Tubular Effects of Insulin

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The direct effect of insulin on NaCl transport in proximal tubules and thick ascending limbs of Henle's loop were examined. First, the effects of insulin on intracellular pH (pHi) in the *in vitro* microperfused rabbit S2 proximal straight tubules (PST) were examined using a fluorescent technique. Addition of insulin to the bath increased pHi in a dose-dependent manner at concentrations ranging from 10^{-11} to 10^{-6} mol/l, and its ED_{50} was 10^{-9} mol/l. The insulin-induced pHi increase was almost completely inhibited by 10^{-3} mol/l amiloride in the lumen, indicating that insulin activates luminal Na/H exchange in PST. Next, the effect of insulin on the transepithelial voltage (V_t) and lumen-to-bath Cl flux (J_{Cl}) were examined in the *in vitro* microperfused rabbit medullary thick ascending limbs of Henle's loop (MTAL). Insulin in the bath increased V_t in a dose-dependent manner, and its ED_{50} was 5×10^{-9} mol/l. Insulin significantly increased J_{Cl} . The insulin-mediated increase in V_t was abolished by ouabain and furosemide. Dibutyryl-cAMP (dbcAMP) increased V_t and J_{Cl} . H-8 abolished the effect of dbcAMP, while it did not inhibit the actions of insulin. Removal of extracellular Ca did not affect the effects of insulin on V_t and J_{Cl} . Chelation of intracellular Ca with BAPTA/AM inhibited the actions of insulin without affecting basal values. Calmodulin (CaM) inhibitors, trifluoperazine and W-7, inhibited the actions of insulin more than 90%. These results indicate that insulin directly increases NaCl reabsorption in the MTAL, which requires the activation of Ca-CaM system, independent of the adenylate cyclase-cAMP-PKA system. In conclusion, insulin directly stimulates NaCl reabsorption in the *in vitro* microperfused rabbit PST and MTAL. (*Hypertens Res* 1996; 19 Suppl. 1: S41-S45)

Key Words: kidney, *in vitro* microperfusion, NaCl, proximal tubule, thick ascending limb

Insulin is considered to have a sodium-retaining action in the kidney, thereby involved in the pathophysiology of hypertension (1, 2). Apparently insulin has a direct effect on tubular transport, as insulin reduces urinary sodium excretion without altering renal hemodynamics or glomerular filtration rate. Moreover, insulin increases free water clearance, indicating that the hormone increases sodium reabsorption in diluting segments (1). As for insulin receptors, specific binding is greatest in the thick ascending limb, distal convoluted tubules and proximal tubules (3).

Baum reported that insulin stimulates reabsorption of fluid, Cl, HCO_3 and probably also Na in isolated perfused rabbit proximal convoluted tubules (4). In proximal tubules, Na reabsorption across the brush border membrane depends on Na/H exchange driven by an electrochemical gradient created by Na/KATPase (5). Renal acidification in this segment is mediated mainly by Na/H exchange in the luminal membrane and Na- HCO_3 cotransporter in the basolateral membrane (6, 7). Na and HCO_3 reabsorptions are mainly accomplished by active transport, which involves Na/H exchange. However, insulin effect on Na/H exchange is controversial. Fine and colleagues demonstrated that insulin increases Na/H exchange in primary cultures of rabbit

proximal tubular cells (8). In contrast, Mellas and co-workers reported that insulin had no stimulatory effect on Na/H exchange in canine proximal tubular cells (9). We here examined whether insulin activates Na/H antiport in proximal tubules and stimulates electrolyte transport by monitoring intracellular pH (pHi) using a fluorescent technique.

Although insulin is suggested to increase Na reabsorption in diluting segments such as the thick ascending limb of Henle's loop (TAL), previous *in vivo* reports did not elucidate whether insulin modulates NaCl absorption by its direct effect on renal tubules or by its effects on local or systemic factors that affected NaCl transport in renal tubules (3, 10). Indeed, Friedberg and associates recently demonstrated in humans that increased Na reabsorption in the diluting segment during insulin infusion is largely secondary to insulin-induced hypokalemia, because Na retention by insulin could be prevented by simultaneous K infusion in an amount sufficient to prevent a fall in plasma K concentration (11). These results contradict the idea that insulin exerts direct tubular effects. Therefore we examined whether insulin directly affects NaCl reabsorption in TAL using isolated perfused tubules.

