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A PUTATIVE SECOND MESSENGER OF INSULIN ACTION REGULATES HEPATIC MICROSOMAL GLUCOSE-6-PHOSPHATASE

Susumu Suzuki, Takayoshi Toyota, Hiroshi Suzuki and Yoshio Goto

Third Department of Internal Medicine, Tohoku University
School of Medicine, Sendai 980, Japan

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SUMMARY. Physiological concentrations of insulin suppressed rat liver microsomal glucose-6-phosphatase activity in vitro. To attest a hypothesis that a putative second messenger of insulin action (insulin mediator) mediated this process, we isolated the low molecular factor from insulin-treated plasma membranes of rat liver, which was acid- and heat-stable substance of a peptide nature. The insulin mediator which was proved to activate the mitochondria pyruvate dehydrogenase suppressed microsomal glucose-6-phosphatase. The insulin mediator was linked to suppression of the gluconeogenic enzyme through the control of non-specific phosphohydroxylase.

A number of recent studies have presented evidence to suggest that the interaction of insulin with its receptor generates a low molecular weight and acid-stable mediator, which modulates the activity of various insulin-sensitive enzymes (1,2). These enzymes which have been shown to be altered by the addition of this mediator in a cell-free system include pyruvate dehydrogenase (1), glycogen synthetase phosphatase, cyclic AMP dependent protein kinase (2), low-Km phosphodiesterase (3), Ca^{++} Mg^{++} ATPase (4), adenylate cyclase (5) and acetyl-CoA carboxylase (6). The putative insulin mediator has been prepared from rat skeletal muscle (2), adipocyte (7), H4-II-EC3' hepatoma cell (8), human IM-9 lymphocyte (9), and isolated from plasma membrane of rat adipocyte (10), liver (11), and human placenta (12). Insulin treatment has shown that an increase in release of this mediator from plasma membrane of adipocyte and liver is dose-dependent (11,13).

Physiological concentrations of insulin suppressed rat liver microsomal glucose-6-phosphatase (G-6-Pase) in vitro (14). We supposed that this

Abbreviations: G-6-Pase, glucose-6-phosphatase; PDH, pyruvate dehydrogenase

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suppressing effect of insulin on G-6-Pase would be mediated by the insulin mediator released from liver plasma membrane in response to insulin bound to its receptor. In the present study, we prepared the insulin mediator extract from insulin-treated liver plasma membrane which was shown to activate mitochondria pyruvate dehydrogenase (PDH). We evaluated the effects of the insulin mediator on G-6-Pase and defined the mechanism of insulin action on G-6-Pase.

METHODS

Materials. Porcine monocomponent insulin was purchased from Novo. [1-^{14}C]-pyruvic acid (8 mCi/mmol) was obtained from New England Nuclear. All other chemicals were analytical grade.

Subcellular Fractionation of Liver. Male Sprague-Dawley rats weighing about 150g were fasted for 14 hr. Rat liver plasma membranes, mitochondria and microsomes were prepared according to the method of Fleischer and Kervina (15), with modifying the homogenized medium by omission of EDTA. Intactness of microsome preparations was assessed by the latency of low-Km mannose-6-phosphate phosphohydroxylase activity, as described by Arion (16). Microsomal preparations were intact when the latency was greater than 90%. Triton X-100 modified microsomes were prepared by the procedure with addition of one volume of 0.2% Triton X-100 in 50 mM Tris-HCl buffer, pH 7.4 to equal volume of intact microsomes (4 mg/ml) at 4°C for 30 min.

Preparation of Insulin Mediator Extracts from Liver Plasma Membranes. Liver plasma membranes (final 0.2 mg/ml) were incubated with or without porcine monocomponent insulin (100 μU/ml) in 50 mM Tris-HCl buffer, pH 7.4, 0.01% BSA at 37°C for 5 min in a volume of 0.5 ml. These incubation mixtures were deproteinized by boiling at 100°C for 5 min and added by 0.5 ml of 2 M formic acid. After centrifugation at 15,000g for 10 min, the supernatants were removed and treated with activated charcoal (final 0.2 mg/ml) at 4°C for 10 min. The supernatants separated from charcoal by centrifugation at 15,000g for 10 min were lyophilized. The lyophilized extracts were resuspended in 250 μl of 2 mM formic acid, of which 50 μl was tested for the ability to stimulate PDH and suppress G-6-Pase.

Measurement of G-6-Pase Activity. Intact or Triton X-100 treated microsomes (200 μg of protein) were preincubated with indicated concentrations of insulin (0-10 mU/ml) in 100 μl of 50 mM Tris-HCl buffer, pH 7.4, 0.01% BSA at 20°C for 5 min. G-6-Pase activity was measured by the method of Harper (17). Briefly, 100 μl of 80 mM glucose-6-phosphate in 0.2 M imidazole-HCl buffer, pH 6.5, was added to the preincubation mixtures at 20°C for 5 min. The final pH was about 6.6. The reaction was terminated by the addition of 1 ml of 10 % trichloroacetic acid and release of inorganic phosphate was assayed according to the method of Chen (18).

