sentido, en la actualidad existen tratamientos farmacológicos con antiinflamatorios o antitumorales cuyo objetivo es revertir la alteración de la función epitelial de barrera (Forster, 2008).

# 2. Eicosanoides

Los eicosanoides son mediadores con acción autocrina o paracrina derivados de la oxidación de los ácidos grasos de veinte átomos de carbono. Su papel en la homeóstasis del epitelio intestinal es muy diverso (Ferrer y Moreno, 2010).

## *2.1. Ácidos grasos poliinsaturados*

Los ácidos grasos poliinsaturados (AGPI) son componentes importantes de la membrana celular ya que determinan su fluidez así como el comportamiento de enzimas y receptores anclados a ésta. De esta forma, intervienen en la regulación de un amplio rango de funciones orgánicas tales como la presión arterial, la coagulación y el desarrollo y función del cerebro y del sistema nervioso y además, son precursores de los eicosanoides (Wall y col., 2010).

Los AGPI se clasifican en función de la posición del primer doble enlace desde el último grupo metil indicado con la letra ω o n seguida de un número. Bajo este criterio, existen tres grandes familias de AGPI: n-3, n-6 y n-9. Las células del organismo disponen de toda una serie de enzimas denominadas elongasas y desaturasas responsables de la elongación y de la introducción de dobles enlaces, respectivamente (Figura 6). Estos procesos se producen a partir de los AG esenciales n-6 linoleico (LA, C18:2) y n-3 α-linolénico (ALA, C18:3). Las células de los mamíferos pueden convertir de esta forma el LA de la dieta en ácido araquidónico (AA, C20:4) y el ALA en ácido eicosapentaenoico (EPA, C20:5) y ácido docosahexaenoico (DHA, C22:6) (Kim y col., 2010).

En general, los n-6 son abundantes en aceites vegetales y los n-3 en productos marinos. En los vegetales, el LA se encuentra principalmente en los aceites de semillas (girasol, maíz, germen de trigo, soja y cacahuete) y en margarinas y el ALA está presente, principalmente, en aceites vegetales como el de soja, colza y linaza y las nueces. Los AGPI EPA y DHA están presentes en pescados con un alto contenido en grasa como el atún, el salmón, la sardina, el arenque y la caballa y en el marisco (Russo, 2009).

El LA y el ALA suponen el 95% de la ingesta de AGPI en la mayoría de dietas occidentales. Dado que el LA y el ALA se metabolizan a través de los mismos enzimas, existe competencia entre ellos. En general, las desaturasas y elongasas presentan mayor afinidad para los AG n-3 que para los n-6 a una relación 1:1-4. Sin embargo, hoy en día, la ingesta de AG en los países occidentales es mayoritaria en LA siendo la relación de AGPI n-6/n-3 entre 5 y 20 (Calder, 2008) y dando lugar, por lo tanto, a una mayor conversión a AA (Wall y col., 2010).

Los eicosanoides se sintetizan en los mamíferos mayoritariamente a partir del AA, si bien también se pueden producir a partir de los AGPI n-3 como el EPA y el DHA dando lugar a eicosanoides de estructura similar (prostanoides de la serie 3 y leucotrienos de la serie 5) pero que pueden tener acciones diferentes a los metabolitos del AA (Eberhart y Dubois, 1995).

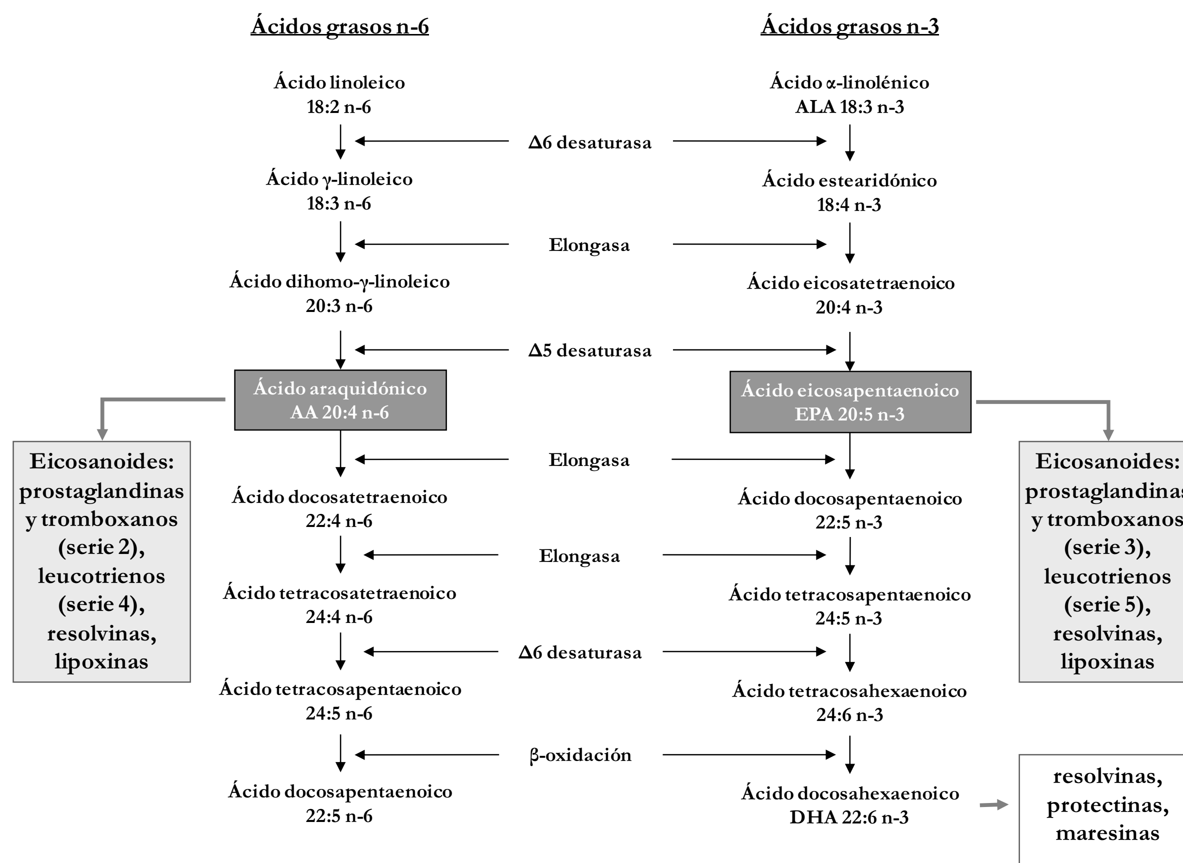


**Figura 6. Metabolismo de los ácidos grasos n-6 y n-3.** Los AGPI n-3 y n-6 son metabolizados a través de diferentes etapas de elongación y desaturación para dar lugar a diferentes AG, entre ellos el AA y el ácido eicosapentaenoico que pueden ser metabolizados por diferentes enzimas para dar lugar a los eicosanoides. Adaptado de Wall y col. (2010).

## *2.2. Cascada del ácido araquidónico*

El AA es un constituyente de las membranas celulares y se encuentra esterificado en la posición sn-2 de los glicerofosfolípidos (Chen y col., 2001). En condiciones fisiológicas, la concentración en el medio interno de AA libre es baja. Cuando los tejidos están expuestos a estímulos fisiológicos o fisiopatológicos como citocinas, factores de crecimiento u hormonas, es liberado de la membrana celular por acción, esencialmente, de fosfolipasa $A_2$ ($PLA_2$). La $PLA_2$ comprende una gran familia de diferentes enzimas que incluyen cuatro grupos principalmente; las secretadas

($sPLA_2$), las citosólicas ($cPLA_2$), las independientes de $Ca^{2+}$ ($iPLA_2$) y las denominadas *platelet activating factor (PAF) acetyl hydrolase/oxidized lipid lipoprotein associated* ($LpPLA_2$) (Burke y Dennis, 2009).

El metabolismo posterior del AA se produce a través de tres vías: la vía de la ciclooxigenasa (COX), la vía de la lipoxigenasa (LOX) y la vía del citocromo P450 monooxigenasa (CYP) (Harizi y col., 2008) que dan lugar a los diferentes eicosanoides (Figura 7).

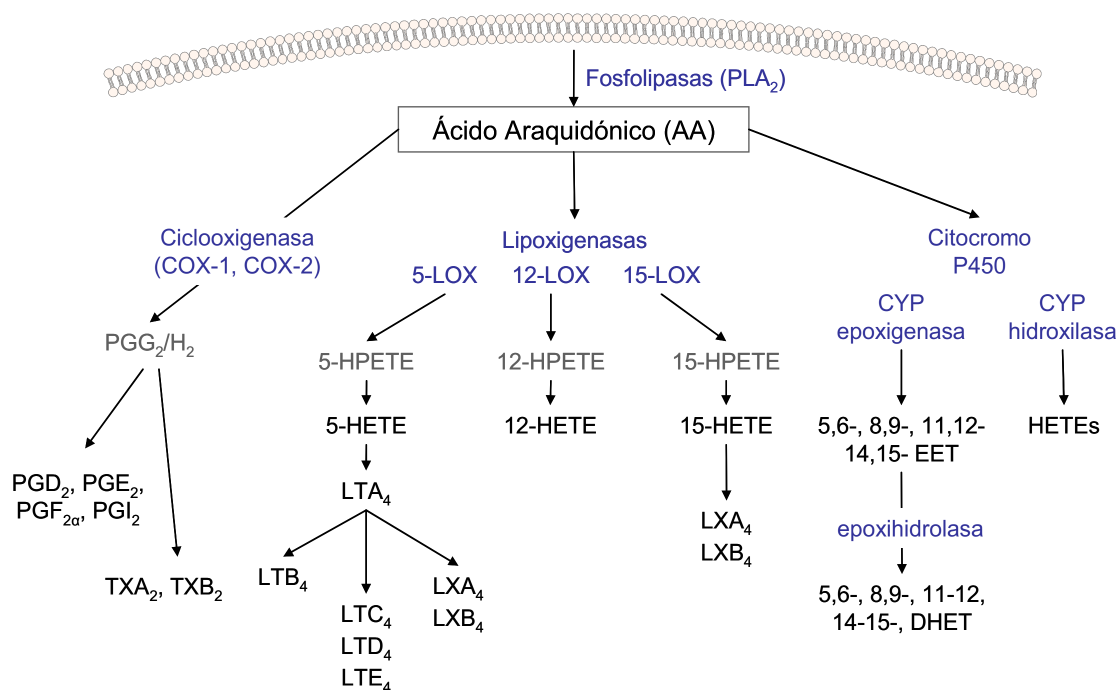


**Figura 7. Cascada del AA.** Una vez librerado de la membrana por la $PLA_2$, el AA puede ser metabolizado a través de 3 vías principales. A través de la vía de la ciclooxigenasa (COX) se producen prostaglandinas (PG), prostaciclina (PGI) y tomboxanos (TX), a través de la vía de la lipoxigenasa (LOX) se producen los ácidos hidroxieicosatetraenoicos (HETE), los leucotrienos (LT) y las lipoxinas (LX) y a través de la vía del citocromo P450 (CYP) se producen los ácidos epoxieicosatrienoicos (EET) y sus metabolitos correspondientes, los ácidos dihidroxieicosatetraenoicos (DHET) y HETEs.

### 2.2.1. Vía de la ciclooxigenasa

El AA se metabolizada por la prostaglandina G/H sintasa o COX dando lugar a la prostaglandina (PG) $G_2$ y posteriormente, a un endoperóxido cíclico inestable denominado $PGH_2$ que a través de diferentes procesos químicos o enzimáticos genera las PG, la prostaciclina (PGI) o los tromboxanos (TX) (Capdevila y col., 2000). Existen cuatro PG: $PGD_2$, $PGE_2$, $PGF_{2\alpha}$ y $PGI_2$ que actúan de forma autocrina y paracrina como mediadores para mantener la homeóstasis local

(Ricciotti y FitzGerald, 2011). La producción de PG depende de la actividad de una enzima, la COX, que puede ejercer tanto la actividad ciclooxigenasa como peroxidasa y que presenta dos isoformas (COX-1 y COX-2). La COX-1 se expresa de forma constitutiva en la mayoría de células y mantiene la concentración fisiológica de PGs. Su expresión regula funciones como, por ejemplo, la citoprotección gástrica y la hemostasia. La actividad de la COX-2 se induce por estímulos inflamatorios, hormonas, factores de crecimiento, lipopolisacáridos, citocinas y promotores de tumores (Harizi y col., 2008; Ricciotti y FitzGerald, 2011). Por ello, es responsable de incrementar la producción de PGs en procesos inflamatorios y enfermedades proliferativas como el cáncer. Durante el proceso inflamatorio, tanto la concentración como el perfil de la producción de PGs cambian drásticamente. Normalmente, la producción de PG es baja en tejidos no inflamados pero aumenta inmediatamente en procesos agudos de inflamación, previo al reclutamiento y a la infiltración de células inmunitarias (Ricciotti y FitzGerald, 2011).

Ambas enzimas son la diana de los fármacos llamados antiinflamatorios no esteroideos (AINE). El ácido acetilsalicílico (AAS) produce la acetilación de una serina en el dominio de unión al substrato y en el caso de la COX-1, es capaz de bloquearlo completamente. En el caso de la COX-2, el AAS es capaz de bloquear la conversión a $PGH_2$ pero la función oxigenasa persiste dando lugar a la conversión al ácido 15-hidroxieicosatetraenoico (15-HETE) (Eberhart y Dubois, 1995).

### 2.2.2. Vía de la lipoxigenasa

Los mamíferos expresan por lo menos tres LOX; la 5-, 12- y 15-LOX que dan lugar a diversos regioisómeros de hidroperóxidos arílicos, los ácidos hidroperoxieicosatetraenoicos (HPETE). La 5-LOX da lugar al 5-HPETE, la 12-LOX al 12-HPETE y la 15-LOX al 15-HPETE. Los HPETE se convierten rápidamente al correspondiente HETE (5-, 12- o 15-HETE). Además cada HETE puede presentar la estereoconfiguración R o S ya que las LOX son esteroespecíficas, las S-LOX predominan en el colon y forman más del 99% de S-HETE y menos del 1% de R-HETE. Las R-LOX no se han detectado en colon si bien los R-HETE pueden metabolizarse por otras vías como la del CYP (Neilson y col., 2012).

La 5-LOX es una dioxigenasa soluble que cataliza la conversión del AA en 5-HPETE mediante la incorporación de un oxigeno en la posición C5 del AG (Figura 8). El 5-HPETE se metaboliza a continuación por la 5-LOX al epóxido inestable $LTA_4$. El $LTA_4$, en función de las enzimas presentes, puede convertirse en el $LTC_4$ -por acción de la $LTC_4$ sintasa u otras enzimas capaces de conjugar el epóxido inestable con glutatión-, en el $LTB_4$ -por acción de la $LTA_4$ hidrolasa- o puede dar lugar a lipoxinas (LX). Una vez liberado al medio extracelular, el $LTC_4$ se

convierte en el $LTD_4$ y a continuación en el $LTE_4$ (Capdevila, 2000; Werz, 2002). En este sentido, la síntesis de los diferentes LT depende de la producción y distribución local de precursores y enzimas específicos en determinadas células (Harizi y col., 2008). Los LT también pueden sintetizarse gracias a la acción de enzimas situadas en diferentes tipos celulares (síntesis transcelular). Así, el $LTA_4$ producido en los neutrófilos puede convertirse en el $LTC_4$ en las plaquetas o en el epitelio vascular y en $LTB_4$ en los eritrocitos.

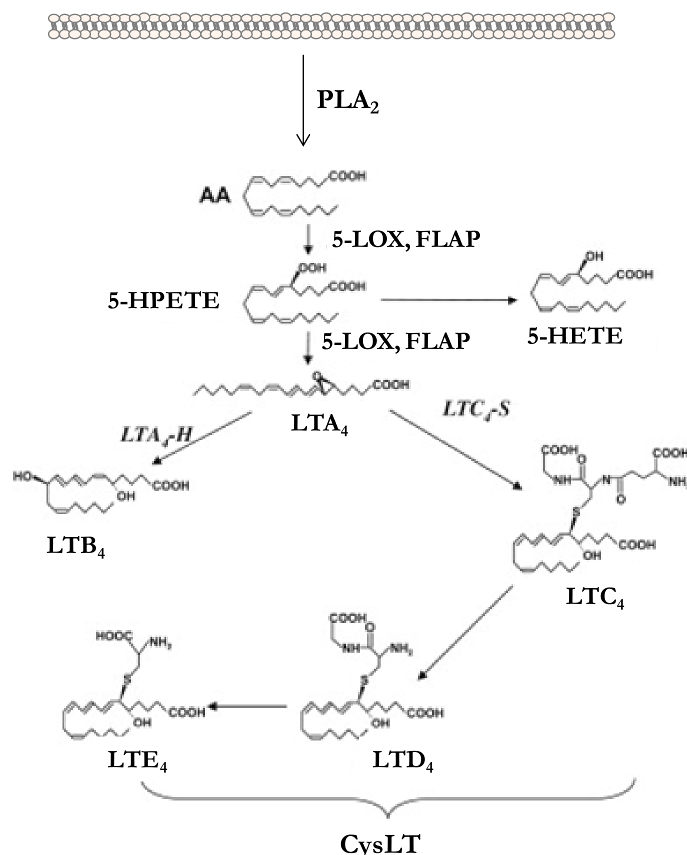


**Figura 8. Esquema de la cascada del AA por la vía de la 5-LOX.** Una vez liberado de la membrana el AA es metabolizado a través de la 5-LOX dando lugar a $LTB_4$ y a los cisteinil leucotrienos. Extraido de Camacho y col. (2012)

La actividad de la 5-LOX se ve incrementada por acción de la proteína activadora de la 5-LOX (*5-lipoxygenase-activating protein,* FLAP), una proteína integral de membrana que a pesar de no tener acción enzimática aumenta la afinidad por el AA (Peters-Golden y Henderson, 2007). La inhibición farmacológica de la FLAP previene la translocación de la 5-LOX desde el citosol a la membrana e impide su activación. Existen diferentes fármacos que interaccionan en diversos puntos de la vía de la LOX. Por ejemplo, los AINE además de inhibir la COX pueden incrementar la producción de LT y los corticoides pueden incrementar la expresión del receptor $BLT_1$ para $LTB_4$ en neutrófilos (Stankova y col., 2002). Existen inhibidores específicos de la 5-LOX como el zileuton cuyo efecto bloquea la

producción de LT aunque su uso es limitado ya que es hepatotóxico y precisa de monitorización de los niveles de enzimas hepáticos.

La enzima 12-LOX cataliza la conversión del AA al 12-HETE y en menor proporción al 15-HETE y la conversión de EPA al ácido 12-(S)-hidroxieicosapentaneoico (12-(S)-HEPE). Se han ensayado diferentes inhibidores de la 12-LOX tanto en animales como en humanos, sin embargo, debido a la falta de eficacia o a los efectos adversos que presentan no han dado lugar a ningún fármaco hasta el momento (Yeung y Holinstat, 2011).

La enzima 15-LOX metaboliza el AA dando lugar al 15-HETE y en menor proporción al 12-HETE y también es capaz de catalizar la conversión del LA al ácido 13-(S)-hidroxioctadecadienoico (13-HODE) y del DHA a resolvinas y protectinas. Los metabolitos de la 15-LOX se consideran con actividad antiinflamatoria, así, tanto el 15-HETE como el 13-HODE son ligandos de los receptores activadores de la proliferación de peroxisomas (*peroxisome proliferator activated receptors*, PPAR) γ cuya activación reduce la expresión de moléculas proinflamatorias como TNF-α, IL-1β y IL-6 (Wittwer y Hersberger, 2007). 15-HETE también puede ser metabolizado posteriormente por la 5-LOX dando lugar a $LXA_4$ y $LXB_4$, también con actividad antiinflamatoria.

### 2.2.3. Vía del citocromo P450

Las enzimas del CYP son oxidasas que catalizan un gran número de reacciones químicas y tienen como sustrato una elevada cantidad de moléculas, entre ellas, AG como el AA. Éste se puede metabolizar a través del CYP con actividad epoxigenasa dando lugar a los ácidos epoxieicosatrienoicos (EET) y a través de la oxidación bis-alílica y de la hidroxilación formando HETE (Fleming, 2007). Se han identificado 57 genes CYP divididos en 15 subfamilias, de los cuales, CYP2C y CYP2J son genes con acción epoxigenasa (y por tanto formadores de EET) (Thomson y col., 2012). Las enzimas del CYP incorporan un átomo de oxígeno a uno de los 4 dobles enlaces del AA dando lugar a: 5,6-EET, 8,9-EET, 11,12-EET y 14,15-EET. Además, cada EET puede presentar la estereoconfiguración S/R o R/S y por tanto los CYP pueden formar ocho potenciales EETs. Sin embargo, no todas las enzimas son capaces de sintetizar todos los EETs, por ejemplo, CYP2C8 produce 14,15-EET y 11,12-EET a una relación de 1,25:1 y CYP2C9 produce 14,15-EET, 11,12-EET y 8,9-EET a una relación de 2,3:1:0,5 (Thomson y col., 2012). Además, pueden formar diferente proporción de enantiómeros, por ejemplo, CYP2C8 produce 11,12-EET R/S en un 82% mientras que CYP2C10 produce S/R en un 69% (Daikh y col., 1994). Los principales eicosanoides producidos por los CYP con actividad epoxigenasa son 14,15-EET y 11,12-EET (Capdevila y col., 2000).

Una vez formados, los EET son metabolizados por diferentes vías siendo la principal vía catabólica la conversión a su ácido dihidroxieicosatrienoico (DHET) correspondiente mediante la enzima epóxido hidrolasa soluble (sEH) (Harizi y col., 2008; Behm y col., 2009). Los DHET se consideran menos activos y son más polares, por lo que difunden rápidamente a otros tejidos. En las células en las que hay baja actividad sEH o se encuentra inhibida, se produce la β-oxidación o la elongación de la cadena. También pueden conjugarse con glutatión u oxidarse por acción de la CYP ω-oxidasa en el caso de 8,9-, 11,12- y 14,15-EET. Los regioisómeros 5,6- y 8,9-EET son también substrato de la COX, dando lugar a análogos de la 5,6-epoxi$PGE_1$ y 11-hidroxi-8,9-EET, respectivamente. Finalmente, son capaces de incorporarse a los fosfolípidos a través de un proceso dependiente de la coenzima A (Zeldin, 2001; Spector y Norris, 2007).

Las enzimas del CYP también median la reacción de oxidación bis-alílica dando lugar a seis HETE diferentes (5-, 8-, 9-, 11-, 12- y 15-HETE) similares a los producidos por la vía de la LOX (Capdevila y Falck, 2002). La actividad hidroxilasa del CYP produce 16-, 17-, 18-, 19- y 20-HETE. Los CYP sintetizan predominantemente R-HETE in vitro (Bylund y col., 1998), aunque la estereoespecificidad parece ser específica del tejido por lo que las ratios de R/S en el colon son desconocidas (Neilson y col., 2012).

## *2.3. Receptores y vías de señalización de los eicosanoides*

Los eicosanoides ejercen sus efectos principalmente a través de la unión a receptores específicos de membrana.

### 2.3.1. Receptores y vías de señalización de las PG y los TX

Se han descrito nueve receptores para las PG: cuatro que reconocen a $PGE_2$ ($EP_1$-$EP_4$), dos que reconocen a $PGD_2$ ($DP_1$ y $DP_2$) y tres que reconocen a $PGF_{2\alpha}$, $PGI_2$ y $TXA_2$ (FP, IP y TP, respectivamente). IP, $DP_1$, $EP_2$ y $EP_4$ actúan a través de una proteína Gs aumentando la concentración de AMPc y en cambio $EP_1$, FP y TP actúan a través de una proteína Gq incrementando el $Ca^{2+}$ intracelular. El receptor $EP_3$ incrementa o disminuye la producción de AMPc a través de la activación de proteínas Gi o Gs y además también actúa a través de la interacción con la proteína G-Rho estimulando la PLC (Harizi y col., 2008).

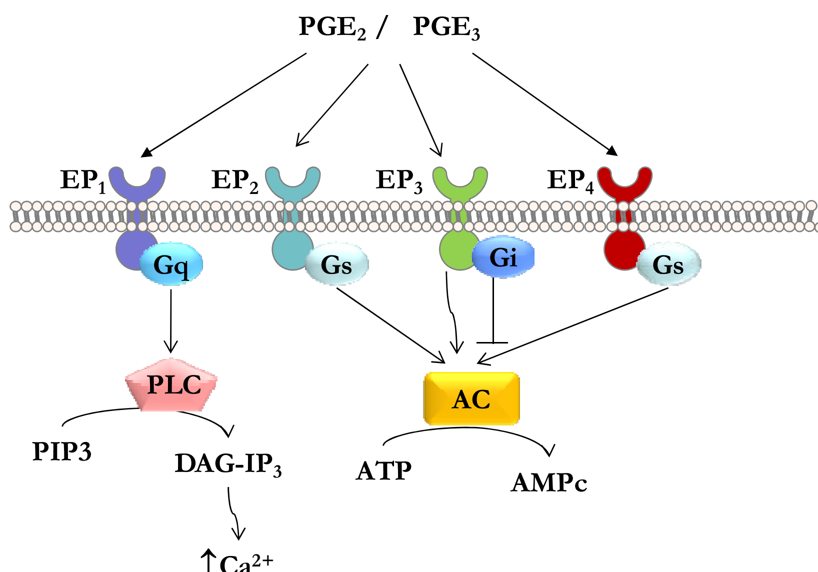


**Figura 9. Receptores de las PGE y sus vías de señalización.** La interacción de la PGE con $EP_1$ produce una activación de fosfolipasa C (PLC) con la consecuente formación de diacilglicerol (DAG) e inositol trifosfato ($IP_3$) elevando la concentración intracelular de $Ca^{2+}$. La interacción de la PGE con los receptores $EP_2$ y $EP_4$ activa adenilato ciclasa (AC) con la consecuente producción de AMPc. $EP_3$ en función de la interacciópn con Gi o Gs inhibe o estimula o la AC dando lugar a una reducción o un incremento de la concentración de AMPc.

### 2.3.2. Receptores y vías de señalización de los LT y los HETE

La acción biológica de los LT está mediada por receptores específicos de la superficie de las células diana. El $LTB_4$ ejerce su efecto a través de la unión a los receptores $BLT_1$ y $BLT_2$ (Figura 10), con diferente distribución y propiedades farmacológicas. El $BLT_1$ es el denominado receptor de alta afinidad que media la mayoría de las acciones quimiotácticas y proinflamatorias, y está relacionado con la aterogénesis, el asma, la glomerulonefritis, la artritis y la IBD. El $LTB_4$ se une al $BLT_1$ y activa la guanilato ciclasa para generar GMPc. Se expresa en leucocitos y está presente en menor proporción en el bazo, el timo y la medula ósea (Owman y col., 1996; Yokomizo y col., 1997). El $BLT_2$ es el receptor de baja afinidad para $LTB_4$ ya que presenta una afinidad veinte veces menor que el $BLT_1$, sin embargo su expresión es más ubicua (Harizi y col., 2008).

Hay dos subtipos de receptores para los cisteinil LT (CysLT: $LTC_4$, $LTE_4$ y $LTD_4$) asociados a proteína Gq: el $CysLT_1R$ y el $CysLT_2R$ (Harizi y col., 2008). El receptor $CysLT_2R$ muestra una mayor distribución que $CysLT_1R$. La afinidad del $CysLT_1R$ es $LTD_4 > LTC_4 > LTE_4$ mientras que la afinidad del receptor $CysLT_2R$ por $LTC_4$ y $LTD_4$ es similar mientras que $LTE_4$ es un agonista débil (Werz y Steinhilber, 2006).

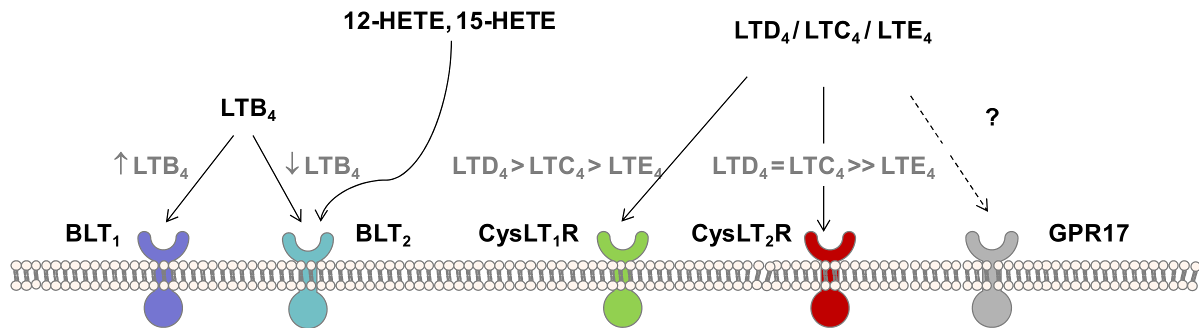


**Figura 10. Receptores de membrana de los LT.** $LTB_4$ se une al receptor de alta afinidad $BLT_1$ y al de baja afinidad $BLT_2$. Los CysLT se pueden unir a $CysLT_1R$ y $CysLT_2R$ con diferente afinidad. También se ha descrito la unión de 12 y 15-HETE a $BLT_2$.

Existen diferentes fármacos antagonistas del receptor $CysLT_1R$ como el notelukast, el zafirlukast y el pranlukast que se usan en el tratamiento del asma en niños y adultos dando lugar a una mejora de la función pulmonar, menos exacerbaciones y en general mayor calidad de vida. También existen estudios con modelos animales de antagonistas del receptor $BLT_1$ pero ninguno ha dado lugar a un uso clínico (Peters-Golden y Henderson, 2007). Hasta el momento, no se han descrito antagonistas específicos para $CysLT_2R$. Ciertas acciones de los LT no se pueden explicar a través de su interacción $CysLT_1R$ o $CysLT_2R$, sugiriéndose la existencia de heterodímeros o de receptores adicionales. En este sentido, candidatos como el receptor el GPR17 ya se han descrito previamente (Peters-Golden y Henderson, 2007).

Tampoco se han propuesto receptores específicos para los HETE, si bien se ha descrito que el 12-HETE y el 15-HETE se pueden unir al $BLT_2$ aunque la función fisiológica de esta interacción es poco conocida (Yokomizo y col., 2001; Peters-Golden y Henderson, 2007). Ambos eicosanoides pueden interaccionar con receptores de elevada afinidad aún no identificados: para el 12-HETE en cultivos de células epidérmicas humanas y para el 15-HETE en cultivos de células de glándula pituitaria de rata (Rabier y col., 1988; Gross y col., 1990). También se ha descrito un receptor de baja afinidad para el 15-HETE en las células inmunitarias PT-18 (Vonakis y Vanderhoek, 1992). Recientemente, se ha asociado un receptor huérfano, el GPR31, con el 12-HETE, demostrando que se trata de un receptor de alta afinidad (Guo y col., 2011).

### 2.3.3. Receptores y vías de señalización de los EET

Aún no se han descrito receptores para los EET y se cree que su acción, al igual que el resto de eicosanoides, está mediada por un receptor de membrana. Se han planteado diversas hipótesis al respecto: la existencia de un receptor putativo de

membrana, la interacción con proteínas transportadoras de AG (FABP) u otras proteínas con dominios de unión a AG (Widstrom y col., 2001; Widstrom y col., 2003) o su incorporación a la membrana plasmática donde se asociarían a efectores como las proteínas G de bajo peso molecular o alterarían la fluidez y el volumen de la bicapa regulando el flujo de iones (como por ejemplo el $Ca^{2+}$). En este sentido, en el músculo cardíaco los EET activan canales de $Ca^{2+}/K^{+}$ a través de una proteína Gs. También se ha sugerido que los PPAR pueden actuar como receptores de los EET y sus metabolitos. Algunos EET, en concreto 11,12- y 14,15-EET, activan varias moléculas de cascadas intracelulares incluyendo tirosina quinasas y fosfatasas como la p38MAP quinasa y la ERK1/2 (*extracellular regulated protein kinase*). Los EETs también son capaces de transactivar el receptor del factor de crecimiento epidérmico (EGF) en células endoteliales (Fleming, 2007). Además, Wong y col. (2000) identificaron un *locus* de unión de gran afinidad en la membrana de células mononucleares para el 14(R),15(S)-EET que daría lugar a un incremento de AMPc y una activación de la PKA.

## *2.4. Eicosanoides y epitelio intestinal*

A lo largo del intestino, la absorción de nutrientes, minerales, electrolitos y agua está altamente regulada. El sistema nervioso entérico y numerosas hormonas regulan el transporte intestinal de agua y electrolitos. Esta regulación además se modula por una gran variedad de substancias entre las que se encuentran los eicosanoides, las citocinas y otros mediadores solubles (Eberhart y Dubois, 1995). Los eicosanoides están implicados en el mantenimiento de la homeóstasis del epitelio intestinal a través de la regulación de diferentes funciones de éste como la proliferación, la diferenciación celular y el mantenimiento de la función barrera. Las células responsables de la producción de eicosanoides son las células inmunitarias de la lámina propia, las células mesenquimales subepiteliales y las células epiteliales (Ferrer y Moreno, 2010).

Las PG estimulan la secreción de agua en el intestino delgado siendo la $PGE_2$ la PG con mayor potencia para inducir la secreción intestinal, seguida de $PGF_{2\alpha}$, $PGD_2$ y $PGI_2$ (Racusen y Binder, 1980; Craven y DeRubertis, 1986). También se ha descrito, tanto *in vivo* como *in vitro*, la capacidad de las PG a dosis farmacológicas para alterar la motilidad intestinal. Así, la $PGE_2$ y la $PGF_{2\alpha}$ son capaces de disminuir el tiempo de tránsito intestinal y producir diarrea (Milton-Thompson y col., 1975; Soffer y Launspach, 1993). Las PG se encuentran a altas concentraciones en la mucosa de pacientes con IBD (Rampton y Hawkey, 1984; Donowitz, 1985).

En general, se ha descrito que durante el proceso inflamatorio los LT producen un incremento de la permeabilidad vascular, los CysLT producen la contracción del

músculo liso y el $LTB_4$ tiene un efecto quimiotáctico sobre neutrófilos, eosinófilos y monocitos, promoviendo la adherencia de los fagocitos a las paredes de los capilares, la degranulación de los neutrófilos y la liberación de aniones superóxido. En cuanto a las LX, controlan la resolución de la inflamación mediante la estimulación de cascadas antiinflamatorias endógenas (Harizi y col., 2008).

