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Insulin-like modulation of Akt/FoxO signaling by copper ions is independent of insulin receptor

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ABSTRACT

Copper ions are known to induce insulin-like effects in various cell lines, stimulating the phosphoinositide 3′-kinase (PI3K)/Akt signaling cascade and leading to the phosphorylation of downstream targets, including FoxO transcription factors. The aim of this work was to study the role of insulin- and IGF1-receptors (IR and IGF1R) in insulin-like signaling induced by copper in HepG2 human hepatoma cells. Cells were exposed to Cu(II) at various concentrations for up to 60 min. While Akt and FoxO1a/FoxO3a were strongly phosphorylated in copper- and insulin-treated cells at all time points studied, only faint tyrosine phosphorylation of IR/IGF1R was detected in cells exposed to Cu(II) by either immunoprecipitation/immunoblot or by immunoblotting using phospho-specific antibodies, whereas insulin triggered strong phosphorylation at these sites. Pharmacological inhibition of IR/IGF1R modestly attenuated Cu-induced Akt and FoxO phosphorylation, whereas no attenuation of Cu-induced Akt activation was achieved by siRNA-mediated IR depletion. Cu(II)-induced FoxO1a nuclear exclusion was only slightly impaired by pharmacological inhibition of IR/IGF1R, whereas insulin-induced effects were blunted. In contrast, genistein, a broad-spectrum tyrosine kinase inhibitor, at concentrations not affecting IR/IGF1R, attenuated Cu(II)-induced Akt phosphorylation, pointing to the requirement of tyrosine kinases other than IR/IGF1R for Cu(II)-induced signaling.

Introduction

Copper ions may interfere with crucial signaling pathways in mammalian cells, resulting in potentially adverse outcomes such as altered gene expression and proliferation [1]. Exposure to copper ions has previously been demonstrated to modulate stress-responsive pathways, such as mitogen-activated protein kinase pathways, and to affect transcription factors such as AP-1 or NF-κB [2–4]. Likewise, insulin signaling in hepatoma cells was shown to be imitated by exposure to copper ions in the absence of insulin: Cu(II) elicited the stimulation of known signaling events downstream of the insulin receptor (IR),[2] e.g. the phosphoinositide 3′-kinase (PI3K)-dependent phosphorylation and activation of the serine/threonine kinase Akt [5]. Moreover, exposure to Cu(II) caused phosphorylation of glycogen synthase kinase 3 (GSK3) as well as of transcription factors of the forkhead box, class O (FoxO) family [6], both of which are known substrates of Akt. Akt-dependent phosphorylation of FoxO proteins leads to their inactivation and nuclear exclusion [7], which was indeed observed in cells expressing EGFP-tagged FoxO1a exposed to Cu(II) [6]. Insulin triggers these same effects, leading to inactivation of FoxOs and attenuation of FoxO-dependent expression of genes, such as those of gluconeogenesis enzymes like the catalytic subunit of glucose 6-phosphatase (G6Pase) [8] or of plasma proteins like selenoprotein P [9,10] and the major copper protein in human plasma, ceruloplasmin [11].

It is currently unclear how Cu(II) induces these described insulin-like signaling effects and what the molecular targets of copper ions in cells are that result in the modulation of signaling events. The molecular targets would be of interest for a definition of the mode of action of copper ions.

Several reasons point to the insulin receptor as an obvious potential target: (i) copper ions stimulate insulin-like signaling (i.e. activation of PI3K/Akt to a comparable extent, followed by comparable FoxO phosphorylation). (ii) Reactive oxygen species (ROS) and several stressful agents, such as quinones, alkylating

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[2] *Abbreviations used:* IR, insulin receptor; PI3K, phosphoinositide 3′-kinase; PTPases, protein tyrosine phosphatases; GSK3, glycogen synthase kinase 3; FoxO, forkhead box, class O; G6Pase, glucose 6-phosphatase; ROS, reactive oxygen species; RTK, receptor tyrosine kinases; IGF1R, insulin-like growth factor-1 receptor; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; HRP, horseradish peroxidase; SDS, sodium dodecyl sulfate; BCA, bicinchoninic acid; Ins, insulin; IB, immunoblotting.

