Anti-Müllerian Hormone Accelerates Pathological Process of Insulin Resistance in Polycystic Ovary Syndrome Patients

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ABSTRACT

Insulin resistance (IR) is one of the most common features of polycystic ovary syndrome (PCOS), which is related to obesity. Whether increased anti-Müllerian hormone (AMH) levels in PCOS are involved in the pathogenesis of insulin resistance remains unclear. We investigated serum levels of leptin and AMH along with basic clinical and metabolic parameters in 114 PCOS patients and 181 non-PCOS women. PCOS patients presented higher fasting blood glucose, insulin concentrations and Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) in addition to body mass index (BMI), lipids profiles and hormone levels. HOMA-IR showed a positive correlation with BMI, AMH, leptin, and low-density lipoprotein-cholesterol (LDL-c) levels. Interestingly, AMH is strongly positively correlated with HOMA-IR and insulin concentrations for 1st and 2nd hours of glucose treatment after fasting. Among PCOS women with BMI ≥ 25 kg/m^2, high AMH level group showed an increased HOMA-IR when compared to normal AMH level. However, among PCOS women with normal BMI, women with high AMH presented an elevated fasting insulin levels but not HOMA-IR when compared to normal AMH group. In vitro treatment of isolated islet cells with high concentration of leptin (200 ng/ml) or high leptin plus high concentration of AMH (1 ng/ml) significantly enhanced insulin secretion. Importantly, co-treatment of AMH plus leptin upregulates the expression of pro-apoptotic proteins, such as Bax, caspase-3, and caspase-8 after incubating with a high level of glucose. These results suggest that AMH may involve in the pathological process of pancreatic β-cells in obese PCOS women.

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Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in reproductive-age women, with a prevalence of 15% depending on the diagnostic criteria used and the population studied [1]. Its clinical manifestations may include menstrual irregularities, signs of hyperandrogenism, and obesity. Insulin resistance (IR) is one of the most common features of PCOS and is associated with an increased risk of type 2 diabetes and cardiovascular events, with a consequent negative effect on long-term health [2]. Although IR is not required for a diagnosis of PCOS, it is clear that intrinsic IR is present in many women with PCOS [3]. However, the underlying etiology and pathogenesis of PCOS remain poorly understood, and there is no effective method of preventing the disease.

As a member of the transforming growth factor-β (TGF-β) family, anti-Müllerian hormone (AMH) is expressed by granulosa cells (GCs) of early developing follicles in the ovary during the reproductive age [4, 5]. AMH is considered a reliable of ovarian reserve in humans and strongly correlate with the number of antral follicles [6, 7]. In the ovary, the main physiologic role of AMH seems to be limited to the inhibition of the early stages of follicular development [8] and preventing the recruitment of non-dominant follicles [9]. Although AMH is mainly thought to play a local role in ovarian follicle recruitment and development [7], it is also available in the peripheral circulation.

IR results from impaired insulin signaling in target tissues that leads to increased levels of insulin required to control plasma glucose levels. IR and pancreatic β-cell dysfunction are two major pathophysiological characteristics of type 2 diabetes mellitus (T_2DM), thus pancreatic β-cells play a crucial role in T_2DM pathogenesis [10, 11]. In the early stage of T_2DM development, the pancreas first increases insulin release by improving the function of the β-cells or increasing their number when IR occurs in peripheral tissues. The β-cell apoptosis caused by high-glucose toxicity results in decreased β-cell function and insulin sensitivity, further reducing insulin secretion in diabetic patients with long-term hyperglycemia.

It is well known that obesity is always related to IR in women with PCOS, while whether the increased AMH levels in PCOS is involved in the pathological process of IR in reproductive-age women with PCOS and whether the increased AMH has an effect on pancreatic β-cells still remains unclear. Hence, the aim of the present study was to investigate the underlying relationship between serum AMH levels and the HOMA-IR, and also with the insulin levels after glucose stimulation. Furthermore, we conducted an in vitro model to examine the effects of high AMH in association with high leptin levels on insulin secretion and the expression of pancreatic β-cell pro-apoptosic genes in the presence of high glucose concentrations.

