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High uric acid directly inhibits insulin signalling and induces insulin resistance

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ABSTRACT

Background and aim: Accumulating clinical evidence suggests that hyperuricemia is strongly associated with abnormal glucose metabolism and insulin resistance. However, how high uric acid (HUA) level causes insulin resistance remains unclear. We aimed to determine the direct role of HUA in insulin resistance *in vitro* and *in vivo* in mice.
Methods: An acute hyperuricemia mouse model was created by potassium oxonate treatment, and the impact of HUA level on insulin resistance was investigated by glucose tolerance test, insulin tolerance test and insulin signalling, including phosphorylation of insulin receptor substrate 1 (IRS1) and Akt. HepG2 cells were exposed to HUA treatment and N-acetylcysteine (NAC), reactive oxygen species scavenger; IRS1 and Akt phosphorylation was detected by Western blot analysis after insulin treatment.
Results: Hyperuricemic mice showed impaired glucose tolerance with insulin resistance. Hyperuricemia inhibited phospho-Akt (Ser473) response to insulin and increased phosphor-IRS1 (Ser307) in liver, muscle and fat tissues. HUA induced oxidative stress, and the antioxidant NAC blocked HUA-induced IRS1 activation and Akt inhibition in HepG2 cells.
Conclusion: This study supplies the first evidence of HUA directly inducing insulin resistance *in vivo* and *in vitro*. Increased uric acid level may inhibit IRS1 and Akt insulin signalling and induce insulin resistance. The reactive oxygen species pathway plays a key role in HUA-induced insulin resistance.

1. Introduction

Uric acid is the end-product of purine metabolism, and in humans, the upper normal range of concentration is 6 mg/dl for women and 7 mg/dl for men [1]. In the last few decades, the prevalence of hyperuricemia has been rapidly increasing worldwide [2,3]. Meanwhile, a large body of evidence has established the association of elevated serum uric acid level and various metabolic disorders, including gout, hypertension, atherosclerosis, chronic renal diseases, and metabolic syndrome [4–6].

Recently, evidence has emerged from several large epidemiological studies that hyperuricemia is related to insulin resistance [7–9]. High-sensitivity C-reactive protein (hs-CRP) level is often higher in hyperuricemic patients than in the general population, hs-CRP level was found to be an independent predictor of homeostatic model assesssment-insulin resistance [10,11]. In addition, soluble uric acid could increase tissue levels of NADPH oxidase and the generation of reactive oxygen species (ROS) in mature adipose tissue [12]; oxidative-stressed adipose tissue shows decreased sensitivity to insulin as a risk factor of insulin resistance. However, the causal mechanism of hyperuricemia in the development of insulin resistance is still unclear.

Some studies have shown that high uric acid (HUA) levels increase oxidative stress in adipocytes, vascular smooth muscle cells and human umbilical vein endothelial cells [13,14]. Our previous study showed increased ROS production with uric acid treatment in β-cells [15]. One attractive hypothesis is that many factors leading to insulin resistance are mediated by the generation

Abbreviations: Akt, protein kinase B; GTT, glucose tolerance test; HUA, high uric acid; IRS, insulin receptor substrate; ITT, insulin tolerance test; NAC, N-acetyl-L-cysteine; ROS, reactive oxygen species.

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of abnormal amounts of ROS. Emerging evidence also supports an important role of ROS in various forms of insulin resistance [16,17].

Insulin resistance is defined as a profound dysregulation of the insulin signalling system and thus represents a state of impaired ability of peripheral tissues to respond to the physiological levels of insulin. Binding of insulin to the insulin receptor (IR) initiates signaling cascades by activating its receptor tyrosine kinase, thus leading to glucose transport activation and other metabolic effects. Most signals of the IR are transmitted through complexes assembled around IR substrate (IRS)-1/2, which is composed of multiple interaction domains and phosphorylation motifs [18,19]. Because IRS1 is centrally located within the insulin signalling pathway, defects in IRS1 function significantly impair downstream responses of the IR [20,21]. In particular, IRS1 Ser phosphorylation leads to decreased Tyr phosphorylation [22], and increased proteasome-mediated degradation [23]. Phosphorylation of human IRS1 (Ser312; corresponding to Ser307 in the rodent form) is a representative molecular marker of insulin resistance [24,25].

Potassium oxonate was previously found to reduce the degradation of uric acid, thereby increasing uric acid level in rodents [26,27]. Therefore, in this study, we used an acute hyperuricemia mouse model to dissect the effect of HUA level on insulin resistance. We investigated the effect of uric acid on glucose tolerance, insulin resistance and insulin signalling, as manifested by changes in the phosphorylation of IRS1 and Akt activity in mouse liver, muscle, and adipose tissues and human HepG2 cells. In view of the potential important role of ROS in insulin resistance, we examined whether antioxidants could prevent insulin resistance induced by HUA.

