Arg^{972} insulin receptor substrate-1 inhibits endothelial nitric oxide synthase expression in human endothelial cells by upregulating microRNA-155

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Abstract. The dysregulation of nitric oxide (NO) synthesis attributable to the abnormal expression/activity of endothelial NO synthase (eNOS) is considered to be a major characteristic of insulin-resistant states, as well as an essential contributor to the pathogenesis of cardiovascular diseases. The Arg^{972} insulin receptor substrate-1 (IRS-1) is associated with insulin resistance. In the present study, we investigated the association between Arg^{972} IRS-1 and eNOS expression/activity in human subjects and in primary cultures of human endothelial cells. Data from 832 human subjects revealed that heterozygous and homozygous Arg^{972} IRS-1 carriers had significantly lower levels of plasma eNOS and nitrite/nitrate than the homozygous wild-type (WT) IRS-1 carriers. Human umbilical vein endothelial cells (HUVECs) established from delivering mothers expressing heterozygous Arg^{972} IRS-1 had significantly lower eNOS expression/activity and higher miR-155 levels than those expressing WT homozygous IRS-1. The overexpression of IRS-1 and Arg^{972} IRS-1 in the HUVECs, respectively, decreased and increased the miR-155 expression level. In addition, the overexpression of IRS-1 in the HUVECs significantly increased eNOS expression; this effect was reversed by transfection with mature miR-155 mimic or treatment with the selective phosphatidylinositol-3 kinase (PI3K) inhibitor, BKM120. On the other hand, the overexpression of Arg^{972} IRS-1 markedly decreased eNOS expression and this effect was reversed by transfection with antagomir-155. On the whole, our *in vivo* data demonstrate that Arg^{972} IRS-1 is associated with decreased plasma eNOS and nitrite/nitrate levels in human subjects. Our *in vitro* data demonstrate that Arg^{972} IRS-1 inhibits eNOS expression in human endothelial cells by upregulating miR-155 expression through the impairment of PI3K signaling. The present study provides new insight into the pathophysiological role of Arg^{972} IRS-1 in cardiovascular diseases.

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Introduction

Nitric oxide (NO) generated and released by endothelial NO synthase (eNOS) exerts multiple beneficial effects on blood vessels and plays a critical role in maintaining cardiovascular homeostasis (1). The dysregulation of NO synthesis attributable to the abnormal activity and/or the expression of eNOS is considered to be a major contributor to the pathogenesis of cardiovascular diseases, such as hypertension and atherosclerosis (2,3). Although the expression of eNOS is regulated at multiple levels, increasing evidence has suggested that post-transcriptional regulation plays an important role in the control of eNOS expression. It has been demonstrated that eNOS expression is mainly regulated through the modification of eNOS mRNA expression through the 3'-untranslated region (3'-UTR) of eNOS mRNA (4-8).

Diabetes is associated with an increased risk of cardiovascular disease due to multiple pathophysiological links between insulin resistance, obesity, hypertension and atherogenic dyslipidemia (9-11). One common characteristic of these conditions is endothelial dysfunction, caused by a relative deficiency of NO (12). Insulin has vasodilator effects, which depend on the production of NO in the vascular endothelium (13,14). Key signaling molecules mediating metabolic actions of insulin, such as the insulin receptor tyrosine kinase, phosphatidylinositol-3 kinase (PI3K) and Akt, are also necessary for insulin to stimulate the production of NO in human vascular endothelial cells (15,16). Thus, a physiological role of insulin may be to couple the regulation of hemodynamic homeostasis with metabolic homeostasis (17).

Previous studies have reported a common polymorphism in the insulin receptor substrate-1 (IRS-1) gene, in which a Gly/Arg substitution occurs at codon 972 (Arg^{972}) (18,19). The presence of Arg^{972} IRS-1 is associated with an impaired IRS-1 ability to activate PI3K, leading to the diminished activity of insulin (18,19). In the present study, we investigated the asso-

ciation between Arg^{972} IRS-1 and eNOS expression/activity in a relatively large sample of human subjects and primary cultures of human endothelial cells.

Materials and methods

Subjects. Between November 2012 and October 2013, 832 subjects randomly selected from a total of 2,672 Han Chinese subjects in the database of the physical examination center at Guangdong Provincial People's Hospital, Guangzhou, China were recruited in the present study. Blood samples were drawn from all subjects within 72 h of enrollment. The characteristics of the subjects are presented in Table I. The present study conforms to the Declaration of Helsinki and was approved by the Ethics Committee of Guangdong Provincial People's Hospital (for the collection of patient blood samples, approval reference number: H40125; and for obtaining umbilical vein endothelial cells, approval reference number: H40126). Written informed consent was obtained from all subjects prior to enrollment.

Plasmids and reagents. A fragment of human genomic DNA containing the entire coding sequence of IRS-1 was cloned and ligated into the pcDNA3.1 expression vector as described in a previous study (20). The Arg^{972} IRS-1 expression vector was constructed as previously described (21). SuperFect and HiPerFect transfection reagents were purchased from Qiagen (Valencia, CA, USA). TRIzol reagent and SYBR-Green Master Mix were purchased from Invitrogen (Carlsbad, CA, USA) and PE Applied Biosystems (Foster City, CA, USA), respectively. Anti-eNOS (49G3; #9586), anti-phospho-eNOS (Ser1177; #9570) and anti-β-actin (8H10D10; #3700) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). The eNOS human ELISA kit (#ab166866) was purchased from Abcam (Cambridge, MA, USA). The nitrite/nitrate colorimetric assay kit (#780001) was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). The selective PI3K inhibitor, BKM120 (#sc-364437A), and anti-IRS-1 (C-20; #sc-559), anti-Akt (5C10; #sc-81434), anti-phospho-Akt (Ser473; #sc-101629) and anti-von Willebrand factor (vWF) (1.B.690; #sc-73268) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mature miR-155 mimic (5'-UUAAUGCUAAUC GUGAUAGGGGUCCCUAUCACGAUUAGCAUUAAUU-3') was purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). Antagomir-155 (#410078-00; miRCURY LNA™ microRNA power inhibitor) was purchased from Exiqon (Woburn, MA, USA). Endothelial cell growth medium (#211-500) was purchased from Cell Applications, Inc. (San Diego, CA, USA). G418, insulin and all other chemicals of reagent grade were purchased from Sigma (St. Louis, MO, USA).

