Insulin-induced activation of glycerol-3-phosphate acyltransferase by a chiro-inositol-containing insulin mediator is defective in adipocytes of insulin-resistant, type II diabetic, Goto–Kakizaki rats

(Intracellular signaling/glycosyl-phosphatidylinositol)

ROBERT V. FARESE†‡, M. L. STANDAERT*, K. YAMADA*, L. C. HUANG‡, C. ZHANG‡, D. R. COOPER*, Z. WANG*, Y. YANG*†, S. SUZUKI‡, T. TOYOTA‡, AND J. LARNER†

*J. A. Haley Veterans’ Hospital, and Departments of Internal Medicine and Biochemistry, University of South Florida College of Medicine, Tampa, FL 33612; †The Third Department of Internal Medicine, Tokohu University School of Medicine, Sendai, Japan; and ‡Department of Pharmacology, University of Virginia Medical School, Charlottesville, VA 22908

Communicated by Mary Ellen Jones, June 24, 1994 (received for review March 14, 1994)

ABSTRACT Type II diabetic Goto–Kakizaki (GK) rats were insulin-resistant in euglycemic–hyperinsulinemic clamp studies. We therefore examined insulin signaling systems in control Wistar and diabetic GK rats. Glycerol-3-phosphate acyltransferase (G3PAT), which is activated by headgroup mediators released from glycosyl-phosphatidylinositol (GPI), was activated by insulin in intact and cell-free adipocyte preparations of control, but not diabetic, rats. A specific chiro-inositol-containing inositol phosphogycycan (IPG) mediator, prepared from beef liver, bypassed this defect and comparably activated G3PAT in cell-free adipocyte preparations of both diabetic GK and control rats. A myo-inositol-containing IPG mediator did not activate G3PAT. Relative to control adipocytes, labeling of GPI by [3H]glucosamine was diminished by 50% and insulin failed to stimulate GPI hydrolysis in GK adipocytes. In contrast to GPI-dependent G3PAT activation, insulin-stimulated hexose transport was intact in adipocytes and soleus and gastrocnemius muscles of the GK rat, as was insulin-induced activation of mitogen-activated protein kinase and protein kinase C. We conclude that (i) chiro-inositol-containing IPG mediator activates G3PAT during insulin action, (ii) diabetic GK rats have a defect in synthesizing or releasing functional chiro-inositol-containing IPG, and (iii) defective IPG-regulated intracellular glucose metabolism contributes importantly to insulin resistance in diabetic GK rats.

Insulin uses multiple signaling systems controlled through insulin receptor tyrosine kinase activation. Tyrosine phosphatases of insulin receptor substrate 1 (IRS-1), epip60 (SHC), and other proteins induces their interaction with src homology region 2-containing proteins—e.g., phosphatidylinositol 3-kinase (3) or growth factor receptor-bound protein 2 (4)—thus sequentially activating SOS (5), ras (6, 7), raf (8), mitogen-activated protein kinase (MAPK) kinase (9), MAPK (10), and insulin-stimulated protein kinase (11). Insulin also stimulates the hydrolysis of glycosyl-phosphatidylinositol (GPI) (12–14) and phosphatidylcholine (PC) (15–19). GPI hydrolysis requires a pertussis toxin-sensitive G protein (15, 20, 21), whereas ras (19, 22) or other factors may be important for PC hydrolysis. As a result of PC or GPI hydrolysis, diacylglycerol increases (12, 15–17, 20, 23) and protein kinase C (PKC) is activated (24–30). GPI hydrolysis also releases inositol phosphogycycan (IPG) headgroups, including those containing chiro-inositol or myo-inositol (14, 31).

With respect to glucose homeostasis, IPG mediators activate glycogen synthase (GS) (32), pyruvate dehydrogenase (PDH) (14, 33), and glycerol-3-phosphate acyltransferase (G3PAT) (21, 34). Thus, insulin may regulate intracellular glucose metabolism through IPGs, which activate protein phosphatases (35). A role for IPG in glucose transport is supported by some (36), but not other (37), findings. Glucose transport may be regulated by protein kinases (PKs) (38)—e.g., PKC (39), MAPK, or other PKs. chiro-Inositol-containing IPG (C-IPG), rather than myo-inositol-containing IPG (M-IPG), may activate GS and PDH through their respective phosphatases (14, 40). As reported here, C-IPG also activates G3PAT, an insulin-sensitive enzyme that (i) controls de novo phospholipid synthesis and (ii) can be conveniently studied in both intact and cell-free systems (21, 34).

