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EL PÉPTIDO IDR-1002 INDUCE LA INHIBICIÓN DE LA ACTIVIDAD DE NF-κB Y PROMUEVE LA ACTIVIDAD DE CREB EN MACRÓFAGOS ESTIMULADOS CON LIPOPOLISACÁRIDO

Tesis que presenta

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A mi familia que me ha forjado en lo que actualmente soy.

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RESUMEN

La inflamación es el principal mecanismo de respuesta del sistema inmune innato, que nos defiende en primera instancia contra los efectos dañinos de microorganismos patógenos. Aunque la respuesta inflamatoria se considera un mecanismo fisiológico que se pone en marcha para proteger la integridad del tejido, su control y resolución son fundamentales para evitar situaciones inflamatorias crónicas que pueden provocar enfermedades degenerativas. En los últimos años el estudio de los mecanismos moleculares que controlan la inflamación ha llegado a ser un tema de frontera que permitirá diseñar fármacos que puedan regular la respuesta inflamatoria. Dentro de estos fármacos se encuentran los péptidos catiónicos sintéticos, denominados IDR (*Innate Defense Regulator*). Uno de estos péptidos, IDR-1002 regula selectivamente el sistema inmune, induce la eliminación eficaz de bacterias como *Staphylococcus aureus* y *Escherichia coli* de los tejidos y controla efectivamente la inflamación. Sin embargo, el mecanismo molecular que IDR-1002 activa para regular la expresión diferencial de citocinas pro- y anti-inflamatorias no ha sido analizado en detalle. Debido a la importancia de los macrófagos en la regulación de la respuesta inflamatoria, decidimos explorar en este tipo celular la participación de CREB y NF-κB en el mecanismo activado por IDR-1002. Los macrófagos estimulados con LPS respondieron con un incremento significativo de TNF-α y COX-2; sin embargo, en presencia de IDR-1002 se observó una disminución significativa de estas dos moléculas pro-inflamatorias. La causa de esta disminución fue que IDR-1002 inhibió la translocación al núcleo de NF-κB, mediante la inhibición de la fosforilación de IκBα. Además, IDR-1002 causó un aumento en la abundancia relativa de CREB fosforilado en Ser133, por un mecanismo dependiente de la activación de p38, ERK1/2 y MSK1. No obstante, esta fosforilación de CREB en el sitio de activación no condujo a la expresión de las citocinas anti-inflamatorias IL-4, IL-10 e IL-13. En conclusión, IDR-1002 presenta un efecto dual al inhibir la activación del factor de transcripción pro-inflamatorio NF-κB y simultáneamente activar al factor de transcripción anti-inflamatorio CREB.

Palabras clave: IDR, inflamación, macrófagos, IκBα, NF-κB, CREB.

ABSTRACT

Inflammation is the main mechanism of the innate immune system against the harmful effects of pathogenic microorganisms. Although inflammation is considered a physiological response that is critical to protect and restore the tissue homeostasis, its control and resolution are fundamental to avoid chronic inflammatory conditions. During the last decade many research groups worldwide have made valuable progress on the molecular mechanisms that regulate inflammation. Their principal goal has been to design anti-inflammatory drugs that specifically inhibit pro-inflammatory molecular targets. The innate defense regulator (IDR) cationic peptides are promising anti-inflammatory drugs because they selectively and specifically modulate the innate immune system, effectively eliminate pathogenic bacteria such as *Staphylococcus aureus* and *Escherichia coli* from the infected tissues, and control inflammation. Although several reports have documented the anti-inflammatory properties of one of these peptides, IDR-1002, the inhibition mechanisms of pro-inflammatory cytokines expression and anti-inflammatory cytokines activation is not known in detail. Due to the important function of macrophages as regulatory cells of the inflammatory response, we decided to explore the role of the pro-inflammatory transcription factor NF-κB and the anti-inflammatory transcription factor CREB in the regulation of the pro- and anti-inflammatory cytokines expression, respectively when macrophages are treated with IDR-1002. We found that macrophages stimulated with lipopolysaccharide (LPS) expressed high amounts of the NF-κB-dependent genes TNF-α and COX-2; however, in the presence of IDR-1002 we observed a significant expression reduction of both pro-inflammatory molecules because this peptide strongly inhibited the IκBα phosphorylation and subsequently the NF-κB nuclear translocation. Furthermore, IDR-1002 induced an increase in the relative abundance of CREB phosphorylated at Ser133 through a mechanism that involved the activation of the protein kinases p38, ERK1/2, and MSK1. Such CREB activation did not lead to expression of the anti-inflammatory cytokines IL-4, IL-6, and IL-13. In conclusion, IDR-1002 inhibits the activity of NF-κB and promotes the activation of CREB in macrophages.

1. MARCO TEÓRICO

1.1. Inmunidad innata e inflamación

La inmunidad innata es la primera línea de defensa contra agentes infecciosos, que consiste de una red interactiva de sistemas moleculares y celulares responsable de reconocer y erradicar microorganismos patógenos. Este tipo de inmunidad incluye una gama amplia de cambios en la célula, cuyo objetivo es producir moléculas que coadyuven en la defensa del hospedero. La habilidad del sistema inmune innato de reconocer y responder a componentes microbianos, se debe a la presencia de receptores tipo I, llamados receptores tipo Toll (TLRs) [1, 2]. Los TLR son expresados tanto en fagocitos profesionales (monocitos, macrófagos y células dendríticas) como en no profesionales como las células epiteliales y endoteliales. Estos receptores de reconocimiento de patógenos (PRRs) son capaces de discriminar entre distintos patrones moleculares asociados a patógenos (PAMPs) que conducen a la activación de una variedad de vías de transducción de señales, las cuales regulan la naturaleza, magnitud y duración de la respuesta inflamatoria [3].

La reacción inflamatoria local desempeña una función muy importante en la respuesta inicial a la infección y en ella participan neutrófilos, monocitos, macrófagos, moléculas del complemento, citocinas, quimiocinas, así como también proteínas y péptidos con actividad antimicrobiana. Todos estos factores en conjunto coadyuvan a eliminar al patógeno del tejido infectado. Aunque la producción de citocinas pro-inflamatorias es importante para mediar la defensa inicial del hospedero contra los patógenos invasores, la incapacidad de regular la intensidad o duración de la respuesta inflamatoria puede ser perjudicial, como en el caso de las enfermedades inflamatorias crónicas [4]. La respuesta inflamatoria es un mecanismo regulado por los factores transcripcionales factor nuclear kappaB (NF-κB) con actividad pro-inflamatoria y el factor de transcripción denominado proteína de unión al elemento de respuesta a AMPc (CREB) con actividad anti-inflamatoria.

1.2. Importancia del complejo NF-κB-IκBα en la inflamación

Se han identificado cinco genes NF-κB/Rel denominados *NF-κB1*, *NF-κB2*, *RelA*, *RelB* y *c-Rel*, que producen siete proteínas diferentes, NF-κB1 (p50 y su precursor p105), NF-κB2 (p52 y su precursor p100), RelA (p65), RelB y c-Rel. Estas proteínas se caracterizan por poseer una secuencia de 300 aminoácidos en la región amino-terminal llamada dominio homólogo a Rel (RHD) y contenida en esta región se encuentra una secuencia de localización nuclear (NLS) que posibilita la translocación de estas proteínas al núcleo. El dominio RHD posibilita además la unión específica al DNA, la formación de homo- o heterodímeros y la interacción con las proteínas inhibidoras κB (IκB) de NF-κB/Rel. El heterodímero que con mayor frecuencia se detecta en una amplia variedad de tipos celulares y por lo consiguiente el más estudiado hasta ahora es el que está constituido por p50 y RelA o p65 [5].

En condiciones basales, NF-κB se encuentra anclado en el citosol por proteínas de la familia IκB [5]. Cuando la célula detecta un estímulo (p. ej. PAMPs), IκBα se fosforila por la cinasa de Iκ (IKK); una vez que IκBα se fosforila, el paso siguiente es su poliubiquitinación, catalizada por una ubiquitín ligasa tipo 3. Esto conduce a la degradación de IκBα por el proteosoma 26S. La degradación de IκBα expone la secuencia de localización nuclear (NLS) de NF-κB, que permite la translocación de este factor de transcripción al núcleo [5-7]. La activación de NF-κB involucra la fosforilación de diversos residuos en el dominio de transactivación de la subunidad p65, entre los cuales Ser536 está bien caracterizado. Una vez en el núcleo, NF-κB puede interaccionar con la proteína de unión a CREB (CBP), activando con ello la transcripción de genes pro-inflamatorios, [8-10].

1.3. Importancia de CREB en el control de la inflamación

La contra-parte de NF-κB es CREB, el cual es un factor de transcripción involucrado en proliferación, diferenciación, sobrevivencia celular, plasticidad sináptica, apoptosis, y recientemente se ha vinculado en la respuesta inmune e inflamación [11-13]. La familia de factores transcripcionales CREB está compuesta por 3 miembros: el elemento modulador de la respuesta al AMPc (CREM), el factor de transcripción activador 1 (ATF-1) y CREB. Los tres se caracterizan por poseer un dominio inducible de cinasa (KID), un dominio de activación rico en glutamina que interacciona con el factor transcripcional TAFII130/135, que es suficiente para reclutar el complejo de RNA polimerasa II [14-16]. También posee un dominio zipper de leucina (bZIP) que permite la unión al DNA y su asociación con coactivadores, y la homo/hetero-dimerización entre los miembros de la familia CREB [16].

El mecanismo de regulación diferencial de la expresión de citocinas con actividad anti-inflamatoria depende de la fosforilación de CREB en Ser133 y de su unión a CBP, que establece de manera competitiva con NF-κB. El incremento de la abundancia relativa de fosfo-CREB-Ser133 le permite a este factor competir con NF-κB por la región de interacción de CBP [17]. Mientras que la interacción de NF-κB-CBP resulta en un aumento en la expresión de citocinas y enzimas con actividad pro-inflamatoria como interleucina (IL)-1, IL-6, IL-12, factor de necrosis tumoral alfa (TNFα), IL-8 y ciclooxigenasa 2 (COX-2) [5,18, 19], la interacción entre CREB y CBP produce un aumento de la expresión de citocinas anti-inflamatorias como IL-4, IL-10 e IL-13 [18-22] (ver Figura 2 en el artículo de divulgación). De lo anterior se deduce que la expresión de citocinas pro- y anti-inflamatorias requiere de un fino equilibrio entre los factores transcripcionales NF-κB y CREB, que involucra la activación de diversas vías de señalización como la constituida por las enzimas fosfatidilinositol 3-cinasa (PI3K), Akt (PKB) y glucógeno sintasa cinasa 3 (GSK3), proteína cinasas activadas por mitógeno (MAPKs), proteína cinasa activada por estrés y mitógeno (MSKs) e IκB [17, 23].

1.4. Activación de la inflamación por estimulación con LPS

Las bacterias Gram negativas (G-) causan choque séptico, que se caracteriza por producir una respuesta inflamatoria generalizada, daño a los tejidos y finalmente fallo en los órganos. El lipopolisacárido (LPS) es ampliamente reconocido como el principal componente estructural de la pared externa de todas las bacterias G- y un potente activador del sistema inmune. LPS es reconocido por TLR4, el cual interaccciona con tres proteínas extracelulares diferentes: la proteína de unión a LPS (LBP), CD14 y la proteína de diferenciación mieloide 2 (MD-2). Esta compleja interacción conduce a la activación de NF-κB y a la producción de citocinas pro-inflamatorias, incluyendo el factor de necrosis tumoral alfa TNFα, diversas interleucinas como IL-1β, IL-6, IL-8, la enzima ciclooxigenasa-2 (COX-2), óxido nítrico (NO) y especies reactivas de oxígeno (ERO) en células epiteliales, neutrófilos, linfocitos y macrófagos [24, 25]. Después de la etapa de inicio y mantenimiento de la respuesta inflamatoria que logra eliminar al agente causal, las células echan a andar un programa genético para controlar y, en última instancia, detener los mecanismos pro-inflamatorios. El estudio de la regulación de genes y de la actividad de enzimas que intervienen para detener la respuesta inflamatoria y devolver la homeostasis a los tejidos afectados es muy importante porque un control inapropiado de la inflamación puede originar padecimientos crónicos y degenerativos. El interés, por lo tanto, se ha enfocado en explicar los mecanismos por el que las células participantes en la respuesta inflamatoria inicial pueden expresar citocinas pro-inflamatorias y más tarde apagar esta expresión e inducir la síntesis de citocinas anti-inflamatorias, controlando con ello la inflamación. De este conocimiento se espera poder diseñar alternativas terapéuticas contra la inflamación causada por infección, lo que ha aumentado notablemente la búsqueda e identificación de moléculas que puedan servir como blancos terapéuticos para el diseño de nuevos fármacos.

A continuación, se describirán brevemente algunas de estas moléculas que forman parte de vías de transducción de señales ampliamente estudiadas y algunas de ellas analizadas experimentalmente en este trabajo.

1.5. Regulación de la inflamación por la vía PI3K-Akt-GSK3

La activación de la vía PI3K-Akt se produce por la interacción de PAMPs (i.e. péptidoglucano, PGN; ácido lipoteicoico, ALT; lipopolisacárido, LPS) con diferentes TLR. Esta interacción promueve la fosforilación de residuos de Tyr en el dominio citosólico de los receptores, lo que genera sitios que son reconocidos por el dominio SH2 de la subunidad reguladora de PI3K p85 e inducen a la subunidad catalítica p110 a producir fosfatidilinositol-3,4,5-trisfosfato (PIP_3). La presencia de PIP_3 en la monocapa interna de la membrana plasmática causa el reclutamiento de Akt. La interacción del dominio homólogo de plekstrina (PH) de Akt con PIP_3 cambia la conformación de Akt y expone sus sitios de fosforilación Thr308 y Ser473, los cuales son fosforilados por PDK1 y PDK2, respectivamente. Una vez activada, Akt fosforila una diversa gama de sustratos, entre los cuales se encuentra GSK3, modulando así una variedad de procesos celulares como apoptosis, proliferación y sobrevivencia celular, e inflamación (Figura 1) [26, 27].

1.6. GSK3 modula la expresión de citocinas con actividad pro- y anti-inflamatoria

GSK3 es una enzima constitutivamente activa muy importante en múltiples procesos biológicos por ser el punto de convergencia de varias vías de señalización [28]. Su secuencia de amino ácidos está altamente conservada en eucariotes y se han caracterizado dos isoformas, α y β, que aparentemente no son redundantes debido a que se ha demostrado que en ciertos procesos GSK3α no puede compensar la pérdida de GSK3β [29]. La actividad de GSK3 está controlada por diferentes mecanismos. Por ejemplo, su actividad puede ser inhibida por la fosforilación de residuos de Ser en la región N-terminal (Ser21 en GSK3 α y Ser9 en GSK3β). Se ha reportado que en monocitos estimulados con LPS la inhibición de GSK3β, pero no de GSK3α, redujo los niveles de las citocinas pro-inflamatorias y aumentó la expresión de la citocina anti-inflamatoria IL-10. El mecanismo propuesto consiste en que la inhibición de GSK3 regula diferencialmente la asociación de NF-κB p65 y CREB con el co-activador nuclear CBP. Es decir, GSK3 en estado inactivo causó un aumento de la actividad de CREB, mientras que redujo apreciablemente

la actividad de NF-κB p65 (Figura 1) [23]. Este mecanismo explica cómo una misma célula puede expresar citocinas pro-inflamatorias o anti-inflamatorias, dependiendo de las concentraciones relativas de NF-B p65 y CREB activados que compiten en el núcleo por la cantidad limitada del co-activador transcripcional CBP.

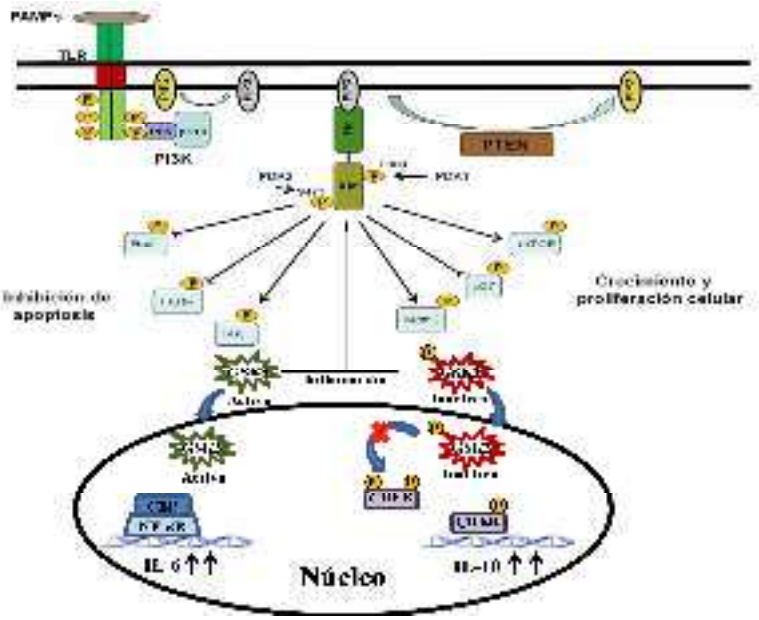

Figura 1. La vía de transducción PI3K-Akt-GSK3 regula diversos procesos celulares, entre ellos la inflamación, por medio de la síntesis de IL-10 e IL-6. La activación de TLR por PAMPs resulta en la auto-fosforilación de residuos tirosina de su dominio citosólico. PI3K es reclutado hacia la membrana para unirse a residuos de fosfotirosina del TLR o adaptadores, a través de uno o varios dominios SH2 en la subunidad adaptadora p85. Esto promueve la activación de la subunidad catalítica p110, que resulta en la producción del segundo mensajero fosfoinositol-3,4,5-trifosfato (PIP_3). La presencia de PIP3 en la membrana causa el reclutamiento de varias proteínas de señalización con dominios de homología a plekstrina (PH), incluyendo PDK1 y Akt. PTEN, es una fosfatasa de PIP_3, la cual regula negativamente la vía PI3K-Akt. Una vez activada, Akt media la activación o inhibición de diversos sustratos que intervienen en la sobrevivencia, crecimiento y proliferación celular e inflamación. GSK3 activa promueve la interacción de NF-κB con CBP y la expresión de IL-6**.** GSK3 inactiva no puede fosforilar a CREB, y como consecuencia este factor puede permanecer asociado al DNA, promoviendo la síntesis de IL-10 (Modificado de Fresno-Vara *et al*., 2004; Beurel *et al*., 2009).

2. IMPORTANCIA DE OTRAS MOLÉCULAS EN LA RESPUESTA INFLAMATORIA

2.1. Proteínas cinasas activadas por mitógeno (MAPKs)

Las MAPKs más estudiadas son ERK1/2, Jun y p38. Las vías de señalización reguladas por MAPKs están involucradas en una gran diversidad de funciones, entre ellas apoptosis,

diferenciación, control celular, e inflamación [30-32]. La unión del ligando al receptor inicia la fosforilación una cascada de cinasas, en la cual la MAPK cinasa cinasa (MAPKKK) activa a la MAPK cinasa (MAPKK) y esta a su vez activa a la MAPK. La activación requiere de la fosforilación dual del motivo Thr-X-Tyr (donde X puede ser Gly, Pro, o Glu) en la región regulatoria, que produce un cambio conformacional y expone el sitio activo que le permite interactuar con su sustrato [33]. Las MAPKs regulan la producción de TNF-α, IL-β, IL-6, COX-2 e iNOS [31, 34, 35].

2.1.1. La cinasa p38

Se han identificado cuatro isoformas de p38: p38α, p38β, p38γ y p38δ, que se caracterizan por compartir un 60% de identidad y son codificadas por genes distintos [35]. En un estado activo, p38 fosforila a MK2, MK3 y a la proteína cinasa activada por estrés y por mitógeno 1/2 (MSK1/2). La activación de estas proteína cinasas promueve la activación de ATF-2, STAT1 y CREB, lo que regula la expresión de genes pro-inflamatorios [36, 37]. Además, se ha reportado que p38 modula la respuesta inflamatoria a nivel post-transcripcional, al alterar la estabilidad del RNA mensajero (RNAm) de algunas citocinas [38].

2.1.2. Las cinasas reguladas por señales extracelulares 1 y 2 (ERK1/2)

Las isoformas ERK1 y ERK2 tienen un 83% de identidad y se expresan en todos los tejidos [39]. Se ha reportado que ERK1/2 son necesarias para desarrollar una respuesta inmune apropiada y durante la inflamación son esenciales en mediar la respuesta inmune antígeno-anticuerpo, al regular la activación de células T, así como inducir la producción de anticuerpos en células B [40]. Además, estas MAPK incrementan la producción de algunas citocinas pro-inflamatorias como IL-1, IL-6 y TNF-α [41]. La activación de NF-κB, ERK1/2 y p38 está mediada por la proteína adaptadora MyD88 acoplada a TLR4 [42]. La

activación de CREB por fosforilación de la Ser133 inducida por LPS y TNF-α está mediada por ERK1/2 y p38. Esta fosforilación no se realiza de manera directa, sino que requiere de MSK1 o MSK2, que son importantes para la expresión de genes dependientes de CREB [43, 44].

3. LAS PROTEÍNAS CINASAS ACTIVADAS POR ESTRÉS Y MITÓGENOS 1 Y 2 (MSK1/2)

Se han identificado dos isoformas de MSK en mamíferos, MSK1 y MSK2, que se caracterizan por poseer dos dominios de cinasa. El uso de inhibidores de la vía de señalización MAPK demostró que ERK1/2 y p38α son importantes en la activación de CREB mediada por MSK1/2 [45, 46]. De manera directa, en fibroblastos de ratones knock-out de MSK1/2 se observó una disminución de la abundancia relativa de fosfo-CREB-Ser133 [46]. Esta vía de señalización es importante para controlar la inflamación porque CREB es un factor de transcripción que induce la expresión de IL-10, una citocina de naturaleza anti-inflamatoria que tiene un papel clave en la retroalimentación que limita la inflamación y previene el daño de tejidos [47]. Un dato interesante es que ERK1/2 y p38 promueven la expresión de tris-tetraprolina, que induce la degradación del ARNm de TNF-α [48].

4. PÉPTIDOS REGULADORES DE LA DEFENSA INNATA

La actividad inmunomoduladora de los péptidos naturales de defensa producidos por el hospedero durante la respuesta inflamatoria inicial ha sido de gran interés científico y tecnológico [49]. La búsqueda de fármacos de tipo químico o peptídico, capaces de modular la respuesta inmune innata y con ello potenciar la capacidad de las células para eliminar a las bacterias causales de una infección, es una de las alternativas que recientemente se ha planteado la industria farmacéutica.

Esfuerzos notables se han hecho para producir en el laboratorio algunos péptidos que imiten la actividad de los péptidos naturales. Sin embargo, esto ha sido problemático

debido a que los péptidos sintéticos frecuentemente son tóxicos o causan apoptosis [50-52]. Una excepción notable son los péptidos catiónicos lineales denominados IDR (*Innate Defense Regulator*), una clase de péptidos que limitan la infección bacteriana al modular selectivamente la inmunidad innata, promoviendo la eliminación de las bacterias patógenas y controlando al mismo tiempo la reacción inflamatoria [53].

Un miembro de esta familia de péptidos es IDR-1002. La secuencia de este péptido de 12 aminoácidos se derivó de la bactenecina. Se ha demostrado que este péptido no es citotóxico para neutrófilos humanos y para monocitos de sangre periférica (PBMC) en concentraciones de hasta 100 y 200 μg/ml. La ausencia de citotoxicidad de IDR-1002 contrasta con la catelecidina LL-37, que muestra citotoxicidad a una concentración de 25μg/ml. IDR-1002 modula selectivamente el sistema inmune y elimina eficazmente a *Staphylococcus aureus* y *E. coli* de los tejidos. Además, es un péptido que induce un aumento en la expresión del antagonista del receptor a IL-1 (IL-Ra) e IL-10, con lo cual coadyuva a controlar efectivamente la inflamación [54]. Estudios realizados en PBMCs de humano reportan que IDR-1002 incrementa los niveles de las proteínas quimioatrayentes de monocitos 1 y 3 (MCP-1, MCP-3), la quimiocina CXCL GRO-α e IL-8, lo que produce un incremento en el número de neutrófilos y monocitos en el sitio de infección [54]. Se ha reportado que IDR-1002 suprime la respuesta inflamatoria en fibroblastos sinoviales [55].

A pesar de los reportes que confirman la actividad anti-inflamatoria de IDR-1002 en varios tipos celulares, no se conoce en detalle el mecanismo por el cual este péptido regula la respuesta inflamatoria. En este trabajo de tesis se presentan evidencias experimentales que demuestran que IDR-1002 inhibe la expresión de TNF-α y COX-2 por su actividad inhibidora de la translocación al núcleo de NF-κB. Además, este péptido indujo la activación de CREB, dependiente de la activación de las proteínas cinasas p38-ERK1/2-MSK1.

5. JUSTIFICACIÓN

IDR-1002 es un péptido que regula la expresión de citocinas involucradas en la inflamación y, aunque hay múltiples trabajos que reportan los efectos benéficos de IDR-1002, el mecanismo molecular inducido por este péptido no ha sido estudiado en detalle. El conocimiento que se obtenga podrá entonces ser usado para diseñar nuevos péptidos con una potencia mayor como anti-inflamatorios. Esto permitiría diseñar biofármacos peptídicos de nueva generación menos citotóxicos y más efectivos a bajas dosis.

6. HIPÓTESIS

El péptido IDR-1002 disminuye la expresión de citocinas pro-inflamatorias y aumenta la expresión de citocinas anti-inflamatorias por un mecanismo dependiente de los factores de transcripción NF-κB y CREB.

7. OBJETIVOS

7.1. Objetivo general

Evaluar en macrófagos estimulados con LPS e IDR-1002 la expresión de citocinas pro-y anti-inflamatorias y su relación con la actividad de NF-κB y CREB.

7.2. Objetivos específicos.

Evaluar el efecto en macrófagos Raw264.7 de IDR-1002 en la expresión de citocinas involucradas en la inflamación.

Determinar la actividad de NF-κB y CREB en macrófagos estimulados con LPS e IDR-1002.

Analizar la expresión de genes dependientes de NF-κB (TNF-α y COX-2) y de CREB (IL-4, IL-10 e IL-13) en macrófagos Raw264.7 estimulados con LPS e IDR-1002.

8. MÉTODOS, RESULTADOS Y DISCUSIÓN

En esta sección se adjuntan los artículos internacionales publicados en revistas indizadas en JCR, que contienen la metodología usada en los experimentos, así como también la discusión correspondiente.

8.1. ARTÍCULO 1: ORIGINAL INTERNACIONAL.

PEPTIDE IDR-1002 INHIBITS NF-κB NUCLEAR TRANSLOCATION BY INHIBITION OF IκBα DEGRADATION AND ACTIVATES p38/ERK1/2-MSK1-DEPENDENT CREB PHOSPHORYLATION IN MACROPHAGES STIMULATED WITH LIPOPOLYSACCHARIDE.

Primer Autor: Alejandro Huante Mendoza.

Este trabajo de investigación publicado en la revista Frontiers in Immunology responde a la hipótesis y los objetivos propuestos en el proyecto. El estudio describe el mecanismo por el cual IDR-1002 inhibe la expresión de TNF-α y COX-2, la fosforilación de IκBα, y la translocación al núcleo de NF-κB en macrófagos estimulados con LPS. Observamos también que el péptido IDR-1002 aumentó la fosforilación de CREB en Ser133, por un mecanismo que involucró la activación de p38/ERK1/2 y MSK1. IDR-1002 inhibió la formación de los complejos CBP-NF-κB y CBP-CREB, lo que explicaría su capacidad para inhibir la expresión de TNF-α/COX-2 y la expresión de citocinas anti-inflamatorias como IL-4, IL-10 e IL-13. Los resultados sugieren que IDR-1002 podría funcionar como un biofármaco coadyuvante para controlar la inflamación.

Mi participación en este trabajo fue obtener la mayoría de los resultados y escribir el primer borrador.

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Peptide IDR-1002 Inhibits NF-κB Nuclear Translocation by Inhibition of IκBα Degradation and Activates p38/ERK1/2–MSK1-Dependent CREB Phosphorylation in Macrophages Stimulated with Lipopolysaccharide

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The inflammatory response is a critical molecular defense mechanism of the innate immune system that mediates the elimination of disease-causing bacteria. Repair of the damaged tissue, and the reestablishment of homeostasis, must be accomplished after elimination of the pathogen. The innate defense regulators (IDRs) are short cationic peptides that mimic natural host defense peptides and are effective in eliminating pathogens by enhancing the activity of the immune system while controlling the inflammatory response. Although the role of different IDRs as modulators of inflammation has been reported, there have been only limited studies of the signaling molecules regulated by this type of peptide. The present study investigated the effect of IDR-1002 on nuclear factor κB (NF-κB) and cAMP-response element-binding protein (CREB) transcription factors that are responsible for triggering and controlling inflammation, respectively, in macrophages. We found that TNF-α and COX-2 expression, IκBα phosphorylation, and NF-κB nuclear translocation were strongly inhibited in macrophages pre-incubated with IDR-1002 and then stimulated with lipopolysaccharide (LPS). IDR-1002 also increased CREB phosphorylation at Ser133 *via* activation of the p38/ERK1/2–MSK1 signaling pathways without detectable expression of the cytokines IL-4, IL-10, and IL-13 involved is suppressing inflammation or alternative activation. Transcriptional activation of NF-κB and CREB is known to require interaction with the transcriptional coactivator CREB-binding protein (CBP). To test for CBP–NF-κB and CBP–CREB complex formation, we performed co-immunoprecipitation assays. These assays showed that IDR-1002 inhibited the interaction between CBP and NF-κB in macrophages stimulated with LPS, which might explain the inhibition of TNF-α and COX-2 expression. Furthermore, the complex between CBP and CREB in macrophages stimulated with IDR-1002 was also inhibited, which might explain why IDR-1002 did not lead to expression of IL-4, IL-10,

and IL-13, even though it induced an increase in phospho-CREB relative abundance. In conclusion, our results indicated that IDR-1002 has a dual effect. On one hand, it inhibited NF-κB nuclear translocation through a mechanism that involved inhibition of IκBα phosphorylation, and on the other, it activated a protein kinase signaling cascade that phosphorylated CREB to selectively influence cytokine gene expression. Based on these results, we think IDR-1002 could be a potential good biopharmaceutical candidate to control inflammation.

