Metal ions induced heat shock protein response by elevating superoxide anion level in HeLa cells transformed by HSE-SEAP reporter gene

Zhanjiang Yu*, Xiaoda Yang a,b,c, Kui Wang a,b

* Department of Chemical Biology, School of Pharmaceutical Sciences, Peking University Health Science Center, Beijing 100083, PR China
b National Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University Health Science Center, Beijing 100083, PR China

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Abstract
The aim of this work is to define the relationship between heat shock protein (HSP) and reactive oxygen species (ROS) in the cells exposed to different concentrations of metal ions, and to evaluate a new method for tracing the dynamic levels of cellular reactive oxygen species using a HSE-SEAP reporter gene. The expression of heat shock protein was measured using a secreted alkaline phosphatase (SEAP) reporter gene transformed into HeLa cell strain, the levels of superoxide anion (•O2−) and hydrogen peroxide (H2O2) were determined by NBT reduction assay and DCFH staining flow cytometry (FCM), respectively. The experimental results demonstrated that the expression of heat shock protein induced by metal ions was linearly related to the cellular superoxide anion level before cytotoxic effects were observed, but not related to the cellular hydrogen peroxide level. The experimental results suggested that metal ions might induce heat shock protein by elevating cellular superoxide anion level, and thus the expression of heat shock protein indicated by the HSE-SEAP reporter gene can be an effective model for monitoring the dynamic level of superoxide anion and early metal-induced oxidative stress/cytotoxicity.

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1. Introduction
Since Ritossa (1962) reported heat shock protein (HSP) as a cellular protective response in Drosophila heated at 42 °C, a number of physical factors and chemical reagents including heavy metals have been reported to induce HSP and a series of other stress proteins known as the cytotoxicity marks for monitoring environment toxins. The mechanism for negative controlling heat shock response has been described by Ananthan et al. (1986) and Hightower (1991). The transcription regulation of HSP was shown to be dependent on the heat shock element (HSE) upstream from the heat shock protein promoter region (Amin et al., 1988). The critical factor controlling the expression of heat shock protein was trans-cytokines which bound to heat shock element and activated the transcription of HSP (Morimoto, 1993). Based on the induction mechanisms, various HSP-responding reporter gene systems have been established for dynamic monitoring of environmental toxins (Ait-Aissa et al., 2003).
Many studies (Becker et al., 1990; Davidson et al., 1996; Flanagan et al., 1998; Marini et al., 1996; Schoeniger et al., 1994) indicated that heat shock protein increased with elevated levels of reactive oxygen species when cells were heated. Schoeniger et al. (1994) found that the expression of HSP72 increased with superoxide anion level in a swine liver ischemia model. Gorman et al. (1999) indicated that HSP27 and HSP70 in the HL-60 cells heated at 40–42°C for 1 h increased with superoxide anion. Therefore, reactive oxygen species induced by heat was regarded to play a key role in the expression of heat shock protein.

Metal ions were shown to induce heat shock protein. Sodium arsenite and CdCl₂ at sub-lethal concentrations were shown to elevate the levels of HSP70 and HSP27 in human proximal tubule cells (Somji et al., 1999a,b, 2002); CuSO₄ induced the expression of HSP70 in rainbow trout hepatocytes (Feng et al., 2003); Hg²⁺ and Ni²⁺ altered the induction of HSP72 in THP-1 human monocytes (Noda et al., 2003); and a series of metal ions (i.e. CdCl₂, HgCl₂, and ZnSO₄) stimulated the level of HSP70 in HeLa cells transfected with HSP70-CAT plasmid (Ait-Aissa et al., 2000). Steiner et al. (1998) demonstrated that the mRNA level of heat shock protein was dependent on the metal ion incubation concentration and exposure time. Madden et al. (2002) indicated that the combination of As³⁺ and Cd²⁺ led to higher expression of HSP60, HSP70 and HSP90. The combination of As and Cd caused a higher lipid peroxide (LPO) levels in the rat’s liver and kidney (Díaz-Barriga et al., 1990). Nonetheless, the mechanism of heat shock protein production and activation of heat shock transcription factor (HSTF) in the presence of heavy metals was not clear.

