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Chromium (VI) induces insulin resistance in 3T3-L1 adipocytes through elevated reactive oxygen species generation

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Abstract
Reactive oxygen species (ROS) have been proposed to be involved in the development of insulin resistance, although the exact molecular link between ROS and insulin resistance remains to be determined. Chromium (Cr(VI)) is known as an inducer of ROS. Therefore, this study examined whether Cr(VI) could induce insulin resistance. It demonstrated that Cr(VI) treatment significantly inhibited insulin-stimulated glucose uptake and attenuated insulin signalling. Moreover, Cr(VI) treatment markedly increased the intracellular levels of superoxide anion, hydrogen peroxide and hydroxyl radical. N-acetylcysteine, superoxide dismutase and catalase can block the ROS generation and alleviate the insulin resistance induced by Cr(VI) treatment. In addition, Cr(VI) treatment induced endoplasmic reticulum (ER) stress and JNK activation and these effects were diminished by N-acetylcysteine. These results suggested that ROS generation through Cr(VI) treatment cause ER stress, JNK activation and insulin resistance in adipocytes. Therefore, the oxidative stress could be a potential interventional target for insulin-resistance related diseases.

Keywords: *Cr(VI), insulin resistance, ROS, ER stress, JNK*

Abbreviations: *CM-DCF-DA, 5 (and 6)-chloromethyl-2′,7′-dichlorofluorescin diacetate; Cr(VI), chromium(VI); DMPO, 5,5-dimethyl-1-pyrroline N-oxide; DPI, diphenyleneiodonium; ER, endoplasmic reticulum; ESR, electron spin resonance; FACS, fluorescence-activated cytometry sorter; FBS, foetal bovine serum; IR, insulin receptor; JNK, c-Jun N-terminal kinase; NAC, N-acetyl-L-cysteine; NADPH, β*-nicotinamide adenine dinucleotide phosphate; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SOD, superoxide dismutase.

Introduction

Insulin resistance is a manifestation of type 2 diabetes mellitus. It usually precedes and predicts the development of type 2 diabetes even under normoglycemic conditions [1]. For example, insulin resistance can be found in lean, normoglycemic offspring of patients with type 2 diabetes and they have a high risk of developing diabetes later in life [2]. Although there are multiple prevailing hypotheses about the cellular/molecular mechanisms of insulin resistance, such as glucolipotoxicity [3,4], inflammation [5,6], adipokine [7], mitochondrial dysfunction [8,9], oxidative stress [10] and ER stress [11,12], etc., the primary cause and cellular mechanisms responsible for this abnormality are still not fully understood. The relative importance of these individual events and the interplay between them remain to be determined.

The reactive oxygen species (ROS) have been proposed to be involved in insulin resistance development. Previous studies showed that the oxidative

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stress markers are up-regulated in diabetes and the depletion of antioxidants is associated with decreased glucose uptake and insulin insensitivity in human populations [13,14]. Conversely, antioxidants, scavengers or over-expression of antioxidant enzymes can improve glucose homeostasis and insulin sensitivity [15–17]. However, the molecular mechanism of how ROS leads to insulin resistance is still unclear.

Many metals and metal-containing particles, such as chromium(VI) (Cr(VI)), nickel, cobalt, cadmium and silica, are known carcinogens [18]. Although the mechanisms of their carcinogenesis are not clear yet, it is generally believed that ROS play a crucial role. The reduction of Cr(VI) to Cr(V) in cells can generate a whole spectrum of ROS including superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$), which in turn elicit DNA damage, lipid peroxidation and activation of nuclear factor-κB (NF-κB) and p53 signalling. The role of Cr(VI) and/or ROS it elaborated in the initiation of insulin resistance is relatively under-documented in the literature. In the present study, we examine whether Cr(VI) can induce the production of free radicals and whether this condition affects the insulin signalling pathway and results in insulin resistance in 3T3-L1 adipocytes. Our results showed that Cr(VI) treatment significantly increased intracellular $O_2^{\bullet -}$, H_2O_2 and $^{\bullet}OH$ generation detected by electron spin resonance (ESR) spin trapping, confocal microscope and fluorescence activated cell sorting (FACS). Meanwhile, insulin stimulated glucose uptake and insulin signalling pathway were both inhibited after Cr(VI) treatment. Furthermore, Cr(VI) also induces ER stress and activates the JNK pathway. All these effects induced by Cr(VI) treatment were diminished by antioxidants including N-acetylcysteine (NAC), superoxide dismutase (SOD), catalase and diphenyleneiodonium (DPI), suggesting that ROS generated through Cr(VI) reduction are responsible to ER stress, JNK activation and insulin resistance in 3T3-L1 adipocytes. These data support the concept that Cr(VI) can induce ROS and cause insulin resistance and provide a potential molecular mechanism by which ROS cause cellular insulin resistance.

Materials and methods

Chemicals and reagents

2-Deoxy-d-[^{3}H]glucose was from GE Healthcare Bio-Sciences (Little Chalfont, UK). 2-Deoxy-d-glucose, potassium dichromate (Cr(VI)), NADPH, sodium formate, insulin, isobutylmethylxanthine, dexamethasone, NAC, DPI, 5, 5-dimethyl-1- pyrroline N-oxide (DMPO), SOD and catalase were from Sigma (St. Louis, MO). CM-DCF-DA and dihydroethidium were from Molecular Probes (Eugene, Oregon). Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS) and calf serum were obtained from Invitrogen (Carlsbad, CA). Antibodies against phospho-IR (Tyr^{1146}), phospho-Akt (Ser^{473}), phospho-Gsk-3β (Ser^{9}), phospho-p70S6K (Thr^{389}), phospho-c-Jun (Ser^{63}), phospho-JNK (Thr^{183}/Tyr^{185}) and phospho-eIF2α (Ser^{51}) were from Cell Signaling Technology (Beverly, MA). Anti Bip antibody was from Stressgen (Victoria, BC, Canada). SP600125 was from Calbiochem (San Diego, CA).

