Insulin Stimulates the Phosphorylation of the 95,000-Dalton Subunit of Its Own Receptor

Abstract. *Cultured human lymphocytes and rat hepatoma cells were labeled with [^{32}P]orthophosphate and the insulin receptor subunits identified by immunoprecipitation and sodium dodecyl sulfate–gel electrophoresis. In both cell types the 95,000-dalton (β) subunit of the insulin receptor was selectively phosphorylated. Phosphorylation was specifically stimulated by insulin in a dose-dependent fashion after 1 and 15 minutes of hormone treatment, whereas human growth hormone was without effect. This phosphorylation may be a very early event in insulin action.*

The insulin receptor is composed of two major subunits (α and β, with molecular weights of approximately 135,000 and 95,000, respectively) linked by disulfide bonds to form the active receptor complex (*1, 2*). Insulin binds to the receptor, and this interaction starts a chain of biochemical events leading to the final effects characteristic of the action of the hormone (*3, 4*). The mechanism responsible for initiating this series of reactions is unknown. However, insulin is known to affect cyclic nucleotide concentrations, ion fluxes, and several membrane enzyme and transport systems as well as phosphorylation of proteins and lipids. Recently insulin has also been shown to generate a low molecular weight peptide that can mimic several insulin actions in broken cell systems (*4, 5*). In the present study, we considered another possibility, namely, that insulin initiates its action by way of phosphorylation of its own receptor.

Cultured human lymphocytes (IM-9) were incubated with [^{32}P]orthophosphate in phosphate-free RPMI medium containing 0.1 percent bovine serum albumin (BSA) at 37°C. After 60 minutes of incubation the cells were exposed to insulin for different periods of time. The reaction was terminated by tenfold dilution with ice-cold RPMI 1640 medium containing 10 m*M* sodium pyrophosphate, 10 m*M* NaF, and 4 m*M* EDTA and washed twice with this same stopping buffer. The ^{32}P-labeled cells were solubilized by 1 percent Triton X-100 containing phenylmethylsulfonyl fluoride (PMSF) and aprotinin to inhibit proteolysis, and the insulin receptor was enriched by chromatography on a wheat germ agglutinin–agarose column and elution with *N*-acetyl glucosamine as previously described (*2*). The insulin receptor in the eluted fractions was quantitatively immunoprecipitated by a serum containing antibody against insulin receptor (serum B-2 at a 1:200 dilution) with addition of staphylococcal protein A (*6, 7*); the immunoprecipitates were analyzed by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and autoradiography. Control immunoprecipitation was produced by using the same technique and a pool of normal human serum.

Antibody against the insulin receptor specifically immunoprecipitated two labeled bands in cells labeled with ^{32}P. These had molecular weights of 95,000 (95K) and 68,000 (68K) and were observed in the cells incubated in both the absence and presence of insulin (Fig. 1). When the cells were incubated with $10^{-6}M$ insulin, the ^{32}P labeling of the 95K band was selectively increased (Fig. 1A, lanes C, D, G, and H). This band corresponds exactly to the position of the β subunit of the insulin receptor in similar gels in which the receptor subunits are labeled either biosynthetically with amino acids or sugars, or by surface labeling (*2, 7*). Analysis of this autoradiograph by scanning densitometry revealed that insulin increased the labeling of the 95K protein by about 250 percent at 1 minute and 350 percent at 15 minutes compared to controls. By contrast, insu-

