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Endothelin-1 exacerbates development of hypertension and atherosclerosis in modest insulin resistant syndrome

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ABSTRACT

Endothelin-1 (ET-1) is known as potent vasoconstrictor, by virtue of its mitogenic effects, and may deteriorate the process of hypertension and atherosclerosis by aggravating hyperplasia and migration in VSMCs. Our previous study demonstrated that insulin infusion caused sequential induction of hyperinsulinemia, hyperendothelinemia, insulin resistance, and then hypertension in rats. However, the underlying mechanism of ET-1 interfere insulin signaling in VSMCs remains unclear. To characterize insulin signaling during modest insulin resistant syndrome, we established and monitored rats by feeding high fructose-diet (HFD) until high blood pressure and modest insulin resistance occurred. To explore the role of ET-1/ET_AR during insulin resistance, ET_AR expression, ET-1 binding, and insulin signaling were investigated in the HFD-fed rats and cultured A-10 VSMCs. Results showed that high blood pressure, tunica medial wall thickening, plasma ET-1 and insulin, and accompanied with modest insulin resistance without overweight and hyperglycemia occurred in early-stage HFD-fed rats. In the endothelium-denuded aorta from HFD-fed rats, ET_AR expression, but not ET_BR, and ET-1 binding in aorta were increased. Moreover, decreasing of insulin-induced Akt phosphorylation and increasing of insulin-induced ERK phosphorylation were observed in aorta during modest insulin resistance. Interestingly, in ET-1 pretreated VSMCs, the increment of insulin-induced Akt phosphorylation was decreased whereas the increment of insulin-induced ERK phosphorylation was increased. In addition, insulin potentiated ET-1-induced VSMCs migration and proliferation due to increasing ET-1 binding. ETAR antagonist reversed effects of ET-1 on insulin-induced signaling and VSMCs migration and proliferation. In summary, modest insulin resistance syndrome accompanied with hyperinsulinemia leading to the potentiation on ET-1-induced actions in aortic VSMCs. ET-1 via ET_AR pathway suppressed insulin-induced AKT activation, whereas remained insulin-induced ERK activation. ET-1 and insulin synergistically potentiated migration and proliferation mainly through ET_AR/ERK dependent pathway, which is dominant in VSMCs during modest insulin resistance syndrome. Therefore, ET-1 and ET_AR are potential targets responsible for the observed synergism effect in the hypertensive atherosclerotic process through enhancement of ET-1 binding, ET-1 binding, ET_AR expression, and ET-1-induced mitogenic actions in aortic VSMCs.

1. Introduction

Insulin resistance, hyperinsulinmia, hyperglycemia, and hypertension are common factors in metabolic syndrome pathogenesis. Hyperinsulinmia companied with insulin signaling abnormality may closely associate within the process of insulin resistance [1] and hypertension [1,2]. Vascular remodeling including vascular smooth muscle cells (VSMCs) hyperplasia and endothelial cell (ECs) dysfunction often observed in the hypertensive animal models [3]. Maintenance of vascular tone blood pressure is responsible by VSMCs in vascular intimal medial layer, and VSMCs are tightly associated within pathological process of hypertension [4,5]. Mitogenic action including intimal-medial thickness, VSMCs

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migration and proliferation often occurred with high blood pressure, insulin resistance, and hyperinsulinemia [6,7]. Previous study show that impaired nitric oxide or PI3K-dependent pathway is often occurred with enhanced high blood pressure in insulin resistant state [8–10]. In addition, the phenomenon that insulin-induced metabolic effect is impaired whereas the mitogenic effect is maintained was observed in insulin resistant model [11]. However, the causative factors and underlying mechanism involved in insulin resistance remains unclear.

Endothelin-1 (ET-1), which is secreted majorly by ECs, is the most potent vessel constrictor and vascular modulator. ET-1 stimulates vasoconstriction through ET subtype A receptor (ET_AR) and vasodilation through ET subtype B receptor (ET_BR) on ECs or VSMCs, respectively [12]. ET-1 enhances vasoconstriction, DNA synthesis, and atherosclerotic actions via ET_AR/ERK dependent pathway [13,14].

