Lack of Insulin Receptor Substrate-2 Causes Progressive Neointima Formation in Response to Vessel Injury

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Background—Insulin resistance is associated with atherosclerosis, but its mechanism is unknown. It has been reported that insulin receptor substrate (IRS)-1 deficient (IRS-1−/−) mice showed insulin resistance without type 2 diabetes, whereas the IRS-2 deficient (IRS-2−/−) mice showed insulin resistance with type 2 diabetes.

Methods and Results—We investigated neointima formation in the IRS-1−/− and IRS-2−/− mice at 8 and 20 weeks. The IRS-2−/− mice showed much greater neointima formation than the IRS-1−/− and wild-type mice at 8 weeks. At 20 weeks, the IRS-2−/− mice had greater neointima formation than the IRS-1−/− mice, which showed more enhanced neointima formation than the wild-type mice. The IRS-1−/− and IRS-2−/− mice had dyslipidemia, hypertension, and insulin resistance. The IRS-2−/− mice had more metabolic abnormalities than the IRS-1−/− mice at 8 and 20 weeks. IRS-2 expression was detected, but IRS-1 expression was not detected in the vessels.

Conclusions—The neointima formation in the IRS-1−/− and IRS-2−/− mice appears to be related to abnormalities induced by the altered metabolic milieu in insulin-resistant states. Moreover, because neointima formation was much greater in the IRS-2−/− mice than in the IRS-1−/− mice at 8 and 20 weeks, it is suggested that a lack of IRS-2 renders the vasculature more susceptible to injury in the abnormal metabolic milieu, and IRS-2 may have a protective effect on neointima formation. We conclude that IRS-2 is protective and retards the development of neointima formation in insulin-resistant states. (Circulation. 2003;107:3073-3080.)

Key Words: atherosclerosis • insulin • vessels • risk factors

The pathogenesis of atherosclerosis involves complex interactions among multiple risk factors. Because insulin resistance is usually seen in patients with multiple risk factors for atherosclerosis, such as hypertriglyceridemia, hypercholesterolemia, type 2 diabetes, and hypertension, it is difficult to evaluate whether insulin resistance directly induces atherosclerosis, even though it has been recognized as an independent risk factor for atherosclerosis.1,2

Transgenic and knockout mouse technology may provide useful models with which to dissect the contribution of insulin resistance to the pathogenic mechanisms that lead to atherosclerosis.3-5 We previously generated two distinct murine models of insulin resistance: a representative murine model of insulin resistance and metabolic syndrome, that is, the insulin receptor substrate-1 deficient (IRS-1−/−) mouse,6 and a representative murine model of insulin resistance and type 2 diabetes, that is, the insulin receptor substrate-2 deficient (IRS-2−/−) mouse.7 However, the IRS-2−/− mice display only insulin resistance without hyperglycemia until 8 to 10 weeks of age. We used a cuff-injured model that has a quantitative and reproducible end point.8 In this model, external vascular cuff placement induces vascular neointima formation without the direct intraluminal injury that occurs, for example, with the balloon model, and this allows assessment of the effect of endothelial factors.

IRS-1 has been identified as a major substrate of both the insulin receptor and insulin-like growth factor-1 receptor tyrosine kinases.9 The IRS-1−/− mice have insulin resistance, hypertension, hypertriglyceridemia, and growth retardation6,10,11 but fail to show type 2 diabetes as the result of compensatory β-cell hyperplasia and hyperinsulinemia. IRS-2 has been identified as an IRS-1–independent pathway for signal transduction of insulin,12-14 and the IRS-2−/− mice have insulin resistance and type 2 diabetes after 8 to 10 weeks.
of age as the result of a combination of insulin resistance and lack of compensatory β-cell hyperplasia. Thus, IRS-1 and IRS-2 play overlapping and distinct roles in the regulation of glucose homeostasis.

To determine the role of IRS-1 and IRS-2 in the development of atherosclerosis, we investigated whether IRS-1<sup>−−</sup> and IRS-2<sup>−−</sup> mice show abnormalities in neointima formation in response to vessel injury.

