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ORIGINAL ARTICLE

Resistin induces insulin resistance by both AMPK-dependent and AMPK-independent mechanisms in HepG2 cells

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Abstract Resistin is a 12.5-KDa cysteine-rich peptide that has been implicated in the impairment of glucose homeostasis via the AMP-activated protein kinase (AMPK) pathway in a rodent model. However, the role resistin plays in humans is controversial. This study investigated the effect of resistin on glucose metabolism and insulin signaling using human recombinant resistin and small interfering RNA (siRNA) against AMPKα2 to treat the human liver HepG2 cells. The mRNA of key genes involved in glucose metabolism and the insulin-signaling pathway were detected by real-time RT-PCR. Phosphorylation levels of Akt and AMPK were measured by western blot. The incorporation of D-[U–^{14}C] glucose into glycogen was quantitated by liquid scintillation counting. The results demonstrate that resistin stimulated expressions of glucose-6-phosphatase (G6Pase), phosphoenolypyruvate carboxykinase (PEPCK), and suppressor of cytokine signaling 3 (SOCS-3), repressed the expressions of insulin receptor substrate 2(IRS-2) and glucose transporter 2(GLUT2). In addition, resistin inhibited the insulin-induced phosphorylation of Akt independent of AMPK. In conclusion, our findings suggest that resistin induces insulin resistance in HepG2 cells at least partly via induction of SOCS-3 expression and reduction of Akt phosphorylation through an AMPK-independent mechanism. Resistin also increases glucose production via AMPK-mediated upregulated expression of the genes encoding hepatic gluconeogenic enzymes, G6Pase, and PEPCK.

Keywords AMPK · Diabetes · Insulin · Resistin · siRNA · HepG2

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Introduction

Obesity is closely correlated with type 2 diabetes mellitus and insulin resistance [1, 2]; however, the underlying mechanisms have not been fully elucidated. There is evidence, both in vitro and in vivo, that adipose tissue can be regarded as a major secretory and endocrine organ and that a variety of factors released by adipose cells potentially mediate insulin resistance [3, 4]. TNFα, leptin, adiponectin, MCP-1, resistin, and several other adipokines are thought to be involved in the regulation of glucose metabolism and insulin sensitivity.

Resistin, as an adipocyte-secreted factor and Fizz3 [5, 6], has been postulated to link obesity and diabetes in a rodent model of disease [7]. Findings from in vivo studies indicate that infusion of resistin dramatically increases glucose production and impairs insulin action in the liver, whereas its specific anti-sense oligodeoxynucleotide reverses these effects [8]. The principal mechanism is resistin acting on the liver to inhibit the phosphorylation state of the fuel-sensing enzyme AMPK and elevate the gene expression of the gluconeogenic enzymes G6Pase and PEPCK [9]. However, in humans, the pathophysiological role of resistin has been controversial [10–13] as the human homolog of resistin shares only 59% amino acid homology with mouse resistin, and the source of resistin appears to differ in humans [7, 14]. Specifically, resistin protein is

almost undetectable in human adipocytes [15]. Macrophages appear to be the principal source [16].

AMPK is known to be an energy sensor that controls glucose and lipid metabolism [17–19]. It is a heterotrimeric enzyme consisting of an α catalytic subunit and noncatalytic regulatory β and γ subunits. Two isoforms have been identified for both the α (α1 and α2) and β subunits (β1 and β2), while three γ subunit isoforms have been detected (γ1, γ2, and γ3) [20]. Regulation of AMPK activity involves allosteric and covalent modification by phosphorylation [21]. Phosphorylation of threonine 172 in the α subunit of AMPK (P-AMPK) is essential for its activation [22], while the activity of AMPKα2, but not that of AMPKα1, is associated with insulin resistance [23, 24].

The serine threonine kinase (Akt) pathway plays an important role in mediating phosphoinositide-3-kinase (PI3 K) signal transduction [25]. Akt thus influences a number of biological processes including metabolism, cell-cycle regulation, and apoptosis [26]. Indeed, Akt has been purported to be involved in insulin resistance (IR) [27]. Three Akt subtypes have been identified: Akt1, Akt2, and Akt3. These subtypes share approximately 80% homology [28]. Akt1 is expressed in most tissues, while Akt2 is expressed mainly in insulin-responsive tissues and Akt3 is highly expressed in the testis and brain tissue [29]. Structurally, Akt is comprised a PH domain at the N terminal, a middle catalytic domain, and regulatory domain at the C terminal [30]. Phosphorylation (mediated by phosphoinositide-dependent kinase 1 and mammalian target of rapamycin complex 2) and subsequent activation occur via phosphorylation sites located in the catalytic and regulatory domains (Thr308 and Ser473) [30]. Maximal Akt activation is only achieved when phosphorylation occurs at both these sites [29, 31].

The aim of this study was to determine whether prolonged exposure to resistin affects glucose metabolism and insulin signaling in the HepG2 human liver cell line and, if so, to elucidate whether the underlying mechanisms are dependent on AMPK by using small interfering RNA (siRNA) against AMPKα2.