Defects in signaling systems that regulate glucose transport or intracellular glucose metabolism may cause insulin resistance in type II diabetes mellitus. Presently, we studied (i) insulin-induced activation of G3PAT to reflect IPG signaling, and (ii) glucose transport and PK signaling in nonobese, type II diabetic, Goto–Kakizaki (GK) rats. The GK genetic rat model is particularly relevant to understanding human type II diabetes since (i) defects in insulin secretion and resistance are both seen at 2–4 weeks of age in the GK rat (41); (ii) the pattern of low chiro-inositol and increased myo-inositol in the urine of the GK rat (42) is similar to that in type II diabetic humans (43) and monkeys (44); (iii) IPG mediator-dependent PDH activation is deficient in GK rat liver membranes (42); (iv) low urinary chiro-inositol excretion in the monkey (44) correlates directly with the severity of insulin resistance as measured by glucose disposal ("M"), GS activation in muscle and fat, and glycogen phosphorylase inactivation in muscle; and (v) C-IPG, but not M-IPG, mediator bioactivity from human hemodialyse, urine, and amputal obesityis is deficient in type II diabetics (45). We now report that (i) insulin-induced activation of G3PAT is defective in the diabetic GK rat, apparently due to deficient synthesis and release of C-IPG; and (ii) a defect in C-IPG-regulated intracellular glucose metabolism, rather than PK-regulated glucose transport, may play a more primary pathogenetic role in insulin resistance in certain tissues of diabetic GK rats.

EXPERIMENTAL METHODS

Except where indicated, fed 200-g male rats were used. Holtzman Wistar rats were used as nondiabetic controls since GK rats were derived from the Wistar strain. [Note that (i) Abbreviations: MAPK, mitogen-activated protein kinase; G3PAT, glycerol-3-phosphate acyltransferase; GPI, glycosyl-phosphatidylinositol; DOG, deoxyglucose; IPG, inositol phosphogycycan; PK, protein kinase; PKC, protein kinase C; GS, glycogen synthase; PDH, pyruvate dehydrogenase; C-IPG, chiro-inositol-containing IPG; M-IPG, myo-inositol-containing IPG; GK, Goto–Kakizaki.]

To whom reprint requests should be addressed: Research Service (VAR 151), J. A. Haley Veterans’ Hospital, 1300 Bruce Downs Boulevard, Tampa, FL 33612.
insulin activated G3PAT comparably in adipocytes from Wistar, Sprague-Dawley, and Zucker rats, and (ii) G3PAT activation in GK adipocytes was all (via mediator) or none (via insulin). Diabetic GK rats were obtained from S. Suzuki and T. Toyoda (Sendai, Japan). Lean (fa/+) (400 g) and obese (fa/fa) (900 g) Zucker, male, nonobese rats were obtained from Charles River Breeding Laboratories.

Adipocytes were prepared by collagenase digestion and suspended in glucose-free Krebs Ringer phosphate medium containing 1% (wt/vol) bovine serum albumin (see ref. 25). Cultured BC3H1 myocytes (a complementary assay system) were placed into phosphate-buffered saline containing 0.1 mM CaC12, 5 mM glucose, and 0.1% bovine serum albumin just before experimental use (see ref. 15).

Methods for G3PAT activation and assay have been described (21, 34). In brief, to study G3PAT activation in intact cells, the cells were equilibrated at 37°C, treated with 10 nM insulin or vehicle for 2 min, and then homogenized in medium containing 10 mM Tris (pH 7.4), 250 mM sucrose, and 0.7 mM dithiothreitol (buffer A). To study G3PAT activation in cell-free homogenates, cells were homogenized in buffer A, and the defatted, postnuclear homogenate was equilibrated at 37°C and treated for 2 min with 10 nM insulin and 2.5 μM IPG mediator or vehicle for 2 min.