Human umbilical vein endothelial cell (HUVEC) culture. Umbilical cords were obtained from mothers delivering at Guangdong Provincial People's Hospital. Primary HUVEC cultures were obtained as previously described (22). Following perfusion of the umbilical cords with 0.1% collagenase at 37˚C, the HUVECs were grown on 0.2% gelatin-coated tissue culture plates in endothelial cell growth medium supplemented with 20% FBS and endothelial cell growth supplement (Cell Applications, Inc.). In all the experiments, the cells were used between the third and the fifth passages. Commercial HUVECs (#200K-05n) were purchased from Cell Applications, Inc. for the stable transfection of IRS-1 and Arg^{972} IRS-1. We confirmed by sequencing that the commercial HUVECs expressed homozygous wild-type (WT) IRS-1 (GG genotype).

Genotyping and determination of eNOS and nitrite/nitrate levels. The Arg^{972} IRS-1 polymorphism was identified by sequencing. Plasma eNOS and nitrite/nitrate levels were respectively determined with an eNOS human ELISA kit (Abcam) and a nitrite/nitrate colorimetric assay kit (Cayman Chemical) according to the manufacturer's instructions. Nitrite/nitrate levels in the HUVEC culture media were normalized to the total proteins of HUVECs.

Transfection. Plasmid constructs were transfected into the HUVECs using SuperFect transfection reagent (Qiagen) according to the manufacturer's instructions. Pools of stable transfectants of IRS-1 and Arg^{972} IRS-1 were generated through selection with G418 (800 μg/ml) as per the manufacturer's instructions. Mature miR-155 mimic or antagomir-155 was transfected into the HUVECs using HiPerFect transfection reagent (Qiagen) according to the manufacturer's instructions.

Reverse transcription-quantitative RT-qPCR. Total RNA from the HUVECs was prepared using TRIzol reagent (Invitrogen) and reverse transcribed into cDNA using SuperScript II reverse transcriptase (Invitrogen) or microRNA TaqMan kit (Applied Biosystems, Foster City, CA, USA). Quantitative PCR (qPCR) was carried out on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems), using the fluorescent dye SYBR-Green Master Mix (PE Applied Biosystems) as per the manufacturer's instructions. The results were normalized to those of the housekeeping gene β-actin in the same sample. The primers used were as follows: eNOS, 5'-GTGGCTGTCTG CATGGACCT-3' (forward) and 5'-CCACGATGGTGACTTT GGCT-3' (reverse); β-actin, 5'-AGCCTCGCCTTTGCCGA-3' (forward) and 5'-CTGGTGCCTGGGGCG-3' (reverse). Stem-loop RT-PCR (TaqMan MicroRNA assays; P/N: 000448 for miR-125a, P/N: 000449 for miR-125b, P/N: 000521 for miR-218, P/N: 000451 for miR-126, P/N: 002623 for miR-155, P/N: 000397 for miR-21, P/N: 000524 for miR-221 and P/N: 000525 for miR-222; Applied Biosystems) was used to quantify the miRNAs according to the manufacturer's instructions. Small unclear RNA U6 was used as an internal control with the following primers: forward, 5'-CTCGCTTCGGCA GCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'. The reverse primer of U6 was used for reverse transcription. Each experiment was repeated 3 times in duplicate.

Western blot analysis. Western blot analysis was carried out as previously described (23). The HUVECs were lysed in 250 μl of 2X SDS loading buffer (62.5 mm Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromphenol blue and 5% 2-mercaptoethanol) and incubated at 95˚C for 10 min. Equal amounts of protein (100 μg) for each sample were separated by 8-15% SDS-polyacrylamide gel and blotted onto polyvinylidene difluoride microporous membranes (Millipore, Billerica, MA, USA). The membranes were incubated for 1 h with a 1:1,000 dilution of primary antibody, and then washed and revealed using

Table I. Characteristics of the 832 study subjects.

Variables	Mean + SD or n (%)	Range
Age (years)	53.5±6.3	33-76
Age group (years)		
30-39	123 (14.8)	-
40-49	189 (22.7)	-
50-59	202 (24.3)	-
60-69	172 (20.7)	-
70-79	146 (17.5)	-
Male	426 (51.2)	-
Smoking	184 (22.1)	-
BMI (kg/m^2)	26.3±4.5	17.9-31.4
Fasting glucose (mmol/l	5.4±2.1	3.1-11.2
Total cholesterol (mmol/l)	5.8±1.7	3.6-9.5
LDL-cholesterol (mmol/l)	3.8±1.5	1.7-6.7
HDL-cholesterol (mmol/l)	1.4±0.6	0.6-2.4
Triglycerides (mmol/l)	2.3±1.3	0.9-6.4
Systolic blood pressure (mmHg)	124.3±19.5	96-185
Diastolic blood pressure (mmHg)	86.7±21.2	59-126
Plasma eNOS (ng/ml)	38.9±12.5	18.2-70.8
Plasma nitrite/nitrate (μM)	9.2±2.7	2.2-12.9
Hyperlipidemia	151 (18.1)	-
Hypertension	225 (27.0)	-
Coronary heart disease	106 (12.7)	-
Type 2 diabetes mellitus	90 (10.8)	-
Kidney disease	52 (6.3)	-
Arg^{972} IRS-1 genotype		
GG	639 (76.8)	-
GA	176 (21.2)	-
AA	17 (2.0)	-

For continuous variables, all values are expressed as the means ± SD. For categorical variables, all values are expressed as n (%). BMI, body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein; eNOS, endothelial nitric oxide synthase; IRS-1, insulin receptor substrate-1.

secondary antibodies with horseradish peroxidase conjugate (1:5,000, 1 h). Peroxidase was revealed using a GE Healthcare ECL kit (GE Healthcare, Shanghai, China).

Immunofluorescence staining. The cells were washed twice with 10 mmol/l sodium phosphate (pH 7.4), 0.14 mol/l NaCl (PBS) and subsequently fixed for 10 min at room temperature with methanol. After fixation, the cells were washed twice with PBS and incubated for 1 h with anti-vWF antibody (Santa Cruz Biotechnology) (1:500 dilution). Subsequently, the coverslips were washed twice with PBS and incubated for 1 h with FITC-conjugated seconday antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA), embedded in mounting medium, and analyzed under a fluorescence microscope (Axioplan 2; Carl Zeiss, Oberkochen, Germany).