Previous studies have suggested that reactive oxygen species (ROS) induced by metal ions may be involved in the production of heat shock protein. Clarifying the correlation between metal-induced reactive oxygen species (ROS) and expression of HSP may not be helpful to elucidate the mechanisms by which metal ions induce HSP production, but also lead to establish novel real-time methods to monitor the dynamic levels of reactive oxygen species in living cells based on HSE response reporter genes. The cellular reactive oxygen species have normally been detected by chemical staining methods (Beccera et al., 2001; Gabriel et al., 1997; Sun et al., 2005), such as NBT reduction (Freire et al., 2003) and DCFH fluorescence assays (Lotem et al., 1996) for superoxide anion/singlet oxygen and hydrogen peroxide/total ROS level, respectively.

In the present study, an HSE response secreted alkaline phosphatase (SEAP) reporter gene from Clontech, a reliable and effective method to detect heat shock protein in real time, was transformed into HeLa cells to investigate the correlation of heat shock protein production with reactive oxygen species upon incubation of cells with a variety of metal ions. HeLa cells exhibited a high level of heat shock protein expression (Ait-Aissa et al., 2000) but little drug metabolizing capacity; therefore, it is a good model for studying the intrinsic response of the compounds as drugs/toxins as well as for establishing high-throughput screening methods. The experimental results have suggested that metal ions induce heat shock protein by elevating cellular superoxide anion, and the HSE response reporter gene assay can be used to monitor at the early stages of the dynamic level of superoxide anion and other metal-induced oxidative stress/cytotoxicity.

2. Materials and methods

2.1. Materials

HeLa cell was kindly provided by the school of basic medical sciences, Peking University; pHSE-SEAP plasmid was purchased from Clontech (Mountain View, CA, USA); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Nitroblue tetrazolium (NBT), Dulbecco’s minimum Eagle’s medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY, USA); Lipofectamine 2000 transfection reagent was purchased from Invitrogen (San Diego, CA, USA); l-homoarginine was purchased from Sigma (New York, NY, USA); all other chemicals were molecular biological or analytical reagent grade.

2.2. MTT assay

The effects of metal ions on the viability of HeLa cells were determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. HeLa cells (100 µL, 2 × 10⁵ cells/mL) were grown in 96-well plate until sub-confluence. After exposure to metals (CdCl₂, HgCl₂, Pb(NO₃)₂, CuCl₂, ZnSO₄, NiSO₄, MnCl₂, Al₂(SO₄)₃; at 0, 1, 5, 10, 25, 50, 100 µM concentrations) in a serum-free DMEM medium for 24 h at 37°C, the cells were washed with D-Hanks and incubated for 4 h at 37°C with 100 µL of 0.5 mg/mL MTT in DMEM medium. MTT was removed and the formazan salts were dissolved in 200 µL of DMSO. Plates were read on a Sunrise (Tecan) microplate reader at the wavelength of 570 nm with the 630 nm as a reference for subtracting the reagent blank (Ait-Aissa et al., 2000).

2.3. Preparation of HeLa cells transformed with the HSE-SEAP plasmid

HeLa cells were grown in 25 cm² culture flask until 80% confluence. The monolayers were washed by D-Hanks, and 6 mL DMEM (serum- and antibiotics-free) added. After 20 min incubation at room temperature, thoroughly mixed HSE-SEAP
and Lipofectamine 2000 (16 μg/20 μL) were added into the cell culture medium. After incubation in 5% CO2, 37 °C for 6 h, the supernatants were removed and the cells grown in complete DMEM for application.

2.4. Treatment of HeLa cells with metals

HeLa cells were seeded into 96-well plate at a concentration of 2 × 10^4 cells/mL, and grown for 24–48 h until 80% confluence. The cells were then washed twice with pre-warmed D-Hanks and allowed to recover of 2 × 10^5 cells/mL, and grown for 24–48 h until 80% confluence. After exposure to metals (CdCl2, HgCl2, Pb(NO3)2, CuCl2, ZnSO4, NiSO4, MnCl2, Al2(SO4)3; at 0, 1, 5, 10, 25, 50, 100 μM concentrations). After incubation at 37 °C for 4 h, the cells were washed with D-Hanks and allowed to recover for 48 h in complete medium. The supernatants were harvested in 0.5 mL plastic tubes and stored at −20 °C. Cytotoxicity was assayed as described previously.