Materials and methods

Cell culture and treatment

Human hepatoma HepG2 cells (Center for Experimental Animals of Sun Yat-sen University, Guangzhou, China) were cultured in DMEM (Dulbecco's Modified Eagle's Medium, GIBCO, Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. The cells were incubated in a humidified atmosphere of 5% CO_2 at 37°C and passaged every 2 days by trypsinization. HepG2 cells between the 20th and 40th passage in an active growing condition were used.

Analysis of phosphorylation level of AMPK in HepG2 cells incubated with various concentrations of resistin

Recombinant human resistin was purchased from Phoenix Pharmaceuticals, Inc. (Burlingame, CA, USA). In order to investigate the dose–response effect of resistin on the phosphorylation state of AMPK, HepG2 cells were grown in six-well plates and incubated with various concentrations of resistin (0, 15, 50, or 100 ng/ml) for 24 h. The phosphorylation levels of AMPK were then measured by western blotting.

siRNA preparation and transfection

siRNA oligos against AMPKα2 were obtained from Ambion and were validated to specifically target the AMPKα2 subunit. The sense sequence was 5′-GGUUUCUUAAA AACAGCUGtt-3′, while the antisense was 5′-CAGCUG UUUUUAAGAAACCtg-3′. The validated siRNA target sequence was submitted to BLAST to compare against the human genome and ensure that the AMPKα2 gene was the only target. *Silencer* Validated siRNA, *Silencer* GAPDH siRNA Control, and *Silencer* CY ™ 3-Labeled Negative siRNA Control were purchased from Ambion (Austin, TX, USA). HepG2 cells were plated at 50–70% confluence and transfected with a validated siRNA, a negative siRNA control, or transfection reagents only (mock transfection) using siPORT™ *NeoFX™* Transfection Agent (Ambion, Austin, TX, USA) according to the manufacturer's instructions. GAPDH siRNA was used for optimizing siRNA transfection conditions. The CY3-labeled negative siRNA was used to monitor transfection efficiency by fluorescence microscopy. The optimal concentration of validated siRNA for transfection was 100 nM giving a transfection efficiency of 92%. The cells were washed after 12 h of transfection and resuspended in DMEM plus 10% FBS and pen-strep for further experiments.

RNA extraction and quantitative real-time RT-PCR

Gene RNA expression levels were determined by quantitative RT-PCR using fluorescent reagents and a temperature cycler (ABI Prism 7000). In brief, total RNA was isolated from HepG2 cells with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA (1 μg) was used as the template for first-strand cDNA synthesis using the SuperScript III first-strand synthesis system (Invitrogen, Carlsbad, CA, USA), and 2 μl of each RT reaction was amplified in a 50-μl PCR system using SYBR Green I PCR Universal Master Mix kit

Table 1 Primers for quantitative real-time PCR

Gene	Accession no.	Forward/reverse primers
AMPKα2	NM_006252	5′-CGGCTCTTTCAGCAGATTCTGT-3′
		5′-ATCGGCTATCTTGGCATTCATG-3′
G6Pase	BC130478	5′-CATTGACACCACACCCTTTGC-3′
		5′-CCCTGTACATGCTGGAGTTGAG-3′
PEPCK	BC023978	5′-GGCTACAACTTCGGCAAATACC-3′
		5′-GGAAGATCTTGGGCAGTTTGG-3′
GLUT2	NM_000340	5′-AATTGCTCCAACCGCTCTCA-3′
		5′-CTAATAAGAATGCCCGTGACGAT-3′
IRS-2	NM_003749	5′-GCAGAACATCCACGAGACCAT-3′
		5-GGAACTCGAAGAGCTCCTTGAG-3′
SOCS-3	NM_003955	5′-GGTCAGCTGGTCTCCTTTTCCTA-3′
		5′-ATCCTCCCGCTCCATCCA-3′
GAPDH	NM_002046	5′-CAATGACCCCTTCATTGACC-3′
		5′-TTGATTTTGGAGGGATCTCG-3′

(Applied Biosystems, Foster City, CA, USA). Samples were incubated in the ABI Prism 7000 for an initial denaturation at 95°C for 10 min, and then 40 PCR cycles were performed under the following conditions: 95°C for 15 s and 60°C for 1 min. Specific transcripts were confirmed by melting-curve profile analysis at the end of each PCR, and the specificity of the PCR products was further verified by subjecting the amplified products to agarose gel electrophoresis. Experimental results were normalized to the threshold cycle (C_T) of GAPDH, referred to as ΔC_T. The fold change in expression of genes in the treated group compared to that in the control group was expressed as $2^{-\Delta\Delta CT}$, in which $-\Delta\Delta CT$ equals the ΔC_T of the treated group minus the ΔC_T of the control group, which was normalized to 1. Primers for real-time PCR were designed using Primer Express 2.0 (Applied Biosystems, USA), and specificity and efficacy were validated before use. Genes in the literature that are impotent in resistin biology were selected for observation. The gene names and corresponding forward and reverse primer pairs are shown in Table 1.