Cell culture

3T3-L1 cells were cultured in DMEM supplemented with 10% calf serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin. Cells were maintained at 37°C in a 10% CO_2 incubator. Differentiation medium (DMEM supplemented with 10% FBS, 0.11 mg/ml isobutylmethylxanthine, 1 μg/ml insulin, 250 nM dexamethasone) was added to cells 2 days after reaching confluence (Day 0). On Day 2, the medium was replaced with DMEM with 10% FBS and 1 μg/ml insulin. After Day 4, cells were maintained in DMEM plus 10% FBS with a media change every other day until experimental treatments were initiated.

Glucose uptake assay

Glucose uptake was determined using the method described previously with minor modifications [19]. In brief, cells were incubated for 20 min at 37°C in the absence or presence of 100 nM insulin in Krebs-Ringer phosphate buffer (128 mM NaCl, 4.7 mM KCl, 5 mM NaH_2PO_4, 5 mM Na_2HPO_4, 1.25 mM $MgSO_4$, 1.25 mM $CaCl_2$, pH 7.4). Then [^{3}H]-2-deoxyglucose and 2-deoxyglucose were added to a final concentration of 1 μCi/ml and 0.1 mM, respectively, and incubated for 10 min. After washing with ice-cold phosphate-buffered saline three times, the cells were lysed with 0.2 M NaOH for 30 min. The radiolabelled glucose in lysates was measured in a high flash-point scintillation cocktail (PerkinElmer) using a liquid scintillation counter.

Western blot

3T3-L1 adipocytes were pre-treated with or without 25 μM Cr(VI) in the presence or absence of the indicated antioxidant for 8 h, then directly harvested to detect the JNK activation or ER stress markers by western blot or then washed twice with DMEM and maintained in the serum-free DMEM to detect insulin signalling. After serum starvation for 2 h, cells were washed with Krebs-Ringer phosphate buffer twice and stimulated with or without insulin (100 nm) for 10 min and then harvested for western blot. Total cell lysates from each sample were resolved by SDS-PAGE and transferred electrophoretically to PDVF membranes. The resulting membranes were

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hybridized with indicated antibodies. After incubation with appropriate horseradish peroxidase-conjugated secondary antibody, the immune complexes were detected using chemiluminescence reagents (Pierce) and autoradiography. Protein quantification was performed using Quantity One software (Bio-Rad).

ESR measurements

ESR spin trapping technique was used to detect short-lived free radical intermediates. This technique involves the addition-type reaction of a short-lived radical with a paramagnetic compound (spin trap) to form a relatively long-lived free radical product (spin adduct), which can then be studied using conventional ESR. The spin trapping is a method of choice for detection and identification of free radical generation because of its specificity and sensitivity. The intensity of the signal is used to measure the amount of short-lived radicals trapped and the hyperfine couplings of the spin adduct are generally characteristics of the original trapped radicals. All measurements were conducted with an EMX-E-8/2.7 ESR spectrometer (Bruker) and a capillary assembly. 3T3-L1 cells at 10^6 cells/ml were mixed with 100 mm DMPO, with or without Cr(VI) and/or the indicated antioxidants as in eppendorf tubes at a final volume of 60 μl and then the samples were immediately transferred to a capillary for ESR measurement. The WIN EPR Acquisition program was employed for data acquisition and analysis.

Cellular superoxide ($O_2^{\bullet -}$) and H_2O_2 assay

CM-DCF-DA and dihydroethidium were used to monitor H_2O_2 and $O_2^{\bullet -}$ levels inside the cells according to previous studies [20,21]. Dihydroethidium and CM-DCF-DA were dissolved in DMSO to the concentration of 2 mM and 5 mM and kept at −20°C. For cellular imaging, the cells (2×10^5/well) were plated onto a glass slip in the 6-well plate. After treated with or without 25 μM Cr(VI) and/or the indicated antioxidants for 15 min, CM-DCF-DA (5 μM) or dihydroethidium (2 μM) was added into the culture medium and incubated at 37°C for another 15 min. Then, the cells were washed once with PBS and fixed with 4% formalin in PBS. The slip was mounted on a glass slide and observed using a confocal microscope. For FACS analysis, about 5×10^6 cells were plated in 10-cm dishes for 16 h and then treated with or without 25 μM Cr(VI) and/or the indicated antioxidants for 15 min. CM-DCF-DA (5 μM) was added into the culture medium and incubated at 37°C for another 15 min, then the cells were washed once with PBS and harvested for FACS analysis.

Statistics

Data are expressed as mean ± SD of at least three independent experiments except indicated. Student's *t*-test was used for statistical analysis. Differences were considered statistically significant at $p < 0.05$.

Results

Cr(VI) treatment reduces insulin-stimulated glucose uptake in 3T3-L1 adipocytes

To test the effect of Cr(VI) on insulin sensitivity, 3T3-L1 adipocytes were treated with or without Cr(VI) for 8 h and then stimulated with insulin. As shown in Figure 1, acute insulin stimulation induced an ~10-fold increase in glucose uptake. When the cells were exposed to different concentration of Cr(VI), glucose uptake was dramatically inhibited. The insulin-dependent glucose uptake was decreased by 50–80%, exhibiting a dose-dependent effect. The diagram shows one representative result of three independent experiments performed in triplicate (Figure 1).

