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LABORATORY STUDY

Protective Effect of $SnCl_2$ on $K_2Cr_2O_7$-Induced Toxicity in LLC-PK1 Cells

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Abstract

The exposure to hexavalent chromium is often known to cause acute renal failure. It has been found that nonenzymatic antioxidants and the induction of heme oxygenase 1 have protective effects against nephrotoxicity induced by potassium dichromate in vivo. In this work, the effect of stannous chloride, an inducer of heme oxygenase 1, on potassium dichromate-induced toxicity in proximal tubular epithelial cells was studied. Hexavalent chromium levels, peroxynitrite content, reduced thiol content, heme oxygenase activity, reactive oxygen species production, and stannous chloride scavenging capacity were measured. It was found that stannous chloride protects proximal tubular epithelial cells from potassium dichromate-induced cell death. The decrease in extracellular and intracellular hexavalent chromium concentration, the induction of heme oxygenase 1, and the ability to scavenge reactive oxygen species and peroxynitrite are involved in the mechanism by which stannous chloride protects proximal tubular epithelial cells from potassium dichromate-induced toxicity.

Keywords: kidney damage, heavy metal toxicity, reactive oxygen species, stannous chloride, heme oxygenase

INTRODUCTION

Hexavalent chromium [Cr(VI)] is an environmental pollutant that is generated by chrome plating, stainless steel, and other industrial processes. Cr(VI) is known to cause allergic dermatitis, carcinogenicity, and acute renal failure (ARF) in humans and in animals.[1,2] Reactive intermediates and free radicals generated during reduction process might be responsible for Cr(VI) toxicity.[3,4] $K_2Cr_2O_7$ induces oxidative stress and nitrosative stress.[2,5,6] Antioxidants prevent the $K_2Cr_2O_7$-induced kidney damage in vivo[7–9] and in vitro.[10] On the other hand, stannous chloride ($SnCl_2$) is a reducing chemical agent used in several man-made products (conserve soft drinks and some fluoride toothpastes). $SnCl_2$ is a potent inductor of heme oxygenase-1 (HO-1) in acute expose in rat and rabbit,[2,5,11] but induce kidney damage in chronic exposure in rabbit.[12] Previous studies have documented induction of HO-1 in proximal tubuli in response to $SnCl_2$ and the protective effect in $K_2Cr_2O_7$-induced ARF in Wistar rats.[2,5] Therefore, in an attempt to go deeper in the understanding about the protection of $SnCl_2$ of Cr(VI) toxicity, in this study proximal tubular epithelial LLC-PK1 cells were used. Hence, the effect of $SnCl_2$ on $K_2Cr_2O_7$-induced cell death, HO activity, cellular Cr(VI) accumulation, reduced thiol content, and superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), and peroxynitrite ($ONOO^-$) production in LLC-PK1 cells as well as the effect of $K_2Cr_2O_7$ on reactive oxygen species (ROS) and Cr(VI) content in culture medium were determined. Furthermore, it was studied whether $SnCl_2$ is able to scavenge $O_2^{\bullet -}$, H_2O_2, $OH^{\bullet}$, and $ONOO^-$.

RESEARCH DESIGN AND METHODS

Reagents

The porcine kidney tubular epithelium cell line (LLC-PK1) was obtained from the American Type

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Culture Collection (ATCC, Manassas, VA, USA). $SnCl_2$, $K_2Cr_2O_7$, hydrogen peroxide (H_2O_2), and glutaraldehyde were obtained from JT Baker (Xalostoc, Edo. México, México). Hemin, stannous (II) protoporphyrin IX (SnPP), and cobalt (II) protoporphyrin IX (CoPP) were obtained from Porphyrin Products Inc. (Logan, UT, USA). Dihydrorhodamine 123 (DHR 123) was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). *N*-acetyl cysteine (NAC), glutathione (GSH), crystal violet, Tris-HCl, ethylenediaminetetraacetic acid (EDTA), Triton X-100, nitroblue tetrazolium (NBT), butylated hydroxytoluene, xylenol orange, ammonium ferrous sulfate, deoxyribose, ascorbic acid, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), diphenyl carbazide (DFC), xanthine oxidase (XO), $FeCl_3$, xanthine, sodium pyruvate ebselen, dimethyl thiourea (DMTU), tempol, and 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Gibco, Invitrogen Corporation (Grand Island, NY, USA).