Western blotting

Cells transfected with control siRNA or AMPKα2 siRNA were grown in six-well plates and then treated with 50-ng/ml resistin for 24 h. Untransfected cells were treated with or without 50 ng/ml resistin under the same conditions and then serum-starved in glucose-free DMEM for 3–5 h in the continued absence or presence of resistin. Cells were then treated with or without insulin (100 nM, Lilly, Fegersheim, France) for 2 h. Plates were washed three times with ice-cold PBS and the following solutions were then added: 200 μl of lysis buffer (135 mM NaCl, 1 mM $MgCl_2$, 2.7 mM KCl, 20 mM Tris, pH 8.0), Triton 1%, glycerol 10%, and protease and phosphatase inhibitors including 0.5 mM Na_3VO_4, 10 mM NaF, 1 μM leupeptin, 1 μM pepstatin, 1 μM okadaic acid, and 0.2 mM PMSF). Lysates were centrifuged (13,000 rpm, 4°C for 10 min), and protein was quantified using Bradford staining (Bio-Rad). Equal amounts (25–50 μg per sample) of protein extracts were then separated by 8–10% SDS-PAGE and electrotransferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Membranes were blocked in 5% skimmed milk dissolved in 1× TBST-T buffer (14 mM Tris, 154 mM NaCl, and 0.5% Tween20, pH 7.5) overnight at 4°C. The primary antibody was incubated for 1 h at room temperature in 5% (w/v) BSA dissolved in 1× TBST-T. The expression levels of AMPKα2, Akt, GAPDH, and β-actin were determined using specific antibodies (1:1,000 dilution for AMPKα2 [Cell Signaling Technology, Danvers, MA, USA] and Akt, 1:3,000 for GAPDH and β-actin rabbit antibody [Abcam, Cambridge, MA, USA]). The phosphorylation levels of AMPK (Thr-172) and Akt (Ser473) were detected using phosphospecific antibodies diluted 1:1,000. Appropriate secondary antibodies conjugated to horseradish peroxidase (1:3,000 dilution, [Cell Signaling Technology, Danvers, MA, USA]) were incubated with respective membranes for 1 h at room temperature. This was followed by five intermittent washes with 1× TBST-T buffer. Visualization was performed with the use of enhanced chemiluminescence (ECL; Cell Signaling Technology). The results were quantified by densitometric analysis using Image-Quant software.

Glycogen synthesis

Glycogen synthesis was measured by assessing the incorporation of D-[U–^{14}C] glucose (Perkin Elmer, Boston, MA, USA) to glycogen. Cells transfected with control siRNA or AMPKα2 siRNA were grown in six-well plates and treated with 50-ng/ml resistin for 24 h [32], while untransfected cells were treated with or without 50-ng/ml resistin under the same condition, followed by serum-starving in glucose-free DMEM for 3–5 h in the continued absence or presence of resistin. Then, cells were treated with 18.5 MBq/l D-[U–^{14}C] glucose in the presence or absence insulin for 2 h. Cells were then washed three times with cold PBS and lysed in 1 M KOH. In order to measure the incorporation of glucose into glycogen, cell lysates underwent overnight glycogen precipitation with ethanol. Precipitated glycogen was then dissolved in water and transferred to scintillation vials for radioactivity counting using a Beckman LS 6500 scintillation counter.

Statistical analysis

The data (from at least three independent experiments) are presented as the mean ± standard deviation. Statistical analyses were carried out by one-way ANOVA followed by Bonferroni's adjustment; insulin and resistin were regarded as two fixed factors in the ANOVA model. The reference condition was set to 1. A value of $P < 0.05$ was considered statistically significant. Statistical analyses were performed using SPSS 15.0 (SPSS Inc., Chicago, IL).

Results

Effects of resistin on the phosphorylation state of AMPK

First, we wished to determine whether prolonged exposure to resistin affects the phosphorylation state of AMPK in HepG2 cells. In a dose–response experiment performed with a 24-h resistin incubation, we found that resistin decreased the phosphorylation level of AMPK in a concentration-dependent fashion, with a significant effect evident for resistin concentrations of 15 ng/ml, 50 ng/ml, and 100 ng/ml (Fig. 1a). For subsequent experiments, 24-h resistin incubations were performed at a concentration of 50 ng/ml unless otherwise stated.

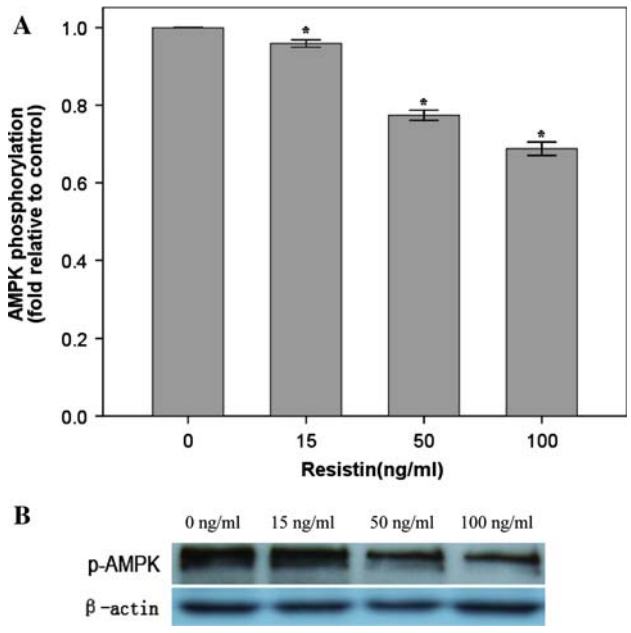

Fig. 1 **a** Dose–response effects of resistin on the phosphorylation state of AMPK in human HepG2 liver cells. Cells were incubated with resistin for 24 h. Bars represent the mean ± standard deviation from three experiments performed in duplicate. * $P < 0.05$ compared with AMPK phosphorylation in the absence of resistin. **b** Representative western blot image

