EGF Receptor (ERBB1) Abundance in Adipose Tissue Is Reduced in Insulin-Resistant and Type 2 Diabetic Women

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Context: Indications of adipose tissue dysfunction correlate with systemic insulin resistance and type 2 diabetes. It has been suggested that a defect in adipose tissue turnover may be involved in the development of these disorders. Whether this dysfunction causes or exacerbates systemic insulin resistance is not fully understood.

Objectives, Participants, and Measures: We tested whether the expression of members of the mitogenic ErbB family was reduced in adipose tissue of insulin-resistant individuals and whether ErbB1 and ErbB2 were involved in adipogenesis. Thirty-two women covering a wide range of body mass index values and insulin sensitivity participated in the cross-sectional portion of this study. We also studied preadipocytes isolated from 12 insulin-sensitive individuals to evaluate the impact of ErbB1 or ErbB2 inhibition on adipogenesis in vitro. For this purpose, we measured phospho-ErbB1 and phospho-ErbB2 levels using ELISA and the expression of peroxisome proliferator-activated receptor γ (PPARγ) and PPARγ-regulated genes by real-time PCR.

Results: Among the ErbB family members, only ErbB1 expression was correlated with insulin sensitivity. Additionally, ErbB1 levels correlated positively with PPARγ and several PPARγ-regulated genes including acyl-coenzyme A synthetase long-chain family member 1 (ACSL1), adiponectin, adipose tissue triacylglycerol lipase (ATGL), diacylglycerol acyl transferase 1 (DGAT1), glycerol-3-phosphate dehydrogenase 1 (GPD1), and lipoprotein lipase (LPL), but negatively with CD36 and fatty acid-binding protein 4 (FABP4). In preadipocyte culture, ErbB1, but not ErbB2, inhibition was associated with a reduction in the expression of all the above-mentioned genes.

Conclusions: These findings demonstrate a key role for ErbB1 in adipogenesis and suggest that lower ErbB1 protein abundance may lead to adipose tissue dysfunction. (J Clin Endocrinol Metab 97: E329–E340, 2012)
Epidemiological studies have established beyond reasonable doubt that obesity is a predominant risk factor for dyslipidemia, hypertension, type 2 diabetes mellitus (T2DM), heart failure, and ultimately premature death (1). Most of the adverse metabolic changes associated with obesity are owed to the progressive development of systemic insulin resistance and hyperinsulinemia (2).

Although epidemiological and interventionalal studies make a clear case for a correlation between obesity and insulin resistance, the molecular mechanisms, especially in humans, are still unclear. There are at least three popular hypotheses: 1) the low-grade inflammation hypothesis; 2) the endocrine hypothesis; and 3) the adipose tissue expandability hypothesis. The low-grade inflammation hypothesis proposes that stressed and/or hypertrophied adipocytes recruit and activate macrophages, which release multiple cytokines that act locally and systemically to cause or exacerbate insulin resistance (3, 4). The endocrine hypothesis suggests that a reduction of the antiinflammatory and insulin-sensitizing adipokine, adiponectin, is central to the development of both inflammation and insulin resistance (5). Finally, the adipose tissue expandability hypothesis supports the idea that a failure of this tissue to accommodate a chronic caloric excess through preadipocyte proliferation and/or differentiation expedites the onset of insulin resistance due to fatty acid efflux to extra-adipose organs (6–8).

In adult humans, adipose cells die at a slow rate, but this rate tends to increase with obesity (9). Because the formation of new fat cells occurs at a rate equivalent to or slightly less than maintenance levels, the number of cells gradually declines from middle to older age (9). Consequently, in the face of caloric excess, adipose tissue expansion primarily occurs through hypertrophy of existing mature adipocytes (10). These enlarged adipocytes have been shown to be insulin resistant and to have an altered adipokine and cytokine secretion profile (11). Cross-sectional and longitudinal studies have also revealed that obese individuals with enlarged adipocytes are more at risk to develop insulin resistance and T2DM (12, 13). Recently, a population of smaller adipocytes, bigger than preadipocytes but apparently unable to adequately store triacylglycerol, have been identified in obese insulin-resistant individuals (14). Overall, these studies suggest that a defect in adipogenesis or lipogenesis can be associated with insulin resistance and that failure to recruit new and competent fat cells may be the cause.

The four ErbB family members, including their prototype that is the epidermal growth factor (EGF) receptor (ErbB1), are membrane-bound tyrosine kinases that play important roles as homo- or heterodimers in cell proliferation lineage determination, differentiation, and tissue remodeling throughout adulthood (15). ErbB1, ErbB3, and ErbB4 bind ErbB ligand family members with differential selectivity (16). ErbB2 does not bind any ligand directly and is therefore dependent on heterodimerization; ErbB3 has no catalytic activity.