Subjects and Methods

Samples collection

The study was approved by the ethical committee of School of Medicine, Zhejiang University, Hangzhou, China. Total of 345 women were recruited from the Reproductive Center of Hangzhou Women's Hospital between 2013 and 2015. Fifty women whose age was more than 37 years and/or with hypertension and other medical diseases were excluded, 295 women including 114 women with PCOS and 181 women without PCOS were enrolled. After stratified by BMI, PCOS women were subgrouped into normal BMI (18.5–24.9 kg/m², n = 73) and high BMI groups (≥ 25 kg/m², n = 41). Among PCOS women with normal BMI, 35 women with normal AMH level and 38 women with high AMH level were analyzed. And among obese PCOS women, 22 women with normal AMH and 19 women with high AMH level were analyzed. The diagnosis of PCOS was made according to the Rotterdam Consensus [12]. Women with hyperprolactinemia, hypothyroidism, androgen-secreting tumors, Cushing syndrome, congenital adrenal hyperplasia, or diabetes were also excluded.

Analysis of serum hormone and biochemical indicators analysis

Basal hormone and metabolic profiles of patients were examined on days 2–5 of menstruation after fasting for 10–12 hours, in subjects who did not take any medications, including hormones, in the past 3 months. The concentrations of basal sex hormone and insulin levels were measured at the clinical chemistry laboratory of Hangzhou Women's Hospital (DX8001; Beckman Instrument). Serum glucose levels were determined using an automated hexokinase method (AU5821; Beckman Instrument), the triglyceride contents were measured by a standard colorimetric assay (AU5821; Beckman Instrument) and the cholesterol levels were measured by enzyme assays (AU5821; Beckman Instrument). The amount of AMH was quantified with automated chemiluminescent immunoassay method (Roche Diagnostics; Basel, Switzerland), measuring a range of 0.01 – 23 ng/ml, the detection sensitivity of 0.3 ng/ml and intra- and inter-assay coefficients of variation < 10%. HOMA-IR index was calculated according to the formula: HOMA-IR = (fasting glucose) (mmol/l) × (fasting insulin) (µU/ml)/22.5 [13].

Pancreatic islet isolation and insulin secretion

In anesthetized 8-week-old rats, 2 mg/ml collagenase (Worthington Biochemical, Lakewood, NJ) was injected into the bile duct as previously described [14, 15]. The pancreas was isolated, with static incubation at 38 °C for 20 minutes followed by shaking incubation at 38 °C for 7 minutes. After ficoll gradient separation, freshly isolated islets of similar size were handpicked under a stereomicroscope. The islets (20 per well) were preincubated in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) washed, and incubated in 500 µl fresh RPMI-1640 containing 11.1 mmol/l glucose, supplemented with 10% fetal calf serum that contained high glucose (25 mmol/l) and this was incubated with different concentration of recombinant rat leptin (L5073, Sigma-Aldrich) and/or r-AMH (CSB-EP001666RA, CUSABIO) for 30 minutes (37 °C). Fifty microliters of the medium were removed for insulin analysis and the islets were extracted and stored (–80 °C) for protein assay and western blotting. After incubation for 24 hours, cells were treated with different concentration of leptin (0, Tris-HCl, 25, 50, 100, 200 ng/ml).

Western blotting

Western blotting was performed as described previously [16]. The islets samples were lysed and centrifuged before measuring the concentration of protein. In brief, the extracted protein was trans-

ferred to polyvinylidene difluoride membranes and incubated overnight at 4 °C with rabbit polyclonal anti-α-caspase-3 (1:1000; Cell Signaling Technology, Boston, CA, USA), anti-α-caspase-8 (1:1000; Cell Signaling Technology, Boston, CA, USA), anti-α-caspase-9 (1:1000; Cell Signaling Technology, Boston, CA, USA), anti-Bax (1:1000; Abcam, Cambridge, MA, USA) and human polyclonal anti-β-actin (1:10 000; Abcam). The samples were then incubated with fluorescence-labeled anti-mouse IgG or anti-rabbit IgG antibody (1:5000, Dylight 680 or 800, KPL) for 1 h at room temperature, and, the protein bands were obtained with enhanced chemiluminescence (Millipore). Image of WB was captured by a CCD camera and analyzed with an Odyssey Imager (Li-cor; Odyssey, NE, USA). By using this software, the intensity of the bands was translated into a numerical value – the brighter the band, the higher the number. The results of western blots were assessed visually by making comparisons between bands in different lanes.