G3PAT activity was assayed by incubating homogenate (100 μg of protein) for 2 min at 37°C in 0.5 ml of buffer containing 250 mM KCl, 50 mM Tris (pH 7.4), 0.7 mM dithiothreitol, 0.4 mM [3H]glycerol 3-phosphate (1 μCi; 37 GBq), 136 μM palmityl-CoA, and 2 mg of bovine serum albumin. Reactions were stopped with H2O-saturated butanol, and the butanol extracts were counted for labeling of phosphatidic acid. As reported (21, 34), (i) the reaction rate is linear, and (ii) insulin activates (via a decrease in Kₘ) G3PAT through a factor that is released from plasma membranes by insulin or phosphatidylinositol-specific phospholipase C and operates through an NAD-reducible enzyme presumably a phosphatase.

IPG mediators were prepared from beef liver (see refs. 14 and 46). The C-IPG mediator was purified by HPLC on a protein Pak 60 column (Waters), using H₂O as a solvent, yielding a single peak of ultraviolet-absorbing (at 195 nm) material that eluted with a retention time of 18 min and coincided with bioactivity. IPG mass was quantitated by its chiro-inositol or myo-inositol content, measured by GC/MS and Dionex HPLC after acid hydrolysis (47). Both IPG mediators are sensitive to nitrous acid deamination and are immunoprecipitated by a specific immunopurified polyclonal anti-IPG antibody (48). Chemical studies (unpublished) demonstrated the formation of (i) 2,5-anhydro[3H]mannitol and myo-inositol following nitrous acid oxidation and reduction of the M-IPG mediator with borotritide, establishing the presence of a myo-inositol-glucosamine moiety; and (ii) 2,5-anhydro[3H]talitol and chiro-inositol from comparable oxidative/reductive treatment of the C-IPG mediator, establishing the presence of a chiro-inositol-galactosamine moiety. Further structural studies will be reported separately.

As described (49, 50), glycolipids were labeled by overnight culture of adipocytes with [3H]glucosamine (also converted to galactosamine and mannosamine), yielding labeled glycolipids that migrated just behind phosphatidylinositol during TLC and decreased rapidly with insulin or phosphatidylinositol-specific phospholipase C treatment.

Total body glucose utilization and hepatic glucose output were quantitated during euglycemic–hyperinsulinemic clamp studies in anesthetized rats, as described (51, 52). Where indicated, [6-3H]glucose accumulation or 3H-labeled 2-deoxyglucose (DOG) uptake in the gastrocnemius muscle was measured during the last 30 or 10 min of clamp studies. 2-[3H]DOG uptake in rat adipocytes and soleus muscles incubated in vitro was measured as described (53).

Adipocyte MAPK activation was measured as described (54), using myelin basic protein as substrate in assays of whole-cell lysates, and, as confirmation, in myelin basic protein-containing gels (i.e., 42-kDa MAPK). PKC-α translocation was measured by Western analysis (see ref. 29).

RESULTS

As in previous studies (21, 34), in both BC3H1 myocytes and control Wistar rat adipocytes, insulin provoked 2- to 3-fold increases in G3PAT activity in intact cells and cell-free homogenates (Fig. 1). Moreover, in cell-free homogenates of both cell types, 2.5 μM C-IPG (from beef liver) provoked 2-fold increases in G3PAT activity (Fig. 1). (Note that this concentration of C-IPG was optimal.) In contrast, M-IPG mediator, at similar concentrations, was inactive in stimulating G3PAT. Nitrous acid treatment led to a complete loss of the C-IPG mediator. As further confirmation that insulin activated G3PAT through an IPG mediator, anti-IPG antiserum (provided by Guillermo Romero; see refs. 48 and 55 regarding epitope specificity) inhibited the G3PAT-activating effects of both insulin and C-IPG in adipocyte homogenates, whereas preimmune serum was without effect (Table 1).

In contrast to control adipocytes, insulin failed to activate G3PAT in intact or cell-free preparations of adipocytes from diabetic GK rats (Fig. 1). This suggested that insulin may not provoke increases in IPG mediators in GK adipocytes. However, to our surprise, C-IPG failed to stimulate G3PAT activity in cell-free preparations of GK adipocytes (Fig. 1), suggesting, as one possibility, the presence of a second defect distal to IPG.