2. Materials and methods

2.1. Reagents

Anti-phospho-Akt (Ser473) and anti-Akt antibodies were from Bioworld (St. Louis Park, MN, USA). Anti-phospho-IRS1 (Ser312; Ser307 in the rodent form) and anti-IRS1 antibodies were from Millipore (Billerica, MA). Rabbit anti-GAPDH antibody was from Abcam. Uric acid, human insulin, hypoxanthine, potassium oxonate, and hydrofluorescein diacetate (DCFH-DA) were from Sigma (St. Louis, MO, USA). N-acetyl-L-cysteine (NAC) was from ENZO Life Sciences (Farmingdale, NY, USA). All other chemicals and solvents were of analytical grade.

For primary buffer, uric acid stock solution was prepared at 15 mg/ml in 0.5 M NaOH. NAC was prepared at 500 mM in ultrapure water.

2.2. Cell culture and treatment

HepG2 cells, a human hepatic adenocarcinoma cell line were obtained from the American Type Cell Collection, grown in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin (complete medium) and maintained at 37 °C in a humidified, 5% CO_2 environment with medium exchange every 2 days. Experiments were typically performed with cells at 80% confluence. To assess IRS1 (Ser) phosphorylation, cells were deprived of serum for 18 h, then exposed to uric acid (0, 5, 15 mg/dl) for 30 min and treated with regular human insulin (100 nmol/l) for 10 min, harvested by scraping, and stored at −80 °C. In experiments with inhibitor, cells were pretreated with antioxidant (NAC, 10 or 20 mM), for 12 h before adding uric acid. To measure intracellular ROS levels, cells were subcultured in 6-well plates (2.5×10^5 cells/well) for treatment: control, vehicle (0.5 M NAOH), HUA (uric acid 15 mg/dl × 30 min), HUA + 10NAC (10 mM × 12 h), HUA + 20NAC (20 mM × 12 h), or NAC (20 mM), then stained with 10 μM DCFH-DA for 30 min at 37 °C [28]. Stained cells were viewed by fluorescence microscopy and analyzed by flow cytometry.

2.3. Animals

Eight-week-old male C57BL/6J mice weighing 20 ± 2 g were purchased from Vital River Laboratories (Beijing) and housed in the Laboratory Animal Center of Shantou University Medical College. Principles of laboratory animal care were followed, and all procedures were approved by the Animal Care Committee of our institute. Mice were fed a standard diet and maintained in individual cages with routine light–dark cycles and allowed to adapt to the laboratory environment for 1 week.

2.4. Hyperuricemia mouse model and treatment

Ten-week-old male C57BL/6J mice were randomly assigned to 3 groups ($n = 4$ each) for treatment: blank, control and HUA ($n = 4$). For HUA treatment, following an 18-h overnight fast, mice received an intraperitoneal (i.p.) injection of potassium oxonate (300 mg/kg) and an intragastric administration of hypoxanthine (500 mg/kg) to create the hyperuricemia model. The volume of drug was based on body weight measured immediately prior to each dose. Then serum uric acid level was inspected at different times by the phosphotungstic acid method [29]. For control and HUA mice, but not blank group, insulin (2 U/kg) was injected 10 min before they were killed by CO_2 inhalation, and the liver, thigh muscle (quadriceps femoris and adductor magnus), and epididymal adipose tissue were carefully excised. All tissue samples were immediately stored in liquid nitrogen.

2.5. Glucose tolerance test

Hyperuricemic (HUA) mice were randomly divided into 2 groups ($n = 6$ each) for treatment: control, the same volume of vehicle; and glucose, intraperitoneal (i.p.) injection of potassium oxonate 30 min (depending on the serum uric acid decay) later, intraperitoneal injection with 10% glucose solution (China Otsuka Pharmaceutical Co., Tianjin, China) at 1 g/kg body weight. Blood glucose levels were measured before and at 15, 30, 60, 90, and 120 min after glucose administration by use of a One-Touch Ultra glucometer (LifeScan) from the tail tip.

2.6. Insulin tolerance test (ITT)

Control and HUA mice ($n = 8$ each), received an intraperitoneal injection of regular human insulin (1.5 U/kg) and 1 g/kg glucose (after 18-h overnight fast for HUA mice). Blood glucose levels were measured before and at 15, 30, 60, and 90 min after insulin administration [30,31].

