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Nickel and copper ion-induced stress signaling in human hepatoma cells: analysis of phosphoinositide 3′-kinase/Akt signaling

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Abstract Nickel compounds may act as carcinogens, affecting both initiation and promotion stages of carcinogenesis due, in large parts, to their capability of inducing DNA damage and of modulating cellular signaling cascades known to affect cellular proliferation, respectively. We have previously demonstrated that the phosphoinositide 3-kinase (PI3K)/Akt signaling cascade is stimulated in cells exposed to copper ions, resulting in phosphorylation and nuclear exclusion of FoxO transcription factors. Here, human hepatoma cells were exposed to nickel or copper ions, followed by comparative analysis of PI3K/Akt-dependent signaling. Exposure of hepatoma cells to copper ions resulted in extensive oxidation of cellular glutathione, while no such effect was detected with nickel ions. Similarly, copper ions were more than 100-fold more toxic to cells than nickel, as deduced from analyses of colony forming abilities. Despite this lack of oxidative and cytotoxic action, exposure of hepatoma cells to Ni^{2+} resulted in a significant activation of Akt that was abrogated by inhibitors of PI3K. Interestingly, activation of Akt—although coincident with a phosphorylation of Akt substrates, such as glycogen synthase kinase-3—did not result in significant nuclear exclusion of FoxO1a. In line with this finding, no significant modulation of the activity of a FoxO-responsive promoter construct was observed in cells exposed to nickel ions. In summary, exposure of HepG2 human hepatoma cells to nickel ions results in stimulation of the Ser/Thr kinase Akt in a PI3K-dependent fashion, activation most likely being independent of oxidative processes. In sharp contrast to copper ions, nickel-induced Akt activation is not propagated further downstream to FoxO-dependent signaling beyond the phosphorylation of FoxO1a and 3a.

Keywords Nickel · Copper · Heavy metal signaling · Insulin signaling · PI3-kinase · FoxO transcription factors

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Introduction

Nickel is a well-established carcinogen to humans. Mechanisms discussed as underlying nickel-induced carcinogenesis not only include the induction of damage to biomolecules such as DNA and the impairment of DNA repair, but also the stimulation of signaling pathways known to be involved in cancer development (Beyersmann and Hartwig 2008; Costa et al. 2005; Denkhaus and Salnikow 2002; Hartwig et al. 2002).

Several of these signaling cascades modulated in cells exposed to Ni^{2+} are stress-responsive cascades

the activation of which results in modulation of transcriptional regulators such as hypoxia-induced factor (HIF)-1α (Maxwell and Salnikow 2004), NF-κB (Goebeler et al. 1995) or AP-1 (Zhang et al. 2007). Nickel-induced modulation of HIF-1α activity was recently demonstrated to occur via phosphoinositide 3′-kinases (PI3K) and the Ser/Thr kinase Akt (Li et al. 2004). The group of transcriptional regulators modulated by Akt also include transcription factors of the FoxO (forkhead box, class O) family, a group of proteins involved in regulation of stress response, glucose metabolism, proliferation and apoptosis (Greer and Brunet 2005), i.e., cellular processes known to affect progression of carcinogenesis.

Of the four FoxO family members in humans, FoxO1a (FKHR), FoxO3a (FKHR-L1) and FoxO4 (AFX) are expressed ubiquitously, whereas FoxO6 is mainly found in developing brain (see Greer and Brunet 2005). Target genes of FoxO proteins involved in conferring stress resistance in response to FoxO activation include those of regulators of cell cycle progression ($p27^{kip}$ Medema et al. 2000), proteins associated with DNA repair (Tran et al. 2002), or the antioxidant enzymes, manganese-superoxide dismutase (Kops et al. 2002), catalase (Nemoto and Finkel 2002) and selenoprotein P (Walter et al. 2008). FoxO proteins are phosphorylated by Akt, which is activated via PI3K by various growth factors and hormones, including insulin (for review, see Barthel et al. 2005; Barthel and Klotz 2005). Insulin- and Akt-dependent phosphorylation results in inactivation and translocation of FoxO into the cytoplasm (Greer and Brunet 2005).

We have recently demonstrated that similar effects are elicited by exposure of cells to stressful stimuli (Barthel and Klotz 2005), such as heavy metal ions: copper and zinc ions strongly stimulate Akt phosphorylation via PI3K in a host of cell types, including human fibroblasts and hepatoma cells, resulting in the phosphorylation, nuclear translocation and transcriptional inactivation of FoxO transcription factors (Barthel et al. 2007; Ostrakhovitch et al. 2002; Walter et al. 2006). As a consequence, the cellular response to these metal ions can be regarded "insulin-mimetic".