In vitro studies performed in rodent-derived preadipocyte cell lines have yielded conflicting results; some revealed stimulatory and other inhibitory actions of ErbB ligands on adipogenesis (17–23). Two studies helped reconcile these observations. Adachi et al. (18) illustrated that EGF exposure can have both effects depending on the stage of differentiation, and Harrington et al. (20) showed that subnanomolar concentrations stimulate whereas higher concentrations of EGF can inhibit adipogenesis. Similar studies using human precursor cells showed that EGF could either inhibit or stimulate differentiation (24, 25).

We hypothesized that ErbB family members mediate the recruitment of new fat cells through proliferation, alternatively differentiation, of preadipocytes. Having found that ErbB1 expression was reduced in insulin-resistant individuals, the initial objective was to investigate the relationship between ErbB1 and markers of proliferation. However, because no marker of proliferation correlated with insulin sensitivity, we explored the roles of ErbB1 and ErbB2 in adipogenesis.

Subjects and Methods

Subjects

All 32 subjects were women from eastern North Carolina, recruited through participating physicians performing abdominal surgery at the Pitt County Memorial Hospital, and had given informed written consent. Their ages ranged from 25 to 61 yr, and their body mass index (BMI) ranged from 20.7 to 54.7 kg/m². The protocol was approved by the East Carolina University Institutional Review Board, and the samples from these subjects were used to investigate gene expression for three independent and parallel projects: 1) handling of and response to fatty acids and their derivatives; 2) buffering and response to reactive oxygen species; as well as 3) inflammation. All subjects were undergoing hysterectomy and were not taking drugs that could affect lipid or glucose homeostasis. BMI was calculated from body mass and height recorded at the time of surgery to the nearest 0.5 kg and centimeter, respectively. Fasting glucose was quantified from blood collected the day of the surgery using the 2300 Stat Plus System (Yellow Springs Instruments, Inc., Yellow Springs, OH). Insulin was quantified using the Beckman-Coulter Access Immunoassay System (Beckman-Coulter, Fullerton, CA). Abdominal sc adipose tissue (outside the fascia superficialis) was chosen because it constitutes the primary site of fatty acid storage and release, and hence would likely be a location where turnover is critical to maintaining fatty acid homeostasis (26). The tissue was immediately frozen in liquid nitrogen, transported to the laboratory, and stored at −80 C. For comparison purposes, the subjects were subdivided into four groups: the insulin-sensitive lean (ISL) [BMI < 30 kg/m², homeostasis model of assessment for insulin resistance (HOMA-IR) < 2.6 (actual highest value was
1.7), fasting insulin <10 μIU/ml, and fasting glucose <110 mg/dl; the insulin-sensitive obese (ISO) (BMI >30 kg/m², HOMA-IR <2.6, fasting insulin <10 μIU/ml, and fasting glucose <110 mg/dl); the insulin-resistant obese (IRO) (BMI >30 kg/m², HOMA-IR >2.6 (actual lowest value was 3.0), fasting insulin >10 μIU/ml, and fasting glucose >126 mg/dl), and the T2DM (fasting glucose >126 mg/dl on two occasions). The HOMA-IR cutoff of 2.6 was determined from our data bank of 38 lean individuals with normal glucose tolerance using the commonly used 75th percentile, and it is in line with published cutoffs (27). For some comparisons, we compared the subjects from the first and fourth quartiles with respect to insulin sensitivity, irrespective of their body weight. Analysis of data from 81 subjects for which we have both HOMA-IR and iv glucose tolerance test minimal model values established that the first and last quartiles of HOMA have a sensitivity of 0.9 to identify subjects belonging to the last and first tertiles of $S_p$, respectively. These are more stringent HOMA-IR cutoffs than our above-described insulin-sensitive and insulin-resistant cutoffs because they correspond to HOMA-IR below 1.2 and above 3.6, respectively. For other comparisons, we compared the subjects from the first and fourth quartiles with respect to ErbB1 protein levels.

