Published in final edited form as:

Mol Cell Endocrinol. 2012 April 4; 351(2): 176–183. doi:10.1016/j.mce.2011.12.003.

Endothelin 1 promotes cytoplasmic accumulation of RIP140 through a ET_A-PLC β -PKC ϵ pathway

Ping-Chih Ho, Yao-Chen Tsui, Yi-Wei Lin, Shawna D. Persaud, and Li-Na Wei

Department of Pharmacology, University of Minnesota Medical School, Minneapolis, MN 55455, USA

Abstract

The physiological signal activating cytoplasmic accumulation of nuclear receptor interacting protein 140 (RIP140) in adipocytes was unclear. We uncover that endothelin 1 (ET-1) promotes cytoplasmic accumulation of RIP140 in 3T3-L1 adipocytes. We determine ET-1's signal transduction pathway in adipocytes, which is by activating ET_A receptor-PLC β -nuclear PKC ϵ . Blocking this pathway in 3T3-L1 adipocyte cultures, by treating cells with an ET_A antagonist, inhibiting PLC β , or silencing PKC ϵ , reduces ET-1-stimulated cytoplasmic accumulation of RIP140. In a HFD-fed obese mouse model, administration of a selective ET_A antagonist, ambrisentan, effectively dampens cytoplasmic accumulation of RIP140 in the epididymal adipose tissue and reduces HFD-caused adipocyte dysfunctions. Importantly, ambrisentan improves blood glucose control and reduces the severity of hepatic steatosis in HFD-fed mice. This study reports a physiological signal that stimulates nuclear export of RIP140 in adipocytes and provides evidence for a strategy using selective ET_A antagonist to treat obesity-induced insulin resistance and, possibly, other metabolic disorders.

Keywords

RIP140; Endothelin-1; Insulin sensitivity; Cytoplasm; ET_A receptor; Ambrisentan

1. Introduction

In recent years obesity has become a worldwide epidemic. A major complication of obesity is type 2 diabetes mellitus (T2DM). The hallmark of this condition is the development of insulin resistance in adipose, muscle and liver (Herman and Kahn, 2006). In healthy individuals, adipocytes take up glucose, store lipid and secrete adipokines, and these adipokines are important in the regulation of metabolism. But in diabetics, adipocytes may become dysfunctional, as shown in the reduction of their glucose uptake ability, change in the profile of secreted adipokines, and increase in lipolysis. These changes may worsen the disease state leading to such complications as hypertension, atherosclerosis and cardiomyopathy (Herman and Kahn, 2006, Guilherme, Virbasius, Puri et al., 2008). Reports have shown that inflammation and endoplasmic reticulum (ER) stress could cause adipocyte dysfunction, but the exact etiology of many of these pathophysiological processes remains to

© 2011 Elsevier Ireland Ltd. All rights reserved.

*Correspondence: Li-Na Wei, Department of Pharmacology, University of Minnesota Medical School, 6-120 Jackson Hall, 321 Church Street SE, Minneapolis, MN 55455-0217, USA, Tel: 612-625-9402, Fax: 612-625-8408, weixx009@umn.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

be determined (Guilherme et al., 2008, Schenk, Saberi and Olefsky, 2008, Wellen and Hotamisligil, 2005).

RIP140 is a nuclear co-regulator for many transcription factors. It plays important roles in various biological processes and affects many disease conditions including the metabolic syndrome (Mostaqul Huq, Gupta and Wei, 2008, Leonardsson, Steel, Christian et al., 2004, Ho, Lin, Tsui et al., 2009, Powelka, Seth, Virbasius et al., 2006). Both its mRNA and protein expression are significantly elevated in fully differentiated, fat-accumulating adipocytes. As adipocytes mature, RIP140 undergoes extensive post-translational modifications (PTMs). These changes can modulate its gene-regulatory activity and facilitate its export to the cytoplasm (Leonardsson et al., 2004, Mostaqul Huq, Gupta, Tsai et al., 2006, Gupta, Ho, Huq et al., 2008, Huq, Tsai, Lin et al., 2007). Recently, we have reported that in 3T3-L1 adipocytes, activated nuclear PKC ϵ phosphorylates RIP140, and the phosphorylated RIP140 is subsequently modified by protein arginine methylation, which enhances the recruitment of exportin 1 to RIP140 for its nuclear export (Mostaqul Huq et al., 2006, Gupta et al., 2008). We have also found that in the primary adipocytes isolated from mice fed a high-fat diet (HFD), RIP140 is increasingly accumulated in their cytoplasm, and that the cytoplasmic RIP140 can negatively regulate basal and insulin-stimulated glucose uptake by decreasing GLUT4 vesicle trafficking (Ho et al., 2009). However, it remains to be determined as to the physiological trigger and/or the extracellular signal for RIP140's nuclear export in adipocytes.