Statistical analyses

All data are presented as mean ± SD or SEM. An independent-sample t-test, the nonparametric test was used to evaluate the statistical significance between two groups. Associations between parameters were assessed using the Pearson correlation coefficient. Multiple linear regression analysis, using HOMR-IR, fasting insulin and insulin levels after oral administration of glucose as dependent variables, and, BMI, serum leptin levels, AMH levels, basal hormone levels, and lipids levels as independent variables, were calculated. SPSS (version 19.0 for Windows) was used for the statistical analysis. p-Values of < 0.05 were considered to be statistically significant.

Results

PCOS exhibit hyperinsulinemia and IR

The flow chart of our prospective cohort study was presented in **Fig. S1**. Among 345 women of reproductive age, despite 50 women with advanced age and/or diabetes, hypertension or other medical diseases, 295 women (114 with PCOS and 181 healthy women) were recruited (87.41 %). Women's age was comparable. Serum metabolic parameters in women with or without PCOS were measured. There were no significant differences in height, basal FSH, PRL, and serum triglyceride (TG) in both groups. However, body weight, body mass index (BMI), serum LH, E_2, total testosterone (TT), Total cholesterol (TC), LDL-c levels were higher and HDL-c was lower in PCOS group (**Table 1**). Furthermore, levels of serum leptin AMH, insulin, fasting glucose, and HOMA-IR were significantly higher in women with PCOS.

Possible correlations between insulin resistance, insulin levels after glucose stimulation, and hormone and lipids parameters in PCOS women

To investigate the risk factors for IR and insulin secretion after glucose stimulation in PCOS patients, we analyzed the correlation coefficients between HOMA-IR, and metabolic and hormone parameters. HOMA-IR was positively correlated to BMI, leptin, AMH, and LDL-c levels (**Table 2**). Glucose treatment for 1st and 2nd hours of fasting markedly increased BMI, insulin and leptin levels. AMH

▶ **Table 1** Serum metabolic parameters between women with and without PCOS.

	Non-PCOS	PCOS	p-Value
No. of subjects	181	114	
Age (years)	27.71 ± 4.17	27.53 ± 4.14	0.372
Height (cm)	157.80 ± 4.79	159.98 ± 4.96	0.095
Weight (kg)	54.61 ± 6.37	62.16 ± 12.45	< 0.0001
BMI (kg/m²)	21.22 ± 2.32	23.82 ± 4.65	< 0.0001
Basal LH level (mIU/ml)	5.81 ± 3.89	10.95 ± 6.65	< 0.0001
Basal FSH level (mIU/ml)	5.84 ± 1.84	5.93 ± 1.46	0.380
Basal E2 level (pmol/l)	82.63 ± 70.28	176.55 ± 90.66	< 0.0001
TT level (nmol/l)	0.63 ± 0.44	2.21 ± 0.91	< 0.0001
Basal PRL level (ng/ml)	13.54 ± 10.14	15.21 ± 8.64	0.143
Serum triglyceride (mM)	1.52 ± 1.39	1.77 ± 1.23	0.079
Serum Cholesterol (mM)	4.73 ± 0.81	4.98 ± 1.08	0.022
Serum HDL-c (mM)	1.58 ± 0.39	1.43 ± 0.45	0.004
Serum LDL-c (mM)	2.55 ± 0.63	2.97 ± 0.90	< 0.0001
Serum leptin level (ng/ml)	5.42 ± 5.31[a]	11.84 ± 11.28[b]	< 0.0001
Serum AMH level (ng/ml)	3.60 ± 1.71	7.66 ± 6.48	< 0.0001
Fasting glucose (mM)	4.46 ± 0.59	4.81 ± 0.84	< 0.0001
Fasting insulin (µU/ml)	6.10 ± 3.77	15.17 ± 8.84	< 0.0001
HOMA-IR	1.21 ± 0.77	2.63 ± 1.50	< 0.0001

Data = mean ± SD, p-values were calculated by t-test. BMI: Body mass index; LH: Luteinizing hormone; FSH: Follicle-stimulating hormone; E2: Estradiol; TT: Total testosterone; PRL: Prolactin; HDL-c: High-density lipoprotein-cholesterol; LDL-c: Low-density lipoprotein-cholesterol; HOMA-IR: Homeostatic Model Assessment for Insulin Resistance. [a] n = 47, [b] n = 91.

showed a significant positive trend in correlation with HOMA-IR and insulin concentrations (▶ **Table 2**). Our present data suggest that high BMI and leptin levels were the main risk factor of IR, while the elevated AMH levels were associated with insulin secretion on the background of glucose stimulation.

