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Heavy metal interference with growth hormone signalling in trout hepatoma cells RTH-149

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Abstract

We have studied the effects of heavy metals (Hg^{2+}, Cu^{2+}, Cd^{2+}) on growth hormone (GH) activation of tyrosine kinase and Ca^{2+} signaling in the trout (*Oncorhynchus mykiss*) hepatoma cell line RTH-149. Molecular cloning techniques using primer designed on *Oncorhynchus* spp. growth hormone receptor (GHR) genes allowed to isolate a highly homologous cDNA fragment from RTH-149 mRNA. Thereafter, cells were analysed by Western blotting or, alternatively, with Ca^{2+} imaging using fura-2/AM. Exposure of cells to ovine GH alone produced a stimulation of the JAK2/STAT5 pathway and intracellular free Ca^{2+} variations similar to what has been observed in mammalian models. Cell pre-exposure to Cu^{2+}, Hg^{2+} or Cd^{2+} affected cell response to GH by enhancing (Cu^{2+}) or inhibiting (Cd^{2+}) the phosphorylation of JAK2 and STAT5. Heavy metals induced the activation of the MAP kinase p38, and pre-exposure to Hg^{2+} or Cu^{2+} followed by GH enhanced the effect of metal alone. Image analysis of fura2-loaded cells indicated that pre-treatment with Hg^{2+} prior to GH produced a considerable increase of the $[Ca^{2+}]_i$ variation produced by either element, while using Cu^{2+} or Cd^{2+} the result was similar but much weaker. Data suggest that heavy metals interfere with GH as follows: Hg^{2+} is nearly ineffective on JAK/STAT and strongly synergistic on Ca^{2+} signaling; Cu^{2+} is activatory on JAK/STAT and slightly activatory on Ca^{2+}; Cd^{2+} is strongly inhibitory on JAK/STAT and slightly activatory on Ca^{2+}; heavy metals could partially activate STAT via p38 independently from GH interaction.

Introduction

A wide variety of metals have been reported to induce a stress syndrome in various organisms, and in particular to act as mutagenic and carcinogenic elements thus presenting a serious hazard to humans due to occupational or residential exposure, and more in general to living organisms due to environmental contamination. Most of these effects seem related to metal interaction with the cell signaling machinery (Harris & Shi 2003; Leonard *et al.* 2004). Several studies indicate that heavy metals can interact with cell plasma membrane receptors (Hansson 1996; Wu *et al.* 1999), thereby activating tyrosine kinase cascades (Lander *et al.* 1992; Rahman *et al.* 1993; Schieven *et al.* 1993; Nakashima *et al.* 1994; Katano *et al.* 1995). Moreover, heavy metals can also induce intracellular free calcium increase by impairing calcium homeostasis systems (Verbost *et al.* 1989; Viarengo & Nicotera 1991) or induce reversible Ca^{2+} release from intracellular stores (McNulty & Taylor 1999; Burlando *et al.* 2003a). The interaction of heavy metals with cell signaling suggests the possibility that metals affect cellular responses mediated by extracellular stimuli like hormones and growth factors acting via analogue signaling pathways. An example is provided by the finding

that estradiol and heavy metals activate a similar mechanism to induce lysosomal membrane destabilisation via $cPLA_2$ activation (Burlando *et al.* 2002; Marchi *et al.* 2004).

In this work, we have investigated the effects of heavy metals on cell response to growth hormone (GH) in the RTH-149 cell line deriving from an adult rainbow trout (*Oncorhynchus mykiss*) hepatoma (Fryer *et al.* 1981). We have chosen a piscine model considering that the aquatic environment provides a sink for many contaminants, thus rendering fish a sensible target of environmental pollution. It has been shown in various studies that heavy metals can affect different signaling pathways in fish hepatocytes (Nathanson *et al.* 1995; Fabbri *et al.* 2003). Moreover, *in vivo* experiments demonstrated that in *Salmo trutta* and in *Oncorhynchus mykiss* the exposure to low levels of heavy metals, and in particular to Cd^{2+}, extremely reduced the potential for growth due to an impairment of the hypothalamo–pituitary–adrenocortical axis (Hontela *et al.* 1996; Norris *et al.* 1999). These observations suggest an effect of these pollutants on the mechanisms involved in the regulation of growth. However, GH is not only involved in the regulation of somatic growth, but in fish it also influences osmoregulation and stimulates gonadal steroidogenesis (Eckert *et al.* 2001). In addition, in rainbow trout GH also acts as a cytokine, enhancing phagocytosis and antibody production by immune system cells (Harris & Bird 2000; Jeay *et al.* 2002). Moreover, it has also been shown that GH can influence neuroendocrine functions related to the production and release of "stress hormones" such as glucocorticoids and prolactin (Bartke *et al.* 2002). It has been suggested, therefore, that GH is involved in maintaining immune system homeostasis in response to environmental change.