According to the dissected intracellular signaling pathway that stimulates RIP140's nuclear export in 3T3-L1 adipocytes, i.e. activated nuclear PKC ϵ that initiates specific phosphorylation on RIP140 to enhance its subsequent arginine methylation for recruiting exportin 1, we investigated several extracellular factors associated with nuclear PKC ϵ activation, and uncovered that endothelin-1 (ET-1) could be such an extracellular trigger. ET-1 is a vasoconstrictor secreted primarily by endothelial cells. It regulates airway and blood vessel tone and wound healing (Potenza, Gagliardi, Nacci et al., 2009, Rehsia and Dhalla, 2010, Schneider, Tilly, Hierl et al., 2002), and is the major therapeutic target in managing chronic thromboembolic pulmonary hypertension, pulmonary arterial hypertension, and pulmonary fibrosis (Humbert, Sitbon and Simonneau, 2004, Braunwald, 2008, Archer, Weir and Wilkins, 2010). Clinical studies have shown that obese and T2DM patients have higher levels of circulating ET-1. As a result, several investigators have proposed that high levels of ET-1 might be an important factor in the observed adipocyte dysfunction (Schneider et al., 2002, Takahashi, Ghatei, Lam et al., 1990, Bedi, Clarke, Dennis et al., 2006, Chai, Chang and Fong, 2009, Ishibashi, Imamura, Sharma et al., 2001). However, the underlying mechanism through which ET-1 modulates adipocyte functions is unclear.

In the current study, we examine the relationship between high levels of ET-1 and cytoplasmic accumulation of RIP140 in 3T3-L1 adipocytes. We find that chronic ET-1 exposure can promote the accumulation of RIP140 in the cytoplasm of adipocytes by activating the ET_A-PLC β -nuclear PKC ϵ pathway. Further, we show that targeting this signaling pathway in 3T3-L1 adipocytes by antagonizing ET-1 receptor, inhibiting PLC β , or silencing PKC ϵ can inhibit cytoplasmic accumulation of RIP140. Importantly, using a selective antagonist of ET_A, the principal ET-1 receptor expressed in adipocytes, we demonstrate that blocking ET-1 signal input to adipocytes in animals indeed can reduce cytoplasmic accumulation of RIP140 in adipocytes. This treatment also reduces adipocyte dysfunctions, improve blood glucose control and ameliorates hepatic steatosis in HFD-fed mice.

2. Material and Methods

2.1. Cell culture, reagents and transfection

3T3-L1 cells and RIP140-null cells were maintained and differentiated as described (Ho, Gupta, Tsui et al., 2008). siRNAs were purchased from Qiagen. ET-1 was from MP Biomedicals. Mouse adiponectin ELISA kit was from Invitrogen. siRNA transfection was conducted using a DeliverX Plus siRNA transfection kit (Panomics). Plasmid transfection was by electroporation with GenePluser (Bio-Rad) using 960 μ F and 200 Ω .

2.2. Endothelin-1 Treatment

For ET-1 treatment, mature 3T3-L1 adipocyte cultures were treated with 50 nM ET-1 for 24 hr and cell lysates were collected for Western blotting.

2.3. Antibodies, Western blotting and cellular fractionation

Anti-RIP140 antibody was from Abcam. Anti-PKC ϵ and anti-lamin antibodies were from Santa Cruz. Anti-alpha-tubulin antibody was from Sigma-Aldrich. Cell lysate was collected as described (Ho et al., 2008) and protein concentration was determined by Bradford method. Immunoblots were quantified by Image J. For nuclear and cytoplasmic isolation, cells were broken by hypotonic buffer (10mM HEPES, pH7.9, 1.5 mM MgCl₂, 10mM KCl with protease inhibitors). Then after centrifugation for 10 min at 1,000g, supernatants (cytosolic fraction) were collected and pellets were lysed in RIPA buffer. Lysates were centrifuged at 14,000 rpm for 15 min. Supernatants were nuclear fraction.

2.4. Adipocyte isolation and glucose uptake assays

Primary adipocyte isolation was conducted as described (Ho et al., 2009). Glucose uptake assay in primary adipocytes was also performed as describe (Ho et al., 2009) and glucose uptake was normalized to the same number of cells.

2.5. PKC ϵ kinase activity and PLC β activity assays

Nuclear PKC ϵ activity was determined using a radiometric kit (Upstate) as described (Ho et al., 2009). PLC β activity assay was conducted as described (O'Carroll, Mitchell, Faenza et al., 2009) with some modification. A 100- μ g lysate was incubated with 300 nmole PLP Thio-PIP₂ (Cayman Chemical) in the reaction buffer at room temperature for 1 hr, supplemented with a 20 μ l DTNB solution (25mM DTNB and 475mM EGTA in 0.5M Tris-HCL) for another 5 min at room temperature, and OD₄₀₅ was determined.

2.6. Mice

Five-week-old male C57BL/6J mice (Jackson Laboratory) were maintained on a 12-hr light/dark photocycle and fed a normal diet containing 5% fat (#2018, Harlan Teklad) or a HFD (60% fat) (#F3282, Bio-Serv). After feeding for 2 weeks, mice were randomly divided into groups for different treatments. Treatments were given daily by oral gavage for another 3 weeks. All the animal experiments were conducted according to procedures approved by University of Minnesota Institutional Animal Care and Use Committee.

2.7. Tissue collection and immunohistochemical staining

Liver and epididymal white adipose tissues and livers were fixed in 10% neutral formalin, embedded in paraffin and sectioned by the Histology & Microscopy Core Facility (University of Minnesota). RIP140 immunohistochemical staining was performed (with antigen retrieval) using Vector[®] Elite ABC kit (Vector Laboratories). The sections were

counterstained with hematoxylin then mounted (Vector Laboratories) for microscopic analysis (Zeiss Axioplan 2).

