Homocysteine Upregulates Resistin Production From Adipocytes In Vivo and In Vitro

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OBJECTIVE—Homocysteine (Hcy) is epidemiologically related to insulin resistance, which has been speculated to be a low-grade systemic inflammatory condition. Resistin acts as a critical mediator of insulin resistance associated with inflammatory conditions. We aimed to determine whether Hcy can induce insulin resistance by directly regulating the expression and secretion of resistin from adipose tissue.

RESEARCH DESIGN AND METHODS—The effect of Hcy on the expression and secretion of resistin and insulin resistance was investigated using primary rat adipocytes and mice with hyperhomocysteinemia (HHcy).

RESULTS—Hcy impaired glucose transport and, particularly, the insulin signaling pathway as shown by decreased insulin-stimulated tyrosine phosphorylation of insulin receptor and insulin receptor substrate (IRS)-1, increased serine phosphorylation of IRS-1, and inhibited Akt phosphorylation both in vitro and in vivo, and these impairments were accompanied by an increase in resistin expression. Compared with normal mice, HHcy mice with a clinically relevant level of plasma Hcy (19 μmol/l) showed significantly increased resistin production from adipose tissue (33.38 ± 3.08 vs. 19.27 ± 1.71 ng/ml, $P < 0.01$). Hcy (300–1000 μmol/l) also increased mRNA expression of resistin in primary rat adipocytes in a time- and concentration-dependent manner, with maximal induction at 24 h of approximately fourfold with 1,000 μmol/l. In addition, Hcy-induced resistin expression attenuated by treatment with reactive oxygen species (ROS) scavengers, protein kinase C (PKC), and nuclear factor (NF)-κB inhibitors implies a role in the process for ROS, PKC, and NF-κB.

CONCLUSIONS—HHcy may promote insulin resistance through the induction of resistin expression and secretion from adipocytes via the activation of the ROS-PKC–NF-κB pathway. *Diabetes* 57:817–827, 2008

Once known as a passive energy reservoir, adipose tissue is now considered an endocrine organ capable of expressing and secreting a variety of bioactive peptides called adipokines. Adipokines are important for the development of obesity-related diseases such as diabetes, cardiovascular diseases, and cancer. Insulin resistance is a hallmark of diabetes, and its development may involve several adipokines including leptin, tumor necrosis factor (TNF)-α, interleukin (IL)-6, adiponectin, plasminogen-activator inhibitor (PAI)-1, and resistin (1,2).

Resistin, a peptide hormone mainly secreted by adipose tissue, has been suggested to be involved in the pathogenesis of insulin resistance and so may link obesity to diabetes (2,3). Resistin plays an important role in insulin resistance and diabetes associated with genetic or diet-induced obesity (4). In rodents, evidence is accumulating that resistin impairs insulin sensitivity. Administration of resistin impaired glucose tolerance and insulin action in mice, whereas neutralization of resistin by anti-resistin antibody improved hyperglycemia and insulin resistance in mice fed a high-fat diet (2). In humans, macrophages, similar in many aspects to adipocytes, have been found to be the predominant source of circulating resistin (5,6) even though human adipocytes express resistin (7). The functions of mouse and human resistin homologues do not differ (8). Resistin, also known as FIZZ3 (found in the inflammatory zone), is closely related to inflammation (9–11). Administration of endotoxin (lipopolysaccharides) to human volunteers greatly increased circulating resistin levels (10,11), which suggests that resistin may act as a critical endocrine or paracrine signal of insulin resistance associated with inflammatory conditions. Many inflammatory factors, such as IL-1, IL-6, TNF-α, and lipopolysaccharides can regulate resistin expression (5,10,12).

Homocysteine (Hcy) is a sulfur-containing amino acid formed during the metabolism of methionine. Hyperhomocysteinemia (HHcy) has been implicated as an independent risk factor for coronary heart disease (13). Hcy is a potent proinflammatory factor and promotes inflammation both in vitro and in vivo (14,15). We previously demonstrated that Hcy induces expression and secretion of proinflammatory factors macrophage chemoattractant protein-1 and IL-8 by the mediation of oxidative stress in human monocytes (16,17). Epidemiological literature suggests that HHcy is associated with insulin resistance, which is considered a chronic inflammatory status (18,19). A significant association of plasma Hcy and insulin levels was shown in healthy and normal-weight subjects (20) and in diabetic and obese subjects (20–22). In vitro studies suggested that the oxidative stress induced by Hcy thiolactone lead to impaired insulin signaling in a rat hepatoma cell line (23,24). However, whether Hcy can promote insulin resistance by directly regulating the expression and secretion of resistin—one of the adipokines that may link inflammation and obesity to insulin resistance—is unknown.

Here we report that Hcy (1) induced resistin expression in cultured adipocytes and in mice with HHcy (2) and

TABLE 1
List and sequences of primers

	Upstream primer (5′-3′)	Downstream primer (5′-3′)	Genebank code
Mouse resistin	TGCTGAATGTCCATCCATGTG	GGATCCTCACACAGGGAGTTG	NM_022984
Rat resistin	CTACATTGCTGGTCAGTCTCC	GCTGTCCAGTCTATGCTTCC	NM_144741
Mouse adiponectin	CAATGTACCCATTCGCTTTACT	CATACACCTGGAGCCAGACT	MMU49915
Rat adiponectin	CAATGTTCCCATTCGCTTTACT	CATACACTTGGAGCCAGACT	NM_144744
Mouse leptin	CCTGTGGCTTTGGTCCTATCTG	AGGCAAGCTGGTGAGGATCTG	NM_008493
Rat leptin	AGGCAAGCTGGTGAGGATCTG	AGGCAAGCTGGTGAGGATCTG	NM_013076
Mouse TNF-α	CGTCGTAGCAAACCACCAAG	GAGATAGCAAATCGGCTGACG	NM_013693
Rat TNF-α	CCAGGTTCTCTTCAAGGGACA	GTACTTGGGCAGGTTGACCTC	X66539
Mouse IL-6	AGTTGTGCAATGGCAATTCTG	GGAAATTGGGGTAGGAAGGAC	NM_031168
Rat IL-6	GCTCTGGTCTTCTGGAGTTCC	GAGTTGGATGGTCTTGGTCCT	NM_012589
Mouse PAI-1	CCTCACCAACATCTTGGATGCT	TGCAGTGCCTGTGCTACAGAGA	M33960
Rat PAI-1	TCTCAGAGGTGGAAAGAGCCAGA	TCCGGAGTGGTGAACTCAGTG	M24067
Mouse β-actin	ATCTGGCACCACACCTTC	AGCCAGGTCCAGACGCA	NM_007393
Rat β-actin	GAGACCTTCAACACCCCAGCC	TCGGGGCATCGGAACCGCTCA	NM_031144

impaired glucose transport both in vitro and in vivo, at least in part, by upregulating resistin from adipocytes.

RESEARCH DESIGN AND METHODS

Materials. MnTMPyP, *N*-acetyl-cysteine (NAC), RO318220, calphostin C, 3-(1-(3-imidazol-1-ylpropyl)-1H-indol-3-yl)-4-anilino-1H-pyrrole-2,5-dione, and MG132 were purchased from CalBiochem (La Jolla, CA). Anti–insulin receptor-β, anti–insulin receptor substrate (IRS)-1, anti-phospho-IRS-1 (Ser307), anti-Akt, anti–phospho-Akt (Ser473), anti–phospho-protein kinase C (PKC) (pan) (βII Ser660), anti–phospho-PKC-α/βII, anti–phospho-IκB kinase (IKK)-α/β (Ser180/181), and anti–nuclear factor-κB (NF-κB) p65 antibodies, all from rabbit source, were purchased from Cell Signaling Technology (Beverly, MA). Mouse anti-phosphotyrosine antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit anti–IκB-α and mouse anti–β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-murine resistin antibody was purchased from CytoLab (Rehovot, Israel). IRDye-conjugated affinity-purified anti-rabbit, -mouse, and -goat IgGs were purchased from Rockland (Gilbertsville, PA). 2-deoxy-D-[1-³H]glucose was purchased from GE Healthcare (Buckinghamshire, UK). All other chemicals and drugs were purchased from Sigma Chemical (St. Louis, MO).

