Roles of Insulin Receptor Substrates in Insulin-Induced Stimulation of Renal Proximal Bicarbonate Absorption

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Insulin resistance is frequently associated with hypertension, but the mechanism underlying this association remains speculative. Although insulin is known to modify renal tubular functions, little is known about roles of insulin receptor substrates (IRS) in the renal insulin actions. For clarifying these issues, the effects of insulin on the rate of bicarbonate absorption (JHCO$_3^-$) were compared in isolated renal proximal tubules from wild-type, IRS1-deficient (IRS1$^{-/-}$), and IRS2-deficient (IRS2$^{-/-}$) mice. In wild-type mice, physiologic concentrations of insulin significantly increased JHCO$_3^-$$. This stimulation was completely inhibited by wortmannin and LY-294002, indicating that the phosphatidylinositol 3-kinase pathway mediates the insulin action. The stimulatory effect of insulin on JHCO$_3^-$ was completely preserved in IRS1$^{-/-}$ mice but was significantly attenuated in IRS2$^{-/-}$ mice. Similarly, insulin-induced Akt phosphorylation was preserved in IRS1$^{-/-}$ mice but was markedly attenuated in IRS2$^{-/-}$ mice. Furthermore, insulin-induced tyrosine phosphorylation of IRS2 was more prominent than that of IRS1. These results indicate that IRS2 plays a major role in the stimulation of renal proximal absorption by insulin. Because defects at the level of IRS1 may underlie at least some forms of insulin resistance, sodium retention, facilitated by hyperinsulinemia through the IRS1-independent pathway, could be an important factor in pathogenesis of hypertension in insulin resistance.

Insulin induces a variety of responses in many cell types, but its primary role is the maintenance of whole-body glucose homeostasis. Insulin action is initiated by activating tyrosine kinase in the β subunit of cell-surface receptor. The receptor then transmits a series of transphosphorylation reactions in several docking proteins, including insulin receptor substrates (IRS), among which IRS1 and IRS2 represent the two major substrates. These tyrosine phosphorylated substrates bind other Src homology 2 proteins, resulting in the activation of mitogen-activated protein kinase (MAPK) as well as phosphatidylinositol 3-kinase (PI3K) cascades. Whereas the activation of MAPK promotes transcription, the activation of PI3K further activates several serine/threonine kinases, including Akt and atypical protein kinase C isoforms, thereby facilitating the translocation of the glucose transporter GLUT4 into the plasma membrane (1).

Insulin resistance, characterized by inappropriate responses of peripheral tissues to a given dose of insulin, is caused by defects in these complex cascades of insulin signaling. As far as the pancreatic β cells can compensate for the insulin-resistant states by augmented secretion of insulin, however, overt type 2 diabetes may not occur. Nevertheless, insulin resistance with hyperinsulinemia is frequently associated with significant morbidity such as hypertension and other coronary risk factors (2,3). Although the precise mechanism by which insulin resistance leads to elevated BP still remains speculative, an attractive hypothesis is proposed that the hyperinsulinemia itself may contribute to hypertension by inducing renal sodium retention (4,5). The antinatriuretic action of insulin was indeed confirmed in human (6). Insulin is known to bind to most of the nephron segments and to modify several functions of renal tubules (7–12). In particular, insulin was shown to stimulate volume and bicarbonate absorption from isolated rabbit renal proximal tubules (13). However, little has been known about the signaling pathways of this insulin action. The most important question—how the antinatriuretic action of insulin could be preserved in insulin resistance—also remains unanswered. To clarify these issues, we compared the effects of insulin on isolated proximal tubules from wild-type (WT) and insulin-resistant mice. For the latter, we used IRS1-deficient (IRS1$^{-/-}$) and IRS2-deficient (IRS2$^{-/-}$) mice, which display insulin resistance through distinct mechanisms (14–16). Of note, IRS1$^{-/-}$ mice show the phenotype similar to type 2 diabetes at the prediabetes stage and are associated with hypertension (17).

