UNIVERSIDAD MICHOACANA DE SAN NICOLÁS DE HIDALGO

FACULTAD DE MEDICINA VETERINARIA Y ZOOTECNIA

CENTRO MULTIDISCIPLINARIO

DE ESTUDIOS EN BIOTECNOLOGÍA

EL PÉPTIDOGLICANO Y LA PROTEÍNA A DE *Staphylococcus aureus* INDUCEN LA FOSFORILACIÓN DE GSK3 POR ACTIVACIÓN DE LA VÍA PI3K-Akt EN CÉLULAS ENDOTELIALES

Tesis que presenta
MAESTRA EN CIENCIAS RICARDA CORTÉS VIEYRA

Para obtener el grado de
DOCTORA EN CIENCIAS BIOLÓGICAS

Opción en
BIOTECNOLOGÍA MOLECULAR AGROPECUARIA

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Morelia, Michoacán, Agosto de 2013

Agradecimientos

Gracias señor, porque al mostrarme el camino de la ciencia, me guiaste hacia la fe. Te pido sabiduría en el desempeño de mi profesión.

Gracias Dr Baizabal, por formarme durante estos años de estudio; por sus consejos, por sus regaños y por compartir conmigo algunos de los secretos que le han permitido a usted ser un exitoso investigador.

Gracias Dr Alejandro, por la asesoría que me ha brindado durante mis estudios como tesista de licenciatura, maestría, y doctorado.

Gracias doctor Juan José, por sus consejos y por enseñarme con su ejemplo, que aún en los momentos difíciles se puede sonreír.

Gracias Dr Rosales, por asistir a cada uno de mis seminarios y por hacer siempre aportaciones importantes a mi trabajo.

Gracias Dr Gutiérrez, por sus consejos en el mejoramiento de mis protocolos de trabajo.

Gracias Dr Cajero, por las conversaciones acompañadas de un rico café y por el apoyo con reactivos y equipo.

Gracias Dr López-Bucio, Dr Joel, Dra Zavala y Lili, por coordinar los trámites administrativos que condujeron a mi examen de grado.

Gracias compañeros: Javier, Alejandro, Humberto, Ariana, Octavio, Roberto, German, Gustavo, Daniela, Elvira, Lulú, Carlos, Indira, Luis, Uriel y Raúl por todas sus enseñanzas, por compartir sus lágrimas de estudiante y por los ratos amenos.

Gracias papá y mamá, porque me dieron la libertad de elegir mi profesión, por su apoyo y porque nunca me pusieron límites.

Gracias hermanas y hermanos, por compartir conmigo sus alegrías y tristezas.

Gracias Sergio, por impulsarme a crecer profesionalmente, por brindarme tu confianza, por aguantarme en los días difíciles y por tu apoyo incondicional.

Gracias mi pequeña Liz, por tu belleza interior, por el placer que me has dado al ser tu mamá, por tus consejos tan acertados y por toda tu paciencia. Te pido perdón por cada

hora en la que no estuve a tu lado, aunque, te aseguro, en mi mente siempre estuviste presente.

Gracias Sr Sergio, mamá, Lupita, Rosy, Alma, Hansel y María Inés, por haber cuidado a mi princesa, en especial, gracias señora Elizabeth por la entrega y el amor al cuidar de Liz.

Gracias tía Adela, tía Meli, tío Jorge y tío Juan por estar al pendiente de mis estudios.

Gracias padre Gerardo, porque con sus homilías tan elocuentes, llenas de sabiduría y fe, usted contribuyó en mi reencuentro con mi señor Jesús.

Gracias amigos: Mago, Vanelly, Richie, Lizeth y Cristi, porque sus cantos, oraciones y sonrisas, hicieron mas ligera mi última etapa del doctorado.

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LISTA DE ABREVIATURAS

AL: Ácido lipoteicoico.

Akt1,2,3/PKB: Cinasa específica de serina treonina/proteína cinasa B 1,2,3.

AP1: Proteína activadora 1.

AT: Ácido teicoico.

BEC. Células endoteliales de bovino.

cSrc-ERK1/2: Sarcoma celular-cinasa regulada por señales extracelulares1/2.

EGFR: Receptor del factor de crecimiento epidérmico

FAK: Cinasa de adhesión focal.

FcγR: Receptor Fc gama.

FnBP: Proteínas de unión a fibronectina.

GSK3α/β: Glucógeno sintasa cinasa 3α/β.

GTPasa RhoA: Guanosina-5'-trifosfatasa A de la familia Ras.

IFN I: Interferón tipo I

IFN-α/β/γ: Interferón α/β/γ.

IFNAR: Receptor del interferón α/β.

IL: Interleucina.

IgG: Inmunoglobulina G.

IRAK: Cinasa asociada al receptor de interleucina 1.

IκBα: Inhibidor α del factor nuclear kappaB.

JAK1: Cinasa 1 de la familia Janus.

JNK: Cinasa c-Jun N-terminal.

IKKα/β: Subunidades α y β de la cinasa del inhibidor del factor nuclear kappaB.

LPS: Lipopolisacárido.

MAPK: Proteína cinasa activada por mitógeno.

MDP: Muramildipéptido.

MLCK: Cinasa de la miosina de cadena ligera.

MRSA: *Staphylococcus aureus* resistente a meticilina.

mTORC2: Complejo blanco de Rapamicina de mamífero.

MyD88: Proteína del gen de respuesta primaria a la diferenciación mieloide 88.

NF-κB: Factor nuclear kappaB

NIK: Cinasa de unión al factor nuclear kappaB.

NK: Célula natural asesina.

NOD: Receptor citoplasmático con dominio de oligomerización de nucleótido.

PAMP: Patrón molecular asociado a patógenos.

PDK1: Cinasa dependiente de fosfoinosítido 1.

PGN: Péptidoglicano.

PGRP: Proteínas de reconocimiento de péptidoglicano.

PRR. Receptor de reconocimiento de patrones moleculares asociados a patógenos.

PI3K: Fosfoinosítido 3-cinasa

PtdIns $(3, 4, 5)_3$: Fosfatidilinositol-3, 4, 5-trifosfato.

ROCK: Cinasa asociada a Rho.

SpA: Proteína A de *Staphylococcus aureus*

SSRs: Secuencias cortas repetidas de la proteína A de *S. aureus*.

STAT1/3: Transductor de señal y activador transcripcional 1/3.

TACE: Enzima convertidora de TNF-α.

TLR: Receptor tipo Toll.

TNF-α: Factor de necrosis tumoral α.

TNFR: Receptor del factor de necrosis tumoral.

sTNFR: Receptor del factor de necrosis tumoral soluble.

TRAF6: Factor 6 asociado al receptor del factor de necrosis tumoral.

TRIF: Dominio conteniendo a la proteína adaptadora TIR, inductora de interferón β.

vWF: Factor von Willebrand.

Xr: Región polimórfica de la proteína A de *Staphylococcus aureus.*

I. RESUMEN

Staphylococcus aureus es un patógeno versátil que provoca diversas enfermedades en humanos y animales. El sello de las enfermedades provocadas por la infección de *S. aureus* es la inflamación, caracterizada por el reclutamiento de leucocitos hacia el tejido infectado. Los mediadores que actúan sobre los leucocitos también actúan sobre las células endoteliales y viceversa, por lo tanto, la inflamación también involucra la activación del endotelio a través de múltiples vías de señalización. *S. aureus* cuenta con un arsenal de factores de virulencia que en su mayoría actúan en conjunto para provocar enfermedad en el huésped. El péptidoglicano (PGN) y la proteína A (SpA) son dos factores de virulencia importantes de *S. aureus* que activan fuertemente la inflamación. En mamíferos, la vía de señalización PI3K/Akt/GSK3 regula diversos procesos celulares esenciales y además es preponderante en la inmunidad innata, debido a que modula la producción de citocinas pro- y anti-inflamatorias. Existen tres isoformas de Akt, denominadas Akt1, Akt2 y Akt3. Sólo las isoformas Akt1 y Akt2, activadas por fosforilación por el complejo mTORC2 a través de la activación de PI3K, en Ser473 y Ser 474, respectivamente, se han asociado con la modulación de la inflamación por estímulos bacterianos. Con respecto a GSK3, existen dos isoformas denominadas GSK3α y GSK3β, las cuales se encuentran constitutivamente activas y pueden ser inhibidas por fosforilación en Ser9 y Ser 21, respectivamente, por Akt. Se ha establecido, principalmente en células del sistema inmune estimuladas con componentes bacterianos, que únicamente la isoforma GSK3β es la responsable de modular la producción de citocinas pro- y anti-inflamatorias. En un estudio previo de nuestro grupo de trabajo se demostró que durante la internalización de *S. aureus* en las células endoteliales de bovino (BEC) se lleva a cabo la fosforilación de GSK3α, GSK3β y NF-κB por activación de PI3K/Akt. Por lo tanto, en este trabajo, analizamos la activación de las BEC estimuladas con PGN y SpA de *S. aureus* a través del análisis de la vía PI3K/Akt/GSK3. Se observó que tanto PGN como SpA inducen la activación de la vía. Sin embargo, se pudo determinar que existen algunas diferencias importantes entre un estímulo y otro. 1) Mientras que PGN indujo la activación de Akt1 por PI3K, SpA de forma dependiente a la concentración y también por activación de PI3K, indujo la activación de Akt1 y Akt2. Cuando se combinaron ambos estímulos, se observó que la fosforilación de Akt2 se incrementó substancialmente en comparación con las células estimuladas solamente con SpA; 2) La activación de Akt1 por PGN provocó la fosforilación de GSK3α y GSK3β; aunque

ambas isoformas se fosforilaron, la fosforilación de GSK3α fue mayor que la fosforilación de GSK3β. Con respecto a SpA, la activación de Akt1 sólo causó la fosforilación de GSK3β en su sitio de inhibición, mientras que la activación de Akt2 no mostró efecto en ninguna de las isoformas de GSK3; 3) La activación de la vía PI3K/Akt1/GSK3α/β por PGN, convergió en la activación del factor transcripcional NF-κB, a través de la fosforilación de Ser536 en su dominio de transactivación. La activación de las vías PI3K/Akt1/GSK3β y PI3K/Akt2 por SpA, aparentemente no indujeron la activación de NF-κB, al menos no por fosforilación en Ser536. La activación de NF-κB por PGN, a través de la vía PI3K/Akt1/GSK3α/β, involucró la activación del receptor TLR-2 y tuvo como consecuencia el incremento en la expresión de la citocina pro-inflamatoria IL-12. Esto es importante porque IL-12 tiene una participación clave en la coordinación de la inmunidad innata y adaptativa, por lo que estos datos sugieren que el endotelio, por medio de la expresión de IL-12 por TLR2/PI3K/Akt1/GSK3α/β/NF-κB puede contribuir en la activación de la respuesta inmune. En conclusión, tanto PGN como SpA de *S. aureus* inducen la activación de la vía PI3K/Akt/GSK3 en células endoteliales, sin embargo, existen algunas diferencias específicas de isoforma en la activación e inhibición de Akt y GSK3, respectivamente, que probablemente conducen a respuestas fisiológicas diferentes. Por lo tanto, estudios posteriores deben enfocarse a establecer estas probables diferencias fisiológicas, lo que permitirá tener una mayor comprensión de la complejidad de la respuesta del endotelio a la infección por *S. aureus*.

II. SUMMARY

Staphylococcus aureus is a versatile pathogen that causes various diseases in humans and animals. The hallmark of the diseases caused by *S. aureus* infection is the inflammation, characterized by leukocyte recruitment to the infected tissue. Mediators of the inflammatory response act on both leukocytes and endothelial cells, activating multiple signaling pathways. *S. aureus* has an arsenal of virulence factors that act together to cause disease in the host. The peptidoglycan (PGN) and the protein A (SpA) are two important virulence factors of *S. aureus* that strongly activate inflammation. In mammals, the PI3K/Akt/GSK3 signaling pathway regulates diverse cellular processes and has an essential role in innate immunity because of its regulatory effect on the production of pro-and anti-inflammatory cytokines. There are three isoforms of Akt, designated Akt1, Akt2 and Akt3. Only the isoforms Akt1 and Akt2, activated by phosphorylation at Ser473 and Ser474 respectively, by mTORC2 complex via activation of PI3K, have been associated with the modulation of inflammation by bacterial stimuli. Regarding to GSK3, there are two constitutively active isoforms denominated GSK3α and GSK3β, which can be inhibited by phosphorylation at Ser 21 and Ser9 respectively, by Akt. It has been established, mainly in immune cells stimulated with bacterial components, that only GSK3β isoform is responsible for modulating the production of pro-and anti-inflammatory cytokines. A previous study of our research group showed that phosphorylation of GSK3α, GSK3β and NF-κB by activation of PI3K/Akt is associated with internalization of *S. aureus* in bovine endothelial cells (BEC). Therefore, we have analyzed the activation of the PI3K/Akt/GSK3 pathway in BEC stimulated with PGN and SpA from *S. aureus*. It was observed that both PGN and SpA induce the activation of the pathway. However, we were able to detect important differences depending on the stimulus. 1) While PGN induced activation of Akt1 by PI3K, SpA in a concentration-dependent manner induced a PI3K-dependent activation of Akt1 and Akt2. When PGN and SpA were added simultaneously we observed that phosphorylation of Akt2 markedly increased compared to to Akt2 from cells stimulated with SpA alone; 2) the activation of Akt1 by PGN caused phosphorylation of GSK3α and GSK3β. Although both isoforms were phosphorylated, GSK3α phosphorylation was higher than that of GSK3β. Activation of Akt1 by SpA, on the other hand, caused phosphorylation of GSK3β, but not GSK3α, whereas activation of Akt2 showed no effect on any of the isoforms of GSK3; 3) activation of PI3K/Akt1/GSK3α/β pathway by PGN

converged on the activation of NF-κB by phosphorylation at Ser536 located in its transactivation domain. Activation of PI3K/Akt1/GSK3β and PI3K/Akt2 pathways by SpA, did not apparently induce activation of NF-κB, at least not through phosphorylation at Ser536. Activation of NF-κB by PGN by PI3K/Akt1/GSK3α/β pathway involved activation of TLR2 and resulted in the increased expression of pro-inflammatory cytokine IL-12p40. This is important because IL-12p40 has a key role in the coordination of innate and adaptive immunity, suggesting that the endothelium, through the expression of IL-12 by TLR2/PI3K/Akt1/GSK3α/β/NF-κB, can contribute to the activation of the immune response. In conclusion, both PGN and SpA from *S. aureus* induce activation of PI3K/Akt/GSK3 pathway in endothelial cells. However, there are some differences in the isoform-specific activation and inhibition of GSK3 and Akt, respectively, which probably leads to various physiological responses. Therefore, future studies should focus on establishing these probable physiological differences, which will enable a greater understanding of the complexity of the endothelial response to infection by *S. aureus*.

III. INTRODUCCIÓN GENERAL

III.I MARCO TEÓRICO

III.IA Inflamación

La inflamación aguda es una respuesta rápida a infecciones microbianas o tejidos dañados que involucra el reclutamiento local y activación de leucocitos, principalmente neutrófilos (Pober y Sessa 2007). Durante la respuesta inflamatoria, el suministro de sangre hacia el área afectada y la permeabilidad capilar se incrementan sustancialmente, permitiendo que los leucocitos migren desde los vasos capilares hacia los espacios intersticiales que rodean el sitio infectado o dañado (Rankin 2004). Si el estímulo no es eliminado, entonces el proceso inflamatorio persiste y evoluciona. La composición de leucocitos infiltrados cambia de neutrófilos a una mezcla de fagocitos mononucleares y células T. La estimulación antigénica prolongada por microbios resistentes o autoantígenos derivados de tejidos dañados conduce a la inflamación crónica (Pober y Sessa 2007). Las moléculas responsables de la comunicación célula-célula, denominadas citocinas, tienen una función extremadamente importante en la modulación del proceso inflamatorio (Rankin 2004, ver Anexo I Cortés-Vieyra *et al.* 2012). La interleucina 12 (IL)-12 es una citocina pro-inflamatoria, especialmente importante porque su expresión durante la infección regula la inmunidad innata y determina el tipo y duración de la respuesta adaptativa (Watford *et al.* 2003). Además de las células del sistema inmune, las células de la microvasculatura endotelial también son participantes activos y reguladores del proceso inflamatorio, cambiando sus fenotipos para soportar las diferentes fases de la inflamación (Pober y Sessa 2007). Por lo tanto, la respuesta inflamatoria representa un complejo proceso biológico y bioquímico que involucra células del sistema inmune, células endoteliales y una plétora de mediadores biológicos (Rankin 2004, Pober y Sessa 2007, Cortés-Vieyra et al., 2012) (Ver Anexo I).

III.IB Características generales de *Staphylococus aureus*

Staphylococcus aureus es una bacteria Gram positiva, caracterizada por ser predominantemente un organismo comensal. Sin embargo, es una bacteria que se adapta al entorno, por lo que está considerada un patógeno facultativo importante (Foster 2005). Las infecciones por *S. aureus* son de gran importancia en la medicina humana y veterinaria (Pantosti 2012). En humanos, la colonización por *S. aureus* de los orificios nasales,

desempeña una función clave en la epidemiologia y patogénesis de la infección, debido a que permite que *S. aureus* sea transmitido entre personas, tanto en hospitales como en la comunidad (Kluytmans *et al.* 1997, Werthein *et al.* 2005). Además, en individuos inmunocomprometidos, el comensalismo nasal puede evolucionar a una variedad de enfermedades que van desde infecciones de la piel o de las mucosas hasta infecciones invasivas que pueden poner en riesgo la vida del huésped tales como sepsis, neumonía, osteomielitis y endocarditis (Kluytmans *et al.* 1997, Lowy 1998). En animales domésticos, *S. aureus* está involucrado principalmente en infecciones intramamarias de hembras lactantes (Sutra y Poutrel 1994). De manera importante, las infecciones en humanos causadas por cepas de *S. aureus* resistentes a antibióticos han alcanzado proporciones epidémicas, en particular las infecciones causadas por cepas de *S. aureus* resistentes a meticilina (MRSA) (Grundmann *et al.* 2006). Las cepas animales de *Staphylococcus* también representan una fuente de MRSA (Pantosti 2012).

El arsenal de factores de virulencia de *S. aureus* responsables de promover el desarrollo de la infección es extenso. Esta bacteria cuenta con componentes estructurales de la pared celular como péptidoglicano (PGN), ácido lipoteicoico (LTA), proteína A (SpA) (Figura 1), colágeno, proteínas de unión a fibronectina (FnBP) etc, y moléculas secretables, por ejemplo las toxinas α, β, δ y γ, así como otros determinantes de virulencia (Gordon y Lowy 2008). Con excepción de las enfermedades provocadas por toxinas específicas, tales como enterotoxinas y toxinas del síndrome de choque tóxico, la virulencia de *S. aureus* es multifactorial, de tal forma que un factor de virulencia puede tener varias funciones en la patogénesis y múltiples factores de virulencia pueden realizar la misma función. Estas funciones múltiples en su virulencia también está asociada con las cepas MRSA (Fournier y Philpott 2005, Gordon y Lowy 2008, Otto 2012).

La mayoría de las manifestaciones de las enfermedades provocadas por *S. aureus* involucran bacterias extracelulares o formación de biopelículas (Fraunholz y Sinha 2012, Savage 2013). Además de estas infecciones, existe evidencia de que *S. aureus* es capaz de sobrevivir dentro de las células del huésped y que puede invadir *in vitro* una variedad de fagocitos no profesionales, incluyendo fibroblastos (Sinha 1999), osteoblastos (Jevon 1999), células endoteliales (Ogawa 1995, Oviedo-Boyso *et al.* 2011) y epiteliales (Dziewanowska 1999), por lo que se le denomina patógeno intracelular facultativo. Las estrategias de

sobrevivencia de *S. aureus* en el interior celular incluyen: escape del fagosoma, subversión de la autofagia, inducción de mecanismos de muerte celular como apoptosis y pironecrosis y la inducción de programas anti-apoptóticos (Fraunholz y Sinha 2012).

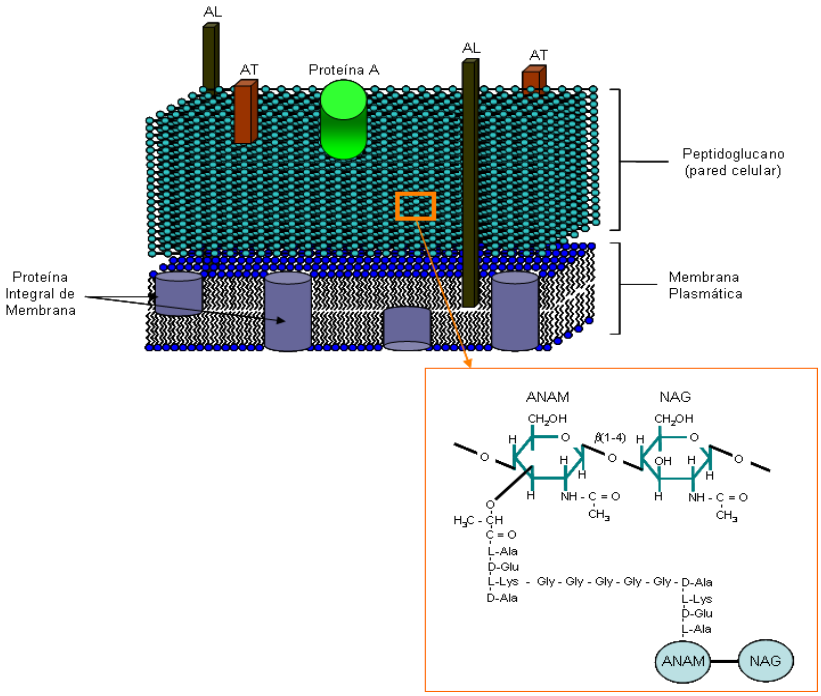

Figura 1. Estructura de la pared celular de *Staphylococcus aureus*. La pared celular de las bacterias Gram positivas como *S. aureus* tienen como componente principal el péptidoglicano. Además del péptidoglicano, en la pared se encuentran otras estructuras tales como el ácido teicoico (AT) y el ácido lipoteicoico (AL). Entre las proteínas de superficie más abundantes de *S. aureus* se encuentra la proteína A (SpA). ANAM, ácido N-acetil murámico; D-Ala, D-Alanina; D-Glu, D-Glutámico; L-Ala, L-Alanina; L-Lys, L-lisina y NAG, *N*-acetilglucosamina. (Tomada de Baizabal-Aguirre *et al.* 2009).