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Methods

In Vitro Microperfusion of Isolated Proximal Tubules

S2 segments of proximal straight tubules (PST) of female Japanese white rabbits were dissected and perfused on the stage of an inverted microscope (IMT-2; Olympus, Tokyo, Japan) as previously described (12). The perfusion chamber was continuously perfused with isotonic standard saline preheated to 38°C. The perfusate and the bathing solution were identical and the composition of the solutions was (in mmol/l), 120 NaCl, 3 KCl, 1.5 CaCl₂, 1 MgCl₂, 2 KH₂PO₄, 25 NaHCO₃, 5.5 glucose and 5 L-alanine gassed with 5% CO₂-95% O₂, or 135 NaCl, 3 KCl, 1.5 CaCl₂, 1 MgCl₂, 2 KH₂PO₄, 10 Hepes, 5.5 glucose and 5 L-alanine, adjusted to pH 7.4 by NaOH and gassed with 100% O₂. The tubules were loaded with the acetoxymethyl derivative of a pH sensitive dye 2',7'-bis-(2-carboxyethyl)-5,6-carboxyfluorescein (BCECF/AM) in 10 μ M from the lumen (12). Fluorescence was measured by a fluorescence microscope equipped with a photomultiplier (OSP-PMU, Olympus) alternately at 490 and 440 nm excitation, and an emission wave length of 530 nm. The excitation ratio was converted to pHi (12).

In Vitro Microperfusion of Isolated Medullary Thick Ascending Limbs

The medullary thick ascending limbs (MTAL) of female Japanese white rabbits were microdissected and microperfused *in vitro* as previously described (13). The perfusate and the bathing solution were identical and the composition of the solutions was (in mmol/l) 120 NaCl, 3 KCl, 1.5 CaCl₂, 1 MgCl₂, 2 KH₂PO₄, 10 Hepes, 5.5 glucose, and 5 L-alanine, adjusted to pH 7.4 by NaOH and gassed with 100% O₂. Then the transepithelial voltage (Vt) and Cl concentration of the perfusate were measured (13). Because TAL is water impermeable, we can calculate J_{Cl} from the decrease in luminal Cl concentration (13).

The present study was carried out in accordance with the Guide for Animal Experimentation, Tohoku University and Tohoku University School of Medicine.

Chemicals

Bovine insulin, dibutyryl-adenosine 3',5'-cyclic monophosphate (dbcAMP), trifluoperazine, and W-7 were purchased from Sigma Chemical (St. Louis, MO, USA). BCECF/AM was from DOJIN Chemical Institute (Kumamoto). H-8 was purchased from Seikagaku (Tokyo). All other chemicals were purchased from Wako Pure Chemical Industries (Osaka).

Statistical Analysis

The data were analyzed using the paired *t*-test. Results are reported as mean $\pm$ SE. A *p* value of < 0.05 was accepted for statistical significant.

Fig. 1. Effects of insulin on pHi of S2 PST. In a CO₂-HCO₃-buffered solution, insulin (10⁻⁶ mol/l) added to the bath gradually increased pHi (thin line). In a Hepes-buffered solution, insulin (10⁻⁶ mol/l) added to the bath immediately increased pHi (thick line). In the presence of amiloride (10⁻³ mol/l) in the lumen, insulin-induced pHi increase was abolished (broken line).

Results

Effect of Insulin on pHi in PST

In a CO₂-HCO₃-buffered solution, the addition of insulin (10⁻⁶ mol/l) to the bath gradually increased pHi from 7.11 $\pm$ 0.02 to 7.26 $\pm$ 0.02 (*n* = 4, *p* < 0.005) at a rate of 0.0272 $\pm$ 0.0069 (dpHi/min), and it reached steady-state within 10 min (Fig. 1). The insulin-induced alkalization of pHi may be the results of various combinations of interactions between stimulating and/or inhibiting acid-base transporters such as Na/H exchanger, Na/HCO₃ cotransporter, Cl/HCO₃ antiporter and H ATPase. In a Hepes-buffered solution, the contribution of transporters involving HCO₃ becomes minimal. In a Hepes-buffered solution, insulin (10⁻⁶ mol/l) rapidly increased pHi from 7.11 $\pm$ 0.04 to 7.26 $\pm$ 0.04 (*n* = 7, *p* < 0.0001) at a rate of 0.416 $\pm$ 0.126 (dpHi/min), which reached steady state within a minute (Fig. 1). Insulin increased pHi in a dose-dependent manner and at physiological concentrations. These results strongly suggest that the transporter contributing to insulin-mediated pHi increase is Na/H exchanger. To confirm this hypothesis, we examined the effects of insulin in the presence of 10⁻³ mol/l amiloride, an inhibitor of Na/H antiport, in the lumen. The increase in pHi by insulin was almost completely inhibited by amiloride (Fig. 1), indicating that the insulin-induced increase in pHi is largely mediated by the stimulation of luminal Na/H exchange.

