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Effects of Insulin on Prenylation as a Mechanism of Potentially Detrimental Influence of Hyperinsulinemia*

BORIS DRAZNIN, PHILIP MILES, YOLANTA KRUSZYNSKA, JERROLD OLEFSKY, JACOB FRIEDMAN, INGA GOLOVCHENKO, RICHARD STJERNHOLM, KEI WALL, MARC REITMAN, DOMENICO ACCILI, ROBERT COOKSEY, DONALD McCLAIN, AND MARC GOALSTONE

Research Service of the Department of Veterans Affairs, Denver Veterans Affairs Medical Center, Department of Medicine, University of Colorado Health Sciences Center (B.D., I.G., R.S., K.W., M.G.), Denver, Colorado 80220; San Diego Veterans Affairs Medical Center, and Department of Medicine, University of California (P.M., Y.K., J.O.), San Diego, California 92161; Department of Nutrition, Case Western Reserve University School of Medicine (J.F.), Cleveland, Ohio 44106; Diabetes Branch (M.R.) and Developmental Endocrinology Branch (D.A.), National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892; and Veterans Affairs Medical Center and Department of Medicine, University of Mississippi Medical Center (R.C., D.M.), Jackson, Mississippi 39216

ABSTRACT

To investigate the cause and effect relationship between hyperinsulinemia and the increased amounts of farnesylated p21Ras, we performed hyperinsulinemic euglycemic clamps in normal weight volunteers as well as in normal mice and dogs. Insulin infusions significantly raised the amounts of farnesylated p21Ras in the white blood cells of humans, in liver samples of mice and dogs, and in aorta samples of mice. Obese hyperinsulinemic individuals and dogs (made hyperinsulinemic by surgical diversion of the pancreatic outflow from the portal vein into the vena cava) displayed increased amounts of farnesylated p21Ras before the hyperinsulinemic clamps. Infusions of insulin did not alter the already increased levels of farnesylated p21Ras in these experimental models.

To further investigate the role of acquired insulin resistance in modulating insulin's effect on p21Ras prenylation, we induced insulin resistance in rats by glucosamine infusion. Insulin-resistant glucosamine-treated animals displayed significantly increased farnesylated p21Ras in response to insulin infusion compared to that in control saline-treated animals. Transgenic models of insulin resistance (heterozygous insulin receptor substrate-1 knockout mice, A-ZIP/F-1 fatless mice, and animals overexpressing glutamine:fructose-6-phosphate amidotransferase) contained increased amounts of farnesylated p21Ras.

We conclude that hyperinsulinemia, either endogenous (a prominent feature of insulin resistance) or produced by infusions of insulin, increases the amounts of farnesylated p21Ras in humans, mice, and dogs. This aspect of insulin action may represent one facet of the molecular mechanism of the potentially detrimental influence of hyperinsulinemia. (*Endocrinology* **141:** 1310–1316, 2000)

HYPERINSULINEMIA is a cardinal feature of insulin resistance, a prevalent metabolic condition that requires higher than normal concentrations of insulin to achieve normal or near-normal utilization of glucose (1). However, the effect of insulin on glucose metabolism is only one of the multiple aspects of insulin action. It is still not clear whether all aspects of insulin action are affected equally by insulin resistance (2–4).

The molecular mechanism of insulin signaling can be arbitrarily divided into two branches: metabolic and mitogenic. Even though there is a certain amount of cross-talk between the two branches, the former appears to involve phosphorylation of the insulin receptor substrate (IRS) family of proteins and activation of phosphatidylinositol 3-kinase and protein kinase B (5, 6). In contrast, the mitogenic branch of insulin action appears to engage the activation of the Ras, Raf, MEK, and mitogen-activated protein kinases (7) and propagate the insulin signaling toward the cell nucleus. Activation of the Ras pathway also leads to the phosphorylation and activation of the prenyl transferases: farnesyltransferase (FTase) and geranylgeranyltransferases I and II (GGTase I and II) (8–10). FTase and GGTase I and II post-translationally modify a variety of cellular proteins, including a large family of small molecular weight GTPases (reviewed in Ref. 11). Post-translational modification of the GTPases is accomplished by the isoprenylation of conserved cysteine residues found on their C-termini (11, 12). Ras and Rho proteins are prenylated with farnesyl or geranylgeranyl on a single cysteine residue of the C-terminal CaaX box (where C = cysteine, a = aliphatic residue, and X = methionine, serine, or glutamine for Ras or leucine for Rho proteins) by FTase and GGTase I, respectively. In contrast, Rab proteins, which terminate with C-terminal cysteine motifs of CC or CxC, are double prenylated with geranylgeranyl by GGTase II (11–14). As a group, prenyl transferases bind iso-

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Address all correspondence and requests for reprints to: Dr. Boris Draznin, Veterans Affairs Medical Center (151), 1055 Clermont Street, Denver, Colorado 80220. E-mail: drazninb@den-res.org.
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prenyl groups to their substrates, so that these GTPases may be anchored to their respective membranes, loaded with GTP, and thereby activated.

We have previously shown that the addition of insulin augmented FTase activity and the amount of farnesylated p21Ras in 3T3-L1 fibroblasts, 3T3-L1 adipocytes, and vascular smooth muscle cells (9, 15, 16). Moreover, our data suggested that hyperinsulinemia, via augmentation of the amounts of farnesylated p21Ras, significantly amplified cellular nuclear responses to other growth factors, such as insulin-like growth factor (IGF-I), epidermal growth factor (EGF), and platelet-derived growth factor (PDGF) (8, 9, 15, 17). This priming effect of insulin required the presence of the intact insulin receptor (9) and was blocked by an inhibitor of FTase (8, 9, 15, 17).

Furthermore, in experiments with hyperinsulinemic animals, such as *ob/ob* mice and *fa/fa* Zucker rats, we found increased amounts of farnesylated p21Ras in liver, aorta, and skeletal muscle (17). In contrast, the levels of farnesylated p21Ras were normal in denervated rat skeletal muscle (17), a model of insulin resistance in the absence of hyperinsulinemia (18). Even though these findings suggested an association of hyperinsulinemia with increased amounts of farnesylated p21Ras, they did not establish a cause and effect relationship between these two variables. The present study was undertaken to confirm the causal role of hyperinsulinemia in both humans and experimental animal models.