Cell culture and differentiation

Human preadipocytes (Zen-Bio, Research Triangle Park, NC) were isolated from the sc adipose tissue of insulin-sensitive nonobese women using collagenase dissociation, and elimination of erythrocytes was performed using an ammonium chloride-based lysis buffer. The cells were grown for 24–48 h in preadipocyte media (PM-1; Zen-Bio) containing DMEM F12 [DMEM/F12 (1:1, vol/vol)], HEPES (pH 7.4), and 10% fetal bovine serum (FBS). Cells were induced to differentiate for 3 d in media containing DMEM/F12 (1:1, vol/vol), HEPES (pH 7.4), 10% FBS, 850 nm insulin, 1 μM dexamethasone, 17 μM pantothenate, 33 μM biotin, and 0.5 mM methylisobutylxanthine. Hereafter, the combination of methylisobutylxanthine, dexamethasone, and insulin will be abbreviated by MDI. This was followed by 3 additional days in media constituted of one third old media and two thirds fresh media containing 10% FBS, 100 nM insulin, 100 nM dexamethasone, 17 μM pantothenate, and 33 μM biotin. Every 3 d, two thirds of the media was exchanged with media containing 10% FBS, 17 μM pantothenate, and 33 μM biotin. Although the omission of a PPARγ agonist reduced the percentage of cells differentiating from 90 to about 60%, we eliminated this additive because it can overcome the effects of ErbB1 inhibition on adipogenesis. In the presence experiments testing the effects of AG1478 (Cell Signaling Technology, Danvers, MA), erlotinib, gefitinib, and lapatinib (LC Laboratories, Woburn, MA), and AG825 (Santa Cruz Biotechnology, Santa Cruz, CA), they were added 30 min before MDI at a concentration of 10 μM.

A figure showing neutral lipid accumulation can be found in Supplemental Fig. 1, published on The Endocrine Society’s Journals Online web site at http://jcem.endojournals.org. This figure additionally shows that the ErbB1 tested prevented neutral lipid accumulation. They were not replenished at the change in media. Therefore, assuming no degradation, the initial 10 μM concentration was reduced to 5 μM 3 d after MDI, then to 1.66 μM. All experiments were performed in pooled preadipocytes from six subjects and repeated at least two times with pools of six additional subjects to validate results and ensure reliability.

RNA isolation, reverse transcription, and quantitative real-time PCR

Adipose tissue dissection and weighing (100 mg) was performed after immersion of the frozen tissue in RNA later ICE (Applied Biosystems, Foster City, CA). Tissue homogenization and RNA isolation were conducted using TriReagent (Sigma Aldrich, St. Louis, MO). For cell culture, RNA isolation was also carried out with TriReagent. First-strand cDNA was synthesized from 5 μg total RNA with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Relative real-time TaqMan PCR was performed with primers and 6-carboxyfluorescein dye-labeled TaqMan probes (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as endogenous control because after comparing GAPDH, HPRT, and POLII, it was found to be the most stable among the samples. Each reaction contained 5 ng cDNA from total RNA, in a total reaction volume of 10 μl. To prevent bias toward a specific group, the relative expression was determined by the comparative threshold method ($\Delta\Delta C_t$) using all samples as reference.

Phospho-ErbB1 and phospho-ErbB2 ELISA

Sandwich ELISA were performed according to the manufacturer’s guidelines (R&D Systems, Minneapolis, MN). The capturing and secondary antibodies were provided as a set (DYC1095 and DYC4438).

Statistical analysis

Data are presented as mean ± SD. The difference between the first and fourth quartiles with respect to EGF receptor (EGFR) expression or insulin sensitivity was performed using a two-tailed unpaired Student’s t test. The variance among ISL, ISO, IRO, and T2DM was tested by one-way ANOVA, followed by Tukey’s multiple comparison tests. For cell culture experiments, one-way repeated measure ANOVA was employed for time course, and regular ANOVA was used for the other. The post hoc analysis was done using Tukey’s multiple comparison tests. Differences were considered significant when $P < 0.05$, and power was calculated with G*Power to be >0.80.

Results

General subject characteristics

Although there is a strong correlation between obesity and insulin resistance, not every obese individual is insulin-resistant, and some obese individuals develop exaggerated insulin resistance for their level of adiposity. Therefore, it is reasonable to propose that some obese individuals are protected or have adapted to their diabetogenic milieu. For this reason, we compared obese individuals that maintained their insulin sensitivity (ISO) to obese individuals that are insulin resistant (IRO) or diabetic (T2DM). Table 1 shows the basal characteristics of our subjects after a 12-h overnight fast. Plasma glucose levels were increased in both the insulin-resistant and diabetic obese compared with ISL individuals, but there was no statistically significant difference between the ISO and IRO groups ($P > 0.05$). Fast-
ErbB1 protein abundance and either age \( r \) or BMI \( r \) shows no correlation observed between these factors and insulin sensitivity was also observed (Fig. 1, C and D). His expression, almost in part, from a lower mRNA level because ErbB1 mRNA and protein correlate \( r \) \( 0.41; P = 0.02 \), and correlation between ErbB1 mRNA and insulin sensitivity was also observed (Fig. 1, C and D). However, there was no correlation observed between ErbB1 protein abundance and either age \( r \) or BMI \( r \) (ErbB2) are the proliferation markers, proliferating cell nuclear antigen (PCNA) and antigen Ki67. Although expression of PCNA and Ki67 were low, Fig. 2, A and C, shows a higher expression of PCNA and Ki67 in subjects belonging to the upper quartile of ErbB1 expression compared with subjects from the lower quartile. However, the expression of PCNA and Ki67 was not correlated with age, BMI, glucose, insulin, or HOMA-IR. The characteristics of the subjects belonging to the first and last quartile of ErbB1 protein expression are found in Table 3.