Intact microsomes were preincubated with extracted insulin mediator preparations and then G-6-Pase was assayed as described above.

Measurement of PDH Activity. Liver mitochondria were preincubated with 250 μM ATP in 50 mM potassium phosphate buffer, pH 8.0, 50 μM $CaCl_2$, 50 μM $MgCl_2$, at 37°C for 5 min. Following incubation, the mitochondria were twice washed with chilled potassium phosphate buffer and nucleotides were discarded by centrifugation. This treatment of mitochondria with ATP resulting 50-80% decrease of PDH activity, was prerequisite to detect the insulin mediator substantially stimulating PDH activity.

Extracted insulin mediator from liver plasma membranes was incubated with ATP-treated mitochondria (500 μg of protein) at 37°C for 5 min in the potassium phosphate buffer in a volume of 100 μl. PDH activity was assayed as follows; 100 μl of 0.5 mM [1-^{14}C]-pyruvic acid, 2 mM dithiothreitol, 0.2 mM CoA, 0.2 mM cocarboxylase and 1 mM β-NAD in the same buffer was added to the incubation mixtures, as described by Seals and Jarett (19). After incubation at 37°C for 5 min, the reaction was terminated by the addition of 200 μl of 8 M H_2SO_4. The release of $^{14}CO_2$ was trapped to 200 μl of phenethylamine and counted by Aloka liquid scintilation counter.

RESULTS. G-6-Pase activities in intact microsomes and Triton X-100 treated microsomes were suppressed by insulin in dose-dependent manner (Fig 1). In Triton X-100 treated microsomes, G-6-Pase activity was higher than the activity in intact microsomes by 74% in insulin-free condition. Treatment of microsome with Triton X-100 at the low concentration of 0.1% disrupted the permeability barrier of microsomal vesicles and released the full activity of G-6-Pase. Insulin suppressed the released full activity of G-6-Pase. From these data, insulin concentration which showed half maximal inhibition of G-6-Pase were 100 μU/ml in intact and detergent modified microsomes.

Fig 2 demonstrates the effects of the formic acid extracts from insulin-treated liver plasma membranes on G-6-Pase and PDH activities. Control

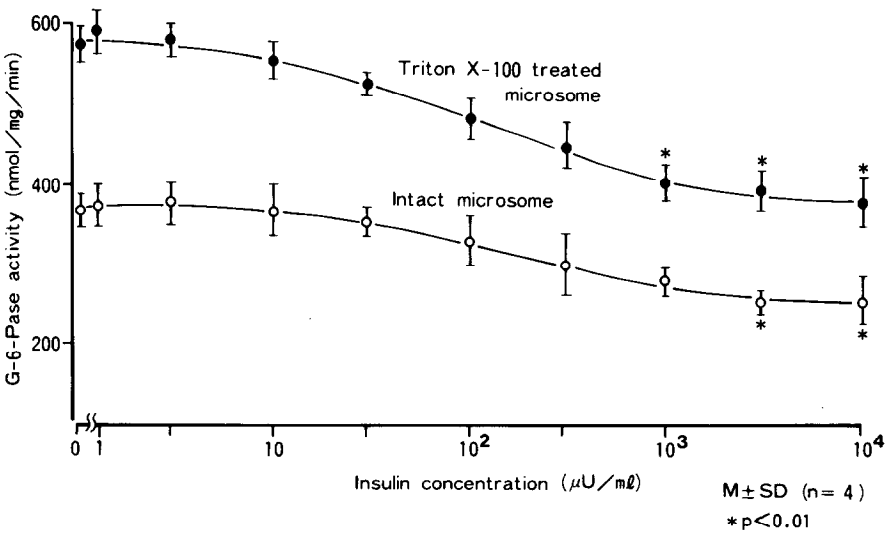

Fig 1. Direct Effects of Insulin on Rat Liver Microsome G-6-Pase.

Intact microsomes (open circle) and Triton X-100 treated microsomes (closed circle) were preincubated with indicated concentrations of insulin (0-10 mU/ml), and then G-6-Pase activity was measured according to METHODS.

