Investigating New Therapeutic Strategies Targeting Hyperinsulinemia's Mitogenic Effects in a Female Mouse Breast Cancer Model

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Epidemiological and experimental studies have identified hyperinsulinemia as an important risk factor for breast cancer induction and for the poor prognosis in breast cancer patients with obesity and type 2 diabetes. Recently it was demonstrated that both the insulin receptor (IR) and the IGF-IR mediate hyperinsulinemia's mitogenic effect in several breast cancer models. Although IGF-IR has been intensively investigated, and anti-IGF-IR therapies are now in advanced clinical trials, the role of the IR in mediating hyperinsulinemia's mitogenic effect remains to be clarified. Here we aimed to explore the potential of IR inhibition compared to dual IR/IGF-IR blockade on breast tumor growth. To initiate breast tumors, we inoculated the mammary carcinoma Mvt-1 cell line into the inguinal mammary fat pad of the hyperinsulinemic MKR female mice, and to study the role of IR, we treated the mice bearing tumors with the recently reported high-affinity IR antagonist-S961, in addition to the well-documented IGF-IR inhibitor picropodophyllin (PPP). Although reducing IR activation, with resultant severe hyperglycemia and hyperinsulinemia, S961-treated mice had significantly larger tumors compared to the vehicle-treated group. This effect maybe secondary to the severe hyperinsulinemia mediated via the IGF-1 receptor. In contrast, PPP by partially inhibiting both IR and IGF-IR activity reduced tumor growth rate with only mild metabolic consequences. We conclude that targeting (even partially) both IR and IGF-IRs impairs hyperinsulinemia's effects in breast tumor development while simultaneously sparing the metabolic abnormalities observed when targeting IR alone with virtual complete inhibition. **(*Endocrinology* 154: 1701–1710, 2013)**

Breast cancer is the second most common cancer and the leading cause of cancer death in women worldwide. The American Cancer Society estimates that breast cancer accounts for almost 30% of all new cancer cases in women (1). Despite intensive research in the last 2 decades, the mechanisms underlying in the initiation of the disease are still not well understood. Epidemiologic and cohort studies have linked type 2 diabetes mellitus (T2DM) with an increased risk for developing breast cancer and higher mortality rates, even in the absence of obesity (2–5). It is believed that endogenous hyperinsulinemia may play a central role in mediating the mitogenicity observed in T2DM. Support for this hypothesis is the fact that no definitive association was found between the risk of breast cancer and type I diabetes (6–8). Furthermore, even in the absence of T2DM, hyperinsulinemia has been reported to increase the risk of breast cancer recurrence and death in women with breast cancer (9, 10). Insulin may directly affect breast tumors by inducing cell proliferation on the one hand, and by inhibiting apoptosis on the other hand (11, 12). In addition, insulin can indirectly affect breast cancer by suppressing insulin-like growth factor binding protein-1, which modifies free IGF-I levels. Furthermore, hyperinsulinemia is able to regulate the production of testosterone and estrogen; both are associated with breast cancer development (13). Insulin receptor (IR) and IGF-

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Abbreviations: IR, insulin receptor; PI3K, phosphatidylinositol 3-kinase; PPP, picropodophyllin; T2DM, type 2 diabetes mellitus.

IR, which belong to the same subfamily of receptor tyrosine kinases, share high sequence homology, especially in the tyrosine kinase domain; moreover, similar signaling pathways, primarily the phosphatidylinositol 3-kinase (PI3K)-Akt pathway and the MAPK pathway, mediate both receptors effects (14–16). Both IR and IGF-IR have been reported to mediate the mitogenic effects of insulin and are frequently overexpressed in breast cancer (17). It was demonstrated that IR overexpression in node-negative breast carcinoma was associated with reduced disease-free survival (18). Furthermore, IR activation by insulin was demonstrated in most breast cancers, thereby enhancing induced cell growth and proliferation, especially in the hyperinsulinemic state (19).

The MKR female mice provide an excellent model to study the association between hyperinsulinemia and breast cancer in the absence of other factors associated with obesity and diabetes. Hyperinsulinemia and severe insulin resistance are the main characteristics detected in these female mice. Blood glucose levels in female MKR mice are only mildly elevated, but importantly, these mice are not obese. Recently, it was demonstrated that hyperinsulinemia induces accelerated mammary gland development in these mice (20). Moreover, inoculation of Mvt-1 cells, a metastatic mammary carcinoma cell line, into the fourth mammary fat pad, forms significantly larger tumors in MKR female mice than in controls (11). Similar results were observed with inoculation of other mammary carcinoma cell lines (7, 21). In these studies, it was demonstrated that IR/IGF-IR mediated hyperinsulinemia's mitogenic effects through the PI3K-Akt pathway, a major axis for delivering insulin's signals. This is not surprising because the PI3K-Akt pathway's role in several types of cancer including breast cancer is well established (22, 23). Using BMS-536924, a nonselective ATP-cleft inhibitor for IR and IGF-IR, Novosyadlyy et al showed significant reduction in breast tumor growth rate in the female MKR mice, despite metabolic abnormalities. These findings highlight the role of IR/IGF-IR in mediating hyperinsulinemia's mitogenic effect (20). However, recent reports have demonstrated that resistance to anti-IGF-IR therapy may be due to IR compensation. Support for this comes from the findings demonstrating improved sensitivity to anti-IGF-IR therapy after tissue-specific IR-knock-out in a mouse model of pancreatic cancer (24, 25).

Until recently, antibodies were the only available antagonists against the IR, and due to the dimeric nature of antibodies, their antagonistic properties depend on the context in which they are used; under certain circumstance they become partial agonists. Recently, Novo-Nordisk has identified S961, a specific antagonist for the IR with low affinity toward the IGF-IR. Unlike IR, several antagonists were developed against the IGF-IR (26); picropodophyllin (PPP), a member of the cyclolignans family, was one of the first to be developed (27).