2.5. Determination of SEAP activity

The activity of SEAP was assayed using the method described previously (Gregory et al., 1994; Schlatter et al., 2002). The supernatants were collected after incubation with metals ions. The samples were heated at 65 °C for 30 min to inactivate endogenous phosphatase, and centrifuged at 5000 × g for 30 min to remove cell debris. Then 40 μL of 2 × SEAP buffers (20 mM L-homoarginine, 1 mM MgCl2, 21% dithanolamine, pH 9.8) and 5 μL of freshly prepared p-nitrophenyl phosphate (PNPP) were added to 96-well plate, and 50 μL of supernatants samples were added. After mixing thoroughly, the plates were read at 405 nm at 5, 10, 15, 20, 25, and 30 min at 37 °C with a Sunrise (Tecan) microplate reader. The slope for each sequence was obtained. SEAP concentration in supernatant that indicated the amount of heat shock protein in HeLa cells was expressed by SEAP activity. The relative SEAP activity was calculated by dividing SEAP concentration by the OD at 570 nm in the corresponding MTT assay.

2.6. The NBT assays

The NBT assays for superoxide anion were carried out according to the method previously described (Bocerra et al., 2001; Freire et al., 2003). HeLa cells (2 × 10^4 cells/mL, 2 mL per well) were grown in 6-well plate until 80% confluence. After exposure to metals (CdCl2, HgCl2, Pb(NO3)2, CuCl2, ZnSO4, NiSO4, MnCl2, Al2(SO4)3) at 0, 1, 5, 10, 25, 50, 100 μM in a serum-free DMEM medium for 4 h at 37 °C. The cells were resuspended in 700 μL of PBS and read in flow cytometer (FCM).

2.7. The DCFH assays

The DCFH assays for hydrogen peroxide were carried out according to the method previously described (Lotem et al., 1996). HeLa cells (2 × 10^4 cells/mL, 2 mL per well) were grown in 6-well plate until 80% confluence. After exposure to metals (CdCl2, HgCl2, Pb(NO3)2, CuCl2, ZnSO4, NiSO4, MnCl2, Al2(SO4)3), at 0, 1, 5, 10, 25, 50, 100 μM in a serum-free DMEM medium for 4 h at 37 °C, the cells were collected after digestion by centrifuging at 1000 × g for 5 min. The cells were resuspended in 1 mL of PBS (20 μM DCFH). After incubation at 37 °C for 30 min, the samples were centrifuged at 1000 × g for 5 min. The cells were resuspended in 700 μL of PBS and read in flow cytometer (FCM).

2.8. Data analysis

All the experiments were performed by triplicate. The averages of six independent measurements were averaged to determine cell viability and SEAP activity. The IC50 (50% Inhibition Concentration) of metal ions was calculated by fitting the data to a Hill Model (Eq. (1)) using a Microcal Origin® program:

\[ V = V_{max} \left( \frac{x^n}{IC_{50} + x} \right) \]

\( V \) represents relative cell viability calculated by dividing the OD570 value of metal-treated samples over that of control, \( s \) is the concentrations of metal ions and \( n \) is Hill constant.

Statistical differences for SEAP activity at \( P < 0.05 \) were determined using the post hoc Student-Newman-Keuls multiple comparison tests. The differences of reactive oxygen species levels were tested by one-way ANOVA.

3. Results

3.1. Induction of SEAP in HSE-SEAP transformed HeLa cell upon heat shock and incubation with CdCl2

Heat shock and CdCl2 treatment are typical stresses inducing HSP expression. As shown in Fig. 1, significant levels of secreted alkaline phosphatase (SEAP) were detected in HeLa cells treated either by a mild heat shock (42 °C) for 1 h or by incubation of cells with 5 μM CdCl2. All treatments resulted in significant increase of SEAP activity; a recovery period of 24–48 h was found to be preferable for the detection of SEAP in the cultural supernatants.