2.8. Blood sample and glucose tolerance test

Blood samples were collected from mice and then analyzed for insulin, adiponectin and endothelin-1 using ELISA kits. For glucose tolerance test, following overnight fasting, blood sugar levels were determined using a glucometer, with a 2-gram/kg-bodyweight D-glucose injection. The blood glucose levels were then monitored at 30, 60 and 120 min after injection.

2.9. Statistical analyses

All immunoblottings were done at least twice, and representative results were shown. All results were presented as the means $\pm$ SD. *P* values were calculated using student *t* test in the two-sided condition. *P* value < 0.05 is considered as statistically significant.

3. Results

3.1. ET-1 promotes cytoplasmic accumulation of RIP140

We found that RIP140 was progressively exported to the cytoplasm in primary adipocytes of HFD animals accompanied by elevated nuclear PKC ϵ activity, but the physiological trigger and/or extracellular signal to activate this pathway in adipocytes were unclear (Ho et al., 2009). Since PKC ϵ is one of the signal mediators of PLC β , which can be activated by numerous extracellular factors including ET-1, we examined if chronic ET-1 treatment could alter the sub-cellular distribution of RIP140 in 3T3-L1 adipocytes. As shown in Fig. 1A, in 3T3-L1 adipocytes, with or without insulin stimulation, ET-1 indeed reduced the nuclear level, and elevated the cytoplasmic level, of RIP140. In adipocytes, endothelin receptor type A (ET_A) is the major form of ET-1 receptor (Bedi et al., 2006). We therefore examined RIP140's sub-cellular distribution in adipocytes under a chronic ET-1 treatment in the absence or presence of an ET_A receptor-selective antagonist, BQ-610 (Fig. 1B). The ET_A antagonist effectively dampened the accumulation of RIP140 in the cytoplasm of ET-1 treated adipocytes. In the absence of ET-1 treatment, BQ-610 had no effect on the accumulation of RIP140 in the cytoplasm. Using immunofluorescence analysis, we further confirmed that ET-1 promoted RIP140 cytoplasmic accumulation in an ET_A receptor-dependent manner (Fig. 1C). These results show that for 3T3-L1 adipocytes, ET-1 can be such an extracellular factor triggering the accumulation of RIP140 in the cytoplasm, which occurs in an ET_A-dependent manner.

3.2. ET-1 increases nuclear PKC ϵ level and promotes nuclear PKC ϵ activity

ET-1 signals, primarily, through G-protein coupled receptor-mediated activation of phospholipase C β (PLC β) (Marasciulo, Montagnani and Potenza, 2006). The activated PLC β converts phosphatidylinositol 4,5-bisphosphate (PIP₂) into inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG), which can activate a spectrum of signal mediators. Among the downstream signal mediators, PKC ϵ is most relevant to RIP140's activity because it can promote nuclear export of RIP140 and insulin resistance in adipocytes (Ho et al., 2009, Gupta et al., 2008). In addition to canonical activation of PLC β , which is activated on the plasma membrane, a nuclear membrane form of PLC β has recently been identified to regulate adipocyte differentiation (O'Carroll et al., 2009). To determine if ET-1 regulates cytoplasmic or nuclear PLC β to elicit the nuclear PKC ϵ activity in our experimental condition, we first monitored the PLC β activity of the nuclear and cytoplasmic fractions of 3T3-L1 adipocytes treated with ET-1 for various durations. The result shows that ET-1 robustly up-regulated the cytoplasmic, but not the nuclear, PLC β activity (Fig. 2A). We

further found that ET-1 indeed enhanced the nuclear PKC ϵ activity (Fig. 2B) and substantially increased the nuclear PKC ϵ level (Fig. 2C). Interestingly, the kinetics of ET-1-promoted accumulation of cytoplasmic RIP140 paralleled that of nuclear PKC ϵ activation (Fig. 2B, right panel). This result suggests that ET-1 stimulates cytoplasmic accumulation of RIP140 through activating nuclear PKC ϵ . Taken together, these data show that ET-1 elevates cytoplasmic PLC β activity and promotes nuclear accumulation of PKC ϵ , accompanied by elevated nuclear PKC ϵ activity in 3T3-L1 adipocytes.