Cell Culture

LLC-PK1 cells were cultured in 10-cm dishes with DMEM, supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C under 5% CO_2 humidified atmosphere. The cells were plated in 96-well plates or 6-cm dishes and incubated for 24 h to confluence, and the experimental measurements were performed.

Study Design

To evaluate the protective effect of $SnCl_2$ on the $K_2Cr_2O_7$-induced toxicity, DMEM without FBS was used. Seven groups of conditions were studied ($n = 3$ per group, the experiments were performed three times at different days): (i) control cells, incubated with medium; (ii) $K_2Cr_2O_7$-treated cells; (iii) $K_2Cr_2O_7$- and $SnCl_2$-treated cells; (iv) $K_2Cr_2O_7$-, $SnCl_2$-, and SnPP-treated cells; (v) $K_2Cr_2O_7$- and SnPP-treated cells; (vi) $SnCl_2$-treated cells; and (vii) SnPP-treated cells. Concentrations used in all different conditions were 30 μM $K_2Cr_2O_7$, 200 μM $SnCl_2$, and 40 μM SnPP. The cells treated with $SnCl_2$ and/or SnPP were incubated for 1 h before the addition of $K_2Cr_2O_7$. The cell groups were further incubated for 24 h with $K_2Cr_2O_7$, and the viability and HO activity were evaluated. Light microscopy micrographs were also taken.

To evaluate the protective effect of antioxidants on $K_2Cr_2O_7$-induced toxicity, DMEM without phenol red and FBS was used. The cells were incubated for 24 h with $K_2Cr_2O_7$ and $SnCl_2$ or $K_2Cr_2O_7$ and with an antioxidant (10 μM NAC or 20 μM GSH), and the experimental measurements were performed. Cell viability, Cr (VI), $ONOO^-$, reduced thiol content, $O_2^{\bullet -}$, H_2O_2, and $OH^\bullet$ in cells, and H_2O_2 in the culture medium were measured. A control group was always included for the studied conditions.

Cell viability measurement

For the determination of cell viability, two methods were carried out; the crystal violet assay is based on the inability of dead cells to remain adherent to cell culture plastic, as previously described.[13] An index of cellular injury was established based on the leakage of lactate dehydrogenase (LDH) from the renal cells into the culture medium. The activity of LDH released from the cells treated with $K_2Cr_2O_7$ was measured using the supernatant obtained after centrifuging the culture medium. LDH activity was determined using a commercial kit (Spinreact, Spain).

A close correlation between both methods (crystal violet and LDH release) for the range of $K_2Cr_2O_7$ concentrations of 0–100 μM ($r = 0.9993$, data not shown) was found. Based on the above information, only the crystal violet method was used to measure cell viability.

ROS and $ONOO^-$ determination

To evaluate ROS or $ONOO^-$ production in our biological model, the culture medium was removed; the cells were washed with 50 mM potassium phosphate buffer, pH 7.0, and briefly sonicated in the same buffer. The cell lysates or the culture medium samples were mixed with the corresponding reaction mixture. The ROS scavenging assays were modified to evaluate ROS production only to measure ROS.

ROS and $ONOO^-$ scavenging activity

Different $SnCl_2$ solutions (0–200 μM) were evaluated for $O_2^{\bullet -}$, H_2O_2, $OH^\bullet$, and $ONOO^-$ scavenging activity. Scavenging capacity was expressed as % and the inhibitory concentration (IC_{50}) value is the concentration of $SnCl_2$ that is able to scavenge or inhibit 50% of the ROS present or generated in each specific assay system.[10]

Superoxide radical

Xanthine–xanthine oxidase system was used to determine the $O_2^{\bullet -}$ in the assay system, and xanthine oxidase activity was measured to evaluate NBT reduction (560 nm) and uric acid production (295 nm), respectively.[10,14] The specificity of the method was validated by adding the $O_2^{\bullet -}$ scavenger tempol (1 mM)[15] to cell lysates or culture medium. In fact, the NBT reduction was completely suppressed by tempol. Scavenging percent was obtained from the optical densities at 560 nm.

Concentrations of $O_2^{\bullet -}$ were determined using a molar extinction coefficient of 24,400 M^{-1} cm^{-1} at 560 nm.[16,17]

Hydrogen peroxide

H_2O_2 was measured by the method described by Long et al.[18] (FOX reagent: butylated hydroxytoluene, xylenol

orange, and ammonium ferrous.). The concentration of H_2O_2 was calculated from a standard curve prepared with increasing H_2O_2 concentrations.[10,14] To validate the specificity of the measurement, the H_2O_2 scavenger sodium pyruvate (1 mM)[19] was added to the standard curve, the cell lysates, and the culture medium. It was found that sodium pyruvate effectively suppressed H_2O_2 detection.

