To examine the impact of homozygous genetic disruption of insulin receptor substrate (IRS)-1 (IRS-1/i−/i) or IRS-2 (IRS-2/−/−) on basal and insulin-stimulated carbohydrate and lipid metabolism in vivo, we infused 18-h fasted mice (wild-type (WT), IRS-1/i−/i, and IRS-2/−/−) with [3-3H]glucose and [2H5]glycerol and assessed rates of glucose and glycerol turnover under basal (0–90 min) and hyperinsulinenic-euglycemic clamp (90–210 min; 5 mM glucose and glycerol turnover) conditions. Both IRS-1/i−/i and IRS-2/−/− mice were insulin-resistant as reflected by markedly impaired insulin-stimulated whole-body glucose utilization compared with WT mice. Insulin resistance in the IRS-1/i−/i mice could be ascribed mainly to decreased insulin-stimulated peripheral glucose metabolism. In contrast, IRS-2/−/− mice displayed multiple defects in insulin-mediated carbohydrate metabolism as reflected by (i) decreased peripheral glucose utilization, (ii) decreased suppression of endogenous glucose production, and (iii) decreased hepatic glycogen synthesis. Additionally, IRS-2/−/− mice also showed marked insulin resistance in adipose tissue as reflected by reduced suppression of plasma free fatty acid concentrations and glycerol turnover during the hyperinsulinenic-euglycemic clamp. These data suggest important tissue-specific roles for IRS-1 and IRS-2 in mediating the effect of insulin on carbohydrate and lipid metabolism in vivo in mice. IRS-1 appears to have its major role in muscle, whereas IRS-2 appears to impact on liver, muscle, and adipose tissue.

Insulin receptor substrate (IRS) proteins mediate the pleiotropic effects of insulin on cellular function, including the regulation of glucose transport and protein metabolism and the control of cell growth and survival (1, 2). A family of at least four IRS proteins has been identified, potentially allowing for a diverse and flexible response to insulin stimulation (3–6). However, the distinct role(s) of the individual IRS proteins have yet to be fully described. To characterize the precise physiological roles of these proteins, we and others have generated mice with targeted disruption of IRS-1, IRS-2, IRS-3, or IRS-4 (3–6), and initial studies are beginning to reveal functional differences between these molecules. For example, IRS-1/i−/i mice are severely growth-retarded, have normal fasting blood glucose concentrations and appear to be mildly insulin-resistant, as suggested by insulin tolerance testing (7, 8). In contrast, IRS-2/−/− mice are normal in size but develop diabetes due to a combination of peripheral and hepatic insulin resistance and a failure in β-cell function (9, 10). Thus, it appears that IRS-1 and IRS-2 are major mediators of insulin action but their relative contribution to the in vivo regulation of carbohydrate and lipid metabolism has not yet been determined.

To examine the tissue-specific roles of IRS-1 and IRS-2 in mediating insulin’s effect in the key insulin-responsive organs (muscle, liver, and adipose), we performed hyperinsulinenic-euglycemic clamps in conscious mice in combination with the infusion of [3-3H]glucose and [2H5]glycerol tracers to assess rates of glucose and glycerol turnover as well as rates of insulin-stimulated liver and muscle glycolysis. These studies demonstrate that, in vivo, IRS-1 appears to have its major role in muscle alone whereas IRS-2 mediates insulin action in liver, fat, and muscle.

**EXPERIMENTAL PROCEDURES**

**Materials**—Unless noted, chemicals were purchased from Sigma. [3H]Glycerol (98 atom % excess) and [2,2-13C]glycerol (99 atom % excess) were purchased from Cambridge Isotopes (Andover, MA). [3-3H]Glucose was purchased from PerkinElmer Life Sciences. Gas chromatography-mass spectrometry supplies were purchased from Hewlett-Packard (Wilmington, DE). Bistrimethylsilyl trifluoroacetamide + 10% trimethylchlorosilane was purchased from Pierce.

