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Short communication

Expression and regulation of glycogen synthase kinase 3 in human neutrophils

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ABSTRACT

Glycogen synthase kinase 3 (GSK-3) is a serine/threonine kinase involved in the regulation of cellular processes ranging from glycogen metabolism to cell cycle regulation. Its two known isoforms, α and β, are differentially expressed in tissues throughout the body and exert distinct but often overlapping functions. GSK-3 is typically active in resting cells, inhibition by phosphorylation of Ser21 (GSK-3α) or Ser9 (GSK-3β) being the most common regulatory mechanism. GSK-3 activity has been linked recently with immune system function, yet little is known about the role of this enzyme in neutrophils, the most abundant leukocyte type. In the present study, we examined GSK-3 expression and regulation in human neutrophils. GSK-3α was found to be the predominant isoform, it was constitutively expressed and cell stimulation with different agonists did not alter its expression. Stimulation by fMLP, LPS, GM-CSF, Fcγ receptor engagement, or adenosine A_{2A} receptor engagement all resulted in phosphorylation of Ser21. The use of metabolic inhibitors revealed that combinations of Src kinase, PKC, PI3K/AKT, ERK/RSK and PKA signaling pathways could mediate phosphorylation, depending on the agonist. Neither PLC nor p38 were involved. We conclude that GSK-3α is the main isoform expressed in neutrophils and that many different pathways can converge to inhibit GSK-3α activity via Ser21-phosphorylation. GSK-3α thus might be a hub of cellular regulation.

1. Introduction

Glycogen synthase kinase-3 (GSK-3) is a multifunctional serine/threonine kinase and a key regulator of numerous signaling pathways involved in a wide range of cellular processes from glycogen metabolism to cell cycle regulation and proliferation. Evidence has accumulated in recent years to link GSK-3 with many facets of the innate and adaptive immune systems, making it a plausible target in the treatment of inflammatory and autoimmune diseases (Beurel et al., 2010; Wang et al., 2011). In mammals, GSK-3 exists in two isoforms, α and β, encoded by distinct genes expressing mature polypeptides of respectively 483 and 433 amino acids. The isoforms are structurally similar but functionally distinct. While ablation of the GSK-3β isoform is lethal in mouse embryos (Hoeflich et al., 2000), mice lacking GSK-3α develop normally but display enhanced glucose tolerance, insulin sensitivity and reduced fat mass (MacAulay et al., 2007). GSK-3 is an unusual kinase, in the sense that it is found activated in resting cells, suppressing cell signaling (Sutherland et al., 1993; Woodgett, 1994). In response to extracellular stimuli, it may be inactivated by phosphorylation at its N-terminal serine residue (Ser21 and Ser9 respectively in GSK-3α and GSK-3β) allowing propagation of signal transduction cascades (Sutherland et al., 1993; Eldar-Finkelman et al., 1995). Depending on cell type and nature of the agonist, this deactivation can be mediated by one or several kinases including among others 90 kDa ribosomal protein S6 kinase (Brady et al., 1998; Saito et al., 1994), protein kinase B (Cross et al., 1995), isoforms of protein kinase C (Fang et al., 2002), and cyclic-AMP-dependent protein kinase (Tanji et al., 2002), suggesting that GSK-3 may be a relatively central molecular switch of cellular activation. It is therefore not surprising that dysregulation of GSK-3 has been linked with the progression of many diseases, including diabetes, Alzheimer's disease, bipolar disorder and cancer (Jope and Johnson, 2004; Jope and Roh, 2006; Jope et al., 2007; Patel and Woodgett, 2008).