La expresión de las LOX y la concentración de los HETE en la mucosa del colon se correlacionan con la inflamación y la hiperproliferación. La mucosa alterada tiene disminuida la expresión de la 15-LOX y aumentada la de la 5- y la 12-LOX (Zijlstra y Wilson, 1991; Neilson y col., 2012). El 15-HETE parece tener actividad antiinflamatoria y anticancerígena mientras que el 5- y el 12-HETE, por el contrario, serian proinflamatorios y procarcinogénicos (van Dijk y col., 1993; Ye y col., 2004). Las concentraciones de HETE en la mucosa del colon son variables, la concentración del 15-HETE es mayor que la del 12-HETE y la del 5-HETE es la que se encuentra en menor proporción (Zijlstra y Wilson, 1991; Zijlstra y col., 1992). Además del tipo de HETE, el perfil de R/S es importante ya que los R-HETE se relacionan con la inflamación y la carcinogénesis (Neilson y col., 2012). Por todo esto se ha postulado que la concentración de HETE en la mucosa del colon puede ser un biomarcador útil en los procesos inflamatorios y en pacientes con riesgo de padecer cáncer de colon (Shureiqi y col., 2010).

Bajo condiciones fisiológicas, los EET son transformados rápidamente al correspondiente DHET por acción de la sEH. La inhibición de sEH o la supresión de su gen estabilizan los EET. El incremento de la concentración de EET da lugar a una disminución en la producción de citocinas proinflamatorias y a una reducción de la infiltración de células inmunitarias. Además su acción sobre el endotelio y su capacidad angiogénica son importantes para los procesos de regeneración que permiten reparar las lesiones inflamadas, particularmente las úlceras y las erosiones típicas de la IBD (Zhang y col., 2012) . Diferentes autores han descrito para sEH un papel en la inflamación en diferentes sistemas (Node y col., 1999; Schmelzer y col., 2005; Smith y col., 2005; Zhang y col., 2012). Zhang y col. (2012) demostraron, en un modelo de IBD en ratones, que la supresión del gen que codifica para sEH reduce la inflamación intestinal.

# 3. Enfermedad inflamatoria intestinal

La IBD incluye un grupo heterogéneo de patologías crónicas como la colitis ulcerosa (*ulcerative colitis*, UC) y la enfermedad de Crohn (*Crohn's disease*, CD), de etiología multifactorial y desconocida que cursa con inflamación crónica y recurrente del tracto digestivo. Las principales manifestaciones de la IBD son diarrea, dolor abdominal, sangrado, anemia y pérdida de peso. La UC se caracteriza por una inflamación difusa e ininterrumpida limitada a la mucosa y restringida al intestino grueso. En el caso de CD, se puede ver afectado cualquier segmento del tracto intestinal aunque comúnmente afecta el íleon y el colon proximal. Se presenta con áreas normales e inflamadas que penetran en capas inferiores a la mucosa dando lugar a úlceras o fístulas y se puede acompañar incluso de manifestaciones extraintestinales (Fiocchi, 1998).

## *3.1. Etiopatogenia de la IBD*

Aunque la etiología de la IBD es poco clara, parece ser que es el resultado de la interacción de tres factores: la susceptibilidad genética, los factores ambientales y la respuesta inmunitaria alterada (Shanahan, 2001). Los factores ambientales incluyen tanto la microbiota intestinal como la dieta. En este sentido, numerosos estudios han relacionado el papel de ciertas dietas en la IBD a raíz de la incidencia de esta enfermedad en los países desarrollados (Bernstein y Shanahan, 2008). En la IBD existen alteraciones de la tolerancia inmunológica a nivel de la mucosa intestinal (Podolsky, 2002).

En los pacientes con IBD se observa un incremento de la PP debido a una alteración de la función barrera del intestino. No se conoce si esta alteración de la función barrera está implicada en el inicio de la enfermedad o es una consecuencia de la inflamación, pero el hecho que los familiares de primer grado de los enfermos de IBD tengan también una permeabilidad alterada sugiere la primera hipótesis (Hollander y col., 1986; Katz y col., 1989; May y col., 1993).

La dieta puede ser un factor que contribuya a la etiopatogenia de la IBD (Campos y col., 2003). Dentro de los nuevos hábitos surgidos en los países desarrollados existe un elevado consumo de azúcares y carbohidratos refinados. Diversos estudios relacionan el consumo de estos productos con la incidencia de la IBD, considerándolos como un factor de riesgo (Martini y Brandes, 1976; Katschinski y col., 1988). Por otra parte, el consumo de cítricos, zumos de frutas y vegetales puede disminuir el riesgo de desarrollar IBD (Tragnone y col., 1995; Russel y col., 1998; Mahmud y Weir, 2001). En el caso de dietas con un elevado contenido en AGPI n-3, como en el caso de los esquimales, también se observa una menor

prevalencia de la IBD (Bang y col., 1980). A partir de estos datos se han realizado numerosos estudios en los que se compara los efectos de los AGPI n-3 y los de los n-6 sobre la inflamación ya que estos últimos y sus metabolitos se han relacionado con el origen de la IBD (Lucendo y De Rezende, 2009). En este sentido, recientemente se ha descrito una mayor concentración de AA, DPA y DHA y una menor de EPA y LA en biopsias de mucosa inflamada de pacientes con CD relacionados con la severidad de la inflamación (Pearl y col., 2013).

Varios estudios relacionan la menor incidencia de UC y CD con la lactancia. Una posible explicación sería el hecho de que la leche materna proporciona protección contra infecciones gastrointestinales, estimula el desarrollo de la mucosa gastrointestinal y de la respuesta inmunitaria (Duffy y col., 1986; Beaudry y col., 1995; Bernt y Walker, 1999) o el hecho de posponer el contacto con la leche de vaca y otros alérgenos o agentes potencialmente infecciosos.

### *3.2. Papel de los eicosanoides en la IBD*

En la mucosa inflamada de enfermos de IBD se ha detectado un incremento en la concentración de $PGE_2$, $TXB_2$, $LTB_4$, $LTC_4$, 5-, 12- y 15-HETE (Donowitz, 1985; Lauritsen y col., 1988; Fretland y col., 1990; Ahrenstedt y col., 1994). Una concentración elevada de $PGE_2$ es característica de muchos procesos inflamatorios y, de hecho, es capaz de inducir en tejidos un número elevado de los signos cardinales de la inflamación. En este sentido, la concentración de la $PGE_2$ en la mucosa inflamada se correlaciona con la actividad de la patología y disminuye con el tratamiento (Smith y col., 1979; Rampton y col., 1980). Existen estudios experimentales en los que se ha investigado la participación de los metabolitos del AA como mediadores de la inflamación en la IBD. Para ello se incubaron resecciones quirúrgicas de mucosa intestinal de pacientes con y sin IBD con AA marcado radioactivamente. En el caso de los pacientes con IBD, el AA se metabolizó por la vía de la LOX a $LTB_4$ y a 5-HETE, por el contrario, en los pacientes sin IBD esto no sucedió (Boughton-Smith y col., 1983). Además, en un estudio similar, también se detectó una concentración más elevada de $LTB_4$, 5-, 12- y 15-HETE en la mucosa de pacientes con IBD (Sharon y Stenson, 1984).

Aún no se sabe si una elevada concentración de eicosanoides está implicada en la etiopatogenia de la IBD. Tanto el $LTB_4$ como el 12-HETE son quimiotácticos para neutrófilos y monocitos y monocitos, respectivamente y esto hace pensar que puedan tener un papel en la infiltración celular en esta enfermedad. Además, ciertos fármacos inhibidores de la producción de eicosanoides como la sulfasalacina, la mesalazina o los glucocorticoides se usan en el tratamiento de la IBD aunque los resultados son variables según el paciente (Eberhart y Dubois, 1995).

### *3.3. Alteración de la función epitelial de barrera en la IBD*

La alteración de la función epitelial de barrera se relaciona con la predisposicón a sufrir enfermedades gastrointestinales como la IBD (Groschwitz y Hogan, 2009). La disrupción de la función barrera puede ser la responsable del incremento de la translocación bacteriana, de la infiltración inmunitaria y del paso de moléculas de elevado peso molecular con capacidad antigénica y, por lo tanto, de la estimulación recurrente del sistema inmunitario. En este sentido, el aumento de la PP contribuye a la patogénesis de la UC (Vermeire y Rutgeerts, 2005) y además los familiares de los pacientes con UC tienen incrementada la permeabilidad intestinal (May y col., 1993).

La alteración de la función epitelial de barrera se asocia a una desestructuración de la TJ que es debida a una modificación de la expresión y la localización de las proteínas o del citoesqueleto. Este efecto puede ser causado por citocinas proinflamatorias y por el daño epitelial, incluyendo apoptosis, erosión y ulceración (Edelblum y Turner, 2009). Son varias las proteínas de la TJ implicadas en la alteración de la función barrera en la IBD. Así, en pacientes con IBD, se observa una reducción de la expresión de ocludina (Heller y col., 2005) aunque los ratones *knockout* para ocludina presentan un función epitelial de barrera normal (Schulzke y col., 2005). Esta discrepancia se podría explicar por la capacidad de la tricelulina para substituir funcionalmente a la ocludina ya que, en cultivos en los que se ha eliminado la expresión de ocludina, la tricelulina se desplaza de las uniones tricelulares a las bicelulares restableciendo la TJ (Ikenouchi y col., 2008). TNF-α produce la redistribución de ocludina, ZO-1 y claudina-1 e induce un incremento de la actividad de la MLCK (Wang y col., 2005). En este sentido, TNF-α es una de las moléculas diana en la lucha contra la enfermedad ya que lo anticuerpos anti-TNF son capaces de restaurar la función barrera en la CD (Suenaert y col., 2002). Además, la expresión y la actividad de la MLCK están incrementadas en la mucosa de pacientes con IBD (Blair y col., 2006) y su inhibición revierte la alteración de la función barrera (Clayburgh y col., 2005). En cuanto a la familia de las claudinas, en el epitelio de pacientes con IBD, se ha detectado un incremento en la expresión de claudina-2 (Prasad y col., 2005) y una disminución de claudina-3, claudina-4, claudina-5 y claudina-8 (Zeissig y col., 2004; Prasad y col., 2005).

### *3.4. Tratamiento de la IBD*

Actualmente el tratamiento de la IBD se aborda, principalmente, a través de tratamientos farmacológicos y a menudo es complementado con intervenciones nutricionales.

### 3.4.1. Tratamiento farmacológico

El tratamiento farmacológico actual es sobretodo sintomático, intenta reducir las recidivas y prevenir las complicaciones. Es importante el control de los periodos de exacerbación de la enfermedad, mantener los de remisión y tratar las complicaciones como las fístulas. Los fármacos de elección se usan solos o combinados y es necesario adecuar el tratamiento a la gravedad, la localización y las complicaciones de la inflamación. Actualmente, los fármacos de elección son los aminosalicilatos como la mesalazina o la sulfasalacina, administrados por vía oral o rectal, solos o en combinación con glucocorticoides para inducir y mantener la remisión. El mecanismo de acción de los aminosalicilatos se basa en la inhibición de la producción de IL-1 y de TNF-α, la inhibición de la vía de la LOX y del NFκB (Pithadia y Jain, 2011). Los glucocorticoides como la hidrocortisona o la prednisolona, administrados por vía oral, rectal o parenteral, son esenciales para el tratamiento de la enfermedad, si bien dan lugar a efectos adversos debidos a la supresión adrenal. Sin embargo, un 15-20% de los pacientes con IBD no responden a este tipo de fármacos (Steinhart y col., 2003).

Para los pacientes que no responden a la mesalazina o a los glucocorticoides, con complicaciones severas o en el tratamiento de CD perianal inoperable, se utilizan tratamientos inmunosupresores con azatioprina o mercaptopurina. Su efecto es significativo a partir de los tres meses de tratamiento y requieren analíticas rutinarias para controlar los posibles efectos adversos como la pancreatitis, (Pearson y col., 1995; Pithadia y Jain, 2011). Se ha observado una mejora en el 70% de los pacientes con IBD severa con el uso del antineoplásico metotrexato (Feagan y col., 2000), sin embargo existen efectos adversos tras su uso continuado como leucopenia y fibrosis hepática, que requieren monitorización. También están indicados en pacientes con UC severa que no responden a glucocorticoides la ciclosporina o el tacrolimus, (Lichtiger y col., 1994). Además, tal y como se ha descrito anteriormente, existen terapias biológicas con anticuerpos contra TNF-α (infliximab, adalimubab o certolizumab pegol) (Bressler y col., 2008; Panaccione y col., 2010; Feagan y col., 2011). Asimismo, para mejorar la calidad de vida de los pacientes también se suele administrar analgésicos, anticolinérgicos, antidiarreicos o antibióticos.

### 3.4.2. Intervenciones nutricionales

Se han descrito diferentes nutrientes y componentes alimentarios capaces de modular la IBD como los antioxidantes (glutatión, vitaminas A, C y E, selenio, cobre, zinc), los AGPI n-3, AG de cadena corta, aminoácidos, prebióticos y probióticos, entre otros.

### 3.4.2.1. AGPI n-3 y n-6

Cambios en la composición lipídica de la dieta conllevan cambios en la composición de los fosfolípidos de la membrana de las células de la mucosa intestinal que, en respuesta a un estímulo proinflamatorio, sintetizan eicosanoides con diferente actividad biológica (Gassull, 2004). Los AGPI n-3, EPA y DHA, presentes fundamentalmente en el aceite de pescado, compiten con el AA tanto para su incorporación a la membrana como para la síntesis de eicosanoides a través de la COX y de la LOX. Existen evidencias de los efectos beneficiosos de los AGPI n-3 en diversas patologías inflamatorias crónicas, en las que se atribuye su efecto antiinflamatorio a la diferente actividad de los eicosanoides sintetizados a partir de EPA o DHA (Gil, 2002). Una de las principales consecuencias de una ingesta rica en AGPI n-3 es una disminución de la producción en la mucosa intestinal de $PGE_2$, $TXA_2$, $LTB_4$ y un incremento de la producción de $PGE_3$, $TXA_3$, $PGI_3$ y $LTB_5$ (Simopoulos, 2002). Se ha descrito que el incremento de AGPI n-3 en la dieta de enfermos de IBD produce una mejoría de la sintomatología que permite la reducción de la dosis de esteroides necesaria para controlar la patología (Aslan y Triadafilopoulos, 1992; Hawthorne y col., 1992; Varnalidis y col., 2011). Sin embargo, también se han detectado casos en los que no se ha observado ninguna mejoría o que incluso describen un incremento de la actividad de la patología (Cabre y col., 2012).

Algo similar ocurre en los modelos experimentales de inflamación intestinal. En este sentido, en un estudio en ratas alimentadas con una dieta rica en AGPI n-3, se observó una reducción de la concentración de $PGE_2$, 6-keto-$PGF_{1\alpha}$, $TXB_2$, $LTB_4$ y $LTC_4$ en la mucosa intestinal y un incremento de la de $PGE_3$ y $LTC_5$ (Guarner y col., 1992). En otro estudio de colitis, se observó cómo una infusión parenteral rica en AGPI n-3, provocó una reducción de la diarrea y de las lesiones de la mucosa, en paralelo a la producción de eicosanoides de la serie 2 (Campos y col., 2002). Además, la suplementación de la dieta con aceite de pescado suaviza las lesiones macroscópicas y microscópicas de la mucosa intestinal, reduce las áreas necróticas y los niveles de $PGE_2$ de la mucosa (Nieto y col., 2002).

# Objetivos

La alteración de la TJ y la pérdida de la función barrera juegan un papel importante en la patogenia de diferentes procesos inflamatorios intestinales como la IBD sugiriendo su participación en la perpetuación de la respuesta inflamatoria y en la pérdida de agua (Bruewer y col., 2006; Turner, 2006). En la mucosa de los pacientes con IBD, la concentración de los eicosanoides como la $PGE_2$, la $PGD_2$ y el $TXB_2$ así como el $LTB_4$, 5-, 12- y 15-HETE se encuentra incrementeda (Boughton-Smith y col., 1983; Eberhart y Dubois, 1995; Krimsky y col., 2003) y por tanto podrían participar en su patogenia (Wang y DuBois, 2007).

En un estudio previo se demostró la capacidad de la $PGE_2$ para inducir una alteración de la PP en una monocapa de células intestinales Caco-2 diferenciadas (Martin-Venegas y col., 2006). Estas células constituyen un modelo ampliamente utilizado para el estudio de la regulación de la permeabilidad de la TJ a través de la medida de la TER y de flujos transepiteliales de substratos específicos de la vía paracelular (Hidalgo y col., 1989).

Considerando estos antecedentes, el objetivo general de este trabajo es estudiar el papel de los eicosanoides representativos de las diferentes vías de la cascada del AA (COX, LOX y CYP450) en la regulación de la función epitelial de barrera utilizando un modelo de células intestinales Caco-2. Los objetivos específicos que se han marcado son:

- estudiar el papel de los principales metabolitos de las diferentes vías de la cascada del AA en la regulación de la PP,
- estudiar los receptores implicados en dicho efecto,
- estudiar las vías de señalización implicadas en la alteración de la función barrera inducida por los eicosanoides, y
- estudiar el efecto de los eicosanoides sobre las proteínas de la TJ ocludina, ZO-1 y claudinas y sobre el anillo de actina

# Resultados

Artículo 1

$PGE_2$ promotes $Ca^{2+}$-mediated epithelial barrier disruption through $EP_1$ and $EP_4$ receptors in Caco-2 cell monolayers.

Rodríguez-Lagunas MJ, Martín-Venegas R, Moreno JJ, Ferrer R.

Am J Physiol Cell Physiol. 2010, 299:C324-C334

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Categoría (posición): Fisiología (19/79)

Los resultados obtenidos han dado lugar a las siguientes comunicaciones a congresos:

- $PGE_2$ receptors involved in the regulation of epithelial barrier function in Caco-2 cell. Rodríguez-Lagunas, M.J; Martín-Venegas, R.; Moreno, J.J.; Ferrer, R. 21th Meeting of the European Intestinal Transport Group, Oberwiesenthal (Alemanya), 2007. *Journal of Physiology and Biochemistry*, 63 (1): 63 (2007)
- $PGE_2$-EP1 interaction in the regulation of epithelial barrier function. M.J. Rodríguez-Lagunas, R. Martín-Venegas, J.J. Moreno and R. Ferrer.
  6th European Mucosal Immunology Group meeting (EMIG), Milano (Italia), 2008

## *Resumen artículo 1*

**Objetivo**: identificar el papel de los receptores de la $PGE_2$ ($EP_1$-$EP_4$) y las vías de señalización implicados en la alteración de la PP inducida por este prostanoide.

**Material y métodos**: la PP se ha estudiado a través de la determinación de la TER y de los flujos de D-manitol en presencia de la $PGE_2$ y de diferentes agonistas y antagonistas de los receptores $EP_1$-$EP_4$ en células Caco-2 cultivadas sobre filtros. La determinación de la $[Ca^{2+}]_i$ se ha realizado por espectrofluorimetría y la localización de las proteínas de la TJ y de los receptores de la $PGE_2$, por immunofluorescencia y posterior análisis por microscopía confocal.

**Resultados:** los resultados de la immunolocalización muestran la expresión de los receptores $EP_1$-$EP_4$ principalmente en la membrana basolateral. Los resultados obtenidos utilizando agonistas y antagonistas de los EP indican que los receptores $EP_1$ y $EP_4$ pero no $EP_2$ ni $EP_3$ están implicados en la alteración de la PP. La interacción de la $PGE_2$ con $EP_1$ induce un incremento de la $[Ca^{2+}]_i$ y la interacción con el receptor $EP_4$ da lugar a un aumento de AMPc. Además, la inhibición de la AC y de la PKA revierte el incremento de la PP inducida por $PGE_2$, confirmando la implicación de estas vías de señalización en la disrupción de la función barrera. La inhibición de una isoforma convencional de la PKC y de la MLCK también previene la alteración de la PP inducida por la $PGE_2$. La $PGE_2$ y los agonistas de $EP_1$ y $EP_4$ provocan una redistribución de la ocludina, además del anillo de actina, sin modificar la de la ZO-1.

**Conclusión**: se ha demostrado la participación de los receptores $EP_1$ y $EP_4$ en la alteración de la PP inducida por $PGE_2$ a través de las vías de señalización PLC-$IP_3$-$Ca^{2+}$/PKC y AMPc-PKA y MLCK. Estos eventos, además, provocan la redistribución de la ocludina de la TJ y del anillo de actina.

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# $PGE_2$ promotes $Ca^{2+}$-mediated epithelial barrier disruption through $EP_1$ and $EP_4$ receptors in Caco-2 cell monolayers

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**Rodríguez-Lagunas MJ, Martín-Venegas R, Moreno JJ, Ferrer R.** $PGE_2$ promotes $Ca^{2+}$-mediated epithelial barrier disruption through $EP_1$ and $EP_4$ receptors in Caco-2 cell monolayers. *Am J Physiol Cell Physiol* 299: C324–C334, 2010. First published May 19, 2010; doi:10.1152/ajpcell.00397.2009.—We recently demonstrated that $PGE_2$ induces the disruption of the intestinal epithelial barrier function. In the present study, our objectives were to study the role of $PGE_2$ receptors ($EP_1$–$EP_4$) and the signaling pathways involved in this event. Paracellular permeability (PP) was assessed in differentiated Caco-2 cell cultures from D-mannitol fluxes and transepithelial electrical resistance (TER) in the presence of different $PGE_2$ receptor agonists (carbacyclin, sulprostone, butaprost, ONO-AE1-259, ONO-AE-248, GR63799, and ONO-AE1-329) and antagonists (ONO-8711, SC-19220, AH-6809, ONO-AE3-240, ONO-AE3-208, and AH-23848). The results indicate that $EP_1$ and $EP_4$ but not $EP_2$ and $EP_3$ might be involved in PP regulation. These effects were mediated through PLC-inositol trisphosphate ($IP_3$)-$Ca^{2+}$ and cAMP-PKA signaling pathways, respectively. We also observed an increase in intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) strengthened by cAMP formation indicating a cross talk interaction of these two pathways. Moreover, the participation of a conventional PKC isoform was shown. The results also indicate that the increase in PP may be correlated with the redistribution of occludin, zona occludens 1 (ZO-1), and the perijunctional actin ring together with an increase in myosin light chain kinase activity. Although the disruption of epithelial barrier function observed in inflammatory bowel disease (IBD) patients has been traditionally attributed to cytokines, the present study focused on the role of $PGE_2$ in PP regulation, as mucosal levels of this eicosanoid are also increased in these inflammatory processes.

paracellular permeability; intestine; tight junctions; eicosanoids; arachidonic acid cascade

THE STRUCTURAL INTEGRITY OF the epithelium is maintained by three distinct adhesion systems: tight junctions (TJ), adherent junctions, and desmosomes. Of these, TJ are the most apical component and are the rate-limiting step for paracellular permeability (PP). In addition, TJ constitute the interface (fence) between apical and basolateral membrane domains (32). TJ are multiprotein complexes composed of transmembrane proteins associated with the cytoskeletal perijunctional ring of actin and myosin and with cytosolic proteins involved in cell signaling and vesicle trafficking. Five transmembrane proteins of the junctional complex have been identified in recent years: occludin, the claudin family, tricellulin, crumbs, and junctional adhesion molecules. These proteins are associated with a wide spectrum of cytosolic proteins, of which zona occludens (ZO) 1, ZO-2, ZO-3, AF6, and cingulin are described as forming the nexus with cytoskeletal proteins (42).

$PGE_2$ is an inflammatory mediator that has pleiotropic effects on signal transduction and exerts its biological action through binding to four specific membrane receptor subtypes, $EP_1$, $EP_2$, $EP_3$, and $EP_4$, which are widely distributed and have different tissue expression. $PGE_2$ stimulation leads to activation of different G proteins, depending on the type of EP subtype engaged, inducing changes in second messengers such as cAMP, $Ca^{2+}$, and inositol phosphates (44). EP expression has been reported in the intestinal epithelium of various species (16). In rat, $EP_1$ is expressed in goblet cells of the small intestine, and it is also expressed in other epithelial cells of the large intestine. In rabbit, it is highly expressed in the brush border membrane of differentiated villous cells. $EP_2$ is expressed in different regions of the intestine, depending on the species, and $EP_3$, in most rodents, is expressed in goblet cells of the small intestine. $EP_4$ expression has been detected in mouse mature enterocytes of ileal villi. In humans, $EP_2$ and $EP_3$ are expressed at the apex of colonic crypts; and $EP_4$, on their lateral side.

Recently, we observed that cell differentiation in intestinal Caco-2 cells induces a decrease in $PLA_2$ activity and cyclooxygenase-2 expression and, consequently, a decrease in arachidonic acid release and $PGE_2$ synthesis in parallel with a reduction in PP. We (30) also demonstrated that the addition of $PGE_2$ to differentiated Caco-2 cells induces an increase in PP. Several intestinal diseases are associated with the disruption of the epithelial barrier function, particularly inflammatory bowel disease (IBD) (49), characterized by increased mucosal $PGE_2$ levels (7, 22). Yu and Chadee (54) demonstrated in T-84 colonic epithelial cells that $PGE_2$ upregulates IL-8 production via $EP_4$ interaction, thus confirming the proinflammatory role of $PGE_2$. In contrast, in $EP_4$ receptor knockout mice, this receptor was described as playing a critical role in keeping mucosal integrity in an experimental model of colitis induced by dextran sodium sulfate, although the role of $EP_4$ in either epithelial or submucosal cells has not been identified (23). Similarly, studies in rats using an $EP_4$ receptor agonist reported the suppression of colitis caused by dextran sodium sulfate treatment through upregulation of an anti-inflammatory cytokine, IL-10 (34). Therefore, the function of EP receptors is still undefined. Dey et al. (16) concluded that $EP_1$, $EP_2$, and $EP_4$ receptors seem to be major determinants in the early stages of intestinal inflammation and also tumorigenesis, whereas $EP_4$ receptors in immune cells may promote the restitution of colitis/inflammation. The main objectives of this study were to study in Caco-2 cells the role of EP receptors in the regulation of epithelial barrier function by $PGE_2$ and their signaling pathways and the contribution of TJ proteins and the perijunctional actin ring to increased PP.

## MATERIALS AND METHODS

*Materials.* DMEM, trypsin, penicillin, and streptomycin were supplied by GIBCO (Paisley, Scotland). Nonessential amino acids, FBS,

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BSA, PBS, D-glucose, HEPES, fura 2-AM, $PGE_2$, butaprost, carbacyclin, inositol trisphosphate ($IP_3$), forskolin, U73122, dantrolene, Gö6983, SQ22,536, KT5720, ML-7, and phalloidin-tetramethylrhodamine B isothiocyanate (TRITC-phalloidin), along with other chemicals, were supplied by Sigma (St. Louis, MO). Dioctanoylglycerol (diC8) was from Molecular Probes (Leiden, The Netherlands). Tissue culture supplies, including Transwells, were obtained from Costar (Cambridge, MA). D-[2-$^3$H]mannitol (specific activity 30 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). Biogreen 3 was supplied by Scharlau Chemie (Barcelona, Spain). ONO-8711, ONO-AE3-240, ONO-AE3-208, ONO-AE1-259, ONO-AE1-329, and ONO-AE3-248 were kindly provided by Ono Pharmaceutical (Osaka, Japan), and AH-6809, AH-23848, and GR63799 by Glaxo Wellcome (Stevenage, United Kingdom).

*Cell culture.* Caco-2 cells were kindly provided by Dr. David Thwaites at the School of Cell and Molecular Biosciences, University of Newcastle-upon-Tyne (United Kingdom), and were cultured as previously described (30). The cells (*passages 107-121*) were routinely grown in plastic flasks at a density of $5 \times 10^4$ cells/cm$^2$ and cultured in DMEM containing 4.5 g/l D-glucose and 2 mM L-glutamine, supplemented with 1% (vol/vol) nonessential amino acids, 10% (vol/vol) heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a modified atmosphere of 5% $CO_2$ in air. Cells grown to ~80% confluence were released by trypsinization and subcultured at a density of $5 \times 10^4$ cells/cm$^2$ in 12-well clusters for intracellular $Ca^{2+}$ determination or at $4 \times 10^5$ cells/cm$^2$ on polycarbonate filters with a pore size of 0.4 μm (Transwells; 12-mm diameter) for PP experiments and confocal immunolocalization. The medium was replaced every 3 days and on the day before the experiment. Experiments were performed in cultures 19–21 days after seeding when cells are differentiated (30).

*PP.* PP was estimated from unidirectional apical-to-basal D-mannitol fluxes and transepithelial electrical resistance (TER) in cells maintained on filters, as described elsewhere (30). After 3-h incubation with $PGE_2$ or $EP_1$–$EP_4$ agonists and antagonists in the apical and basolateral compartments, monolayers were washed with modified Krebs buffer (room temperature), which had 137 mM NaCl, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.0 mM $MgSO_4$, 0.3 mM $NaH_2PO_4$, 10 mM D-glucose, and 10 mM HEPES/Tris (pH 7.4). The filters were then placed in culture wells containing 1.5 and 0.7 ml of modified Krebs buffer in the basolateral and apical compartments, respectively, and TER was determined by a Millicell-ERS Voltohmmeter (Millipore, Bedford, MA). Results are expressed as ohms per centimeter squared monolayer surface area. The resistance of the supporting membrane in filters was subtracted from all readings before calculations. After TER determination, apical medium was replaced by the same volume of modified Krebs buffer containing 0.2 mCi/ml D-[2-$^3$H]mannitol, and the cells were incubated for 5 min at 37°C. At the end of the incubation, basolateral medium was withdrawn, and radioactivity was counted in a scintillation counter (1500 Tri-Carb; Packard, Downers Grove, IL). The concentrations of the $PGE_2$ antagonists tested were chosen while taking into account their reported inhibitor constant ($K_i$) values for $PGE_2$ receptor interaction.

*Confocal immunolocalization.* Immunolocalization was performed as described elsewhere (36). Caco-2 control or treated monolayers grown on filters were gently washed with PBS and fixed in 3% paraformaldehyde and 2% sucrose in 0.1 M PBS (pH 7.4) for 15 min at room temperature. Cells were washed twice for 5 min in 10 mM PBS containing 20 mM glycine (PBS-glycine) and permeabilized with 0.2% (vol/vol) Triton X-100 for 10 min at room temperature. Cells were washed twice in PBS-glycine and blocked for 20 min in PBS-glycine containing 1% BSA (incubation solution). As primary antibodies, mouse monoclonal anti-occludin (1:500 dilution; Zymed, South San Francisco, CA), rabbit polyclonal anti-ZO-1 (1:250 dilution; Zymed), and rabbit polyclonal anti-$EP_1$, -$EP_2$, -$EP_3$, and -$EP_4$ (1:500 dilution; Cayman, Ann Arbor, MI) were used. Cells were incubated with the primary antibodies for 1 h at 37°C and washed twice in PBS-glycine for 5 min at room temperature. Monolayers were then incubated for 1 h at 37°C with Alexa dye-conjugated secondary antibodies (Molecular Probes) and Hoechst 33258 (1:2,000; Sigma) to visualize the nuclei. Finally, the cells were washed for 10 min at room temperature in PBS, mounted in Mowiol (Calbiochem, San Diego, CA), and examined with a confocal laser scanning microscope (TCS 4D; Leica Lasertechnik). Images were taken using a ×63 (numerical aperture 1.3, phase 3, oil) Leitz Plan-Apochromatic objective. Background absorbance (measured by secondary antibody labeling only) was subtracted from all samples. To view the actin subapical ring, cell monolayers were fixed and permeabilized as described above and incubated with TRITC-phalloidin for 1 h at 37°C (1:1,000 dilution).

*Intracellular calcium concentration.* Intracellular calcium concentration ($[Ca^{2+}]_i$) was monitored using the selective fluorescent $Ca^{2+}$ indicator, fura 2-AM. Caco-2 cells grown on clusters were loaded with 25 μM fura 2-AM in DMEM for 1 h at 37°C. The preloaded monolayers were then washed in modified Krebs buffer (pH 7.4) at 37°C and incubated for 1 h at 37°C to allow fura 2-AM deesterification. The monolayers were washed again to ensure removal of all unloaded indicator and antagonists, and inhibitors were added to the respective wells. Continuous fluorescent signal was monitored with excitation wavelengths of 340 and 380 nm and an emission at 510 nm using a fluorescent microplate reader (FLUOstar OPTIMA; BMG Labtech) before and after the injection of $PGE_2$, $PGE_2$ agonists, $IP_3$, or forskolin. Cells were maintained throughout the experiment at a temperature of 37°C. At the end of the incubation period, the maximal and minimal intracellular probe fluorescent signals were determined by the addition of cell lysis buffer and 20 mM EDTA in Krebs, respectively. $[Ca^{2+}]_i$ was calculated following Grynkiewicz et al. (20) from 340:380 ratio by using a dissociation constant of 224 nM.

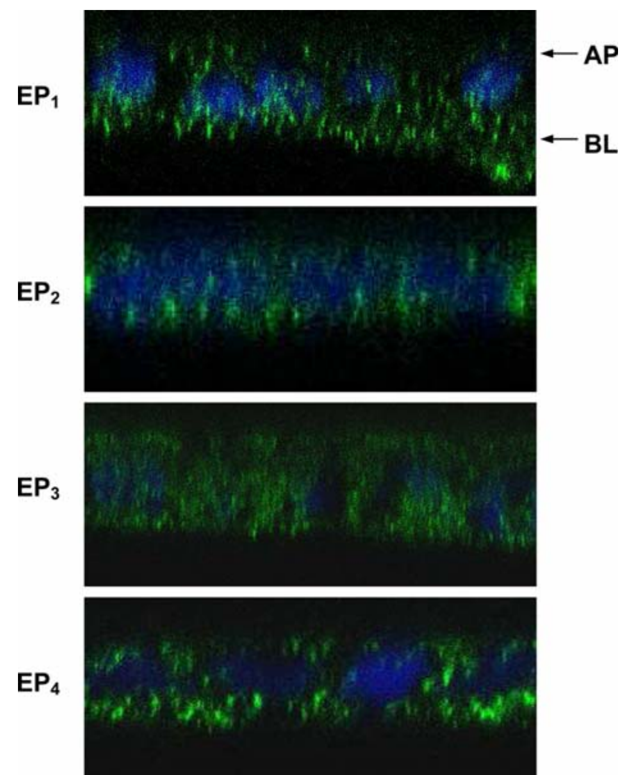


Fig. 1. Immunolocalization of $PGE_2$ receptors $EP_1$–$EP_4$ by confocal analysis. Specific anti-$EP_1$–$EP_4$ antibodies were used, as described under MATERIALS AND METHODS, in differentiated Caco-2 cells maintained in filters. Hoechst staining (blue) was used to visualize nuclei. In each case, a representative *x-z* image of sections is shown. Apical (AP) and Basolateral (BL) membrane borders are indicated with arrows.

