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Insulin Binding and Effects of Insulin on Glucose Uptake and Metabolism in Cultured Rabbit Coronary Microvessel Endothelium (42137)

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Abstract. Microvascular endothelial cells were isolated from rabbit cardiac tissue, and cultivated by standard tissue culture techniques. The conversion of [U-^{14}C]glucose to CO_2 and lipids was significantly enhanced by insulin treatment. Insulin stimulated uptake of 2-[^{3}H]deoxyglucose and enhanced the transport of 3-*O*[^{3}H]methyl-D-glucose. Specific binding of [^{125}I]insulin to RCME cells, displaceable by cold insulin, was also observed. These data demonstrate that insulin is capable of regulating metabolic activities in coronary microvascular endothelium. © 1985 Society for Experimental Biology and Medicine.

Surface binding sites for insulin have been described in endothelium derived from human and rodent blood vessels (1, 2) and differential binding sites for insulin have been described in human arterial and venous endothelium (3). Recently, several groups have suggested that receptors for insulin may be identified in microvessel endothelium (4, 5) and that isolated microvessels exhibit several biological responses to insulin, including enhanced [U-^{14}C]glucose oxidation to CO_2 (5). In the present study, we report on the presence of receptors for insulin in cultured rabbit coronary microvessel endothelial (RCME) cells, and the effects of insulin on [U-^{14}]glucose metabolism, 3-*O*-[^{3}H]methylglucose transport and 2-[^{3}H]deoxyglucose uptake in rabbit coronary microvascular endothelial cells.

Materials and Methods. *Preparation of rabbit coronary microvascular endothelium.* Coronary microvessel endothelial cells were isolated and cultivated from rabbit coronary microvessels by a modification of the original procedure of Simionescu and Simionescu (6) as detailed in a previous communication (7). The cells were routinely cultivated in 95% air, 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 15% fetal calf serum, and supplemental L-glutamine. Media were replaced every 2–3 days and cells were used within 1–3 days of attaining confluency. Cells were subcultivated by brief trypsin-EDTA treatment and 1:4 dilution. Cells used in the present study were obtained from the second to fifth passages of several different cell lines, and 90–95% of the cells were endothelial cells, as assessed by factor VIII staining.

[^{125}I]Insulin binding. All binding studies were performed on adherent monolayer cultures; methods used were those described by Bar *et al.* (3) with some modifications. Confluent cells from the second to fifth passages were used. For this series of studies, cells were cultivated in Linbro 24-well "space saver" culture dishes. The culture media from each culture well was aspirated and the cells (~200,000 cells/well) washed twice with 1 ml of Krebs–Ringer phosphate buffer containing 1% bovine serum albumin, pH 7.8 (incubation buffer). For the binding studies, cells were incubated with 7.2×10^{-11} *M* [^{125}I]insulin and increasing concentrations of unlabeled insulin (8.2×10^{-11}–10^{-6} *M*) in a final volume of 0.6 ml per dish. After 60 min at 22°C or 4 hr at 4°C, the fluid was removed and the cells washed rapidly with cold (4°C) incubation buffer. Cells were dissolved in 1 ml of 1.0% sodium dodecyl sulfate (SDS), and cell associated radioactivity counted in a Searle gamma counter, at 78% efficiency. Binding of [^{125}I]insulin in the presence of 1.67×10^{-5} *M* insulin was termed "nonspecific" binding, and was subtracted from total binding to calculate specific binding.

Glucose oxidation. RCME cells were grown on Cytodex 3 microcarrier beads in suspension using a Techne microcarrier stirrer apparatus. The cytodex beads were prepared in phosphate-buffered saline (8), autoclaved, and