**In Vivo Studies**—Male mice (wild type, IRS-1/i−/i, or IRS-2/−/−) were received from Dr. Morris F. White (Joslin Diabetes Center, Boston, MA). Catheters were implanted in the jugular vein of 5–6-week-old mice, approximately 5 days prior to the study. Mice were fasted 18 h and infused with [2H5]glycerol (3 μmol·kg−1·min−1) to estimate rates of lipolysis and [3-3H]glucose (1 μCi·kg−1·min−1) to estimate rates of glucose production and utilization. The experimental design consisted of a 90-min basal period followed by a 2-h hyperinsulinenic-euglycemic clamp. A primed-continuous infusion of insulin (Humulin, Eli Lilly, Indianapolis, IN) was given (5 milliunits·kg−1·min−1). Glucose (20% w/v) was infused to maintain blood glucose at ~6 mM. Blood (~40 μl) was sampled via tail-tip bleeds at 70 and 90 min (basal) and at 190 and 210 min (clamp) for determination of the rate of appearance of glucose and glycerol and the concentration of plasma glycerol and free fatty acids. Additional blood samples (~20 μl) were obtained at 30-min intervals (i.e., 120, 150, and 180 min) for determination of blood glucose concentration. Total blood loss was approximately 20% of the estimated blood volume. At the end of the experiment the liver and hind-limb muscles (mixed muscle from the
TABLE I

| Carbohydrate and lipid metabolism in wild type, IRS-1<sup>−/−</sup> and IRS-2<sup>−/−</sup> mice. All rates are expressed in μmol·kg<sup>−1</sup>·min<sup>−1</sup>; data are presented as the mean ± S.E. For glucose data n = 11, 9, and 8 each for WT, IRS-1<sup>−/−</sup>, and IRS-2<sup>−/−</sup>, respectively. For glycerol and FFA data n = 8, 9, and 8 each for WT, IRS-1<sup>−/−</sup>, and IRS-2<sup>−/−</sup>, respectively. |
|-----------------|-----------------|-----------------|
|                 | Basal           | Hyperinsulinemic-euglycemic clamp |
|                 | WT              | IRS-1<sup>−/−</sup> | IRS-2<sup>−/−</sup> |
| Glucose (nmol)  | 5.98 ± 0.29     | 6.16 ± 0.35      | 7.16 ± 0.73      |
| Glucose production rate | 147 ± 11    | 143 ± 11         | 139 ± 15         |
| Glucose utilization rate | 147 ± 11   | 143 ± 11         | 139 ± 15         |
| Glucose infusion rate | 426 ± 40   | 369 ± 45         | 406 ± 55         |
| Glycerol production rate | 118 ± 9     | 117 ± 9          | 104 ± 12         |
| FFA (mM)        | 1.41 ± 0.19     | 0.74 ± 0.11<sup>a</sup> | 0.68 ± 0.07<sup>a</sup> |

<sup>a</sup>p < 0.05 versus basal for a group.

<sup>b</sup>p < 0.05 versus WT.

whole leg) were quick-frozen in liquid nitrogen.

**Analytical**—Plasma samples were processed for <sup>3</sup>H counting as follows. Briefly, a 10-μl aliquot of plasma was deproteinized by adding 20 μl of 10% trichloroacetic acid. A 15-μl portion of the supernatant was dried to remove <sup>3</sup>H<sub>2</sub>O. The residue was dissolved in 100 μl of water. Samples were counted after addition of 2 ml of scintillation fluid (Ultima Gold, Packard Instrument Co., Meriden, CT).

**GC-MS analyses** were conducted using electron impact ionization on a Hewlett-Packard 5973 MSD equipped with a Hewlett-Packard 6890 GC. All samples were analyzed using split injection (10:1) on a HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm) maintained at a constant helium flow (1.2 mllmin<sup>−1</sup>). Glycerol and free fatty acids were determined as follows. A 15-μl plasma sample was added to 15 μl of internal standard mixture containing [2-<sup>13</sup>C<sub>5</sub>]glycerol and heptadecanoic acid (C17:0). To this sample was added 200 μl of methanol. The supernatant was evaporated to dryness and reacted at room temperature with 65 μl of trimethylchlorosilane reagent. Plasma glycerol concentration was calculated from the m/z (205 + 208)/206 signal, and <sup>2</sup>H-enrichment was calculated from the m/z (208/208) signal. Plasma free fatty acid concentration was calculated from the ratio of C17:0 (m/z 327) to the sum of C16:0 (m/z 313), C18:0 (m/z 341), C18:1 (m/z 339), and C18:2 (m/z 357).

