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Chronic insulin treatment causes insulin resistance in 3T3-L1 adipocytes through oxidative stress

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Abstract
Insulin resistance and hyperinsulinemia are commonly present in obesity and pre-diabetes, and hyperinsulinemia is both a marker and a cause for insulin resistance. However, the molecular link between hyperinsulinemia and insulin resistance remains elusive. The present study examined the effect of chronic insulin treatment on the reactive oxygen species (ROS) production, insulin signalling and insulin-stimulated glucose uptake in 3T3-L1 adipocytes. The results showed that chronic insulin treatment significantly increased the intracellular generation of superoxide anion, hydrogen peroxide and hydroxyl radical. ROS induced by chronic insulin treatment inhibited insulin signalling and glucose uptake, induced endoplasmic reticulum (ER) stress and JNK activation. Furthermore, these effects were reversed by antioxidants N-acetylcysteine, superoxide dismutase or catalase. These results suggested that ROS, ER stress and JNK pathway are involved in insulin resistance induced by chronic insulin treatment. Therefore, oxidative stress could be a potential interventional target for hyperinsulinemia-induced insulin resistance and related diseases.

Keywords: *Insulin resistance, diabetes, ROS, ER stress, JNK*

Abbreviations: *CM-DCF-DA, 5-(and-6)-chloromethyl-2′,7′-dichlorofluorescen diacetate; 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; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SOD, superoxide dismutase*

Introduction

Changes in lifestyle, such as consumption of a high-calorie diet and lack of physical activity, have increased the global prevalence not only of obesity but also of diabetes. Obese individuals develop resistance to the cellular actions of insulin (insulin resistance), characterized by an impaired ability of insulin to inhibit glucose output from the liver and to promote glucose uptake in fat and muscle tissues. Obesity-associated insulin resistance is a major risk factor for cardiovascular disease and type 2 diabetes mellitus, which has reached epidemic proportions [1]. Thus, a better understanding of the molecular mechanisms underlying insulin resistance will be required to combat the epidemics of type 2 diabetes and cardiovascular disease that are fuelled by obesity-associated insulin resistance.

The cellular/molecular mechanisms of insulin resistance have been under intensive study and multiple hypotheses have been proposed, such as glucotoxicity, lipotoxicity, inflammation, oxidative stress and

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endoplasmic reticulum (ER) stress. The involvement of these individual events has been established. However, the relative importance and the interplay between them are still elusive.

Systemic oxidative stress, defined as a persistent imbalance between the production of highly reactive molecular species and antioxidant defenses, correlates with fat accumulation and elevated insulin level in mice and humans [2–4]. Previous studies have shown that reversal of the imbalance between reactive oxygen species (ROS) and antioxidants improves insulin sensitivity in mice and humans, suggesting that oxidative stress may be a causal factor in the development of insulin resistance [5–8]. Numerous studies about the relationship between oxidative stress and insulin resistance focus on the generation of ROS by hyperglycemia in diabetes, implicating ROS as a consequence of diabetes-induced hyperglycemia [9]. Since insulin resistance is evident long before the development of chronic hyperglycemia, it is unlikely that insulin resistance at the pre-diabetic stage is a result of the oxidative stress triggered by hyperglycemia *per se*. It remains to be understood how insulin resistance is developed under conditions of nearly normal glucose in the blood. On the other hand, elevation of serum insulin level (hyperinsulinemia) usually occurs earlier before hyperglycemia [10–12] and is usually associated with insulin resistance in obesity and early development of type 2 diabetes [13,14]. Moreover, recent evidence indicates that insulin can lead to ROS production in cells and affect the insulin signalling pathway [15,16].

Based on the previous findings, we postulated that hyperinsulinemia might induce insulin resistance through oxidative stress and antioxidants could block this effect. We tested this hypothesis and found that chronic insulin treatment significantly increased intracellular generation of superoxide radical anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$) in 3T3-L1 pre-adipocytes. Meanwhile, chronic insulin treatment can inhibit insulin signalling and insulin-stimulated glucose uptake, induce ER stress and activate c-Jun N-terminal kinase (JNK) pathway in 3T3-L1 adipocytes. All these effects can be diminished by antioxidants including N-acetylcysteine (NAC), superoxide dismutase (SOD), catalase and diphenylene iodonium (DPI, an inhibitor of NADPH oxidase). Therefore, ROS may be underlying the chronic insulin treatment induced ER stress, activation of JNK pathway and insulin resistance in 3T3-L1 adipocytes. Taken together, the present work indicates the hyperinsulinemia-elicited ROS production may be a cause of insulin resistance and provides a potential target for prevention and treatment of 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, 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).