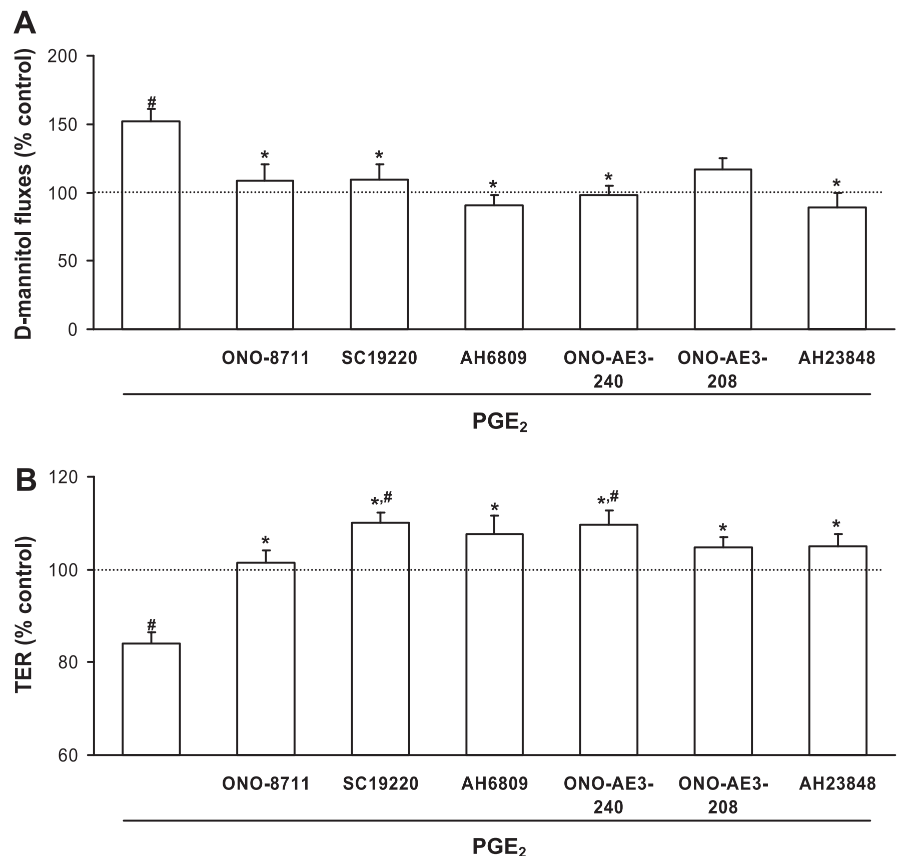


Fig. 2. Effect of $EP_1$–$EP_4$ antagonists on epithelial barrier disruption induced by $PGE_2$. D-mannitol fluxes (*A*) and transepithelial electrical resistance (TER; *B*) were determined in differentiated Caco-2 cell monolayers, as described under MATERIALS AND METHODS. Cells were incubated for 3 h with $PGE_2$ (3 nM) and $PGE_2$ plus ONO-8711 (250 nM), plus SC-19220 (300 nM), plus AH-6809 ($3 \times 10^3$ nM), plus ONO-AE3-240 (2 nM), plus ONO-AE3-208 (2 nM), or plus AH-23848 (200 nM) in the apical and basolateral compartments. Results were expressed as the percentage of D-mannitol fluxes and TER values obtained in control conditions ($4.8 \pm 0.48$ fmol/cm$^2$ and $350 \pm 32.7$ $\Omega \cdot cm^2$). The data were means ± SE of $n = 6–8$. $*P < 0.05$ vs. $PGE_2$; $\#P < 0.05$ vs. control.

*Data analysis.* Results were expressed as means ± SE. Data were analyzed by one-way analysis of variance followed by Dunnett post hoc test using SPSS software (SPSS, Chicago, IL). $P < 0.05$ was considered to denote significance.

## RESULTS

First, the immunolocalization of EP receptors was performed, and the results show the presence of the four receptors in differentiated Caco-2 cells (Fig. 1). Then, to investigate the EP receptors involved in the regulation of epithelial barrier function, the ability of different receptor antagonists to prevent the effects of $PGE_2$ and $PGE_2$ analogs on D-mannitol fluxes and TER was tested. The results shown in Fig. 2, *A* and *B*, confirm previous data (30), indicating that $PGE_2$ induces an increase in D-mannitol fluxes and a reduction in TER values. The results also show that all the antagonists tested prevented the effects of $PGE_2$ on both variables, leading to values no different from those detected under control conditions, except in the case of SC-19220 and ONO-AE3-240, which reverted TER to significantly higher values. Since ONO-8711 and SC-19220 are specific for $EP_1$ and AH-23848 for $EP_4$ (Table 1), these results suggest the participation of these two receptors in these events. Nevertheless, since the other antagonists tested (AH-6809, ONO-AE3-240, and ONO-AE3-208) show cross-reaction interactions (Table 1), the results do not completely clarify the contribution of $EP_2$ and $EP_3$. Therefore, the effect of all these antagonists on specific $EP_1$–$EP_4$ agonists was further investigated.

First, carbacyclin and sulprostone were tested, and the results show that these $PGE_2$ analogs significantly increased PP, but to a lesser extent than $PGE_2$ (Fig. 3, *A* and *B*). The data also show that the effects of carbacyclin, an $EP_1$ agonist but with some $EP_3$ activity (Table 1), were prevented by $EP_1$ antagonists (ONO-8711 and SC-19220), whereas an $EP_3$ antagonist (ONO-AE3-240) was unable to modify the variables studied. The effects of sulprostone, an $EP_3$ agonist but with some $EP_1$ activity, were not significantly affected by ONO-AE3-240, whereas a significant recovery was observed for ONO-8711 and SC-19220. Therefore, all these results obtained for $PGE_2$ and $PGE_2$ analogs suggest the participation of $EP_1$ but not of $EP_3$ in PP regulation by $PGE_2$.

As for $EP_2$ and $EP_4$ participation, butaprost was tested as an $EP_2$ agonist but with some $EP_4$ activity. In this case again, the effect of this analog on PP was lower than that observed for

Table 1. *$PGE_2$ receptor ($EP_1$–$EP_4$) agonists and antagonists tested*

| | Agonist | Antagonist |
|---|---|---|
| $EP_1$ | Carbacyclin ($EP_1 > EP_3$)[25] | ONO-8711[51]<br>SC-19220[18] |
| $EP_2$ | Butaprost ($EP_2 > EP_4$)[5,44]<br>ONO-AE1-259[44] | AH-6809 ($EP_1 \approx EP_2$)[52] |
| $EP_3$ | Sulprostone ($EP_3 > EP_1$)[1,44]<br>ONO-AE-248[44]<br>GR63799[1] | ONO-AE3-240 ($EP_3 > EP_4$)[3,44] |
| $EP_4$ | ONO-AE1-329[44] | ONO-AE3-208 ($EP_4 > EP_3$)[14,44]<br>AH-23848[1,14] |

$>$, Higher affinity than; $\approx$, similar affinity to. Superscripted numbers correspond to references.

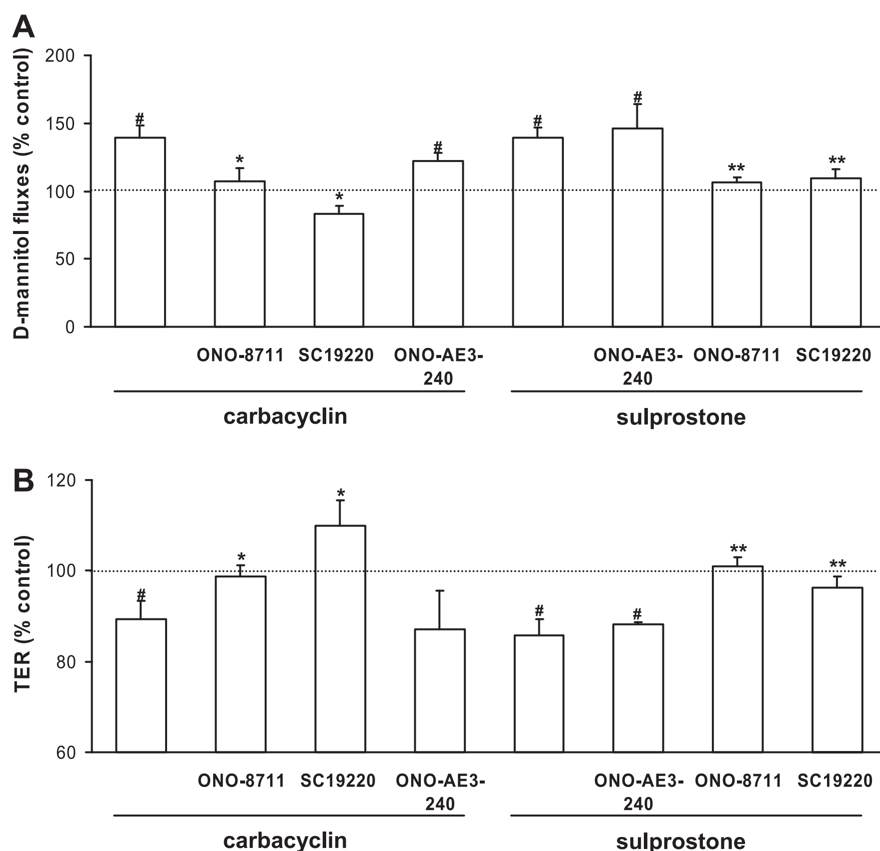


Fig. 3. Effect of $EP_1$ and $EP_3$ antagonists on epithelial barrier disruption induced by carbacyclin and sulprostone. D-mannitol fluxes (*A*) and TER (*B*) were determined in differentiated Caco-2 cell monolayers, as described under MATERIALS AND METHODS. Cells were incubated for 3 h with carbacyclin (300 nM) or sulprostone (2 nM) plus ONO-8711 (250 nM), plus SC-19220 (300 nM), or plus ONO-AE3-240 (2 nM) in the apical and basolateral compartments. Results were expressed as the percentage of D-mannitol fluxes and TER values obtained in control conditions ($3.8 \pm 0.38$ fmol/cm$^2$ and $314 \pm 20.3$ $\Omega \cdot$cm$^2$). The data were means $\pm$ SE of $n = 6$–8. *$P < 0.05$ vs. carbacyclin; **$P < 0.05$ vs. sulprostone; #$P < 0.05$ vs. control.

PGE$_2$ (Fig. 4, *A* and *B*). The results revealed no effect of AH-6809, here used as an $EP_2$ antagonist, but did show the capacity of two $EP_4$ antagonists (ONO-AE3-208 and AH-23848) to completely prevent these effects. To confirm $EP_4$ participation, increasing concentrations of a specific agonist for this receptor (ONO-AE1-329: 10, 100, $10^3$, and $10^4$ nM) were tested (Fig. 5, *A* and *B*), and the results showed an increase in D-mannitol fluxes for the highest concentration, although no effects on TER values were detected. Finally, to confirm the lack of $EP_2$ and $EP_3$ involvement, the effect of increasing concentrations of an $EP_2$ (ONO-AE1-259: 10, 100, $10^3$, and $10^4$ nM) and two $EP_3$ agonists (ONO-AE-248: 10, 100, $10^3$, and $10^4$ nM and GR63799: 2, 20, 200, and $2 \times 10^3$ nM) were determined. The results obtained revealed no effect on D-mannitol fluxes or on TER values for either of the concentrations tested. Figure 5, *A* and *B*, shows the values obtained for the highest concentration tested. Therefore, all these results together suggest $EP_1$ and $EP_4$, but not $EP_2$ and $EP_3$, involvement in PP regulation by PGE$_2$.

The participation of $EP_1$ and $EP_4$ was further investigated by analyzing the signaling pathways involved, especially the changes in $[Ca^{2+}]_i$. $EP_1$ activates a $G_q$ protein that induces PLC activation. PLC cleaves phosphatidylinositol 4,5-bisphosphate into diacylglycerol (DAG) and inositol trisphosphate ($IP_3$) and the interaction of $IP_3$ with its receptors at the endoplasmic reticulum results in an increase in $[Ca^{2+}]_i$ (44). Therefore, coupling of PGE$_2$ to $EP_1$ results in PKC activation. In contrast, $EP_4$ activates a $G_s$ protein, which stimulates cAMP formation by adenylate cyclase (44). The results shown in Fig. 6*A* confirm the capacity of PGE$_2$ to increase $[Ca^{2+}]_i$ in Caco-2 cell cultures, an effect that was prevented by ONO-8711, U73122 (PLC inhibitor), and dantrolene (an inhibitor of intracellular $Ca^{2+}$ release from the endoplasmic reticulum). The changes in $[Ca^{2+}]_i$ induced by carbacyclin were not so pronounced, and the maximum obtained for this variable was delayed (Fig. 6*B*). This effect was also prevented by ONO-8711, U73122, and dantrolene. $IP_3$ was tested, too, and the results also revealed a delayed peak but a similar increase in $[Ca^{2+}]_i$ as in PGE$_2$-treated cells.

Phosphorylation of $IP_3$ receptors by PKA is considered an important locus for cross talk between PLC-$IP_3$-$Ca^{2+}$ and cAMP-PKA pathways and has been put forward as having major functions in diverse $Ca^{2+}$-regulated events, such as neural activity, epithelial cell fluid secretion, and modulation of insulin secretion (11, 13, 21, 37, 46). For this reason, the effect of butaprost on $[Ca^{2+}]_i$ was also tested. Interestingly, the results revealed a significant increase in this variable (Fig. 6*C*), an effect that was mimicked by forskolin (adenylate cyclase activator) and prevented, in both cases, by dantrolene. The addition of KT5720 (PKA inhibitor) to butaprost and to PGE$_2$ was also tested and, after 5-min treatment, $[Ca^{2+}]_i$ was reduced by 84 and 64%, respectively (data not shown).

The participation of both transduction pathways in PGE$_2$-induced epithelial barrier disruption was also tested. The results revealed that the increase in D-mannitol fluxes and the reduction in TER induced by PGE$_2$ were prevented by U73122, dantrolene, and Gö6983 (PKC inhibitor; Fig. 7, *A* and *B*). Moreover, $IP_3$ and diC8 (a DAG analog) significantly increased D-mannitol fluxes and reduced TER values.

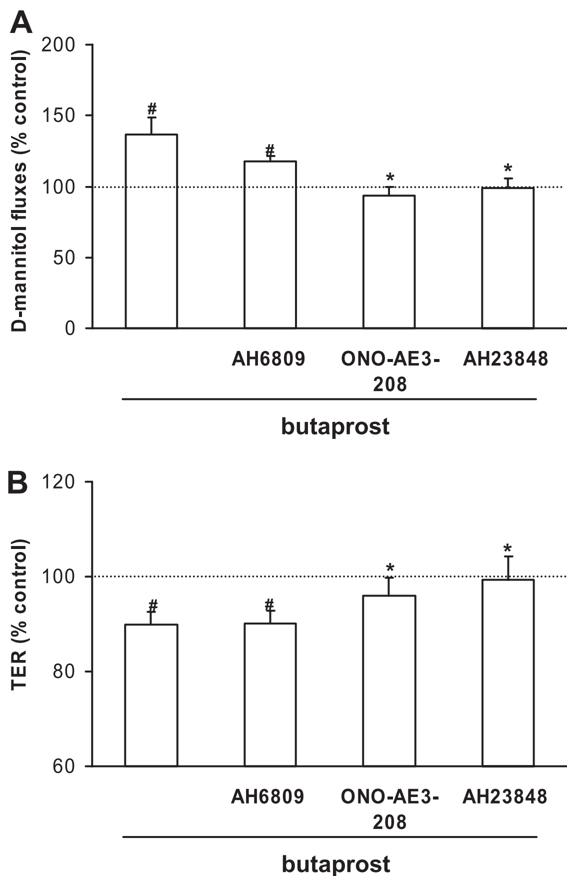


Fig. 4. Effect of $EP_2$ and $EP_4$ antagonists on epithelial barrier disruption induced by butaprost. D-mannitol fluxes (*A*) and TER (*B*) were determined in differentiated Caco-2 cell monolayers, as described under MATERIALS AND METHODS. Cells were incubated for 3 h with butaprost (250 nM) and butaprost plus AH-6809 ($3 \times 10^3$ nM), plus ONO-AE3-208 (2 nM), or plus AH-23848 (200 nM) in the apical and basolateral compartments. Results were expressed as the percentage of D-mannitol fluxes and TER values obtained in control conditions (3.7 ± 0.3 fmol/cm² and 295 ± 28.4 Ω·cm²). The data were means ± SE of $n = 6$–12. *$P < 0.05$ vs. butaprost; #$P < 0.05$ vs. control.

Regarding $EP_4$ participation, the effect of butaprost on D-mannitol fluxes was also prevented by dantrolene, although no effects on TER values were detected (Fig. 8, *A* and *B*). Moreover, $PGE_2$ effects on both D-mannitol fluxes and TER were prevented by the addition of SQ22,536 (adenylate cyclase inhibitor) and KT5720, thus suggesting cAMP involvement associated with $Ca^{2+}$ mobilization. Forskolin also induced a significant increase in PP, an effect again prevented by the addition of dantrolene.

The contribution of TJ proteins and cytoskeletal actin to PP regulation by $PGE_2$ was also investigated. The results of TJ protein immunofluorescent staining show in control conditions occludin and ZO-1 mainly located at the cell border (Fig. 9). The treatment of Caco-2 cell monolayers with $PGE_2$, carbacyclin, or butaprost resulted in a redistribution of occludin with adjacent diffuse intracellular staining and granular appearance, mainly in carbacyclin-treated cells. The effect on ZO-1 location was not so pronounced; only the presence of cytosolic diffuse fluorescence was detected in carbacyclin- and butaprost-treated cells. Morphological assessment of subapical actin showed characteristic perijunctional rings in control monolayers. Treatment with $PGE_2$ induced a complete disorganization of the F-actin belt. In the case of carbacyclin and butaprost, the images revealed a general reduction in fluorescent signal, more pronounced in the case of carbacyclin, showing brighter foci alternating with areas of reduced labeling, accompanied by the presence of adjacent diffuse intracellular fluorescent material.

Myosin light chain (MLC) kinase (MLCK) activation induces the phosphorylation of the regulatory MLC and thus the contraction of the subapical actomyosin ring leading to an increase in PP (29). The results shown in Table 2 revealed that ML-7, an MLCK inhibitor, completely prevented the effects of $PGE_2$ and butaprost on D-mannitol fluxes and TER, thus suggesting MLCK involvement.

## DISCUSSION

Cultures of differentiated Caco-2 cells form a highly polarized epithelium with many of the properties of the intestinal villous absorptive cells and constitute an in vitro experimental model, currently used to evaluate intestinal epithelial PP. Human colon cancer cells express EP receptors. In this sense, Shoji et al. (43) analyzed $EP_1$–$EP_4$ expression in different cell lines including Caco-2 cells and observed the presence of $EP_1$, $EP_2$, and $EP_4$ in most of them. Accordingly, Löffler et al. (27)

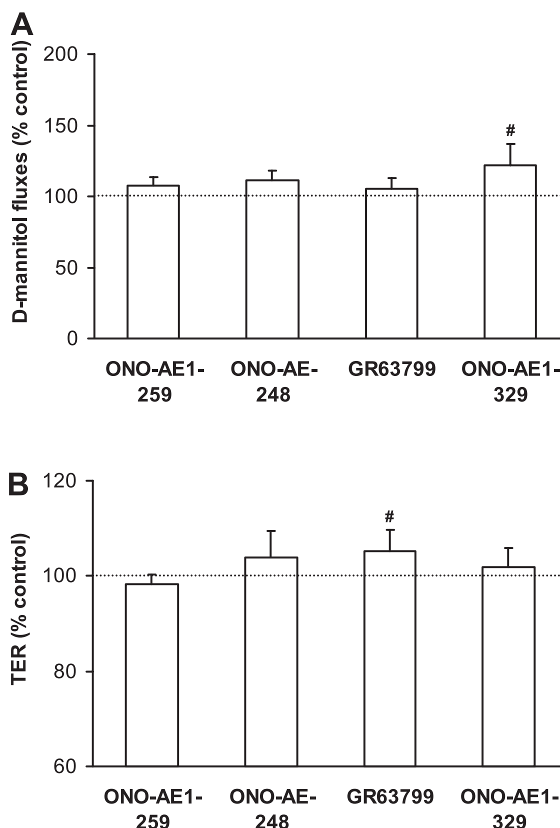


Fig. 5. Effect of $EP_2$, $EP_3$, and $EP_4$ agonists on paracellular permeability (PP). D-mannitol fluxes (*A*) and TER (*B*) were determined in differentiated Caco-2 cell monolayers, as described under MATERIALS AND METHODS. Cells were incubated for 3 h with ONO-AE1-259 ($10^4$ nM), ONO-AE-248 ($10^4$ nM), GR63799 ($2 \times 10^3$ nM), or ONO-AE1-329 ($10^4$ nM) in the apical and basolateral compartments. Results were expressed as the percentage of D-mannitol fluxes and TER values obtained in control conditions (3.2 ± 0.2 fmol/cm² and 254 ± 11 Ω·cm²). The data were means ± SE of $n = 5$–11. #$P < 0.05$ vs. control.

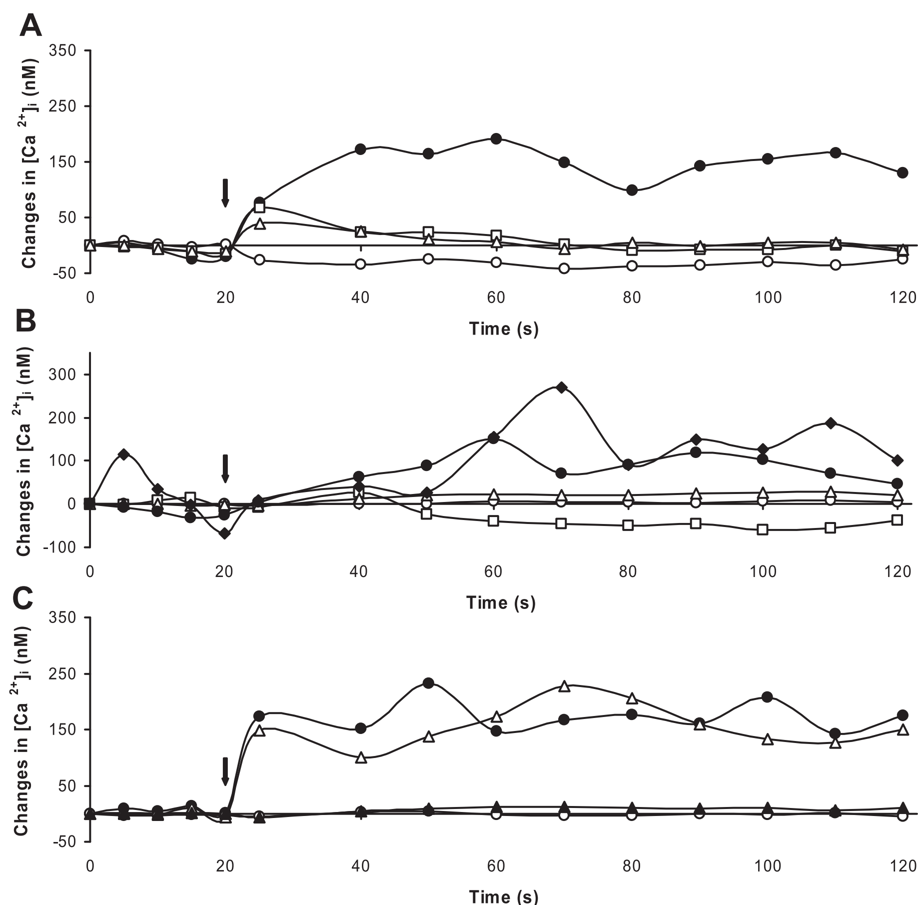


Fig. 6. Changes in intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) induced by PGE$_2$ and PGE$_2$ agonists. Changes in $[Ca^{2+}]_i$ were determined in differentiated Caco-2 cell monolayers using fura-2 AM, as described under MATERIALS AND METHODS. Cells were incubated for 120 s in the presence of *A*: PGE$_2$ (●; 13 μM) and PGE$_2$ plus ONO-8711 (□; 250 nM), plus U73122 (△; 0.1 μM), or plus dantrolene (○; 50 μM); *B*, inositol trisphosphate (IP$_3$; ♦; 45 μM), carbacyclin (●; 70 μM), carbacyclin plus ONO-8711 (□; 250 nM), plus U73122 (△; 0.1 μM), or plus dantrolene (○; 50 μM); and *C*: butaprost (●; 250 μM), butaprost plus dantrolene (○; 50 μM), forskolin (△; 450 μM), or forskolin plus dantrolene (▲; 50 μM). Each plot corresponds to a representative profile obtained for $n = 3$. The arrow indicates the injection of PGE$_2$ or PGE$_2$ agonist, IP$_3$, or forskolin. Antagonists and inhibitors were preincubated for 30 min.

detected the expression of these EP receptors in intestinal HT-29 cells. Here, we demonstrate the presence of EP$_1$–EP$_4$ receptors in differentiated Caco-2 cells, suggesting a higher basolateral localization. Moreover, EP receptors were described to be involved in the regulation of various cellular processes in Caco-2 cells (cell proliferation and adhesion, interleukin synthesis, and PP) (15, 26, 27, 31, 43, 45).

The first step to investigate the involvement of PGE$_2$ receptors in the regulation of epithelial barrier function was to test the capacity of different receptor antagonists to prevent PGE$_2$ effects. ONO-8711 and SC-19220 are specific antagonists for EP$_1$, and AH-23848 is for EP$_4$; therefore, their capacity to prevent PGE$_2$ epithelial barrier disruption was considered the first proof of EP$_1$ and EP$_4$ participation in these events. The results obtained with AH-6809 also suggest the participation of EP$_1$, although this antagonist has greater affinity for EP$_2$. Moreover, the capacity of ONO-AE3-240 and ONO-AE3-208 to prevent PGE$_2$ effects may indicate the participation of EP$_3$ and/or EP$_4$. Thus our first hypothesis was to consider the participation of at least EP$_1$ and EP$_4$. Nevertheless, the cross-reactivity of some of the antagonists used led us to test the effect of these antagonists on changes in PP induced by other, more specific agonists than PGE$_2$. Carbacyclin and sulprostone were used to test the contribution of EP$_1$ and EP$_3$, and butaprost of EP$_2$ and EP$_4$. The results obtained show the capacity of ONO-8711 and SC-19220 to prevent both carbacyclin and sulprostone effects, and no changes were detected for ONO-AE3-240, which in this case only interacts with EP$_3$. These results may allow both the rejection of EP$_3$ involvement and confirmation of EP$_1$ contribution. As for butaprost results, ONO-AE3-240 and AH-23848 were found to prevent the changes induced by PGE$_2$ in PP, which remained unchanged with AH-6809 (used in this case as an EP$_2$ antagonist), thus suggesting the lack of EP$_2$ participation while confirming EP$_4$ involvement. The participation of EP$_4$ and the lack of EP$_2$ and EP$_3$ involvement were further confirmed with specific receptor agonists such as ONO-AE1-259 for EP$_2$, ONO-AE-248 and GR63799 for EP$_3$, and ONO-AE1-239 for EP$_4$.

Recently, Tanaka et al. (45) investigated the mechanisms underlying the effect of PGE$_2$ on PP, but they did so in nondifferentiated Caco-2 cells, using a high PGE$_2$ concentration that is hard to reach in physiological/pathological conditions. They conclude that EP$_1$ and EP$_2$ were involved in PGE$_2$ effects from the results obtained with a specific EP$_1$ agonist and butaprost, respectively. However, they did not consider that butaprost is also an EP$_4$ agonist (44), and they did not test any PGE$_2$ antagonist. Therefore, these data in addition to our results support EP$_1$ participation and the lack of EP$_3$ contribution, but different conclusions can be drawn concerning EP$_2$ and/or EP$_4$ involvement.

We must consider that PP change induced by PGE$_2$ could be consequence of PGE$_2$ binding to other prostanoid receptors such as DP, FP, IP, and TP. In this sense, PGD$_2$ is a prostanoid showing higher affinity for DP and FP receptors than PGE$_2$ (9, 39, 40), very low and similar affinity as PGE$_2$ for IP and TP receptors (33, 39), and very low affinity for EP receptors (39,

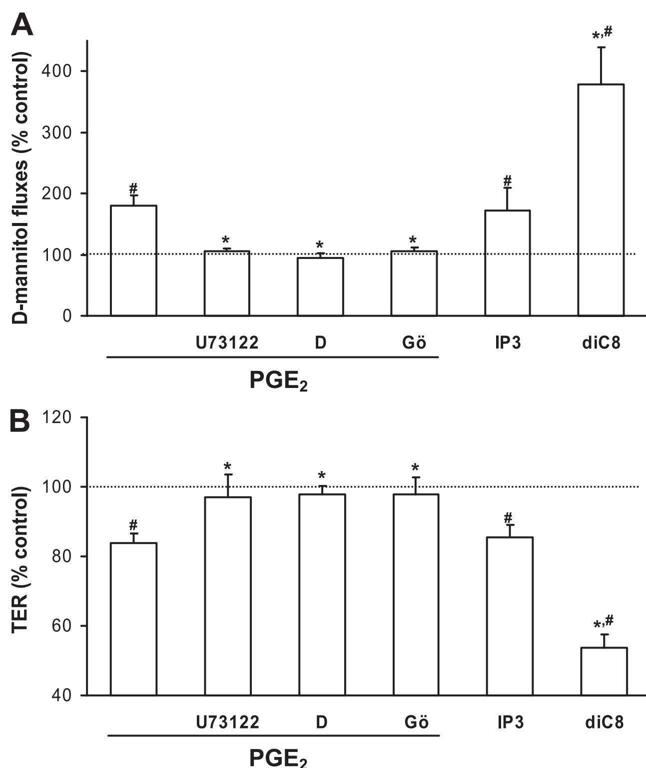


Fig. 7. PLC-IP$_3$-Ca$^{2+}$-PKC pathway involvement in epithelial barrier disruption induced by PGE$_2$. D-mannitol fluxes (*A*) and TER (*B*) were determined in differentiated Caco-2 cell monolayers, as described under MATERIALS AND METHODS. Cells were incubated for 3 h with PGE$_2$ (3 nM) or PGE$_2$ plus U73122 (0.1 μM), plus dantrolene (D; 50 μM), or plus Gö6983 (Gö; 1 μM), IP$_3$ (18 μM), or dioctanoylglycerol (diC8; 0.5 mM) in the apical and basolateral compartments. Results were expressed as the percentage of D-mannitol fluxes and TER values obtained in control conditions (4.0 ± 0.24 fmol/cm$^2$ and 302 ± 47 Ω·cm$^2$). The data were means ± SE of $n$ = 5–11. *$P < 0.05$ vs. PGE$_2$; #$P < 0.05$ vs. control.

44). However, we have observed that PGD$_2$ did not have any effect either on D-mannitol fluxes (control: 100 ± 7.7, PGD$_2$: 116.4 ± 22.9%, $n$ = 4–5; $P > 0.05$) or TER (control: 100 ± 1.6, PGD$_2$: 101.4 ± 10.2%, $n$ = 4–5; $P > 0.05$). Consequently, these findings report that, in our experimental conditions, a DP/FP/IP/TP agonist such as PGD$_2$ did not modify PP, suggesting that the effect of PGE$_2$ on PP is not mediated by its interaction with the above mentioned prostanoid receptors.

The participation of EP$_1$ and EP$_4$ in epithelial barrier function regulation was further investigated through the study of intracellular signaling pathways, especially changes in $[Ca^{2+}]_i$. As expected from PGE$_2$ interaction with EP$_1$ receptor, an increase in $[Ca^{2+}]_i$ was observed after the treatment of the cells with this prostanoid, an effect that was prevented by an EP$_1$ antagonist. The results also indicate the participation of the PLC-IP$_3$-Ca$^{2+}$ signaling pathway, since both $[Ca^{2+}]_i$ and PP were increased by IP$_3$. Moreover, the increase in $[Ca^{2+}]_i$ and PP induced by PGE$_2$ and carbacyclin was prevented by an EP$_1$ antagonist and by the inhibition of PLC or intracellular Ca$^{2+}$ release from the endoplasmic reticulum.

PKC consists of a family of Ser/Thr-specific kinases that includes 12 known isozymes that can be classified into 3 subfamilies: conventional (α, β$_1$, β$_2$, and γ), novel (δ, ε, θ, η, and μ), and atypical (λ, τ, and ζ). Conventional isoforms are both Ca$^{2+}$- and DAG-dependent, novel isoforms are Ca$^{2+}$-independent but DAG-dependent, whereas atypical PKC isoforms are both Ca$^{2+}$- and DAG-independent (19). Although contradictory results are reported on PKC contribution to the regulation of epithelial barrier function, it is accepted that conventional isoforms participate in TJ disassembly, whereas novel isoforms regulate TJ formation (4). The results reported here indicate that the increase in PP induced by PGE$_2$ was prevented by Gö6983, which is described as a pan-PKC inhibitor (19). Given the results obtained with dantrolene, indicating the contribution of Ca$^{2+}$ to PKC activation and the capacity of IP$_3$ and diC8 to increase PP, the participation of a conventional PKC isoform in the disruption of epithelial barrier function by PGE$_2$ should also be considered.

As for EP$_4$ underlying intracellular mechanisms, the protective effect detected by adenylate cyclase and PKA inhibition confirm the expected participation of the cAMP-PKA pathway. Nevertheless, a significant increase in $[Ca^{2+}]_i$ was observed after butaprost incubation, an effect that was mimicked by forskolin and prevented, in both cases, by the addition of dantrolene, thus suggesting the involvement of intracellular Ca$^{2+}$ stores. Interestingly, the profile of $[Ca^{2+}]_i$ changes shows a rapid and sustained increase for PGE$_2$, butaprost, and forskolin, whereas a more delayed peak was observed for carbacyclin and IP$_3$. Therefore, the profile of $[Ca^{2+}]_i$ changes is different, whether cAMP is involved or not.