It is currently assumed that tyrosine phosphorylation is an early signaling mechanism for GH, which binds to a couple of GH receptors (GHRs) with no intrinsic tyrosine kinase, causing their dimerization and recruitment of the JAK2 tyrosine kinase (Argetsinger *et al.* 1993). Upon its activation, JAK2 phosphorylates STAT5, which plays an essential role in the effect of GH on the genome (Udy *et al.* 1997; Teglund *et al.* 1998; Herrington & Carter-Su 2001). Taking into account that there was no information about the presence of a GHR in RTH-149 cells we have preliminary identified the presence of GHR in these cells by RT-PCR analysis. We have then studied the effects of Hg^{2+}, Cd^{2+} or Cu^{2+} on GH signaling. Mercury and cadmium are xenobiotic metals with no known physiological functions that produce several mechanisms of cytotoxicity (Misra *et al.* 2002; Kim & Sharma 2004). Copper is an essential metal that become toxic at excess concentrations by inducing oxidative stress (Stohs & Bagchi 1995; Viarengo *et al.* 2002). Moreover, it has been demonstrated that Hg^{2+} and Cu^{2+} induce tyrosine phoshorylation in RTH-149 cells (Burlando *et al.* 2003b). By using Western immunoblotting, total protein tyrosine phosphorylation, as well as the phosphorylation state of JAK2 and STAT5, have been analysed in cells treated with GH, heavy metals, or pre-treated with each metal and then exposed to the hormone. Metal and GH effects on the phosphorylation of the p38 MAP kinase have also been studied. This latter kinase is involved in cell response to stress, but is also activated by GH in a JAK2-dependent manner and mediates the hormone control of cell proliferation and cytoskeleton re-organization (Zhu & Lobie 2000). Furthermore, considering that GH is also able to activate Ca^{2+} signaling (Billestrup *et al.* 1995; Marrero *et al.* 1996), metal interferences on GH-dependent Ca^{2+} mobilization have been studied in fura2-loaded cells.

Methods

Materials

Ovine GH was kindly provided by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDKD, Torrance, CA). Fura2/AM was from Molecular Probes (IL). Anti-phosphotyrosine, anti-phospho p38, anti-phospho JAK2 and anti-phospho STAT5 antibodies were from Upstate Biotechnology Inc. (Lake Placid, NY). All other reagents were from Sigma Chemical Co. (St. Louis, MO). Metals were used as chloride salts.

Cell culture and treatments

RTH-149 cells were obtained from ATCC (Rockville, MD). The cell line, with an epithelial morphology, is the first established from fish liver (Fryer *et al.* 1981). Cells were routinely cultured

at 21 °C in Basal Eagle's medium (with Earl's salts and 25 mM Hepes) with 10% FBS. The medium was supplemented with non-essential amino acids, 1 mM L-glutamine, 10 mM sodium pyruvate, 10,000 units/ml penicillin, 10 mg/ml streptomycin, and 250 μg/ml amphoterycin B. Prior to treatments, cells were starved at 0.2% heat inactivated serum (56 °C) for 24 h in Basal Eagle's medium. The use of mammalian GH is widely reported in literature for studies on both growth and metabolism of various fish cell types (Seidelin & Madsen 1999). The use of ovine GH is justified here by the fact that this hormone, despite structural differences with respect to fish GH, is known to bind specifically to the receptor of its homologue in salmonid teleosts thereby producing similar qualitative osmoregularory effects as teleost hormones (Sakamoto & Hirano 1991; Yao *et al.* 1991).

Molecular cloning of a putative GH receptor mRNA fragment

Total RNA was extracted from approximately 10^6 cells by the acid phenol extraction method (Chomczynski & Sacchi 1987) using the TriReagent (Sigma–Aldrich). cDNA was obtained from 1 μg of total RNA, by reverse transcription in a 20 μl reaction mixture containing 200 U of M-MLV H- reverse transcriptase (Fermentas, Vilnius, Lithuania), 0.5 μg anchored oligo $dT_{(19)}V$, following the manufacturer instructions. The RT mixture (1 μl) was subjected to 35 cycles of amplification using a 2400 Perkin Elmer Apparatus. The 25 μl reaction mixture contained: 50 ng RNA reverse-transcribed cDNA; 0.020 U/μl Platinum Taq polymerase (Invitrogen); 1 × Taq Buffer; 0.2 mM dNTPs; 1.5 mM $MgCl_2$; 0.5 μM sense primer (5′-CTATACCCAGGT-GAGCGAGG-3′) and 0.5 μM antisense primer (5′GCTTCAGAAGGAGGCTGTTC-3′). Thermal parameters in a iCycler PCR apparatus (Bio-Rad Laboratories, Milan, I) were as follows: 5 min initial denaturation at 95 °C followed by 35 cycles: 30 s at 95 °C, 30 s at 50 °C, 30 s at 72 °C, followed by extra 10 min at 72 °C. The PCR product was ligated into the PCR2.1-Topo plasmid (Invitrogen), and four independent clones were sequenced using the dideoxynucleotide dye method at the MWG-Biotech AG (Ebersberg, D) sequencing facility.

Western blot analysis

After serum starvation, cells were subjected to treatments and lysed in boiling Laemmli SDS-buffer. Equal amounts of protein in each sample were electrophoresed under reducing conditions (SDS-PAGE) (Laemmli 1970) and blotted to Hybond ECL filters (Amersham Pharmacia Biotech, Uppsala, Sweden) in transfer buffer (25 mM Tris/192 mM glycine, 20% methanol, pH 8.3) for 1.5 h at 290 mA using a Mini Trans Blot device (Bio Rad Laboratories, Hercules, CA). Blots were then probed with specific antibodies in TBS with 3.5% BSA. Binding of antibodies was visualized by enhanced chemiluminescence (ECL) detection system (Roche).