Polymorphonuclear neutrophils, typically the first and most abundant cell type to migrate to injury sites, play important roles in host defense. These dedicated phagocytes engulf microbial cells or cellular debris into phagosomes which rapidly fuse with the various granule subsets. Engulfed particles are degraded by proteases, phospholipases, cationic peptides, defensins, and cathelicidins introduced into the phagolysosome, and reactive oxygen and reactive nitrogen species are generated to contribute to the

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destruction (Segal, 2005). In addition, neutrophils in general produce an array of mediators of inflammation, including eicosanoids such as leukotriene B_4 and prostaglandin E_2, as well as several cytokines and chemokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-8, and macrophage inflammatory peptides (Nathan, 2006). When their response is properly regulated and agonist-specific, neutrophils play a pivotal role in orchestrating the early stages of host-defense and inflammatory responses.

Surprisingly little is known about GSK-3 function in neutrophils. In the present study, we sought to shed light on this subject by examining isoform expression and regulation using an array of agonists and metabolic inhibitors, and then delineating the intracellular signaling pathways that regulate GSK-3 activity. The results point to a central role for GSK-3α in regulating neutrophil function.

2. Materials and methods

2.1. Reagents

Dextran T-500 was purchased from Pharmacia Biotech (Dorval, Québec, Canada) and Ficoll-Paque from Wisent (St-Bruno, Québec, Canada). Formyl-methionyl-leucyl phenylalanine (fMLP), wortmannin (from Penicillium fumiculosum) and PI3K inhibitor AS605240 (5-(6-quinoxalinylmethylene)-2,4-thiazolidinedione) were obtained from Sigma–Aldrich (St. Louis, MO). H89 and SB203580 were obtained from Biomol Research Laboratories (Plymouth Meeting, PA). Lipopolysaccharide (LPS from *Escherichia coli* 0111:B40), U0126 (1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio) butadiene), Gö6976, PP2 (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo(3,4-d-pyrimidine)), PP3 (4-amino-7-phenylpyrazol(3,4-d-pyrimidine)), p90 ribosomal S6 kinase inhibitor SL0101 (kaempferol-3-O-(3″,4″-di-O-acetyl-α-L-rhamnopyranoside)), and Akt-1/2 inhibitor VIII (1,3-dihydro-1-(1-((4-(6-phenyl-1H-imidazo[4,5-g]quinoxalin-7-yl)phenyl)methyl)-4-piperidinyl)-2H-benzimidazol-2-one) were purchased from Calbiochem (San Diego, CA). Recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) was purchased from PeproTech (Rocky Hill, NJ, USA). Sp-cAMPS-AM (Adenosine-3′,5′-cyclic monophosphorothioate acetoxymethyl ester, Sp-isomer, as well as its inactive Rp-isomer, were purchased from Biolog (Bremen, Germany). CGS-21680 (4-[2-[[6-Amino-9-(*N*-ethyl-β-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl]benzene-propanoic acid) was obtained from Cedarlane (Burlington, Canada).

2.2. Antibodies

Anti-phospho (Ser21/9) GSK-3α/β was purchased from cell signaling technology. Anti-GSK-3α/β (0011-A) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary anti-mouse (#NXA931) and anti-rabbit (#NA934V) antibodies were obtained from Amersham Biosciences (Baie d'Urfé, Québec, Canada).

2.3. Human neutrophil isolation

The Université Laval ethics committee approved all experiments involving human tissues. Informed consent was obtained in writing. Polymorphonuclear leukocytes were isolated as originally described (Boyum, 1968), with modifications (Pouliot et al., 2002). Briefly, venous blood from healthy volunteers was collected in isocitrate anticoagulant solution and centrifuged at 250 × *g* for 10 min. Leukocytes were obtained from the pellet following erythrocyte sedimentation in 2.0% Dextran T-500 (Sigma–Aldrich). Granulocytes were then separated from other leukocytes by centrifugation on a cushion (10 mL) of lymphocyte separation medium (Wisent, St-Bruno, QC, Canada). Contaminating erythrocytes were removed by 15 s of hypotonic lysis. Purified granulocytes (>95% neutrophils, <5% eosinophils) contained less than 0.1% monocytes, as determined by esterase staining. Viability was greater than 98%, based on tryptan blue dye exclusion. The entire procedure was carried out at room temperature under sterile conditions. Neutrophils were re-suspended in Hank's balanced salt solution (HBSS; 37 °C) containing 10 mmol L^{-1} HEPES pH 7.4, 1.6 mmol L^{-1} Ca^{2+}, 10 μg mL^{-1} leupeptin, 10 μg mL^{-1} aprotinin (anti-proteases) and no Mg^{2+}.