**Table 1.** Characteristics of the subjects from the ISL, ISO, IRO, and type 2 diabetes mellitus groups. The reduction in ErbB1 protein levels was approximately 50% in the insulin-resistant quartile compared with the insulin-sensitive quartile (Fig. 1B). The characteristics of the subjects belonging to these quartiles are described in Table 2. The reduction in ErbB1 protein seems to originate, at least in part, from a lower mRNA level because ErbB1 mRNA and protein correlate \( r \) \( 0.41; P = 0.02 \), and correlation between ErbB1 mRNA and insulin sensitivity was also observed (Fig. 1, C and D). However, there was no correlation observed between ErbB1 protein abundance and either age \( r \) or BMI \( r \) (ErbB2) are the proliferation markers, proliferating cell nuclear antigen (PCNA) and antigen Ki67. Although expression of PCNA and Ki67 were low, Fig. 2, A and C, shows a higher expression of PCNA and Ki67 in subjects belonging to the upper quartile of ErbB1 expression compared with subjects from the lower quartile. However, the expression of PCNA and Ki67 was not correlated with age, BMI, glucose, insulin, or HOMA-IR. The characteristics of the subjects belonging to the first and last quartile of ErbB1 protein expression are found in Table 3.

**ErbB1 protein expression correlates with insulin level and insulin sensitivity**

The initial purpose of the present study was to evaluate whether in obesity there is a correlation between ErbB family member’s expression and systemic insulin sensitivity. Therefore, we measured ErbB1-Erbb4 mRNA using real-time PCR and ErbB1 and ErbB2 protein abundance by ELISA in sc adipose tissue from women of various ages, BMI, and insulin sensitivities. We found that ErbB1 expression, at the protein level, correlated strongly with fasting glucose levels \( r \) \( -0.57; P = 0.0006 \), insulin levels \( r \) \( -0.59; P = 0.0004 \), and HOMA-IR \( r \) \( -0.59; P = 0.0003 \). As illustrated in Fig. 1A, receptor abundance was clearly reduced in IRO donors and those that developed T2DM. The protein level of the receptor was reduced approximately 50% in the insulin-resistant quartile compared with the insulin-sensitive quartile (Fig. 1B). The characteristics of the subjects belonging to these quartiles are described in Table 2. The reduction in ErbB1 protein seems to originate, at least in part, from a lower mRNA level because ErbB1 mRNA and protein correlate \( r \) \( 0.41; P = 0.02 \), and correlation between ErbB1 mRNA and insulin sensitivity was also observed (Fig. 1, C and D). However, there was no correlation observed between ErbB1 protein abundance and either age \( r \) or BMI \( r \) (ErbB2) are the proliferation markers, proliferating cell nuclear antigen (PCNA) and antigen Ki67. Although expression of PCNA and Ki67 were low, Fig. 2, A and C, shows a higher expression of PCNA and Ki67 in subjects belonging to the upper quartile of ErbB1 expression compared with subjects from the lower quartile. However, the expression of PCNA and Ki67 was not correlated with age, BMI, glucose, insulin, or HOMA-IR. The characteristics of the subjects belonging to the first and last quartile of ErbB1 protein expression are found in Table 3.

**ErbB1 protein expression correlates with markers of proliferation, but these do not relate to insulin sensitivity**

Two canonical pathways downstream of ErbB1 and of its heterodimerization partner ErbB2 are the proliferation markers, proliferating cell nuclear antigen (PCNA) and antigen Ki67. Although expression of PCNA and Ki67 were low, Fig. 2, A and C, shows a higher expression of PCNA and Ki67 in subjects belonging to the upper quartile of ErbB1 expression compared with subjects from the lower quartile. However, the expression of PCNA and Ki67 was not correlated with age, BMI, glucose, insulin, or HOMA-IR.
upper ErbB1 protein expression quartile (Fig. 4A). ACSL1 expression was also reduced in insulin-resistant individuals (Fig. 4B). The expression of adiponectin was also correlated with ErbB1 protein level ($r = 0.45; P = 0.01$). Adiponectin expression was 120% higher in the upper quartile of ErbB1 expression (Fig. 4C) and was lower in insulin-resistant and T2DM obese (Fig. 4D). Next, we looked at adipose tissue triacylglycerol lipase (ATGL) mRNA. The expression of ATGL was increased 90% in the upper quartile of ErbB1 expression (Fig. 4E) and was reduced in IRO subjects (Fig. 4F). CD36, another PPAR-regulated transcript, was surprisingly decreased by 43% in the high ErbB1-expressing subjects (Fig. 4H); this decrease was highly significant because the correlation between ErbB1 and CD36 was $r = -0.53$ ($P = 0.002$). The expression of diacylglycerol acyl transferase 1 (DGAT1) was 150% higher in the upper quartile of ErbB1 expression (Fig. 4I) and was correlated with PPAR expression ($r = 0.44; P = 0.01$). DGAT1 expression was also reduced in insulin-resistant and T2DM individuals (Fig. 4J). FABP4 was decreased 32% in the upper quartile of ErbB1