Expression of AMPKα2 after treatment with AMPKα2 siRNA

In this study, it was first necessary to determine whether siRNA could efficiently transfect HepG2 cells, hence Cy3-labeled nontargeting siRNA was performed. Fluorescence microscopy revealed that more than 90% of HepG2 cells were Cy3-labeled positive and that the siRNA remained in the cells throughout the culture period despite the removal of the siRNA after 48 h (Fig. 2). This indicates that siRNA-induced gene knockdown may continue for an extended period following a relatively short exposure to the siRNA oligos.

Following this, to test whether siRNA could modulate AMPKα2 expression in HepG2 cells, we treated HepG2 cells with siRNA oligos against the AMPKα2 subunit for 12 h. AMPKα2 expression was analyzed by real-time RT-PCR, and western blotting. HepG2 cells collected at different intervals after a 12-h transfection with 100-nM AMPKα2-specific siRNA exhibited reduced AMPKα2 mRNA expression levels compared with those in transfection reagent-exposed cells (mock). AMPKα2 mRNA expression was reduced by 52.3% 24 h after transfection and was similarly reduced at 72 h (Fig. 3a). AMPKα2 protein levels were reduced by 48.1% at 24 and were not further reduced at 72 h (Fig. 3b). Moreover, the transfection reagent alone and the control siRNA transfection had no significant effect on the expression of AMPKα2. Therefore, control siRNA was used in further experiments.

Effect of resistin on gene expression in HepG2 cells

Resistin has been implicated in the regulation of hepatic glycogen synthesis and insulin resistance [8, 33, 34]. In order to investigate the effect of resistin on glucose metabolism and the insulin-signaling pathway, the mRNA expression levels of genes encoding AMPKα2, G6Pase, PEPCK, GLUT2, IRS2, SOCS-3, and GAPDH were determined by real-time RT-PCR. The results shown in Fig. 4 demonstrate that resistin decreased AMPKα2, GLUT2, and IRS2 mRNA expression levels in both basal and insulin-stimulated conditions, while increasing mRNA expression levels of G6Pase, PEPCK, and SOCS-3 under the same condition. Interestingly, as shown in Fig. 4e and f, the mRNA levels of IRS2 and SOCS-3 were not different between the resistin group and the AMPKα2 siRNA-treated group (in the presence of resistin). In contrast, those of G6Pase, PEPCK, and GLUT2 were significantly different between the aforementioned two groups. The results indicate that resistin induced insulin resistance in HepG2 cells at least partly via the induction of SOCS-3 expression and the reduction of IRS2 via an AMPK-independent mechanism. Resistin increased glucose production via upregulating the expression of genes

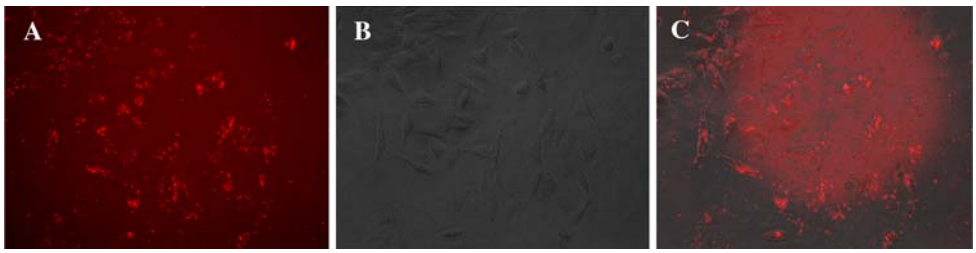

Fig. 2 Assessment of siRNA delivery efficiency in HepG2 cells in vitro using nontarget Cy3-labeled control siRNA. **a** Transfected HepG2 cells as seen with a fluorescence microscope. **b** The same cells as seen with an optical microscope. **c** Merged cells

