Enhanced Insulin Signaling via Shc in Human Breast Cancer

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Insulin is a mild mitogen and has been shown to potentiate mitogenic influence of other growth factors. Because hyperinsulinemia and/or overexpression of insulin receptors have been linked to development, progression, and outcome of breast cancer, we attempted to evaluate the mechanism of these associations. We have compared the expression of insulin receptors and the magnitude of insulin signaling in breast tumors and adjacent normal mammary tissue samples obtained from 20 patients. We observed that insulin binding more than doubled in the tumors as compared with the normal tissue ($P < .01$ by paired *t* test). Insulin signaling to Shc, judged by the magnitude of its phosphorylation, was also significantly enhanced in the tumors. In contrast, the phosphorylation of the insulin-receptor substrate-1 (IRS-1), Akt, and mitogen-activated protein (MAP) kinase were identical in the tumorous and normal mammary tissues. Finally, tumors displayed significantly increased amounts of farnesylated p21 Ras and geranylgeranylated Rho-A ($P < .01$), consistent with Shc-dependent activation of farnesyl (FTase) and geranylgeranyl transferases (GGTase) in the tumor tissue. We conclude that the mechanism of the mitogenic influence of insulin in breast cancer may include increased expression of insulin receptors, preferential hyperphosphorylation of Shc, and increased amounts of prenylated p21 Ras and Rho-A in tumor tissue as compared with adjacent normal mammary tissue.

INSULIN, A MAJOR metabolic hormone, is also a key anabolic hormone, which belongs to a family of growth factors that include insulin-like growth factors-1 and 2 (IGF-1 and IGF-2).[1] Insulin action is initiated by its interaction with a specific cell surface receptor, a member of the tyrosine kinase family of receptors.[2,3] Intracellularly, insulin signal is mediated by a cascade of tyrosine and then serine/threonine phosphorylation and dephosphorylation steps involving multiple intermediates.[4,5] The initial steps are believed to be the phosphorylation of the insulin-receptor substrates 1/2 (IRS-1/2) and Shc.[4-6] It appears that the phosphorylation of IRS proteins engages both metabolic and mitogenic responses to insulin, whereas the phosphorylation of Shc initiates mitogenic responses only.[7]

The role of insulin in cancerogenesis has been debated since Heuson et al[8] described insulin-induced proliferation of rat mammary carcinoma cells in 1967 and Kessler[9] implicated insulin in the pathogenesis of cancer in 1971. Since that time, a number of epidemiologic and case-control studies corroborated animal and in vitro observations consistently showing an increase in breast, pancreatic, colorectal liver, endometrial, and prostate cancer in hyperinsulinemic individuals.[10-15] However, the molecular mechanisms of insulin effect on cancer development and/or progression remains enigmatic.

Insulin exerts a significant mitogenic action in normal mammary tissue and breast cancer cells in culture.[16,17] Many human breast cancers overexpress the insulin receptor.[16-19] Additionally, breast cancer cells fail to downregulate the insulin receptor in the presence of hyperinsulinemia.[20] Even though some reports have suggested that overexpression of insulin receptors on breast cancer cells may convey a favorable prognosis (possibly reflecting better differentiation of tumors expressing insulin receptors), at least one recent study demonstrated that women with insulin receptor-positive breast cancer have worse prognosis than women with insulin receptor-negative tumors.[16]

We have recently identified a novel aspect of insulin action: its ability to stimulate the prenylation of the small molecular weight guanine-triphosphatases (GTPases), such as p21 Ras and Rho-A.[21-25] We have demonstrated that insulin promotes the phosphorylation of the α-subunit of farnesyl transferase (FTase) and geranylgeranyl transferase I (GGTase I) that is common for these 2 enzymes.[26,27] We have further demonstrated that insulin-induced increases in the amounts of farnesylated p21 Ras and geranylgeranylated Rho-A augmented mitogenic responsiveness of numerous tissues to various growth factors, including IGF-1, epderman growth factor (EGF), platelet-derived growth factor (PDGF), and lysophosphatidic acid (LPA).[22,23,25,26] Insulin signaling to FTase and GGTase I appears to be mediated via Shc and MAP kinase and is completely independent of IRS-1.[24]

In this prospective study, we compared expression of insulin receptors and the state of insulin signaling in breast cancer specimens and in normal adjacent mammary tissue of 20 women undergoing surgery for breast cancer at the University of Colorado Health Sciences Center (UCHSC).