### Table 2. Characteristics of the subjects from the first and fourth quartiles of insulin sensitivity

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<tr>
<td>Age (yr)</td>
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<td>44.1 ± 11.6</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>25.9 ± 4.7</td>
<td>43.6 ± 7.7***</td>
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<tr>
<td>Glucose (mg/dl)</td>
<td>91.3 ± 10.0</td>
<td>124.3 ± 18.4***</td>
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<tr>
<td>Insulin (μU/ml)</td>
<td>3.2 ± 1.3</td>
<td>19.6 ± 6.9***</td>
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<td>HOMA</td>
<td>0.7 ± 0.3</td>
<td>6.0 ± 2.1***</td>
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$^a$ Data are presented as mean ± SD. Statistical analysis for this table and all subsequent results are described in Subjects and Methods. Difference between quartiles were compared using Student’s $t$ test for unpaired samples. $***, P < 0.001$.

FIG. 1. ErbB1 expression is lower in IRO and T2DM. ErbB1 protein abundance was quantified by ELISA (A and B). EGFR protein level was lower in IRO and T2DM compared with ISL and ISO individuals (A). EGFR protein level was also higher in the first quartile compared with the fourth quartile of insulin sensitivity (B). ErbB1 and ErbB2 mRNA levels quantified through real-time PCR using GAPDH as the endogenous control (C–F). ErbB1 expression was reduced in IRO compared with ISL and ISO individuals (C). ErbB1 expression was higher in the first quartile compared with the fourth quartile of insulin sensitivity (D). There was no difference in ErbB2 expression between groups (E and F). Results are expressed as means ± SD of 10 (ISL), 11 (ISO), six (IRO), and five (T2DM) individuals or eight subjects per quartile. Tukey’s multiple comparison tests were considered significant when $P < 0.05$; the same letter means that there were no statistically significant differences (A, C, E). Differences between quartiles were compared using Student’s $t$ test for unpaired samples. $**, P = 0.01$ (B, D, F).

FIG. 2. Although the expression of markers of cellular proliferation was correlated with ErbB1 protein levels, it was not correlated with insulin sensitivity. PCNA (A) and Ki67 (C) expression was higher in the high ErbB1-expressing subjects (Fig. 4H); this decrease was highly significant because the correlation between ErbB1 and CD36 was $r = -0.53$ ($P = 0.002$). The expression of diacylglycerol acyl transferase 1 (DGAT1) was 150% higher in the upper quartile of ErbB1 expression (Fig. 4I) and was correlated with PPAR expression ($r = 0.44; P = 0.01$). DGAT1 expression was also reduced in insulin-resistant and T2DM individuals (Fig. 4J). FABP4 was decreased 32% in the upper quartile of ErbB1
expression (Fig. 4K), slightly less than CD36, but still significantly \( (P < 0.05) \). FABP4 expression was increased in insulin-resistant individuals (Fig. 4L). Glycerol-3-phosphate dehydrogenase 1 (GPD1) expression was doubled in the upper quartile of ErbB1 protein expression (Fig. 4M) and reduced in IRO individuals (Fig. 4N). Finally, Fig. 4O shows that lipoprotein lipase (LPL) expression was three times higher in the upper quartile of ErbB1 protein level \( (P < 0.001) \), and Fig. 4P shows that LPL expression was decreased in both IRO and T2DM subjects.

**ErbB1 phosphorylation increased during in vitro stimulated adipogenesis, and ErbB1 kinase activity is involved in the differentiation process**

Our results presented here suggest that ErbB1 is involved in adipogenesis. Because ErbB1 can work as homodimers or as ErbB1:ErbB2 heterodimers, we next sought to determine whether ErbB1 and ErbB2 play functional roles in human preadipocyte differentiation. Human sc preadipocytes stimulated with 0.5 mM isobutylmethylxanthine, 1 μM dexamethasone, and 850 nM insulin (MDI) differentiate into adipocytes. Figure 5, A and B, shows a modest increase in ErbB1 autophosphorylation that lasted at least 6 h during differentiation while ErbB2 phosphorylation levels did not change. Five-minute incubations with TGFα and heparin-binding EGF (HB-EGF) were used as positive controls for ErbB1 and ErbB2 activation, whereas 5-min incubations with amphiregulin were used as a positive control for ErbB1 activation. Total ErbB1 and ErbB2 protein levels did not change significantly (Fig. 5, C and D). Next, we empirically determined the concentration of AG1478, an inhibitor that displays a marked (40-fold) selectivity for ErbB1 over ErbB2, sufficient to inhibit MDI-induced ErbB1 phosphorylation. Figure 5E shows that 10 μM AG1478 totally prevented ErbB1 autophosphorylation. We also tested two clinically used ErbB1 inhibitors, erlotinib (100-fold selectivity) and gefitinib (100-fold selectivity), as well as the dual ErbB1/