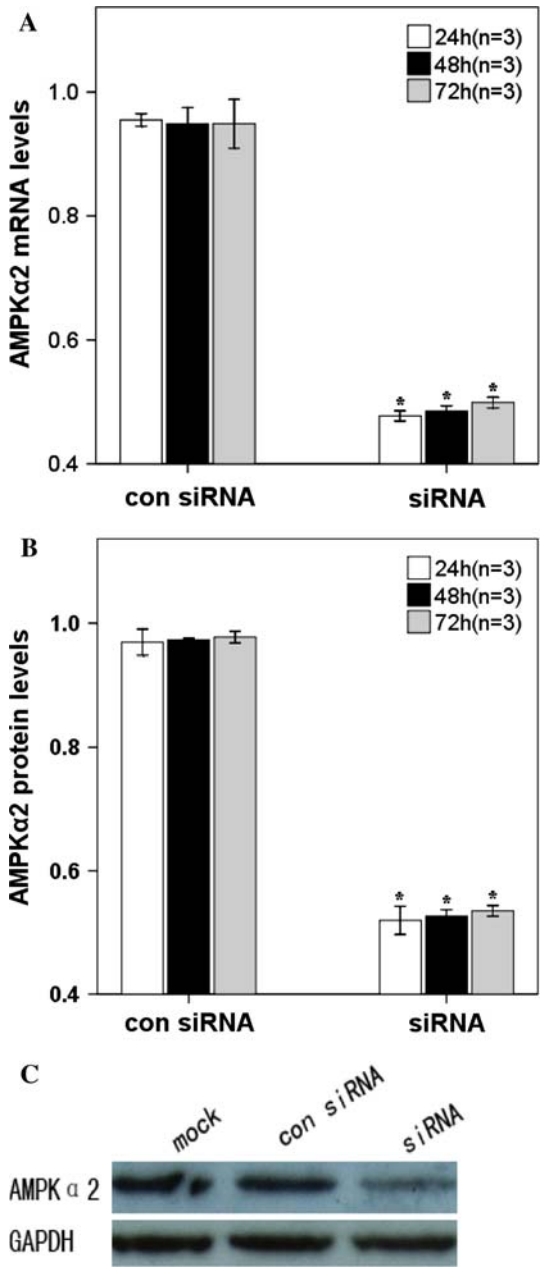

Fig. 3 AMPKα2 mRNA (**a**) and protein (**b**) expression levels 24, 48, and 72 h after transfection with AMPKα2 siRNA. Data are shown as the mean ± standard deviation from three independent experiments. * Indicates a statistically significant difference, siRNA versus control ($P < 0.05$). **c** Representative western blot images from the 24-h time point

encoding hepatic gluconeogenic enzymes G6Pase and PEPCK. This was mediated by AMPK.

Effect of resistin on the protein content and phosphorylation state of AMPK-α and Akt in HepG2 cells

Having determined the effect of resistin on mRNA expression levels of the genes encoding AMPKα2, G6Pase, PEPCK, GLUT2, IRS2, and SOCS-3 by real-time RT-PCR, we next examined the effect of resistin on the protein content and phosphorylation states of AMPK-α and Akt in HepG2 cells. We found that resistin decreased the phosphorylation level of AMPK in both the basal and insulin-stimulated conditions. Resistin also reduced the total Akt content and level of Akt phosphorylation under the insulin-stimulated condition (Fig. 5). There was a significant difference in AMPKα phosphorylation content between the resistin group and the AMPKα2 siRNA-treated group. There was a significant difference in the total Akt content and level between the basal and insulin-stimulated group. However, there was no significant difference between the resistin group and the AMPKα2 siRNA-treated group. There was no significant difference in Akt phosphorylation between the resistin group and the AMPKα2 siRNA-treated group.

Effect of resistin on the regulation of glycogen synthesis

Next, we investigated the effects of resistin on the regulation of glycogen synthesis. The results shown in Fig. 6 demonstrate that both basal and insulin-stimulated glycogen synthesis were decreased in cells treated with resistin. Consistent with the GLUT2 mRNA findings, glycogen synthesis in the AMPKα2 siRNA-treated group (in the presence of resistin) was lower than that in the resistin group.

Discussion

The liver is the central metabolic organ of glucose metabolism. Hepatic insulin resistance is presumed to be