In the present study we took advantage of both S961 and PPP to identify new therapeutic strategies for breast cancer in the hyperinsulinemic state and to explore the role of both IR and IGF-IR in mediating hyperinsulinemia's mitogenic effects. We inoculated Mvt-1 cells in to the fourth mammary fat pad of the hyperinsulinemic MKR female mice and examined separately the effect of S961 and PPP on tumor growth and metabolic state and the signaling pathways mediating these effects.

Materials and Methods

Cell culture

Mouse mammary cancer cell line, Mvt-1, was derived from an explant culture of an MMTV c-Myc/Vegf transgenic female mouse as previously described (28). Cells were grown in monolayer culture in DMEM (Biological Industries, Beit Haemek, Israel) supplemented with 10% fetal bovine serum (Biological Industries) and antibiotics (penicillin:streptomycin; Biological Industries) at 37°C in a humidified atmosphere consisting of 5% CO_2 and 95% air.

Animals

The generation of the MKR mice, a transgenic mouse with a dominant-negative insulin-like growth factor-I receptor specifically targeted to the skeletal muscle, was previously described (29). Mice were kept on a 12-hour light/dark cycle with access to standard mouse chow and fresh water ad libitum. Mice studies were performed according to the protocol approved by the Technion Animal Inspection Committee. The Technion holds an NIH animal approval license number A5026-01.

Protein extraction and Western blot analysis

Cellular extraction was carried out as follows. Cells were washed in PBS and lysed in lysis buffer (10 mmol/L Tris-HCl at pH 7.5, 150 mmol/L NaCl, 10 mmol/L sodium pyrophosphate, 1.0 mmol/L β-glycerophosphate, 1.0 mmol/L sodium orthovanadate [Na_3VO_4], 50 mmol/L sodium fluoride [NaF], 1.25% CHAPS [3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate]) and Protease Inhibitor Cocktail (Complete; Roche Diagnostics, Mannheim, Germany). Protein from homogenized tissues was extracted in a similar fashion. After incubation on ice for 30 minutes and centrifugation at 13 000 rpm for 10 minutes, the supernatants were collected and the protein concentrations were determined with the Protein Assay Kit (Bio-Rad, Richmond, California). Protein was electrophoresed through a 10% polyacrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad) by electroblotting. The membranes were immunoblotted with the desired antibody, followed by a matched secondary antibody conjugated with horseradish peroxidase (Jackson Laboratories, Bar Harbor, Maine).

The following antibodies, phospho-IR$\beta^{Y1150/51}$/IGF-IR$\beta^{Y1135/36}$, IRβ, IGF-IR, Phospho-Akt$^{(Thr\ 308)}$, total Akt, phospho-p44/42$^{(Thr202/Tyr204)}$ MAPK (ERK 1/2), total p44/42-

MAPK, and β-actin were purchased from Cell Signaling Technology (Danvers, Massachusetts). Membranes were developed by enhanced chemiluminescence (GE Healthcare Biosciences, Piscataway, New Jersey) and analyzed using luminescent image analyzer LAS-4000 (Fujifilm, Tokyo, Japan). Densitometry analysis was performed using ImageQuant software (GE Healthcare Biosciences).

Immunoprecipitation

Tissue lysates (0.5 mg) were mixed with anti-IRβ (2 μg) or anti-IGF-IR (2 μg) (Santa Cruz Biotechnology, Santa Cruz, California) antibodies at 4°C overnight with continuous agitation. Protein A-Sepharose beads (Santa Cruz Biotechnology) were added and incubated at 4°C for an additional 4 hours. The beads were washed 3 times with the lysis buffer. Laemmli sample buffer (5% β-mercaptoethanol, 2% sodium dodecyl sulfate, 62.5 mmol/L Tris-HCl, 1 mmol/L EDTA, 10% glycerol) elutes were analyzed as described above.

Metabolic assays

Body weights and blood glucose levels were measured weekly. Blood glucose levels were measured in the nonfasting state with an automated glucometer (Accu-Check Preforma System; Roche Diagnostics). At the end of the study, mice were anesthetized and blood samples were taken from the heart and used for insulin levels measurement by ELISA kit (cat. no. EZRMI-13K; Millipore, Billerica, Massachusetts) according to the manufacturer's instructions.

Syngeneic orthotopic tumor models

Mvt-1 cells were grown until 90% confluence and detached by trypsin solution (Biological Industries). Injected into the left inguinal mammary fat pad (no. 4) of 8-week-old female MKR mice were 100,000 Mvt-1 resuspended in 100 μL PBS. Tumor volume was monitored once a week with calipers; volume was calculated in cubic millimeters by the formula: $(\text{width}^2 \times \text{length} \times 0.5)$.

S961 and PPP treatment

Two weeks after cell inoculation, treatment groups were set with matched tumor size between groups. Mice were administered for 3 weeks by 100 μL ip injections twice daily with either the high-affinity insulin receptor antagonist S961 (400 μg/kg body weight; a gift from Novo Nordisk, Maaloev, Denmark) or the IGF-IR inhibitor PPP (20 mg/kg body weight; a gift from Dr Ada Girnita, Cellular and Molecular Tumor Pathology, Department of Oncology and Pathology, Cancer Centre Karolinska R8:04, Karolinska Hospital, Sweden). Control mice were administered an equal volume of vehicle (sterile PBS). At the end of the study, mice were euthanized, and tumors were removed and immediately snap frozen for immunoblot analyses.

Statistical analysis

All data are presented as mean ± SEM. The Mann-Whitney test was used for statistical analyses, with P values less than .05 considered statistically significant.