Hydroxyl radical

The $OH^{\bullet}$ was generated with the H_2O_2–EDTA– $FeCl_3$ system and was measured using the degradation of deoxyribose.[20] The degradation of deoxyribose was measured in terms of thiobarbituric acid reactive substances formation.[21] The degradation of deoxyribose was completely suppressed by the $OH^{\bullet}$ scavenger DMTU (50 mM). The specificity of $OH^{\bullet}$ measurement in cell lysates or culture medium was evaluated by the addition of this $OH^{\bullet}$ scavenger.[22] The tubes were read at 532 nm against a standard curve of tetramethoxypropane.[21]

Peroxynitrite

Peroxynitrite was synthesized as previously described.[23] Peroxynitrite scavenging activity was measured by monitoring the oxidation of DHR 123 by a modified method of Haddad et al.[10,14,24] The specificity of $ONOO^-$ measurement in cell lysates was evaluated by the addition of ebselen.[25] In fact, the DHR oxidation was completely suppressed by ebselen. Concentrations of $ONOO^-$ were determined using a molar extinction coefficient of 78,000 M^{-1} cm^{-1} at 500 nm.[24]

Heme oxygenase enzyme activity

Cells microsomes were prepared and used for determination of enzyme activity as described.[2]

Cr(VI) content

Cr(VI) content was determined with diphenyl carbazide (1 volume of 100 mg DFC in 95% ethanol and 1 volume of 10% H_2SO_4) in cell homogenates or culture medium (incubated with or without cells) at 540 nm. For calibration, a commercial 0.1% (w/v) Cr(VI) stock solution was used as standard. The reaction was linear up to 50 nmol Cr(VI).[26]

Reduced thiol content

The cells were sonicated with 0.1 M phosphate buffer, pH 8. For determination of reduced thiol, cellular samples were mixed with 1 mM DTNB, and immediately the optical density at 412 nm was recorded (extinction coefficient of thiol compounds derivates by DTNB used was 13.2 $mM^{-1}cm^{-1}$).[26,27]

Protein content

The protein content was determined according to Lowry, as previously described.[2]

Statistical Analysis

Results are expressed as mean ± SE. Data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett or Bonferroni multiple comparisons post-test as appropriate using the software Prism 3 (GraphPad, San Diego, CA, USA). p-Value <0.05 was considered significant.

RESULTS

Effect of $SnCl_2$ on $K_2Cr_2O_7$-Induced Toxicity on LLC-PK1 Cells

Previous in vivo studies had been shown that $K_2Cr_2O_7$ induced necrosis in 50% of tubuli for 24 h after its administration [2]. In vitro studies found that incubation of LLC-PK1 cells for 24 h with 30 μM $K_2Cr_2O_7$ induced 50% of death. Therefore, this concentration was used to perform this study.

LLC-PK1 cells were incubated for 24 h with 30 μM $K_2Cr_2O_7$ and 200 μM $SnCl_2$. $SnCl_2$ prevented $K_2Cr_2O_7$-induced cell death (Figures 1 and 2B and D). $SnCl_2$-induced HO-1 activity which was blocked by SnPP, an inhibitor of the HO activity (HO-1 and HO-2) (Figure 1). SnPP was able to partially block the protective effect induced by $SnCl_2$ (Figure 1), suggesting that HO-1 is involved, at least in part, in the protective effect against Cr(VI). We evaluated the potential protective effect of CoPP, a more specific HO-1 inductor. It was found that the protection was very small, and the addition of SnPP was able to partially block this small protective effect (data not shown).

Effect of $SnCl_2$ on the $K_2Cr_2O_7$-Induced Changes in LLC-PK1 Cells

The data were compared with two well-known antioxidants (NAC and GSH). It was found that $SnCl_2$ was able to prevent cell death, $ONOO^-$ formation, and the increase in reduced thiol content (Figure 3A). In fact, the amount of