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suspended in DMEM with 15% calf serum. Cells from primary cultures or first passage were removed by trypsin-EDTA treatment and placed immediately into the stir bottles containing the beads. The beads were stirred at a low speed ("30" setting on Techne microcarrier stirrer) using the interval stirring mode. Cells grew rapidly on the beads and were used for studies when greater than 90% of beads examined were completely covered with endothelial cells as assessed by phase contrast microscopy. Prior to use in the following experiment beads were transferred to plastic conical centrifuge tubes and washed with 5–6 vol of Krebs–Ringer phosphate buffer (pH 7.4). The beads were then suspended in 1 ml of Krebs–Ringer phosphate buffer containing 2% bovine serum albumin. The tubes were capped tightly with a rubber stopper fitted with a three-way stop cock and two syringes from which a 1 × 2-cm strip of NCS (Amersham) coated filter paper was suspended. The reaction was initiated by the injection of D-[1-^{14}C]glucose (0.2 μCi/tube) in Krebs–Ringer phosphate (vol 500 μl) into each tube and incubation at 37°C. The reaction was stopped by the injection of 1 ml of 1 N H_2SO_4 into each tube and a further 15 min incubation at 37°C. The filter paper containing trapped $^{14}CO_2$ was transferred to liquid scintillation vials, neutralized by the addition of 1 N HCl and 10 ml scintillation fluid added (Liquiscint, National Diagnostics). Radioactivity was determined in a Searle beta-scintillation counter, Model 1185. Tissue quantities were normalized (using weight of beads used/tube). Preliminary experiments indicated an initial lag of $^{14}CO_2$ production, followed by a progressive linear increase in $^{14}CO_2$ formation for 30–120 min. Experiments were therefore performed comparing $^{14}CO_2$ production after 120 min by cells pretreated with 1.6×10^{-10} M insulin with untreated (control) cells.

Lipogenesis. Conversion of D-[U-^{14}C]glucose into total cell lipids was measured in samples prepared as above. Following completion of the glucose oxidation studies, samples were extracted as described by Rodbell (9). An aliquot of the organic phase was mixed with 4 ml of OCS (Amersham) and counted in a liquid scintillation counter. Blank samples were run without tissue.

Studies of 2-deoxyglucose uptake and 3-O-methyl-D-glucose transport. Confluent RCME cells (cultivated in 24-well culture plates (Falcon)) were washed twice with 1 ml of sterile PBS, 1% bovine serum albumin. To avoid the influence of insulin or insulin-like peptides in the calf serum, RCME cells were preincubated 16 hr at 37°C (in 5% CO_2 in air) in DMEM containing 1% bovine serum albumin (BSA) and 10 mM Hepes (DMEM/BSA/Hepes buffer). At the end of the preincubation period, the DMEM was removed, the cells were washed three times with 1 ml PBS; fresh glucose-free DMEM/BSA/Hepes buffer was added and incubated with or without insulin for 3 hr unless otherwise indicated. The cells were preequilibrated to 23°C and the monolayers were pulsed with either [2-^{3}H]deoxyglucose (0.2 μCi, 5×10^{-3} M final concentration) or 3-O-methylglucose (0.2 μCi, 5×10^{-5} M final concentration) for the indicated time at 23°C. The monolayers were washed four times with 2 ml of ice-cold PBS and all associated radioactivity was determined by dissolving the cells in 1 ml of 0.1 M NaOH in 0.1% sodium dodecyl sulfate (SDS) and adding the SDS solution to 4 ml of liquiscint and radioactivity counted in a Searle scintillation counter. In other experiments, the nature of the labeled sugar in the RCME cells was determined as described by others (10, 11). Cells were incubated as above with 2-[^{3}H]deoxyglucose (15 mM) for 30 min. The cells were washed three times with 1 ml of cold PBS/BSA buffer. Boiling water (1 ml) was added containing 0.2% Triton X-100. The dissolved cells were heated to 100°C for 5 min. The suspension was extracted with chloroform and the aqueous phase passed over a Dowex-1×8 chloride column in a Pasteur pipet. The column was washed with 4.5 ml of H_2O and the fractions were counted. Phosphorylated 2-deoxyglucose was removed from the column by washing with 9 ml of 0.2 M formic acid/0.5 M ammonium acetate pH 9. Recovery of labeled sugar was 91%.

Materials. Crystalline porcine insulin, bovine insulin, human insulin, and porcine proinsulin was kindly provided by Dr. Mary Root of Eli Lilly and Company, Indianapolis, Indiana. Receptor grade [^{125}I]insulin (300 μCi/ug) was provided by New England Nuclear. [2-^{3}H]Deoxyglucose and 3-O-

[^{3}H]methyl-D-glucose was purchased from Amersham. 2-Deoxyglucose and 3-*O*-methyl-D-glucose were purchased from Sigma. Dulbecco's modified Eagle's medium, MEM amino acid, and vitamins were purchased from GIBCO.

