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#### Research Article

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## Differential Responsiveness to Insulin of Endothelial and Support Cells from Micro- and Macrovessels

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ABSTRACT The pathologies of diabetic micro- and macroangiopathy are different, suggesting that diabetes affects these two types of vascular tissue in a dissimilar manner. We have compared insulin receptors and the effects of insulin on cultured endothelium from calf retinal capillaries and aorta, and the vascular supporting cells, retinal pericytes, and aortic smooth muscle cells. <sup>125</sup>I-insulin binds to high affinity insulin receptors on all four cell types. Receptor concentrations were similar except for aortic smooth muscle cells, which have 10-fold fewer receptors than the other cell types. Insulin at a concentration of 10 ng/ ml stimulated [14C]glucose incorporation into glycogen in retinal endothelial cells and pericytes and aortic smooth muscle cells, but had no effect on aortic endothelium. Insulin over a concentration range of 10 ng/ml-10  $\mu$ g/ml, stimulated [<sup>3</sup>H]thymidine incorporation into the DNA of retinal pericytes, and endothelial cells and aortic smooth muscle cells but had no effect on aortic endothelial cells. These data suggested that a differential response to insulin may exist between endothelium of micro- and macrovasculature, and suggest that retinal capillary endothelium and retinal pericytes are both very insulin-sensitive tissues.

#### INTRODUCTION

Endothelial cells have been implicated in the pathogenesis of diabetic complications in the development of both micro- and macroangiopathy (1-3). Studies of cells in culture have demonstrated differences in the growth requirements for endothelium and other vascular cells of large vessels and capillaries, although detailed characterization of these differences has not been established (4-7). Similarly, the effects of insulin,

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a pivotal hormone in the regulation of cellular metabolism and growth in diabetics and nondiabetics, have not been investigated in detail (8–10), although some studies have shown that insulin receptors exist on endothelial cells from large blood vessels and isolated brain microvessels (11–12).

In the present report, we have established cultures of endothelial cells from bovine retinal capillaries and aorta and characterized the insulin receptor, and insulin's metabolic and growth-promoting effect on these cells. In addition, similar studies were performed on the vascular supporting cells, i.e., capillary pericytes and aortic smooth muscle cells. These studies suggested that insulin may have a role both in the metabolism and in the growth of these cells. In addition, cells of the retinal capillary and aorta appear to be different in their responsiveness to hormonal stimulation.

#### METHODS

Isolation and culturing of cells. For retinal capillary endothelium and pericyte cultures, pure endothelium and pericytes, respectively, were harvested from calf retinal capillaries as previously described (6). Briefly, whole retinas from newborn calves were obtained by dissection and were then homogenized in phosphate-buffered saline (PBS), pH 7.4. The mixture was filtered through three nylon screens of decreasing mesh sizes from 88 to 5.3 µM. Retinal capillaries retained by the last nylon screen were incubated in PBS, pH 7.4, containing 0.75% collagenase (Worthington Biochemical Corp., Freehold, NJ) and 0.5% bovine serum albumin (Sigma Chemical Co., St. Louis, MO) for 30 min at 37°C. Vascular cells and partially digested capillaries were collected by centrifugation at 800 g for 10 min and cultured on gelatin-coated tissue culture dishes (Costar, Data Packaging, Cambridge, MA). Endothelial cells were cultured in conditioned Dulbecco's modified Eagle's medium (DMEM)<sup>1</sup> with penicillin, 200 U/ml, and 10% calf serum (Gibco Lab-

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<sup>&</sup>lt;sup>1</sup>Abbreviation used in this paper: DMEM, Dulbecco's modified Eagle's medium.

oratories, Grand Island Biological Co., Grand Island, NY). The DMEM had been previously conditioned by being cultured in mouse sarcoma 180 tumor cells (6). For pericytes, "conditioned" DMEM with 10% calf serum was used.

After 2-3 d, individual cells and small colonies of two or three cells were identified under phase-contrast microscopy. Unwanted cells were removed with a finely tapered Pasteur pipette attached to a micromanipulator (Brinkmann Instruments, Inc., Westbury, NY) as described by Folkman et al. (7). The culture was washed twice with Eagle's balanced salt solution, pH 7.4, and fed with growth media as described before. This weeding procedure was performed daily until pure cultures were obtained. The cells were then harvested by treatment with 1 ml of 0.05% trypsin (Sigma Chemical Co.), with 0.2% EDTA (Gibco Laboratories) for 3 min. The suspended cells were replated in gelatin-coated six-well cluster plates (Costar) and maintained in growth media as described.