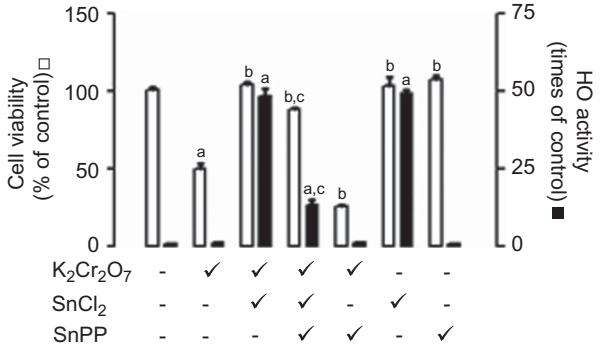

Figure 1. Effect of stannous chloride ($SnCl_2$) on potassium dichromate ($K_2Cr_2O_7$)-induced toxicity and on HO activity in LLC-PK1 cells. $K_2Cr_2O_7$ (30 μM), $SnCl_2$ (200 μM), and stannous protoporphyrin (SnPP, 40 μM) were added for 24 h as indicated above. At the end of this period, viability or HO activity were measured. Data are expressed as mean ± SE, $n = 9$. [a]$p < 0.001$ versus control, [b]$p < 0.001$ versus $K_2Cr_2O_7$, [c]$p < 0.001$ versus $K_2Cr_2O_7$ + $SnCl_2$.

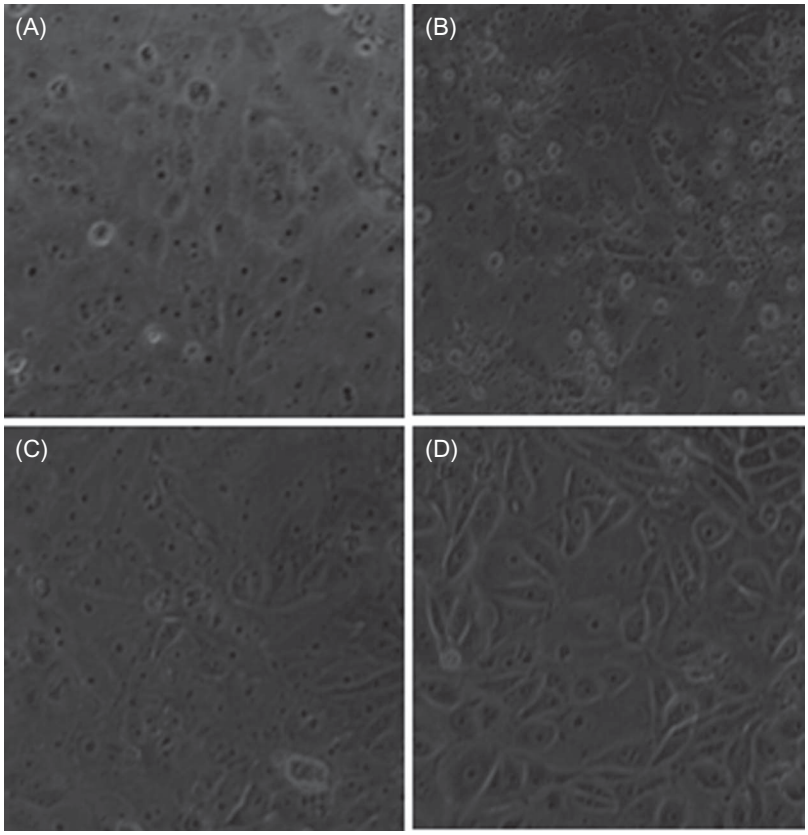

Figure 2. Effect of stannous chloride ($SnCl_2$) on potassium dichromate ($K_2Cr_2O_7$)-induced toxicity on LLC-PK1 cells. The cells were incubated without or with treatment for 24 h at the end of which light microscopy micrographs were taken (200×). (A) Control cells; (B) $K_2Cr_2O_7$ (30 μM)-treated cells; (C) $SnCl_2$ (200 μM)-treated cells; (D) $K_2Cr_2O_7$- and $SnCl_2$-treated cells.

reduced thiol is even lower when cells were co-incubated with $K_2Cr_2O_7$ and $SnCl_2$. $SnCl_2$ was able to prevent the Cr (VI) entrance to the cells (Figure 3A). However, the fact that $SnCl_2$ decreases completely Cr(VI) levels in the culture medium incubated without or with cells (Figure 3B) was a relevant finding. These protective effects were reproduced by NAC and GSH.