The failure to observe G3PAT activation in GK adipocytes with either insulin or C-IPG led us to question whether either defect could be reversed by insulin treatment [e.g., protein

![](image)
Table 1. Inhibition of G3PAT-stimulating effects of insulin and C-IPG by anti-IPG antiserum

<table>
<thead>
<tr>
<th>Antiserum added</th>
<th>G3PAT activity, cpm per mg of protein per min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (10 nM)</td>
</tr>
<tr>
<td>None</td>
<td>5738 ± 38</td>
</tr>
<tr>
<td>Preimmune</td>
<td>6116 ± 356</td>
</tr>
<tr>
<td>Anti-IPG</td>
<td>6120 ± 348</td>
</tr>
</tbody>
</table>

Homogenates were prepared from adipocytes of nonobese diabetic Wistar rats. Antisera were added at the start of a 5-min equilibration period, after which treatments were added as indicated, and incubation was continued for 2 min. See Fig. 1 legend for other details of G3PAT assay. Values are the means ± SE of three determinations.

phosphatase activity is reversibly decreased in diabetic rats (56), and a deficiency of this or a similar protein phosphatase could account for the poor activation of G3PAT by IPG. We therefore treated GK rats with daily s.c. injections of 2 units of Humulin NPH insulin (Eli Lilly) for 6 days, causing serum glucose concentrations to decrease modestly to 239 ± 5 mg/dl (mean ± SE; n = 17), as measured 16–20 hr after the last injection, vs. 297 ± 6 mg/dl (n = 88) in untreated diabetic GK rats and 148 ± 2 mg/dl (n = 16) in nonobese diabetic Wistar rats. After insulin priming of GK rats in vivo, subsequent G3PAT activation in cell-free homogenates of adipocytes was, indeed, readily elicited by C-IPG, but, again, not by insulin (Fig. 2). Moreover, the stimulatory effects of C-IPG on G3PAT in insulin-primed diabetic GK rat adipocyte preparations were comparable to those of insulin and C-IPG in nondiabetic Wistar rat adipocyte preparations. Thus, insulin priming fully reversed a defect distal to C-IPG but had no effect on a defect proximal to C-IPG.

As in adipocytes of older 200-g diabetic GK rats, insulin failed to activate G3PAT in adipocytes of younger, 100-g, diabetic GK rats. With hyperinsulinemic Zucker rats, insulin provoked comparable 2.5-fold increases in G3PAT activity in adipocytes of both obese 900-g (fa/fa) and nonobese (fa/+) rats. When Wistar and GK adipocytes were cultured for 20 hr in DMEM (see refs. 49 and 50), insulin activated G3PAT nearly 2-fold in cultured Wistar, but not diabetic GK, adipocytes. Thus, the defect in insulin-induced G3PAT activation in diabetic GK rat adipocytes is present at an early age, is not due to hyperinsulinemia alone, and is not reversed by short-term culture ex vivo.

Because insulin failed to release a functional IPG mediator in GK adipocytes, we questioned whether there were defects in the biosynthesis or stimulated hydrolysis of glycolipid precursors of IPGs. As compared to controls, overnight [3H]glucosamine labeling of glycolipids (presumably GPIs) was diminished by 50%, and, moreover, insulin failed to stimulate GPI hydrolysis in GK rat adipocyte preparations (Fig. 3).

In contrast to G3PAT activation, insulin effects (peak 2-fold increases at 10 min) on MAPK activity in adipocytes of 120-g GK rats were indistinguishable from effects observed in adipocytes of 120-g control Wistar rats (younger rats were used for MAPK activation, as older rats were less responsive to insulin). Similarly, insulin at 10–20 min provoked comparable increases in membrane PKC-α in control Wistar (62% ± 12%; n = 14) and GK (59% ± 9%; n = 16) rat adipocytes. Thus, insulin-induced activation of MAPK and PKC was not compromised in GK adipocytes.

To verify that GK rats were insulin-resistant, we studied glucose metabolism during euglycemic–hyperinsulinemic clamp conditions. At two insulin infusion rates producing moderate and marked hyperinsulinemia (see ref. 52), total glucose utilization was decreased by 29% and 42%, and hepatic glucose output was increased by 69% and 72%, in diabetic GK rats, as compared to control Wistar rats (Table 2).