Recently, several authors described the cross-talk relationship between IP$_3$ receptors/Ca$^{2+}$ release and the cAMP-PKA signaling pathway. Phosphorylation of IP$_3$ receptors by PKA is the locus where these two signal transduction pathways converge and is involved in diverse Ca$^{2+}$-regulated physiological processes, such as neuronal activity, epithelial fluid secretion, and modulation of insulin secretion (11, 13, 21, 37, 46). These authors have demonstrated that PKA phosphorylation results in a significant enhancement of IP$_3$-induced $[Ca^{2+}]_i$ from either extracellular or intracellular origins. Chaloux et al. (13) showed that PKA enhances IP$_3$-induced Ca$^{2+}$ release in endocrine cells by increasing IP$_3$ binding affinity. More recently, Wagner et al. (50) demonstrated that PKA phosphorylation increases the sensitivity of the IP$_3$ receptor to IP$_3$. They explained this effect as the ability of IP$_3$ to gate the channel by increasing Ca$^{2+}$ sensitivity of the receptor. Thus our results show the capacity of butaprost and forskolin to increase $[Ca^{2+}]_i$ in the absence of any IP$_3$-stimulating Ca$^{2+}$ release stimulus. In line with this, Chaloux et al. (13) found that forskolin also increased $[Ca^{2+}]_i$ in the absence of any Ca$^{2+}$-mobilizing agonist. They ascribed this phenomenon to the ability of basal intracellular levels of IP$_3$ to simulate IP$_3$ receptors. Therefore, this effect was exerted by low IP$_3$ doses and, accordingly, they found that a maximum IP$_3$ dose was unable to modify Ca$^{2+}$ release. Thus our hypothesis to explain the capacity of butaprost and forskolin to enhance $[Ca^{2+}]_i$ is that basal IP$_3$ levels in Caco-2 cell monolayers induce Ca$^{2+}$ release under conditions in which cAMP levels were increased. This proposed mechanism may explain the ability of dantrolene to prevent the increase in PP induced by PGE$_2$, butaprost, and forskolin. These results corroborate those of Gu et al. (21), who also described such an effect for PGE$_2$ in cultured rat vagal sensory neurons. Therefore, the effect of PGE$_2$ on PP mediated through EP$_4$ interaction may result, in Caco-2 cells, in increased $[Ca^{2+}]_i$ strengthened by cAMP formation, thus

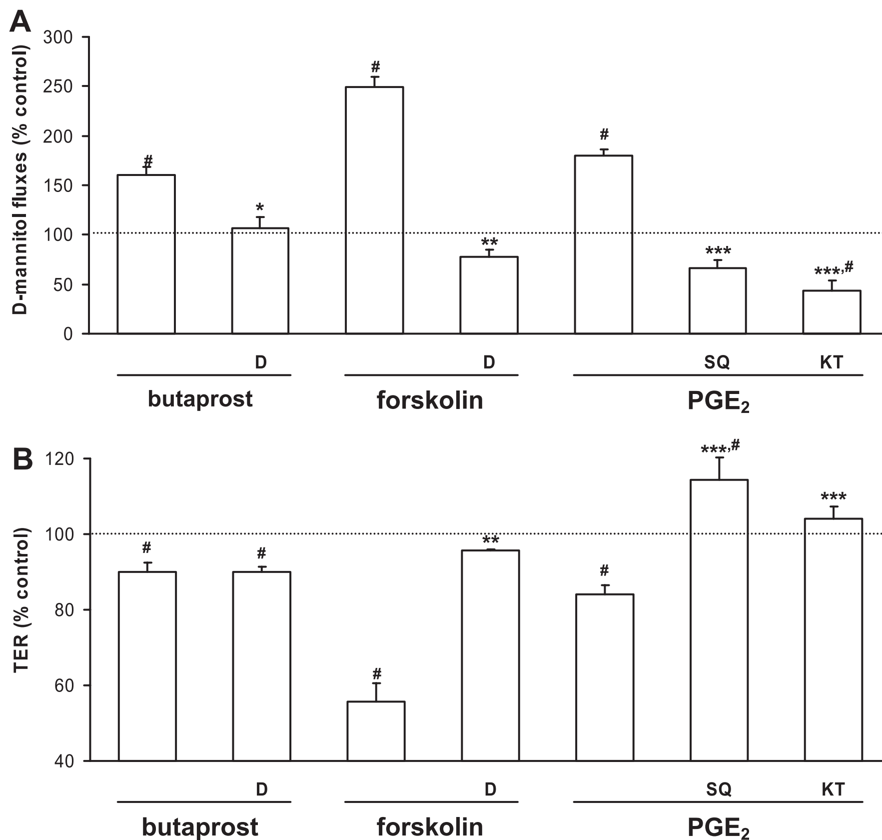


Fig. 8. cAMP-PKA pathway involvement in epithelial barrier disruption induced by PGE$_2$. D-mannitol fluxes (*A*) and TER (*B*) were determined in differentiated Caco-2 cell monolayers, as described under MATERIALS AND METHODS. Cells were incubated for 3 h with butaprost (250 nM), butaprost plus dantrolene (D; 50 μM), forskolin (10 μM), forskolin plus dantrolene (D; 50 μM), PGE$_2$, or PGE$_2$ plus SQ22,536 (SQ; 10 μM) or plus KT5720 (KT; 10 μM) in the apical and basolateral compartments. Results were expressed as the percentage of D-mannitol fluxes and TER values obtained in control conditions (3.0 ± 0.28 fmol/cm$^2$ and 456 ± 24.3 Ω·cm$^2$). The data were means ± SE of $n$ = 4–7. *$P$ < 0.05 vs. butaprost; **$P$ < 0.05 vs. forskolin; ***$P$ < 0.05 vs. PGE$_2$; #$P$ < 0.05 vs. control.

confirming the cross talk interaction of these two signaling pathways in the intestinal epithelium.

Previous studies indicated that MLCK plays an important role in the regulation of intestinal TJ permeability. The increase in PP induced by TNF-α, IFN-γ, IL-1β, bile acids, cytochalasins, ethanol, $Na^+$-glucose transport, and extracellular $Ca^{2+}$ has been described as mediated by increased MLCK activity (2, 6, 28, 41, 48, 53). Moreover, the overexpression of MLCK in Caco-2 cells induces the reorganization of perijunctional actin and increases PP (41). In fact, MLCK expression is significantly enhanced in the mucosa of IBD patients (8). In addition, the protective action of glucocorticoids on epithelial barrier function has been ascribed to the suppression of TNF-α-induced increase in MLCK activity (10). Our results showing the ability of an MLCK inhibitor to prevent the effects induced on both D-mannitol fluxes and TER by PGE$_2$ and butaprost confirm these data. Since MLCK is activated by the $Ca^{2+}$-calmodulin complex (19), these results also confirm the above data by suggesting that the butaprost effect may be mediated by an increase in $[Ca^{2+}]_i$. In this respect, the reduction in TER induced by bile acids in Caco-2 cells through increased MLCK activity was described as mediated at least in part by an increase in cyclooxygenase and PKC activities (6). It is not the first time that a relationship between PKC and MLCK has been posited in TER regulation, but different conclusions were drawn. Turner et al. (47) found in Caco-2 cells that MLCK can be inhibited via PKC phosphorylation, thus reducing MLC phosphorylation and increasing TER. In contrast, the increase in PP induced in T-84 cells by infection with enterohemorrhagic *Escherichia coli* and in brain endothelial cells with HIV-1 envelope glycoprotein gp120 was described by a process that includes the activation of both PKC and MLCK (24, 35), as appears to be the case of PGE$_2$ in our experimental conditions.

Confocal microscopy findings revealed an alteration in occludin, ZO-1, and actin distribution. ZO-1 constitutes the bridge between the perijunctional actin ring and TJ occludin (17). Therefore, a correlation between the disturbance of perijunctional actin filaments and occludin and ZO-1 location may be established. Nevertheless, ZO-1 is the TJ protein that shows the fewest alterations in distribution; the main change observed after incubation with carbacyclin and butaprost was the presence of cytosolic fluorescence. Similarly, Bruewer et al. (12) detected that, despite the dramatic redistribution of TJ transmembrane proteins following IFN-γ exposure, ZO-1 was only minimally affected, and most of it remained at the TJ. Moreover, Tanaka et al. (45) found that the incubation of nondifferentiated Caco-2 cells with PGE$_2$ significantly affected E-cadherin, β-catenin, claudin-1, and occludin distribution but was unable to alter ZO-1 location. Regarding the formation of fluorescent clumps, Ma et al. (28) attributed their formation in Caco-2 cells treated with ethanol to a multifocal aggregation of cytoskeletal elements, including actin. They also suggested a central role for actin-myosin contraction in the formation of these aggregates (29). In this respect, the presence of cytosolic occludin is associated with protein internalization by endocytosis. In the case of barrier loss induced by TNF-α, occludin internalization was described as a MLCK-dependent process (38). The results obtained here in Caco-2 cells indicate that the increase in PP may correlate with the redistribution of TJ

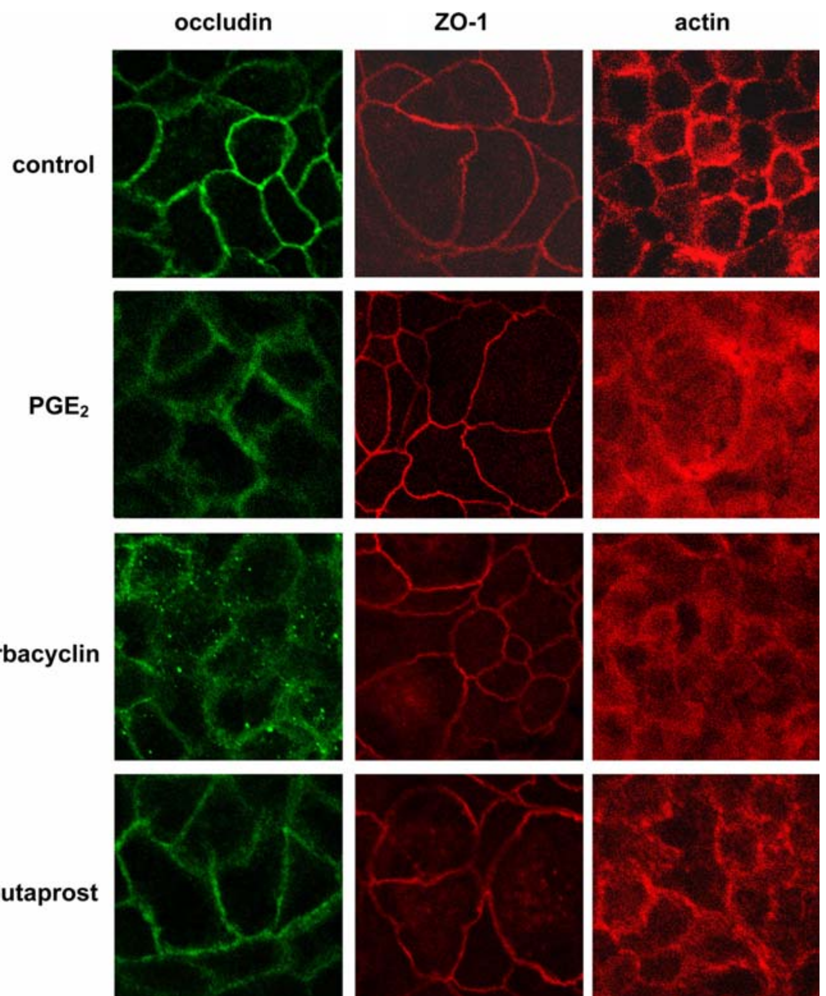


Fig. 9. Changes in occludin, zona occludens 1 (ZO-1), and perijunctional actin distribution induced by PGE$_2$ agonists. Confocal analysis was performed using specific occludin and ZO-1 antibodies and phalloidin-tetramethylrhodamine B isothiocyanate (TRITC-phalloidin), as described under MATERIALS AND METHODS, in cells incubated for 3 h with PGE$_2$ (3 nM), carbacyclin (300 nM), or butaprost (250 nM). In each case, a representative *x*-*y* image of sections close to the apical cell side is shown.

proteins (mainly occludin), the perijunctional actin ring, and MLCK activation.

All the results obtained lead us to conclude that the regulation of the epithelial barrier function by PGE$_2$ is mediated by interaction with EP$_1$ and EP$_4$ receptors, which activate PLC-IP$_3$-Ca$^{2+}$ and cAMP-PKA pathways, respectively, and lead to a common increase in [Ca$^{2+}$]$_i$. Moreover, PKC and MLCK may be involved in the regulation of PP by PGE$_2$. Furthermore, these events are accompanied by the redistribution of TJ

Table 2. *Effect of ML-7 on changes induced by PGE$_2$ or butaprost on* D*-mannitol fluxes and TER*

| | D-Mannitol Fluxes, % Control | TER, % Control |
|---|---|---|
| PGE$_2$ | 162.7 ± 24.3‡ | 84 ± 2.6‡ |
| PGE$_2$ + ML-7 | 77.4 ± 18.6* | 100.2 ± 4.6* |
| Butaprost | 136.9 ± 11.9‡ | 89.1 ± 3.57‡ |
| Butaprost + ML-7 | 89.5 ± 35.9† | 111.1 ± 3.4† |

Values are means ± SE of $n$ = 3–5. D-Mannitol fluxes and transepithelial electrical resistance (TER) were determined in differentiated Caco-2 cell monolayers, as described under MATERIALS AND METHODS. Cells were incubated for 3 h with PGE$_2$ (3 nM), PGE$_2$ plus myosin light chain kinase inhibitor ML-7 (50 μM), butaprost (250 nM), or butaprost plus ML-7 (50 μM). Results are expressed as the percentage of D-mannitol fluxes and TER values obtained in control conditions (3.5 ± 0.4 fmol/cm$^2$ and 326 ± 66 Ω·cm$^2$). *$P$ < 0.05 vs. PGE$_2$; †$P$ < 0.05 vs. butaprost; ‡$P$ < 0.05 vs. control.

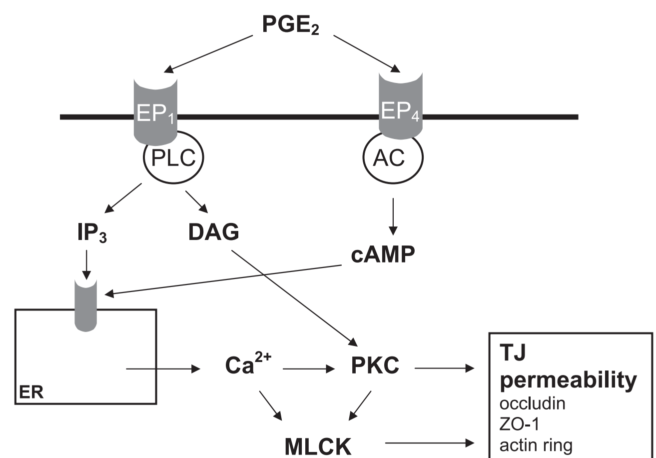


Fig. 10. Mechanisms involved in the regulation of PP by PGE$_2$. PGE$_2$ interaction with EP$_1$ activates PLC, which induces IP$_3$ and diacylglycerol (DAG) formation. IP$_3$ induces Ca$^{2+}$ release from endoplasmic reticulum (ER). Moreover, PGE$_2$ binding to EP$_4$ induces cAMP synthesis by adenylate cyclase (AC). The enhancement of cAMP levels stimulates IP$_3$-induced Ca$^{2+}$ release. These events are involved in PP enhancement associated with an increase in myosin light chain kinase (MLCK) activity and changes in the distribution of the tight junction (TJ) proteins, occludin and ZO-1, and the perijunctional actin ring.

proteins and the perijunctional actin ring (Fig. 10). Although the disruption of epithelial barrier function observed in IBD patients has been traditionally attributed to the effect of TNF-α and IFN-γ, the present study goes deeper into the function of $PGE_2$ in the inflammatory process, thus opening up new therapeutic strategies.

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## DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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Artículo 2

Effect of eicosapentaenoic acid derived prostaglandin $E_3$ on intestinal epithelial barrier function.

Rodríguez-Lagunas MJ, Ferrer R, Moreno JJ

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Los resultados obtenidos han dado lugar a las siguientes comunicaciones a congresos:

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### *Resumen artículo 2*

**Objetivo**: investigar el efecto de la $PGE_3$ sobre la PP e identificar los receptores y las vías de señalización implicados.

**Material y métodos**: la PP se ha estudiado a través de la determinación de la TER y de los flujos de dextrano en presencia de la $PGE_3$ y de diferentes agonistas y antagonistas de los receptores $EP_1$-$EP_4$ en células Caco-2 cultivadas sobre filtros. La determinación de la $[Ca^{2+}]_i$ se ha realizado por espectrofluorimetría, los niveles de AMPc y de NFκB se han cuantificado por enzima inmuno ensayo y el estado de las proteínas de la TJ se ha determinado por inmunofluorescencia.

**Resultados**: la presencia de $PGE_3$ en cultivos de células Caco-2 induce un incremento de la PP. Dicha alteración se previene al preincubar las células con antagonistas de los receptores $EP_1$ y $EP_4$. Además, la inhibición de la PKC y de la MLCK previenen la alteración de la PP inducida por la $PGE_3$. Por otra parte, la $PGE_3$ induce un incremento de la $[Ca^{2+}]_i$ y del AMPc, confirmando la participación de $EP_1$ y $EP_4$, respectivamente. Los resultados de immunofluorescencia muestran como la presencia de $PGE_3$ es capaz de inducir la redistribución de las proteínas de la TJ ocludina y claudina-4, además del anillo de actina, sin modificar la ZO-1, la claudina-1 o -2.

**Conclusiones**: el hecho que la $PGE_3$ sea capaz de incrementar la PP hace reconsiderar el rol de la relación $PGE_2$/$PGE_3$ en los efectos beneficiosos de la suplementación dietética con aceite de pescado en la disrupción de la función barrera.

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# Effect of eicosapentaenoic acid-derived prostaglandin $E_3$ on intestinal epithelial barrier function

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ABSTRACT

Prostaglandins (PG) are inflammatory mediators derived from arachidonic or eicosapentaenoic acid giving rise to the 2-series or the 3-series prostanoids, respectively. Previously, we have observed that $PGE_2$ disrupts epithelial barrier function. Considering the beneficial effect of fish oil consumption in intestinal inflammatory processes, the aim of this study was to assess the role of $PGE_3$ on epithelial barrier function assessed from transepithelial electrical resistance and dextran fluxes in Caco-2 cells. The results indicate that $PGE_3$ increased paracellular permeability (PP) to the same extent as $PGE_2$, through the interaction with $EP_1$ and $EP_4$ receptors and with intracellular $Ca^{2+}$ and cAMP as the downstream targets. Moreover, we observed a redistribution of tight junction proteins, occludin and claudin-4. In conclusion, $PGE_3$ is able to increase PP thus leading to reconsider the role of $PGE_2/PGE_3$ ratio in the beneficial effects of dietary fish oil supplementation in the disruption of barrier function.

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## 1. Introduction

Many mediators of inflammation including prostaglandins (PG), leukotrienes (LT), and other oxygenated derivatives are synthesized from arachidonic acid (AA), an omega-6 (ω-6) polyunsaturated fatty acid (PUFA) highly abundant in all mammalian cells. PG are involved in numerous physiological and biochemical processes and altered production is associated with a variety of disorders, including acute and chronic inflammation and colon cancer [1].

Nutritional regulation of PG generation may be modulated by dietary enrichment with ω-3 fatty acids present in fish oil such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). EPA can be incorporated into cell membrane phospholipids [2] and can also act as a substrate for cylooxygenase (COX), giving rise to the 3-series prostanoids [3]. Although similar in structure and stability [4], mediators formed from EPA are believed not to be as potently inflammatory as those formed from AA [5]. In addition to reducing the concentrations of the proinflammatory 2-series PG, increased consumption of ω-3 PUFA also results in a 10- to 50-fold increase in 3-series PG, thus reducing the $PGE_2/PGE_3$ ratio [6]. Along these lines, fish oil supplementation of the human diet has been used as a preventive measure against a number of diseases including coronary heart disease, cancer, and inflammatory bowel disease (IBD) [7]. However, to the best of our knowledge, no studies directly comparing the effects of the two series of PG on intestinal cellular functions have been reported.

The gastrointestinal epithelium functions as a selective barrier that allows the absorption of nutrients, electrolytes and water but restricts the passage of larger, potentially toxic compounds into systemic circulation. This characteristic of the intestinal epithelium, which has been referred to as selective permeability, is maintained by three distinct adhesion systems: tight junctions (TJs), adherent junctions, and desmosomes. Of these, TJs are the most apical component and are the rate-limiting step for paracellular permeability (PP). In addition, TJs constitute the interface (fence) between the apical and basolateral membrane domains [8]. TJs are multiprotein complexes composed of transmembrane proteins associated with the cytoskeletal perijunctional ring of actin and myosin. They also contain cytosolic proteins involved in cell signaling and vesicle trafficking. Several transmembrane proteins of the junctional complex have been identified: occludin, the claudin family, tricellulin, crumbs, and junctional adhesion molecules, among others. These proteins are associated with a wide range of cytosolic proteins, of which zona occludens (ZO), i.e. ZO-1, ZO-2, ZO-3, AF6, and cingulin are described as forming the nexus with cytoskeletal proteins [9].

Epithelial barrier function can be modulated by a number of factors under physiological or pathophysiological conditions. Intestinal diseases are associated with disruption of epithelial barrier function, particularly IBD [10]. The levels of several eicosanoids, such as $PGE_2$, $PGD_2$, thromboxane $B_2$, 5-, 12- and 15-hydroxyeicosatetraenoic acid, and leukotriene (LT) $B_4$ were

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found to be elevated in the mucosa of IBD patients [11]. It has been reported that fish oil decreases colonic inflammation compared with an ω-6 PUFA-rich diet, associated with a reduction in the production of AA-derived eicosanoids in IBD experimental models. Moreover, some benefits in human trials of fish oil consumption in IBD have been reported [12].

We recently observed that the addition of $PGE_2$ to differentiated intestinal Caco-2 cells induces an increase in PP, an effect mediated by the interaction of $PGE_2$ with $EP_1$ and $EP_4$ receptors [13]. The main objective of this study was to assess in Caco-2 cells whether the 3-series PG effects on epithelial barrier function could explain, at least in part, the beneficial effects of fish oil consumption in IBD.

## 2. Methods and materials

### *2.1. Materials*

DMEM, trypsin, penicillin, and streptomycin were supplied by GIBCO (Paisley, Scotland). Non-essential amino acids, fetal bovine serum (FBS), bovine serum albumin (BSA), phosphate buffered saline (PBS), D-glucose, HEPES, Fura-2 acetoxymethyl ester (Fura-2-AM), fluorescein isothiocyanate-dextran (FD-4, average molecular weight 3000–5000), $PGE_2$, $PGE_3$, U73122, dantrolene, Gö6983, and phalloidin-tetramethylrhodamine B isothiocyanate (TRITC-phalloidin), 1-(5-iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine hydrochloride (ML-7), along with other chemicals, were purchased from Sigma-Aldrich (St. Louis, MO). Tissue culture supplies, including Transwells, were obtained from Costar (Cambridge, MA). ONO-8711 and ONO-AE3-240 were kindly provided by Ono Pharmaceutical Co. Ltd. (Osaka, Japan); and AH23848, by Glaxo-Wellcome (Stevenage, UK).

### *2.2. Cell culture*

Caco-2 cells were provided by the American Type Cell Collection and cultured as previously described [14]. Cells (passages 53–65) were routinely grown in plastic flasks at a density of $5 \times 10^4$ cells/cm$^2$ and cultured in DMEM containing 4.5 g/L D-glucose and 2 mM L-glutamine, supplemented with 1% (v/v) non-essential amino acids, 10% (v/v) heat-inactivated FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a modified atmosphere of 5% $CO_2$ in air. Cells grown to approximately 80% confluence were released by trypsinization and subcultured at a density of $4 \times 10^5$ cells/cm$^2$ on polycarbonate filters with a pore size of 0.4 μm (Transwells, 12 mm diameter) for PP experiments and at a density of $5 \times 10^4$ cells/cm$^2$ in 12-well clusters for intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) determination, in 24-well clusters containing coverslips for immunofluorescent microscopy, or in 75 cm$^2$ flasks for cAMP determination. For all experiments the same PGs concentration and incubation period were assayed as previously described for $PGE_2$ [13]. The medium was replaced every three days and on the day before the experiment. Experiments were performed in cultures 19–21 days after seeding, once the cells had differentiated [14].

### *2.3. Paracellular permeability*

PP was estimated from transepithelial electrical resistance (TER) and transepithelial FD-4 fluxes. After 3 h incubation with the PGs in DMEM in the apical and basolateral compartments, TER was then determined at 37 °C using a Millicell-ERS voltohmmeter (Millipore, Bedford, MA). The results were expressed as Ω cm$^2$ monolayer surface area. The resistance of the supporting membrane was subtracted from all readings before calculations. After TER determination, 1 mg/mL of FD-4 was added to the apical compartment and cells were incubated for 1 h at 37 °C. After incubation, the basolateral medium was removed and fluorescence was determined in a Fluorostar Optima (BMG Labtech, Germany) at excitation and emission wavelengths of 485 nm and 544 nm, respectively.

### *2.4. Intracellular $Ca^{2+}$ concentration*

$[Ca^{2+}]_i$ was monitored using the selective fluorescent $Ca^{2+}$ indicator Fura 2-AM as previously described [13]. Cells grown in clusters were loaded with 25 μM Fura 2-AM in DMEM for 1 h at 37 °C. The preloaded monolayers were then washed in modified Krebs buffer (pH 7.4) at 37 °C and incubated for 1 h at 37 °C to allow Fura 2-AM de-esterification. The monolayers were washed again to ensure the removal of all unloaded indicator, and eicosanoids and inhibitors were added to the respective wells. The fluorescent signal was continuously monitored with excitation wavelengths of 340 and 380 nm and emission at 510 nm using a fluorescent microplate reader (Fluorostar Optima, BMG Labtech, Germany) before and after injection of the eicosanoids. Cells were maintained at 37 °C throughout the experiment. At the end of the incubation period, the maximum and minimum intracellular probe fluorescent signals were determined by the addition of cell lysis buffer and 20 mM EDTA in Krebs, respectively. $[Ca^{2+}]_i$ was calculated following Grynkiewicz et al. [15] from a 340/380 ratio using a dissociation constant of 224 nM.

### *2.5. Intracellular cAMP concentration*

cAMP determination was performed using a competitive enzyme immunoassay (EIA) Kit (Cayman, Ann Arbor, MI) following the manufacturer's instructions. Briefly, cells maintained in flasks were incubated for 5, 15, and 30 min at 37 °C with a range of PGs concentrations. They were then incubated for 20 min at room temperature with 2.5 mL of 0.1 N HCl and harvested and homogenized. The homogenate was then centrifuged (1000 g, 10 min) and the supernatant was assayed following the acetylation procedure (sensitivity 0.1 pM).

### *2.6. NFκB activation*

Cytosolic IκB proteins bind to the NFκB/Rel transcription factor complex to maintain its inactive state. For NFκB to become activated, it must first dissociate from the inhibitor IκB, thereby enabling NFκB to translocate into the nucleus to modulate gene expression. This is induced by phosphorylation of IκB at Ser32 and Ser36 in response to various extracellular signals, including inflammatory cytokines, growth factors, and chemokines. Therefore, NFκB activation was evaluated by measuring total and phosphorylated IκB using a competitive EIA Kit (eBioscience, San Diego, CA) following the manufacturer's instructions. Briefly, cells grown in clusters were incubated for 5 and 15 min and 3 h at 37 °C with $PGE_2$ and $PGE_3$ (3 nM) and TNF-α (100 ng/mL) as a positive control.

### *2.7. Immunofluorescent staining of TJ proteins*

Control or treated Caco-2 monolayers grown on coverslips were washed gently with PBS and fixed in iced methanol or 3% paraformaldehyde (PF) and 2% sucrose in 0.1 M PBS (pH 7.4) for 15 min at −20 °C or room temperature, respectively. Cells were washed twice for 5 min in 10 mM PBS containing 20 mM glycine (PBS–glycine) and then permeabilized with 0.2% (v/v) Triton X-100 for 10 min at room temperature. Cells were washed twice in PBS-glycine and blocked for 20 min in PBS–glycine containing 1%

BSA (incubation solution). Mouse monoclonal anti-occludin (1:500 dilution; Zymed, South San Francisco, CA), rabbit polyclonal anti-ZO-1 (1:250 dilution; Zymed) and mouse polyclonal anti-claudin-2 (1:250 dilution; Invitrogen, San Diego, CA) were used as primary antibodies. Cells were incubated with the primary antibodies for 1 h at 37 °C and washed twice in PBS–glycine for 5 min at room temperature. Monolayers were then incubated for 1 h at 37 °C with Alexa dye-conjugated secondary antibodies (1:500 dilution, Molecular Probes, Leiden, The Netherlands). Finally, cells were washed for 10 min at room temperature in PBS, mounted in Mowiol (Calbiochem, San Diego, CA) and examined under an immunofluorescent microscope (BX 41, Olympus Barcelona, Spain). Images were taken using a 60x (numerical aperture 1.25, phase 3, oil) Olympus UPlanFL N objective. To view the actin subapical ring and claudin-1 and -4, coverslips were fixed in PF or methanol, respectively, then permeabilized as described above and incubated with TRITC-phalloidin for 1 h at 37 °C (1:1000 dilution) or direct-labeled claudin (1:250 dilution).

### 2.8. Data analysis

The results are expressed as mean ± SE. Data were analyzed by one-way analysis of variance followed by Dunnett's *post hoc* test using SPSS® software (SPSS Inc. Chicago, IL, USA). $P<0.05$ was considered to denote significance.

## 3. Results

Here we have examined the effect of $PGE_3$ – a COX metabolite derived from EPA – on PP using differentiated Caco-2 cell monolayers. As observed in Fig. 1A, the extent to which ω-3 prostanoid decreased TER values was comparable to that of $PGE_2$. Treatment of Caco-2 cell monolayers with $PGE_3$ in the presence of $EP_1$ and $EP_4$ receptor antagonists, (ONO-8711 and AH-23848, respectively [16,17]), resulted in the reversion of the TER values whereas the $EP_3$ antagonist (ONO-AE3-240 [18]) did not significantly modify the TER values in comparison to $PGE_3$ alone.

Moreover, we observed that the effect induced by $PGE_3$ also altered TJ permeability by increasing FD4 fluxes to a similar level as when $PGE_2$ was used (Fig. 1B). In agreement with the results for TER, the incubation of $EP_1$ and $EP_4$ antagonists led to a decrease in FD4 permeability induced by $PGE_3$. Treatment with $EP_3$ antagonist also modified this value but to a lesser extent and without significant differences in comparison to $PGE_3$.

The ability of Gö6983, a pan-PKC inhibitor [19], and ML-7, a myosin light chain kinase (MLCK) inhibitor, to prevent $PGE_3$-induced PP alteration was also assessed. The results in Fig. 1 show that both Gö6983 and ML-7 were able to revert the changes in the values of TER and FD4 fluxes induced by $PGE_3$ to control conditions.

As shown in Fig. 2A, the addition of $PGE_3$ to Caco-2 cultures resulted in a concentration-dependent increase in the levels of $[Ca^{2+}]_i$ as when $PGE_2$ was added. Furthermore, the presence of U73122, a phospholipase C (PLC) inhibitor [20], and dantrolene, an inhibitor of intracellular $Ca^{2+}$ released from the endoplasmic reticulum [21], prevented this increase in $[Ca^{2+}]_i$ (Fig. 2B). The changes in $[Ca^{2+}]_i$ induced by $PGE_3$ were also reverted when $Ca^{2+}$ was withdrawn from the incubation media (data not shown).

For the cAMP pathway, the results shown in Fig. 3 reveal that $PGE_2$ and $PGE_3$ were able to induce an increase in cAMP.

The phosphorylation of IκB allows NFκB to translocate into the nucleus to modulate gene expression. To assess whether NFκB is activated in cells treated with $PGE_3$, IκB phosphorylation was determined. The results show that neither ω-6 nor ω-3 prostaglandins were able to increase the amount of phosphorylated IκB compared to control conditions in any of the incubation periods assayed (5 min, 15 min, and 3 h), whereas TNF-α, which was used as a positive control, was able to significantly increase IκB phosphorylation (Fig. 4).

The contribution of TJ proteins and cytoskeletal actin to PP regulation by $PGE_3$ was also investigated. The results of TJ protein immunofluorescent staining show that in control conditions occludin and ZO-1 are located mainly at the cell border. The

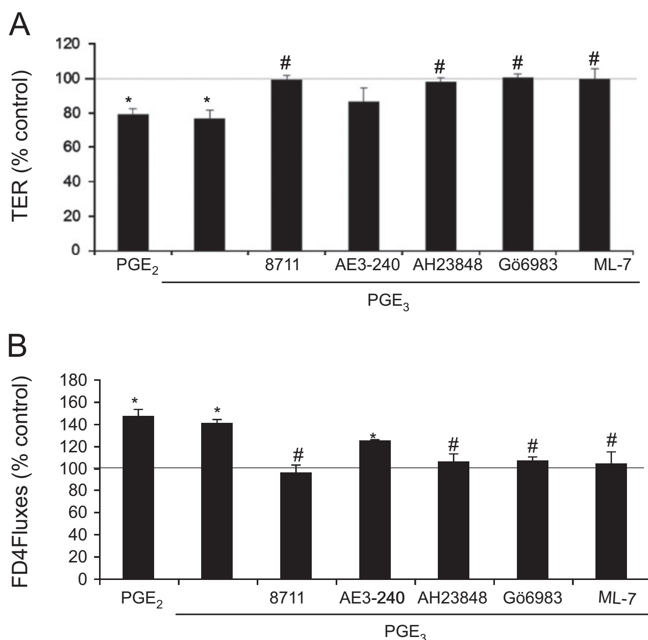


**Fig. 1.** Effect of $PGE_2$ and $PGE_3$ on epithelial barrier function TER (A) and FD-4 fluxes (B) were determined in differentiated Caco-2 cell monolayers, as described in "Materials and Methods". Cells were incubated for 3 h with PGs (3 nM) and $PGE_3$ plus ONO-8711 (250 nM), plus ONO-AE3-240 (2 nM), plus AH-23848 (200 nM), plus Gö6983 (1 μM), or plus ML7 (0.05 μM), in the apical and basolateral compartments. Results are expressed as the percentage of FD-4 fluxes and TER values obtained in control conditions (0.31 ± 0.02 ng/μL and 1689.69 ± 59.87 Ω cm²), respectively). Data are means ± SE of $n=6$–8 experiments. $^*P<0.05$ vs. control conditions. $^{\#}P<0.05$ vs. $PGE_3$.

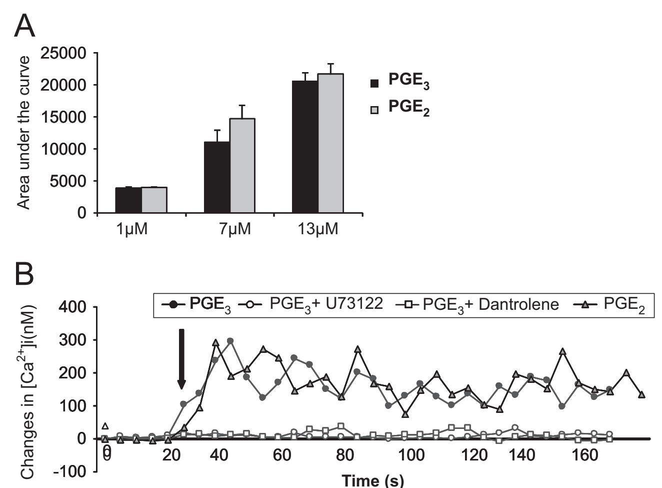


**Fig. 2.** Changes in $[Ca^{2+}]_i$ induced by $PGE_2$ and $PGE_3$. $[Ca^{2+}]_i$ were determined in differentiated Caco-2 cell monolayers using Fura-2 AM, as described in "Materials and Methods". (A) Cells were incubated for 180 s in the presence of PGs at different concentrations. Results are expressed as area under the curve (AUC). Data are means ± SE of $n=3$ experiments. (B) Cells were incubated in the presence of PGs (13 μM) and $PGE_3$ plus U73122 (0.1 μM) or plus dantrolene (50 μM). The arrow indicates the injection of PGs. Inhibitors were pre-incubated for 30 min. Each plot corresponds to a representative profile obtained for $n=3$ experiments.