**TABLE 3. Characteristics of the subjects from the first and fourth quartiles of Erbb1 protein abundance**

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<tr>
<td>BMI (kg/m²)</td>
<td>43.3 ± 6.3</td>
<td>33.6 ± 9.0*</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>137.1 ± 20.4</td>
<td>92.6 ± 10.8***</td>
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<tr>
<td>Insulin (μU/ml)</td>
<td>16.0 ± 8.6</td>
<td>6.1 ± 2.2**</td>
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<tr>
<td>HOMA</td>
<td>5.1 ± 2.4</td>
<td>1.4 ± 0.6***</td>
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\( a \) Data are presented as mean ± SD. Statistical analysis for this table and all subsequent results are described in Subjects and Methods. Difference between quartiles were compared using Student’s \( t \) test for unpaired samples. *, \( P \leq 0.05; **, \( P \leq 0.01; *** \), \( P \geq 0.001 \).

**FIG. 3.** PPARγ and C/EBPα, but not C/EBPβ, mRNA expression was correlated with ErbB1 protein level and insulin sensitivity. PPARγ (A) and C/EBPα (C) expression was higher in the high ErbB1-expressing individuals. PPARγ (B) and C/EBPα (D) expression was lower in IRO and T2DM compared with ISL and ISO individuals. There was no significant difference in C/EBPβ (E) expression between the low and high ErbB1-expressing individuals. There was no significant difference in C/EBPβ (F) expression between the ISL, ISO, IRO and T2DM individuals. Results are expressed and analyzed as in Fig. 2 ***, \( P \leq 0.01 \) (A and C).

ErbB2 inhibitor lapatinib and the ErbB2-selective (50-fold) inhibitor AG825 (28). Figure 5F shows that 10 μM gefitinib reduced ErbB1 phosphorylation by 40%, which resembles the reduced level of ErbB1 measured from the tissue of insulin-resistant individuals. Lapatinib was not more efficacious than AG1478, and AG825 had no impact on ErbB1 phosphorylation levels.

The next question was whether inhibition of ErbB1 or ErbB2 kinase activities prevents adipogenesis. Therefore, we evaluated the impact of full ErbB1 inhibition with AG1478, partial ErbB1 inhibition with gefitinib, or ErbB2...
inhibition with AG825 on the expression of PPARγ, C/EBPα, and C/EBPβ. We found that both AG1478 and gefitinib, but not AG825, decreased MDI-induced PPARγ expression measured at d 9 by 40–85% (Fig. 6, A and B). None of the inhibitors affected C/EBPβ expression measured 3 h after MDI (Fig. 6C). AG1478 inhibited all PPARγ-regulated genes measured at d 9, including CD36 (Fig. 6, D–I). Gefitinib also reduced the expression of most PPARγ-dependent genes, in particular ATGL (Fig. 6F), CD36 (Fig. 6G), and GPD1 (Fig. 6J). The exception was DGAT1, which was less impacted in the presence of AG1478 (Fig. 6H). AG825 did not affect the expression of any of the genes in this study (Fig. 6, D–I). Altogether, these data show that ErbB1 participates in adipogenesis.

FIG. 4. ACSL1, adiponectin, ATGL, DGAT1, GPD1, and LPL mRNA expression was positively correlated, whereas CD36 and FABP4 expression was negatively correlated, with insulin sensitivity and ErbB1 protein level. ACSL1 (A and B), adiponectin (C and D), ATGL (E and F), DGAT1 (I and J), GPD1 (M and N), and LPL (O and P) expression was higher in insulin-sensitive and in high ErbB1-expressing individuals. CD36 expression was higher in insulin-resistant and low ErbB1 expressing subjects (G and H), as was also the case for FABP4 (K and L). Results are expressed and analyzed as in Fig. 2. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001 (A and C).
This study is the first to examine the relationship between adipose tissue ErbB family member expression and systemic insulin sensitivity. We used HOMA-IR to describe subjects as insulin-sensitive or insulin-resistant. The use of HOMA-IR is clearly not the “gold standard.” However, using value below the 25th percentile (first quartile) and above the 75th percentile (fourth quartile) should be stringent enough to greatly diminish the chances of mislabeling individuals as insulin-sensitive and insulin-resistant, respectively (27). These cutoff values corresponded to HOMA-IR values of 1.1 and 3.6, respectively. Our main finding is that ErbB1 protein levels are lower in both insulin-resistant and T2DM obese women. Additionally, we demonstrated that inhibition of ErbB1 kinase activity impedes adipogenesis in human preadipocytes in vitro. This indicates that ErbB1 may be important for the maintenance of adipose tissue function in ISO patients.

