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CEREBRAL CORTICAL MICROVESSELS: AN INSULIN-SENSITIVE TISSUE+

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Cerebral cortical microvessels were isolated from bovine brains and incubated in the presence or absence of porcine insulin. The conversion of D-glucose to CO_2 and lipids was increased significantly in insulin-treated microvessels. Insulin also caused a significant increase in the activity of cyclic-AMP phosphodiesterase in these microvessels. Specific binding of ^{125}I-insulin to isolated brain microvessels, displaceable by cold insulin was also observed. The effects of insulin reported in this communication mark the first direct evidence that insulin is capable of regulating the metabolic activity of cerebral cortical microvascular tissue. The possible physiological significance of this finding is discussed.

INTRODUCTION

Insulin plays a key role in glucose homeostasis throughout the body and in particular in target organs such as the liver, muscle and adipose tissue. The hormone binds to high-affinity receptors on the cell surface and causes a variety of metabolic changes, including accelerated D-glucose uptake, protein synthesis and the activation of key enzymes such as glycogen synthetase, pyruvate dehydrogenase and cyclic-AMP phosphodiesterase (1).

The relationship between insulin and the metabolic activity of vascular tissue has only recently been explored. The incidence of atherosclerosis and ischaemic vascular disease is increased among patients with diabetes mellitus, as shown by several prevalence studies in large communities (2-4). Diabetics also have a higher incidence of retinopathy, neuropathy and peripheral vascular disease, thus exhibiting microangiopathy as well as macroangiopathy. Despite this epidemiologic data to suggest that vascular tissue throughout the

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body may be adversely affected by alterations in the level of insulin, very little rigorous biochemical data has been accumulated concerning the effects of insulin on the metabolism of vascular tissue.

Evidence from several laboratories has indicated that arteries are insulin-sensitive tissues (5-7), further strengthening the association between vascular disease and diabetes mellitus. Most metabolic studies of vascular tissue have concentrated on arterial tissue since this was the easiest portion of the cardiovascular system to isolate. Recent advances have been made which now allow mild homogenization followed by sieving techniques to be used in the isolation of microvessels from brain cortical tissues (8, 9) and thus permit characterization of the effects of insulin on metabolic activities in these vessels.

MATERIALS AND METHODS

Tissue Isolation - Bovine brain cortical microvessels were isolated as described by Brendel, et al.(8, 9). Pieces of cortical tissue were removed from the brain with forceps and placed in a glass hand-held homogenizer equipped with a Teflon pestle. Gentle homogenization with 10 up and down strokes in Krebs-Ringer phosphate buffer disrupted the majority of nonvascular tissues. The homogenate was poured over a 153 µm pore-size nylon sieve, which retained the vascular elements but not the disrupted nonvascular material. Washing with buffer followed by rehomogenization of the material originally retained by the sieve and then re-sieving provided a highly purified preparation of brain microvessels. Routinely, two bovine brains were obtained from a local slaughterhouse for each experiment and were used to prepare microvessels, which were used immediately. Several experiments have also been carried out with microvessels isolated from a newborn porcine brain by the same techniques. Rat adipocytes were isolated as previously described (10).

Glucose Oxidation - Cerebral microvessels were diluted to an appropriate volume with Krebs-Ringer phosphate buffer and 0.1 ml was distributed into plastic test tubes containing D-[U-^{14}C]glucose (0.2 mM, 0.5 µCi/ml) in a total volume of 0.4 ml of Krebs-Ringer phosphate buffer containing 2-3% of bovine serum albumin (Fraction V, Sigma Chemical Co.) as described previously for rat adipocytes (10). Samples run without tissue served as blanks and the counts measured in these samples, which never exceeded 0.5% of the radioactivity present, were subtracted from the experimental values obtained with samples containing tissue. Preliminary experiments showed a linear production of CO_2 during 30-120 minutes of incubation following an initial lag. Tissue quantities were measured as wet weights (5 to 15 mg/tube) and results are normalized to account for differences between samples. Representative samples were saved for determination of protein levels.

Lipogenesis - Conversion of D-glucose into lipid was measured in tissue samples prepared as described above for glucose oxidation studies. Following completion of the glucose oxidation study, samples were frozen and stored at -20°C for up to two weeks. Samples were thawed and lipids were extracted by the technique described by Rodbell (11) using the solvents of Dole and Meinertz (12). An aliquot (0.5 ml) of the upper phase was then mixed with 3.5 ml Econofluor (Beckman Instrument Co.) and counted in a liquid scintillation

counter. Blank samples were run without tissue and the values obtained, which never exceeded 0.1% of the total radioactivity in the tube were subtracted from all other samples.