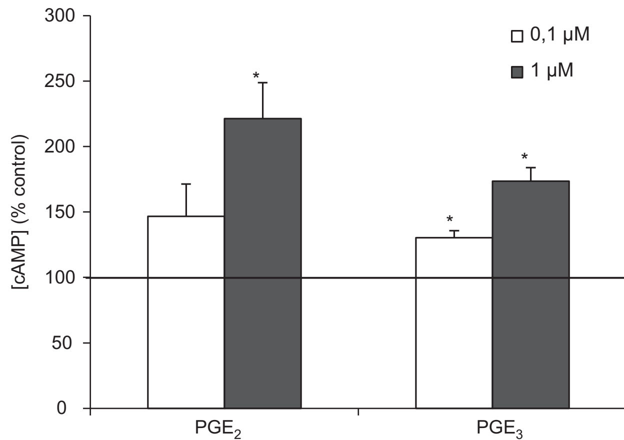


**Fig. 3.** Effect of $PGE_2$ and $PGE_3$ on intracellular cAMP concentration. cAMP was determined in differentiated Caco-2 cell, as described in "Materials and Methods". Cells were incubated for 5 min with $PGE_3$ and $PGE_2$ at two concentrations (0.1 µM, white bars and 1 µM black bars). Results are expressed as the percentage of cAMP values obtained in control conditions (23.54 ± 1.53 nM). The data are means ± SE of $n$=3–5 experiments. $^{*}P < 0.05$ vs. control conditions.

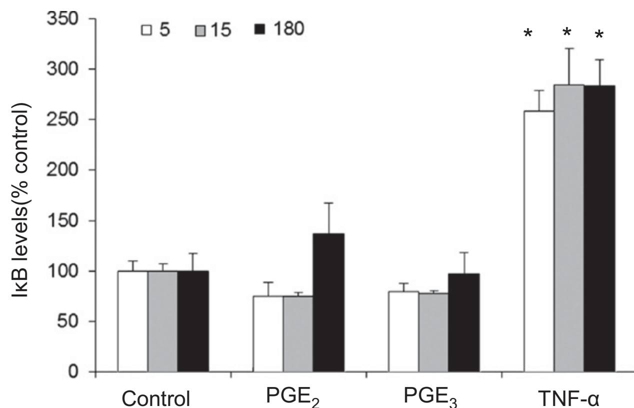


**Fig. 4.** Effect of $PGE_2$ and $PGE_3$ on phosphorylated IκB levels. Phosphorylated IκB levels were determined in differentiated Caco-2 cell monolayers as described in "Materials and Methods". Cells were incubated for 5 min, 15 min, or 3 h in the presence of the $PGE_3$ and $PGE_2$ (3 nM) or TNF-α (100 ng/mL) as a positive control. The results are expressed as the percentage with respect to control conditions. Data are mean ± SE of $n$=4–6 experiments. $*P < 0.05$ vs. control conditions.

treatment with $PGE_3$ resulted in a redistribution of occludin with adjacent diffuse intracellular staining and granular appearance (Fig. 5). No effect on ZO-1 location was observed (data not shown). The morphological assessment of subapical actin showed characteristic perijunctional rings in the control monolayers. The treatment with $PGE_3$ induced a complete disorganization of the F-actin belt. As seen in the control monolayers, claudin-4 was predominantly present at TJs and a weak cytoplasmic localization was also observed (Fig. 6). However, in response to either $PGE_2$ or $PGE_3$, claudin-4 markedly dissociated from the TJ to form protein clumps whereas the localization of claudin-1 and claudin-2 was not affected (data not shown) in neither of the PGs. Effects of $PGE_3$ on occludin and claudin-4 were partially prevented by $EP_1$ and $EP_4$ antagonists (ONO-8711 and AH-23848) as well as MLCK inhibitor (ML7) (Figs. 5 and 6).

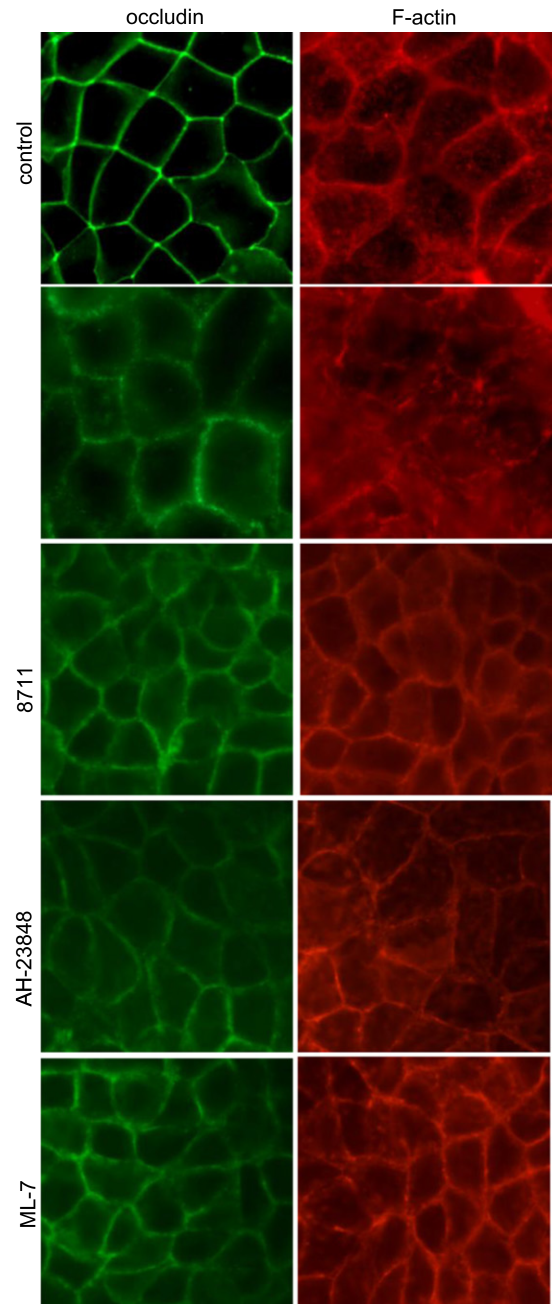


**Fig. 5.** Changes in occludin and perijunctional actin distribution induced by $PGE_3$. Cells were incubated for 3 h with $PGE_3$ (3 nM) and $PGE_3$ plus ONO-8711 (250 nM), plus AH-23848 (200 nM) or plus ML7 (0.05 µM). Fluorescent analysis was performed in cells incubated for 3 h with $PGE_3$ (3 nM) using specific occludin and ZO-1 antibodies and TRITC-phalloidin, as described in "Materials and Methods". In each case, a representative $x-y$ image of sections close to the apical cell side is shown.

## 4. Discussion and conclusions

The disruption of the intestinal TJ barrier results in an increase in paracellular permeability and is now believed to be involved in a variety of gastrointestinal disorders such as IBD, irritable bowel syndrome and celiac disease [22,23]. Previous findings indicate that the AA cascade is activated in intestinal mucosa in IBD patients [24–26]. In relation to this, we previously reported that the enhancement of $PGE_2$ levels increases PP in differentiated Caco-2 cell cultures [14]. Moreover, we found that activation of $EP_1$ and $EP_4$ receptors, which activate the PLC-$IP_3$-$Ca^{2+}$ and cAMP-PKA pathways, respectively is involved in these events [13]. However, the beneficial effect of an enriched ω-3 diet on symptom alleviation in intestinal inflammatory diseases is still controversial and to date no studies of nutritional interventions in

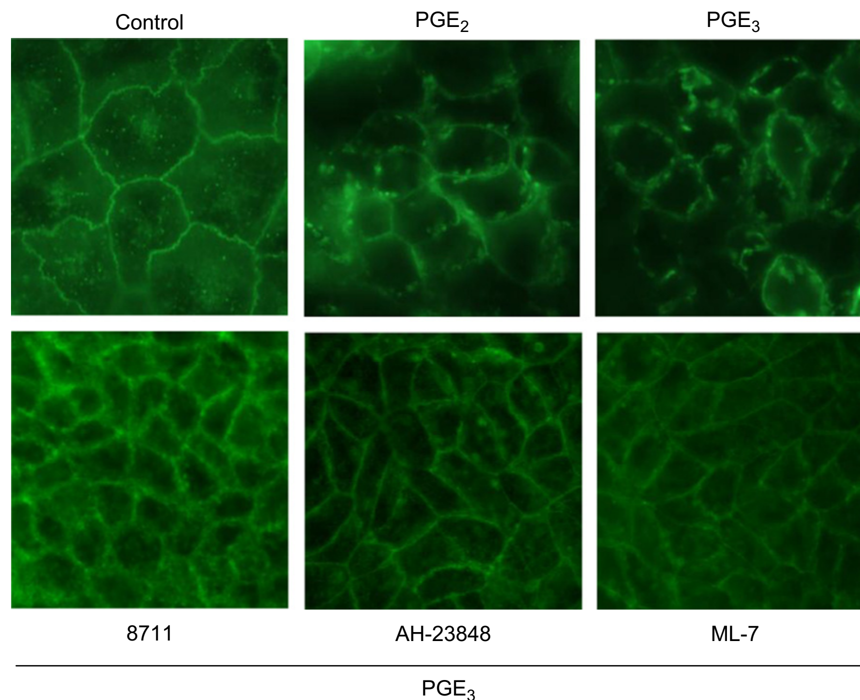


**Fig. 6.** Changes in claudin-4 distribution induced by $PGE_2$ and $PGE_3$. Fluorescent analysis was performed in cells incubated for 3 h with the PGs (3 nM) and $PGE_3$ plus ONO-8711 (250 nM), plus AH-23848 (200 nM) or plus ML7 (0.05 μM) using specific antibodies as described in "Materials and Methods". In each case, a representative $x-y$ image of sections close to the apical cell side is shown.

humans have been conclusive [5]. Taking into account all these data, the aim of this study was to assess the role of EPA-derived $PGE_3$ in the regulation of intestinal epithelial barrier function. Considering that EP receptors localization is mainly on the basolateral membrane [13], cells cultured on filters or clusters were incubated with different PGs concentrations.

Bagga et al. [27] compared the effects of $PGE_2$ and $PGE_3$ on COX-2 gene and protein expression in fibroblasts and found that both induced an increase in COX-2 mRNA. The present study demonstrates the activation of PLC-$IP_3$-$Ca^{2+}$ and cAMP-PKA pathways, through $EP_1$ and $EP_4$ interaction, respectively, as we previously reported for $PGE_2$ [13].

Willemsen et al. [28] found that the addition of EPA or DHA to intestinal cell line monolayers (T84) resulted in enhanced basal barrier integrity (TER) and in the reversion of IL-4 mediated increased permeability (FD4 fluxes). Nevertheless, there are several studies of ω-3 PUFAs such as DHA [29,30] or EPA [30,31] where they are described as being able to disrupt the epithelial barrier function of the Caco-2 cell monolayer, an effect mediated by PG formation since indomethacin, a COX inhibitor, reverted the increase in PP [30–32], thus indirectly indicating the participation of $PGE_3$ in this action. This would therefore be consistent with the results shown here, which demonstrate for the first time that $PGE_3$ is also able to induce the disruption of the epithelial barrier to an extent that is similar to $PGE_2$. Thus, EPA would have a role in PP disruption due to the action of the COX pathway metabolites. Nevertheless, we must consider that other eicosanoids such as LTs may be involved in the beneficial effects of EPA on inflammatory processes since we previously observed that EPA-derived $LTB_5$ does not have proinflammatory effects as seen with $LTB_4$ [33].

PUFA are postulated to modify intracellular signaling and several reports have been published indicating their ability to activate PKC [34,35]. Our results show that the PKC inhibitor Gö6983 was able to revert the effects induced by $PGE_3$, indicating the participation of this kinase in the downstream regulation of the TJ function.

NFκB is activated as a result of a signaling cascade triggered by extracellular inflammatory stimuli such as INF-γ, TNF-α, and IL-1β, on epithelial barrier disruption in intestinal Caco-2 cell cultures [36]. Some authors suggest a direct effect of ω-3 PUFAs on inflammatory gene expression via the inhibition or activation of the transcription factor, NFκB. In this regard, NFκB activation was also evaluated to assess its possible involvement in the $PGE_3$-induced PP events. However, we did not observe any alteration in IκB levels, indicating that the NFκB transcription factor is not a downstream target of either $PGE_2$ or $PGE_3$ in Caco-2 cells.

The delocalization of the TJ proteins, occludin and ZO-1, from TJs is associated with epithelial barrier dysfunction and increased PP [37]. Immunofluorescent examination of Caco-2 cell cultures treated with $PGE_3$ showed evidence of changes in the cellular distribution of occludin and actin through $EP_1$ and $EP_4$, while ZO-1 was not modified. Similarly, despite the dramatic redistribution of TJ proteins following IFN-γ exposure, it has been reported that ZO-1 is only minimally affected and that most of it remains at the TJ [38]. The formation of fluorescent clumps in Caco-2 cells has been attributed to a multifocal aggregation of cytoskeletal elements, including actin [39]. The same authors also proposed a central role for actomyosin contraction in the formation of these aggregates [40]. In this respect, the presence of cytosolic occludin is associated with protein internalization by endocytosis. In the case of barrier loss induced by TNF-α, occludin internalization has been described as an MLCK-dependent process [41]. Thus, the increase in PP induced by some cytokines, short chain fatty acids, ethanol, and extracellular $Ca^{2+}$ is mediated by an increase in MLCK activity [39,42–45]. Moreover, MLCK overexpression in Caco-2 cells induces the reorganization of perijunctional actin and thus an increase in PP [44]. In fact, MLCK expression is significantly enhanced in the mucosa of IBD patients [46]. Our results indicate that the increase in PP induced by $PGE_3$ correlates with the redistribution of TJ proteins (mainly occludin) and the contraction of the perijunctional actin ring through $EP_1$ and $EP_4$ interaction, and MLCK activation since PGE receptor antagonists

and ML-7 treatment was able to prevent the disruption of epithelial barrier function and changes in TJ structure. Recently, the pathophysiological relevance of claudins in the intestine has also been highlighted, as claudin-2 expression has been described to be elevated in colon epithelia of patients suffering from IBD. In contrast, the expression of claudin-4 was reduced and this protein was redistributed [47]. In general, the overexpression of claudin-4 localized within the TJ has been associated with an improvement in epithelial barrier function [48]. Nevertheless, Takehara et al. [49] found that claudin-4 overexpression in Caco-2 cells impairs barrier function. Our results showing the redistribution of claudin-4 and no effect on claudin-1 and claudin-2 by $PGE_2$ and $PGE_3$ are in accordance with Lejeune et al. [48], who observed a similar effect induced by $PGE_2$ produced by *Entamoeba histolytica.* Moreover, we demonstrated that these effects were reverted by $EP_1$ and $EP_4$ antagonists and MLCK inhibitor as we have above mentioned for occludin and actin.

On the basis of our findings, we can conclude that either ω-6- or ω-3-derived prostanoids $PGE_2$ and $PGE_3$ contribute to the regulation of epithelial barrier function through a similar mechanism [13]. Thus the previously described beneficial effect of EPA on IBD might not be attributed to the reduction in $PGE_2$/$PGE_3$ ratio as both PG has a deleterious effect on epithelial barrier function. Therefore, these findings may be taken into account for the future development of new nutritional interventions for IBD.

## Acknowledgments

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Artículo 3

5- hydroxyeicosatetraenoic acid and leukotriene D4 increase intestinal epithelial paracellular permeability

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Los resultados obtenidos han dado lugar a las siguientes comunicaciones a congresos:

- Effect of lipoxygenase pathway metabolites on intestinal epithelial barrier function. J.J. Moreno, M.J. Rodríguez-Lagunas, and R. Ferrer. 10th World Congress on Inflammation, París, 2011. *Inflammation Research* 60 (1): S243 (2011)
- Role of n-6 PUFA derived lipid mediators on epithelial barrier function in intestinal Caco-2 cell monolayers. M.J. Rodríguez-Lagunas, R. Martín-Venegas, J.J. Moreno and R. Ferrer. 3rd International Immunonutrition Workshop, Platja d'Aro, 2009. *Proceedings of the Nutrition Society*, 69 (OCE3): E307 (2010)

***Resumen artículo 3***

**Objetivo**: determinar el papel de los eicosanoides producidos por la vía de la 5-LOX ($LTD_4$, $LTB_4$ y 5-HETE) en la regulación de la PP.

**Material y métodos**: la PP se ha estudiado a través de la determinación de la TER y de flujos de dextrano en presencia de diferentes agonistas y antagonistas de los receptores de los CysLT en células Caco-2 cultivadas sobre filtros. La determinación de la $[Ca^{2+}]_i$ se ha realizado por espectrofluorimetría, los niveles de AMPc y de NFκB se han cuantificado por enzima inmuno ensayo, la fosforilación de la MLC a través de western blot y el estado de las proteínas de la TJ se ha determinado por inmunofluorescencia.

**Resultados**: los resultados indican que tanto el $LTD_4$ como el 5-HETE son capaces de incrementar la PP modificando los flujos de dextrano y la TER, mientras que el $LTB_4$ no ha tenido ningún efecto sobre estas variables. El estudio de la alteración de la PP inducida por el $LTD_4$ y el 5-HETE en presencia de antagonistas de los receptores de los CysLT y del BLT indica que en el caso de $LTD_4$ el receptor implicado en dicho efecto es el $CysLT_1R$, mientras que para el 5-HETE no se ha podido identificar ningún candidato. En ambos casos, se observa un incremento de la $[Ca^{2+}]_i$ y de la concentración intracelular de AMPc y de NFκB, demostrado la participación de la vía de la PLC-$Ca^{2+}$/PKC y la de la PKA independiente de AMPc. También se observa la redistribución de la ocludina y de la claudina-4 sin deslocalización de las proteínas ZO-1, claudina-1 o -2. Además, sólo en el caso de $LTD_4$, se observa la desorganización del anillo subapical de actina provocada por la fosforilación de la MLC inducida por la MLCK.

**Conclusiones**: algunos de los metabolitos de la vía de la 5-LOX como $LTD_4$ y 5-HETE participan en la disrupción de la barrera epitelial.

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# 5-Hydroxyeicosatetraenoic acid and leukotriene $D_4$ increase intestinal epithelial paracellular permeability

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ABSTRACT

The loss of epithelial barrier function plays a crucial role in the pathogenesis of inflammatory bowel disease, with levels of 5-lipoxygenase metabolites being increased in the mucosa of these patients. The objective of this study was to determine the effect of the eicosanoids produced by the 5-lipoxygenase pathway, leukotriene $B_4$ and $D_4$, and 5-hydroxyeicosatetraenoic acid on epithelial barrier function. Paracellular permeability was estimated from fluorescein isothiocyanate–dextran fluxes and transepithelial electrical resistance in differentiated Caco-2 cells. Our results suggest that leukotriene $D_4$ and 5-hydroxyeicosatetraenoic acid altered both parameters. Identification of the receptors involved in these changes indicated that cysteinyl-leukotriene receptor 1 participates in the effects of leukotriene $D_4$. For both eicosanoids, these effects were mediated by activation of the phospholipase C/$Ca^{2+}$/protein kinase C pathway, in addition to cAMP-independent protein kinase A activation. Furthermore, we observed a correlation between increased paracellular permeability and the redistribution of occludin, and for leukotriene $D_4$, the disorganization of the subapical actin ring and myosin light chain kinase activation. In conclusion, on the basis of our results, we propose that 5-lipoxygenase pathway metabolites participate in the disruption of epithelial barrier function that is characteristic of inflammatory bowel disease.

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## 1. Introduction

Upon release from cell membranes, arachidonic acid (AA) is metabolized into a range of eicosanoids through three major enzymatic pathways: cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P-450 monooxygenase. The LOX pathway involves the stereospecific enzymes 5-, 12-, and 15-LOX. 5-LOX incorporates molecular oxygen at position C5 of the fatty acid, yielding 5-hydroperoxyeicosatetraenoic acid, which is further metabolized to 5-hydroxyeicosatetraenoic acid (HETE) and to the instable epoxide leukotriene (LT)$A_4$. The intermediate $LTA_4$ can be further converted into $LTB_4$ by $LTA_4$ hydrolase (Haeggstrom, 2004) or into cysteinyl-LT (CysLT) $LTC_4$ by $LTC_4$ synthase and microsomal glutathione S-transferase, which can conjugate $LTA_4$ with glutathione yielding $LTC_4$ (Schroder et al., 2003). Release of $LTC_4$ into the extracellular milieu and successive amino acid cleavage yields $LTD_4$ and then $LTE_4$ (also CysLT) via the action of a dipeptidase.

Mediators of the 5-LOX pathway are released by a variety of cells in response to specific cellular stimuli that result in cell activation. LTs have also been implicated in the pathophysiology of both acute and chronic inflammatory diseases including asthma, arthritis, psoriasis, and inflammatory bowel disease (IBD) (Wang and DuBois, 2007). LTs exert their biological effect by binding to distinct receptor subtypes, but which receptors participate in the effect of 5-HETE is not yet known. There are two G-protein-coupled receptors (GPCR) for $LTB_4$, the high affinity $BLT_1$ and the low affinity $BLT_2$ (Yokomizo et al., 2000b). CysLTs bind to at least two distinct receptor subtypes, also belonging to the GPCR family, namely $CysLT_1R$ and $CysLT_2R$ (Matuk et al., 2004). $CysLT_1R$ is thought to mediate bronchospasm, plasma exudation, vasoconstriction, mucus secretion, and eosinophil recruitment (El Miedany et al., 2006; Kefalakes et al., 2009). $CysLT_2R$ is less well defined, due to a lack of specific agonists and antagonists, but it is thought to mediate some of the vascular effects attributed to CysLTs (Wang and DuBois, 2007).

The gastrointestinal epithelium is a selective barrier that allows the absorption of nutrients, electrolytes and water, but restricts the passage of larger potentially toxic compounds into the circulation,

*Abbreviations:* AA, arachidonic acid; $[Ca^{2+}]_i$, intracellular $Ca^{2+}$ concentration; COX, cyclooxygenase; CysLT, cysteinyl-leukotriene; CysLTR, cysteinyl-leukotriene receptor; FD-4, fluorescein isothiocyanate–dextran; Fura 2-AM, fura-2 acetoxymethylester; GPCR, G-protein-coupled receptor; HETE, hydroxyeicosatetraenoic acid; IBD, inflammatory bowel disease; $IP_3$, inositol trisphosphate; LOX, lipoxygenase; LT, leukotriene; MLCK, myosin light chain kinase; NF-κB, nuclear transcription factor κB; PP, paracellular permeability; PL, phospholipase; $PGE_2$, prostaglandin $E_2$; PK, protein kinase; TBS, Tris-buffered saline; TER, transepithelial electrical resistance; TJ, tight junction; TRITC-phalloidin, phalloidin-tetramethylrhodamine B isothiocyanate.

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thereby preventing bacterial translocation and systemic infection. The structural integrity of the epithelium is guaranteed by three adhesion systems: tight junctions (TJs), adherent junctions and desmosomes. Of these, TJs form a selective barrier that restricts paracellular diffusion, being the rate-limiting step for paracellular permeability (PP). Moreover, TJs, which are the most apical intercellular junctions, form a barrier between apical and basolateral membrane domains (Shin et al., 2006). TJs are multiprotein complexes composed of transmembrane proteins that interact with the actin cytoskeleton and with cytosolic proteins involved in the regulation of signaling cascades. In recent years, various transmembrane proteins of the junctional complex have been identified: occludin, claudins, tricellulin, crumbs, and junctional adhesion molecules, among others. These proteins are associated with the cytoplasmatic plaque, which comprises a wide spectrum of adaptor and scaffold proteins, of which ZO-1, ZO-2, ZO-3, AF6, and cingulin are known to form the nexus with cytoskeletal proteins such as the subapical actin ring (Mitic and Anderson, 1998).

Many lines of evidence indicate that the disruption of TJs and loss of epithelial barrier function play a crucial role in the pathogenesis of gastrointestinal disorders, such as IBD, alcoholic endotoxemia, infectious enterocolitis, celiac disease, and necrotizing enterocolitis (Laukoetter et al., 2006; Oberhuber and Vogelsang, 1998; Pravda, 2005; Rao, 2008). With regard to IBD, the increase in PP in response to injurious factors has been identified as the mechanism responsible for the perpetuation of the inflammatory response (Turner, 2006). Several eicosanoids, such as $PGE_2$, $PGD_2$, and thromboxane $B_2$, 5-, 12- and 15-HETE, as well as $LTB_4$, were found to be increased in the mucosa of IBD patients (Boughton-Smith et al., 1983; Eberhart and Dubois, 1995; Krimsky et al., 2003) and to be involved in this pathology (Wang and DuBois, 2007). In this regard, we previously observed that the addition of $PGE_2$ to differentiated intestinal Caco-2 cells induces an increase in PP, an effect mediated by the interaction of $PGE_2$ with $EP_1$ and $EP_4$ receptors (Rodriguez-Lagunas et al., 2010).

Disease exacerbation has been reported in IBD patients treated with conventional non-steroidal anti-inflammatory drugs or COX-2 inhibitors (El Miedany et al., 2006; Kefalakes et al., 2009; Matuk et al., 2004). For this reason, dual inhibition of the COX and 5-LOX pathways (5-aminosalicylic acid or steroids) is currently used for the treatment of IBD (Krimsky et al., 2003). Blocking only COX pathway might divert the AA cascade to the production of LOX metabolites and therefore worsen inflammation. In this context, and given the lack of information about the effect of 5-LOX pathway metabolites on the state of TJs, our main objective was to elucidate the effect of 5-LOX pathway metabolites on PP to clarify the mechanism involved in the beneficial effects of dual inhibition in IBD treatment.

## 2. Materials

DMEM, trypsin, penicillin, and streptomycin were supplied by GIBCO (Paisley, Scotland). Non-essential amino acids, FBS, BSA, PBS, D-glucose, HEPES, Fura-2 acetoxymethylester (Fura-2-AM), fluorescein isothiocyanate–dextran (FD-4, average mol wt 3000–5000), U73122, dantrolene, Gö6983, SQ22,536, KT5720, ML-7, ketoprofen, and phalloidin-tetramethylrhodamine B isothiocyanate (TRITC-phalloidin), along with other chemicals, were purchased from Sigma–Aldrich (St. Louis, MO). 5-HETE, $LTD_4$, $LTB_4$, LY171883, Bay u9773, U75302, LY255283, and MK571 were purchased from Cayman (Ann Arbor, MI). Tissue culture supplies, including Transwells, were obtained from Costar (Cambridge, MA).

### 2.1. Cell culture

Caco-2 cells were provided by the American Type Cell Collection and cultured as previously described (Martin-Venegas et al., 2006). Cells (passages 53–65) routinely grown to approximately 80% confluence were released by trypsinization and subcultured at a density of $4 \times 10^5$ cells/cm$^2$ on polycarbonate filters with a pore size of 0.4 μm (Transwells, 12 mm diameter) for PP experiments and at a density of $5 \times 10^4$ cells/cm$^2$ in 12-well clusters for intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) determination, in 24-well clusters containing coverslips for immunofluorescent microscopy, or in 75 cm$^2$ flasks for cAMP determination. The medium was replaced every 3 days and on the day before the experiment (19–21 days after seeding).

### 2.2. Paracellular permeability

PP was estimated from transepithelial electrical resistance (TER) and transepithelial FD-4 fluxes. After 3 h incubation with the eicosanoids in DMEM in the apical and basolateral compartments, TER was determined at 37 °C using a Millicell-ERS voltohmmeter (Millipore, Bedford, MA). The results were expressed as $\Omega$ cm$^2$ monolayer surface area. The resistance of the supporting membrane was subtracted from all readings before calculations. After TER determination, 1 mg/mL of FD-4 was added to the apical compartment and after incubation period (1 h at 37 °C), the basolateral medium was removed and fluorescence was determined in a Fluorostar Optima (BMG Labtech, Germany) at excitation and emission wavelengths of 485 nm and 544 nm, respectively.

### 2.3. Western blot analysis

Cells grown in plastic dishes were washed twice with ice-cold PBS, scrapped of into PBS, and pelleted. The pellets were then sonicated in lysis buffer containing 2 mM sodium EDTA, 20 μg/mL aprotinin, 20 μg/mL leupeptin, 20 μg/mL PMSF, 200 μg/mL diethyldithiocarbamic acid, 50 mM Tris–HCl, 150 mM NaCl, 0.5% Igepal CA-630 and 1 mM DTT. After lysis 30 μg of protein from cell lysate was mixed with a reducing buffer containing 0.5 M Tris–HCl, 10% glycerol, 10% SDS, 2% β-mercaptoethanol and 0.5% blue bromopherol and heated at 100 °C for 5 min. Samples were separated by 12% SDS-PAGE gel and blotted for 2 h at voltage of 100 V and constant amperage of 400 mA onto a nitrocellulose membrane (Trans-Blot, 0.45 μm pore size, Bio-Rad) using a Mini protean II system (Bio-Rad). A prestained SDS-PAGE protein standard (Bio-Rad) was used as molecular weight marker to check transfer efficiency. Membranes were blocked with 5% non-fat milk power in Tris-buffered saline (TBS) 0.1% Tween-20 (TBS-T20) for 1 h. A rabbit polyclonal antiserum directed against myosin light chain 2 (MLC) or p-MLC (Cell Signaling Technology) was applied at a dilution of 1:1000 for 1 h. Blots were washed several times with TBS-T20 and incubated with goat anti-rabbit antibody at a 1:100,000 dilution for 1 h. Antibody binding was visualized by an enhanced chemical luminescence technique using SuperSignal West Femto Maximun Sensitivity Substrate (Pierce) and Kodak X-OMAT film (Rochester, NY).

### 2.4. Intracellular $Ca^{2+}$ concentration

$[Ca^{2+}]_i$ was monitored using the selective fluorescent $Ca^{2+}$ indicator Fura 2-AM as previously described (Rodriguez-Lagunas et al., 2010). Cells grown on clusters were loaded with 25 μM Fura 2-AM in DMEM for 1 h at 37 °C, then washed in modified Krebs buffer (pH 7.4) at 37 °C and incubated for 1 h at 37 °C to allow Fura 2-AM de-esterification. The monolayers were washed again to ensure the removal of all unloaded indicator, and eicosanoids and inhibitors were added. The fluorescent signal was continuously monitored with excitation wavelengths of 340 and 380 nm and emission at 510 nm using a fluorescent microplate reader (Fluorostar Optima, BMG Labtech, Germany) before and after injection

of the eicosanoids. Cells were maintained at 37 °C throughout the experiment. At the end of the incubation period, the maximum and minimum intracellular probe fluorescent signals were determined by the addition of cell lysis buffer and 20 mM EDTA in Krebs, respectively and $[Ca^{2+}]_i$ was calculated following (Grynkiewicz et al., 1985).

### 2.5. cAMP determination

cAMP determination was performed using a competitive EIA Kit (Cayman, Ann Arbor, MI) following the manufacturer's instructions. Briefly, cells maintained in flasks were incubated for 5, 15, and 30 min at 37 °C with a range of eicosanoid concentrations. They were then incubated for 20 min at room temperature with 2.5 mL of 0.1 N HCl and harvested and homogenized. The homogenate was then centrifuged (1000 × *g*, 10 min) and the supernatant was assayed following the acetylation procedure (sensitivity 0.1 pM).

### 2.6. NFκB determination

Cytosolic IκB proteins bind to the NFκB/Rel transcription factor complex to maintain its inactive state. For NFκB to become activated, it must first dissociate from the inhibitor IκB, thereby enabling NFκB to translocate into the nucleus to modulate gene expression. This is induced by phosphorylation of IκB at Ser32 and Ser36 in response to various stimuli. Therefore, NFκB activation was performed by measuring total and phosphorylated IκB using a competitive EIA Kit (eBioscience, San Diego, CA) following the manufacturer's instructions. Briefly, cells grown on clusters were incubated for 5 and 15 min and 3 h at 37 °C with $LTD_4$ and 5-HETE (1 μM) and TNF-α (100 ng/mL) as a positive control.

### 2.7. Immunofluorescent staining of TJ proteins

Caco-2 monolayers grown on coverslips were washed gently with PBS and fixed in iced methanol or 3% paraformaldehyde (PF) and 2% sucrose in 0.1 M PBS (pH 7.4) for 15 min at −20 °C. Cells were washed twice for 5 min in 10 mM PBS containing 20 mM glycine (PBS-glycine) and then permeabilized with 0.2% (v/v) Triton X-100 for 10 min at room temperature. Cells were washed twice in PBS-glycine and blocked for 20 min in PBS-glycine containing 1% BSA (incubation solution). Mouse monoclonal anti-occludin (1:500 dilution; Zymed, South San Francisco, CA) and rabbit polyclonal anti-ZO-1 (1:250 dilution; Zymed) and mouse polyclonal anti-claudin-2 (1:250 dilution; Invitrogen, San Diego, CA) were used as primary antibodies. Cells were incubated with the primary antibodies for 1 h at 37 °C and washed twice in PBS-glycine for 5 min at room temperature. Monolayers were then incubated for 1 h at 37 °C with Alexa dye-conjugated secondary antibodies (1:500 dilution, Molecular Probes, Leiden, The Netherlands). Finally, cells were washed for 10 min at room temperature in PBS, mounted in Mowiol (Calbiochem, San Diego, CA) and examined under an immunofluorescent microscope (BX 41, Olympus, Japan). Images were taken using a 60× (numerical aperture 1.25, phase 3, oil) Olympus UPlanFL N objective. To view the actin subapical ring, coverslips were fixed in paraformaldehyde and permeabilized as described above and incubated with TRITC-phalloidin or direct-labeled claudin-1 and -4 (1:250 dilution; Invitrogen) for 1 h at 37 °C (1:1000 dilution). TJ and cytosolic fluorescence intensity was quantified by using the ImageJ software analysis (US National Institutes of Health, Bethesda, MD, USA, http://rsbweb.nig.gov/ij/) along two horizontal axis for each image. The results were expressed as ratio TJ/cytosolic fluorescence.

### 2.8. Data analysis

The results are expressed as mean ± SE. Data were analyzed by one-way analysis of variance followed by Dunnett's post hoc test using SPSS® software (SPSS Inc. Chicago, IL, USA). $P < 0.05$ was considered to denote significance.

## 3. Results

We first tested the effect of the representative eicosanoids produced in the intestinal mucosa by the 5-LOX pathway that are involved in IBD initiation and/or perpetuation on PP. 5-HETE and $LTD_4$ (Fig. 1) but not $LTB_4$ (data not shown) induced a significant increase in FD-4 fluxes and a decrease in TER values.

We examined the receptors involved in these effects of $LTD_4$ and 5-HETE through the capacity of various receptor antagonists to prevent the action of these eicosanoids on FD-4 fluxes and TER. The increase in FD-4 fluxes and the reduction in TER values induced by $LTD_4$ were prevented by LY171883 and MK 571, both $CysLT_1R$ antagonists (Fleisch et al., 1985; Martin et al., 2001) and by Bay u9773, a non-selective CysLTR antagonist (Nothacker et al., 2000) (Fig. 1A and B). These results suggest that $CysLT_1R$ participates in the effects induced by $LTD_4$. Since specific $CysLT_2R$ antagonists are not available we cannot rule out the participation of this receptor in these events.