Obesity is defined as an increase in adipose tissue mass. The correlation between adipose tissue mass and several metabolic disorders, including insulin resistance and T2DM, has led to the assumption that these disturbances stem from adipose tissue expansion. Nevertheless, how adipose expansion causes insulin resistance remains unclear, and individuals with similar adiposity display a wide range of insulin sensitivity. It has been postulated that adipose tissue dysfunction, or adiposopathy, rather than adipose tissue enlargement is the causal link between obesity and insulin resistance or diabetes (29).

Among the dysfunctions that have been suggested to be of causality are reductions in the rates of fatty acid uptake and esterification, as well as a reduction in adiponectin secretion (5, 30). These dysfunctions, present in most individuals, have been suggested to be a consequence of a decline in adipocyte turnover that, in turn, underlie hypertrophy of existing mature adipocytes (6–8).

Our hypothesis was that ErbB family members are part of a paracrine axis leading to the recruitment of new adipocytes primarily through proliferation, alternatively differentiation, of preadipocytes. This hypothesis stemmed from empirical observations that addition of ErbB family ligands in human preadipocyte propagation media promotes proliferation and lipogenesis (31, 32). Insulin-resistant individuals had lower ErbB1 protein abundance, which correlated with PCNA and Ki67 expression, yet these mitogenic genes were not correlated with insulin sensitivity. This may be due to the fact that PCNA and Ki67 gene expression was very low or elevated in a subset of cells only, which would be consistent with the findings of Spalding et al. (9), who showed that less than 10% of adipocytes are renewed annually. Alternatively, it is also possible that we were underpowered to see small, but physiologically significant differences.

An alternative mechanism by which ErbB1, alone or through transactivation of ErbB2, could rescue adipose
FIG. 6. ErbB1, but not ErbB2, tyrosine kinase inhibition impaired adipogenesis. Inhibition of ErbB1 reduced PPARγ (A) and C/EBPα (B), but not C/EBPβ (C) induction by MDI. Inhibition of ErbB1 reduced PPARγ-regulated genes like ACSL1 (D), adiponectin (E), ATGL (F), CD36 (G), DGAT1 (H), FABP4 (I), GPD1 (J), and LPL (K); induction was measured by real-time PCR. Results are expressed as means and SEM of two independent experiments using six pooled individuals. For these experiments, one-way ANOVA were followed by Tukey’s multiple comparison tests. *, P ≤ 0.05; **, P ≤ 0.01; and ***, P ≤ 0.001 compared with respective control; –, P ≤ 0.05; –, P ≤ 0.01; and —, P ≤ 0.001 compared with MDI.
tissue from the deleterious effects of our obesigenic environment is through the promotion of adipogenesis. In that respect, our results show a strong correlation between ErbB1 protein levels and PPARγ expression as well as the expression of at least six PPARγ-regulated genes. Although our observations could be fortuitous in nature, our findings that inhibition of ErbB1 kinase activity in human preadipocytes dampened PPARγ induction strengthen the relationship found in our cross-sectional study. However, because ErbB1 phosphorylation waxed and waned before PPARγ induction, we can only speculate that an ErbB1 signaling-dependent PPARγ gene transactivation pathway is involved. Because a decreased PPARγ expression in adipose tissue of insulin-resistant individuals has been documented (33, 34), a decreased ErbB1 activity may be the cause of this reduced PPARγ expression. Interestingly, reduction in PPARγ expression in adipose tissue of obese individuals has been proposed as a protective mechanism to prevent further adipose tissue expansion (35). In our study, we did not find such a correlation between PPARγ and BMI \( (r = 0.15; \ P = 0.40) \). This is mainly because we selected 11 ISO and 11 insulin-resistant individuals (six IRO and five T2DM subjects), which is obviously not the natural ratio of occurrence. Our results suggest the alternative hypothesis that failure to maintain PPARγ activity, due to reduced ErbB1 levels, allows some obese individuals to develop insulin resistance.

ErbB1 expression correlated with the targets of PPARγ in vivo, and inhibition of ErbB1 activity in vitro dampened ACSL1, adiponectin, ATGL, CD36, C/EBPα, DGAT1, FABP4, GPD1, and LPL expression. Reduction of adipose tissue ACSL1 activity could be deleterious in many ways: 1) it could reduce fatty acid β-oxidation (36); and 2) it could reduce triacylglycerol synthesis (37). Loss of either process could lead to increased fatty acid outflow from adipose tissue and insulin resistance. We believe that it’s the first report of decreased expression of ACSL1 in the adipose tissue of IRO.