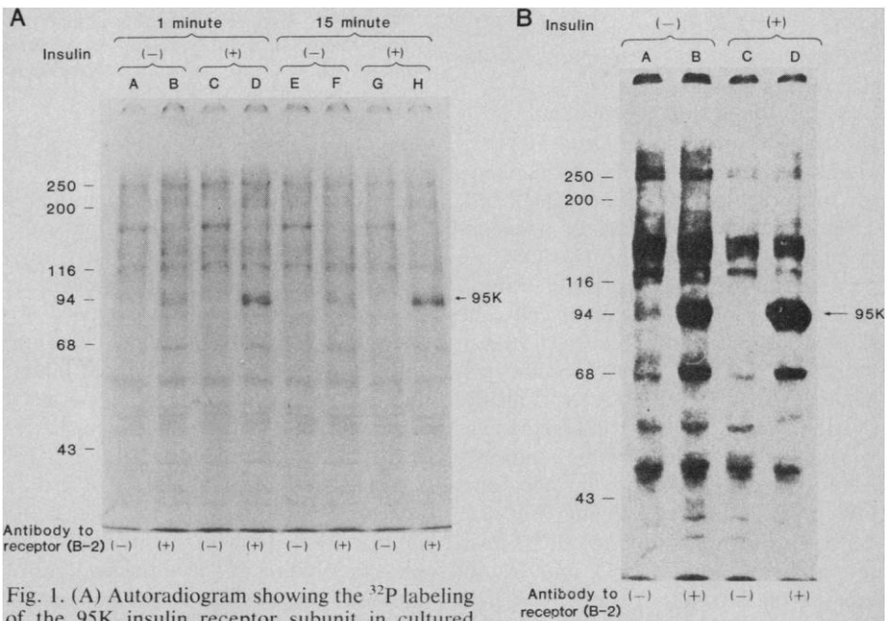

Fig. 1. (A) Autoradiogram showing the ^{32}P labeling of the 95K insulin receptor subunit in cultured human lymphocytes (IM-9). The lymphocytes were grown in RPMI medium with 10 percent fetal calf serum. Cells (8×10^8) were washed two times in medium without phosphate containing 0.1 percent BSA, *p*H 7.4, and incubated with 1 mCi of [^{32}P]orthophosphate in 20 ml of RPMI medium without phosphate for 60 minutes at 37°C. Cells were divided into four 5-ml portions. Insulin ($10^{-6}M$) was added to two samples and each pair of samples was incubated for either 1 or 15 minutes. Reactions were stopped by adding 45 ml of stopping solution, washed two times at 4°C, and solubilized with 1 percent Triton X-100 in stopping solution containing phenylmethylsulfonyl fluoride (PMSF) (2 m*M*) and aprotinin (1000 trypsin inhibitor units per milliliter). The insulin receptor was enriched by chromatography on a wheat germ agglutinin–agarose column and elution with *N*-acetyl glucosamine (*2, 7*). Eluted fractions (800 μl) were quantitatively immunoprecipitated by serum containing antibody against insulin receptor (serum B-2 at a 1:200 dilution) or normal control serum after addition of protein A (*7*). The precipitates were washed twice with 1 percent Triton and 0.1 percent SDS, solubilized by boiling for 3 minutes in 2 percent SDS, 0.1*M* dithiothreitol, 0.01 percent bromophenol blue, and 10 m*M* sodium phosphate (*p*H 7.0), and electrophoresed in 7.5 percent polyacrylamide slab gels in the presence of 0.1 percent SDS (*2*). After electrophoresis, the slab gels were stained, destained, dried, and autoradiographed (*2, 7*). Subunit molecular weights were calculated with standards as previously described (*7*). (B) Autoradiogram showing insulin-stimulated ^{32}P labeling of insulin receptor subunit in a rat hepatoma cell line (Fao). The cells were cultured in modified Ham's F12 medium with 5 percent fetal calf serum in Falcon 75-cm^2 flasks (*11*). Confluent cells (six flasks) were washed twice with phosphate-free Krebs-Ringer bicarbonate buffer with 0.1 percent BSA and incubated at 37°C in this buffer with [^{32}P]orthophosphate (0.1 mCi/ml) in a humidified atmosphere containing 5 percent CO_2. After 60 minutes, insulin ($10^{-6}M$) was added to half of the flasks and incubations were continued for an additional 15 minutes at 37°C. The reactions were stopped with cold Krebs-Ringer bicarbonate buffer containing 10 m*M* sodium pyrophosphate, 10 m*M* NaF, and 4 m*M* EDTA and washed two times and processed as described above.

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lin produced no significant increase in the ^{32}P incorporated into total trichloroacetic acid precipitable proteins.

To evaluate in more detail the mechanism by which insulin stimulates the phosphorylation of the 95K protein we first labeled cells with [^{32}P]orthophosphate and then incubated them with several concentrations of porcine insulin, guinea pig insulin, and human growth hormone (Fig. 2). Porcine insulin stimulated covalent ^{32}P labeling of the 95K protein in a dose-dependent fashion. Labeling was doubled by a concentration of $10^{-9}M$ insulin and was increased about fivefold at the highest insulin concentration ($10^{-6}M$). Guinea pig insulin, which is about 2 percent as potent as porcine insulin in stimulating glucose metabolism (*8*), was about 2 percent as potent in stimulating the phosphorylation. Human growth hormone ($10^{-6}M$), although it binds to its own receptor in these cells (*9*), had little effect on the phosphorylation of this protein.