No specific receptors have been identified for 5-HETE to date. Nevertheless, it has been reported that HETEs can interact with the $LTB_4$ receptor, $BLT_2$ (Yokomizo et al., 2001). To test the role of these receptors in 5-HETE-induced barrier disruption, we used U75302 and LY255283 as selective $BLT_1$ and $BLT_2$ receptor antagonists, respectively (Yokomizo et al., 2000a), as well as Bay u9773 as a non-selective CysLTR antagonist. The results indicate that none of these antagonists was able to prevent the effects of 5-HETE on PP (Fig. 1C and D).

Some eicosanoids such as $LTD_4$ and 5-HETE are able to induce $PGE_2$ production in intestinal epithelial cells (Di Mari et al., 2007; Ohd, 2000; Ohd et al., 2000). Given that $PGE_2$ disrupts PP (Martin-Venegas et al., 2006; Rodriguez-Lagunas et al., 2010), we must consider that the increase in PP induced by $LTD_4$ or 5-HETE might be attributable instead to the COX metabolites induced by these eicosanoids. To test this hypothesis, we tested the effect of ketoprofen, a COX inhibitor (Sanchez and Moreno, 1999), on PP effects induced by $LTD_4$/5-HETE. The addition of ketoprofen did not significantly modify the increase in FD-4 fluxes induced by $LTD_4$ (Fig. 1A) or 5-HETE (Fig. 1C) although the TER values did not differ significantly from control values.

Next, we addressed the participation of several signaling pathways in the disruption of PP by eicosanoids. The results revealed that the increase in FD-4 fluxes induced by $LTD_4$ (Fig. 2A) was prevented by U73122, a phospholipase C (PLC) inhibitor (Smith et al., 1990); dantrolene, an inhibitor of intracellular $Ca^{2+}$ release from the endoplasmic reticulum (Van Winkle, 1976); Gö6983, a pan protein kinase (PK) C inhibitor (Gonzalez-Mariscal et al., 2008); KT5720, a PKA inhibitor (Doherty et al., 1995) and ML7, a myosin light chain kinase (MLCK) inhibitor (Park et al., 2002). However, SQ22,536, an adenylate cyclase inhibitor (Harris et al., 1979), had no effect. The TER values showed a similar profile, with the exception of that for SQ22,536, which did not differ significantly from control conditions (Fig. 2B). In the case of 5-HETE, the effect on FD-4 fluxes was reverted by U73122, dantrolene, Gö6983, and KT5720, but not by SQ22,536 or ML7 (Fig. 2C). Again, TER values (Fig. 2D) matched the profile of FD-4 fluxes, with the exception of that for SQ22,536.

We further examined the participation of myosin light chain in $LTD_4$-treated cells. As observed on western blot analysis of MLC phosphorylation (Fig. 3), there is a significant increase in the ratio

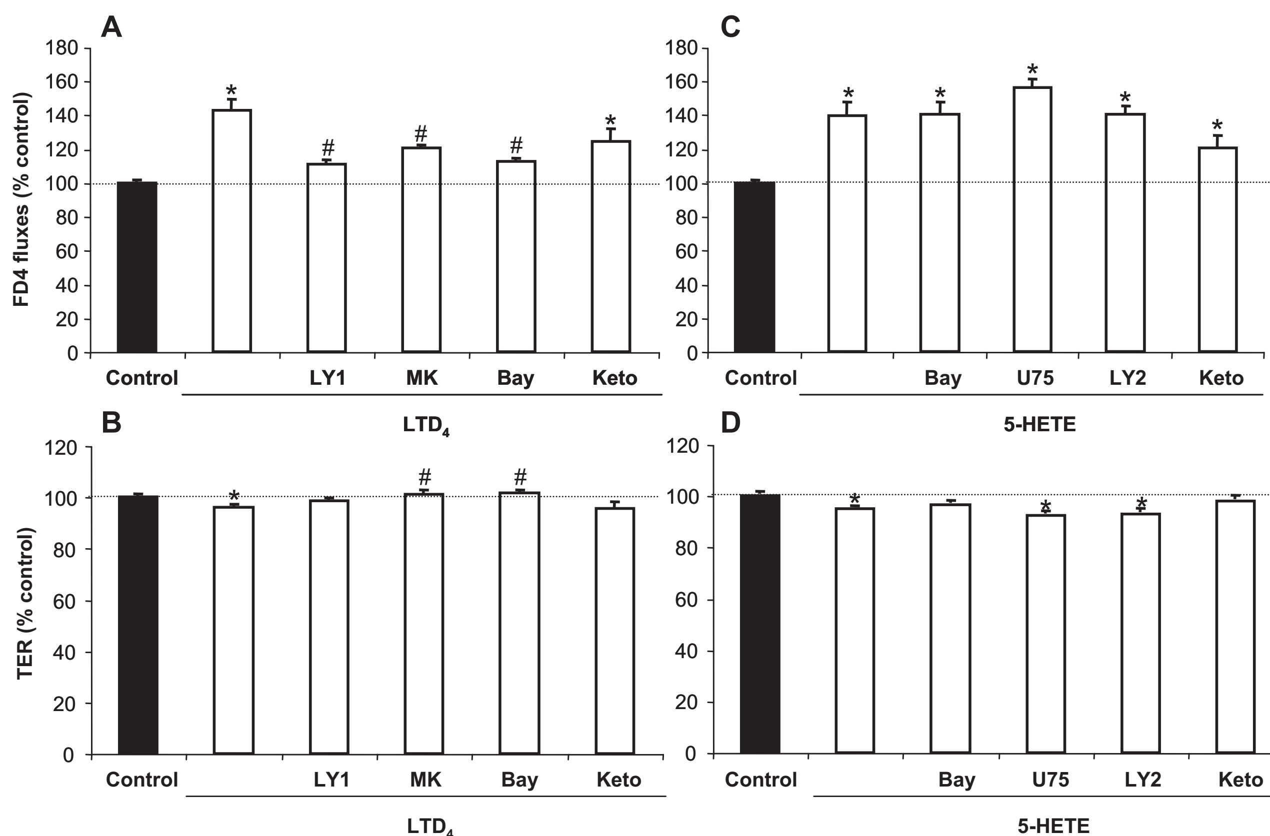


**Fig. 1.** Effect of LT receptor antagonists or COX inhibitor on epithelial barrier disruption induced by $LTD_4$ or 5-HETE. FD-4 fluxes (A and C) and TER (B and D) were determined in differentiated Caco-2 cell monolayers, as described in Section 2. Cells were incubated for 3 h with $LTD_4$ (A and B) (0.1 μM) and $LTD_4$ plus LY171883 (LY1, 25 μM), MK 571 (MK, 25 μM), Bay u9773 (Bay, 1 μM), or ketoprofen (Keto, 5 μM), or 5-HETE (C and D) (0.1 μM) and 5-HETE plus Bay u9773 (Bay, 1 μM), U75302 (U75, 5 μM), LY255283 (LY2, 50 μM), or ketoprofen (Keto, 5 μM) in the apical and basolateral compartments. Results are expressed as the percentage of FD-4 fluxes and TER values obtained in control conditions (0.33 ± 0.01 ng/μL and 1720.65 ± 68.49 Ω cm$^2$, respectively). Data are means ± SE of $n$ = 6–10 experiments. $^*P < 0.05$ vs. control and $^\#P < 0.05$ vs. $LTD_4$ (A and B) or 5-HETE (C and D).

p-MLC/MLC in cells treated with $LTD_4$ but not with 5-HETE. Furthermore when cells are treated with ML7 in the presence of $LTD_4$, the ratio significant reverts to control conditions.

Our results also show the capacity of $LTD_4$ and 5-HETE to increase $[Ca^{2+}]_i$, an effect that was prevented by U73122 and dantrolene (Fig. 4). The increase in $[Ca^{2+}]_i$ was also prevented when $Ca^{2+}$ was withdrawn from the incubation media. Regarding cAMP levels, $PGE_2$ increased cAMP, which reached the highest concentration after 5 min of incubation. However, neither $LTD_4$ nor 5-HETE modified this variable at either of the concentrations tested (0.1 and 1 μM) (Fig. 5).

The phosphorylation of IκB at Ser32 and Ser36 allows the dissociation of the complex IκB-NFκB/Rel, thereby enabling NFκB to translocate into the nucleus to modulate gene expression. We wished to know whether NFκB is activated by $LTD_4$ or 5-HETE, and for this purpose IκB phosphorylation was determined. The results show that neither $LTD_4$ nor 5-HETE were able to increase the amount of phosphorylated IκB with respect to control conditions in any of the incubation periods assayed (5 min, 15 min, and 3 h), whereas TNFα, which was used as a positive control, was able to increase IκB phosphorylation (Table 1).

Finally, we studied the contribution of TJ proteins and cytoskeletal actin to the increase in PP induced by these eicosanoids. TJ protein immunofluorescent staining in control conditions showed occludin and ZO-1 located mainly at the cell border (Fig. 6A). Treatment with the eicosanoids that disrupted barrier function, namely $LTD_4$ and 5-HETE, resulted in a redistribution of occludin with adjacent diffuse intracellular staining and a granular appearance. However, neither $LTD_4$ nor 5-HETE had a significant effect on ZO-1 location. Morphological assessment of subapical actin showed the characteristic perijunctional ring in control conditions. Treatment with $LTD_4$ but not 5-HETE induced complete disorganization of the actin belt. In control monolayers, claudin-4 was predominantly present at TJs and a weak cytoplasmic localization was also observed. However, in response to either 5-HETE or $LTD_4$, claudin-4 markedly dissociated from the TJ to form protein clumps, being this effect more pronounced in $LTD_4$-treated cells. The localization of claudin-1 and claudin-2 was also assessed but no difference was found with respect to control monolayers (data not shown). Moreover, the images show that PLC and MLCK inhibitors were able to prevent the redistribution of occludin, actin and claudin-4 in $LTD_4$-treated cells (Fig. 6B). In the case of 5-HETE-treated cells, the addition of PLC inhibitor also prevented occludin and claudin-4 redistribution (data not shown).

**Table 1**
Effect of 5-HETE and $LTD_4$ on NFκB pathway.

| | 5 min | 15 min | 3 h |
|---|---|---|---|
| Control | 100 ± 10.52 | 100 ± 7.59 | 100 ± 17.74 |
| 5-HETE | 73.60 ± 10.27 | 86.28 ± 14.82 | 95.51 ± 13.30 |
| $LTD_4$ | 102.09 ± 15.75 | 82.80 ± 23.27 | 95.10 ± 13.82 |
| TNFα | 259.12 ± 20.15* | 285.42 ± 36.29* | 284.64 ± 26.68* |

Changes in phosphorylated IκB levels were determined in differentiated Caco-2 cell monolayers as described in Section 2. Cells were incubated for 5 min, 15 min, or 3 h in the presence of the eicosanoids (1 μM) or TNF-α as a positive control (100 ng/mL) and the results are expressed as the % with respect to control conditions. Data are mean ± SE of $n$ = 4–6 experiments.

* $P < 0.05$ vs. control.

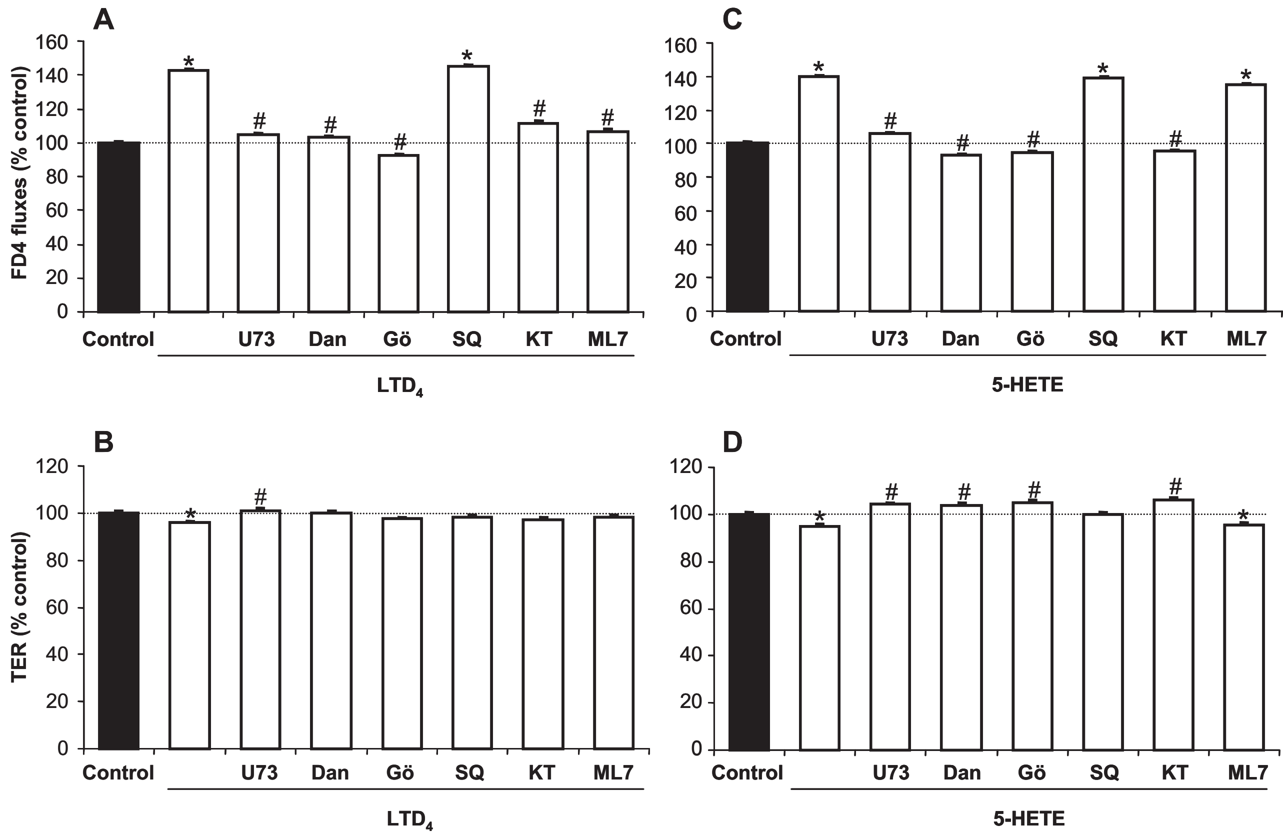


**Fig. 2.** Cell signaling pathways involved in the effects exerted by $LTD_4$ and 5-HETE on PP. FD-4 fluxes (A and C) and TER (B and D) were determined in differentiated Caco-2 cell monolayers, as described in Section 2. Cells were incubated for 3 h with $LTD_4$ (A and B) (0.1 μM) or 5-HETE (C and D) (0.1 μM) plus U73122 (U73, 0.1 μM), dantrolene (Dan, 50 μM), Gö6983 (Gö, 1 μM), SQ22,536 (SQ, 10 μM), KT5720 (KT, 1 μM), or ML7 (0.05 μM) in the apical and basolateral compartments. Results are expressed as the percentage of FD-4 fluxes and TER values obtained in control conditions (0.42 ± 0.04 ng/μL and 1750.40 ± 89.81 Ω cm$^2$, respectively). Data are means ± SE of $n$ = 6–10 experiments. $^*P < 0.05$ vs. control and $^\#P < 0.05$ vs. $LTD_4$ (A and B) or vs. 5-HETE (C and D).

## 4. Discussion

Increased PP of the intestinal epithelium is now believed to be involved in the pathophysiology of a variety of gastrointestinal disorders (Clayburgh et al., 2004). In IBD, altered PP increases the entrance of pro-inflammatory stimuli to the underlying immune cells, thereby triggering further cytokine-induced changes to TJs and a vicious cycle of mucosal barrier dysfunction and activation of the mucosal immune response (Barbara, 2006; Bruewer et al., 2006; Mankertz and Schulzke, 2007). On the basis of previous

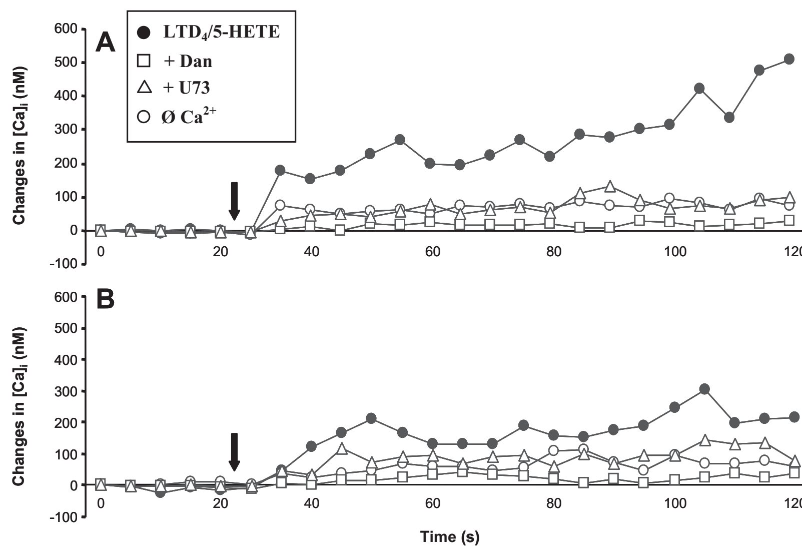


**Fig. 4.** Changes in $[Ca^{2+}]_i$ induced by $LTD_4$ and 5-HETE. $[Ca^{2+}]_i$ were determined in differentiated Caco-2 cell monolayers using Fura-2 AM, as described in Section 2. Cells were incubated for 120 s in the presence of $LTD_4$ (1 μM) (A) or 5-HETE (1 μM) (B) (●) plus dantrolene (□, 50 μM) or U73122 (△, 0.1 μM), or in the absence of extracellular $Ca^{2+}$ (○). The arrow indicates the injection of $LTD_4$ or 5-HETE. Inhibitors were pre-incubated for 30 min. Each plot corresponds to a representative profile obtained for $n$ = 3 experiments.

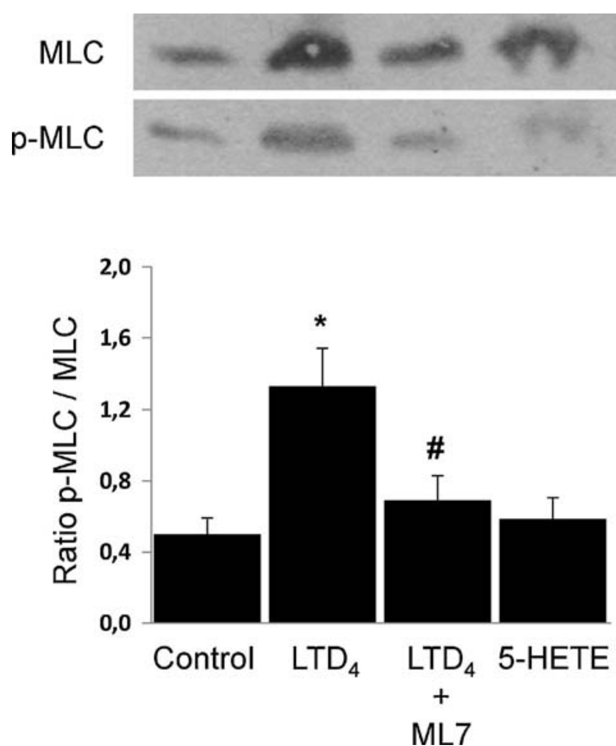


**Fig. 3.** Western blot of p-MLC in the presence of $LTD_4$. Cells were incubated with $LTD_4$ (0.1 μM) ± ML7 (0.05 μM) or 5-HETE (0.1 μM) and MLC and p-MLC were determined using specific antibodies. Values are mean ratio p-MLC/MLC ± SE ($n$ = 4–5). $^{*}P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. $LTD_4$.

findings indicating that the AA cascade is activated in intestinal mucosa in IBD (Boughton-Smith et al., 1983; Eberhart and Dubois, 1995; Krimsky et al., 2003), we hypothesized that eicosanoids could be involved in the regulation of intestinal epithelial barrier function in the above-mentioned pathophysiological processes. In this regard, we previously reported that the enhancement of $PGE_2$ levels increases PP in differentiated Caco-2 cell cultures (Martin-Venegas et al., 2006). Given that 5-LOX metabolites are also increased in the mucosa of IBD patients, and that 5-LOX pathway inhibition is effective in the clinical treatment of IBD, we studied the effect of these eicosanoids on intestinal PP. The addition of representative 5-LOX pathway metabolites such as $LTD_4$ and 5-HETE, at concentrations reached in the inflamed intestinal mucosa (Wardle et al., 1993; Zijlstra and Wilson, 1991; Zijlstra et al., 1992), disrupt barrier function, while $LTB_4$ had no effect.

To study the cell signaling involved in the effect of $LTD_4$ and 5-HETE on intestinal PP, the first step was to identify the receptors involved. $LTD_4$ binds to $CysLT_1$ and $CysLT_2$, both of which are

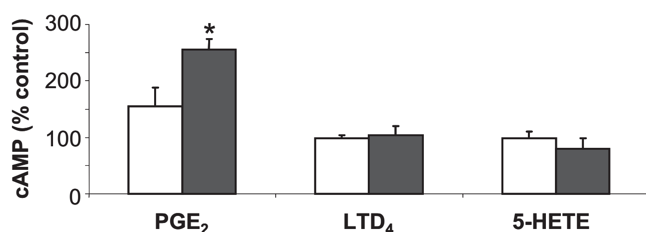


**Fig. 5.** Effect of $LTD_4$ and 5-HETE on intracellular cAMP concentration. cAMP was determined in differentiated Caco-2 cell, as described in Section 2. Cells were incubated for 5 min with $LTD_4$ and 5-HETE at two concentrations (0.1 μM, white bars and 1 μM black bars). $PGE_2$ was used as a positive control. Results are expressed as the percentage of cAMP values obtained in control conditions (22.59 ± 1.64 nM). The data are means ± SE of $n$ = 3–5 experiments. $^{*}P < 0.05$ vs. control.

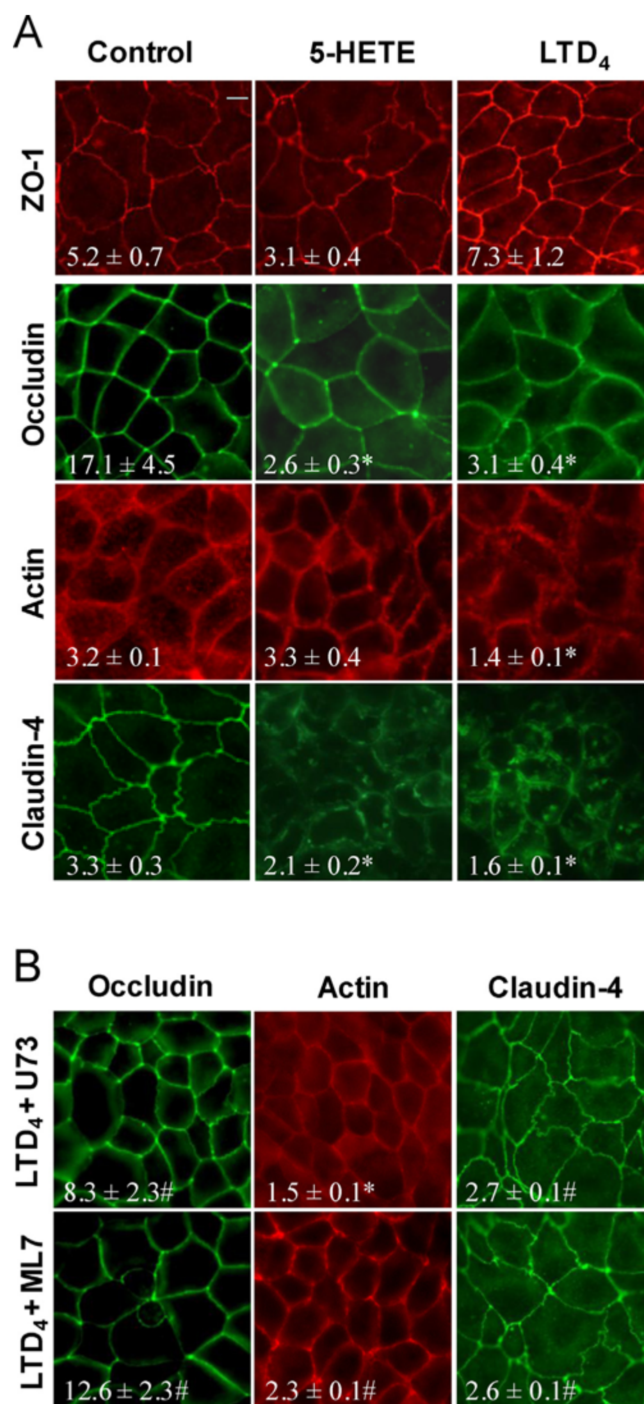


**Fig. 6.** Changes in TJ proteins induced by eicosanoids. Fluorescent analysis was performed in cells incubated for 3 h with different eicosanoids (0.1 μM) and inhibitors using specific ZO-1, occludin and claudin-4 antibodies and TRITC-phalloidin, as described in Section 2. In each case, a representative $x$–$y$ image of sections close to the apical cell side is shown. (A) ZO-1, occludin, perijunctional actin and claudin-4 distribution in cells treated with $LTD_4$ and 5-HETE. (B) Effect of U-73122 (U73, 0.1 μM) or ML7 (0.05 μM) on occludin, perijunctional actin and claudin-4 distribution in $LTD_4$-treated cells. All the images are shown at the same magnification. White numbers indicated on each image corresponds to the ratio TJ/cytosolic fluorescence intensity. The results are means ± SE of $n$ = 2 horizontal axis from 3 images. $^{*}P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. $LTD_4$.

expressed in Caco-2 cells (Magnusson et al., 2007; Nielsen et al., 2005). Our results indicate for the first time that the $LTD_4$–$CysLT_1R$ interaction participates in the regulation of intestinal barrier function. In this regard, $LTD_4$–$CysLT_1R$ was recently reported to be involved in the regulation of other intestinal epithelial functions,

such as cell survival and proliferation (Paruchuri et al., 2006) and to participate in plasma protein extravasation in inflammatory conditions (Beller et al., 2004).

Specific HETE receptors have not been identified to date. However, it has been reported that 12-(S) and 15-(S)-HETE bind to $LTB_4$ receptor, $BLT_2$ (Yokomizo et al., 2001). However a $BLT_2$ receptor antagonist (LY255283) did not affect the increased PP induced by 5-HETE. Moreover, given that $BLT_1$ is expressed in Caco-2 cells (Ihara et al., 2007), a specific antagonist of the high affinity $BLT_1$ receptor (U75302) was used to rule out the participation of this $LTB_4$ receptor in these events, and again no effect was observed. In addition, the results obtained with a non-selective CysLTR antagonist (Bay u9773) indicate the lack of participation of these receptors. Therefore, no candidate receptor involved in PP regulation has thus far been identified for 5-HETE.

It has recently been reported that $LTD_4$ and 5-HETE induce the expression of COX-2, and consequently $PGE_2$ production (Di Mari et al., 2007; Krimsky et al., 2003). Therefore, it is possible that the effects of these eicosanoids on PP may be due indirectly to an increase in $PGE_2$ production. Considering that we previously demonstrated that $PGE_2$ induces the disruption of epithelial barrier function (Rodriguez-Lagunas et al., 2010), we assayed whether COX inhibition reverses the effect of $LTD_4$ and 5-HETE on PP. Our results suggest that the formation of $PGE_2$ does not play a pivotal role in the effects exerted by $LTD_4$ and 5-HETE on intestinal epithelial barrier function.

The mechanism by which 5-HETE and $LTD_4$ induce epithelial barrier disruption was further studied by characterization of the intracellular signaling pathways involved. According to recent data, $CysLT_1R$ activates a G protein that induces PLC activation and consequently diacylglycerol and inositol trisphosphate ($IP_3$) release. Thus, $LTD_4$ coupled to $CysLT_1R$ results in an increase in $[Ca^{2+}]_i$ and PKC activation in various cell types (Profita et al., 2008; Singh et al., 2010; Suzuki et al., 2008; Woszczek et al., 2008). Our results revealed that the increase in PP induced by $LTD_4$ was prevented by the inhibition of PLC, by the reduction of $Ca^{2+}$ release from the endoplasmic reticulum, and by PKC inhibition. Moreover, we also observed that the enhancement of $[Ca^{2+}]_i$ induced by $LTD_4$ was prevented by PLC inhibition as well as by the inhibition of $Ca^{2+}$ release from intracellular stores, and by the withdrawal of extracellular $Ca^{2+}$. Therefore, our results suggest that intracellular and extracellular $Ca^{2+}$ participate in these events. Furthermore, we should also consider that PKA inhibition prevented the increase in PP induced by $LTD_4$ and 5-HETE, although cAMP levels were not modified by either eicosanoid. Even though PKA is commonly activated by cAMP, some cAMP-independent mechanisms of activation have been reported in various cell types including epithelial cells (Howe, 2004; Kohr et al., 2010; Niu et al., 2001). NFκB activation mediates the effects of several stimuli on epithelial barrier disruption in intestinal Caco-2 cell cultures (Al-Sadi, 2009). Moreover, it should also be considered that NFκB-dependent PKA activation has been previously described (Gambaryan et al., 2010). In this regard, NFκB activation was also evaluated as a possible way of cAMP-independent PKA activation in $LTD_4$/5-HETE-treated cells. As phosphorylated IκB levels were not increased we suggest that the NFκB transcription factor is not a downstream target of either $LTD_4$ or 5-HETE. Therefore, other cAMP and NFκB-independent PKA activation pathways should be further considered as it has been described before in different cell types (Kohr et al., 2010; Niu et al., 2001).

The delocalization of occludin and ZO-1 from TJs is associated with epithelial barrier dysfunction and increased epithelial permeability (Harhaj and Antonetti, 2004). Our findings revealed a change in occludin and actin distribution, while ZO-1 was not modified when cells were incubated with $LTD_4$. Similarly, despite the dramatic redistribution of TJ proteins following IFN-γ exposure, it has been reported that ZO-1 is only minimally affected and that most of it remains at the TJ (Bruewer et al., 2003). Recently, the pathophysiological relevance of claudins in the intestine has been highlighted as claudin-2 expression has been described to be elevated in colon epithelium of patients suffering from IBD (Amasheh et al., 2011; Prasad et al., 2005). In contrast, the expression of claudin-4 was reduced and this protein was redistributed (Prasad et al., 2005). In general, overexpression of claudin-4 localized within the TJ has been associated to an improvement in epithelial barrier function (Suzuki and Hara, 2009; Vreeburg et al., 2012). For the moment, little information is available on claudin redistribution induced by 5-LOX pathway derived eicosanoids. Although, the redistribution of claudin-4 without effect on claudin-1 and claudin-2 observed in microorganism-induced intestinal inflammation is in agreement with our data (Hering et al., 2011; Lejeune et al., 2011).

The presence of cytosolic occludin is associated with protein internalization by endocytosis. In the case of barrier loss induced by TNF-α, occludin internalization has been described as a MLCK-dependent process (Schwarz et al., 2007). In this regard, previous studies indicate that MLCK is crucial for the regulation of intestinal TJ permeability. Thus, the increase in PP induced by several proinflammatory cytokines, short chain fatty acids, ethanol, and extracellular $Ca^{2+}$ is mediated by an increase in MLCK activity (Al-Sadi et al., 2008; Ma et al., 1999; Ohata et al., 2005; Shen et al., 2006; Ye et al., 2006). In fact, MLCK expression is significantly enhanced in the mucosa of IBD patients (Blair et al., 2006). Our results indicate that the increase in PP induced by $LTD_4$ correlates with the redistribution of TJ proteins (mainly occludin) and the contraction of the perijunctional actin ring through MLCK activation and subsequent increase in MLC phosphorylation. In contrast, the effects induced by 5-HETE on occludin redistribution may be mediated by a mechanism other than that induced by $LTD_4$. In this case, the contraction of the actin subapical ring through MLCK activation and MLC phosphorylation is not involved.

On the basis of our findings, we can conclude that $LTD_4$ effects on TJ permeability are mediated by the interaction with $CysLT_1R$, which activates the PLC-$Ca^{2+}$-PKC and MLCK pathways. Moreover, cAMP-independent PKA activation may also be involved in these events. Furthermore, these events are accompanied by the redistribution of occludin and claudin-4 and the contraction of the perijunctional actin ring. Regarding the effects of 5-HETE on PP, we did not identify any receptor nevertheless, a common intracellular signaling pathway to $LTD_4$ can be predicted, excluding the redistribution of the subapical actin ring. Interestingly, our findings provide a highly plausible explanation for the negative effect of COX inhibition on PP in IBD attributable to the enhancement of 5-LOX pathway eicosanoids synthesis.

Given the involvement of eicosanoids in the disruption of the homeostasis of intestinal barrier function in inflammatory processes such as IBD (Ferrer and Moreno, 2010), our findings provide a valuable basis on which to perform research into the interrelation between 5-LOX pathway activation, PP changes, and the initiation/perpetuation of IBD. Moreover, these findings may be useful to understand the effectivity of dual COX/LOX inhibitors and for the future development of new diagnostic tools and therapeutic strategies for IBD.

## Acknowledgments

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Artículo 4

12- and 15-HETE disrupt intestinal epithelial barrier function

Rodríguez-Lagunas MJ, Moreno JJ, Ferrer R.

Manuscrito pendiente de enviar

Los resultados obtenidos han dado lugar a las siguientes comunicaciones a congresos:

- Role of 12 lipoxygenase derived eicosanoids on epithelial barrier function in intestinal Caco-2 cells. M.J. Rodríguez-Lagunas, J.J. Moreno and R. Ferrer. 6th International Immunonutrition Workshop, Palma de Mallorca, 2012. *Proceedings of the Nutrition Society*, 72 (OCE1): E60 (2013)

### *Resumen artículo 4*

**Objetivo**: determinar el efecto de los eicosanoides producidos por las vías de la 12- y 15-LOX y del citocromo P450 en la función epitelial de barrera.

**Material y métodos**: la PP se ha medido en filtros a partir de la determinación de la TER y de flujos de dextrano en presencia de diferentes eicosanoides. La determinación de la $[Ca^{2+}]_i$ se ha realizado por espectrofluorimetría, la concentración de AMPc y de NFκB se ha determinado mediante enzima immuno ensayo y las proteínas de la TJ se han estudiado mediante inmunofluorescencia.

**Resultados**: los resultados indican que el 20-HETE, los 11,12- y 14,15-EET, los 11,12 y 14,15-DHETE, el 13(S)-HODE y el 12-HEPE no tienen efecto sobre la PP mientras que 12-(R)-HETE, 12-(S)-HETE y 15-HETE han sido capaces de alterar la barrera epitelial modificando los flujos de dextrano y la TER. Los efectos del 12- y del 15-HETE están mediados a través del incremento de la $[Ca^{2+}]_i$ junto con la producción de AMPc, en el caso de 12-(S)-HETE. Además, se ha observado una correlación entre el incremento de la PP y la redistribución de la ocludina, la activación de la MLCK y la desorganización del anillo de actina.

**Conclusiones**: el 12- y 15-HETE participan en la disrupción de la barrera epitelial en un modelo de células intestinales Caco-2.