Using normoinsulinemic controls and models of acquired or genetic insulin resistance, we demonstrate that hyperinsulinemia, resulting from either infusions of insulin or endogenous metabolic insulin resistance, increases the prenylation of p21Ras, thus creating a favorable background for enhanced mitogenic responsiveness of these tissues to various growth factors.

Materials and Methods

Materials

All standard chemicals were obtained from Sigma (St. Louis, MO), anti-Ras monoclonal antibody (Y13–259) was purchased from Transduction Laboratories (Lexington, KY), and anti-Rho-A antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All supplies and reagents for SDS-PAGE were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA), and the enhanced chemiluminescence kit was obtained from Amersham Pharmacia Biotech (Arlington Heights, IL).

Insulin infusion in mice

Male C 57BL/6 mice, aged 10–14 weeks, were fitted with chronic indwelling jugular catheters according to the method of Ren *et al.* (19) as previously described (20). Insulin (20 mU/kg·min) was infused for 230 min while a steady state blood glucose level was maintained. Animals were then killed by an overdose of anesthetic, and the liver and aorta were removed, frozen immediately in liquid nitrogen, and stored at −70 C. An equal number of animals was killed before insulin infusion, and they served as controls.

Insulin infusion in dogs

Studies were conducted in 14 male mongrel dogs, weighing 20.8–28 kg. Animals were housed and operated upon as described previously (21). Eight animals were made hyperinsulinemic by diverting the veins of the pancreas from the portal vein to the vena cava (PVD dogs) (21). A sham operation was performed on the control group of dogs (n = 6). Hyperinsulinemic euglycemic clamp studies were performed 1 month postoperatively. Infusion of insulin (7.2 nmol/kg·min) was continued for 160 min while maintaining steady state levels of glycemia. Liver samples were obtained by biopsy before and at the conclusion of the insulin infusion. Tissue was frozen in liquid nitrogen and stored at −70 C.

Hyperinsulinemic euglycemic clamps in normal and obese humans

Initially, white blood cells (WBC; buffy coat) were obtained from 6 normal weight [4 men and 2 women; body mass index (BMI), 23.8 ± 0.66] and 21 obese (17 men and 4 women; BMI, 36.17 ± 1.25) individuals (aged 30–50 yr) from an overnight fasting sample. Subsequently, 4 normal weight and 4 obese individuals underwent infusions of insulin (80 mU/m^2·min) for 180 min. None of these individuals received any medications, none was hypertensive, and none had a family history of diabetes. WBC were obtained before and at the end of the insulin infusion. The cells were frozen immediately and stored at −70 C until measurements of farnesylated p21Ras were performed.

Glucosamine infused in rats

Animals were infused with either saline (0.5 ml/h) or glucosamine (6.5 mg/kg·min) for 6.5 h as described previously (22). Their preglucosamine or presaline insulin level was 10.0 ± 3.0 μU/ml and was not increased by the infusion of glucosamine (11.5 ± 1.5 μU/ml). At 5 h, a hyperinsulinemic (4 mU/kg·min; to yield a plasma concentration of ~100 μU/ml) euglycemic glucose clamp was performed for 90 min. The animals were anesthetized, and tissue and blood samples were collected for determinations of prenylated p21Ras, glycemia, and insulinemia (22).

Transgenic models of insulin resistance

We have used three transgenic models of insulin resistance developed, characterized, and previously described by the authors of the current study. The heterozygous insulin receptor (IR)/IRS-1 knockout mice (50% reduction) have been developed in the laboratory of Dr. Accilli (23) by breeding heterozygous IR knockout ($IR^{+/-}$) mice with heterozygous IRS-1 knockout ($IRS\text{-}1^{+/-}$) mice. The latter were obtained from Dr. C. R. Kahn's laboratory (24). The A-ZIP/F-1 mice were developed in the laboratories of Drs. Reitman and Vinson (25), and the glutamine:fructose-6-phosphate amidotransferase (GFA)-overexpressing mice were developed in the laboratory of Dr. McClain (26). All three models have been shown to be resistant to the metabolic actions of insulin (23–27). Liver samples were used in the experiments with IRS-1 knockout and A-ZIP/F-1 mice. The GFA-overexpressing mice were infused with insulin for 60 min, and the hind limb muscle samples were collected for measurements of prenylated p21Ras as described below. A-ZIP/F-1 mice were studied at birth and 3 weeks of age, GFA-overexpressing mice were 6–10 weeks old, and $IRS\text{-}1^{+/-}$ and $IR/IRS\text{-}1^{+/-}$ mice were 6 months of age at the time of study.

Determinations of farnesylated p21Ras and geranylgeranylated Rho-A

Tissue samples were homogenized in lysis buffer (150 mM NaCl, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonylfluoride, 1 mM dithiothreitol, 1 mM sodium vanadate, 1 mM sodium phosphate, 1% Triton X-100, 0.5% SDS, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 50 mM HEPES, pH 7.5). Crude lysates were sonicated and centrifuged at 10,000 rpm. Total protein from the resultant supernatant was determined by the bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL) and diluted to 1 mg/ml. Equal volumes of lysate and 4% Triton X-114 were combined in a borosilicate glass tube, vortexed, and incubated at 37 C for 3 min. Solutions were kept at room temperature until phases had separated. Equal volumes from each phase were placed into separate 1.5-ml microcentrifuge tubes, and p21Ras or Rho-A was immunoprecipitated using a monoclonal anti-Ras antibody (Y13–259) or a monoclonal anti-Rho-A antibody, respectively. Prenylated p21Ras and Rho-A were recovered in the detergent phase (8, 9). Increases in the amounts of prenylated proteins have been previously shown to reflect and closely correlate with increases in the activity of prenyl transferases (8–10, 16).

Statistical analysis

All statistics were analyzed using Student's t test, with $P < 0.05$ considered significant.