Cell culture

3T3-L1 cells were cultured in DMEM with 10% calf serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37°C in a 10% CO_2 incubator. The cell culture medium was changed to differentiation medium (DMEM supplemented with 10% FBS, 0.11 mg/ml isobutylmethylxanthine, 1 μg/ml insulin, 250 nM dexamethasone) 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 with the method described previously [17]. In brief, cells were incubated 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) at 37°C for 20 min. 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 lysates containing radiolabelled glucose were 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 10 nM insulin 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 washed twice with DMEM and maintained in serum-free DMEM to detect insulin signalling. After serum deprivation 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 hybridized with indicated antibodies and appropriate horseradish peroxidase-conjugated secondary antibody. The immune complexes were finally detected by chemiluminescence reagents (Pierce) and autoradiography. Protein quantification was performed using Quantity One software (Bio-Rad).

Electron spin resonance (ESR) measurement

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 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 from Bruker and a capillary assembly. 3T3-L1 cells at 10^6 cells/ml were mixed with 100 mM DMPO, with or without 5 μM insulin and/or the indicated antioxidants 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

Dihydroethidium and CM-DCF-DA used to monitor intracellular $O_2^{\bullet -}$ and H_2O_2 level were dissolved in DMSO to the concentration of 2 mM and 5 mM, respectively, 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 treating with or without 5 μM insulin and/or the indicated antioxidants for 15 min, CM-DCF-DA or dihydroethidium was added into the culture medium to a final concentration of 5 μM or 2 μM, respectively, 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, $\sim 5 \times 10^6$ cells were plated in 10-cm dishes for 16 h and then treated with or without 5 μM insulin and/or the indicated antioxidants for 15 min. CM-DCF-DA was added into the culture medium to a final concentration of 5 μM and incubated at 37°C for another 15 min. Subsequently, the cells were washed once with PBS and harvested for FACS analysis.

Statistical analysis

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

Chronic insulin treatment reduces insulin-stimulated glucose uptake in 3T3-L1 adipocytes

It has been reported that insulin at physiological concentration could establish the insulin resistant state and treatment with 10 nM insulin for 6–8 h could achieve the maximal desensitization in 3T3-L1 adipocytes [18]. So we treated 3T3-L1 adipocytes with 10 nM insulin for 8 h to induce insulin resistance. As shown in Figure 1, acute insulin stimulation at 100 nM induced an ~10-fold increase in glucose uptake in 3T3-L1 adipocytes. When the cells were pre-treated with 10 nM insulin for 8 h and treated with acute insulin stimulation at 100 nM, insulin-dependent glucose uptake was decreased ~50%. This result showed that chronic insulin treatment could lead to insulin resistance.

Chronic insulin treatment inhibits insulin signalling pathway in 3T3-L1 adipocytes

To further investigate whether chronic insulin treatment affects insulin signalling pathway, 3T3-L1 adipocytes pre-treated with or without 10 nM insulin for 8 h were stimulated with insulin and then harvested for western blot. As shown in Figure 2A, acute insulin stimulation activated its signalling

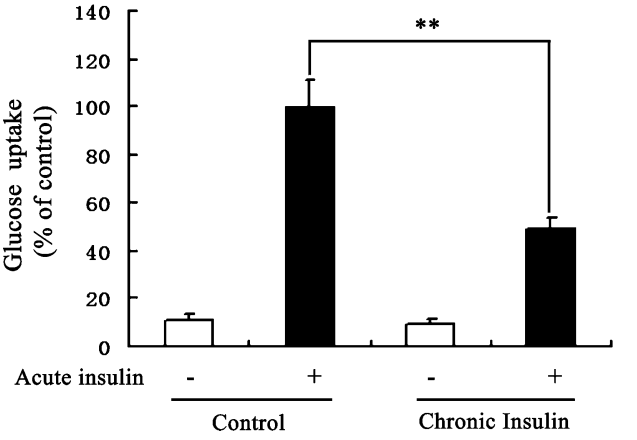

Figure 1. Chronic insulin treatment inhibited insulin-stimulated glucose uptake in 3T3-L1 adipocytes. Basal or acute insulin-stimulated glucose uptake in 3T3-L1 adipocytes with or without 10 nM insulin pre-treatment for 8 h (Chronic Insulin) were determined by glucose uptake assay. ** $p < 0.01$. In this and all other figures, error bars represent SD.