Hence, the exposure of cells to heavy metal ions in doses that are not acutely toxic may affect cellular signaling networks regulating proliferation, apoptosis and metabolic processes, resulting in an interference of metals with, and a metal ion-induced modulation of, cellular metabolism.

Here, we aimed at analyzing the capability of nickel ions of modulating PI3K/Akt signaling and the activity of FoxO transcription factors. We demonstrate that exposure of human hepatoma cells to nickel ions results in stimulation of the PI3K/Akt cascade already in non-toxic concentrations but does not appear to significantly affect FoxO-dependent signaling. We compare these effects with those of copper ions that were previously established as potent activators of Akt signaling.

Materials and methods

Reagents and plasmids

All chemicals were from Sigma (Steinheim, Germany) or Merck (Darmstadt, Germany), if not mentioned otherwise. Inhibitors of PI3K, LY294002 or wortmannin, were from Merck/Calbiochem (San Diego, CA, USA) and were held as stock solutions in DMSO and diluted into cell culture media for use. Cells were preincubated with the inhibitors for 30 min prior to exposure to metal ions, which was in the continued presence of the inhibitors. DMSO was used as vehicle control. The glucose 6-phosphatase (G6Pase) promoter construct, G6Pase-luc (−1227/+57), as well as FoxO1a expression plasmids (Guo et al. 1999; Schmoll et al. 2000) were a kind gift of Dr. Dieter Schmoll (Sanofi-Aventis, Frankfurt, Germany), the FoxO1a-EGFP expression plasmid (Kortylewski et al. 2003) was kindly provided by Dr. Andreas Barthel (Endokrinologikum, Bochum, Germany).

Cell culture and fluorescence microscopy analyses

HepG2 human hepatoma cells were a kind gift of Dr. Johannes Bode (Heinrich-Heine-Universität Düsseldorf) and were held in Dulbecco's modified Eagle's medium (DMEM, with 4.5 g/l glucose, PAA, Pasching, Austria) supplemented with 10% (v/v) fetal calf serum (PAA), 2 mM glutamax (Invitrogen, Karlsuhe, Germany), non-essential amino acids (PAA) and penicillin/streptomycin (PAA), at 37°C in a humidified atmosphere with 5% (v/v) CO_2.

HepG2 cells were grown to near confluence prior to exposure to metal ions. Cells were then held in serum-free medium for another 24 h prior to treatment, washed once with PBS and incubated in the presence of $CuSO_4$, $NiSO_4$, $NiCl_2$ or insulin diluted into Hanks' balanced salt solution (HBSS, Sigma). HeLa human cervix carcinoma cells (European Collection of Cell Cultures, Salisbury, UK) and A431 human squamous carcinoma cells (kindly provided by Prof. Peter Brenneisen, Düsseldorf) were cultured in DMEM (low glucose, PAA), supplemented with 10% (v/v) fetal calf serum, 2 mM glutamax, and penicillin/streptomycin, at 37°C in a humidified atmosphere with 5% (v/v) CO_2.

For fluorescence microscopy analyses, cells were grown to approximately 70% confluence in 9 cm^2 cell culture dishes and were transfected with 3 μg of FoxO1a-EGFP plasmids using the Nanofectin transfection kit (PAA) according to the manufacturer's instructions. Fluorescence microscopy of cells expressing EGFP-tagged FoxO was performed on an Axiovert observer D1 fluorescence microscope (Zeiss, Göttingen, Germany). 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).

Cell viability and colony formation

Cell viabilities after exposure to copper or nickel salts were assessed by staining viable cells with neutral red solution (3.3 g/l neutral red in PBS) for 1 h. Cells were washed carefully with PBS twice, and neutral red that had been incorporated by cells was extracted from cells with ethanol:water:acetic acid (50:49:1 v/v/v) for at least 4 h at 4°C prior to analysis of neutral red content in extracts at 405 nm (reference wavelength: 550 nm). For analysis of the capability of cells to attach to cell culture dishes and proliferate (analysis of colony forming ability), cells were trypsinized from cell culture dishes after exposure to copper or nickel salts, counted and seeded at dilutions ranging from 1:10 to 1:1,000. Cells were grown under the above-mentioned cell culture conditions for 11 days with one change of media after 6 days. Cells were washed with PBS, followed by staining with crystal violet solution [0.2% (w/v) in 20% (v/v) ethanol] for 30–90 min at room temperature. The staining solution was discarded and cell culture dishes washed with water, air dried and stained colonies counted.