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agents and ultraviolet radiation, have previously been shown to trigger activation of receptor tyrosine kinases (RTK) [12–18]. (iii) Copper ions are redox-active entities potentially triggering ROS formation that could elicit RTK activation [1]. (iv) Insulin receptor is a RTK whose activation may be modulated by ROS, such as hydrogen peroxide [19].

Therefore, we set out to investigate whether copper imitates insulin by acting on the insulin receptor (IR) and the related insulin-like growth factor-1 receptor (IGF1R), thereby causing stimulation of downstream signaling. We here demonstrate that copper ions strongly stimulate insulin-like signaling in a fashion independent of IR and IGF1R.

Materials and methods

Reagents and plasmids

All chemicals were from Sigma–Aldrich (Oakville, ON, Canada), if not mentioned otherwise. The insulin receptor tyrosine kinase inhibitor, linsitinib (OSI-906), was from Selleckchem (Burlington, ON, Canada) and the general tyrosine kinase inhibitor, genistein, was from LKT Laboratories (St. Paul, MN, USA). Inhibitors were held as stock solutions in DMSO and diluted into serum-free cell culture media for use. The FoxO1a-EGFP expression plasmid [20] was kindly provided by Dr. Andreas Barthel (Endokrinologikum, Bochum, Germany).

Cell culture and fluorescence microscopy analyses

HepG2 human hepatoma cells were purchased from the German collection of microorganisms and cell cultures (DSMZ, Braunschweig, Germany) and were held in Dulbecco's modified Eagle's medium (DMEM, with 4500 mg/l glucose and 2 mM glutamine, Sigma–Aldrich) supplemented with 10% (v/v) fetal calf serum (FCS) (PAA, Etobicoke, ON, Canada), 1% penicillin/streptomycin (Life Technologies, Burlington, ON, Canada) and 1% non-essential amino acids (Sigma–Aldrich), at 37 °C in a humidified atmosphere with 5% (v/v) CO_2.

Cell viability was assessed using neutral red uptake. HepG2 cells were grown to 60–70% confluence in 24 well-plates, treated with copper for 1 h, washed with PBS and subsequently held in serum-free medium for another 24 h. Cells were then incubated for 2 h with neutral red solution (Sigma–Aldrich; 4 ml of 3.3 g neutral red/l PBS in 100 ml serum-free DMEM). Cells were washed twice with PBS, followed by extraction of neutral red from viable cells by incubation with an ethanol/water/acetic acid (50:49:1, v/v/v) solution under gentle shaking at room temperature for 2 h. The dye-containing solution was then centrifuged and the absorbance of the cell-free supernatant was measured at 550 nm (with 405 nm as reference).

For treatment of cells with copper ions or other agents, HepG2 cells were grown to near confluence, held in serum-free medium for 24 h, followed by the respective treatment. If indicated, cells were preincubated with an inhibitor (genistein or linsitinib) for 60 min prior to the respective treatment with copper or insulin, which was in the continued presence of the inhibitor. DMSO was used as vehicle control. For exposure to copper ions or insulin, cells were washed once with PBS and incubated for 30–60 min in the presence of various concentrations of Cu(II) sulfate or insulin diluted into Hanks' balanced salt solution (HBSS, Sigma–Aldrich). For exposure to hydrogen peroxide, cells were washed once with PBS and incubated for 30 min in the presence of various concentrations of H_2O_2 in HBSS.