### Methods

#### Animals

The IRS-1<sup>−−</sup> and IRS-2<sup>−−</sup> mice were maintained on the original C57BL6/CBA hybrid background. Two weeks after cuff placement, femoral arteries were fixed in situ with 10% formalin and embedded in paraffin. Continuous cross sections (5 μm) were then cut from one edge to the other edge of the cuffed portion. Morphometric analyses were performed on tissue stained for elastic fibers and with hematoxylin and eosin, as previously described, with some modifications.

#### Cuff Placement, Tissue Harvesting, and Morphometry

The experiment in this study was performed on 8- and 20-week-old male mice. Femoral artery cuff (polyethylene tube; inner diameter, 0.58 mm; length, 2 mm) placement was performed as previously described, with some modifications. Two weeks after cuff placement, femoral arteries were fixed in situ with 10% formalin and embedded in paraffin. Continuous cross sections (5 μm) were then cut from one edge to the other edge of the cuffed portion. Morphometric analyses were performed on tissue stained for elastic fibers and with hematoxylin and eosin, as previously described, with some modifications.

#### Antibody

Immunohistochemical staining was performed with an anti-α smooth muscle actin antibody (DAKO), anti–von Willebrand factor antibody (DAKO), anti–IRS-1 antibody (Santa Cruz Biotechnology, Inc), and anti–IRS-2 antibody (Santa Cruz Biotechnology, Inc).

#### Western Blot Analysis of the Aorta

For Western blot analysis of the aorta, the aorta was rapidly excised and freed of surrounding fat and connective tissue. It was then homogenized, and samples were precipitated with anti–IRS-1 antibody (Upstate Biotechnology) or anti–IRS-2 antibody (Upstate Biotechnology) for 1 hour. Immunoprecipitated fractions were separated by 6% or 7.5% SDS-PAGE and transferred to PVDF membranes. Blots were first incubated with anti–IRS-1 antibody or anti–IRS-2 antibody for 1 hour at room temperature, and detection was performed by immuno blotting with the antibody.

#### Poly(A)<sup>+</sup> RNA Preparation and RT-PCR

Poly (A)<sup>+</sup> RNA extraction was performed by an mRNA kit (Invitrogen Co). RT-PCR was performed in accordance with standard practices, using poly (A)<sup>+</sup> RNA from liver and artery. RT was performed for 50 minutes at 42°C with Super Script II (Invitrogen Co). RT-PCR was replicated a minimum of 5 times.

#### Blood Sample Assay

Fasting blood glucose was measured with an automatic glucometer (Glutest Ace, Sanwa Chemical Co). Plasma insulin (Shibayagi Co, Ltd), triglyceride, total cholesterol, free fatty acids, and HDL (Wako Pure Chemical Industries, Ltd) were assayed by enzymatic methods.

### Measurement of Blood Pressure and Heart Rate

Systolic blood pressure and pulse rate were measured with an automatic sphygmomanometer by the tail-cuff method in unanesthetized animals.

### Measurement of Isometric Force

The thoracic aorta of the IRS-1<sup>−−</sup>, IRS-2<sup>−−</sup>, and wild-type mice at 8 and 20 weeks was removed. It was placed in oxygenated and modified Krebs-Henseleit solution (KHS), as previously described. The tissue was placed in a well-displacement transducer (TB-612T; Nihon-Kohden). The relaxation response to acetylcholine (Ach, Daiichi Pharmaceutical Co, Ltd) and sodium nitroprusside (SNP, Sigma Chemical Co) was expressed as percentage decrease in the tension of contractile force induced by PGE<sub>2</sub> (Ono Pharmaceutical Co, Ltd).

### Statistical Analysis

Values are expressed as mean±SEM. Statistical analyses were performed by ANOVA, and post hoc analysis was performed by the Bonferroni/Dunn method. Probability values of <sup>P</sup>≤0.05 were considered statistically significant.