Our previous study demonstrated that insulin infusion cause sequential induction of hyperinsulinemia, hyperendothelinemia, insulin resistance, and then hypertension in rat model [15]. In addition, ET-1, and ET_AR are associated with plasma insulin [16,17] and elevated in insulin resistant models [18]. However, whereby ET-1 interferes insulin signaling and underlying mechanisms in VSMCs during insulin resistance remain unclear. We hypothesize that ET-1 may be a modulator to regulate insulin signaling, and hyperendothelinemia and hyperinsulinemia may cooperate to induce the process of hypertensive atherosclerosis by enhancing the migration and hyperplasia of VSMCs. We used insulin resistant and hypertensive animal model and VSMCs line to clarify the role of ET-1/ET_AR on aortic disorder in modest insulin resistance.

2. Materials and methods

2.1. Animal model

Sprague–Dawley (SD) rats, purchased from the Animal Center of the National Yang-Ming University, at the age of 6 week (weighing 200–250 g), were housed in a temperature- and light-controlled room (20 ± 5 °C; 12-h light/dark cycle). All animal procedures were conformed to the guidelines and approved by the Animal Welfare Committee of Taipei Veterans General Hospital. SD rat and randomly divided into two groups: normal chow diet (NCD) group or high fructose diet (HFD) group, which fed with 60% HFD (Harlan Laboratories, Madison, WI). The data of systolic blood measured by programmable sphygmomanometer (BP-98A; Softron, Tokyo, Japan) were collected each week. Blood samples for glucose, insulin, and triglycerides were collected each two weeks at the 0 min of oral glucose tolerance test (OGTT) after 12 h-fasting. Plasma of OGTT (0, 30, 60, 90, 120 min) was separated and frozen until analysis. In order to assess insulin resistance of animals, homeostasis model assessment-insulin resistance (HOMA-IR) were determined at the end of experiment. To confirm the insulin-induced signaling in aorta, some rats were performed10-min administration of insulin (0.75 U/kg, iv) before sacrificing. In the aortic study, thoracic descending aorta was isolated and prepared by method previous described [19]. All aortae used in this study were referred to endothelium-denuded unless specified otherwise.

2.2. Cell culture

A-10 cells possessing the nature of VSMCs were purchased from the Bioresource Collection and Research Center, Taiwan and cultured in DMEM (Gibco BRL, MD, USA) containing 10% fetal bovine serum (Biological Industries, Kebbutz beit haimek, Israel) plus 100 U/ml penicillin, 100 μg/ml streptomycin, and 25 mM HEPES (pH7.4) at 37 °C in a humidified incubator with 5% CO_2. Cells were grown to 80% confluence and split into 12-well plates for binding assay or 6-cm dishes for protein harvest. Cells were serum free for 6 h prior to treatment of ET-1 (Sigma, MO, USA) or insulin (Sigma, MO, USA), and some cells were subjected to 30-min pre-treatment by using inhibitors: BQ610 (antagonist for ET_AR, 10 μM, Phoenix Pharmaceutical, CA, USA), BQ788 (antagonist for ET_BR, 10 μM, Phoenix Pharmaceutical, CA, USA), PD98059 (inhibitor for ERK, 5 μM Sigma, MO, USA), Wortmannin (inhibitor for Akt, 1 μM, Sigma, MO, USA) individually.

2.3. Total membrane protein extraction and binding assay

Total membranes of aorta from SD rats were harvested and analyzed by using previously described method [19].

2.4. ^{125}I-ET-1 specific binding

To examine the effects of insulin on ET-1 binding, A-10 VSMCs were cultured for 24 h in the presence of insulin (10^{-7} M). The ET-1 binding was done in binding buffer (DMEM with 25 mM HEPES, pH 7.4, 0.1% BSA, and 5.5 mM glucose) containing 20 pM ^{125}I-ET-1 (specific activity at 5 mCi/mmol, New England Nuclear, Amersham, Aylesbury, UK) with various concentrations of unlabeled ET-1 (Peptide Institute, Osaka, Japan), and then incubated for 1 h at 37 °C. Cells were then washed twice with ice-cold phosphate buffer saline (PBS) and solubilized by incubation in 1 N NaOH for 3 h at room temperature. The bound ^{125}I-ET-1 was measured with γ-counter. Nonspecific binding as determined in the presence of 1×10^{-6} M unlabeled ET-1, was subtracted from the total bind to give specific binding.