Cr(VI) treatment inhibits insulin signalling pathway in 3T3-L1 adipocytes

To further investigate whether Cr(VI) treatment affects insulin signalling pathway, 3T3-L1 adipocytes pre-treated with or without 25 μM Cr(VI) for 8 h were stimulated with insulin and then harvested for western blot. As shown in Figure 2A, insulin stimulated its signalling including the phosphorylation of IR, Akt, Gsk-3β and p70S6K and Cr(VI) treatment significantly inhibited phosphorylation of IR, Akt, Gsk-3β and p70S6K stimulated by insulin.

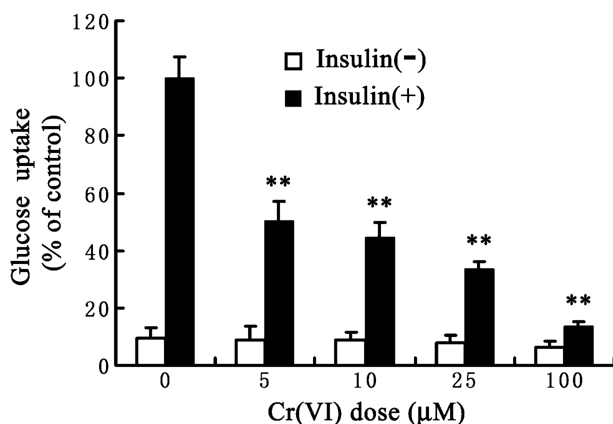

Figure 1. Cr(VI) inhibited insulin-stimulated glucose uptake in 3T3-L1 adipocytes. Basal or insulin-stimulated glucose uptake in 3T3-L1 adipocytes with or without Cr(VI) pre-treatment at the indicated concentrations for 8 h were determined by glucose uptake assay. **$p < 0.01$, compared to the insulin-stimulated cells without Cr(VI) pre-treatment. In this and all other figures, error bars represent SD.

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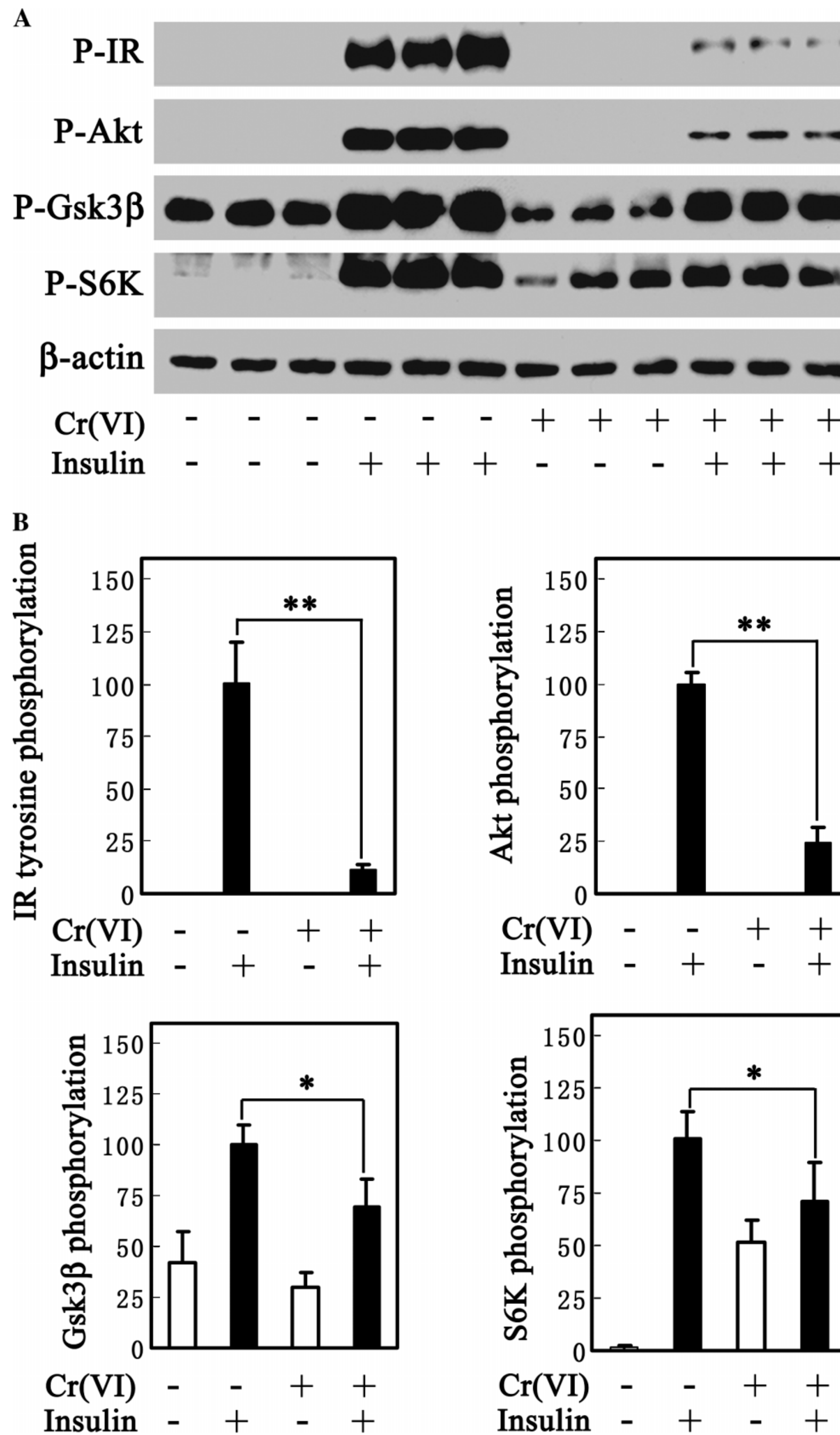

Figure 2. Cr(VI) attenuated insulin signalling in 3T3-L1 adipocytes. (A) Cr(VI) significantly inhibited insulin signalling, including the phosphorylation of IR, Akt, Gsk-3β and S6K, in 3T3-L1 adipocytes. After pre-treatment with or without 25 μM Cr(VI) for 8 h, the cells were stimulated with insulin for 10 min and then harvested for western blot. (B) Signals were quantified by densitometry and normalized to β-actin levels. *$p < 0.05$, **$p < 0.01$.