Materials and Methods

Animals

WT, IRS1$^{-/-}$, and IRS2$^{-/-}$ mice were prepared by heterozygote intercrosses and were maintained on the original C57BL6/CBA hybrid background as described (14,15,18). Mice were housed on a 12-h light-
dark cycle and were given ad libitum access to regular diet, and male mice of 7 to 9 wk of age were used in this study. All experimental procedures were performed in accordance with the local institutional guidance.

**Microperfusion Technique**

Mice were anesthetized with pentobarbital sodium, and the thin sections from the left kidney were obtained and stored in ice-cold Ringer solution. Proximal tubules (S2 segment) were microdissected manually without collagenase treatment and then microperfused according to the method described by Burg et al. (19) with a modified version of the perfusion and sampling capillary system (20–22). The tubular lumen was perfused with Ringer solution, which contained 25 mmol/L HCO$_3^-$ and 40 mmol/L raffinose. DMEM that contained 1 μmol/L norepinephrine was used as bath perfusate, which had been shown to improve maximally the functions of isolated proximal tubules (20,23–25). The experimental chamber was perfused continuously at a rate of approximately 10 ml/min with prewarmed (37°C) and gas-equilibrated (5% CO$_2$/ 95% O$_2$) bath perfusate for 30 min, and the measurements of bicarbonate absorption rate (JHCO$_3^-$) were started. In preliminary experiments, we also tried to perfuse the initial segment of proximal convoluted tubules (S1 segment). However, the calculated JHCO$_3^-$ values varied considerably from tubules to tubules. We speculated that this could be due to the very high metabolic rates of S1 segment, which might somehow interfere with the complete preservation of tubular functions even in our improved in vitro incubation conditions. Therefore, only the S2 segment was used in this study.

**Determination of JHCO$_3^-$**

We used the stop-flow microfluorometric technique, which had been shown to be applicable for both rabbit and mouse proximal tubules (20–22). In brief, isolated tubules were mounted on the stage of an inverted epifluorescence microscope (IMT-2; Olympus, Tokyo, Japan), and a pH-sensitive fluorescence dye BCECF was added to the luminal perfusate. Luminal pH (pH$_L$) was monitored by a microspectrofluorometer system (OSIF-10; Olympus), which alternately illuminates the preparation with light of 440 and 490 nm and measures emission at the 530-nm wavelength. For determining JHCO$_3^-$, the rapid (approximately 80 nl/min) luminal perfusion was stopped abruptly by suddenly reducing the perfusion pressure from approximately 18 to 0 cmH$_2$O. After stop-flow, pH$_L$ fell from 7.4 to values near 6.8 within 30 s, where it remained virtually constant. This decrease in pH$_L$ reflects the gradual absorption of HCO$_3^-$ and the attainment of a steady-state zero net volume flux that develops because of the presence of poorly absorbable raffinose in the luminal perfusate as described (20). The decay in luminal HCO$_3^-$ concentration ([HCO$_3^-$]$_L$) was calculated from the changes in pH$_L$ and JHCO$_3^-$ was calculated from the following equation:

$$JHCO_3^- = \pi \times r^2 \times \left( [HCO_3^-]_L - [HCO_3^-]_0 \right) \times k$$

where $r$ is the luminal radius before stop-flow, [HCO$_3^-$]$_L$ and [HCO$_3^-$]$_0$ are [HCO$_3^-$]$_L$ before stop-flow and in the steady-state, respectively, and $k$ is the rate constant of [HCO$_3^-$]$_L$ decline. The correction for volume loss into the pipettes during stop-flow was achieved by using the decaying 440 nm (pH-insensitive) fluorescence signals as a marker of the residual luminal volume as described previously (20).