2.5. MTT assay

Cells were seeded in 96-well plates at 1×10^4 cells per well in 200 μl of DMEM, and cultured for 24 h to allow adherence. After further incubation for 24 h with insulin or ET-1, 100 μl of MTT (0.5 g/L in PBS, Calbiochem, CA, USA), which is tetrazolium dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), was added to each well and the plates were incubated at 37 °C for 2 h. To each well 150 μl of dimethyl sulfoxide was added, and the plates were agitated on a plate shaker for 10 min. Optical density at 570 nm was read with an microplate reader in 48 h.

2.6. Migration assay

To measure the effect of insulin and ET-1 with/without inhibitors on cell migration, A-10 VSMCs were transfected with green fluorescent protein (GFP) maker and assayed by Platypus' Oris™ Cell migration Assay Protocol accordingly. After A-10 VSMCs were seeded at 1×10^4 cells and allowed to adhere in 96-well plate, stoppers remained in designated wells until assay readout to serve as pre-migration references. Stoppers were removed to reveal a 2 mm diameter exclusion zone in the center of 96-well plate. And then the treatment of ET-1(10^{-7} M) and/or insulin(10^{-7} M), with/without inhibitors added as description above, and intensity of GFP was confirmed using optical plate reader. Cell migration data were captured from the microplate reader in 48 h.

2.7. Immunoblotting

Total protein lysates were prepared from A-10 VSMCs or endothelium-denuded aorta from SD rats. Immunoblotting was performed using previously described method [19]. The primary antibody of ET_AR (Merk Millipore, MA, USA), ET_BR (GeneTex, CA, USA), pERK, ERK, pAKT, and AKT (Santa Cruz Biotechnology, CA,

USA) were used. α-tublin (Merk Millipore, Billerica, MA, USA) or β-actin (Sigma–Aldrich, St. Louis, MO, USA) was used as the internal control.

2.8. Data analysis

Experiments were repeated at least four times and the results expressed as means ± SD of the numbers of observations. Statistical significance was assessed by one-way analysis of variance or Student's *t*-test. A *P* value of 0.05 was considered statistically significant.

3. Results

3.1. Modest insulin resistance, hypertension, hyperinsulinemia, hyperendothelia, and tunica medial thickening in HFD-fed rats

To monitor the development of insulin resistance accompanied with hypertension and atherosclerosis, we establish insulin resistant syndrome model by feeding rat with HFD. Data of blood samples, body weight, and systolic blood pressure were collected and compared between HFD group and NCD group during the 8-week process (supplementary). Insulin resistant was occurred at 5th week, and high plasma insulin was induced at the 6th week in HFD-group determined by OGTT test. At the end of monitoring time, systolic blood pressure, triglyceride, HOMA-IR index, area under curve of OGTT, plasma insulin and ET-1 levels, as well aortic tunica medial thickening were increased in HFD group (Table 1), whereas plasma glucose and body weight weren't significantly increased in HFD group. These data indicated that modest insulin resistance occurred in the insulin resistant syndrome model (HFD group), accompanied with insidious processing of hypertension and atherosclerosis.

3.2. ^{125}I-ET-1 binding capacity and affinity and ETRs expression in aorta

Examining the effect of ET-1 and ET-1 receptors (ETR) required determining whether the ET-1 binding sites or affinity in HFD-group was enhanced during modest insulin resistance. Aortic ^{125}I-ET-1 binding and ETR expression were measured at the end of monitoring. Scatchard plot analysis revealed that aorta from HFD led to a 8-fold increase in receptor binding sites, as calculated using Bmax (HFD 71.03 ± 0.62 × 10^8 site/cell vs. NCD 8.78 ± 0.92 × 10^8 site/cell, $p < 0.05$) (Fig. 1A inset). Moreover, aorta from HFD led to a 6-fold increase in receptor binding affinity, as calculated from the dissociation constant (Kd) (HFD 1.79 ± 0.53 pM vs. 6.95 ± 0.86 pM, $p < 0.05$, Fig. 1A inset).

To clarify whether ET_AR or ET_BR is dominated in insulin resistant syndrome, ETRs protein expressions were examined by immunoblotting. Results showed that ET_AR, but not ET_BR, was increased on aorta from HFD group comparing to NCD group (Fig. 1B), indicating that HFD-enhanced ET-1 binding occurs mainly through ET_AR on aorta. We next investigated whether the ET-1 binding was enhanced by insulin. Similar to the data from binding assays of aorta, ET-1 binding levels were enhanced by insulin pretreatment in A-10 VSMCs (Fig. 1C). These data indicated that not only ET-1 binding capacity and affinity increased, but also ET_AR expression may be enhanced in modest insulin resistance.