### Results

#### Increased Neointima Formation Is Induced by Vessel Injury in the IRS-2<sup>−−</sup> Mice

We placed a cuff around the femoral artery to induce inflammation of the adventitia and neointima formation. The IRS-1<sup>−−</sup>, IRS-2<sup>−−</sup>, and wild-type mice had no visible intima at baseline, and sham-operated vessels did not show evidence of intimal proliferation at 8 and 20 weeks (data not shown). At 8 weeks, the intimal thickness 2 weeks after cuff placement was greater in the IRS-2<sup>−−</sup> mice than in the IRS-1<sup>−−</sup> or wild-type mice. The intimal thickness in the IRS-1<sup>−−</sup> mice was not significantly different from that in the wild-type mice, but there was a trend toward less intimal thickness in the IRS-1<sup>−−</sup> mice (Figure 1A, a through f, and Figure 2A, b). There were no significant differences in medial thickness among the IRS-1<sup>−−</sup>, IRS-2<sup>−−</sup>, and wild-type mice at 8 weeks (Figure 2A, c). At 8 weeks, the intima (I)/media (M) volume ratio was much greater in the IRS-2<sup>−−</sup> mice (60±8%) than in the IRS-1<sup>−−</sup> (32±4%) or wild-type mice (40±5%). The I/M volume ratio in the IRS-1<sup>−−</sup> mice was not significantly different from that in the wild-type mice, but there was a trend toward a lower I/M volume ratio in the IRS-1<sup>−−</sup> mice (Figure 2A, d). There were also no significant differences in medial thickness among the IRS-1<sup>−−</sup>, IRS-2<sup>−−</sup>, and wild-type mice at 20 weeks (Figure 2B, c). At 20 weeks, the intimal thickness was significantly greater in the IRS-1<sup>−−</sup> and IRS-2<sup>−−</sup> mice than in the wild-type mice (95±17%) than in the IRS-1<sup>−−</sup> mice (67±8%, <sup>P</sup>≤0.08) and it was significantly greater in the IRS-1<sup>−−</sup> mice than in the wild-type mice (41±7%, <sup>P</sup>≤0.04) (Figure 2B, d). At 8 weeks, staining for von Willebrand factor as an endothelial cell marker was positive only in the endothelial cells in the cuff-injured vessels of the IRS-1<sup>−−</sup>, IRS-2<sup>−−</sup>, and wild-type mice (Figure 3, a through f). The majority of cells in the neointima of the cuff-injured vessels, as well as the cells in the media, were stained positive for α-smooth muscle actin at 8 weeks (Figure 3, a through f).
3, g through l). These findings suggest that the cells in the neointima may have migrated from the smooth muscle cells in the media. At 8 weeks, intimal thickness assessed by α-smooth muscle actin staining increased in the IRS-2−/− mice as compared with the IRS-1−/− and wild-type mice, whereas medial thickness was similar in all three genotypes.