MATERIALS AND METHODS

Patients

The age of patients ranged from 40 to 75 years, and the majority of patients presented in stages I, II, and IIA of breast cancer (Table 1). Two patients were in stage IIIA and 1 patient in stage IV. Patients underwent either lumpectomy, modified radical mastectomy, or total mastectomy as was clinically indicated. None of the patients had diabetes. Protocols were approved by the UCHSC Institutional Review Board. Tumor and normal tissue samples were frozen immediately until

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Table 1. Clinical Characteristics of 20 Patients Enrolled in Study

Patient No.	Age	Cancer Stage	Tumor Size (cm)	Mets	Nodes	ER	PR	Insulin (μU/mL)	IGF-1 (ng/mL)
1	51	II	1.5	—	Neg	+	+	5.8	144
2	64	I	1.2	—	Neg	+	+	7.1	120
3	43	IV	2.7	Lung	10/13	+	+	5.8	126
4	69	IIA	1.0	—	2/21	+	+	3.8	105
5	42	IIA	3.9	—	Neg	+	+	5.6	129
6	75	IIA	1.0	—	1/2	Neg	Neg	4.7	119
7	49	IIIA	5.0	—	27/34	+	+	13.2	201
8	65	I	1.5	—	Neg	+	+	4.3	116
9	47	IIA	3.5	—	Neg	Neg	Neg	17.9	235
10	68	II	2.6	—	Neg	Neg	Neg	3.4	75
11	67	I	1.5	—	Neg	+	+	9.0	99
12	49	IIIA	6.3	—	1/1	+	+	4.7	204
13	59	II	1.4	—	2/19	+	+	9.8	117
14	75	IIA	2.2	—	Neg	+	+	3.6	58
15	64	I	1.5	—	Neg	+	+	3.0	121
16	52	I	1.8	—	Neg	+	+	6.0	124
17	49	I	1.7	—	Neg	+	+	16.0	118
18	40	IIA	1.2	—	2/2	Neg	Neg	4.0	121
19	58	IIA	6.0	—	3/17	+	+	7.0	129
20	57	IIA	1.3	-1/3	+	+	7.0	118	
Mean±SEM								7.1 ± 0.9	129 ± 9.3

Abbreviations: Mets, metastases; ER, estrogen receptor; PR, progesterone receptor; Neg, negative.

subsequent use. Blood samples were taken for insulin and IGF-1 determinations. Patients were followed up for 2 years from surgery.

Measurements of IRS-1, Shc, Grb2, mitogen-activated protein (MAP) kinase, Akt, prenylated p21 Ras, and Rho-A were performed by Western blot with appropriate antibodies as described in our previous publications.[23-25] Phosphorylation of IRS-1 and Shc was assessed by Western blot with p20 antibody of corresponding immunoprecipitates. Phospho-MAP kinase and phospho-Akt were determined with antiphospho-antibodies. Prenylated p21 Ras and Rho-A were separated from nonprenylated proteins by detergent extraction as previously described.[21-26] Insulin binding was assessed by incubating crude plasma membrane fractions (100,000 $\times$ *g* pellet) with 33.3 pmol/L ^{125}I-insulin. Nonspecific binding was determined in the presence of 2 μmol/L unlabeled insulin and subtracted from each point.

RESULTS

Characteristics of 20 patients aged 40 to 75 years involved in the study are summarized in Table 1. Only 1 patient had distant metastasis, 4 patients were estrogen receptor negative, and 4 patients progesterone receptor negative. Nine patients had positive nodes. Levels of insulin were elevated in 3 patients (above 10 μU/mL), and all patients had normal IGF-1 levels. There were no correlations between the stage of cancer, the size of tumor or presence of positive nodes, and the levels of either insulin or IGF-1.

Insulin binding was significantly increased in the tumor tissue as compared with control tissue from the same breast (Fig 1). Percent of insulin binding per 10 μg protein was 3.1 $\pm$ 0.6 in tumor versus 1.3 $\pm$ 0.3 in control samples ($P < .01$). K_D of insulin binding was identical in both tissues (0.67 nmol/L).