ErbB1 expression correlated with adiponectin, and inhibition of ErbB1 reduced adiponectin expression. Although adiponectin is the most abundant transcript in adipose tissue, its expression is known to be reduced in IRO subjects (38). Because maintaining adiponectin expression in obese individuals could preserve insulin sensitivity (39), decreased adiponectin expression could be a link between reduced ErbB1 function and insulin resistance. Nevertheless, although our in vivo correlation was very strong, inhibition of ErbB1 in vitro only marginally prevented adiponectin expression. This may stem from the very complex nature of the adiponectin promoter, which integrates signals emanating from at least a dozen other transcription factors (40).

ErbB1 expression correlated with ATGL, and inhibition of ErbB1 blunted ATGL induction. Reduction in ATGL expression in IRO, which is a pure PPARγ target, has been reported before (41). It was suggested that because insulin is a negative regulator of ATGL expression, insulin resistance is the cause rather than the consequence of ATGL down-regulation. Our in vitro data clearly show that preventing ErbB1 kinase activity reduced ATGL induction by 84%. This raises the possibility that a reduction in ErbB1 expression is, like insulin resistance itself, a way to refrain lipolysis and, by doing so, be a protective mechanism against further dyslipidemia and insulin resistance.

In adipose tissue, DGAT1 catalyzes the final step of triacylglycerol synthesis, and its activity has been shown to be reduced in larger adipocytes (42). DGAT1 expression was shown to be reduced in insulin-resistant individuals and up-regulated by PPARγ agonists (43). This, coupled to our in vitro data, suggests that ErbB1 promotes DGAT1 expression through PPARγ induction, which would be another way to divert fatty acids from overflowing extra-adipose tissues.

GPD1 in our study served as the canonical marker of adipogenesis and PPARγ-regulated genes GPD1 is also a gene that was shown to be reduced in insulin-resistant individuals and increased by PPARγ agonist treatment (44). ErbB1 regulation of GPD1 is an additional way in which the receptor could favor adipose tissue storage of triacylglycerol.

LPL, one of the earlier markers of adipocyte differentiation, is the enzyme responsible for the hydrolysis of triacylglycerol from chylomicron and very low-density lipoproteins (45, 46). The observed decrease in LPL expression may be deleterious because it would favor uptake of fatty acids by extra-adipose tissues. Therefore, ErbB1 regulation of LPL expression may be another way that the receptor prevents extra-adipose lipotoxicity.

Admittedly, we did not attempt to elucidate through which molecular mechanisms ErbB1 leads to PPARγ induction, nor did we demonstrate that ErbB1 promotes the expression of these PPARγ-dependent genes via PPARγ. We can only speculate on their likeliness.

Finally, our in vitro data show that inhibition of ErbB1 leads to a decrease in CD36 and FABP4 expression by more than 90%. This is in contrast to our in vivo data showing a negative correlation between ErbB1 and CD36 or FABP4. A possible reason for this discrepancy is that these two proteins are highly expressed in macrophages (47, 48). Therefore, the increase in CD36 and FABP4 seen in insulin-resistant individuals may be an indication of macrophage infiltration.

In summary, we established an important role for ErbB1 activity in human preadipocytes because inhibition
of the kinase hinders the adipogenic process. This in vitro response seems to involve the modulation of PPARγ expression, which may explain why ErbB1 expression in adipose tissue correlates with expression of genes that are believed to be important for proper adipocyte function. Finally, our study revealed a lower expression of ErbB1 in sc adipose tissue of obese women that are insulin-resistant or diabetic. Future research will show whether or not ErbB1 signaling pathways can be harnessed to protect or rescue adipose tissue from our obesigenic environment.

Acknowledgments

We thank Dr. David Taylor and Dr. James Gibson for their critical review of the manuscript.

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Funding for this work was from start-up funds (to J.R.) from East Carolina University, Brody School of Medicine, and a grant from the Brody Brothers Foundation.

B.B., C.R., F.M., H.B., and M.A.M. collected the data and revised the manuscript; B.D. and K.W.D. provided technical assistance and revised the manuscript; S.C. gave expert guidance and revised the manuscript; J.R. conceived and designed the study, collected the data, analyzed and interpreted the data, and wrote the manuscript.

Preliminary data from this study were presented in abstract form at the 69th Scientific Sessions of the American Diabetes Association, New Orleans, Louisiana, June 5–9, 2009.

Disclosure Summary: B.B. and K.W.D. are Zen-Bio employees. The other authors have nothing to declare.

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