Blot stripping and reprobing

When required, blots were stripped of the primary antibody-secondary antibody complex by incubating them in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris–HCl, pH 6.7) for 30 min at 50 °C. Blots were incubated with blocking buffer and reprobed with a different antibody according to the procedures described above.

Densitometric analysis

Densitometry of ECL immunoblots was performed using a Fluor-S Max fluorimager (Bio-Rad) or by scanning (GS-710, Bio Rad) of films (Hyperfim, Amersham). Quantitation was performed by a PC-compatible image analysis program (Quantitiy One, Bio Rad).

Calcium measurements

Cells were cultured for 24 h at 21 °C on glass coverslips, incubated in loading buffer (30 mM Hepes, 0.5 mM sucrose, 2.5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 125 mM NaCl, 2.5 mM KCl, pH 7.3) containing 4 μM fura-2/AM for 30 min at 21°C, rinsed with buffer to remove extracellular dye, and treated or not with heavy metals and/or GH. Cells were then observed under an Olympus IMT-2 inverted microscope equipped with an IMT2-RFL fluorescence attachment (Olympus Optical Co., Germany) and with an MTI SIT 68 intensified camera (Oatencourt Ltd., England). Images were acquired every minute using the CUE2 RMS 4.0

imaging system (Galai Production Ltd., Israel). Background fluorescence was subtracted before analysis. Fura-2 calibration was achieved by the equation reported by Grynkiewicz *et al.* (1985):

$$[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F)Sf2/Sb2,$$

where $K_d = 224$ nM, F_{max} and F_{min} are maximum and minimum fluorescence intensities, measured after cell treatment with 50 μM digitonin and 5 mM EGTA, respectively, and Sf2/Sb2 is the ratio between the excitation efficiencies of free probe and Ca^{2+}-bound probe at 380 nm.

Statistics

ANOVA and post hoc test of multiple comparisons (Tukey test) were performed using GraphPad InStat version 3.05, GraphPad Software, San Diego California USA.

Results

Identification of GHR

Using primers designed on the *Oncorhynchus* spp. GHR sequences, available from the non-redundant NCBI nucleotide database, we isolated a cDNA partial fragment of 410 bp from RTH-149 mRNA (NCBI accession number: AY663793). As expected, this sequence showed 97% identity with the *Oncorhynchus masou* GHR mRNA (AB071216), 96% identity with the *Oncorhynchus kisutch* GHR isoform 2 mRNA (AF403540), and 86% identity for the *O. kisutch* GHR isoform 1 mRNA (AF403539). The conceptual translation of the novel cDNA fragment gives rise to a polypeptide displaying the highest homology (94% identity) with the *O. kisutch* GHR isoform 2 (Figure 1). This finding suggests that RTH-149 cells actually express a GHR.

GH signaling in RTH-149 cells

Whole lysates from GH-exposed RTH-149 cells were analysed by Western blotting using monoclonal phosphotyrosine antibody. After a set of analyses aimed at finding non-saturating GH doses and optimal exposure time, we found fair western blot detection of phosphotyrosine activation with a GH dose of 0.5 nM for 10 min. Under these conditions, we also found that a 15 min preincubation with 100 μM genistein abolished the tyrosine phosphorylation induced by GH (Figure 2).

We also analysed the effect of GH on the JAK/STAT system, by using phosphospecific antibodies against mammalian JAK2 and STAT5. Western blot analysis using an anti-phospho JAK2 antibody revealed that GH induces a marked increase in the phosphorylation of a putative homologue of JAK2 (Figure 3). Accordingly, by probing cell lysates with anti-phospho STAT5, a couple of intensely stained bands were obtained from GH-exposed cells (Figure 4), possibly corresponding to the STAT5a and STAT5b isoforms of mammals (Hoey & Schindler 1998).

Effects of heavy metals and GH on JAK2, STAT5 and p38 phosphorylation

In these experiments we made treatments of 10 min (unless otherwise indicated) using each of the following: 0.5 nM GH, 0.5 μM Hg^{2+}, 5 μM Cd^{2+}, 5 μM Cu^{2+}; we also made 10 min

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                 ...|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         430       440       450       460       470       480       490
RTH-149 GHR       --YTQVSE VRPTGEVLLM PEEQRKVEKD AEEKAKEGKE KAKPRKEFQL LVVNADGGGY TTELDAGKMS
O.kisutch GHR     DLYTQVSE VRPTGEVLLM PEEQSKVEKD AEEKAKEEMG KKKPRKEFQL LVVNADRGGY TTELDAGKMS
Clustal Co          ****** ********** **** ***** *******    * ******** ****** *** **********

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         500       510       520       530       540       550       560
RTH-149 GHR      AKLPTGRGSQ PAPTEDSRLV QGQPFGEYQS LYFEAEMPPI PPASPVSSLL PLSVYTMVEG VDRQNSLLLK
O.kisutch GHR    AKLPTGRGSQ PAPTEDSRLV QGQPFGEYQS LYFEAEMPPI PPTSPVSPLL PLSVYTMVEG VDRQNSLLLK
Clustal Co       ********** ********** ********** ********** **:****.** ********** **********
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Figure 1. Alignment of the putative RTH-149 GHR polypeptide. The polypeptide obtained from conceptual translation of the 410 bp partial cDNA fragment isolated from RTH-149 cells was aligned against the *O. kisutch* GHR isoform 2 (NCBI accession number: AAK95625) using the Clustal-W algorithm (Thompson *et al.* 1994), showing 94% identity.