Animals and treatments. Male C57BL/6J mice, 6 weeks old, were fed standard mouse chow with or without 1.8 g/l DL-Hcy added to the drinking water for 4 weeks, as previously described (17). For the oral glucose tolerance tests, mice were fasted for 12 h before the gastric administration of glucose (3 g/kg body wt) by gavage. For insulin tolerance test, mice were fasted for 4 h, followed by intraperitoneal injection of 2 IU/kg. Blood was drawn from a cut at the tip of the tail at 0, 30, 60, 90, and 120 min, and blood glucose concentrations were detected immediately. After 3 days' adaptation, mice were killed; blood and epididymal fat pads were taken. All animal protocols were approved by the Animal Care and Use Committee of Peking University.

Preparation of adipose tissue extracts and Hcy measurement. Mouse epididymal adipose tissue (~200 mg) was homogenized as previously described (25); the tissue lysate was centrifuged at 12,000 rpm for 15 min twice at 4°C. The clear lysate fraction was collected for Hcy and protein measurements. Total Hcy level in plasma and adipose tissue extracts was quantified by gas chromatography–mass spectrometry (17).

Measurements of plasma lipids, glucose, resistin, insulin, and malondialdehyde. Triglycerides, total cholesterol and high-density lipoprotein cholesterol contents in plasma were measured using commercial kits from Biosino Biotechnology and Science (Beijing, China). Total blood glucose content was measured with Glucotrend from Roche Diagnostics (Mannheim, Germany). Resistin levels in plasma were assayed using the murine resistin EIA kit from Cayman Chemical (Ann Arbor, MI), which cross-reacts at 100% with murine resistin and shows <0.01% cross-reactivity with recombinant murine RELM-α and -β (data from Cayman Chemical). Insulin levels in plasma were assayed with the ELISA insulin kit from Linco Bioscience Institute (St. Charles, MO). Malondialdehyde contents in plasma and adipose tissue extracts were measured using commercial kits from Tianlai Biotechnology and Science (Beijing, China).

Isolation and culture of primary rat adipocytes. Mature adipocytes were isolated from epididymal fat pads of Sprague-Dawley rats (aged 6–8 weeks; 160–200 g) as previously described (26). Packed adipocytes were diluted in serum-free Dulbecco's modified Eagle's medium (DMEM) to generate a 10% (vol/vol) cell suspension. After being incubated at 37°C for 1 h, adipocytes were treated with Hcy (30–1,000 μmol/l) for 8–24 h. For the inhibition experiments, primary rat adipocytes were pretreated with the indicated inhibitors for 1 h before stimulation with Hcy at 500 μmol/l for 24 h.

Differentiation of rat preadipocytes. Adipose precursor cells were isolated from epididymal fat pads of 6-week-old Sprague-Dawley rats (~150 g) and differentiated into adipocytes for 3 days in serum-free DMEM-F12 (1:1) medium supplemented with 5 μg/ml insulin, 33 μmol/l biotin, and 200 pmol/l triiodothyronine (26). Differentiated adipocytes were then incubated in serum-free DMEM-F12 (1:1) medium for another 2 days before treatment.

Measurement of 2-deoxy-D-[1-³H]glucose uptake. Following treatment, differentiated rat adipocytes were glucose starved for 30 min. Insulin (100 nmol/l) was then added for 30 min, followed by the addition of labeled glucose (0.33 μCi of 2-deoxy-D-[1-³H]glucose/ml) in the continued presence of insulin for another 2 h. After terminating the reaction with three washes of ice-cold PBS, the plate was set in the Filtermate Havester (Packard Bioscience, Meriden, CT) and tritium counts were obtained.

RNA extraction and quantitative real-time PCR analysis. Total RNA from primary rat adipocytes or mouse epididymal adipose tissue was isolated by use of Trizol reagent (Promega, Madison, WI) and reverse transcribed using the reverse transcription system (Promega, Madison, WI). Then the reaction mixture underwent PCR. The amount of PCR products formed in each cycle was evaluated on the basis of SYBR Green I fluorescence. Primers for the rat and mouse resistin, adiponectin, leptin, TNF-α, IL-6, PAI-1, and β-actin are in Table 1. All amplification reactions were performed using the Mx3000 Multiplex Quantitative PCR System (Stratagene, La Jolla, CA). Results were analyzed with Stratagene Mx3000 software.

Preparation of cytosolic and nuclear proteins. Following treatment, primary rat adipocytes were packed and homogenized in ice-cold fractionation buffer (26). The cell lysate was incubated on ice for 15 min and then centrifuged at 20,000g for 30 min at 4°C. The cytosolic fraction was collected. Nuclear proteins were extracted with use of NE-PER reagents (Pierce, Rockford, IL).

Western blot analysis. Proteins were subjected to SDS-PAGE with a 10% running gel and then transferred to a polyvinylidene fluoride membrane. The membrane was incubated successively with 0.1% bovine serum albumin in tris-Tween-buffered saline at room temperature for 1 h, with different antibodies at 4°C for 12 h, and then with IRDye-conjugated second antibody for 1 h. A specific immunofluorescence band was detected by the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE).

Measurement of intracellular reactive oxygen species generation. Following treatment, differentiated rat adipocytes were incubated with a reactive oxygen species (ROS) probe dye 2′, 7′-dichlorofluorescin diacetate (20 μmol/l). Determination of intracellular oxidant production was based on the oxidation of dichlorofluorescin diacetate by intracellular ROS, resulting in the formation of the fluorescent compound 2′, 7′-dichlorofluorescin (16). Dichlorofluorescin fluorescence was monitored by confocal laser scanning microscopy (Leica, Germany).

Measurement of NF-κB DNA-binding activity. NF-κB DNA-binding activity was measured using a colorimetric transcription factor assay kit (Chemicon, Temecula, CA) according to the manufacturer's instructions (17). In brief, double-stranded biotinylated oligonucleotide containing the flanked DNA binding consensus sequence for NF-κB was mixed with nuclear extract. The bound NF-κB subunit, p65, was detected with a specific primary antibody, a rabbit anti–NF-κB p65. The horseradish peroxidase–conjugated secondary antibody was then used for spectrophotometric detection.

TABLE 2
Biometric and biochemistry indexes for normal and HHcy mice

	Normal mice	HHcy mice
Plasma Hcy (μmol/l)	7.76 ± 0.81	19.05 ± 1.18*
Adipose tissue Hcy (μmol/g protein)	6.24 ± 2.80	45.87 ± 7.69*
Body weight (g)	26.70 ± 0.64	26.18 ± 0.55
Triglycerides (mmol/l)	2.22 ± 0.21	2.02 ± 0.18
Total cholesterol (mmol/l)	1.55 ± 0.11	2.40 ± 0.15*
HDL cholesterol (mmol/l)	0.56 ± 0.04	0.56 ± 0.03
Insulin (μIU/ml)	15.88 ± 2.56	21.67 ± 3.04
HOMA-IR	4.10 ± 1.15	6.75 ± 1.28†
Resistin (ng/ml)	19.27 ± 1.71	33.38 ± 3.08*
Plasma MDA (nmol/l)	6.62 ± 0.83	12.86 ± 0.82*
Adipose tissue MDA (μmol/g protein)	43.69 ± 3.07	62.64 ± 2.54*

Data are means ± SEM. $n = 8$–16. *$P < 0.01$ compared with control; †$P < 0.05$. HOMA-IR, homeostasis model assessment of insulin resistance; MDA, malondialdehyde.