3.3. ET-1 modulates RIP140's sub-cellular distribution through the PLC β -nuclear PKC ϵ signaling pathway

To determine the functional roles for the major signaling players in the PLC β -nuclear PKC ϵ pathway, we used inhibitor and siRNA to manipulate the players of this signaling pathway in adipocytes. We treated mature 3T3-L1 adipocytes with ET-1 in the absence or presence of a PLC β inhibitor, U73122 (Bedi et al., 2006). Indeed, PLC β inhibitor effectively reduced nuclear PKC ϵ activity as compared to the control treatment (Fig. 3A). This result supports that ET-1 promotes nuclear PKC ϵ activity via a PLC β -dependent manner. This was further confirmed by the finding that ET-1 failed to increase the cytoplasmic accumulation of RIP140 as well as nuclear PKC ϵ level in the presence of the PLC β inhibitor (Fig. 3B). To investigate if PKC ϵ is required for ET-1-stimulated cytoplasmic accumulation of RIP140, we knocked down PKC ϵ in ET-1-treated cells and monitored the distribution of RIP140 (Fig. 3C). Indeed, there was a decrease in the cytoplasmic accumulation of RIP140 and an increase in the nuclear RIP140 in PKC ϵ -silenced adipocytes as compared to the control group. Our previous study identified that cytoplasmic RIP140 can interact with AS160 to diminish glucose uptake (Ho et al., 2009). We then further examined the functional role for RIP140, particularly its cytoplasmic form, in modulating ET-1 stimulated glucose uptake by rescuing RIP140 null adipocytes. We treated three groups of RIP140 null adipocytes with ET-1: i) RIP140-null adipocytes rescued with control vector (Ctrl), ii) RIP140 null adipocytes rescued with wild type RIP140 (Wt), and iii) RIP140 null adipocytes rescued with mutant RIP140 (CN, a phosphor-deficient mutant that loses cytoplasmic localization and activity), and monitored their insulin-stimulated glucose uptake. In agreement with previous reports showing that chronic ET-1 could decrease glucose uptake in adipocytes by dampening insulin-stimulated Akt signaling (Ishibashi et al., 2001), we also detected a reduction in glucose uptake following ET-1 treatment in all three groups (Fig. 3D). However, comparing to the group rescued with the control vector, the group rescued with wild type RIP140 showed a significantly greater reduction in glucose uptake, whereas the group rescued with the CN mutant (that lost its cytoplasmic activity) had their glucose uptake reduced to a level comparable to that in the control rescue group. This result not only supports our conclusion that RIP140 plays a role in modulating ET-1's effect, but also validates that it is the cytoplasmic accumulation of RIP140 that plays such a role in dampening glucose uptake. Taken together, the data show that ET-1 modulates RIP140's sub-cellular localization by activating PLC β -PKC ϵ pathway, and targeting this pathway can effectively block ET-1-promoted accumulation of cytoplasmic RIP140.

3.4. Ambrisentan, ET $_A$ Antagonist, blocks HFD-triggered cytoplasmic RIP140 accumulation in epididymal adipose tissues

Our data of 3T3-L1 adipocytes suggest that the ET-1-triggered signaling pathway could elevate the level of cytoplasmic RIP140 in adipocytes. We next examined whether this signaling pathway is active in the context of whole animals. Previously, we showed that RIP140 accumulated in the epididymal adipose tissue in mice after feeding with HFD for 5 weeks (Ho et al. 2009). We treated HFD-fed mice with a selective ET $_A$ antagonist that has been approved for animal and clinical uses, ambrisentan (10 mg/kg or 30 mg/kg), for three weeks. Since ambrisentan is a potent vasodilator, we therefore included hydralazine (10 mg/

kg) as a control treatment to rule out the potential effects of vasodilation (Marasciulo et al., 2006, Ellershaw and Gurney, 2001, Casserly and Klinger, 2009, Shetty and Derk, 2011). HFD indeed elevated plasma ET-1 levels in mice (Fig. 4A). Of note, ambrisentan treatment enhanced the circulating ET-1 level in ND-fed mice, which could be caused by certain feedback mechanism to maintain the ET-1 affected vascular tone. Further, both hydralazine and a high dose of ambrisentan decreased the body weights of those mice fed with HFD, which might be due to side effects caused by vasodilation, such as a reduction in appetite (Fig. 4B) (Marasciulo et al., 2006). Because sub-cellular fractionation of primary adipose tissues is not as reliable for examining a protein's sub-cellular localization, we then used an established immunohistochemical method for detecting RIP140 (Ho et al. 2009) to examine the sub-cellular distribution of endogenous RIP140 in the epididymal tissue collected from these animals. As expected, the epididymal adipocytes significantly accumulated RIP140 in their cytoplasm under a HFD as compared to the control (Fig. 4C, HFD-Ctrl versus ND-Ctrl). Interestingly, ambrisentan treatment, in either a high or low dose, significantly reduced cytoplasmic accumulation of RIP140 in the epididymal adipose tissue of HFD-fed mice (Fig. 4C, HFD-Am10 and HFD-Am30). In contrast, hydralazine could not reduce cytoplasmic accumulation of RIP140 (Fig. 4C, HFD-Hydra). Moreover, these treatments did not affect the total RIP140 level in the epididymal adipose tissue (Fig. 4D). Since cytoplasmic RIP140 can retard GLUT4 vesicle trafficking to reduce glucose uptake in adipocytes (Ho et al. 2009), we then examined if glucose uptake in primary adipocytes was also increased in the ambrisentan-treated mice. As shown in Fig. 4E, HFD animals had a defect in insulin stimulated glucose uptake into their primary adipocytes (comparing HFD Ctrl to the ND Ctrl). Interestingly, a low dose ambrisentan readily rescued this defect and increased glucose uptake in the primary adipocytes of treated animals (comparing HFD Am-10 to HFD Ctrl). Of note, a high dose of ambrisentan also increased the basal level of glucose uptake; this may be due to the effect of ET-1 on the expression of GLUT1 (Ishibashi, Imamura, Sharma et al., 2000). A low dose of ambrisentan did not affect body weight but significantly reduced the cytoplasmic RIP140 level and improved glucose uptake. These results support the notion that the ET_A antagonist, ambrisentan, can prevent certain pathological conditions caused by HFD, including increased cytoplasmic accumulation of RIP140 and reduced glucose uptake in epididymal adipocytes. These animal data also support our prediction based on the studies of cell cultures, that ET-1 can promote the accumulation of RIP140 in the cytoplasm of adipocytes through an ET_A-mediated pathway.