Furthermore, $SnCl_2$ was able to prevent $K_2Cr_2O_7$-induced $O_2^{\bullet -}$ and H_2O_2 production and to ameliorate $OH^{\bullet}$ production (Figure 4A) in LLC-PK1 cells. NAC was also able to prevent H_2O_2 and $OH^{\bullet}$ formation but unable to prevent $O_2^{\bullet -}$ formation (Figure 4A). GSH was able to prevent H_2O_2, $O_2^{\bullet -}$, and $OH^{\bullet}$ formation (Figure 4A). $SnCl_2$ ameliorates extracellular H_2O_2 formation, and NAC and GSH were able to completely prevent extracellular H_2O_2 formation (Figure 4B). The protective effect of $SnCl_2$ against extracellular H_2O_2 formation was evident even in the absence of cells (Figure 4B).

Scavenging Activity of $SnCl_2$

$SnCl_2$ was able to scavenge $O_2^{\bullet -}$, H_2O_2, $OH^{\bullet}$, and $ONOO^-$ in a concentration-dependent way (Figure 5). IC_{50} values of $SnCl_2$ were the following: 2.19 μM for $O_2^{\bullet -}$, 8.37 μM for H_2O_2, 9.08 μM for $OH^{\bullet}$, and 40.10 μM for $ONOO^-$.

DISCUSSION

$K_2Cr_2O_7$ was able to induce cell death, ROS, and $ONOO^-$ production, suggesting that LLC-PK1 cells may be a suitable model to study the mechanism by which cells may be protected against $K_2Cr_2O_7$-induced toxicity. $SnCl_2$ was able to protect LLC-PK1 cells against $K_2Cr_2O_7$-induced cell death. In spite the fact that $SnCl_2$ was able to increase HO activity, the inhibition of this enzyme with SnPP was unable to completely prevent the protection induced by $SnCl_2$, suggesting that additional mechanisms of protection are involved in this particular cell type of porcine origin. The pre-induction of HO-1 prevents the kidney damage in rat.[2,5] In contrast, in this work was found that HO-1 induction is partially involved in the protective effect of $SnCl_2$, which was confirmed using the specific HO-1 inductor CoPP. This may be due to the differences in both studies, that is, in vivo versus in vitro conditions and the species studied, that is, rat versus pig. In addition, it is possible that products of HO activity (such as biliverdin/bilirubin and carbon monoxide) generated in proximal tubuli in the in vivo study may exert beneficial effects in another cell types in kidney inducing recovery of glomerular function of rats.[2,5] In contrast, this type of cooperation is not possible in the cells in culture.

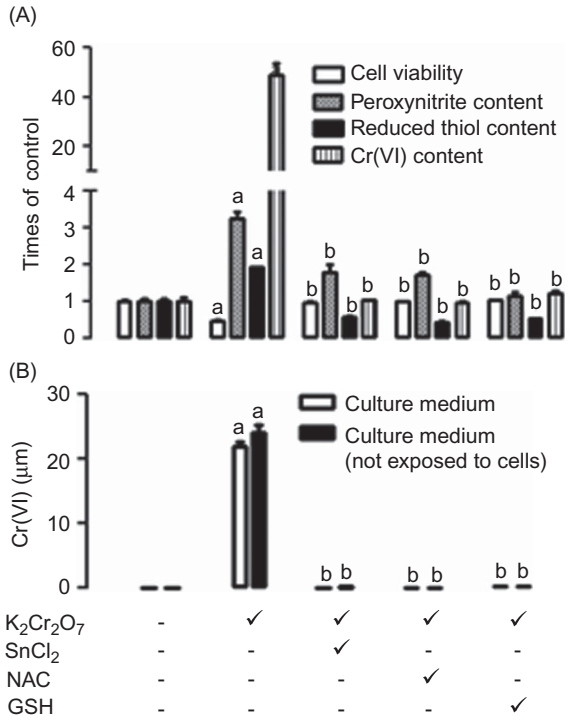

Figure 3. Comparative effect of stannous chloride ($SnCl_2$, 200 μM), *N*-acetyl cysteine (NAC, 10 μM), and glutathione (GSH, 20 μM) on potassium dichromate ($K_2Cr_2O_7$, 30 μM) induced changes on (A) cell viability, (A) peroxynitrite production, (A) reduced thiol content, (A) intracellular content of Cr(VI), (B) extracellular concentration of Cr(VI) in culture medium and (B) concentration of Cr(VI) in culture medium not exposed to cells. Data are expressed as mean ± SE, $n = 9$. [a]$p < 0.001$ versus control, [b]$p < 0.001$ versus $K_2Cr_2O_7$.