Induction of the heat shock protein promoted by cadmium chloride was time and concentration dependent (Fig. 1b, Fig. 3a). The SEAP activity was found to increase with incubation time (Fig. 1b); this is in consistency with previous studies (Ait-Aissa et al., 2000, 2003). Therefore, the results above suggested that the
Fig. 2. Effect of metal ions on the viability of HeLa cells. The cells were exposed to serum-free DMEM containing 0, 1, 5, 10, 25, 50, 100, 200 μM metal ions for 24 h. The cytotoxicity was determined by MTT assay as described in Section 2. The results were expressed as mean ± S.D. from six independent experiments.

3.4. Reactive oxygen species induced by metals

Superoxide anion induction was found to vary among the metals tested. CdCl₂, HgCl₂, Pb(NO₃)₂, and CuCl₂ caused significant increase of superoxide anion levels with the elevated concentration (Fig. 4a). CdCl₂ had the strongest effect on superoxide anion induction. No significant changes were observed for cells incubated with MnCl₂, ZnSO₄, NiSO₄, and Al₂(SO₄)₃ (data not shown).

As shown in Fig. 4b, hydrogen peroxide increased significantly when cells were incubated with CdCl₂, HgCl₂, or Pb(NO₃)₂ at concentrations above 25 μM. No significant elevation of hydrogen peroxide level was observed in cells treated by CuCl₂, Al₂(SO₄)₃ and MnCl₂ and ZnSO₄ at 0–200 μM. Above 25 μM CdCl₂, HgCl₂, Pb(NO₃)₂ caused obvious cell damage with apoptotic cell morphology.

3.5. Effect of hydrogen peroxide on SEAP

To clarify whether hydrogen peroxide can induce heat shock protein in HeLa cells, various concentrations of hydrogen peroxide (0–100 μM, which is within the sublethal concentrations determined by MTT assay) were tested for the induction of HSE-SEAP. No SEAP induction was observed after 4 h incubation at 37 °C (data not shown), suggesting that hydrogen peroxide may not directly cause the heat shock response.
4. Discussion

Heat and cadmium chloride treatment have been reported to significantly increase cellular heat shock protein (Ritossa, 1962; Somji et al., 1999a, 2002; Yiangou et al., 1991). Both treatments were used to validate the effectiveness and reliability of reporter gene method for monitoring heat shock protein (Ait-Aissa et al., 2000). In the present paper, a model was established by transforming a commercial available HSE-SEAP plasmid into HeLa cells. The tests of SEAP induction upon heat shock or incubation with cadmium chloride (Fig. 1) demonstrated that the present model was feasible for dynamic tracing the level of heat shock protein by determining the activity of SEAP in the culture supernatant; the optimal experimental conditions for the study of heat shock protein activation by metal ions were thus chosen.

Comparing the concentration dependency of metal-induced HSP induction (Fig. 3), cell viability (Fig. 2), and ROS induction (Fig. 4), it can be noted that the decrease of cell viability is related with the increase of cellular hydrogen peroxide; while the expression of HSP increased with the elevation of superoxide anion level. It is also noted that both metal-induced induction of HSP and elevating of superoxide anion level go up quickly with the increase of metal concentration and approaching a plateau before the decrease of cell viability. Hence, the experimental results suggested that induction of HSP by metal ions was an event below the sub-lethal concentration of metal ions and metal-induced HSP induction was closely related to elevated cellular ROS levels.

Among metal ions tested (Cd\(^{2+}\), Hg\(^{2+}\), Cu\(^{2+}\), Al\(^{3+}\), Ni\(^{2+}\), Zn\(^{2+}\) and Mn\(^{2+}\)), the capacity for metal ions to induce HSP production was shown to vary. Cd\(^{2+}\) was shown to be the strongest HSP inducer; Hg\(^{2+}\) and Pb\(^{2+}\) also induced significant HSP elevation. The effects of Cu\(^{2+}\), Al\(^{3+}\) and Ni\(^{2+}\) were relative weak; Zn\(^{2+}\) or Mn\(^{2+}\) were not observed to induce HSP production even up to 200 µM. Hence, no physicochemical properties of metal ions could explain well this difference among the metals, which mechanism should be an interesting topic for the further studies. In addition, it was observed that a slightly decrease of SEAP activity at the concentration of ∼25 µM (Fig. 3a and c), at which the cell viability
Fig. 4. Level of reactive oxygen species in the HeLa cells upon incubation with metal ions for 4 h at 37°C. (a) Level of superoxide anion determined by NBT reduction method; (b) level of hydrogen peroxide determined by DCFH staining flow cytometry. Results were average of three independent experiments. Data with significant difference from the control (P<0.05) were marked with *.