The amounts of IRS-1, Shc, MAP kinase, and Akt were similar and not significantly different in tumor and control samples (not shown). While the phosphorylation of IRS-1 was also similar between the 2 groups of tissue, there was a statistically significant increase in the phosphorylation of Shc (particularly p52 Shc) ($P < .01$) in the tumor tissue (Fig 2). There was no difference in the phosphorylation of either MAP Kinase or Akt between the control and tumor tissues. There was a tendency towards increased association of Grb-2 with Shc in the tumor tissue, but it did not reach statistical significance (Fig 2). Tumor tissue also contained significantly increased amounts of farnesylated p21 Ras ($P < .01$) and geranylgeranylated Rho-A ($P < .01$) than control samples (Fig 3), indicating enhanced prenylation of these proteins in the tumors.

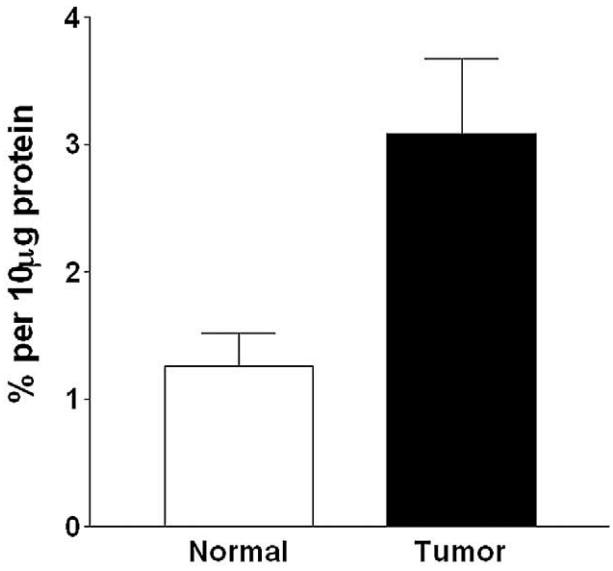

Fig 1. Insulin binding in tumor and adjacent normal mammary tissue of 20 patients with breast cancer. Results are expressed as the mean ± SEM; *$P < .01$.