Total liver and muscle glycogen concentration was determined according to the method of Walaas and Walaas (13) with minor modifications. A portion of the hydrolyzed glycogen was also used to determine the total counts of <sup>3</sup>H.

Plasma insulin was assayed using the rat radioimmunoassay from Linco, with the mouse standard.

**Calculations**—The rate of appearance of glucose and glycerol were calculated from: tracer infusion rate/labeling of plasma pool. In the case of [3-<sup>2</sup>H<sub>1</sub>glucose the tracer infusion rate is in dpmmol<sup>−1</sup>·min<sup>−1</sup> and labeling of plasma pool is in dpmmol glucose<sup>−1</sup>. In the case of [3-<sup>2</sup>H<sub>1</sub>glycerol the tracer infusion rate is in μmolkg<sup>−1</sup>·min<sup>−1</sup>, and the labeling of plasma pool is the <sup>2</sup>H-enrichment of plasma glycerol.

The rate of hepatic glycogen synthesis was estimated from μmol of glucose equivalent/g wet weight/120 min clamp. The rate of muscle glycogen syntheses was estimated from dpmmol wet weight/minute activity<sub>plasma glucose</sub> × 120 min clamp. Data are presented as the mean ± S.E. Statistics were calculated using one-way analysis of variance, Tukey’s post hoc testing was used to determine significance. Where paired data were analyzed, a t test was used.

**RESULTS**

Insulin action on glucose and lipid metabolism in vivo was examined before and during a 2-h hyperinsulinemic-euglycemic clamp in conscious WT, IRS-1<sup>−/−</sup>, and IRS-2<sup>−/−</sup> mice, and before the development of fasting hyperglycemia in the IRS-2<sup>−/−</sup> animals. Basal plasma glucose concentrations were similar in mice from all three genotypes (Table I). Likewise glucose production rates were comparable in all three groups during the basal period (Table I). From separate studies we have determined that fasting (18 h) insulin levels in 4-week-old mice were WT, 10.5 ± 0.79 microunits·ml<sup>−1</sup>; IRS1<sup>−/−</sup>, 54 ± 7 microunits·ml<sup>−1</sup>; and IRS2<sup>−/−</sup>, 30 ± 2.5 microunits·ml<sup>−1</sup> mice (n = 6 for each group). The observation of basal hyperinsulinemia in IRS-1<sup>−/−</sup> and IRS-2<sup>−/−</sup> mice (p < 0.05 for each versus WT) confirm that both IRS-1<sup>−/−</sup> and IRS-2<sup>−/−</sup> mice are insulin-resistant. Also, IRS-1<sup>−/−</sup> are insulin-resistant as compared with IRS-2<sup>−/−</sup> mice (p < 0.05).

During the clamp WT mice exhibited a 70 ± 20% increase in their rate of whole-body glucose utilization. Data are expressed as the percent increase in the rate of whole-body glucose utilization, as compared with basal in each group. The effect of hyperinsulinemic-euglycemic clamp on the rate of endogenous glucose production. Data are expressed as the percent decrease in the endogenous glucose production, as compared with basal in each group. *p < 0.05 versus basal.
confirm that IRS-2^{-/-} mice are markedly insulin-resistant and demonstrate for the first time that IRS-1^{-/-} mice, studied in vivo using a hyperinsulinemic-euglycemic clamp, also display significant insulin resistance.