**12- and 15-HETE disrupt intestinal epithelial barrier function**

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Abbreviations: AA arachidonic acid; AUC, area under the curve; $[Ca^{2+}]_i$, intracellular $Ca^{2+}$ concentration; COX, cyclooxygenase; CYP450, cytochrome P450 monooxygenase; DHET, dihydroxyeicosatrienoic acid ; EET, epoxyeicosatrienoic acid; FD-4, fluorescein isothiocyanate–dextran; Fura 2-AM, fura-2 acetoxymethylester; HETE, hydroxyeicosatetraenoic acid; HPETE hydroperoxyeicosatetraenoic acid; IBD, inflammatory bowel disease; $IP_3$, inositol trisphosphate; LOX, lipoxygenase; MLCK, myosin light chain kinase; NFκB, nuclear transcription factor κB; PP, paracellular permeability; PL, phospholipase, $PGE_2$, prostaglandin $E_2$; PK, protein kinase; TER, transepithelial electrical resistance; TJ, tight junction; TRITC-phalloidin, phalloidin-tetramethylrhodamine B isothiocyanate;

**Abstract** Here we studied the effect on epithelial barrier function of the eicosanoids produced by the lipoxigenase pathway: hydroxyeicosatetraenoic acids (HETEs) and by the cytochrome P450 monooxygenase pathway: 20-HETE, epoxyeicosatrienoic acids (EETs) and dihydroxyeicosatrienoic acids (DHETs). Paracellular permeability (PP) was estimated from fluorescein isothiocyanate–dextran fluxes and transepithelial electrical resistance in differentiated Caco-2 cells. Our results suggest that 20-HETE and EETs/DHETs have no effect on PP whereas 12-(R)-HETE, 12-(S)-HETE and 15-HETE increased this parameter. For all 12- and 15-LOX HETE, these effects were mediated through the increase of intracellular $Ca^{2+}$ concetration and in the case of 12-(S)-HETE with cAMP activation. Furthermore, we observed a correlation between increased PP and the redistribution of occludin and the disorganization of the subapical actin ring. In conclusion, on the basis of our results, we propose that 12 and 15 lipoxygenase pathway metabolites participate in the disruption of epithelial barrier function characteristic of inflammatory diseases such as inflammatory bowel disease.

**Supplementary key words** paracellular permeability - tight junctions - eicosanoids – hydroxyeicosatetraenoic acids- epoxyeicosatrienoic acid

Upon release from cell membranes, arachidonic acid (AA) is metabolized into a range of eicosanoids through three major enzymatic pathways: cyclooxygenase (COX), lipooxygenase (LOX) and cytochrome P450 monooxygenase (CYP450). The LOX pathway involves the stereospecific enzymes 5-, 12-, and 15-LOX. These generate the corresponding hydroperoxyeicosatetraenoic acids (HPETEs), which are rapidly metabolized to 5-, 12- or 15-hydroxyeicosatetraenoic acids (HETEs). The CYP450 pathway includes a wide range of enzymes with epoxygenase and ω/ω-1 hydroxylase activity, among others. The hydroxylase pathway produces subterminal and terminal HETEs (Imig. 2000) including 20-HETE, while the epoxygenase pathway generates regioisomeric (5,6-, 8,9-, 11,12-, 14,15-) epoxyeicosatrienoic acids (EETs) (Capdevila 2000). Each EET can be further converted to the corresponding dihydroxyeicosatrienoic acids (DHETs) by soluble epoxide hydrolase (Natarajan and Reddy. 2003).

The gastrointestinal epithelium provides a barrier which allows nutrients, electrolytes and water to be absorbed, while preventing the passage of larger potentially toxic compounds into the bloodstream, thereby preventing bacterial translocation and systemic infection. Three adhesion systems guarantee the structural integrity of the epithelium: tight junctions (TJs), adherent junctions and desmosomes. Of these, TJ seal the paracellular space between epithelial cells, thus preventing paracellular diffusion and being the rate-limiting step for paracellular permeability (PP). Moreover, TJs, which are the most apical intercellular junctions, form a barrier between apical and basolateral membrane domains (Shin, Fogg and Margolis. 2006). TJs are multiprotein complexes composed of several adhesion molecules that link the neighbouring cells and interact with the actin cytoskeleton and with cytoplasmic scaffolding proteins involved in the regulation of signaling cascades. TJ contain tetraspan proteins sucha as occludin and claudins which

form the paracellular permeability barrier and determine the paracellular diffusion and single-span transmembrane. These proteins are associated with the cytoplasmic plaque, which comprises a wide range of proteins (ZO-1, ZO-2, ZO-3, among others) and forms an interface between the junctional membrane and the cytoskeleton and regulates adhesion, paracellular permeability and cellular processes such as migration and gene expression (Balda and Matter. 2008).

The disruption of TJs and the loss of epithelial barrier function increase the intestinal permeability to injurious factors leading to inflammation and mucosal injury. Many lines of evidence indicate that these effects play a crucial role in the pathogenesis of gastrointestinal disorders, such as inflammatory bowel disease (IBD), alcoholic endotoxemia, infectious enterocolitis, celiac disease and necrotizing enterocolitis (Laukoetter, Bruewer and Nusrat. 2006, Oberhuber and Vogelsang. 1998, Pravda. 2005, Rao. 2008). With regard to IBD, the increase in PP to injurious factors has been identified as the mechanism responsible for the activation of the inflammatory response (Turner. 2006). Several eicosanoids, such as $PGE_2$, $PGD_2$ and $TXB_2$, $LTB_4$, 5-, 12- and 15-HETE were found to be increased in the mucosa of IBD patients (Boughton-Smith, Hawkey and Whittle. 1983, Eberhart and Dubois. 1995, Krimsky et al. 2003). In contrast, there are no data on AA-derived CYP450 metabolites such as 20-HETE, EETs and DHETs. In this regard, we previously observed that the addition of different eicosanoids to differentiated intestinal Caco-2 cells induces an increase in PP, this is the case for $PGE_2$ (Rodriguez-Lagunas et al. 2010) and the 5-LOX derived eicosanoids $LTD_4$ and 5-HETE (Rodriguez-Lagunas et al. 2013).

In IBD patients treated with conventional non-steroidal anti-inflammatory drugs or COX-2 inhibitors it has been described a disease exacerbation (El Miedany et al. 2006, Kefalakes et al. 2009, Matuk et al. 2004). Since COX and

LOX metabolites are believed to be involved in the progression of inflammation in IBD (Eberhart and Dubois. 1995), blocking only one of these pathways might result in increased inflammation, i.e. COX inhibition might divert the AA cascade to the production of LOX metabolites which may worsen inflammation.

Taking into account these data, here we extended our previous research on the effects of $PGE_2$ and 5-LOX pathway derived eicosanoids on PP to those of the metabolites of the AA cascade produced by 12- and 15-LOX and CYP450 pathways. Our results suggest that only 12-HETE and 15-HETE are involved in the regulation of intestinal epithelial barrier function.

**Materials**

DMEM, trypsin, penicillin and streptomycin were supplied by GIBCO (Paisley, Scotland). Non-essential amino acids, FBS, BSA, PBS, D-glucose, HEPES, Fura-2 acetoxymethylester (Fura-2-AM), fluorescein isothiocyanate–dextran (FD-4, average mol wt 3,000-5,000), phalloidin-tetramethylrhodamine B isothiocyanate (TRITC-phalloidin), along with other chemicals, were purchased from Sigma-Aldrich (St. Louis, MO). 12-(S)-, 12-(R)-, 15- and 20-HETE, 11,12- and 14,15-EET, 11,12-, 14,15-DHET, were from Cayman (Ann Arbor, MI). Tissue culture supplies, including Transwells, were obtained from Costar (Cambridge, MA).

**Cell Culture**

Caco-2 cells were provided by American Type Cell Collection and cultured as previously described (Martin-Venegas et al. 2006). Cells (passages 53-65) were routinely grown in plastic flasks at a density of $5 \cdot 10^4$ cells/cm$^2$ and cultured in DMEM containing 4.5 g/L D-glucose and 2 mM L-glutamine, supplemented with 1% (v/v) non-essential amino acids, 10% (v/v) heat-inactivated FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37ºC in a modified atmosphere of 5% $CO_2$ in air. Cells grown to approximately 80% confluence were released by trypsinization and subcultured at a density of $4 \cdot 10^5$ cells/cm$^2$ on polycarbonate filters with a pore size of 0.4 µm (Transwells, 12 mm diameter) for PP experiments and at a density of $5 \cdot 10^4$ cells/cm$^2$ in 12-well clusters for intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$) determination, in 24-well clusters containing coverslips for immunofluorescent microsopy or in 75 cm$^2$ flasks for cAMP determination. The medium was replaced every 3 days and on the day before the experiment. Experiments were performed in cultures 19–21 days after seeding, when cells had differentiated.

**Paracellular permeability**

PP was estimated from transepithelial electrical resistance (TER) and transepithelial FD-4 fluxes. After a 3 h incubation with the eicosanoid in the apical and basolateral compartments, TER was determined at 37 ºC by a Millicell-ERS voltohmmeter (Millipore, Bedford, MA). Results were expressed as $\Omega \cdot cm^2$ monolayer surface area. The resistance of the supporting membrane was subtracted from all readings before calculations. After TER determination, 1 mg/mL of FD-4 was added to the apical compartment and cells were incubated for 1 h at 37ºC. At the end of the incubation, basolateral medium was withdrawn and fluorescence was determined in a Fluorostar Optima (BMG Labtech, Germany) at excitation and emission wavelengths of 485 nm and 544 nm, respectively.

**Intracellular $Ca^{2+}$ concentration**

$[Ca^{2+}]_i$ was monitored using the selective fluorescent $Ca^{2+}$ indicator Fura 2-AM as previously described (14). Cells grown on clusters were loaded with 25 μM Fura 2-AM in DMEM for 1 h at 37°C. The preloaded monolayers were then washed in modified Krebs buffer (pH 7.4) at 37°C and incubated for 1h at 37ºC to allow Fura 2-AM de-esterification. The monolayers were washed again to ensure removal of all unloaded indicator, and eicosanoids and inhibitors were added to the respective wells. Continuous fluorescent signal was monitored with excitation wavelengths of 340 and 380 nm and an emission at 510 nm using a fluorescent microplate reader (Fluorostar Optima, BMG Labtech, Germany) before and after the injection of the eicosanoids. Cells were maintained throughout the experiment at 37°C. At the end of the incubation period, the maximal and minimal intracellular probe fluorescent signals were determined by the addition of cell lysis buffer and 20 mM EDTA in Krebs, respectively. $[Ca^{2+}]_i$ was calculated following Grynkiewicz et al., (Grynkiewicz, Poenie and Tsien. 1985) from a 340/380 ratio using a dissociation constant of 224 nM.

**cAMP determination**

cAMP determination was performed using a competitive EIA Kit (Cayman, Ann Arbor, MI) following the manufacturer's instructions. Briefly, cells maintained in flasks were incubated for 5, 15 and 30 min at 37ºC with a range of eicosanoid concentrations. After they were incubated for 20 min at room temperature with 2.5 mL of 0.1 N HCl and then harvested and homogenized. The homogenate was then centrifuged (1,000 g, 10 min) and the supernatant was assayed following the acetylation procedure (sensitivity 0.1 pM).

**NFκB activation**

Cytosolic IκB proteins bind to the NFκB/Rel transcription factor complex to maintain its inactive state. For NFκB to become activated, it must first dissociate from the inhibitor IκB, thereby enabling NFκB to translocate into the nucleus to modulate gene expression. This is induced by phosphorylation of IκB at Ser32 and Ser36 in response to various extracellular signals, including inflammatory cytokines, growth factors, and chemokines. Therefore, NFκB activation was evaluated by measuring total and phosphorylated IκB using a competitive EIA Kit (eBioscience, San Diego, CA) following the manufacturer's instructions. Briefly, cells grown in clusters were incubated for 5 and 15 min and 3 h at 37ºC with $PGE_2$ and $PGE_3$ (3 nM) and TNF-α (100 ng/mL) as a positive control.

**Immunofluorescent staining of TJ proteins**

Caco-2 control or treated monolayers grown on coverslips were washed gently with PBS and fixed in iced methanol for 15 min at -20ºC. Cells were washed twice for 5 min in 10 mM PBS containing 20 mM glycine (PBS-glycine) and then permeabilized with 0.2% (v/v) Triton X-100 for 10 min at room temperature. Cells were washed twice in PBS-glycine and blocked for 20 min in PBS-glycine containing 1% BSA (incubation solution). Mouse monoclonal anti-occludin (1:500 dilution; Zymed, South San Francisco, CA) and rabbit

polyclonal anti-ZO-1 (1:250 dilution; Zymed) were used as primary antibodies. Cells were incubated with the primary antibodies for 1 h at 37°C and washed twice in PBS-glycine for 5 min at room temperature. Monolayers were then incubated for 1 h at 37°C with Alexa dye-conjugated secondary antibodies (1:500 dilution, Molecular Probes, Leiden, The Netherlands). Finally, cells were washed for 10 min at room temperature in PBS, mounted in Mowiol (Calbiochem, San Diego, CA) and examined with an immunofluorescent microscope (BX 41, Olympus Barcelona, Spain). Images were taken using a 60x (numerical aperture 1.25, phase 3, oil) Olympus UPlanFL N objective). To view the actin subapical ring, coverslips were fixed and permeabilized as described above and incubated with TRITC-phalloidin for 1 h at 37ºC (1:1,000 dilution).

**Data analysis**

Results were expressed as mean ± SE. Data were analyzed by one-way analysis of variance followed by Dunnett's *post hoc* test using SPSS® software (SPSS Inc. Chicago, IL, USA). $P < 0.05$ was considered to denote significance.

## Results

Here we have examined the effect of the HETEs produced by 12- and 15-LOX as well as CYP450 pathway metabolites (20-HETE, EET and DHET) on PP using differentiated Caco-2 cell monolayers. We observed that 12-HETE and 15-HETE altered TJ permeability by increasing FD4 fluxes (**Figure 1A**) and decrease TER (**Figure 1B**). Moreover, the results also shown that both 12-HETE enantiomers disrupted PP. This was not the case for the CYP hydroxilase- derived 20-HETE which did not alter these parameters. For the other CYP epoxigenase-derived eicosanoids, neither the EET (11,12-, and 14,15-EET) nor the DHET (11,12-, and 14,15-DHET) assayed induced an increase in FD4 fluxes neither an impairment of TER, suggesting that these eicosanoids are not involved in the regulation of TJ permeability in Caco-2 cells.

We have previously observed that the EPA-derived prostanoid $PGE_3$ was able to induce an increase in PP to the same extent as $PGE_2$ (Rodriguez-Lagunas, Ferrer and Moreno. 2013). Here we have observed that the addition of 12-Hydroxy-eicosapentaenoic acid (12-HEPE, a 12-LOX EPA-derived eicosanoid) to Caco-2 cell monolayers did not modify neither FD4 fluxes nor TER values (data not shown). This is also the case for the LA-derived eicosanoid 13-(S)-HODE which did not alter PP (data not shown).

In addition, our results also show the capacity of 12-(R)-, 12-(S)- and 15-HETE to increase $[Ca^{2+}]_i$, at a concentration of 1 μM (**Table 1**). Two main candidate calcium sources contribute to the intracellular calcium increase, either intracellular or extracellular. To study the role of extracellular calcium, the same experiment was assessed by using a calcium free medium. External calcium entry did not appear to be important as the increase in $[Ca^{2+}]_i$ induced by the above mentioned eicosanoids was not prevented when $Ca^{2+}$ was

withdrawn from the incubation media (**Figure 2A-C**). Moreover, preincubation with dantrolene, an inhibitor of intracellular $Ca^{2+}$ release from the endoplasmic reticulum (Van Winkle. 1976) and U73122, a phospholipase C (PLC) inhibitor (Smith et al. 1990) prevented the increase in $[Ca^{2+}]_i$ induced by 12-(R)-, 12-(S)- and 15-HETE (**Figure 2A-C**).

No specific receptors have been identified for 12- or 15-HETE to date. Nevertheless, it has been reported that HETEs can interact with the $LTB_4$ receptor, $BLT_2$ (Yokomizo et al. 2001). In our case, the addition of U75302 and LY255283 as selective $BLT_1$ and $BLT_2$ receptor antagonists, respectively (Yokomizo et al. 2000), as well as Bay u9773 as a non-selective CysLTR antagonist (Nothacker et al. 2000) did not prevent the increase in $[Ca^{2+}]_i$ induced by 12 and 15-HETE (data not shown).

Regarding cAMP levels, only 12-(S)-HETE increased cAMP, at all the concentrations assayed. However, neither 12-(R)- nor 15-HETE modified this variable at either of the concentrations tested (0.1 and 1 μM) (**Table 1**). Thus suggesting a different role or intensity for both 12-HETE enantiomers.

Moreover, we have studied the ability of the 12- and 15-LOX AA metabolites to activate NFκB. None of them was able to increase the phosphorilated levels of IϰB (data not shown).

Finally, we studied the contribution of TJ proteins and cytoskeletal actin to the increase of PP induced by these eicosanoids. TJ protein immunofluorescent staining in control conditions showed occludin and ZO-1 located mainly at the cell border (**Figure 3**). The treatment with the eicosanoids that disrupted barrier function, namely 12-(R), 12-(S) and 15-HETE, resulted in a redistribution of occludin with adjacent diffuse intracellular staining and granular appearance, an effect that was more pronounced for the two 12-HETE enantiomers. However, no significant effect was observed on ZO-1 location for any of the eicosanoids.

Morphological assessment of subapical actin showed the characteristic perijunctional ring in control conditions. Treatment with all the eicosanoids that were able to alter PP, induced a complete disorganization of the actin belt.

## Discussion

There are a variety of gastrointestinal disorders, such as IBD, irritable bowel syndrome, celiac disease, and the early stages of colon cancer development (Clayburgh, Shen and Turner. 2004) with characteristic increased PP of the intestinal epithelium. In IBD, altered PP leads to the passage of pro-inflammatory stimuli to the underlying immune cells, thereby triggering a vicious cycle of mucosal barrier dysfunction and the activation of mucosal immune response (Bruewer, Samarin and Nusrat. 2006, Mankertz and Schulzke. 2007). Taking into account that the AA cascade is activated in intestinal mucosa in IBD (Boughton-Smith, Hawkey and Whittle. 1983, Eberhart and Dubois. 1995, Krimsky et al. 2003), we hypothesized that these eicosanoids would be involved in the regulation of intestinal epithelial barrier function in the above mentioned pathophysiological processes. In this regard, we previously reported that eicosanoids such as $PGE_2$, $PGE_3$, $LTD_4$ and 5-HETE increase PP in Caco-2 cells (Rodriguez-Lagunas et al. 2010, Rodriguez-Lagunas et al. 2013, Rodriguez-Lagunas, Ferrer and Moreno. 2013). 12- and 15-LOX pathways, both with pivotal roles in the regulation of intestinal function (Ferrer and Moreno. 2010), are synthetized in IBD (Mankertz and Schulzke. 2007, Zijlstra and Wilson. 1991, Zijlstra et al. 1992). Here we demonstrate that the addition of 12-, and 15-HETE at concentrations reached in the inflamed intestinal mucosa (Wardle, Hall and Turnberg. 1993, Zijlstra and Wilson. 1991, Zijlstra et al. 1992, Zijlstra et al. 1992) induce marked epithelial barrier disruption. However, in contrast to our findings, Ohata et al. (Gambaryan et al. 2010) reported that the addition of 5-, 12- and 15-HETE to Caco-2 cells reduces PP. Nevertheless, this effect was observed for a much higher HETE concentration (20 μM) than that tested in our study (0.1 μM).

The CYP450 pathway eicosanoids 20-HETE and EETs were previously reported to modify glomerular and endothelial permeability, respectively (Alvarez, Gjerde and Townsley. 2004, Sharma. 2006). In our case, no effect on intestinal PP was induced after exposure to physiological/physiopathological concentrations of EET, DHET or 20-HETE.

Our results revealed that the enhancement of $[Ca^{2+}]_i$ induced by 12- and 15-HETE was prevented by the inhibition of $Ca^{2+}$ release from intracellular stores, but not by the withdrawal of extracellular $Ca^{2+}$. Therefore, our results suggest that intracellular but not extracellular $Ca^{2+}$ participate in these events. Furthermore, we should also consider that PLC inhibition did not prevent the increase in $[Ca^{2+}]_i$ and cAMP levels were not modified by 12-(R) or 15-HETE, however 12-(S)-HETE increased cAMP levels. NFκB activation mediates the effects of several stimuli, such as INF-γ, TNF-α and IL-1β, on epithelial barrier disruption in intestinal Caco-2 cell cultures (Al-Sadi. 2009). The current data suggest that this is not the mechanism for neither the enantiomers of 12-HETE nor 15-HETE.

TJ proteins are pivotal for the maintenance of the epithelial permeability. Delocalization of occludin and ZO-1 from TJs is associated with epithelial barrier dysfunction and increased epithelial permeability (Harhaj and Antonetti. 2004). Immunofluorescent examination of Caco-2 cell cultures treated with the eicosanoids that altered PP showed TJ proteins redistribution. Thus, our findings revealed an alteration in occludin and actin distribution, while ZO-1 was not modified when cells were incubated with both the enantiomers of 12-HETE or 15-HETE. Similarly, despite the dramatic redistribution of TJ proteins following IFN-γ exposure, it has been reported that ZO-1 is only minimally affected and most of it remains at the TJ (Bruewer et al. 2003). This finding could be attributed to the observation that PP to ions and solutes is believed to be controlled mainly by the expression of

a range of transmembrane TJ proteins rather than ZO-1 protein (Krause et al. 2008). The formation of fluorescent clumps, in Caco-2 cells has been attributed to a multifocal aggregation of cytoskeletal elements, including actin (Ma et al. 1999). These authors also proposed a central role for actin-myosin contraction in the formation of these aggregates (Ma et al. 2000). In this respect, the presence of cytosolic occludin is associated with protein internalization by endocytosis.

On the basis of our findings, here we provide the first report on the relationship between signaling induced by 12/15-LOX pathway metabolites and the disruption of epithelial barrier function, and the first demonstration of a physiological function of these AA metabolites in the regulation of TJ permeability. Furthermore, our findings also provide a highly plausible explanation for the negative effect of COX inhibition on PP in IBD. This negative effect would be attributable to the synthesis of LOX pathway eicosanoids, which have a high capacity to disrupt TJ.

Given the involvement of eicosanoids in inflammatory processes such as IBD, our findings provide a valuable basis on which to perform a new line of research into the interrelation between AA cascade activation, PP changes, and the initiation/perpetuation of IBD (Turner. 2006). A dual inhibition of the COX and 5-LOX pathways (5-aminosalicylic acid or steroids) is currently used for the treatment of IBD (Krimsky et al. 2003). However, to date, no information is available on the inhibition of 12- or 15-LOX in IBD. For this reason, these findings may be useful for the future development of new diagnostic tools and therapeutic strategies for IBD.

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## Figure legends

**Fig. 1.** Effect of eicosanoids on PP. FD-4 (A) fluxes and TER (B) were determined in differentiated Caco-2 cell monolayers, as described in "Materials and Methods". Cells were incubated for 3 h with the eicosanoids (0.1 μM) in the apical and basolateral compartments. Results are expressed as the percentage of FD-4 fluxes and TER values obtained in control conditions (0.36 ± 0.01 ng/μL and 2081.69 ± 151.05 $\Omega \cdot cm^2$, respectively). Data are means ± SE of n = 7-9 experiments. *P < 0.05 vs control.

**Fig. 2.** Changes in $[Ca^{2+}]_i$ induced by 12-(R), 12-(S) and 15-HETE. $[Ca^{2+}]_i$ was determined in differentiated Caco-2 cell monolayers using Fura-2 AM, as described in "Materials and Methods". Cells were incubated for 160 s in the presence of the 12-(R), 12-(S) and 15-HETE (1 μM) (●) (A, B and C, respectively) plus dantrolene (□, 50 μM) or U73122 (Δ, 0.1 μM) and in the absence of extracellular $Ca^{2+}$ (Ø) The arrow indicates the injection of the eicosanoids. Inhibitors were pre-incubated for 30 min. Each plot corresponds to a representative profile obtained for n = 3 experiments.

**Fig. 3.** Changes in ZO-1 and occludin and perijunctional actin distribution induced by the eicosanoids. Fluorescent analysis was performed in cells incubated for 3 h with different eicosanoids (0.1 μM) using specific occludin and ZO-1 antibodies and TRITC-phalloidin, as described in "Materials and Methods". In each case, a representative *x-y* image of sections close to the apical cell side is shown.

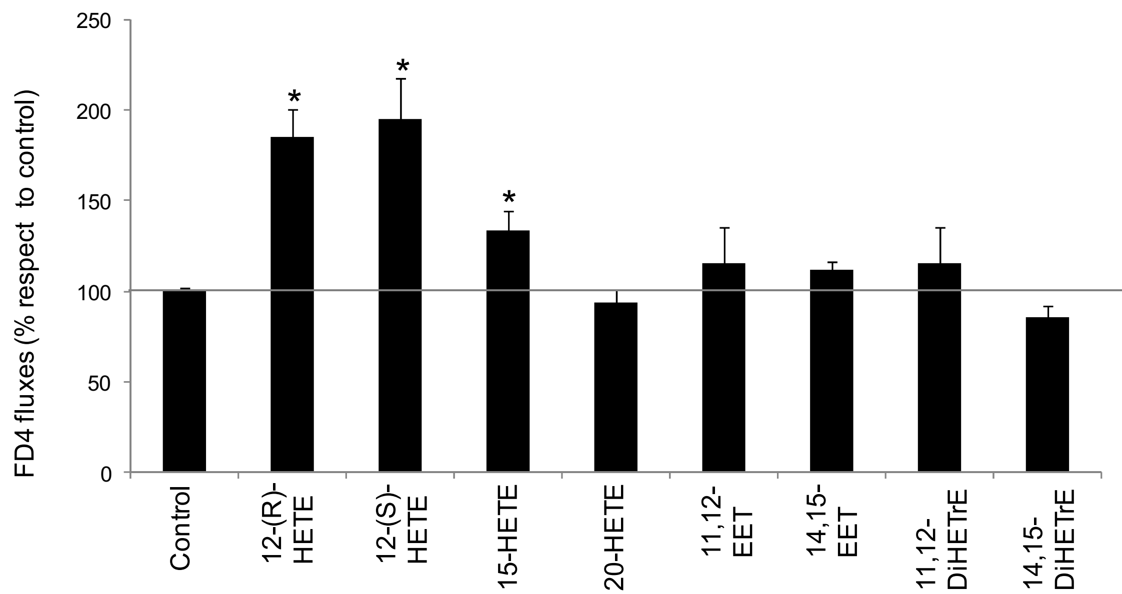
**Table 1.** Effect of 12/15-LOX pathway metabolites on $[Ca^{2+}]_i$ and intracellular cAMP concentration.

| | 12-(R)-HETE | | 12-(S)-HETE | | 15-HETE | |
|---|---|---|---|---|---|---|
| | **0.1 µM** | **1 µM** | **0.1 µM** | **1 µM** | **0.1 µM** | **1 µM** |
| $[Ca^{2+}]_i$ (AUC) | 1381 ± 202 | 29791 ± 5833 * | 3189 ± 597 | 16982 ± 2504 * | 3627 ± 591 | 12628 ± 2730 * |
| **cAMP** (% respect to control) | 78.55 ± 5.54 | 113.59 ± 18.21 | 157.14 ± 11.27 # | 128.09 ± 7.88 # | 104.47 ± 14.38 | 114.35 ± 15.19 |

Changes in $[Ca^{2+}]_i$ and cAMP levels were determined in differentiated Caco-2 cell monolayers as described in "Materials and Methods". For $Ca^{2+}$ determination, cells were incubated for 120 s in the presence of 12-(R)-, 12-(S)- and 15-HETE at two concentrations (0.1 µM and 1 µM) and the results are expressed as the area under the curve (AUC). cAMP was quantified in cells incubated for 5 min with the eicosanoids. Results are expressed as the percentage of cAMP values obtained in control conditions (22.59 ± 1.64 nM). Data are mean ± SE of n = 3-5 experiments. $^{\#}P<0.05$ vs control and $^{*}P<0.05$ vs 0.1 µM.

**Figure 1**

**A**




**B**

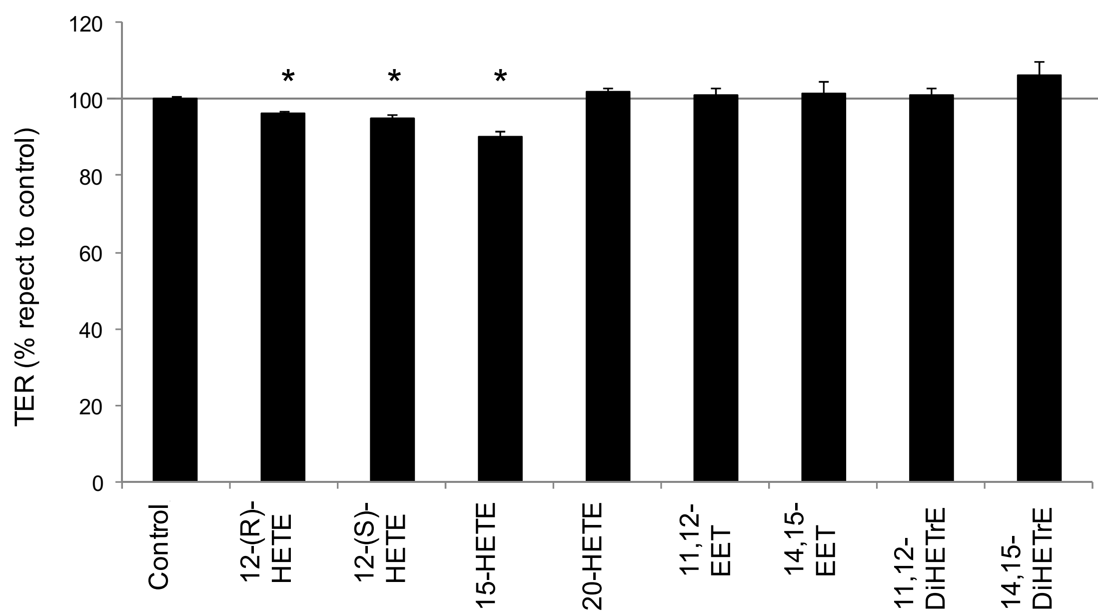
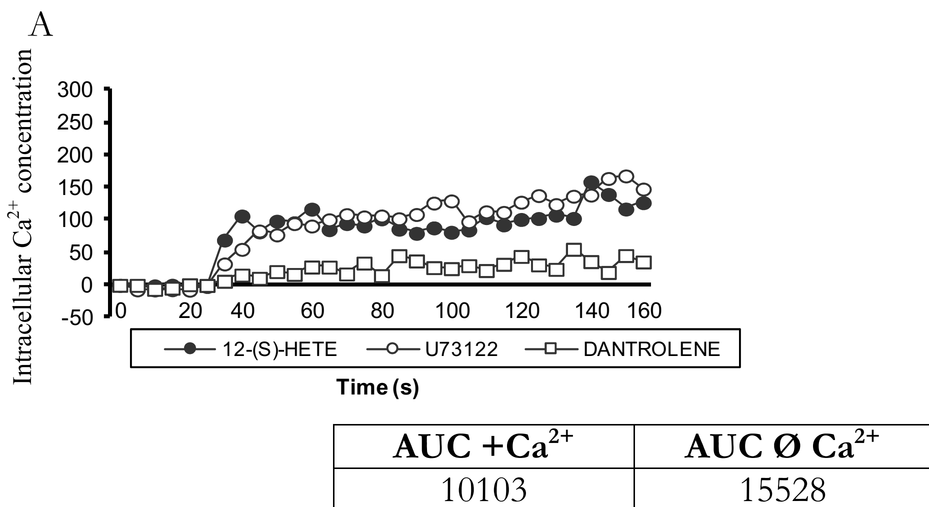


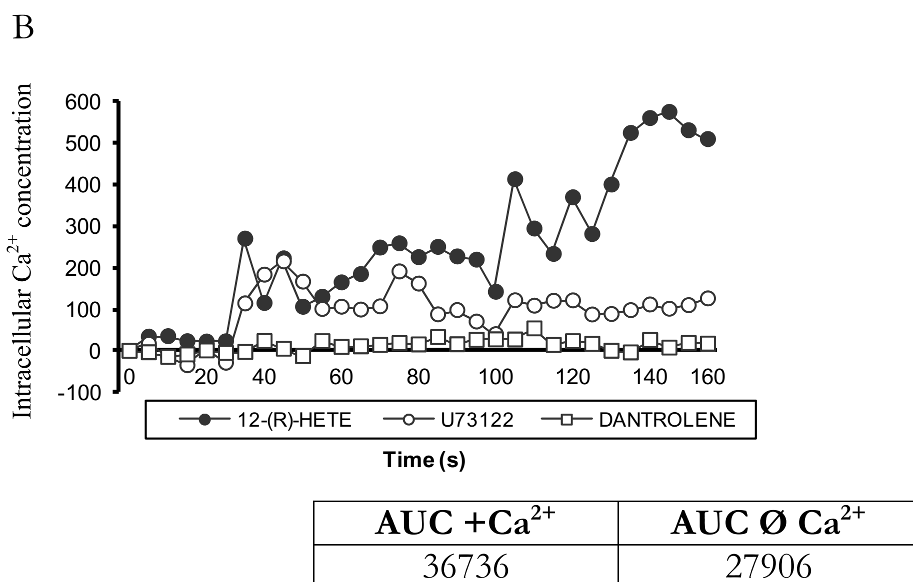
Figure 2

A



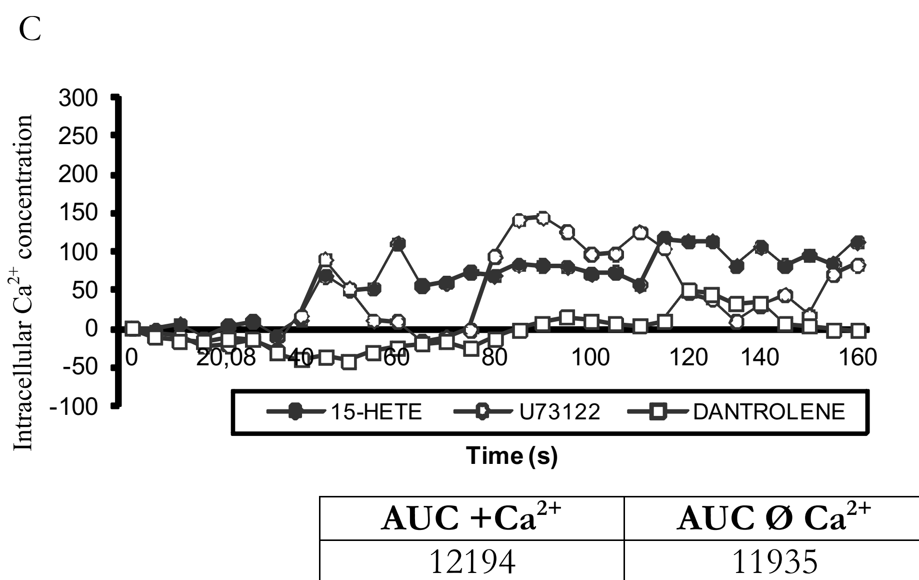

| AUC $+Ca^{2+}$ | AUC Ø $Ca^{2+}$ |
|---|---|
| 10103 | 15528 |

B



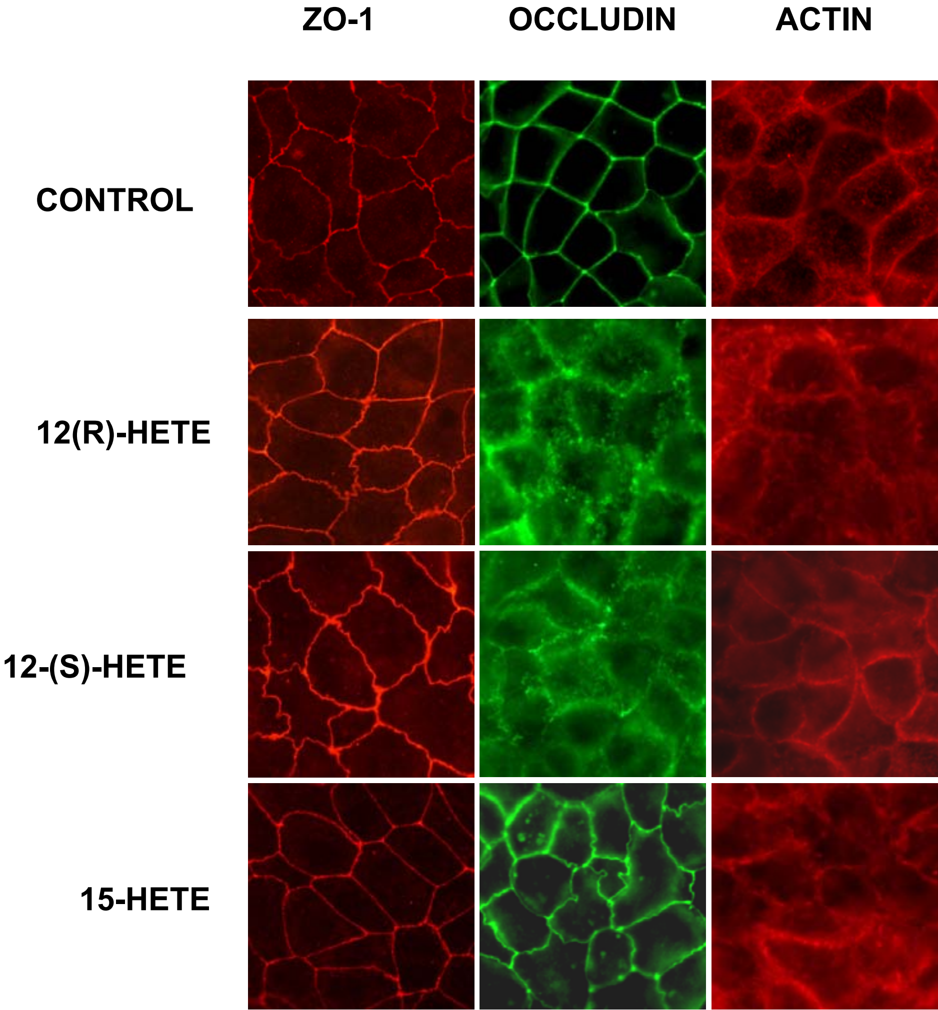

| AUC $+Ca^{2+}$ | AUC Ø $Ca^{2+}$ |
|---|---|
| 36736 | 27906 |

C




| AUC $+Ca^{2+}$ | AUC Ø $Ca^{2+}$ |
|---|---|
| 12194 | 11935 |

## Figure 3



***Resumen global de los resultados***

Los resultados muestran la implicación de diferentes eicosanoides producidos por las diferentes vías del metabolismo del AA (COX, LOX y CYP 450) en la regulación de la PP. Se ha demostrado como la $PGE_2$, la $PGE_3$, el $LTD_4$ y los 5-, 12-(R)-, 12-(S)- y 15-HETE son capaces de romper la función barrera, mientras que la $PGD_2$, el $LTB_4$, el 13-HODE, el 20-HETE, los 11,12- y 14,15-EET, los 11,12- y 14,15-DHETE y el 12-HEPE no presentan este efecto.