Calf aortic and endothelial cells. Calf aortas were obtained from a local slaughterhouse. The intimal aspect of calf aorta was exposed and incubated in 0.1% collagenase (Worthington Biochemical Corp.) for 30 min at room temperature. The aorta was washed with PBS at pH 7.4. The aorta was then gently scraped once by a cotton swab, and the cells on the swab were plated in culture dishes. Occasionally, weeding procedures were necessary to isolate and purify endothelial cells from contaminating smooth muscle cells as described above for retinal capillary cells, except aortic endothelial cells were grown in M199 media with 20% fetal bovine serum (Gibco Laboratories).

Calf aortic smooth muscle cells. Aortic smooth muscle cells were harvested from calf aorta by using the same procedure as the isolation of endothelial cells except that the aorta was scraped vigorously with a cotton swab several times (13). The cells were washed off the swab into a Costar culture dish in M199 media with 10% fetal bovine serum. After the cells had covered the bottom of the plate, they were incubated with 0.1% trypsin for 2 min and loose cells were rinsed from the plates. The cells that were enriched for smooth muscle cells remained attached and were fed with M199 media with 20% calf serum.

Human endothelial cells. Primary human endothelial cells from umbilical veins were a generous gift of Dr. M. Gimbrone, Jr. (14). The cells were propagated by the method of Maciag et al. (15) by using partially purified endothelial growth factor from bovine hypothalamus.

Cell identification. The identity of the cells was confirmed by morphologic criteria with light and transmission electron microscope (6). In addition, all the cells were examined for the presence of Factor VIII by using indirect immunofluorescence as described previously with a monospecific bovine Factor VIII antibody, a gift of J. Brown, University of California, San Diego (6). Angiotensin converting enzyme activity in cultured cells was determined by using a radioassay (Ventrex, Portland, ME). The endothelial cells from the retinal capillary and the aorta were positive for Factor VIII and any iontensin converting enzyme activity, whereas pericytes and smooth muscle cells were not. By using these biochemical and morphological tests, the cultures were determined to be free of contaminating cells.

<sup>125</sup>I-insulin binding. [<sup>125</sup>I]iodoinsulin with specific activity of 100-200  $\mu$ Ci/ $\mu$ g was prepared by chloramine T oxidation as described by Freychet et al. (16). All <sup>125</sup>I-insulin binding was performed on cells still attached to 35-mm multiwell plates. Incubation was at 15°C for 4 h in Hepes binding buffer (pH 8.0, 0.1 M Hepes, 0.12 M NaCl, 5 mM KCl, 1.2 mM MgSO<sub>4</sub>, 8 mM glucose, and 1% bovine serum

albumin). Concentrations of unlabeled insulin were added as noted in the figures. After binding, the cells were washed with PBS at 4°C and solubilized with 0.1% sodium dodecyl sulfate (11). The samples were counted in a Tracor 1190 gamma counter (Tracor Analytic Inc., Elk Village, IL) at 80% efficiency. Cells were counted either by using a calibrated ocular recticle for monolayer cells or by hemocytometer on suspended cells after trypsin digestion.

Protein assays were performed by the method of Lowry et al. with bovine serum albumin as standards (17).

Biological effects of insulin. For measurements of [14C]glucose incorporation, incorporation of [14C]glucose was measured by a modification of the method of Cuendat et al. (18). Confluent cells were preincubated with glucose-free M199 media for 24 h. Pork insulin (Elanco Products Co., Indianapolis, IN) and [14C-(U)]glucose (New England Nuclear, Boston, MA; 250 mCi/mMol sp act) at 1  $\mu$ Ci/ml were added simultaneously and incubated for 1 h at 37°C. The incubation medium was Krebs-Ringer bicarbonate buffer containing 118 mM NaCl, 5 mM KCl, 1.3 mM CaCl<sub>2</sub>, 1.3 mM MgSO<sub>4</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 2.5 mM NaHCO<sub>3</sub>, and 0.3 mM glucose at pH 7.4. The reaction was stopped by washing three times with PBS at 4°C. Cells were solubilized with 0.1% SDS and precipitated with 100% (CH2OH at -20°C after addition of 1 mg of glycogen to each sample as a carrier. The pellet was washed repeatedly with 66% CH<sub>2</sub>OH at -20°C. The final pellet was dissolved in H<sub>2</sub>O and transferred to counting vials with 10 ml of aqueous counting solution (Amersham Corp., Arlington Heights, IL) and counted in a liquid scintillation counter (LKB 1215, Sweden).

[<sup>3</sup>H]Thymidine incorporation into DNA. Cells were grown to near confluence at 37°C in six-well cluster plates (Costar). The growth media was replaced with serum-free M199 media containing 2.5 mg/ml bovine serum albumin at pH 7.4 for 2-3 d. Indicated concentrations of insulin were added to each well for an 18-h preincubation, after which the cells were pulsed with [<sup>3</sup>H]thymidine, 2  $\mu$ Ci/ml, for 30 min at 37°C. The cells were solubilized with 0.1% sodium dodecyl sulfate, and the DNA precipitated with 10% trichloracetic acid at 4°C as previously described (19, 20).