Table 1
Comparison of metabolic and hypertensive parameters on high fructose diet (HFD) and normal chow diet (NCD) group.

	NCD (n = 6)	HFD (n = 6)
Body weight (g)	336.70 ± 16.81	348.88 ± 11.03
Fasting insulin (μU/mL)	5.79 ± 2.16	10.66 ± 2.73*
Fasting glucose (mg/dL)	71.17 ± 7.36	80.50 ± 9.04
OGTT AUC120		
Insulin (mU/mL•120 min)	2.15 ± 0.88	3.18 ± 0.85*
Glucose (g/dL•120 min)	21.00 ± 0.44	25.02 ± 1.01
OGTT $\triangle AUC_{120}$		
Insulin (mU/mL•120 min)	0.73 ± 0.34	1.07 ± 0.33*
Glucose (g/dL•120 min)	7.38 ± 0.18	8.73 ± 0.36
HOMAIR Index	1.07 ± 0.42	2.54 ± 0.41*
HOMAB Index	190.10 ± 68.79	260.58 ± 71.49
TG (mg/dL)	32.02 ± 6.53	57.46 ± 18.08*
ET-1 (pg/mL)	9.03 ± 0.52	14.23 ± 1.02*
SBP (mmHg)	107.74 ± 7.67	134.47 ± 5.49*
TMT (μm)	80.23 ± 4.05	115.57 ± 3.64*

Footnote: NCD: Normal Chow Diet, HFD: High Fructose Diet, OGTT: Oral glucose tolerance, AUC: Area under curve, $HOMA_{IR}$: Homeostasis model assessment of insulin resistance, $HOMA_B$: Homeostasis model assessment of β-cell function, ET-1: Endothelin-1, SBP: Systolic blood pressure, TMT: Tunica Medial Thickness. Value were mean ± SD for six experiments. *$p < 0.05$ vs. control (NCD).

3.3. Insulin-induced phosphorylation of AKT and ERK in NCD and HFD groups

To clarify insulin signaling during modest insulin resistance, rats from NCD and HFD group were assay by the 10-min-insulin administration before sacrificing. Results showed phosphorylation of AKT and ERK were remained induced by insulin comparing with the basal levels within group. Interestingly, the increment of insulin-induced AKT phosphorylation was decreased (Fig. 2A), but the increment of insulin-induced ERK phosphorylation was remained high in HFD group comparing to NCD group (Fig. 2C). These data indicated that insulin signaling selectively resistance on AKT pathway in aorta from HFD rats.

To clarify weather ET-1 interfered insulin signaling in VSMCs, the ET-1 24-h-pretreatment was performed in A-10 VSMCs. Results showed that phosphorylation of AKT and ERK were induced by insulin comparing with basal levels. Notably, the increment of insulin-induced AKT phosphorylation was decreased by ET-1 pretreatment (Fig. 2B), whereas the increment of insulin-induced ERK phosphorylation was remained high by ET-1 pretreatment (Fig. 2D). Furthermore, the induction patterns of insulin signaling were similar in aorta from HFD group and in A-10 VSMCs pretreated by ET-1 (Fig. 2A vs. 2B, Fig. 2C vs. 2D). These data indicated that ET-1 may cause insulin resistance selectively on AKT pathway but not on ERK pathway.

3.4. Association of ET-1/ET_AR pathway with reducing insulin/AKT activation and inducing insulin/ERK activation

To further clarify the hypothesis that ET-1 leads to insulin resistance selectively, BQ610 and BQ788, specific antagonist for ET_AR and ET_BR, were used in the pretreatment of ET-1. After co-incubating with BQ610, not BQ788, ET-1-reduced AKT activation induced by insulin was reversed (Fig. 3A). Notably, BQ610 also reversed the effects of ET-1 on insulin-induced ERK activation (Fig. 3B). Neither BQ610 nor BQ788 per se affected actions of insulin on AKT or ERT. These data showed that ET-1/ET_AR signaling caused insulin resistance selectively on insulin-induced AKT pathway, whereas ET-1/ET_AR signaling remained the activation of insulin-induced ERK pathway.