Keywords: IDR, peptides, inflammation, macrophages, IκBα, NF-κB, CREB, TNF-α

INTRODUCTION

The inflammatory response is one of the main defense mechanisms of the innate immune system that is activated by host infection with microbial pathogens or tissue damage. This response is fundamental to identify and neutralize the causative agent and restore tissue homeostasis (1). However, in some cases, inflammation can be detrimental, especially when the control or resolution mechanisms fail, leading to chronic inflammatory conditions such as arteriosclerosis, insulin resistance, rheumatoid arthritis, and Alzheimer's disease (2).

The balance between the inflammatory response and its resolution is maintained in part by the activity of two transcription factors with opposite roles, the nuclear factor κB (NF-κB) and the cAMP-response element-binding protein (CREB). Activation of NF-κB induces expression of pro-inflammatory genes (e.g., TNF-α, IL-6, IL-8, and COX-2), while activation of CREB leads to gene expression of cytokines that have an anti-inflammatory role (e.g., IL-4, IL-10, and IL-13) (3, 4). NF-κB has five subunits but the heterodimer of NF-κB, constituted of p65 and p50 subunits, is one of the most studied active forms of this family of transcription factors (5, 6). When cells are not stimulated, NF-κB is anchored in the cytosol to IκBα protein. After stimulation, IκBα becomes phosphorylated, ubiquitinated, and degraded by the 26S proteosome. Degradation of IκBα releases NF-κB that is translocated to the nucleus where it initiates a complex transcriptional response (7). Once in the nucleus NF-κB can interact with a transcriptional coactivator CREB-binding protein (CBP) and initiate a distinct transcriptional pattern. Activation of NF-κB can also involve phosphorylation of several residues in the transactivation domain of p65 subunit, among which Ser536 is well characterized (8–10).

cAMP-response element-binding protein is a cellular transcription factor that belongs to the basic leucine-zipper domain family of proteins (11). This family is formed by dozens of proteins, including CREB, the cAMP-response element modulator (CREM), and the activating transcription factor-1 (ATF-1). In cells activated by growth factors, hormones, lipopolysaccharide (LPS), and other stimuli (12, 13), CREB is phosphorylated at Ser133 by protein kinase A, protein kinase B (Akt), MSK, or other enzymes (14, 15). Phosphorylation at this residue promotes CREB binding to transcriptional coactivator CBP, which can lead to displacement of NF-κB from the same interaction domain on CBP (16, 17). Formation of CREB–CBP complex promotes the expression of cytokines, typically involved in anti-inflammatory events or alternative activation of monocytes, thereby suppressing expression of pro-inflammatory cytokines activated by the NF-κB–CBP complex (18). Although this mechanism has been widely proposed (19–22), other groups have reported no expression of genes when CREB is phosphorylated at Ser133 downstream of mitogen-activated protein kinase (MAPK) pathways (15, 23, 24). It may imply that control of inflammation by CREB phosphorylation does not necessarily involve the expression of anti-inflammatory genes induced by this transcription factor.

Innate defense regulator (IDR) peptides were designed from the natural theme of cationic host defense peptides (25) as an alternative to traditional anti-inflammatory and antimicrobial drugs. The immunomodulatory effects of IDRs have attracted the attention because of the worldwide problem of multiple antibiotic resistance in bacteria (26–28). In addition to downregulation of the pro-inflammatory response to bacterial ligands, IDRs stimulate protective immunity (enhancing cellular recruitment and promoting differentiation of immune cells) leading to lowering of the bacterial burden without any direct interaction with bacteria (26–28). In addition to their anti-infective and anti-inflammatory effects due to modulation of the immune response, these peptides may also be considered good potential therapeutic candidates to combat chronic inflammation associated with autoimmune diseases (29, 30). One of these peptides, IDR-1002, efficiently enhanced the immune response to eliminate *Staphylococcus aureus* and *Escherichia coli* from infected tissues, even when these bacterial pathogens were multi-resistant to antibiotics (26, 27). Moreover, in synovial fibroblasts, IDR-1002 effectively controlled inflammation by selectively modulating the expression of interleukin (IL)-1Ra and IL-10 and reducing NF-κB p50 nuclear translocation induced by IL-1β (29).

Although several studies have previously reported the potential beneficial effects of IDR-1002, the molecular transduction mechanisms involved in the IDR-1002 activity have not been well described to date (26, 27, 31, 32). Therefore, we focused our attention on the NF-κB and CREB transcription factors, because they are important in triggering and resolving the inflammatory response, respectively. We found that, in RAW264.7 macrophages, stimulated with LPS IDR-1002 was able to inhibit NF-κB nuclear translocation by inhibiting IκBα degradation, leading to a reduction of cycloooxygenase-2 (COX-2), and TNF-α expression. Co-immunoprecipitation analysis of CBP–NF-κB molecular complex showed that IDR-1002 inhibited the

interaction between CBP and NF-κB induced by LPS. We also observed that IDR-1002 induced high levels of CREB phosphorylation at Ser133 through activation of p38/ERK1/2–MSK1/2 signaling pathways. Interestingly, we could not detect expression of the anti-inflammatory cytokines IL-4, IL-10, and IL-13, which are known to be regulated by CREB. By applying a similar co-immunoprecipitation analysis, the molecular complex between CBP and CREB was not detected in macrophages stimulated with IDR-1002, which may explain the lack of anti-inflammatory gene expression.

MATERIALS AND METHODS

Media and Chemicals

Innate defense regulator peptides (IDR) 1002 (VQRWLIVWRIRK-NH_2), HH2 (VQLRIRVAVIRA-NH_2), and 1 (KSRIVPAIPVSLL-NH_2) were synthesized using solid phase F-moc chemistry by CPC Scientific, Inc. (Sunnyvale, CA, USA) and were >95% pure. RPMI 1640 cell culture medium, Bradford reagent, bovine serum albumin (BSA), Wortmannin (inhibitor of PI3K), Akt IV (inhibitor of Akt), Rapamycin (inhibitor of p70S6K), SB216763 (inhibitor of GSK3), PD98059 (inhibitor of ERK1/2), and Ro-318220 (inhibitor of MSK1) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). SB203580 (inhibitor of p38) and SP600125 (inhibitor of JNK) were purchased from Calbiochem (San Diego, CA, USA). Lymphoprep™ was purchased from Axis-Shield PoC AS (Oslo, Norway). Upon arrival, all inhibitors were solubilized in DMSO and stored in aliquots at −20°C. Newborn calf serum was acquired from Hyclone Laboratories, Inc. (South Logan, UT, USA). Penicillin G and streptomycin were purchased from Gibco-BRL (Gaithesburg, MD, USA). EDTA-free protease inhibitor cocktail (50×) was purchased from Roche Applied Science (Manheim, Germany). Antibodies against β-actin (Sc-47778), COX-2 (Sc-1747), GAPDH (Sc-25778), laminin A/C (6215), IκBα (Sc-203), and phospho-IκBα (Sc-52943) for the detection of phosphorylated Ser32 and Ser36, CREB-1 (Sc-186), NF-κB p65 (Sc-8008), CBP (Sc-7300), anti-rabbit IgG-HRP (Sc-2004), rabbit anti-goat IgG-HRP (Sc-2768), and goat anti-mouse IgG-HRP (Sc-2031) were acquired from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against the phosphorylated forms of CREB at Ser133 (9196), p38 at Thr180/Tyr182 (4511), NF-κB p65 at Ser536 (3033), GSK3α at Ser21 (9316), and GSK3β at Ser9 (9336) were purchased from Cell Signaling Technology (Boston, MA, USA). Antibodies to detect phosphorylated MSK1 at Thr581 (AF2518) were purchased from R&D Systems (Minneapolis, MN, USA). Antibodies to detect the phosphorylated forms of IKKα and β at Thr23 were obtained from Santa Cruz Biotechnology (Cat. Sc-21660, Santa Cruz, CA, USA). Non-fat dry milk was acquired from Bio-Rad (California, CA, USA) and Luminol reagent from Millipore (Billerica, MA, USA).

Cell Line and Culture Conditions

The mouse leukemic monocyte macrophage cell line RAW 264.7 used was obtained from the American Type Culture Collection (ATCC). This immortalized cell line was grown and maintained in RPMI medium supplemented with 10% FCS, unless otherwise noted.

Isolation of Human Peripheral Blood Mononuclear Cells

Venous blood (20 ml) from healthy human donors was collected in tubes containing EDTA in accordance with the approval and ethical guidelines of the Universidad Michoacana de San Nicolás de Hidalgo. Blood was diluted in the same volume of RPMI medium. Then, 20 ml of diluted blood was carefully added to a tube containing 10 ml of Lymphoprep™ and centrifuged at 800 × *g* for 20 min at room temperature (23°C). The phase containing peripheral blood mononuclear cells (PBMC) was separated and washed 3× with enough PBS to complete 50 ml for each wash and centrifuged at 250 × *g* for 10 min. After washing, PBMC were resuspended in RPMI medium and seeded in six-well plate at 2×10^6 cells/well. Cells were left resting for at least 3 h before the experiments.

Gene Knockdown of MSK1 and MSK2

MSK1 and MSK2 gene expression were silenced with siRNA acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The siRNA for MSK1 (sc-35978), MSK2 (sc-75837), and control siRNA (sc-37007) were added to macrophages at the final concentration of 40 nM for 48 h, according to the manufacturer's instructions. To test for MSK1/MSK2 participation in CREB phosphorylation at Ser133, macrophages were first gene silenced for MSK1 and/or MSK2 and then stimulated with 20 μg/ml of IDR-1002 for 15 min.

Protein Extraction and Western Blot Analysis

The relative abundance of phosphorylated and non-phosphorylated proteins was evaluated in protein extracts from macrophages grown in six-well culture plates to ~90% confluence before serum starvation for at least 4 h. For each assay, total protein (cytosolic plus nuclear from control and treated cells) was obtained by washing cells with cold PBS (2×) and lysing them with 80 μl of a cold lysis buffer containing 20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Igepal CA-930, 10 mM Na-pyrophosphate, and 50 mM NaF, supplemented with 1 mM Na-orthovanadate and 1× protease inhibitor cocktail that was added immediately before use. The lysates were centrifuged at 13,000 × *g* for 20 min at 4°C, and the supernatant was transferred to ice-cold Eppendorf tubes. Nuclear and cytoplasmic fractions were prepared from cells using the NE-PER Reagent kit from Pierce (Rockford, IL, USA), and stored in aliquots at −80°C. Protein concentration was measured by the Bradford method (33) using BSA as standard. Then, 40 μg of protein was separated by electrophoresis in 10% sodium dodecyl sulfate polyacrylamide gels and electroblotted in a wet chamber to 0.45 μm nitrocellulose membrane (Bio-Rad) at 250–300 mA for 1 h. Membranes were then probed with the indicated antibody and the abundance of the phosphorylated and unphosphorylated forms of each protein was detected using the Immobilon Western Chemiluminescent HRP substrate kit from

Millipore (Billerica, MA, USA). Membranes were exposed to an X-ray film (Kodak) with two intensifying screens (DuPont) at room temperature (23°C).

Co-Immunoprecipitation Assays

Macrophages Raw 264.7 were seeded in 10-cm cell plate culture until confluence, left in serum-free media for 4 h, and then stimulated as indicated. For immunoprecipitation technique, we use the kit Pierce™ Protein A/G Magnetic Beads (Thermo Fischer Scientific). In brief, ~1×10^7 cells were lysed in washing/lysis buffer supplemented with 1× protease inhibitor cocktail, and cells debris were collected by centrifugation at 18,000 × *g* for 10 min at 4°C. The supernatant was collected and quantified for protein concentration. Then, 4 µg of each immunoprecipitation antibody (CBP or IκBα) was added to 500 µg of protein extract and the volume adjusted to 500 µl. The mixture was incubated with continuous agitation all night at 4°C. Next day, 25 µg of magnetic beads were added and incubated 1 h in agitation at room temperature (23°C); beads were washed 2× with washing buffer and 1× with water. The target antigen was eluted with lane marker buffer 1× plus 50 mM DTT in the final volume of 100 µl. For immunoblots, 20 µl of target antigen in elution buffer was used and detected as described in the western blot analysis section.

Cytokine Quantitation

Mouse cytokines, IL-4, IL-6, IL-10, IL-13, TNF-α, and PGE2 levels, were measured by ELISA, according to the manufacturer's instructions (R&D, Minneapolis, MN, USA).

Cytokine Array

Macrophages were incubated for 1 h with 20 µg/ml of IDR-1, IDR-HH2, or IDR-1002 and then stimulated for 2–24 h with 10 ng/ml of LPS. Supernatants were collected and cytokines were detected using a mouse cytokine antibody pair-based array spotted on a membrane (Abcam, ab133993) according to the manufacturer's instructions (Cambridge, UK). Briefly, analysis of cytokines present in the macrophages supernatant is carried out by chemiluminescent western blot assay, using biotinylated detector antibodies and streptavidin HRP. Targets of this ELISA-like array are granulocyte-colony stimulating factor (GCSF), granulocyte-macrophage colony stimulating factor (GM-CSF), IL-2, 3, 4, 5, 6, 9, 10, 12 p40/p70, 12p70, 13, 17, interferon gamma (IFN-γ), monocyte chemoattractant protein 1 and 5 (MCP-1 and MCP-5), regulated on activation normal T cell expressed and secreted (RANTES), stem cell factor (SCF), soluble tumor necrosis receptor factor 1 (sTNFR1), tumor necrosis factor alpha (TNF-α), thrombopoietin, and vascular endothelial growth factor (VEGF).

Statistical Analysis

Statistical significance was evaluated using Student's paired *t*-test using the SIGMASTAT program version 3.0 (SPSS Inc., Chicago, IL, USA). Band densitometric analysis was performed using the Image Processing and Analysis module in Java Program ImageJ (http://rsbweb.nih.gov/ij).

RESULTS

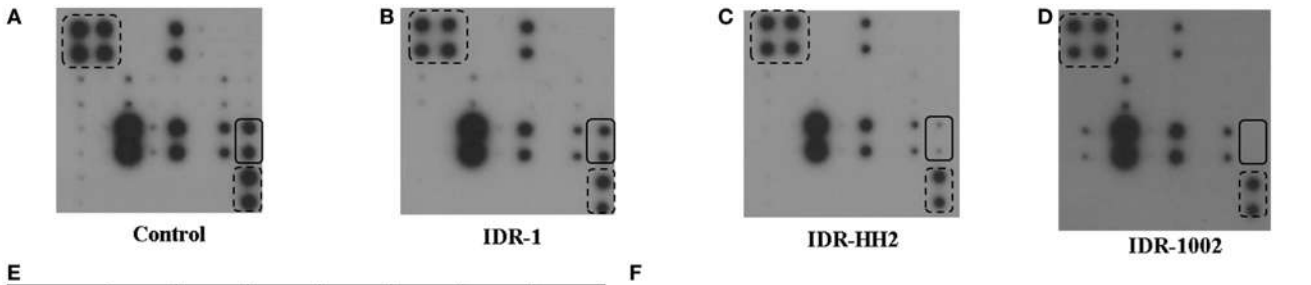
IDR-1002 Reduced the LPS-Induced Expression of TNF-α and COX-2

To explore the effect of IDR-1, IDR-HH2, and IDR-1002 on cytokine expression, we used an antibody array of 22 cytokines. After 12 h of incubation with each of the IDR peptides, we evaluated the level of cytokine expression in the supernatants. Compared to control (**Figure 1A**), IDR-1 had a modest effect on TNF-α expression (**Figure 1B**). However, when macrophages were treated with IDR-HH2 or IDR-1002, the basal levels of TNF-α were significantly reduced. We observed that IDR-1002 was a somewhat stronger inhibitor than IDR-HH2 (**Figures 1C,D**). A map of the cytokine array is shown in **Figure 1E**. To determine the kinetics of the effect of IDR-1002 on TNF-α production, macrophages were pre-incubated with IDR-1002 for 1 h and then stimulated, for periods of 2–24 h, with LPS from *E. coli*, an agonist that induced a strong release of TNF-α. We observed that production of TNF-α activated by LPS was strongly reduced in the presence of IDR-1002 (**Figure 1F**). These results indicate that IDR-1002 had an inhibitory effect on the basal and LPS-induced expression of TNF-α.

Another important gene expressed in the inflammatory response and induced by LPS is COX-2. This enzyme catalyzes the conversion of arachidonic acid into prostaglandins and thromboxane, playing an important role in the inflammatory response (34). To determine the effect of IDR-1002 on COX-2 production, macrophages were pre-incubated with IDR-1002 for 1 h and then stimulated with LPS for a period of 2–24 h. Under this condition, we observed that COX-2 expression was strongly and significantly reduced in the presence of IDR-1002 (**Figure 2A**). Incubation of macrophages with IDR-1002 alone for 2, 4, and 8 h did not have any effect on COX-2 (**Figure 2B**). These results indicate that IDR-1002 had an inhibitory effect on the LPS-induced expression of COX-2.

IDR-1002 Reduced the Phosphorylation of NF-κB p65 Subunit at Ser536 and Its Nuclear Translocation

Activation of NF-κB stimulates its nuclear translocation leading to the expression of many pro-inflammatory genes, among which TNF-α and COX-2 play important roles in the inflammatory response. To explore if IDR-1002 inhibited TNF-α and COX-2 expression by inhibiting NF-κB activity, we evaluated phosphorylation of the p65 subunit at Ser536 (a residue located in the transactivation domain), and its nuclear relative abundance. An ~20–40% reduction of p65 phosphorylation at Ser536 was observed when macrophages were incubated with IDR-1002 for 5, 30, or 60 min (**Figure 3A**). Next, we investigated the relative abundance of p65 in the cytoplasm and nucleus of macrophages incubated with LPS and IDR-1002. From data shown in **Figure 3B**, it was clear that LPS stimulated p65 nuclear translocation, which was inhibited by IDR-1002. The co-immunoprecipitation of NF-κB and coactivator CBP was detected exclusively in macrophages stimulated with LPS, but not in those stimulated with IDR-1002 followed by LPS or IDR-1002 alone (**Figure 3C**). These



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Pos	Pos	Neg	Neg	GCSF	GM-CSF	IL-2	IL-3
Pos	Pos	Neg	Neg	GCSF	GM-CSF	IL-2	IL-3
IL-4	IL-5	IL-6	IL-9	IL-10	IL-12 p40p70	IL-12p70	IL-13
IL-4	IL-5	IL-6	IL-9	IL-10	IL-12 p40p70	IL-12p70	IL-13
IL-17	IFN-γ	MCP-1	MCP-5	RANTES	SCF	sTNFRI	TNF-α
IL-17	IFN-γ	MCP-1	MCP-5	RANTES	SCF	sTNFRI	TNF-α
Trombopoietin	VEGF	BLANK	BLANK	BLANK	BLANK	BLANK	Pos
Trombopoietin	VEGF	BLANK	BLANK	BLANK	BLANK	BLANK	Pos

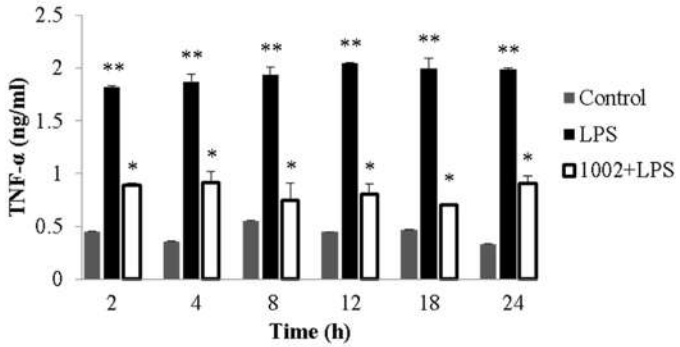

FIGURE 1 | **IDR peptides reduced the expression of TNF-α in RAW 264.7 macrophages**. Macrophages were left unstimulated (control) **(A)** or stimulated with 20 μg/ml of IDR-1 **(B)**, IDR-HH2 **(C)**, or IDR-1002 **(D)** for 12 h. Conditioned media were then collected and centrifuged at 18,800 × *g* for 10 min. The supernatants were transferred to ice-cold tubes and stored at −80°C. Cytokine expression was evaluated with an antibody base-paired array kit (Abcam ab133993), in which antibodies against 22 different cytokines were spotted on a membrane. **(E)** Cytokines array map. In each array, the position of TNF-α is indicated with continuous-line rectangles while positive controls are indicated with dashed-line rectangles. **(F)** Cells were left unstimulated (control), stimulated with 10 ng/ml LPS, or pretreated with 20 μg/ml IDR-1002 for 1 h followed by incubation with 10 ng/ml LPS for 2–24 h. Supernatants were collected and treated as described above. TNF-α quantitation was performed by ELISA (R&D), according to the manufacturer's instructions. Data represent means ± SEM ($n = 3$). $^{**}P < 0.05$ for LPS assays compared with the unstimulated control value. $^{*}P < 0.05$ for 1002 + LPS assays compared with LPS treatment.

results indicate that IDR-1002 reduced the relative abundance of p65 phosphorylated at Ser536 and its LPS-induced nuclear translocation and interaction with CBP.

IDR-1002 Inhibited the Phosphorylation and Degradation of IkBα Induced by LPS

In unstimulated cells, NF-κB is anchored to IκBα in the cytoplasm. Release of NF-κB requires phosphorylation of IκBα, which marks it to become ubiquitinated and then degraded in the proteosome. This mechanism implies that if phosphorylation of IκBα is inhibited, then NF-κB would not be released and its nuclear translocation would not occur. Our data indicate that IDR-1002 strongly inhibited the LPS-induced phosphorylation of IκBα (**Figure 4A**) and its degradation (**Figure 4B**). This effect of IDR-1002 on IκBα seemed to be independent of the stimulus because inhibition of IκBα phosphorylation and degradation by IDR-1002 was equally effective in macrophages stimulated with TNF-α or IL-1β (**Figures 4A,B**). Interaction of NF-κB p65 and IκBα was stabilized as a consequence of IDR-1002 inhibition of IκBα phosphorylation and degradation. **Figure 4C** shows that there was an increase in co-immunoprecipitation NF-κB p65 bound to IκBα in macrophages stimulated with IDR-1002. These data may explain the inhibitory effect exerted by IDR-1002 on TNF-α and COX-2 expression.

Activation of CREB Phosphorylation at Ser133 by IDR-1002 Was Dependent on ERK1/2, p38, and MSK1 Activity

A major mechanism for controlling the inflammatory response is the suppression of pro-inflammatory cytokine expression, which can be promoted when NF-κB forms a molecular complex with CBP (16, 17). This process takes place during the inflammatory response when CREB is also activated by phosphorylation at Ser133. Phosphorylation of CREB enables it to compete with NF-κB for the same interaction domain on CBP (18). The consequence of NF-κB separation from CBP is a reduction in inflammatory cytokine expression and simultaneously an increment, in many but not all cases, of anti-inflammatory cytokine expression due to CREB activity. To explore if IDR-1002 activated CREB phosphorylation at Ser133, macrophages were incubated with IDR-1002 for 5– 60 min. Under these conditions, a significant increase in CREB phosphorylation was observed for up to 45 min of incubation with IDR-1002 (**Figure 5A**). Because CREB phosphorylation can be mediated by Akt (PKB), ERK1/2,

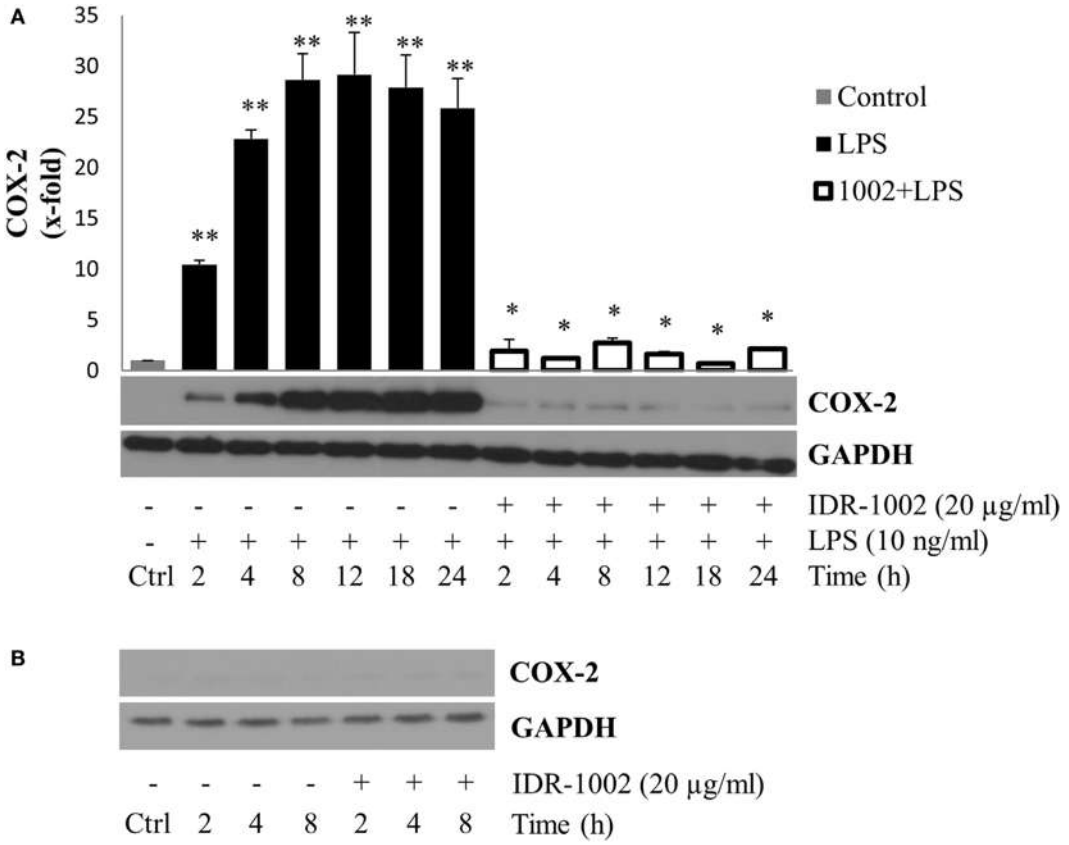

FIGURE 2 | **IDR-1002 inhibited the LPS-induced COX-2 expression in RAW 264.7 macrophages**. **(A)** Macrophages Raw 264.7 were left unstimulated (Ctrl), stimulated with 10 ng/ml of LPS, or pretreated with 20 μg/ml of IDR-1002 for 1 h followed by incubation with 10 ng/ml of LPS for 2–24 h. At the end of each incubation period, COX-2 was analyzed by Western blotting. Protein from Ctrl cells were collected at 4 h. A representative immunoblot of three independent experiments is shown. GAPDH detection was used as control to ensure equal protein loading. **(B)** At each indicated incubation time, COX-2 expression was measured in untreated control and IDR-1002-treated cells. Values were normalized with GAPDH as protein loading control. This graph is representative of two independent experiments. Data represent means ± SEM ($n = 3$). $^{**}P < 0.05$ for LPS compared with the unstimulated control value. $^{*}P < 0.05$ for 1002 + LPS compared with LPS treatment.

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p38, and MSK1/2, we performed experiments utilizing specific inhibitors for each one of these enzymes. Macrophages stimulated with IDR-1002 showed an increased relative abundance of CREB phosphorylated at Ser133 and this was reduced by specific inhibitors of p38 and ERK1/2 (**Figures 5B,C**). Correspondingly, IDR-1002 induced a strong phosphorylation of ERK1/2 and p38 (Figures S1A,B in Supplementary Material), as described previously (27). However, specific inhibitors of PI3K, GSK3, and Akt did not have any effect on CREB phosphorylation (Figures S2A,B in Supplementary Material).

Other authors have reported that ERK1/2 and p38 do not directly phosphorylate CREB but instead they first phosphorylate MSK1/2, which in turn phosphorylates CREB at Ser133 (35). To test if MSK1/2 linked the activation of ERK1/2 and p38 with CREB phosphorylation, we silenced the MSK1 and MSK2 genes using siRNA. When these genes were independently or simultaneously silenced, a significant reduction in CREB phosphorylation was observed (**Figure 6A**), which indicated that both MSK1 and MSK2 could participate. However, only MSK1 (**Figure 6B**), but not MSK2 (data not shown), phosphorylation was detected in macrophages stimulated with IDR-1002. Evidence of MSK1 gene knockout by siRNA is presented in **Figure 6A**. Furthermore, incubation of macrophages with either ERK1/2- or p38-specific inhibitors almost completely prevented phosphorylation of MSK1 (**Figure 6B**).

However, although CREB was strongly phosphorylated, we observed no increase in the anti-inflammatory cytokines IL-4, IL-10, and IL-13 as measured by ELISA (data not shown). The lack of expression of these cytokines associated with M2 macrophage response might be due to the absence of interaction between phosphorylated CREB and CBP. The co-immunoprecipitation of CREB with CBP from macrophages stimulated with IDR-1002 showed that no complex was formed between CBP and CREB (**Figure 6C**). Together, these genetic and biochemical data indicated that IDR-1002-induced phosphorylation of CREB at Ser133 depended on the ERK1/2/p38–MSK1 signaling pathway, and that, at least in our system, phosphorylation of CREB by MSK1 did not promote interaction between CREB and CBP.