III.IC Modulación de la inflamación por estimulación con PGN de *S. aureus*

PGN es un componente bacteriano esencial que provee rigidez y estructura a la pared celular. Todas las bacterias contienen PGN, pero la cantidad, localización y composición específica varía (Schleifer y Kandler 1972). En la pared de bacterias Gram positivas como *S. aureus* el componente dominante es PGN (Baizabal-Aguirre *et al.* 2009) (Fig. 1). PGN (típicamente de 20-30 nm de grosor) es un polímero insoluble, heterogéneo y altamente entrecruzado, lo que le permite funcionar como una barrera protectora, así como un andamio para la de unión de proteínas de superficie y matrices extracelulares, las cuales son requeridas para la morfogénesis, división celular y la patogénesis (Giesbrecht *et al.* 1998).

PGN se ensambla a partir de una unidad repetida que consiste de un disacárido o hebra de glicanos, un tallo y un puente. El disacárido, N-Acetilglucosamina y ácido N-

Acetilmurámico unido mediante enlaces β-1,4, está conservado en todas las bacterias, pero las estructuras del tallo y del puente varían de un organismo a otro (Rogers *et al.* 1980). En *S. aureus*, el tallo es el pentapéptido L-alanina-D-iso-glutamina-L-lisina-D-alanina-D-alanina y el puente es un segmento pentaglicil (Fig. 1) (Sharif *et al.* 2009). En algunas especies de patógenos bacterianos las hebras de glicanos de PGN son modificadas, deacetiladas y/o O-acetiladas, después de su inserción en la pared celular. Estas alteraciones afectan el reconocimiento de las bacterias por el huésped y contribuyen a la resistencia de la bacteria a factores de defensa (Vollmer 2008). *S. aureus* es altamente resistente a lisozimas y el mecanismo principal para esta resistencia es la modificación de su PGN por O-acetilación en la posición C-6 del ácido N-Acetilmurámico (Bera *et al.* 2005).

La virulencia de patógenos bacterianos depende en gran medida de su habilidad para producir toxinas. Las toxinas bacterianas pueden ser componentes estructurales de la pared (endotoxinas) o proteínas secretadas (exotoxinas). Se ha sugerido que la extensión sistémica de las toxinas, más que la bacteremia en sí misma, es el evento crucial en el desarrollo de sepsis y choque séptico (Grandel y Grimminger 2003). Estudios recientes, han mostrado un incremento en la tasa de bacteremias, provocadas tanto por bacterias Gram positivas, siendo *S. aureus* la causa dominante (Gould 2013), como por bacterias Gram negativas (Hagel *et al.* 2013). En las bacteremias provocadas por Gram negativas, el lipopolisacárido (LPS) es ampliamente reconocido como la endotoxina clásica, responsable de provocar una respuesta inflamatoria sistémica que acompaña la sepsis (Peters *et al.* 2003). Por otro lado, en modelos de cultivos de células del sistema inmune innato, células epiteliales, fibroblastos y microglía de humano se ha demostrado que PGN estimula la producción de citocinas pro-inflamatorias, tales como IL-1α/β, IL-6, IL-8 y TNFα (McDonald *et al.* 2005, Chiu *et al*. 2009, Lin *et al*. 2010). Se ha observado *in vivo* que durante las infecciones por bacterias Gram positivas, PGN puede entrar a torrente sanguíneo, provocando inflamación sistémica, por lo cual se ha aceptado que PGN también debe ser considerado como una endotoxina (Myhre *et al* 2006).

La razón por la cual algunos investigadores han refutado que PGN tenga la capacidad de provocar una fuerte inflamación, responsable de provocar sepsis, se debe a que los roedores desafiados con PGN mostraron una patología no severa en comparación con roedores desafiados con LPS (De Kimpe *et al*. 1995, Wray *et al.* 2001). Actualmente, se sabe que esto se debe a que en contraste con las células del sistema inmune innato de humanos, los

macrófagos de ratón tienen una respuesta mínima a la estimulación con PGN. La razón de la diferencia entre especies aún no es clara, aunque puede ser explicada en parte por el hecho de que PGN se une débilmente a los macrófagos de ratón (Iyer *et al.* 2010).

Además, también se ha sugerido que la actividad biológica de PGN de debe a la contaminación con agonistas de TLR y que PGN es prácticamente inocuo (Travassos *et al.* 2004, Volz *et al.* 2010). El descubrimiento de los receptores citoplasmáticos con dominio de oligomerización de nucleótido (NOD) ha hecho más complejo el estudio de PGN. Se ha propuesto que algunos monómeros derivados del polímero de PGN, denominados muramildipéptidos (MDPs), son internalizados y reconocidos por receptores NOD, induciendo la activación de NF-κB y la expresión de moléculas pro-inflamatorias (Marina-García *et al.* 2009; Lee *et al.* 2009, Humann y Lenz 2009, Kaparakis *et al.* 2010). Sin embargo, otros estudios recientes han demostrado que el polímero de PGN, altamente purificado, estimula la producción de citocinas pro-inflamatorias en células del sistema inmune innato y que la naturaleza polimérica del PGN es esencial para llevar a cabo la inducción de citocinas (Langer *et al.* 2008, Iyer *et al.* 2010).

A pesar de que también existe controversia acerca de la activación del receptor extracelular TLR-2 por PGN (Travassos *et al.* 2004), diversos estudios, incluyendo un análisis de co-localización con PGN libre de lipoproteínas, han demostrado que en células del sistema inmune innato y en otros tipos celulares PGN induce la respuesta pro-inflamatoria, principalmente por activación del receptor TLR-2 (Chen *et al*. 2004, Chen *et al*. 2009, Chiu *et al*. 2010, Lin *et al*. 2010, Müller-Anstett *et al*. 2010). Además de TLR-2, PGN se une a las proteínas de reconocimiento de PGN (PGRP), una clase PGRPs, denominadas PGLYRP-2, son N-acetilmuramoil-L-alanina amidasas que hidrolizan PGN y reducen su actividad pro-inflamatoria (Dziarski y Gupta 2006). Se ha demostrado que PGN activa receptores exclusivos de las células del sistema inmune innato (monocitos, macrófagos, neutrófilos), tales como CD14, que también se une a LPS y FcγR, activando fuertemente la inflamación y contribuyendo a la sepsis (Dziarski *et al* 1998, Sun *et al.* 2012). En el caso del receptor FcγR, caracterizado por ser altamente fagocítico, se propuso que puede funcionar como el receptor extracelular de PGN, permitiendo su internalización y estimulando la producción de citocinas, después de que la degradación lisosomal ha generado los ligandos que interactúan con NOD (Iyer *et al.* 2010, Sun *et al.* 2012).

La inducción de moléculas pro-inflamatorias por PGN requiere la activación de NF-κB mediada por múltiples moléculas de señalización dependientes de TLR-2, como la proteína de diferenciación mieloide (MyD88), la cinasa asociada a IL-1R (IRAK), el factor 6 asociado al receptor del factor de necrosis tumoral (TRAF6), la cinasa de unión a NF-κB (NIK) y la cinasa IκB (IKK) (Wang *et al* 2001, Xu *et al* 2001). La inducción de la inflamación por PGN también se lleva a cabo a través de las MAPKs, incluyendo ERK, JNK/SAPK y p38, resultando en la activación de NF-κB y AP1 (Beutler 1992, Gupta *et al* 1995, In su *et al* 2007). Recientemente, se ha observado en células estimuladas con PGN que la vía TLR2/PI3K/Akt es responsable de inducir la expresión de moléculas pro-inflamatorias, a través de la activación de los factores transcripcionales NF-κB (Chen *et al.* 2009, Lin *et al.* 2010) y AP-1 (Chiu *et al.* 2009) (Fig. 2). Sin embargo, no se ha asociado la participación de la cinasa GSK3, como parte de la vía PI3K-Akt en la modulación de la inflamación en células activadas con PGN.

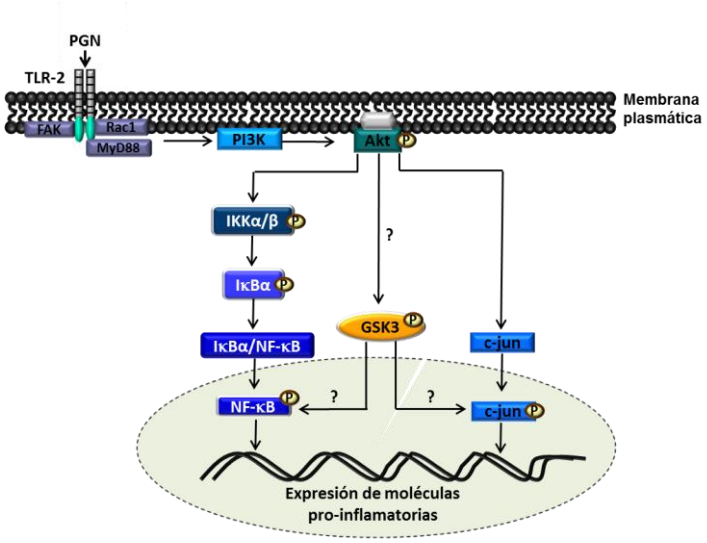

Figura 2. Inducción de la inflamación por activación de la vía PI3K-Akt en células estimuladas con péptidoglicano (PGN) de *Staphylococcus aureus*. La unión de PGN al receptor TLR-2 puede inducir la activación de MyD88 y Rac1, responsables de inducir la activación de la vía de señalización PI3K-Akt. La vía PI3K-Akt puede incrementar la activación de IKKα/β y la degradación de IκBα, provocando la fosforilación de p65 y la transactivación de NF-κB. La unión de PGN al receptor TLR-2 también puede inducir la activación de PI3K-Akt a través de la activación de FAK, provocando un incremento en la unión de c-jun al sitio AP-1. La transactivación de NF-κB y c-jun inducen la expresión de moléculas pro-inflamatorias. Hasta ahora no se ha demostrado si GSK3, como parte de la vía PI3K-Akt, también participa en la fosforilación y transactivación de NF-κB y AP-1 en células estimuladas con PGN. PGN, péptidoglicano; TLR-2, receptor tipo Toll 2; FAK, cinasa de adhesión focal; Rac1; MyD88, proteína del gen de respuesta primaria a la diferenciación mieloide 88; PI3K, fosfoinosítido 3-cinasa; Akt/PKB, proteína cinasa B; GSK3, glucógeno sintasa cinasa 3; IKKα/β, subunidades α y β de la cinasa del inhibidor del factor nuclear kappa-B; IκBα, inhibidor del factor nuclear kappa-B; NFκB, factor nuclear kappa de cadena ligera-potenciador de células B activadas; c-jun; cinasa N-terminal; P, grupo fosfato.

III.ID Modulación de la inflamación por estimulación con proteína A (SpA) de *S. aureus*

La proteína A (SpA) es un componente de superficie abundante (constituye aproximadamente el 7% de la pared de *S. aureus*) presente en todas las cepas de *S. aureus* (Fig. 1) (Cheung *et al.* 1997, Martin *et al.* 2009). Además de ser una proteína de superficie, SpA es secretada activamente en el sobrenadante de algunos cultivos en crecimiento (Cheung *et al.* 1997). SpA es una proteína de aproximadamente 42 kDa constituida por 5 dominios E, D, A, B y C de unión a la inmunoglobulina G (IgG), que están localizados en la región amino terminal, seguidos por una región polimórfica llamada Xr, la cual contiene un número variable (1-22) de secuencias cortas repetidas de 24-bp que son la base de un esquema internacional de tipificación epidemiológica y una región carboxilo terminal de anclaje a la pared bacteriana (Fig. 3) (Martin *et al.* 2009, Garofalo *et al.* 2012).

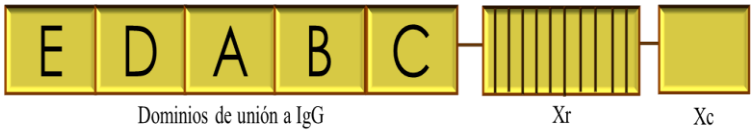

Figura 3. Estructura de la proteína A (SpA) de *Staphylococcus aureus*. E, D, A, B, C: dominios conservados de unión a IgG; Xr: región de secuencias repetidas cortas; Xc: región de anclaje a la pared bacteriana. Modificada de Martin *et al.* (2009).

Debido a que SpA es la principal proteína de superficie encontrada en *S. aureus*, especialmente en aislados respiratorios, y debido a su habilidad para imitar la activación del receptor de TNF-α, TNFR1, SpA está considerado el factor estafilocócico pro-inflamatorio principal de los pulmones. La activación de TNFR1 por SpA induce la expresión de la citocina pro-inflamatoria IL8 por la activación de los factores transcripcionales NF-κB y AP-1 (Fig. 4) (Gómez *et al.* 2004). Además, la interacción de SpA con TNFR1 también induce la expresión de la citocina moduladora de la inflamación, IL-6, así como liberación a la superficie celular de la forma soluble de TNFR1 (sTNFR1) por la enzima convertidora de TNF-α (TACE), provocando una respuesta anti-inflamatoria (Fig. 4) (Gómez *et al.* 2004, Gómez *et al.* 2006). La liberación de sTNFR1 por TACE depende de la activación del receptor del factor de crecimiento epidérmico (EGFR), el cual induce la fosforilación de TACE, a través de la vía de señalización cSrc-ERK1/2 (Fig. 4) (Gómez *et al.* 2007). La interacción de SpA con TNFR1 y EGFR también activa a la GTPasa RhoA, la cinasa asociada a Rho (ROCK) y la cinasa de la

miosina de cadena ligera (MLCK), alterando el citoesqueleto de células epiteliales de pulmón y permitiendo la invasión de *S. aureus* a monocapas de células epiteliales polarizadas, vía las uniones paracelulares (Soong et al. 2011).

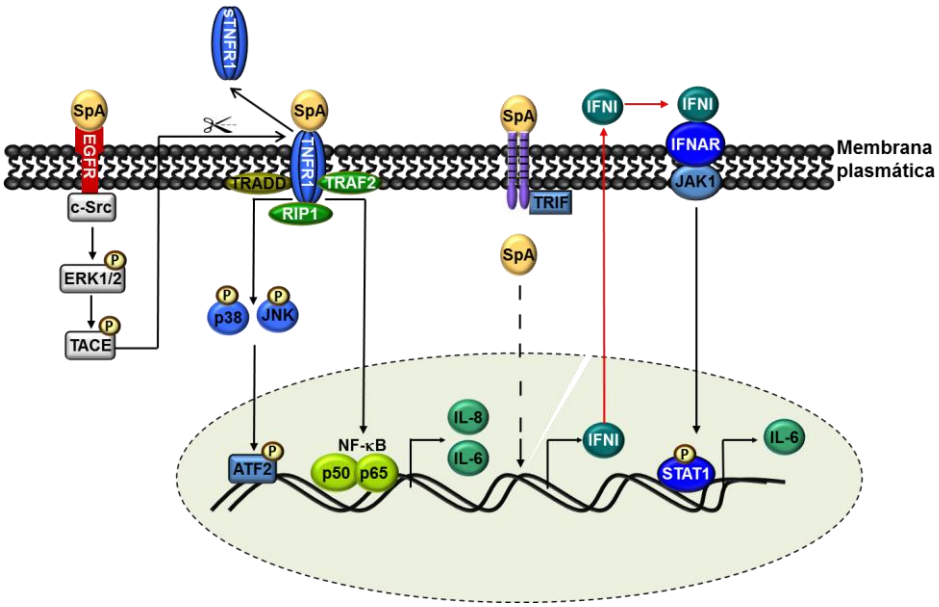

Figura 4. Modulación de la inflamación en células estimuladas con proteína A (SpA) de *Staphylococcus aureus.* Al unirse SpA a TNFR1 induce el reclutamiento de las proteínas adaptadoras TRADD, TRAF2 y RIP1, conduciendo a la translocación al núcleo y activación de NF-κB. Simultáneamente, la interacción de SpA con TNFR1 induce la activación de las cinasas p38 y JNK, responsables de inducir la translocación al núcleo y la activación de ATF2. La activación de los factores transcripcionales NF-κB y ATF2 induce la expresión de la interleucina pro-inflamatoria IL-8 y de la interleucina pro y anti-inflamatoria IL-6. SpA es capaz de inducir una disminución de la respuesta pro-inflamatoria inducida por la activación de TNFR1, al unirse al receptor EGFR. La unión de SpA con EGFR activa la vía de señalización c-Src-ERK1/2 que induce la fosforilación y activación de TACE, encargada de liberar a TNFR1 de la membrana plasmática en forma soluble (sTNFR1). SpA también es capaz de internalizarse, probablemente a través de la activación de un receptor TLR, provocando la expresión del IFNI. Entonces, IFNI de manera autócrina activa la vía de señalización IFNAR-JAK1-STAT1, induciendo la producción de IL-6. SpA, proteína A de *S. aureus*; TNFR1, receptor del factor de necrosis tumoral 1; TRADD, proteína de dominio de muerte asociada al receptor del factor de necrosis tumoral 1; TRAF2, factor 2 asociado al receptor TNF; p38 y JNK (cinasa N-terminal c-Jun), proteínas cinasas activada por mitógeno; ATF2, factor de la activación de la transcripción 2; NFκB, factor nuclear kappa de cadena ligera-potenciador de células B activadas; TLR, receptor tipo Toll; TRIF, dominio que contiene el adaptador TIR (dominio del receptor Toll de interleucina 1) inductor del interferón β; IFN1, interferón tipo I; IFNAR, receptor del interferón α/β; JAK, cinasa Janus 1; STAT1, transductor de señal y activador de la transcripción 1; P, grupo fosfato.

Cada uno de los dominios de unión a IgG de SpA puede afectar múltiples repuestas inmunológicas de forma independiente, por ejemplo pueden activar la inflamación a través de TNFR1, bloquear la opsonización al unirse fuertemente al dominio Fc de las IgGs y promover la unión bacteriana al factor von Willebrand (vWF) inmovilizado. Los 5 dominios de unión a IgG, E-C, son necesarios para frenar la inflamación a través de la liberación de sTNFR1 (Gómez *et al.* 2006, O´Seaghdha *et al.* 2006). Por otra parte, la región polimórfica Xr de SpA modula la inflamación por activación de la vía de señalización del IFN tipo I. Después del

reclutamiento de la proteína adaptadora TRIF en células epiteliales, inducida por la región Xr, SpA es endocitada y con ello se induce la producción de INF-β, el cuál activa de manera autócrina la vía de señalización IFNAR-JAK1-STAT1, responsable de inducir la expresión de IL-6 (Fig. 4) (Martin *et al.* 2009). Existe una aparente correlación entre el número de SSRs en la región Xr y la fuerza de la respuesta inflamatoria. Además, también se ha asociado la longitud de la región Xr con la influencia de SpA en el desarrollo de enfermedades inflamatorias crónicas y agudas (Garofalo *et al.* 2012).

III.IE Modulación de la inflamación por la enzima glucógeno sintasa cinasa 3

La glucógeno sintasa cinasa 3 (GSK3) es una cinasa de Ser/Thr multifuncional presente en eucariotes (Doble and Woodgett *et al.* 2003). En mamíferos existen dos isoformas principales de GSK3, denominadas GSK3α y GSK3β (Woodgett 1990). GSK3 regula numerosas vías de señalización involucradas en varias funciones celulares esenciales, que van desde la regulación del metabolismo de glucosa hasta la modulación del sistema inmune innato y adaptativo (Beurel *et al.* 2010). Se ha observado en células del sistema inmune innato y en otros tipos celulares que la isoforma GSK3β es importante durante la infección bacteriana, especialmente en la respuesta inflamatoria que acompaña a este proceso, debido a que principalmente regula la actividad del factor nuclear kappaB (NFκB). (Fig. 5) (Anexo I) (Cortés-Vieyra *et al.* 2012). GSK3β también tiene una participación prominente en la regulación de la proliferación de las células T, a través de la modulación de la expresión de IL-12 (Ohteki *et al.* 2000). Con respecto a GSK3α, no se ha estudiado su participación en la modulación de la inflamación durante infecciones bacterianas. A diferencia de la mayoría de las proteínas cinasas, GSK3 está constitutivamente activa en células no estimuladas y su actividad se inhibe por fosforilación durante ciertas respuestas celulares (Doble and Woodgett 2003).