**Immunoblotting and Immunoprecipitation**

Immunoblotting and immunoprecipitation were performed as described previously with some modifications (26,27). For detection of Akt phosphorylation, thin slices of kidney cortex were obtained from mice. They were divided into pieces of small bundles, consisting mostly of proximal tubules. These samples were incubated at 37°C for 40 min in DMEM under 5% CO$_2$. After insulin was added for the indicated time, the samples were homogenized in ice-cold buffer A (25 mmol/L Tris-HCl [pH 7.4], 10 mmol/L sodium orthovanadate, 10 mmol/L sodium pyrophosphate, 100 mmol/L sodium fluoride, 10 mmol/L EDTA, 10 mmol/L EGTA, and 1 mmol/L PMSF) and centrifuged. Equal amounts (approximately 20 μg) of protein samples were obtained from the supernatants, separated by SDS-PAGE, and transferred to a nitrocellulose membrane. The membrane was incubated with anti-Akt or anti–phospho-Akt (Ser473) antibodies (Cell Signaling Technology, Beverly, MA) and then with horseradish peroxidase–conjugated anti-rabbit IgG. The signals were detected by an ECL Plus system (Amersham, Aylesbury, UK). In some experiments, isolated proximal tubules were used instead of kidney cortex samples. In this case, proximal tubules of approximately 1-mm length were dissected manually from thin kidney slices, and each sample contained approximately 100 tubules. For immunoprecipitation, kidney cortex samples were treated as described above and the supernatants were obtained. Equal amounts (approximately 200 μg) of protein samples were incubated with anti-IRS1 (α-IRS1) or anti-IRS2 (α-IRS2) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), followed by the addition of Protein G-Sepharose. The immunoprecipitates were washed with 1% Nonidet P-40–buffer A three times, then subjected to immunoblotting using α-IRS1, α-IRS2, or anti-phosphotyrosine (Upstate Biotechnology, Lake Placid, NY) antibodies as the primary antibody. For detection of Na$^+$/HCO$_3^-$ co-transporter NBC1, membrane-enriched fractions were obtained as described (27) from insulin-treated kidney cortex samples. Equal amounts (approximately 50 μg) of protein samples were incubated with anti-NBC1 (Chemicon, Temecula, CA) or anti–β-actin (Santa Cruz Biotechnology) antibodies as the primary antibody.

**Statistical Analyses**

The data were represented as mean ± SEM. Significant differences were determined by applying the paired or unpaired t test as appropriate.

**Results**

**Insulin Effects in WT Mice**

We first examined the effects of insulin on bicarbonate absorption from renal proximal tubules of WT mice. The JHCO$_3^-$ was measured by the stop-flow microspectrofluorometric method, which was shown to be applicable to both rabbit and mouse proximal tubules (20,22). Baum (13) reported that >10$^{-10}$ mol/L insulin had the stimulatory effect on volume and bicarbonate absorption from isolated rabbit proximal tubules that were bathed in Ringer solution. When isolated mouse proximal tubules were bathed in Ringer solution, however, we could not detect the stimulatory effect of insulin because of the rapid deterioration of JHCO$_3^-$ as reported (22). When tissue culture medium (DMEM), instead of Ringer, was used as peritubular perfusate, the rapid deterioration of JHCO$_3^-$ was prevented, but the stimulatory effect of insulin again was undetected. We therefore used DMEM that contained norepinephrine as peritubular perfusate, which has been
shown to improve maximally the functions of isolated proximal tubules (20,23–25). In this improved incubation condition, the addition of physiologic concentrations (10^{-10} and 10^{-9} mol/L) of insulin for 15 min indeed increased JHCO_{3^-} as shown in Figure 1A. Time control experiments without insulin showed that JHCO_{3^-} did not change during this period. Unlike in rabbit tubules (13), however, higher (>10^{-8} mol/L) concentrations of insulin did not increase JHCO_{3^-} in mice tubules. To examine the effect of insulin on the expression of basolateral Na^+HCO_{3^-} co-transporter NBC1, we performed immunoblotting analysis. However, incubation with either 10^{-9} or 10^{-7} mol/L insulin for 15 min did not significantly change the expression level of NBC1, as shown in Figure 1B.

To examine the signaling pathways of insulin in proximal tubules, we tested two different PI3K inhibitors, wortmannin and LY-294002 (28,29). The addition of 100 nmol/L wortmannin did not change the basal JHCO_{3^-} values (17.3 ± 1.2 versus 17.0 ± 1.3 pmol/cm per s; n = 5, NS). Similarly, the addition of 50 μmol/L LY-294002 did not change the basal JHCO_{3^-} values (16.5 ± 1.1 versus 16.8 ± 1.4 pmol/cm per s; n = 5, NS). However, both inhibitors completely inhibited the stimulatory effect of 10^{-9} mol/L insulin, as shown in Figure 1C. These results indicate that the PI3K pathway plays a major role in the stimulation of JHCO_{3^-} by insulin.