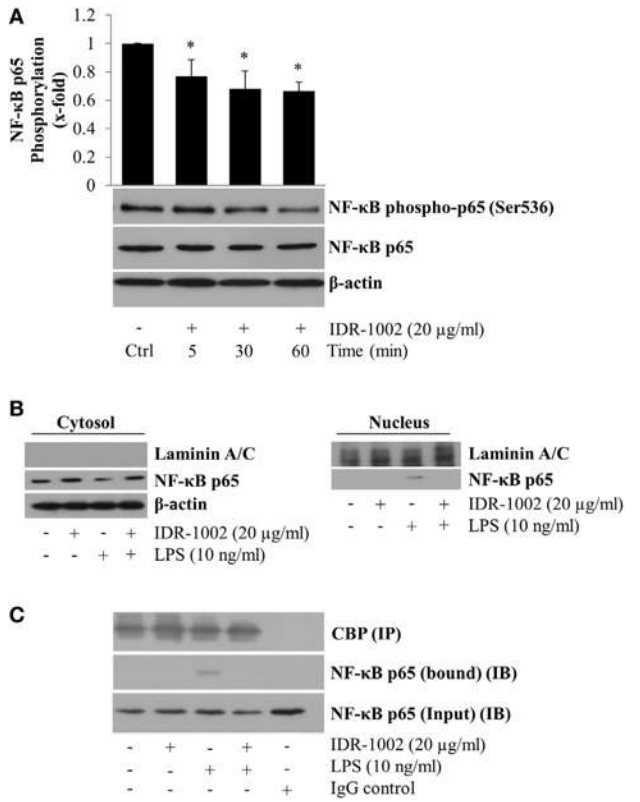

FIGURE 3 | IDR-1002 reduced NF-κB p65 phosphorylation at Ser536 and inhibited its LPS-activated nuclear translocation in RAW264.7 macrophages. **(A)** Macrophages were left unstimulated (Ctrl) or stimulated with 20 μg/ml of IDR-1002 for 5–60 min. Phosphorylation of NF-κB p65 subunit was detected by Western blotting. Detection of β-actin and unphosphorylated p65 in each sample was performed to ensure equal protein loading. **(B)** Macrophages were pretreated with 20 μg/ml of IDR-1002 for 1 h and then with 10 ng/ml of LPS for 2 h. Non-phosphorylated NF-κB p65 subunit was detected by Western blotting in nuclear and cytosolic protein enriched fractions. Laminin A/C and β-actin were detected as controls for nuclear and cytosolic fractions, to observe equal protein loading and no cross-contamination between nuclear and cytosolic fractions, respectively. **(C)** Macrophages were treated as described in **(B)**. Eluates from CBP immunoprecipitates (IP) were subjected to Western blot analysis with NF-κB p65 antibody to detect the p65 bound to CBP and its amount present in the input (IB). Control with isotype IgG was also included. Detection of CBP was performed to show equal protein loading. *$P < 0.05$ for IDR-1002 compared with the unstimulated control value.

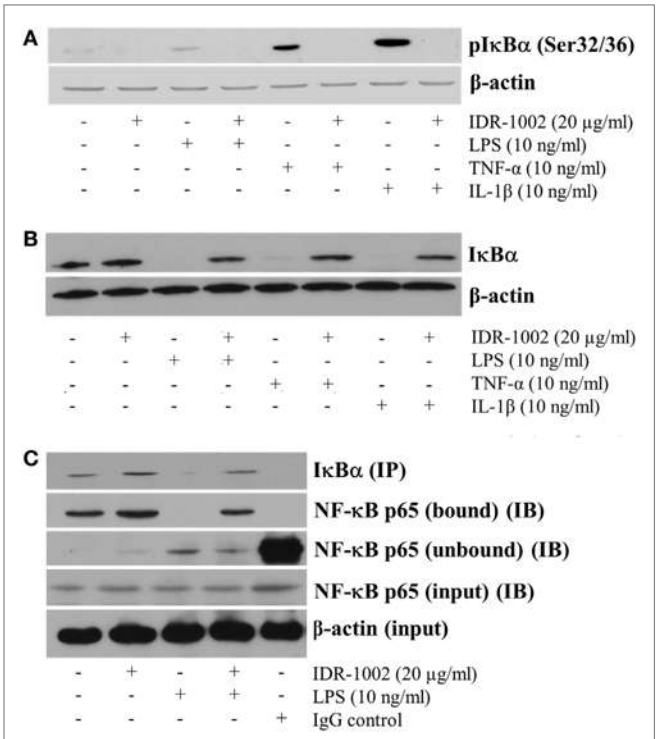

FIGURE 4 | IDR-1002 inhibited IκBα phosphorylation and degradation in RAW 264.7 macrophages. **(A)** Macrophages were left unstimulated or pretreated with 20 μg/ml of IDR-1002 for 1 h. Then, macrophages were stimulated for 10 min with either 10 ng/ml of LPS, 10 ng/ml of TNF-α, or 10 ng/ml of IL-1β. Phosphorylated IκBα was detected by Western blot analysis and β-actin was detected to ensure equal protein loading. **(B)** Macrophages were treated as in **(A)** except that incubation with LPS, TNF-α, or IL-1β was for 30 min. Total IκBα was detected by Western blot analysis in total cell lysates extracts. β-actin detection was performed to ensure total protein loading. **(C)** Macrophages were left unstimulated or stimulated with 20 μg/ml of IDR-1002 for 1 h followed by stimulation with 10 ng/ml of LPS for 10 min. IκBα was immunoprecipitated (IP) and eluates were subjected to Western blot to detect NF-κB p65 in the immunoprecipitates of IκBα (bound) or supernatants (unbound) samples (IB). β-actin detection was performed in the input cell extracts to ensure equal protein.

DISCUSSION

Data presented here demonstrated that in RAW264.7 macrophages/monocytes stimulated with LPS, IDR-1002 was able to inhibit IκBα phosphorylation and degradation, completely blocking NF-κB p65 nuclear translocation. This would explain the inhibition of the interaction between NF-κB and CBP, and partially inhibition of NF-κB p65 Ser536-phosphorylation, which can enhance transactivation. These effects of IDR-1002 on NF-κB p65, together with others previously reported (36, 37), were sufficient to reduce the expression of TNF-α and COX-2. In addition, IDR-1002 induced a significant increase in CREB phosphorylation at Ser133 through the activation of p38/ERK1/2 and MSK1/2. Although the relative abundance of phosphorylated CREB was higher in macrophages stimulated with IDR-1002 than in untreated controls, no interaction between CREB and CBP occurred, and thereby preventing the enhanced expression of cytokines IL-4, IL-10, and IL-13.

Inhibition of TNF-α expression in RAW264.7 macrophages has been demonstrated by *in vitro* administration of different compounds in response to LPS from *E. coli* (38–41). For example, the anti-inflammatory effect of extracellular phospho-ceramide analog-1 (PCERA-1) was found to depend on suppression of TNF-α production, an increase in cAMP levels, and increased expression of IL-10 (38). The low molecular weight chemical compounds madecassic and asiatic acid, and the isoflavone genistein potently inhibited the expression of TNF-α, COX-2, NO, prostaglandin E_2, IL-1β, and IL-6 by blocking both IκBα

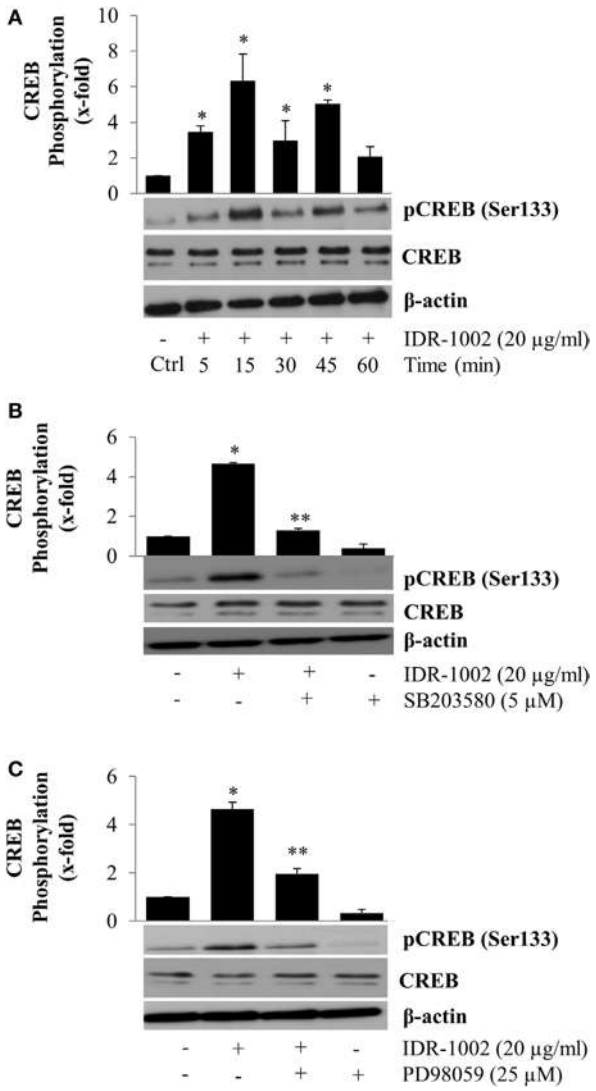

FIGURE 5 | Inhibition of p38 and ERK1/2 activity reduces CREB phosphorylation at Ser133 in Raw 264.7 macrophages activated by IDR-1002. (A) Macrophages were left unstimulated (Ctrl) or stimulated with 20 μg/ml of IDR-1002 for 5–60 min. After stimulation, CREB phosphorylation was detected by Western blotting. **(B)** Cells were left untreated (–) or treated with 5 μM of SB203580 (SB, a p38 inhibitor) for 30 min and then stimulated with 20 μg/ml of IDR-1002 for 15 min. **(C)** Cells were left untreated (–) or treated with 25 μM of PD98059 (PD, an ERK1/2 inhibitor) for 30 min and then stimulated with 20 μg/ml of IDR-1002 for 15 min. Additional controls in which cells were treated with 5 μM of SB or 25 μM of PD alone were also included. Phosphorylation of CREB was analyzed by Western blotting. Detection of β-actin and unphosphorylated CREB were performed to ensure equal protein loading. Blots are representative of three independent experiments. The data represent means ± SEM ($n = 3$). *$P < 0.05$ for IDR-1002 compared with the unstimulated control value. **$P < 0.05$ for inhibitor + IDR-1002 assays compared with the IDR-1002 assay.

degradation and NF-κB nuclear translocation (39–41). The effects in macrophages induced by IDR-1002 on TNF-α/COX-2 expression, and NF-κB inhibition, resemble those triggered by the compounds mentioned above, suggesting that parallel mechanisms mediate the effect of IDR-1002 to control inflammation. Specifically, we found in RAW 264.7 macrophages that IDR-1002 was able to inhibit LPS-induced phosphorylation of IKKα and IKKβ, which may explain the inhibitory effect of this peptide on IκBα phosphorylation (Figure S3 in Supplementary Material). Recently, Liu et al. (42) discovered that a long non-coding RNA inhibits IκB phosphorylation by forming a ternary complex with NF-κB and IκBα, which caused a strong inhibition of metastasis in breast cancer. Based on these results, it is possible that IDR-1002, as an inhibitor of IKK and IκBα phosphorylation, may be a good candidate for metastasizing cancers. We are currently performing experiments to test IDR-1002 in various cancer cell lines.

COX-2 is important in the control of inflammation, pain, fever, and other physiological processes. Inhibition of COX-2 activity by non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin has been the clinicians' choice to alleviate medical conditions. However, NSAIDs induce tolerance over time (43) and administration for even short periods can cause gastrointestinal and renal secondary effects in ~25% of individuals and almost 5% of them suffer serious health problems (44). Because of these detrimental effects induced by NSAIDs, a search for new anti-inflammatory drugs without side effects is of primary importance in the pharmaceutical industry. The strong inhibition by IDR-1002 of LPS-induced COX-2 expression observed in our study suggests that IDR peptides may be a viable alternative. However, more experiments *in vivo* and chemical modification/formulation of IDR-1002 to increase its potency, specificity, and half-life time must be considered before its administration to human beings.

Apart from the inhibitory effect of IDR-1002 on LPS-induced NF-κB and COX-2, this cationic peptide induced the phosphorylation/activation of p38, ERK1/2, and MSK1, which led to the phosphorylation of CREB at its Ser133 activation site. The contribution of the signaling pathway PI3K/Akt/GSK3 as an effector of IDR-1002-induced CREB phosphorylation was ruled out (Figure S2 in Supplementary Material). According to one current model, once CREB becomes phosphorylated at Ser133, it is able to interact with CBP and induce expression of anti-inflammatory cytokine genes, among which IL-4, IL-10, and IL-13 are the most important for reducing inflammation (23). However, it has been documented that in some cases phosphorylation of CREB at Ser133 by MSK1/2 is not absolutely required to promote recruitment of CBP and, consequently, expression of some CREB target genes does not take place (15). Similarly, activation of T-cell receptor (TCR) promotes a strong phosphorylation of CREB at Ser133 without formation of CREB–CBP complex, unless low amounts of cAMP were present as a costimulus (24). In our case, IDR-1002-activated MSK1-dependent phosphorylation of CREB did not promote the interaction of CREB with CBP, which might explain lack of effects on gene expression of IL-4, IL-10, and IL-13. This conclusion was drawn after several attempts to co-immunoprecipitate CBP with CREB in protein extracts recovered from macrophages stimulated with IDR-1002. More experiments to find out the

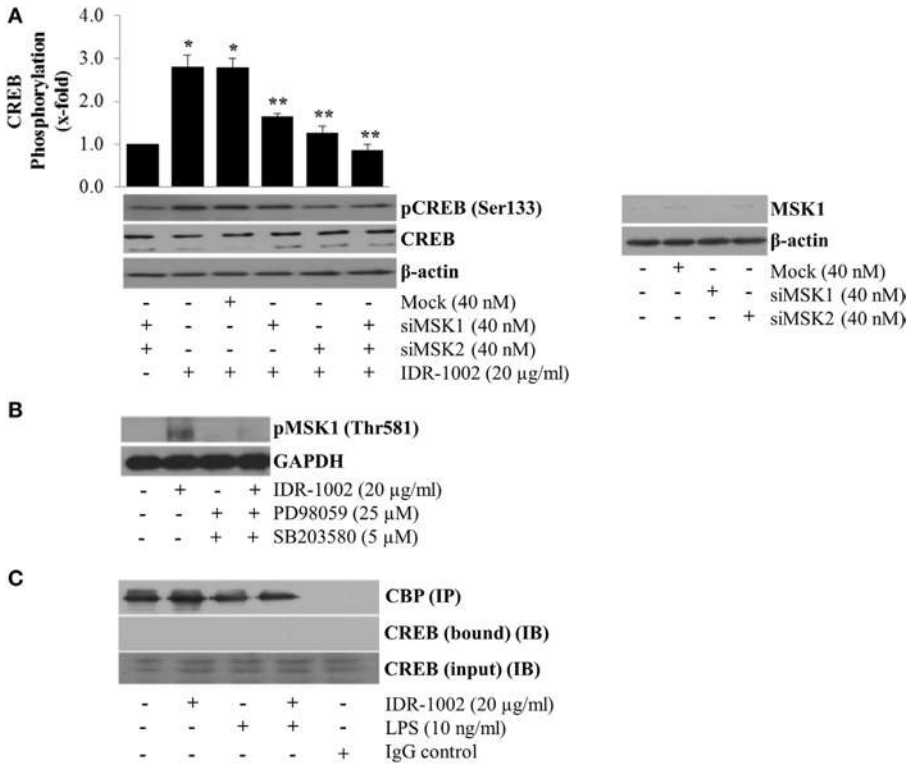

FIGURE 6 | **MSK1-mediated CREB phosphorylation at Ser133 in RAW 264.7 macrophages stimulated with IDR-1002**. **(A)** Macrophages were transfected with non-specific siRNA control (Mock), MSK1 (siMSK1), MSK2 (siMSK2) or MSK1, and MSK2 simultaneously for 48 h. Cells were then stimulated with 20 μg/ml of IDR1002 for 15 min. CREB phosphorylation was analyzed by Western blotting. Detection of β-actin and unphosphorylated CREB in each sample was performed to ensure equal protein loading. The right panel shows detection of MSK1 gene expression when macrophages were treated with MSK1-specific siRNA. In **(B)**, macrophages were left unstimulated (–) or pretreated with 25 μM of PD98059 (PD, an ERK1/2 inhibitor) and 5 μM of SB203580 (SB, a p38 inhibitor) for 30 min, and then stimulated with IDR-1002 for 15 min. Additional controls with 25 μM of PD and 5 μM of SB alone were also included. Under these conditions, phosphorylation of MSK1 was detected by Western blotting. **(C)** Macrophages were left unstimulated or stimulated with 20 μg/ml of IDR-1002 followed by stimulation with 10 ng/ml of LPS. Immunoprecipitation was performed as previously described and eluates from CBP immunoprecipitates (IP) were subjected to Western blot analysis with CREB antibodies. Then, we detected CREB immunoprecipitated (bound) with CBP and in the input (IB). Control with isotype IgG was also included. Detection of CBP was performed to show equal protein loading. The blots are representative of three independent experiments. Data represent means ± SEM ($n = 3$). $^{*}P < 0.05$, compared with the unstimulated control value. $^{**}P < 0.05$ for siRNA treatment compared with the IDR-1002 value.

molecular details of this mechanism are currently underway in our laboratory.

The experimental evidence obtained in RAW 264.7 macrophages indicating that IDR-1002 inhibits LPS-induced COX-2 expression, activates CREB phosphorylation at Ser133, and inhibits LPS-induced IκBα degradation was confirmed in PBMC (Figures S4A–C in Supplementary Material). This novel spatiotemporal combination of both effects (inhibition of NF-κB signaling and activation of CREB phosphorylation) makes IDR-1002 an attractive anti-inflammatory pharmaceutical drug.

AUTHOR CONTRIBUTIONS

AH-M performed most of the experiments and helped with the writing of the manuscript. OS-G performed many experiments. JO-B helped to perform some experiments. RH financially supported the research (provided the peptide IDR-1002) and critically reviewed the manuscript. VMB-A financially supported most of the supplies and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2016.00533/full#supplementary-material.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

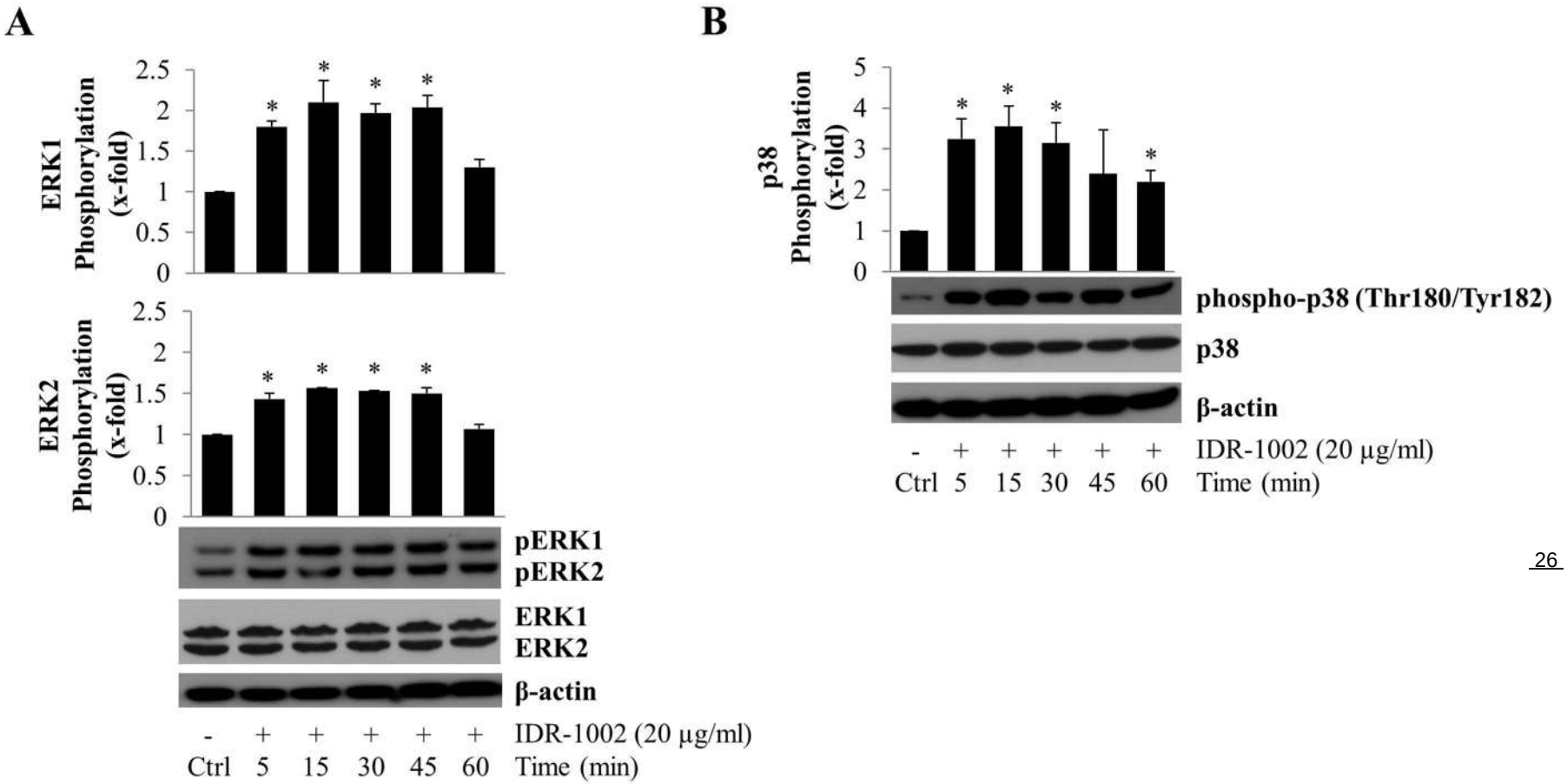

FIGURE S1. IDR-1002 induced ERK1/2 and p38 phosphorylation in Raw 264.7 macrophages . Macrophages were left unstimulated (Ctrl) or stimulated with 20 µg/ml of IDR-1002 for 5-60 min. Then, phosphorylation of ERK1/2 (A) and p38 (B) were detected by Western blotting. The relative abundances of β–actin , unphosphorylated ERK1/2 and unphosphorylated p38 were evaluated to ensure equal protein loading. Data represent means ± SEM (n = 3). *P < 0.05, compared with the unstimulated control value.

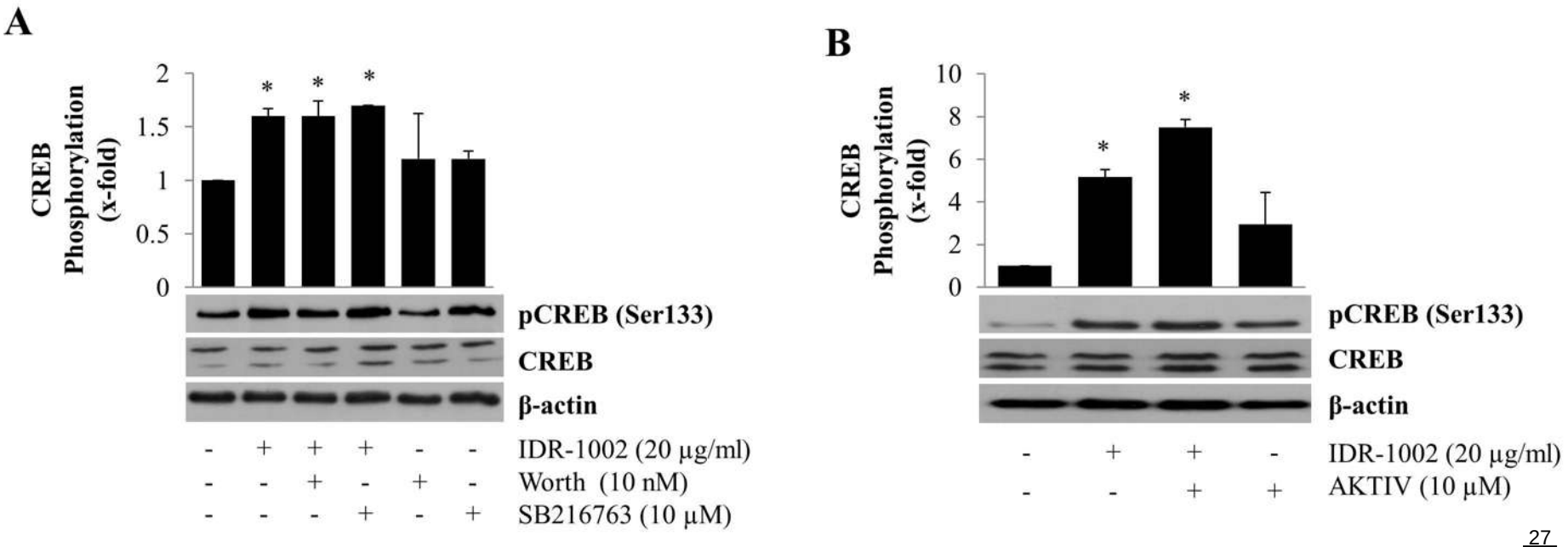

FIGURE S2. CREB phosphorylation activated by IDR-1002 was not mediated by AKT, PI3K and GSK3 in RAW 264.7 macrophages. (A) Macrophages were incubated with 10 nM of Worthmanin (Worth), or 10 µM of SB212763 (a GSK3 inhibitor). (B) Macrophages were incubated with 10 µM of AKTIV (an AKT inhibitor). In all cases, control cells were left unstimulated (-) or incubated for 30 min with the inhibitor and then stimulated with 20 µg/ml of IDR-1002 for 15 min. Each experiment also includes a control treatment of macrophages with the inhibitor alone. Phosphorylation of CREB was detected by Western blotting. Detection of β-actin and unphosphorylated CREB in each sample was performed to ensure equal protein loading. The blots are representative of three independent experiments. Data represent means ± SEM (n = 3). *P < 0.05 for IDR-1002 compared with the control value.

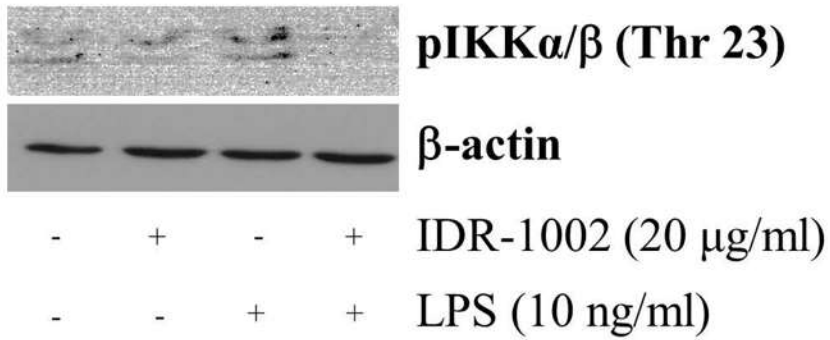

FIGURE S3. Inhibition of IKKα and IKKβ phosphorylation by IDR-1002 in RAW 264.7 macrophages stimulated with LPS. Macrophages were left untreated or incubated with 20 μg/ml of IDR-1002 for 60 min and then stimulated with 10 ng/mL of LPS for 10 min. Phosphorylation of IKKα/β was detected by Western blotting. Detection of β-actin was performed to ensure equal protein loading. The blot is representative of two independent experiments.

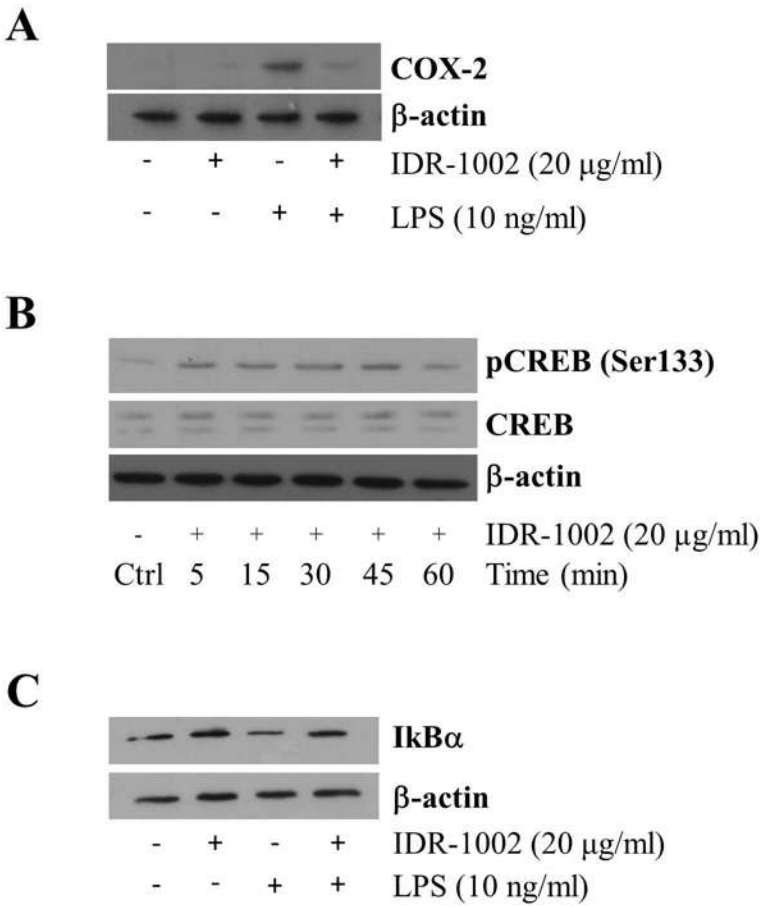

FIGURE S4. The effects induced by IDR-1002 on human PBMC were similar to those observed in RAW 264.7 macrophages. Isolated PBMC from healthy donors were left unstimulated or stimulated with 20 μg/ml of IDR-1002 for 60 min followed by stimulation with 10 ng/ml of LPS for 10 min (A and C). CREB phosphorylation at Ser133 (B) was detected in untreated (Ctrl) and treated macrophages with 20 μg/ml of IDR-1002 from 5 to 60 min. Total CREB (B) and β–actin (A and C) detections were performed to ensure equal protein loading.

8.2. ARTÍCULO 2: DIVULGACIÓN

LA ENZIMA GLUCÓGENO SINTASA CINASA 3 REGULA LA ACTIVIDAD DE LOS FACTORES DE TRANSCRIPCIÓN NF-κB Y CREB DURANTE LA RESPUESTA INFLAMATORIA CAUSADA POR *Staphylococcus aureus.*

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En la primera parte de este trabajo aceptado para su publicación en la Revista de Educación Bioquímica se explicaron brevemente algunos de los principales conceptos de la respuesta inflamatoria, para después discutir la función de GSK3 como una enzima mediadora importante de la vía de transducción PI3K/Akt. Con esta información se abordaron con mayor detalle algunos aspectos estructurales y mecanísticos de GSK3, lo que dio lugar a entender mejor la regulación que ejerce esta enzima sobre NF-κB y CREB en la respuesta inflamatoria. Por último, se describirán algunos resultados de nuestro laboratorio ya publicados, donde evaluamos la regulación de la expresión de citocinas por GSK3 y una de las perspectivas de investigación que se tiene en el laboratorio a corto y mediano plazo.

Mi participación en este trabajo fue conjuntar la información, ordenarla y escribir el primer borrador.