3.5. Ambrisentan, ET_A Antagonist, increases circulating adiponectin levels, improves blood glucose control and ameliorates hepatic steatosis in HFD-fed mice

Recently, we have shown that cytoplasmic RIP140 also contributed to adipocyte dysfunctions such as an increase in lipolysis and a decrease in GLUT4 vesicle trafficking and adiponectin secretion (Ho et al., 2009, Ho, Chuang, Hung et al., 2011, Ho and Wei, 2012). We therefore also monitored the circulating adiponectin levels and crown-like structure (CLS), a proinflammatory sign, in the epididymal adipose tissue of ND- or HFD-fed mice treated with ambrisentan. We found that HFD indeed lowered the circulating adiponectin levels. Feeding with ambrisentan, but not hydralazine, for three weeks, enhanced the circulating adiponectin levels, which suggests that ambrisentan promotes adiponectin secretion, but this is not caused by its vasodilating effect (Fig. 5A). Additionally, ambrisentan decreased the number of CLS in the adipose tissue, indicating that ambrisentan also improves chronic inflammation (Shi, Kokoeva, Inouye et al., 2006) (Fig. 5B and 5C). Taken together, these animal studies show that the ET_A antagonist, ambrisentan, can prevent certain pathological conditions caused by HFD, such as reduction in adiponectin secretion and the accumulation of inflammatory macrophages in the adipose tissue.

We then examined the effect of ambrisentan on the fasting blood glucose level. It appears that none of these treatments altered the fasting blood glucose levels in the ND group, but ambrisentan effectively reduced the fasting blood glucose levels in HFD animals (Fig. 5D) without significantly changing their plasma insulin levels (Fig. 5E), ruling out the effect through altering the circulating insulin levels. In glucose tolerance test (GTT), ambrisentan improved the glucose clearance rate of HFD-fed mice to a level similar to that of the ND group (Fig. 5F and 5G). Furthermore, ambrisentan administration to HFD animals also decreased their hepatic steatosis (Fig. 5H), accompanied by a dose-dependent reduction in liver TG content (Fig. 5I). Together, these results show that ET_A antagonist, ambrisentan, is beneficial for the maintenance of systemic glucose homeostasis, and can be protective against liver steatosis induced by HFD.

4. Discussion

We have previously shown that a HFD increases the levels of RIP140 in the cytoplasm of epididymal adipocytes (Ho et al., 2009), but the physiological trigger, or extracellular signal, for this phenomenon in adipocytes was unclear. In the current study, we extend these findings and demonstrate that ET-1 can be such a physiological/extracellular signal, and that the effect of ET-1 to stimulate RIP140's nuclear export in adipocytes is mediated by the ET_A-PLC β -nuclear PKC ϵ signaling cascade (Fig. 6). Using a diet-induced obese mouse model, we further provide a proof-of-concept that, it might be useful to exploit this signaling pathway to manage adipocyte dysfunction, such as to improve their glucose uptake. This is supported by the effectiveness of using the ET_A antagonist, ambrisentan, to block cytoplasmic accumulation of RIP140 and improve glucose uptake in adipocytes. However, considering that PLC β can affect multiple downstream PKC isoforms and cytoplasmic accumulation of RIP140 is specifically triggered by the nuclear PKC ϵ , it could be even more specific and efficient to reduce cytoplasmic accumulation of RIP140 by targeting PKC ϵ . Of note, ET_A antagonists are widely used for pulmonary hypertension and other vascular diseases, and it has been suggested that ET_A antagonists can provide beneficial effects to reduce diabetes-caused complications (Rehsia and Dhalla, 2010, Braunwald, 2008, Marasciulo et al., 2006). There are also increasing studies showing that ET-1 can modulate adipocyte function, including adipokine secretion, lipid metabolism and insulin sensitivity. It is important in future studies to examine the beneficial effects of administration of ET_A antagonists to ameliorate certain complications affected by HFD.

In recent years, our lab has revealed the importance of PTMs of RIP140 in modulating RIP140's activity and nuclear export (Mostaqul Huq et al., 2008, Mostaqul Huq et al., 2006, Gupta et al., 2008, Huq et al., 2007). Although we have identified that cytoplasmic RIP140 can block glucose uptake by impeding GLUT4 trafficking and enhance lipolysis by interacting with perilipin (Ho et al., 2009, Ho et al., 2011), the full spectrum of cytoplasmic RIP140's activities remains to be examined. Nevertheless, these limited studies have begun to illustrate that cytoplasmic RIP140 can also be involved in regulating lipid and glucose metabolism in response to an alteration in the nutrient status or pathological conditions. Determining the interacting proteins of cytoplasmic RIP140 should be helpful to the elucidation of the full spectrum of cytoplasmic RIP140's activities (Mostaqul Huq et al., 2008). On the same note, it may also be interesting to determine whether RIP140 can accumulate in the cytoplasm of other cell types and, if so, it will be important to identify the physiological or pathological stimuli for nuclear export of RIP140 in various cell types. Our limited studies already show that there are extrinsic (circulating ET-1) (this current study) and intrinsic (lipid content within adipocyte) (Ho et al. 2011) mechanisms to modulate RIP140's sub-cellular distribution in adipocytes.