3.5. The impact of ET-1 and insulin co-incubation on VSMC migration and proliferation

To clarify the physiological outcomes of hyperendothelinemia and hyperinsulinemia coexist, the impact of ET-1 and insulin co-incubation on migration and proliferation of VMSCs were

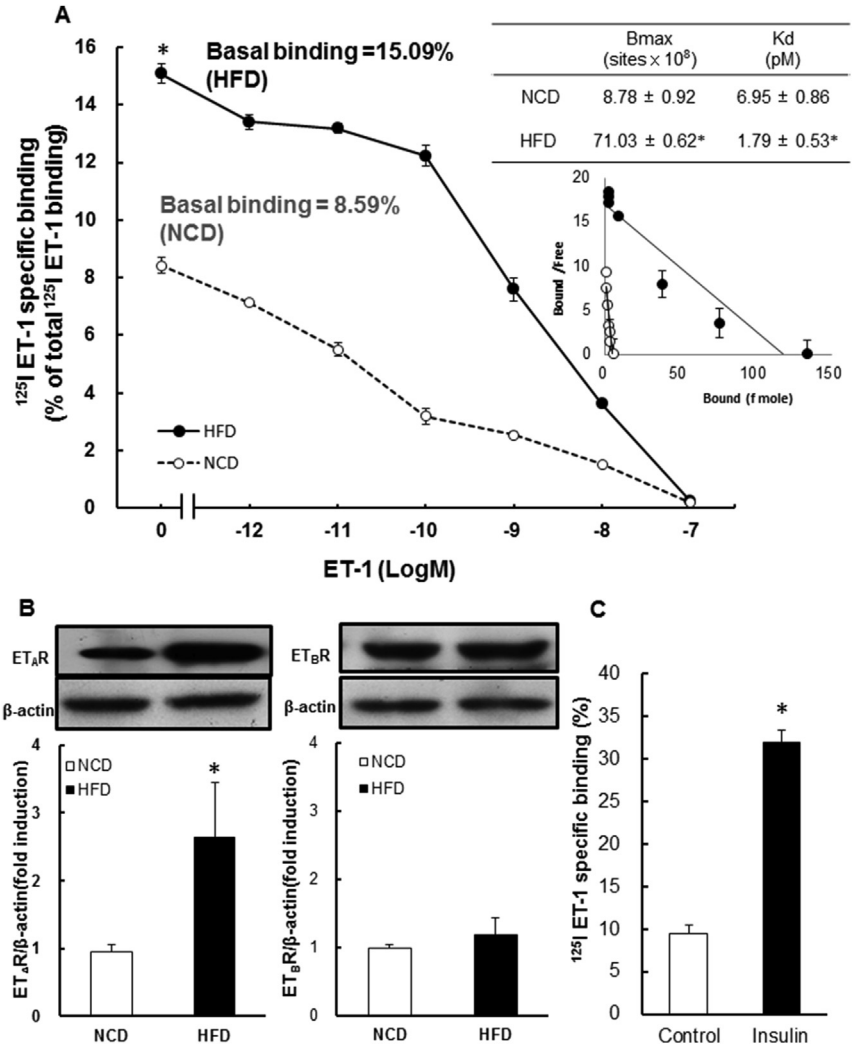

Fig. 1. Enhanced ET-1 binding and ETAR expression were observed in aortae from HFD rats. (A) Competitive binding curve and Scatchard Plot (inset) for ET-1 binding to cells with/without HFD. (B) ET_AR, not ET_BR, protein levels were increased in aortae from HFD rats. (C) Insulin pretreatment increased ET-1 binding in A-10 VSMCs. Values were mean ± SD for six experiments. $^*p < 0.05$ vs. corresponding control.

determined. Results demonstrated that insulin or ET-1 alone stimulated VSMCs migration (Fig. 3C). ET-1 alone stimulated VSMCs proliferation, and insulin alone stimulated VSMCs proliferation mildly (Fig. 3D). In addition, in co-incubation with insulin, the ET-1-stimulated migration and proliferation of VSMCs increased. Moreover, BQ610 and PD98059 blocked the VSMCs migration (Fig. 3C) and proliferation (Fig. 3D), whereas BQ788 or Wortmannin didn't affect functions of VSMCs. Inhibitors per se didn't affect cell migration and proliferation. These data indicated that insulin is a mild mitogen, but it potentiated the ET-1-stimulated VSMCs proliferation and migration due to enhancement of ET-1 binding (Fig. 1C). Coexist of insulin and ET-1 potentiated VSMCs functions through ET_AR/ERK dependent pathway.