Cyclic-AMP Phosphodiesterase Activity - Cerebral microvessels were isolated by sieving and resuspended in Krebs-Ringer phosphate buffer, (pH 7.4). The vessels (0.1 ml) were then incubated in plastic test tubes for 15 min at 37°C with or without the addition of insulin. The blood vessels were then washed and resuspended in 1.0 ml of 0.25 M sucrose, 0.01 M Tris-HCl buffer (pH 7.4). Vessels were then disrupted (Tissue-Mizer, Tekmar Co., setting 6 for 10 seconds) followed by centrifugation at 2,250 x g for 5 min to remove cellular debris and unbroken cells. The low Km form of cyclic-AMP phosphodiesterase was measured by a modification of the procedure of Kono, et al. (13). Samples run without tissue served as blanks and the amount of radioactivity obtained with these samples was subtracted from all samples containing tissue. Triplicate aliquots of the tissue extract were taken and results are expressed as mean ± S.E.M. in pmoles [^{3}H]-adenosine formed/10 min/mg protein.

^{125}I-Insulin Binding - Porcine crystalline insulin was generously supplied by Dr. Ronald Chance of the Eli Lilly Laboratories. [^{125}I]-insulin (60-90 μC_i/mg) was purchased from New England Nuclear. Bovine serum albumin (BSA) was Fraction V from Sigma Chemical Co. Cerebral microvessels were diluted as described for glucose oxidation experiments and 0.1 ml distributed into iced Beckman microfuge tubes. Tubes were centrifuged at 10,000 xg for 1 minute and the supernatant solutions discarded. The pellets were resuspended in 200 µl assay buffer consisting of Krebs-Ringer phosphate, pH 7.6 containing 1.0% BSA. Nonspecific binding was determined in the presence of 10 µg/ml unlabelled insulin. The reactions were started by the addition of ^{125}I-insulin in a final concentration of 2.5 ng/ml (0.1 μC_i). All incubations were carried out for 75 minutes at room temperature and continuously shaken to prevent settling of the microvessels.

The reactions were terminated by transferring the reaction mixtures to microfuge tubes containing 500 µl dinonylphthalate/dibutylphthalate oil (1:2 ratio) and immediately centrifuging at 10,000 xg for 1 minute. The supernatant solutions were aspirated from the top of the oil layers and saved. The oil was then aspirated, the pellets drained, and the tube tips containing the tissue pellets cut off and placed into counting tubes. After counting, the tissue pellets were solubilized in 0.1 N NaOH at 50°C for 60 minutes, neutralized with 0.1 N HCl, and then assayed for protein using the Biorad protein dye reagent. Individual pellets contained 100-200 µg protein. Insulin degradation was monitored by precipitation of the incubation medium in 10% trichloroacetic acid (TCA). Less than 2.0% degradation occurred during incubation for 75 minutes at room temperature, as determined by the amount of soluble radioactivity remaining following TCA-precipitation.

RESULTS

It has been shown previously (8) that isolated cerebral microvessels are capable of metabolizing D-glucose to CO_2. The data presented in Fig. 1 indicates that porcine insulin, at physiological levels, can increase the rate of D-glucose oxidation by cerebral microvessels when compared to untreated tissue. The data in Fig. 1 also include a representative experiment with rat adipocytes, in which the number of adipocytes was adjusted to yield approximately the same amount of glucose oxidation as routinely obtained with

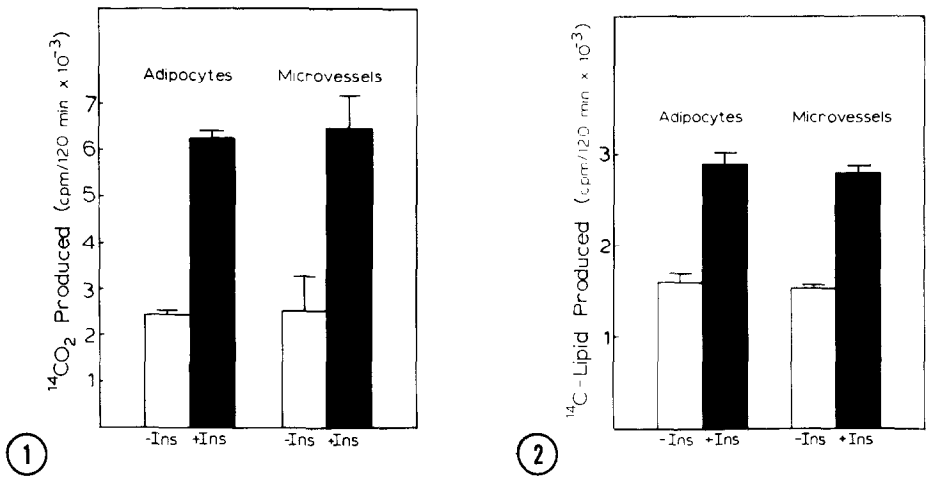

Fig. 1 Glucose oxidation by rat adipocytes and bovine cerebral microvessels. Tissues were isolated as described and adipocytes (1 to 2 x 10^{-5} cells) or microvessels (5 to 15 mg wet weight, 0.2 to 0.4 mg protein) were incubated at 37°C for 120 min with D-[U-^{14}C] glucose (0.2 mM, 0.5 µCi) in the presence or absence of 257 µU/ml insulin. Radioactive CO_2 was trapped on filter papers soaked in Hyamine Hydroxide. Data represent the mean ± S.E.M. of triplicate determinations.