Results

Specific inhibition of the insulin receptor after S961 treatment

To examine S961 efficiency in specifically blocking the IR, we first validated that S961 blocked insulin-induced Akt phosphorylation. S961 treatment for 30 minutes before insulin stimulation caused a dose-dependent decrease in Akt phosphorylation in a similar fashion to wortmannin, a specific PI3K inhibitor (Figure 1, A and B). Next, we validated that S961 does not affect signaling via the IGF-IR. Hence, Mvt-1 cells were stimulated with IGF-I after 30 minutes of treatment with S961. Interestingly, not only did Akt phosphorylation not decrease after S961 treatment, but also an increase in Akt phosphorylation was observed after S961 treatment. As expected, Akt phosphorylation was blocked with the treatment of wortmannin (Figure 1, C and D). Thus, S961 specifically inhibits IR, and the hyperphosphorylation of Akt observed when cells treated with S961 and IGF-1 might suggest hyperactivation of the IGF-IR as a compensatory mechanism when IR is inhibited.

PPP inhibits both IR and IGF-IR activation in Mvt-1 cells

We examined the PPP effect on both IR and IGF-IR in a similar manner to S961, as described above. Cells treated with PPP for 60 minutes before IGF-1 stimulation had a moderate but significant decrease in Akt phosphorylation, unlike wortmannin that abrogates the IGF-1 effect (Figure 2, A and B). Surprisingly, Mvt-1 cells that were treated with insulin after 60 minutes of PPP treatment had the same reduction in Akt phosphorylation as observed with IGF-I stimulation (Figure 2, A and C), suggesting that PPP is not specific to the IGF-1R.

S961 induces hyperglycemia and severe hyperinsulinemia in MKR female mice

To study the role of the IR and IGF-IR in tumor progression, we used the nonobese female MKR mouse, which is characterized by marked hyperinsulinemia but only mild dysglycemia. We orthotopically inoculated MKR mice with Mvt-1 cells, and 2 weeks after injection of the cells, the mice were divided into 3 treatment groups that were matched for respective tumor size. Mice were treated with S961 (10 μg/kg) or PPP (20 mg/kg) twice a day by ip injections or the same volume of vehicle. Treatment was continued for 3 weeks. We evaluated the metabolic state during and after the treatment with the inhibitors. After 3 weeks of treatment, significant hyperglycemia developed in the S961-treated group. Unlike S961, PPP treatment had no effect on blood glucose