Akt is one of the main downstream effectors of the PI3K pathway and is shown to be involved in the PI3K-mediated stimulation of NHE3, the apical Na^+H^+ exchanger expressed in renal and intestinal epithelia (30–32). Therefore, we next examined the phosphorylation status of Akt by immunoblotting analysis. As shown in Figure 2A, the addition of insulin for 5 min dose-dependently induced Akt phosphorylation in the kidney cortex tissues. Densitometric analysis confirmed that >10^{-10} mol/L concentrations of insulin significantly enhanced the phosphorylation of Akt (Figure 2B). The Akt phosphorylation was very prominent at 5 min but was slightly attenuated at 15 min after the addition of insulin (Figure 2C). The insulin-induced Akt phosphorylation was almost completely inhibited by wortmannin (Figure 2D) or LY-294002 (data not shown), consistent with the results obtained by microperfusion experiments. To examine the origin of phosphorylated Akt in the kidney cortex, we performed a similar immunoblotting analysis using microdissected proximal tubules. As shown in Figure 2E, we confirmed that insulin indeed induced Akt phosphorylation in proximal tubules.

Roles of IRS in Insulin Signaling

The results thus far suggest that the stimulatory effect of insulin on bicarbonate absorption is mediated by the classical pathway involving IRS and PI3K. To examine the roles of IRS1 and IRS2 in insulin signaling in this segment, we performed immunoprecipitation followed by immunoblotting analysis on the kidney cortex of WT, IRS1^{-/-}, and IRS2^{-/-} mice. As shown in Figure 3A, the expression of IRS1 (approximately 175 kD) was confirmed in WT and IRS2^{-/-} but not in IRS1^{-/-} mice. In addition, the expression of IRS2 (approximately 190 kD) was confirmed in WT and IRS1^{-/-} mice but not in IRS2^{-/-} mice, as shown in Figure 3B. In anti-IRS1 immunoprecipitates, insulin induced only a faint approximately 175-kD band corresponding to tyrosine-phosphorylated IRS1 in WT and IRS2^{-/-} mice (Figure 3A). In anti-IRS2 immunoprecipitates, however, insulin induced a prominent approximately 190-kD band corresponding to tyrosine-phosphorylated IRS2 in WT and IRS1^{-/-} mice (Figure 3B).

To clarify further the relative importance of IRS1 and IRS2, we next compared the effects of insulin on proximal tubules from IRS1^{-/-} and IRS2^{-/-} mice using DMEM that contained norepinephrine as peritubular perfusate. The basal JHCO_{3^-} values of these mice were 17.2 ± 0.5 pmol/cm per s for IRS1^{-/-} mice (n = 31) and 16.9 ± 0.6 pmol/cm per s for IRS2^{-/-} mice (n = 30). These values were statistically not different from that
of WT mice (17.0 ± 0.5 pmol/cm per s; n = 41). As shown in Figure 4A, IRS1−/− mice showed very similar responses to insulin as WT mice, and the stimulatory effect was detectable at both 10−10 and 10−9 mol/L insulin. As shown in Figure 4B, by contrast, IRS2−/− mice showed attenuated responses to insulin as compared with WT mice, and the stimulatory effect was detectable only at 10−9 mol/L insulin. We confirmed that 100 nmol/L wortmannin completely inhibited the stimulatory effect of 10−9 mol/L insulin in both IRS1−/− (n = 5) and IRS2−/− mice (n = 5). Figure 4C compares the stimulatory effect of 10−9 mol/L insulin in WT, IRS1−/−, and IRS2−/− mice. The percentage of stimulation in IRS1−/− mice (26 ± 5%) was not significantly different from that in WT mice (26 ± 4%). However, the percentage of stimulation in IRS2−/− (14 ± 3%) was significantly less than that in WT (P < 0.05).