Statistical analysis. Data are expressed as means ± SEM. Data analysis used GraphPad Prism software. One-way ANOVA, Student-Newman-Keul's test (comparisons between multiple groups), or unpaired Student's t test (between two groups) was used as appropriate. $P < 0.05$ was considered significant.

RESULTS

Hcy promoted insulin resistance in vivo. The HHcy mice model was confirmed by a significant increase of Hcy concentration (2.4-fold) in plasma, accompanied by an even higher increase in adipose tissue (7.4-fold) (Table 2). Homeostasis model assessment of insulin resistance, but not the body weight, plasma lipid, and insulin levels in HHcy mice, was significantly high (Table 2). Two weeks of Hcy supplementation significantly increased blood glucose levels during feeding, an effect observed up to 4 weeks, whereas blood glucose levels during fasting were not affected (Fig. 1A). We further examined insulin sensitivity by performing oral glucose tolerance test and insulin tolerance test. HHcy mice demonstrated severe hyperglycemia upon administration of glucose, as well as impaired glucose tolerance, with typical glucose disposal curves of insulin resistance (Fig. 1B). Similarly, the insulin-stimulated glucose disposal curves in HHcy mice markedly shifted to the right relative to normal mice (Fig. 1C).

We next examined whether the signaling capacity of insulin receptor in adipose tissues of HHcy mice was impaired. HHcy mice showed significant reduction in tyrosine phosphorylation of insulin receptor and IRS-1 (Fig. 1D and E), a significant increase in serine phosphorylation of IRS-1 (Fig. 1E), and a decrease in insulin-induced Akt phosphorylation, a downstream molecule of insulin receptor signaling (Fig. 1F). All these data suggest that HHcy may promote insulin resistance of adipose tissues in vivo.

Hcy promoted insulin resistance in vitro. We then examined the effect of Hcy on insulin resistance by assaying the insulin-stimulated glucose uptake in cultured differentiated rat adipocytes. No significant change was found in the lactate dehydrogenase release rate from the adipocytes in response to Hcy treatment (11.01 ± 1.21 vs. 11.59 ± 0.64%, $P = 0.323$), which indicated no significant cytotoxicity. Hcy (100–1000 μmol/l) impaired insulin-mediated 2-deoxy-D-[1-³H]glucose uptake in a time- and concentration-dependent manner, with maximal inhibition at 24 h and maximal inhibition of ~70% found with 1,000 μmol/l Hcy (Fig. 2A and B), which suggests that Hcy impairs insulin sensitivity of rat adipocytes in vitro.

To investigate whether Hcy interferes with insulin action, we then examine the effect of Hcy on insulin-induced phosphorylation events in primary rat adipocytes. Hcy significantly decreased insulin-stimulated tyrosine phosphorylation of insulin receptor and IRS-1 (Fig. 2C and D), increased the serine phosphorylation of IRS-1 (Fig. 2D), and inhibited the Akt phosphorylation (Fig. 2E).

To determine whether the Hcy-mediated impairment of insulin sensitivity in adipocytes is an autocrine/paracrine effect, primary rat adipocytes or Cos-7 cells were treated with Hcy for 24 h and then conditioned media were collected and used to treat differentiated rat adipocytes. After another 24 h incubation, adipocytes treated with the conditioned medium from Hcy-treated adipocytes showed significant reduction in insulin-stimulated glucose uptake (Fig. 2F), which indicates that Hcy promotes insulin resistance through autocrine or paracrine effects.

Hcy induced resistin expression in vivo and in vitro. We next examined whether Hcy induces the expression of inflammation-related adipokines. Epididymal adipose tissue of HHcy mice showed increased mRNA and protein levels of resistin relative to that in controls (Fig. 3A and B), accompanied by elevated plasma resistin level (Table 2). The mRNA expression of TNF-α and PAI-1 in HHcy mice was higher but that of leptin was lower than that in control mice, while adiponectin and IL-6 mRNA expressions remained unchanged (Fig. 3A).

Exposure of primary rat adipocytes to Hcy (500 μmol/l) for 24 h also induced a significant increase in resistin mRNA (Fig. 3C). Contrary to the in vivo results, adiponectin expression decreased significantly, with leptin, TNF-α, IL-6, and PAI-1 expression showing no change (Fig. 3C). Hcy at 500 μmol/l induced resistin mRNA expression in a time-dependent manner in primary rat adipocytes. Elevated resistin mRNA level occurred as early as 8 h and remained increased up to 24 h in these adipocytes (Fig. 3D). Hcy treatment (300–1000 μmol/l) for 24 h also caused a concentration-dependent increase in resistin mRNA expression (Fig. 3E), with maximal induction of approximately fourfold found with 1,000 μmol/l Hcy. Considering that Hcy promoted insulin resistance through the autocrine or paracrine effect of the induced adipokine(s), resistin may be an important target for Hcy-induced insulin resistance.

ROS participated in Hcy-induced resistin expression. ROS has been suggested to mediate the expression of a few cytokines induced by Hcy in monocytes (16,17). We therefore examined whether Hcy also induced ROS pro-
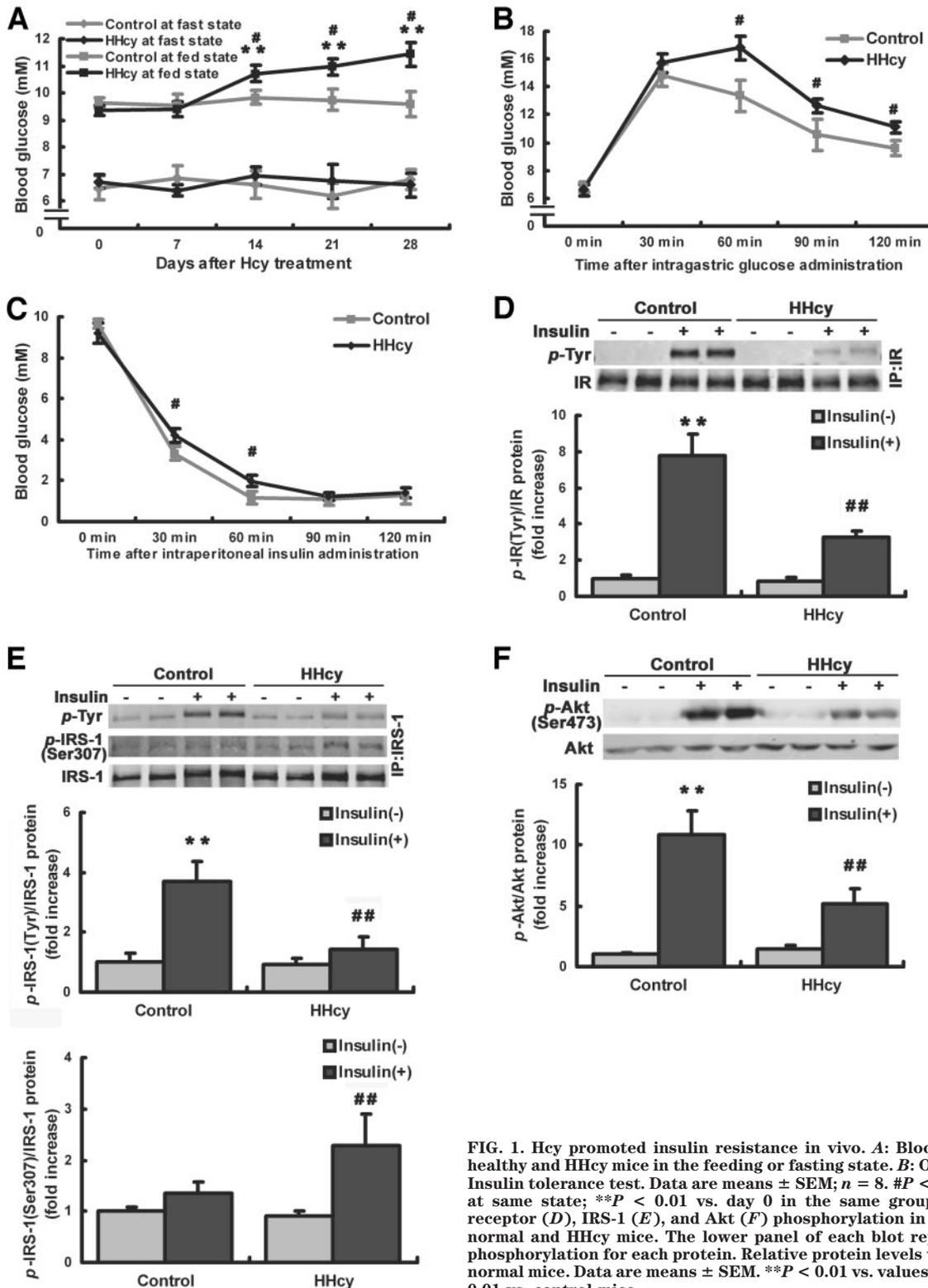
FIG. 1. Hcy promoted insulin resistance in vivo. *A*: Blood glucose concentrations in healthy and HHcy mice in the feeding or fasting state. *B*: Oral glucose tolerance test. *C*: Insulin tolerance test. Data are means ± SEM; $n = 8$. #$P < 0.05$ vs. normal control mice at same state; **$P < 0.01$ vs. day 0 in the same group. Insulin-stimulated insulin receptor (*D*), IRS-1 (*E*), and Akt (*F*) phosphorylation in epididymal adipose tissue of normal and HHcy mice. The lower panel of each blot represents the quantitation of phosphorylation for each protein. Relative protein levels were normalized to levels for normal mice. Data are means ± SEM. **$P < 0.01$ vs. values for absence of insulin; ##$P < 0.01$ vs. control mice.