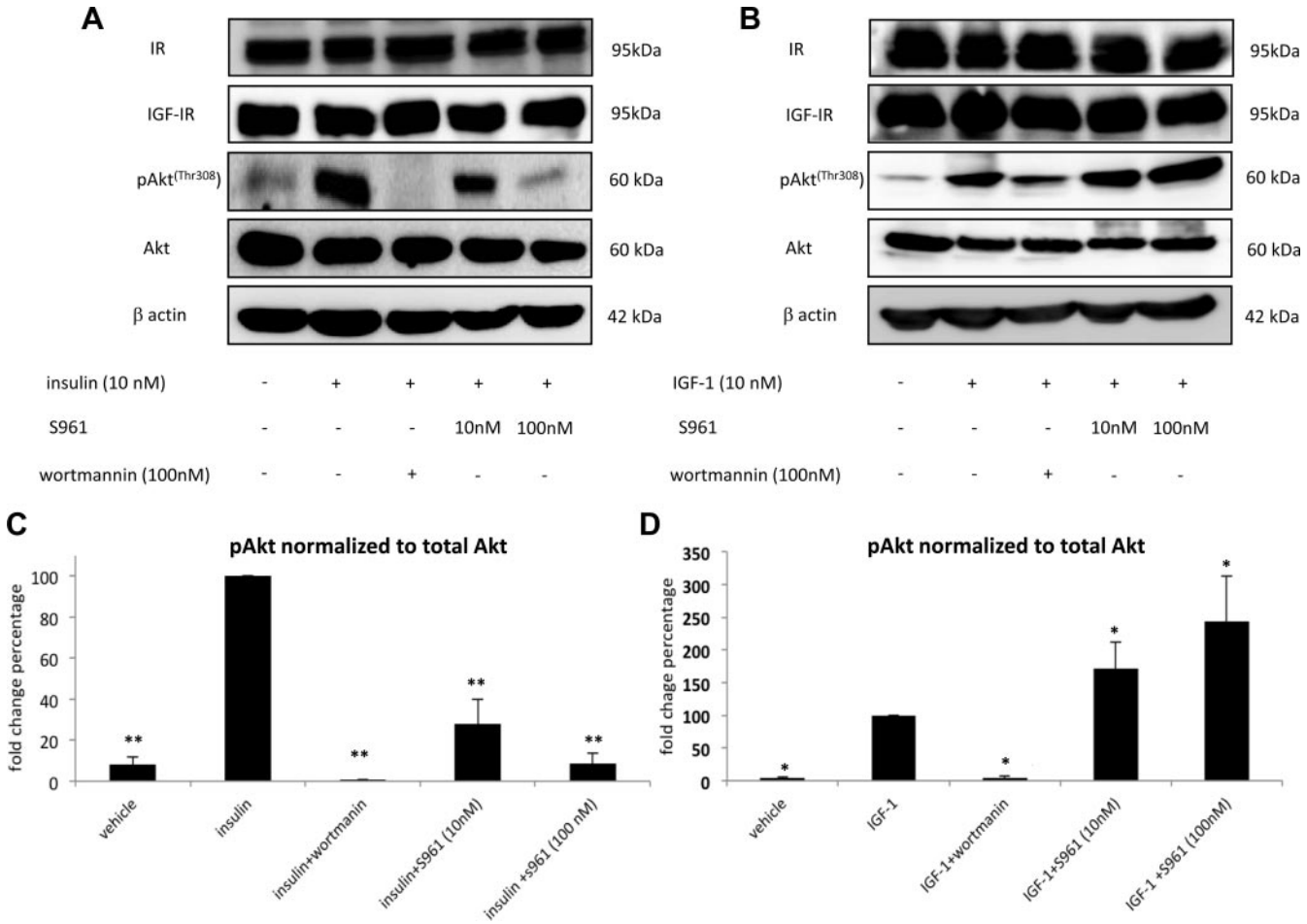

Figure 1. Specific inhibition of the insulin receptor after S961 treatment in Mvt-1 cells. Mvt-1 cells were starved with serum-free medium for 16 hours and then treated for 30 minutes with the insulin receptor inhibitor S961 (10 nM and 100 nM) or the PI3K inhibitor wortmannin (100 nM) as a control before treatment with 10 nM insulin (A) or IGF-I (B). Cellular lysates were separated by SDS-PAGE, and the phosphorylation and protein levels were assessed using specific antibodies by Western blotting. Protein expression was quantified by densitometric analysis; pAkt levels were normalized to Akt expression, and relative phosphorylation for insulin treated cells (C) and IGF-I-treated cells (D) was determined. Equal loading of proteins was demonstrated by immunoblotting with an antibody directed against β-actin. The Mann-Whitney test was performed to compare the difference from the insulin or IGF-1-treated cells. $*P < .05$; $**P < .01$.

levels (Figure 3A). IR is generally accompanied by a compensatory rise in the insulin level. Therefore, we examined the effect of both inhibitors on plasma insulin levels. Severe hyperinsulinemia was observed after S961 treatment compared to the control group. PPP induced a slight but significant increase in the plasma insulin levels (Figure 3B). A very small but significant increase in body weight was detected only in the S961-treated mice (Figure 3C).

S961 induces enhanced tumor progression, whereas PPP impairs tumor progression in MKR female mice

To examine the effect of the inhibitors on tumor growth rate, interval measurements of tumor volume over the treatment period were performed in addition to measurements of tumor weight at the end of the study. Tumor volumes were significantly higher in the MKR mice treated with S961 compared with the MKR vehicle-treated group. In contrast, tumor volumes of mice treated with PPP were significantly lower compared with the MKR vehicle-treated group (Figure 4A). Similarly, although tumor weights were significantly higher after S961 treatment, tumor weights were significantly reduced in the PPP-treated mice (Figure 4B).

S961 impairs IR activation, whereas PPP partly inhibits both IR and IGF-IR activation and significantly reduces both receptors expression in mammary tumors

To confirm the in vivo efficiency of both S961 and PPP, tumor tissues were extracted from PPP, S961, and vehicle-treated groups; lysates were subjected to immunoprecipitation of the β-subunits of IR (IRβ) and IGF-1R (IGF-IRβ) and immunoblotted with an antiphosphotyrosine antibody. As expected, S961 treatment significantly inhibited IR activation as shown by reduced tyrosine phosphoryla-