Skeletal muscle, liver, and fat are the major sites of insulin action. To gain an insight into tissue-specific differences in IRS function underlying the observed insulin resistance in IRS-1^{-/-} and IRS-2^{-/-} mice, muscle and liver glycogen metabolism, hepatic glucose production, and whole-body lipid turnover were analyzed. Following the hyperinsulinemic-euglycemic clamp there were no significant differences in muscle glycogen content among the three genotypes, although there was a trend to lower muscle glycogen stores in the IRS-1^{-/-} mice compared with wild-type animals (WT mice: 0.57 \pm 0.07 mmol glucose equivalents\textpercm\textpercm g wet weight^{-1}; IRS-1^{-/-} 0.43 \pm 0.03 mmol glucose equivalents\textpercm\textpercm g wet weight^{-1}, p = 0.084 versus WT; IRS-2^{-/-} 0.71 \pm 0.17 mmol glucose equivalents\textpercm\textpercm g wet weight^{-1}, p = not significant versus WT). In WT mice the rate of [H]glucose incorporation into muscle glycogen was 0.3 \mu mol\textpercm kg\textpercm min^{-1}. Both IRS-1^{-/-} and IRS-2^{-/-} mice synthesized significantly less muscle glycogen during the clamp (IRS-1^{-/-} 0.08 \mu mol\textpercm kg\textpercm min^{-1}, p < 0.05 versus WT and IRS-2^{-/-} 0.15 \mu mol\textpercm kg\textpercm min^{-1}, p < 0.05 versus WT). Furthermore, IRS-1^{-/-} mice synthesized significantly less glycogen than IRS-2^{-/-} mice (p < 0.05) reflecting a more significant impairment in insulin action in the muscle of these mice.

To analyze the contribution of defective insulin signaling in the liver to insulin resistance in IRS-1^{-/-} and IRS-2^{-/-} mice, hepatic glucose production was determined. In the basal state, endogenous glucose production was similar in animals of all three genotypes (Table I). During the hyperinsulinemic-euglycemic clamp, we observed a 59 \pm 11\% decrease in endogenous glucose production in WT mice (p < 0.05 versus basal glucose production rate) (Fig. 1B). Likewise, IRS-1^{-/-} mice displayed a 34 \pm 12\% decrease in endogenous glucose production (p < 0.05 versus basal) (Fig. 1B), achieving a similar level of suppression to that observed in WT mice. In contrast, there was no significant effect of the hyperinsulinemic-euglycemic clamp on the rate of endogenous glucose production in IRS-2^{-/-} mice (12 \pm 9\% decrease in glucose production, p = not significant versus basal) (Fig. 1B).

Analysis of hepatic glycogen metabolism in the three groups of mice revealed differences in the roles of IRS-1 and IRS-2 in liver. IRS-1^{-/-} mice had a slight reduction in hepatic glycogen content compared with WT animals (WT, 3.58 \pm 1.27 mmol glucose equivalents\textpercm\textpercm g wet weight^{-1} versus IRS-1^{-/-} mice, 2.31 \pm 0.90 mmol glucose equivalents\textpercm\textpercm g wet weight^{-1}, p = not significant). In contrast, IRS-2^{-/-} mice had markedly reduced hepatic glycogen (0.52 \pm 0.13 mmol glucose equivalents\textpercm\textpercm g wet weight^{-1}, p = 0.05 versus WT mice and IRS-1^{-/-} mice). Extrapolating from the glycogen content at the end of the clamp and during the clamp, we estimated rates of hepatic glycogen synthesis. The rate of insulin-stimulated hepatic glycogen synthesis in WT mice was 30 \pm 11 \mu mol\textpercm kg\textpercm g^{-1} \textpercm min^{-1}. The rate of glycogen synthesis in IRS-1^{-/-} mice was 19 \pm 8 \mu mol\textpercm kg\textpercm g^{-1} \textpercm min^{-1}, which was not significantly different from WT mice. In contrast, the rate of insulin stimulated hepatic glycogen synthesis was significantly reduced in IRS-2^{-/-} mice (4 \pm 1 \mu mol\textpercm kg\textpercm g^{-1} \textpercm min^{-1}, p < 0.05 versus WT mice or IRS-1^{-/-} mice). These findings demonstrate marked defects in insulin action in vivo in the liver of the IRS-2^{-/-} mice.

To determine the effects of deletion of IRS-1 and IRS-2 on lipid metabolism in vivo, we analyzed plasma glycerol and FFA concentrations and the rate of glycerol production both basally and during the clamp. Basal plasma glycerol and the basal rate of glycerol production were similar in mice from each group (Table I). In addition in mice of all three genotypes there was no change in the concentration of plasma glycerol in response to insulin. During the hyperinsulinemic-euglycemic clamp there was a comparable reduction in the rate of production of glycerol in WT mice and in IRS-1^{-/-} mice (WT mice, 28 \pm 9\% reduction; IRS-1^{-/-} mice, 30 \pm 12\% reduction; p < 0.05 versus basal period for each group). In contrast, we observed no reduction in the rate of production of glycerol in IRS-2^{-/-} mice during the hyperinsulinemic-euglycemic clamp with only a 5 \pm 6\% decrease from the basal period being achieved (p = not significant versus basal period).