Results

Effect of hyperinsulinemia on the amount of farnesylated p21Ras in humans

In the initial experiments we measured the amounts of farnesylated p21Ras in WBC of obese hyperinsulinemic individuals. Circulating monocytes possess IR (28) and represent a target tissue for insulin action on FTase. The obese individuals had significantly greater BMIs and levels of insulinemia than the age-matched lean controls (Fig. 1, A and B) and displayed significantly ($P < 0.05$) increased amounts of farnesylated p21Ras in their WBC (Fig. 1C; 53.8 ± 2.4% *vs.* 45.2 ± 1.7%).

Even though we have previously demonstrated that insulin increased the amounts of farnesylated p21Ras in cells in culture, the cause and effect relationship between these two variables has not been established *in vivo*. To assess whether elevations in the amounts of farnesylated p21Ras are caused by hyperinsulinemia, we measured farnesylated p21Ras content in WBC of four normal weight and four obese individuals before and after a 3-h hyperinsulinemic euglycemic clamp. Insulin infusion raised the amounts of farnesylated p21Ras in all normal weight subjects from 48 ± 5% to 61 ± 4%, but failed to further augment already increased amounts of farnesylated p21Ras (62 ± 6% *vs.* 61 ± 6%) in obese individuals (Fig. 2).

Effect of hyperinsulinemia on the amount of farnesylated p21Ras in mice and dogs

To confirm that insulin augments the amount of farnesylated p21Ras in other tissues as well, we performed hyperinsulinemic euglycemic clamps in two animal models: mice and dogs. In normal mice, an infusion of insulin significantly ($P < 0.05$) raised the amounts of farnesylated p21Ras in aorta and liver (Fig. 3). Similarly, an infusion of insulin raised the amounts of farnesylated p21Ras in the livers of normal dogs (Fig. 4).

Recently, Miles *et al.* (21) developed an interesting model of insulin resistance caused by chronic hyperinsulinemia in otherwise normal dogs. Chronic hyperinsulinemia (16.5 ± 2.7 *vs.* 7.7 ± 1.0 μU/ml) has been produced by surgically diverting the venous pancreatic outflow from the portal vein to the vena cava. Pancreatic venous diversion (PVD) caused a marked peripheral insulin resistance characterized by a decreased insulin-

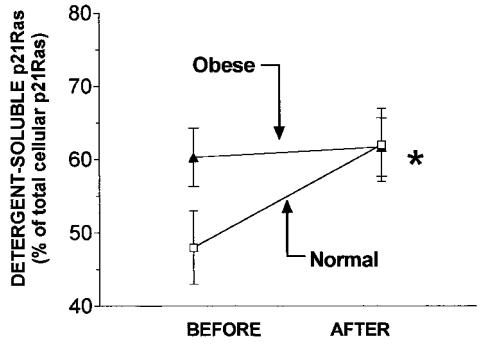

FIG. 2. Amounts of farnesylated p21Ras in WBC in control and obese individuals before and after hyperinsulinemic euglycemic clamps. Results represent the mean ± SEM of four individuals per group. *, $P < 0.05$.

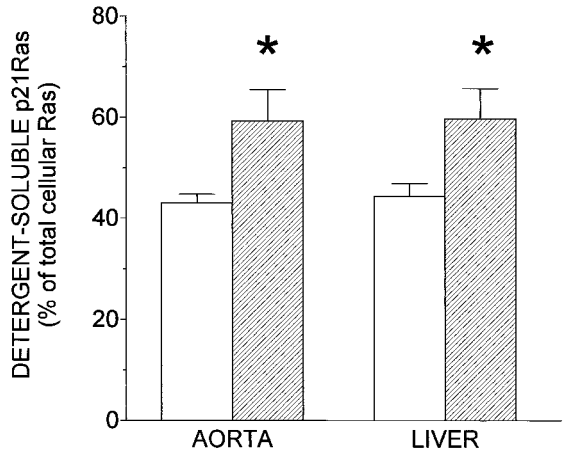

FIG. 3. Amounts of farnesylated p21Ras in aorta and liver samples of normal mice. Four mice were killed after the hyperinsulinemic euglycemic clamp (*hatched bars*) as described in *Materials and Methods*. Four littermate animals served as controls (*open bars*). Results represent the mean ± SEM. $P < 0.05$.

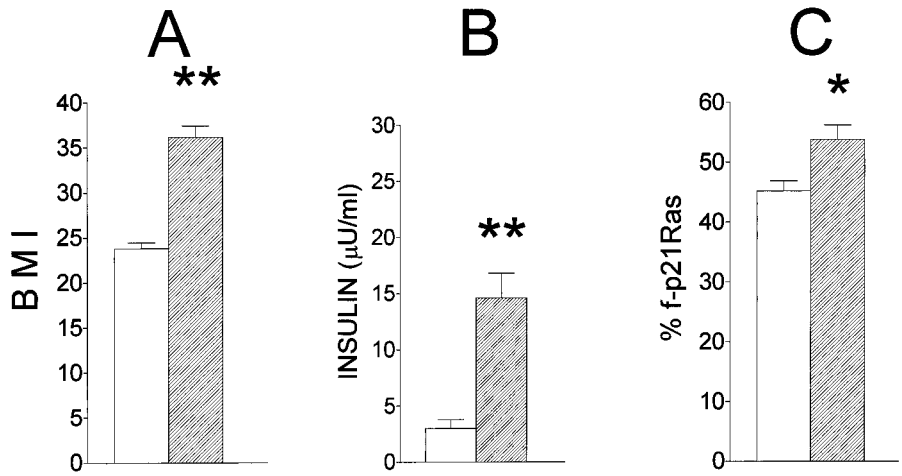

FIG. 1. BMI (A), insulinemia (B), and amounts of farnesylated p21 Ras (C) in normal weight (*open bars*) and obese (*hatched bars*) individuals. Blood samples for measurements of insulinemia and farnesylated p21 Ras in WBC were obtained after an overnight fast in 6 control and 21 obese patients. Results represent the mean ± SEM. *, $P < 0.05$; **, $P < 0.01$.

stimulated glucose disposal rate (21). However, like hyperinsulinemic humans, hyperinsulinemic PVD dogs displayed increased amounts of farnesylated p21Ras that no longer responded to an additional infusion of insulin (Fig. 4).