By quantification, the phosphorylation levels of IR, Akt, GSK-3β and p70S6K were all significantly decreased, as shown in Figure 2B. These results indicated that the Cr(VI) treatment impairs the insulin signalling pathway in 3T3-L1 adipocytes.

Cr(VI) induces the generation of $^{\bullet}OH$, $O_2^{\bullet -}$ *and* H_2O_2

Previous studies have shown that after entering the cell through an anion transport system, Cr(VI) is able to generate a whole spectrum of ROS [22,23]. We therefore examined ROS generation in Cr(VI)

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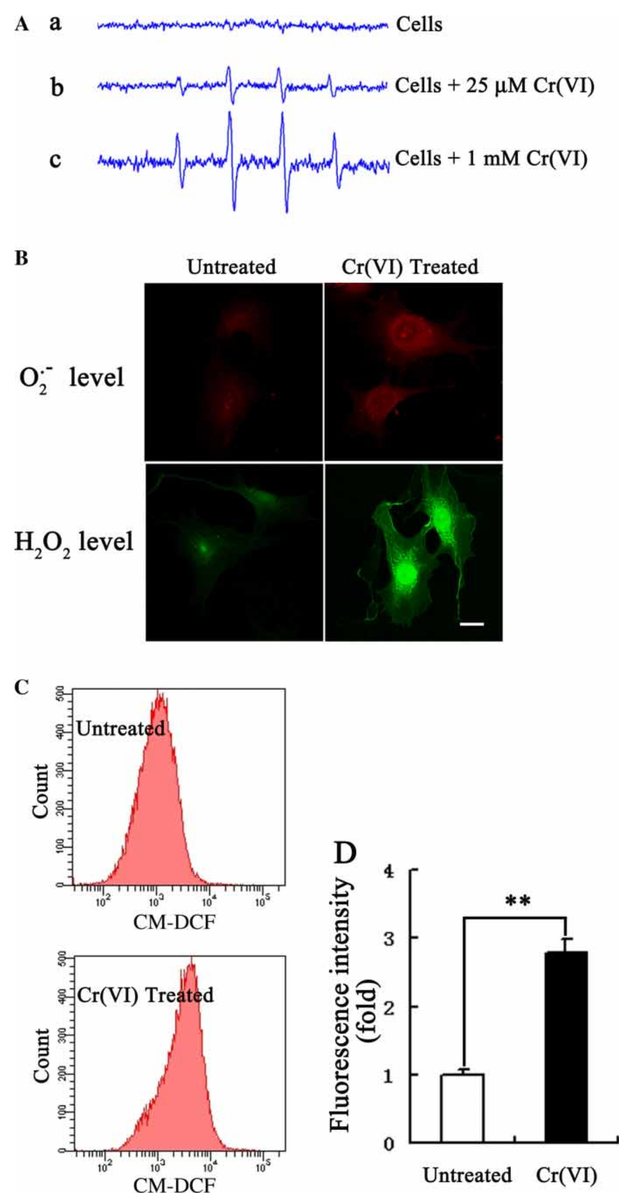

Figure 3. Measurements of Cr(VI)-induced ROS generation in 3T3-L1 cells. (A) Cr(VI) induced the generation of •OH in 3T3-L1 cells. ESR spectrum was recorded 2 min after reaction initiation from a mixture containing 1×10^6 3T3-L1 cells/ml and 100 mM DMPO, with or without Cr(VI) as indicated. DMPO was employed as the trapping reagent for •OH. (B) Cr(VI) stimulated the generation of $O_2^{\bullet -}$ and H_2O_2 in 3T3-L1 cells. To detect the intracellular level of $O_2^{\bullet -}$ and H_2O_2, 3T3-L1 cells were loaded with dihydroethidium or CM-DCF-DA and treated with or without Cr(VI) for 30 min. The fluorescence intensity in the cells observed under a confocal microscope represents the level of $O_2^{\bullet -}$ or H_2O_2. Scale bar, 20 μM. (C) The effect of Cr(VI) on intracellular H_2O_2 level was further confirmed by FACS analysis. (D) Quantification of the fluorescence intensity corresponding to (C). $^{**}p < 0.01$.

treated 3T3-L1 cells by several methods. The generation of •OH radicals induced by Cr(VI) was examined using an ESR spin trapping method. Figure 3A(b) show a typical ESR spectrum generated by 3T3-L1 cells with Cr(VI) treatment compared with the cells without Cr(VI) treatment (Figure 3A(a)). The spectrum consists of an 1:2:2:1 quartet. Based on the 1:2:2:1 line shape [22], this spectrum was assigned to the DMPO/•OH adduct, which is evidence of •OH radical generation. When the concentration of Cr(VI) was increased to 1 mM, the generation of •OH was also enhanced as indicated by the heightening of the spikes (Figure 3A(c)). The ability of Cr(VI) to generate $O_2^{\bullet -}$ and H_2O_2 in 3T3-L1 cells was analysed by fluorescent dye hydroethidine and CM-DCF-DA, respectively. Both $O_2^{\bullet -}$ and H_2O_2 were observed inside the untreated cells which indicated the basal generation of ROS (Figure 3B, untreated). In the presence of Cr(VI), both $O_2^{\bullet -}$ and H_2O_2 levels were dramatically elevated (Figure 3B, Cr(VI) treated). The generation of H_2O_2 was also quantified by flow cytometry analysis of the cells stained with CM-DCF-DA. The fluorescence intensity of positive stained cells increased significantly after Cr(VI) treatment (Figure 3C). The fluorescence mean value increased ~2-fold after Cr(VI) treatment (Figure 3D). These data demonstrated that Cr(VI) treatment induced the ROS generation in 3T3-L1 cells.