...started to decrease. This decrease of HSP expression may be due to data fluctuation; however, it is also possible that the potential change of cell viability might constrict the expression of HSE-SEAP plasmid, which mechanism needs to be further investigated.

It has been proposed that reactive oxygen species are closely associated with the induction of heat shock protein (Flanagan et al., 1998; Gorman et al., 1999). In cells treated with heat stress, good correlation between induction of HSP and level of superoxide anion was observed (Marini et al., 1996; Schoeniger et al., 1994). As described above, metal ions induced HSP production and elevated the levels of both superoxide anion and hydrogen peroxide. However, hydrogen peroxide was shown to be related with the decrease of cell viability and exhibited no significant effects on HSP production. To test the possible correlation, the metal-induced HSP was plotted against the level of superoxide anion (Fig. 5). It was shown that under the sub-lethal concentrations of metal ions (<50 μM), HSP induction exhibited a linear relationship with the level of superoxide anion and the overall correlation coefficient was 0.8690 (P<0.05) (the correlation coefficient was greater than 0.95 for Cd²⁺, Hg²⁺, Pb²⁺ and Cu²⁺ if the metals were plotted individually). Hence, it is confirmed that superoxide anion was an important factor involved in metal-induced heat shock protein and HSP may be an indicator for early stage elevation of superoxide anion.

Three possible mechanisms accounting for the correlation of HSP with superoxide anion were: (i) metal ions induce superoxide anion (\(\cdot O_2^-\)), and then superoxide anion induces HSP; (ii) metal ions induce one unknown cell response, which stimulate HSP and superoxide anion in the same time; (iii) metal ions induce superoxide anion and meanwhile some induce HSP. The third hypothesis can be excluded because a negative correlation would be expected by this mechanism. With the second hypothesis, an unknown cell response would be expected to simultaneously stimulate a protective action (HSP) and a damage action (\(\cdot O_2^-\)); thus, this hypothesis was also unreasonable. Therefore, it is most possible that metal ions may induce heat shock protein by elevating cellular superoxide anion level.

It would be an interesting topic for further investigation that how metal ions induce elevating cellular superoxide anion level at the sub-lethal concentrations. Since superoxide anion has been reported to be primarily generated by electron leaking from mitochondrion respiratory chain (Genova et al., 2001; Raha and Robinson, 2000; Turrens, 2003) and decomposed by the action of superoxide dismutase (SOD) enzymes (Tyler, 1975), e.g. Cu, Zn-SOD, it is possible that metal ions may inhibit SOD activity or bind to mitochondrial respiratory enzymes to stimulate electron leakage. If this is true,
then the induction of HSP may serve as a mark for early mitochondrial damage.

In recent years, reporter genes have received considerable attention for their high sensitivity, reliability, convenience, dynamic detection and adaptability to high-throughput assays (Naylor, 1999). The linear correlation of HSP and superoxide anion (Fig. 5) indicated the great potential to establish novel methods based on the HSE-SEAP and other HSP-responding reporter genes system for monitoring the dynamic level of superoxide anion at a very early stage before decrease of cell viability was observed. Since secreted alkaline phosphatase was detected in the cultural supernatants (Gregory et al., 1994; Schlatter et al., 2002), cells breakage step can be skipped in HSE-SEAP-based assays. This should give additional merits over the chemical staining methods for superoxide anion.

In summary, the effects of metal ions on HSP and ROS induction were studied based on the HeLa cell model transformed by an HSE-SEAP reporter gene. It was shown that metal ions induced HSP in both time- and concentration-dependent manner, but the capacity was different for individual metal ions. The HSP induced by metal ions was found to linearly relate to the cellular superoxide anion before cytotoxic effects were observed, suggesting that metal ions might induce heat shock protein by elevating cellular superoxide anion level. The present work supports use of HSE-SEAP system (or other HSP-responding reporter genes) for monitoring the dynamic level of superoxide anion as well as metal-induced oxidative stress at early stages of metal cytotoxicity.

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References


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