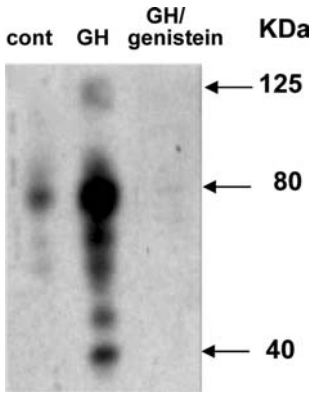

Figure 2. GH-stimulated protein tyrosine phosphorylation from RTH-149 cell lysates. Cell lysates were analysed by 9–14% gradient SDS-PAGE, blotted to nitrocellulose and probed with antiphosphotyrosine antibody. This Western blot figure and the following ones are representative results from 4 separate experiments, performed at 21 °C after cell starvation at 0.2% serum for 24 h, as described in the Methods.

pre-treatments with each metal followed by wash out and a 10 min treatment with GH. Analysis with anti-phospho JAK2 indicated that Hg^{2+} alone produces no significant activation and that Hg^{2+} pre-treatment prior to GH did not alter significantly the stimulation of JAK2 evoked by GH (Figure 3). The use of Cd^{2+} alone produced no activation of JAK2, while Cd^{2+} pre-treatment had a strong inhibitory effect on the activation induced by GH (Figure 3). In contrast to the former metals, the use of Cu^{2+} alone produced a significant activation of JAK2, and moreover Cu^{2+} pre-treatment induced a significant increase of the activation induced by GH (Figure 3).

The use of phosphospecific STAT5 antibody in a set of parallel experiments gave consistent results: treatment with Hg^{2+} was stimulatory while pre-treatment with Hg^{2+} prior to GH did not enhance the activation of STAT5 induced by GH alone; pre-treatment with Cd^{2+} abolished the effect of GH; treatment with Cu^{2+} was strongly stimulatory on STAT5, while Cu^{2+} pre-treatment followed by GH only produced a slight increase of STAT5 stimulation (Figure 4).

Blot incubation with phosphospecific p38 antibody revealed a slight phosphorylation of a putative homologue of mammalian p38 after treatment with GH only. Treatment with Hg^{2+} induced a significant activation, while the highest activation level was obtained with Hg^{2+}

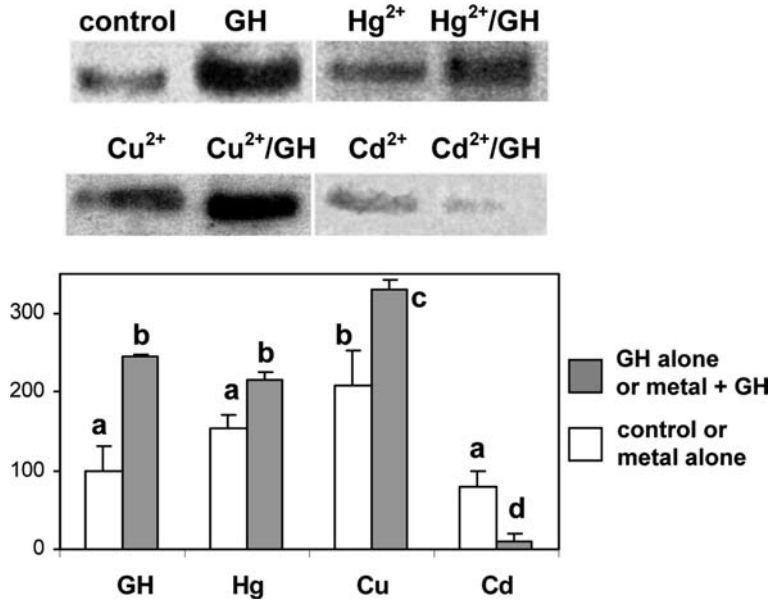

Figure 3. Effects of GH, heavy metals, and heavy metals followed by GH on JAK2 phosphorylation. Upper panels: Western blot analysis obtained using a phosphospecific JAK2 antibody; see text for the indication of exposure times and concentrations. Lower panel: densitometric analysis of bands ($n = 4$); data are expressed as mean percent variations respect to control. Differences among means were analysed using ANOVA followed by the Tukey multiple comparison test, and denoted by letters on bars. The mean of a group is significantly different from any other group with a different letter ($P < 0.05$) while it shows no differences with respect to groups with the same letter.