Results. *Binding studies.* The binding of [^{125}I]insulin to RCME cells was time and temperature dependent. Maximum specific binding was reached at 30 min of incubation at 23°C and remained at this level for at least 1 hr. At 4°C, maximum specific binding occurred after 4 hr of incubation (Fig. 1). The optimum pH range for binding was 7.7–8.0. All subsequent studies were performed at 23°C, pH 7.8, for an hour of incubation. Under these conditions, degradation of [^{125}I]insulin (precipitation with 5% trichloroacetic acid) was less than 2%. Nonspecific binding represented 30–35% of total binding.

The competition curve for [^{125}I]insulin binding to RCME cells is presented in Fig. 2. Fifty percent of maximum specific binding was achieved with 1×10^{-9} *M* insulin, and more than 70% displacement of specific insulin binding occurred with 5×10^{-8} *M* insulin. The Scatchard analysis of the binding isotherm is presented in Fig. 3. The binding of [^{125}I]insulin was reversible, as shown in the dissociation study in Fig. 4. The RCME cells exhibited specificity of binding. Thus, much higher concentrations of porcine proinsulin were required to displace [^{125}I]porcine insulin (Fig. 5). Fifty percent inhibition of maximum binding was achieved with 10^{-9} *M* human insulin, 2×10^{-10} *M* bovine insulin, and 2×10^{-8} *M* porcine proinsulin.

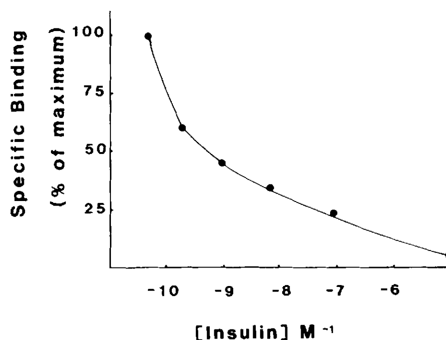

FIG. 2. Displacement of [^{125}I]insulin by porcine insulin. RCME cells were incubated with 750 pg of [^{125}I]insulin, in the presence of increasing concentrations of insulin. Results are plotted as the percentage of maximum specific binding. Each point is the mean of eight experiments (maximum cpm [^{125}I]insulin specifically bound = 1129 ± 30).

Glucose oxidation and glucose conversion into lipids. Insulin stimulated the conversion of [U-^{14}C]glucose to $^{14}CO_2$ (Fig. 6) and to lipids (Fig. 7) at physiological levels (1.6×10^{-10} *M*). The effects of insulin on hexose transport were determined using two analogs, 2-deoxyglucose and 3-*O*-methylglucose and

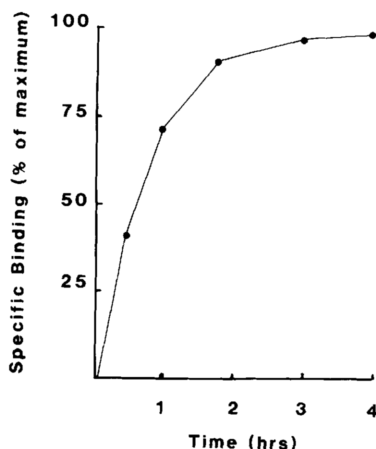

FIG. 1. Time course of association of [^{125}I]insulin at 4°C. The results are plotted as the percentage of the maximum specific binding. Each point is the mean of four experiments (maximum cpm [^{125}I]insulin specifically bound 1300 ± 112).

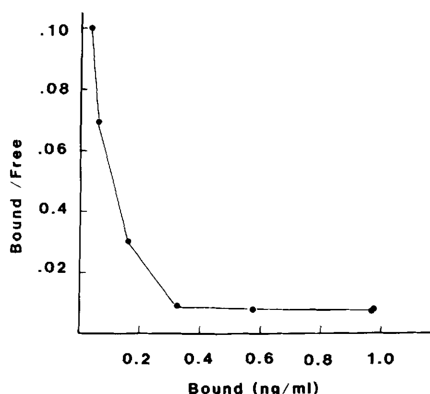

FIG. 3. Displacement of [^{125}I]insulin binding by porcine insulin. The bound/free ratio is plotted as a function of insulin bound (Scatchard plot). Each point is the mean of eight experiments (maximum cpm [^{125}I]insulin specifically bound = 1129 ± 30).