IRS-1-associated PI3K activity assay. IRS-1-associated PI3K activity was determined as previously described (24). Briefly, 700 μg of total protein were immunoprecipitated with anti-IRS-1 antibody (Santa Cruz Biotechnology), and kinase activity was detected by the appearance of radiolabeled ^{32}P-labeled phosphatidylinositol 3-phosphate ([^{32}P]PI-3-P) following thin-layer chromatography (TLC) as previously described (25). Autoradiographic signals were quantified using NIH Image software version 1.63.

Statistical analysis. Statistical analyses were carried out using SPSS 15.0 software (IBM, Chicago, IL, USA). All continuous variable values are expressed as the means ± SD. Comparisons of the means between 2 independent groups were performed using the Student's t-tests. A stepwise multi-linear regression model was used to determine which variables explained unique variance in plasma eNOS levels. All variables listed in Table I were tested in the multi-linear regression model. Comparisons of the means among multiple groups were performed using one-way ANOVA followed by post hoc pairwise comparisons using Tukey's tests. Categorical variables were compared using χ^2 tests. A value of p=0.05 was considered to indicate a statistically significant difference in the present study.

Results

Association between Arg^{972} IRS-1 and plasma eNOS and nitrite/nitrate levels in human subjects. The dysregulation of NO synthesis attributable to the abnormal expression and activity of eNOS is considered to be a major contributor to the pathogenesis of vascular diseases (2,3). The results from one of our pilot *in vitro* studies suggested that Arg^{972} IRS-1 exerts significant regulatory effects on eNOS expression in human endothelial cells. In the present study, we first genotyped 832 randomly selected human subjects for the Arg^{972} IRS-1 polymorphism and determined their plasma eNOS and nitrite/nitrate levels. As shown in Table I, 76.8% of the total subjects carried the homozygous WT IRS-1 (GG genotype), 21.2% carried the heterozygous Arg^{972} IRS-1 (GA genotype) and only 2% carried the homozygous Arg^{972} IRS-1 (AA genotype). The A allele frequency was found to be 12.6% in our study population. Multivariate regression analysis revealed that after adjustment for all variables except for the plasma nitrite/nitrate level (which highly correlated with the plasma eNOS level) listed in Table I, the copy number of the Arg^{972} IRS-1 A allele, age, body mass index (BMI), smoking and blood fasting glucose levels were independent predictors of the plasma eNOS levels, collectively explaining 50.8% of the total variance (Table II). Analyses of the subject characteristics by the Arg^{972} IRS-1 genotypes revealed that the Arg^{972} IRS-1 heterozygous and homozygous carriers had significantly higher blood pressure and a prevalence of hyperlipidemia, coronary heart disease and type 2 diabetes mellitus (T2DM), but lower levels of plasma eNOS and nitrite/nitrate than the homozygous WT IRS-1 carriers (Table III). These findings indicate that Arg^{972} IRS-1 is associated with decreased eNOS expression and activity in the human body.

eNOS expression/activity and eNOS activation/phosphorylation in HUVECs expressing homozygous WT IRS-1 or

Table II. Multivariate regression analysis of independent predictors of plasma endothelial nitric oxide synthase (eNOS) level.

Dependent variable	Independent variable	Partial r^2	Total r^2	β co-efficient	P-value
Plasma eNOS (ng/ml)	Copy number of Arg^{972} IRS-1 A allele	0.197	0.197	-0.274	0.011
	Age	0.140	0.337	-0.228	0.015
	BMI	0.082	0.419	-0.202	0.016
	Smoking	0.055	0.474	-0.141	0.040
	Fasting glucose	0.034	0.508	-0.095	0.047

Stepwise multilinear regression analysis was performed using data from the entire cohort (n=832) of study subjects. IRS-1, insulin receptor substrate-1; BMI, body mass index.

Table III. Characteristics of study subjects by Arg^{972} insulin receptor substrate-1 (IRS-1) genotypes.

	Arg^{972} IRS-1 genotype			
Variables	GG (n=639)	GA (n=176)	AA (n=17)	P-value
Age (years)	53.3±6.0	53.8±6.5	57.9±6.8	0.862
Smoking (n=184)	136 (21.3)	44 (25.0)	4 (23.5)	0.569
BMI (kg/m^2)	26.2±4.3	26.7±4.6	25.9±4.7	0.905
Fasting glucose (mmol/l)	5.1±1.8	6.3±2.5	7.4±2.8	0.033[a]
Total cholesterol (mmol/l)	5.8±1.6	5.8±1.9	5.8±2.1	0.946
LDL-cholesterol (mmol/l)	3.7±1.3	4.1±1.8	4.5±1.9	0.083
HDL-cholesterol (mmol/l)	1.5±0.6	1.1±0.9	0.7±0.4	0.021[a]
Triglycerides (mmol/l)	2.0±1.0	3.2±1.6	4.3±1.9	0.007[a]
Systolic blood pressure (mmHg)	120.5±18.7	136.2±22.1	143.9±25.7	0.010[a]
Diastolic blood pressure (mmHg)	84.5±20.3	93.1±25.5	103.1±21.6	0.015[a]
Plasma eNOS (ng/ml)	41.2±10.4	32.7±15.8	27.0±16.9	0.019[a]
Plasma nitrite/nitrate (μM)	9.8±2.6	7.1±3.0	5.0±2.9	0.029[a]
Hyperlipidemia (n=151)	101 (15.8)	39 (22.2)	11 (64.7)	<0.001[a]
Hypertension (n=225)	170 (26.6)	47 (26.7)	9 (52.9)	0.054
Coronary heart disease (n=106)	67 (10.5)	29 (16.5)	10 (58.8)	<0.001[a]
Type 2 diabetes mellitus (n=90)	62 (9.7)	25 (14.2)	13 (76.5)	<0.001[a]
Kidney disease (n=52)	34 (5.3)	12 (6.8)	6 (35.3)	<0.001[a]

For continuous variables, all values are expressed as the means ± SD. For categorical variables, all values are expressed as n (%). BMI, body mass index; [a]P<0.05, indicating statisitical significance. LDL, low-density lipoprotein; HDL, high-density lipoprotein; eNOS, endothelial nitric oxide synthase.