La fosforilación e inhibición de GSK3α en Ser 21 y GSK3β en Ser9 puede llevarse a cabo por la proteína cinasa B (Akt/PKB) mediante la activación de la fosfoinosítido 3-cinasa (PI3K) (Fig. 5) (Jope y Jhonson 2004). Existen tres isoformas de Akt estrechamente relacionadas, denominadas Akt1 (PKBα), Akt2 (PKBß), y Akt3 (PKBγ) que están codificadas por genes independientes. Akt1 se expresa en la mayoría de los tejidos (Chen *et al.* 2001), Akt2 se expresa principalmente en órganos que responden a insulina (Garofalo *et al.* 2003) y

Akt3 se expresa principalmente en cerebro y testículos (Easton *et al.* 2005). Akt se activa por unión con fosfatidilinositol 3,4,5-trifosfato (PtdIns 3,4,5)$_3$), que es sintetizado por la enzima PI3K y por la fosforilación de Akt1 en Thr308 y Ser473, Akt2 en Thr309 y Ser474 y Akt3 en Thr305 y Ser472 por la cinasa PDK1 (Alessi *et al.* 1997, Frodin *et al.* 2002) y mTORC2 (Fig. 5) (Sarbassov *et al.* 2005, Hresko *et al.* 2005). En macrófagos estimulados con LPS se demostró que la isoforma Akt1, a través de la activación de PI3K, es la responsable de fosforilar e inhibir a la isoforma GSK3β, modulando negativamente la inflamación (Martin *et al.* 2005, Androulidaki *et al.* 2009). En contraste, en un modelo de gastroenteritis aguda por *Salmonella*, se demostró que la isoforma Akt2 es esencial en la protección contra inflamación y apoptosis (Kum *et al.* 2011).

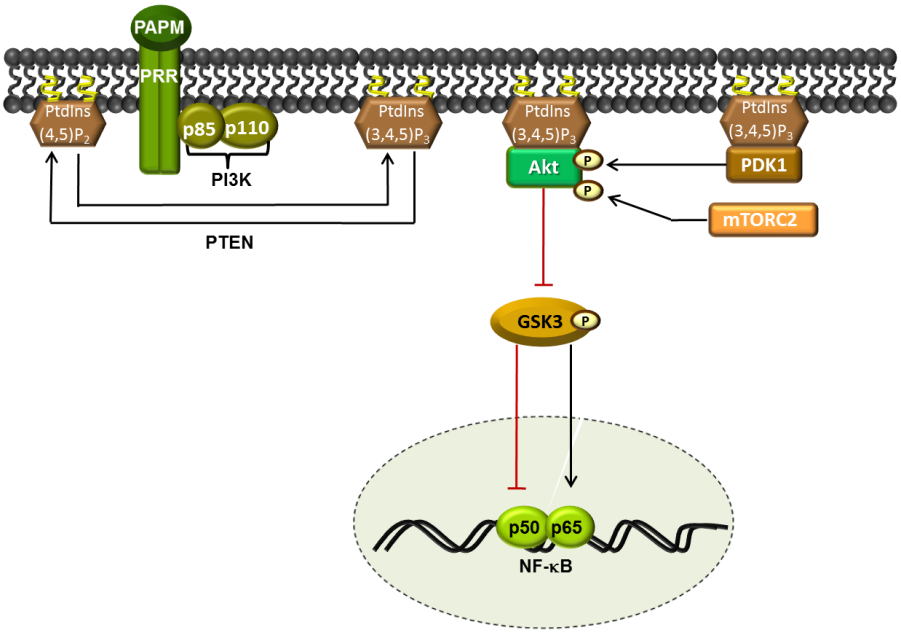

Figura 5. Modulación de la inflamación por la de glucógeno sintasa cinasa 3 (GSK3). La activación de un PRR por un algunos PAMPs bacterianos induce la unión de la subunidad reguladora p85 de PI3K al receptor activado, permitiendo que la subunidad catalítica p110 de PI3K catalice la conversión de fosfatidilinositol-4,5-difosfato (PtdIns $(4,5)_2$) a fosfatidilinositol-3,4,5-trifosfato (PtdIns $(4,5)_3$). La reacción reversible de conversión de PtdIns $(3,4,5)_3$ a PtdIns $(4,5)_2$ es llevada a cabo por la fosfatasa PTEN. Los PtdIns $(3,4,5)_3$ funcionan como segundos mensajeros, induciendo el reclutamiento de las proteínas cinasas Akt y PDK1 a través de sus dominios con homología a plecstrina. La completa activación de Akt se lleva a cabo por su fosforilación en un residuo de Treonina y en un residuo de Serina por las cinasas PDK1 y mTORC2, respectivamente. Akt puede fosforilar e inactivar a GSK3. La inactivación máxima de GSK3 puede tener como consecuencia la activación o inactivación del factor transcripcional NFκB, dependiendo del estímulo, del tipo celular y probablemente del estado fisiológico de la célula estimulada. PAMP, patrón molecular asociado a patógenos; PRR, receptor de reconocimiento de patógenos; PI3K, fosfoinositido-3-cinasa; PTEN, fosfatasa y homólogo de tensina; Akt/PKB/RAC, proteína cinsasa B; PDK1, cinasa dependiente de fosfoinositido 1; mTORC2, complejo blanco de rapamicina 2.

III.II JUSTIFICACIÓN

En general, la habilidad de los patógenos microbianos para causar enfermedad en el hospedero se debe a la evasión y manipulación del sistema inmune innato y adaptativo (Finlay y McFadden 2006). Los neutrófilos son las células más prominentes del sistema inmune innato porque activan la inflamación y proveen una defensa primaria esencial contra patógenos bacterianos, tales como *S. aureus* (Rigby y DeLeo, 2012). Debido a que la inflamación involucra el rápido reclutamiento de neutrófilos y la activación de células endoteliales (Pober y Sessa 2007), es evidente que durante la infección con *S. aureus*, un gran número de moléculas estafilocócicas interactúan con las células del sistema inmune innato y el endotelio para activar la inflamación, sugiriendo una compleja interacción entre *Staphylococcus* y estas células eucariotas (Fournier y Philpott 2005). Sin embargo, la excesiva o prolongada inflamación puede dañar al hospedero y provocarle enfermedad (Lawrence y Gilory 2007). Dado que el endotelio, con su diversidad de funciones fisiológicas es el principal blanco de toxinas bacterianas (Grandel y Grimminger 2003), se ha propuesto que la disfunción endotelial resultante de la interacción con toxinas bacterianas contribuye al desarrollo patológico de la inflamación y al daño de órganos (Grandel y Grimminger 2003, Pober y Sessa 2007)

El péptidoglicano (PGN), es reconocido por los PRRs de células del sistema inmune innato y de células endoteliales debido a que es una molécula estructural de la pared celular que está conservada entre especies bacterianas y que no se encuentra en las células animales (Fournier y Philpott 2005). Se ha reportado que provoca una fuerte respuesta inflamatoria (McDonald *et al.* 2005), por lo que se le considera una endotoxina (Myhre *et al.* 2006). La proteína de superficie, proteína A (SpA), presente en casi todas las cepas de *S. aureus* (Cheung *et al.* 1997, Martin *et al.* 2009) es también un importante inductor de la inflamación (Bien *et al.* 2011), mostrando la capacidad multifactorial de *S. aureus* para activar la inflamación en el hospedero (Gordon y Lowy 2008) y dejando claro que el sello de las infecciones de *S. aureus* es la inflamación (Martin *et al.* 2009).

Se ha observado en ciertos tipos celulares de mamíferos que la proteína cinasa multifuncional GSK3, cuya actividad es regulada por la vía PI3K-Akt (Jope y Johnson 2004), es un importante modulador de la inflamación durante las infecciones bacterianas (Cortés-Vieyra *et al.* 2012) (ver Anexo I). Es importante destacar que existen dos principales

isoformas de GSK3, α y β, pero la isoforma GSK3β es la que principalmente se ha asociado con infecciones bacterianas (Cortés-Vieyra *et al.* 2012). Se ha demostrado en macrófagos, microglía y fibroblastos que PGN induce la activación de los factores transcripcionales NF-κB y AP-1 y la expresión de moléculas pro-inflamatorias por activación de la vía PI3K-Akt (Chen *et al.* 2009, Chiu *et al.* 2009, Lin *et al.* 2010), pero hasta ahora no se ha establecido en células estimuladas con PGN si la actividad de la isoforma GSK3α o GSK3β, inhibida por la actividad de PI3K-Akt, modula la actividad de NF-κB. Con respecto a SpA, se han publicado diversos estudios en células epiteliales de pulmón de humano estimuladas con este factor de virulencia, para establecer las vías de transducción responsables de inducir la expresión de citocinas pro-inflamatorias (Gómez *et al.* 2004, Gómez *et al.* 2006, Gómez *et al.* 2007, Martin *et al.* 2009). Sin embargo, hasta ahora no se ha estudiado la activación de la vía PI3K-Akt-GSK3α/β por SpA.

Recientemente, nuestro grupo de trabajo demostró en endotelio bovino que la actividad de la vía PI3K-Akt y la fosforilación de GSK3α, GSK3β y NF-κB es importante para la internalización de *S. aureus* (Oviedo-Boyso *et al.* 2011) (Anexo II). Es probable que durante la internalización de *S. aureus* por las células endoteliales, algunos de los componentes de la bacteria activen un proceso inflamatorio. Por lo tanto, es interesante estudiar en células endoteliales si PGN o SpA de *S. aureus* son responsables de inducir la activación de NF-κB, por activación la vía PI3K-Akt-GSK3α/β y su efecto en la inflamación. Un marcador de la inflamación es la IL-12, debido a que es una molécula pro-inflamatoria moduladora de la inmunidad innata y adaptativa (Watford *et al.* 2003). El establecimiento de las vías de señalización responsables de activar las células del hospedero por componentes de *S. aureus* permitirá tener un esquema más amplio de la patogenicidad de esta bacteria y en un futuro diseñar estrategias biotecnológicas para eliminar o reducir los efectos inflamatorios adversos que se presentan en las infecciones de diversos tejidos por esta bacteria.

IV. HIPÓTESIS

El péptidoglicano (PGN) y la proteína A (SpA) de *Staphylococcus aureus* modulan la actividad del factor transcripcional NF-κB por la vía PI3K/Akt/GSK3 en células endoteliales de bovino (BEC).

V. OBJETIVOS

V.I Objetivo general

Evaluar si el estímulo de las BEC con PGN o SpA de *S. aureus* modulan la actividad del factor transcripcional NF-κB por la vía PI3K/Akt/GSK3.

V.II Objetivos específicos

V.II A

Analizar el nivel de fosforilación de Akt, GSK3 y NF-κB en BEC estimuladas con PGN o SpA de *S. aureus*.

V.II B

Demostrar que el estado de fosforilación de NF-κB depende de la activación de la vía PI3K/Akt/GSK3 en BEC estimuladas con PGN o SpA de *S. aureus*.

V.II C

Analizar la expresión de la citocina pro-inflamatoria IL-12 en BEC estimuladas con PGN o SpA de *S. aureus* y demostrar que su expresión depende de la activación de la vía PI3K/Akt/GSK3/ NF-κB.

VI. RESULTADOS

El cumplimiento de los objetivos planteados permitió obtener los resultados que a continuación se presentan en dos capítulos. En el capítulo I se abordó en BEC estimuladas con PGN de *S. aureus*, el análisis de la vía TLR2/PI3K/Akt/GSK3 y su efecto sobre el factor transcripcional NF-κB y la expresión de la citocina pro-inflamatoria IL-12p40. En el capítulo II se estudió la respuesta del endotelio frente al estímulo con SpA de *S. aureus*, también a través del análisis de la vía PI3K/Akt/GSK3 y de NF-κB. Sin embargó, aunque se observó la activación de la vía y del regulador positivo de la inflamación, NF-κB, la activación de NF-κB no dependió de la activación de PI3K/Akt, por lo tanto, en BEC estimuladas con SpA, no se analizó si la expresión de citocinas pro-inflamatorias, tales como IL-12-p40.

Peptidoglycan from *Staphylococcus aureus* Activates Interleukin (IL)-12p40 Expression in Endothelial Cells by Phosphoinositide 3-kinase/Akt-Dependent Glycogen Synthase Kinase 3 (GSK3) Inhibition

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Abstract

Glycogen synthase kinase 3β (GSK3β) is an important modulator of the inflammatory response caused by bacterial pathogens. It is known that inhibition of GSK3β by bacterial virulence factors inhibits the expression of pro-inflammatory cytokines. We have demonstrated that internalization of *Staphylococcus aureus* by bovine endothelial cells (BEC) was associated with activation of the PI3K/Akt/GSK3α/β signaling pathway. Because peptidoglycan (PGN) is the most abundant cell-wall structure of Gram-positive bacteria, and it is also an important inducer of inflammation and an activator of the PI3K/Akt pathway, we decided to analyze whether inhibition of GSK3α/β activity by phosphorylation via PGN regulates the expression of IL-12p40, an important pro-inflammatory cytokine. We found that treatment of BEC with PGN from *S. aureus* triggered a TLR2/PI3K/Akt-dependent phosphorylation of GSK3α and GSK3β at their inhibitory residues Ser21 and Ser9 respectively, and that NF-κB p65 subunit (p65) was also phosphorylated at Ser536, a residue located at the transactivation domain. Incubation of BEC with 10 μg/mL of PGN for 30 min induced a maximum phosphorylation of GSK3α, GSK3β and p65, which was dependent on PI3K activity and Akt phosphorylation at Ser473. Interestingly, phosphorylation of GSK3α was greater than that of GSK3β in BEC stimulated with PGN. The increase of IL-12p40 expression was inhibited in BEC pre-treated with the Akt inhibitors (SH-5 and Akt-inhibitor IV), and increased after treatment with GSK3 inhibitors (LiCl or SB216763). Furthermore, treatment of BEC with an anti-TLR2 antibody, addressed against the extracellular domain of the receptor, inhibited activation of the PI3K/Akt/GSK3 signaling pathway and blocked the expression of IL-12p40. Importantly, opposite to the model in which inhibition of GSK3β by PI3K/Akt in macrophages stimulated with LPS and other TLR ligands inhibits IL-12p40 expression, our

results suggest that PGN inhibits GSK3α/β activity via TLR2/PI3K/Akt, and this activates the production of IL-12p40 in endothelial cells.

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] by activating the PI3K/Akt signaling pathway. In this context, it is known that binding of PGN to TLR2 in macrophages RAW264.7 induces COX2 expression, and IKKα/β-NF-κB activation through the Rac1/PI3K/Akt and Ras/Raf1/Erk1-2 signaling pathways [3, 4]. In BV-2 microglia, PGN binds to TLR2 and activates the 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 [5]. Moreover, binding of PGN to TLR2 of fibroblasts activates FAK/PI3K/Akt and AP-1, increasing the expression of IL-6 [6].

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-trisphosphate (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]. There are two major mammalian GSK3 protein isoforms, α and β [14], that 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,

71 embryonic development, cell cycle progression, apoptosis [16, 17] and in the inflammatory
72 response caused by bacterial infection through the regulation of NF-κB activity [13, 15]. In
73 regard to GSK3α most of the glucose/glycogen homeostasis appears to depend mainly on
74 this isoform, with a minor contribution of GSK3β in skeletal muscle [18-20]. Also, GSK3α
75 plays a potential role as a regulatory enzyme of the central nervous system [21]. This
76 isoform, but not GSK3β, has recently been identified in the maintenance and/or
77 proliferation of Th17 cells stimulated with the pro-inflammatory cytokine IL-1 [22].
78 However, participation of GSK3α in the modulation of inflammation, triggered by
79 microbial products has not been documented. Interestingly, studies in murine models have
80 indicated that both isoforms of GSK3 are not physiologically redundant [16, 18, 23].
81 Furthermore, we have previously shown that internalization of *S. aureus* by endothelial
82 cells is associated with the PI3K/Akt activity and higher phosphorylation of GSK3α at
83 Ser21 than GSK3β at Ser9 [24]. Thus, it is likely that GSK3α has regulatory functions in
84 the inflammatory response induced by bacterial pathogens.

85 Interleukin (IL)-12 is an important pro-inflammatory cytokine because its
86 expression during bacterial infection regulates the innate response and determines the type
87 and duration of the adaptive immune response [25, 26]. Structurally, this cytokine is a
88 heterodimer composed of two subunits designated p35 and p40 linked by disulfide bonds
89 [27, 28]. The *IL-12p40* gene is highly inducible by microbial products such as
90 lipopolysaccharides (LPS), lipoteichoic acid (LTA) and PGN via Toll-like receptor
91 signaling and NF-κB activation [26]. Antigen-presenting cells and phagocytic cells are the
92 primary producers of IL-12 [26], although human endothelial cells also produce it [29].
93 Although IL-12 is vital 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 [30, 31].

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 both the activity of NF-κB and the expression of IL-12p40 through the inhibition of GSK3β [30]. Data presented in this study indicate that stimulation of bovine endothelial cells (BEC) with PGN from *S. aureus* induced the expression of IL-12p40 both at the mRNA and protein level. Interestingly, this expression was associated with inhibition of GSK3α, and to a lesser extent GSK3β, and phosphorylation of NF-κB p65 subunit (p65) at Ser536 by a mechanism involving the TLR2/PI3K/Akt signaling pathway. In this context, it is now accepted that NF-κB can be activated by phosphorylation of p65 at residues located in the transactivation domain, without promoting IκB degradation, nuclear translocation or DNA binding [32, 33]. One of these residues, Ser536 is phosphorylated by activation of the PI3K/Akt signaling pathway [34]. This work demonstrates, for the first time, that inhibition of GSK3 activity as a result of the PI3K/Akt pathway activation induces the production of IL-12p40 through NF-κB p65 phospho-activation at Ser536.

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*, Wortmannin (Wort), Akt-inhibitor IV or benzimidazolium, LY294002 (LY), SB216763 (SB), NaCl, LiCl 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-SH-5 (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.

Antibodies

Rabbit polyclonal antibodies against the extracellular domain of TLR2 (N-17; sc-8689) 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 p65 were purchased from Cell Signaling Technology (Boston, MA, USA).

Cell Line and Culture Conditions

The endothelial cell line used 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) [35]. 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.

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, 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 [36] 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 to 0.45 µm nitrocellulose membrane (Bio-Rad) at 250-300 mA for 1 h. Membranes were then probed with polyclonal antibodies to phosphorylated forms of Akt, GSK3α, GSK3β, or

p65. Then, membranes were stripped, reprobed with 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, immediately 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, stimulated 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, 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 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 [37]. 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 IL12p40 mRNA were calculated with the delta-delta C_t method, using β-actin as the reference gene.

Measurement of IL-12p40 protein levels

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

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 one. These intensities were referred to a value of 1.0 that was arbitrarily assigned to the untreated control. The statistical significance of triplicate blots was evaluated with 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

PGN Induces Phosphorylation of Akt, GSK3α, GSK3β, and NF-κB in BEC

PGN is able to activate the transcriptional factor NF-κB by phosphorylation of p65 at Ser536 and induce the expression of pro-inflammatory molecules via the PI3K/Akt signaling pathway [4, 5]. However, the role that GSK3α and GSK3β play on the regulation

of NF-κB phosphorylation induced by PGN during the inflammatory response is not clear. To test whether GSK3α/β isoforms affect the phosphorylation of p65 at Ser536, BEC were stimulated with 1, 10, 20 or 30 μg/mL of PGN for 30 min. At these various concentrations of PGN, we first analyzed Akt phosphorylation at Ser473 because activation of this enzyme catalyzes GSK3 phosphorylation at its inhibitory sites. We observed an increase in Akt phosphorylation at 10, 20 and 30 μg/mL PGN **(Fig. 1A)** with the highest phosphorylation found at 15-30 min post-treatment that decreased to the level of untreated control at 60 min **(Fig. 1B)**. Interestingly, analysis of GSK3α/β phosphorylation indicated that stimulation of BEC with 10 μg/ml of PGN for 15, 30 and 60 min induced a slight increase of GSK3β phosphorylation at Ser9, compared with the stronger increase of GSK3α phosphorylation at Ser21. Phosphorylation of both isoforms declined at 120 min, reaching a value similar to the untreated control **(Figs. 1C and D)**. In addition, phosphorylation of p65 at Ser536 reached its highest value at 30-60 min, returning to basal levels at 120 min **(Fig. 1E)**. These results suggest that phosphorylation of Akt, GSK3, and NF-κB is temporally correlated in BEC stimulated with PGN.

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

Dependency of Akt phosphorylation on PI3K activity was tested in BEC pre-treated with LY (an inhibitor of PI3K) for 30 min and then incubated for the same time with PGN. A complete loss of Akt phosphorylation was observed **(Fig. 2A)**. Inhibition of PI3K and Akt with Wort and SH-5 respectively for 30 min, caused a significant decrease in GSK3α/β

and p65 phosphorylation in BEC stimulated for 30 min with PGN **(Figs. 2B-D)**. These data indicate that phospho-inhibition of GSK3α/β and phospho-activation of p65 depends on PI3K and Akt activity.

Phosphorylation of NF-κB at Ser536 Depends on GSK3 Inhibition in BEC Stimulated with PGN

To test if PGN also induced the inhibition of GSK3 activity we evaluated the phosphorylation of glycogen synthase (GS) at Ser641. Incubation of BEC with PGN inhibited 40% and 60% GS phosphorylation at 30 and 60 min respectively, compared with the untreated control (**Fig. 3A**). 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 GSK3 activity (**Fig. 3B**). Dependency of p65 phosphorylation on GSK3 inhibition was tested in BEC pretreated with LiCl and/or stimulated with PGN. It was observed an ~5 fold increase in p65 phosphorylation when BEC were stimulated with PGN alone and an even stronger increase (~9 fold) was detected when pretreatment with LiCl was included **(Fig. 3C)**. Pretreatment of BEC with NaCl was included to discard any osmolarity effect. Furthermore, we found that treatment of BEC with Wort or SH-5 led to an increase of CREB phosphorylation at Ser133 **(Fig. S1A)** while treatment with LiCl returned phosphorylated CREB to basal levels **(Fig. S1B)**, suggesting that GSK3 activity causes activation of this transcription factor. These data indicate that the PI3K/Akt-dependent phosphorylation of GSK3 in BEC stimulated with PGN decreased GSK3's activity.