LA ENZIMA GLUCÓGENO SINTASA CINASA 3 REGULA LA ACTIVIDAD DE LOS FACTORES DE TRANSCRIPCIÓN NF-κB Y CREB DURANTE LA RESPUESTA INFLAMATORIA CAUSADA POR *Staphylococcus aureus**

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RESUMEN

La enzima glucógeno sintasa cinasa 3 (GSK3) es una proteína cinasa de Ser/Thr, que regula la expresión de citocinas durante la respuesta inflamatoria. GSK3 modula la actividad del factor nuclear κB (NF-κB) y de la proteína de unión al elemento de respuesta de AMPc (CREB). En esta revisión se presenta uno de los mecanismos reguladores de la actividad de GSK3 y su efecto en las infecciones por *Staphylococcus aureus*.

PALABRAS CLAVE:
GSK3, NFκB, CREB, inflamación, *Staphylococcus aureus*.

ABSTRACT

The enzyme glycogen synthase kinase 3 (GSK3) is a Ser/Thr kinase that regulates the expression of cytokines during the inflammatory response by modulating the activity of nuclear factor κB and cAMP response element-binding protein (CREB). This review presents one of the mechanisms that regulate GSK3 activity and its effect on *Staphylococcus aureus* infections.

KEY WORDS:
GSK3, NF-κB, CREB, inflammation, *Staphylococcus aureus*.

INTRODUCCIÓN

La inmunidad innata es la primera línea de defensa contra agentes infecciosos, y consiste de una red interactiva de sistemas moleculares y celulares responsable de reconocer y eliminar microorganismos patógenos. Este tipo de inmunidad incluye una amplia variedad de cambios en la célula, cuyo objetivo es producir moléculas que coadyuven a eliminar al patógeno (1). La habilidad del sistema inmune innato de reconocer y responder a componentes microbianos se debe a la presencia de receptores tipo Toll (TLRs) (2, 3). Los TLR están presentes tanto en fagocitos profesionales (monocitos, macrófagos y células dendríticas) como en no profesionales como las células epiteliales y endoteliales. Estos receptores pueden discriminar entre distintos patrones moleculares asociados a patógenos (PAMPs) que conducen a la activación de una variedad de vías de transducción de señales, las cuales regulan la naturaleza, magnitud y duración de la respuesta inflamatoria (4). La reacción inflamatoria es muy importante en la respuesta inicial a la infección y en ella participan neutrófilos, monocitos, macrófagos, moléculas del complemento, citocinas, quimiocinas, así como también proteínas y péptidos con actividad antimicrobiana, que contribuyen coordinadamente a eliminar al patógeno del tejido infectado. Aunque la producción de citocinas pro-inflamatorias es importante para regular la defensa inicial del hospedero contra los patógenos invasores, la incapacidad de modular la intensidad o duración de la respuesta inflamatoria puede ser perjudicial y dar lugar a enfermedades inflamatorias crónicas (5).

En la última década, el interés se ha enfocado en explicar el mecanismo por el que las células participantes en la respuesta inflamatoria inicial

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expresan citocinas pro-inflamatorias (p.ej. interleucina 8, IL-8) y más tarde apagan esta expresión y activan la síntesis de citocinas anti-inflamatorias (p.ej. IL-10), disminuyendo con ello la inflamación. En este sentido, se ha observado que uno de los principales mecanismos moduladores de la expresión de citocinas con actividad pro- y anti-inflamatoria es el que está mediado por la vía de señalización constituida por las enzimas fosfatidilinositol 3-cinasa (PI3K), Akt (denominada también proteína cinasa B, PKB) y glucógeno sintasa cinasa 3 (GSK3), activada por TLR (6, 7). Se ha propuesto que la activación de GSK3 es fundamental para inducir la producción de citocinas que promueven la inflamación (IL-8) o la disminuyen (IL-10), por medio de un mecanismo que involucra la participación de los factores de transcripción factor nuclear-kappa B (NF-κB) y la proteína de unión al elemento de respuesta de AMPc (CREB), respectivamente (7).

En este trabajo se explicarán brevemente algunos de los principales conceptos de la respuesta inflamatoria, para después discutir la función de GSK3 como una enzima mediadora importante de la vía de transducción PI3K/Akt. Con esta información abordaremos con mayor detalle algunos aspectos mecanísticos de GSK3, lo que dará lugar a entender mejor la regulación que ejerce esta enzima sobre NF-κB y CREB en la respuesta inflamatoria. Por último, se describirán algunos resultados de nuestro laboratorio, donde evaluamos la regulación de la expresión de citocinas por GSK3 y una de las perspectivas de investigación que tenemos a mediano plazo.

LA RESPUESTA INFLAMATORIA

La inflamación es el primer proceso fisiológico de respuesta a un daño o infección de un tejido y es fundamental tanto para la inmunidad innata como la adaptativa. Cuando un tejido es infectado por bacterias patógenas se activa un programa de respuesta que incluye la expresión de citocinas, quimiocinas, mediadores lipídicos y aminas bioactivas por diversos tipos de células como monocitos, células dendríticas, células asesinas naturales ("*natural killer cells*") y mastocitos. Entre las múltiples funciones de estos factores, una muy importante es el incremento de la permeabilidad vascular que conduce a la extravasación de leucocitos polimorfonucleares al sitio de infección. La mayor cantidad de información molecular que se tiene hasta ahora sobre el proceso inflamatorio proviene de estudios sobre componentes de las vías de transducción activados por el receptor al factor de necrosis tumoral (TNFR), el receptor a IL-1 y los TLR (8). Las citocinas pro-inflamatorias predominantemente producidas son el factor de necrosis tumoral (TNF), IL-1, IL-8, IL-12, IL-15 IL-18, IL-23 e IL-27 (9). Estos péptidos activan procesos citotóxicos, celulares, humorales y alérgicos, que en conjunto tienen la finalidad de eliminar al agente causal del daño al tejido.

Es importante mencionar que la respuesta inflamatoria está sujeta a una regulación estricta, de tal forma que, una vez cumplido el objetivo de eliminar la causa de la inflamación, las mismas células involucradas cambian su programa genético para expresar ahora mediadores anti-inflamatorios, cuyo objetivo es restaurar la homeostasis. Cuando este programa anti-inflamatorio falla puede haber daño al tejido, lo que en ocasiones deriva en inflamación crónica y enfermedad (10). Se han identificado citocinas que tienen principalmente una función anti-inflamatoria, por ejemplo el antagonista del receptor a IL-1 (IL-1Ra), el factor de crecimiento transformante beta (TGF-β), IL-10 e IL-35 (11).

Tal cambio de actividad celular pro-inflamatoria a anti-inflamatoria resulta relativamente fácil de entender si echamos andar nuestra lógica al formular preguntas como ¿qué sucedería si la respuesta inflamatoria continuara indefinidamente? La respuesta creo que es obvia, nuestros órganos se alterarían a tal grado que se perderían las funciones que mantienen al organismo en homeostasis. Para explicar el tránsito de mecanismos pro-inflamatorios a anti-inflamatorios, lo primero que se pensó fue que a medida que transcurría la inflamación, el tejido iba recuperando la homeostasis porque los mediadores pro-inflamatorios se iban degradando. Es decir, no se consideraba que las mismas células iniciadoras de la inflamación pudieran ser capaces de inhibir la expresión de moléculas pro-inflamatorias y activar la expresión y secreción de moléculas anti-inflamatorias. Esta forma de conceptualizar la respuesta inflamatoria ha cambiado y ahora se acepta que hay moléculas que son críticas para controlar el cambio de actividad pro- a anti-inflamatoria. Una de estas moléculas es la enzima GSK3 descubierta en 1980 por su efecto regulador de la síntesis de glucógeno (12). Sin embargo, la evidencia acumulada indica sin lugar a dudas que GSK3 tiene múltiples funciones, como su participación en el control de la inflamación (13). Durante la respuesta inflamatoria la activación de Akt regula la actividad de GSK3, por lo que a continuación estudiaremos un poco acerca de la vía de transducción que activa a Akt.

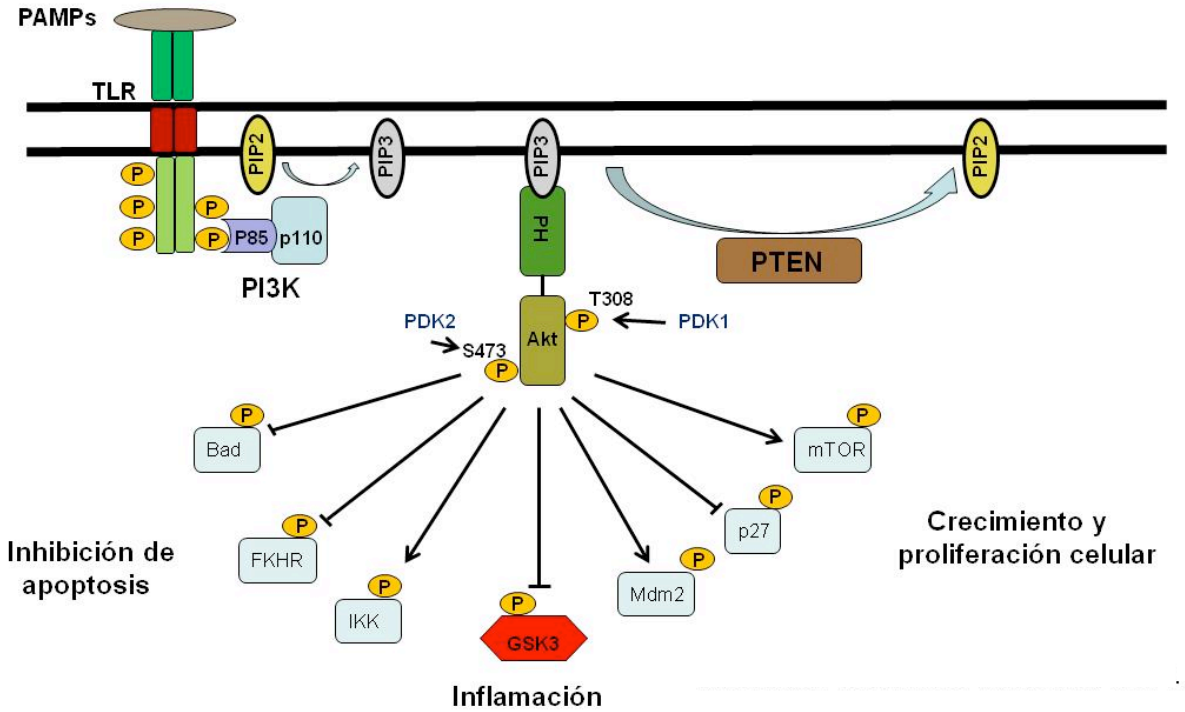

Figura 1. Modificado de referencia 14

CARACTERÍSTICAS Y MECANISMO DE ACTIVACIÓN DE LA VÍA DE TRANSDUCCIÓN PI3K/Akt/GSK3

La enzima PI3K es un heterodímero constituido por una subunidad catalítica de 110 kDa (p110) y una subunidad reguladora de 85 kDa (p85). De acuerdo con su estructura y mecanismo de activación las PI3K se clasifican en varias familias. Las enzimas de la clase IA (principales responsables de la producción de 3'-fosfoinosítidos) son activadas por receptores que tienen actividad de tirosina cinasa (RPTKs) y las que pertenecen a la clase IB por receptores acoplados a proteínas G (GPCR). Dentro de la clase IA se han caracterizado tres isoformas de p110, denominadas α, β y γ, y siete isoformas de p85 que se sintetizan por procesamiento alternativo de tres genes, *p85*α, *p85*β y *p85*γ (14). La segunda enzima de la vía, denominada Akt/PKB, fue caracterizada como un homólogo del oncogén viral *v-akt*, que se considera responsable de causar leucemia en ratones (15). Debido a que *v-akt* y su contraparte en humanos presentan similitud con PKA y PKC, a esta nueva proteína cinasa de Ser/Thr se le llamó inicialmente PKB. Existen tres isoformas de Akt, Akt1, Akt2 y Akt3, con un 80% de homología entre ellas, aunque son codificadas por genes diferentes. Las tres Akt tienen en su región N-terminal un dominio de unión a plekstrina (PH) de aproximadamente 100 aminoácidos, que le permite anclarse a la membrana plasmática mediante su interacción con 3'-fosfoinosítidos. Para activarse al 100%, Akt debe ser fosforilada en Thr 308 (localizada en su dominio catalítico) y en Ser 473 (localizada en su región C-terminal). La interacción de factores de crecimiento con RPTKs y de PAMPs (p.ej. péptidoglicano, PGN; ácido lipoteicoico, LTA y lipopolisacárido, LPS) con TLRs da lugar a la activación de Akt (16, 17).

La activación de la vía PI3K-Akt se inicia cuando un estímulo (p.ej. factores de crecimiento o PAMPs) promueve la fosforilación de residuos de Tyr en el dominio citosólico de los receptores (Fig. 1). Estas fosfo-Tyr son reconocidas por el dominio SH2 de p85, lo que trae como consecuencia la activación alostérica de p110 y la producción de fosfatidilinositol-3,4,5-trisfosfato (PIP_3). La presencia de PIP_3 en la capa interna de la membrana plasmática funciona como un mediador esencial para el funcionamiento de la vía PI3K-Akt, en la que participan PI3Ks de la clase IA. En esta vía, las dos enzimas que contienen dominios PH y, por lo tanto, pueden reconocer a PIP_3 son la cinasa de Ser/Thr dependiente de 3'-fosfoinosítido (PDK1), constitutivamente activa, y Akt. La interacción del dominio PH de Akt con PIP3 cambia la conformación de Akt y expone sus sitios de fosforilación Thr308 y Ser473, los cuales son fosforilados por PDK1 y PDK2 (denominada también complejo diana de rapamicina de mamíferos, mTORC2), respec-

tivamente. Una vez activada, Akt fosforila una diversa variedad de sustratos, entre los cuales se encuentra GSK3, modulando así una variedad de procesos celulares como apoptosis, proliferación y sobrevivencia celular, e inflamación.

GSK3 MODULA LA EXPRESIÓN DE CITOCINAS CON ACTIVIDAD PRO- Y ANTI-INFLAMATORIA

GSK3 es una enzima constitutivamente activa muy importante en múltiples procesos biológicos, debido a que es el punto de convergencia de varias vías de señalización y porque regula la actividad de más de 50 sustratos (18). Su secuencia de aminoácidos está altamente conservada en eucariontes y se han caracterizado dos isoformas α y β, que aparentemente no son redundantes porque la interrupción de la isoforma β en ratones resulta en letalidad embrionaria; esto significa que GSK3α es incapaz de compensar la pérdida de GSK3β (19). Debido a la amplia influencia que GSK3 tiene en diversas funciones celulares, la actividad de GSK3 está regulada por diferentes mecanismos. Por ejemplo, su actividad puede ser inhibida por la fosforilación de residuos de Ser en la región N-terminal (Ser21 en GSK3α y Ser9 en GSK3β). Además, la actividad de GSK3 también está controlada por su asociación con proteínas de anclaje y su localización intracelular (6). Se ha demostrado que la activación o inhibición de GSK3 tiene una función importante en la regulación de la respuesta inmune al ejercer un control, mediado por TLRs, de la producción de citocinas con actividad anti- y pro-inflamatoria (7).

Las vías de señalización activadas por TLR2, TLR4, TLR5 y TLR9 son fundamentales para regular la producción de las citocinas pro-inflamatorias (p.ej. IL-1β, TNF-α, IL-8) y anti-inflamatoria (p.ej. IL-4, IL-10) (20, 7, 21). El mecanismo por el que estos TLR regulan la expresión de citocinas que intervienen en la inflamación se estudió en monocitos estimulados con LPS (7). Los datos obtenidos indicaron que la inhibición de GSK3β, pero no de GSK3α, redujo los niveles de las citocinas pro-inflamatorias y aumentó la expresión de IL-10, en respuesta a los agonistas de TLR, LTA (TLR2), lípido A sintético (TLR4), flagelina (TLR5) y CpG (TLR9). La inhibición de GSK3 ejerció un efecto regulador diferencial en la asociación de NF-κB (promueve la expresión de citocinas pro-inflamatorias) y CREB (promueve la expresión de citocinas anti-inflamatorias) con el co-activador nuclear, proteína de unión a CREB (CBP), es decir, GSK3 inactiva causó un aumento en la actividad de unión de CREB al DNA y, al mismo tiempo, inhibió apreciablemente la actividad nuclear de NF-κB (7). De esta manera, la interacción de NF-κB y CREB con CBP, regulada por la actividad de GSK3, ocupa un lugar preeminente con respecto a la naturaleza y magnitud de la producción de citocinas pro- y anti-inflamatorias, lo que resulta en la modulación adecuada y precisa de la inflamación.

GSK3α Y GSK3β REGULAN DIFERENCIALMENTE LA EXPRESIÓN DE CITOCINAS EN CÉLULAS ENDOTELIALES INFECTADAS CON *Staphylococcus aureus*

Staphylococcus aureus es una bacteria Gram$^+$ causante de importantes enfermedades infecciosas en seres humanos y animales. Esta bacteria expresa una amplia variedad de factores de virulencia y PAMPs que inducen inflamación y son responsables de dañar al tejido (22). Las células del hospedero reconocen los PAMPs de *S. aureus* por medio del receptor TLR2. La interacción de estos PAMPs con TLR2 activa la vía de señalización PI3K/Akt/GSK3, lo cual da lugar a cierto número de respuestas celulares tales como proliferación, diferenciación, sobrevivencia, apoptosis e inflamación (23).

La participación de GSK3α y GSK3β en infecciones por *Staphylococcus aureus* se observó por primera vez cuando la internalización de esta bacteria en células endoteliales produjo un incremento en la fosforilación e inhibición de la actividad de ambas isoformas de GSK3 (24). Un aspecto interesante de ese estudio fue que GSK3α alcanzó un nivel de fosforilación y de inhibición mayor que GSK3β. Esta evidencia experimental indicó que no solo GSK3β está involucrada en procesos de infección e inflamación causados por bacterias patógenas, como se ha descrito ampliamente en la literatura (13), sino que también GSK3α podría tener una función preponderante. Más adelante se pudo demostrar que ambas isoformas de GSK3 regulan la expresión de IL-12p40 en células endoteliales estimuladas con PGN de *S. aureus* (25). Esta citocina es muy importante porque controla la respuesta inmune innata durante la infección bacteriana y determina el tipo y duración de la respuesta inmune adaptativa. IL-12p40 es altamente inducible por LPS, LTA y PGN, por su interacción con TLRs y activación de la vía NF-κB (26). Aunque la función de IL-12p40 promueve un desarrollo apropiado de la respuesta inflamatoria inicial, su sobreexpresión es perjudicial para el tejido. Por ello, las células contrarrestan los efectos de esta citocina produciendo IL-10, que disminuye la actividad de NF-κB y aumenta la actividad de CREB. Los datos de Cortés-Vieyra et al. (25) indicaron que la vía PI3K/Akt/GSK3 se activó por efecto de PGN, lo que se correlacionó con cambios en la actividad de GSK3α y GSK3β. Los autores pudieron establecer que la inhibición de la actividad de GSK3α indujo un incremento en la expresión de IL-12p40, mientras

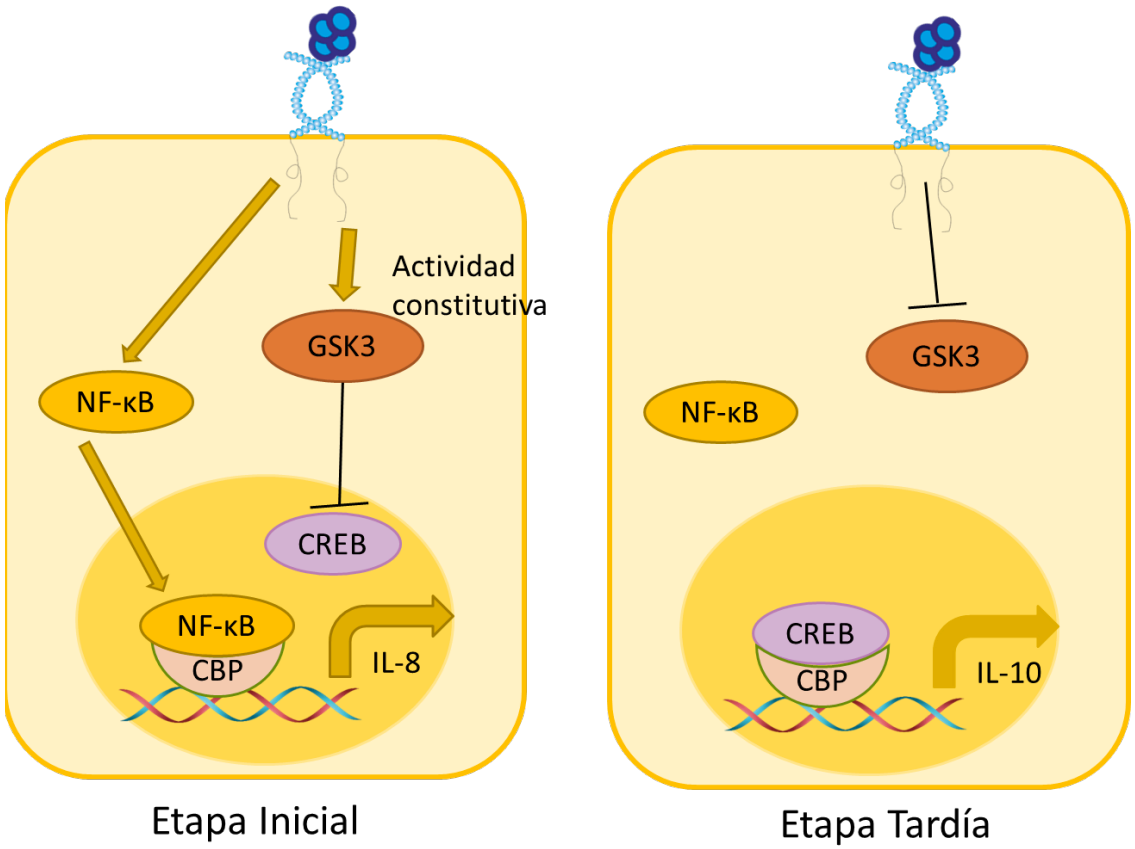

Figura 2.

que la inhibición de la actividad de GSK3β resultó en una expresión disminuida de esta citocina.

Los resultados más recientes obtenidos en nuestro grupo (Silva-García O y Baizabal-Aguirre VM, manuscrito enviado) han mostrado que la actividad de GSK3α y, en menor grado, GSK3β en células endoteliales infectadas por *S. aureus* durante 60 min ejerció un efecto inhibidor de la activación de CREB, limitando con ello su actividad transcripcional y promoviendo la actividad de NF-κB, el cual a su vez indujo la expresión de la citocina pro-inflamatoria IL-8. Cuando se ensayaron tiempos de infección de 120 min observamos que GSK3α/β alcanzó su fosforilación e inhibición máxima por Akt, lo cual inhibió indirectamente la actividad transcripcional de NF-κB. Esto causó un aumento en la actividad transcripcional de CREB, lo que estimuló la expresión de la citocina anti-inflamatoria y tolerogénica IL-10, y disminuyó simultáneamente la expresión de IL-8 (Fig. 2). Con base en estos resultados creemos que este mecanismo de "*switch*" molecular de la activación de un factor transcripcional pro-inflamatorio (NF-κB) en la fase inicial de la infección, a la activación de un factor transcripcional anti-inflamatorio (CREB) en la fase tardía de la infección por *S. aureus* explicaría en parte por qué las infecciones causadas por esta bacteria transitan de una fase de inflamación aguda a una fase de inflamación crónica.

Estos datos plantean la posibilidad de que GSK3α y GSK3β ejerzan una regulación diferencial de la expresión de genes pro- y anti-inflamatorios, por medio de la modulación de la actividad transcripcional de NF-κB, CREB y probablemente otros factores, dependiendo del tipo de célula infectada y de la bacteria en turno. En este contexto, uno de los objetivos a mediano plazo en nuestro laboratorio es realizar un análisis transcriptómico y proteómico global de los genes regulados por cada una de las isoformas de GSK3 cuando *S. aureus* infecta células endoteliales y epiteliales de humano. Esta evaluación *ómica* se combinará también con la identificación de factores de virulencia expresados por la bacteria y que son los responsables de activar NF-κB en fase temprana y CREB en fase tardía de la infección. Toda esta información que se genere sentará las bases para diseñar una estrategia biotecnológica cuya finalidad será eliminar *S. aureus* y promover la recuperación de la homeostasis celular.

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9. NOTAS FINALES

El descubrimiento de la penicilina en 1928 inició la era de los antibióticos y generó la búsqueda de nuevas drogas que eliminaran a los microorganismos patógenos. Durante los primeros años que los humanos recibieron dosis de penicilina y otros antibióticos relacionados estructuralmente, se observó una disminución notable en el número de personas infectadas. Sin embargo, el uso indiscriminado de antibióticos causó que las bacterias se adaptaran genéticamente, lo que ha resultado en resistencia múltiple a la mayoría de los antibióticos disponibles en el mercado. El problema recurrente de resistencia bacteriana ha provocado que la investigación en salud pública de un salto de calidad a una "*era post-antibióticos*", en la cual los antibióticos convencionales, y aún los de "*nueva generación*", están dejando de ser efectivos. Esto ha motivado la necesidad de encontrar alternativas para controlar a los microorganismos invasores y al mismo tiempo también controlar los daños generados por la inflamación que se presenta en los tejidos infectados. Una alternativa terapéutica son los péptidos antimicrobianos de origen natural; sin embargo, el problema es que son citotóxicos cuando se aplican directamente a las células infectadas. Por lo tanto, la búsqueda y el desarrollo de análogos sintéticos que carezcan de efectos adversos se ha convertido en una alternativa prometedora [56].

Los avances en bioinformática han permitido el desarrollo de nuevas estrategias al uso tradicional de anti-inflamatorios y antibióticos convencionales. En este sentido, se ha documentado en animales modelo que los péptidos catiónicos reguladores de la defensa inmune innata (IDR, por sus siglas en inglés), modulan la respuesta inmunitaria que coadyuva a eliminar al microorganismo patógeno y además reduce la severidad de la inflamación. Por ejemplo, se ha reportado que algunos de estos péptidos como IDR-1 e IDR-1002, pueden regular la actividad de moléculas con actividad pro-inflamatoria [57]; sin embargo, los detalles sobre el mecanismo molecular no son claros. En nuestro trabajo observamos que IDR-1002 indujo la fosforilación de p38, ERK1/2, y MSK1, lo cual incrementó la fosforilación de CREB en su sitio de activación Ser133 (Figura 2). Por otra parte, también se pudo demostrar que la vía de señalización PI3K-Akt-GSK3 no es relevante para esta fosforilación de CREB. A pesar del incremento en la fosforilación de CREB, no pudimos detectar un aumento en

la expresión de citocinas de naturaleza anti-inflamatoria (IL-4, IL-10 e IL-13). En otros trabajos también se ha reportado que la fosforilación de CREB en Ser133 por la vía p38/ERK1/2-MSK1 no promueve la interacción de CREB con CBP, que es fundamental para la expresión de genes [43].

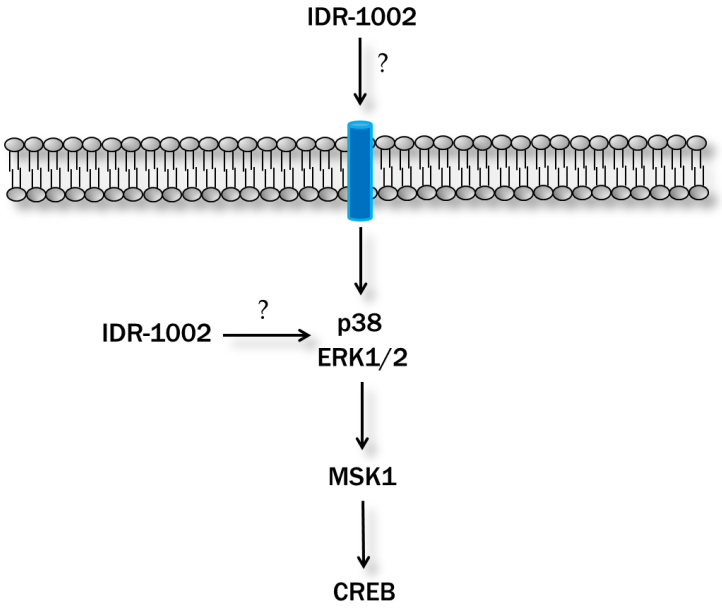

Figura 2. IDR-1002 induce la fosforilación de la vía ERK1/2/p38-MSK1-CREB. Se desconoce hasta ahora si IDR-1002 induce la activación de las proteínas cinasas ERK1/2, p38 y MSK1 mediante su interacción con un receptor de membrana plasmática o por interacción directa/indirecta en el citosol con ERK1/2 y p38.

La inflamación constante es un problema que puede causar daño fisiológico que de no resolverse satisfactoriamente puede llegar a causar el desarrollo de enfermedades crónico degenerativas como artritis y enfermedades cardiovasculares [58-60]. Varios padecimientos crónico-degenerativos como psoriasis, artritis reumatoide y aterosclerosis cursan con una expresión descontrolada de TNFα [61-63]. La búsqueda incesante para neutralizar a TNFα ha conducido al descubrimiento de CLP-19, un péptido sintético derivado del factor anti-LPS presente en los hemocitos del quelicerado marino *Limulus polyphemus.* Este péptido neutraliza la citotoxicidad de LPS debido a que inhibe la producción de TNFα inducida por LPS *in vitro* (PBMC) e *in vivo* (ratones) [64]. Se ha demostrado también que la catelecidina humana CAP18 y la catelicidina de cerdo CAP11 suprimen la expresión de TNFα en macrófagos Raw 264.7 estimulados con LPS [65]. En nuestro trabajo, se observó que IDR-1002 inhibió la expresión de COX-2 y TNFα inducida por LPS y que este mecanismo es dependiente de la inhibición de la fosforilación de IκBα, con lo cual se inhibe su degradación y se

bloquea translocación de NF-κB al núcleo inducida por LPS. Esto imposibilita la unión de NF-κB a CBP y se inhibe la expresión de COX-2 y TNFα.