Basal plasma FFA concentrations were similar in each group (Table I). During the hyperinsulinemic-euglycemic clamp there was a 48 \pm 3\% reduction in the concentration of FFA in WT mice and a 53 \pm 4\% reduction in the concentration of FFA in IRS-1^{-/-} mice (p < 0.05 versus basal concentration in each). In contrast, we observed only a minor reduction in the concentration of plasma FFA in IRS-2^{-/-} mice during the hyperinsulinemic-euglycemic clamp (i.e. only a 17 \pm 7\% decrease, p = not significant from basal concentrations). Taken together these findings suggest that IRS-2 plays a major role in regulating lipid metabolism in vivo in mice.

**DISCUSSION**

The role of IRS proteins as mediators of insulin signaling is well established (1, 2). However, despite the structural similarities between IRS-1 and IRS-2 and their co-expression in insulin-sensitive tissues, it has been shown that disruption of these proteins in mice yields distinct phenotypes (7–10). While the development of diabetes in IRS-2^{-/-} mice at least in part reflects the role of IRS-2 in beta cell function, it is less clear whether IRS-1 and IRS-2 play redundant or selective roles in mediating insulin action in insulin-sensitive tissues in vivo (9, 10). Thus in this study we determined the insulin responsiveness of carbohydrate and lipid metabolism in WT, IRS-1^{-/-} and
normoglycemic IRS-2⁻/⁻ mice in vivo to evaluate the functional roles of IRS-1 and IRS-2 in muscle, liver, and fat. Our findings demonstrate that in vivo IRS-1 mediates insulin action in skeletal muscle, while IRS-2 plays important roles in liver, muscle, and adipose tissue.

The deletion of IRS-1 or IRS-2 causes profound resistance to insulin-stimulated whole-body glucose utilization in vivo (Fig. 1A). Deletion of IRS-1 causes a marked defect in insulin-stimulated muscle glycogen synthesis. This is consistent with the previously reported finding of a 50% reduction in insulin-stimulated glucose transport in isolated skeletal muscle preparations (14). In contrast, deletion of IRS-2 has a small yet significant effect on insulin-stimulated muscle glycogen synthesis. We have previously shown that in vitro the absence of IRS-2 does not significantly impair insulin-stimulated glucose uptake in muscle (15). Although those observations were made in a distinct experimental model (15), they suggest that IRS-2 has a role in regulating glycogen synthesis distinct from mediating insulin-stimulated glucose transport in skeletal muscle. Taken together, our previous findings (14, 15), and our current observations, suggest that in vivo IRS-1 has a more important role than IRS-2 in the overall regulation of carbohydrate metabolism in muscle. Consistent with these observations Kido et al. (16) have recently demonstrated that mice with a combined heterozygous disruption of the insulin receptor and IRS-1 (IR⁻/⁻/IRS-1⁻/⁻) develop severe muscle insulin resistance and a reduction in insulin-stimulated phosphatidylinositol 3-kinase activation, the major signaling mediator of glucose transport and glycogen synthesis (16). In contrast, the muscle defects in IR⁻/⁻/IRS-2⁻/⁻ mice in these parameters are less marked (16).

It was well established that the regulation of hepatic glucose production by insulin is a major determinant of blood glucose concentrations (17). To further dissect the nature of the insulin resistance in IRS-1⁻/⁻ and IRS-2⁻/⁻ mice, we examined hepatic carbohydrate metabolism. Basal endogenous glucose production was similar in WT, IRS-1⁻/⁻, and IRS-2⁻/⁻ mice (Table I). Endogenous glucose production decreased by approximately 60% during the infusion of 5 milliunits of insulin-kg⁻¹min⁻¹ in overnight fasted WT mice (Fig. 1B). These data are consistent with the results of Shen et al. (18) who reported a dose response of insulin to glucose fluxes in awake mice.