250 Phosphorylation of Akt, GSK3α and GSK3β in BEC Stimulated with PGN is TLR2-
251 Dependent

252 It is known that PGN is able to induce the expression of pro-inflammatory
253 molecules by activation of the PI3K/Akt signaling pathway in a TLR2 dependent manner
254 [3-6]. Therefore, we decided to explore the involvement of TLR2 in the PI3K/Akt-
255 dependent inhibition of GSK3 that leads to the p65 activation in BEC stimulated with PGN.
256 We first observed that the relative abundance of TLR2 did not change in BEC control and
257 stimulated with PGN **(Fig. 4A)**. However, the relative abundance of phosphorylated Akt,
258 GSK3α and GSK3β decreased to the level observed in unstimulated control when BEC
259 were pre-treated with anti-TLR2 **(Figs. 4B-D)**, indicating that PGN activates
260 PI3K/Akt/GSK3 signaling pathway by binding to TLR2.

261

262 PGN Induced the Expression of IL-12p40 through a Mechanism that Involves Binding to
263 TLR2, Akt Activation and GSK3 Inhibition in BEC

264 When transcript levels of IL-12p40 were analyzed in BEC stimulated with PGN we
265 observed an increase in IL-12p40 mRNA at 4 h, which decreased to the control level at 8 h
266 **(Fig. 5A)**. Then, BEC were pre-treated with SH-5, LiCl or SB, and stimulated with PGN
267 for 4 h, to determine if IL-12p40 mRNA expression depended on Akt and GSK3 activity.
268 The expression of IL-12p40 mRNA strongly decreased in the presence of SH-5. In contrast,
269 when BEC were pre-treated with SB, a significant increase of IL-12p40 mRNA expression
270 was obtained compared to the PGN-treated and untreated BEC **(Fig. 5B)**. A similar increase
271 of IL-12p40 mRNA expression was observed in BEC pretreated with LiCl and stimulated

272 with PGN compared with the values obtained in BEC stimulated with PGN **(Fig. 5B)**. As a
273 control for the LiCl treatment, the expression of IL-12p40 mRNA was not altered in BEC
274 pretreated with NaCl **(Fig. 5B)**. Furthermore, expression of IL-12p40 mRNA depended on
275 PGN binding to TLR2 because pretreatment with anti-TLR2 blocked it **(Fig. 5C).** These
276 results suggest that IL-12p40 mRNA expression was induced in BEC by binding of PGN to
277 TLR2, Akt activation and GSK3 inhibition.

278 Next, we tested IL-12p40 protein levels by ELISA assays. Data on Figure 5D
279 indicate that PGN enhanced the levels of IL-12p40 protein (~2 fold). These protein levels
280 were significantly increased by treatment of BEC with the GSK3 inhibitors LiCl or SB (~6-
281 7 fold) and reduced to the control level by the Akt inhibitor Akt-i IV or antibodies against
282 the extracellular domain of TLR2. These results indicate that GSK3 inhibition via
283 TLR2/PI3K/Akt activation leads to NF-κB activation and production of IL-12p40.

284

285 **Discussion**

286 Endothelial cell activation during an inflammatory process may be divided into
287 rapid and slow responses that are independent and dependent on new gene expression,
288 respectively [38]. TLRs of endothelial cells play a fundamental role in the regulation of the
289 inflammatory response upon exposure to any of the currently known TLR ligands [39].
290 PGN, one of the major cell-wall structures of *S. aureus*, is an important inducer of the
291 inflammatory response [40]. Several authors have demonstrated that Ser536 of the p65
292 subunit, an important amino acid residue of the transactivation domain that positively
293 controls NF-κB transcriptional activity [32, 33, 34], is phosphorylated in response to a
294 variety of pro-inflammatory stimuli, including PGN [4, 5, 41]. Although, it is also 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 [4, 5], no report has shown that GSK3 inhibition increases pro-inflammatory cytokine expression in response to PGN. Data presented in this work, using BEC as a model cell stimulated with PGN from *S. aureus*, are the first to show that phospho-inhibition of both GSK3α and GSK3β, but predominantly GSK3α, links PI3K and Akt with phospho-activation of the NF-κB p65 subunit, thereby promoting the production of IL-12p40 **(Fig. 7)**, a multifunctional cytokine with important functions in the innate and adaptive immune responses [25, 26].

TLR2 plays a crucial role in the host response against *S. aureus* because knockout mice deficient in TLR2 are highly susceptible to staphylococcal infections [42]. However, the specificity of TLR2 for PGN is still an issue of debate. According to Travassos et al. (2004) highly purified PGN did not activate TLR signaling [43]. In contrast, several other authors have proposed that PGN interacts with TLR2 [3-6, 44] and studies with PGN from *S. aureus* lacking lipidated prelipoproteins have co-localized it 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 [45]. Data in this study support the notion that PGN interacts with TLR2 from BEC because we observed that blocking TLR2 with a TLR2 specific antibody inhibited activation of the PI3K/Akt/GSK3α/β signaling pathway and expression of IL-12p40 **(Fig. 6)**. Satta et al. (2008) detected an induction of TLR2 expression in human endothelial cells that served to amplify the inflammatory response to lipopeptides [46]. We obtained evidence indicating that levels of TLR2 protein in BEC are not modified by PGN

318 treatment. These results indicate that an increase in the TLR2 protein is not a requirement
319 for IL-12p40 expression in BEC stimulated with PGN.

320 Although it is well established that Akt phosphorylates and inactivates GSK3α and
321 GSK3β, as we have observed in this study, Gulen et al. (2012) showed that GSK3α, but not
322 GSK3β, can reversely phosphorylate and suppress Akt activation in resting Th17 cells.
323 These authors also demonstrated that activation of Th17 treated with IL-1 leads to an
324 increase of IKKi activity and GSK3α phosphorylation at Ser21, promoting Akt-mTOR
325 activation [22]. We have ruled out a reverse phosphorylation reaction from GSK3 to Akt in
326 BEC stimulated with PGN because pharmacological inhibition of PI3K and Akt completely
327 blocked PGN-induced GSK3 phosphorylation. Previous evidence of GSK3α/β
328 phosphorylation, as a consequence of *S. aureus* internalization in BEC, was obtained in our
329 laboratory [24]. We found that both isoforms of GSK3 may be involved in the
330 internalization process and perhaps the inflammatory response caused by this bacterium.
331 Although IL-12p40 expression by CD154 stimulation was already detected in endothelium
332 [29], we are the first to demonstrate expression of this cytokine in endothelial cells
333 stimulated with a bacterial structure. Other authors have detected expression of IL-12-
334 related molecules, but not expression of IL-12p40, in human intestinal microvascular
335 endothelial cells stimulated with pro-inflammatory compounds (TNF-α, IFN-γ, IL-1β) and
336 microbial antigens [LPS, LTA, PGN, CpG-DNA, flagellin, and poly(I:C)] [47].

337 Data in this study are different from those obtained by Martin et al. (2005), in regard
338 to phosphorylation and inhibition of GSK3 with bacterial components different from PGN
339 [30]. They found that inhibition of only GSK3β activity by treatment of macrophages with
340 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 [30]. In contrast, our data clearly indicate that inhibition of GSK3 by treatment of BEC with PGN from *S. aureus* increased IL-12p40 expression. Furthermore, PGN not only activated phosphorylation of the inhibitory amino acid residues in GSK3α (Ser21) and GSK3β (Ser9) but also phosphorylation of GSK3α was greater than that of GSK3β. This suggests that although GSK3β is the isoform generally associated with the inflammatory response to bacterial infections [13, 30], GSK3α may also play an important role in this process, as a modulator of NF-κB activity and cytokine expression. It is likely that the type of cell used and the stimulus applied explain the mechanistic differences between our results and those reported by Martin et al. (2005) [30].

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 production and increases cytotoxicity activity as well as proliferation of these cells [25, 26]. 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 [48, 49]. 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 [50]. Thus, 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. Future experiments will be conducted to demonstrate the autocrine or paracrine production of IFN-γ due to secretion of IL-12p40 by endothelial cells.

In conclusion, the novel findings of this work can be summarized as follows: First, we have shown that GSK3 is a modulator of the inflammatory response in endothelial cells activated with PGN. Although previous reports have documented that PGN activates PI3K/Akt and induces pro-inflammatory molecules production [4-6], they did not explore how this was correlated with the change of GSK3 activity state. Second, although it is accepted that GSK3β is the only isoform that mediates inflammation in cells stimulated with bacterial virulence factors and other stimuli [13] we found that PGN induces a marked increase in the level of phosphorylated GSK3α (higher than that of GSK3β), which suggests that it might be participating in the regulation of IL-12p40 expression. Third, the current model that explains how GSK3β regulates NF-κB and CREB activities was established in macrophages stimulated with LPS and other bacterial structures [30]. In this model, activation of GSK3β leads to NF-κB activation and expression of IL-12p40. In contrast, our data indicate that inhibition of GSK3 activity results in activation of NF-κB and transcriptional expression of IL-12p40. Fourth, this inhibition of GSK3 was shown to be linked to the interaction of PGN with TLR2, and activation of the PI3K/Akt signaling pathway.

Acknowledgments

The authors wish to thank Dr. Carlos Rosales Ledezma and Dr. Sergio Gutiérrez Castellanos for valuable suggestions to this work, and Dr. Wanyin Deng for critical comments to the manuscript.

385 **Author Contributions**

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

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Figure Legends

Figure 1. Peptidoglycan (PGN) induces phosphorylation of Akt, GSK3α, GSK3β, and NF-κB in bovine endothelial cells (BEC). A) BEC were left unstimulated (0) or stimulated with 1, 10, 20 or 30 μg/mL of peptidoglycan (PGN) from *S. aureus* for 30 min. Protein extracts were analyzed by western blot and probed with a monoclonal antibody against the phosphorylated form of Akt1 (pAkt Ser473). B-E) BEC were left unstimulated (0) or stimulated with 10 μg/mL 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 Akt1 (pAkt Ser473), GSK3α (pGSK3α Ser21), GSK3β (pGSK3β Ser9) or p65 (NF-κB p65 Ser536). To verify that equal amount of proteins was loaded in each lane, blots were stripped and reprobed with antibodies that recognize the nonphosphorylated forms of Akt (A and B), GSK3β (C and D) or the NF-κB p65subunit (E). 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.

Figure 2. PGN induces PI3K/Akt-dependent phosphorylation of GSK3α, GSK3β, and NF-κB in BEC. A-D) BEC were left untreated and unstimulated (U), untreated (-) or treated with 10 μM LY294002 (LY), 100 nM Wortmannin (Wort) or 10 μM SH-5 for 30 min, and then stimulated with 10 μg/mL PGN for 30 min. Protein extracts were analyzed by western blot and probed with monoclonal antibodies against the phosphorylated forms of Akt1 (pAkt Ser473), GSK3α (pGSK3α Ser21), GSK3β (pGSK3β Ser9) or 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 Akt (A), GSK3β (B and C) or p65 (D). 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.

Figure 3. PGN induces GSK3-dependent phosphorylation of NF-κB in BEC. A) BEC were left untreated and unstimulated or stimulated with 10 μg/mL of PGN for 30 and 60 min. B) BEC were left untreated and unstimulated, or treated for 60 min with 10 mM NaCl, 10 mM LiCl or 10 μM SB and then stimulated with 10 μg/mL PGN for 30 min. Treatments for 60 min with LiCl or SB alone were also included as controls. C) BEC were left untreated and unstimulated, or treated for 60 min with 10 mM NaCl or 10 mM LiCl and then stimulated with 10 μg/mL PGN for 30 min. Treatment for 60 min with 10 mM LiCl was also included as control. Protein extracts were analyzed by western blot and probed with a monoclonal antibody against the phosphorylated form of glycogen synthase (GS Ser641) or with a monoclonal antibody against the phosphorylated form of p65 (NF-κB p65 Ser536). To verify that equal amount of proteins was loaded in each lane, blots were stripped and reprobed with antibodies that recognize the nonphosphorylated forms of GSK3β (A) or p65 (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.

Figure 4. PGN interaction with TLR2 leads to phosphorylation of Akt, GSK3α and GSK3β in BEC. A) BEC were left unstimulated (U), or stimulated with 10 μg/mL PGN for

15, 30, 60, 120 or 240 min. B-D) BEC were left untreated and unstimulated, stimulated with 10 µg/mL PGN for 30 min, treated with 5 µg/mL anti-TLR2 for 60 min and stimulated with 10 µg/mL PGN for 30 min or treated with 5 µg/mL anti-TLR2 for 60 min. Protein extracts were analyzed by western blot and probed with a polyclonal antibody against TLR2 or 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 recognize β-actin (A) or with antibodies that recognize the nonphosphorylated forms of Akt (B), GSK3α (C) or Akt (D) 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; **p <0.01 compared with the unstimulated control.

Figure 5. PGN induces IL-12p40 expression through TLR2/Akt activation and GSK3 inhibition in BEC. A) BEC were left untreated and unstimulated (U) or stimulated with 10 µg/mL PGN for 2, 4 or 8 h; B) BEC were left untreated and unstimulated (U), stimulated with 10 µg/mL PGN for 4h, treated with 10 µM SH-5 for 30 min and then stimulated with 10 µg/mL PGN for 4 h, treated with 10 mM NaCl for 60 min, treated with 10 mM LiCl for 60 min and then stimulated with 10 µg/mL PGN for 4 h, or treated with 10 µM SB 216763 (SB) for 30 min and then stimulated with 10 µg/mL PGN for 4 h. C) BEC were left untreated and unstimulated (U), stimulated with 10 µg/mL PGN for 4 h, treated with 5 µg/mL anti-TLR2 for 60 min, or treated with 5 µg/mL anti-TLR2 for 60 min and then stimulated with 10 µg/mL PGN for 4 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. D) BEC were left untreated and unstimulated (U), or treated for 60 min with 5 μg/mL anti-TLR2, 1 μM Akt iIV, 10 mM LiCl or 10 μM SB and then stimulated with 10 μg/mL PGN for 9 h. Cell-free supernatants were analyzed by ELISA for production of IL-12p40. Results are expressed as the mean ± S.E.M. (n = 3). In A-C, *p <0.05; **p <0.01. In D, *p <0.05; **p <0.001. All data were compared with the untreated and unstimulated control.

Figure 6. Schematic diagram of the signaling pathway involved in PGN-induced IL-12p40 expression in bovine endothelial cells (BEC). PGN induces the activation of the PI3K/Akt/GSK3 pathway, which in turn induces NF-κB p65 subunit phosphorylation in its transactivation domain, resulting in IL-12p40 expression. Anti-TLR2 blocks the activation of the pathway and inhibits the expression of IL-12p40. Akt inhibitors SH-5 or Akt inhibitor IV (Akt-i) blocks while LiCl or SB-216763 increases the expression of IL-12p40. The question mark indicates a proposed and an unidentified enzyme that is inhibited when GSK3 is active.

Figure S1. PGN induces PI3K/Akt/GSK3-dependent inhibition of phosphorylation of CREB in BEC. A) BEC were left untreated and unstimulated (U), untreated (-) or treated with 100 nM Wortmannin (Wort) or 10 μM SH-5 for 30 min, and then stimulated with 10 μg/mL PGN for 30 min. B) BEC were left untreated and unstimulated, treated with 10 mM NaCl for 60 min, treated with 10 mM LiCl for 60 min and then stimulated with 10 μg/mL of PGN for 30 min or stimulated with 10 μg/mL PGN for 30 min. Protein extracts were analyzed by western blot and probed with a monoclonal antibody against the

phosphorylated form of CREB (pCREB Ser133). An antibody that recognizes the nonphosphorylated form of p65 was used to verify equal amount of protein in each lane. 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 unstimulated control.

VI.II Capítulo II

Protein A from *Staphylococcus aureus* induces phosphoinositide 3-kinase-dependent phosphorylation of Akt1 and Akt2 in endothelial cells

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ABSTRACT

We have shown that internalization of *Staphylococcus aureus* in bovine endothelial cells (BEC) was associated with phosphoinositide 3-kinase (PI3K)/Akt phosphorylation of GSK3β at Ser9 and phosphorylation of the NF-κB p65 subunit (p65) at Ser536. In this work, we present data indicating that protein A (SpA) from *S. aureus*, an important inducer of inflammation, stimulated a PI3K-dependent phosphorylation of Akt1 and Akt2 in BEC. Time-kinetics assays of BEC incubated with low concentration (0.5 μg/mL) of soluble SpA (sSpA) produced a PI3K-dependent phosphorylation of Akt1 and GSK3β, and a PI3K-independent phosphorylation of NF-κB p65, whereas high concentration (25 μg/mL) induced phosphorylation of both Akt1 and Akt2. Interestingly, Akt2 phosphorylation induced by high concentration of sSpA did not activate phosphorylation of GSK3β or p65. Infection of BEC with a wild-type *S. aureus* strain caused an increase in Akt1 and Akt2 phosphorylation, which was higher than the level of Akt1/2 phosphorylation observed from BEC infected with a SpA-null mutant *S. aureus* strain. Simultaneous incubation of BEC with high concentration of sSpA and peptidoglycan (PGN), a known inducer of PI3K/Akt1 activation, strongly increased the phosphorylation of Akt2 without any additive effect on Akt1. These results suggest that sSpA induces the activation of PI3K/Akt1/Akt2, and the activation of PI3K/Akt1, but not PI3K/Akt2, induces phosphorylation of GSK3β. Furthermore, these data suggest that other component of *S. aureus*, such as PGN may contribute to enhance the phosphorylation of Akt2 induced SpA.

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 (Pantosti *et al.* 2012). Staphylococcal protein A (SpA) is a conserved cell wall component found in all *S. aureus* strains. Apart from being an integral protein of the cell wall, SpA is sometimes actively secreted into the supernatant of growing cultures (Cheung *et al.* 1997). It is now known that the IgG binding domain, located at the amino terminal of SpA, induces inflammation by binding to TNFR1 and activation of TRAF2, p38/JNK1-2, and NF-κB (Gómez *et al.* 2004). This conserved domain of SpA also binds to EGFR and elicits an anti-inflammatory response through the activation of tumor necrosis factor α converting enzyme (TACE), which breaks soluble TNFR1 (Gómez *et al.* 2006, Gómez *et al.* 2007). Interestingly, the polymorphic region of SpA named Xr, which contains a variable number (1-22) of 24 to 26-bp short sequence repeats (SSRs), modulates inflammation by activating the type I IFN cascade (Martin *et al.* 2009), with an apparent correlation between the number of SSRs and the strength of the inflammatory response (Garofalo *et al.* 2012).

The phosphoinositide 3-kinase/Akt (PI3K/Akt) signaling pathway mediates a variety of cellular responses such as survival, proliferation, differentiation, apoptosis, and inflammation (Liu *et al.* 2009, Wang *et al.* 2011). There are three closely related Akt isoforms named Akt1 (PKBα), Akt2 (PKBß), and Akt3 (PKBγ) encoded by independent genes. Activation of PI3K/Akt pathway leads to the PI3K-dependent synthesis of phosphatydilinositol-3,4,5-triphosphate (PIP_3) and phosphorylation of Akt1 at Thr308 and Ser473, Akt2 at Thr309 and Ser474, and Akt3 at Thr305 and Ser472 by the constitutively active PDK1 (Alessi *et al.* 1997, Frodin *et al.* 2002) and mTORC2 (Sarbassov et al. 2005, Hresko and Mueckler 2005). Activation of Akt regulates the activity of a wide range of substrates, among which glycogen synthase kinase 3 (GSK3) is important in the modulation of inflammatory response (Cortés-Vieyra *et al.* 2012, Wang *et al.* 2011). There are two major mammalian GSK3 protein isoforms, α and β (Woodgett 1990), which can be inactivated by phosphorylation at Ser21 (GSK3α) or Ser9 (GSK3β) by Akt (Jope and Johnson 2004). Since its discovery, GSK3β has been characterized in a number of cellular

processes such as the inflammatory response caused by bacterial infection through the regulation of NF-κB activity (Jope and Johnson 2004, Cortés-Vieyra *et al.* 2012).

Despite all the works done on the pro-inflammatory role of SpA from *S. aureus*, no report has evaluated the importance of the activation of PI3K/Akt pathway, and the modulation that this pathway may exert on the inhibition of GSK3β and NF-κB phosphorylation in its transactivation domain in cells stimulated with this virulence factor. A role of PI3K/Akt activation in bovine endothelial cells (BEC) infected with *S. aureus*, leading to NF-κB p65 subunit (p65) at Ser536 and GSK3β phospho-inactivation at Ser9 was previously reported (Oviedo-Boyso *et al.* 2011). Due to the prominent role of SpA as a pro-inflammatory factor we decided to analyze if the stimulation of BEC with soluble SpA (sSpA) activates the PI3K/Akt-dependent phosphorylation of GSK3β and p65. Our data show that sSpA induces the activation of PI3K/Akt1 and PI3K/Akt2 pathways and that GSK3β phosphorylation, but not p65, is modulated only by the PI3K/Akt1 pathway. Furthermore, the phosphorylation pattern of Akt1 and Akt2 in BEC stimulated with sSPA was similar to the phosphorylation pattern observed in BEC infected with a wild-type *S aureus* strain, but not in BEC infected with a SpA-null mutant *S. aureus* strain. Interestingly, our results also indicate that there is an additive effect on the phosphorylation of Akt2 when BEC were stimulated with a high concentration of sSpA and peptidoglycan (PGN).