In order to determine the interaction between obesity and elevated AMH levels on insulin resistance, we further analyzed the metabolic and hormone parameters among PCOS women after stratified by BMI (**Fig. S1** and ▶ **Table 3**). No statistic correlation between leptin and AMH levels was found (data not shown). Surprising, PCOS women in high AMH level group showed an increased HOMA-IR when compared to normal AMH level group among obese PCOS women (3.96 ± 1.27 vs. 3.02 ± 1.42). The HOMA-IR in obese PCOS women with high AMH was the highest among the four subgroups (▶ **Table 3**). However, among PCOS women with normal BMI, women with high AMH presented an obviously elevated fasting insulin levels when compared to normal AMH group (20.14 ± 11.96 vs. 10.71 ± 4.98). But there was no difference in HOMA-IR between normal and high AMH groups among PCOS women with normal BMI (▶ **Table 3**).

▶ **Table 2** Correlation coefficients between insulin resistance, insulin levels after glucose stimulation, and hormone and lipids parameters in PCOS women.

Parameters	HO-MA-IR (n = 74)	Fasting insulin (n = 74, mmol/l)	1 hour insulin (n = 42, µU/ml)	2 hours insulin (n = 42, µU/ml)
BMI (kg/m^2)	0.227#	0.157	0.341#	0.326#
Leptin levels (ng/ml)	0.326*	0.192	0.315#	0.239
AMH levels (ng/ml)	0.222#	0.211#	0.506†	0.468*
Basal LH level (mIU/ml)	0.107	−0.127	0.082	−0.321#
Basal FSH level (mIU/ml)	0.014	0.010	0.287#	0.150
TT (nmol/l)	−0.058	0.094	0.060	0.217
Serum TG (mmol/l)	0.069	0.102	−0.039	−0.106
Serum TC (mmol/l)	0.048	−0.001	0.173	0.234
Serum HDL (mmol/l)	−0.242#	−0.284#	−0.161	−0.335#
Serum LDL (mmol/l)	0.248#	0.139	0.133	0.287#

HDL: High-density lipoprotein; HOMA: Homeostatic Model Assessment; and LDL: Low-density lipoprotein. † $p < 0.001$; * $p < 0.01$; # $p < 0.05$.

Co-treatment of high levels of AMH plus leptin increases insulin secretion in vitro

Insulin itself appears to be a proximate and important quantitative contributor to insulin resistance [17]. In order to verify the direct effect of AMH on insulin secretion, isolated pancreatic (identified by incubating with dithizone, ▶ **Fig. 1a**) were cultured for 2 hours in a high glucose medium (25 mmol/l) and treated with different AMH (0.25 ng/ml and 1 ng/ml) concentrations, or with AMH (1 ng/ml) plus leptin (200 ng/ml) concentrations. We found a significant increase in levels of insulin secretion in the group treated with high levels of AMH plus leptin, compared to the group treated with AMH only (▶ **Fig. 1b**). The results indicate that a slight increase in AMH concentrations may accelerate pancreatic islets insulin secretion.

High AMH upregulated the expression of pro-apoptotic proteins in pancreatic islet cells in association with high leptin levels

To further examine whether cell death may happen in in vitro model, we tested the expression of pro-apoptotic genes in pancreatic islets treated with leptin (200 ng/ml) or leptin plus high concentration of AMH (1 ng/ml) for 2 hours was developed in a medium containing a high level of glucose. Our results showed that isolated islet cells exposure to high AMH (1 ng/ml) plus leptin levels (200 ng/ml) increased the expression levels of pro-apoptotic pro-

▶ **Table 3** Serum metabolic parameters between PCOS women with normal and high AMH levels stratified by BMI.