These results suggest that IRS1 but not IRS2 may be dispensable for the insulin-induced activation of the PI3K-Akt pathway in proximal tubules. To test for this view, we compared the insulin-induced Akt phosphorylation in kidney cortex of these mice. As shown in Figure 5A, the Akt phosphorylation by 10−7 mol/L insulin was comparable in WT and IRS1−/− mice. However, it was significantly attenuated in IRS2−/− mice. Densitometric analysis confirmed that the Akt phosphorylation was preserved in IRS1−/− mice but was reduced by approximately 50% in IRS2−/− mice compared with that in WT mice (Figure 5B). We also confirmed that the Akt phosphorylation by 10−9 mol/L insulin was similarly preserved in IRS1−/− mice but was significantly attenuated in IRS2−/− mice compared with that in WT mice (Figure 5C).

Discussion

In this study, we showed that physiologic concentrations of insulin stimulated bicarbonate absorption from isolated mouse renal proximal tubules. This stimulation was completely inhibited by the two different PI3K inhibitors, wortmannin and LY-294002. In addition, insulin induced the significant Akt phosphorylation in both kidney cortex tissues and pure proximal tubular samples, indicating that the PI3K pathway mediates the insulin action. It should be mentioned, however, that the stimulatory effect of insulin was detected only in the presence of norepinephrine. We showed previously that norepinephrine has a permissive role for the inhibitory effects of dopamine on proximal transport (25). Norepinephrine may also have a permissive role for insulin actions on transport process, at least in these experimental conditions. Whereas the stimulation of bicarbonate absorption by insulin was completely preserved in IRS1−/− mice, it was significantly attenuated in IRS2−/− mice. The insulin-induced Akt phosphorylation was similarly preserved in IRS1−/− mice but was markedly attenuated in IRS2−/− mice, suggesting that IRS1 but not IRS2...
might be dispensable in the activation of PI3K in proximal tubules. The comparison of insulin-induced tyrosine phosphorylation of IRS1 and IRS2 also supported this view. Unlike IRS1 mice, which show insulin resistance without diabetes (14,33), IRS2 mice show both insulin resistance and type 2 diabetes (15,34). However, blood glucose of our IRS2−/− mice does not significantly elevate until 10 wk of age (15), ruling out the influence of hyperglycemia. Although kidney development in IRS1−/− mice was found to be somewhat affected, especially in female mice (35), the relative roles of IRS1 and IRS2 in insulin modification of renal tubular functions had not been determined. Our study has revealed, for the first time to our knowledge, that IRS2 plays a major role in the stimulation of renal proximal absorption by insulin. Previous studies reported that Akt plays a critical role in the PI3K-mediated translocation of NHE3 into the apical plasma membrane in several cultured cells (30–32). Because a highly specific Akt inhibitor is not yet available, however, future studies using Akt1−/− (36) or Akt2-deficient mice (37) would be required to determine definitely the role of Akt in insulin-induced stimulation of bicarbonate absorption. It is currently unknown whether NHE3 undergoes the significant translocation in response to insulin in intact renal proximal tubules.