Amounts of farnesylated p21Ras in experimental models of insulin resistance induced by glucosamine

Our current (Figs 1 and 4) and previously published (16) data indicate that the tissues of insulin-resistant animals and individuals contain increased amounts of prenylated p21Ras. We postulated that insulin resistance to the metabolic aspects of insulin action augments the influence of the ensuing hyperinsulinemia on prenylation of the Ras family of proteins. To confirm this hypothesis experimentally, we examined the amount of prenylated p21Ras in tissues of animals with acquired and genetic insulin resistance.

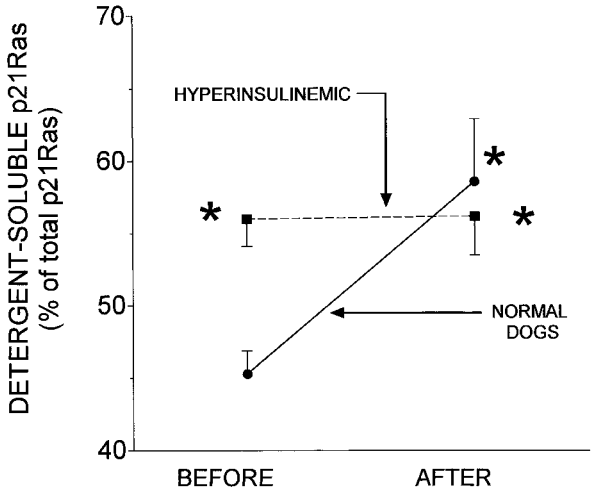

FIG. 4. Amounts of farnesylated p21 Ras in liver samples of normal and hyperinsulinemic PVD dogs before and after the hyperinsulinemic euglycemic clamp. Results represent the mean ± SEM. *, $P < 0.05$ *vs.* controls (n = 6).

To induce insulin resistance experimentally, we infused male Sprague Dawley rats (150–175 g) with either saline (0.5 ml/h) or glucosamine (6.5 mg/kg·min) for 6.5 h followed by infusion of insulin (4 mU/kg·min) for 180 min (euglycemic clamp) as previously described (22). Glucosamine infusion resulted in diminution of glucose disposal as measured by euglycemic clamp (22). However, 6.5 h of glucosamine infusion did not produce endogenous hyperinsulinemia (11.5 ± 1.5 *vs.* 10 ± 3 μU/ml in saline-infused rats) and did not affect the amount of farnesylated p21Ras (48 ± 3.2% *vs.* 46 ± 3.8%). The influence of glucosamine-induced insulin resistance became apparent after the infusion of insulin. Despite the metabolic insulin resistance, the insulin-induced increments in the amount of farnesylated p21Ras were significantly ($P < 0.005$) greater in glucosamine-treated animals than in saline-treated controls (28 ± 5% *vs.* 12 ± 3%) compared to preinsulin infusion levels (Fig. 5A).

Our second approach was to measure the amount of farnesylated p21Ras in transgenic animals that overexpressed GFA, an enzyme responsible for the production of endogenous glucosamine (reviewed in Ref. 29). These animals have been previously shown to be insulin resistant in terms of glucose transport and utilization (26, 30). As shown in Fig. 5B, insulin infusion for 60 min significantly ($P < 0.05$) increased the amount of farnesylated p21Ras in the skeletal muscle of wild-type mice. In contrast, in GFA-overexpressing transgenic mice (basal insulinemia, 39 ± 2 *vs.* 16.7 ± 2 μU/ml in control mice), the basal amount of farnesylated p21Ras was significantly ($P < 0.05$) greater than that in control wild-type mice, and insulin infusion no longer influenced the amount of farnesylated p21Ras.

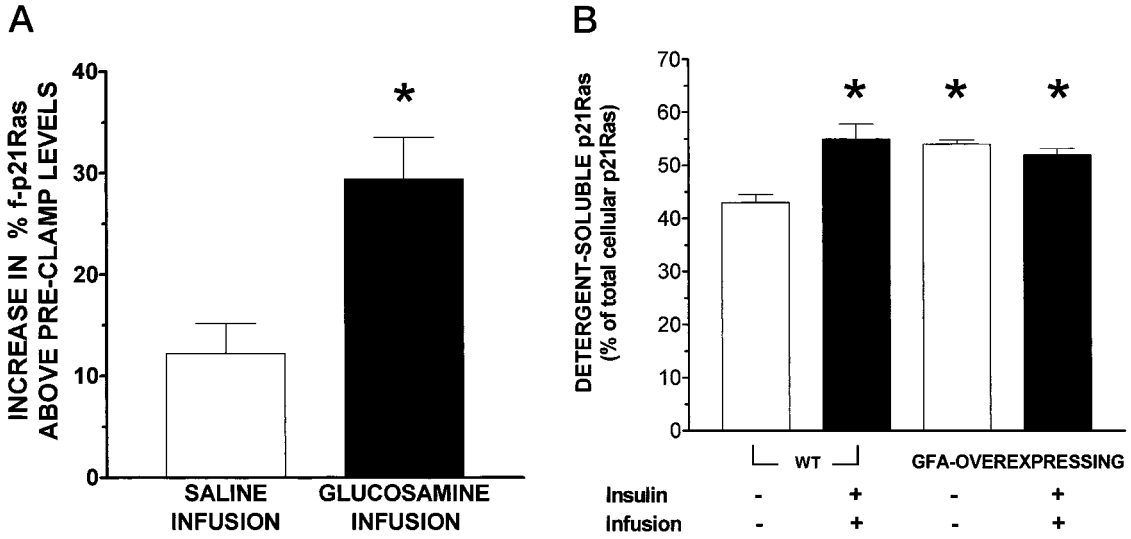

FIG. 5. Effect of glucosamine on the amounts of farnesylated p21Ras in rat liver and skeletal muscle tissue. A, Rats were infused with saline (0.5 ml/h) or glucosamine (6.5 mg/kg·min) for 6.5 h, followed by an 180-min infusion of insulin (4 mU/kg·min; euglycemic clamp). Liver samples were homogenized, normalized for protein, and assayed for farnesylated p21Ras. Results are expressed as the percent increase in farnesylated p21Ras above preclamped levels and represent the mean ± SEM of three experiments. *, $P < 0.05$ *vs.* saline controls (n = 12). B, Control (WT) and transgenic, GFA-overexpressing mice were infused without (*open bars*) or with insulin (20 mU/kg·min; *closed bars*) for 60 min. Skeletal muscle was collected from each animal, homogenized, normalized for protein, and assayed for the amount of farnesylated p21Ras. Results are expressed as the amount of farnesylated p21Ras as a percentage of total cellular p21Ras and represent the mean ± SEM. *, $P < 0.05$ (n = 4).