heterozygous Arg^{972} IRS-1. To further examine the association between Arg9^{72} IRS-1 and eNOS expression/activity, we used HUVEC cultures established from delivering mothers expressing homozygous WT IRS-1 (GG genotype) or heterozygous Arg^{972} IRS-1 (GA genotype). HUVEC cultures expressing homozygous Arg^{972} IRS-1 (AA genotype) were not included for the following reasons: i) the low frequency of homozygous Arg^{972} IRS-1 in this study (Han Chinese) population (Table I); ii) heterozygous Arg^{972} IRS-1 carriers showed significantly decreased plasma eNOS and nitrite/nitrate levels compared to the homozygous WT IRS-1 carriers (Table III). Based on a two-sided p=0.05, power=0.80 and an effect size=1.6 (based on data from our pilot *in vitro* study), a sample size of 8 was calculated for the comparison of eNOS expression between 2 HUVEC groups (26). As shown in Fig. 1A, the majority of the cultured HUVECs (~90%) displayed typical endothelial morphology and immunofluorescence staining for the endothelial cell marker, vWF. Without insulin stimulation, the HUVECs showed no significant differences in eNOS expression and nitrite/nitrate levels; we examined different methods of insulin stimulation, and found that treatment with 10^{-8} M of insulin for 24 h had the strongest stimulatory effects on eNOS expression and nitrite/nitrate levels in the HUVECs (data not shown). Thus, in all subsequent experiments in the present study, the HUVECs were pre-stimulated with 10^{-8} M of insulin for 24 h. Following insulin stimulation, HUVECs expressing heterozygous Arg^{972} IRS-1 (GA group) showed a 38% decrease in the eNOS mRNA level compared with those expressing homozygous WT IRS-1 (GG group) (Fig. 1B). Western blot analyses revealed that compared with the

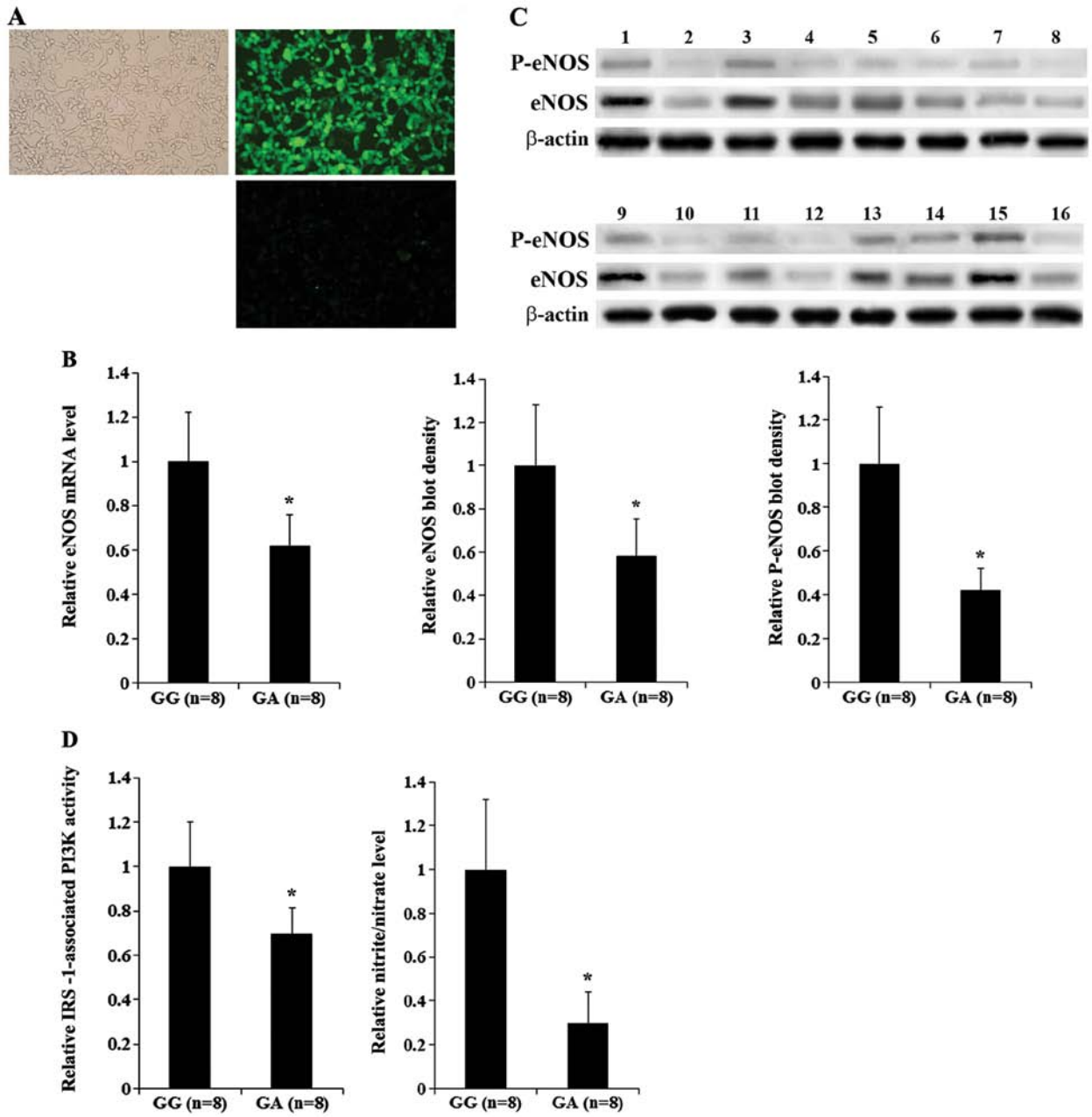

Figure 1. Endothelial nitric oxide synthase (eNOS) expression and activity in human umbilical vein endothelial cells (HUVECs) expressing homozygous wild-type (WT) insulin receptor substrate-1 (IRS-1) or heterozygous Arg^{972} IRS-1. HUVECs were established from delivering mothers expressing homozygous WT IRS-1 (GG group; n=8) or heterozygous Arg^{972} IRS-1 (GA group; n=8) and stimulated with insulin (10^{-8} M) for 24 h. (A) HUVEC cultures were stained for von Willebrand factor (vWF), an endothelial cell marker. Phase contrast Images of (left panel) and immunofluorescence staining for vWF (upper right panel) of the same field of view are shown. A negative staining control lacking the primary antibody for vWF is included (lower right). (B) The eNOS mRNA level was determined by RT-qPCR and shown as a fold change to that of the GG group (designated as 1). (C) The eNOS protein level and eNOS phosphorylation at serine 1177 (p-eNOS) were determined by western blot analyses in the GG group (n=8, lanes 1, 3, 5, 7, 9, 11, 13 and 15) or the GA group (n=8, lanes 2, 4, 6, 8, 10, 12, 14 and 16). β-actin was used as a loading control. Density of the eNOS blot was normalized to that of β-actin to obtain a relative blot density. The mean relative eNOS blot density of the GA group was expressed as a fold change to that of the GG group (designated as 1). Density of the p-eNOS blot was normalized to that of eNOS and β-actin to obtain a relative blot density. The mean relative p-eNOS blot density of the GA group was expressed as a fold change to that of the GG group (designated as 1). (D) IRS-1-associated phosphatidylinositol-3 kinase (PI3K) activities and nitrite/nitrate levels in the GA group were expressed as fold changes to those of the GG group (designated as 1). Data values are expressed as the means + SD. *P<0.05 vs. GG.