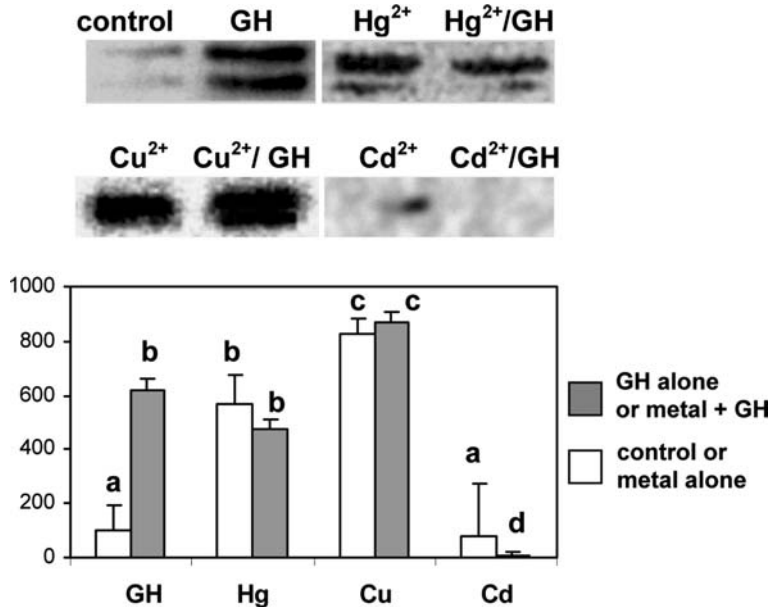

Figure 4. Effects of GH, heavy metals, and heavy metals followed by GH on STAT5 phosphorylation. Upper panels: Western blot analysis obtained using a phosphospecific STAT5 antibody; exposure times and concentrations as in Figure 3. Lower panel: densitometric analysis of bands ($n = 4$); data and statistics as in Figure 3.

pre-incubation followed by exposure to GH (Figure 5). A similar trend was found using Cu^{2+} instead than Hg^{2+}, though in this case the levels of phosphorylation of p38 were lower (Figure 5). Cd^{2+} alone produced a strong rise in p38 phosphorylation, similar to that induced by

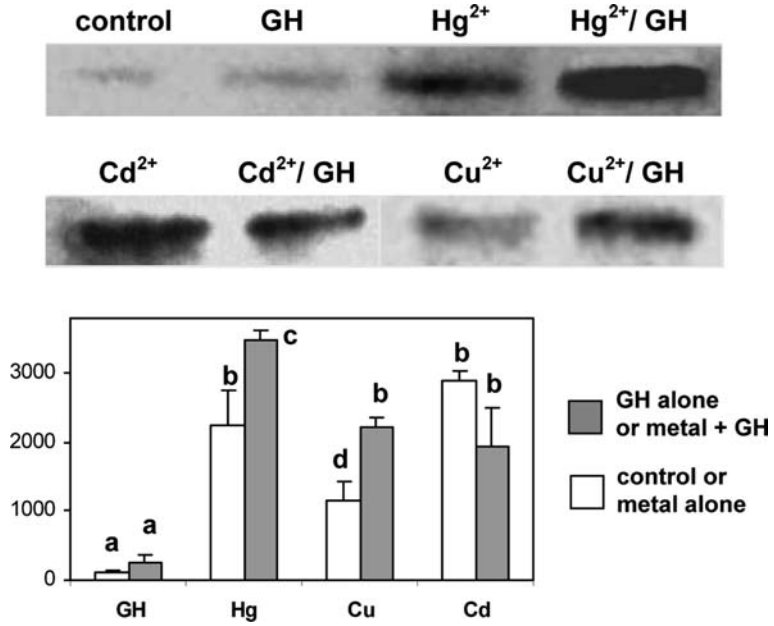

Figure 5. Effects of heavy metals alone or heavy metals followed by GH on the phosphorylation of p38. Upper panels: Western blot analysis obtained using a phosphospecific p38 antibody; exposure times and concentrations as in Figure 3. Lower panel: densitometric analysis of bands ($n = 4$); data and statistics as in Figure 3.

Hg^{2+}, and there was no further activation by GH (Figure 5).

Ca^{2+} signaling induced by GH

Variations of $[Ca^{2+}]_i$ were recorded in fura 2-loaded cells as described in the Methods. Quantifications of $[Ca^{2+}]_i$ revealed a basal value of about 80 nM in control cells, the occurrence of Ca^{2+} oscillations in cells exposed to 0.5 nM GH, a higher Ca^{2+} transient at 1.25 nM, and a sustained variation reaching a plateau of about 400–500 nM $[Ca^{2+}]_i$ at a GH concentration of 2.5 nM (Figure 6A). Omission of Ca^{2+} from the extracellular medium reduced, but did not abolish, the excursion of the GH-induced $[Ca^{2+}]_i$ variation