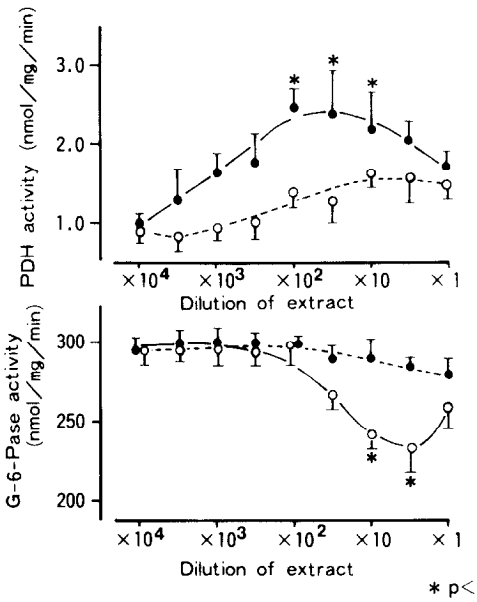

Fig 2. Effects of Dilutions of Insulin Mediator Extracts from Liver Plasma Membranes on the Stimulation of PDH and the Suppression of G-6-Pase.

Insulin mediator (closed circle) and control extracts (open circle) were prepared from insulin-treated and untreated liver plasma membranes respectively, as described in METHODS. Dilutions of each extract were prepared using the incubation buffer as the diluent, and 50 µl of each sample was tested in quadricate for its ability to stimulate PDH and suppress G-6-Pase.

extracts produced the slight stimulation of PDH and the slight inhibition of G-6-Pase. Insulin mediator extracts stimulated PDH activity and suppressed G-6-Pase activity significantly compared to the control extracts. The effects of the insulin mediator on PDH and G-6-Pase were biphasically dose-dependent; The suppression of G-6-Pase and the activation of PDH were increased in proportion to the concentration of insulin mediator, but higher concentrations of insulin mediator caused a reversal of the effects.

The insulin mediator extracts suppressing G-6-Pase and stimulating PDH were chromatographied on a Sephadex G-25 column (0.9x50 cm) and an aliquot of each fraction was tested for its ability to inhibit G-6-Pase and stimulate PDH. Data in Fig 3 show that aliquots of the fraction number 44-45 of the insulin mediator extracts activated PDH and suppressed G-6-Pase, as compared to those of the control extracts. Insulin mediator has the effects on both PDH and G-6-Pase. The insulin mediator from rat liver plasma membrane had an approximate molecular weight of 1,500 as indicated by the molecular weight

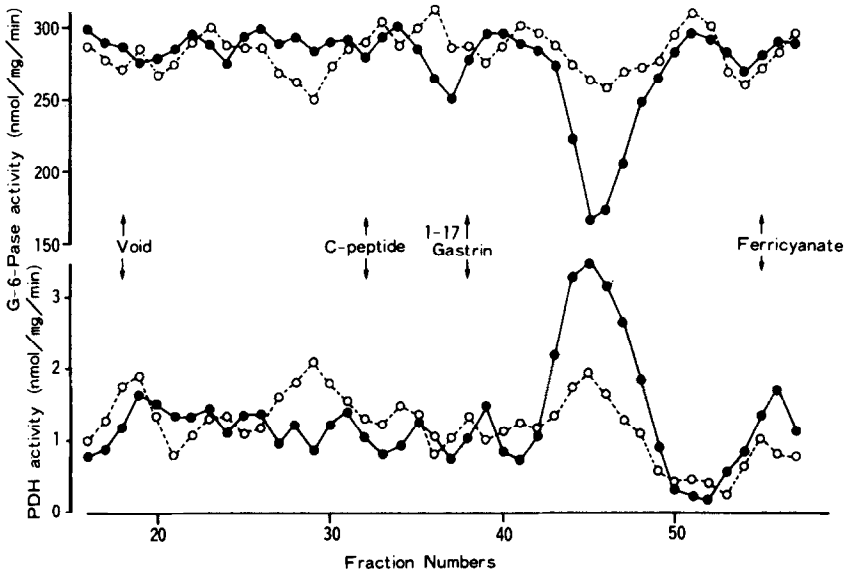

Fig 3. Gel-Chromatography of Insulin Mediator Extracts from Liver Plasma Membranes.

Liver plasma membranes (1 mg of protein) were incubated with or without insulin (100 μU/ml) at 37°C for 5 min, and insulin mediator (closed circle) and control extracts (open circle) were prepared as described in METHODS. Both extracts were chromatographied through a Sephadex G-25 column (0.9 x 50 cm) run in 50 mM formic acid. 1 ml of fractions were collected and 50 μl of samples were tested for the ability to stimulate PDH and suppress G-6-Pase. Markers used to calibrate the column were blue dextran (void), C-peptide (3,000), $^{1-17}$gastrin (2,100) and ferricyanate (329).

markers. After digestion of the insulin mediator in the fraction number 44-45 by trypsin (final 0.1 mg/ml) at 37°C for 5 min, the activation of PDH and the suppression of G-6-Pase were abolished (Data not shown).