duction in adipocytes. Since malondialdehyde is an index of lipid peroxidation, increase malondialdehyde content is usually used as a marker of oxidative injury. HHcy mice showed a significant increase of malondialdehyde level (1.9-fold) in plasma, accompanied by a similar increase in adipose tissue (Table 2). Increased intracellular ROS level in differentiated rat adipocytes occurred at 15 min and peaked at 60 min after the addition of 500 μmol/l Hcy (Fig. 4*A*). To determine whether ROS participated in Hcy-

induced resistin expression, primary rat adipocytes were pretreated for 1 h with the ROS scavengers NAC (2 mmol/l) or MnTMPyP (50 μmol/l) and showed significantly attenuated resistin expression induced by 500 μmol/l Hcy for 24 h (Fig. 4*B*).

PKC mediated Hcy-induced resistin expression. Adipocytes express all PKC isoforms except PKC-γ (27). HHcy mice showed significantly increased phosphorylation of PKCs in epididymal adipose tissue (Fig. 5*A*).
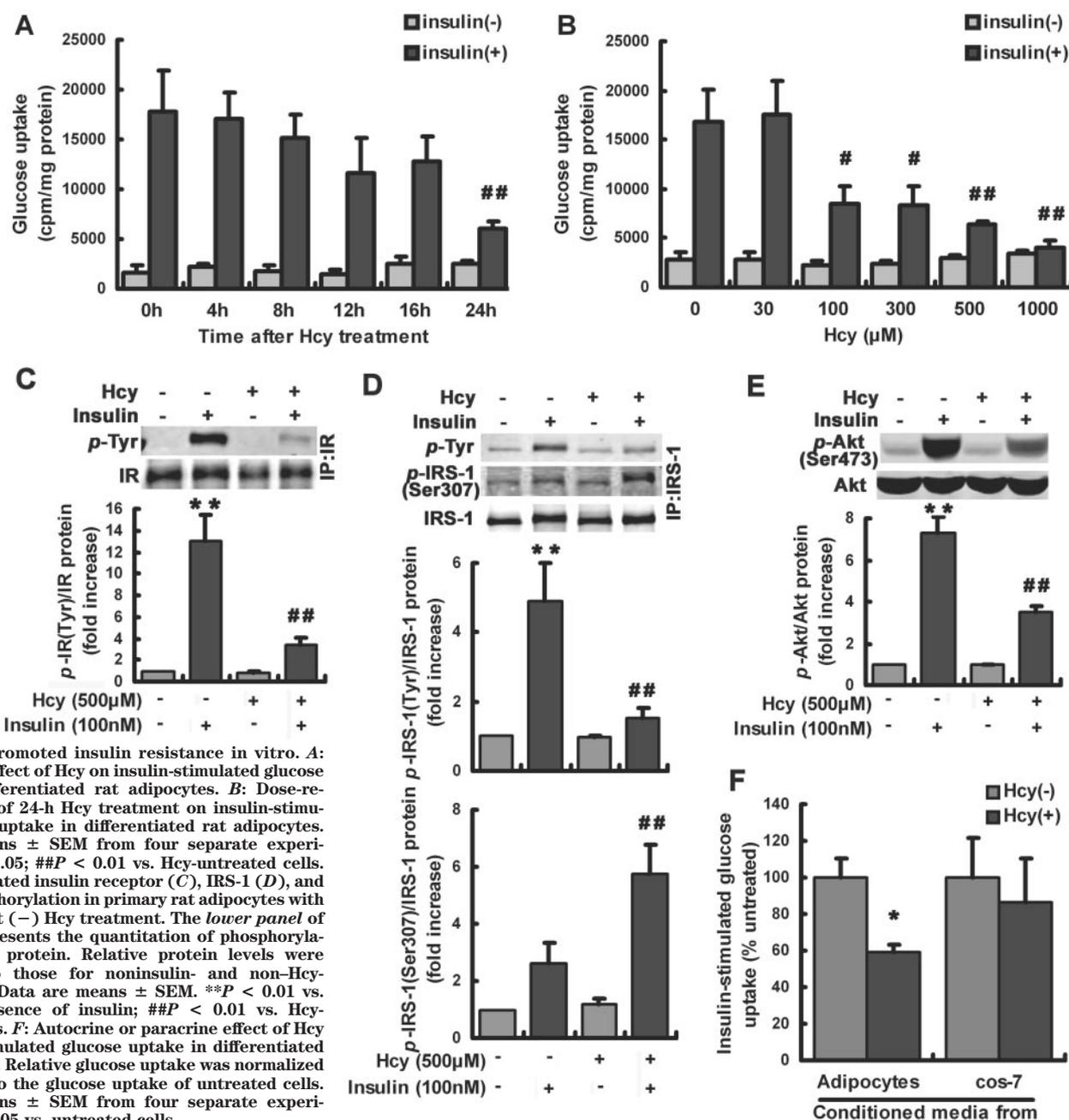
FIG. 2. Hcy promoted insulin resistance in vitro. *A*: Time course effect of Hcy on insulin-stimulated glucose uptake in differentiated rat adipocytes. *B*: Dose-response effect of 24-h Hcy treatment on insulin-stimulated glucose uptake in differentiated rat adipocytes. Data are means ± SEM from four separate experiments. #$P < 0.05$; ##$P < 0.01$ vs. Hcy-untreated cells. Insulin-stimulated insulin receptor (*C*), IRS-1 (*D*), and Akt (*E*) phosphorylation in primary rat adipocytes with (+) or without (−) Hcy treatment. The *lower panel* of each blot represents the quantitation of phosphorylation for each protein. Relative protein levels were normalized to those for noninsulin- and non–Hcy-treated cells. Data are means ± SEM. **$P < 0.01$ vs. values for absence of insulin; ##$P < 0.01$ vs. Hcy-untreated cells. *F*: Autocrine or paracrine effect of Hcy on insulin-stimulated glucose uptake in differentiated rat adipocytes. Relative glucose uptake was normalized with respect to the glucose uptake of untreated cells. Data are means ± SEM from four separate experiments. *$P < 0.05$ vs. untreated cells.