Antioxidants inhibit ROS generation

NAC, SOD and catalase are established antioxidants. When the antioxidants including NAC, SOD, catalase and sodium formate (a scavenger of •OH radical), were applied to 3T3-L1 cells concomitantly with Cr(VI), the intensity of the spin adduct signals in the Cr(VI)-treated cells was diminished (Figure 4A(a–f)). NADPH oxidase is usually involved in ROS generation. In the presence of DPI, an inhibitor of NADPH oxidase, the intensity of the spin adduct signals in the Cr(VI)-treated cells was also diminished (Figure 4A(g)). NADPH, a cofactor of flavoenzymes including NADPH oxidase, markedly enhanced the generation of the •OH radical (Figure 4A(h)). These data showed that Cr(VI) induced •OH radical generation can be scavenged by antioxidants and could be mediated by NADPH oxidase.

When the antioxidant NAC was coexistent with Cr(VI), the levels of both $O_2^{\bullet -}$ and H_2O_2 upregulated by Cr(VI) treatment were also dramatically decreased (Figure 4B). When the H_2O_2 level was analysed by flow cytometry analysis with the fluorescent probe CM-DCF-DA, the fluorescence in the Cr(VI)-treated cells was almost abolished by NAC (Figure 4C and D). The results further confirmed that Cr(VI) induced ROS generation can be scavenged by antioxidants.

Antioxidants reverse insulin resistance induced by Cr(VI) treatment

The previous sections have shown that Cr(VI) treatment is able to induce insulin resistance in 3T3-L1 adipocytes and ROS levels are increased in 3T3-L1

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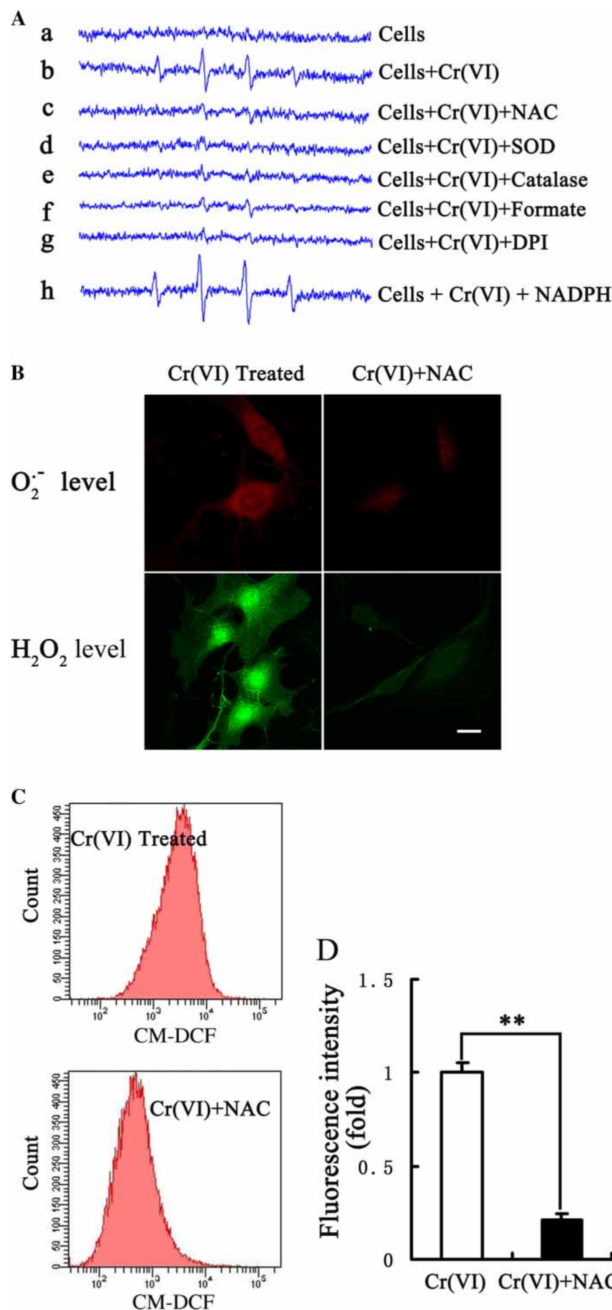

Figure 4. Cr(VI)-induced ROS generation was inhibited by antioxidants. (A) ESR spectrum was recorded 2 min after reaction initiation from a mixture containing 1×10^6 3T3-L1 cells/ml and 100 mM DMPO, with or without 25 μM Cr(VI) and different antioxidants or reagents as indicated. NAC (10 mM), SOD (1000 units/ml), catalase (5000 units/ml), sodium formate (2 mM), DPI (20 μM) and NADPH (1 mM) were used to pre-treat the cells just before the addition of Cr(VI). (B) The generation of $O_2^{\bullet -}$ and H_2O_2 induced by Cr(VI) in 3T3-L1 cells was blocked by NAC. Dihydroethidium and CM-DCF-DA were applied to detect the $O_2^{\bullet -}$ and H_2O_2 levels, respectively. Scale bar, 20 μM. (C) The effect of NAC on H_2O_2 level induced by Cr(IV) was further confirmed by FACS analysis. (D) Quantification of the fluorescence intensity corresponding to (C). $^{**}p < 0.01$.

cells after Cr(VI) treatment. Next, the role of ROS in Cr(VI)-induced insulin resistance were examined in this experimental system. When antioxidants such as NAC, SOD, catalase or DPI were applied to 3T3-L1 adipocytes concomitantly with Cr(VI), the acute insulin-stimulated glucose uptake was partially restored to the level of control (Figure 5A–D). The results suggested that the insulin sensitivity of 3T3-L1 can be reversed when ROS are depleted by antioxidants or ROS production is reduced by specific inhibitors despite the presence of Cr(VI).