DISCUSSION. Speth and Schulze reported that physiological concentrations of insulin suppressed rat liver microsome G-6-Pase in vitro (14). This effect of insulin may be caused by a putative second messenger of insulin released from liver plasma membrane. The present study showed that microsomal G-6-Pase is apparently modulated to suppression not only by insulin molecule itself (Fig 1) but also by its putative second messenger substance (Fig 2,3). Therefore it is clear that suppressing effect of insulin on G-6-Pase is mediated by the insulin mediator.

G-6-Pase exists in a two-component system, consisting of a glucose-6-phosphate specific transportor which mediates the movement of glucose-6-

phosphate from the cytosol to the lumen of the endoplasmic reticulum (microsomal vesicle), and a non-specific phosphohydroxylase localized within luminal surface of endoplasmic reticulum (20). Treatment with Triton X-100 disrupted the permeability barrier of microsomal vesicles and released the full activity of non-specific phosphohydroxylase. Because the phosphohydroxylase activity in Triton X-100 treated microsome was suppressed by insulin, the action of insulin on G-6-Pase seems to be regulated at the step of non-specific phosphohydroxylase component.

G-6-Pase can be added to the list of insulin-sensitive enzymes whose activities are modulated by the insulin mediator. Several enzymes, such as glycogne synthase, cyclic AMP dependent protein kinase (2) and PDH (21), appear to be regulated by phosphorylation-dephosphorylation reactions. Several lines of evidence demonstrated the possibility of G-6-Pase by phosphorylation-dephosphorylation process (22,23). The action of insulin on G-6-Pase might be occurred by this mechanism.

REFERENCES

1. Jarett,L. and Seals,J.R. (1979) Science 206, 1407-1408.
2. Larner,J., Galasko,G., Cheng,K., Depaoli-Roach,A.A., Huang,L., Daggy,P. and Kellogg,J. (1979) Science 206, 1408-1410.
3. Kiechle,F.L. and Jarett,L. (1981) FEBS letter 133, 279-282.
4. McDonald,J.M., Pershadsingh,H.A., Kiechle,F.L. and Jarett,L. (1981) Biochem. Biophys. Res. Commun. 100, 857-864.
5. Saltiel,A.R., Siegel,M.I., Jacobs,S. and Cuatrecasas,P. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 3513-3517.
6. Satiel,A.R., Doble,A., Jacobs,S. and Cuatrecasas,P. (1983) Biochem. Biophys. Res. Commun. 110, 789-795.
7. Kiechle,F.L., Jarett,L., Popp,D.A. and Kotagal,N. (1980) Diabetes 29, 852-855.
8. Parker,J.C., Kiechle,F.L. and Jarett,L. (1982) Arch. Biochem. Biophys. 215, 339-344.
9. Jarett,L., Kiechle,F.L., Popp,D.A., Kotagal,N. and Gavin,J.R.III. (1980) Biochem. Biophys. Res. Commun. 96, 735-741.
10. Seals,J.R. and Czech,M.P. (1980) J. Biol. Chem. 255, 6529-6531.
11. Saltiel,A., Jacobs,S., Siegel,M. and Cuatrecasas,P. (1981) Biochem. Biophys. Res. Commun. 102, 1041-1047.
12. Sakamoto,Y., Kuzuya,T. and Sato,J. (1982) Biomedical Res. 3, 599-605.
13. Seals,J.R. and Czech,M.P. (1981) J. Biol. Chem. 256, 2894-2899.
14. Speth,M. and Schulze,H-U. (1981) Biochem. Biophys. Res. Commun. 99, 134-141.
15. Fleischer,S. and Kervina,M. (1974) Methods Enzymol. 31, 6-41.
16. Arion,W.J., Ballas,L.M., Lange,A.J. and Wallin,B.K. (1976) J. Biol. Chem. 251, 4901-4907.
17. Harper,A.E. (1962) Methoden der Enzymatischen Analyse, pp. 788-792, Bergmeyer,H-U. ed., Verlagchemie, Weiheim/bergstr., FRG.

18. Chen,P.S.Jr., Toribara,T.Y. and Warner,H. (1956) Anal. Chem. 28, 1756-1758.
19. Seals,J.R. and Jarett,L. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 77-81.
20. Arion,W.J., Wallin,B.K., Lange,A.J. and Ballas,L.M. (1975) Mol. Cell. Biochem. 6, 75-83.
21. Popp,D.A., Kiechle,F.L., Kotagal,N. and Jarett,L. (1980) J. Biol. Chem. 255, 7540-7543.
22. Burchell,A. and Burchell,B. (1980) FEBS letter 118, 180-184.
23. Begley,P.J. and Craft,J.A. (1981) Biochem. Biophys. Res. Commun. 103, 1029-1034.