MATERIALS AND METHODS

Media and chemicals. F-12 Ham (HF-12) of Dulbecco´s modified Eagle´s medium, bovine serum albumin (BSA), trypsin-EDTA, sSpA from *S. aureus* (P6031), Igepal CA-930, Wortmannin (Wort) and Bradford reagent 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-SH-5 (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). All other reagents were acquired from Sigma-Aldrich.

Antibodies. Rabbit polyclonal antibody against phospho-Akt1/2/3 (Ser473/Ser474/Ser472) and goat anti-rabbit IgG-HRP were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). Rabbit monoclonal antibodies against, phospho-GSK3β (Ser9), phospho-p65 (Ser536), Akt1, GSK3β, and p65 were purchased from Cell Signaling Technology (Boston, MA, USA).

Cell line and culture conditions. 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) (Cajero-Juárez *et al.* 2002). 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.

Protein extraction and western blot analysis. To test for the relative abundance of phosphorylated and non-phosphorylated proteins, BEC were grown in six-well culture plates 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 added immediately before lysing the 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 (Bradford 1996) using BSA as standard. Then 30-40 µg of protein was separated by electrophoresis in 10% sodium dodecyl sulfate-polyacrylamide gels and electroblotted to 0.45 µm nitrocellulose membrane (Bio-Rad) in a wet chamber at 250-300 mA for 1 h. Membranes were then probed with polyclonal antibodies to phosphorylated forms of Akt1/2/3, GSK3α, GSK3β, or p65. Then, membranes were stripped, reprobed with monoclonal antibodies to the non-phosphorylated form of Akt1 or polyclonal antibodies to

GSK3β or p65, and detected with the Immobilon Western Chemiluminescent HRP substrate kit from Millipore (Billerica, MA, USA).

Densitometric and statistical analysis. Densitometry analysis was performed 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 band. The intensity obtained from different experimental conditions was referred to a value of 1.0 that was arbitrarily assigned to the untreated control, except for the intensity of Akt2 obtained under different assay conditions in Figs. 1B-C; 2A-B, in which a value of 1.0 was assigned because we were unable to detect any phosphorylation of this isoform in the untreated control. The statistical significance of triplicate blots was evaluated with One-Way analysis of variance (ANOVA) by using the SIGMASTAT program version 3.0 (SPSS Inc., Chicago, IL, USA.

RESULTS

sSpA induces a PI3K-dependent phosphorylation of Akt1 and Akt2 in BEC. Stimulation of BEC with 0.5-25 μg/mL of sSpA for 5 min induced a marked increase in phosphorylation of Akt1 at Ser473, while 5-100 μg/mL activated a strong phosphorylation of Akt2 at Ser474 **(data not shown)**. Based on this result, we performed time-kinetics assays with low (0.5 μg/mL) or high (25 μg/mL) concentrations of sSpA. At low concentration sSpA caused Akt1 phosphorylation with a maximum at 5 and 10 min that decreased at longer incubation times **(Fig. 1A)**. In contrast, high concentration of sSpA induced a slight increase in Akt1 phosphorylation at 5-10 min while Akt2 phosphorylation was stronger at 5-20 min and decreased at 40 min **(Fig. 2B)**. No phosphorylation of any isoform of Akt was detected when BEC were incubated for 6 hours with 200 μg/mL of sSpA **(data not shown)**, which are the conditions previously established by Gómez *et al.* 2004 for the TNFR1-dependent activation of NF-κB in lung epithelial cells stimulated with sSpA. To show that phosphorylation of Akt1 and Akt2 by sSpA was dependent on PI3K activity, BEC were pre-incubated with Wort and then stimulated with a high concentration of sSpA for 10 min. Under these conditions, this inhibitor significantly reduced phosphorylation of both Akt isoforms **(Fig. 1C)**.

Treatment of BEC with sSpA plus PGN or infection of BEC with a wild-type strain of *S. aureus* increases Akt phosphorylation. Incubation of BEC with 10 μg/mL of PGN plus a high concentration of sSpA for various times caused a strong activation of Akt2 phosphorylation at 15 and 30 min, compared with the level of phosphorylation obtained at 30 min with either stimuli **(Fig. 2A)**. At 60 min, phosphorylation of Akt2 decreased to a value similar to that observed when BEC were stimulated with sSpA alone **(Fig. 2A)**. To confirm the effects of sSpA on Akt phosphorylation, we performed BEC infections with a SpA-null mutant strain. Interestingly, we observed that phosphorylation of Akt1 and Akt2 was reduced compared with the levels of Akt phosphorylation detected in BEC infected with a wild-type strain of *S. aureus* **(Fig. 2B)**

sSpA induced the PI3K/Akt1-dependent phosphorylation of GSK3β in BEC. SpA from *S. aureus* induces the expression of pro-inflammatory molecules in airway epithelial cells through the activation of p38, JNKs, and NF-κB (Gómez *et al.* 2004). Regulation of NF-κB activity can also be achieved by activation of the PI3K/Akt/GSK3β signaling pathway in response to bacterial stimuli (Cortés-Vieyra *et al.* 2012). Therefore, we asked whether SpA was able to activate this signaling pathway and induce phosphorylation of p65 in its transactivation domain in BEC. We first analyzed the time-kinetics of GSK3β and p65 phosphorylation in BEC stimulated with low and high concentrations of sSpA because we found that these conditions activated phosphorylation of Akt1 and Akt2 **(Figs. 1A and 1B)**. Stimulation of BEC with a low concentration of sSpA induced GSK3β and p65 phosphorylation **(Figs. 3A and 3B)**. Phosphorylation of p65 was high at 10-20 min post-stimulation with a low concentration of sSpA and returned to the basal level at 40 min **(Fig. 3B)**. GSK3β phosphorylation in BEC stimulated with a low concentration of sSpA depended on the activation of PI3K/Akt1 signaling pathway, because pre-incubation with Wort or SH-5 decreased its level of phosphorylation **(Fig. 3C)**. Interestingly, phosphorylation of p65 in BEC stimulated with a low concentration of sSpA did not depend on the activation of the PI3K/Akt1/GSK3 signaling pathway because there was not significant decrease in the phosphorylation of p65 when BEC were pre-incubated with Wort or SH-5, compared with BEC stimulated with sSpA alone **(Fig. 3D)**. In contrast,

When BEC were stimulated with a high concentration of sSpA, no increase in phosphorylation of GSK3β or p65 was observed **(Figs. 4A and 4B)**.

DISCUSSION

Interaction of some bacterial components with different TLRs of mammal cells activates the PI3K/Akt/GSK3 signaling pathway, which can merge in NF-κB activation or inactivation, triggering or inhibiting the inflammatory response respectively (Cortés-Vieyra *et al.* 2012). Phosphorylation of p65 at Ser536, an important amino acid residue of the transactivating domain that positively controls NF-κB transcriptional activity (Jefferies and O'Neill 2000, Sizemore *et al.* 1999), is physiologically activated in response to a variety of pro-inflammatory stimuli (Buss *et al.* 2004, Chen *et al.* 2009, Lin *et al.* 2010) and through activation of the PI3K/Akt pathway (Madrid *et al.* 2001). Previous evidence of PI3K/Akt/GSK3 activation was obtained in our laboratory when we studied the internalization of *S. aureus* in BEC. We were able to demonstrate that p65 phosphorylation at Ser536 depended on PI3K activation (Oviedo-Boyso *et al.* 2011). Among the pro-inflammatory virulence factors of *S. aureus*, SpA is a conserved and a major surface component of all *S. aureus* strains (Forsgren 1969, Martin *et al.* 2009) and one of the strongest inducer of inflammation (Gómez *et al.* 2004, Martin *et al.* 2009, Garofalo *et al.* 2012). Therefore, we explored if SpA could activate the phosphorylation of p65 at Ser536 through the PI3K/Akt/GSK3 signaling pathway.

Our results indicate that different concentrations of sSpA caused a differential phosphorylation of Akt1 and Akt2, and that activation of these two Akt isoforms was dependent on the active state of PI3K. A low concentration of sSpA (0.5 μg/mL) induced an increase in GSK3β and p65 phosphorylation. Interestingly, phosphorylation of GSK3β at Ser9, but not of p65 at Ser536, depended on the activation of Akt1. Moreover, although high concentration of sSpA (25 μg/mL) activated the phosphorylation of Akt1 and Akt2, this phosphorylation did not change the phosphorylation state of GSK3β and p65. We corroborated that SpA is capable of inducing the phosphorylation of Akt1 and Akt2 in BEC, because we observed a similar pattern of phosphorylation of the Akt isoforms when

BEC were either stimulated with a high concentration of sSPA or infected with a wild-type *S. aures* strain but not when infected with a SpA-null mutant strain of *S. aureus*.

We believe that Akt1 and Akt2 phosphorylation at low and high concentrations of sSpA might be the consequence of the interaction of sSpA with two distinct cell surface receptors. If this is the case, one of the receptors should have a high affinity for sSpA (activated with a low concentration of sSpA) while the other should have a low affinity for sSpA (activated with a high concentration of sSpA). Alternatively, BEC might also be able to endocytise sSpA, as it was demonstrated in epithelial cells by Martin et al. 2009, and triggers from inside the activity of PI3K/Akt through an intracellular receptor (Zhao *et al.* 2008). If the PI3K/Akt2 pathway is activated by sSpA through an intracellular receptor, it is likely that PGN somehow increases the ability of BEC to endocytise sSpA because we have observed that Akt2 was strongly phosphorylated when BEC were stimulated with sSpA plus PGN. Regarding to the partial inhibition of p65 phosphorylation in BEC stimulated with a low concentration of sSpA, it is likely that a different signaling pathway could also be actively phosphorylating p65 at Ser536. A different, but not less interesting explanation, is that activation of the PI3K/Akt1/GSK3β signaling pathway by a low concentration of sSpA inhibits the inflammatory response through inhibition of NF-κB activity, as it has been demonstrated in other systems in which different bacterial virulence factors interact with mammal cells and inhibit NF-κB activity through this pathway (Cortés-Vieyra *et al.* 2012).

In conclusion, this study shows that sSpA from *S. aureus* induces the activation of the signaling pathways PI3K/Akt1 and PI3K/Akt2. Moreover, low concentration of sSpA was able to activate a PI3K/Akt1-dependent phosphorylation of GSK3β **(Fig. 5)**. Future experiments are needed to identify the receptors involved in BEC stimulated with low or high concentrations of sSpA and the Akt2-downstream substrates and to establish the physiological consequences of Akt1 and Akt2 activation in BEC stimulated with SpA.

ACKNOWDLEDGMENTS

The authors wish to thank Dr. Carlos Rosales Ledezma and Dr. Sergio Gutiérrez Castellanos for valuable suggestions to this work. This study was supported by grants from Coordinación de la Investigación Científica of the Universidad Michoacana de San Nicolás de Hidalgo, and Consejo Nacional de Ciencia y Tecnología (CONACYT, México), grant number 152518 to VMBA. RCV, OSG, and AHM are receiving master and doctoral scholarships from CONACYT, México.

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FIGURE LEGENDS

Figure 1. Soluble protein A (sSpA) induces PI3K-dependent phosphorylation of Akt1 and Akt2 in BEC. A-C) BEC were left unstimulated (U) or stimulated with 0.5 µg/mL (A) or 25 µg/mL (B) of sSpA for 5, 10, 20 or 40 min or pretreated with 100 nM of Wortmannin (Wort) for 30 min, and then stimulated with 25 µg/mL of sSpA for 10 min (C). Protein extracts were analyzed by western blot and probed with a polyclonal antibody against the phosphorylated forms of Akt1 62 kDa (pAkt1 Ser473) and Akt2 56 kDa (pAkt2 Ser474). To verify that equal protein amount was loaded in each lane, blots were stripped and reprobed with an antibody that recognize the nonphosphorylated form of Akt1. The blots are representative of three independent experiments. Graphs indicate the band intensity obtained by densitometric analysis of each assay compared with the unstimulated control for pAkt1 or compared with BEC stimulated with sSpA for 10 min for pAkt2. Results are expressed as the mean ± S.E.M. (n = 3). *p <0.05; **p <0.01, compared with the untreated and unstimulated control.

Figure 2. Infection of BEC with a wild-type strain of *S. aureus* or treatment with SpA plus PGN increases de phosphorylation of Akt. A) BEC were left uninfected (U), infected with a strain wild-type of *S. aureus* or infected with a strain protein A-null mutant of *S. aureus* for 40 min, following the protocol of infection of Oviedo-Boyso *et al.* (20119). B) BEC were left unstimulated (U) or stimulated with 25 µg/mL of sSpA for 30 min, 10

μg/mL of PGN for 30 min or at the same time with 25 μg/mL of sSpA plus 10 μg/mL of PGN for 15, 30 or 60 min. Protein extracts were analyzed by western blot and probed with a polyclonal antibody against the phosphorylated forms of Akt1 62 kDa (pAkt1 Ser473) and Akt2 56 kDa (pAkt2 Ser474). To verify that equal protein amount was loaded in each lane, blots were stripped and reprobed with an antibody that recognize the nonphosphorylated form of Akt1. The blots are representative of three independent experiments. Graphs indicate the band intensity obtained by densitometric analysis of each assay compared with the unstimulated control for pAkt1 (A, B), compared with BEC infected with the strain wild-type for Akt2 (A) or compared with BEC stimulated with 25 μg/mL of sSpA for 30 min for Akt2 (B). Results are expressed as the mean ± S.E.M. (n = 3). *p <0.05; **p <0.01, compared with the untreated and unstimulated control.

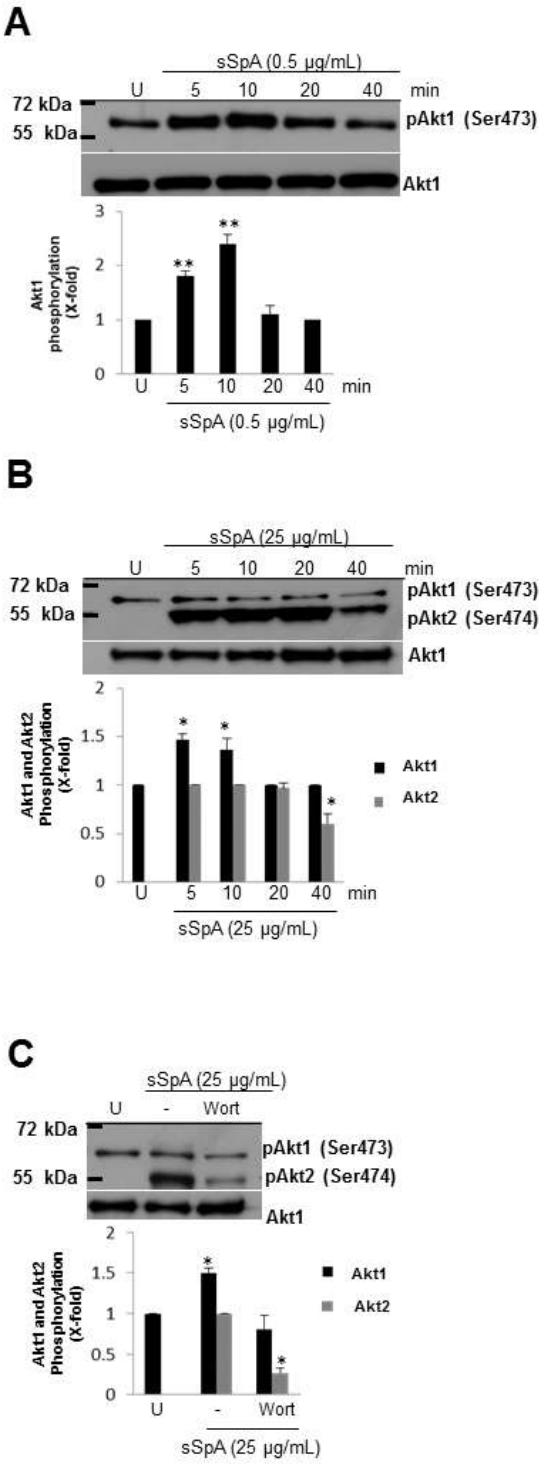
Figure 3. sSpA induces a PI3K/Akt1-dependent phosphorylation of GSK3β in BEC. A-B) BEC were left unstimulated (U) or stimulated with 0.5 μg/mL of sSpA from *S. aureus* for 5, 10, 20, and 40 min C-D) BEC were left untreated and unstimulated (U), untreated (-) or treated with 100 nM of Wortmannin (Wort), or 10 μM of SH-5 for 30 min, and then stimulated with 0.5 μg/mL of sSpA for 10 min. Protein extracts were analyzed by western blot and probed with antibodies against the phosphorylated forms of, GSK3β (pGSK3β Ser9) and NF-κB p65 subunit (NF-κB p65 Ser536). To verify that equal protein amount was loaded in each lane, blots were stripped and reprobed with an antibody that recognizes the nonphosphorylated forms of GSK3β and p65. Blots are representative of three independent experiments. Graphs indicate the band intensity obtained by densitometric analysis of each assay compared with the unstimulated control. Results are expressed as the mean ± S.E.M. (n = 3). *p <0.05; **p <0.01, compared with the untreated and unstimulated control.

Figure 4. sSpA induces a PI3K/Akt2-independent phosphorylation of GSK3β in BEC. A-B) BEC were left unstimulated (U) or stimulated with 0.5 μg/mL of sSpA from *S. aureus* for 5, 10, 20, and 40 min C-D) BEC were left untreated and unstimulated (U), untreated (-) or treated with 100 nM of Wortmannin (Wort), or 10 μM of SH-5 for 30 min, and then

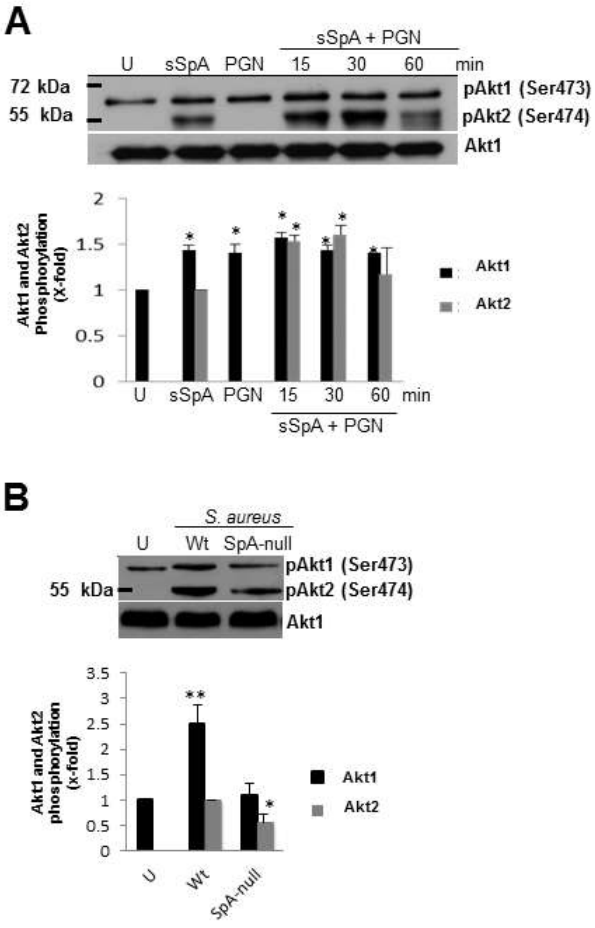
stimulated with 0.5 µg/mL of sSpA for 10 min. Protein extracts were analyzed by western blot and probed with antibodies against the phosphorylated forms of, GSK3β (pGSK3β Ser9) and NF-κB p65 subunit (NF-κB p65 Ser536). To verify that equal protein amount was loaded in each lane, blots were stripped and reprobed with an antibody that recognizes the nonphosphorylated forms of GSK3β and p65. Blots are representative of three independent experiments. Graphs indicate the band intensity obtained by densitometric analysis of each assay compared with the unstimulated control. Results are expressed as the mean ± S.E.M. (n = 3).

Figure 5. Diagram that shows the PI3K/Akt1 and PI3K/Akt2 signaling pathways in BEC stimulated with sSpA from *S. aureus*. sSpA induces phosphorylation of Akt1 and Akt2 by activation of PI3K. Phosphorylation of Akt1 leads to GSK3β, while Akt2 activation has no effect on GSK3β. The site of each inhibitor used (Wortmannin and SH-5) in this study is indicated. Question marks indicate molecules not identified in this study.