GG group of HUVECs, the GA group showed a 42% decrease in the eNOS protein level, and a 58% decrease in eNOS phosphorylation at serine 1177 (Ser1177), which is required for the insulin-stimulated activation of eNOS activity (Fig. 1C) (15). In addition, IRS-1-associated PI3K activity and eNOS activity (measured by the nitrite/nitrate level in the cell culture media) in the GA group decreased by approximately 30 and 70%, respectively (Fig. 1D).

miRNA levels in HUVECs expressing homozygous WT IRS-1 or heterozygous Arg^{972} IRS-1. A recent study demonstrated that eNOS expression can be regulated by microRNAs (miRNAs or

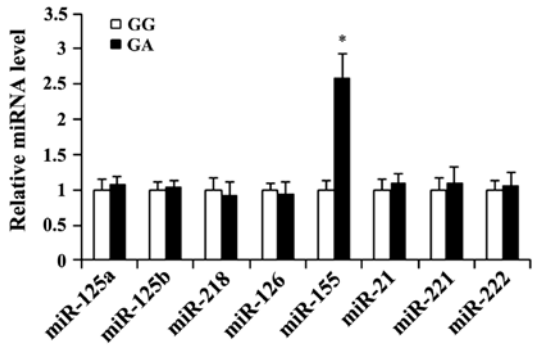

Figure 2. MicroRNA (miRNA or miR) expression levels in human umbilical vein endothelial cells (HUVECs) expressing homozygous wild-type (WT) insulin receptor substrate-1 (IRS-1) or heterozygous Arg^{972} IRS-1. HUVECs were established from delivering mothers expressing homozygous WT IRS-1 (GG group; n=8) or heterozygous Arg^{972} IRS-1 (GA group; n=8) and stimulated with insulin (10^{-8} M) for 24 h. Expression levels of the indicated miRNAs were determined by RT-qPCR and normalized to those of small unclear RNA U6 to obtain a relative miRNA level. The mean relative miRNA level of the GA group was expressed as a fold change to that of the GG group (designated as 1). Data values are expressed as the means + SD. *P<0.05 vs. GG.

miRs) (27). Thus, in the HUVECs, we examined the expression levels of miRNAs reportedly involved in regulating vascular tone (miR-125a, miR-125b, miR-218, miR-126, miR-155 and miR-21) (28) or eNOS expression in endothelial cells (miR-155, miR-221 and miR-222) (27), particularly miR-155, which has been shown to inhibit eNOS expression by directly targeting the 3'-untranslated region (3'-UTR) of eNOS mRNA (27). Following insulin stimulation, the expression level of miR-155 in the GA (heterozygous Arg^{972} IRS-1) group of HUVECs was increased approximately 2.5-fold compared with that in the GG (homozygous WT IRS-1) group of HUVECs, while there were no significant group differences observed in the expression level of the other miRNAs (Fig. 2).

eNOS expression/activity and eNOS activation/phosphorylation in HUVECs overexpressing IRS-1 or Arg^{972} IRS-1 in the presence or absence of miR-155 or antagomir-155. All the above findings suggested that Arg^{972} IRS-1 decreases eNOS expression and activity in endothelial cells by upregulating miR-155. Thus, we examined eNOS expression/activity and miR-155 expression levels in HUVECs stably overexpressing IRS-1 or Arg^{972} IRS-1 in the presence or absence of miR-155 or a functional inhibitor of miR-155 (antagomir-155). As shown in Fig. 3A, compared with the controls, the stable overexpression of IRS-1 in the insulin-stimulated HUVECs decreased the miR-155 expression level by approximately 60%; this effect was reversed by transfection with mature miR-155 mimic or by treatment with the selective PI3K inhibitor, BKM120 (50 μM), for 60 min. On the other hand, the stable overexpression of Arg^{972} IRS-1 increased the miR-155 expression level by 1.55-fold; this effect was reversed by transfection with antagomir-155. Notably, antagomir-155 decreased the miR-155 expression level, possibly by inhibiting the quantitative RT-PCR reaction by competing with the reverse transcription (RT) primer to bind miR-155 during reverse transcription. As shown in Fig. 3B and C, the overexpression of IRS-1 in the HUVECs increased eNOS expression by approximately 1.5-fold; this effect was reversed by transfection with mature miR-155 mimic or treatment with BKM120. On the other hand, the overexpression of Arg^{972} IRS-1 decreased eNOS expression by approximately 50%, which was reversed by transfection with antagomir-155. Transfection with mature miR-155 mimic or treatment with BKM120 alone decreased eNOS expression by approximately 80 and 70%, respectively, while transfection with antagomir-155 alone increased eNOS expression by over 1.6-fold. In addition, mature miR-155 mimic, antagomir-155 and BKM120 showed no significant effect on IRS-1 expression in the HUVECs (Fig. 3C), indicating that their effects on eNOS expression are not mediated by altering the expression of IRS-1. Taken together, these findings indicate that Arg^{972} IRS-1 inhibits eNOS expression in endothelial cells by upregulating miR-155. Of note, the overexpression of IRS-1 and Arg^{972} IRS-1 increased and decreased eNOS phosphorylation at Ser1177 by 4.4-fold and 75%, respectively; these effects were much more pronounced than their effects on eNOS expression and were only partially reversed by miR-155 and antagomir-155, respectively (Fig. 3C). By contrast, the effects of mature miR-155 mimic and antagomir-155 on eNOS phosphorylation at Ser1177 were similar to those on eNOS expression (Fig. 3C).

Arg^{972} IRS-1 is reportedly associated with impaired insulin/IRS-1 signaling to activate the PI3K/Akt pathway, which is required to activate eNOS activity by phosphorylating eNOS at Ser1177 (15). Thus, we examined IRS-1-associated PI3K activity, Akt activation/phosphorylation and eNOS activity (measured by the nitrite/nitrate level in the cell culture media) in the HUVECs stably overexpressing IRS-1 or Arg^{972} IRS-1 in the presence or absence of miR-155 or antagomir-155.