En conclusión nuestros resultados indican que IDR-1002 inhibe la translocación nuclear de NF-κB inducida por LPS, mediante un mecanismo que involucra la inhibición de la fosforilación de IκBα. Este trabajo sienta las bases para seguir investigando los mecanismos antiinflamatorios de estos péptidos con la idea de desarrollar péptidos propios más potentes como una alternativa comercial al tratamiento de enfermedades crónico-degenerativas, inflamación e infecciones bacterianas.

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11. ANEXOS

11.1. ARTÍCULO 3: ORIGINAL INTERNACIONAL

THE GLYCOGEN SYNTHASE KINASE 3α AND β ISOFORMS DIFFERENTIALLY REGULATES INTERLEUKIN-12P40 EXPRESSION IN ENDOTHELIAL CELLS STIMULATED WITH PEPTIDOGLYCAN FROM *Staphylococcus aureus*.

Co-Autor 4: Alejandro Huante Mendoza.

El principal objetivo de este trabajo de investigación publicado en la revista Plos One fue evaluar si la expresión de la citocina IL-12p40 está regulada por la actividad enzimática de las isoformas GSK3α y GSK3β en células endoteliales estimuladas con péptidoglicano (PGN) de *Staphylococcus aureus*. Uno de los principales hallazgos fue confirmar que la abundancia relativa de GSK3α fosforilada en Ser21 fue consistentemente mayor que la isoforma GSK3β fosforilada en Ser9. Este resultado había sido obtenido previamente en el laboratorio y fue parte de un artículo publicado en la revista Infection and Immunity, del cual también soy co-autor y que se detalla más adelante en esta tesis. Los datos originales de este estudio indicaron que PGN indujo la fosforilación de GSK3α, GSK3β y NF-κB, mediante la activación de la vía TLR2/PI3K/Akt. La participación de cada isoforma de GSK3 por silenciamiento del gen respectivo con siRNA específico indicó que la expresión de IL-12p40 fue diferente dependiendo de la isoforma silenciada. Se observó que al silenciar el gen de GSK3α hubo un incremento significativo de la expresión de IL-12p40, mientras que el silenciamiento de GSK3β dio lugar a una disminución de la expresión de esta interleucina.

Mi participación en este trabajo de investigación consistió en la obtención de algunos resultados de fosforilación de Akt, GSK3α y GSK3β, incluidos como parte de la Figura 1.

PLOS | ONE

RESEARCH ARTICLE

The Glycogen Synthase Kinase 3α and β Isoforms Differentially Regulates Interleukin-12p40 Expression in Endothelial Cells Stimulated with Peptidoglycan from *Staphylococcus aureus*

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Abstract

Glycogen synthase kinase 3 (GSK3) is a constitutively active regulatory enzyme that is important in cancer, diabetes, and cardiovascular, neurodegenerative, and psychiatric diseases. While GSK3α is usually important in neurodegenerative and psychiatric diseases GSK3β is fundamental in the inflammatory response caused by bacterial components. Peptidoglycan (PGN), one of the most abundant cell-wall structures of Gram-positive bacteria, is an important inducer of inflammation. To evaluate whether inhibition of GSK3α and GSK3β activity in bovine endothelial cells (BEC) regulates the expression of the pro-inflammatory cytokine IL-12p40, we treated BEC with SDS-purified PGN from *Staphylococcus aureus*. We found that PGN triggered a TLR2/PI3K/Akt-dependent phosphorylation of GSK3α at Ser21, GSK3β at Ser9, and NF-κB p65 subunit (p65) at Ser536, and the phosphorylation of GSK3α was consistently higher than that of GSK3β. The expression of IL-12p40 was inhibited in BEC stimulated with PGN and pre-treated with a specific neutralizing anti-TLR2 antibody that targets the extracellular domain of TLR2 or by the addition of Akt-i IV (an Akt inhibitor). Inhibition of GSK3α and GSK3β with LiCl or SB216763 induced an increase in IL-12p40 mRNA and protein. The effect of each isoform on IL-12p40 expression was evaluated by siRNA-gene expression silencing of GSK3α and GSK3β. GSK3α gene silencing resulted in a marked increase in IL-12p40 mRNA and protein while GSK3β gene silencing had the opposite effect on IL-12p40 expression. These results indicate that the TLR2/PI3K/Akt-dependent inhibition of GSK3α activity also plays an important role in the inflammatory response caused by stimulation of BEC with PGN from *S. aureus*.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

Staphylococcus aureus causes important infectious diseases in animals and humans because it expresses a wide arrange of virulence factors and cell-wall associated structures that are responsible for damaging tissues [1]. One of the main cell wall structures of *S. aureus* is the peptidoglycan (PGN) that activates the innate immune system of the host and promotes inflammation [2] via the PI3K/Akt signaling pathway [3–5]. While it has been proposed that the intracellular receptors NOD1/2, but not TLR2, are the main proteins involved in PGN signaling [2], some studies have shown that TLR2 is a major receptor that senses PGN. For example, treatment of RAW264.7 macrophages with PGN induced the TLR2-dependent recruitment of p85α, Rac1 and Ras that mediates IKKα/β-NF-κB activation, and COX2 expression through the Rac1/PI3K/Akt and Ras/Raf1/Erk1-2 signaling pathways [3, 6]. Also, in BV-2 microglia, PGN activates the TLR2/MyD88/PI3K/Akt pathway, which leads to IκBα degradation, phosphorylation of NF-κB p65 subunit (p65) at Ser536, and expression of pro-inflammatory cytokines, iNOS, and COX2 [4]. Moreover, PGN binds to TLR2 in fibroblasts and activates the FAK/PI3K/Akt signaling and the transcription factor AP-1, leading to an increase in IL-6 expression [5].

The phosphoinositide 3-kinase/Akt (PI3K/Akt) signaling pathway mediates a variety of cellular responses such as survival, proliferation, differentiation, apoptosis, and as mentioned above, inflammation [7, 8]. Activation of PI3K/Akt pathway leads to the PI3K-dependent synthesis of phosphatydilinositol-3,4,5-triphosphate (PIP_3) and phosphorylation of Akt at Thr308 and Ser473 by the constitutively active PDK1 [9, 10] and mTORC2 [11, 12]. Akt in turn regulates the activity of a wide range of substrates, among which glycogen synthase kinase 3 (GSK3) is important in the modulation of the inflammatory response [8, 13]. GSK3 refers to two mammalian paralogs that are commonly called GSK3α and GSK3β isoforms [14], which are constitutively active and can be inactivated by phosphorylation at Ser21 (GSK3α) or Ser9 (GSK3β) by Akt [15]. Since its discovery, GSK3β has been shown to be involved in the regulation of many cellular functions including growth, differentiation, embryonic development, cell cycle progression, apoptosis [16, 17] and in the inflammatory response caused by bacterial infection through the regulation of NF-κB activity [13, 15]. In regard to GSK3α most of the glucose/glycogen homeostasis appears to depend mainly on this isoform, with a minor contribution of GSK3β in skeletal muscle [18–20]. Also, GSK3α plays a potential role as a regulatory enzyme of the central nervous system [21]. This isoform, but not GSK3β, has recently been identified in the maintenance and/or proliferation of Th17 cells stimulated with the pro-inflammatory cytokine IL-1 [22]. However, participation of GSK3α in the modulation of inflammation, triggered by microbial products has not been well documented. Studies in murine models indicate that both isoforms of GSK3 are not physiologically redundant [16, 18, 23]. Recently, it was found that LiCl inhibition of GSK3α in lipopolysaccharide (LPS)-activated neutrophils and in the murine dorsal air-pouch model lead to a large increase in TNF-α secretion by affecting the translational mechanism of the TNF-α protein without altering its mRNA levels [24]. Furthermore, in a previous report we demonstrated that internalization of *S. aureus* by endothelial cells is associated with the PI3K/Akt activity [25]. Although the correlation between *S. aureus* internalization and GSK3α or GSK3β activity was not analyzed in that report, we indeed observed a higher phosphorylation of GSK3α at Ser21 than GSK3β at Ser9 [25]. Thus, it is likely that GSK3α has also regulatory functions in the inflammatory response induced by *S. aureus*.

Interleukin (IL)-12 is an important pro-inflammatory cytokine because its expression during bacterial infection regulates the innate response and determines the type and duration of the adaptive immune response [26, 27]. Structurally, this cytokine is a heterodimer composed of two subunits designated p35 and p40 linked by disulfide bonds [28, 29]. The *IL-12p40* gene is highly inducible by microbial products such as LPS, lipoteichoic acid (LTA) and PGN via

Toll-like receptor signaling and NF-κB activation [27]. Antigen-presenting cells and phagocytic cells are the primary producers of IL-12 [27], although human endothelial cells also produce it [30]. Despite IL-12 is essential for host defense, its overexpression can cause persistent inflammation giving rise to autoimmune disorders. To counterbalance the action of IL-12, immune cells produce IL-10 that decreases NF-κB and AP-1 activity, and at the same time increases CREB activity [31, 32].

Stimulation of human monocytes and peripheral blood mononuclear cells (PBMCs) with agonists of TLR2 (LTA from *Streptococcus pneumoniae*), TLR4 (LPS or synthetic lipid A), TLR5 (flagellin from *Salmonella* Typhimurium), or TLR9 (human CpG), reduced the expression of IL-12p40 through the inhibition of GSK3β with no participation of the GSK3α isoform [32]. In contrast, data presented in this work indicate that stimulation of bovine endothelial cells (BEC) with PGN from *S. aureus* modulates the expression of IL-12p40 through the inhibition of GSK3α and GSK3β. Interestingly, inhibition of GSK3α with pharmacological drugs or its gene expression silencing with interference RNA in BEC stimulated with PGN produced a marked increase in the expression of IL-12p40. In similar experiments, directed to GSK3β, we observed a reduced expression of IL-12p40. In both cases the mechanism involved the activation of the TLR2/PI3K/Akt signaling pathway. Altogether, the biochemical evidence presented indicates that both isoforms of GSK3 differentially modulate the expression of IL-12p40, a pro-inflammatory cytokine.

Materials and Methods

Media and Chemicals

F-12 Ham (HF-12) of Dulbecco's modified Eagle's medium, bovine serum albumin (BSA), trypsin-EDTA, Igepal CA-930, PGN from *S. aureus* 77140, Wortmannin (Wort), Akt-i IV, LY294002 (LY), SB216763 (SB), NaCl, LiCl, puromycin, and Bradford reagents were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Fetal calf serum (FCS) was acquired from Equitech-Bio, Inc. (Kerrville, TX, USA). A cocktail of sodium penicillin G, streptomycin sulfate, and amphotericin B was purchased from Gibco-BRL (Gaithesburg, MD, USA). Akt Inhibitor II, D-3-Deoxy-2-O-methyl-myo-inositol 1-[(R)-2-methoxy-3-(octadecyloxy) propyl hydrogen phosphate (SH-5) was acquired from Calbiochem (Darmstadt, Germany). Halt Phosphatase inhibitor cocktail was purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA). Protease inhibitor cocktail was acquired from GE Healthcare Bio-sciences (Little Chalfont, UK). Trizol reagent and EXPRESS One-Step SYBR GreenER Universal Kit were purchased from Invitrogen (Carlsbad, CA, USA). Bovine Interleukin 12 (IL-12/p40) TSZ ELISA kit was purchased from Biotang (Massachusetts, USA). All other reagents were acquired from Sigma-Aldrich.

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Antibodies

Rabbit polyclonal antibodies against the extracellular domain of TLR2 (N-17; sc-8689), the isotype unspecific IgG, and goat anti-rabbit IgG-HRP were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Rabbit polyclonal antibodies against phospho-glycogen synthase (Ser641) and rabbit monoclonal antibodies against phospho-Akt (Ser473), phospho-GSK3α (Ser21), phospho-GSK3β (Ser9), phospho-p65 (Ser536), Akt, GSK3β, and NF-κB p65 subunit were purchased from Cell Signaling Technology (Boston, MA, USA).

Cell Line and Culture Conditions

The endothelial cell line used as a model cell in this study was obtained from bovine umbilical veins and immortalized by transfection with an expression vector containing the E6-E7

oncogenes of human papillomavirus 16 (BVE-E6E7) [33]. This immortalized bovine endothelial cell line, called BEC in this study, was grown and maintained in HF-12 supplemented with 10% FCS and a cocktail of sodium penicillin G, streptomycin sulfate, and amphotericin B, unless otherwise noted.

Purification of PGN

We eliminated lipopeptides from commercial PGN preparations from *S. aureus* (Sigma-Aldrich) as previously described by Dziarski and Gupta (2005) [34]. Briefly, 2 mg of PGN per mL were treated with 8% sodium dodecyl sulfate (SDS) at 90°C for 30 min, followed by 10 washes with H_2O to remove SDS. The concentration of purified PGN was calculated spectrophotometrically at 450 nm by adjusting the value of the dispersion to the same value obtained from commercial PGN that was prepared at 1 μg/mL.

BEC Transfection

BEC (2×10^5) were grown in six-well culture plates with HF-12K without serum and antibiotics for 24 h. Then, each well was transfected with the X-tremeGENE HP DNA Transfection Reagent kit (Roche). We used either 3 μg of siRNA-GSK3α plasmids (Sigma Aldrich, clone IDs, pLKO.1-GSK3α1: NM_019884.1-948s1c1; pLKO.1-GSK3α2: NM_019884.1-567s1c1, and pLKO.1-GSK3α3: NM_019884.2-1207s1c1) or siRNA-GSK3β plasmids (pLKO.1-GSK3β1 and pLKO.1-GSK3β2) that were gifts from Alex Toker (Addgene plasmid # 32496 and # 32497). Control cells were transfected with 3 μg of the pLKO.1 plasmid (pLKO.1) that was a kind gift from Bob Weinberg (Addgene plasmid # 8453). For GSK3α the targeting sequences were: 5'-TACATCTGTTCTCGCTACTA-3' (nucleotides 1535–1554, pLKO.1-GSK3α1); 5'-CCAGGACAAGAGGTTCAAGAA-3' (nucleotides 1153–1173, pLKO.1-GSK3α2); 5'-CCTGGACAAAGGTGTTCAAAT-3' (nucleotides 1788–1808, pLKO.1-GSK3α3); 5'-GAAGTCAGCTATACAGACACT-3' (nucleotides 587–607, pLKO.1-GSK3β1) and 5'-GAAAGCTAGATCACTGTAACA-3' (nucleotides 735–755, pLKO.1-GSK3β2). After 24 h, the culture medium was changed to HF-12K with serum plus 0.8 μg/mL of puromycin and cells were incubated for 15 days to select for stable transfections. BEC were recovered and sub-cultured in six-well culture plates in HF-12K without serum, incubated for 24 h and stimulated with PGN, as described below.

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Protein Extraction and Western Blot Assays

To test for the relative abundance of phosphorylated and non-phosphorylated proteins, BEC were grown in six-well tissue culture plates (Ultra Cruz) to approximately 90% confluence before serum starvation for at least 4 h. Total protein (cytosolic plus nuclear) from control and treated cells was obtained by washing the cells 2X with cold PBS and lysing them with 100 μl of a cold lysis buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Igepal CA-930, 10 mM Na-pyrophosphate, 50 mM NaF and 1 mM Na-orthovanadate supplemented with 1X protease inhibitor cocktail and 1X phosphatase inhibitor cocktail, which were added immediately before lysing the endothelial cells. The lysates were centrifuged at 16,000 xg for 20 min at 4°C and the supernatant was transferred to ice-cold Eppendorf tubes. Protein concentration was measured by the Bradford method [35] using BSA as standard. Then, 30–40 μg of protein was separated by electrophoresis in 10% sodium dodecyl sulfate-polyacrylamide gels and electroblotted in a wet chamber onto 0.45 μm nitrocellulose membrane (Bio-Rad) at 250–300 mA for 1 h. Membranes were probed with primary polyclonal antibodies to phosphorylated forms of Akt, GSK3α, GSK3β, or p65. Then, membranes were stripped, reprobed with secondary monoclonal antibodies to the non-phosphorylated form of Akt or polyclonal antibodies to GSK3β or

p65 as controls of protein loading, and detected with the Immobilon Western Chemiluminescent HRP substrate kit from Millipore (Billerica, MA, USA).

RNA Extraction and qRT-PCR

To analyze the relative expression of IL-12p40 mRNA, BEC were grown in six-well culture plates to approximately 90% confluence before serum starvation for at least 4 h. Then 10 μg/mL of PGN was added to the cultured cells, centrifuged at 130 xg for 5 min and incubated for 2, 4 or 8 h at 37°C in 5% CO_2, or pretreated with 10 μM SH-5, 10 μM SB216763, 10 mM NaCl, 10 mM LiCl or 5 μg/mL anti-TLR2 for 1 h, or with Akt-i IV for 0.5 h followed by washing with HF-12 without serum and stimulation with 10 μg/mL PGN, centrifuged at 130 xg for 5 min and incubated for 4 h at 37°C in 5% CO_2. At the end of the incubation period, BEC were washed 2X with cold PBS and total RNA was extracted using 1 mL Trizol of reagent following the isolation procedure described by the supplier. One-step reverse transcription and real-time quantitative PCR (qRT-PCR) was performed using the EXPRESS One-Step SYBR GreenER Universal Kit and the real-time StepOnePlus thermocycler from Applied Biosystems. Each reaction was performed with 100 ng/μL of RNA under the standard 20 μL reaction provided by Invitrogen. The one-step cycling program conditions was: 58°C (for *IL12p40*) and 50°C (for *β-actin*) for 5 min (cDNA synthesis); 95°C for 2 min; 40 cycles at 95°C for 15 s and 55°C (for *IL12p40*), 60°C (for *β-actin*) for 1 min. The oligonucleotide primers used were based on the sequences published by Konnai et al. 2003 [36]. Amplification of the expected single products (186 pb for *IL12p40* and 227 pb for *β-actin*) was confirmed by visualization on 1% agarose gels stained with ethidium bromide. Relative transcript levels of IL-12p40 mRNA were calculated with the delta-delta C_t method, using β-actin as the reference gene.

Measurement of IL-12p40 protein levels

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Bovine IL-12p40 protein in culture supernatants was measured by sandwich ELISA, according to manufacturer instructions (Biotang).

Statistical analysis

The relative abundance of phosphorylated proteins was quantitated by densitometric analysis with the Image Processing and Analysis in Java Program ImageJ (http://rsbweb.nih.gov/ij). To calculate the densitometric values, the intensity of the phosphorylated band was divided by the intensity of the non-phosphorylated ones. These intensity values were referred to a value of 1.0 that was arbitrarily assigned to the untreated control. The statistical significance of triplicate blots, IL-12p40 mRNA levels and IL-12p40 protein levels were evaluated with the Tukey multiple-comparison test and One-Way analysis of variance (ANOVA) by using the SIGMASTAT program version 3.0 (SPSS Inc., Chicago, IL, USA). P values <0.05 or <0.01 or <0.001 were considered statistically significant.

Results

Phosphorylation of Akt, GSK3α and GSK3β in BEC Stimulated with PGN Depends on TLR2

In macrophages, microglia and fibroblasts PGN induces the expression of pro-inflammatory molecules by activation of the PI3K/Akt signaling pathway in a TLR2-dependent manner [3–6], and Akt-dependent inhibition of GSK3β by phosphorylation at Ser9 [15]. Therefore, we first decided to explore the involvement of TLR2 in the phosphorylation of Akt, GSK3α and GSK3β in BEC stimulated with PGN. We observed that in BEC stimulated with 10 μg/mL of

PGN for 15 min (S1A and S1B Fig), the relative abundance of phosphorylated Akt (∼2-fold), GSK3α (∼6-fold) and GSK3β(∼3-fold) were inhibited in BEC pre-treated with a neutralizing antibody against the extracellular domain of TLR2 (Fig 1A, 1B and 1C). Phosphorylation of Akt was not affected in BEC pre-treated with an unspecific isotype IgG (Fig 1D). It has been reported that PGN induces the expression of TLR2 in microglia and fibroblasts [4, 5]. To rule out this effect, we evaluated the relative abundance of TLR2 in BEC stimulated with PGN at different times and no change was observed in the levels of TLR2 (S1C Fig). A complete loss of Akt phosphorylation was observed when BEC were pre-treated with LY-294002 (LY, an inhibitor of PI3K) and then stimulated with PGN (S1D Fig). Analysis of GSK3α/β phosphorylation confirmed that stimulation of BEC with 10 μg/mL of PGN for 15 to 60 min induced a strong increase in GSK3α phosphorylation at Ser21 compared with the minor increase in GSK3β phosphorylation at Ser9 (S2A and S2B Fig). These results indicate that 1) PGN induces TLR2-dependent phosphorylation of Akt, GSK3α and GSK3β in endothelial cells and 2) GSK3α was consistently the isoform with the highest level of phosphorylation.

PGN Activates the PI3K/Akt-Dependent Phosphorylation of GSK3α and GSK3β in BEC

Next, we tested if phosphorylation of GSK3α and GSK3β in endothelial cells stimulated with PGN required activation of PI3K and Akt. Pre-treatment of BEC with LY or Wortmannin (Wort, an inhibitor of PI3K) and SH-5 (an inhibitor of Akt) and stimulated with PGN induced a significant decrease in GSK3α and GSK3β phosphorylation (Fig 2A and 2B). No significant changes in phosphorylation levels were observed when cells were treated with the inhibitors for PI3K (LY), Akt (Akt-i IV) and GSK3 (SB-216763, SB) alone (S3A–S3C Fig). These data indicate that phospho-inhibition of GSK3α and GSK3β in BEC depends on the PI3K and Akt activity.

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Phosphorylation of GSK3α and GSK3β by Akt in BEC Stimulated with PGN Inhibited the Ability of both Isoforms to Phosphorylate Glycogen Synthase

To test if phosphorylation of GSK3α at Ser21 and GSK3β at Ser9 resulted in the inhibition of their enzymatic activity, we evaluated the phosphorylation of glycogen synthase (GS) at Ser641, one of the downstream targets of both isoforms. Incubation of BEC with PGN caused the inhibition of 40% to 60% GS phosphorylation, compared with the untreated control (Fig 3). Pre-treatment of BEC with LiCl or SB (two inhibitors of GSK3), SB plus PGN or LiCl plus PGN caused an even stronger reduction in GS phosphorylation. These effects were not due to an osmolarity effect because treatment of BEC with 10 mM NaCl did not change the phosphorylation level of GS in cells not stimulated or stimulated with PGN (Fig 3). These data indicate that phosphorylation of GSK3 in BEC stimulated with PGN induced a significant reduction of GSK3 activity.

PGN Regulates the Expression of IL-12p40 through a Mechanism that Involves TLR2/Akt Activation and GSK3 Inhibition

One of the most important cytokines produced during the initial stages of the inflammatory response is the IL-12p40. The expression of this cytokine in macrophages stimulated with different PAMPs is down-regulated due to the inhibitory effect on GSK3β activity [32]. Therefore, we determined if IL-12p40 expression is regulated in a similar manner in endothelial cells stimulated with PGN from *S. aureus*. When transcript levels of IL-12p40 were analyzed in BEC stimulated with PGN we observed an increase in IL-12p40 mRNA at 4 h, which decreased to

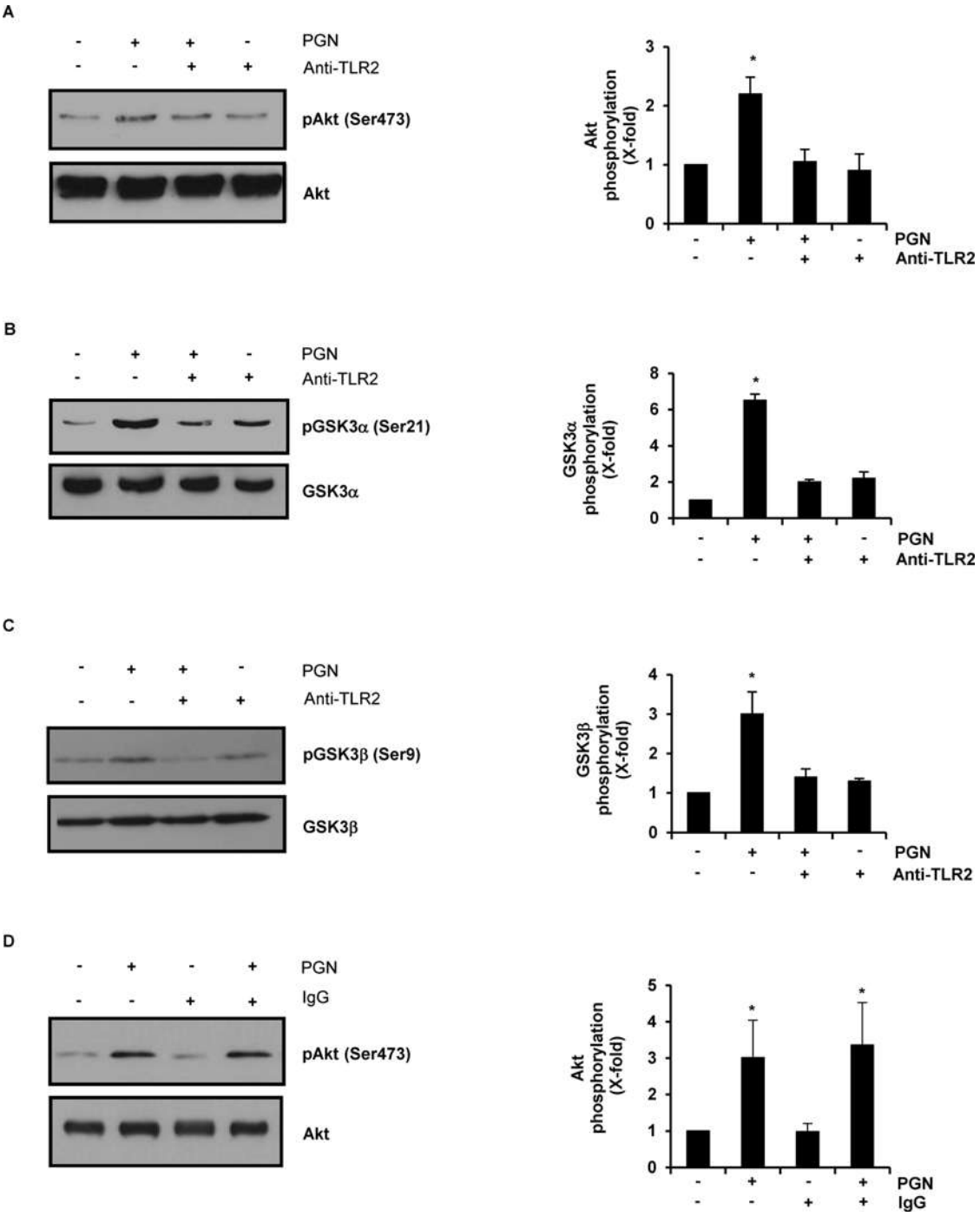

Fig 1. Peptidoglycan (PGN) induces phosphorylation of Akt, GSK3α and GSK3β in bovine endothelial cells (BEC) through TLR2 activation. A, B, C) BEC were either stimulated with 10 μg/mL of PGN for 30 min, pre-incubated with 5 μg/mL of neutralizing antibody against the extracellular domain of TLR2 (anti-TLR2) for 60 min and stimulated with 10 μg/mL of PGN for 30 min or pre-incubated with 5 μg/mL of anti-TLR2 for 60 min. D) BEC were either stimulated with 10 μg/mL of PGN for 30 min, pre-incubated with 5 μg/mL of an isotype anti-IgG for 60 min or pre-incubated with 5 μg/mL of anti-IgG for 60 min and stimulated with 10 μg/mL of PGN for 30 min. As control in all cases A-D, BEC were left untreated (-). After treatments, protein extracts were analyzed by western blot and probed with monoclonal antibodies against the phosphorylated forms of Akt1 (pAkt Ser473), GSK3α (pGSK3α Ser21) or GSK3β (pGSK3β Ser9). Blots were stripped and reprobed with an antibody that recognizes the nonphosphorylated forms of Akt (A, and D), GSK3α (B) or GSK3β (C) to verify equal protein loading. Blots are representative of three independent experiments. Graphs on the right panel indicate the band intensity obtained by densitometric analysis. Results are expressed as the mean ± S.E.M. ($n = 3$). *$p < 0.05$, compared with the untreated control.

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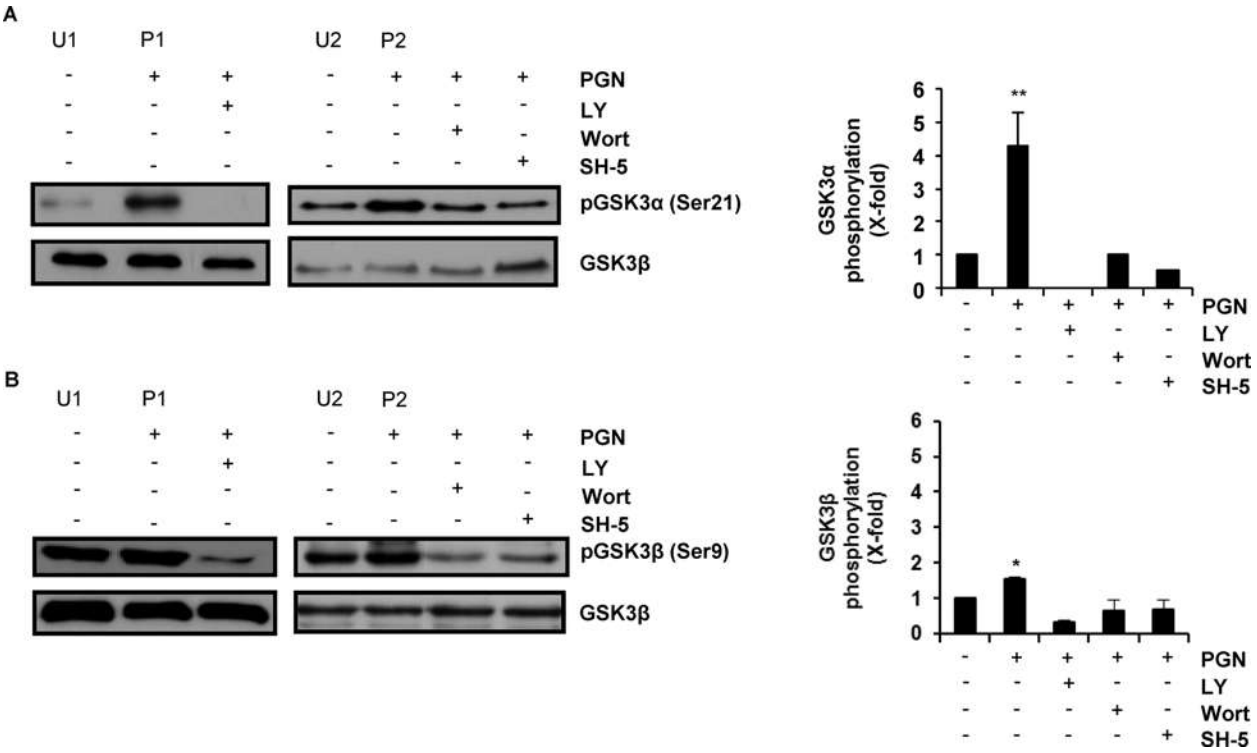

Fig 2. PGN induces PI3K/Akt-dependent phosphorylation of GSK3α and GSK3β in BEC. A-B) BEC were untreated and unstimulated (U1 and U2), stimulated with 10 μg/mL of PGN for 30 min (P1 and P2) or treated with either 10 μM of LY294002 (LY), 100 nM of Wortmannin (Wort) or 10 μM of SH-5 for 30 min, and then stimulated with 10 μg/mL of PGN for 30 min. Protein extracts were analyzed by western blot and probed with monoclonal antibodies against the phosphorylated forms of GSK3α (pGSK3α Ser21) or GSK3β (pGSK3β Ser9. To verify for equal amount of proteins, blots were stripped and reprobed with an antibody that recognizes the nonphosphorylated form of GSK3β. Blots are representative of three independent experiments. Graphs indicate the band intensity obtained by densitometric analysis. In each graph, the densitometric control values plotted were the average of U1 + U2 while the values plotted for the PGN-stimulated cells were the average of P1 + P2. Results are expressed as the mean ± S.E.M. ($n = 3$). *$p < 0.05$; **$p < 0.01$, compared with the unstimulated control.