Luciferase reporter gene assays

HepG2 cells were grown to approximately 60% confluency in 9 cm^2 cell culture dishes and transfected using Polyfect reagent (Qiagen, Hilden, Germany). G6Pase-luc (0.75 μg) was cotransfected with 0.1 μg of renilla luciferase control plasmid (pRL-SV40, Promega, Mannheim, Germany) and 0.75 μg of FoxO1a expression plasmids. All transfections were performed in serum-free medium (containing glutamax, penicillin/streptomycin and non-essential amino acids). Cells were exposed to metal ions or insulin 24 h after transfection. For the respective treatment, cells were washed once with PBS and held in serum-free medium containing Cu^{2+} (10 μM), Ni^{2+} (100 μM) or insulin (100 nM) for 18 h prior to determination of luciferase activities using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions.

Determination of glutathione and glutathione disulfide

Gluathione (GSH) and glutathione disulfide (GSSG) were determined enzymatically according to Anderson (1985) with minor modifications (Abdelmohsen et al. 2003). Briefly, cells on 79 cm^2 cell culture dishes were lysed by scraping them in 500 μl of ice-cold HCl (10 mM) followed by one freeze/thaw cycle, brief sonication on ice, and centrifugation at 20,000*g* for 10 min to remove cell debris. Aliquots of the supernatants were kept for protein determination in a bicinchoninic acid (BCA)-based protein assay (Pierce/Thermo Scientific, Bonn, Germany). For GSH/GSSG determination, protein was precipitated from the supernatant with 5% (w/v; final concentration) 5-sulfosalicylic acid on ice. Samples were vortexed and centrifuged at 20,000*g* for 10 min. Total glutathione (GSH plus GSSG) and, after blocking thiols with 2-vinylpyridine, GSSG were determined from the supernatant using 5,5′-dithionitrobenzoic acid in the presence of NADPH and glutathione reductase (Anderson 1985).

Western blotting

For analysis of Akt, FoxO1a, FoxO3a, GSK-3, GAPDH levels or modifications, cells were lysed in 2× SDS–PAGE buffer [125 mM Tris/HCl, 4% (w/v) SDS, 20% glycerol, 100 mM dithiothreitol and 0.02% (w/v) bromphenol blue, pH 6.8] after treatment, followed by brief sonication. Samples were applied to SDS–polyacrylamide gels of 10% (w/v) acrylamide, followed by electrophoresis and blotting onto PVDF or nitrocellulose membranes. Immunodetection was performed using the following antibodies: anti-phospho-Akt (S473), anti-phospho-Akt (T308), anti-Akt, anti-phospho-GSK-3 α/β (S21/S9), anti-phospho-FoxO1a/FoxO3a (T24/T32) rabbit polyclonal antibodies were from Cell Signaling Technology (Danvers, MA, USA). Anti-phospho-FoxO3a (T32) and anti-GAPDH mouse monoclonal antibodies were from Millipore (Billerica, MA, USA). A rabbit polyclonal anti-FoxO1a antibody was generated and used as described previously (Barthel et al. 2001; Walter et al. 2006). Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and anti-mouse IgG secondary antibodies were from Dianova (Hamburg, Germany) and Amersham (Munich, Germany), respectively.

All antibody incubations were in 5% (w/v) non-fat dry milk in Tris-buffered saline containing 0.1% (v/v) Tween-20 (TBST), except for detection of phospho-FoxO1a/FoxO3a (T24/T32), which was performed in 5% (w/v) BSA in TBST.

Results and discussion

Nickel and copper ions: oxidative and cytotoxic action

Both nickel and copper ions are well known to be capable of generating ROS intracellularly (for a recent overview, see Beyersmann and Hartwig 2008), resulting in oxidative damage of biomolecules, including—at toxic doses of the metals—DNA (Dally and Hartwig 1997; Schwerdtle et al. 2007). Indeed, cellular glutathione was extensively oxidized to GSSG in cells exposed to copper ions at doses that, although not acutely (i.e., within 1 h following commencement of exposure) cytotoxic, resulted in a severe loss of cell viability (detected 24 post-exposure) and colony forming ability (Fig. 1). Interstingly, nickel ions emerged as neither causing GSSG formation (Fig. 1a) nor toxic to human hepatoma cells at concentrations of up to 1 mM (Fig. 1b, c).