2.7. Western blot analysis

Liver, muscle, and adipose tissue (30 mg) and cells (5×10^5) were sonicated in 300 μl RIPA buffer supplemented with protease and phosphatase inhibitor (3 μl), NaF (1 mM 3 μl), sodium orthovanadate (1 mM 3 μl), PMSF (1 mM 3 μl), homogenized, then underwent centrifugation (12000 g for 5 min). The supernatant was used for protein determination by the BCA Protein Assay Kit (Pierce, IL, USA). Equal amounts of total protein underwent 8–10% SDS–PAGE, then were transferred to polyvinylidene difluoride membranes (Millipore, Shanghai), which were blocked with 5% non-fat milk and incubated with primary antibodies for phosphorylated and total IRS1 or Akt (1:1000 dilution), then

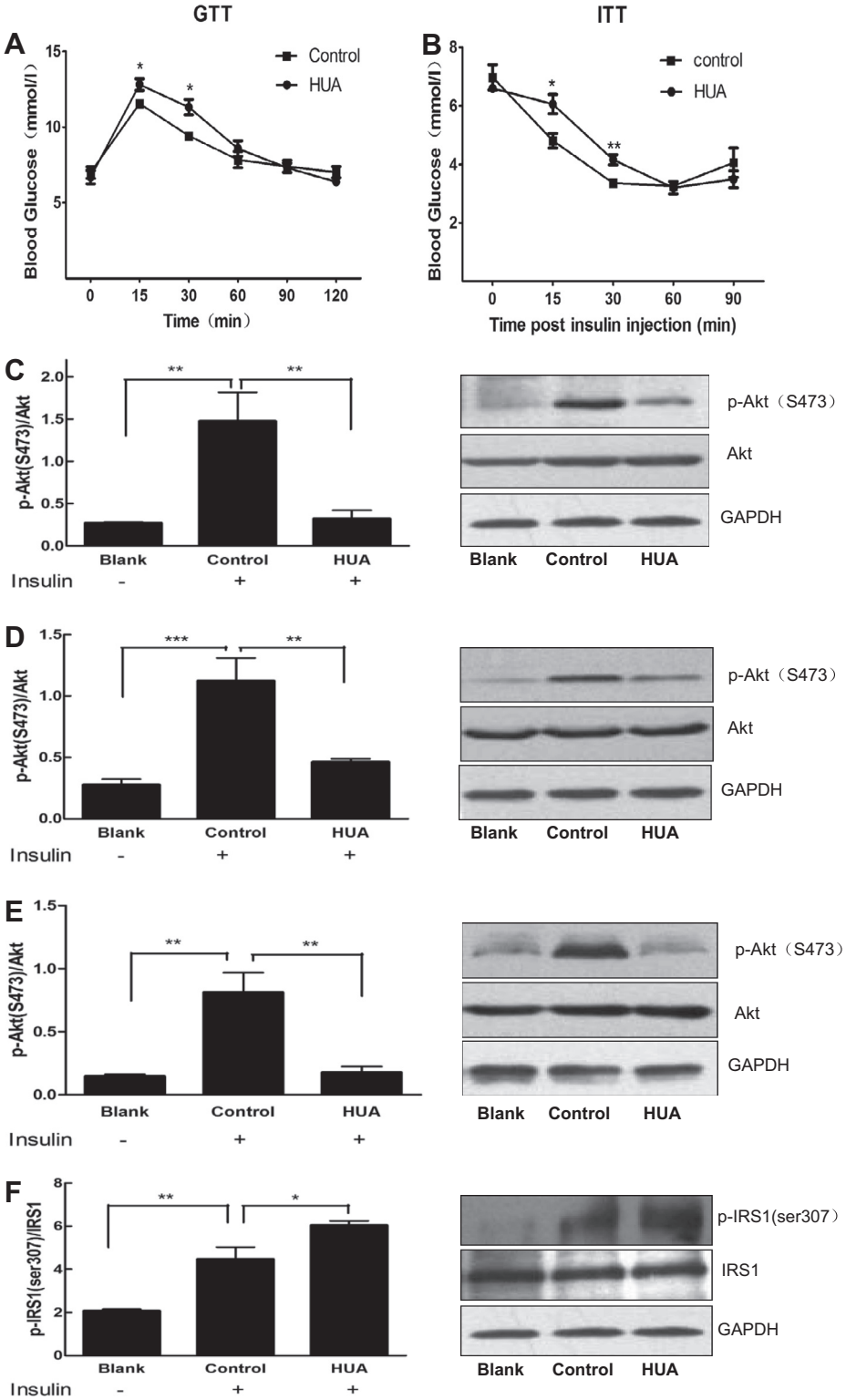

Fig. 1. (A and B) Impaired glucose tolerance, insulin resistance in hyperuricemic mice (with high uric acid [HUA] level). Glucose tolerance test (GTT): C57BL/6J mice were induced to hyperuricemia for 30 min before GTT with glucose, 1 g/kg intraperitoneally (i.p.). Glucose in whole blood samples from tail incision was measured at baseline, 15, 30, 60 and 90 min in HUA mice and controls ($n = 6$ each). Insulin tolerance test (ITT): regular human insulin (1.5 U/kg) was administered i.p. to HUA and control mice ($n = 8$ each) and glucose was measured. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control. (C, D and E) Inhibition of insulin-induced increase in phospho-Akt (Ser473) level by hyperuricemia (HUA) in mouse liver, muscle, and adipose tissue. HUA mice were treated with regular insulin (2 U/kg i.p.). Western blot analysis of phospho-Akt (Ser473) and total Akt production in liver (C), muscle (D) and adipose tissue (E). Data are mean ± SEM from 3 independent experiments; $^{**}p < 0.01$, $^{***}p < 0.001$. (F, G and H) Production of phospho-IRS1 (Ser307) increased with induced HUA in mouse liver, muscle, and adipose tissue. HUA mice were treated with regular insulin (2 U/kg i.p.) 10 min before death. Western blot analysis of phospho-IRS1 (Ser307) in liver (F), muscle (G) and adipose tissue (H). Data are mean ± SEM from 3 independent experiments; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