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the control level at 8 h (Fig 4A). This increase was dependent on TLR2 activation because treatment of BEC with a neutralizing anti-TLR2 reduced the IL-12p40 to the level of control mRNA (Fig 4B and 4C). Inhibition of Akt activity strongly reduced the IL-12p40 protein levels (Fig 4D). In contrast, inhibition of GSK3 activity with LiCl or SB caused a strong increase in IL-12p40 protein amounts (Fig 4E and 4F). These results indicate that IL-12p40 expression in BEC stimulated with PGN was associated with activation of TLR2/Akt and inhibition of GSK3.

GSK3 can both positively and negatively affect different transcription factors such as NF-κB and CREB that are responsible for the regulation of pro- and anti- inflammatory cytokine production, respectively [32]. Thus, we tested the relative abundance of NF-κB p65 subunit phosphorylation at Ser536 (one of the phosphorylation sites at the transactivation domain) when both isoforms of GSK3 were inhibited with LiCl and stimulated with PGN. Data obtained show a ∼5-fold increase in p65 phosphorylation in BEC stimulated with PGN alone and an even stronger increase (∼9 fold) in BEC pre-treated with LiCl (S4A Fig). Pretreatment of BEC with NaCl was included to rule out any osmolarity effect (S4A Fig). Next, we performed GSK3α/β gene silencing. Surprisingly, siRNA gene silencing of either GSK3α or GSK3β in BEC stimulated with PGN produced a strong increase in p65 phosphorylation (S4B Fig). We reproducibly observed less phospho-p65 relative abundance when expression of GSK3β was silenced by siRNA. Perhaps, this may imply that phosphorylation at Ser536 contributes differentially not only to the expression of IL-12p40 but also to other NF-κB-regulated genes.

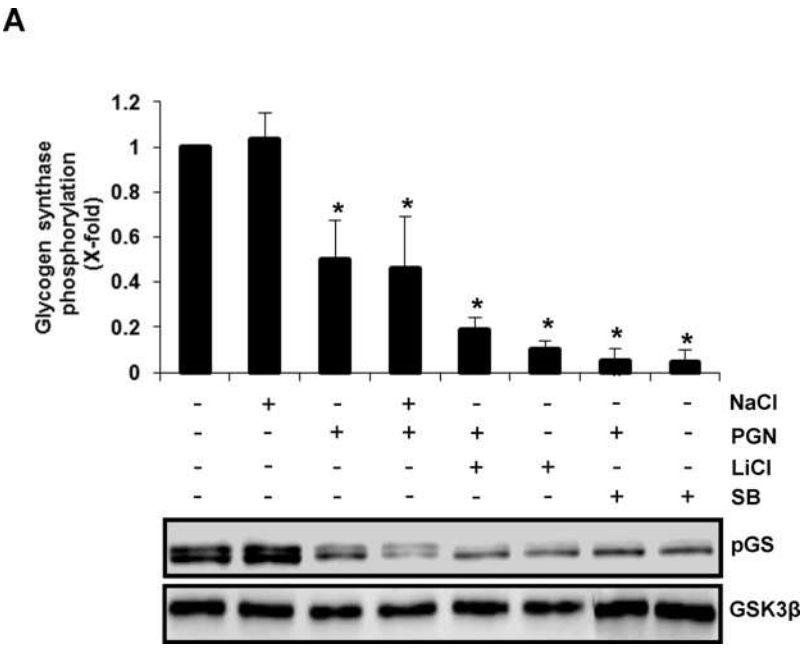

Fig 3. Inhibition of glycogen synthase phosphorylation at Ser641 in BEC treated with PGN, LiCl or SB, and stabilization of β-catenin levels by GSK3α or GSK3β gene silencing. A) BEC were either stimulated with 10 μg/mL of PGN for 30 min, treated with 10 mM NaCl for 60 min, 10 mM LiCl or 10 μM SB and then stimulated with 10 μg/mL of PGN for 30 min. As controls BEC were left untreated (-) or incubated with NaCl, LiCl or SB alone for 60 min. B) BEC were transfected with siRNA targeting GSK3α (siRNA GSK3α), siRNA targeting GSK3β (siRNA GSK3β), control siRNA (siRNA control) or left untransfected (-) for 16 days. After transfection, BEC were incubated for 24 h. Protein extracts were analyzed by western blot and probed with a monoclonal antibody against the phosphorylated form of glycogen synthase (GS Ser641) or the nonphosphorylated form of β-catenin. To verify that equal amount of protein was loaded in each lane, blots were stripped and reprobed with antibodies that recognize the nonphosphorylated forms of GSK3β (A) or β-actin (B). Blots are representative of three independent experiments. Graphs indicate the band intensity obtained by densitometric analysis. Results are expressed as the mean ± S.E.M. ($n = 3$). *$p<0.05$, compared with the untreated (A) or untransfected (B) control.

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Inhibition of GSK3α Activity is Responsible for IL-12p40 Increase

A number of reports have confirmed that GSK3β is the isoform involved in the regulation of pro- and anti-inflammatory cytokines expression. Such experimental evidence, along with the fact that in our case we consistently observed that GSK3α was the isoform more phosphorylated when BEC were stimulated with PGN, prompted us to explore which GSK3 isoform was responsible for the IL-12p40 increase. First, we obtained the evidence of gene silencing for each isoform (Fig 5A). Next, as another control of GSK3α/β gene silencing, we measured the total (cytoplasmic and nuclear) relative abundance of β–catenin because it is known that in resting cells the constitutive activity of GSK3 negatively regulates the Wnt/β-catenin signaling. That is GSK3-dependent phosphorylation of β-catenin promotes its ubiquitylation and subsequent degradation by the proteasome 26S [20]. Thus, when the activity of GSK3 isoforms is inhibited by chemical compounds or the genes of the isoforms are silenced by siRNA treatment, the phosphorylation of β-catenin is blocked and this in turn induces an intracellular accumulation of β-catenin. Transfection of BEC with siRNA targeting GSK3α (siRNA GSK3α) or with siRNA targeting GSK3β (siRNA GSK3β) affected the activity of GSK3 because we detected an increase in total β-catenin levels compared with un-transfected or transfected BEC with control siRNA (siRNA control) (Fig 5B, S1 Dataset). These data demonstrate that the activity GSK3α

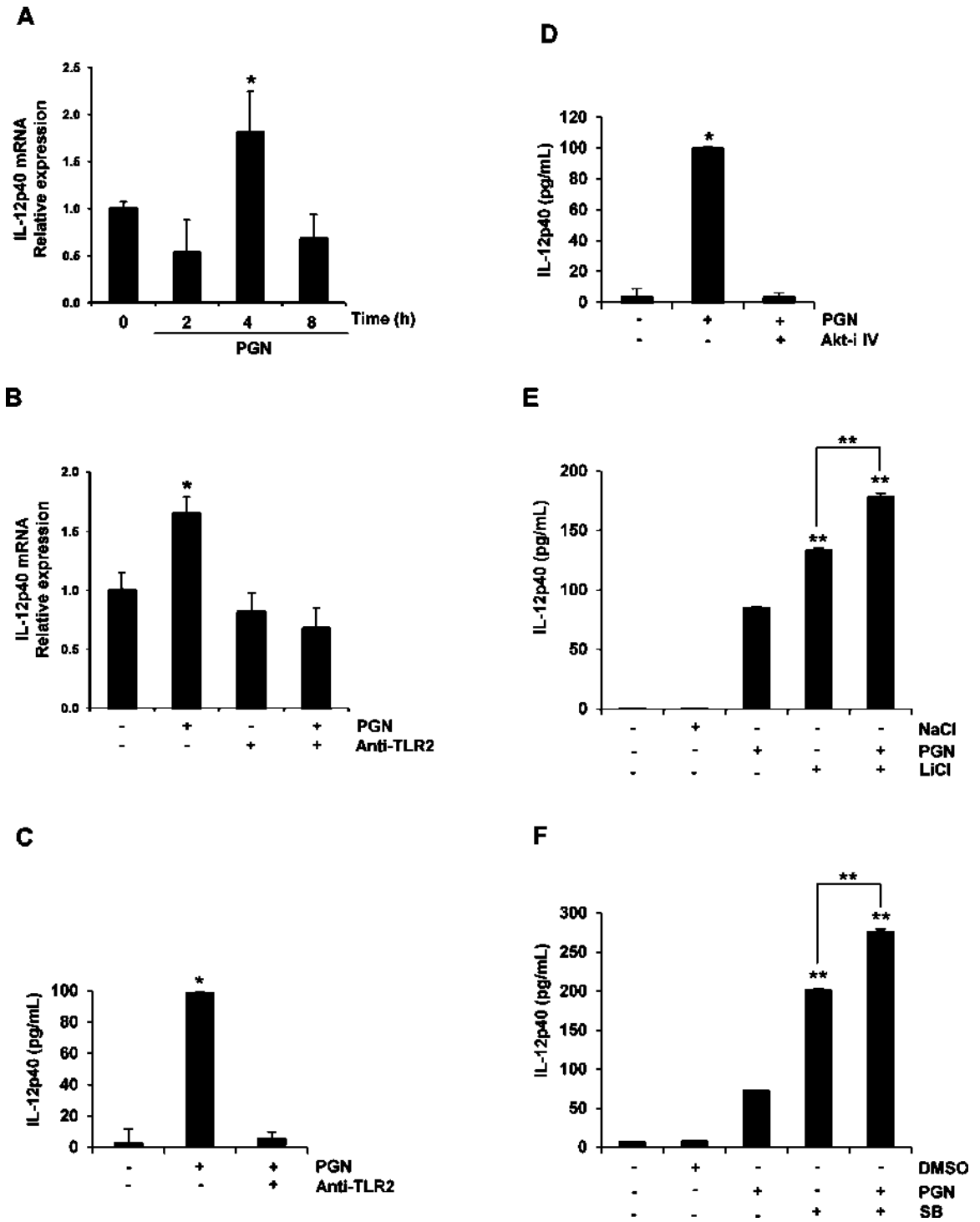

Fig 4. PGN induces IL-12p40 expression through TLR2/Akt activation and GSK3 inhibition in BEC. A) BEC were left untreated and unstimulated (0) or stimulated with 10 μg/mL of PGN-prep for 2, 4 or 8 h. B) BEC were stimulated with 10 μg/mL of PGN for 4 h, treated with 5 μg/mL of neutralizing anti-TLR2 for 60 min, or treated with 5 μg/mL of anti-TLR2 for 60 min and then stimulated with 10 μg/mL of PGN for 4 h. C) BEC were stimulated with 10 μg/mL of PGN for 9 h or treated with 5 μg/mL of anti-TLR2 for 60 min and then stimulated with 10 μg/mL of PGN for 9 h. D) BEC were stimulated with 10 μg/mL of PGN for 9 h or pretreated with 1 μM of Akt inhibitor IV (Akt-i IV) for 30 min and then stimulated with 10 μg/mL for 9 h. E) BEC were treated with 10 mM NaCl for 60 min, stimulated with 10 μg/mL of PGN for 9 h, treated with 10 mM of LiCl for 60 min or treated with 10 mM of LiCl for 60 min and then stimulated with 10 μg/mL of PGN for 9 h. F) BEC were treated with 10 μM of DMSO, stimulated with 10 μg/mL of PGN for 9 h, treated with 10 μM of SB 216763 (SB) for 30 min or treated with 10 μM of SB 216763 (SB) for 30 min and then stimulated with 10 μg/mL of PGN for 9 h. As controls, in B-F BEC were left untreated and stimulated (-). Total RNA was extracted and relative transcript level of IL-12p40 was quantitated by qRT-PCR using the delta-delta Ct method, and amplification of β-actin

as a reference gene (A-B). Cell-free supernatants were analyzed by ELISA for production of IL-12p40 (C-F). Results are expressed as the mean ± S.E.M. ($n = 3$). *p <0.05; **p <0.01. All data were compared with the untreated and unstimulated controls.

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and GSK3β regulates the stability of β–catenin in BEC, as it has been confirmed in other types of cells [20].

Analysis of IL-12p40 expression at the level of mRNA and protein showed a significant increase in BEC transfected with siRNA GSK3α and stimulated with PGN, compared with BEC stimulated with PGN alone or BEC transfected with siRNA control and stimulated with PGN (Fig 5C and 5D, S2 Dataset). On the other hand, and according to Martin et al. (2005) [32], a significant down-regulation of IL-12p40 was obtained in BEC transfected with siRNA GSK3β and stimulated with PGN (Fig 6A and 6B, S3 Dataset). To rule out any off-target effect of the siRNA sequence used, we tested other siRNA sequences for each GSK3 isoform. Quantitation of the IL-12p40 protein levels after silencing GSK3α or GSK3β with different siRNA sequences gave comparable values to those previously obtained (S5 Fig, and S4 Dataset). Altogether these results indicate that both GSK3α and GSK3β differentially modulate the expression of IL-12p40 in endothelial cells stimulated with PGN from *S. aureus*.

Discussion

The novel findings of this work can be summarized as follows (Fig 7): 1) GSK3 functions as a modulator of the inflammatory response in endothelial cells stimulated with PGN. Previous reports have documented that PGN activates TLR2/PI3K/Akt and induces the expression of pro-inflammatory molecules [3–5]; however, they did not explore how this activation and production of pro-inflammatory cytokines was correlated with changes in GSK3 activity; 2) although it has been accepted that GSK3β is the only isoform that mediates inflammation in cells stimulated with bacterial virulence factors and other stimuli [13] we have found that both GSK3α and GSK3β regulate IL-12p40 expression in endothelial cells stimulated with PGN; 3) the effect of the isoforms on IL-12p40 was different because inhibition of GSK3α activity resulted in the increased expression of IL-12p40 while inhibition of GSK3β activity lead to a decreased expression of the same cytokine and 4) the inhibition of GSK3α and GSK3β was linked to the activation of the TLR2/PI3K/Akt signaling pathway.

Endothelial cell activation during an inflammatory process may be divided into rapid and slow responses that are independent and dependent on new gene expression, respectively [37]. TLRs of endothelial cells play a fundamental role in the regulation of the inflammatory response upon exposure to any of the currently known TLR ligands [38]. PGN, one of the major cell-wall structures of *S. aureus*, is an important inducer of the inflammatory response [39]. Although, it is known that activation of the TLR2/PI3K/Akt signaling pathway by PGN induces the activation of NF-κB and the expression of pro-inflammatory molecules such as cytokines, COX2 and iNOS (3, 4), no report has shown that GSK3α or GSK3β inhibition by this signaling pathway regulates pro-inflammatory cytokine expression in response to PGN from *S. aureus*. Our findings, using BEC as a model cell, are the first to show that phospho-inhibition of both GSK3α and GSK3β, but predominantly GSK3α, links the TLR2/PI3K/Akt signaling pathway activation with phosphorylation of the NF-κB p65 subunit at Ser536. Furthermore, the inhibition of both isoforms is associated with a differential regulation of IL-12p40 expression (Figs 4–6), a multifunctional cytokine with important tasks in the innate and adaptive immune responses [26, 27].

A number of reports have documented that TLR2 plays a crucial role in the host response against *S. aureus* because knockout mice deficient in TLR2 are highly susceptible to

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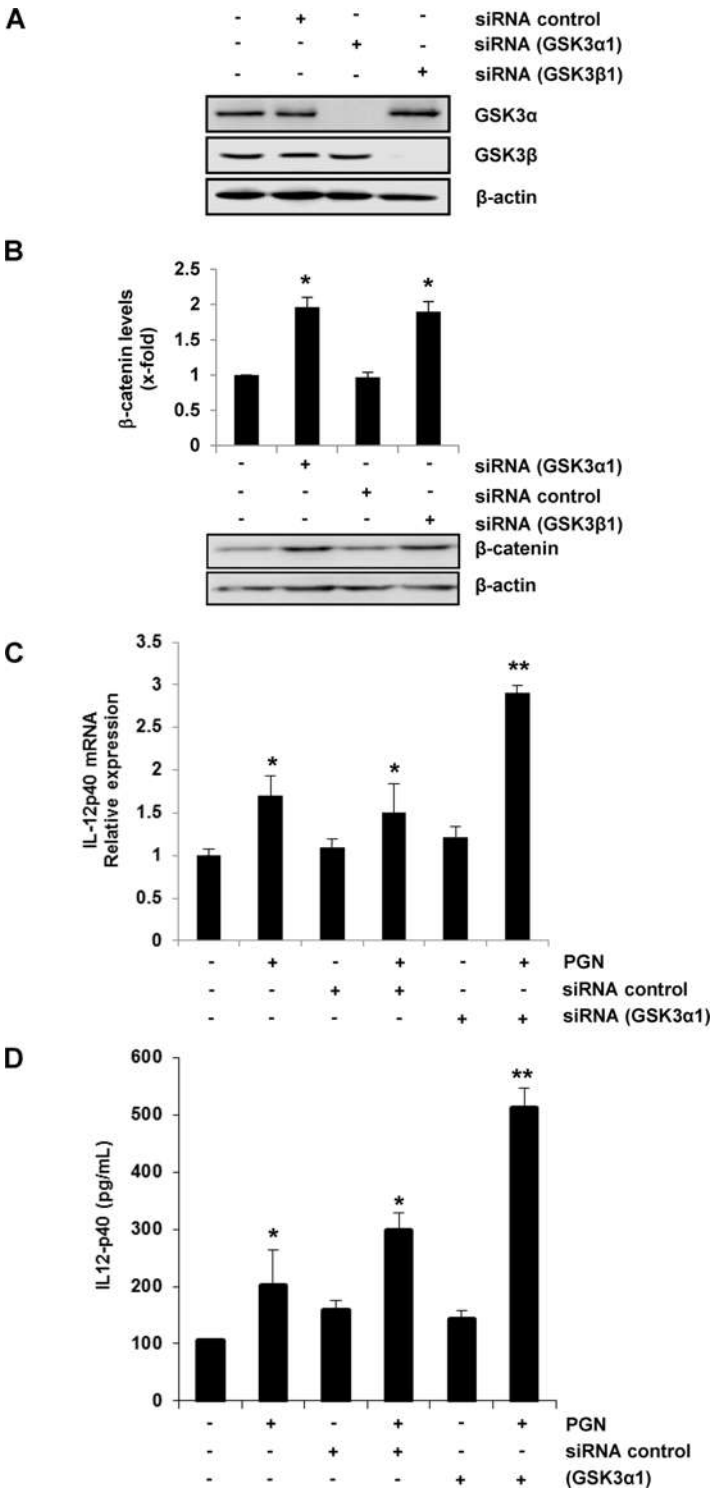

Fig 5. GSK3α inhibition up-regulates IL-12p40 expression in BEC stimulated with PGN. A) BEC were left untransfected (-), transfected with control siRNA (siRNA control), transfected with siRNA targeting GSK3α (siRNA GSK3α1) or transfected with siRNA targeting GSK3β (siRNA GSK3β1). B) BEC were left untransfected and unstimulated (-), stimulated with 10 μg/mL of PGN for 4 h, transfected with control siRNA (siRNA control), transfected with control siRNA and then stimulated with 10 μg/mL PGN for 4 h, transfected with siRNA targeting GSK3α (siRNA GSK3α1) or transfected with siRNA targeting GSK3α (siRNA GSK3α1) and then stimulated with 10 μg/mL of PGN for 4 h. C) BEC were left untransfected and unstimulated (-),

stimulated with 10 μg/mL of PGN for 9 h, transfected with control siRNA (siRNA control), transfected with control siRNA and then stimulated with 10 μg/mL of PGN for 9 h, transfected with siRNA targeting GSK3α (siRNA GSK3α1) or transfected with siRNA targeting GSK3α1 and then stimulated with 10 μg/mL of PGN for 9 h. Protein extracts were analyzed by western blot and probed with a monoclonal antibody against the phosphorylated forms of GSK3α and GSK3β (A). To verify that equal amount of proteins was loaded in each lane, blots were stripped and reprobed with an antibody that recognizes the nonphosphorylated form of β-actin (A). Blots are representative of three independent experiments. Total RNA was extracted and relative transcript level of IL-12p40 was quantitated by qRT-PCR, using the delta-delta Ct method and amplification of β-actin as a reference gene (B). Cell-free supernatants were analyzed by ELISA for production of IL-12p40 (C). Results are expressed as the mean ± S.E.M. ($n = 3$). *p <0.05; **p <0.01. All data were compared with the untreated and untransfected controls.

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staphylococcal infections [40]. However, the specificity of TLR2 for PGN is still an issue of debate. According to Travassos et al. (2004) [41] highly purified PGN did not activate TLR signaling. In contrast, several other authors have proposed that PGN activates TLR2 [3–6, 42] and studies with PGN from *S. aureus* lacking lipidated prelipoproteins have co-localized them with Nod2, TLR2 and TLR4 in keratinocytes from murine oral epithelium and HEK293/hTLR2 cells, demonstrating that staphylococcal PGN, and not the associated lipoproteins, is able to trigger a TLR2 specific immune response [43]. Our data support the notion that PGN activates signaling that are associated with TLR2 activation in BEC because blocking of TLR2 with a TLR2 specific neutralizing antibody inhibited the phosphorylation of Akt, phospho-inhibition of GSK3α and GSK3β (Fig 1) and expression of IL-12p40 (Fig 4). Moreover, the PGN used in this study to stimulate BEC was purified with hot SDS, which eliminates the lipopeptides [39]. In agreement with our work, Zhang et al. (2012) [44] observed that TLR2 was activated in dendritic cells stimulated with heat-killed *Brucella abortus*, and this was an essential step for IL-12p40 induction through the activation of subpathways that regulate TLR9 signaling. In a different report, Satta et al. (2008) [45] detected an induction of TLR2 expression in human endothelial cells that served to amplify the inflammatory response to lipopeptides. This was not the case in our study because levels of TLR2 protein in BEC were not modified by PGN treatment, which means that an increase in TLR2 abundance is not a requirement for IL-12p40 expression in BEC stimulated with PGN from *S. aureus*.

Although it is well established that Akt phosphorylates and inactivates GSK3α and GSK3β, as we have observed in this study, Gulen et al. (2012) [22] showed that GSK3α, but not GSK3β, can reversely phosphorylates and suppresses Akt activation in resting Th17 cells. These authors also demonstrated that activation of Th17 treated with IL-1 leads to an increase of IKKi activity and GSK3α phosphorylation at Ser21, promoting Akt-mTOR activation [22]. Previous evidence from our lab indicated that GSK3α and GSK3β phosphorylation, as a consequence of BEC infected by *S. aureus*, may be involved in the internalization process and perhaps the inflammatory response caused by this bacterium [25].

In the last few years experimental evidence on the different functions of GSK3α and GSK3β is accumulating [14]. Our data demonstrate that inhibition of both GSK3α and GSK3β activity exerts an opposed function on IL-12p40 expression and this is in part different from data obtained by Martin et al. (2005) [32]. These authors found that inhibition of GSK3β, but no GSK3α, activity by treatment of macrophages with LPS or synthetic lipid-A as specific ligands of TLR4 or LTA from *S. pneumoniae* as a specific ligand of TLR2, reduced the expression of IL-12p40 [32]. In contrast, our data clearly indicate that phospho-inhibition of GSK3α by treatment of BEC with PGN from *S. aureus* increased IL-12p40 expression. This suggests that although GSK3β is the isoform generally associated with the inflammatory response to bacterial infections, as reported by several authors [13, 32], GSK3α may also play an important role in this process through the regulation of pro-inflammatory cytokine expression. Evidence that

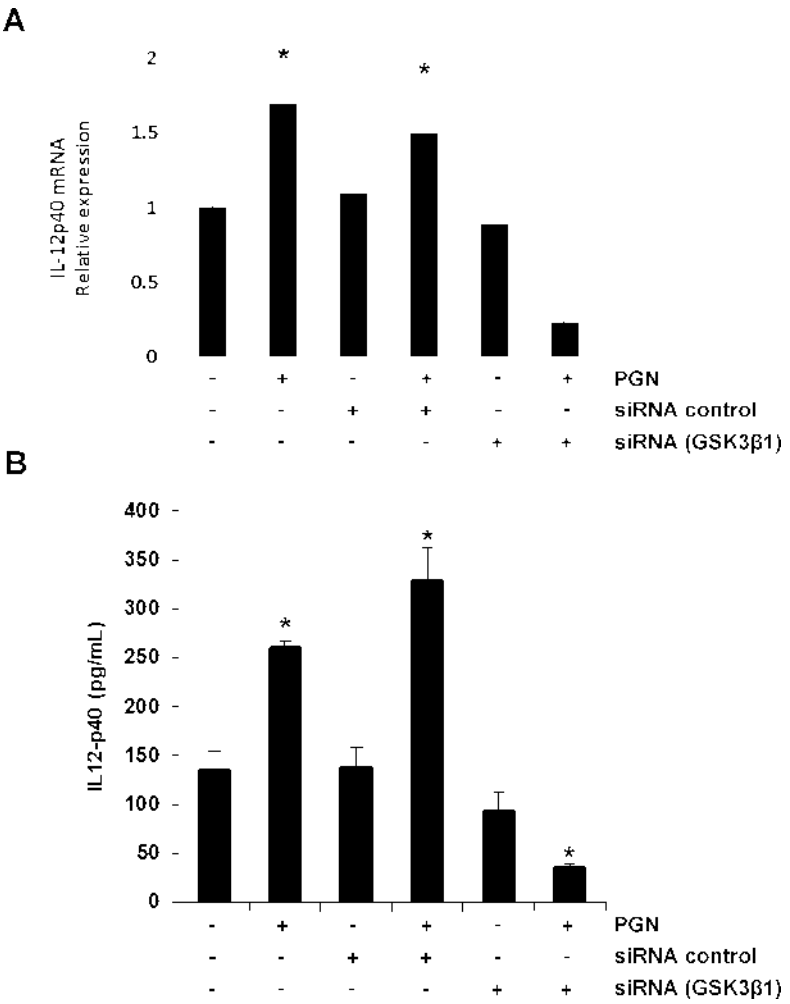

Fig 6. GSK3β inhibition down-regulates IL-12p40 expression in BEC stimulated with PGN. A) BEC were left untransfected and unstimulated (-), stimulated with 10 μg/mL of PGN for 4 h, transfected with control siRNA (siRNA control), transfected with control siRNA and then stimulated with 10 μg/mL of PGN for 4 h, transfected with siRNA targeting GSK3β (siRNA GSK3β1) or transfected with siRNA targeting GSK3β (siRNA GSK3β1) and then stimulated with 10 μg/mL of PGN for 4 h. B) BEC were left untransfected and unstimulated (-), stimulated with 10 μg/mL of PGN for 9 h, transfected with control siRNA (siRNA control), transfected with control siRNA and then stimulated with 10 μg/mL of PGN for 9 h, transfected with siRNA targeting GSK3β (siRNA GSK3β1) or transfected with siRNA targeting GSK3β (siRNA GSK3β1) and then stimulated with 10 μg/mL of PGN for 9 h. Total RNA was extracted and relative transcript level of IL-12p40 was quantitated by qRT-PCR, using the delta-delta Ct method, and amplification of β-actin as a reference gene (A). Cell-free supernatants were analyzed by ELISA for production of IL-12p40 (B). Results are expressed as the mean ± S.E.M. ($n = 3$). *$p < 0.05$. All data were compared with the untreated and untransfected controls.