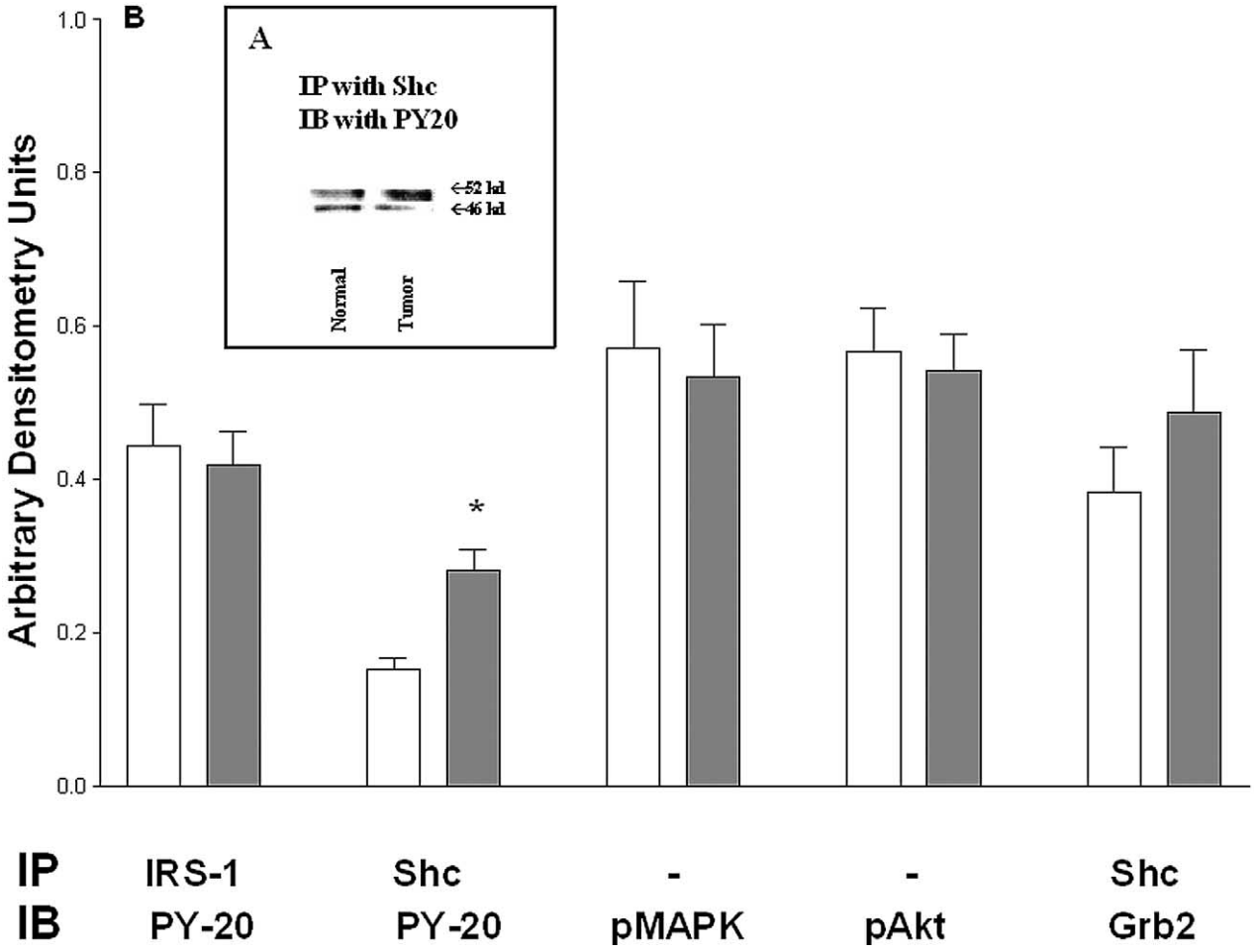

Fig 2. Phosphorylation of IRS-1, Shc, MAP kinase, Akt, and coimmunoprecipitation of Grb2 with Shc in tumor (closed bars) and adjacent normal mammary tissue (open bars) of 20 breast cancer patients. Insert (A) A representative experiment determining the phosphorylation of Shc. (B) Results expressed as the mean ± SEM; *$P < .01$.

DISCUSSION

The association of insulin resistance and hyperinsulinemia with breast cancer has been consistently observed (reviewed in Gupta et al[28]), but its underlying mechanism remains incompletely understood. Insulin resistance implies an inadequate ability of insulin to promote its effects in insulin target tissues. Clinically, the term "insulin resistance" is taken to mean impairment in the metabolic action of insulin, namely, an inability of insulin to lower blood glucose levels. To compensate for this inadequate response, insulin-resistant individuals develop various degrees of hyperinsulinemia in an attempt to normalize glucose utilization.

How, then, can the inability of insulin to promote adequate utilization of glucose become mitogenic and potentiate either the development or progression of breast cancer? The answer to this important question arose from the recent realization that the "mitogenic signaling" of insulin remains unaffected by the state of "metabolic insulin resistance." The insulin receptor is structurally and functionally normal in almost all patients with insulin resistance. Insulin action on glucose uptake and utilization is impaired at the postreceptor level. The immediate postbinding events include the activation of the tyrosine kinase of the insulin receptor and the phosphorylation of 2 signaling intermediates, IRS and Shc proteins.[4-7] Metabolic insulin action is mediated by insulin signaling via the IRS-1-phosphatidylinositol (PI)-3 kinase pathway, whereas mitogenic insulin action relies mainly upon the proper functioning of the Shc-Ras-MAP kinase branch of insulin signaling, which is not involved in the mechanism of the metabolic action of insulin.[4-7] In the state of metabolic insulin resistance, there is diminished insulin signaling along the IRS-1/PI-3 kinase pathway, but normal or even augmented insulin signaling along the Shc-Ras-MAP kinase pathway. In fact, several investigators have now shown that the inability of insulin to activate PI-3 kinase coexists with normal activation of MAP kinase in the same tissue.[29-31] Physiologically, these observations imply that normal mitogenic action of insulin is retained in the presence of the metabolic insulin resistance.