Amount of farnesylated p21Ras in transgenic animals with metabolic insulin resistance

In these sets of experiments, we measured the amount of farnesylated p21Ras in transgenic animals with well characterized metabolic insulin resistance: 50% reduction in IRS-$1^{+/-}$, IR/IRS-$1^{+/-}$ mice (23, 24), and A-ZIP/F-1 fatless mice (25). The IRS-$1^{+/-}$ mice (basal insulinemia ranged from 40–180 μU/ml) had significantly ($P < 0.05$) increased amounts of farnesylated p21Ras compared with wild-type mice (Fig. 6a). Mice that had a concomitant reduction in the number of IR (IR/IRS-$1^{+/-}$ double heterozygotes: IR/IRS-$1^{+/-}$; basal insulinemia was not different from the IRS-$1^{+/-}$ mice) displayed normal amounts of farnesylated p21Ras (Fig. 6a).

A-ZIP/F-1 mice are born normoinsulinemic (23.6 ± 3 *vs.* 20.2 ± 4 μU/ml in wild-type mice) and have normal amounts of farnesylated p21Ras at birth (Fig. 6b). However, by 3 weeks of age these animals become hyperinsulinemic (249.5 ± 22 *vs.* 24.4 ± 3 μU/ml in wild-type mice) and insulin resistant and display significantly ($P < 0.05$) increased amounts of farnesylated p21Ras (Fig. 6b).

Effect of hyperinsulinemia on the amount of geranylgeranylated Rho-A in dogs

As the α-subunit of FTase is also a component of GGTase I (31), an enzyme that prenylates Rho proteins, it is conceivable that insulin also increases the activity of GGTase I. To examine this possibility, we measured the amount of geranylgeranylated Rho-A in liver samples from normal dogs. We observed that insulin infusion significantly increased the amount of geranylgeranylated Rho-A in these animals (Fig. 7). In contrast, the PVD dogs had increased basal levels of geranylgeranylated Rho-A that were not significantly increased by insulin infusion.

Discussion

This work clearly demonstrates three salient features. For the first time there is an unequivocal indication that infusions of insulin in humans, mice, and dogs augment the amount of farnesylated p21Ras in various insulin target tissues. Second, this is the first demonstration of the ability of insulin to influence the amount of farnesylated p21Ras in humans. Third, the experiments with either genetic or acquired insulin resistance strongly indicate that the resistance to the metabolic aspects of insulin action is accompanied by enhanced prenylation of p21Ras in response to hyperinsulinemia.

These experiments complement our previous findings of increased amounts of farnesylated p21Ras in hyperinsulinemic *ob/ob* mice and Zucker *fa/fa* rats (17). However, we have also demonstrated that the amount of farnesylated p21Ras remained normal in denervated rat skeletal muscle (17), a model of insulin resistance not associated with hyperinsulinemia (18). The latter data suggested that hyperinsulinemia and not insulin resistance was responsible for the increased amount of farnesylated p21Ras. Current studies confirm this suggestion by demonstrating that insulin infusions *in vivo* cause prompt and significant increases in the amount of farnesylated p21Ras in humans, mice, and dogs, thus supporting the causal role of hyperinsulinemia in this process.

We have previously shown that insulin promotes the phosphorylation and activation of the prenyl transferases (8–10). Increased activity of prenyl transferases parallels the increases in prenylated GTPases (8–10, 16, 32). Therefore, insulin-induced changes in the amount of prenylated proteins reflect the influence of insulin on the prenyl transferases. The reduction of hyperinsulinemia and concomitant amelioration of insulin resistance in *fa/fa* Zucker rats by exercise resulted in decreases in the amount of farnesylated p21Ras toward normal levels (17). However, we were unable to correlate the level of insulinemia with the amount of farnesylated p21Ras. Conceivably, the length of time that hyperinsulinemia is present is more important than single measurements of ambient insulinemia. Alternatively, it is possible that the current methodology for measurement of farnesylated p21Ras is not sensitive enough to appreciate minor differences. Finally, one should keep in mind that