IRS-1-associated PI3K activity, Akt activation/phosphorylation and eNOS activity in HUVECs overexpressing IRS-1 or Arg^{972} IRS-1 in the presence or absence of miR-155 or antagomir-155. As shown in Fig. 4A, compared with the controls, the stable overexpression of IRS-1 in the insulin-stimulated HUVECs increased the IRS-1-associated PI3K activity by 2-fold, which was abolished by treatment with BKM120, but not by transfection with mature miR-155 mimic. On the other hand, the stable overexpression of Arg^{972} IRS-1 decreased the IRS-1-associated PI3K activity by approximately 40%, which was not significantly altered by transfection antagomir-155. Mature miR-155 mimic and antagomir-155 showed no significant effects on the IRS-1-associated PI3K activity. A similar data trend was observed with Akt phosphorylation at serine 473 (ser473) (Fig. 4B), which is required for the activation of Akt by PI3K (29). As shown in Fig. 4C, the overexpression of IRS-1 and Arg^{972} IRS-1 increased and decreased the nitrite/nitrate level by 3.9-fold and 70%, respectively; these effects were much more pronounced than their effects on eNOS expression (Fig. 3C) and were only partially reversed by transfection with miR-155 and antagomir-155, respectively. By contrast, the effects of mature miR-155 mimic and antagomir-155 on the nitrite/nitrate level were similar to those on eNOS expression (Fig. 3C). Taken together, our findings indicate that under insulin stimulation, Arg^{972} IRS-1 inhibits eNOS activity by two different means: i) by downregulating eNOS expression through the upregulation of miR-155; and ii) by inhibiting eNOS activation through the decrease in the PI3K-mediated phosphorylation of eNOS at Ser1177.