**IRS-2 Is More Abundantly Expressed in Mouse Vessel Tissue Than IRS-1**

To investigate expression of IRS-1 and IRS-2 in vessel tissue, we performed immunohistochemical staining and Western blot analysis in the IRS-1−/−, IRS-2−/−, and wild-type mice. Staining of vessel tissue with anti–IRS-1 antibody was similar to staining with control serum in all three mouse genotypes (Figure 4, b through d), whereas the liver of the wild-type mice clearly stained with anti–IRS-1 antibody (Figure 4, a). Consistent with this, Western blot analysis with antibody for IRS-1 showed that IRS-1 was undetectable not only in the vessel tissue of the IRS-1−/− mice, as predicted, but in that of the IRS-2−/− and wild-type mice as well (Figure 4, q). RT-PCR with IRS-1 primers, however, showed that a small amount of IRS-1 mRNA was detectable in the vessel tissue of the IRS-2−/− and wild-type mice as well (data not shown). No staining of vessel tissue with anti–IRS-2 antibody was observed in the IRS-2−/− mice, but staining was clearly detected in the IRS-1−/− and wild-type mice (Figure 4, j through l). Western blot analysis with anti–IRS-2 antibody clearly detected IRS-2 in the vessel tissue of the IRS-1−/− and wild-type mice as well as in the liver of the wild-type mice but not in the vessel tissue of the IRS-2−/− mice (Figure 4, r). These findings indicate that IRS-2 is much more abundantly expressed in vessel tissue, including endothelial cells, than IRS-1. Jiang et al reported that both IRS-1 and IRS-2 were expressed in rat aorta. They made comparisons of IRS
between lean and obese animals but did not compare expression of IRS-1 and IRS-2 in the same animals. Our results clearly show that IRS-2 is much more abundantly expressed in mouse aorta than IRS-1. We compared IRS-2 expression in vessel tissue at 8 weeks with those at 20 weeks by immunohistochemical staining, Western blot analysis, and RT-PCR. Immunohistochemical staining and RT-PCR showed that IRS-2 expression was similar in the wild-type, IRS-1+/−, and IRS-2+/− mice. b, Intimal thickness 2 weeks after cuff placement. At 8 and 20 weeks, intimal thickness had increased significantly in IRS-2−/− mice compared with wild-type and IRS-1−/− mice. c, Medial thickness 2 weeks after cuff placement. Medial thickness did not differ among wild-type, IRS-1−/−, and IRS-2−/− mice at 8 and 20 weeks. d, I/M 2 weeks after cuff placement. At 8 weeks, I/M ratio showed significantly increased neointima formation in IRS-2−/− mice (I/M, 66.5±8%) compared with wild-type mice (I/M, 40.7±5%) or IRS-1−/− mice (I/M, 32.4%). At 20 weeks, I/M ratio showed significantly increased neointima formation in IRS-1−/− mice (I/M, 66.5±8%) and IRS-2−/− mice (I/M, 94.5±17%) compared with wild-type mice (I/M, 40.7±7.4%). *P<0.05; **P<0.005. A, Wild-type, n=17; IRS-1+/−, n=11; IRS-2+/−, n=10; B, wild-type, n=8; IRS-1−/−, n=13; IRS-2−/−, n=9.

IRS-1−/− and IRS-2−/− Mice Exhibit Hyperinsulinemia, Increased Free Fatty Acid, and Triglyceride Levels, and Hypertension

Blood glucose levels after a 16-hour fast did not differ significantly among the IRS-1−/−, IRS-2−/−, and wild-type mice at 8 weeks (Figure 6A, a) but were significantly higher in the IRS-2−/− mice than in the IRS-1−/− or wild-type mice at 20 weeks, indicating that the IRS-2−/− mice developed type 2 diabetes as previously described (Figure 6B, a). Plasma insulin levels were significantly higher in the IRS-1−/− and IRS-2−/− than in the wild-type mice at 8 and 20 weeks, indicating that the IRS-1−/− and IRS-2−/− mice were insulin-resistant (Figure 6A, b, and Figure 6B, b). Plasma free fatty acid levels were higher in the IRS-1−/− and IRS-2−/− mice than in the wild-type mice. However, they did not differ significantly between the IRS-1−/− and IRS-2−/− mice at 8 weeks (Figure 6A, c) or among the IRS-1−/−, IRS-2−/−, and wild-type mice at 20 weeks (Figure 6B, c). Plasma triglyceride levels were significantly higher in the IRS-1−/− and IRS-2−/− mice than in the wild-type mice at 8 weeks (Figure 6A, d) and were significantly higher in the IRS-2−/− mice than in the wild-type mice at 20 weeks. In addition, they tended to be higher in the IRS-1−/− mice than in the wild-type mice at 20 weeks (Figure 6B, d). Plasma total cholesterol levels were higher in the
IRS-2 mice than in the IRS-1 or wild-type mice at 8 and 20 weeks (Figure 6A, e, and Figure 6B, e), primarily as the result of increased plasma HDL levels (Figure 6A, f, and Figure 6B, f). Systolic blood pressure was significantly higher in the IRS-1 and IRS-2 mice than in the wild-type mice at 8 and 20 weeks (Figure 6A, g, and Figure 6B, g). Pulse rates did not differ significantly among the IRS-1, IRS-2, and wild-type mice at 8 and 20 weeks (Figure 6A, h, and Figure 6B, h).