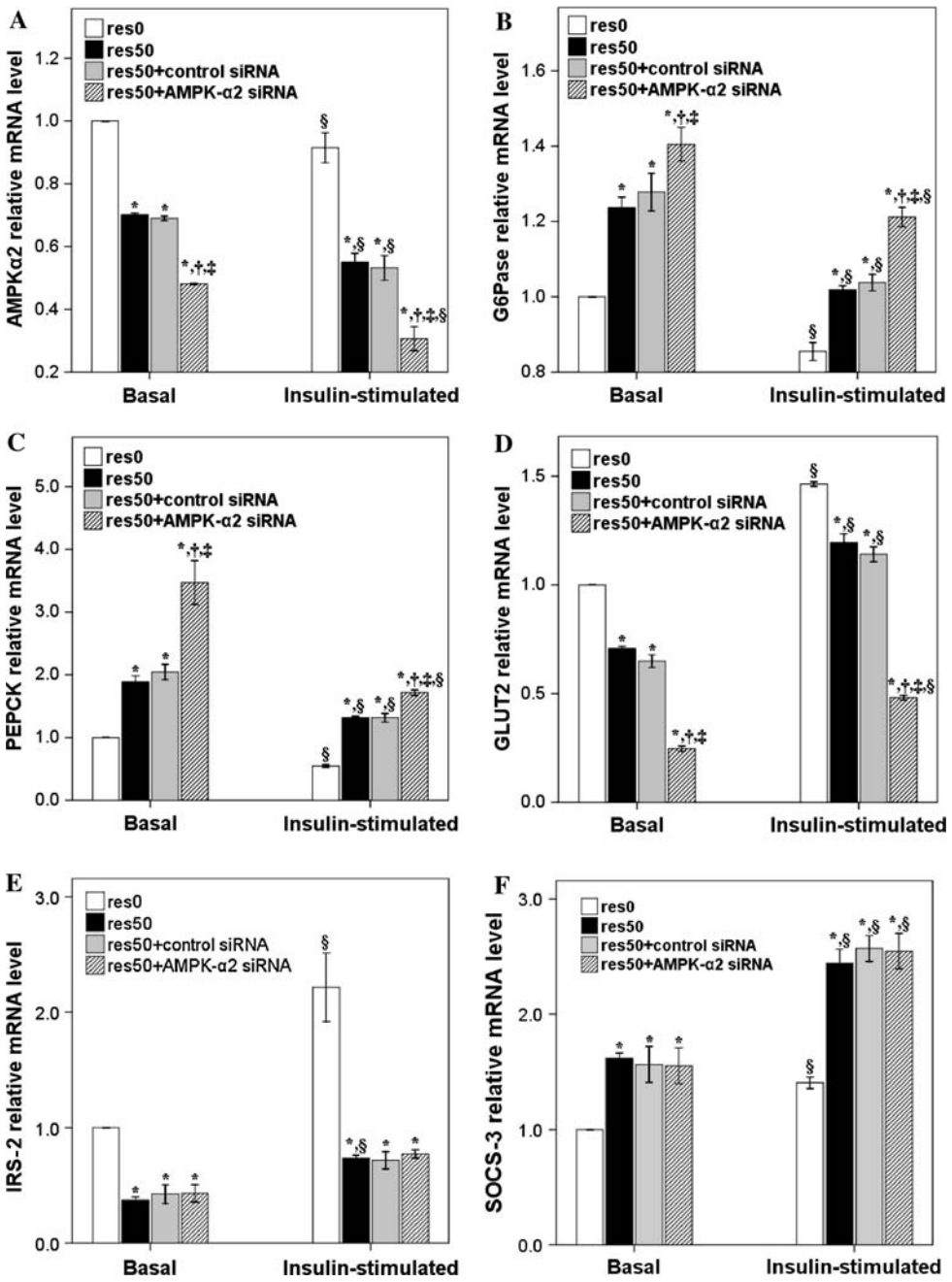

Fig. 4 Relative gene expression levels of AMPKα2 **a**, G6Pase **b**, PEPCK **c**, GLUT2 **d**, IRS2, **e** and SOCS-3 **f** in HepG2 cells following transfection with control siRNA (resistin 50 ng/ml + control siRNA ▭) or AMPKα2 siRNA (resistin 50 ng/ml + siRNA ▨), then serum-starving in glucose-free media, and finally treatment with or without insulin. Data for identically treated untransfected control cells with (resistin 50 ng/ml ■) or without resistin (▭) are also shown. mRNA expression levels are relative to GAPDH expression levels. Data are shown as the mean ± standard deviation from more than three independent experiments. * $P < 0.05$ indicates a statistically significant difference to the untransfected cells without 50 ng/ml resistin. † $P < 0.05$ indicates a statistically significant difference to the untransfected cells exposed to 50 ng/ml resistin. ‡ $P < 0.05$ indicates a statistically significant difference to cells transfected with control siRNA exposed to 50 ng/ml resistin. § $P < 0.05$ indicates a statistically significant difference to the basal group at each treatment level

the primary cause leading to the development of type 2 diabetes [35]. In this study, we used HepG2 cells as a model to investigate the effect of resistin on glucose metabolism and insulin signaling. HepG2 cells have been shown to express various genes involved in the insulin-signaling pathway and glucose metabolism via AMPK [36, 37]. These cells are a useful tool in the analyses of glucose and lipid metabolism, and hepatic insulin resistance [34, 38].

Resistin, so named because it plays a putative role in mediating insulin resistance in rodents [7], can affect glucose metabolism in the liver by attenuating the action of insulin [8, 39]. This suggested mechanisms include decreasing the phosphorylation of AMPK [7, 8], increasing SOCS-3 expression [40], decreasing activation of PPAR gamma [41], and regulating NF-κB expression [42]. Despite intense investigation, the relative contribution of these various mechanisms to glucose metabolism and insulin resistance remains controversial. In this study, we aimed first to explore whether prolonged exposure to resistin affected glucose metabolism and insulin signaling in the human liver HepG2 cells via AMPK. After 24 h of resistin incubation, we found that resistin decreased the phosphorylation level of AMPK in a concentration-