The cultured human lymphocyte is generally regarded as an insulin-insensitive cell, although insulin induces receptor down regulation in these cells (*7, 10*). To evaluate possible receptor phosphorylation in an insulin-sensitive cell, we performed similar experiments using a well-differentiated hepatoma cell line (Fao) derived from the Reuber H-35 hepatoma (*11*). As with the lymphocytes, insulin receptor antibody specifically precipitated one major band (molecular weight 95,000) and one minor band (molecular weight 68,000) in the hepatoma cells after phosphate labeling (Fig. 1B, lanes A and B). Addition of $10^{-6}M$ insulin to the medium for 15 minutes increased the labeling of the 95K protein by about 2.5-fold and was without effect on the labeling of the 68K protein (Fig. 1B, lanes C and D).

Phosphorylation of proteins is an important regulatory mechanism in hormone action. Insulin has effects on both phosphorylation and dephosphorylation of membrane, mitochondrial, and ribosomal proteins (*12*). The present study demonstrates that insulin specifically stimulates the phosphorylation of a 95K protein. This protein has been tentatively identified as the β subunit of insulin receptor by its immunoprecipitation by antibody to the insulin receptor and by the fact that it migrates in the same position in SDS–polyacrylamide gel electrophoresis as the β subunit of the receptor (*2, 7*) in reduced gels and as a protein of molecular weight of > 300,000 in nonreduced gels (data not shown). In addition, serum samples from several different patients containing antibodies to insulin receptor (*2*) immunoprecipitate this protein, and immunoprecipitation is decreased in the presence of excess unlabeled insulin (*2*) (data not shown). A phosphorylated 68K band was also observed in the gels of the immunoprecipitates. However, insulin did not inhibit the immunoprecipitation of this labeled material. The identity of this band remains to be determined (*13*).

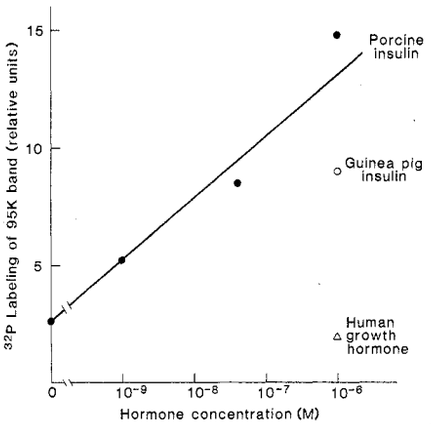

Fig. 2. Effect of porcine insulin, guinea pig insulin, and human growth hormone on the ^{32}P labeling of the 95K protein in cultured human lymphocytes. Lymphocytes were labeled with [^{32}P]orthophosphate as described in legend to Fig. 1A and incubated with the indicated concentrations of porcine insulin, guinea pig insulin, and human growth hormone at 37°C for 15 minutes. The autoradiographs were scanned in a Joyce-Loebl microdensitometer and the peak areas corresponding to the 95K band were calculated and expressed in arbitrary units.

Since the biological effects of insulin appear to be initiated by the interaction of the hormone with its receptor, the phosphorylation of one of the subunits of the insulin receptor may have important physiological significance. This phosphorylation could not only be an early step in the pathway of signaling involved in insulin's metabolic action, but could also play a role in the regulation of receptor affinity or in "down regulation" of insulin receptor number. Rappoport and co-workers (*14*) have presented some indirect data suggesting that adenosine diphosphate ribosylation may also be involved in insulin receptor down regulation in IM-9 lymphocytes, and our own recent studies of receptor turnover in which we used biosynthetic and surface labeling have suggested that down regulation is due to accelerated receptor degradation (*7*).

Cohen *et al.* (*15*) have shown that epidermal growth factor (EGF) can stimulate the phosphorylation of a tyrosine residue in its receptor protein in membrane fractions. If one considers that both insulin and EGF have major effects on cell growth, it is also possible that phosphorylation of this 95K receptor subunit is somehow associated with the growth-promoting effect of these hormones.

Our results demonstrate a direct biochemical covalent modification of the insulin receptor as a consequence of insulin-receptor complex formation. It seems likely that this phosphorylation of the insulin receptor may be an early event in insulin action. Phosphorylation could act as a direct signal, could alter the rate of internalization of the receptor (and hormone), could modify the activity of a membrane protease which generates a peptide second messenger, or could initiate a cascade of phosphorylation and dephosphorylation reactions that are responsible for the effects of insulin.