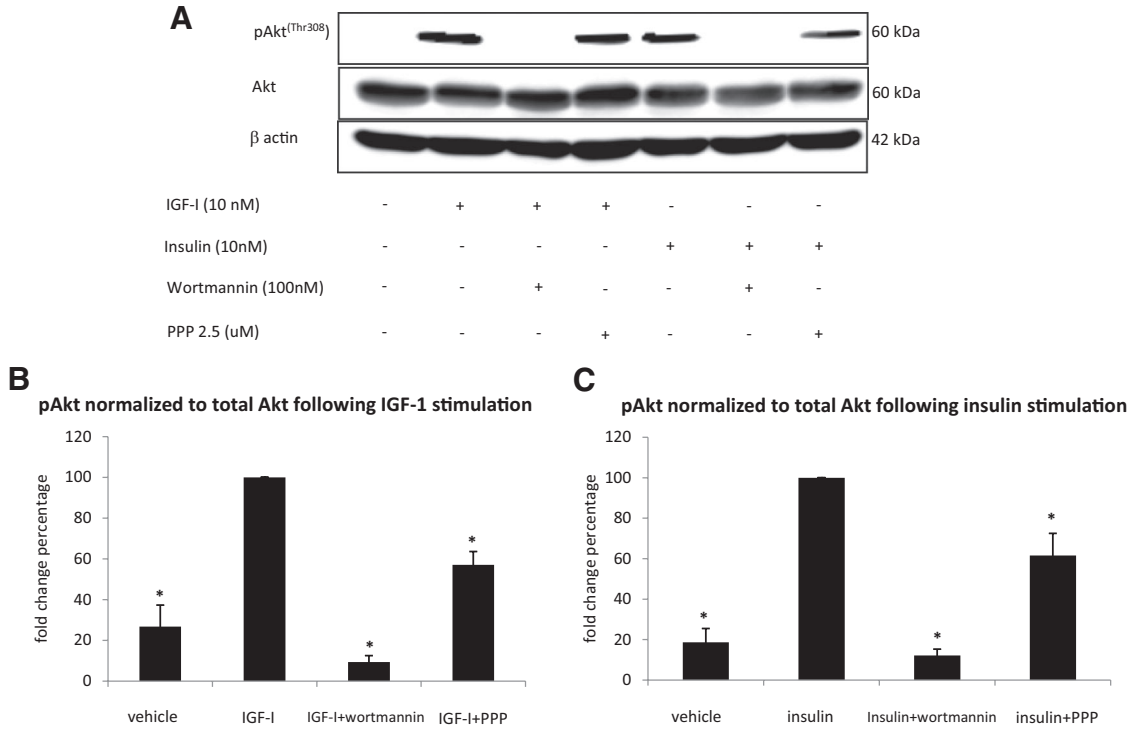

Figure 2. PPP inhibits both IR and IGF-IR activation in Mvt-1 cells. (A) Mvt-1 cells were starved with serum-free medium for 16 hours and then treated for 1 hour with PPP (2.5 mM) or wortmannin (100 nM) for 30 minutes as a control before treatment with 10 nM IGF-I or insulin. Cellular lysates were separated by SDS-PAGE, and the phosphorylation and protein levels were assessed using specific antibodies by Western blotting. Protein expression was quantified by densitometric analysis; pAkt levels were normalized to Akt expression and relative phosphorylation for IGF-I treated cells (B) and insulin-treated cells (C) was determined. Equal loading of proteins was demonstrated by immunoblotting with an antibody directed against β-actin. The Mann-Whitney test was performed to compare the difference from the insulin or IGF-1 treated cells. $*P < .05$.

tion compared to the control group; this effect was not observed for the IGF-IR. In accordance with its in vitro effect, PPP led to significant reductions of both IR and IGF-IR phosphorylation (Figure 5, A and B). In addition, S961 moderately affects IGF-IR expression but had no effect on IR expression. Tumors from PPP-treated mice had a reduction in both IR and IGF-IR expression (Figure 5, C and D).

S961 induces tumor growth by enhancing of Akt signaling in the MKR mouse

To identify potential mechanisms to explain the more rapid tumor growth rate when IR is inhibited, we examined the 2 primary intracellular signal pathways mediated by IR and IGF-IR: the PI3K pathway and the MAPK pathway. S961 treatment resulted in sustained activation of the PI3K, as demonstrated by significant hyperphosphorylation of Akt. On the other hand, S961 had no significant effect on the MAPK pathway as demonstrated by ERK1/2 phosphorylation levels. Both pathways were unaffected after PPP treatment; Akt and ERK1/2 phosphorylation levels were similar between vehicle-treated and PPP-treated mice (Figure 6, A and B).

Discussion

The association between T2DM and cancer was observed more than 100 years ago (30).

Recently, an increasing number of studies have shown an increased incidence of breast cancer and poorer outcomes in T2DM patients. Although T2DM is accompanied by obesity and inflammation, which both can promote cancer, it was also shown that hyperinsulinemia alone may mediate mitogenic effects in T2DM (4, 5, 31, 32). Insulin can directly promote cancer growth by induction of cell proliferation, cell division, migration, and inhibition of apoptosis (33, 34). It can also promote cancer and especially breast cancer growth indirectly, by reducing IGF-binding proteins production, thus increasing free IGF-1 availability (35), and hyperinsulinemia may also result in the increased estrogen production and inhibition of hepatic production of sex hormone binding globulin (13, 36, 37). Both IGF-1 and estrogen mediate mammary gland development and are associated with breast cancer (38, 39). Insulin binds with high affinity to its cognate IR but at high concentrations insulin can associate and activate the IGF-IR (40). In addition, both receptors were shown to mediate the mitogenic effects of hyperinsulin-

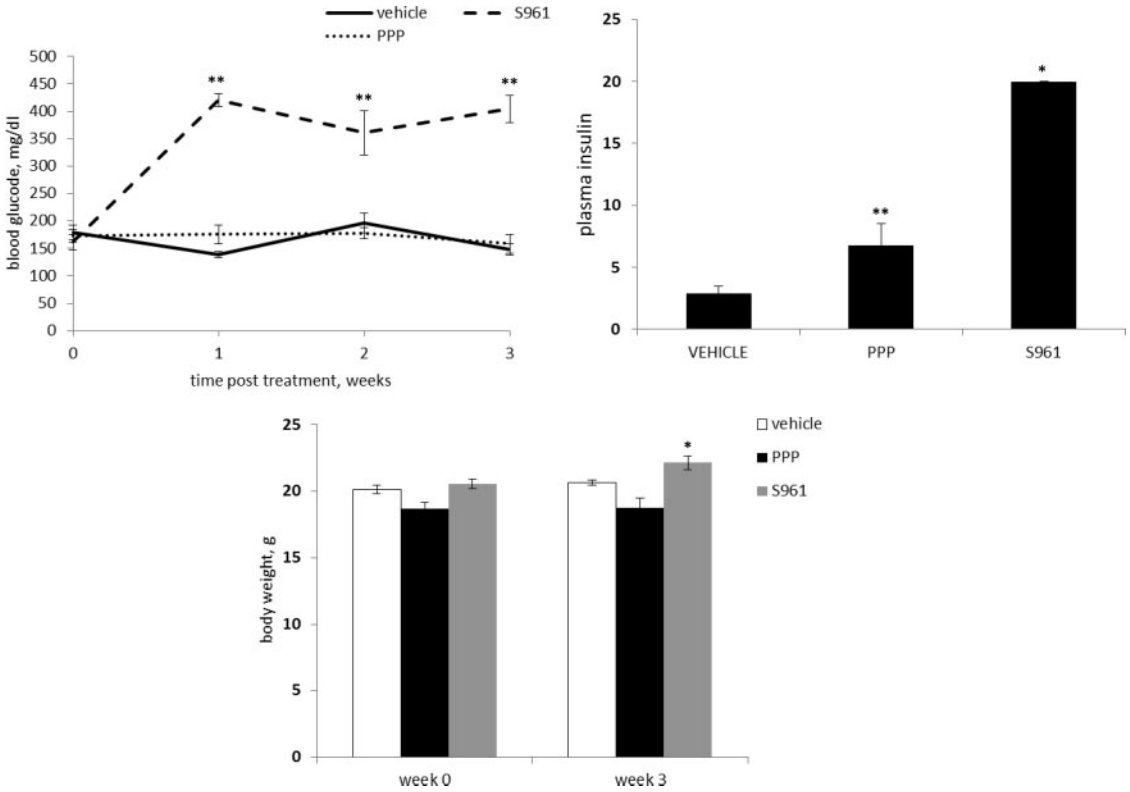

Figure 3. S961 induces hyperglycemia and severe hyperinsulinemia in MKR female mice. (A) Blood samples were obtained from the tail vein, and glucose concentrations were determined before, during, and after treatment. (B) Plasma insulin concentrations were measured at the end of the study. (C) Total body weight was measured before treatment (week 0) and after treatment (week 3). The Mann-Whitney test was performed to compare metabolic parameter before, after, and during the experiments. $*P < .05$; $**P < .01$. n = 6–7 mice per group.