Figure 1. Cortés-Vieyra *et al.* 2013



443 **Figure 2. Cortés-Vieyra *et al*. 2013**



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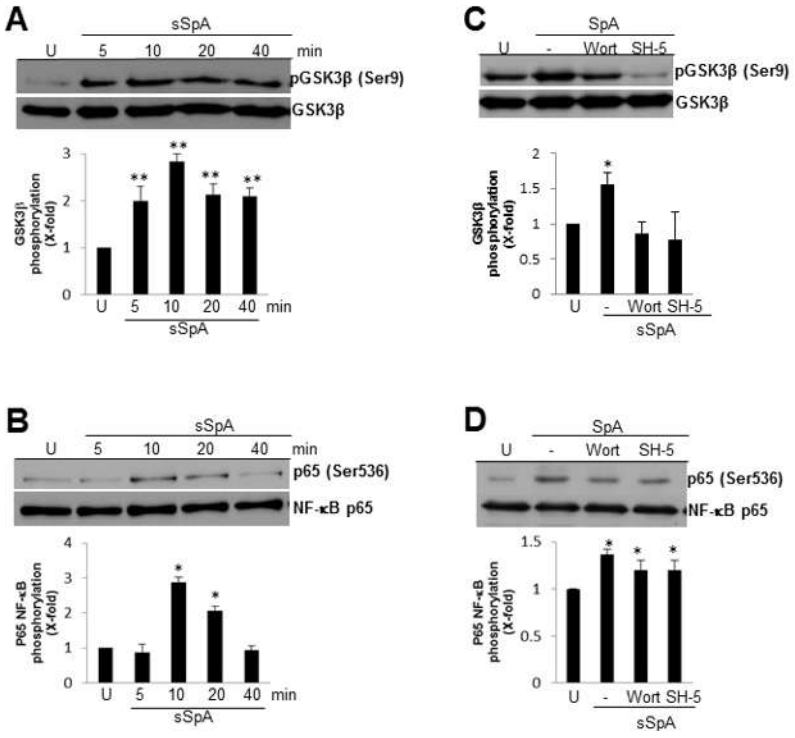
446 **Figure 3. Cortés-Vieyra *et al*. 2013**



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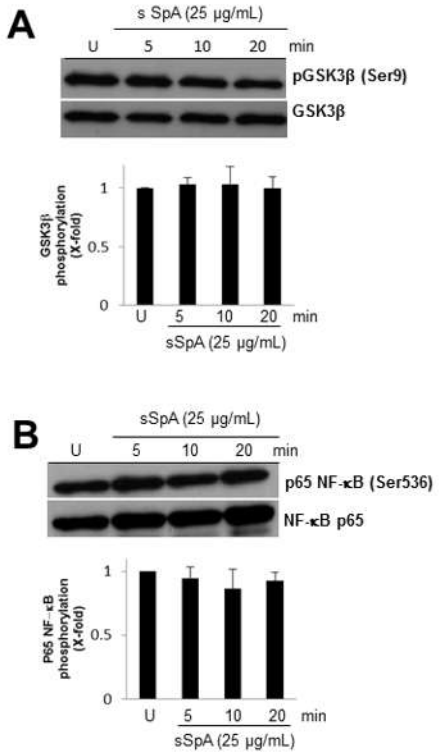
449 **Figure 4. Cortés-Vieyra *et al*. 2013**



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452 **Figure 5. Cortés-Vieyra *et al*. 2013**

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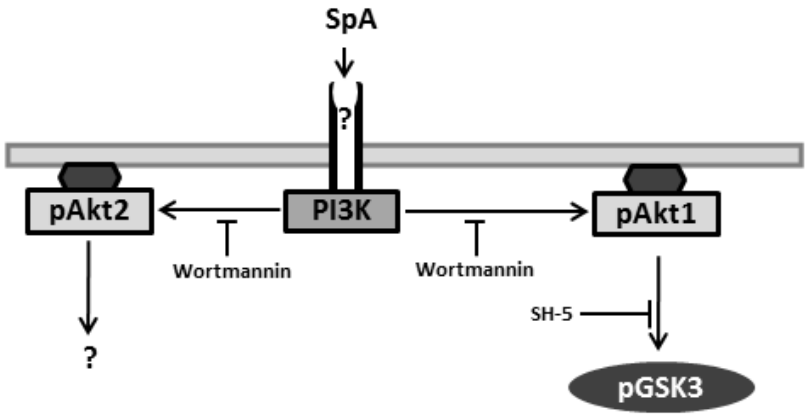

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VII. DISCUSIÓN GENERAL

Staphylococcusaureus es una bacteria piogénica que induce fuertemente el reclutamiento de neutrófilos hacia el sitio de la infección, activando la inflamación (Fournier 2013) y provocando enfermedad tanto en humanos como en animales (Pantosti2012). Generalmente, la patogenicidad de *S. aureus* se debe a la actividad coordinada de varios factores de virulencia (Fournier y Philpott 2005, Gordon y Lowy 2008). Por lo tanto, en este trabajo analizamos la respuesta del endotelio bovino a dos componentes pro-inflamatorios de *S. aureus*, PGN y SpA. De manera importante, por primera vez, se observó que tanto PGN como SpA inducen la fosforilación de la cinasa multifuncional y moduladora de la inflamación, GSK3, por activación de la vía de transducción PI3K/Akt [([a]Cortés-Vieyra*et al*., 2013) (Capítulo I); ([b]Cortés-Vieyra*et al*., 2013)(Capítulo II)]. Aunque las dos estructuras promueven la respuesta inflamatoria, se pudieron establecer tres diferencias entre un estímulo y otro: 1) PGN indujo la activación de la isoforma Akt1, mientras que SpA indujo la activación de Akt1 y Akt2. Un resultado interesante e inesperado fue observar que la estimulación de BEC con PGN y SpA causó un efecto aditivo en la fosforilación de la isoforma Akt2; 2) La activación de la vía PI3K/Akt1 por PGN activó principalmente la fosforilación de la isoforma GSK3α en su sitio de inhibición Ser21 y en menor proporción la fosforilación de GSK3β en su sitio de inhibición Ser9. SpA, sólo indujo la fosforilación de GSK3β en Ser9 por medio de la activación de PI3K/Akt1; 3) La activación de la vía PI3K/Akt1 por PGN activó a NF-κB; en contraste, la activación de las vías PI3K/Akt1 y PI3K/Akt2 por SpA, no indujeron la activación de NF-κB.

El estudio de la activación de la vía PI3K/Akt/GSK3 en células endoteliales, por componentes de *S. aureus*, es importante debido a que el endotelio tiene una función preponderante durante el proceso inflamatorio (Pober y Sessa 2007). Además, la mayoría de la información de las vías de señalización activadas por componentes bacterianos ha sido obtenida de un limitado número de tipos celulares, especialmente de células del sistema inmune; sin embargo, se ha observado que el tipo de respuesta depende estrictamente del tipo celular involucrado (Cortés-Vieyra*et al.* 2012). De hecho, nosotros observamos en las células endoteliales de bovino estimuladas con PGN, que la activación de la vía PI3K/Akt/GSK3 no resultó en una respuesta anti-inflamatoria como lo reportó Martin *et al.* (2005) en monocitos

humanos estimulados con LPS, sino en una respuesta pro-inflamatoria ([a]Cortés-Vieyra*et al*. 2013).

Los receptores tipo Toll (TLRs) son la clase más importante de receptores de respuesta inmune innata de reconocimiento de patrones (PRRs), a través de los cuales las células inmunes (fagocitos profesionales) y no inmunes (fagocitos no profesionales) del huésped son capaces de reconocer los patrones moleculares asociados a patógenos (PAMPs) (Pietrocola*et al.* 2011). El receptor TLR2 puede reconocer PAMPs con estructuras diversas, tales como lipoproteínas/lipopéptidos, PGN, glicopolímeros, ácido lipoteicoico, porinas, capsides virales etc (Fournier 2013). El reconocimiento de diversos ligandos por TLR2 se debe en parte a su hetereodimerización con co-receptores que le permiten expandir el espectro de estímulos reconocidos, sin alterar las vías de señalización activadas (Farhat*et al.* 2008). TLR2 es crucial en la respuesta del huésped a *S. aureus*. Prueba de esto es que los ratones deficientes en TLR2 son altamente susceptibles a infecciones estafilocócicas (Pietrocola*et al.* 2011). En este trabajo, al igual que en el de otros investigadores, se observó que TLR2 es activado por PGN de *S. aureus* (Dziarski y Gupta 2005,Chen*et al.* 2009, Chiu*et al.* 2009, Lin*et al.* 2010, Müller-Anstett et al. 2010). En BEC, la activación de TLR2 por PGN promovió la expresión de la interleucina pro-inflamatoria IL-12p40, por activación de NF-κB a través de la vía PI3K/Akt1/GSK3([a]Cortés-Vieyra*et al*. 2013). Es probable que también SpA active las vías PI3K/Akt1 y PI3K/Akt2 por TLRs porque se ha demostrado que además de activar los receptores TNFR-1 y EGFR, responsables de activar a las MAPKs JNK, p38 y ERK (Gómez *et al.* 2004, Gómez *et al.* 2007) (Fig. 4), también induce el reclutamiento de la proteína adaptadora de TLRs, TRIF ([b]Cortés-Vieyra*et al*. 2013).

Las isoformas existentes de Akt (Akt1, Akt2 y Akt3) son esenciales en la regulación de la supervivencia, la proliferación, la inflamación y otros múltiples procesos celulares. Las tres cinasas son muy similares en secuencia, parecen ser reguladas de manera similar y tienen funciones redundantes (González y McGraw 2009). Sin embargo, análisis fenotípicos en ratones knockout mostraron que las funciones de estas cinasas no son completamente redundantes y que la señalización específica de cada isoforma contribuye a la diversidad de actividades dependientes de Akt (Maroulakou*et al.* 2007, Gonzalez y McGraw 2009,Arranz *et al.* 2012). Por ejemplo, en macrófagos estimulados con LPS, la inhibición del fenotipo pro-inflamatorio, que contribuye al desarrollo de tolerancia a un subsecuente estímulo con esta

endotoxina, depende exclusivamente de la activación de Akt1 (Martin *et al.* 2005, Androulodaki*et al.* 2009, Arranz et al., 2012). En contraste, en un modelo de gastroenteritis aguda causado por *Salmonella*, la isoforma Akt2 mostró una función esencial en la protección contra la inflamación (Kum*et al.* 2011). De manera sorprendente se ha observado que las isoformas Akt1 y Akt2 pueden tener funciones opuestas en ratones, pues mientras que Akt1 inhibe, Akt2 acelera el desarrollo de adenocarcinomas mamarios (Maroulakou*et al.* 2007). Aunque en BEC estimuladas con SpA no evaluamos un fenotipo, tal como la inflamación, probablemente la activación de Akt1 y Akt2 por SpA no tienen un efecto redundante y probablemente las dos isoformas de Akt modulan un mismo proceso de forma opuesta ([b]Cortés-Vieyra*et al*. 2013).

En células del sistema inmune innato, se ha establecido que la vía PI3K/Akt y su molécula efectora río abajo, GSK3, tienen una participación esencial en la regulación de la producción de citocinas pro- y anti-inflamatorias (Wang *et al.* 2011). En mamíferos, existen dos principales isoformas de GSK3, α y β, las cuáles son codificadas por distintos genes (Woodgett 1984). Hasta ahora, la modulación de la inflamación por GSK3 en diferentes tipos celulares, durante un estímulo bacteriano, sólo había sido atribuida a la isoforma GSK3β (Cortés-Vieyra*et al.* 2012) (Anexo I). Inclusive, aunque nosotros no medimos la respuesta inflamatoria de CEB estimuladas con SpA de *S. aureus*, observamos que SpA induce fuertemente la fosforilación de GSK3β (Ser9) ([b]Cortés-Vieyra*et al*. 2013). También observamos que la modulación de la inflamación en las BEC estimuladas con PGN de *S. aureus* involucró principalmente la fosforilación de la isoforma GSK3α en Ser 21, con una mínima fosforilación de GSK3β en Ser9([a]Cortés-Vieyra*et al*. 2013). Estos resultados son congruentes con lo reportado por Oviedo-Boyso*et al.* (2011)(Anexo II), en donde se reportó que durante la internalización de *S. aureus* en las BEC, se fosforila GSK3α (Ser21) en mayor proporción que la fosforilación de GSK3β (Ser9) por activación de PI3K/Akt.

Las citocinas coordinan todos los aspectos de la respuesta inmune, incluyendo inflamación, diferenciación, tolerancia y memoria (Watford 2003,Steinke y Borish 2006). En particular, la interleucina 12 (IL-12) es una citocina multifuncional que induce inmunidad celular, promoviendo la producción de interferón γ (IFN-γ), la proliferación y actividad citotóxica en las células naturales asesinas (NK) y células T (Trinchieri 2003). IL-12 es producida principalmente por células dendríticas, monocitos, macrófagos, neutrófilos y células

B, (Watford 2003), así comopor células endoteliales (Lienenlüke*et al.* 2000). Existen dos subunidades de IL-12, denominadas p35 y p40, las cuáles pueden formar el heterodímero p70 o el homodímero $p40_2$ (Gately*et al*. 1991,Gately*et al*. 1996). Dado que la expresión de IL-12 es inducida por productos microbianos y regula el desarrollo de células inmunes adaptativas, IL-12 es una citocina pro-inflamatoria que coordina la inmunidad innata, así como el tipo y duración de la inmunidad adaptativa (Trinchieri 2003, Watford 2003).

Martin *et al.* (2005) demostró que GSK3 a través de PI3K-Akt modula negativamente la expresión de IL-12. Debido a que nosotros observamos la activación de NF-κB por la vía PI3K/Akt/GSK3 en BEC estimuladas con PGN ([a]Cortés-Vieyra*et al*. 2013), consideramos importante analizar la expresión de IL-12. En contraste con Martin *et al.* (2005), observamos que la vía modula positivamente la actividad de NF-κB y la expresión de la subunidad p40 de IL-12 ([a]Cortés-Vieyra*et al*. 2013). Esto es de gran importancia en endotelio, porque se ha propuesto que IL-12p40 funciona como quimioatrayente de macrófagos (Ha *et al.* 1999). Por lo tanto, estos resultados sugieren que el endotelio estimulado con PGN de *S. aureus* puede inducir el reclutamiento de macrófagos a través de la expresión de IL-12p40 por activación de PI3K/Akt/GSK3.El incremento en la expresión de IL-12 por la inactivación de GSK3, también ocurre en linfocitos $CD8^+$ T por estimulación antígeno-específica, lo que induce su proliferación (Ohteki*et al.* 2000). Dada la estrecha comunicación que existe entre el endotelio y los linfocitos (Strasly et al. 2001, Mitola*et al.* 2003), probablemente la expresión de IL-12 por endotelio estimulado con PGN, también activa la proliferación de linfocitos, contribuyendo así a la activación de la inmunidad adaptativa.

Finalmente, nuestros datos sugieren que las terapias propuestas para contrarrestar la inflamación en tejidos infectados, que tienen como blanco la inhibición de GSK3 (Martin *et al.* 2005), deben ser terapias isoforma y tejido específicas. Además, debido al involucramiento de GSK3 en múltiples procesos fisiológicos, es importante valorar en el huésped los efectos de la inhibición o activación de esta molécula.

En conclusión, en este trabajo observamos que PGN y SpA de *S. aureus*, dos estímulos ampliamente reconocidos como pro-inflamatorios, indujeron la activación de la vía PI3K/Akt/GSK3 en células endoteliales. Sin embargo, sólo PGN indujo la activación de NF-κB y la expresión de la citocina pro-inflamatoria IL12, por activación de TLR2/PI3K/Akt/GSK3. Importante también fue el hallazgo de que en las CEB estimuladas

con SPA, la activación de NF-κB por fosforilación en el residuo Ser536 de su dominio de transactivación, no depende de la activación de PI3K/Akt/GSK3.

VIII. PERSPECTIVAS

Futuros experimentos deben ser diseñados para definir la participación de la isoforma GSK3α en la modulación de la inflamación en células estimuladas con componentes de *S. aureus* y en células estimuladas con otros estímulos pro-inflamatorios. Es importante observar si efectivamente, la expresión de IL-12p40 por activación de PI3K/Akt/GSK3 en endotelio estimulado con PGN, tiene como consecuencia el reclutamiento de macrófagos e incluso la proliferación de linfocitos. También, debe ser analizada la naturaleza de la respuesta inflamatoria en CEB estimuladas con SpA de *S. aureus* por activación de PI3K/Akt1 y PI3K/Akt2, así como el receptor involucrado en la activación de las dos vías. Finalmente, sería interesante analizar cómo se lleva a cabo el efecto aditivo en la fosforilación de Akt2 en CEB estimulada con PGN y SPA.

IX. BIBLIOGRAFÍA COMPLEMENTARIA

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X. ANEXO

x.i Role of glycogen synthase kinase-3 beta in the inflammatory response caused by bacterial pathogens.

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JOURNAL OF INFLAMMATION

REVIEW Open Access

Role of glycogen synthase kinase-3 beta in the inflammatory response caused by bacterial pathogens

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Abstract

Glycogen synthase kinase 3β (GSK3β) plays a fundamental role during the inflammatory response induced by bacteria. Depending on the pathogen and its virulence factors, the type of cell and probably the context in which the interaction between host cells and bacteria takes place, GSK3β may promote or inhibit inflammation. The goal of this review is to discuss recent findings on the role of the inhibition or activation of GSK3β and its modulation of the inflammatory signaling in monocytes/macrophages and epithelial cells at the transcriptional level, mainly through the regulation of nuclear factor-kappaB (NF-κB) activity. Also included is a brief overview on the importance of GSK3 in non-inflammatory processes during bacterial infection.

Keywords: GSK3β, NF-κB, Inflammation, Virulence factors, Bacterial infection

Background

Glycogen synthase kinase 3 (GSK3), in its two isoforms GSK3α and GSK3β, is a multifunctional Ser/Thr kinase found in eukaryotes [1]. This enzyme phosphorylates and regulates the function of more than 50 substrates [2] and it is a point of convergence for numerous cell-signaling pathways involved in various essential cellular functions, such as glycogen metabolism, cell cycle control, apoptosis, embryonic development, cell differentiation, cell motility, microtubule function, cell adhesion and inflammation [1-3]. The view of GSK3β has changed from an obscure metabolic kinase to an enzyme that profoundly regulates many components of the innate and adaptive immune systems. The broad array of immune actions affected by GSK3β is partly attributable to the remarkable number of crucial transcription factors that it regulates [4]. The main objective of this review is to show the importance of GSK3β in innate immunity against bacterial infections through regulation of the inflammatory response induced by virulence factors.

General properties of GSK3

There are two major mammalian GSK3 protein isoforms (α and β) encoded by two distinct genes (*gsk3α* and *gsk3β*) [5] that are highly homologous within their kinase domains (approximately 98% of identity), but with only 36% identity in the last 76 C-terminal amino acid residues [5]. Both isoforms are structurally similar but not functionally identical because ablation of the GSK3β isoform in mice resulted in embryonic lethality via hepatocyte apoptosis. The inability of GSK3α to rescue the GSK3β-null mice indicates that the degenerative liver phenotype arises specifically from the loss of the beta isoform. Although severe hepatocyte cell death could be due to β–catenin inhibition of NF-κB, increased amount of β–catenin in GSK3β (-/-) cells was not found [6]. Physical inhibitory interaction between β–catenin and NF-κB is likely a mechanism for tumor size progression mediated by β–catenin [7]. Alternatively, GSK3α knockout mice are viable but display enhanced glucose and insulin sensitivity accompanied by reduced fat mass [8]. Mechanisms that regulate GSK3 activity are not yet fully understood. The precise control

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appears to be achieved by a combination of intracellular localization, phosphorylation, and interactions with GSK3 binding proteins [2]. In this regard, GSK3 has been traditionally considered a cytosolic protein; however, it is also present in the nucleus and mitochondria, where it is highly active compared with the cytosolic form [9].

The crystal structure of GSK3β has provided insight into both the regulation of its activation and its inhibition by phosphorylation [1]. GSK3 is activated by phosphorylation of Tyr216 (GSK3β) or Tyr279 (GSK3α) and it is inactivated by phosphorylation of Ser9 (GSK3β) or Ser21 (GSK3α). Several protein kinases can phosphorylate Ser9 and Ser21, including the protein kinase B (PKB/Akt), protein kinase A (PKA), protein kinase C (PKC) and ribosomal protein 6 kinase (S6K) [2,10]. The inactivation of GSK3β by phosphorylation, carried out mainly by Akt, may result in the activation of transcription factors such as AP-1 (Jun family), cAMP-response element binding protein (CREB), signal-transducer and activator of transcription 1-3 (STAT1-3), β-catenin, and nuclear factor-kappaB (NF-κB) in response to bacterial infections [2,3] (Figure 1).

NF-κB plays a critical role in the inflammatory response and it has been traditionally used as an indicator of pro-inflammatory gene expression in cells exposed to bacterial infections. When an inflammatory stimulus induces the phosphorylation of IκB by the IκB kinase (IKK) complex, the NF-κB heterodimer (p50/p65) is free to translocate to the nucleus and activates pro-inflammatory gene expression. GSK3β is important for the modulation of NF-κB because p65 (RelA), p105 (NF-κB1) and B-cell lymphoma 3-encoded protein (BCL-3) (a transcriptional co-activator of NF-κB p50 homodimer) are phosphorylated *in vitro* by this kinase [12,14]. GSK3β promotes a rapid NF-κB activation wave by targeting the TNFα-/p65-dependent pathway and limiting NF-κB activation in BCL-3-dependent pathways [10] stabilizing and preventing p105 degradation in unstimulated cells [15]. However, GSK3β also catalyzes the phosphorylation of p105, which in turn activates the phosphorylation and degradation of IKK upon tumor necrosis factor alpha (TNF-α) treatment [15].Therefore, in basal or stimulated cells GSK3β plays a double function upon p105 [15]. Moreover, GSK3 plays distinct roles in the regulation of NF-κB, depending on the physiological state of the cell. This enzyme promotes survival and stimulates the activity of NF-κB in cells treated with TNF-α or in tumor cells in which the NF-κB pathway is constitutively active. In contrast, in quiescent cells GSK3 suppresses the expression of growth factor-inducible genes and induces apoptosis or cell cycle arrest by inhibiting both the IKK-phosphorylation of IκBα and the nuclear translocation of p50 and p65 subunits of NF-κB [16].