Exposure of primary rat adipocytes to 500 μmol/l Hcy also caused a significant increased phosphorylation of PKC (Fig. 5*C*), especially the α/βII isoform (Fig. 5*B* and *D*). Treatment with the PKC activator phorbol myristate acetate (20 nmol/l) for 24 h directly elevated resistin expression in these cells (Fig. 5*E*). In addition, 500 nmol/l phorbol myristate acetate added to cells for 20 h to exhaust PKCs or pretreatment with the PKC inhibitors RO 318220 (1 μmol/l), calphostin C (200 nmol/l), or the specific PKC-βII inhibitor 3-(1-(3-imidazol-1-ylpropyl)-1H-indol-3-yl)-4-anilino-1H-pyrrole-2,5-dione (1 μmol/l) for 1 h significantly attenuated the Hcy-induced resistin expression (Fig. 5*F–H*). Inhibition of cAMP-dependent protein kinase (rp-cAMPs) or cGMP-dependent protein kinase (KT 5823) produced no effect (Fig. 5*G*). Furthermore, Hcy-induced PKC activation was inhibited by pretreatment of cells with the ROS scavenger MnTMPyP (50 μmol/l) or NAC (2 mmol/l) for 1 h (Fig. 5*I*). These results

strongly indicate that PKC-βII may act downstream ROS to regulate the Hcy-induced resistin expression in primary rat adipocytes.

NF-κB involved in Hcy-induced resistin expression.
Since the resistin promoter contains NF-κB binding sites and the activation of NF-κB might be regulated by ROS in adipocytes (28), we next studied whether NF-κB is involved in Hcy-induced resistin expression. HHcy mice significantly induced phosphorylation of IKKs (Fig. 6*A*) in epididymal adipose tissue, followed by decreased I-κB protein level (Fig. 6*B*). Exposure of primary rat adipocytes to 500 μmol/l Hcy caused the nuclear translocation of the p65 subunit of NF-κB between 1 h and 6 h (Fig. 6*C*). Hcy also significantly enhanced NF-κB DNA-binding activity (Fig. 6*D*). The NF-κB inhibitors MG 132 (10 μmol/l) and pyrrolidine dithiocarbamate (10 μmol/l) significantly attenuated Hcy-induced resistin mRNA expression in adipocytes (Fig. 6*E*). In addition, adipocytes infected with
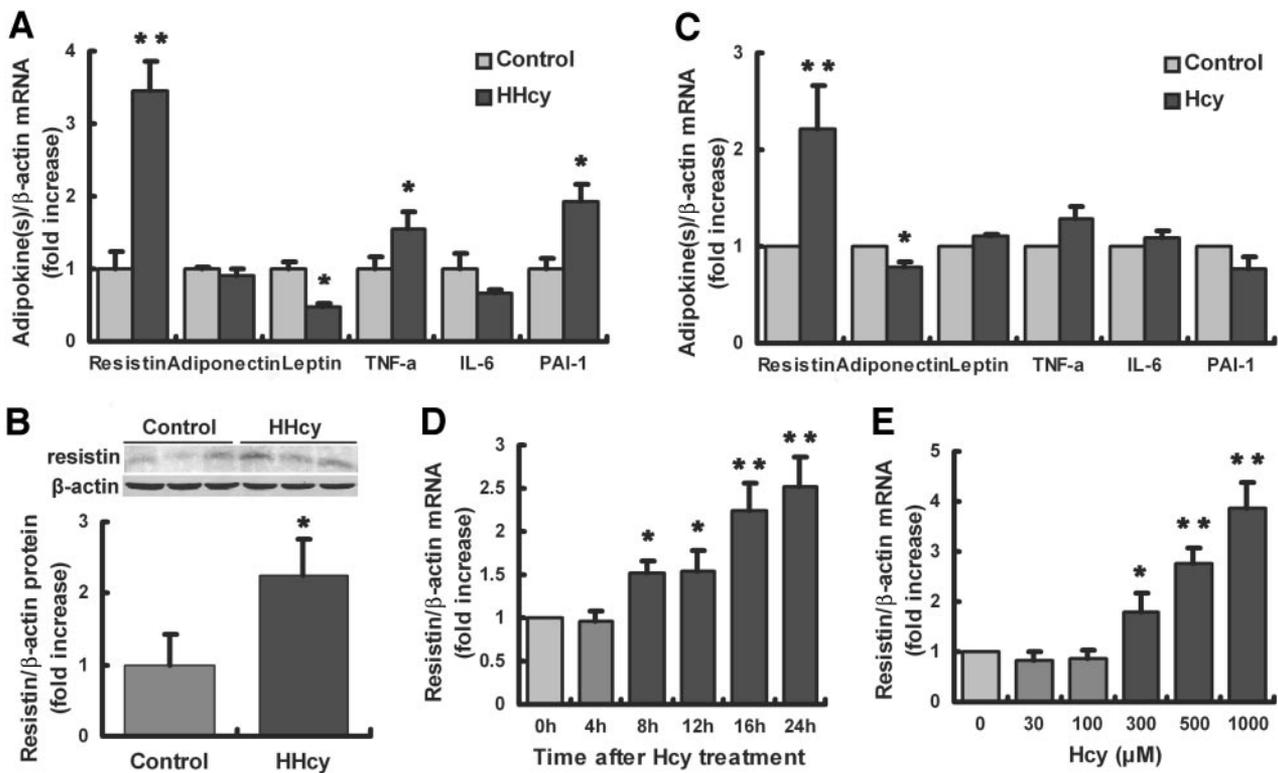
FIG. 3. Hcy induced resistin expression both in vivo and in vitro. *A*: Resistin, adiponectin, leptin, TNF-α, IL-6, and PAI-1 mRNA levels in epididymal adipose tissue of normal and HHcy mice. Relative mRNA levels were normalized to the levels for normal mice. Data are means ± SEM; $n = 8$. *$P < 0.05$; **$P < 0.01$ vs. normal control mice. *B*: Resistin protein level in epididymal adipose tissue of normal and HHcy mice. The *lower panel* represents the summary results of resistin protein level. Relative protein levels were normalized to the levels for normal mice. Data are means ± SEM. *$P < 0.05$ vs normal control mice. *C*: Effect of 500 μmol/l Hcy treatment for 24 h on resistin, adiponectin, leptin, TNF-α, IL-6, and PAI-1 mRNA expression in primary rat adipocytes. *D*: Time course of 500 μmol/l Hcy treatment on resistin mRNA levels in primary rat adipocytes. *E*: Dose-response effect of 24-h Hcy treatment on resistin mRNA levels in primary rat adipocytes. Relative mRNA levels were normalized to that of untreated cells. Data are means ± SEM from four separate experiments. *$P < 0.05$; **$P < 0.01$ vs. untreated cells.

recombinant adenovirus-expressing I-κB showed a significant decrease in Hcy-induced resistin expression (Fig. 6*F*). Furthermore, pretreatment of adipocytes with MnT-MPyP (50 μmol/l) or calphostin C (200 nmol/l) significantly attenuated the nuclear translocation (Fig. 6*G*) and DNA-binding activity of NF-κB (Fig. 6*H*), which indicates that the ROS-PKC–NF-κB pathway is involved in Hcy-induced resistin expression in primary rat adipocytes.