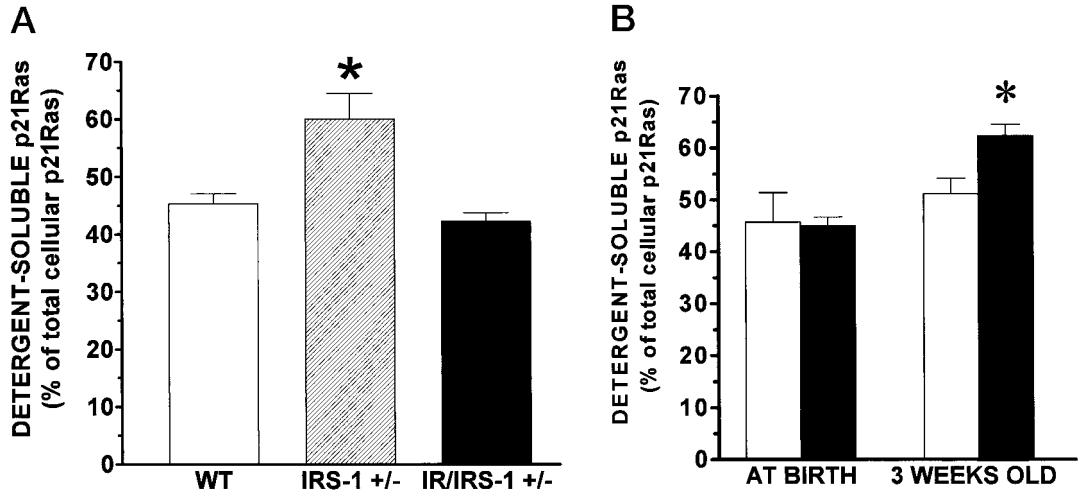

FIG. 6. Amount of farnesylated p21Ras in liver tissue of transgenic mice. A, Wild-type (IRS-$1^{+/+}$, *open bars*) and transgenic (IRS-$1^{+/-}$, *hatched bars*; IR/IRS-$1^{+/-}$, *closed bars*) mice were killed at 6 months, and liver samples were homogenized, normalized for protein, and assayed for farnesylated p21Ras (n = 2 animals/group). B, Livers were dissected from control (*open bars*) and transgenic (*closed bars*) A-ZIP/F-1 fatless mice at birth and 3 weeks of age, homogenized, normalized for protein, and assayed for amounts of farnesylated p21Ras (n = 4). Results are expressed as the amount of farnesylated p21Ras as a percentage of the total cellular p21Ras and represent the mean ± SEM. *, $P < 0.05$.

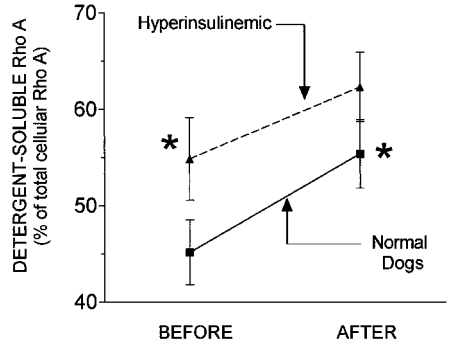

FIG. 7. Amounts of geranylgeranylated Rho-A in the liver samples of normal and PVD dogs. Results represent the mean ± SEM. *, $P < 0.05$ *vs.* controls (n = 6).

maximally activated FTase may not be further augmented by even higher concentrations of insulin. This might explain the failure of insulin infusions to further augment already increased levels of farnesylated p21Ras in obese individuals, hyperinsulinemic dogs, and mice overexpressing GFA.

The present results are in agreement with our previous observations that insulin increases the activity of FTase in tissue culture and in hyperinsulinemic animals (8, 9, 15–17). Insulin-induced phosphorylation of the α-subunit of FTase appears to be responsible for increased FTase enzymatic activity (32). The effects of insulin on the phosphorylation and activation of FTase appear to be specific and were not mimicked by IGF-I, EGF, or PDGF (9). Furthermore, we have shown that inhibition of phosphatidylinositol 3-kinase activity (a key signaling intermediate of the metabolism aspects of insulin action) did not alter insulin effects on FTase (32). In contrast, inhibition of the Ras-mitogen-activated protein kinase pathway completely blocked the insulin effect on FTase (32), yet did not affect the metabolic aspects of insulin action.

Even though the increase in the absolute amount of farnesylated p21Ras is modest (~25%), it produces a significantly greater effect downstream in the signaling cascade. These increases in prenylated p21Ras result in greater than 2-fold increases in the magnitude of Ras-GTP loading (15) and the Ras-mediated influence of PDGF on vascular endothelial growth factor (VEGF) messenger RNA levels (16).

The term insulin resistance generally implies the reduced ability of insulin to promote normal glucose disposal. Other aspects of insulin action may or may not be affected or may be affected to a different degree (3). Several lines of experimental evidence suggest that insulin resistance at the metabolic branch of insulin action is not accompanied by resistance at the mitogenic branch. Recently, Koopmans *et al.* (4) found that metabolic insulin resistance (diminished glucose uptake) in chronically hyperinsulinemic rats coexisted with increased effects of insulin on *de novo* lipogenesis. Our current observations strongly suggest that hyperinsulinemia that develops as a compensatory feature of the metabolic insulin resistance increases the activity of the prenyl transferases and the amounts of prenylated p21Ras and Rho-A.

We observed that transgenic animals with insulin resistance at the level of glucose disposal (such as IRS-$1^{+/-}$, A-ZIP/F-1 fatless mice, and GFA-overexpressing mice) have

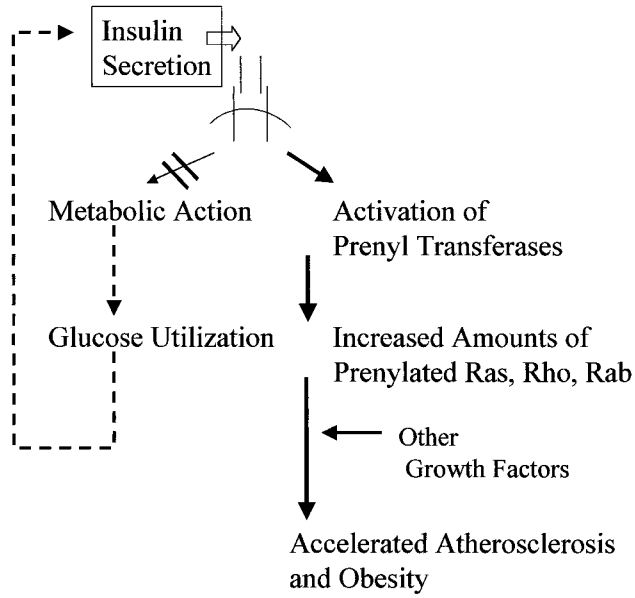

FIG. 8. Resistance to the metabolic effects of insulin at the postreceptor level results in compensatory hyperinsulinemia and overstimulation of the prenylation of the Ras family of GTPases. Cells with increased amounts of prenylated proteins have greater nuclear and other mitogenically relevant responses to other growth factors, resulting in the acceleration of several pathophysiological conditions that frequently accompany hyperinsulinemia.