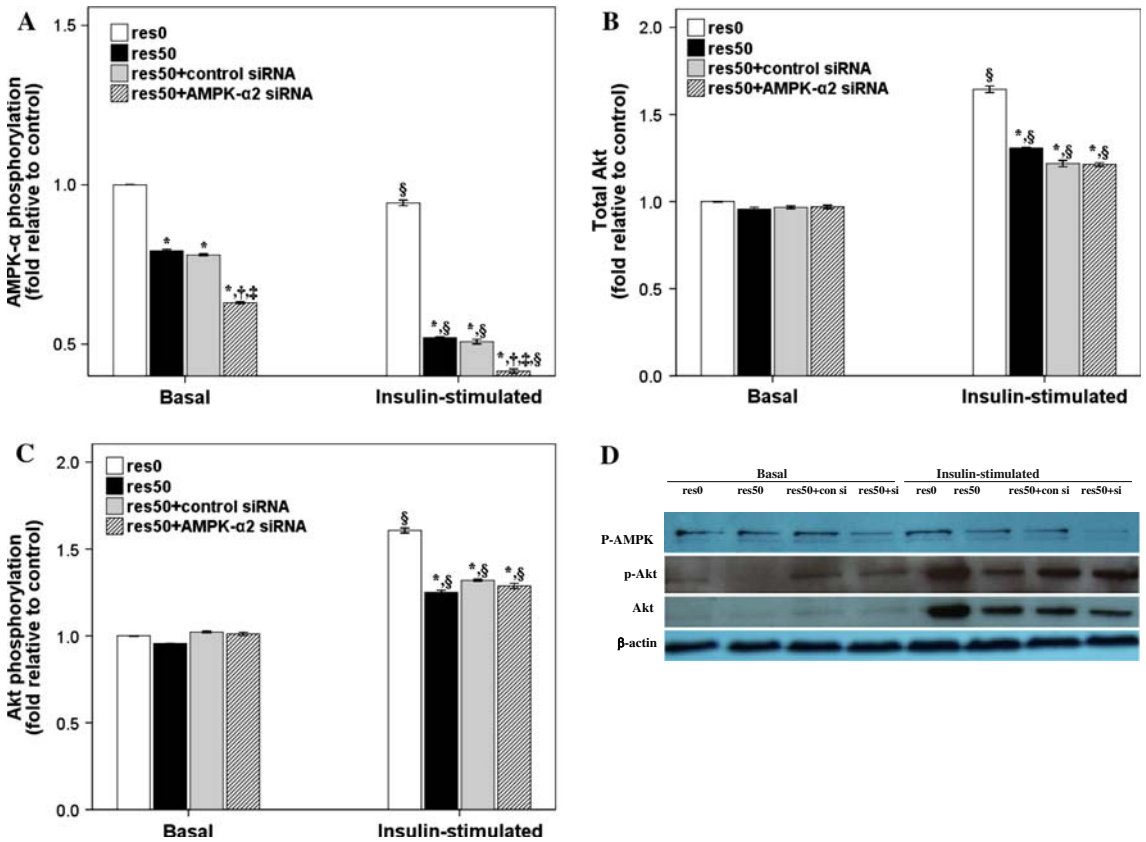

Fig. 5 Effect of resistin on the protein content and phosphorylation states of AMPK-α (**a**) and Akt (**b**, **c**) in HepG2 cells following transfection with control siRNA (resistin 50 ng/ml + control siRNA □) or AMPKα2 siRNA (resistin 50 ng/ml + siRNA ▨), then serum-starving in glucose-free media, and finally treatment with or without insulin. Data for identically treated untransfected control cells with (resistin 50 ng/ml ■) or without resistin (□) are also shown. Data are shown as the mean ± standard deviation from more than three independent experiments. Representative blots for phospho-AMPKα-Thr172 (pAMPK) and Akt as well as Phospho-Akt-Ser473 (pAkt) are shown (**d**). * $P < 0.05$ indicates a statistically significant difference to the untransfected cells without 50 ng/ml resistin. † $P < 0.05$ indicates a statistically significant difference to the untransfected cells exposed to 50 ng/ml resistin. ‡ $P < 0.05$ indicates a statistically significant difference to cells transfected with control siRNA exposed to 50 ng/ml resistin. § $P < 0.05$ indicates a statistically significant difference to the basal group at each treatment level

dependent fashion. This findings are consistent with those from previous reports [7, 8, 43].

In order to further elucidate the role of AMPK in resistin-mediated glucose metabolism and insulin signaling, we used siRNA to inhibit the expression of AMPKα2 in HepG2 cells. AMPK is a phylogenetically conserved intracellular energy sensor that has been implicated in the regulation of glucose and lipid homeostasis [21, 44, 45]. We are tempted to speculate that in hepatocytes, inhibition of AMPK phosphorylation by resistin is the primary mechanism of insulin resistance. However, to date, no specific inhibitors of AMPK have been reported. In an attempt to address this problem, we sought to develop an approach that pitted a siRNA against AMPKα2, thus allowing us to determine directly whether AMPK is involved in the induction of resistin-mediated gene expression in hepatocytes. Our results revealed that AMPKα2 was remarkably inhibited in HepG2 cells following AMPKα2 siRNA treatment, indicating that the corresponding mRNA sequence for AMPKα2 siRNA is a specific RNAi target. Furthermore, we showed that prolonged exposure to resistin decreases glycogen synthesis via AMPK under both basal and insulin-stimulated conditions in HepG2 cells. These results suggest that resistin inhibits the phosphorylation of AMPK even in the absence of insulin, and that this effect is independent of the insulin signaling described by Viana et al. [46]. The hyperglycemic effect of resistin is consistent with the elevation of endogenous glucose production, as indicated by the upregulated, insulin-independent expression of genes encoding the hepatic gluconeogenic enzymes G6Pase and PEPCK in liver cells [47].