ROS induced by Cr(VI) treatment cause the dysfunction of insulin signalling pathway

The above work showed that there is a connection between ROS and insulin sensitivity in 3T3-L1 adipocytes under chronic Cr(VI) treatment. Then we checked the effect of blocking ROS production with NAC on the insulin signalling pathway. In agreement with the above work, activations of insulin receptor, Akt, Gsk-3β and p70S6K were all suppressed after chronic Cr(VI) treatment. When NAC was applied to 3T3-L1 adipocytes together with Cr(VI), the following acute insulin-stimulated activation of the above four key proteins was significantly restored (Figure 6A). Figure 6B shows the quantification results of Figure 6A.

ER stress and JNK activation are involved in the insulin resistance induced by Cr(VI) treatment

The above results established the relationship between ROS and insulin resistance in 3T3-L1 cells. However, the mechanistic link between ROS and insulin resistance remains undetermined. It has been shown recently that ER stress caused hyperactivation of JNK is underlying obesity-related insulin resistance. JNK is known to be activated by oxidative stress [24] and involved in insulin resistance [25] and inhibition of JNK activity through genetic knockout [26], inhibitory peptides [27], antioxidant [16] or other ways [12] improves insulin sensitivity both *in vivo* and *in vitro*. Therefore, we hypothesize that ROS-induced insulin resistance may also be mediated by JNK and tested the JNK activity in our experimental system. Our data showed that JNK and c-Jun were activated in response to Cr(VI) treatment and that these effects were reversed by the antioxidant NAC (Figure 7A and B). Furthermore, Bip and phosphorylated eIF2α, two markers of ER stress, were markedly increased by Cr(VI) treatment and NAC treatment also significantly inhibited the expression of Bip and the phosphorylation of eIF2α (Figure 7A and B). In addition, inhibition of JNK with an inhibitor SP600125 significantly attenuated the effect of Cr(VI) on glucose uptake (Figure 7C). Taken together, our results demonstrated that Cr(VI)-induced ROS lead to ER stress and JNK activation which might be the mechanism of ROS induced insulin resistance.

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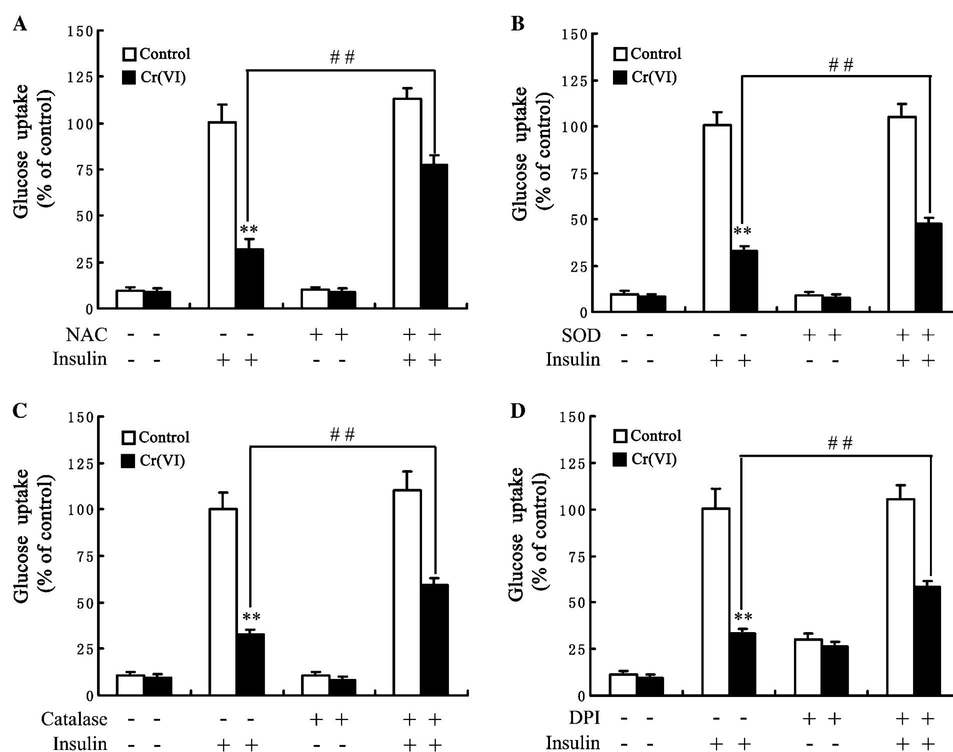

Figure 5. The effect of antioxidants on Cr(VI)-induced insulin resistance. Restoration of Cr(VI)-induced insulin resistance by 10 mm NAC (A), 1000 units/ml SOD (B), 5000 units/ml Catalase (C) and 20 μm DPI (D). After pre-treated with or without 25 μm Cr(VI) and/or the indicated antioxidants for 8 h, basal or insulin-stimulated glucose uptake in 3T3-L1 adipocytes were determined by glucose uptake assay. $^{**}p < 0.01$, compared to the cells only treated with insulin. $^{\#\#}p < 0.01$, significant difference between two groups as indicated.