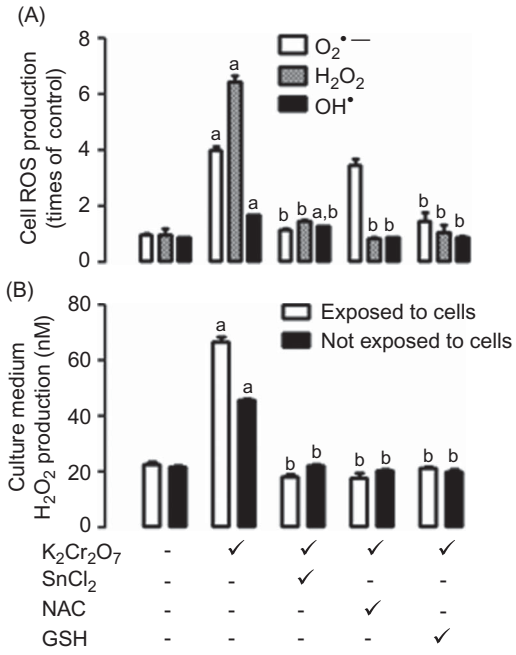

Figure 4. Comparative effect of stannous chloride ($SnCl_2$, 200 μM), *N*-acetyl cysteine (NAC, 10 μM), and glutathione (GSH, 20 μM) on potassium dichromate ($K_2Cr_2O_7$, 30 μM) induced changes on (A) superoxide ($O_2^{\bullet -}$) production, (A) hydrogen peroxide (H_2O_2) production, (A) hydroxyl radical ($OH^{\bullet}$) production, (B) extracellular hydrogen peroxide (H_2O_2) production, and (B) hydrogen peroxide (H_2O_2) production in culture medium not exposed to cells. Data are expressed as mean ± SE, $n = 9$. [a]$p < 0.001$ versus control, [b]$p < 0.001$ versus $K_2Cr_2O_7$.

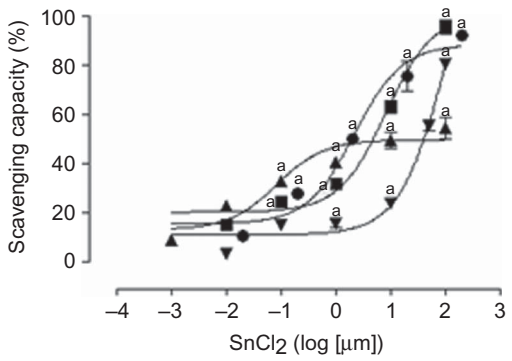

Figure 5. Scavenging activity of $SnCl_2$. ●, superoxide; ■, hydrogen peroxide; ▲, hydroxyl; ▼, peroxynitrite. Data are expressed as mean ± SE, $n = 9$. [a]$p < 0.001$ versus 0.

The $K_2Cr_2O_7$-induced increase in intracellular ROS and $ONOO^-$ in LLC-PK1 is associated with the increase in the intracellular Cr(VI) content and its decrease in the culture medium. Furthermore, ROS increase is associated with the increase in reduced thiol content.

The protective effect of $SnCl_2$ was associated with its ability to decrease extracellular and intracellular Cr(VI) concentrations as well as with the prevention of ROS and $ONOO^-$ production and in the increase in intracellular reduced thiol content. The prevention of intracellular Cr (VI) concentration is secondary, in all probability, to $SnCl_2$-induced decrease in extracellular Cr(VI) concentration. We are tempting to speculate that $SnCl_2$ reduces Cr(VI)-generating non-cell membrane permeant chromium species. In fact, it has been shown that reduced species of chromium such as Cr(III) are non-cell membrane permeant.[27,28] In addition, we are tempting to speculate that the preventive effect of $SnCl_2$ on $K_2Cr_2O_7$-induced ROS production may be secondary to its ability to decrease intracellular Cr(VI) content. $SnCl_2$ was also able to prevent extracellular H_2O_2 production, which may also be involved in its protective effect. However, H_2O_2 generated in the culture medium in our assay conditions is not enough to induce cell death; in fact, damage to LLC-PK1 cells has been observed with 100-fold concentrations of H_2O_2.[29,30] In addition, for the first time, this work showed that the ROS scavenging ability of $SnCl_2$ also may contribute to decrease ROS levels.

In addition, it was found that the antioxidants NAC and GSH induced similar effects to that observed with $SnCl_2$.

In conclusion, the decrease in extracellular and intracellular Cr(VI) concentrations, the induction of HO-1, and the ability to scavenge ROS and $ONOO^-$ are involved in the mechanism by which $SnCl_2$ protects LLC-PK1 cells from $K_2Cr_2O_7$-induced toxicity.

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