Furthermore, we have mapped out another branch of insulin action that leads to the activation of the prenyl transferases

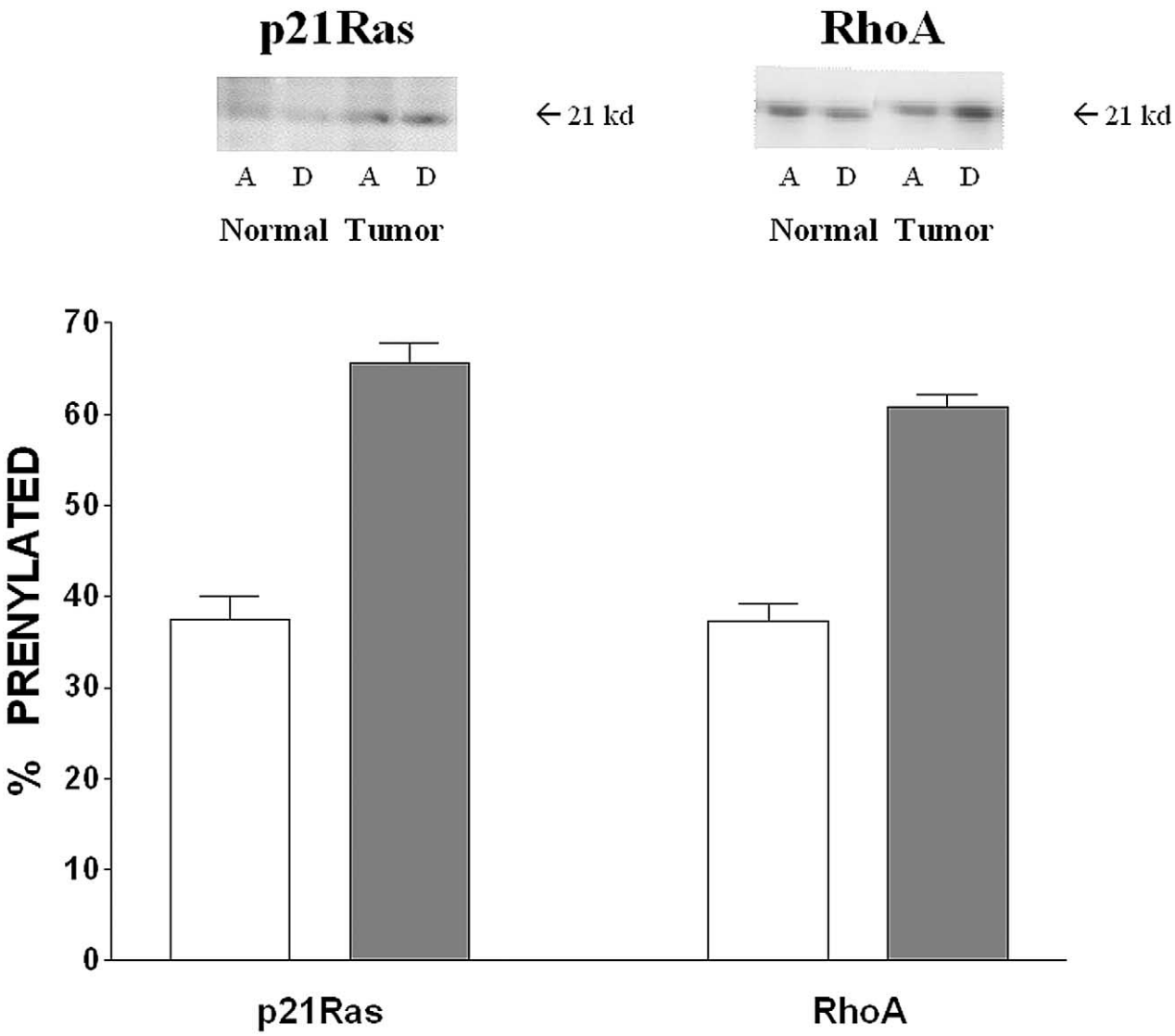

Fig 3. Amounts of farnesylated p21 Ras and geranylgeranylated Rho-A in tumor (closed bars) and adjacent normal mammary tissue (open bars) of 20 women with breast cancer. Representative blots for p21 Ras and Rho-A are displayed above (A, aqueous phase; D, detergent phase). Results are expressed as the mean ± SEM; *$P < .01$.