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Discussion

Reactive oxygen species have been typically viewed as the toxic by-products of metabolism. However, an emerging body of evidence has indicated that reactive species serve as signalling molecules that are involved in the regulation of cellular function [28]. The chronic increased production of these reactive oxygen species and/or a reduced capacity for their elimination, termed oxidative stress, can lead to abnormal changes in intracellular signalling and ultimately result in insulin resistance [14,29]. Lines of evidence show that oxidative stress has been linked to insulin resistance *in vivo*. Furthermore, recent studies have found that this association is not restricted to insulin resistance in type 2 diabetes, but is also evident in obese, non-diabetic individuals [30–32] and patients with metabolic syndrome [6,14,33]. An increased concentration of reactive molecules triggers the activation of serine/threonine kinase cascades such as JNK and NF-κB that in turn phosphorylate multiple targets, including the insulin receptor and the insulin receptor substrate (IRS) proteins. Increased serine phosphorylation of IRS reduces its ability to undergo tyrosine phosphorylation and may accelerate the degradation of IRS-1, offering a feasible explanation for the molecular basis of oxidative stress-induced insulin resistance [34,35].

Cr(VI) compounds are highly toxic and carcinogenic [36]. Several epidemiologic studies have investigated the association between cancer risk and employment in chromium producing and using industries [37,38]. Workers employed in chromate pigment production and possibly spray painters of chromate pigment paints appear to be at excess risk of respiratory cancer. The evidence indicates that the hexavalent form of chromium is the primary agent of chromium carcinogenesis. Previous studies have shown that in cellular systems, Cr(VI) is reduced by certain flavoenzymes such as glutathione reductase to generate Cr(V) [39–41]. During this process, molecular oxygen is reduced to $O_2^{\bullet -}$, which generates H_2O_2 via dismutation. The resultant Cr(V) reacts with H_2O_2 to generate $^{\bullet}OH$ radical via a Fenton-like reaction. Thus, during the one-electron reduction of Cr(VI), a whole spectrum of ROS are generated. To find the direct evidence of the cause–effect relationship between ROS and insulin resistance, Cr(VI) was utilized as a model to generate ROS in the present study. We found that adipocytes treated chronically with Cr(VI) were insulin-resistant as evidenced by impaired glucose uptake and decreased activation of insulin receptor, Akt, Gsk-3β and p70S6K, which is consistent with the results observed previously in 3T3-L1 adipocytes with other inducers [42–44].

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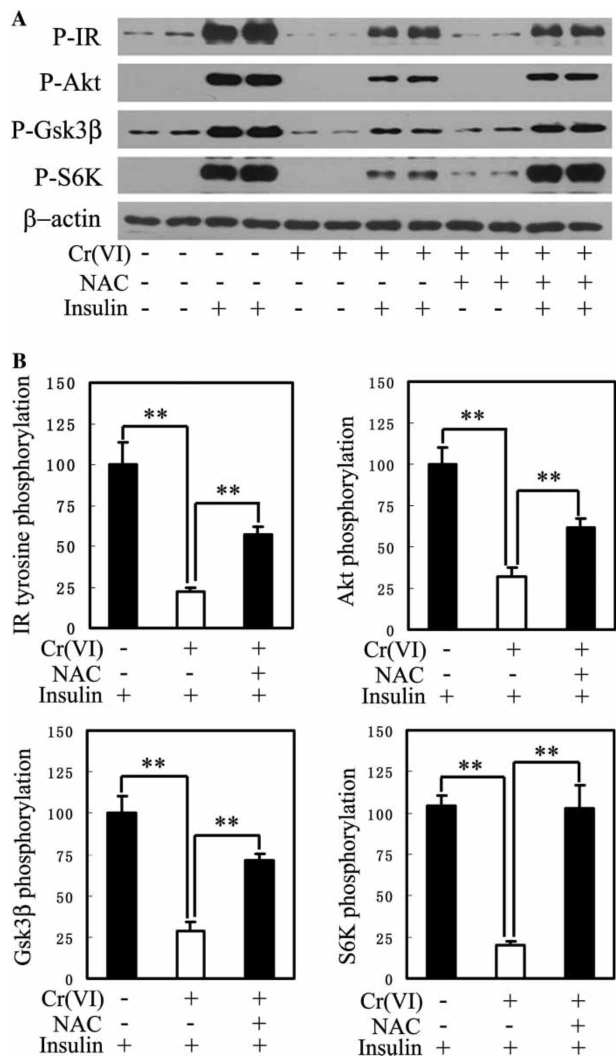

Figure 6. NAC treatment improved insulin signalling affected by Cr(VI). (A) NAC improved insulin signalling, including the phosphorylation of IR, Akt, Gsk-3β and S6K, in 3T3-L1 adipocytes treated with Cr(VI). After pre-treatment with or without 25 μM Cr(VI) and/or 10 mM NAC for 8 h, the cells were stimulated with insulin for 10 min and then harvested for western blot. (B) Signals were quantified by densitometry and normalized to β-actin levels. $^{**}p < 0.01$.