Glucose accumulation in the gastrocnemius muscle during the final 30 min of the clamp was decreased by 69% in the GK rat (Table 2). This defect in glucose accumulation contrasts with normal uptake of 2-DOG (i.e., transport and trapping as nonmetabolizable 2-DOG-6-P) during a 10-min period in comparable clamp conditions (Table 2; see below). Presumably, the decrease in glucose accumulation in the GK gastrocnemius reflects that, after uptake, glucose that is not stored or oxidized may be metabolized and released as pyruvate/lactate/alanine, particularly in type II diabetes (57).

Since insulin failed to activate G3PAT in adipocytes of diabetic GK rats, it was surprising to find that insulin was very effective in stimulating hexose transport (2-[3H]DOG

![Fig. 2. Effects of insulin (INS) and C-IPG mediator (MED) on G3PAT activation in cell-free homogenates of insulin-treated diabetic GK rats. Experiments were conducted as in Fig. 1, except that these GK rats were treated in vivo with subcutaneous injections of 2 units of NPH insulin for 6 consecutive days prior to experimental use in vitro. The values shown are the means ± SE of (n) assays.](image1)

![Fig. 3. Effects of insulin on [3H]glucosamine-labeled GPI in cell-free adipocyte preparations of control Wistar rats (●) and diabetic GK rats (○). After overnight labeling, adipocytes were washed, homogenized, and incubated as in G3PAT activation experiments (see Experimental Methods) with insulin for the indicated times. [3H]labeled GPIs were purified by TLC. The values shown are the means ± SE of five experiments. P was determined by the t test.](image2)
Table 2. Effects of insulin on glucose utilization, hepatic glucose output, and hexose uptake in nondiabetic Wistar and diabetic GK rats during euglycemic–hyperinsulinemic clamp studies

<table>
<thead>
<tr>
<th>Group</th>
<th>Parameter</th>
<th>0 mU/min</th>
<th>4 mU/min</th>
<th>40 mU/min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wistar</td>
<td>GK</td>
<td>Wistar</td>
<td>GK</td>
</tr>
<tr>
<td>A</td>
<td>Glucose utilization, mg/min/kg of body wt</td>
<td>ND</td>
<td>3.49 ± 0.31 (6)</td>
<td>2.49 ± 0.23* (5)</td>
</tr>
<tr>
<td>B</td>
<td>Hepatic glucose output, mg/min/kg of body wt</td>
<td>ND</td>
<td>1.38 ± 0.21 (6)</td>
<td>2.33 ± 0.24† (5)</td>
</tr>
<tr>
<td>C</td>
<td>2-DOG uptake in gastrocnemius muscle, nmol/g of muscle/10 min</td>
<td>168 ± 22 (7)</td>
<td>387 ± 25 (4)</td>
<td>461 ± 46‡ (4)</td>
</tr>
<tr>
<td>D</td>
<td>Glucose accumulation in gastrocnemius muscle, nmol/g of muscle/30 min</td>
<td>270 ± 27 (4)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Food was withheld for 16–20 hr before the experiment. Rats were anesthetized with isoflurane, and body temperature was maintained at 37°C. Physiological saline (along with n-[6-3H]glucose for groups A and B) was infused at constant rates to deliver 0, 4, or 40 milliliters of insulin per min, as indicated. (Note that GK rats were not studied at the 0 insulin infusion rate, as these rats are hyperglycemic and moderately hyperinsulinemic in their “basal” fasting state, and large amounts of insulin are required to render them euglycemic.) In all groups, glucose (measured every 5–10 min) was brought to 150 mg/dl (± 10%) and held constant at this level for at least 60 min prior to, and during, a 30-min sampling period, by varying the rate of glucose infusion, which, at equilibrium, was generally 3-fold greater in control Wistar rats, as compared to diabetic GK rats. The total duration of each study was 3–4 hr. In groups A and B, the measured serum glucose-specific activities were constant during the final 30 min (glucose utilization was therefore calculated by dividing the [3H]glucose infusion rate by the final serum glucose specific 3H radioactivity). In group C, 2-DOG uptake (i.e., transport and conversion to nonmetabolizable 2-DOG-6-PO4) (calculated by dividing 3H radioactivity in muscle by the specific 3H radioactivity of serum hexose) was measured during the final 10 min of the sampling period, after bolus injection of 10 µCi of 2-[3H]DOG, along with 0.5 µCi of L-14C glucose to correct for nonspecific radioactivity in muscle samples. In group D, glucose accumulation (i.e., net balance of transport metabolism, storage, and release of metabolites) was measured during the final 30 min of the sampling period, after bolus injection of 10 µCi of D-[6-3H]glucose and 0.5 µCi of L-[14C]glucose to correct for nonspecific radioactivity in muscle samples. Values are means ± SE of (n) determinations. ND, Not determined; wt, weight; mU, milliliters.