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GSK3α is important in an inflammatory process was recently reported by Giambelluca et al. (2014) [24]. They found that in human neutrophils, in which the main isoform is GSK3α, the addition of LiCl resulted in a significant postranscritptional up-regulation of TNF-α secretion [24]. Interestingly, our results point out that inhibition of GSK3α activity resulted in a marked transcriptional up-regulation of IL-12p40. One explanation for these opposite results may be that active GSK3β phosphorylates NF-κB at its transactivation domain allowing the expression of IL-12p40. In a different scenario and when the activity of GSK3β is inhibited by phosphorylation at Ser9 or gene silencing, this isoform is unable to phosphorylate and activates NF-κ,

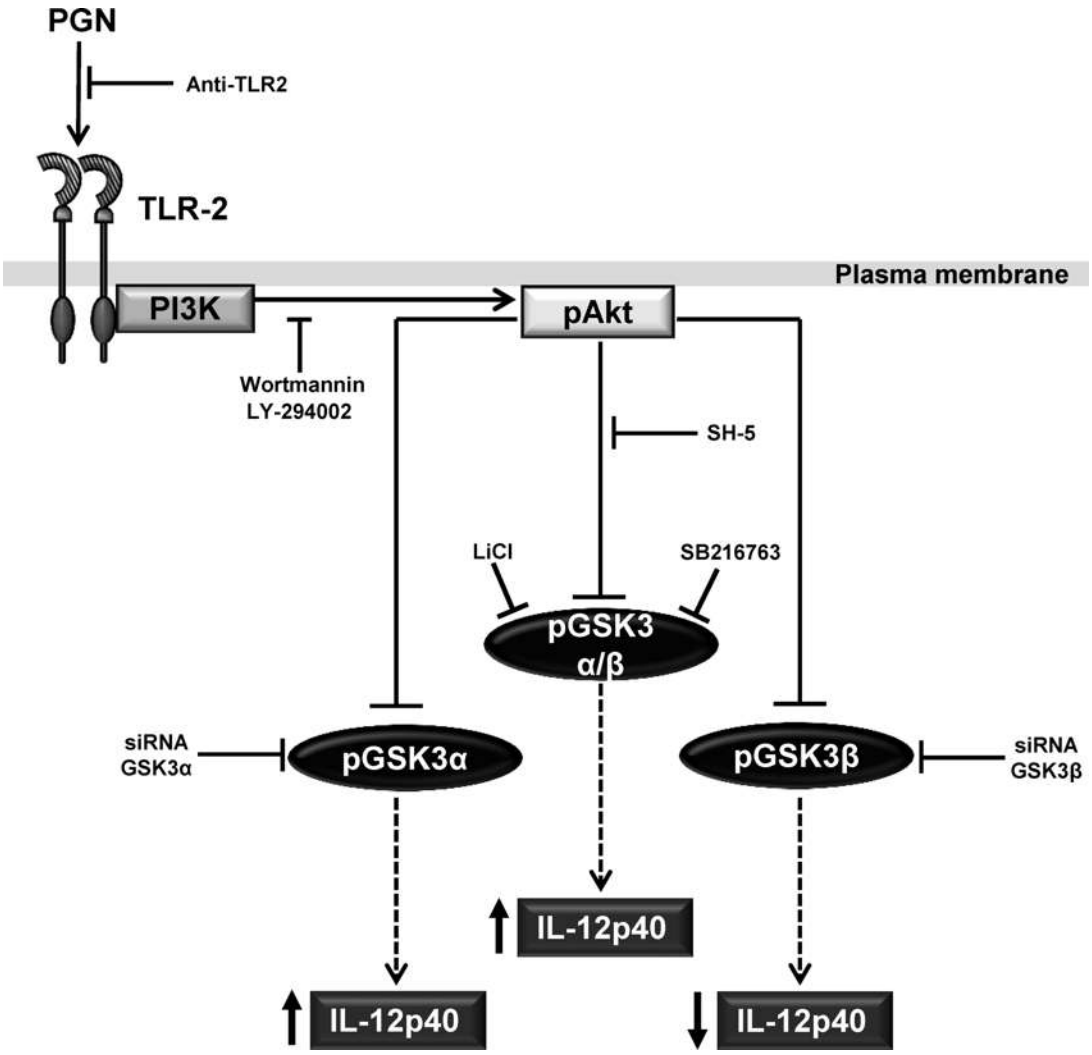

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Fig 7. A working model of the signaling pathway involved on the IL-12p40 expression induced by PGN in BEC. PGN induces the activation of the TLR2/PI3K/Akt pathway, which in turn induces GSK3α and GSK3β phosphorylation/inhibition and this in turn up- or down-regulates IL-12p40 expression. Anti-TLR2, Wortmannin (PI3K inhibitor), LY-294002 (PI3K inhibitor) or SH-5 (Akt inhibitor) blocks the phosphorylation/inhibition of GSK3α and GSK3β. LiCl (GSK3 inhibitor), SB-216763 (GSK3 inhibitor) or siRNA GSK3α increases the expression of IL-12p40 while siRNA GSK3β decreases the expression of IL-12p40. Not represented in the diagram is the decrease in IL-12p40 expression when TLR2 is blocked with anti-TLR2 and when Akt is inhibited with Akt-i IV.

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causing the decrease in IL-12p40 levels. It is also likely that the type of cell used, the stimulus applied, and even the type of cytokine evaluated may explain the mechanistic differences between our results and those reported by Martin et al. (2005) [32] and Giambelluca et. al. (2014) [24]

IL-12 is a cytokine required for innate immune defense and adaptive immunity to pathogens because stimulation of peripheral blood lymphocytes and NK cells with IL-12 produced as a result of infection, induces IFN-γ secretion and increases the cytotoxicity activity as well as proliferation of these cells [26, 27]. Interestingly, it has been proposed the existence of an IL-12-regulated circuit between endothelium and lymphocytes through IFN-γ, resulting in a reciprocal modulation of cellular responses [46, 47]. Moreover, it is likely that IL-12p40 produced by endothelium recruits macrophages to the site of infection because this cytokine has been shown to have chemotactic properties [48]. Although IL-12p40 expression by CD154

stimulation was already detected in endothelium [30], we are the first to demonstrate expression of this cytokine in endothelial cells stimulated with a bacterial structure. Other authors have detected expression of IL-12-related molecules, but not expression of IL-12p40 in human intestinal microvascular endothelial cells stimulated with pro-inflammatory compounds (TNF-α, IFN-γ, IL-1β) and microbial structures [LPS, LTA, PGN, CpG-DNA, flagellin, and poly(I:C)] [49]. In this context, our data suggest that production of IL-12p40 by endothelial cells stimulated with PGN might reflect innate and adaptive immune roles of the endothelium in response to Gram positive microbial antigens. More importantly, the fine modulation in the IL-12p40 expression is of paramount importance because this cytokine is critical for host defense; however, excessive increase in its production can cause severe inflammatory disorders [31]. Therefore, it is likely that a switch in the up- and down-regulation of IL-12p40 expression should co-exist, which might depend on the differences in spatio-temporal participation of the two isoforms of GSK3 and the diverse mechanisms controlling their activity such as phophorylation, subcellular distribution and formation of molecular complexes [14, 15]. More experiments will be undoubtedly needed to clarify the mechanistic details of the differential actions of GSK3α and GSK3β on the phosphorylation of NF-κB and the expression of IL-12p40 and other cytokines during the inflammatory response caused by pathogenic bacteria.

Supporting Information

S1 Fig. PGN does not induce changes in the expression of TLR2 but activates PI3K-dependent phosphorylation of Akt in BEC. A) BEC were left untreated and unstimulated (0) or stimulated with 10 μg/mL of PGN for 15, 30, 60, 120 or 240 min. B) BEC were left unstimulated (U) or stimulated with 1, 10, 20 or 30 μg/mL of PGN for 30 min. C) BEC were left unstimulated (U) or stimulated with 10 μg/mL of PGN for 15, 30, 60 or 120 min. D) BEC were left untreated and unstimulated (U), untreated (-) or treated with 10 μM of LY294002 (LY) for 30 min and then stimulated with 10 μg/mL of PGN for 30 min. Protein extracts were analyzed by western blot and probed with a polyclonal antibody against TLR2 (A) or the phosphorylated form of Akt1 (pAkt Ser473) (B-D). To verify that equal amount of proteins was loaded in each lane, blots were stripped and reprobed with antibodies that recognize β-actin (A) or the nonphosphorylated form of Akt (B-D). Blots are representative of three independent experiments. Graphs on the right indicate the band intensity obtained by densitometric analysis. Results are expressed as the mean ± S.E.M. (n = 3). $^{*}p < 0.05$, compared with the unstimulated control.
(PDF)

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S2 Fig. Temporal course of GSK3α and GSK3β phosphorylation induced by PGN in BEC. A and B) BEC were left unstimulated (0) or stimulated with 10 μg/mL of PGN for 15, 30, 60 or 120 min. Protein extracts were analyzed by western blot and probed with monoclonal antibodies against the phosphorylated forms of GSK3α (pGSK3α Ser21) or GSK3β (pGSK3β Ser9). To verify equal protein loading, blots were stripped and reprobed with an antibody that recognizes the nonphosphorylated form of GSK3β. Blots are representative of three independent experiments. Graphs on the right indicate the band intensity obtained by densitometric analysis. Results are expressed as the mean ± S.E.M. (n = 3). $^{*}p < 0.05$; $^{**}p < 0.01$, compared with the unstimulated control.
(PDF)

S3 Fig. Phosphorylation of Akt at Ser473, GSK3α at Ser21 and GSK3β at Ser9 in BEC treated with inhibitors. BEC were left untreated, treated with 10 μM of LY294002 (LY) for 30 min, treated with 1 μM of Akt inhibitor IV (Akt-i IV) for 30 min or treated with 10μM of SB216763 (SB) for 30 min. Untreated cells were incubated with 10 μM of DMSO. Then, total

protein from untreated and treated cell was obtained. A) Phosphorylation of Akt at Ser473; B) Phosphorylation of GSK3α at Ser21; C) Phosphorylation of GSK3β at Ser9. Detection of β–actin and GAPDH were used as control of protein loading. Data presented are representative of two independent experiments.
(PDF)

S4 Fig. GSK3 inhibition induces phosphorylation of NF-κB. A) BEC were left untreated and unstimulated (-), treated for 60 min with 10 mM of NaCl, stimulated with 10 μg/mL of PGN for 30 min, treated for 60 min with 10 mM of LiCl and then stimulated with 10 μg/mL of PGN for 30 min or treated for 60 min with 10 mM of LiCl. B) BEC were transfected with control siRNA (siRNA control), transfected with siRNA control and then stimulated with 10 μg/mL of PGN for 30 min, transfected with siRNA targeting GSK3α (siRNA GSK3α), transfected with siRNA GSK3α and then stimulated with 10 μg/mL of PGN for 30 min, transfected with siRNA targeting GSK3β (siRNA GSK3β) or transfected with siRNA GSK3β and then stimulated with 10 μg/mL of PGN for 30 min. Protein extracts were analyzed by western blot and probed with monoclonal antibodies against the phosphorylated forms of p65 (NF-κB p65 Ser536). To check for equal amount of proteins, blots were stripped and reprobed with antibodies that recognize the nonphosphorylated forms of p65 (A) or β-actin (B). Blots are representative of three independent experiments. Graphs indicate the band intensity obtained by densitometric analysis. Results are expressed as the mean ± S.E.M. (n = 3). $^{*}p < 0.05$; $^{**}p < 0.01$, compared with the unstimulated control.
(PDF)

S5 Fig. GSK3α and GSK3β gene silencing differentially regulates IL-12p40 expression in BEC stimulated with PGN. BEC were left untransfected and unstimulated (-), stimulated with 10 μg/mL of PGN for 9 h, transfected with control siRNA (siRNA control), transfected with control siRNA and then stimulated with 10 μg/mL of PGN for 9 h, transfected with siRNA targeting GSK3α (siRNA GSK3α2 or siRNA GSK3α3) or transfected with siRNA targeting GSK3β (siRNA GSK3β1 or siRNA GSK3β2) and then stimulated with 10 μg/mL of PGN for 9 h. A) Cell-free supernatants were analyzed by ELISA for production of IL-12p40 and B) Protein extracts were analyzed by western blot and probed with a monoclonal antibody against the phosphorylated forms of GSK3α and GSK3β. To verify that equal amount of protein was loaded in each lane, blots were stripped and reprobed with an antibody that recognizes the nonphosphorylated form of β-actin. Results are expressed as the mean ± S.E.M. (n = 3). $^{*}p < 0.05$.
(TIF)

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S1 Dataset. Raw values used to analyze and construct the graph.
(XLSX)

S2 Dataset. Raw values used to analyze and construct the graph.
(XLSX)

S3 Dataset. Raw values used to analyze and construct the graph.
(XLSX)

S4 Dataset. Raw values used to analyze and construct the graph.
(XLSX)

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Author Contributions

Conceived and designed the experiments: RCV OSG VMBA. Performed the experiments: RCV OSG JOB AHM. Analyzed the data: RCV OSG JOB. Contributed reagents/materials/analysis tools: ABP JJVA JOB BBF VMBA. Wrote the paper: RCV VMBA.

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11.2. ARTÍCULO 4: ORIGINAL INTERNACIONAL

THE PHOSPHOINOSITIDE-3-KINASE-Akt SIGNALING PATHWAY IS IMPORTANT FOR *Staphylococcus aureus* INTERNALIZATION BY ENDOTHELIAL CELLS.

Co-Autor 3: Alejandro Huante Mendoza.

En este trabajo de investigación publicado en la revista Infection and Immunity se demostró que la internalización de *Staphylococcus aureus* por células endoteliales está asociada a la activación de PI3K/Akt, lo que da lugar a la activación de NF-κB por fosforilación en Ser536 e inactivación de las isformas GSK3α y GSK3β por fosforilación de Ser21 y Ser9, respectivamente.

Mi participación en este trabajo fue obtener los resultados de la Figura 5 sobre el número de *S. aureus* internalizados en células endoteliales. Esta cuantificación se realizó en células modificadas genéticamente por transfección independiente con vectores que contienen el gen de Akt dominante negativo y constitutivamente activo.

Infection and Immunity

The Phosphoinositide-3-Kinase–Akt Signaling Pathway Is Important for Staphylococcus aureus Internalization by Endothelial Cells

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The Phosphoinositide-3-Kinase–Akt Signaling Pathway Is Important for *Staphylococcus aureus* Internalization by Endothelial Cells[▽]

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Internalization of *Staphylococcus aureus* in bovine endothelial cells (BEC) is increased by tumor necrosis factor alpha stimulation and NF-κB activation. Because the phosphoinositide-3-kinase (PI3K)–Akt signaling pathway also modulates NF-κB activity, we considered whether the internalization of *S. aureus* by BEC is associated with the activity of PI3K and Akt. We found a time- and multiplicity of infection-dependent phosphorylation of Akt on Ser473 in BEC infected with *S. aureus*. This phosphorylation was inhibited by LY294002 (LY), indicating the participation of PI3K. Inhibition of either PI3K with LY or wortmannin, or Akt with SH-5, strongly reduced the internalization of *S. aureus*. Transfection of BEC with a dominant-negative form of the Akt gene significantly decreased *S. aureus* internalization, whereas transfection with the constitutively active mutant increased the number of internalized bacterium. Inhibition of PDK1 activity with OSU-03012 did not affect the level of *S. aureus* internalization, demonstrating that phosphorylation of Akt on Thr308 is not important for this process. Compared to the untreated control, the adherence of *S. aureus* to the surface of BEC was unaltered when cells were transfected or incubated with the pharmacological inhibitors. Furthermore, Akt activation by internalized *S. aureus* triggered a time-dependent phosphorylation of glycogen synthase kinase-3α (GSK-3α) on Ser21 and GSK-3β on Ser9 that was partially inhibited with SH-5. Finally, treatment of BEC with LY prior to *S. aureus* infection inhibited the NF-κB p65 subunit phosphorylation on Ser536, indicating the involvement of PI3K. These results suggest that PI3K-Akt activity is important for the internalization of *S. aureus* and phosphorylation of GSK-3α, GSK-3β, and NF-κB.

Staphylococcus aureus is a Gram-positive bacterium widely distributed among humans and animals. In humans, *S. aureus* causes a variety of illnesses ranging from minor skin and soft tissue infections to life-threatening diseases such as endovascular infections, pneumonia, septic arthritis, endocarditis, osteomyelitis, and sepsis (21). This bacterium can also infect animals that serve as reservoirs with zoonotic implications (34). In bovine cattle, *S. aureus* is the main pathogenic bacterium causing mastitis, a disease characterized by mammary gland inflammation (45, 46) that causes important economic losses to dairy producers and represents a risk to the consumers because of contaminated milk.

S. aureus has been traditionally considered an extracellular bacterium, but different reports have demonstrated its ability to invade an array of nonprofessional phagocytic cells such as bovine epithelial cells (5), human and bovine endothelial cells (27, 42, 47), and human osteoblasts (9). This intracellular location potentially contributes to bacterial persistence in different diseases, evasion of the immune response, and protection from antibiotics activity. Efforts have been made to elucidate the molecular mechanisms that *S. aureus* uses to be internalized by its host cells. Different reports have pointed out that binding of the *S. aureus* fibronectin-binding protein to the integrin dimer α5β1 plays an important role in the internalization process (40, 49, 57). The data obtained with kidney epithelial cells and fibroblasts showed that *S. aureus* internalization requires polymerization of the actin cytoskeleton and the activation of the enzymes focal adhesion kinase (FAK) and Src (1, 2, 24). Moreover, internalized *S. aureus* has been shown to cause the recruitment of Rab5 in a cyclic and alternating manner, as well as the participation of tensin (54). Although the signaling pathway downstream of FAK activation has not been studied in detail, autophosphorylation of FAK on Y397 induced by *S. aureus* is the binding site for phosphoinositide-3-kinase (PI3K) and Src enzymes through their Src-homology 2 (SH2) domain (16).

The PI3K-Akt is a signaling pathway that is important in phagocytosis, regulation of the inflammatory response, and other activities, including vesicle trafficking and cytoskeletal reorganization (22, 63). PI3K is a heterodimeric protein with lipid kinase activity constituted by a catalytic subunit of 110 kDa (p110) and a regulatory subunit of 85 kDa (p85). When a ligand (i.e., growth factors or a bacterial molecular structure) binds to the cognate plasma membrane receptor, the SH2 domain of p85 recognizes the phosphorylated tyrosines on the

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cytosolic domain of the receptor. This causes an allosteric activation of p110 and the production of phosphatidylinositol-3,4,5-trisphosphate (PIP_3) that is recognized by the enzymes Akt and the constitutively active 3′-phosphoinositide-dependent kinase 1 (PDK1) through their plekstrin homology domains (25). The interaction of Akt with PIP_3 causes a change in the Akt conformation and phosphorylation of the residues Thr308 and Ser473 by PDK1 (4) and rictor-mTOR complex (53), respectively. Phosphorylation of these two residues causes the activation of Akt which in turn phosphorylates, among other substrates, the enzyme glycogen synthase kinase-3 (GSK-3). This enzyme is present in two constitutively active isoforms GSK-3α and GSK-3β that are structurally related but functionally nonredundant (17). Inactivation of GSK-3 is observed when the residues Ser21 in GSK-3α or Ser9 in GSK-3β, located in their regulatory N-terminal domains, are phosphorylated by Akt and other kinases (6, 7). Inhibition of GSK-3 by phosphorylation is important for the modulation of the inflammation and phagocytosis processes (13, 39).

Although several studies using different bacteria or bacterial virulence factors have documented the activation of the PI3K-Akt signaling pathway (3, 7, 20, 30, 33, 38, 50, 60), NF-κB (18, 20, 30, 38), and more recently GSK-3 (11, 18, 43, 44), none of them has reported the participation of the PI3K-Akt signaling pathway in the internalization of *S. aureus*. We have recently demonstrated that internalization of *S. aureus* by bovine endothelial cells (BEC) was increased by the soluble proinflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β) through a process associated with the NF-κB activity state (47). However, the signaling pathway activated during the internalization of *S. aureus* was not elucidated. We show here that the number of *S. aureus* internalized by BEC decreased when cells were pretreated with specific inhibitors of PI3K and Akt, implying that activation of both enzymes is required for *S. aureus* internalization without affecting its adherence to the cell surface. In addition, confirmation of the results observed with the pharmacological inhibitors was obtained in BEC expressing a dominant-negative form of the Akt gene. Interestingly, activation of the PI3K-Akt signaling pathway by *S. aureus* produced a time-dependent phosphorylation of GSK-3α/GSK-3β and NF-κB p65 subunit that was blocked by specific inhibitors of Akt and PI3K, respectively. The data presented in this study indicate that internalization of *S. aureus* by BEC is a process associated with the activation of the PI3K-Akt signaling pathway, as well as with the PI3K-Akt-dependent phosphorylation of GSK-3α, GSK-3β, and NF-κB.

MATERIALS AND METHODS

Media and chemicals. Ham F-12 (HF-12), Dulbecco modified Eagle medium, trypsin-EDTA, wortmannin (W), LY294002 (LY), Bradford reagent, bovine serum albumin (BSA), and lysostaphin were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Fetal calf serum (FCS) was acquired from Equitech-Bio, Inc. (Kerrville, TX). Penicillin G, streptomycin, and reduced serum Opti-MEM I medium were purchased from Gibco-BRL (Gaithersburg, MD). SH-5 was acquired from Enzo Life Sciences (Plymouth, PA), and OSU-03012 (OSU) was purchased from Cedarlane Labs (Burlington, NC). FuGENE transfection reagent and the 50× EDTA-free protease inhibitor cocktail were purchased from Roche Applied Science (Manheim, Germany).

Antibodies and plasmids. Rabbit monoclonal antibodies against phospho-Akt (Ser473 and Thr308), phospho-GSK-3α (Ser21), and GSK-3β, as well as the polyclonal antibodies against phospho-GSK-3β (Ser9), NF-κB p65, and phospho-p65 (Ser536) were purchased from Cell Signaling Technology (Boston, MA). Antibodies against hemagglutinin (HA) tag (rat) and calnexin (rabbit) were acquired from Roche and Sigma, respectively. pCMV5 and plasmids containing the constitutively active (pCMV5-Akt-CA) and dominant-negative (pCMV5-Akt-DN) forms of the Akt gene were purchased from Addgene (Cambridge, MA).

Bacterial strain, cell line, and culture conditions. The strain of *S. aureus*, isolated from a clinical case of bovine mastitis, was obtained from the American Type Culture Collection (ATCC 27543). Bacteria were cultured overnight in 3 ml of Luria-Bertani (LB) medium at 37°C with continuous agitation. The inoculum for infection assays was prepared by adding 1 ml of this preculture to 49 ml of LB medium and grown at 37°C until the culture reached the initial-middle log phase (optical density at 600 nm of 0.3).

The endothelial cell line used was obtained from bovine umbilical veins and immortalized by transfection with an expression vector containing the E6E7 oncogenes of human papillomavirus 16 (BVE-E6E7) (14). This immortalized bovine endothelial cell line, referred to as BEC here, was grown and maintained in HF-12 supplemented with 10% FCS unless otherwise noted.

Bacterial internalization and attachment assays. Quantitative analysis of intracellular *S. aureus* was done essentially as described by Oviedo-Boyso et al. (47) with minor modifications. In brief, 0.5×10^6 cells per well were seeded in 24-well plates (Corning-Costar, Inc., Corning, NY) in a medium containing 2 ml of HF-12 supplemented with 10% FCS, 100 U of penicillin G/ml, and 100 μg of streptomycin/ml and then cultured at 37°C in 5% CO_2 and 95% air up to a confluence of 90 to 100%. For internalization assays, BEC were washed three times with phosphate-buffered saline (PBS) to remove FCS and antibiotics and then infected with 10^7 CFU of *S. aureus*/ml, which gives a multiplicity of infection (MOI) of 20. Then, infected cells were immediately centrifuged at $130 \times g$ for 5 min, followed by incubation at 37°C for 40 min. After infection, extracellular noninternalized *S. aureus* was killed by incubation of BEC with 5 μg of lysostaphin/ml for 20 min, and the cells were washed three times with PBS, lifted with 250 μl of 0.25% trypsin–0.5 mM EDTA, and recovered by centrifugation at 3,500 rpm for 12 min in an Eppendorf centrifuge. The supernatant was discarded, and BEC were lysed by hypotonic shock in 250 μl of sterile deionized water containing 0.1% Triton X-100. Intracellular bacteria were cultured in LB agar at 37°C for 19 to 24 h, and the number of *S. aureus* CFU/ml was calculated by the counting plate technique. For the adherence assays, the procedure was identical except that the incubation of BEC with lysostaphin was omitted. Because in this case the number of CFU represented internalized and adherent *S. aureus*, we calculated the number of adherent bacteria by subtracting the number of intracellular ones to the total CFU counted for each condition tested. To test the effect of inhibitors on the internalization and adherence of *S. aureus*, BEC were preincubated for 30 min with LY294002 (LY), W, and SH-5 and for 15 min with OSU and then infected with bacteria in the presence of the inhibitors. The intracellular and adherent bacteria were recovered, cultured, and calculated according to the procedure described. The viability of BEC, evaluated by the trypan blue technique, was >95% in the presence of 50 μM LY, 100 nM W, 10 μM SH-5, or 2 μM OSU.

Transient transfection of BEC. Cells were grown in 24-well plates to 60 to 70% confluence, and the culture medium was changed to HF-12 plus 10% FCS. Then, in order to have a similar protein expression 5 ng of pCMV5-Akt-CA or 200 ng of pCMV5-Akt-DN in 1.2 μl of FuGENE transfection reagent (ratio, 4:1 [FuGENE-plasmid]) were added to BEC in reduced serum Opti-MEM I medium according to the manufacturer's instructions. As a control, BEC were transfected with 300 ng of pCMV5. To maintain a constant amount of DNA in transfection assays, pCMV5 was added to pCMV5-Akt-CA or pCMV5-Akt-DN transfection mixtures to have a final amount of 300 ng of total DNA. After 24 h of incubation at 37°C in 5% CO_2, we performed the internalization and the Western blot assays to quantitate the number of intracellular *S. aureus* and the level of expression of the two Akt mutants, respectively.

Protein extraction and Western blot analysis. To test for the relative abundance of phosphorylated and nonphosphorylated proteins, BEC were grown in six-well culture plates to ca. 90% confluence before serum starvation for at least 4 h. In control and treated cells, the total protein (cytosolic plus nuclear) was obtained by washing the cells twice with cold PBS and lysing them with 80 μl of a cold lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Igepal CA-930, 10 mM sodium pyrophosphate, and 50 mM NaF, supplemented with 1 mM sodium orthovanadate and 1× protease inhibitor cocktail added immediately before lysing the cells. The lysates were centrifuged at $13,000 \times g$ for 20 to 30 min at 4°C, and the supernatant was transferred to ice-cold Eppendorf tubes. The protein concentration was measured by the Bradford method (10) using BSA as standard. Then, 40 to 60 μg of protein was separated by electrophoresis in 10% sodium dodecyl sulfate-polyacrylamide gels and electroblotted to a 0.45-μm-pore-size nitrocellulose membrane (Bio-Rad) in a wet chamber at 250 to 300

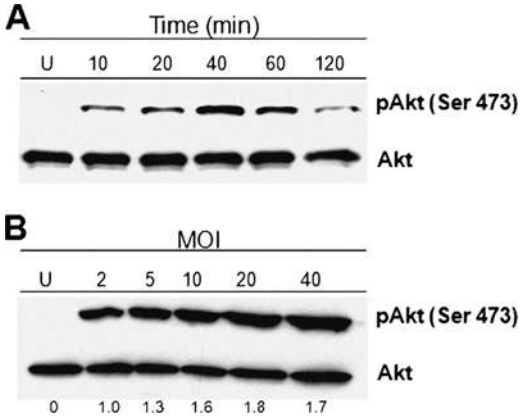

FIG. 1. *S. aureus* activates the phosphorylation of Akt on Ser473 in BEC. (A) Cells were left uninfected (U) or infected with *S. aureus* at an MOI of 20 for 10 to 120 min. (B) Cells were left uninfected (U) or infected with *S. aureus* at MOIs of 2 to 40 for 40 min. After infection, the phosphorylation of Akt was analyzed by Western blotting. Detection of Akt isoforms 1 to 3 in each sample was performed to ensure equal protein loading. Blots are representative of three (A) and two (B) independent experiments. The numbers at the bottom of panel B indicate the relative band intensities obtained by densitometric analysis of each assay compared to the uninfected control.

mA for 1 h. The membranes were then probed with the indicated antibody, and the abundance of the phosphorylated forms of Akt, GSK-3α, or GSK-3β, and the NF-κB p65 subunit was detected with the Immobilon western chemiluminescent HRP substrate kit from Millipore (Billerica, MA). Membranes were exposed to an X-ray film (Kodak) with two intensifying screens (DuPont) at room temperature.

Statistical analysis. For internalization and adherence, the data were normalized by calculating the ratio of *S. aureus* CFU/ml to the number of BEC/ml for each condition tested. In each experiment, the ratio obtained for each condition was referenced to the control condition that was arbitrarily assigned a value of 100%. For each condition, the error standard of the mean ($n = 3$) was calculated. The statistical significance was evaluated with the *t* test paired analysis by using the SigmaStat program (version 3.0; SPSS, Inc., Chicago, IL). Densitometric analysis of the bands was performed with the Image Processing and Analysis in Java Program ImageJ (http://rsbweb. nih.gov/ij).

RESULTS

Internalization of *S. aureus* by BEC involves the PI3K-dependent phosphorylation of Akt. To investigate the host-cell signaling events involved in the internalization of *S. aureus* by BEC, we explored the role of the PI3K-Akt signaling pathway because of its well-known function in diverse cellular processes, including inflammation and cytoskeleton rearrangement. Since we have previously reported that *S. aureus* internalization by BEC is strongly inhibited by cytochalasin D (47), we determined whether *S. aureus* internalization in these cells

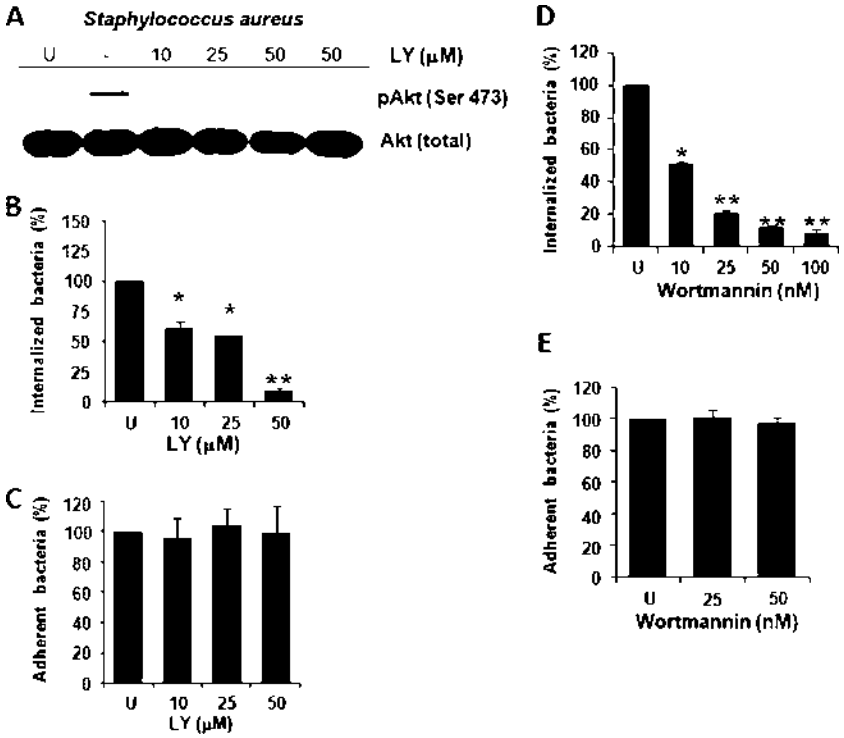

FIG. 2. Inhibition of PI3K activity abolishes phosphorylation of Akt and reduces *S. aureus* internalization by BEC. (A) Cells were left untreated and uninfected (U), were untreated (−), or were treated with 10, 25, or 50 μM LY294002 (LY) for 30 min and then infected with *S. aureus* at an MOI of 20 for 40 min. A control in which cells were only treated with 50 μM LY for 30 min was also included. After infection, the phosphorylation of Akt was analyzed by Western blotting. Detection of the Akt isoforms 1 to 3 in each sample was performed to ensure equal protein loading. The blot is representative of three independent experiments. (B and D) The number of internalized *S. aureus* was determined in untreated cells (U) or in cells treated with the indicated concentrations of LY and wortmannin (W). Extracellular *S. aureus* was killed by lysostaphin treatment. (C and E) The number of adherent *S. aureus* was analyzed in untreated cells (U) or in cells treated with the indicated concentrations of LY and W. In this case, the cells were washed three times with PBS to eliminate nonadherent bacteria. Untreated controls contained 0.1% dimethyl sulfoxide (DMSO). Bacteria were recovered from hypotonically lysed cells, cultured on LB agar for 19 to 24 h at 37°C, and counted. The data represent means ± the standard error of the mean (SEM; $n = 3$). *, $P < 0.05$; **, $P < 0.001$ (compared to the untreated control value).

causes the activation of PI3K and the subsequent phosphorylation of Akt. The data shown in Fig. 1A indicated that *S. aureus* was able to induce a time-dependent Akt phosphorylation on Ser473 with a maximum at 40 min postinfection, followed by a gradual decrease with longer infection times of 60 and 120 min. A gradual increase in Akt phosphorylation was also observed when BEC were infected with different MOI values reaching the maximum activation at an MOI of 20 (Fig. 1B). These results allowed us to establish the conditions (MOI of 20 and infection time of 40 min) to evaluate the involvement of the PI3K-Akt signaling pathway in the internalization of *S. aureus* by BEC.