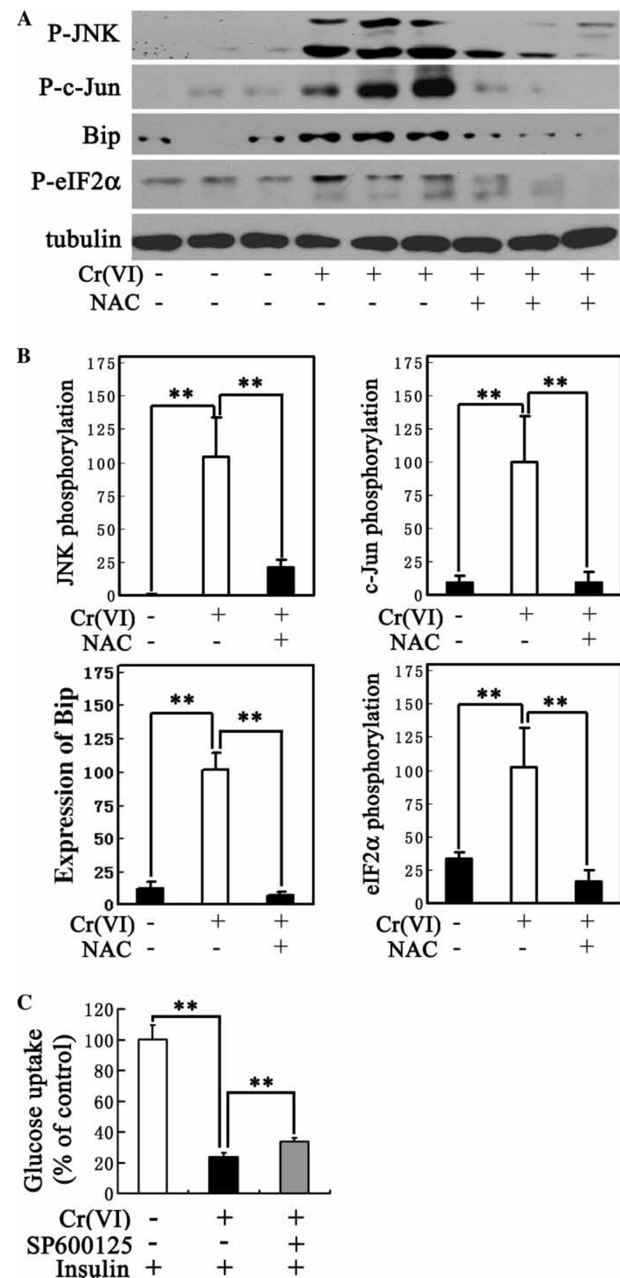

Figure 7. Cr(VI) induced JNK activation and ER stress in 3T3-L1 adipocytes and this effect was inhibited by NAC. (A) Cr(VI) induced the JNK activation including the phosphorylation of JNK and c-Jun and the ER stress as evidenced by the increased expression of Bip and phosphorylation of eIF2α. This effect was blocked by NAC treatment. After pre-treatment with or without Cr(VI) in the presence or absence of 10 mM NAC for 8 h, the cells were harvested for western blot. (B) Signals were quantified by densitometry and normalized to tubulin levels. $^{**}p < 0.01$. (C). Inhibition of JNK attenuated the effect of Cr(VI) on glucose uptake. After 3T3-L1 adipocytes were pre-treated with or without JNK inhibitor SP600125 at 25 μM for 1 h, the cells were treated with or without Cr(VI) in the presence or absence of 25 μM SP600125 for 8 h. Then insulin-stimulated glucose uptake was determined by glucose uptake assay. $^{**}p < 0.01$.

In 3T3-L1 adipocytes, Cr(VI) treatment significantly increased the generation of $O_2^{\bullet -}$, H_2O_2 and $^{\bullet}OH$ (Figure 3). To test whether the insulin resistance induced by Cr(VI) treatment is the result of either ROS or Cr(VI) *per se*, several antioxidants and/or scavengers were co-applied with Cr(VI) in the experimental system. As expected, administration of antioxidants such as NAC, SOD and catalase not only reduced the ROS generation in the Cr(VI)-treated cells (Figure 4), but also reversed the impaired glucose uptake and insulin signalling (Figures 5 and 6). Furthermore, addition of DPI, a potent inhibitor for NADPH oxidase, produced the similar effects of antioxidants (Figures 4 and 5). All these results indicated that Cr(VI) induced insulin resistance in 3T3-L1 adipocytes is caused by ROS.

It was reported that hydrogen peroxide, TNF-α and hyperglycemia could all induce oxidative stress and insulin resistance *in vitro* and/or *in vivo* [45]. In the present work, we found that chronic Cr(VI)

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treatment can also induce oxidative stress and insulin resistance in 3T3-L1 adipocytes *in vitro*. Furthermore, we found that hyperinsulinemia can produce similar effects as Cr(VI) (unpublished data). Recently, it was shown that chronic inflammation may be involved in the pathogenesis of insulin resistance, type 2 diabetes and metabolic syndrome [33,46,47]. It is well recognized that inflammation is one manifestation of oxidative stress and the pathways that generate the mediators of inflammation, such as adhesion molecules and interleukins, can all be induced by oxidative stress. Taken together, it seems that oxidative stress is a common factor underlying insulin resistance, type 2 diabetes and metabolic syndrome and may explain the presence of inflammation in all these conditions.

Although it is believed that oxidative stress can induce the activation of serine/threonine kinase cascades such as JNK and inhibit the insulin signalling pathway, the exact molecular link between oxidative stress and insulin resistance is largely unknown. In the present study, we found that chronic Cr(VI) treatment can initiate JNK activation and ER stress in 3T3-L1 adipocytes that can be reversed by antioxidants. In addition, inhibition of JNK attenuated the effect of Cr(VI) on insulin-stimulated glucose uptake. These data are consistent with recent reports that ER stress is associated with insulin resistance in many conditions *in vivo* and *in vitro*, including genetic obesity and diabetes, and diet-induced obesity and diabetes. These results suggested that oxidative stress probably induce insulin resistance through JNK activation and ER stress pathway.

In summary, the present study illustrates that increased ROS is a characteristic of insulin resistance induced by chronic Cr(VI) treatment in adipocytes. A causal role for ROS in the development of insulin resistance is shown by the observation that pharmacological interventions designed to decrease ROS levels substantially prevent the development of insulin resistance. ER stress and JNK activation may be involved in ROS induced insulin resistance which can be reversed and inhibited by antioxidants. These observations suggested that oxidative stress, ER stress and JNK pathway may play a crucial role in the progression of insulin resistance induced by chronic Cr(VI) treatment. Epidemicological evidence and animal studies are needed to investigate whether metals and metal-containing particles, such as chromium(VI), nickel, cobalt and cadmium can cause diabetes and other insulin-resistant related diseases.

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