Fig. 2 Glucose conversion to lipids by rat adipocytes and bovine cerebral microvessels. Tissues were isolated and incubated for 120 min at 37°C in the presence or absence of 257 µU/ml insulin as described in Fig. 1. Lipids were extracted with an acidic hexane-heptane solution and an aliquot was taken to determine the amount of D-[U-^{14}C] glucose that had been converted to a form that would associate with the lipid phase. Data represent the mean ± S.E.M. for triplicate determinations.

5 to 15 mg of brain microvessels. The magnitude of the stimulatory effect of insulin on glucose oxidation varied considerably with different microvessel preparations, ranging from 20-30% to as much as 10-fold, and rat adipocytes also showed a variability in the response to insulin, although adipocytes occasionally showed far greater stimulation, 50 to 100 fold, than brain microvessels. Preliminary studies with several insulin-like agents such as H_2O_2, concanavalin A and diamide indicate that these reagents also stimulate glucose oxidation in isolated cerebral microvessels (Pillion and Meezan, submitted for publication). The effect of insulin on brain microvessel sugar metabolism was also measured as glucose conversion to lipids. The data depicted in Fig. 2 demonstrate that physiological levels of insulin stimulate glucose conversion to lipids in bovine cerebral microvessels as well as in rat adipocytes. Once again there was variability in the magnitude of the hormone effect on each individual tissue preparation.

TABLE I. ^{125}I-INSULIN BINDING TO ISOLATED BOVINE CEREBRAL MICROVESSELS

Unlabelled Insulin (ng/ml)	Specific Bound (ng ^{125}I-insulin/mg protein)	% Initial ^{125}I-insulin Bound
0	.151	100
9	.104	68.9
100	.036	23.8
1,000	.017	11.3

Cerebral cortical microvessels (0.1-0.2 mg protein) were incubated with 2.5 ng/ml ^{125}I-insulin and various concentrations of unlabelled insulin for 75 min at 22°C. Nonspecific binding was determined in the presence of 10 µg/ml unlabelled insulin and subtracted from all binding values to yield specific binding.

The action of insulin in cerebral microvessels was next studied by measuring the activity of cyclic-AMP phosphodiesterase, an enzyme not immediately linked to the glucose transport system. The activity of this enzyme is also increased in homogenates from microvessels treated with insulin for 15 minutes at 37°C before disruption. Untreated microvessels had a cyclic-AMP phosphodiesterase activity of 48.6 ± 6.0 pmol [^{3}H] adenosine formed/mg protein/10 min, while insulin-treated microvessels had an activity of 120.6 ± 16.8 pmol [^{3}H] adenosine formed/mg protein/10 min. Studies are currently in progress to characterize the dose-response relationship, temperature and time required for the hormone to activate this enzyme.

Specific binding of ^{125}I-insulin to isolated cerebral microvessels could be demonstrated, and such binding was inhibited in the presence of unlabelled insulin as shown by the data in Table I. These data indicate an affinity of binding of ^{125}I-insulin to brain microvascular receptors in the order of 10^{-9} M.

DISCUSSION

Investigations of insulin action have, in the past, concentrated on the three primary tissues known to respond to physiological concentrations of the

hormone, i.e., adipose, liver and muscle. The data presented in this communication is the first direct evidence of an effect of insulin on cerebral microvessel metabolism, with glucose utilization and cyclic-AMP phosphodiesterase activity both significantly increased following brief exposure to physiological levels of hormone. Cerebral microvessels were first shown to be capable of glucose oxidation by Brendel, et al. (8). Since that time, several investigators have focused attention on the brain as a site of insulin binding (14-17) and glucose uptake (18,19). In our laboratory, preliminary experiments (20) indicate that ^{125}I-insulin binds to these cerebral microvessels, with many of the same binding characteristics described for other tissues which are known to be insulin sensitive. Specific binding of ^{125}I-insulin to brain microvessels isolated by a procedure based on that used in this study has been recently reported (17).

Diabetes mellitus of long-standing is associated with a high incidence of retinopathy and other microvascular and macrovascular complications. Preliminary studies with retinal microvessels in this laboratory (Pillion and Meezan, submitted for publication) indicate that this tissue shows a response to insulin similar to the response of cerebral microvessels observed in Figs. 1 and 2. Several questions remain to be answered before the effect of exogenous insulin on microvessel glucose utilization observed in the present investigation can be related to diabetic vascular disease. The present studies employed isolated microvessels exposed to insulin at the anti-lumenal surface, rather than the in vivo condition of hormone circulating in the bloodstream. The in vitro accessability of lumenal cerebral microvascular hormone receptors to insulin must be proven by autoradiographic or histochemical means. In addition, the acute administration of insulin in these studies is not comparable to the long-term effects on vascular integrity resulting from periods of hypoinsulinemia and hyperinsulinemia in diabetics. The effect of chemically-induced diabetes mellitus on cerebral microvessel metabolism and hormone-sensitivity will be of interest in answering this question more rigorously. The present study serves to document for the first

time that cerebral microvessels are an insulin-sensitive tissue and focuses attention on the possible hormonal regulation of metabolic activity in this organ.

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