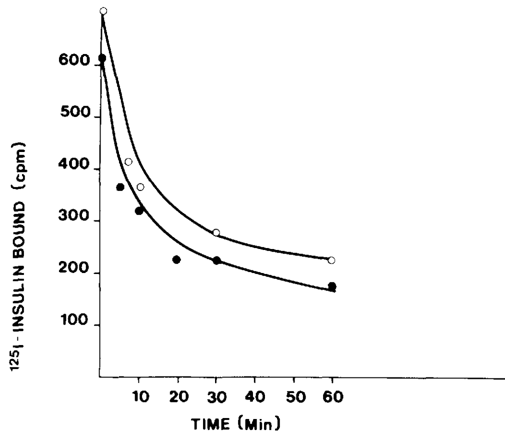

FIG. 4. Dissociation curves of [^{125}I]insulin in the absence (O) and presence (●) of 1.67×10^{-5} *M* insulin. Dissociation was conducted after RCME cells had been incubated with [^{125}I]insulin (750 pg) at 22°C for 1 hr. Cells were then washed twice with 1 ml of 4°C Krebs–Ringer phosphate buffer, containing 1% bovine serum albumin and incubated at the indicated times. Each point is the mean of four experiments.

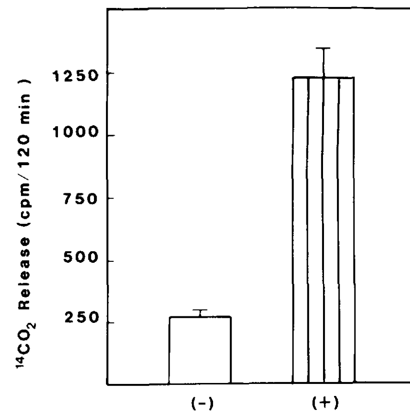

FIG. 6. Stimulation of [U-^{14}C]glucose metabolism to $^{14}CO_2$ by porcine insulin (1.6×10^{-10} *M*). (□) Incubation in the absence of insulin; (▥) incubation in the presence of insulin. Each value is the mean of 20 experiments. *$P < 0.05$ (ANOVA).

porcine insulin. Initial experiments indicated variable effects of insulin if cells were tested immediately after removal of culture media. Overnight incubation in insulin-free incubation media resulted in reproducible and consistent effects of insulin on glucose transport. Uptake of 3-*O*-methylglucose, a transported analog of glucose that is not further metabolized (12), showed a rapid time course at 23°C (Fig. 8). Preincubation of RCME cells for 3 hr with insulin significantly enhanced 3-*O*-methylglucose uptake determined at 15 sec (Fig. 9).

Further studies of insulin's action on hexose uptake were determined using 2-deoxyglucose. An incubation of at least 60 min with insulin was necessary to demonstrate enhancement of 2-deoxyglucose uptake (Fig. 10) or 3-*O*-methylglucose uptake (data not shown). The dose–response curve for insulin

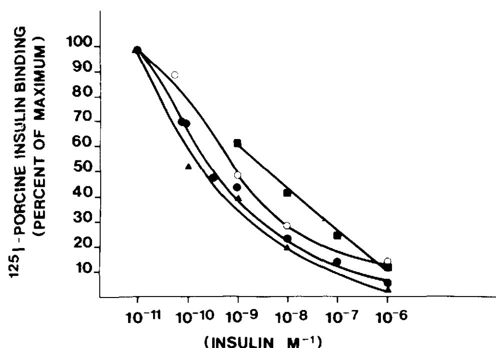

FIG. 5. Displacement of [^{125}I]insulin by porcine insulin (▲), bovine insulin (●), human insulin (O), and proinsulin (■). Each point is the mean of four experiments (maximum cpm [^{125}I]insulin specifically bound = 1103 ± 6 cpm).

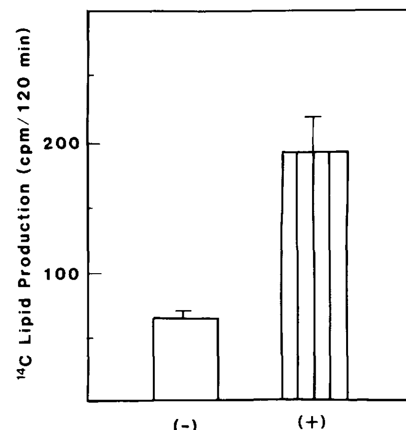

FIG. 7. Stimulation of [U-^{14}C]glucose metabolism to [^{14}C]lipids by porcine insulin (1.6×10^{-10} *M*). (□) Incubation in the absence of insulin, (▥) incubation in the presence of insulin. Each value is the mean of 20 experiments. *$P < 0.05$ (ANOVA).