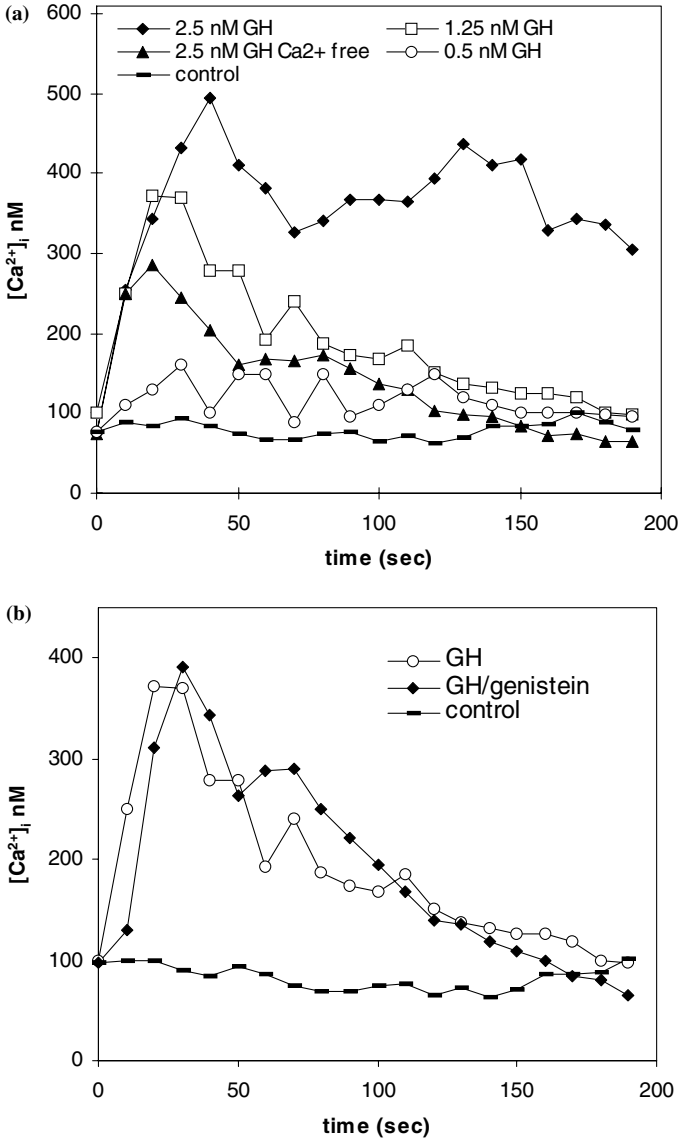

Figure 6. $[Ca^{2+}]_i$ variations induced by GH. Cells were loaded with the fura-2 Ca^{2+} probe and analyzed by fluorescence microscopy digital imaging as described in the Methods; each trace represents the Ca^{2+} variation recorded in a single cell every 10 s, and is representative of 5 experiments (20 cells). Panel a. cell exposures to GH in the range 0.5–2.5 nM induce $[Ca^{2+}]_i$ variations consisting of oscillations at the lowest dose, and transients of increasing amplitude for increasing GH concentrations. In a Ca^{2+}-free medium, the $[Ca^{2+}]_i$ rise induced by 2.5 nM GH is reduced. Panel b. preincubation with 100 μM genistein does not modify the $[Ca^{2+}]_i$ variations induced by 1.25 nM GH.

(Figure 6a), thus indicating a contribution from both Ca^{2+} entry and Ca^{2+} release.

A comparison of Ca^{2+} measurements and Western blot data reveals that the effects of GH on intracellular Ca^{2+} and on phosphorylation processes are not correlated. Cell pre-treatment with 100 μM genistein, which abolished the GH-induced tyrosine phosphorylation did not alter the GH-dependent $[Ca^{2+}]_i$ variation (Figure 6b).

Effects of heavy metals on the GH-induced $[Ca^{2+}]_i$ variation

When cells were exposed to the metal doses used in our study the effects on Ca^{2+} were very limited. A low Ca^{2+} transient was only recorded with 0.5 μM Hg^{2+}, while 5 μM Cd^{2+} or 5 μM Cu^{2+} were totally ineffective (Figure 7). Conversely, cell pre-exposure to Hg^{2+} followed by GH produced a strong $[Ca^{2+}]_i$ transient. Such a combined effect resulted more than additive respect to the effects of each reagent used separately (Figure 7a). A similar trend was found by using pre-treatments with Cd^{2+} or Cu^{2+} prior to GH (Figure 7b and c), but in these cases the $[Ca^{2+}]_i$ transients obtained as a combined effect were lower than that recorded with Hg^{2+} plus GH.

Discussion

GH signaling without heavy metals

The presence of the GHR had been already assessed in fish of the genus *Oncorhynchus* (Yao *et al.* 1991), as shown by full length mRNA sequences at the NCBI database (http://www.ncbi.nlm.nih.gov). Our molecular data suggest that a component of the GHR family is also expressed in the RTH-149 cell line deriving from *O. mykiss*. Accordingly, Western blot analyses have shown that in these cells GH activates a mechanism of signal transduction involving the JAK2/STAT5 system, as demonstrated in mammalian cells (Carter-Su *et al.* 1996). Moreover, it has been found that GH induces intracellular calcium mobilisation, and also in this case we have found patterns that are typical of the Ca^{2+} response of hepatocytes to various hormones, consisting of Ca^{2+} oscillations at low doses (Kawanishi *et al.* 1989; Rooney *et al.* 1989) and higher, non-oscillatory Ca^{2+} variations at high doses (Somogyi & Stucki 1991). In addition, pre-treatment of cells with 100 μM genistein abolishes the GH-induced tyrosine phosphorylation but does not alter the GH-dependent $[Ca^{2+}]_i$ increase, suggesting a differently switched mechanism of activation for GH-dependent tyrosine and Ca^{2+} signalling. This latter process probably does not involve GHR dimerization and tyrosine phosphorylation cascade, but instead an alternative signal transduction pathway involving PLC activation and $InsP_3$ (Billestrup *et al.* 1998; Gaur *et al.* 2000; Marrero & Deniz 2004). Stimulation of tyrosine phosphorylation by GH was well documented over the past years, in various cell lines (Moller *et al.* 1992) with GHR dimerization recognized as the first step (Zhang *et al.* 1999) in activating multiple downstream signaling cascades. Out of these cascades, however, GH-dependent calcium signaling is the only one that was supposed not to require the GHr/JAK2 pathway (Billestrup *et al.* 1995; Negatu & Meier 1995).