	Normal BMI (18.5–24.9 Kg/m^2)		p-Value	High BMI (≥ 25 Kg/m^2)		p-Value
	Normal AMH	High AMH		Normal AMH	High AMH	
No. of subjects	35	38		22	19	
Age (years)	26.94 ± 4.38	27.73 ± 4.22	0.229	26.95 ± 5.54	26.71 ± 4.86	0.442
Height (cm)	159.50 ± 5.22	160.26 ± 4.47	0.274	159.43 ± 5.12	161.31 ± 5.27	0.141
Weight (kg)	55.41 ± 8.60	55.12 ± 6.28	0.433	75.40 ± 9.63	74.03 ± 10.17	0.331
BMI (kg/m^2)	20.94 ± 3.15	21.20 ± 2.20	0.345	29.22 ± 3.34	28.42 ± 2.80	0.209
Serum AMH level (ng/ml)	3.18 ± 1.57	10.95 ± 4.69	<0.001	3.20 ± 1.92	14.48 ± 8.75	<0.001
Basal LH level (mIU/ml)	12.51 ± 7.82	12.05 ± 7.25	0.427	9.33 ± 4.60	8.21 ± 4.71	0.261
Basal FSH level (mIU/ml)	6.07 ± 1.51	5.92 ± 1.11	0.375	5.66 ± 1.44	6.04 ± 1.89	0.266
Basal E2 level (pmol/l)	180.22 ± 93.14	189.14 ± 76.16	0.381	170.65 ± 100.26	162.62 ± 94.05	0.414
TT level (nmol/l)	2.01 ± 0.87	2.79 ± 0.83	0.003	1.96 ± 0.81	2.47 ± 1.06	0.700
Basal PRL level (ng/ml)	14.83 ± 8.57	15.14 ± 10.21	0.456	14.97 ± 7.56	16.83 ± 9.89	0.283
Serum triglyceride (mM)	1.77 ± 0.99	1.53 ± 0.98	0.174	1.86 ± 1.16	2.08 ± 2.01	0.331
Serum Cholesterol (mM)	5.08 ± 1.11	4.72 ± 1.57	0.091	5.04 ± 1.15	5.17 ± 1.04	0.352
Serum HDL-c (mM)	1.58 ± 0.39	1.41 ± 0.35	0.066	1.40 ± 0.36	1.20 ± 0.35	0.041
Serum LDL-c (mM)	3.17 ± 0.77	2.61 ± 0.94	0.006	3.03 ± 0.85	3.16 ± 1.02	0.335
Fasting glucose (mM)	4.94 ± 0.66	4.63 ± 0.94	0.059	4.99 ± 0.83	4.77 ± 0.94	0.211
Fasting insulin (µU/ml)	10.71 ± 4.98	20.14 ± 11.96	<0.001	11.98 ± 6.95	13.32 ± 9.40	0.302
HOMA-IR	2.53 ± 1.19	2.46 ± 1.95	0.471	3.02 ± 1.42	3.96 ± 1.27	0.028

Data = mean ± SD; p-values were calculated by t-test. BMI: Body mass index; LH: Luteinizing hormone; FSH: Follicle-stimulating hormone; E2: Estradiol; TT: Total testosterone; PRL: Prolactin; HDL-c: High-density lipoprotein-cholesterol; LDL-c: Low-density lipoprotein-cholesterol; HOMA-IR: Homeostatic Model Assessment for Insulin Resistance.
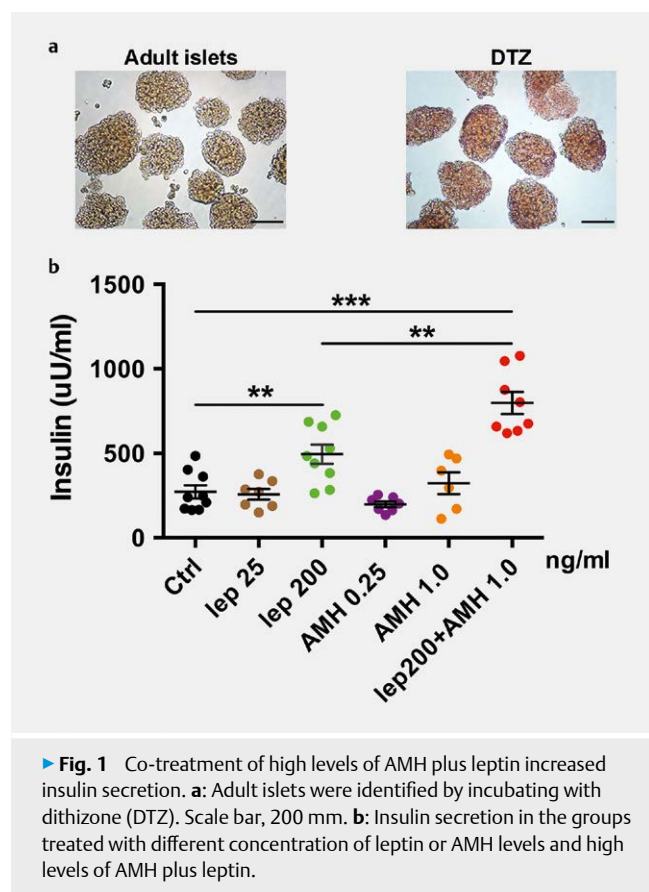
▶ Fig. 1 Co-treatment of high levels of AMH plus leptin increased insulin secretion. **a**: Adult islets were identified by incubating with dithizone (DTZ). Scale bar, 200 mm. **b**: Insulin secretion in the groups treated with different concentration of leptin or AMH levels and high levels of AMH plus leptin.