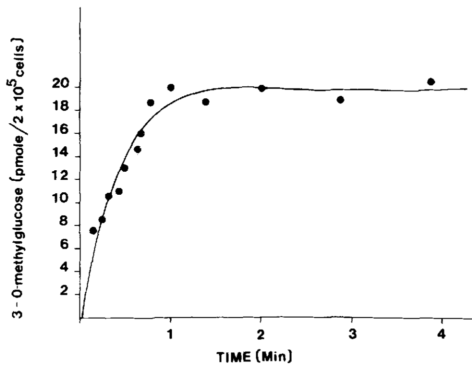

FIG. 8. Time course for uptake at 23°C of 50 μM 3-*O*-methyl-D-glucose. Data shown are the average of duplicate determinations.

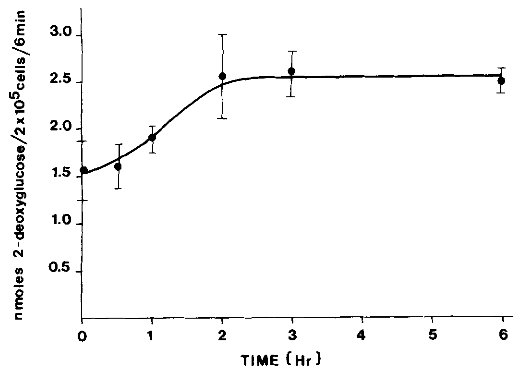

FIG. 10. Time course of insulin stimulation of 2-deoxyglucose uptake. After preincubation in 1 M serum-free incubation media, RCME cells were incubated with 8×10^{-9} M insulin for the indicated periods of time before 2-deoxyglucose uptake was determined. Data shown are the $X \pm$ SEM of four experiments.

effects on 2-deoxyglucose uptake is given in Table I. Significant effects of insulin were observed at 10^{-10} M insulin and the maximum effective dose was 10^{-8} M insulin.

The distribution of [2-^{3}H]deoxyglucose in the cell was initially determined by washing the cells in PBS followed by dissolving the cells in 2% Triton X-100. After a 30-min incubation with 2-deoxyglucose, this method indicated 61 ± 4% of the intracellular-labeled sugar was 2-deoxyglucose and 39 ± 4% (n = 4) was present as 2-deoxyglucose 6-phosphate. However, Betz *et al.* (13) found rapid dephosphorylation of 2-deoxyglucose 6-phosphate in brain capillaries unless the cells were boiled. Boiling the RCME cells prior to separation of labeled sugar indicated that 60–90% of the labeled sugar was phosphorylated and 20–40% was free 2-deoxyglucose.

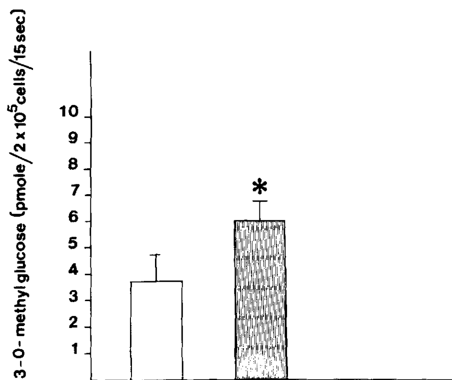

FIG. 9. Effects of insulin on 3-*O*-methyl-D-glucose uptake. RCME cells were preincubated in incubation medium as described in the methods, then incubated 3 hr with or without 8×10^{-9} M insulin for 3 hr. Uptake of 3-*O*-[^{3}H]methyl-D-glucose at 15 sec (23°C) was then determined. Data shown are the $X \pm$ SEM of 12 determinations. *$P < 0.05$; two-way ANOVA followed by Dunnett's t test. □ Control; ▥ + insulin.