4. Discussion

Our previous studies by using HFD model or insulin-infusion model have demonstrated that plasma ET-1 elevation and aortic ET_AR elevation contribute to the insulin resistance syndrome [15,16,18]. Another previous study demonstrated that ET-1 antagonist pretreatment rescue the impairment of insulin-mediated vasodilation in a type 2 diabetes animal model [20]. The key findings of this study explore the potential role of ET-1/ET_AR on early process of hypertension and atherosclerosis accompanied with modest insulin resistance. Our data reveal that ET-1 binding and ET_AR expression were elevated due to hyperinsulinemia during modest insulin resistance. ET-1 via ET_AR pathway suppressed insulin-induced AKT activation, whereas remained intact insulin-induced ERK activation. ET-1 and insulin synergistically potentiated migration and proliferation mainly through ET_AR/ERK dependent pathway, which is dominant in aortic VSMCs observed in insulin resistance syndrome. Thus, ET-1 and ET_AR are potential targets responsible for the observed synergism effect with insulin in the hypertensive atherosclerotic process through upregulating ET-1 binding, ET_AR expression in aortic VSMCs.

Previous studies show that high insulin concentration stimulates ET-1 expression *in vitro* [17]; however, hyperinsulinemia is not associated with increased plasma ET-1 levels in healthy subjects *in vivo* [21–23]. Plasma ET-1 levels are not significantly elevated in non-insulin-dependent diabetes mellitus (NIDDM) with insulin resistance syndrome comparing to NIDDM without insulin resistance syndrome [22]. Our data demonstrated that ET-1 binding and ET_AR expression in aorta were elevated in early-stage insulin resistance. In addition, hyperinsulinemia increased ET-1 binding