*, P < 0.05 vs. Wistar control; †, P < 0.001 vs. Wistar control; ‡, P < 0.025 vs. Wistar control; §, P not significant vs. Wistar control; ††, P < 0.005 vs. Wistar control as determined by the t test.

Discussion

The present findings provide direct evidence that insulin activates G3PAT through an IP3 mediator released from GPI in the plasma membrane. Previously, we reported that both insulin and phosphatidylinositol-specific phospholipase C stimulate the release of a (M, < 5000) water-soluble substance that stimulates microsomal G3PAT (21, 34). Presently we found that a purified C-IPG mediator from beef liver directly activated G3PAT in cell-free preparations of BC3H1 myocytes and Wistar rat adipocytes. The exact chemical structure of this C-IPG mediator awaits further study.

Most importantly, adipocytes from GK rats manifested a marked deficiency in insulin-stimulated G3PAT activation but responded well with respect to hexose transport. Thus, certain insulin-sensitive signaling pathways were deficient in the GK rat, whereas other signaling pathways were preserved. More specifically, insulin-induced hydrolysis of GPI was markedly impaired, in the face of normal activation of both MAPK and PKC in adipocytes of the diabetic GK rat.

There were at least two defects in the GPI hydrolysis signaling pathway in GK rat adipocytes. One, more distal, involved the response of G3PAT to C-IPG and was reversible with 6-day insulin priming. The other, more proximal, was not reversed by insulin but was fully bypassed by exogenously added C-IPG mediator and was best explained by a failure of insulin to synthesize and/or release the C-IPG mediator.

Along with impaired G3PAT activation, [3H]glucosamine labeling of GPI precursors for IP3 mediators was deficient in GK adipocytes, and insulin failed to stimulate GPI hydrolysis in GK adipocyte preparations. Further studies will be required to determine whether these abnormalities in the GK rat are due to decreased substrate availability or a defective biosynthetic enzyme(s), GPI-specific phospholipase C coupling factor, or other factor.

It was surprising that insulin resistance in the GK rat did not involve a defect in insulin-stimulated hexose transport, at least as measured in adipocytes, soleus muscles in vivo, and gastrocnemius muscle in vivo, at moderate to saturating insulin concentrations. Thus, hyperglycemia in the GK rat appears to be due to combined defects in insulin secretion and diminished...
insulin effects on both hepatic glucose production and non-hepatic intracellular glucose metabolism. Indeed, in the gastrocnemius muscle, we observed decreased accumulation of glucose, most likely reflecting storage defects, in the face of normal glucose transport. Accordingly, in addition to G3PAT, insulin-induced activation of adipocyte PDE in vitro (42) and muscle GS and GS phosphatase in vivo (58) are impaired in the diabetic GK rat. This is not to say that insulin-regulated glucose transport is not diminished in the diabetic GK rat, but such diminution may reflect insulin deficiency or feedback from intracellular defects and could also be apparent at lower insulin concentrations or in tissues not presently studied.

In summary, a purified C-IPG mediator, like insulin, activated G3PAT in cell-free preparations of Wistar rat adipocytes and BC3H1 myocytes. This suggests that insulin-induced hydrolysis of GPI and release of IPG mediators from the plasma membrane is largely responsible for the activation of microsomal G3PAT. In addition, insulin-induced activation of G3PAT, but not heoxose transport, was severely compromised in adipocytes of the diabetic GK rat. Moreover, this defect in G3PAT activation was fully bypassed by addition of a C-IPG mediator to cell-free preparations of GK rat adipocytes. Thus, a specific insulin-regulated signaling pathway—GPI synthesis and hydrolytic release of C-IPG mediators—is defective and contributes importantly to insulin resistance in the type II diabetic GK rat.

This research work was supported by funds from the Research of the Department of Veterans' Affairs; National Institutes of Health Grants DK-38079, DK14334, and F30DK38942; Insimed Pharmaceuticals Company, Charlottesville, VA; and J. Bishop and The Center for Innovative Technology, Herndon, VA.