Discussion. Several recent studies have indicated that the microvasculature may be an important site for insulin action. Brendel *et al.* (14) initially demonstrated the ability of cerebral microvessels to metabolize glucose. The observations were verified by other investigators using coronary (15), and retinal (16) microvessels. Recent reports (4, 5) have

TABLE I. UPTAKE OF 2-[^{3}H]DEOXYGLUCOSE INTO RCME CELLS ($\bar{X} \pm$ SEM, n = 4)

Incubation conditions	2-Deoxyglucose uptake (nmole/6 min)
Control	0.97 ± 0.24
10^{-11} M Insulin	1.19 ± 0.18
10^{-10} M Insulin	2.83 ± 0.58*
10^{-9} M Insulin	2.71 ± 1.67
10^{-8} M Insulin	3.32 ± 0.78*
10^{-7} M Insulin	3.29 ± 0.66*

Note. Final concentration of 2-deoxyglucose, 5 mM. RCME cells were incubated overnight in incubation media, followed by a 3 hr incubation in media with or without insulin at the indicated concentrations.

* Denotes values significantly different from control group ($P < 0.05$; ANOVA and post hoc Dunnett's t test).

described specific binding of [^{125}I]insulin to brain microvessels, indicating cerebral cortical microvessels may be an insulin-dependent tissue. A recent study by King *et al.* (17) recently reported marked stimulation by insulin of [^{14}C]glucose incorporation into glycogen in endothelial cells cultured from retinal capillaries, although they did not observe similar effects of insulin on cells cultured from large caliber vessels (bovine aorta and human umbilical vein).

The results of the present study are the first indication of specific binding of insulin to and the ability of insulin to alter glucose uptake and metabolism in cultured coronary microvascular endothelium. These findings are supported by an earlier report of insulin receptors in microvessels of the intact heart (18). The binding of insulin to the RCME cells was rapid, specific for insulin, and reversible. The characteristics of insulin binding to the RCME cells were similar to those of human umbilical artery and vein endothelial cells (3). The maximum metabolic effects of porcine insulin were observed at concentrations less than 10^{-9} *M* insulin, although the displacement curve indicated that at this concentration binding was only 50% of maximum. This discrepancy may be explained by either invoking the spare receptor theory and/or the difference in temperatures and experimental conditions between binding and metabolic studies. The sensitivity of the metabolic effects (i.e., carbohydrate and lipid metabolism) occurs over a similar range of insulin concentrations, and the concentrations of insulin required to evoke these effects are well within the physiological range. Initial experiments evaluating the effects of insulin on hexose transport in RCME cells cultured in DMEM with 15% fetal calf serum yielded variable results. Similar variable effects of insulin on glucose transport in cultured fibroblasts were initially reported by Howard and co-workers (19). The possibility that insulin stimulation of hexose transport is influenced by nutritional and hormonal factors has been suggested in other cell systems (20). Preincubation in a culture medium devoid of serum was found to optimize insulin's effects on glucose entry in cultured human fibroblasts (19). Use of these methods with cultured RCME cells yielded similar results, i.e., reproducible, significant effects of insulin in 3-*O*-methyl glucose transport and 2-deoxyglucose uptake. The time course of insulin action on hexose transport is much longer than that required in other systems, such as the isolated adipocyte (11). Ishibashi *et al.* (21) also found that the effects on insulin on hexose uptake in fibroblasts required 30–60 min. It is not known if these observations imply a different mechanism of insulin action on hexose transport in the RCME cell or fibroblasts and must be further explored.

Our data on the uptake and metabolism of 2-deoxyglucose in RCME cells indicate that substantial dephosphorylation of the 2-deoxyglucose 6-phosphate can occur unless the cells are boiled prior to separation of labeled sugars. Similar artifacts resulting from rapid dephosphorylation of 2-deoxyglucose 6-phosphate have been reported for rat kidney slices (22) and isolated rat brain capillaries (13).

An additional role that has been proposed for insulin association with endothelial cells is that of internalization, metabolism, and/or transport of insulin from the blood to the surrounding tissue. A study by Bar (18) indicated that the amount of insulin retained in capillary endothelium of the heart could reach levels as high as 20% relative to an area of interstitium and muscle six to seven times the size and, in addition, the authors found at least 1% of infused [^{125}I]insulin was retained in intact form within the cardiac microvascular system. Further support for an insulin transport role of endothelial cells was provided by the recent report of Jialal *et al.* (23) who observed rapid internalization of [^{125}I]insulin and later release (with minimal degradation) in bovine aortic endothelial cells.

Diabetes mellitus has been associated with microangiopathy in several organs, including the retina, skeletal muscle and kidney. Although the coronary microvasculature is not recognized as a site of "significant" microangiopathy, the results from the present study indicate that the coronary microvascular endothelium is an insulin-sensitive tissue and may be a useful model for studies concerning the effects of a diabetic microenvironment.

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