In view of the contrasting effects that GSK3 plays as a functional regulator of the cell activity, the following sections of this review discuss our current knowledge about the importance of GSK3β as a regulator of the inflammatory process triggered by bacterial virulence factors. Also, in the last section a brief overview on the non-inflammatory phenomena induced by bacteria is presented, which are correlated with the activity of GSK3.

The inflammatory response

Inflammation is the body's primary response to infection or injury and is critical for both innate and adaptive immunity. Upon infection, a variety of cytokines, chemokines, lipid mediators and bioactive amines are secreted by resident tissue cells, primarily macrophages, dendritic cells, natural killer cells, and mast cells. These factors immediately trigger a local increase of blood flow, capillary permeability and recruitment of additional circulating leukocytes via extravasation. This acute inflammatory response is characterized by the arrival of neutrophils, monocytes that differentiate into macrophages at the site of inflammation, and dendritic cells. This process is complex and involves many different signaling pathways. Most of our knowledge about pro-inflammatory signaling pathways has been obtained from studying the molecules of signaling pathways that are initiated by the activation of tumor necrosis factor receptor (TNFR), interleukin 1 receptor (IL1R), and Toll-like receptors (TLRs) [17]. Activation of TLRs by a variety of pathogen associated molecular patterns (PAMPs) or virulence factors can induce the expression of inflammatory cytokines and other molecules that help to eliminate pathogens and instruct pathogen-specific adaptive immune responses [18]. Cytokines, primarily derived from mononuclear phagocytic cells and other antigen-presenting cells (APCs), are effective in promoting the cellular infiltrate and tissue damage characteristic of inflammation. Monocytes are potently triggered to produce cytokines through the stimulation of pattern recognition receptors (PRRs). The pro-inflammatory cytokines predominantly produced by monocytes include TNF, IL-1, IL-6, CXCL8 (IL-8) and other members of the chemokine family IL-12, IL-15, IL-18, IL-23 and IL-27 [19].

During inflammation, leukocytes amplify the response but excessive or prolonged inflammation may cause damage to the host. In normal circumstances, the immune system has several mechanisms to resolve the inflammatory responses that require the termination of pro-inflammatory signaling pathways and clearance of inflammatory cells, allowing the restoration of normal tissue function. Failure of these mechanisms may lead to chronic inflammation and disease [20]. In addition to cytokines that stimulate cytotoxic, cellular, humoral, and allergic inflammation, several cytokines have predominantly anti-inflammatory effects, including IL-1Ra, TGF-β, IL-10 and IL-35 [21]. Recently, a number of reports have documented that GSK3β activity is crucial to regulate the

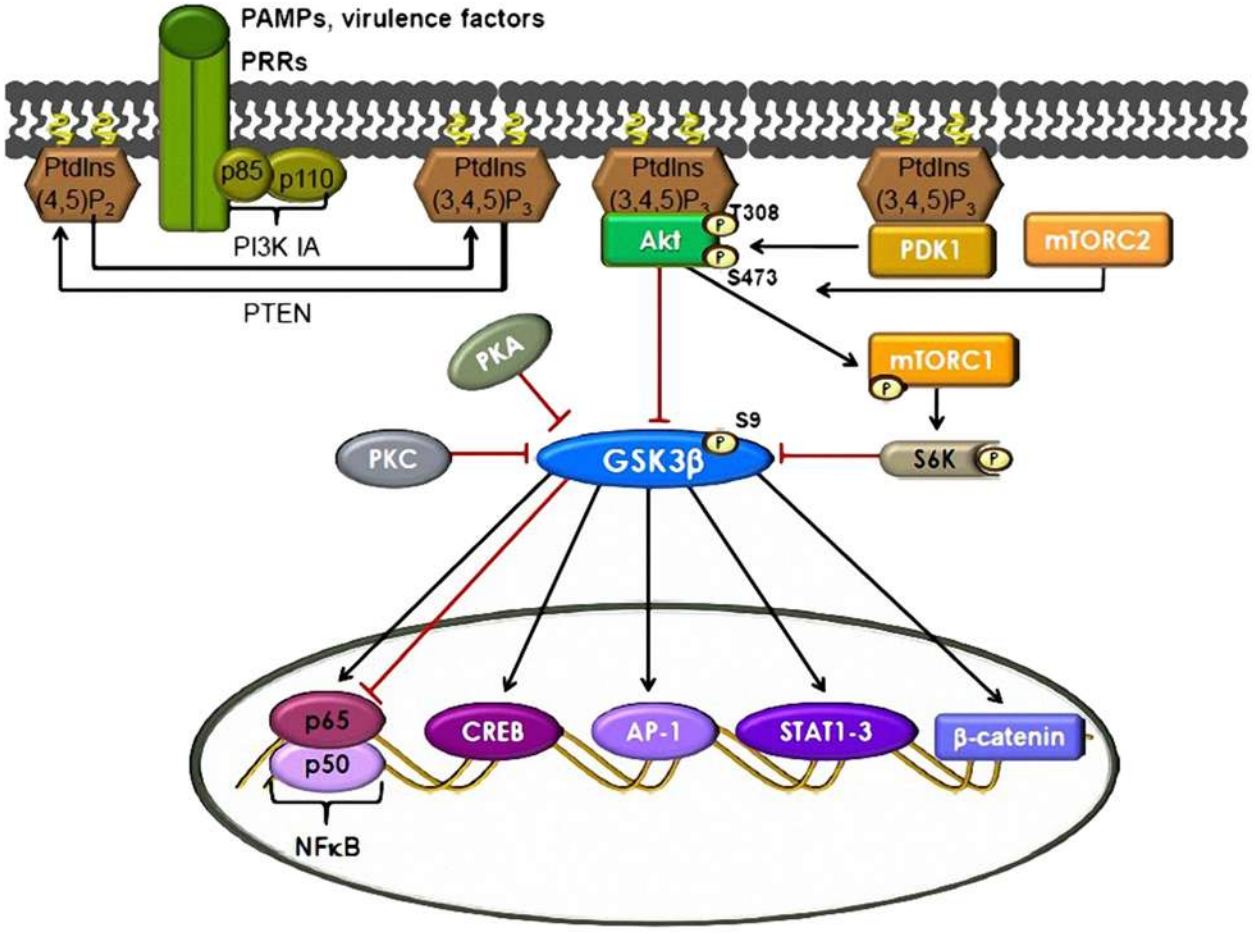

Figure 1 GSK3β regulation of transcription factors activity is important for the modulation of inflammatory responses. Pattern ecognition receptors (PRRs) activation by pathogen-associated molecular patterns (PAMPs) or virulence factors recruits class IA of phosphoinositide 3-kinases [PI3K IA (p85–p110)] to the membrane by direct interaction of the p85 subunit with the activated receptors or by interaction with adaptor proteins associated with the receptors [3,11]. The activated p110 catalytic subunit converts phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P_2] to phosphatidylinositol-3,4,5-trisphosphate [PtdIns(3,4,5)P_3], providing docking sites for the signaling proteins 3'-phosphoinositide-dependent kinase 1 (PDK1) and protein kinase B (PKB/Akt) that have pleckstrin homology domains [11]. Phosphatase and tensin homologue (PTEN) antagonizes the PI3K action by dephosphorylating [PtdIns(3,4,5)P_3] [11]. Akt is phosphorylated and activated by PDK1 and the mammalian target of rapamycin complexes 2 (mTORC2) at Thr308 and Ser473, respectively, and then is able to phosphorylate and inactivates glycogen synthase kinase-3 beta [GSK3β (S9)] [11]. GSK3β can be also phosphorylated and inactivated by protein kinase A/C (PKA/C) and by the mammalian target of rapamycin complexes 1 (mTORC1) through ribosomal protein 6 kinase (S6K) [3,10]. Inactivation of GSK3β results in the activation of transcription factors such as nuclear factor-κB (NF-κB), cAMP-response element binding protein (CREB), activator protein 1 (AP-1), signal transducers and activators of transcription 1-3 (STAT1-3) and β-catenin that are involved in the regulation of the inflammatory responses [3,12,13]. GSK3β regulation on NF-κB is complex due to cell-, stimulus-, and promoter-selective interactions that might be stimulatory or inhibitory [4].

inflammatory response either promoting or inhibiting the process through the expression of pro- or anti-inflammatory cytokines.

Inhibition of inflammation by inhibition of the GSK3β activity

Several studies have demonstrated that inflammation is regulated by the TLR-dependent activation of PI3K-Akt signaling pathway [3,22-26]. A breakthrough paper by Martin et al. [27] established that the PI3K-Akt-dependent inhibition of GSK3β activity in human monocytes, stimulated with lipopolysaccharide (LPS), differentially affected the nature and magnitude of the inflammatory response through the activation of TLR2. This in turn resulted in the production of the anti-inflammatory cytokine IL-10, while production of pro-inflammatory cytokines IL-1β, IL-6, TNF, IL-12 and IFN-α fell substantially. Inhibition of GSK3β negatively modulated the inflammatory response because it differentially affected the nuclear activity of NF-κB (p65 subunit) and CREB through the interaction with the co-activator CREB-binding protein (CBP) [27]. In a recent

study carried out in monocytes stimulated with LPS, it was established that the mammalian target of rapamycin complex 1 (mTORC1) regulates the activity of GSK3β through the activation of S6K, affecting the inflammatory response by inactivation of GSK3β. Furthermore, the inhibition of GSK3β by mTORC1 affected the association of NF-κB (p65 subunit) and CBP [10].

GSK3β activity negatively regulated the level of the anti-inflammatory cytokine IL-1Ra while concurrently increased the levels of IL1β in LPS-stimulated human monocytes. The PI3K-Akt-dependent inhibition of GSK3 increased the production of IL-1Ra due to its ability to modulate the activity of extracellular-signal-regulated kinase 1/2 (ERK1/2) [28]. These results and the fact that IL-1Ra counteracts the inflammatory properties of IL-1β [29] showed that in LPS-stimulated human monocytes the inhibition of GSK3β increases the production of anti-inflammatory cytokines and reduces the expression of pro-inflammatory cytokines, confirming the model proposed by Martin et al. [27], in which GSK3β in its active form acts as a positive regulator of inflammation.

In a study with *Mycobacterium bovis* BCG as a *Mycobacterium* model, it was demonstrated that GSK3β inhibition through the PI3K-Akt signaling increased the production of IL-10 in primary human blood monocytes (PHBM) [30]. Among the cytokines induced by BCG in PHBM, IL-10 was the key factor suppressing the production of interferon-γ (IFN-γ) in response to mycobacterial infection. Moreover, IL-10 expression induced by BCG was able to suppress the IFN-γ-dependent expression of HLA-DR, an inducible MHC class II molecule whose primary function is to present peptide antigens to the immune system. These findings suggest a significant role for GSK3β in guarding against mycobacterial evasion of host immunity, via IL-10 expression.

The PI3K-Akt signaling pathway activation following the nucleotide oligomerization domain 2 (Nod2) recognition of the agonist muramyldipeptide (MDP), a structure from peptidoglycan (PGN), negatively regulates the NF-κB pathway and interleukin (IL)-8 expression through inactivation of GSK3β. These results suggest that the PI3K-Akt-GSK3β pathway may be involved in the resolution of inflammatory responses induced by Nod2 activation [31].

Lipoteichoic acid (LTA) is a membrane-bound cell wall component of Gram-positive bacteria and is believed to be the equivalent of LPS of Gram-negative bacteria. Treatment of human gingival fibroblasts (HGFs) with LTA activated Akt which in turn inactivated GSK-3 and promoted the accumulation of β-catenin, resulting in an increase of connexin43 expression [32]. Given that the interaction of β-catenin with NF-κB leads to a decrease of the NF-κB ability to bind DNA and induce gene expression [7,33], it is likely that the accumulation of β-catenin in LTA-stimulated HGFs causes a negative regulation of the NF-κB activity and that this gives rise to a decrease of the pro-inflammatory cytokines production [32]. It is also likely that GSK-3β inactivation might be able to modulate the transcription of specific pro-inflammatory genes containing a T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) binding site in their promoter. In this regard, it was recently demonstrated that β-catenin induces pro- and anti-inflammatory responses simultaneously as a result of differential gene expression carried out by Wnt/β-catenin signaling through a TCF/LEF consensus sequence and NF-κB modulation in the context of liver cancer-related inflammation [34].

Innate immunity and inflammatory responses play central roles in the pathophysiology of myocardial ischaemia/reperfusion (I/R) injury and heart failure. In this context, it was observed that PGN administration induced cardio protection in hearts of mice subjected to ischaemia, followed by reperfusion. Activation of the PI3K-Akt-GSK3β signaling pathway and reduction of the NF-κB nuclear translocation were the main factors responsible for the protection [35]. Although one may assume that reduction of NF-κB nuclear translocation decreased swelling, this waits further demonstration.

Inhibition of inflammation by activation of GSK3β

In neonatal mouse cardiomyocytes and heart tissue culture, LPS increased the activity of GSK3β and its inhibition with chemical and genetic inhibitors enhanced LPS-induced p65 phosphorylation at the residue Ser536 and increased TNFα expression [36]. Furthermore, in line with GSK3β dephosphorylation at Ser9, Akt phosphorylation at Thr308 was reduced in LPS-treated cardiomyocytes and chemical inhibition of PI3K-Akt attenuated LPS-induced TNFα expression. These results suggest that PI3K-Akt-dependent inactivation of GSK3β plays an important function in LPS-induced TNF-α expression.

Induction of inflammation by inhibition of GSK3β activity

The production of pro- and anti-inflammatory cytokines by activation of TLR2 and TLR4 in macrophages is dependent upon signaling events initiated by the adaptor molecules TIR-domain-containing adaptor protein (TIRAP) and myeloid differentiation primary response gene 88 (MyD88) [13]. In contrast, inactivation of GSK3β by phosphorylation at Ser9 in macrophages occurred in the absence of MyD88 [32]. In this case, GSK3β activity was a critical component of the regulatory mechanism that controlled the levels of IFNβ in TLR4-stimulated cells both *in vitro* and *in vivo* [37]. In particular, it was shown that inhibition of GSK3β activity augmented the levels of IFNβ in LPS-stimulated

macrophages whereas the ectopic expression of a constitutively active GSK3β mutant caused a reduction of the IFNβ production. Interestingly, inhibition of GSK3β controlled the cellular levels of the transcription factor c-Jun that turned out to be necessary for GSK3-mediated IFNβ production. The conclusion from these results is that GSK3β acts as a critical regulatory kinase that modulated the MyD88-independent synthesis of IFNβ and of MyD88-dependent production of pro- and anti-inflammatory cytokines, demonstrating the existence of a cross-talk signaling network between these two pathways with GSK3β as a central kinase [37].

The intracellular infection of monocytes and macrophages with *Burkholderia cenocepacia*, a Gram-negative bacterium associated with exacerbated inflammation [38], caused the activation of PI3K-Akt signaling that in turn inactivated GSK3β and enhanced NF-κB activity, with the subsequent production of pro-inflammatory cytokines such as, TNFα, IL-6 and IL-8. Interestingly, NF-κB activation did not require the activation of IKK or NF-κB p65 phosphorylation, indicating that the inactivation of GSK3β was the major mechanism by which PI3K-Akt modulated the NF-κB activity without affecting *B. cenocepacia* uptake or survival [38].

Induction of inflammation by activation of GSK3β

A model in which IFN-γ specifically inhibits TLR2-dependent production of IL-10 in macrophages by increasing the activity of GSK3α/β, and decreasing the expression and activity of CREB and AP-1 proteins has been established [39]. Moreover, at the same time of IL-10 suppression, IFN-γ induced the expression of TNFα. In this study GSK3 and CREB/AP-1 were key players in the signaling activated by the IFN-γ receptor and TLR2.

Microglial inflammation caused by pathogenic *S. aureus* occurred through modulation of GSK3β activity that positively regulated the NF-κB-dependent production of TNFα and nitric oxide (NO) [35]. GSK3β negatively regulated IL-10 production, and this inhibition affected the protection against heat-inactivated *S. aureus*-induced microglial inflammation [40]. These authors showed that TNFα acted upstream of NO production and that inhibition of GSK3β blocked heat-inactivated *S. aureus*-induced NF-κB p65 nuclear translocation.

In the study of the mechanisms by which GSK3β positively modulates the inflammatory response in LPS-stimulated microglia, Wang et al. (2010) [41] showed that inhibition of GSK3β activity by selective pharmacological inhibitors or its gene silencing by small interfering RNA suppressed TNFα production by blocking the NF-κB p65 transactivation activity through deacetylation of p65 at Lys310. In addition, these authors also demonstrated that inhibition of GSK3β blocked mixed lineage kinase 3 (MLK3) activity leading to a reduction of TNFα expression.

The role of GSK3β in modulating the β-catenin response in colon inflammation caused by pathogenic *Salmonella* Typhimurium was examined by using a streptomycin-pretreated mouse model [33]. *S.* Typhimurium induced an increase in β-catenin phosphorylation by augmenting GSK3β activity, reducing total β-catenin expression and compromising the physical cytoplasmic interaction between β-catenin and NF-κB. IκBα, the well-established negative regulator of NF-κB, was degraded in a similar manner as β-catenin after *Salmonella* infection. Following β-catenin and IκBα degradation, released NF-κB translocated to the nucleus and stimulated the production of the pro-inflammatory cytokines IL-6 and IL-8 [33]. The results of this study suggest a novel role for β-catenin as a negative regulator of NF-κB activity *in vivo*. Altogether, these data suggest that inhibition of GSK3β as well as β-catenin and IκBα stabilization provides important control points in the inflammatory cascade of colonic epithelial cells.

The mechanisms by which IFN-γ synergizes with LPS to induce iNOS/NO (important inductors in inflammatory cytokine production) biosynthesis in macrophages involve GSK3β-dependent inhibition of CREB activity and IL-10 expression [42]. IFN-γ co-administration with LPS was also used to study the inflammatory responses modulated by GSK3 in mouse primary glia cultures [43]. In this case, active GSK3 decreased the expression of chemokine CXCL2/MIP-2 and increased the expression of pro-inflammatory molecules CXCL1/KC, IL-12p40, CCL9/MIP-1γ, CCL2/MCP-1, P-Selectin and CCL5/RANTES. However and most prominently, active GSK3 promoted IL-6 expression due to the cooperative actions of STAT3 and GSK3 during neuro-inflammation. The production of IL-6 by glia was largely blocked by inhibiting the activity of STAT3 or GSK3β, revealing the strong dependence of IL-6 production on these signaling molecules [43]. These data reflect the cell's ability to hyper-response to TLR-induced IFN-γ production regulated by GSK3β, resulting in a synergism of the inflammatory response.

The opposing functions of GSK3β in the inflammatory response described in the text are summarized in Table 1.

GSK3 regulation of non-inflammatory cellular processes activated by bacterial components

The *Helicobacter pylori* virulence factor VacA is one of the most important toxins that contributes to the pathogenesis and severity of gastric injury in infected humans [44]. Although it is still controversial whether cross-talk exists between the PI3K-Akt and Wnt pathways [45], the work of Nakayama et al. [46] showed that VacA induced two effects on β-catenin in gastric epithelial AZ-521 cells. The first one was the activation and nuclear

Table 1 GSK3β modulation of the inflammatory response caused by bacterial stimuli

Bacterium or bacterial PAMP	Type of cell	GSK3β Inhibition: + pSer9 GSK3β Activation: - pSer9 or + pTyr216	NF-κB Inhibition ↓ NF-kB Activation ↑ Not tested -	Pro or anti- inflammatory molecules Expressed	Pro or anti-inflammatory molecules Inhibited	Refs.
LPS	Human monocytes	+pSer9	↓	IL-10	IL-1β, IL-6, TNF, IL-12, IFN-α	[10,27]
LPS	Human monocytes	+pSer9	-	IL-1Ra	IL-1β	[28]
Mycobacterium bovis	Primary human monocytes	+pSer9	-	IL-10	IFN-γ	[30]
Muramyl dipeptide	Human embryonic kidney epithelial cells	+pSer9	↓	-	IL-8	[31]
LPS	Neonatal mouse cardiac cells		↓	-	TNF-α	[36]
LPS	Mice macrophages	+ pSer9	-	IFNβ	-	[37]
Burkholderia cenocepacia	Human monocytes and mouse macrophages	+ pSer9	↑	TNF-α, IL-6, IL-8	-	[38]
Pam_3Cys^b and IFN-γ	Human macrophages	- pSer9	-	TNFα	IL-10	[39]
Staphylococcus aureus	Murine microglia	+ pTyr216	↑	TNF-α, NO	IL10	[40]
LPS	Murine microglia	A	↑	TNF-α	-	[41]
Salmonella typhimurium	Mouse colonic epithelial cells	- pSer9; + pTyr216	↑	TNF-α, IL-6	-	[33]
IFN-γ and LPS	Murine macrophages	+ pTyr216	-	iNOS, NO	IL-10	[42]
IFN-γ and LPS	Mouse primary microglia and astrocytes	A	-	IL-6, CXCL1, IL-12p40, CCL9, CCL2/MCP-1, P- Selectin,CCL5	CXCL2, MIP2	[43]

[A] GSK3β phosphorylation at Ser9 or Tyr216 was not analyzed.
[b] Synthetic PAMP.

accumulation of β-catenin following a short incubation with VacA, a process dependent on an active PI3K-Akt pathway and an inactive GSK3β. The second effect was that prolonged incubation with VacA resulted in inactivation of Akt and activation of GSK3β, which then down-regulated β-catenin activity. It was evident in this study that Wnt signaling, modulated by PI3K-Akt-GSK3β played a role in the pathogenesis of *H. pylori* infection, including the development of gastric cancer [46].