DISCUSSION

In the present study, we have demonstrated for the first time that Hcy increases resistin expression and secretion from adipocytes via the ROS-PKC–NF-κB pathway and subsequently promotes insulin resistance both in vitro and in vivo.

Insulin resistance is speculated to be associated with a low-grade systemic inflammatory condition (29). The close relation between inflammation and diabetes is supported by the observation that stimulation of the innate immune response by bacterial endotoxin results in insulin resistance (30,31). The interaction between inflammation and insulin signaling is also suggested by the ability of aspirin to improve insulin resistance, in part by preventing the antagonistic effects of fatty acids and cytokines (32). Hcy is considered to be a potent proinflammatory factor. In vitro studies in rat hepatoma cells transfected with human insulin receptors suggested that the oxidative stress produced by Hcy thiolactone inhibits insulin receptor tyrosine kinase activity, which results in decreased phosphatidyl-inositol 3-kinase activity and inhibition of glycogen syn-

thesis, thereby impairing insulin signaling (23,24). Hcy is therefore proposed to be a new mediator of oxidative stress–induced insulin resistance.

In the present study, HHcy mice manifested glucose intolerance, insulin resistance, and impaired insulin signaling pathway, which is consistent with a previous report of Sprague-Dawley rats (33) and epidemiological studies (18–22), despite other epidemiological studies finding no association between plasma Hcy level and insulin resistance (34,35). Besides finding the direct impairment of Hcy on the conventional insulin signaling pathway in adipocytes, we found Hcy to induce insulin resistance in differentiated adipocytes possibly in an autocrine/paracrine manner: adipocytes treated with the conditioned medium from Hcy-treated adipocytes also showed a significant reduction in insulin-stimulated glucose uptake, which indicates that Hcy might promote insulin resistance through induced adipokine(s).

Resistin levels are increased in insulin-resistant mice and humans (36,37). Administration of endotoxin to human volunteers was associated with greatly increased circulating resistin levels (10,11); thus, resistin seems to act as a critical mediator of the insulin resistance associated with sepsis and possibly other inflammatory conditions. A strong increase of resistin mRNA by proinflammatory factors also supports the hypothesis that resistin may play a role in the link between inflammation and obesity-associated insulin resistance (5). Our research and other previous studies have demonstrated the effect of Hcy-induced chemokine formation in
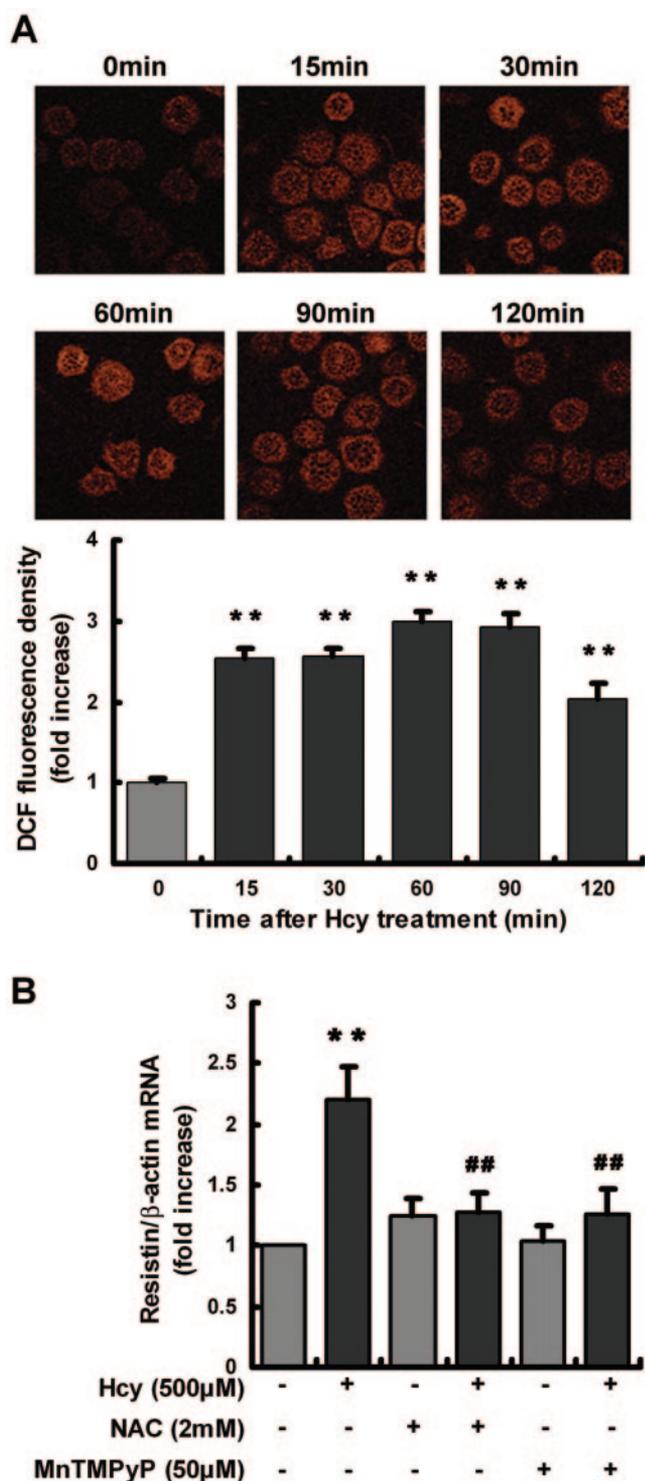
FIG. 4. ROS participated in Hcy-induced resistin expression. *A*: Schematic presentation of the effect of Hcy on ROS formation in differentiated rat adipocytes. Results of representative experiments are shown in the *top panel*. The *lower panel* shows fluorescence intensity of Hcy-induced ROS formation, normalized to the intensity of untreated cells. *B*: Attenuation of Hcy-induced resistin expression by ROS scavengers in primary rat adipocytes. Relative resistin mRNA levels were normalized to that of untreated cells. Data are means ± SEM from four separate experiments. **$P < 0.01$ vs. untreated cells; ##$P < 0.01$ vs. Hcy treatment alone.

induce the expression and secretion of adipokines. Here, we show that Hcy is highly accumulated in adipose tissue (3.1 times that in plasma); moreover, increased expression and secretion of resistin is observed in HHcy mice (19 μmol/l in plasma). Short-term treatment with Hcy (300–1,000 μmol/l for up to 24 h) markedly increased resistin expression in primary rat adipocytes. The plasma concentration of Hcy in patients with homozygous homocystinuria was as high as 500 μmol/l (39), and patients with plasma Hcy level of ~15–30 μmol/l are considered to have moderate HHcy (40). Since this is the range used in the present in vitro and in vivo study, our results have important clinical implications.