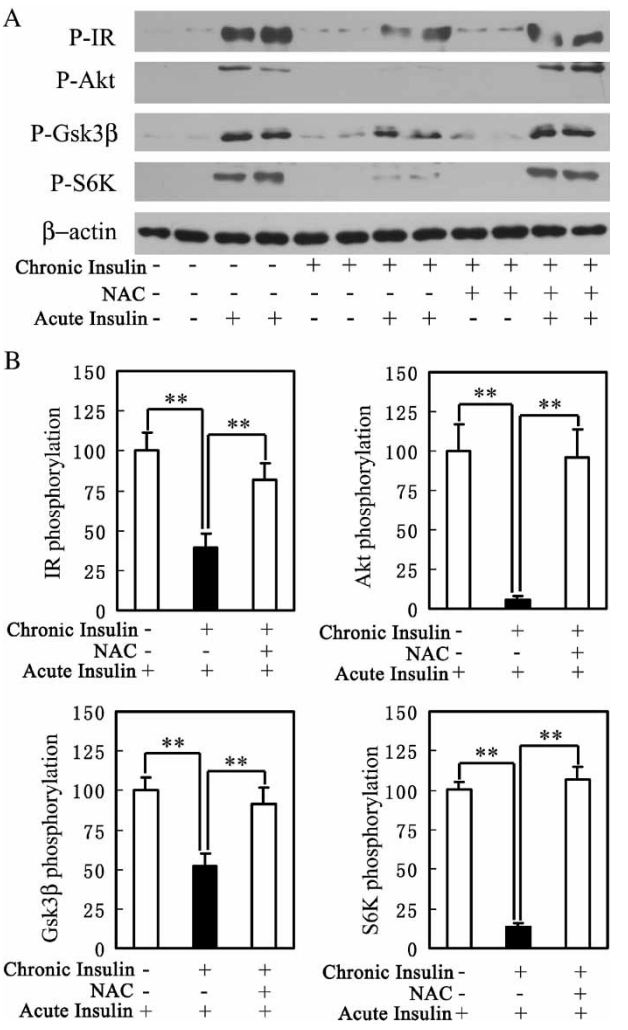

Figure 2. Chronic insulin treatment attenuated insulin signalling in 3T3-L1 adipocytes. (A) Chronic insulin treatment significantly inhibited insulin signalling, including the phosphorylation of IR, Akt, Gsk-3β and S6K, in 3T3-L1 adipocytes. After pre-treatment with or without 10 nM insulin for 8 h (Chronic Insulin), the cells were maintained in serum-free medium for 2 h and then stimulated with 100 nM insulin for 10 min (Acute Insulin) and then harvested for western blot. (B) Quantification of the protein levels corresponding to (A) by densitometry and normalized to β-actin levels. ** $p < 0.01$.

including the phosphorylation of IR, Akt, Gsk-3β and p70S6K and chronic insulin treatment significantly inhibited phosphorylation of IR, Akt, Gsk-3β and p70S6K induced by acute insulin stimulation. By quantification, the phosphorylation levels of IR, Akt, GSK-3β and p70S6K were all significantly decreased, as shown in Figure 2B. These results demonstrated that the chronic insulin treatment impaired the insulin signalling pathway in 3T3-L1 adipocytes.

Insulin induces the generation of $^{\bullet}OH$, $O_2^{\bullet -}$ and H_2O_2

Previous studies suggested that insulin can induce ROS production in several cell lines *in vitro* [15,16]. We therefore examined ROS generation in insulin-treated 3T3-L1 cells by several methods. The generation of $^{\bullet}OH$ radicals induced by insulin was examined using an ESR spin trapping method. Figure 3A(b) shows a typical ESR spectrum generated by 3T3-L1 cells with insulin treatment compared with the cells without insulin treatment (Figure 3A(a)). The spectrum consists of a 1:2:2:1 quartet. Based on the 1:2:2:1 line shape, which is the evidence of $^{\bullet}OH$ radical generation, this spectrum was assigned to the