Next, we aimed to investigate the mechanism whereby prolonged exposure to resistin affects insulin signaling. We examined the mRNA expression levels of IRS2 and SOCS-3, and the protein content and phosphorylation levels of AMPK-α and Akt in HepG2 cells. We found that resistin

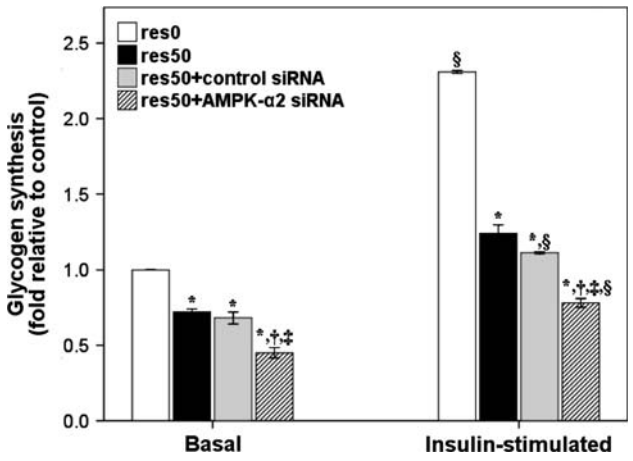

Fig. 6 Effect of resistin on the regulation of glycogen synthesis in HepG2 cells following transfection with control siRNA (resistin 50 ng/ml + control siRNA ▨) or AMPKα2 siRNA (resistin 50 ng/ml + siRNA ▨), then serum-starving in glucose-free media, and finally treatment with D-[U–^{14}C] glucose in the presence or absence of insulin. Data for identically treated untransfected control cells with (resistin 50 ng/ml ■) or without resistin (□) are also shown. Data are shown as the mean ± standard deviation from more than three independent experiments. * $P < 0.05$ indicates a statistically significant difference to the untransfected cells without 50 ng/ml resistin. † $P < 0.05$ indicates a statistically significant difference to the untransfected cells exposed to 50 ng/ml resistin. ‡ $P < 0.05$ indicates a statistically significant difference to cells transfected with control siRNA exposed to 50 ng/ml resistin. § $P < 0.05$ indicates a statistically significant difference to the basal group at each treatment level

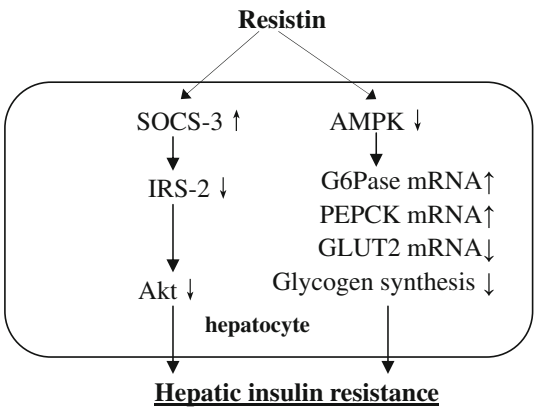

Fig. 7 Proposed model for resistin-induced insulin resistance in HepG2 cells. The up- or downregulation of metabolic pathways is indicated by arrows (↑ for upregulation and ↓ for downregulation)

decreased mRNA expression of genes encoding AMPKα2 and IRS2, and the phosphorylation levels of AMPK under both basal and insulin-stimulated conditions. The total Akt content and level of Akt phosphorylation were also decreased under insulin-stimulated conditions only. However, resistin increased the mRNA expression levels of SOCS-3 under both basal and insulin-stimulated conditions. Moreover, similar to that observed for IRS2 and SOCS-3 mRNA expression, there was no significant difference in the total Akt content and level of Akt phosphorylation between the resistin group and the AMPKα2 siRNA-treated group (in the presence of resistin). These data indicate that resistin-induced insulin resistance in HepG2 cells is due at least in part to induction of SOCS-3 expression and reduction of IRS-2 and Akt phosphorylation by an AMPK-independent mechanism.

SOCS-3 belongs to the SOCS protein family, which is composed of eight members, all possessing a common structure that displays a variable N-terminal region, a central SH2 domain, and a C-terminal tail, named the SOCS box motif [40]. SOCS-3 binds to phosphorylated-insulin receptors and competitively interferes with the binding of other SH2 domain-containing proteins [40]. In addition, the induction of SOCS-3 in the liver is an important mechanism for interleukin-6-mediated insulin resistance [48]. Based on the previous reports [49, 50], we speculated that resistin would upregulate SOCS-3; then, SOCS-3 would competitively bind to insulin receptors and inhibit IRS-2 and insulin-induced phosphorylation of Akt. This may be another mechanism underlying resistin-mediated insulin resistance in HepG2 cells.

In conclusion, our findings suggest that resistin induces insulin resistance in HepG2 cells, at least in part through the induction of SOCS-3 expression and the suppression of Akt phosphorylation via an AMPK-independent mechanism. Resistin increases hepatic glucose production by upregulating the expression of genes encoding the hepatic gluconeogenic enzymes G6Pase and PEPCK, which are mediated by AMPK (Fig. 7).

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