La $PGE_2$ es capaz de alterar la función barrera a través de su interacción con los receptores $EP_1$ y $EP_4$. La interacción con el receptor $EP_1$ activa la vía PLC-$IP_3$-$Ca^{2+}$, mientras que la interacción con $EP_4$ incrementa la concentración de AMPc y activa la vía AMPc-PKA. Estos cambios están inducidos también por la $PGE_3$ con la implicación de los mismos receptores y vías de señalización. En el caso de $LTD_4$ se observa que el receptor implicado en la disrupción de la función barrera es $CysLT_1R$. Además, se ha demostrado la participación de la vía de la PLC-$IP_3$-$Ca^{2+}$/PKC y PKA independiente de AMPc y de NFκB para $LTD_4$ y 5-HETE. El 12- y 15-HETE también inducen un incremento de la $[Ca^{2+}]_i$ mientras que no han modificado la concentración de AMPc a excepción de 12-(S)-HETE.

A partir del estudio de la localización de las proteínas de la TJ, se observa que los eicosanoides que alteran la función epitelial de barrera modifican la distribución de la ocludina y la claudina-4, sin modificar la de la ZO-1. La desorganización del anillo de actina y el incremento de la actividad de la MLCK se ha inducido por todos los eicosanoides disruptores de la función epitelial de barrera a excepción de 5-HETE.

# Discusión

La alteración de la función barrera del epitelio intestinal se produce en diversas enfermedades gastrointestinales como la IBD, el síndrome del intestino irritable (*Irritable Bowel Sindrome*, IBS) y la enfermedad celíaca (Clayburgh y col., 2004; Ma y col., 2004; Martinez y col., 2012). En la IBD, el incremento de la PP permite la entrada de estímulos proinflamatorios que activan las células inmunitarias y la secreción de citocinas, hecho que produce cambios en el estado de la TJ, dando lugar a un círculo vicioso de disfunción de la barrera epitelial y de activación de la respuesta inmunitaria mucosal (Barbara, 2006; Bruewer y col., 2006; Mankertz y Schulzke, 2007). Por otra parte, teniendo en cuenta que estudios previos indican que la cascada del AA está activada en la mucosa de pacientes con IBD (Boughton-Smith y col., 1983; Eberhart y Dubois, 1995; Krimsky y col., 2003) nos planteamos demostrar la hipótesis de que los eicosanoides puedan estar implicados en la homeóstasis del epitelio intestinal y que puedan tener un papel relevante en la alteración de la función epitelial de barrera (Ferrer y Moreno, 2010).

El incremento de la síntesis de la $PGE_2$ se ha relacionado con un incremento en la PP en un cultivo de células Caco-2 diferenciadas (Martin-Venegas y col., 2006). Para profundizar en el mecanismo por el cual este eicosanoide ejerce su efecto, en primer lugar se identificaron los receptores de la $PGE_2$ implicados en la alteración de la función barrera. La $PGE_2$ interacciona con cuatro receptores de membrana EP asociados a proteína G ($EP_1$-$EP_4$) (Harizi y col., 2008) que se expresan en las células Caco-2 (Shoji y col., 2004) y que hemos observado que se localizan principalmente en la membrana basolateral. Los antagonistas de $EP_1$ y $EP_4$ previenen la disrupción de la función epitelial de barrera inducida por $PGE_2$, sugiriendo la participación de dichos receptores. Recientemente, Tanaka y col. (2008) han estudiado el mecanismo implicado en el efecto de la $PGE_2$ sobre la PP en células Caco-2 no diferenciadas, usando altas concentraciones difíciles de alcanzar en condiciones fisiológicas o fisiopatológicas. Su conclusión es que los receptores implicados en dicho efecto son $EP_1$ y $EP_2$. Sin embargo, sus resultados se obtuvieron utilizando únicamente un agonista específico $EP_1$ y butaprost, un agonista $EP_2$, sin considerar que éste es también agonista $EP_4$ (Sugimoto y Narumiya, 2007). Por lo tanto, estos resultados corroboran la participación de $EP_1$ y probablemente $EP_4$.

La interacción de la $PGE_2$ con $EP_1$ produce una activación de la PLC, la PKC y la elevación de la concentración de mediadores secundarios como el $IP_3$, el DAG y el $Ca^{2+}$ (Dey y col., 2006). En nuestras condiciones experimentales, la interacción de la $PGE_2$ con el receptor $EP_1$, incrementa la $[Ca^{2+}]_i$. Nuestros resultados también

indican la participación de la vía PLC-$IP_3$-$Ca^{2+}$, ya que tanto la $[Ca^{2+}]_i$ como la PP aumentan al tratar las células Caco-2 con $IP_3$. El incremento en la $[Ca^{2+}]_i$ y la alteración de la PP inducidos por la $PGE_2$ y un agonista $EP_1$ como la carbaciclina (Lawrence y col., 1992) no se produce cuando las células se preincuban con un antagonista $EP_1$, con un inhibidor de la PLC o con un inhibidor de la liberación de $Ca^{2+}$ del retículo endoplasmático como el dantroleno (Van Winkle, 1976), sugiriéndose, pues, la participación del $Ca^{2+}$ intracelular.

La interacción de la $PGE_2$ con el $EP_1$ produce una activación de la PKC (Dey y col., 2006). En este sentido, el incremento de la PP inducido por esta PG en cultivos de células Caco-2 se revierte al añadir un inhibidor de la PKC convencional, el Gö6983 (Gonzalez-Mariscal y col., 2008). De hecho, las quinasas de esta familia que son dependientes de $Ca^{2+}$ y de DAG contribuyen a la alteración de la TJ (Andreeva y col., 2006).

La interacción de la $PGE_2$ con el $EP_4$ activa la AC incrementando la concentración de AMPc y dando lugar a la activación de la PKA (Dey y col., 2006). Así, la $PGE_2$ induce un incremento de la concentración intracelular de AMPc en las células Caco-2. Cuando estas células se preincuban con un inhibidor de la AC o con un inhibidor de la PKA, los efectos sobre la PP se previenen, demostrándose la participación de una PKA dependiente de AMPc en la disrupción de la función barrera inducida tras la interacción $PGE_2$ - $EP_{4.}$

Sorprendentemente, la incubación de las células Caco-2 con un agonista $EP_4$ produjo un incremento de la $[Ca^{2+}]_i$, siendo dicho efecto similar al inducido por la forskolina. Además, en ambos casos este efecto se previene al preincubar las células con dantroleno. Recientemente varios autores han descrito una relación entre la vía de señalización $IP_3$-$Ca^{2+}$ y la vía de la AMPc-PKA que da lugar a la regulación de la $[Ca^{2+}]_i$. Así, el punto en el que convergen ambas vías de señalización es la fosforilación de los receptores de $IP_3$ por la PKA. Este mecanismo está implicado en diferentes procesos fisiológicos como la actividad neuronal, la secreción de fluidos en el epitelio y la modulación de la secreción de insulina (Bruce y col., 2002; Gu y col., 2003; Tang y col., 2003; Chaloux y col., 2007; Schmidt y col., 2008). Estos trabajos demuestran que la activación de la PKA da lugar a un incremento significativo de la $[Ca^{2+}]_i$ de origen extracelular o intracelular mediado por $IP_3$. Varios autores demostraron que este efecto se debe a un incremento de la afinidad del receptor por $IP_3$ (Chaloux y col., 2007; Wagner y col., 2008). La hipótesis para explicar la capacidad del butaprost (agonista $EP_2$/$EP_4$) y de un activador de la AC para incrementar la $[Ca^{2+}]_i$ en células Caco-2 se sustenta en que los niveles basales

de $IP_3$ ya son suficientes para inducir la liberación de $Ca^{2+}$ en condiciones en las que la concentración de AMPc está incrementada, hechos que también pueden ocurrir tras la interacción de la $PGE_2$ con el $EP_4$. Este mecanismo podría explicar la capacidad del dantroleno para prevenir la alteración de la PP inducida no sólo por la interacción de la $PGE_2$ con el $EP_4$ sino también por un agonista $EP_4$ y por un activador de la AC. En este sentido, esta interacción entre ambas vías de señalización ya se ha descrito previamente para la $PGE_2$ en un cultivo de neuronas sensoriales del nervio vago de rata (Gu y col., 2003).

La deslocalización de las proteínas de la TJ, ocludina y ZO-1, se asocia a la disrupción de la función epitelial de barrera (Harhaj y Antonetti, 2004). La $PGE_2$ altera la distribución celular de la ocludina y del anillo subapical de actina sin cambios importantes en la localización de la ZO-1. La presencia de la ocludina en el citosol está asociada con la internalización de dicha proteína por endocitosis. En el caso de la pérdida de la función barrera inducida por TNF-α y etanol, la internalización de la ocludina se asocia con la contracción del anillo subapical de actina (Ma y col., 1999; Schwarz y col., 2007). La ZO-1 constituye un puente entre el anillo subapical de actina y las proteínas de la TJ como la ocludina (Fanning y col., 1998). Cambios en la localización de las proteínas de la TJ sin afectación de la ZO-1 ya se han descrito previamente por otros autores al exponer células intestinales a IFN-γ (Bruewer y col., 2003) o $PGE_2$ (Tanaka y col., 2008).

La MLCK juega un papel importante en la regulación de la función epitelial de barrera. Concretmante, la sobreexpresión de la MLCK en células Caco-2 induce la reorganización de la actina e incrementa la PP (Shen y col., 2006). El incremento de la PP inducido por diversas citocinas (TNF-α, IFN-γ y IL-1β), ácidos grasos de cadena corta y etanol se ha relacionado con el incremento de la actividad de la MLCK (Ma y col., 1999; Ohata y col., 2005; Shen y col., 2006; Ye y col., 2006; Al-Sadi y col., 2008). De hecho, la expresión de MLCK en pacientes con IBD está incrementada (Blair y col., 2006). Además, la acción protectora de los glucocorticoides sobre la disrupción de la función epitelial de barrera inducida por TNF-α se ha relacionado con la reducción de la actividad MLCK (Boivin y col., 2007). En las células Caco-2 tratadas con $PGE_2$ se observa la deslocalización del anillo subapical de actina así como la capacidad de un inhibidor de la MLCK para prevenir los efectos de esta PG sobre la función epitelial de barrera. Este hecho indica la participación del citoesqueleto en la regulación de la PP en estas condiciones experimentales. Además, la reducción de la TER inducida por los ácidos biliares en células Caco-2 -a través del incremento de la actividad MLCK- se relaciona con el aumento de la actividad de la COX y de la PKC (Araki y col.,

2005). Previamente, se ha relacionado la PKC con la MLCK en la regulación de la PP con resultados contradictorios. Así, (Turner y col., 2000) encontraron en células Caco-2 que la MLCK se inhibe a través de la fosforilación de la PKC dando lugar a un incremento de la TER. Por el contrario, el incremento de la PP inducido por una infección con *Escherichia coli* en células T-84 y por la glicoproteína gp120 de HIV-1 en células endoteliales de cerebro se debe a la activación de la PKC y la MLCK (Philpott y col., 1998; Kanmogne y col., 2007). Nuestros resultados coinciden con estos últimos autores y es la primera vez que se describe el papel de la PKC y de la MLCK en la disrupción de la PP inducida por $PGE_2$ en células Caco-2.

Recientemente, se ha constatado la relevancia de las claudinas en la disrupción de la función epitelial de barrera. Así, en los enfermos de IBD se ha detectado un incremento de la expresión de claudina-2 y una disminución de la de claudina-4 (Amasheh y col., 2011). En cambio, Takehara y col. (2009) observan que la sobreexpresión de la claudina-4 en células Caco-2 altera la función barrera. Nuestros resultados muestran la alteración de la localización de la claudina-4 en presencia de la $PGE_2$ sin modificación de las claudinas-1 y -2. Estos cambios en las proteínas de la TJ son similares a los inducidos por la $PGE_2$ producida en infecciones por *Entamoeba histolytica* (Lejeune y col., 2011).

El enriquecimiento de los medios de cultivo con los AGPI n-3, como el DHA y el EPA, ha demostrado ser capaz de alterar la función epitelial de barrera en células Caco-2 (Usami y col., 2001; Usami y col., 2003; Roig-Perez y col., 2004). Se ha de tener en cuenta que el EPA es también substrato de la COX-2 dando lugar a prostanoides de la serie 3 como la $PGE_3$. El efecto de estos AGPI sobre la función barrera se ha relacionado con la formación de PG, ya que la indometacina, un inhibidor de la COX, es capaz de prevenir el incremento de la PP inducido por estos AG (Usami y col., 2001; Usami y col., 2003; Roig-Perez y col., 2010), sugiriendo la participación de la $PGE_3$ en dicha acción. La $PGE_3$ interacciona con los mismos receptores que la $PGE_2$, es decir, $EP_1$-$EP_4$ (Wada y col., 2007). Hemos observado, por primera vez, un efecto de la $PGE_3$ sobre la función barrera similar al de la $PGE_2$, debido a la interacción con los receptores $EP_1$ y $EP_4$ y a la activación de las mismas vías de señalización y a los mismos cambios en la TJ. Por todo ello, si bien se ha descrito un efecto beneficioso de los AGPI de origen marino en pacientes con IBD, éste no puede ser atribuido a la reducción de la relación $PGE_2/PGE_3$ ya que ambas PG tienen el mismo efecto disruptor de la función barrera.

Además de las PG, se ha descrito que algunos metabolitos de la vía de la LOX como el 5-, 12- y 15-HETE así como el $LTB_4$ están incrementados en la mucosa de pacientes con IBD (Boughton Smith, 1983; Eberhart y Dubois, 1995). Los tratamientos tradicionales con AINE pueden exacerbar la enfermedad (Stenson, 1990). En este sentido, la inhibición de la vía de la COX daría lugar a un aumento de la proporción de eicosanoides de la vía de la LOX, que podría estar relacionado con la exacerbación de la enfermedad. Por ello, existen tratamientos clínicos efectivos basados en la inhibición de la vía de la LOX como la sulfasalazina o la mesalazina aunque su efecto también se basa en la inhibición de la producción de TNF-α e IL-1, entre otros mecanismos (Pithadia y Jain, 2011). Sin embargo, los inhibidores de la 5-LOX como el zileuton no producen un efecto terapéutico suficiente en pacientes con UC (Werz y Steinhilber, 2006). Esto podría ser debido a que si bien la síntesis de los metabolitos de la vía de la 5-LOX como el $LTB_4$, el $LTD_4$ y el 5-HETE estarían disminuidos, la síntesis de los metabolitos de las otras LOX no se verían afectados o estarían incrementados, como en el caso del 12- o 15-HETE. Por todo ello creímos interesante estudiar el efecto de estos eicosanoides sobre la regulación de la PP. Los resultados muestran cómo metabolitos representativos de las LOX como el $LTD_4$ y el 5-, 12- y 15-HETE -a concentraciones alcanzadas en la mucosa intestinal inflamada (Zijlstra y Wilson, 1991; Zijlstra y col., 1992; Wardle y col., 1993)- alteran la función epitelial de barrera en cultivos diferenciados de células Caco-2, mientras que el $LTB_4$ no tiene ningún efecto. Así, la alteración de la PP inducida por los eicosanoides derivados de la LOX refuerza la hipótesis que los efectos negativos de la inhibición de la COX en pacientes con IBD puede ser debido al aumento de la síntesis de los eicosanoides de la vía de la LOX.

El $LTD_4$ se une al $CysLT_1R$ y al $CysLT_2R$ que se expresan en células Caco-2 (Nielsen y col., 2005; Magnusson y col., 2007). Nuestros resultados muestran la participación de $LTD_4$-$CysLT_1R$ en la regulación de la función epitelial de barrera. En este sentido, $LTD_4$-$CysLT_1R$ se ha relacionado con la regulación de otras funciones del epitelio intestinal como la proliferación (Paruchuri y col., 2006) y con la extravasación de proteínas plasmáticas durante la inflamación (Beller y col., 2004). Si bien los receptores para los LT están bien caracterizados, no se han identificado todavía receptores específicos para los HETE. Sin embargo, se ha descrito que 12-(S)- y 15-(S)-HETE se unen al receptor $BLT_2$ de baja afinidad para $LTB_4$ (Yokomizo y col., 2001). Nuestros resultados descartan la participación tanto del $BLT_1$ como del $BLT_2$ -ambos expresados en células epiteliales intestinales (Tager y Luster, 2003; Ihara y col., 2007)- en el efecto del 5-HETE sobre la función barrera. Además, los resultados obtenidos con un antagonista receptorial no

selectivo del CysLTR indican que estos receptores tampoco participan en su efecto sobre la PP.

La unión de $LTD_4$ a $CysLT_1R$ activa una proteína G que induce la activación de la PLC con la consecuente liberación de DAG e $IP_3$. Así, cuando el $LTD_4$ se une al $CysLT_1R$ se produce un aumento de la $[Ca^{2+}]_i$ y la activación de la PKC, tal y como ha sido descrito anteriormente (Profita y col., 2008; Suzuki y col., 2008; Woszczek y col., 2008; Singh y col., 2010). Nuestros resultados muestran cómo la inhibición de la PLC, la reducción de la liberación de $Ca^{2+}$ del retículo endoplasmático y la inhibición de la PKC previenen la alteración de la PP inducida por el $LTD_4$ y el 5-HETE. Además, también se ha observado cómo el incremento de la $[Ca^{2+}]_i$ inducido por estos eicosaniodes se previene, al igual que en el caso de la $PGE_2$, por inhibidores de la PLC o de la liberación de $Ca^{2+}$ intracelular, así como al eliminar el $Ca^{2+}$extracelular del medio, resultados que sugieren la participación del $Ca^{2+}$intra y extracelular en este efecto. Por el contrario, el incremento de la PP inducido por 12- y 15-HETE se puede asociar sólo a un incremento de la $[Ca^{2+}]_i$ proveniente de las reservas intracelulares ya que éste no se revirtió al eliminar el $Ca^{2+}$ del medio.

La presencia de un inhibidor de la PKA previno el incremento de la PP inducido por el $LTD_4$ y el 5-HETE a pesar de que ninguno de ellos alteró la concentración intracelular de AMPc. Aunque la PKA se activa normalmente por el AMPc, se han descrito diversos mecanismos de activación independientes del AMPc en diversos tipos celulares (Niu y col., 2001; Howe, 2004; Kohr y col., 2010). Un ejemplo es la activación de la PKA dependiente del NFκB (Gambaryan y col., 2010). El NFκB es activado por diferentes estímulos que producen la disrupción de la función epitelial de barrera en células intestinales Caco-2 (Al-Sadi, 2009). Por ello, la activación del NFκB se contempló como un posible mecanismo de activación de la PKA independiente de AMPc. Sin embargo, los niveles de IκB fosforilados no se incrementaron al tratar las células con ambos eicosanoides, descartando que la activación de la PKA se produzca por esta vía. Por lo tanto, otros mecanismos de activación de la PKA independientes de AMPc y de NFκB deben de estar implicados en estos efectos. En este sentido, se ha descrito la activación de la PKA por una proteína efectora de la subunidad Gα en células neuronales (Niu y col., 2001) o por peroxinitrito en cardiomiocitos (Kohr y col., 2010). Del resto de eicosanoides de la vía de la LOX estudiados sólo 12-(S)-HETE incrementó la concentración de AMPc, de manera que en este caso la activación de la PKA por AMPc podría estar implicada en la alteración de la PP mientras que 12-(R)- o 15-HETE podrían compartir el mismo mecanismo el descrito para 5-HETE.

El EPA y el DHA también son substratos de las mismas enzimas que metabolizan el AA. Así, el EPA da lugar a las PG de la serie 3, a los LT de la serie 5 y a los HEPE. En este sentido, y teniendo en cuenta el efecto de la $PGE_3$ sobre la PP se estudió el papel de un HEPE representativo, el 12-HEPE, en la regulación de la función epitelial de barrera. A diferencia del 12-HETE (derivado del AA) el 12-HEPE no alteró la PP. Además se ha descrito que las dietas ricas en aceite de pescado reducen los niveles de ambos S- y R-HETE. Una posible explicación es el hecho que el EPA compite con el AA como substrato de las LOX y además parece reducir la expresión de LOX (Neilson y col., 2012). Todo esto podría explicar el efecto beneficioso del EPA en pacientes con IBD -que podría ser atribuido a la generación de 12-HEPE que no altera la PP al contrario que 12-HETE- Así pues, estos datos deberán tenerse en cuenta para el desarrollo de nuevas terapias nutricionales en el tratamiento de enfermedades inflamatorias como la IBD que además podrían combinarse con inhibidores de las vías de la cascada del AA.

Al igual que la $PGE_2$ y la $PGE_3$, el $LTD_4$, el 12- y el 15-HETE también producen una deslocalización de la ocludina, de la claudina-4 y del anillo de actina. Sin embargo, no se produce ninguna alteración en la localización de la ZO-1, la claudina-1 y la claudina-2. En el caso del 5-HETE se observa sólo la deslocalización de la ocludina y de la claudina-4. Existe poca información sobre la alteración de las claudinas inducida por metabolitos de la 5-LOX, aunque la redistribución de la claudina-4 sin efectos sobre la -1 y la -2 se ha observado recientemente en modelos de inflamación intestinal inducida por microorganismos (Hering y col., 2011; Lejeune y col., 2011), lo que concuerda con nuestros resultados. Tal y como hemos observado para la $PGE_2$ y la $PGE_3$, la alteración de la PP inducida por el $LTD_4$, así como la desorganización del anillo de actina, se correlacionan con la activación de la MLCK y la consecuente fosforilación de la MLC. Sin embargo, estos eventos no se observan en el caso del 5-HETE.

El AA puede metabolizarse a través de las enzimas del CYP: el CYP epoxigenasa da lugar a los EET y el CYP hidroxilasa forma HETE (Fleming, 2007). Una vez formados, los EET son inestables porque se metabolizan rápidamente a su correspondiente DHET a través de la enzima sEH (Spector y Norris, 2007). Concentraciones fisiológicas de EET inhiben la inflamación al reducir la expresión de las moléculas de adhesión celular en el endotelio e inhibir NFκB e Iκκ (Node y col., 1999). Así, la inhibición de la sEH da lugar a un incremento de la concentración de EET y por tanto un aumento de su actividad antiinflamatoria (Inceoglu, 2007). Sin embargo, se ha sido descrito que el 20-HETE es capaz de alterar la permeabilidad glomerular (Sharma, 2006) y los EET la endotelial (Alvarez

y col., 2004). En nuestro modelo experimental, el 20-HETE, 11,12- y 14,15-EET y sus respectivos metabolitos 11,12- y 14,15–DHET no han modificado la PP.

A pesar de que la disrupción de la función epitelial de barrera observada en pacientes con IBD se ha atribuido tradicionalmente al efecto de citocinas proinflamatorias como TNF-α e IFN-γ, la presente tesis ha aportado evidencias del papel de los eicosanoides en la permeabilidad de la barrera epitelial, abriendo posibilidades de nuevas estrategias terapéuticas que podrían estar basadas en la inhibición de la vía de la LOX (no sólo la 5-LOX), tratamientos duales de inhibición de la COX y la LOX o la inhibición de la fosforilación de la MLC, entre otros.

# Conclusiones

- Del estudio del papel de la $PGE_2$ y sus receptores ($EP_1$-$EP_4$) en la regulación de la PP, así como de las vías de señalización implicadas se puede concluir que (Figura 11):
    - la acción de la $PGE_2$ sobre la función barrera está mediada principalmente por la interacción con sus receptores $EP_1$ y $EP_4$, situados principalmente en la membrana basolateral. Dicha interacción, a su vez, activa las vías PLC-$IP_3$-$Ca^{2+}$ y AMPc-PKA, respectivamente.
    - existe una relación entre la activación de la MLCK, la alteración del anillo de actina y el incremento de la PP en células tratadas con $PGE_2$.
    - las proteínas de la TJ ocludina y claudina-4 están implicadas en la alteración de la función barrera inducida por la $PGE_2$, mientras que no se han observado cambios en la ZO-1 ni en las claudinas-1 y -2.

- Del estudio de los efectos de los eicosanoides derivados del EPA, principalmente de la $PGE_3$, sobre la función epitelial de barrera y sus vías de activación se puede concluir que (Figura 11):
    - la $PGE_3$ es capaz de alterar la PP, al contrario que otros metabolitos del EPA como 12-HEPE.
    - la acción de la $PGE_3$ sobre la función barrera está mediada, al igual que en el caso de la $PGE_2$, por la interacción con los receptores $EP_1$ y $EP_4$, que a su vez activan las vías PLC-$IP_3$-$Ca^{2+}$ y AMPc-PKA, respectivamente.
    - existe una relación entre la activación de la MLCK, la alteración del anillo de actina y la regulación de la PP en células tratadas con $PGE_3$.
    - la ocludina y la claudina-4 están implicadas en la alteración de la función barrera inducida por la $PGE_3$, mientras que no se han observado cambios en la ZO-1 ni en las claudinas-1 y -2.

- Del estudio del papel de los metabolitos de la vía de la LOX y de la vía del CYP sobre la PP y sus vías de activación se puede concluir que (Figura 11):

– los eicosanoides $LTD_4$, 5-, 12- y 15-HETE son capaces de alterar la PP, a diferencia de $PGD_2$, $LTB_4$, 11,12-, 14,15-EET, 11,12-, 14,15-DHET, 12-HEPE, 13-HODE y 20-HETE. En el caso del 12-HETE, ambos enantiómeros han alterado la PP.

– la acción del $LTD_4$ sobre la función barrera está mediada por el $CysLT_1R$ que activa la vía PLC-$Ca^{2+}$/PKC, la MLCK y la PKA independiente de AMPc y de NFκB.

– las proteínas de la TJ ocludina y claudina-4 y el anillo de actina están implicados en la regulación de la PP inducida por los eicosanoides que han demostrado alterar la PP, a excepción de 5-HETE para el que el anillo de actina no se ve modificado.

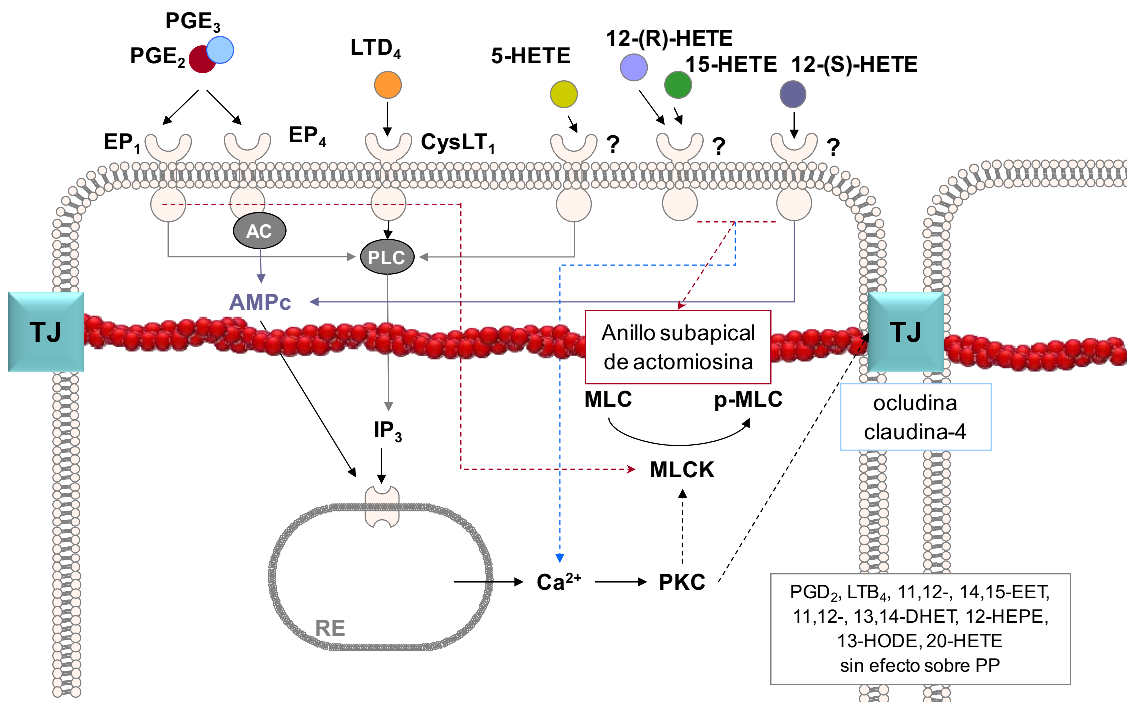



**Figura 11. Receptores y vías de señalización implicados en la alteración de la función epitelial de barrera inducida por los eicosanoides.**

- From the study of the role of $PGE_2$ and its receptors ($EP_1$-$EP_4$) in the regulation of PP and the underlying pathways we can conclude that:
    - the action of $PGE_2$ on epithelial barrier function is regulated through the interaction with $EP_1$ and $EP_4$ which are localized mainly in the basolateral membrane. This interaction activates the PLC-$IP_3$-$Ca^{2+}$ and cAMP-PKA pathways, respectively.
    - there is a relation between in MLCK activation and the subsequent alteration of the subapical actin ring and the increase in PP on Caco-2 cells exposed to $PGE_2$.
    - the TJ proteins occludin and claudin-4 are involved in the alteration of epithelial barrier function induced by $PGE_2$, whereas there is no alteration on neither ZO-1 nor claudin-1 or -2 TJ proteins.

- From the study of the EPA derived eicosanoids -mainly $PGE_3$- on the epithelial barrier function and the underlying events we can conclude that:
    - $PGE_3$ is able to increase PP, on the contrary, the EPA-derived 12-HEPE do not alter epithelial barrier function.
    - Similar to $PGE_2$, $PGE_3$ induced PP is due to $EP_1$ and $EP_4$ interaction and further PLC-$IP_3$-$Ca^{2+}$ and cAMP-PKA pathways activation, respectively.
    - there is also a relation between MLCK activation and the subsequent alteration of the subapical actin ring and the increase in PP on Caco-2 cells treated with $PGE_3$.
    - the TJ proteins occludin and claudin-4 are involved in the alteration of PP induced by $PGE_3$, whereas there is no alteration on neither ZO-1 nor claudin-1 or -2 TJ proteins.

- From the study of the role of the metabolites from LOX and CYP pathways on PP and the underlying pathways we can conclude that:
    - the eicosanoids $LTD_4$, 5-, 12- and 15-HETE increase PP whereas $PGD_2$, $LTB_4$, 11,12-, 14,15-EET, 11,12-, 14,15-DHET, 12-HEPE, 13-HODE and 20-HETE do not alter epithelial barrier function. In the case of 12-HETE, both enantiomers increase PP.

- the increase in PP induced by $LTD_4$ is mediated through $CysLT_1R$ interaction which in turns activates the PLC-$Ca^{2+}$-PKC pathway, MLCK and cAMP and NFκB indepent PKA.
- the TJ proteins occludin and claudin-4 and the subapical actin ring are involved in the increase in PP induced by the eicosanoids assayed except for 5-HETE which did not alter the actin ring.

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Bibliografía

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