increased amounts of farnesylated p21Ras in their tissues. Furthermore, an induction of insulin resistance either by glucosamine infusion (30, 33, 34) or by diverting the pancreatic outflow into the inferior vena cava (21) was accompanied by significant increments in the amount of prenylated p21Ras. Experiments with insulin infusions in normal humans, mice, and dogs indicate that insulin *per se* is responsible for the increases in prenylation. Interestingly, insulin-resistant mice, heterozygous for IRS-1 knockout, do not display increased levels of farnesylated p21Ras if they have a concomitant reduction in the number of IRs (*i.e.* double heterozygotes, IR/IRS-$1^{+/-}$), confirming the requirement for the IR for insulin's effect on prenylation and that this effect does not involve the IGF-I receptor (9). Insulin's effect on prenylation is mediated exclusively via an intact IR (9). Thus, insulin failed to stimulate the prenylation of p21Ras in cells overexpressing either the intact IGF-I receptor or a chimeric insulin/IGF-I receptor (9). In cells overexpressing IGF-I receptors, IGF-I had no effect on either phosphorylation of the α-subunit of FTase or the amount of farnesylated p21Ras (9).

We suggest that in the state of insulin resistance at the metabolic branch of insulin action, the ensuing hyperinsulinemia augments prenylation of Ras and Rho proteins (Fig. 8). This increase in the magnitude of insulin action on prenylation, in the presence of its diminished influence on glucose disposal, is analogous to the pathophysiology of congenital adrenal hyperplasia; when an enzymatic block in one branch of adrenal steroidogenesis results in augmentation of the effects of ACTH on an unaffected branch(s). The precise molecular locus of insulin resistance is still unknown. However, the inability of insulin to assure normal glucose uptake and utilization leads to compensatory hyperinsulinemia that

may or may not fully correct glucose disposal. Because insulin's ability to stimulate prenyl transferases is not impaired, hyperinsulinemia increases the amounts of prenylated proteins in various tissues.

Prenylation of Ras and Rho proteins is a prerequisite for their subsequent activation by GTP loading (11, 35, 36). Insulin appears to stimulate the activities of prenyl transferases and increase the amounts of prenylated Ras and Rho proteins in various cell lines and tissues (8–10, 15–17, 32). Increased amounts of farnesylated p21Ras and geranylgeranylated Rho-A could result in enhanced cellular responsiveness to various growth-promoting agents whose actions are mediated by Ras and Rho. Thus, we recently demonstrated that hyperinsulinemia potentiated the effects of EGF, IGF-I, and PDGF on DNA synthesis and the effect of PDGF on VEGF gene expression (9, 17). This priming effect of insulin was blocked by an inhibitor of FTase (8, 9, 17, 32). Our preliminary data also indicate that insulin potentiates the Rho-A-mediated nuclear effects of lysophosphatidic acid and angiotensin II (Chappell, J., I. Golovchenko, R. Stjernholm, M. Goalstone, and B. Draznin, unpublished), suggesting that insulin creates a new background for the cellular responsiveness to various growth factors that exert their nuclear effects via the Ras and Rho pathways.

In summary, our results demonstrate that hyperinsulinemia, either exogenous (infusions of insulin to normal humans, mice, and dogs) or endogenous (insulin resistance in obese humans, PVD dogs, glucosamine-infused rats, IRS-$1^{+/-}$ mice, A-ZIP/F-1 mice, and GFA-overexpressing mice), activates the prenyl transferases in various tissues and increases the amounts of farnesylated p21Ras and geranylgeranylated Rho-A. The ability of insulin to increase the cellular pool of prenylated Ras and Rho proteins may play a critical role in the mechanism of the potentially detrimental influence of hyperinsulinemia.