The present study also addresses the signaling pathways responsible for Hcy-induced resistin expression in adipocytes. Oxidative stress was observed in HHcy mice, as manifested by increased malondialdehyde levels in both plasma and adipose tissue. Hcy also increased intracellular ROS significantly, and Hcy-induced expression of resistin was inhibited by the antioxidants in adipocytes, which is consistent with previous reports that exposure of adipocytes to hyperglycemic conditions reduces insulin sensitivity and increases ROS levels in vitro (41) and that Hcy induces ROS formation in monocytes followed by chemokine formation (16,17) but argues against other results that ROS is not involved in Hcy-induced secretion of chemokines from endothelial cells (38). Moreover, our results do not support the observation that oxidative stress, from either short-term exposure to high concentrations of H_2O_2 or sustained exposure to low concentrations of H_2O_2, significantly decreases resistin expression and secretion in 3T3-L1 adipocytes (42). Increased ROS levels are an important trigger for insulin resistance in numerous settings (43); adipocytes isolated from animals with high-fat diet–induced diabetes display significantly elevated ROS levels, together with reduced insulin-mediated glucose uptake (44). The fact that ROS mediates elevated resistin level may give a new mechanism for oxidative damage–induced insulin resistance.

Primary cultured rat adipocytes express all PKC isoforms except PKC-γ (27). Our results show that in adipocytes, PKCs, most likely PKC-βII, are significantly activated with Hcy stimulation both in vivo and in vitro. In addition, Hcy-induced resistin expression is PKC dependent, since resistin expression is stimulated directly by the PKC activator and significantly attenuated if PKCs are depleted or blocked. Furthermore, PKCs act downstream of ROS in the Hcy-responsive signal pathway in adipocytes, which agrees with their pattern in hepatoma cells, thus showing that ROS plays a central role in mediating 12-*O*-tetradecanoylphorbol-13-acetate–triggered PKC activation (45). Inactivation of PKCs, most likely PKC-βII, elevates glucose uptake (46), and release of PKC-λ from PKC-βII is an important mechanism in the regulation of glucose transporter distribution in 3T3-L1 adipocytes (47). Our results that PKCs participate in the Hcy-induced resistin expression suggest that PKCs are involved in insulin resistance.

The superoxide radical activates the redox-sensitive proinflammatory transcription factor NF-κB, which increases the transcription of most proinflammatory genes. We have reported a determinant role of oxidative stress in the stimulatory effect of Hcy on chemokine expression in human monocytes, most likely via activation of NF-κB (16,17). Hcy also activates NF-κB in endothelial cells via oxidative stress (48). NF-κB is one of the important signal

many cell types, such as monocytic cell lines (16,17), vascular smooth muscle cells (14), and endothelial cells (38). However, little is known about whether Hcy can
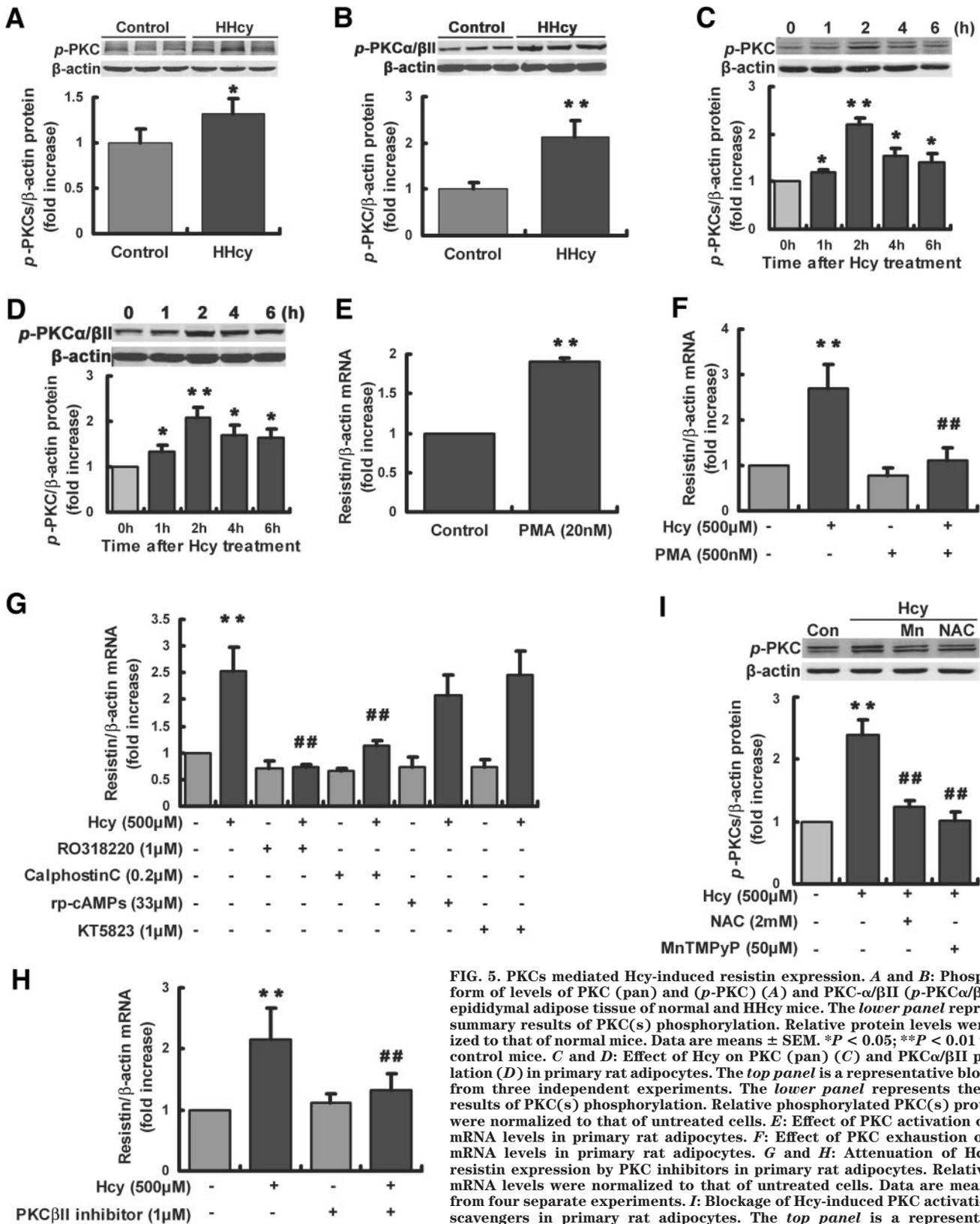
FIG. 5. PKCs mediated Hcy-induced resistin expression. *A* and *B*: Phosphorylated form of levels of PKC (pan) and (*p*-PKC) (*A*) and PKC-α/βII (*p*-PKCα/βII) (*B*) in epididymal adipose tissue of normal and HHcy mice. The *lower panel* represents the summary results of PKC(s) phosphorylation. Relative protein levels were normalized to that of normal mice. Data are means ± SEM. *$P < 0.05$; **$P < 0.01$ vs. normal control mice. *C* and *D*: Effect of Hcy on PKC (pan) (*C*) and PKCα/βII phosphorylation (*D*) in primary rat adipocytes. The *top panel* is a representative blot obtained from three independent experiments. The *lower panel* represents the summary results of PKC(s) phosphorylation. Relative phosphorylated PKC(s) protein levels were normalized to that of untreated cells. *E*: Effect of PKC activation on resistin mRNA levels in primary rat adipocytes. *F*: Effect of PKC exhaustion on resistin mRNA levels in primary rat adipocytes. *G* and *H*: Attenuation of Hcy-induced resistin expression by PKC inhibitors in primary rat adipocytes. Relative resistin mRNA levels were normalized to that of untreated cells. Data are means ± SEM from four separate experiments. *I*: Blockage of Hcy-induced PKC activation by ROS scavengers in primary rat adipocytes. The *top panel* is a representative blot obtained from three independent experiments. The *lower panel* represents the summary results of PKC(s) phosphorylation. Relative phosphorylated PKC(s) protein levels were normalized to that of untreated cells. *$P < 0.05$; **$P < 0.01$ vs. untreated cells; ##$P < 0.01$ vs. Hcy treatment alone.