Nickel ion-induced Akt activation

Despite the lack of toxicity of Ni^{2+} in concentrations up to 1,000 μM, nickel ions were capable of inducing Akt activation in HepG2 human hepatoma cells exposed to 10, 50 or 100 μM of $NiSO_4$ dissolved in Hanks' balanced salt solution (HBSS) (Fig. 2a). Although copper ions much more potently stimulate Akt already at 10 μM, the fact that Ni^{2+} stimulates this signaling cascade does have mechanistical implications: it is highly unlikely that nickel-induced formation of reactive oxygen species (ROS) is a prerequisite for its effect on Akt (no detectable oxidation of cellular glutathione, Fig. 1a), which is in line with previous findings on Cu^{2+}- or Zn^{2+}-induced stimulation of Akt that were both suggested to be independent of the formation of ROS (Barthel et al. 2007; Ostrakhovitch et al. 2002). These findings are in line also with reports on the potentially detrimental interaction of nickel ions with protective cellular enzymes even at non-toxic concentrations, as demonstrated for the DNA repair machinery in HeLa cells (Dally and Hartwig 1997).

To confirm that the activation of Akt induced by $NiSO_4$ is due to the metal cation, we examined the effect of $NiCl_2$, which was equally effective in inducing Akt phosphorylation (data not shown). Enhanced Akt phosphorylation was seen already after 5 min of treatment, with most prominent activation visible only after 15–30 min of exposure due to initially high basal Akt phosphorylation following change of incubation media to HBSS (Fig. 2b). Thr-308, a second residue in addition to Ser-473 critical for Akt to be fully activated, was also phosphorylated in Ni^{2+}-treated cells (Fig. 2c).

We have recently demonstrated that exposure of mammalian cells to other heavy metal ions results in stimulation of the PI3K/Akt cascade, the most potent activators of the cascade being $Cu^{2+} > Zn^{2+} > Cd^{2+}$ in various cell types (Barthel et al. 2007; Walter et al. 2006). Different from these ions, no stimulation of Akt was found with Mn^{2+}, Fe^{2+}, Co^{2+}, Pb^{2+} in the cell types tested (Barthel et al. 2007; Walter et al. 2006). The strong Cu^{2+}-induced activation of Akt