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References

1. **Kruszynska YT, Olefsky JM** 1996 Cellular and molecular mechanisms of non-insulin dependent diabetes. J Invest Med 44:413–428
2. **Reaven GM** 1988 Role of insulin resistance in human disease. Diabetes 37:1595–1607
3. **Blankard WG, Clore JN** 1997 Insulin effects on substrate metabolism. In: Draznin B, Rizza R (eds) Clinical Research in Diabetes and Obesity. Humana Press, Totowa, pp 205–220
4. **Koopmans SJ, Kushwaha RS, DeFronzo R** 1999 Chronic physiologic hyperinsulinemia impairs suppression of plasma free fatty acids and increases de novo lipogenesis, but does not cause dyslipidemia in conscious normal rats. Metabolism 48:330–337
5. **Cheatham G, Kahn CR** 1995 Insulin action and the insulin signaling network. Endocr Rev 16:117–142
6. **White MG** 1998 The IRS-signaling system: a network of docking proteins that mediate insulin action. Mol Cell Biochem 182:3–11
7. **Sasaoka T, Draznin B, Leitner JW, Langlois WJ, Olefsky JM** 1994 Shc is the predominant signaling molecule coupling insulin receptors to activation of guanine nucleotide releasing factor and p21ras-GTP formation. J Biol Chem 269:10734–10738
8. **Goalstone ML, Draznin B** 1996 Effect of insulin on farnesyltransferase activity in 3T3–L1 adipocytes. J Biol Chem 271:27585–27589
9. **Goalstone ML, Leitner JW, Wall K, Dolgonos L, Rother KI, Accili D, Draznin B** 1998 Insulin's effect on farnesyltransferase: specificity of insulin action and potentiation of nuclear effects of IGF-I, EGF, and PDGF. J Biol Chem 273:23892–23896
10. **Goalstone ML, Leitner JW, Golovchenko I, Stjernholm MR, Cormont M, Le Marchand-Brustel Y, Draznin B** 1999 Insulin promotes phosphorylation and activation of geranylgeranyltransferase. II. Studies with geranylgeranylation of Rab-3 and Rab-4. J Biol Chem 274:2880–2884
11. **Zhang FL, Casey PJ** 1996 Protein prenylation molecular mechanisms and functional consequences. Annu Rev Biochem 65:241–269
12. **Schaber MD, O'Hara UB, Garsky VD, Mosser SD, Bergstrom JD, Moores SL, Marshall MS, Friedman PS, Dixon RAF, Gibbs JB** 1990 Polyisoprenylation of Ras *in vitro* by a farnesyl-protein transferase. J Biol Chem 265:14701–14704
13. **Seabra MC, Brown MS, Slaughter CA, Sudhof TC, Brown JL** 1992 Purification of component A of Rab geranylgeranyltransferase possible identity with the choroideremia gene product. Cell 70:1049–1057
14. **Armstrong SA, Hannah VC, Goldstein JL, Brown MS** 1995 CAAX geranylgeranyltransferase transfers farnesyl as efficiently as geranylgeranyl to Rho B. J Biol Chem 270:7864–7868
15. **Leitner JW, Kline T, Carel K, Goalstone M, Draznin B** 1997 Hyperinsulinemia potentiates activation of p21Ras by growth factors. Endocrinology 138:2211–2214
16. **Goalstone ML, Natarajan R, Standley PR, Walsh MF, Leitner JW, Carel K, Scott S, Nadler J, Sowers JR, Draznin B** 1998 Insulin potentiates platelet-derived growth factor action in vascular smooth muscle cells. Endocrinology 139:4067–4072
17. **Goalstone ML, Wall K, Leitner JW, Kurowski T, Ruderman N, Pan SJ, Ivy JL, Moller DE, Draznin B** 1999 Increased amounts of farnesylated p21Ras in tissues of hyperinsulinemic animals. Diabetologia 42:310–316
18. **Saha AK, Kurowski TG, Ruderman NB** 1995 A malonyl-CoA fuel sensing mechanism in muscle effects of insulin, glucose, and denervation. Am J Physiol 269:E283–E289
19. **Ren JM, Marshall BA, Mueckler MM, McCaleb M, Amatruda JM, Shulman GI** 1995 Overexpression of GLUT-4 protein in muscle increases basal and insulin-stimulated whole body glucose disposal in conscious mice. J Clin Invest 95:429–432
20. **Liu S, Cromiger C, Hirada-Shiba M, Ren JM, Poli V, Hanson RW, Friedman JE** 1999 Disruption of the gene for CCAAT/Enhancer binding protein (C/EBP) results in hypoglycemia and impaired glucagon-stimulated hepatic glucose production. J Clin Invest 103:207–213
21. **Miles PD, Li S, Hart M, Romeo O, Cheng J, Cohen A, Raafat K, Moossa AR, Olefsky JM** 1998 Mechanism of insulin resistance in experimental hyperinsulinemic dogs. J Clin Invest 101:202–211
22. **Miles PD, Higo K, Romeo OM, Lee M-K, Raafat K, Olefsky JM** 1998 Troglitazone prevents hyperglycemia-induced but not glucosamine-induced insulin resistance. Diabetes 47:395–400
23. **Accili D, Drago J, Lee EJ, Johnson MD, Cool MH, Salvatore P, Asico LD, Jose PA, Taylor SI, Westphal H** 1996 Early neonatal death in mice homozygous for a null allele of the insulin receptor gene. Nat Genet 12:106–109
24. **Araki E, Lipes MA, Patti M-E, Brûning JC, Haag III B, Johnson RS, Kahn CR** 1994 Alternative pathway of insulin signaling in mice with targeted disruption of the IRS-1 gene. Nature 372:186–189
25. **Moitra J, Mason MM, Olive M, Krylov D, Gavrilova O, Marcus-Samuels B, Geigenbaum L, Lee E, Aoyama T, Eckhaus M, Reitman ML, Vinson C** 1998 Life without fat: a transgenic mouse. Genes Dev 12:3168–3181
26. **Hebert LFJ, Daniels MC, Zhou J, Crook ED, Turner RL, Simmons ST, Neidigh JC, Zhu JS, Baron AD, McClain DA** 1996 Overexpression of glutamine fructose-6-phosphate amidotransferase in transgenic mice leads to insulin resistance. J Clin Invest 98:930–936
27. **Bruning JC, Winnay J, Bonner-Weir S, Taylor Si, Accili D, Kahn CR** 1997 Development of a novel polygenic model of NIDDM in mice heterozygous for IR and IRS-1 null alleles. Cell 88:561–572
28. **Olefsky JM, Reaven GM** 1977 Insulin binding in diabetes Relationships with plasma insulin levels and insulin sensitivity. Diabetes 26:680–688
29. **McClain DA, Crook ED** 1996 Hexosamines and insulin resistance. Diabetes 45:1003–1008
30. **Cooksey, RC, Hebert LFJ, Zhu J-H, Wofford P, Garvey T, McClain DA** 1999 Mechanism of hexosamine-induced insulin resistance in transgenic mice overexpressing glutamine:fructose-6-phosphate amidotransferase: decreased glucose transporter GLUT4 translocation and reversal by treatment with thiazolidinedione. Endocrinology 140:1151–1157
31. **Seabra MC, Reiss Y, Casey PJ, Brown MS, Goldstein JL** 1991 Protein farnesyltransferase and geranylgeranyltransferase share a common α subunit. Cell 65:429–434
32. **Goalstone M, Carel K, Leitner JW, Draznin B** 1997 Insulin stimulates the phosphorylation and activity of farnesyltransferase via the Ras-mitogen-activated protein kinase pathway. Endocrinology 138:5119–5124
33. **Kim Y-B, Zhu J-S, Zierath JR, Shen H-Q, Baron AD, Kahn BB** 1999 glucosamine infusion in rats rapidly impairs insulin stimulation of phosphoinositide 3-kinase but does not alter activation of Akt/protein kinase B in skeletal muscle. Diabetes 48:310–320
34. **Rossetti L, Hawkins M, Chen W, Gindi J, Barzilai N** 1995 *In vivo* glucosamine infusion induces insulin resistance in normoglycemic but not in hyperglycemic conscious rats. J Clin Invest 96:132–140
35. **Kikuchi A, Williams LT** 1994 The post-translational modification of ras p21 is important for Raf-1 activation. J Biol Chem 269:20054–20059
36. **Porfiri E, Evans T, Chardin P, Hancock JF** 1994 Prenylation of ras proteins is required for efficient hSOS1-promoted guanine nucleotide exchange. J Biol Chem 269:22672–22677