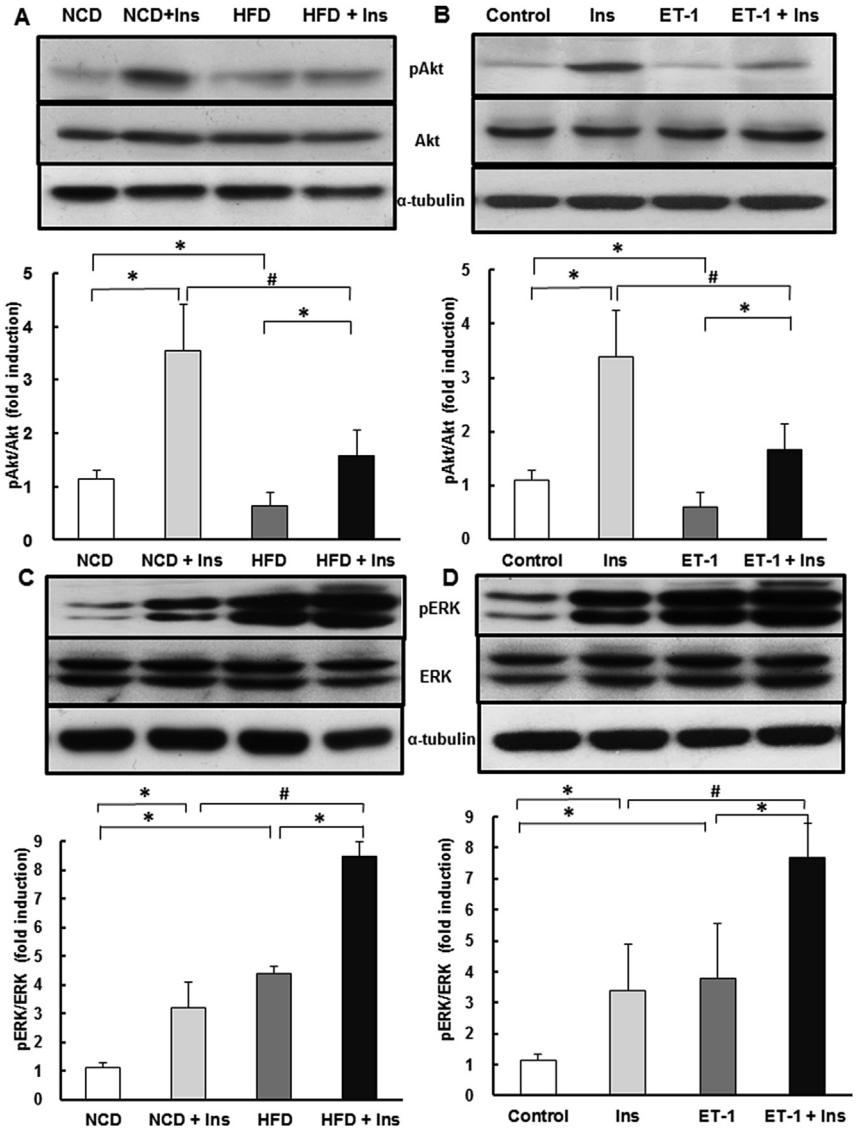

Fig. 2. Insulin-induced increment of pAkt/Akt protein levels were decreased in aortae from HFD rats (A) or ET-1 pretreatment in A-10 VSMCs (B), whereas insulin-induced increment of pERK/ERK levels were remained high in aortae from HFD rats (C) or ET-1 pretreatment in A-10 VSMCs (D). Values were mean ± SD for all four experiments. *$p < 0.05$ vs. corresponding control, #$p < 0.05$ vs. NCD + insulin or insulin alone.

and mainly through ET_AR dependent pathway in VSMCs. These data indicated that ET-1 and ET_AR effects on aortic VSMCs may be emphasized by hyperinsulinemia even while plasma ET-1 levels were not increase largely.

Our data showed the co-incubation of insulin and ET-1 potentiated cell migration and proliferation, but insulin alone didn't induce VSMCs proliferation significantly. Moreover, the effects of ET-1 plus insulin were blunted by inhibition of ET_AR and ERK. Notably, other hormone, such as angiotensin II (Ang II), promotes ET-1-induced long lasting vasoconstriction through upregulating ET_AR expression and ET-1 binding in VSMCs, although Ang II itself induces short-term vasoconstriction [19]. These data suggest insulin may act as a priming effector to potentiate ET-1 mitogenic function through enhancing ET-1/ET_AR binding and ERK activation in VSMCs.

Previous study reveals ET-1 binding is decreased by ET-1 pretreatment due to lysosomal degredation [24,25]. Our data demonstrated that ET_AR levels and ET-1 binding potency including capacity or affinity were increased due to hyperinsulinemia during modest insulin resistance. Previous studies show β-arrestins act as receptor desensitizers and endocytosis adaptors, and association of β-arrestins and ET_AR may be a key modulatory mechanism [26,27]. ET_AR is preferential binding to β-arrestin-2 and by desensitizing followed by rapid recycling [28]. Deficiency of β-arrestin-2 is observed in the insulin resistant model and type 2 diabetes patients [29]. Moreover, insulin decreases cellular β-arrestin content due to ubiquitination and proteosome-mediated degradation [30]. It is likely protein expression and binding potency of ET_AR can be enhanced by hyperinsulinemia or insulin resistance through decreasing of β-arrestins.

Previous study demonstrated that IRS-1/SHP2 interaction acts as a switching machinery between differentiation and proliferative status of VSMCs. When alternative IRS-1 phosphorylation sites acts, IRS-1 activates more Grb2 downstream ERK pathway [31]. In addition, ERK switches insulin signaling by modulation of IRS-1 tyrosine phosphorylation [32,33]. Our data indicated ET_AR/ERK pathway was dominant in modest insulin resistance and ET-1-treated VSMCs, which caused an imbalance