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References and Notes

1. S. Jacobs, E. Hazum, Y. Schechter, P. Cuatrecasas, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4918 (1979); C. C. Yip, C. W. T. Yeung, M. L. Moule, *Biochemistry* **19**, 70 (1980); J. Massague, P. F. Pilch, M. P. Czech, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 7137 (1980); M. Kasuga, E. Van Obberghen, K. M. Yamada, L. C. Harrison, *Diabetes* **30**, 354 (1981).
2. E. Van Obberghen, M. Kasuga, A. LeCam, A. Itin, J. A. Hedo, L. C. Harrison, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 1052 (1981); J. A. Hedo, M. Kasuga, E. Van Obberghen, J. Roth, C. R. Kahn, *ibid.*, p. 1052.
3. I. Pastan, J. Roth, V. Macchia, *ibid.* **56**, 1908 (1966); T. Kono and F. W. Barham, *J. Biol. Chem.* **246**, 6210 (1971); P. Cuatrecasas and M. D. Hollenberg, *Adv. Protein Chem.* **30**, 252 (1976); C. R. Kahn, *Trends Biochem. Sci.* **263**, 4 (1979).
4. M. P. Czech, *Annu. Rev. Biochem.* **46**, 359 (1977).
5. J. R. Seals and L. Jarett, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 77 (1980); J. Larner, G. Galasko, K. Cheng, A. A. DePaoli-Roach, L. Huang, P. Daggy, J. Kellogg, *Science* **206**, 1408 (1979).
6. L. C. Harrison, J. S. Flier, J. Roth, F. A. Karlsson, C. R. Kahn, *J. Clin. Endocrinol. Metab.* **48**, 59 (1979).
7. M. Kasuga, C. R. Kahn, J. A. Hedo, E. Van Obberghen, K. M. Yamada, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6917 (1981).
8. P. Freychet, J. Roth, D. M. Neville, Jr., *ibid.* **68**, 1833 (1971).
9. M. A. Lesniak, P. Gorden, J. Roth, J. R. Gavin, III, *J. Biol. Chem.* **249**, 1661 (1974).
10. J. R. Gavin, III, J. Roth, D. M. Neville, Jr., P. De Meyts, D. N. Buell, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 84 (1974).
11. J. Deschatrette, E. E. Moore, M. Dubois, D. Cassio, M. C. Weiss, *Somat. Cell Genet.* **5**, 697 (1979); Y. Iwamoto, K. Y. Wong, I. D. Goldfine, *Endocrinology* **108**, 44 (1981).
12. J. Avruch, G. R. Leone, D. B. Martin, *J. Biol. Chem.* **251**, 1511 (1976); G. J. Benjamin and N. C. Clayton, *ibid.* **253**, 1700 (1978); R. J. Marchmont and M. D. Houslay, *Nature (London)* **286**, 904 (1980); J. R. Seals, J. M. McDonald, L. Jarett, *J. Biol. Chem.* **254**, 6991 (1979); C. J. Smith, P. J. Wejksnora, J. R. Warner, S. Rubin, O. M. Rosen, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 2725 (1979).
13. A faint specific band of protein with a molecular weight of about 210,000 was also observed in several gels. This phosphorylated protein may be an unreduced heterodimer of the α and β subunits of insulin receptor or a third subunit of insulin receptor as previously described (*2, 7*).

14. S. Filetti, N. A. Takai, B. Rappoport, *Endocrinology* **108**, 2409 (1981).
15. L. E. King, Jr., G. Carpenter, S. Cohen, *Biochemistry* **19**, 1524 (1980); S. Cohen, G. Carpenter, L. E. King, Jr., *J. Biol. Chem.* **255**, 4834 (1980); H. Ushiro and S. Cohen, *J. Biol. Chem.* **255**, 8363 (1980).
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Origin of the Cholecystokinin-Containing Fibers in the Rat Caudatoputamen

Abstract. *Large amounts of cholecystokinin-octapeptide (CCK) are present in the rat caudatoputamen. The peptide occurs in axons and nerve endings but not in perikarya. The origin of CCK in the caudatoputamen was investigated with the use of immunocytochemistry and a radioimmunoassay specific for CCK. Although a small amount of CCK (approximately 30 percent) originates in the amygdaloid complex, the bulk of the peptide (approximately 70 percent) occurs in processes of neurons located ventral to the caudatoputamen, that is, the claustrum or the piriform cortex. The claustrum and piriform cortex receive inputs from various cortical areas and the olfactory system, respectively, and may process information and relay it to the caudatoputamen. Thus CCK may be the transmitter in the final common pathway linking various cortical areas and the olfactory system to the caudatoputamen.*

Several neurotransmitters have been found in the caudate nucleus (*1*). Some of these are made by neurons located within the nucleus itself (for example, acetylcholine, γ-aminobutyric acid, substance P, and enkephalin); others are made in processes of neurons that are found elsewhere in the brain (for example, dopamine and serotonin). Numerous studies of the interactions among neurons that innervate and constitute the caudate nucleus have contributed to a better understanding of its role in somatic motor function and the pathophysiology of Parkinson's and Huntington's diseases (*2*).