teins, such as Bax, caspase-3, and caspase-8, compared to the group treated with leptin only (▶ **Fig. 2a–e**).

Discussion

Our results illustrate that the possible average value for basal hormonal and lipids levels, HOMA-IR as well as insulin levels for both fasting and after glucose stimulation are significantly higher in PCOS patients compared to the controls. Multiple linear regression analysis showed that HOMA-IR was positively correlated to BMI and leptin levels, which indicated that PCOS women with high leptin level were susceptible to IR. Furthermore, elevated AMH levels were related to fasting insulin level and continuously insulin release after glucose administration, and, HOMA-IR was the highest in obese PCOS women with high AMH levels, which implied that high AMH might promote the intolerance of glucose and pathological process of IR.

IR is highly prevalent among PCOS patients, independent of obesity [18]. Hyperinsulinemia seems to be associated with hyperandrogenism, since insulin acts in the theca interna cells, potentiating the effects of luteinizing hormone (LH) on steroidogenesis and reducing hepatic production of sex-hormone-binding globulin, resulting in higher concentrations of free androgens [19]. In our daily clinical practice, AMH is widely used to observe ovarian response and oocyte yield. PCOS patients had significantly higher AMH levels compared to healthy fertile women [20]. Serum AMH level increased with

antral follicle count (AFC), LH, T, and E_2 in PCOS women and decreased with BMI, age, and FSH in infertile control women [21]. Circulating AMH was well known to be play important role in ovarian follicle development and was considered to be a marker of reproductive ageing. Recent epidemiological investigation was surveyed in 3293 female participants and it was observed that lower age-specific AMH levels were associated with a higher risk of type 2 diabetes in women [22]. However, whether AMH is involved in part of the pathological process of IR remains unclear. It has been reported that there was a significant positive correlation between AMH levels and IR in non-obese adolescent females with PCOS [23]. But most studies have shown that women with PCOS and IR presented high AMH levels [24, 25]. Hyperinsulinemia resulting from IR appears to increase the premature differentiation of the granulosa cells, suggesting that IR plays some role in AMH secretion by these cells. Caglar et al. [26] designed a study to evaluate the correlation between AMH levels and IR in normal-weight PCOS patients. Although the PCOS patients were not subdivided according to different phenotypes, no correlation was found between AMH levels and IR. Later on, Tian et al. [27] conducted a more comprehensive study involving more patients, and according to the results, no correlation was observed between AMH and indices of IR among all phenotypes of PCOS cases. In contrast, La Marca et al. [28] found a significant positive association between AMH and IR, as well as an association between androgens and AMH in women with PCOS. Nevertheless, in relation to IR and PCOS, a direct correlation has been found between antral follicle count, ovarian volume and hyperinsulinemia [24, 29]. So, it is reasonable to suggest that a high AMH level is a risk factor of IR. In our study, we found that women with high serum AMH levels positively correlated to fasting insulin and insulin levels of glucose tolerance, and the patients with high AMH levels presented higher insulin levels as well.