Finally, in obese and diabetic patients, increased secretion of ET-1 from endothelial cells has been considered as an important factor contributing to vascular dysfunction. Although we show that targeting ET-1 signaling by ET_A antagonist can be beneficial, such as improving glucose control and hepatic steatosis, it remains unclear if adipose tissue is the key target through which ET_A antagonists produce these beneficial effects, because ET-1 is also the strongest vasoconstrictor and is involved in vascular tone and other physiological homeostasis (Marasciulo et al., 2006). In our study, a high dose ambrisentan indeed reduced body weight gain (Fig. 4B), which might be caused by ambrisentan's side effects. Understanding the essential target of ET-1 blockage that produces these beneficial effects will help to design better treatments with fewer side effects such as hypotension.

Taken together, we report that ET_A antagonist ambrisentan exerts beneficial effects in controlling the progression of diet-induced diabetes, suggesting that blockade of ET-1 signaling would attenuate the vicious crosstalk between endothelial and adipocyte dysfunctions in diabetes progression. Moreover, this study reveals a pathological signal crosstalk between the vascular system and the adipose tissue through ET-1. This signal crosstalk provides points of intervention to develop novel therapeutic/preventive strategies for obesity- and diabetes-related metabolic syndromes.

Acknowledgments

This work is supported by NIH grants DK54733, DK054733-09S1, DK60521, DK060521-07S1, DA11190, K02-DA13926 and the Distinguished McKnight University Professorship to LNW. P.-C. H. is also supported by American Heart Association predoctoral fellowship. We thank A. Smith, C.-H. Hung, Y.-S. Chuang, F. A. Beyan and S.-C. Luo for technical help, and M Parker for RIP140-null MEF.

Abbreviations

RIP140	Receptor-interacting protein 140
HFD	High-fat diet
ET-1	Endothelin-1
PKCε	Protein kinase C epsilon
PTM	post-translational modification

References

- Archer SL, Weir EK, Wilkins MR. Basic science of pulmonary arterial hypertension for clinicians: new concepts and experimental therapies. *Circulation*. 2010; 121:2045–66. [PubMed: 20458021]
- Bedi D, Clarke KJ, Dennis JC, Zhong Q, Brunson BL, Morrison EE, Judd RL. Endothelin-1 inhibits adiponectin secretion through a phosphatidylinositol 4,5-bisphosphate/actin-dependent mechanism. *Biochem Biophys Res Commun*. 2006; 345:332–9. [PubMed: 16682005]
- Braunwald E. Biomarkers in heart failure. *N Engl J Med*. 2008; 358:2148–59. [PubMed: 18480207]
- Casslerly B, Klinger JR. Ambrisentan for the treatment of pulmonary arterial hypertension. *Drug Des Devel Ther*. 2009; 2:265–80.
- Chai SP, Chang YN, Fong JC. Endothelin-1 stimulates interleukin-6 secretion from 3T3-L1 adipocytes. *Biochim Biophys Acta*. 2009; 1790:213–8. [PubMed: 19162127]
- Ellershaw DC, Gurney AM. Mechanisms of hydralazine induced vasodilation in rabbit aorta and pulmonary artery. *Br J Pharmacol*. 2001; 134:621–31. [PubMed: 11588117]
- Guilherme A, Virbasius JV, Puri V, Czech MP. Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. *Nat Rev Mol Cell Biol*. 2008; 9:367–77. [PubMed: 18401346]