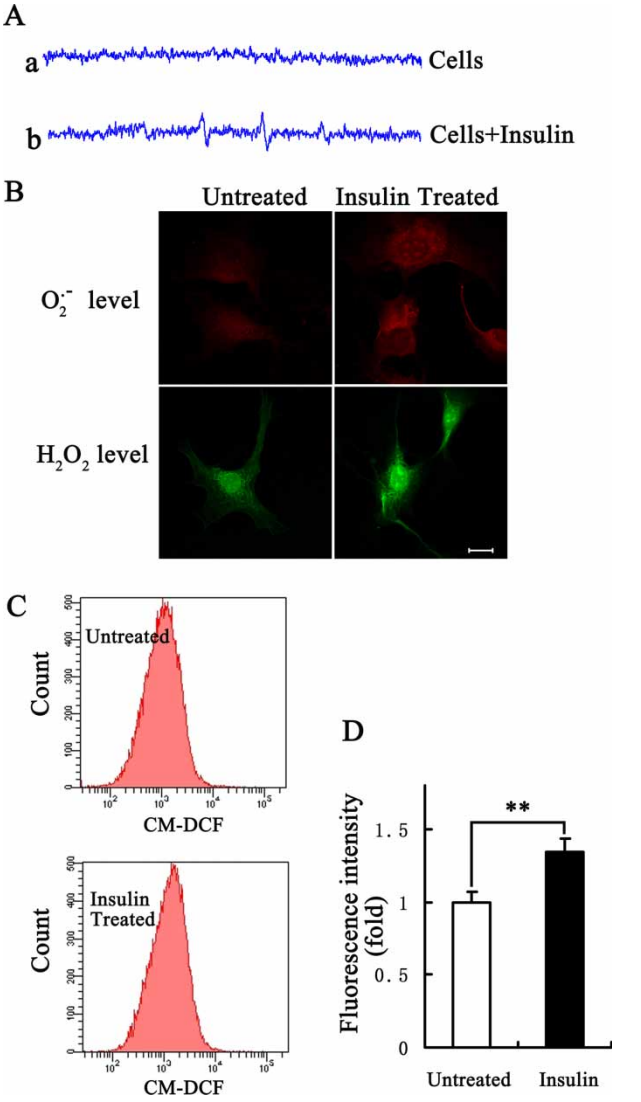

Figure 3. Measurements of insulin treatment-induced ROS generation in 3T3-L1 cells. (A) Insulin treatment induced the generation of $^{\bullet}OH$ in 3T3-L1 cells. ESR spectrum was recorded 2 min after reaction initiation from a mixture containing 1×10^6 3T3-L1 cells/ml and 100 mM DMPO, with or without insulin as indicated. DMPO was employed as the trapping reagent for $^{\bullet}OH$. (B) Insulin treatment 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 insulin 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 insulin on intracellular H_2O_2 level was further confirmed by FACS analysis. (D) Quantification of the fluorescence intensity corresponding to (C). ** $p < 0.01$.

DMPO/•OH adduct. The ability of insulin treatment to generate $O_2^{\bullet -}$ and H_2O_2 in 3T3-L1 cells was analysed by fluorescent dye dihydroethidium 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 insulin, both $O_2^{\bullet -}$ and H_2O_2 levels were dramatically elevated (Figure 3B, Insulin-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 the positive stained cells increased significantly after insulin treatment (Figure 3C). The fluorescence mean value increased to 1.4-fold after insulin treatment (Figure 3D). These data demonstrated that insulin treatment induced the ROS generation in 3T3-L1 cells.

Antioxidants inhibit ROS generation induced by insulin

NAC, SOD and catalase are established antioxidants. When the antioxidants including NAC, SOD and catalase were applied to 3T3-L1 cells concomitantly with insulin, the intensity of the spin adduct signals in the insulin-treated cells was diminished (Figure 4A(a–e)). 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 insulin-treated cells was also diminished (Figure 4A(f)). These data showed that insulin-induced •OH radical generation can be scavenged by antioxidants and could be mediated by NADPH oxidase.

When the antioxidant NAC was coexistent with insulin, the levels of both $O_2^{\bullet -}$ and H_2O_2 upregulated by insulin 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 insulin-treated cells was almost abolished by NAC (Figure 4C and D). The results further confirmed that insulin induced ROS generation can be scavenged by antioxidants.

Antioxidants reverse insulin resistance induced by chronic insulin treatment

To test the role of ROS in insulin resistance induced by chronic insulin treatment, antioxidants such as NAC, SOD, catalase or DPI were applied to 3T3-L1 adipocytes concomitantly with 10 nM insulin for 8 h. As shown in Figure 5A–D, glucose uptake in 3T3-L1 adipocytes with chronic insulin treatment was partially or completely restored to the level of control. The results suggested that the insulin sensitivity of 3T3-L1 adipocytes can be reversed when ROS are depleted by antioxidants or ROS production is reduced by specific inhibitors despite of chronic insulin treatment.