emia on breast tumor development in the hyperinsulinemic female MKR mice (20). Overexpression of both IR and IGF-IR was found in breast cancer tissues and breast cancer cell lines (41, 42), and IR overexpression has been associated with decreased disease-free survival (18). Identification and understanding the importance of each receptor in mediating the hyperinsulinemic mitogenic effect associated with breast cancer are vital for developing better therapeutic strategies.

Here, we took advantage of 2 newly characterized inhibitors. First, we tested the IR antagonist, S961. This compound has been shown to be IR specific in vitro and in vivo without having any effect on IGF-IR and therefore making it suitable for use in our system (43, 44). We con-

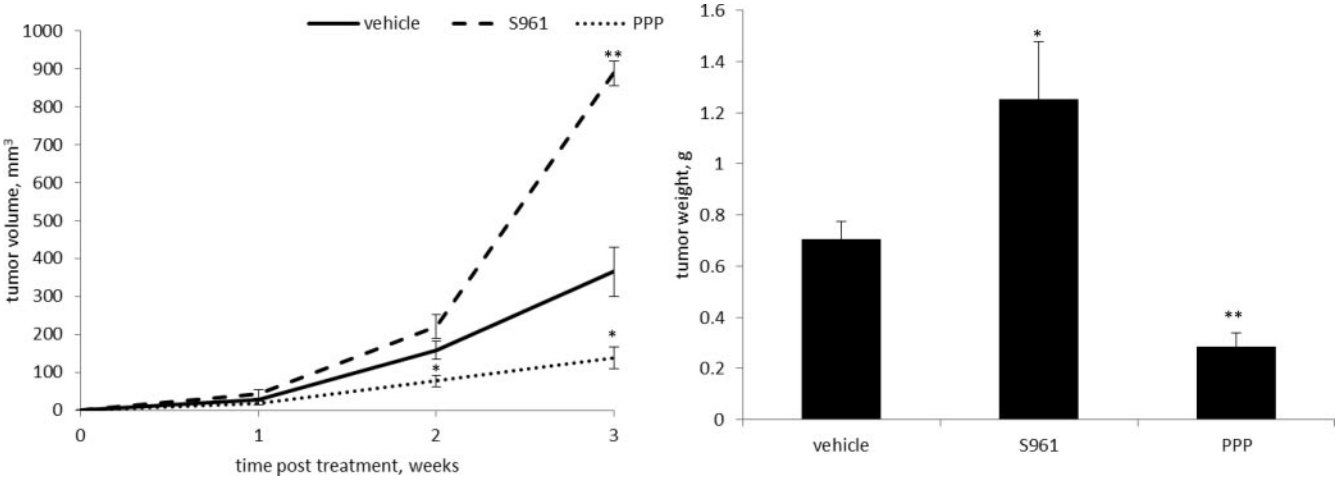

Figure 4. S961 induces enhanced tumor progression, whereas PPP impairs tumor progression in MKR female mice. (A) Mvt-1 cells were injected into the fourth mammary fat pad of 8-week-old virgin MKR mice. Treatment with vehicle or S961 began 2 weeks after cell injections. Tumor volume was measured each week during vehicle or S961 treatments. (B) Tumor weight was measured at necropsy. The Mann-Whitney test was performed to compare the difference between the groups, $*P < .05$; $**P < .01$. n = 6–7 mice per group.