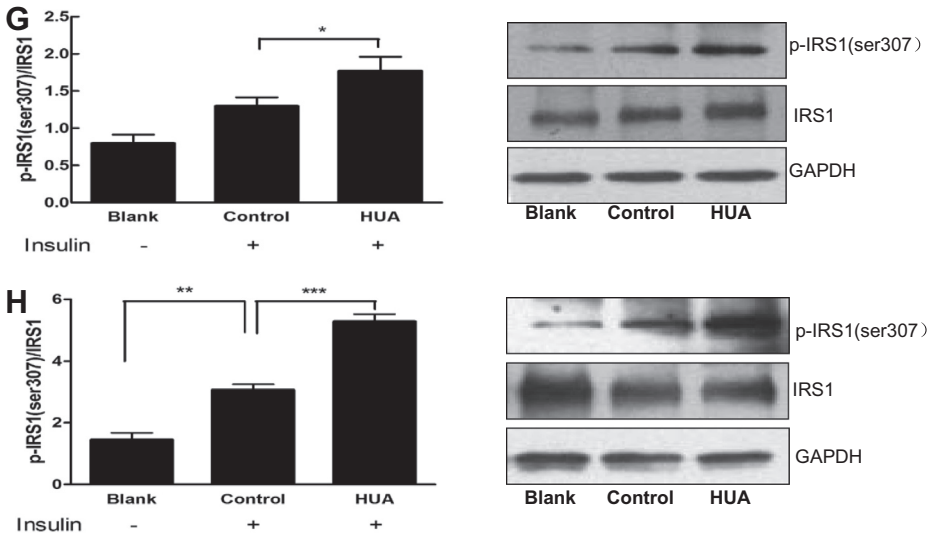

Fig. 1 (*continued*)

horseradish peroxidase-conjugated secondary antibody (1:10,000 dilution). Signals were developed by enhanced chemiluminescence (Amersham, Piscataway, USA). Images of blots were acquired by a digital image processing system (Universal HoodII76S/0608, Bio-Rad, Hercules, CA) and quantified by use of Quantity One (Bio-Rad).

2.8. Statistical analysis

Data are expressed as mean ± SEM. Data were analyzed by unpaired Student's *t* test, comparisons between groups involved one-way ANOVA with Tukey–Kramer post hoc testing. Statistical analysis involved use of SPSS 17.0 (SPSS Inc., Chicago, IL). $P < 0.05$ was considered statistically significant. Figures were obtained by use of GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Impaired glucose tolerance with insulin resistance in hyperuricemia mouse model

To examine the effect of hyperuricemia on glucose tolerance, we used the experimental mouse model of acute hyperuricemia. Glucose load (1 g/kg intraperitoneally) significantly differed between HUA mice and control mice ($p < 0.05$ for 15 and 30 min; Fig. 1A). At 15 min, glucose peaked at a mean of 12.82 ± 0.95 mmol/l in HUA mice and 11.55 ± 0.65 mmol/l in controls, from similar fasting levels (control group: 7.00 ± 0.94 mmol/l; HUA group: 6.70 ± 1.08 mmol/l).

Following regular human insulin injection (1.5 U/kg intraperitoneally) blood glucose declined slower in HUA than control mice ($p < 0.05$ at 15 min, and $p < 0.01$ at 30 min) (Fig. 1B). In controls, blood glucose decreased more than 52%, from a mean of 6.98 ± 1.22, to 3.36 ± 0.41 mmol/l at 30 min. In contrast, in HUA mice, insulin injection did not produce hypoglycaemia; the decrease in glucose level was 36% at 30 min, with mean baseline blood glucose of 6.60 ± 0.39 mmol/l decreasing to 4.16 ± 0.48 mmol/l ($p < 0.01$). ITT experiments suggested that acute hyperuricemia weakened insulin sensitivity.