Cholecystokinin (CCK), a peptide hormone that stimulates pancreatic enzyme secretion and contracts the gall bladder, has been found in high concentrations in the caudatoputamen (*3*). Binding sites for this hormone have also been found there and appear to be on cells that are intrinsic to the nucleus (*4*). No CCK-positive neuronal perikarya have been found in the rat caudatoputamen (*5*), but a somewhat dense plexus of axons and nerve endings has been visualized. Thus it seemed that another structure must provide the caudate with CCK.

The caudate is innervated by the amygdala, ventral tegmental area, substantia nigra, dorsal raphe nucleus, thalamus, and several cortical areas (*6*). Except for the thalamus all of these areas have CCK-containing perikarya (*5*) and could potentially provide the caudate with CCK. We therefore examined the effect on the CCK content of lesions that selectively destroy caudate afferents. In addition, we used immunocytochemistry to visualize CCK-containing projections to the nucleus.

Unilateral brain lesions were made in female Osborne-Mendel rats anesthetized with ether. These lesions removed inputs to the caudate nucleus from the substantia nigra, amygdaloid complex, dorsal cerebral cortex, insular cortex, and the claustrum-piriform cortex. Seven days later the rats were killed by decapitation. The brains were quickly removed and coronal sections 2 mm in thickness were made with razor blades and a template. The part of the caudatoputamen approximately 7 mm rostral to the interauricular line was dissected from the sections and CCK was extracted and measured by radioimmunoassay (*3*).

A surgical lesion of the nigrostriatal pathway (*7*) caused the dopamine to disappear from the caudatoputamen (*8*) but did not decrease the total CCK-concentration (Table 1). This indicates that neurons in the substantia nigra contribute little CCK to the caudatoputamen (*9*). Similarly, neither separating the insular cortex (*10*) from the caudate nor removing those parts of the frontal, parietal, and occipital cortex that are accessible after a dorsal craniotomy (*11*) had any effect on the CCK concentration in the caudatoputamen (Table 1).

In contrast, a knife cut severing afferents from the amygdala reduced the CCK concentration in the caudatoputamen by approximately 30 percent (Table

Table 1. The effect of several knife cuts on the concentration of CCK in the head of caudate nucleus. The concentration of CCK (mean ± standard error) is given in nanograms per milligram of protein. The number of animals in each group appears in parentheses. Protein was measured according to Lowry *et al.* (*16*). The lesioned side was compared to the unlesioned side of the same animals (paired Student's *t*-test); N.S., not significant.

Area severed	Lesioned side	Control side	*P*
Substantia nigra	11.28 ± 2.22 (8)	6.34 ± 0.94 (8)	N.S.
Amygdaloid complex	5.55 ± 0.94 (11)	7.77 ± 0.79 (11)	.05
Dorsal cortex	9.1 ± 1.0 (5)	7.1 ± 1.0 (5)	N.S.
Insular cortex	4.98 ± 0.52 (8)	6.49 ± 0.57 (8)	N.S.
Claustrum-piriform cortex	2.63 ± 0.79 (11)	7.38 ± 1.61 (11)	.005

Fig. 1. Brain sections showing the distribution of CCK. Immunocytochemistry was performed according to Sternberger (*17*). Twenty-four hours after the injection of 25 μg of colchicine into the caudatoputamen (in 2 μl of vehicle; 9.2 mm anterior to the interauricular line, 2.5 mm lateral to the midline; 6.2 mm ventral to the dural surface, with the plane angle 5° down) rats were fixed with 4 percent paraformaldehyde in 0.1*M* sodium phosphate buffer (*p*H 7.35). The brains were removed and placed in fixative for 12 hours. After immersion in 30 percent sucrose for 48 hours 20- to 25-μm sections were cut on a cryostat or on a Vibratome. The sections were incubated with the immunoglobulin G fraction of an antiserum to CCK8-sulfate, conjugated to bovine serum albumin by glutaraldehyde which had been preabsorbed with liver acetone powder (dilution 1:300; incubation time 24 hours at room temperature). For staining, 3,3'-diaminobenzidine (5 mg/ml) was used in the presence of 0.01 percent hydrogen peroxide. In preabsorption control experiments antiserum (1:300) was first incubated with 3.75 μg of CCK8 per milliliter for 24 hours. This procedure abolished all staining. (A) A section showing the piriform cortex (*CPF*), claustrum (*CL*), and caudatoputamen (*CP*) (×38). (B) A section showing the claustrum with the perikarya staining for CCK (×220).

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