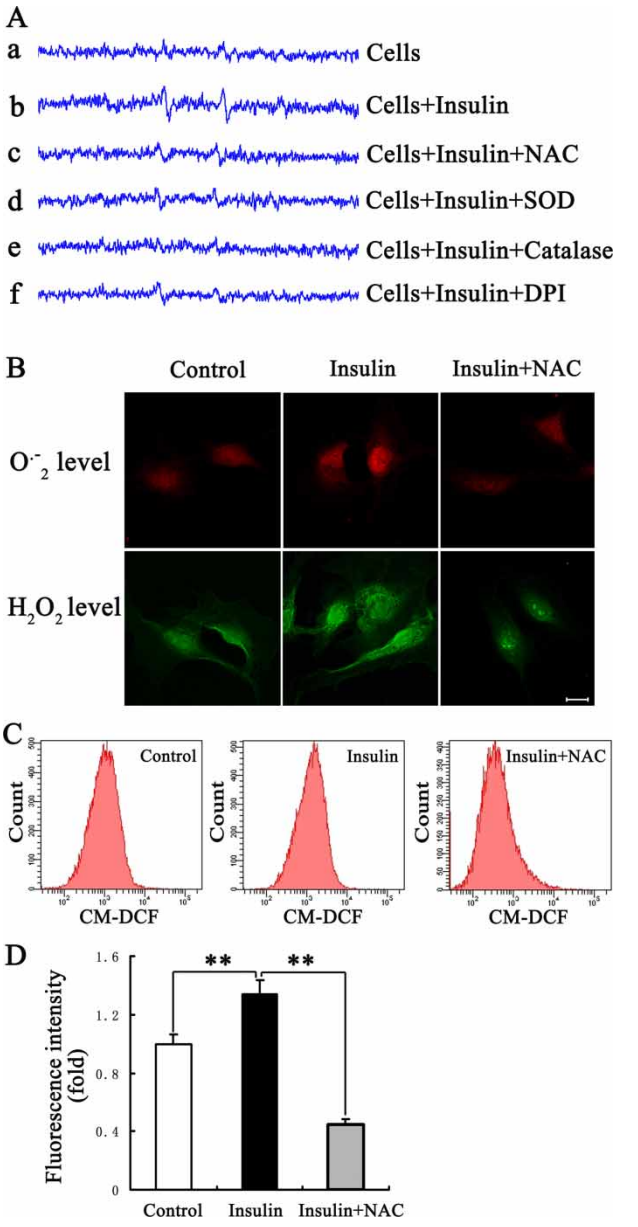

Figure 4. Insulin-induced ROS generation was inhibited by antioxidants. (A) ESR spectrum was recorded 2 min after reaction initiation from a mixture containing 1×10^6 3T3-L1 cells/ml and 100 mM DMPO, with or without insulin and different antioxidants or reagents as indicated. NAC (10 mM), SOD (1000 units/ml),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

ROS induced by chronic insulin 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 insulin treatment. Then we examined the effect of blocking ROS production