Considering that excess body fat is the primary predictor of insulin resistance in PCOS subjects [30], we then analyzed the insulin levels and HOMA-IR between PCOS women with normal or high AMH levels stratified by BMI. Indeed, women with BMI ≥ 25 Kg/m² showed higher HOMA-IR then women with normal BMI. Importantly, PCOS women with BMI ≥ 25 Kg/m² and high AMH presented a higher HOMA-IR as well. But in PCOS women with normal BMI, women with high AMH showed an increased fasting insulin level, which indicated that AMH might promote the insulin secretion and played a role in the initial compensatory phase of diabetes, and once women underwent overweight or obesity, high AMH would play a very important role in the process of developing insulin resistance.

In addition, there are some indications that AMH may be directly involved in the physiology of target organs and increased the risk of disease [31, 32]. In vitro studies have confirmed that elevated AMH can affect the primary-cultured human luteinizing granulosa cells to attenuate FSH-induced estradiol and progesterone production [33]. High AMH level had an inhibitory effect on early human ovarian follicular development and suppressed the initiation of primordial follicle growth [34]. Taking these studies into account, we speculate that high peripheral AMH may exert an influence on pancreatic islets function and may increase the risk of IR and diabetes later in life, especially in obese PCOS women.

Using in vitro model, we demonstrated that r-leptin and r-AMH exert effects on the primary islets. Isolated islet cells treatment with
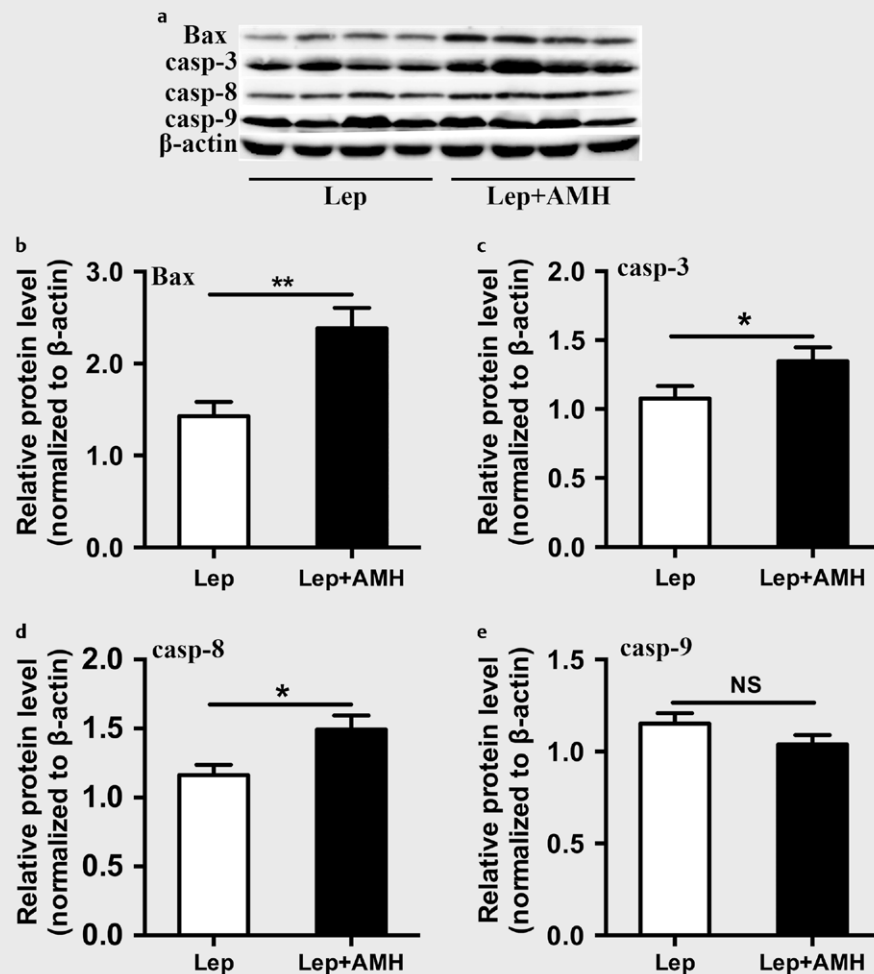
▶ **Fig. 2** High AMH promotes apoptosis in pancreatic islet cells in association with high leptin levels. **a**: Protein expression of BAX, caspase-3, caspase-8 and caspase-9 in pancreatic islet cells treated with leptin (200 ng/ml) or leptin plus high concentration of AMH (1 ng/ml) for 2 hours (n = 4, respectively). This figure shows one representative experiment among three separate experiments. **b**: Relative protein levels of BAX normalized to β-actin. **c**: Relative protein levels of caspase-3 normalized to β-actin. **d**: Relative protein levels of caspase-8 normalized to β-actin. **e**: Relative protein levels of caspase-9 normalized to β-actin. The values are expressed as mean ± SEM. * $p < 0.05$, * * $p < 0.01$.