- Gupta P, Ho PC, Huq MD, Khan AA, Tsai NP, Wei LN. PKCepsilon stimulated arginine methylation of RIP140 for its nuclear-cytoplasmic export in adipocyte differentiation. *PLoS One*. 2008; 3:e2658. [PubMed: 18628823]
- Herman MA, Kahn BB. Glucose transport and sensing in the maintenance of glucose homeostasis and metabolic harmony. *J Clin Invest*. 2006; 116:1767–75. [PubMed: 16823474]
- Ho PC, Gupta P, Tsui YC, Ha SG, Huq M, Wei LN. Modulation of lysine acetylation-stimulated repressive activity by Erk2-mediated phosphorylation of RIP140 in adipocyte differentiation. *Cell Signal*. 2008; 20:1911–9. [PubMed: 18655826]
- Ho PC, Lin YW, Tsui YC, Gupta P, Wei LN. A negative regulatory pathway of GLUT4 trafficking in adipocyte: new function of RIP140 in the cytoplasm via AS160. *Cell Metab*. 2009; 10:516–23. [PubMed: 19945409]
- Ho PC, Chuang YS, Hung CH, Wei LN. Cytoplasmic receptor-interacting protein 140 (RIP140) interacts with perilipin to regulate lipolysis. *Cell Signal*. 2011; 23:1396–1403. [PubMed: 21504789]
- Ho PC, Wei LN. Negative regulation of adiponectin secretion by receptor interacting protein 140 (RIP140). *Cell Signal*. 2012; 24:71–6. [PubMed: 21872658]
- Humbert M, Sitbon O, Simonneau G. Treatment of pulmonary arterial hypertension. *N Engl J Med*. 2004; 351:1425–36. [PubMed: 15459304]
- Huq MD, Tsai NP, Lin YP, Higgins L, Wei LN. Vitamin B6 conjugation to nuclear corepressor RIP140 and its role in gene regulation. *Nat Chem Biol*. 2007; 3:161–5. [PubMed: 17277785]
- Ishibashi K, Imamura T, Sharma PM, Ugi S, Olefsky JM. The acute and chronic stimulatory effects of endothelin-1 on glucose transport are mediated by distinct pathways in 3T3-L1 adipocytes. *Endocrinology*. 2000; 141:4623–8. [PubMed: 11108276]
- Ishibashi KI, Imamura T, Sharma PM, Huang J, Ugi S, Olefsky JM. Chronic endothelin-1 treatment leads to heterologous desensitization of insulin signaling in 3T3-L1 adipocytes. *J Clin Invest*. 2001; 107:1193–202. [PubMed: 11342583]
- Leonardsson G, Steel JH, Christian M, Pocock V, Milligan S, Bell J, So PW, Medina-Gomez G, Vidal-Puig A, White R, Parker MG. Nuclear receptor corepressor RIP140 regulates fat accumulation. *Proc Natl Acad Sci U S A*. 2004; 101:8437–42. [PubMed: 15155905]
- Marasciulo FL, Montagnani M, Potenza MA. Endothelin-1: the yin and yang on vascular function. *Curr Med Chem*. 2006; 13:1655–65. [PubMed: 16787211]
- Mostaqul Huq MD, Gupta P, Wei LN. Post-translational modifications of nuclear co-repressor RIP140: a therapeutic target for metabolic diseases. *Curr Med Chem*. 2008; 15:386–92. [PubMed: 18288993]
- Mostaqul Huq MD, Gupta P, Tsai NP, White R, Parker MG, Wei LN. Suppression of receptor interacting protein 140 repressive activity by protein arginine methylation. *EMBO J*. 2006; 25:5094–104. [PubMed: 17053781]
- O'Carroll SJ, Mitchell MD, Faenza I, Cocco L, Gilmour RS. Nuclear PLCbeta1 is required for 3T3-L1 adipocyte differentiation and regulates expression of the cyclin D3-cdk4 complex. *Cell Signal*. 2009; 21:926–35. [PubMed: 19385066]
- Potenza MA, Gagliardi S, Nacci C, Carratu MR, Montagnani M. Endothelial dysfunction in diabetes: from mechanisms to therapeutic targets. *Curr Med Chem*. 2009; 16:94–112. [PubMed: 19149564]
- Powelka AM, Seth A, Virbasius JV, Kiskinis E, Nicoloso SM, Guilherme A, Tang X, Straubhaar J, Cherniack AD, Parker MG, Czech MP. Suppression of oxidative metabolism and mitochondrial biogenesis by the transcriptional corepressor RIP140 in mouse adipocytes. *J Clin Invest*. 2006; 116:125–36. [PubMed: 16374519]
- Rehsia NS, Dhalla NS. Potential of endothelin-1 and vasopressin antagonists for the treatment of congestive heart failure. *Heart Fail Rev*. 2010; 15:85–101. [PubMed: 19763821]
- Schenk S, Saberi M, Olefsky JM. Insulin sensitivity: modulation by nutrients and inflammation. *J Clin Invest*. 2008; 118:2992–3002. [PubMed: 18769626]
- Schneider JG, Tilly N, Hierl T, Sommer U, Hamann A, Dugi K, Leidig-Bruckner G, Kasperk C. Elevated plasma endothelin-1 levels in diabetes mellitus. *Am J Hypertens*. 2002; 15:967–72. [PubMed: 12441216]

- Shetty N, Derk CT. Endothelin receptor antagonists as disease modifiers in systemic sclerosis. *Inflamm Allergy Drug Targets*. 2011; 10:19–26. [PubMed: 21184655]
- Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H, Flier JS. TLR4 links innate immunity and fatty acid-induced insulin resistance. *J Clin Invest*. 2006; 116:3015–3025. [PubMed: 17053832]
- Takahashi K, Ghatei MA, Lam HC, O'Halloran DJ, Bloom SR. Elevated plasma endothelin in patients with diabetes mellitus. *Diabetologia*. 1990; 33:306–10. [PubMed: 2198188]
- Wellen KE, Hotamisligil GS. Inflammation, stress, and diabetes. *J Clin Invest*. 2005; 115:1111–9. [PubMed: 15864338]

Highlights

ET-1 stimulates nuclear export of RIP140 in adipocytes.

ETA activates a signal transduction pathway through PLC β .

PLC β elicits nuclear PKC ϵ activity.

PKC ϵ activation initiates nuclear export of RIP140.

Ambrisentan reduces cytoplasmic accumulation of RIP140 in primary adipocytes in animals and reduces severity of metabolic syndromes.

Fig. 1.

Chronic endothelin-1 treatment promotes cytoplasmic accumulation of RIP140. A, Sub-cellular distribution of RIP140 in 3T3-L1 adipocytes stimulated with or without insulin (6 hr) in the absence or presence of 50 nM ET-1 for 24 hr. Lamin and tubulin show the loading control and fractionation markers for nucleus and cytoplasm, respectively. Nu.: nuclear fraction; Cyto.: cytoplasmic fraction. B, Effect of ET_A antagonist (BQ-610) on sub-cellular distribution of RIP140 in 3T3-L1 adipocytes under chronic ET-1 treatment. Left: 3T3-L1 adipocytes were pretreated with BQ-610 for 1 h and then incubated with ET-1 for another 24 h. Right: 3T3-L1 adipocytes were treated with BQ-610 25 h. C, Representative confocal images showing RIP140 distribution in 3T3-L1 adipocytes treated as indicated. DAPI shows nucleus staining.