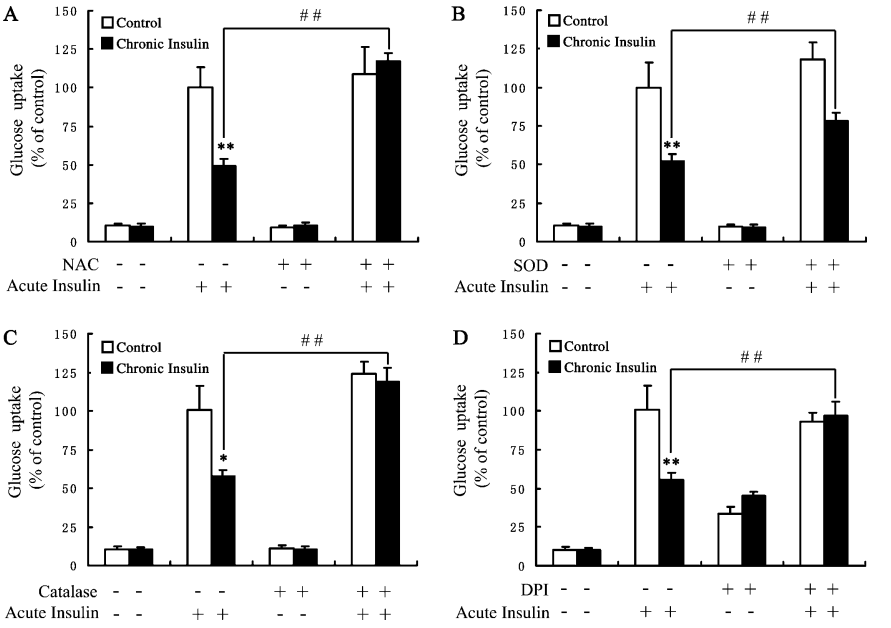

Figure 5. The effect of antioxidants on chronic insulin treatment-induced insulin resistance. Restoration of chronic insulin treatment-induced insulin resistance (decrease of acute insulin-stimulated glucose uptake) by 10 mM NAC (A), 1000 units/ml SOD (B), 5000 units/ml Catalase (C) and 20 μM DPI (D). After pre-treated with or without 10 nM insulin and/or the indicated antioxidants for 8 h, basal or acute insulin-stimulated glucose uptake in 3T3-L1 adipocytes was determined by glucose uptake assay. * $p < 0.05$, ** $p < 0.01$, compared to the cells only acutely treated with insulin. ## $p < 0.01$.

with NAC on insulin signalling pathway. In agreement with the above work, activations of insulin receptor, Akt, Gsk-3β and p70S6K were all suppressed after chronic insulin treatment. When NAC was applied to 3T3-L1 adipocytes together with chronic insulin treatment, the following acute insulin-stimulated activation of the above four key proteins was significantly restored (Figure 6A). Figure 6B showed the quantification results of Figure 6A.

ER stress and JNK activation are involved in the insulin resistance induced by chronic insulin 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 hyperactivation of JNK caused by ER stress is underlying obesity-related insulin resistance [19]. JNK is known to be activated by oxidative stress and involved in insulin resistance [20] and the inhibition of JNK activity improves insulin sensitivity both *in vivo* [21] and *in vitro* [22]. 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 chronic insulin 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 chronic insulin treatment and NAC treatment also significantly inhibited the expression of Bip and the phosphorylation of eIF2α (Figure 7A and B). Taken together, our results demonstrated that chronic insulin treatment-induced ROS lead to ER stress and JNK activation which might be the mechanism of ROS-induced insulin resistance.

Discussion

Insulin resistance and hyperinsulinemia are commonly present in obesity and pre-diabetes. Although it is still controversial about how much the hyperinsulinemia and reduced insulin sensitivity contribute to the pathogenesis of diabetes, it is generally believed that both of these factors play important roles [12,23,24]. Insulin resistance is known to induce hyperinsulinemia. A high level of insulin is also known to induce insulin resistance. These are well established in both cellular and animal models. *In vivo*, insulin injection induces insulin resistance in humans [10], dogs [25] and rats [26]. *In vitro*, insulin at physiological (< 5 nM) or pharmacological (100 nM) levels induces insulin resistance in adipocytes and muscle cells [18,27]. At the molecular level, chronic insulin treatment leads to a reduction in the activity of insulin signalling pathway components, such as IR, IRS-1, PI3K, Akt and GLUT4 proteins, in 3T3-L1 adipocytes. We found that chronic insulin treatment inhibits glucose uptake and insulin signalling pathway. All these observations support that hyperinsulinemia may contribute to insulin resistance.