Effects of heavy metals on GH signaling

The above data confirmed that in RTH-149 cells GH activates a mechanism of signal transduction similar to that observed in mammalian cells, providing an essential basis for the study of the possible interactions of heavy metals with GH signaling. The concentrations of GH and of heavy metals used to study interactive effects have been chosen by first keeping in mind the need of using a hormone concentration close to physiological values and metal doses mimicking tissue accumulation from environmental contamination. The adopted concentrations have allowed an optimal detection of GH/metal interactions with the employed techniques. Cells have been pre-exposed to each metal prior to GH in order to reconstruct as close as possible the cell responses to the hormone that could be produced by organisms under metal contamination. The effects of metals on GH have been explored by taking into account the two pathways characterized previously, viz. JAK/STAT and Ca^{2+} signaling, and in addition the p38 MAP kinase that as said above, is linked to GH signaling through JAK2.

Western blot data indicate that a submicromolar concentration of Hg^{2+} has a slight,

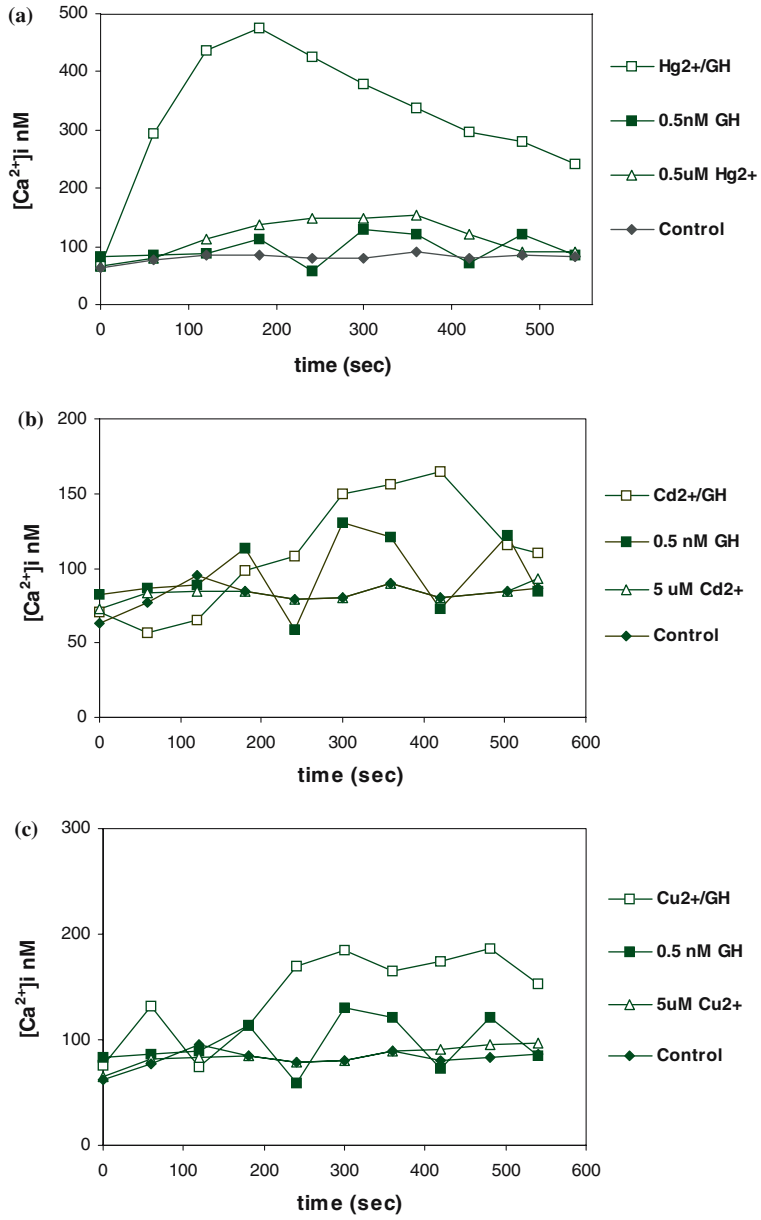

Figure 7. Effects of heavy metals alone or heavy metals prior to GH on $[Ca^{2+}]_i$. Data have been collected, analysed, and reported as in Figure 6, except that Ca^{2+} has been measured every 60 s. Also in these experiments Ca^{2+} oscillations are detected in cells exposed to GH alone. In contrast, Hg^{2+} alone (a) produces a single Ca^{2+} transient followed by recovery, while Cd^{2+} (b) and Cu^{2+} (c) alone are ineffective. In case of metal pre-treatment followed by GH, Hg^{2+} produces a marked Ca^{2+} transient, while Cu^{2+} and Cd^{2+} abolish the oscillatory pattern typical of GH alone and produce a limited increase of Ca^{2+}.

but non-significant effect on the activation of JAK2, while the effect on STAT5 is significant. However, the pre-incubation with Hg^{2+} followed by GH does not produce any significant variation in the phosphorylation of JAK2 induced by GH alone, suggesting a scarce effect of Hg^{2+} on the GHr-dependent signaling pathway. As previously found in these and other cells (Burlando *et al.* 2003b; Kim & Sharma 2004), Hg^{2+} activates p38, and even though the activation induced by GH per se at the adopted concentration is minimum, when Hg^{2+} and GH are combined together there is a

significant synergistic effect. This could depend on the stimulation by Hg^{2+} and/or GH of a complex of p38-activating factors, including the stress-dependent MKK3/6 cascade (Widmann *et al.* 1999), the JAK2 kinase (Bode *et al.* 1999), and intracellular Ca^{2+} (Elzi *et al.* 2001). The activation of p38 by Hg^{2+} could also explain the higher activation exerted by this metal alone on STAT5 compared to JAK2, as STAT5 seems to be also activated via p38 (Bode *et al.* 1999).