3.2. HUA levels inhibited phospho-Akt (Ser473) response to insulin in mouse liver, muscle, and adipose tissue

Insulin is an important regulatory hormone that mediates energy uptake by inhibiting glucose production in liver and by increasing glucose uptake into peripheral tissues [32]. We examined insulin signalling in liver, muscle, and adipose tissues of mice. Insulin (2 U/kg) was intraperitoneally injected in HUA and control mice 10 min before they were killed. Western blot analysis revealed reduced phospho-Akt (Ser473) level in HUA mouse liver (Fig. 1C), muscle (Fig. 1D), or adipose tissue (Fig. 1E) as compared with control mice, with no change in total Akt level, which demonstrates a profound effect of HUA on downstream insulin signalling ($p < 0.01$).

3.3. HUA levels increased phospho-IRS1 (Ser307) in mouse liver, muscle, and adipose tissue

As compared with other phosphorylations of IRS1, which are activating, phospho-IRS1 (Ser307) acts to inhibit downstream insulin signalling. Western blot analysis demonstrated that acute hyperuricemia increased phospho-IRS1 (Ser307) level in mouse liver, muscle, and adipose tissues (Fig. 1F, G, H). Thus, via increasing phospho-IRS1 (Ser307) level, HUA could inhibit insulin signalling.

3.4. HUA levels induced oxidative stress in HepG2 cells and NAC inhibited ROS

To understand the underlying mechanism of HUA inhibiting insulin signalling, we examined whether HUA induces ROS production in HepG2 cells. Cells were subcultured in 6-well plates and ROS production was measured by DCFH-DA staining. Treating HepG2 cells with 15 mg/dl uric acid for 30 min strongly increased ROS levels as compared with untreated or controls (Fig. 2A); moreover, pretreatment with NAC completely scavenged ROS levels increased by HUA. DCFH-DA–positive cells were increased by 6.9-fold with HUA treatment as compared with controls (Fig. 2B and C). Thus, increased HUA level induced oxidative stress in HepG2 cells.

3.5. Antioxidant NAC blocks HUA-induced IRS1 activation and Akt inhibition in HepG2 cells

To determine whether HUA inhibits insulin signalling by increasing oxidative stress, HepG2 cells were exposed to different levels of uric acid (0, 5, 15 mg/dl) for 30 min, and then regular insulin (100 nmol/l) for 10 min, insulin induced phospho-Akt (Ser473) level as compared with controls, with a marked decrease in level