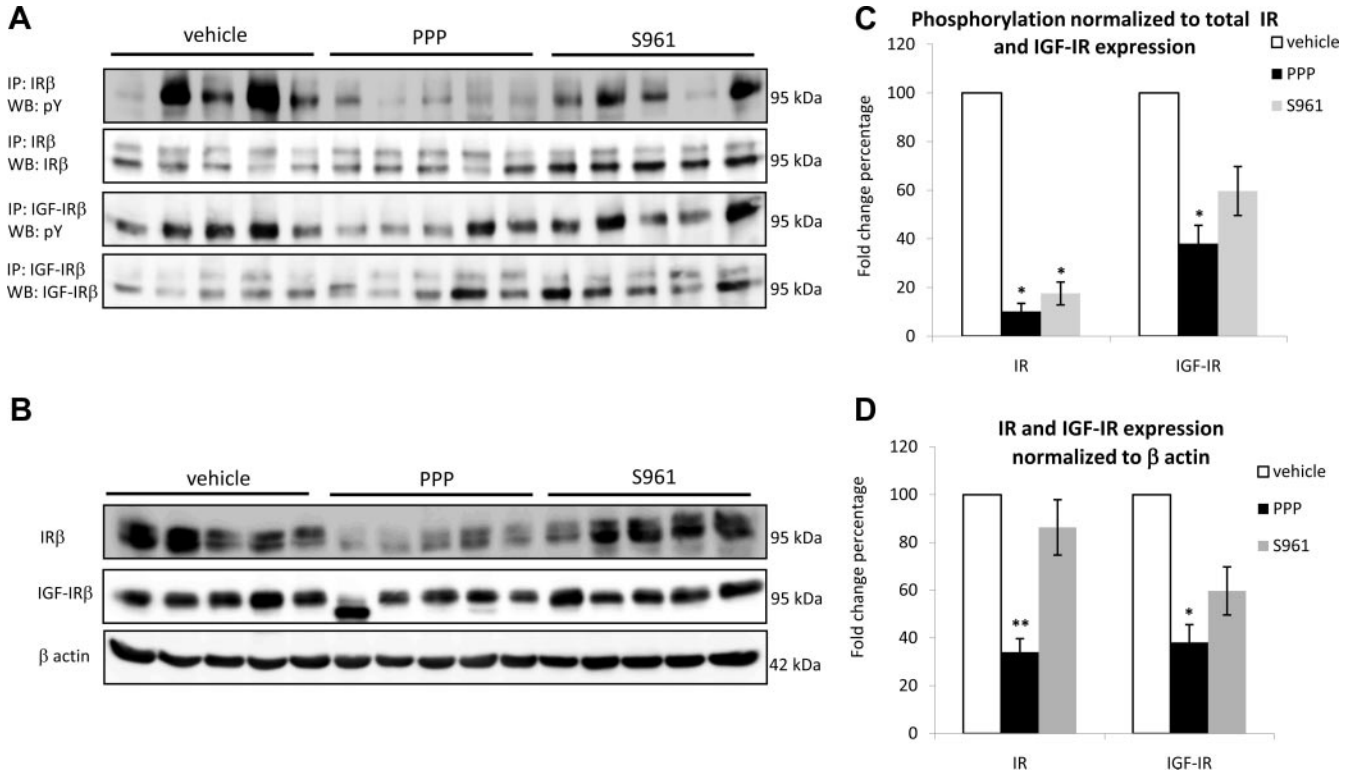

Figure 5. S961 impaired IR activation, whereas PPP partly inhibited both IR and IGF-IR activation and significantly reduced both receptors expression in breast tumors. (A) At necropsy, tumors were homogenized in detergent lysis buffer followed by immunoprecipitation (IP) of IRβ or IGF-IRβ. After SDS-PAGE separation, tyrosine phosphorylation of the precipitated proteins was evaluated by Western blotting analysis with antiphosphotyrosine antibody (anti-pY). Equal loading of proteins was ensured by reprobing the immunoblots with the antibodies used for immunoprecipitation. (B) IR/IGF-IR expression was evaluated by Western blotting analysis with anti-IR and anti-IGF-IR antibodies. Equal loading of proteins was demonstrated by immunoblotting with an antibody directed against β-actin. (C) IR/IGF-IR phosphorylation (normalized to IR and IGF-IR, respectively) was quantified by densitometric analysis and is presented as a fold change compared with the vehicle-treated control group. (D) IR/IGF-IR expression was quantified relative to actin expression. The Mann-Whitney test was performed to compare the difference between groups. $*P < .05$; $**P < .01$.

firmed the specificity of S961 for the IR on the mammary carcinoma Mvt-1 cell line. Second, we examined PPP, a cell-permeable *cis*-cyclolignan compound that was discovered as an inhibitor for tyrosine phosphorylation of the IGF-1R (27). Using the Mvt-1 cell line, we demonstrated that PPP inhibits both IR and IGF-IR in a similar fashion. Unlike S961, PPP induced only partial inhibition (<50%) of both receptors. These findings are in accord with a recent report showing PPP impairs cell proliferation of both IGF-IR-deficient and IGF-IR-positive cells, suggest-

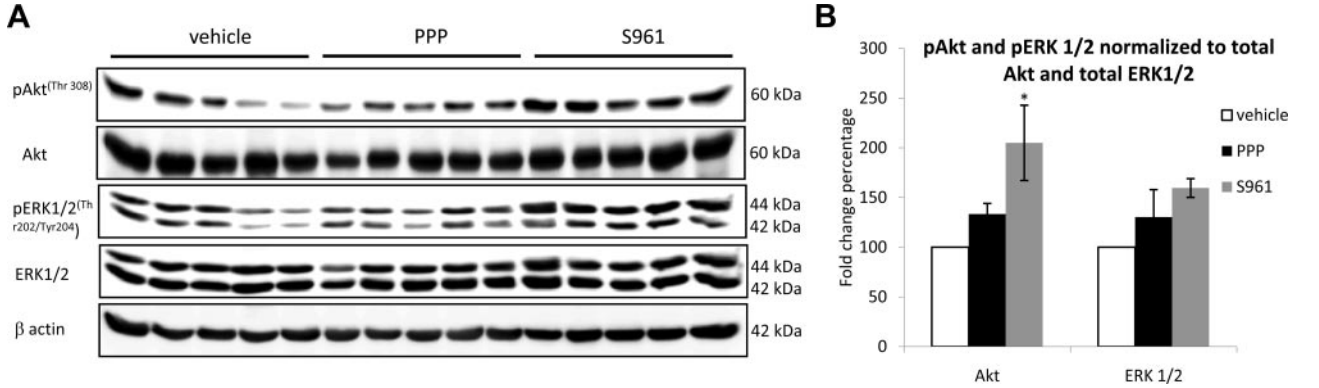

Figure 6. S961-induced tumor growth by enhancing of Akt signaling in the MKR mouse. (A) Tumors lysates were separated by SDS-PAGE, and the phosphorylation and protein levels were assessed using specific antibodies by Western blotting. (B) Relative expression was quantified by densitometric analysis and is presented as a fold change compared with the vehicle-treated control group. pAkt levels were normalized to Akt expression, and phospho-ERK1/2 levels were normalized to ERK1/2. Equal loading of proteins was demonstrated by immunoblotting with an antibody directed against β-actin. The Mann-Whitney test was performed to compare the difference between groups. $*P < .05$; $**P < .01$.

ing PPP also affects other receptor tyrosine kinases, such as the IR (45).