Endothelium-Dependent Vascular Relaxation Is Impaired in 20-Week-Old IRS-1 and IRS-2 Mice
When PGF-induced (10^-6 to 3 x 10^-6 mol/L) contraction of aortic strips from the three mouse genotypes reached a plateau, ACh (10^-5 to 10^-7 mol/L) was added cumulatively and caused concentration-dependent relaxation of the aortic strips. The ACh-induced relaxation did not differ significantly among the IRS-1, IRS-2, and wild-type mice at 8 weeks (Figure 7, a), but at 20 weeks, the endothelium-dependent relaxation induced by ACh (10^-8 to 10^-7 mol/L) was significantly impaired in the IRS-2 mice compared with the IRS-1 or wild-type mice. The endothelium-dependent relaxation by ACh (10^-8 to 10^-5 mol/L) was also significantly impaired in the IRS-1 mice compared with the wild-type mice (Figure 7, c). By contrast, endothelium-independent relaxation induced by SNP (10^-10 to 10^-7 mol/L) did not differ significantly among the IRS-1, IRS-2, and wild-type mice at 8 and 20 weeks (Figure 7, b and d).

Discussion
In this study, we carried out cuff placement as an atherosclerotic model. This enabled us to quantify neointima formation reproducibly and differs from other atherosclerotic models, such as a filament model or a balloon injury model, in several important ways. The latter two models involve
removal of the endothelial layer, whereas in the cuff model, endothelial cells are not directly manipulated or removed, thereby allowing study of the effect of individual endothelial factors, as previously described.8 We do not consider the mouse cuff model to be an ideal model of human atherosclerosis; however, we believe it to be close to the initial lesion of human atherosclerosis (type 1 lesion). Neointima formation mainly consists of cell components and does not include lipid

Figure 4. Expression of IRS-1 and IRS-2 in wild-type, IRS-1−/−, and IRS-2−/− mice at 8 weeks. a through h, Immunohistochemical staining for IRS-1 (a through d) and its negative controls (e through h), i through p, Immunohistochemical staining for IRS-2 (I through l) and its negative controls (m through p). q, Western blot analysis for IRS-1; r, Western blot analysis for IRS-2. IRS-2 predominates over IRS-1 in vessel tissues, whereas IRS-1 and IRS-2 were expressed in the liver. Images (a through p) are at the same magnification (×400). q and r, Wild-type, n=10; IRS-1−/−, n=15; IRS-2−/−, n=10.

Figure 5. Expression of IRS-2 at 8 and 20 weeks in wild-type and IRS-1−/− mice. a, Immunohistochemical staining for IRS-2; b, Western blot analysis for IRS-2; c, RT-PCR with IRS-2 primers.
deposition. It is a simple lesion but not a complex one. We believe cuff-induced neointima formation is associated with inflammation of the adventitia. This is not an exact model of restenosis after angioplasty because it avoids direct injury of endothelial cells. However, the restenosis after angioplasty is thought to be mainly due to an increased cell component rather than lipid deposition, and the cuff model can be investigated for a potential link between insulin resistance and restenosis after percutaneous coronary intervention.

This is the first study to demonstrate that IRS-2 is protective against vascular neointima formation in response to vessel injury. At 8 weeks, although both IRS-1 and IRS-2 mice had similar levels of fasting blood glucose, fasting plasma insulin, fasting plasma free fatty acid, fasting plasma triglyceride, fasting plasma total cholesterol, fasting plasma HDL, systolic blood pressure, and pulse rate, IRS-2 mice had significantly lower fasting plasma insulin levels than IRS-1 mice (*P<0.05; **P<0.005; ***P<0.0001).