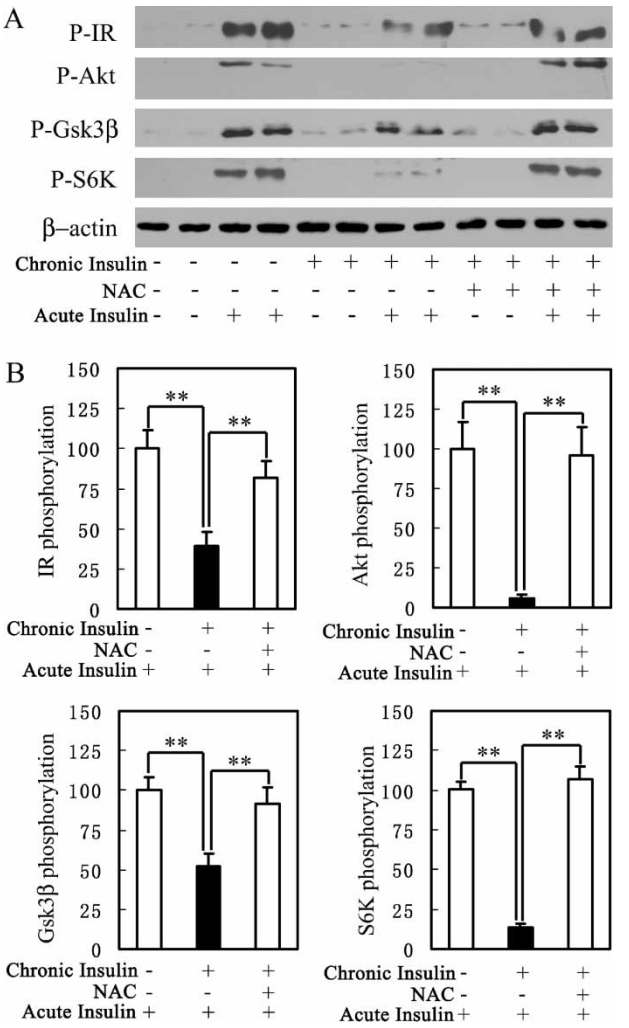

Figure 6. NAC treatment improved insulin signalling affected by chronic insulin treatment. (A) NAC improved insulin signalling, including the phosphorylation of IR, Akt, Gsk-3β and S6K, in 3T3-L1 adipocytes chronically treated with insulin. After pre-treatment with or without 10 nM insulin and/or 10 mM NAC for 8 h, the cells were maintained in serum-free medium for 2 h and stimulated with 100 nM insulin for 10 min and then harvested for western blot. (B) Quantification of the protein levels corresponding to (A) by densitometry and normalized to β-actin levels. ** $p < 0.01$.

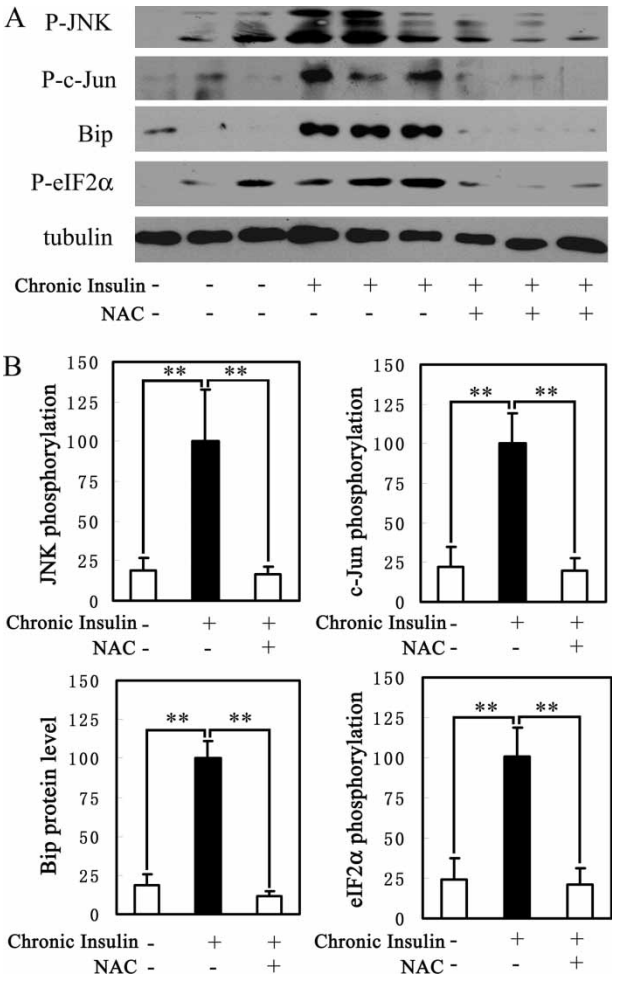

Figure 7. Chronic insulin treatment induced JNK activation and ER stress in 3T3-L1 adipocytes and this effect was inhibited by NAC. (A) Chronic insulin treatment induced the JNK activation including the phosphorylation of JNK and c-Jun and the ER stress as evidenced by the increased expression of Bip and phosphorylation of eIF2α. This effect was blocked by NAC treatment. After pre-treatment with or without 10 nM insulin in the presence or absence of 10 mM NAC for 8 h, the cells were harvested for western blot. (B) Quantification of the protein levels corresponding to (A) by densitometry and normalized to tubulin levels. ** $p < 0.01$.

Oxidative stress is associated with a wide variety of pathologies, including diabetes, cardiovascular disease, cancer and neurodegenerative diseases [28]. In diabetes, hyperglycemia results in the generation of ROS, ultimately leading to oxidative stress in a variety of tissues. In the absence of an appropriate compensatory response from the endogenous antioxidant network, over-production of ROS leads to the activation of stress-sensitive intracellular signalling pathways [9] and induces the production of gene products that cause cellular damage and are ultimately responsible for the late complications of diabetes. In addition to playing a key role in the aetiology of diabetic complications, oxidative stress has also been linked to insulin resistance [29,30]. Furthermore, some studies have found that this association is not restricted to insulin resistance in diabetes, but is also evident in obese, non-diabetic individuals [31,32]. These findings suggest that oxidative stress could be an early event in the pathology of diabetes, but not simply a consequence of chronic hyperglycemia. Previous studies also showed that the production of reactive molecules and the ensuing oxidative stress could originate from sources other than hyperglycemia, including increased free fatty acids or hyperinsulinemia [33,34]. Our work demonstrates that chronic exposure to physiological concentration of insulin can also induce ROS production, disrupt insulin signaling pathway, and induce insulin resistance in 3T3-L1 adipocytes and that all these effects can be reversed by antioxidants. These results may partially explain how the development of insulin resistance can precede that of hyperglycemia in