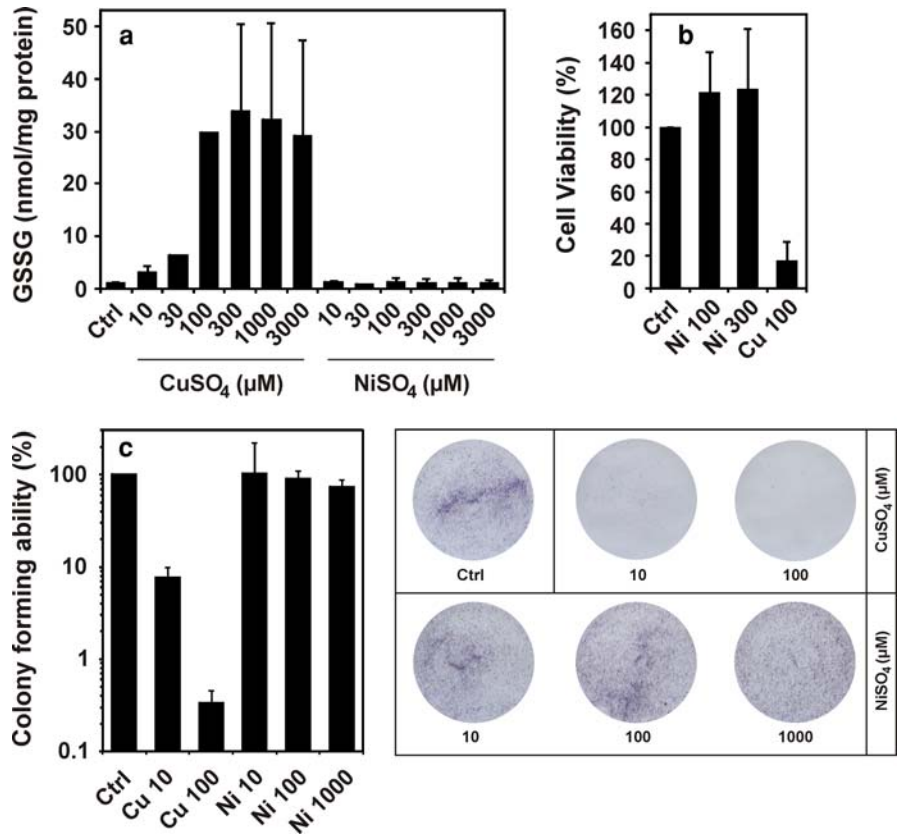

Fig. 1 Glutathione oxidation in cells exposed to nickel and copper ions, viability and clonal survival. HepG2 human hepatoma cells were grown to near confluence, then held in serum-free medium for 18 h, washed with PBS and exposed to the given concentrations of $NiSO_4$ or $CuSO_4$ in Hanks' balanced salt solution (HBSS) for 60 min. **a** Glutathione disulfide levels were analysed in cells immediately following 60 min of exposure to metal ions. Data are given as means of three (30 μM Ni/Cu and 100 μM Cu:$n = 2$) independent experiments ± SD. **b** Viabilities of hepatoma cells were analysed by neutral red staining of cells that were exposed to Ni or Cu salts (100 or 300 μM) for 60 min, washed and held in serum-free cell culture medium for another 24 h. Data are means ± SD ($n = 3$). **c** Hepatoma cells exposed to Ni or Cu salts (10, 100 or 1,000 μM) for 60 min were trypsinized, counted, replated in several dilutions and cultured for 11 days. Hepatoma cell clones were then stained with crystal violet and counted. Pictures of representative culture dishes are shown on the *right*. Relative numbers of developing clones are given as means ± SEM ($n = 3$)

was also detected in HeLa cervix carcinoma and A431 squamous carcinoma cells (Fig. 2d). Interestingly, however, and for reasons yet unknown, Akt activation upon exposure to Ni^{2+} was detected exclusively in HepG2 cells—Akt phosphorylation was seen neither in HeLa nor A431 cells (Fig. 2d). Insulin signaling was intact in these cells, as deduced from the insulin-induced stimulation of Akt phosphorylation, which was as strong as that induced by Cu^{2+} (Fig. 2d). It is therefore unlikely that deficiencies in the insulin signaling cascade leading to Akt activation might be the reason for the activation of Akt by Ni^{2+} apparently being specific to HepG2 cells.

As expected for an insulin-mimetic activator of Akt, the stimulation of Akt phosphorylation by Ni^{2+} was via PI3K: LY294002 (Fig. 3) and wortmannin (data not shown), two structurally unrelated PI3K inhibitors, abrogated both nickel and copper ion-induced Akt activation.

Phosphorylation of Akt substrates in cells exposed to nickel ions

Glycogen synthase kinase-3 (GSK3) is a substrate of Akt in vivo and in vitro. Phosphorylation by Akt inhibits the enzyme and its action on downstream targets such as glycogen synthase or β-catenin (Doble

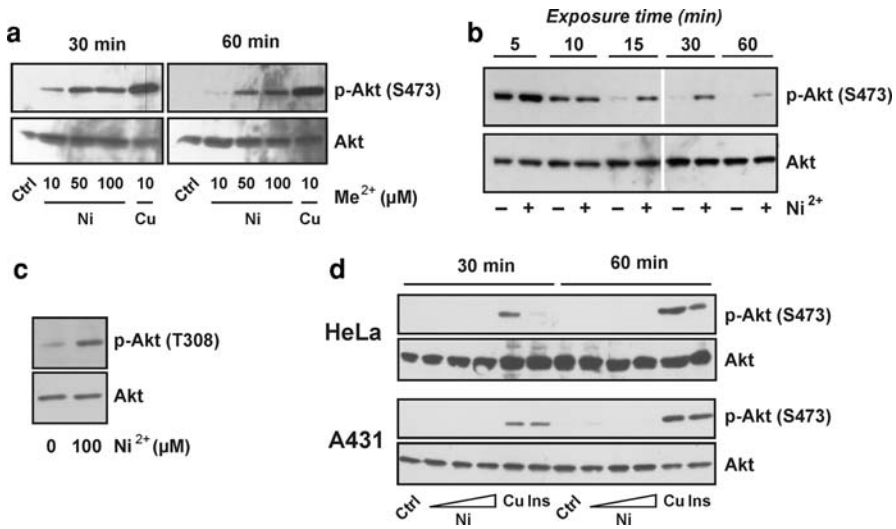

Fig. 2 Phosphorylation of Akt in cells exposed to nickel and copper ions. **a** HepG2 human hepatoma cells were grown to near confluence, then held in serum-free medium for 18 h, washed with PBS and exposed to the given concentrations of $NiSO_4$ or $CuSO_4$ in Hanks' balanced salt solution (HBSS) for 30 or 60 min. Akt phosphorylation at Ser-473 was analyzed by Western blotting and immunodetection using a phospho-specific antibody. **b** HepG2 cells were grown as above and exposed to $NiSO_4$ (100 μM) for the indicated times prior to lysis and Western blotting analysis for phosphorylation of Akt. **c** HepG2 cells were exposed to $NiSO_4$ (100 μM) for 15 min, followed by analysis of Akt phosphorylation at Thr-308. **d** Analysis of Akt phosphorylation in HeLa human cervix carcinoma and A431 human squamous carcinoma cells were treated with $NiSO_4$ (10, 50, and 100 μM), $CuSO_4$ (10 μM) or insulin (100 nM) as in (**a**) without prior incubation in serum-free media. The experiments shown are representative of two (**c**, **d**) and three (**a**, **b**) independent experiments with similar results