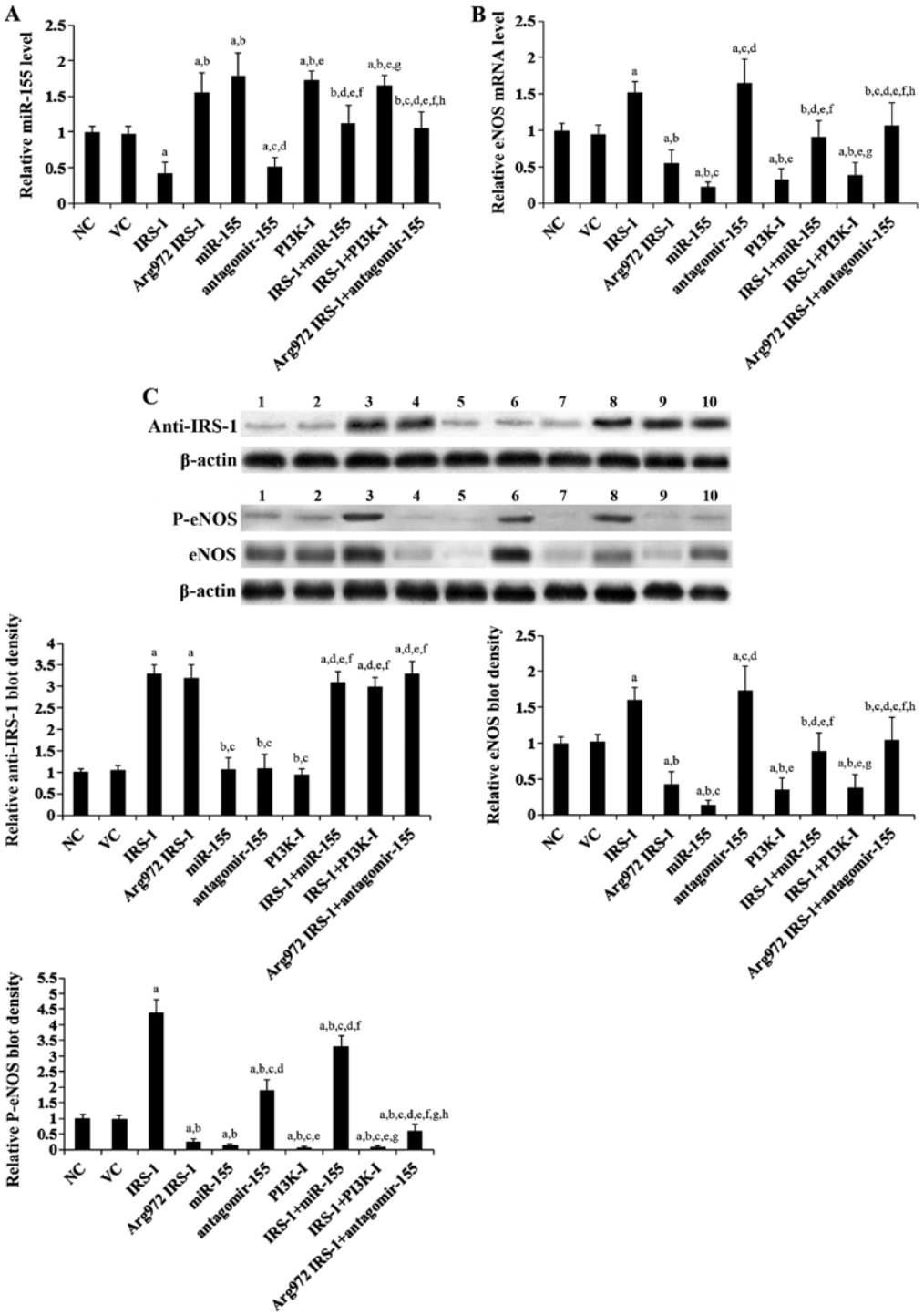

Figure 3. Endothelial nitric oxide synthase (eNOS) and miR-155 levels in human umbilical vein endothelial cells (HUVECs) overexpressing wild-type (WT) receptor substrate-1 (IRS-1) or Arg^{972} IRS-1 in the presence or absence of the overexpression or inhibition of miR-155. In HUVECs stimulated with insulin (10^{-8} M) for 24 h, the expression of (A) miR-155 and (B) eNOS mRNA in normal control cells (NC), cells stably transfected with empty pcDNA3 vector (VC), cells stably transfected with IRS-1 with or without miR-155 treatment, cells stably transfected with Arg^{972} IRS-1 with or without antagomir-155 treatment, cells treated with selective phosphatidylinositide 3-kinase (PI3K) inhibitor BKM120 (50 μM) (PI3K-I) for 60 min, and cells stably transfected with IRS-1 plus BKM120 (50 μM) treatment was analyzed by RT-qPCR. The miR-155 and the eNOS mRNA levels were expressed as fold changes to those of NC (designated as 1), respectively. (C) In HUVECs stimulated with insulin (10^{-8} M) for 24 h, western blot analyses were performed to examine IRS-1/Arg^{972} IRS-1, eNOS and phosphorylated eNOS (at serine 1177) (P-eNOS) levels in normal control cells (NC, lane 1), cells stably transfected with empty pcDNA3 vector (VC, lane 2), cells stably transfected with IRS-1 (lane 3), cells stably transfected with Arg^{972} IRS-1 (lane 4), cells transfected with miR-155 (lane 5), cells transfected with antagomir-155 (lane 6), cells treated with BKM120 (50 μM) for 60 min (PI3K-I, lane 7), cells stably transfected with IRS-1 plus miR-155 treatment (IRS-1 + miR-155, lane 8), cells stably transfected with IRS-1 plus BKM120 (50 μM) treatment (IRS-1 + PI3K-I, lane 9) and cells stably transfected with Arg^{972} IRS-1 plus antagomir-155 treatment (Arg^{972} IRS-1 + antagomir-155, lane 10). β-actin was used as a loading control. Density of the anti-IRS-1 and the eNOS blots was respectively normalized to that of β-actin to obtain a relative blot density, which was expressed as a fold change to the relative anti-IRS-1 blot density and the relative eNOS blot density of NC (designated as 1), respectively. Density of the p-eNOS blot was normalized to that of eNOS and β-actin to obtain a relative blot density, which was expressed as a fold change to the relative P-eNOS blot density of NC (designated as 1). Three independent experiments were performed for each western blot analysis. Data values are expressed as the means + SD. [a]P<0.05 vs. NC or VC; [b]P<0.05 vs. IRS-1; [c]P<0.05 vs. Arg^{972} IRS-1; [d]P<0.05 vs. miR-155; [e]P<0.05 vs. antagomir-155; [f]P<0.05 vs. PI3K-I; [g]P<0.05 vs. IRS-1 + miR-155; [h]P<0.05 vs. IRS-1 + PI3K-I.