leptin (200 ng/ml) or leptin plus AMH (1 ng/ml) significantly promoted insulin secretion. Interestingly, however, high levels of AMH non-significantly increase insulin secretion, compared to a group co-treated leptin plus AMH. Based on these results, we may suggest that high AMH in association with leptin might have a role in aggravating the impairment of pancreatic islet function. In order to overcome insulin resistance, the pancreatic islet β-cells releases more insulin initially to prevent hyperglycemia [35]. However, with the elevation of glucose continues to rise, this hyperactivity causes β-cell dysfunction that results in cell death [36]. For further analysis, we subjected the pancreatic islets to r-leptin or r-leptin plus r-AMH to study the effect of high AMH on apoptosis of islets under the condition of high glucose. Caspases (cysteine-aspartic proteases, cysteine aspartases or cysteine-dependent aspartate-directed proteases) are a family of protease enzymes playing essential roles in programmed cell death (including apoptosis, pyroptosis, and

necroptosis) and inflammation. Caspase-3 is a converging point of the apoptotic pathway [37], and its peptide inhibitors have been shown to prevent islet apoptosis [38, 39]. Conversely, over-activation of some caspases such as caspase-3 can lead to excessive programmed cell death.

As another pro-apoptotic protein, BAX plays a critical role in promoting apoptosis [40]. Bax can translocate from the cytosol to mitochondria in response to pro-apoptotic stimuli, resulting in mitochondrial cytochrome-c release and activation of caspase-9 and caspase-3 [41]. In addition, activated caspase-8 can cleave Bid, which in turn triggers the release of cytochrome-c from mitochondria and the activation of caspase-9 and caspase-3 [42]. Hyperglycemia-induced β-cell apoptosis has been implicated and has been studied mainly in T_2DM [36]. We found that co-treatment of leptin plus AMH significantly increased the expression of Bax, caspase-3, and caspase-8 in the pancreatic islets isolated from rats.

The strength of our study is its prospective nature. All of the Rotterdam criteria were met for each PCOS diagnosis. To our knowledge, the study was designed for the first time to evaluate the association between AMH and IR in reproductive PCOS women. However, there are a few limitations that need to be addressed. First, the number of subjects was small in this study and thus do not wholly represent the general population. Second, more detail information, such as ovarian size and follicle count were not determined. Third, we only found co-treatment of high levels of AMH plus leptin increases insulin secretion and upregulated the expression of pro-apoptotic proteins when continuous incubating with high glucose level. The in vitro experiment is insufficient to state the relation of the pathogenesis of insulin resistance (IR) to high AMH. Therefore, a well-designed case-control or cohort study needs to be performed and more experiments for investigating the relationship between AMH and apoptosis were needed in the future.

Conclusions

In obese PCOS patients, high levels of AMH may present an increased risk for IR. In vitro study showed that increasing AMH levels in pancreatic β-cells may trigger the secretion of insulin, and high AMH level in association with leptin promoted hyperglycemia-induced β-cell pro-apoptotic proteins expression. However, further study needs to fully clarify the molecular mechanism of AMH-induced activation of apoptosis.

Author Contributions

X.H.L and H.F.H conceived, designed, and supervised the study as well as critically revising the manuscript; X.J.L and H.W collected the blood samples and wrote the manuscript; T.T.Y and K.U carried out the in vitro experiments; X.Y.S and H.Y.S did the hormone, lipids, and other blood tests; X.Y.F and Z.Y.L helped for the follow-up study and discussions.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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