In the case of Ca^{2+}, the strong synergistic effect produced by the combination of Hg^{2+} and GH, can be easily explained by considering that Hg^{2+} has been found to activate the $InsP_3$-dependent Ca^{2+} signaling in RTH cells (Burlando *et al.* 2003a) and to act on intracellular Ca^{2+} stores in skate hepatocytes (Nathanson *et al.* 1995), and moreover that GH is known to activate the $InsP_3$ pathway in rat hepatocytes (Marrero & Deniz 2004). Therefore, it is likely that a convergent action of the two elements, mediated by some G protein-linked receptor, is able to produce a strong amplification of the Ca^{2+} signal, that in our experiments went much beyond the sum of the Ca^{2+} variations induced by each element alone. Ca^{2+}-induced Ca^{2+} release could also play a role in the amplification of Ca^{2+} response, as in these cells this mechanism mediates Hg^{2+}-dependent Ca^{2+} signalling due to a combination of Ca^{2+} entry and intracellular release (Burlando *et al.* 2003a), and in addition the use of GH in Ca^{2+} free medium suggests the involvement of Ca^{2+} entry and release.

The use of Cd^{2+} alone has no effect on JAK2, thus being similar to Hg^{2+}, but conversely to this latter Cd^{2+} is also ineffective on STAT5. Moreover, cell pre-exposure to Cd^{2+} has shown that this metal completely abolishes the GH activation of the JAK2/STAT5 system by acting at the level of JAK2 or upstream. The silencing of GH signalling by Cd^{2+} is quite consistent with field studies demonstrating that this metal inhibits the growth of the brown trout (Norris *et al.* 1999). The activatory effect of Cd^{2+} on p38 is in line with its known ability of strongly activating this MAP kinase (Harris & Shi 2003; Waisberg *et al.* 2003), but in contrast to Hg^{2+} there is no significant variation in the p38 activation when Cd^{2+} is used in combination with GH. This is probably due to the lack of JAK2 activation in the presence of Cd^{2+} and also on the low synergistic effect of Cd^{2+} and GH on Ca^{2+} that would render negligible any GH contribution to the effect of Cd^{2+} on p38.

The main result obtained with Cu^{2+} alone is a level of activation of JAK2 similar to that of GH alone, and an even higher activation of STAT5. An additive effect of Cu^{2+} and GH has been recorded for the activation of JAK2 but not of STAT5, whose activation is already at its highest level with Cu^{2+} alone. Such a strong effect of Cu^{2+} could be related to its known ability of inducing oxidative stress (Stohs & Bagchi 1995) that is also a well known activator of the JAK/STAT system (Simon *et al.* 1998; Carballo *et al.* 1999). In the case of Cu^{2+}, a contribution of p38 in the activation of STAT5, as previously assumed for Hg^{2+}, is unlikely since the activation of Cu^{2+} on this MAPK is weak. However, there is a synergistic effect between Cu^{2+} and GH on p38, which could be mediated by JAK2 and would thus reflect the synergism observed on this latter kinase. The role of Ca^{2+} should instead be scarcely relevant, because similar to what has been found with Cd^{2+}, the Ca^{2+} response obtained with Cu^{2+} alone or followed by GH is weak, at least at the concentrations used in our experiments.

In conclusion, our study has demonstrated that different heavy metals can specifically interact with GH signaling through a variety of activatory or inhibitory mechanisms: (1) GH alone independently activates JAK/STAT, possibly via GHR, and Ca^{2+} signaling, possibly via PLC/$InsP_3$; (2) the interaction of Hg^{2+} with GH is nearly ineffective on the JAK/STAT pathway, whereas a strong synergistic effect is found on Ca^{2+}; (3) the interaction with Cu^{2+} is activatory on JAK/STAT and slightly activatory on Ca^{2+}; (4) the interaction with Cd^{2+} is strongly inhibitory on JAK/STAT and slightly activatory on Ca^{2+}; (5) GH activation of p38 could be increased by Cu^{2+} via JAK2 and by Hg^{2+} via Ca^{2+}, while Hg^{2+} could partially activate STAT5 via p38.

Considering that these results have been obtained using low metal and GH doses, we can infer that also in intact organisms heavy metal overload can seriously affect cellular responses to hormones, thereby producing signaling disruption. This can be viewed like the two sides of a coin. On one side the interaction between heavy metals and signaling mechanisms results in an amplification of the sensitivity of cells to metals, which could also be

promoted by metal binding to plasma membrane receptors, thus bypassing intracellular metal buffering systems. On the other side, this can produce an impairment of cell responses to hormones or other physiological signals that govern the organism's homeostasis and the responses to environmental stimuli. It can be even argued that much of the noxious effects produced by the low heavy metal doses that are typical of environmental contamination could be mediated by this kind of processes, a possibility that would deserve to be explored by further research effort in this direction.

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