Fig. 2. Endothelin-1 increases cytoplasmic PLCβ and nuclear PKCε activity. A, PLCβ activity of the nuclear or cytoplasmic fraction of 3T3-L1 adipocytes stimulated by ET-1 for the indicated time period. All values show the means $\pm$ SD., n=3. B, Left: Quantification of nuclear PKCε activity of 3T3-L1 adipocytes stimulated by ET-1 for the indicated time period. Right: Cytoplasmic level of RIP140 in 3T3-L1 adipocytes stimulated by ET-1. C, Sub-cellular distribution of PKCε in 3T3-L1 adipocytes stimulated by ET-1. All values show the means $\pm$ SD., n=3; *: $P < 0.05$ compared to 2 hr treatment.

Fig. 3. Endothelin-1 promotes cytoplasmic accumulation of RIP140 in adipocytes via the PLC β -PKC ϵ pathway. **A**, PLC β inhibitor, U73122, blocks nuclear PKC ϵ activity under chronic ET-1 treatment. 3T3-L1 adipocytes were pretreated with U73122 for 1 h and then incubated with ET-1 for another 24 h. The nuclear fraction was collected and subjected into PKC ϵ activity assay. All values show the means $\pm$ SD., $n=3$; *: $P < 0.05$ compared to ctrl treatment. **B**, PLC β inhibitor decreases ET-1-mediated cytoplasmic accumulation of RIP140 in 3T3-L1 adipocytes. Nuclear and cytoplasmic fractions were collected in the same procedure as described in Fig. 2C. **C**, Western blot analyses of RIP140 in the nuclear and cytoplasmic fractions from ctrl- or PKC ϵ -silenced 3T3-L1 adipocytes after chronic ET-1 treatment. Knockdown efficiency was monitored by western blot analyses of PKC ϵ in whole cell extract. Nu.: nuclear fraction; Cyto.: cytoplasmic fraction; WCE: whole cell extract. **D**, Basal and insulin-stimulated glucose uptake of RIP140-null adipocytes rescued with control vector (Ctrl), wild type RIP140 (Wt) or phosphor-deficient mutant (CN) RIP140 under a control or chronic ET-1 treatment. Glucose uptake was measured. The relative fold was determined by normalization to the control vector group under the control treatment that was arbitrarily set at the value of 1. All values represent the means $\pm$ SD., $n=4$; *: $P < 0.05$.

Fig. 4. ET_A antagonist, ambrisentan, decreases cytoplasmic RIP140 accumulation in epididymal adipose tissue of HFD-fed mice. Mice were fed a normal diet (ND) or a high-fat diet (HFD) for two weeks and then treated, daily, as indicated for three more weeks. A, Plasma ET-1 levels were determined by ELISA. B, Body weight of mice from indicated group. C, Representative immunohistochemical staining of RIP140 in epididymal adipose tissue of the ND or HFD animals. D, The expression level of total RIP140 in epididymal adipose tissue of indicated group of mice. E, Basal and insulin-stimulated glucose uptake of primary adipocytes isolated from epididymal adipose tissues. Glucose uptake, per 10^6 cells, was measured. The relative fold was obtained by normalization to the control of the ND group that was arbitrarily set at the value of 1. All values represent the means $\pm$ SD, $n=4-7$; *: $P < 0.05$. Hydra.: hydralazine (10 mg/kg); Am-10: ambrisentan (10 mg/kg); Am-30: ambrisentan (30 mg/kg).

Fig. 5.

ET_A antagonist, ambrisentan, ameliorates metabolic dysfunctions in HFD-fed mice. A, Plasma adiponectin levels of mice fed a normal (ND) or high-fat diet (HFD) were determined by ELISA. All values represent the means $\pm$ SD, $n=4-7$; *: $P < 0.05$. B, H&E staining of epididymal adipose tissue to detect crown-like structures. C, Quantitative analysis of crown-like structures in adipose tissue. All values represent the means $\pm$ SD, $n=5-7$ random images from 3 different mice in the same group; *: $P < 0.05$. D, Fasting blood glucose. E, plasma insulin levels from overnight fasted mice fed with a ND or HFD. F, Glucose tolerance test. G, The area under curve (AUC) in glucose tolerance tests. H, Upper panel: Representative images of liver appearance. Lower panel: Representative images of H&E staining of liver sections. I, Triglyceride (TG) contents in animal livers. All values represent the means $\pm$ SD, $n=4-7$; *: $P < 0.05$. Hydra.: hydralazine (10 mg/kg); Am-10: ambrisentan (10 mg/kg); Am-30: ambrisentan (30 mg/kg).

Fig. 6.

Proposed model for ET-1-mediated nuclear export of RIP140. ET-1 binds with ET_A receptor on adipocytes and then activates cytoplasmic PLCβ. Activated PLCβ promotes nuclear accumulation of PKCε, that initiates the PTMs of RIP140 and its nuclear export.