Table 1
List of inhibitors used.

Inhibitor	Concentration	Targeted enzyme
Wortmannin (PI3K inh I.)	200 nmol L^{-1}	Phosphoinositide-3-kinase (PI3K)
AS605240 (PI3K inh II.)	10 μmol L^{-1}	PI3K
Inhibitor VIII	10 μmol L^{-1}	Protein kinase B (Akt)
U0126	10 μmol L^{-1}	Extracellular signal-regulated kinase (ERK1/2)
SL0101	10 μmol L^{-1}	Ribosomal S6 kinase (RSK)
U73122	2 μmol L^{-1}	Phospholipase C (PLC)
Gö6976	1 μmol L^{-1}	Protein kinase C (PKC)
SB 203580	10 μmol L^{-1}	p38
PP2	10 μmol L^{-1}	Src kinase
PP3	10 μmol L^{-1}	Inactive PP2 analog
H89	10 μmol L^{-1}	cAMP-dependent protein kinase A (PKA)

2.4. Study of pathways leading to GSK-3 phosphorylation

Neutrophils were pretreated for 60 min with one of the inhibitors included in Table 1. The cells were then stimulated with one of the following agonists: fMLP (100 nmol L^{-1}), GM-CSF (1.4 nmol L^{-1}), LPS (100 ng mL^{-1}), CGS 21680 (1 μmol L^{-1}), Sp-cAMPS-AM (50 μmol L^{-1}), or its inactive isomer Rp-cAMPS-AM (50 μmol L^{-1}). The concentrations used are based on previous studies (Pouliot et al., 1998; Leclerc et al., 2008). For FcγR cross-linking, neutrophils were pre-incubated with monoclonal anti-FcγRIIa (1 μg mL^{-1}) and anti-FcγRIIIb (4 μg mL^{-1}), followed by anti-mouse IgG $F(ab')_2$ fragment (Fc-specific, 12 μg mL^{-1}), as described by Marois et al. (2011). The exposure times ranged from 0 to 240 min (please see Fig. 2 for details). Experiments were conducted at 37 °C.

For Western blotting, reactions were stopped by adding 200 μL of cell suspension to the same volume of 2× Laemmli sample buffer (1× composition: 62.5 mmol L^{-1} Tris–HCl, pH 6.8, 4% SDS, 5% β-mercaptoethanol, 8.5% glycerol, 10 mmol L^{-1} p-nitrophenylphosphate, 0.025% bromophenol blue). The samples were boiled for 7 min, and then stored on ice until loading onto SDS gels.

2.5. Western blot analysis

Proteins were separated by SDS-PAGE on 10% acrylamide gel. Separated proteins were transferred from the gels to polyvinylidene difluoride membrane (Immobilon Membranes, Millipore, Bedford, MA, USA). Blotted membranes were blocked with non-fat milk solution (5% in TBS-Tween) and then kept at 4 °C overnight in contact with primary antibody (1:1000 in TBS Tween) directed against p-GSK-3 or total GSK-3. TBS-Tween: (in deionized water) 25 mmol L^{-1} Tris–HCl, 190 mmol L^{-1} NaCl, 0.15% Tween 20, pH 7.8. The membranes were then rinsed three times in TBS-Tween and incubated with an HRP-labeled secondary antibody for 60 min at 37 °C. A chemiluminescence kit (ECL; Amersham Bioscience Corp., Baie d'Urfé, Quebec, Canada) was used according to the manufacturer's instructions.