Figure 6. Comparison of risk factors for atherosclerosis. A, 8 weeks; B, 20 weeks; a, fasting blood glucose levels; b, fasting plasma insulin levels; c, fasting plasma free fatty acid levels; d, fasting plasma triglyceride levels; e, fasting plasma total cholesterol levels; f, fasting plasma HDL levels; g, systolic blood pressure; h, pulse rate. *P<0.05; **P<0.005; ***P<0.0001.

Figure 7. Vascular reactivity in the aortic strip. Relaxation response to ACh (left) and SNP (right) is expressed as a percentage of decreased tension of contractile force induced by 10^{-6} to 3×10^{-5} mol/L PGF_{2α}. Relaxation by ACh did not differ significantly among IRS-1, IRS-2, and wild-type mice at 8 weeks (a) but at 20 weeks was impaired at concentrations of 10^{-5} to 10^{-4} mol/L in IRS-2 mice compared with the IRS-1 and wild-type mice. At 20 weeks, relaxation by ACh (10^{-7.5} to 10^{-7} mol/L) was significantly impaired in IRS-1 mice compared with wild-type mice (c). Endothelium-independent vascular relaxation did not differ among IRS-1, IRS-2, and wild-type mice at 8 and 20 weeks (b and d). *P<0.05; **P<0.005; ***P<0.0001, IRS-1 and IRS-2 vs wild-type mice; †P<0.05; ††P<0.005; †††P<0.0001, IRS-1 vs IRS-2 mice. ACh: 8 weeks; wild-type, n=8; IRS-1, n=11; IRS-2, n=11; 20 weeks; wild-type, n=12; IRS-1, n=11; IRS-2, n=11; SNP: 8 weeks; wild-type, n=8; IRS-1, n=11; IRS-2, n=11; 20 weeks; wild-type, n=12; IRS-1, n=11; IRS-2, n=11.
IRS-2−/− mice showed hypertriglyceridemia, hypertension, and hyperinsulinemia, the metabolic abnormalities were greater in the IRS-2−/− mice than in the IRS-1−/− mice and the IRS-2−/− mice exhibited greater neointima formation than the IRS-1−/− and wild-type mice. At 20 weeks, the IRS-2−/− mice but not the IRS-1−/− mice had type 2 diabetes and the IRS-2−/− mice had greater neointima formation than the IRS-1−/− and wild-type mice. The IRS-1−/− mice failed to show greater neointima formation than the wild-type mice at 8 weeks but did show greater neointima formation at 20 weeks. Thus, the increased neointima formation in the IRS-1−/− and IRS-2−/− mice is likely to be related to abnormalities induced by the altered metabolic milieu in insulin-resistant states. Nevertheless, we cannot completely exclude the possibility that IRS-2 has a direct protective effect on neointima formation because IRS-2 was much more abundantly expressed than IRS-1 in the vessels and IRS-2 expression tended to be lower at 20 weeks than at 8 weeks in the IRS-1−/− mice. These findings collectively indicate that a lack of IRS-2 renders the vasculature more susceptible to injury in the abnormal metabolic milieu and that IRS-2 may have a protective effect on neointima formation.

Hypothesizing that the vascular endothelial cell dysfunction precedes the progression of atherosclerosis, we compared the endothelium-dependent vascular relaxation response among the IRS-1−/−, IRS-2−/−, and wild-type mice. The results showed that endothelium-dependent vascular relaxation was impaired in the IRS-2−/− mice at 20 weeks but not at 8 weeks, when the neointima of the IRS-2−/− mice began to be formed. There was mild impairment of endothelium-dependent vascular relaxation in the IRS-1−/− mice at 20 weeks, even though the IRS-1−/− mice did not show increased neointima formation at 8 weeks. On the basis of these observations, the endothelial cell dysfunction may not precede the formation of neointima, and it may be a consequence of the continued constellation of multiple risk factors rather than the lack of IRS-1 or IRS-2 in the vessels.

In summary, this study provides the first evidence that IRS-2 is protective and retards the development of neointima formation that occurs in response to vessel injury in insulin-resistant states.

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