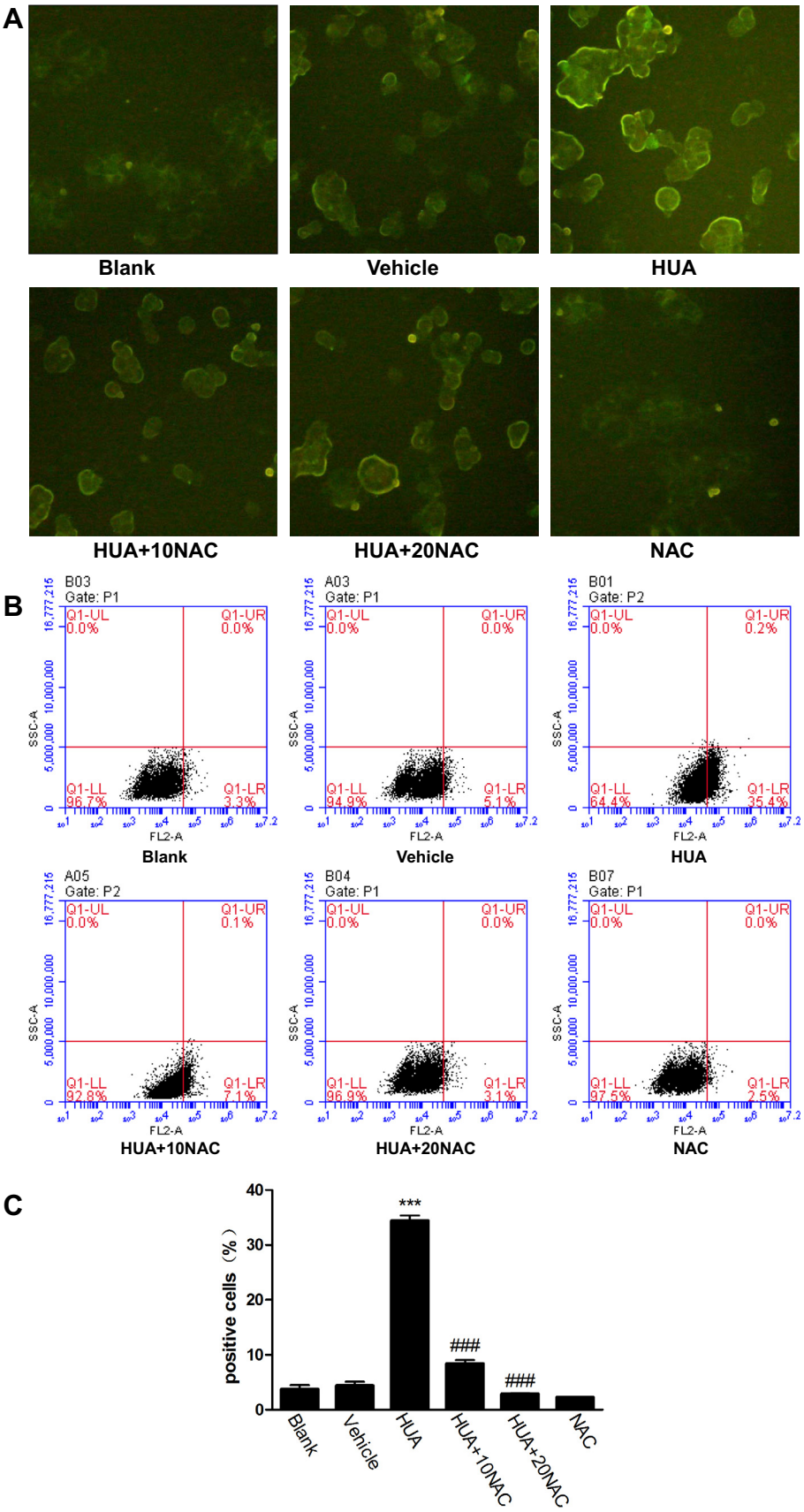

Fig. 2. Effect of HUA on reactive oxygen species production in HepG2 cells. HepG2 cells were divided into 6 groups and stained with DCFH-DA for fluorescence microscopy (A, magnification × 100), and flow cytometry (B). (C) Intracellular ROS levels following HUA exposure, and pretreatment with N-acetyl-L-cysteine (NAC; 10 or 20 mM). Data are mean ± SEM of DCFH-DA-positive cells from 3 independent experiments. $^{***}p < 0.001$ vs. vehicle, $^{###}p < 0.001$ vs. HUA.