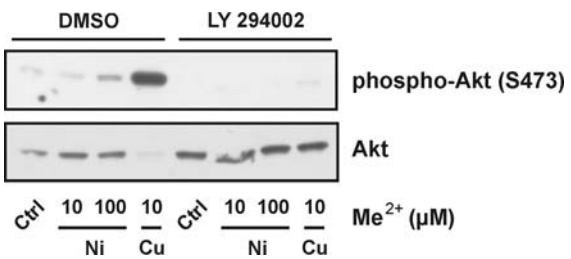

Fig. 3 Activation of Akt by nickel and copper ions is via phosphoinositide 3′-kinase (PI3K). HepG2 human hepatoma cells were grown to near confluence. Cells were held in serum-free medium for 18 h, followed by incubation in the presence of the PI3K inhibitor LY294002 (20 μM) for 30 min prior to the addition of $CuSO_4$ or $NiSO_4$ and incubation for another 30 min in the continued presence of the inhibitor. Dimethyl sulfoxide (DMSO) was used as solvent control. The experiment shown is representative of three independent experiments with identical results

and Woodgett 2003). To further verify the functional significance of Akt phosphorylation in cells exposed to nickel ions, we examined whether GSK3 also became phosphorylated following treatment. Using an antibody specific for the phosphorylated forms of GSK3, we observed an increase in the amounts of phosphorylated GSK3-α and GSK3-β after treatment. The time course of induction of GSK3 phosphorylation was similar to that of Akt phosphorylation (Fig. 4a). As in the case of Akt phosphorylation, both Cu^{2+} and insulin-induced GSK3 phosphorylation was much stronger than that induced by nickel ions (Fig. 4a). Further Akt substrates include transcription factors of the FoxO family (see above). Indeed, both FoxO1a and FoxO3a were phosphorylated at Thr-24 and Thr-32, respectively, in insulin-treated cells. Similarly, Cu^{2+} caused a strong phosphorylation in these positions. However, only a slight and hardly detectable phosphorylation was induced in HepG2 cells by exposure to Ni^{2+} (Fig. 4b), despite a significant nickel-induced Akt phosphorylation. Yet nickel-induced Akt activation is much less potent than phosphorylation induced by copper ions or insulin and appears to be too weak to significantly modulate the extent of FoxO phosphorylation.

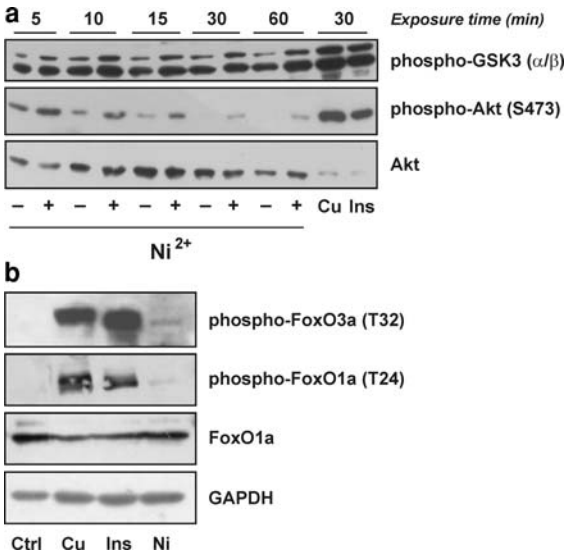

Fig. 4 Nickel and copper ion-induced phosphorylation of Akt substrates. HepG2 cells were grown to near confluence, held in serum free medium for 18 h, washed with PBS and exposed to $CuSO_4$ (10 μM), insulin (Ins, 100 nM) or $NiSO_4$ (100 μM) in HBSS for the indicated periods of time (**a**) or 30 min (**b**). Phosphorylation of GSK3 α and β at Ser-21 and Ser-9, respectively, or of FoxO1a and FoxO3a at Thr-24 and Thr-32, respectively, was analyzed by Western blotting and immunodetection employing phospho-specific antibodies. The experiments shown are representative of three independent experiments with similar results