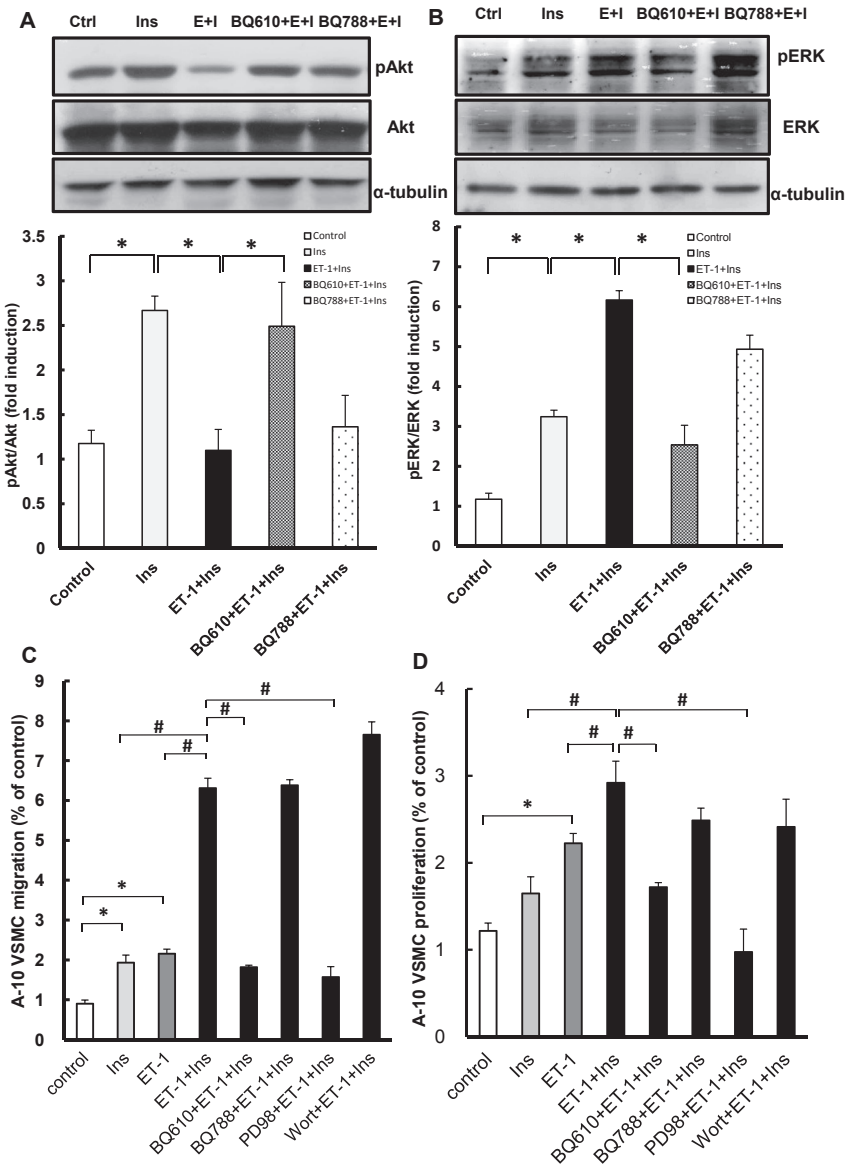

Fig. 3. Inhibitors of ET_AR and ERK reversed ET-1-interfered insulin signaling and mitogenic functions in VSMCs. ET_AR receptor (BQ610, 10^{-5} M), but not ET_BR receptor (BQ788, 10^{-5} M), reversed the effect of ET-1 pretreatment on insulin-induced AKT activation (A). BQ610 also reversed effect of ET-1 pretreatment on insulin-induced ERK activation (B). Inhibitor of ET_AR or ERK blocked VSMC migration (C) or proliferation (D), but inhibitor of ET_BR or AKT didn't block VSMC migration or proliferation. Values were mean ± SD for all four experiments.*$p < 0.05$ vs. corresponding control, #$p < 0.05$ vs. ET-1 + Insulin.

between insulin-induced AKT and ERK. It is likely ET_AR/ERK pathway may modulate IRS-1 tyrosine phosphorylation in ET-1-stimulated VSMCs. However, we cannot rule out the possibility that negative feedback of AKT pathway on IRS-1 or other epigenetic actions. Nevertheless, insulin-stimulated ERK activation is enhanced by ET-1/ET_AR signaling in insulin resistance syndrome.

These findings provide novel insights to reveal the interaction of ET-1 with insulin in modest insulin resistance syndrome. Our data indicate that hyperinsulinemia may amplify ET-1 binding and ET-1-stimulated VSMCs migration and proliferation via ET_AR/ERK pathway, whereas insulin-induced AKT signaling and insulin resistance may be deteriorated by ET-1. Taken together, ET-1/ET_AR pathway may exacerbate the process of insulin resistance, atherosclerosis, and hypertension through suppressing insulin-induced Akt pathway and activating insulin-induced ERK to make a cross-link between metabolic and mitogenic actions in vascular system.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.017.

Conflicts of interest

The manuscript is written and formatted in according with the instruction for Authors. All authors, including **Y**an-Jie Lin,

Chi-Chang Juan, Ching-Fai Kwok, Yung-Pei Hsu, Kuang-Chung Shih, Chin-Chang Chen, and Low-Tone Ho., concur with the submission and that none of the data have been previously reported or are under consideration for publication elsewhere. All authors have no conflict of interests.

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