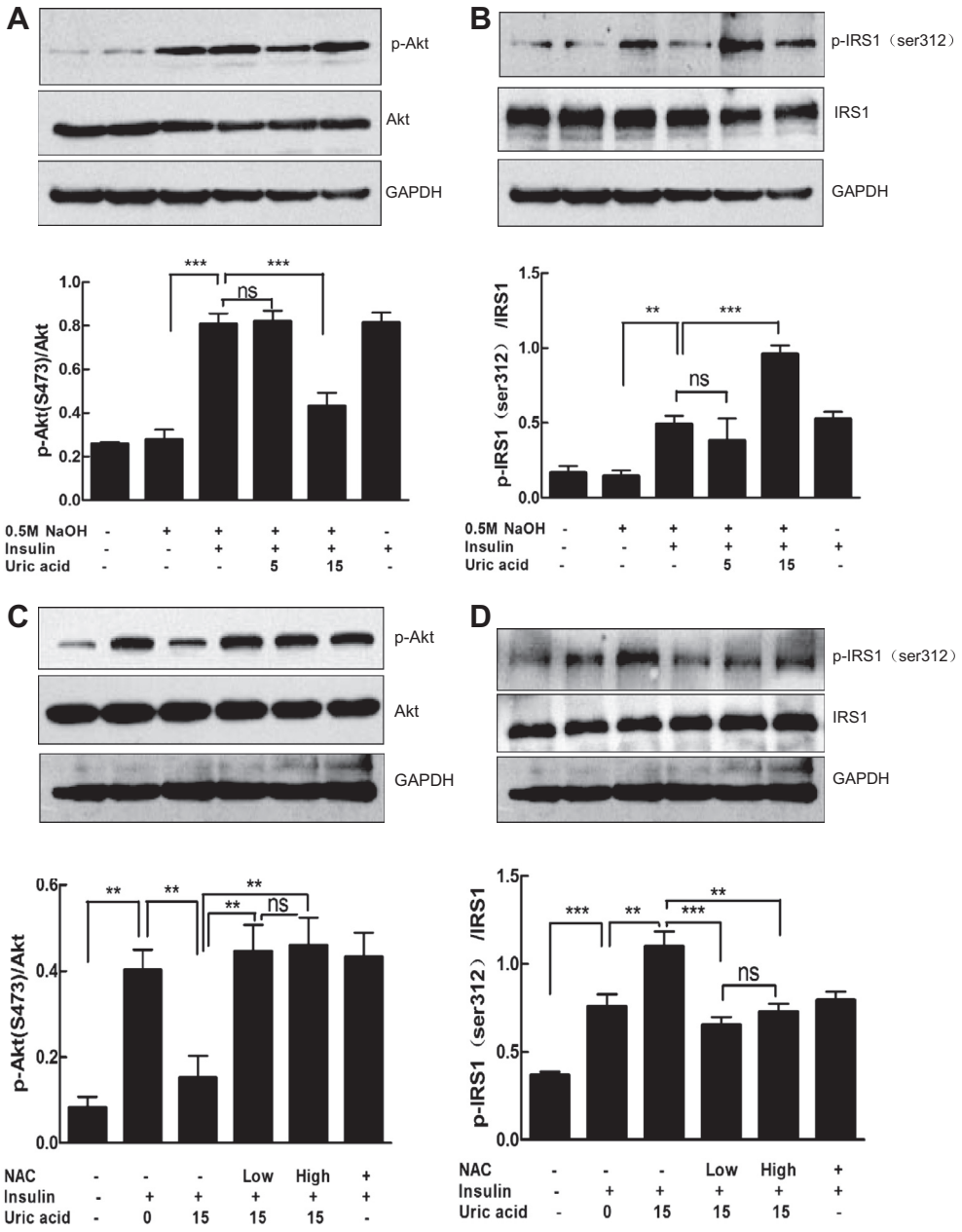

Fig. 3. Insulin signalling is impaired following HUA in HepG2 cells and is recovered by elimination of HUA-induced ROS. (A) Cells were treated with different concentrations of uric acid for 30 min and insulin (100 nmol/l) for 10 min. Western blot analysis of phospho-Akt (Ser473) and (B) Phospho-IRS1 (Ser312). (C) Cells were treated with HUA (30 min) and NAC (10 or 20 mM, 12 h) for measuring phospho-Akt (Ser473) and (D) phospho-IRS1 (Ser312). Data are mean ± SEM from 3 independent experiments; $^{**}p < 0.01$, $^{***}p < 0.001$.

with 15 mg/dl uric acid as compared with 0 or 5 mg/dl uric acid (Fig. 3A). Furthermore, phospho-IRS1 (Ser307) level was increased after exposure to uric acid (Fig. 3B). These results are similar to findings in HUA mice. Next, we measured insulin signaling in HepG2 cells pretreated with NAC (10 or 20 mM), for 12 h before adding HUA. NAC administration recovered the HUA-impaired insulin-dependent Akt phosphorylation (Fig. 3C) and completely decreased HUA-induced phospho-IRS1 (Ser307) level (Fig. 3D). ROS may act as a key signaling molecule for inhibiting insulin signalling under HUA treatment.

4. Discussion

A positive relationship between hyperuricemia and insulin resistance has been reported in several cross-sectional studies [7–9]. However, the underlying mechanism by which hyperuricemia is linked to an increased risk for insulin resistance is still unknown. In this study, we investigated the mechanism of HUA leading to insulin resistance. HUA impaired glucose tolerance with insulin resistance and inhibited insulin signalling *in vivo*; HUA induced oxidative stress *in vitro*, and the antioxidant NAC blocked the HUA-induced insulin signalling impairment. The finding that level of phospho-IRS1 (Ser307) was increased by HUA treatment provides insight into one mechanism of HUA exposure impairing insulin signalling. Because phospho-IRS1 (Ser307) inhibits the downstream transduction of insulin signalling, reduced phosphorylation of Akt (Ser473) follows (Fig. 4). This is the first report of the direct effects of HUA on glucose intolerance, insulin resistance, and inhibition of insulin signaling *in vivo* in a mouse model and human HepG2 cells. Our results suggest that hyperuricemia is an independent risk factor of insulin resistance.