Insulin-mediated suppression of endogenous glucose production was similar in WT and IRS-1⁻/⁻ mice (Fig. 1B). In contrast, there was virtually no response to endogenous glucose production in IRS-2⁻/⁻ mice (Fig. 1B). We have recently reported (9) a normal response of endogenous glucose production in IRS-2⁻/⁻ mice, infused with 2.5 milliunits of insulin-kg⁻¹min⁻¹, as compared with virtually no response to endogenous glucose production in IRS-2⁻/⁻ mice. In that study (9) we observed that endogenous glucose production was almost completely suppressed at 20 milliunits of insulin-kg⁻¹min⁻¹ in IRS-2⁻/⁻ mice, suggesting that the insulin resistance can be overcome at supraphysiologically insulin concentrations.

Coupled with our previous data (9), our current findings suggest that deletion of IRS-2 profoundly impairs hepatic carbohydrate metabolism in vivo. Furthermore our current observations on hepatic glycogen metabolism show defects in glycogen synthesis that are significantly more marked in IRS-2⁻/⁻ mice compared with the relatively mild abnormalities in the IRS-1⁻/⁻ mice. These findings are consistent with our analysis of phosphatidylinositol 3-kinase activation in the livers of IR⁻/⁻/IRS-2⁻/⁻ and IR⁻/⁻/IRS-1⁻/⁻ mice and the analysis of IR-deficient hepatocyte cell lines (16, 19).

There is increasing evidence that adipose tissue (e.g., presumably the release of free fatty acids) may modulate the functions of muscle, liver, and beta cell (20, 21). Indeed we have recently shown that lipid abnormalities impair insulin action in muscle (22, 23). The data presented here show that plasma FFA and glycerol concentrations and glycerol production were comparable in WT, IRS-1⁻/⁻, and IRS-2⁻/⁻ mice, suggesting that basal lipolysis rates were similar in animals from all three genotypes (Table I). In contrast, during the hyperinsulinemic-euglycemic clamp IRS-2⁻/⁻, but not IRS-1⁻/⁻, mice displayed an attenuated suppression of lipolysis suggesting an important role for IRS-2 in adipose tissue function. Isolated IRS-1⁻/⁻ adipocytes show that insulin-stimulated glucose transport is attenuated in vitro (7, 8). Additionally we have observed that isolated IRS-2⁻/⁻ adipocytes do not display significant defects in glucose transport. Taking together these observations suggest that IRS-2, but not IRS-1, may be required for different “anti-lipolytic” responses to insulin. For example, IRS-1 may be more important for re-esterification (perhaps via insulin-mediated effects on adipose tissue glucose utilization), whereas IRS-2 may be more important for the direct inhibition of lipolysis (perhaps via effects on hormone-sensitive lipase). Our experimental design only permits an assessment of lipolysis, we are unable to comment on the role of IRS-1 or IRS-2 with regards to adipose tissue glucose utilization in vivo.

Analysis of the phenotype of the IRS-1⁻/⁻ and IRS-2⁻/⁻ mice has suggested that IRS-1 and IRS-2 display unique roles in vivo. However, previous in vivo studies on IRS-1⁻/⁻ and IRS-2⁻/⁻ mice involved treating animals with large doses of insulin, administered to the intraperitoneal cavity, to examine signaling events in liver and muscle. Such “supraphysiologica” doses of insulin may potentially activate IGF-1 receptors (10, 16). The current study utilized a euglycemic clamp at physiological concentrations of insulin, thereby enabling us to develop a clearer picture of the role(s) of IRS-1 and IRS-2 in insulin-mediated metabolism. Thus our results demonstrate for the first time in vivo tissue-specific differences in the metabolic pathways regulated by IRS-1 and IRS-2. These signaling molecules appear to mediate divergent insulin-dependent metabolic events. Deletion of either IRS-1 or IRS-2 causes insulin resistance, which largely resides in skeletal muscle in IRS-1⁻/⁻ mice, whereas IRS-2⁻/⁻ mice have significant abnormalities in liver, muscle, and adipocyte function. The presence of marked skeletal muscle resistance, but not diabetes, in the IRS-1⁻/⁻ mice again demonstrates that the development of glucose intolerance requires defects in multiple sites, including the muscle, liver, and adipose tissue (17, 24). Our results emphasize the critical role of IRS-2 in integrating metabolic responses to insulin in liver, muscle, and fat and thus identifies IRS-2 and its downstream effectors as potential common pathway in the pathogenesis of Type 2 diabetes.

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