Effect of Insulin on Electrolyte Transport of MTAL

The addition of insulin (10⁻⁶ mol/l) to the bath gradually increased Vt from 3.1 $\pm$ 0.3 to 5.7 $\pm$ 0.3 mV (*n* = 12, *p* < 0.0001, Fig. 2). The effect of insulin was observed in a dose-dependent manner and at physiological concentrations and its ED₅₀ was 5 $\times$ 10⁻⁹ mol/l. Moreover, insulin (10⁻⁶ mol/l) increased J_{Cl} from 143 $\pm$ 15 to 292 $\pm$ 37 pmol·mm⁻¹·min⁻¹ (*n* = 5, *p* < 0.005), indicating that Vt reflects increase in NaCl reabsorption.

Several peptide hormones such as vasopressin, calcitonin, glucagon, and β -adrenergic agonists have been shown to increase NaCl reabsorption by stimu-

Fig. 2. Effects of insulin on V_t of MTAL. Insulin (10^{-6} mol/l) added to the bath gradually increased V_t , which reached a maximal value within 20 min. Elimination of insulin gradually decreased V_t to the control level.

lating adenylate cyclase in TAL. Therefore we examined whether cAMP also contributes to the effect of insulin in MTAL. Insulin further increased V_t in the presence of dbcAMP (control, 3.3 ± 0.3 mV; dbcAMP in 5×10^{-4} mol/l, 4.8 ± 0.1 mV; dbcAMP + insulin in 10^{-6} mol/l, 6.6 ± 0.4 mV; $n = 4$, $p < 0.005$). In addition, a selective inhibitor of cAMP-dependent protein kinase (PKA), H8 (5×10^{-5} mol/l), did not inhibit the insulin stimulated increase in V_t , whereas it inhibited the increase in V_t by dbcAMP, suggesting that the effect of insulin on NaCl transport in MTAL is not mediated by adenylate cyclase-cAMP-PKA cascade.

As Ca is suggested to be involved in the signal transduction of insulin in glucose transport in adipocytes, we examined the role of Ca in insulin-stimulated Cl transport in MTAL. Removal of extracellular Ca with EGTA did not affect the insulin-stimulated increase in V_t . In addition, when intracellular Ca ($[Ca^{2+}]_i$) was measured using fura-2, insulin did not change $[Ca^{2+}]_i$. However, chelation of intracellular Ca with BAPTA/AM inhibited the actions of insulin without affecting basal values. Furthermore, calmodulin (CaM) inhibitors, trifluoperazine and W-7 inhibited the actions of insulin more than 90%. These results indicate that Ca-CaM system is important in the actions of insulin in MTAL.

Discussion

In the present study we examined the direct effects of insulin on electrolyte transport in the isolated *in vitro* microperfused PST and MTAL.

Effects of Insulin on pH_i of PST

The results indicate that the insulin-induced pH_i increase is largely dependent on the stimulation of luminal Na/H exchange, which is the key transporter in Na, Cl and HCO_3^- reabsorption in the proximal tubules. Brush border Na/H exchange is regulated by various hormones and pathophysiological states. For example, glucocorticoids (14), thyroid hormones (15) and metabolic acidosis (16) increased Na/H exchange in brush border membrane vesicles isolated from rats. Insulin and prostaglandin E_1 stimulated Na/H exchange in cultured proximal tubular cells (8). Gesek and coworkers demon-

strated that insulin and hormones that stimulate Na/H exchange (α_1 adrenoceptor agonists, α_2 adrenoceptor agonists and angiotensin II) had no synergistic effects; in addition, with hormones that inhibit Na/H exchange (parathyroid hormone, dopamine), insulin stimulation was decreased (17). In our study, the interaction between insulin and other hormones that modulate Na/H exchange was not examined.

The apical membrane as well as the basolateral membranes of the proximal tubules have insulin receptors (18, 19). We also tested the effect of insulin in the lumen and obtained the result that pH_i was unchanged (data not shown). Our data are in good agreement with that by Baum (4). Therefore, insulin binding capacity of brush border membranes may indicate the presence of its clearance receptor and that luminal insulin plays no significant role on water and electrolyte transport of proximal tubules (20).