For analysis of FoxO1a-EGFP subcellular localization by fluorescence microscopy, HepG2 cells were grown to approximately 60% confluence in 9 cm^2 cell culture dishes and transfected with 3 μg FoxO1a-EGFP expression plasmid in serum-free DMEM for 24 h using Nanofectin transfection reagent as described by the manufacturer (PAA). Following transfection, cells were washed with PBS, then incubated in the presence of copper(II) sulfate or insulin for 60 min. Where applicable, cells were incubated in the presence of linsitinib as described above. Fluorescence microscopy of cells expressing EGFP-tagged FoxO1a was performed on an Axiovert Observer.A1 fluorescence microscope (Zeiss, Göttingen, Germany) coupled to an AxioCam MRm camera (Zeiss) using suitable filters. Analysis of EGFP-positive cells was done by counting and separating cells into three categories with respect to the major localization of FoxO1a-EGFP (nuclear, cytosolic or both nuclear/cytosolic). For each determination, approximately 200 cells were counted.

Western blotting

For analysis of IR, Akt, FoxO1a, FoxO3a and beta-actin levels or modifications, cells were lysed in 2× SDS–PAGE buffer [125 mM Tris/HCl, 4% (w/v) SDS, 20% (w/v) glycerol, 100 mM dithiothreitol and 0.02% (w/v) bromophenol blue, pH 6.8], followed by brief sonication. Samples were applied to SDS–polyacrylamide gels of 10% (w/v) acrylamide, electrophoretically separated and blotted onto nitrocellulose membranes. Membranes were blocked in 5% non-fat dry milk in Tris–buffered saline containing 0.1% (v/v) Tween-20 (TBST) and probed with primary antibody overnight at 4 °C, followed by washing, incubation with secondary antibody [horseradish peroxidase (HRP)-conjugated anti-rabbit IgG or HRP-coupled anti-mouse IgG, GE-Healthcare (Piscataway, USA)] and detection using chemiluminescent HRP substrate. The following primary antibodies were used: anti-phospho-IR-β/IGF1R-β (Y1150/1151)/(Y1135/1136), anti-total-IR-β, anti-phospho-Akt (Ser473), anti-total-Akt, anti-phospho-FoxO1a/FoxO3a (T24/T32), anti total FoxO1a (all from Cell Signaling Technology, Danvers, MA, USA), anti-β-actin (Sigma–Aldrich) and GAPDH (Millipore, Billerica, MA, USA). All primary antibody incubations were in 5% (w/v) BSA in TBST, and all secondary antibody incubations were in 5% (w/v) non-fat dry milk in TBST. General tyrosine phosphorylation was detected using a mouse monoclonal anti-phosphotyrosine antibody, "4G10 Platinum", which is a mixture of two anti-pY antibody clones, 4G10 and PY20 (Millipore).

Test for protein tyrosine phosphatase (PTPase) inhibition

Tyrosine phosphorylation of IR/IGF1R was stimulated by incubation of cells with insulin (100 nM) for 30 min. Insulin treatment was in the absence or presence of the known phosphatase inhibitor, vanadate (sodium orthovanadate at a final concentration of 1 mM) or the compound(s) of interest whose PTPase inhibitory activity was being investigated. To prevent any further autophosphorylation, cells were treated with 10 μM linsitinib. After 5 min, medium was quickly removed, cells washed with PBS and lysed in 2× SDS–PAGE sample buffer, followed by detection of phospho-IR-β/IGF1R-β (Y1150/1151)/(Y1135/1136) and β-actin by Western blotting.

Immunoprecipitation

For immunoprecipitation, cells were grown to 70% confluence in 58 cm^2 culture dishes as described above. After treatment, cells were washed once with PBS and lysed in 500 μl RIPA buffer (1% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl, 50 mM Tris–HCl (pH 8), 5 mM sodium fluoride, 1 mM sodium vanadate, 1 mM β-glycerophosphate, 2.5 mM sodium pyrophosphate, 1 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA and 1 mM DTT), followed by brief sonication. Insoluble material was removed by centrifugation