To investigate the role of IR and IGF-IR in mediating the specific effect of hyperinsulinemia on breast cancer progression, we used these 2 inhibitors in vivo in the syngeneic Mvt-1 orthotopic model in MKR female mice. These mice represent a "prediabetic" model with virtually isolated hyperinsulinemia and therefore are appropriate to determine the mechanisms underlying hyperinsulinemia in mediating breast cancer development (20). Mammary gland development was shown to be enhanced in the hyperinsulinemic MKR female mice; in addition, hyperinsulinemia in these mice induced accelerated growth of breast tumors in both orthotopic and genetic models of breast cancer (11, 20, 21, 46). The present study was undertaken to determine which receptor was more responsible for the hyperinsulinemic effect on tumor growth.

Our results demonstrate that IR blockade via ip injections of S961 induced severe hyperglycemia and hyperinsulinemia, thus clearly demonstrating that S961 strongly affects IR signaling in all peripheral metabolic tissues (Figure 3, A and B). Surprisingly, S961 significantly enhanced mammary tumor growth rate despite a reduction in activated IR levels, which leads us to speculate that the resultant markedly elevated insulin levels may now be promoting tumor growth via the IGF-1R expressed by the tumors that are not blocked by S961. Whether the hyperinsulinemia is now acting via the IGF-1 receptor directly or increasing IGF-1 bioavailability remains to be determined. To examine whether S961 treatment resulted in activation of the IGF-1R, we tested the acute effect of S961 on the activation of both IR and IGF-R, and activation of the downstream PI3K-Akt signaling pathway. In support of our hypothesis, S961 acute treatment induced IGF-IR activation that was sufficient to significantly activate the PI3K-Akt signaling pathway ~3-fold compared to vehicle treatment in breast tumors from MKR female mice inoculated with Mvt-1 cells (data not shown).

The PI3K-Akt pathway regulates key cellular events, such as cell survival, cell proliferation, apoptosis, and cytoskeletal rearrangements. Therefore it is not surprising that deregulation of the PI3K-Akt pathway is frequently observed in human cancers (23, 47). IR/IGF-IR was found to mediate the hyperinsulinemic effect on mammary tumor progression through activation of the PI3K-Akt pathway (20). It was demonstrated that inhibition of the PI3K pathway reduced the accelerated tumor growth rate in female MKR mice, while worsening the metabolic state of the mice (21, 48). Our current results highlight the importance of the PI3K-Akt pathway in breast cancer development as demonstrated by significantly higher levels of pAkt in mammary tumors from the S961-treated mice. Although IR binds insulin with higher affinity, it was previously shown that high levels of insulin can activate the IGF-IR (49). Yee et al showed that IGF-IR knockdown increases insulin sensitivity, as demonstrated by higher levels of pAkt in IGF-IR knockdown LCC6 cell line (50). These results, and the results of our current study, suggest that IR blockade with S961, which induced severe hyperinsulinemia, led to high pAkt levels through IGF-IR activation. The MAPK cascade, which is also activated by insulin and other growth factors, has been shown to be involved in cancer development in both mice and humans (51, 52). However, as shown in other breast cancer models in the MKR mice, our results demonstrate that the MAPK pathway is apparently not mediating the mitogenic effects of the hyperinsulinemia.

PPP has been shown to induce anticancer effects in several mouse models (27, 53, 54), and PPP is currently being tested in a phase II study on patients with squamous nonsmall-cell lung carcinoma (55). Here, we demonstrate that PPP treatment for 3 weeks had no effect on blood glucose levels and only very minor effects on insulin levels. Moreover, PPP treatment significantly reduced tumor development in the female MKR mice. The PPP in vivo blockade effect was similar to its in vitro effect; PPP partially blocked both the IGF-IR and the IR activation to a similar degree. These results suggest that partial inhibition of both IR and IGF-IR may abrogate insulin's mitogenic effect while only mildly affects the metabolic state. It was previously shown that IGF-I binds to hybrid receptors with the same affinity to the IGF-IR, whereas insulin has much lower affinity for IR/IGF-IR hybrids. Thus, hybrid receptors may increase the IGF-1 mitogenic signals (49, 56). Tumors from PPP-treated mice had a significant reduction in both IR and IGF-IR expression; a direct consequence of this may be the reduction in IR/IGF-IR hybrid formation, which could be another possible mechanism by which PPP acts to reduce tumor development in the female MKR mice. PPP treatment did not induce significant changes in the activation of both the PI3K and the MAPK pathways. This may be due to other endogenous factors that could contribute to activation of both pathways (57–59).

In conclusion, our study demonstrates that complete blockade of IR by itself in the whole animal results in a worsening of the insulin resistance and metabolic abnormalities that may have secondary effects on tumor growth. We also confirmed that hyperinsulinemia induces tumor growth primarily through the PI3K-Akt pathway. Thus, we hypothesize that IR blockade in the hyperinsulinemic state induces enhanced insulin binding to and activation of the IGF-IR, which may then contribute to insulin's mitogenic effects. Our results raise the possibility that tissue-specific IR blockade is required for better understanding

of the mechanisms linking IR, hyperinsulinemia, and breast cancer, and recent published data support this approach (25, 60). The present study has clinical implications; although most attention has been directed toward targeting the IGF-1R, we demonstrate here that dual (and even partial) inhibition of both IR and IGF-IRs impairs hyperinsulinemia-mitogenic effects in breast tumor development, with minimal metabolic consequences.

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