Subcellular localisation of FoxO1a in cells exposed to nickel ions

Thr-24 (Thr-32 in FoxO3a) is one of the three amino acid residues phosphorylated by Akt the phosphorylation of which is linked to nuclear exclusion of FoxO transcription factors (see Greer and Brunet 2005 for review). Exposure to copper ions or to insulin causes extensive phosphorylation of Akt and of FoxO transcription factors (see above). Accordingly, FoxO factors would be expected to be located primarily outside the nucleus. Indeed, treatment of cells expressing EGFP-tagged FoxO1a with either Cu^{2+} or insulin causes the nuclear exclusion of FoxO1a-EGFP (Fig. 5a, b): FoxO1a-EGFP is predominantly cytosolic in ~90% of the treated cells, whereas under control conditions FoxO1a-EGFP is both cytosolic and nuclear in most cells (Fig. 5a). To test whether the observed minute FoxO phosphorylation in cells exposed to Ni^{2+} suffices to cause detectable changes in subcellular localisation, HepG2 cells expressing FoxO1a-EGFP were held in the presence of Ni^{2+} under conditions allowing for Akt phosphorylation (100 μM $NiSO_4$ for 30 min). No more than a tendency towards nuclear exclusion was detected in nickel ion-exposed cells (Fig. 5a): Both the numbers of cells with FoxO1a-EGFP being predominantly nuclear and those with the protein being mostly cytosolic are only slightly changed versus control. Accordingly, distribution of EGFP fluorescence in Ni^{2+}-exposed FoxO1a-EGFP expressing cells is nearly indistinguishable from that in control cells (Fig. 5b).

Phosphorylation by Akt and nuclear exclusion of FoxO transcription factors renders them transcriptionally inactive. To test for effects of metal ion exposure on the activity of a FoxO-responsive promoter region, cells were cotransfected with a FoxO1a expression plasmid and a luciferase reporter plasmid coding for the promoter region of the G6Pase gene, a reportedly FoxO-responsive promoter (Schmoll et al. 2000). As expected for a strong activator of Akt-dependent FoxO phosphorylation and as expected from previous work (Walter et al. 2006) the G6Pase promoter-driven luciferase production induced by FoxO1a overexpression was significantly attenuated by insulin treatment (Fig. 5c). Similarly, Cu^{2+} (10 μM) strongly attenuated promoter activity. Although it was previously demonstrated that this copper ion-induced attenuation of G6Pase promoter activity is largely FoxO-independent (Walter et al. 2006), this effect is in line with Cu^{2+} being a potent stimulator of stress signaling cascades, including the PI3K/Akt cascade. Nickel ions, however, do not attenuate G6Pase promoter activity (Fig. 5c), suggesting that neither Akt nor FoxO activities are modulated to extents that allow for significant effects on FoxO-dependent gene expression.

Conclusions

This work investigates the mechanisms by which nickel ions activate stress-responsive signaling cascades. Specifically, it was found that Ni^{2+} causes the activation of the Ser/Thr-kinase Akt in a PI3K-dependent manner, at subcytotoxic doses and most likely independent of the formation of ROS. The cellular response to Ni^{2+} under these conditions is