transduction pathways linking inflammation and insulin resistance (49). Hcy significantly activates IKKs, which then phosphorylates the natural inhibitor of NF-κB, IκB, resulting in the degradation of IκB. Present in vitro results show that Hcy increases NF-κB nuclear translocation and

DNA binding activity, and inhibition of NF-κB significantly attenuates Hcy-induced resistin expression in primary rat adipocytes. Elimination of ROS or inhibition of PKCs impairs the Hcy-induced NF-κB nuclear translocation and DNA-binding activity and thus affects resistin expression,
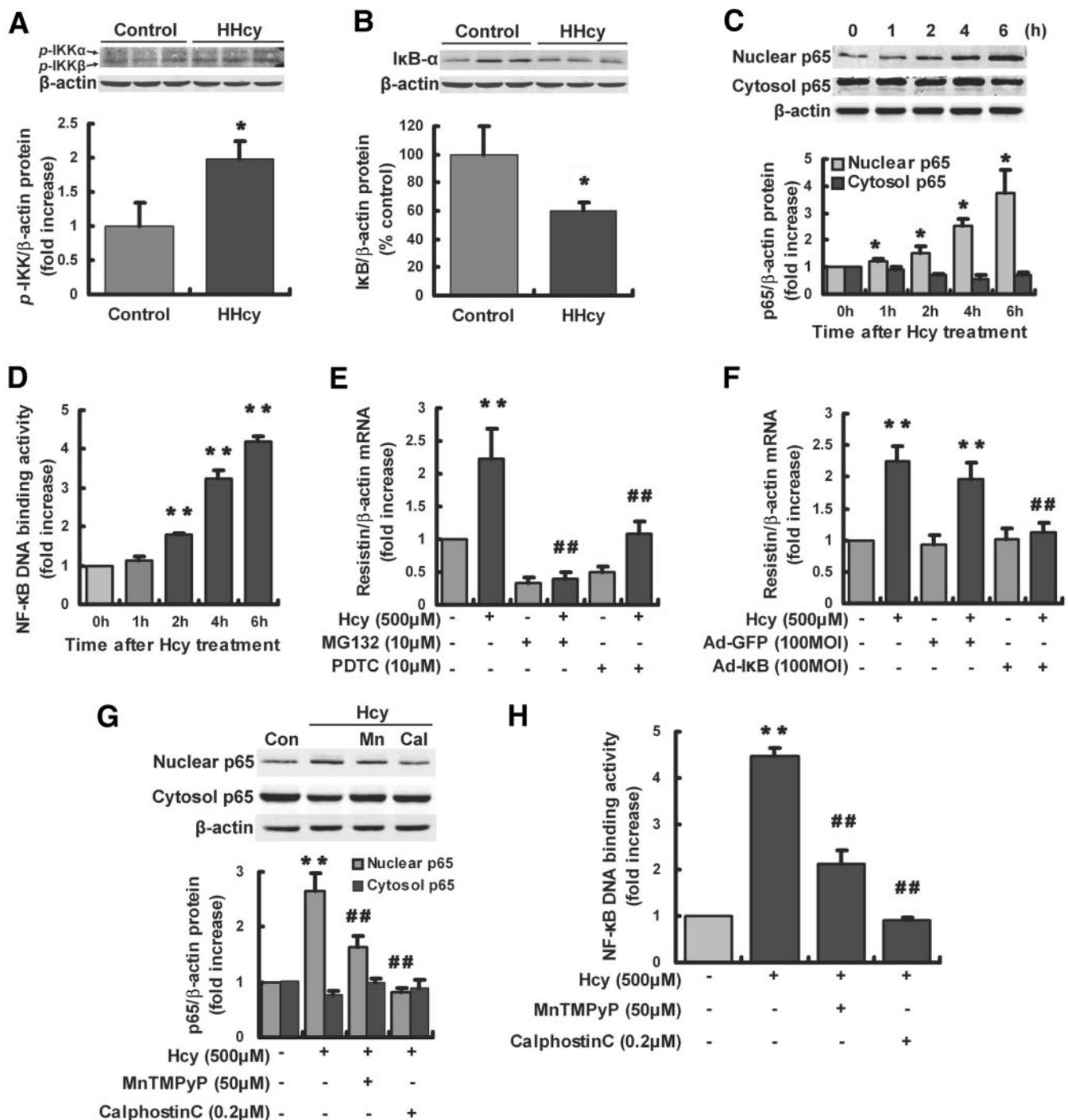
FIG. 6. NF-κB involved in Hcy-induced resistin expression. *A*: Phosphorylated form of IKKα/β (*p*-IKKα/β) level in epididymal adipose tissue of normal and HHcy mice. The *lower panel* represents the summary results of IKK phosphorylation. *B*: IκB-α protein level in epididymal adipose tissue of normal and HHcy mice. The *lower panel* represents the summary results of IκB-α protein level. Relative protein levels were normalized to that of normal mice. Data are means ± SEM. *$P < 0.05$ vs. normal control mice. *C*: Effect of Hcy on nuclear translocation of NF-κB p65 subunit in primary rat adipocytes. The *top panel* is a representative blot obtained from three independent experiments. The *lower panel* represents the summary results of p65 nuclear translocation. *D*: Effect of Hcy on NF-κB DNA-binding activity in primary rat adipocytes. *E*: Attenuation of Hcy-induced resistin expression by NF-κB inhibitors in primary rat adipocytes. *F*: Attenuation of Hcy-induced resistin expression by overexpression of I-κB in primary rat adipocytes. Relative resistin mRNA levels were normalized to that of untreated cells. Data are means ± SEM from four separate experiments. *G*: Blockage of Hcy-stimulated nuclear translocation of NF-κB p65 subunit by ROS scavenger and PKC inhibitor in primary rat adipocytes. The *top panel* is a representative blot obtained from three independent experiments. The *lower panel* summarizes the results of p65 nuclear translocation. Relative p65 protein levels were normalized to that of untreated cells. *H*: Blockage of Hcy-stimulated NF-κB DNA-binding activity by ROS scavenger and PKC inhibitor in primary rat adipocytes. Relative NF-κB DNA-binding activities were normalized to those of untreated cells. **$P < 0.01$ vs. untreated cells; ##$P < 0.01$ vs. Hcy treatment alone.

suggests that NF-κB is downstream the Hcy-induced ROS-PKC pathway. These results confirm that resistin acts as an inflammatory adipokine that impairs the insulin action.

Further study on the promoter activity of resistin will reveal the role of NF-κB in resistin transcription.

Significant induction of TNF-α and PAI-1 and inhibition

of leptin was detected in HHcy mice, although we found no change in level of these adipokines under Hcy stimulation in vitro or secretion of these cytokines in vivo or in vitro (data not shown). Overexpression of resistin augments the secretion of inflammatory cytokines such as TNF-α, macrophage chemoattractant protein-1, and IL-6 in 3T3-L1 cells (50). Differences in cell types and experimental condition may account for these different observations, since we induced the intrinsic expression and secretion of resistin by Hcy rather than overexpression by lentivirus (50). The effects of these adipokines in Hcy-induced insulin resistance cannot be totally excluded. Experimental observations in mice do not always hold true for humans; therefore, further investigation is still needed.

In conclusion, this study demonstrates that Hcy significantly enhances adipose resistin production both in vitro and in vivo and, subsequently, promotes insulin resistance. The intracellular oxidative products and subsequent activation of multiple signaling mediators, including PKCs and NF-κB, are involved in Hcy effects in adipocytes. These findings reveal a novel target of Hcy-induced insulin resistance with the direct regulation of the expression and secretion of resistin from adipose tissue.

ACKNOWLEDGMENTS

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