(FTase and GGTase I) and the accumulation of farnesylated p21 Ras and geranylgeranylated Rho-A.[21-26] This process is also unaffected by the presence of metabolic insulin resistance.[31] In fact, activation of the prenylation process is increased in the presence of the insulin resistance and compensatory hyperinsulinemia.[31] Increased amounts of prenylated p21 Ras and Rho-A magnify the cellular responsiveness of these tissues to other growth factors, which exert their mitogenic influence via the Ras- and Rho-dependent pathways.[21-26,32] Thus, metabolic insulin resistance coexists with the enhanced mitogenic responsiveness of insulin-resistant tissues to hyperinsulinemia and to other growth factors.

Studies in MDA-MBI57, MCF-10A, and in IRS-1-deficient ZR-75-1 breast cancer cell lines convincingly[33] demonstrated that insulin may stimulate cell growth via PI-3 kinase-dependent and PI-3 kinase-independent pathways. These findings correlate well with our own observations,[25] suggesting that a direct (mild) mitogenic action of insulin in MCF-7 cells is PI-3 kinase and cyclin D-dependent, while its potentiating action is Rho and cyclin E-dependent. Cyclin D appears to be regulated posttranscriptionally by the PI-3 kinase-dependent pathway.[34,35] Insulin augments the amounts of geranylgeranylated Rho-A[25,32] and activation of the latter leads to increased expression of cyclin E.[36,37]

Insulin has been shown[38] to act synergistically with estrogen receptors in human neuroblastoma cells. These effects appeared to be related to an insulin-dependent activation of the unliganded estrogen receptor. The investigators also demonstrated that p21 Ras is essential for insulin and estrogen receptor interaction, while PI-3 kinase activation did not contribute to this crosstalk.

Together these data support a hypothesis of a 2-sided mechanism of the mitogenic action of insulin. One is a direct mitogenic effect of insulin mediated via the PI-3 kinase-dependent branch of its intracellular signaling. Another aspect of the mitogenic action of insulin is much more robust and is mediated by the ability of insulin to augment the amounts of prenylated p21 Ras and Rho-A in tumor tissue, thus potentiating mitogenic effectiveness of various growth promoting factors.

In many instances, insulin, particularly in higher concentrations, may act via the IGF-1 receptors. Also, both hormones promote the phosphorylation of IRS-1.[1] However, insulin effect on the prenyl transferases requires the presence of the intact insulin receptor and is not mimicked by IGF-1.[22] Even hybrid insulin/IGF-1 receptors were unable to mediate insulin's effect on prenylation. Furthermore, insulin signaling to the prenyl transferases does not involve the phosphorylation of IRS-1.[22] Instead, 2 independent signals, one from MAP kinase and another from Shc independently of MAP kinase, are required for insulin to promote the phosphorylation and activation of prenyl transferases.[24] The nature of the Shc-associated signal leading to activation of prenyl transferases is still unknown.

Our study demonstrates an increased expression of the insulin receptor in mammary tumor tissue as compared with adjacent normal tissue (Fig 1). We also observed increased phosphorylation of Shc and not IRS-1 in the tumors. This preferential activation of the Shc in tumor tissue remains unexplained, even though we believe this can lead to activation of prenyl transferases in cancerous tissue. The mechanism of this preferential activation of Shc should be explored in future studies.

If the mechanism of the potentiating influence of hyperinsulinemia involves activation of the prenyl transferases, then inhibitors of these enzymes should block the detrimental influence of hyperinsulinemia. Even though FTase inhibitors are now in clinical trials,[39] we are not aware of their efficacy in hyperinsulinemic, insulin-resistant patients. Based upon our preclinical data, we would expect this to be the case.

It has also been shown that certain Ras proteins may escape the influence of the FTase inhibitors and become geranylgeranylated,[40] thus negating the therapeutic effectiveness of FTase inhibitors. To avoid this problem, we have recently developed a dominant negative mutant of the α-subunit of both FTase and GGTase I.[41-43] Forced expression of this subunit blocked the ability of insulin to stimulate both enzymes in MCF-7, vascular smooth muscle cells, and 3T3 LI preadipocytes.[41-43] The effectiveness of overexpression of this subunit in vivo remains to be determined.

In summary, human breast cancer tissue expresses increased amounts of insulin receptors, increased phosphorylation of Shc, increased association of Shc with Grb2, and increased amounts of prenylated p21 Ras and Rho-A. The latter are proposed to be an important element in the mechanism of the mitogenic influence of insulin. Amelioration of insulin resistance may be an important adjunct in the therapy of breast cancer.

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