It has been shown that insulin stimulates Na/K-ATPase in the basolateral membrane of the rabbit renal cortex (21) and dog kidney (22). Feraille *et al.* have demonstrated that insulin increases the initial rate of ouabain-sensitive ^{86}Rb uptake in rat proximal convoluted tubule (23). Therefore insulin-induced stimulation of Na/H exchange may be the result of Na/K-ATPase activation. However, Hunley and coworkers demonstrated that insulin-induced stimulation of proximal tubule volume reabsorption in rabbits is not associated with changes in cAMP or Na/K-ATPase activity (24). Gesek and Schoolworth demonstrated in rat proximal tubule suspension that insulin stimulated amiloride-sensitive Na uptake in the presence of a Na/K-ATPase inhibitor. Therefore an Na/K-ATPase-independent mechanism can not be ruled out. In contrast to the results of our study, in micropunctured proximal tubules hyperinsulinemia decreased Na reabsorption (2) or had no effect on proximal tubule fluid or Cl reabsorption under sustained volume expansion in euglycemic rats (25). This discrepancy may be due to initial insulin level and nephron heterogeneity as well as to differences in species and experimental conditions. The existence of other hormones may also account for the difference.

Effect of Insulin on Electrolyte Transport of MTAL

The isolated perfused tubule is a preferred model for examining the direct effects of insulin on renal tubules, because this preparation allows examination without influence of *in vivo* conditions. In TAL, transepithelial Cl reabsorption occurs as a secondary active process via an apical Na-K-2Cl cotransporter. As shown by our data, both furosemide in the lumen and ouabain in the bath abolished the effect of insulin on V_t , indicating that insulin stimulates transcellular Cl reabsorption. The present data, however, do not provide information about which transporter insulin primarily activates. Feraille and associates demonstrated in rat kidney that insulin inhibited the rate of ouabain-sensitive ^{86}Rb uptake in the TAL by 44% indicating the inhibitory effect of insulin on Na/K-ATPase (23). It seems unlikely that the inhibition of Na/K-ATPase results in

the stimulation of transcellular NaCl reabsorption. At present, we do not know whether this is also the case in rabbit MTAL.

A PKA inhibitor did not inhibit the actions of insulin on V_t or J_{Cl} , but did inhibit the effect of dbcAMP, indicating that the effect of insulin is independent of adenylate-cyclase cAMP-PKA system. In agreement with the present results, Morel reported that insulin did not affect adenylate cyclase activity in the rabbit nephron (26). It has been demonstrated that various short-term effects of insulin are mediated either by protein phosphorylation brought about by activation of the intrinsic tyrosine kinase activity of the insulin receptors or by intracellular production of phosphatidylinositol glycan and diacylglycerol from membrane glycopospholipids (27-29). In our preliminary study, genistein, a tyrosine kinase inhibitor, inhibited the effects of insulin in MTAL.

We have demonstrated that neither extracellular Ca removal nor verapamil affects the actions of insulin in MTAL (30), suggesting that extracellular Ca or Ca influx is not necessary for the actions of insulin. The antagonizing effects of the Ca channel blocker on insulin antinatriuresis may be mediated by changes in renal medullary hemodynamics. The renal medullary hemodynamics may be important for insulin antinatriuresis in addition to the direct tubular effect of insulin. Although extracellular Ca or Ca influx is not necessary for insulin-stimulated NaCl transport in MTAL, it is possible that the actions of insulin are due to the involvement of intracellular Ca. Direct measurements of $[Ca^{2+}]_i$ with fura-2 showed that insulin did not change $[Ca^{2+}]_i$ in MTAL cells, but chelation of intracellular Ca by BAPTA/AM inhibited insulin-stimulated V_t , indicating that intracellular Ca is required for the actions of insulin in MTAL. Furthermore, two chemically dissimilar CaM inhibitors inhibited the actions of insulin. Previous reports have provided evidence of interaction between insulin receptor and CaM. Insulin directly stimulates CaM binding to plasma membranes (31). The insulin receptor contains a CaM-binding domain (32). CaM enhances insulin-stimulated phosphorylation of the β subunit of the insulin receptor, and insulin also stimulates phosphorylation of CaM (33). Our results suggest that CaM mediates insulin receptor or post-receptor signal transduction.

In summary, insulin stimulates Na reabsorption by activating luminal Na/H exchange in rabbit S2 PST. Insulin directly increases NaCl reabsorption in MTAL, which requires the activation of intracellular Ca-CaM system, independent of the adenylate cyclase-cAMP-PKA system.

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