Renal proximal tubules reabsorb approximately 60% of the glomerular ultrafiltrate, which may have significant impacts on water and salt balance. The volume absorption in this segment is coupled to the active sodium and bicarbonate absorption. This process is accomplished by the coordinated operation of the apical Na+/H+ exchanger NHE3 and the basolateral Na+/HCO3− co-transporter NBC1, whereas their electrochemical driving forces are created by the Na+/K+ ATPase (38,39). Although the signaling pathways have not been clarified completely, insulin has been reported to stimulate all of the transporters involved in this transport process (8–10). Consistent with these stimulatory effects of insulin, Baum (13) reported that insulin stimulated volume and bicarbonate absorption from rabbit proximal tubules. In this study, we confirmed that the physiologic concentrations (10−10 and 10−9 mol/L) of insulin stimulated bicarbonate absorption in mouse proximal tubules. Because insulin acts on proximal tubules only from the basolateral (blood) side (13), these results indicate that changes in blood insulin levels may have significant influence on renal proximal transport in vivo. Unlike in rabbit proximal tubules, however, the higher concentrations (>10−8 mol/L) of insulin, despite the definite activation of PI3K-Akt pathway, no longer stimulated bicarbonate absorption in mouse proximal tubules.
In this regard, insulin is known to activate NHE1 and/or intracellular Na⁺ concentrations, which originate from either species difference or different metabolic conditions of isolated tubules (21,23–25), could potentially explain the diverse responses to the higher concentrations of insulin. Another possible explanation is that the pharmacologic concentrations of insulin might activate not only the PI3K pathway but also other unknown inhibitory pathways in mouse proximal tubules. It is interesting that a similar dual effect of IGF, involving both ERK-dependent MAPK and protein tyrosine kinase, on the apical K⁺ channel was reported recently in the thick ascending limb of rat kidney (41). Although insulin could cross-react on the receptors for IGF, IGF-I was reported to have no effects on proximal bicarbonate absorption (42). However, insulin is also known to stimulate NaCl transport in isolated medullary thick ascending limb of Henle (11). In this segment, however, much higher concentrations (>10⁻⁷ mol/L) of insulin were required to elicit the definite stimulation (11).

Recent progress in knockout technology has revealed that IRS1 and IRS2, the two major IRS, are not functionally interchangeable in many tissues (14,15,33). For example, IRS2 may have a major role in hepatic insulin action and pancreatic β cell development, whereas IRS1 may have a major role in glucose uptake in skeletal muscle and adipose tissue (26,43,44). It is interesting that accumulating evidence suggests that defects at the level of IRS1 frequently underlie some forms of insulin resistance. Thus, in skeletal muscle from obese subjects, a significant reduction was observed in IRS1 content, insulin-stimulated IRS1 phosphorylation, and PI3K activation (45). Impairment in insulin-stimulated IRS1 phosphorylation was also reported in skeletal muscle from pregnant obese women with and without gestational diabetes (46). This defect could be due to decreased expression of IRS1, whereas IRS2 expression seemed to be increased (46). In isolated adipocytes from patients with type 2 diabetes and insulin resistance, IRS1 expression was reduced, whereas IRS2 levels remained unchanged (47). In addition, low IRS1 gene and protein expression in adipocytes was found in approximately 30% of two groups of healthy individuals who were at high risk for type 2 diabetes: Those of first-degree relatives of patients with type 2 diabetes and another group with morbid obesity (48). From these and other observations, some investigators consider low expression of IRS1 in target tissues of insulin action as a molecular marker of insulin-resistant states such as obesity and type 2 diabetes (44). At present, little is known about the renal expression levels of IRS1 and IRS2 in individuals with insulin resistance. However, the IRS1-independent stimulation of renal proximal absorption, identified in our study, could provide a novel mechanism by which hyperinsulinemia promotes sodium retention, at least in some forms of insulin resistance. Multiple factors may be involved in the development of hypertension associated with insulin resistance. For instance, impairment of endothelium-dependent vascular relaxation was reported in both IRS1⁻/⁻ and IRS2⁻/⁻ mice, which might partly contribute to hypertension in these mice (17,18). Activation of the renin-angiotensin and sympathetic nervous systems also could be involved in obesity-associated hypertension (49), and the renal hemodynamic effects of angiotensin II seemed to be enhanced in type 2 diabetes (50). Nevertheless, sodium retention, facilitated by elevated blood insulin levels within the physiologic ranges, could be another important factor in the pathophysiology of hypertension. Consistent with this view, the antinatriuretic action of insulin was reported to be preserved in humans with insulin resistance (4,5).

In summary, we identified that IRS2 plays a major role in insulin stimulation of renal proximal transport. The IRS1-independent stimulation of proximal absorption may contribute, at least partially, to sodium retention in insulin-resistant states
and could be a potential therapeutic target in the prevention of hypertension.

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References
34. Withers DJ, Gutierrez JS, Towery H, Burks DJ, Ren JM, Previs S, Zhang Y, Bernal D, Pons S, Shulman GI, Bonner-Weir S, White MF: Disruption of IRS-2 causes type 2 diabetes in mice. Nature 391: 900–904, 1998