To determine whether *S. aureus* mediates Akt phosphorylation via PI3K activity, we incubated BEC with increasing concentrations of LY, a specific inhibitor of PI3K. We observed that LY completely abolished the phosphorylation of Akt induced by infection of BEC with *S. aureus* (Fig. 2A). We next sought to determine whether the inhibition of PI3K with LY or W affected the internalization and adherence of *S. aureus* to BEC. Inhibition of PI3K with increasing concentrations of both chemicals caused a concentration-dependent reduction of *S. aureus* internalization with maximal decreases of 90 and 95% observed in the presence of 50 μM and 100 nM LY and W, respectively (Fig. 2B and D). Although internalization of *S. aureus* by BEC was significantly reduced in the presence of LY and W, the adherence was not affected even at the highest concentrations used for both inhibitors (Fig. 2C and E). These data suggest that *S. aureus* is able to induce the PI3K-dependent phosphorylation of Akt on Ser473 and that PI3K activity is involved in the internalization of this bacterium.

The Akt activity is important for *S. aureus* internalization by BEC. To explore if the activity of Akt is involved in *S. aureus* internalization, we incubated BEC with SH-5, a specific inhibitor of Akt, and evaluated both the phosphorylation level of Ser473 and the number of intracellular bacteria. Our results indicated that 5 μM SH-5 inhibited the phosphorylation of Akt by 35%, whereas 10 μM caused a reduction of 86% with respect to the untreated control (−) (Fig. 3A). No effect on Akt phosphorylation was observed when BEC were incubated with 10 μM SH-5 alone (Fig. 3A). *S. aureus* internalization was 95% inhibited compared to the untreated control when BEC were incubated with SH-5 (Fig. 3B). In contrast, the adherence of *S. aureus* to BEC was unaffected in the presence of this inhibitor (Fig. 3C), as was the case when LY or W was added to inhibit the activity of PI3K.

It is known that Akt becomes fully activated when the residues Thr308 and Ser473 are phosphorylated by PDK1 and rictor-mTOR complex, respectively (4, 53). To determine whether phosphorylation of Thr308 is important for *S. aureus* internalization, we incubated BEC with 0.5 or 2 μM OSU, an inhibitor of PDK1 that specifically inhibits the phosphorylation of Akt on Thr308 (66), prior to infection with *S. aureus*. As expected, OSU did not inhibit the phosphorylation of Ser473 induced by *S. aureus* even at the highest concentration used, whereas it completely abolished the phosphorylation of Thr308 (Fig. 4A and B). Interestingly, neither internalization nor adherence of *S. aureus* was affected in BEC pretreated with OSU (Fig. 4C and D).

To confirm that Akt plays a central role in the *S. aureus* internalization, we expressed the constitutively active (CA) and dominant-negative (DN) forms of the Akt gene in BEC. Cells were transfected with pCMV5 plasmids containing the Akt-CA or Akt-DN mutants tagged with HA and after 24 h of incubation, the presence of the corresponding proteins was detected by Western blotting with antibodies that recognize the HA tag. We observed similar expression levels for the CA and DN mutants, while no signal was detected when BEC were left untransfected (U) or transfected with the vector alone (pCMV) (Fig. 5A). Internalization of *S. aureus* was significantly reduced (~46%) or increased (~65%) in BEC expressing the Akt-DN or Akt-CA, respectively (Fig. 5B). The adherence of *S. aureus* to the BEC surface remained unaltered in transfected cells compared to the values of the untreated and pCMV controls (Fig. 5C). Altogether, these results demonstrated that Akt activity is associated with *S. aureus* internalization and that phosphorylation of Akt on Ser473, but not on Thr308, appears to be essential for this process.

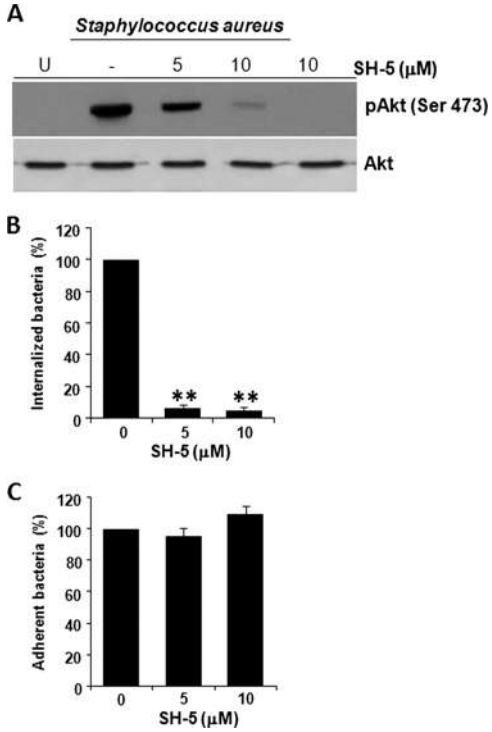

FIG. 3. Inhibition of Akt phosphorylation on Ser473 reduces the *S. aureus* internalization by BEC. (A) Cells were left untreated and uninfected (U), were left untreated (−), or were treated with 5 or 10 μM SH-5 for 30 min and then infected with *S. aureus* at an MOI of 20 for 40 min. A control in which cells were only treated with 10 μM SH-5 for 30 min was also included. After infection, the phosphorylation of Akt was analyzed by Western blotting. Detection of the Akt isoforms 1 to 3 in each sample was performed to ensure equal protein loading. The blot is representative of three independent experiments. (B) The number of internalized *S. aureus* was analyzed in untreated cells (column 0) or in cells treated with the indicated concentrations of SH-5. Extracellular *S. aureus* was killed by lysostaphin treatment. (C) The number of adherent *S. aureus* was analyzed in nontreated cells (column 0) or in cells treated with the indicated concentrations of SH-5. In this case the cells were washed three times with PBS in order to eliminate nonadherent bacteria. Untreated controls contained 0.1% DMSO. Bacteria were recovered from hypotonically lysed cells, cultured on LB agar for 19 to 24 h at 37°C, and counted. The data represent means ± the SEM ($n = 3$). **, $P < 0.001$ (compared to the untreated control value).

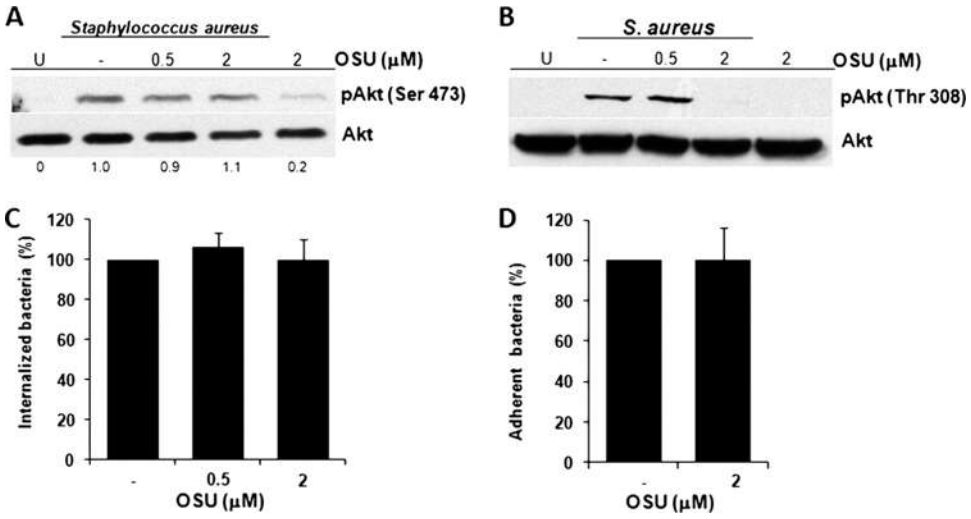

FIG. 4. Inhibition of Akt phosphorylation on Thr308 does not reduce the *S. aureus* internalization or adherence by BEC. (A and B) Detection of Akt phosphorylated on Ser473 or Thr308 in cells left untreated and uninfected (U), in untreated cells (−), or in cells treated with 0.5 or 2 μM OSU-03012 (OSU) for 15 min and then infected with *S. aureus* at an MOI of 20 for 40 min. A control in which cells were only treated with 2 μM OSU for 15 min was also included. After infection, the phosphorylation of Akt was analyzed by Western blotting. Detection of the Akt isoforms 1 to 3 in each sample was performed to ensure equal protein loading. The blots are representative of three independent experiments. (C) The number of internalized *S. aureus* was evaluated in untreated cells (column −) or in cells treated with the indicated concentrations of OSU. Extracellular *S. aureus* was killed by lysostaphin treatment. (D) The number of adherent *S. aureus* was evaluated in untreated cells (column −) or in cells treated with the indicated concentrations of OSU. In this case, the cells were washed three times with PBS in order to eliminate nonadherent bacteria. Bacteria were recovered from hypotonically lysed cells, cultured on LB agar for 19 to 24 h at 37°C, and counted. The data represent means ± the SEM ($n = 3$). The numbers at the bottom of panel A indicate the relative band intensities obtained by densitometric analysis of each assay compared to the uninfected control.

Phosphorylation of GSK-3α and GSK-3β in BEC infected with *S. aureus* depends on the Akt activity. Akt phosphorylates many substrates that modulate diverse cellular functions such as proliferation, differentiation, and apoptosis (37). One of those substrates is GSK-3 that, among its multiple functions, is involved in the regulation of the inflammatory process caused by whole bacteria or bacterial virulence factors (7). Because GSK-3 is a constitutively active enzyme present in two isoforms whose activities are inhibited by Akt phosphorylation (32), we sought to determine whether the activation of Akt by *S. aureus* leads to the phosphorylation of GSK-3α and GSK-3β. When BEC were infected with *S. aureus* at various times, we observed the phosphorylation of GSK-3α and GSK-3β (Fig. 6A and C) with maxima at 40 to 60 min for GSK-3α and 40 min for GSK-3β, a finding that is in agreement with the time for maximal phosphorylation of Akt (see Fig. 1A). *S. aureus*-induced phosphorylation of GSK-3α and GSK-3β was strongly reduced by treatment of BEC with SH-5 prior to *S. aureus* infection (Fig. 6B and D). Compared to the uninfected and untreated (U) control, a slight decrease in the phosphorylation of both isoforms were observed when BEC were treated with SH-5 alone, suggesting an inhibition of basal Akt activity. These data indicate that phosphorylation of the isoforms GSK-3α and GSK-3β induced by *S. aureus* proceeds via Akt.

Phosphorylation of the NF-κB p65 subunit in BEC infected with *S. aureus* depends on the PI3K activity. Full transactivation potential of NF-κB, one of the main transcription factors involved in the inflammation process, is the result of its phosphorylation and subsequent translocation from the cytoplasm to the nucleus. Moreover, in certain cell types the activation of the PI3K–Akt–GSK-3 signaling pathway leads to the regulation of NF-κB activity (39). To explore whether the activation of PI3K and Akt induced by *S. aureus* causes the phosphorylation of NF-κB, BEC were infected with *S. aureus* for 10 to 120 min and phosphorylation of p65 on Ser536 was detected by Western blotting. The phosphorylation of Ser536 reached the maximum level at 40 min postinfection, followed by a marked decrease at 120 min (Fig. 7A). Phosphorylation of p65 on Ser536 was dependent on the PI3K activity because incubation of BEC with LY for 30 min prior to infection with *S. aureus* caused a significant reduction of phosphorylation (Fig. 7B). However, no effect on the nuclear translocation of p65 was observed when BEC were infected with *S. aureus* (data not shown). These results suggest that *S. aureus* caused a PI3K-dependent NF-κB p65 subunit phosphorylation on Ser536 without affecting its nuclear translocation.

DISCUSSION

The PI3K-Akt signaling pathway integrates a variety of extracellular signals for the control of diverse cellular responses such as cell growth and proliferation, glucose homeostasis, survival, and apoptosis. Immune responses in cancer are also regulated by the PI3K-Akt signaling (65). Regulation of actin cytoskeleton rearrangement by PI3K has also been shown to play an essential role in the phagocytosis of pathogenic microorganisms by macrophages (63), formation of pseudopods, and phagosome maturation (59, 64). Furthermore, a correlation of PI3K-Akt signaling pathway activity with the cellular internalization of *Chlamydia pneumonia*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Helicobacter pylori*, *Legionella pneumophila*, *Salmonella* spp., and *Streptococcus pyogenes* has been

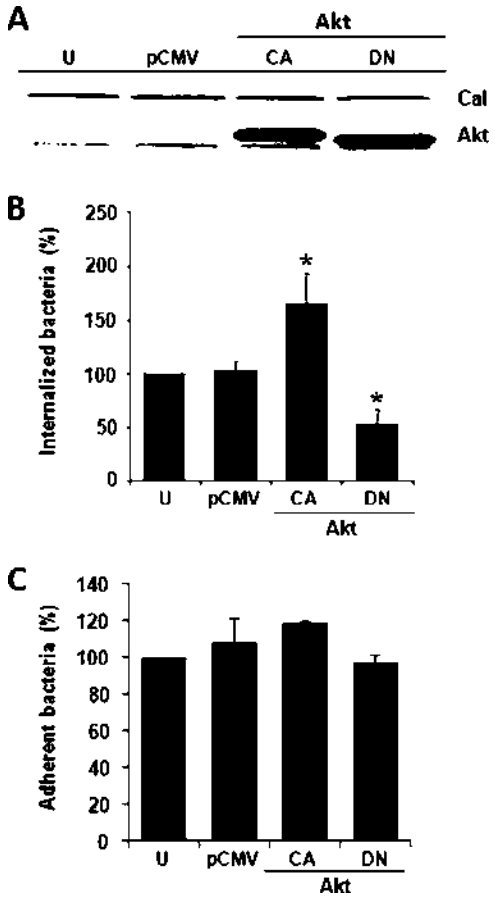

FIG. 5. The number of internalized but not adherent *S. aureus* was altered in BEC transfected with mutants of Akt. (A) Cells were left untransfected (U), were transfected with pCMV5 vector (300 ng) only (pCMV), or were transfected with pCMV5 containing the constitutively active (CA) Akt (5 ng) or the dominant-negative (DN) Akt (200 ng) gene forms. The amount of total DNA transfected was kept constant at 300 ng by the addition of 295 ng of pCMV5 to the Akt-CA or 100 ng of pCMV5 to the Akt-DN. Expression of Akt-CA and Akt-DN (Akt) was analyzed 24 h after transfection by Western blotting with an antibody against the HA tag. Evidence of equal protein loading was obtained by detection of calnexin (Cal). (B) The number of internalized *S. aureus* was quantitated in untransfected cells (U) or in cells after 24 h of transfection as described in panel A. Extracellular *S. aureus* was killed by lysostaphin treatment. (C) The number of adherent *S. aureus* was quantitated in untransfected cells (U) or in cells after 24 h of transfection as described in panel A. In this case, the cells were washed three times with PBS in order to eliminate nonadherent bacteria. Bacteria were recovered from hypotonically lysed cells, cultured on LB agar for 19 to 24 h at 37°C, and counted. The data represent means ± the SEM ($n = 3$). *, $P < 0.05$ (compared to the untransfected control value).

established (3, 19, 33, 43, 50, 56, 60). We propose the addition of *S. aureus* to this list because we have obtained evidence indicating that internalization of this bacterium by endothelial cells activates the PI3K-Akt signaling pathway, which in turn leads to the phosphorylation of GSK-3 and NF-κB.

Several reports have demonstrated that *S. aureus* is able to invade nonprofessional phagocytic cells such as epithelium and endothelium (5, 9, 27, 42, 47). The data in the present study indicated that internalization of *S. aureus* was associated with a time- and MOI-dependent Akt phosphorylation on Ser473 (Fig. 1A and B) that was mediated by the activity of PI3K because treatment of endothelial cells with LY abolished Akt phosphorylation (Fig. 2A). We also observed that treatment of BEC with LY and W caused a significant reduction of *S. aureus* internalization by BEC without altering its adherence to the cell surface (Fig. 2). These results demonstrate that the activity of PI3K-Akt pathway in BEC is only involved in the internalization but not the adherence of *S. aureus* to the cell surface. Similar data have been obtained with *Cronobacter sakazakii*, an opportunistic pathogenic bacterium that causes neonatal sepsis and meningitis. The infection of human brain microvascular endothelial cells (HBMEC) with this bacterium causes an increase in Akt phosphorylation and its invasion is blocked by treatment of HBMEC with PI3K inhibitors (36). The molecular mechanism of internalization used by *S. aureus* is also similar to that used by *C. pneumonia*, an intracellular pathogen. The invasion but not the binding to the surface of HEp2 epithelial cells by *C. pneumoniae* requires Akt phosphorylation, which reaches a maximum level at 40 min postinfection and is mediated by the activity of PI3K (19). Likewise, PI3K activity is essential for *Campylobacter jejuni* invasion of the human embryonic intestinal cell line INT407 (29) and for *L. pneumophila* invasion of macrophages (60).

Evidence of Akt participation in the *S. aureus* internalization by BEC was obtained by incubating the endothelial cells with SH-5, a specific inhibitor of the Akt activity. We found that SH-5 caused a significant reduction of Akt phosphorylation on Ser473 induced by *S. aureus*, and this reduction correlated with a strong decrease in the internalization of *S. aureus*, without altering its adherence to the cell surface (Fig. 3). To confirm the results obtained with SH-5, we genetically modified BEC by overexpressing the CA and DN forms of Akt. The expression of the Akt-DN caused a marked reduction of *S. aureus* internalization, while expression of Akt-CA significantly increased it without affecting the adherence (Fig. 5). Similarly, the internalization, but not the adherence, of *P. aeruginosa* was reduced when MDCK cells were pretreated with SH-5 or HeLa cells were transfected with small interfering RNAs specific to silence the Akt gene (33). Taken together, these results indicate that PI3K-Akt is one of the main signaling pathways involved in the internalization of several pathogenic bacteria in phagocytic and nonphagocytic cells.

Full activation of Akt is achieved when both Ser473 and Thr308 are phosphorylated (22). We have demonstrated that *S. aureus* is able to induce Akt phosphorylation on these residues (Fig. 4A and B) and that treatment of BEC with OSU effectively blocks the phosphorylation of Thr308 without affecting the phosphorylation of Ser473. More importantly, inhibition of Thr308 phosphorylation does not affect the internalization or adherence of *S. aureus* (Fig. 4C and D), indicating that internalization of this bacterium is associated with phosphorylation of Akt on Ser473 but not on Thr308. Although differential phosphorylation of these Akt residues has been observed in acute myeloid leukemia (26), this is the first report in which the phosphorylation of only one Akt residue is important for the internalization of a pathogenic bacterium. Interestingly, Jacinto et al. (31) have proposed that dual Akt phosphorylation is not necessarily required for all Akt functions. Further studies are necessary to precisely define the physiological importance of each Akt phosphorylation site and

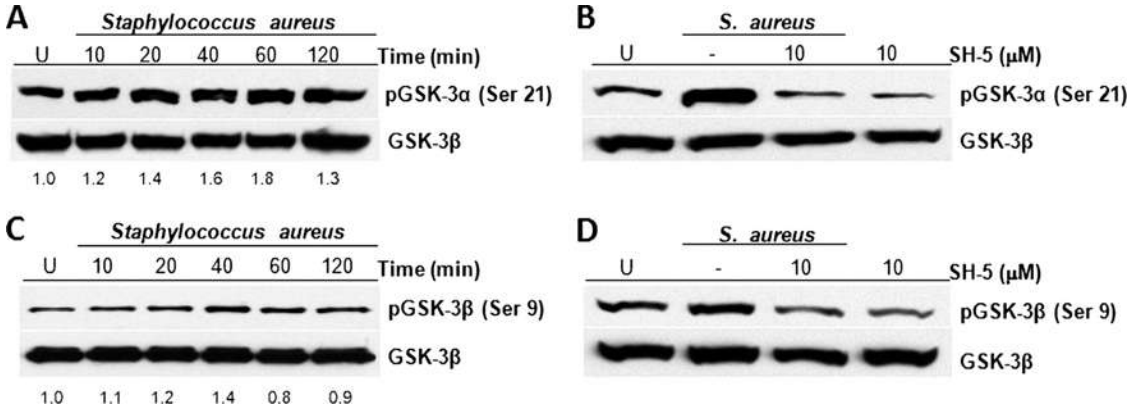

FIG. 6. *S. aureus* activates the phosphorylation of GSK-3α and GSK-3β in BEC through the activation of Akt. (A and C) Cells were left uninfected (U) or were infected with *S. aureus* at an MOI of 20 for 10 to 120 min. (B and D) Cells were left untreated and uninfected (U), were untreated (−), or were treated with 10 μM SH-5 for 30 min and then infected with *S. aureus* at an MOI of 20 for 40 min. A control in which cells were only treated with 10 μM SH-5 for 30 min was also included. After infection, the phosphorylation of GSK-3α on Ser-21 or GSK-3β on Ser-9 was analyzed by Western blotting. Detection of total GSK-3β in each sample was performed to ensure equal protein loading. The blots are representative of two (A and C) or three (B and D) independent experiments. Numbers at the bottom of panels A and C indicate the relative band intensities obtained by densitometric analysis of each assay compared to the uninfected control.

in particular the participation of phosphorylated Thr308 in different cellular processes.

The Akt activated by phosphorylation gives rise to downstream phosphorylation and inactivation of GSK-3, a constitutively active kinase involved in diverse cellular functions such as metabolism and regulation of the innate immune response (7, 12, 35). In the present study we found that *S. aureus* caused a time-dependent phosphorylation of GSK-3α and GSK-3β (Fig. 6A and C) with time peaks similar to the time for maximal Akt phosphorylation (Fig. 1). Moreover, treatment of BEC with SH-5 revealed that phosphorylation of both GSK-3 isoforms was dependent on Akt activity (Fig. 6B and D). Phosphorylation of paxillin by ERK/GSK-3 and rearrangement of actin cytoskeleton has already been observed (13, 41). It is likely that when *S. aureus* is internalized by BEC, the Akt-dependent phosphorylation of GSK-3 may lead to phosphorylation of paxillin and reorganization of the actin cytoskeleton, which favors the entry of bacteria. GSK-3 has also been associated with the regulation of pro- and anti-inflammatory cytokines production through TLR signaling (39). Interestingly, phosphorylation of GSK-3 by Akt turns off its catalytic activity, resulting in the activation of pathways that are normally repressed by GSK-3 (15). In this context, Cheng et al. (18) reported that inhibition of GSK-3β blocks NF-κB activation, TNF-α production, and iNOS/NO biosynthesis but increases IL-10 production when the microglia is stimulated with heat-inactivated *S. aureus*.

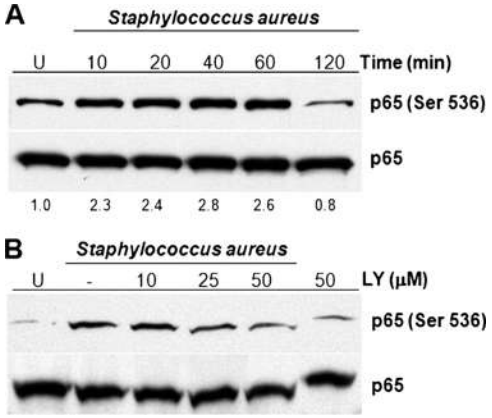

FIG. 7. Inhibition of PI3K activity reduces the level of phosphorylation of NF-κB p65 on Ser536 in BEC infected with *S. aureus*. (A) Cells were left uninfected (U) or were infected with *S. aureus* at an MOI of 20 for 10 to 120 min. (B) Cells were left untreated and uninfected (U), were untreated (−), or were treated with 10, 25, or 50 μM LY294002 (LY) for 30 min, and then infected with *S. aureus* at an MOI of 20 for 40 min. A control in which cells were only treated with 50 μM LY for 30 min was also included. After infection, the phosphorylation of NF-κB p65 on Ser536 was analyzed by Western blotting. Detection of total NF-κB p65 in each sample was performed to ensure equal protein loading. The blots are representative of three independent experiments. Numbers at the bottom of A indicate the relative band intensity obtained by densitometric analysis of each assay compared to the uninfected control.

NF-κB is one of the major transcriptional factors that regulate the inflammatory response (8), and its activity is modulated by microbial pathogens (51). The transactivation potential of this factor could be enhanced by phosphorylation of p65 (48, 52, 55). We found here that *S. aureus* caused the phosphorylation of p65 on Ser536 as early as 10 min postinfection and that this phosphorylation was partially inhibited by LY, indicating a dependency on PI3K (Fig. 7A and B). Several bacteria interfere with the activation of NF-κB as one of the strategies to evade the immune response and guarantee their intracellular survival (62). Phosphorylation of p65 on Ser536 has also been observed in gastric epithelial cells infected with *H. pylori* (61), and it has recently been shown that inhibition of NF-κB activity is associated with a reduction of *P. aeruginosa* internalization into human respiratory epithelial cell lines and HeLa cervical cancer cells (23). Taking into consideration that inhibition of the Akt activity also diminishes *P. aeruginosa* internalization (33), it is likely that a link between the PI3K-Akt signaling pathway and NF-κB activation may exist and that they coordinately function during bacteria internalization. A role of GSK-3β in NF-κB function was demonstrated during

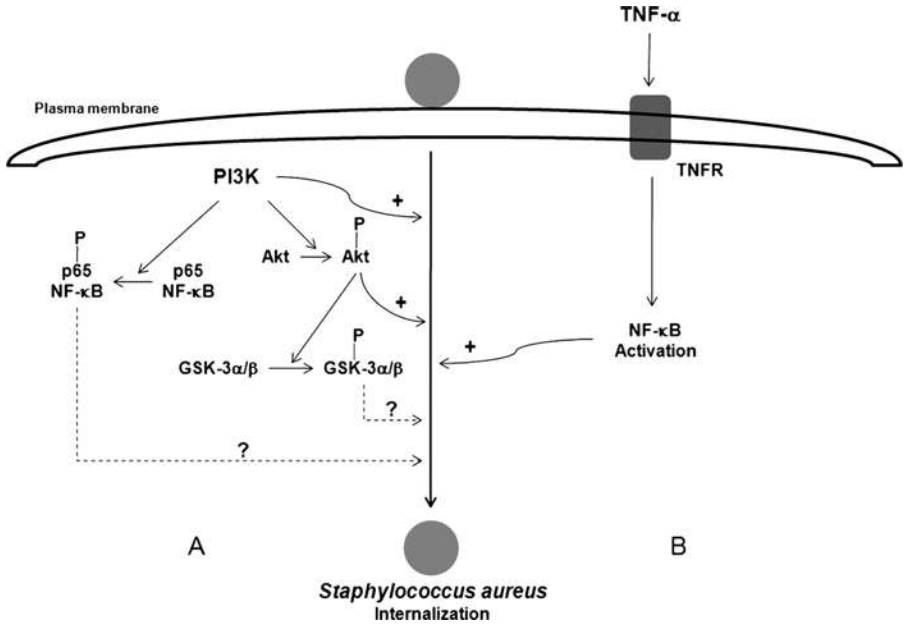

FIG. 8. Schematic diagram summarizing the main results obtained in the present study (A) and in a previous report by Oviedo-Boyso et al. in 2008 (47) (B).

mammalian development by disrupting the GSK-3β gene in murine embryonic stem cells (28). However, Steinbrecher et al. (58) did not observe any TNF-α-induced change in nuclear localization of p65 associated with a GSK-3β activity loss in GSK-3-null mouse embryonic fibroblasts. In the present study we observed that, although *S. aureus* internalization activated the PI3K-dependent phosphorylation of p65, it did not apparently affect cytoplasmic and nuclear distribution of this NF-κB subunit (data not shown). Thus, it is likely that nuclear translocation of the NF-κB p65 subunit in BEC is not a process directly related to the phosphorylation inactivation of GSK-3 caused by *S. aureus*.

Based on the results presented here and data published elsewhere (47), we propose that the PI3K-Akt signaling pathway activated by *S. aureus* and the NF-κB nuclear translocation induced by TNF-α are important for *S. aureus* internalization by BEC. In the diagram shown in Fig. 8, positive symbols indicate activation of *S. aureus* internalization, whereas question marks indicate an as-yet-undefined role on *S. aureus* internalization of the PI3K- and Akt-dependent phosphorylation of the NF-κB p65 subunit and GSK-3 isoforms. The role of the NF-κB p65 subunit and GSK-3α/GSK-3β phosphorylation in the internalization of this bacterium was not determined here. Future experiments will be designed to clarify this point.

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