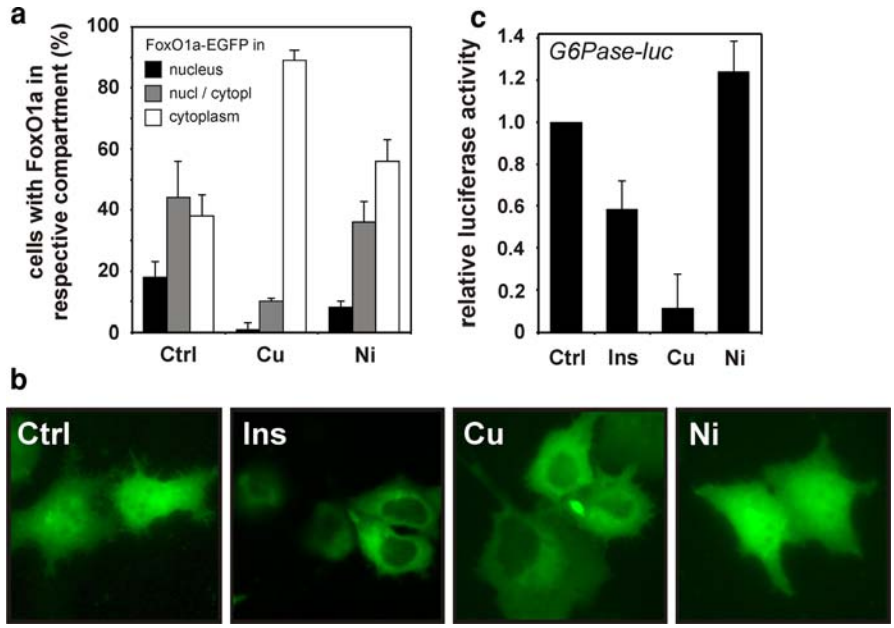

Fig. 5 Subcellular localization of FoxO1a-EGFP and activity of a FoxO-responsive promoter in HepG2 cells exposed to nickel or copper ions. **a**, **b** HepG2 human hepatoma cells were transfected with a FoxO1a-EGFP expression plasmid as described in "Materials and methods." Cells were exposed to HBSS (control), $CuSO_4$ (10 μM), insulin (Ins, 100 nM) or $NiSO_4$ (100 μM) in HBSS for 30 min. Localization of FoxO1a-EGFP was analyzed by fluorescence microscopy and cells categorized according to FoxO1a-EGFP localization. Data are given as means of three independent experiments ± SD. **c** HepG2 cells were transfected with a glucose 6-phosphatase promoter-luciferase reporter gene construct (G6Pase-luc). Twenty-four hours later, cells were exposed to serum-free medium or $CuSO_4$ (10 μM), insulin (Ins, 100 nM) and $NiSO_4$ (100 μM) in serum-free medium for another 18 h, followed by analysis of luciferase activity in cell lysates. Data are given as means of three independent experiments ± SD

not unique to this ion; indeed, copper ions induce a similar stress signaling cascade within the cell (Fig. 2, Walter et al. 2006). However, the effects mediated by these metals are not uniform: Cu^{2+} more potently activates these stress responses and copper ion-induced Akt activation is propagated further downstream than in the case of Ni^{2+}. While Akt activation and FoxO phosphorylation is robust in cells exposed to Cu^{2+}, causing changes in subcellular localization of FoxO1a, exposure to Ni^{2+} affects the activity of Akt but no detectable changes in FoxO localization occur (Fig. 5). Furthermore, whereas nickel appears to exclusively mediate the observed effects in HepG2 cells, Cu^{2+} ubiquitously activates these cascades in all cell types studied thus far (Barthel et al. 2007; Ostrakhovitch et al. 2002; Ostrakhovitch and Cherian 2004; Walter et al. 2006, 2008). Zn^{2+} and Cd^{2+}, like Cu^{2+} and Ni^{2+}, also stimulate the PI3K/Akt cascade, whereas no activation was detected with Mn^{2+}, Fe^{2+}, Co^{2+}, Pb^{2+} (Barthel et al. 2007). The follwing approximate order in potency of activation resulted from such comparative studies: $Cu^{2+} > Zn^{2+} > Cd^{2+} \approx Ni^{2+}$. Thus, although the activation of the PI3K/Akt cascade may well be a generalized stress response, it is not a general response to metal exposure of cells.

The mechanisms of metal ions inducing PI3K-dependent signaling processes are presently unclear. Although both copper and nickel ions are known to generate ROS intracellularly, and although ROS are well-known stimulators of PI3K/Akt signaling (Barthel and Klotz 2005), we have previously demonstrated that Cu-induced Akt activation does not require the formation of ROS—which is in line with the fact that a redox-inert metal ion such as Zn^{2+} is capable of strongly stimulating the PI3K/Akt cascade (Barthel et al. 2007). Rather, it was proposed for copper ions that an interaction with essential thiols (e.g., of protein tyrosine phosphatases involved in controlling PI3K/Akt signaling) may initiate signaling processes (Barthel et al. 2007 and references therein). For nickel ions similar

interaction might occur, but as nickel ions less avidly interact with thiol ligands than Cu^{+} (which is the form of copper that is generated intracellularly after uptake of Cu^{2+}), less extensive modulation of signaling processes would be expected, which is in line with the observed lack of nickel ion-induced modulation of FoxO signaling. In summary, Ni^{2+} stimulates Akt in human hepatoma cells in a fashion similar to insulin or Cu^{2+}. Different from these latter stimulators, however, nickel ion-induced Akt activation does not translate into modulation of FoxO activity. It is concluded that while stimulation of Akt signaling may indeed explain some of the biological effects of copper intoxication, such as insulin-like effects (Barthel et al. 2007), this is not the case for nickel: stimulation of stress-responsive Akt-dependent signaling is of minor significance to nickel toxicity. Finally, it may further be concluded that FoxO transcription factors, although generally stress-modulated, are not among the major nickel ion-responsive gene expression regulators: a recent report on transcriptional profiling analysis in human keratinocytes exposed to nickel indeed revealed that NF-κB, AP-1, Myc family transcription factor and aryl hydrocarbon receptor binding sites were present in a large fraction of genes whose expression was shown to be modulated upon exposure to nickel (Gazel et al. 2008).

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