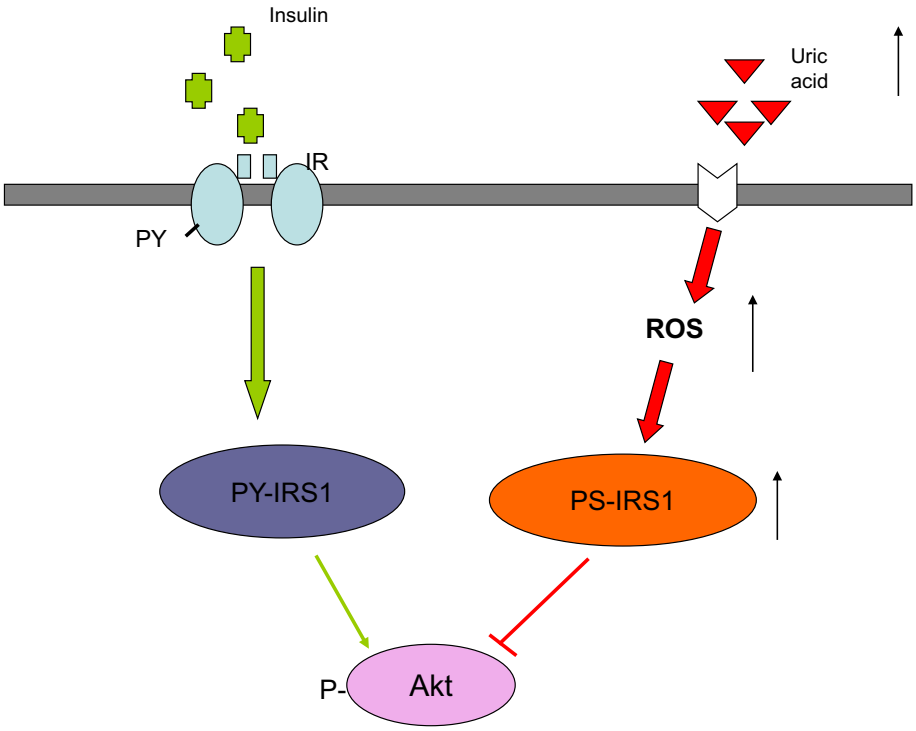

Fig. 4. Schematic representation of HUA-mediated hepatic insulin resistance. Following treatment with HUA, oxidative stress is increased, which activates serine (rat Ser307, human Ser312) phosphorylation of IRS-1. This activity impairs Akt phosphorylation, thus resulting in acute insulin resistance in liver following HUA. IR, insulin receptor; PY, tyrosine phosphorylation; PS, serine phosphorylation.

The causal relationship between hyperuricemia and insulin resistance has not been clearly established and is under investigation. Insulin resistance was found associated with reduced levels of nitric oxide, and increased serum urate concentration can reduce nitric oxide levels [5]; other reports suggested that uric acid induced insulin resistance by increased tissue NADPH oxidase or hs-CRP level [10–12]. Our study focused on the generation of ROS following HUA exposure and found that HUA could increase intracellular ROS level and that lowering ROS improved insulin resistance. These results provide evidence for HUA causing insulin resistance mediated by the generation of abnormal amounts of ROS.

Insulin is an important regulatory hormone that mediates energy uptake by inhibiting glucose production in liver and by increasing glucose uptake into peripheral tissues [32]. In this study, we checked the phosphorylation level of IRS1 and Akt in mouse liver, muscle, and adipose tissue and found inhibited insulin signalling in the hyperuricemic mice, which led to glucose intolerance with insulin resistance. Our study used an acute hyperuricemia mouse model, but human hyperuricemia is a chronic process. So further *in vivo* study with a chronic hyperuricemia mouse model may be needed to clarify the association of hyperuricemia and insulin sensitivity.

In the current study, HepG2 cells showed increased production of ROS with high uric acid treatment. Uric acid, although an antioxidant in some cases, may direct pro-oxidative effects in different microenvironments. The many sources of cellular ROS include NADPH oxidase, mitochondria and glutathione peroxidase [33]. Some studies showed that NADPH oxidase is a major source of increased ROS level induced by uric acid [34,35]. Our data confirmed that increased uric acid leads to ROS production, which caused oxidative damage to insulin signalling in cells.

The insulin receptor and the IRS proteins might be counterregulated by degradation, differential expression, or modification by Ser/Thr phosphorylation [23,36–38]. Increased Ser phosphorylation of IRS-1 is a common finding during insulin resistance [39]. The finding that phosphorylation of Ser307 on IRS1 was increased by HUA provides insight into one mechanism whereby HUA exposure can impair insulin signalling. Since phospho-IRS1 (Ser307/312) inhibits the downstream transduction of insulin signalling, phosphorylation of Akt (Ser473) is reduced (Fig. 4). In that the physiological levels of insulin stimulate Tyr phosphorylation of IRS-1, recent experimental work found that with insulin treatment, phospho-IRS1 (Ser307/312) was increased as compared with no treatment; the explanation may be that insulin could by a negative feedback partially stimulate Ser phosphorylation of IRS1; however, the phosphorylation of IRS1 (Ser307) markedly increased with increased HUA as compared with the control (insulin treatment), confirmed that phospho-IRS1 (Ser307/312) level was increased following HUA exposure.

In conclusion, our study elucidated the direct effect of HUA on insulin resistance. HUA induced oxidative stress and increased ROS levels, which subsequently activated phospho-IRS1 (Ser307/312), then inhibited phospho-Akt (Ser473), which led to insulin resistance and could contribute to abnormal glucose metabolism. Future work may provide additional insights into HUA-induced ROS mechanisms that contribute to activating phospho-IRS1 (Ser307/312).

Acknowledgments

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The experiments were mainly carried out in the Laboratory of Molecular Cardiology, First Affiliated Hospital, Shantou University Medical College.

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