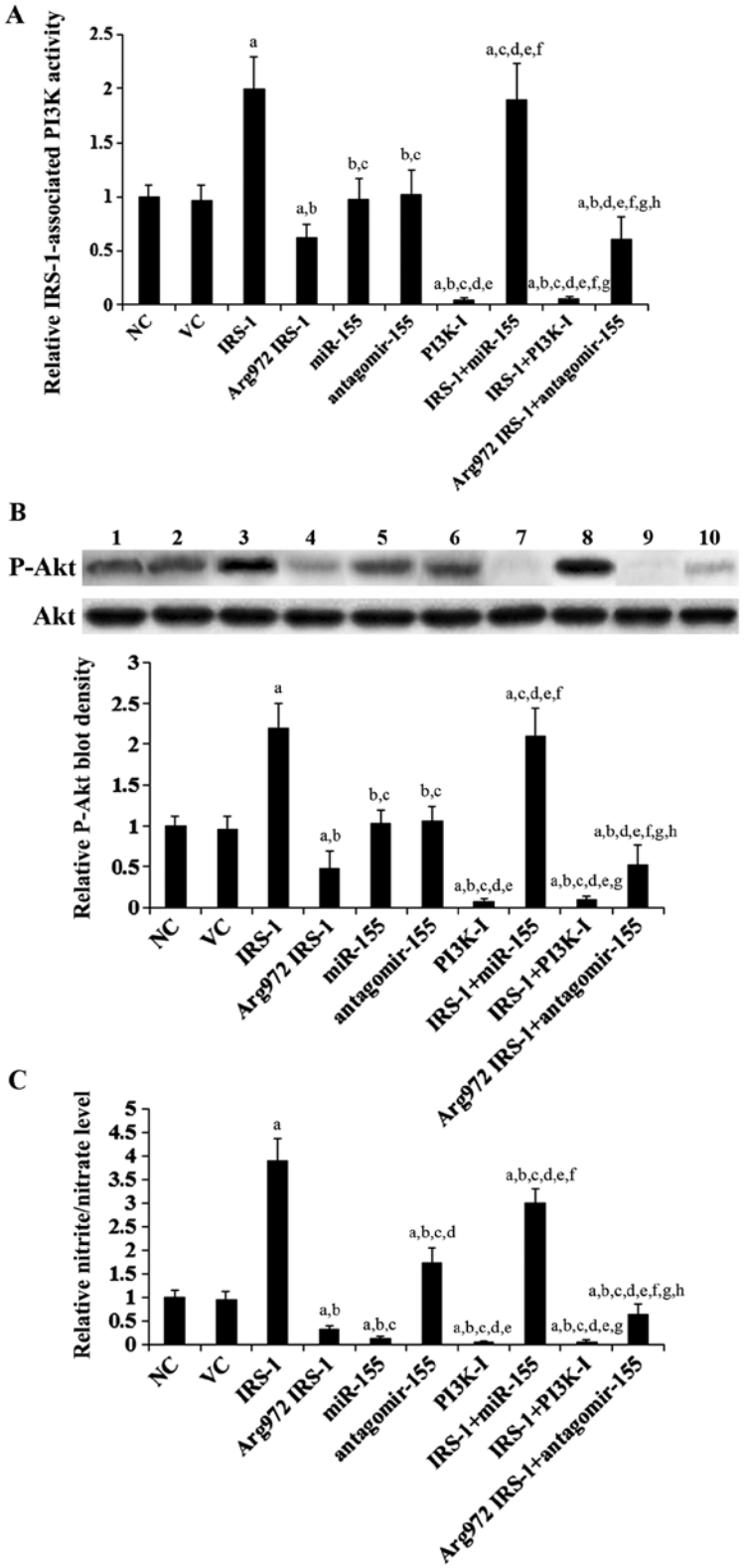

Figure 4. Insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositide 3-kinase (PI3K) activity and phosphorylated Akt (P-Akt) level in human umbilical vein endothelial cells (HUVECs) overexpressing wild-type (WT) IRS-1 or Arg^{972} IRS-1 in the presence or absence of the overexpression or inhibition of miR-155. In HUVECs stimulated with insulin (10^{-8} M) for 24 h, (A) IRS-1-associated PI3K activity, (B) total Akt and p-Akt (at serine 473) levels and (C) nitrite/nitrate levels in normal control cells (NC, lane 1), cells stably transfected with empty pcDNA3 vector (VC, lane 2), cells stably transfected with IRS-1 (lane 3), cells stably transfected with Arg^{972} IRS-1 (lane 4), cells transfected with miR-155 (lane 5), cells transfected with antagomir-155 (lane 6), cells treated with selective PI3K inhibitor BKM120 (50 μM) (PI3K-I, lane 7) for 60 min, cells stably transfected with IRS-1 plus miR-155 treatment (IRS-1 + miR-155, lane 8), cells stably transfected with IRS-1 plus BKM120 (50 μM) treatment (IRS-1 + PI3K-I, lane 9) and cells stably transfected with Arg^{972} IRS-1 plus antagomir-155 treatment (Arg^{972} IRS-1 + antagomir-155, lane 10) were determined. In (B) western blot analyses, the density of the p-Akt blot was normalized to that of total Akt to obtain a relative blot density, which was expressed as a fold change to the relative p-Akt blot density of NC (designated as 1). Three independent experiments were performed for each western blot analysis. (A) IRS-1-associated PI3K activity and (C) nitrite/nitrate levels were expressed as fold changes to those of NC (designated as 1), respectively. Data values are expressed as the means + SD. ^{a}P<0.05 vs. NC or VC; ^{b}P<0.05 vs. IRS-1; ^{c}P<0.05 vs. Arg^{972} IRS-1; ^{d}P<0.05 vs. miR-155; ^{e}P<0.05 vs. antagomir-155; ^{f}P<0.05 vs. PI3K-I; ^{g}P<0.05 vs. IRS-1 + miR-155; ^{h}P<0.05 vs. IRS-1 + PI3K-I.

Discussion

The dysregulation of NO synthesis attributable to the abnormal expression/activity of eNOS is considered to be a major feature of insulin-resistant states (30,31), as well as an essential contributor to the pathogenesis of cardiovascular diseases (2,3). Arg^{972} IRS-1 is reportedly associated with impaired IRS-1 ability to activate PI3K, leading to insulin resistance (18,19). In the present study, to the best of our knowledge, we provide the first evidence that Arg^{972} IRS-1 inhibits eNOS expression in human endothelial cells by upregulating miR-155.

Multivariate regression analysis using data from 832 human subjects revealed that Arg^{972} IRS-1 was an independent predictor of the human plasma eNOS level after adjustment for multiple potential confounders, which was in agreement with our results that heterozygous and homozygous Arg^{972} IRS-1 carriers had significantly lower levels of plasma eNOS and nitrite/nitrate than homozygous WT IRS-1 carriers. This was corroborated by our findings that HUVECs derived from delivering mothers expressing heterozygous Arg^{972} IRS-1 had significantly lower expression levels of eNOS and nitrite/nitrate than those expressing homozygous WT IRS-1.

A recent study demonstrated that miR-155 inhibits eNOS expression in human endothelial cells by directly targeting the eNOS mRNA (27). In the present study, the overexpression of IRS-1 and Arg^{972} IRS-1, respectively, upregulated and downregulated miR-155 in human endothelial cells by altering PI3K signaling. This provides a mechanistic explanation for the enhanced and inhibited eNOS expression induced by the overexpression of IRS-1 and Arg^{972} IRS-1, respectively. Since miR-155 and its inhibitor, antagomir-155, respectively, reversed the effects of the overexpression of IRS-1 and Arg^{972} IRS-1 on eNOS expression, it can be concluded that Arg^{972} IRS-1 inhibits eNOS expression in endothelial cells by upregulating miR-155. Although our findings indicate that Arg^{972} IRS-1 upregulates miR-155 in endothelial cells by impairing PI3K signaling, the mechanisms through which PI3K signaling alters the miR-155 level in endothelial cells remain unclear. We aim to elaborate on this issue in our future studies.

We noted that compared with their effects on eNOS expression, the overexpression of IRS-1 and Arg^{972} IRS-1 exerted much more pronounced effects on eNOS activity, which were only partially reversed by miR-155 and antagomir-155, respectively. This may be explained by the fact that Arg^{972} IRS-1 inhibited eNOS activity both by downregulating eNOS expression through the upregulation of miR-155, and by inhibiting eNOS activation through the impairment of the PI3K signaling-mediated phosphorylation of eNOS (Ser1177), which is required to activate eNOS (15).

Federici *et al* (22) reported that Arg^{972} IRS-1 inhibited eNOS expression in insulin-stimulated endothelial cells *in vitro*. In the present study, we uncovered a mechanism responsible for this phenomenon by demonstrating that Arg^{972} IRS-1 inhibits eNOS expression through the upregulation of miR-155 in endothelial cells. Moreover, our *in vivo* data from a relatively large sample of human subjects support the *in vitro* inhibitory effects of Arg^{972} IRS-1 on eNOS expression; this increases the generalizability of our findings. In addition, our findings that Arg^{972} IRS-1 both downregulates eNOS expression and inhibits PI3K-mediated eNOS activation/phosphorylation account for the marked inhibitory effects of Arg^{972} IRS-1 on eNOS activity *in vitro*. In view of the close association of eNOS activity/NO production with cardiovascular diseases, such as hypertension and atherosclerosis (2,3), our *in vitro* findings were also in line with the data on human subjects showing that heterozygous and homozygous Arg^{972} IRS-1 carriers had significantly higher blood pressure and a prevalence of coronary heart disease than homozygous WT IRS-1 carriers. Thus, the present study provides both *in vivo* and *in vitro* evidence supporting an important pathophysiological role for Arg^{972} IRS-1 in cardiovascular diseases.

In conclusion, our *in vivo* data demonstrate that Arg^{972} IRS-1 is associated with decreased plasma eNOS and nitrite/nitrate levels in human subjects. Our *in vitro* data demonstrate that Arg^{972} IRS-1 inhibits eNOS expression in human endothelial cells by upregulating miR-155 through the impairment of PI3K signaling; additionally, Arg^{972} IRS-1 exerts marked inhibitory effects on eNOS activity by inhibiting both eNOS expression and eNOS activation/phosphorylation through the impairment of PI3K signaling. The present study provides new insight into the pathophysiological role of Arg^{972} IRS-1 in cardiovascular diseases.

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