obesity and pre-diabetic state. Our results also suggest that hyperinsulinemia itself may be a causal factor of oxidative stress.

As to the source of ROS, some evidence indicates that NADPH oxidase is a potential mediator of insulin-stimulated ROS [2,35]. NADPH oxidase catalyses the reduction of oxygen to a superoxide radical: $2O_2 + NADPH \rightarrow 2O_2^{\bullet -} + NADP^+ + H^+$. Superoxide anion itself can convert spontaneously or by superoxide dismutase in the cell to generate H_2O_2 [36]. $^{\bullet}OH$ radical generation involves H_2O_2 via a Fenton-like reaction. Recently, some evidence from human studies suggests that hyperinsulinemia and free fatty acids may contribute to oxidative stress in metabolic syndrome patients through activation of NADPH oxidase [2,4,37]. In the present study, we showed that ROS production and insulin resistance induced by chronic insulin exposure can be inhibited by the NADPH oxidase inhibitor DPI. These results suggest that prolonged treatment of insulin can induce ROS production and insulin resistance through activation of NADPH oxidase in 3T3-L1 adipocytes.

It has been reported that ER stress is involved in insulin resistance. Once ER stress is provoked in the cells, various pathways are activated, including JNK [38]. Many studies suggest that JNK is a major mediator of the insulin resistance induced by TNF-α [22], free fatty acids [39] and ER stress [40]. To test whether ER stress and JNK activation are involved in ROS induced insulin resistance in 3T3-L1 adipocytes, we detect ER stress markers and JNK activation levels in chronic insulin-treated cells. The results show that both ER stress and JNK activation are induced by chronic insulin exposure and that they can be reversed by co-treatment with the antioxidant NAC. Previous work has demonstrated that ROS and oxidative stress are capable of modulating JNK activation through multiple signalling pathways [41], whereas our data may provide a novel link from hyperinsulinemia and oxidative stress to JNK activation and insulin resistance—the ER stress. This concept was recently proposed by Muoio and Newgard [42] and Houstis et al. [6], although we could not exclude the possibility of other mechanisms involved.

Chronic hyperinsulinemia is both a marker and a cause for insulin resistance. In genetically pre-disposed subjects, the combination of excess caloric intake and relatively scarce physical activity, with the likely consequence of obesity, can induce hyperinsulinemia and/or a state of resistance to the action of insulin [11]. Regarding the relationship of insulin resistance and hyperinsulinemia, there has been a debate about which occurs first in the obese condition. The hyperinsulinemia of obesity has generally been regarded as a compensatory adaptation to the peripheral insulin resistance that is characteristic of the obese state. Other studies have shown that, in the initial stage of obesity, hyperinsulinemia is not due to insulin resistance, but constitutes a primary metabolic alteration caused by beta-cell dysfunction or dysregulation. On the basis of our work and others, no matter what the case is, long-term increase in insulin can increase ROS production and oxidative stress, as well as activate stress-sensitive pathways. This can worsen insulin action and induce insulin resistance, while insulin resistance in turn exacerbates chronic hyperinsulinemia. This vicious cycle will accelerate the progression to type 2 diabetes. So the results from us and others [43,44] may suggest that chronic hyperinsulinemia can initiate a vicious cycle to the development of insulin resistance and diabetes.

In summary, our results indicate that increased levels of ROS are a characteristic of insulin resistance induced by chronic insulin exposure. A causal role for ROS in the development of insulin resistance is demonstrated by the observation that several pharmacological interventions designed to decrease ROS levels substantially prevent the development of insulin resistance. ER stress and JNK activation are involved in ROS-induced insulin resistance which can be inhibited by antioxidants. These observations suggest that oxidative stress, ER stress and JNK pathway play crucial roles in the development of insulin resistance induced by physiological inducer of oxidative stress—hyperinsulinemia and thus could be a potential interventional target for the prevention of diabetes and other insulin resistant-related diseases.

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***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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