The lethal toxin (LeTx), produced by *Bacillus anthracis*, has been regarded as a key virulence factor in the pathogenesis of anthrax, causing immune paralysis, cell cycle arrest and cell death in host immune cells. These effects could contribute to the survival and proliferation of *B. anthracis* within the host. LeTx is a binary A:B toxin comprising protective antigen (PA) and lethal factor (LF) [47]. PA is a molecular transporter that allows receptor-mediated entry and release of LF into the cytosol. LF is a zinc metalloprotease that cleaves and inactivates the N-terminal region of the mitogen-activated protein kinase (MAPK) kinases MEKs1-4 and 6-7, resulting in the inactivation of most of their downstream signaling substrates [47]. In non-dividing cells (human peripheral blood mononuclear cells or mouse primary peritoneal macrophages) brief exposure to LeTx induced the cleavage of MEKs by LF, generating cell cycle arrest in G0-G1 phase by rapid down-regulation of cyclin D1/D2 and checkpoint kinase 1 [47]. LF also prevented TNF production in response to LPS. However, it was observed that recovery from the effects of LeTx can be facilitated by activating the PI3K-Akt-GSK3β signaling-mediated adaptive responses, indicating that modulation of this pathway can be beneficial against LeTx in cells depending on basal MEK1 activity for proliferation [47].

The inhibition of GSK3 via PI3K-Akt pathway has been identified in bacterial internalization processes in several host cells. For example, the invasion of HeLa cells by group B streptococcus (GBS) was associated with the activation of the PI3K and Akt kinases and GSK3α/β phosphoinibition [48]. One of the two type III secretion systems (TTSSs) of *Salmonella enterica* serovar Typhimurium triggers bacterial internalization through activation of PI3K-Akt [49]. Among the effectors proteins translocated by this TTSS, the GTPase modulator SopE/E2 and the phosphoinositide phosphatase SigD are known to play key roles in the process [49]. Using a reverse-phase protein array technology in HeLa, it was reported the SigD-dependent phosphorylation of Akt and its target GSK3β, demonstrating the importance of phosphoinhibition of GSK3β during host cell signaling events through bacterial infection [50]. Recently, the participation of PI3K-Akt-GSK3α/β pathway in *Staphylococcus aureus* internalization by endothelial cells was demonstrated. Although the role of the PI3K- Akt- dependent phosphorylation of GSK3α/β in the internalization of this bacterium was not determined in this study, phosphorylation of GSK3β at Ser9 and GSK3α at Ser21 was clearly associated with the invasion of *S. aureus* to the endothelial cells [51]. It is likely that in the internalization of GBS and *Salmonella enterica* by HeLa cells and *S. aureus* by endothelial cells, GSK3 functions by regulating the cytoskeletal rearrangement, as it was observed in macrophages RAW264.7 in which phosphorylation of paxillin at Ser126 and 130 was mediated by an ERK/GSK3 dual-kinase mechanism [52].

Conclusions

The experimental evidence accumulated so far indicates that GSK3β plays an essential role in the regulation of the inflammatory response during the interaction between pathogenic bacteria and animal cells. The opposing effects of GSK3β on the inflammation is dependent upon the bacterium or virulence factor (*Mycobacterium bovis*, *Burkholderia cenocepacia, Staphylococcus aureus, Salmonella typhimurium,* LPS, MDP), the type of cell (epithelial cells, monocytes, cardiomyocytes, macrophages, microglia or astrocytes and fibroblasts) and probably on the physiological state of the cell [16]. Although activated NF-κB induces an inflammatory response, the active or inactive state of GSK3β modulates the activity of NF-κB, either promoting or inhibiting an inflammatory response.

Apart from its fundamental regulatory role on the inflammatory response, GSK3 is associated with bacterial internalization [48,50,51] and other processes related to the pathogenesis of the infection [46,47]. However, more studies are needed to clarify the details about the mechanisms that GSK3 employs to control the bacterial internalization, the pathogenesis of infection and the expression of genes with pro- or anti-inflammatory function.

Abbreviations

AP-1: Activator protein 1; APCs: Antigen-presenting cells; BCL3: B-cell lymphoma 3-encoded protein; CREB: cAMP-response element binding protein; ERK1/2: Extracellular-signal-regulated kinase 1/2; GSK3α/β: Glycogen synthase kinase-3 alpha/beta; HLA-DR: Major histocompatibility complex, MHC class II, cell surface receptor; LTA: Lipoteichoic acid; IFN-γ/β: Interferon-γ/β; IL1R: Interleukin 1 receptor; IKK: IκB kinase; MAPK: Mitogen-activated protein kinase; MLK3: Mixed lineage kinase 3; mTORC1/2: The mammalian target of rapamycin complexes 1/2; MDP: muramyldipeptide; MyD88: Myeloid differentiation primary response gene 88; NF-κB: Nuclear factor-κB; NO: Nitric oxide; iNOS: inducible Nitric oxide synthase; Nod2: Nucleotide oligomerization domain 2; PAMPs: Pathogen associated molecular patterns; PDK1: 3'-phosphoinositide-dependent kinase 1; PKA/C: Protein kinase A/C; PKB: Protein kinase B; PI3K IA: Class IA of phosphoinositide 3-kinases; PTEN: Phosphatase and tensin homologue; PRRs: Pattern recognition receptors; S6K: Ribosomal protein 6 kinase; STAT1-3: Signal-transducer and activator of transcription 1-3; TCF/LEF: T-cell factor/lymphoid enhancer-binding factor; TIR: Toll/Interleukin-1 receptor; TIRAP: TIR-domain-containing adaptor protein; TLRs: Toll-like receptors; TRIF: TIR-domain-containing adapter-inducing interferon-β; TNFα: Tumor necrosis factor alpha; TNFR: Tumor necrosis factor receptor.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions
RCV and VMBA conceived of the review, designed, and wrote the manuscript. ABP, JJVA, MCJ and BBF contributed to critical reading and comments of the manuscript. All authors read and approved of the final manuscript.

Acknowledgements and funding
The authors thank Alejandro Huante-Mendoza and Octavio Silva-García for their valuable comments to the manuscript. Work on the corresponding author's laboratory is supported by Coordinación de la Investigación Científica of the Universidad Michoacana de San Nicolás de Hidalgo and Consejo Nacional de Ciencia y Tecnología (CONACYT-México), grant number 152518, and Programa Integral de Fortalecimiento Institucional (PIFI-2011-2012). VMB-A was a recipient of a sabbatical scholarship from CONACYT-México, file 118602. B.B.F. is a CIHR Distinguished Investigator, a Howard Hughes Medical Institute International Research Scholar, and UBC Peter Wall Distinguished Professor. RC-V is receiving a doctoral scholarship from CONACYT.

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Received: 12 January 2012 Accepted: 22 May 2012
Published: 12 June 2012

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doi:10.1186/1476-9255-9-23
Cite this article as: Cortés-Vieyra *et al.*: Role of glycogen synthase kinase-3 beta in the inflammatory response caused by bacterial pathogens. *Journal of Inflammation* 2012 **9**:23.

INFECTION AND IMMUNITY, Nov. 2011, p. 4569–4577 Vol. 79, No. 11
0019-9567/11/$12.00 doi:10.1128/IAI.05303-11

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

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Received 25 April 2011/Returned for modification 30 May 2011/Accepted 6 August 2011

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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▽ Published ahead of print on 15 August 2011.

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

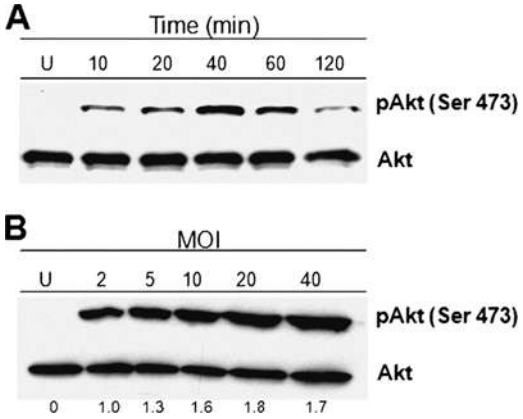

FIG. 1. *S. aureus* activates the phosphorylation of Akt on Ser473 in BEC. (A) Cells were left uninfected (U) or infected with *S. aureus* at an MOI of 20 for 10 to 120 min. (B) Cells were left uninfected (U) or infected with *S. aureus* at MOIs of 2 to 40 for 40 min. After infection, the phosphorylation 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

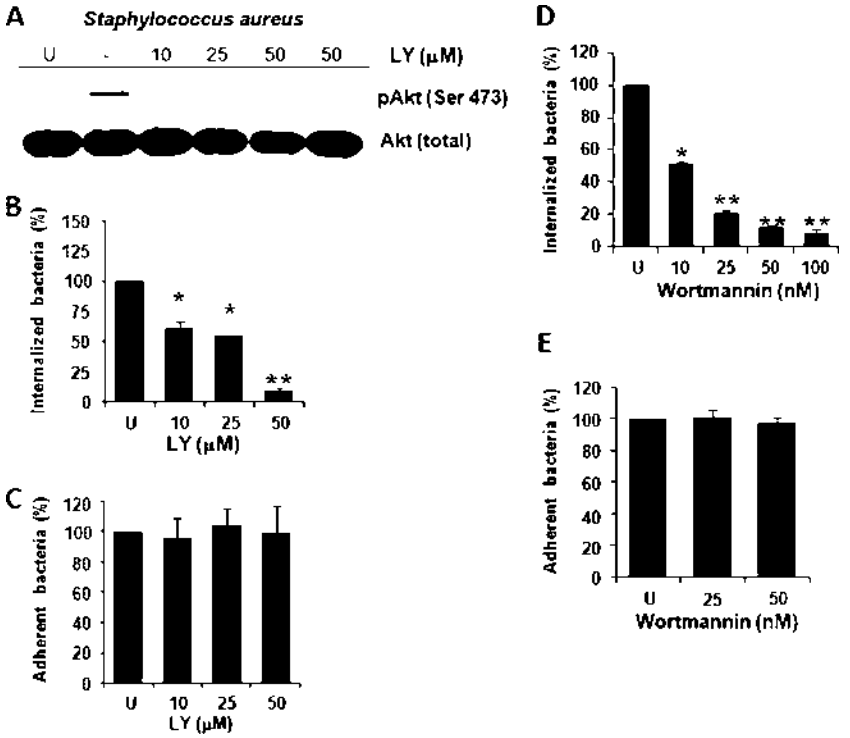

FIG. 2. Inhibition of PI3K activity abolishes phosphorylation of Akt and reduces *S. aureus* internalization by BEC. (A) Cells were left untreated and uninfected (U), were untreated (−), or were treated with 10, 25, or 50 μM LY294002 (LY) for 30 min and then infected with *S. aureus* at an MOI of 20 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.

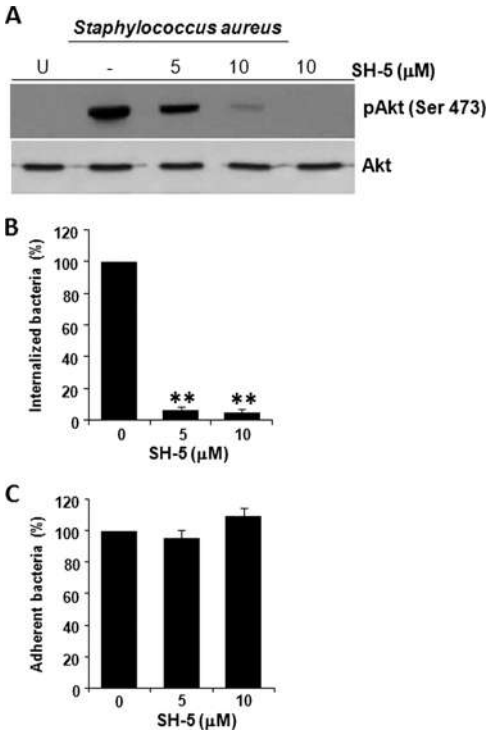

FIG. 3. Inhibition of Akt phosphorylation on Ser473 reduces the *S. aureus* internalization by BEC. (A) Cells were left untreated and uninfected (U), were left untreated (−), or were treated with 5 or 10 μM SH-5 for 30 min and then infected with *S. aureus* at an MOI of 20 for 40 min. A control in which 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).

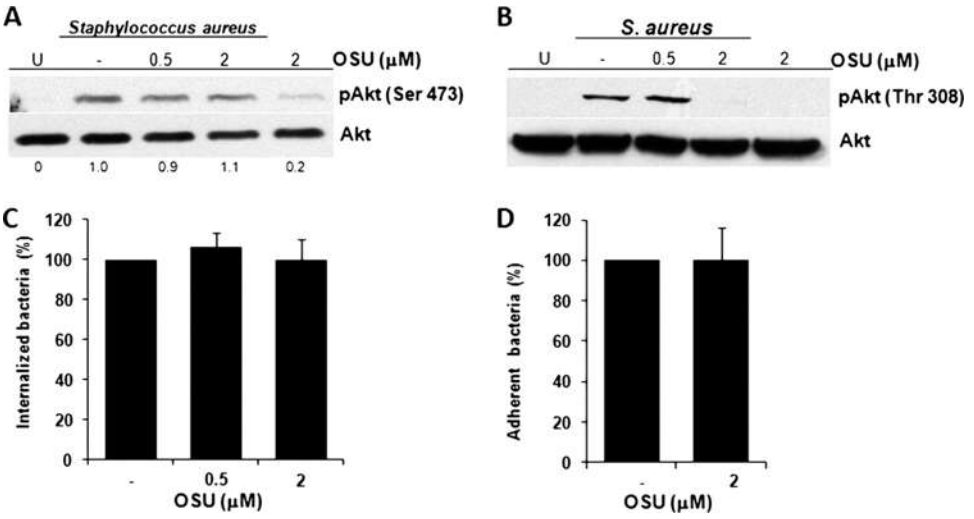

FIG. 4. Inhibition of Akt phosphorylation on Thr308 does not reduce the *S. aureus* internalization or adherence by BEC. (A and B) Detection of Akt phosphorylated on Ser473 or Thr308 in cells left untreated and uninfected (U), in untreated cells (−), or in cells treated with 0.5 or 2 μM OSU-03012 (OSU) 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

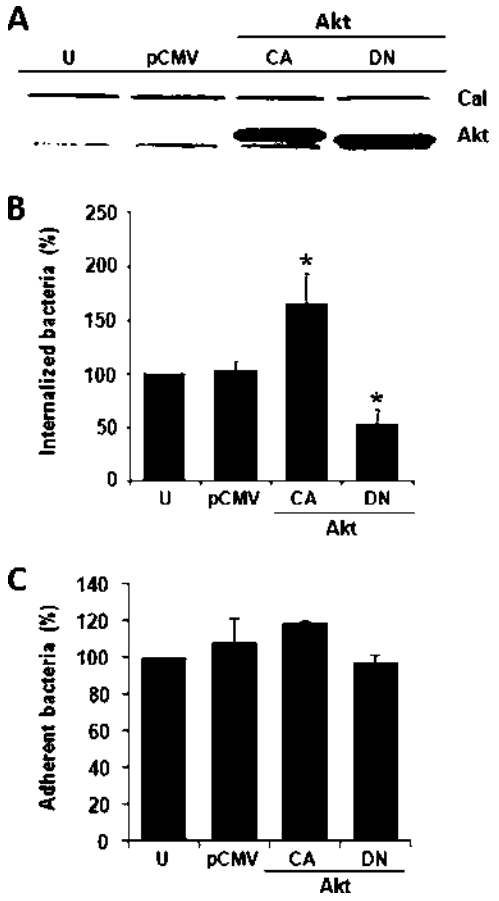

FIG. 5. The number of internalized but not adherent *S. aureus* was altered in BEC transfected with mutants of Akt. (A) Cells were left untransfected (U), were transfected with pCMV5 vector (300 ng) only (pCMV), or were transfected with pCMV5 containing the constitutively active (CA) Akt (5 ng) or the 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

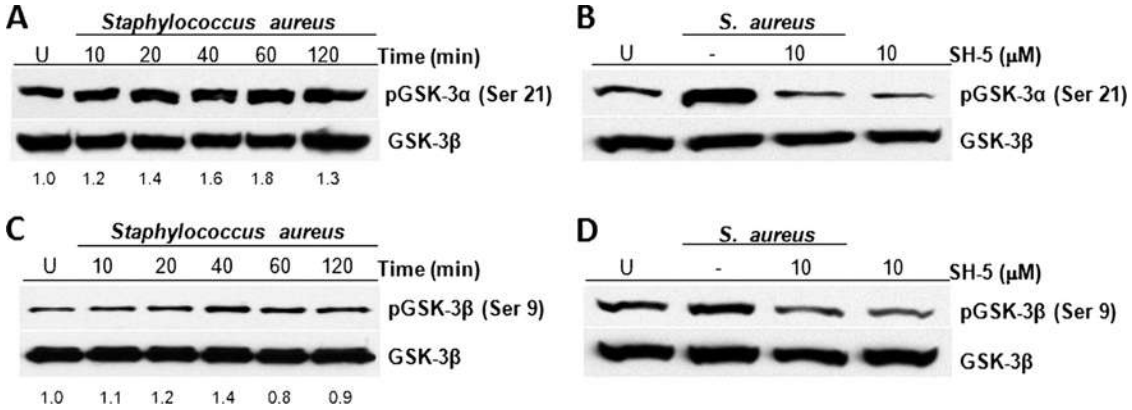

FIG. 6. *S. aureus* activates the phosphorylation of GSK-3α and GSK-3β in BEC through the activation of Akt. (A and C) Cells were left uninfected (U) or were infected with *S. aureus* at an MOI of 20 for 10 to 120 min. (B and D) Cells were left untreated and uninfected (U), were untreated (−), or were 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*.

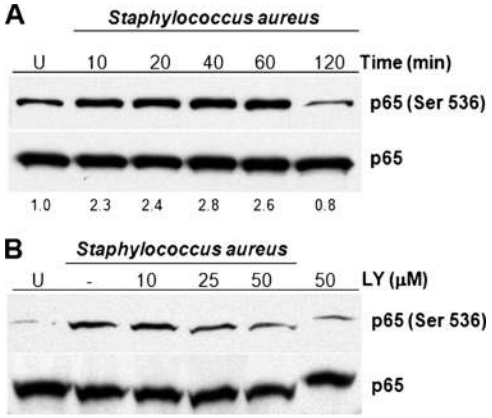

FIG. 7. Inhibition of PI3K activity reduces the level of phosphorylation of NF-κB p65 on Ser536 in BEC infected with *S. aureus*. (A) Cells were left uninfected (U) or were infected with *S. aureus* at an MOI of 20 for 10 to 120 min. (B) Cells were left untreated and uninfected (U), were untreated (−), 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

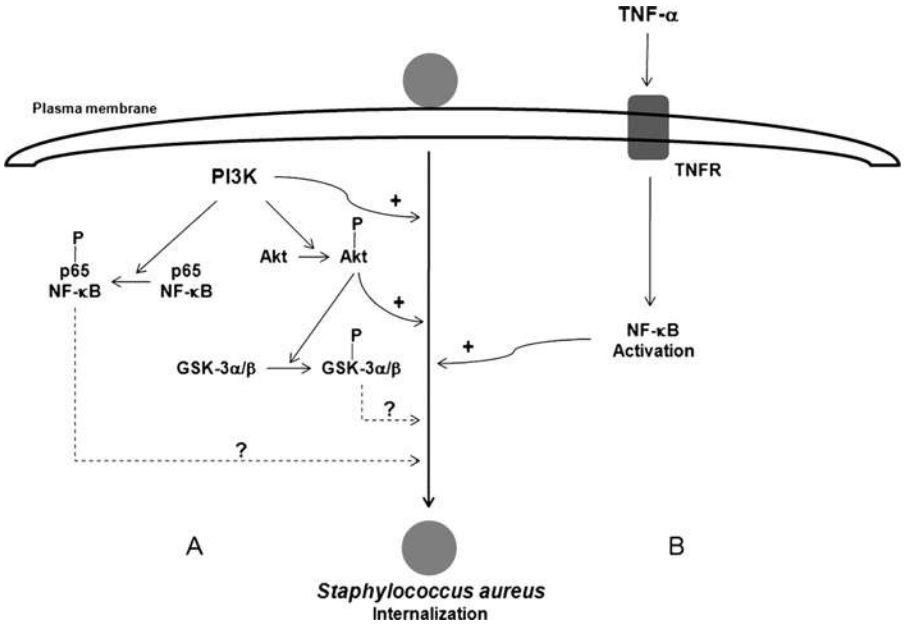

FIG. 8. Schematic diagram summarizing the main results obtained in the present study (A) and in a previous report by Oviedo-Boyso et al. in 2008 (47) (B).

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.

ACKNOWLEDGMENTS

We thank Wanyin Deng and Amit Bahvsar for critical reading and valuable suggestions to the manuscript. We also thank Inimex Pharmaceuticals, Inc., for sharing part of their research on *S. aureus*.

This study was supported by grants from Coordinación de la Investigación Científica, Universidad Michoacana de San Nicolás de Hidalgo, to V.M.B.-A. and by grants from the Canadian Institutes for Health Research (CIHR) to B.B.F. B.B.F. is a CIHR Distinguished Investigator, a Howard Hughes Medical Institute International Research Scholar, and UBC Peter Wall Distinguished Professor. V.M.B.-A. was a recipient of a sabbatical scholarship from Consejo Nacional de Ciencia y Tecnología (CONACYT), Mexico. R.C.-V. and A.H.-M. are receiving doctoral scholarships from CONACYT. H.B.Y. is supported by a CIHR postdoctoral fellowship.

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Editor: J. B. Bliska