#### RESULTS

<sup>125</sup>I-Insulin binding. Using <sup>125</sup>I-insulin, specific insulin binding was found on both retinal and aortic endothelial cells, retinal pericytes, and aortic smooth muscle cells (Figs. 1 and 2). Endothelial cells from retinal capillary and aorta showed similar high affinity receptors. The concentration of unlabeled insulin that inhibited the binding of <sup>125</sup>I-insulin by 50% was 1.5 ng/ml ( $2.5 \times 10^{-10}$  M) for both types of endothelial cells, although the retinal cells had higher binding of the <sup>125</sup>I-insulin tracer. At 0.2 ng/ml of <sup>125</sup>I-insulin, endothelial cells from retinal capillary bound 7.2% per 10<sup>6</sup> cells, whereas aortic endothelial cells bound only 3.8% per 10<sup>6</sup> cells. (Fig. 1). Nonspecific binding accounted for <10% of the total binding.

Scatchard analyses of the competition curves for endothelial cells were curvilinear, similar to those for insulin receptors in other tissues (11, 14) and parallel to each other, indicating that the insulin receptors on



FIGURE 1 A  $^{125}$ I-insulin binding to endothelium, competition curves with unlabeled insulin. B Scatchard analysis of the competition curves on the left. The binding studies were performed at 15°C for 4 h on monolayer cells on culture dishes. Details of the assays are described in Methods.

endothelial cells of aorta and retinal capillary had comparable affinities. The two curves intersected with the abscissa at different points, however, indicating that the greater total binding exhibited by the retinal endothelial cells was due to a greater number of insulin receptors rather than a change in receptor affinity (Fig. 1).

For retinal pericytes and aortic smooth muscle cells, the analysis of <sup>125</sup>I-insulin bound and the apparent affinity of binding revealed differences both in the amount of <sup>125</sup>I-insulin bound and the apparent affinity of binding. (Fig. 2). At tracer concentration, pericytes bound 9% per  $10^6$  cells, whereas smooth muscle cells bound only 0.3% per  $10^6$  cells. The concentrations of unlabeled insulin needed to decrease maximal binding by 50% were 3 ng/ml ( $6 \times 10^{-10}$  M) and 15 ng/ml ( $2.5 \times 10^{-9}$  M) for pericytes and smooth muscle cells, respectively, suggesting some difference in receptor affinity. Scatchard analysis showed the typical curvilinear pattern observed for insulin receptors, with a major difference of the abscissal intercepts, indicating that the greater total binding of the pericytes was also due to a higher receptor number. Although the cells differ in size (the endothelial cells are smaller than the peri-



FIGURE 2 A <sup>125</sup>I-insulin binding to vascular supporting cells of retinal pericytes and aortic smooth muscle cells, competition curves with unlabeled insulin. B Scatchard analysis using competition curves from the left. The conditions of the binding are as described in Fig. 1 and Methods. The percentage of binding for the aortic smooth muscle cells has been expanded by a factor of 10 as indicated.

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cytes and smooth muscle cells), the normalization of <sup>125</sup>I-insulin binding by protein did not change the relative order of binding among these cells. With the limitation of accuracy as posed by a curvilinear Scatchard curve, we approximated that retinal pericytes and endothelial cells have 4.5 and  $1.2 \times 10^4$  receptors per cell, respectively. For the aortic endothelial cells and smooth muscle cells, 1 and  $0.5 \times 10^4$  receptors were calculated.

Stimulation of [<sup>14</sup>C]glucose incorporation into glycogen. In the basal state, endothelial cells from retinal capillaries incorporated [<sup>14</sup>C]glucose into glycogen at the rate of  $17\pm 2$  nmoles/mg protein per h. Insulin at concentrations of 10 ng/ml ( $1.7 \times 10^{-9}$  M) and 10 µg/ ml ( $1.7 \times 10^{-6}$  M) increased the rate of [<sup>14</sup>C]glucose incorporation by 95 and 100%, respectively. In contrast, aortic endothelium and human umbilical vein endothelial cells did not respond to insulin at either concentrations, although the basal rate of glucose incorporation was similar to that in endothelial cells from retinal capillaries (Fig. 3).

The basal rates of glycogen synthesis in retinal pericytes and aortic smooth muscle cells were ~10-50% that of the endothelial cells. In the basal state, these cells incorporated [<sup>14</sup>C]glucose at the rate of  $1.6\pm0.2$ and  $11\pm2$  nmoles/mg protein per h, respectively. With the addition of 10 ng/ml ( $1.7 \times 10^{-9}$  M) and 10  $\mu$ g/ml ( $1.7 \times 10^{-6}$  M) of insulin, both cells increased the rate of [<sup>14</sup>C]glucose incorporation (Fig. 3 C and D). At a concentration of 10  $\mu$ g/ml, insulin increased glucose incorporation by 50% for pericytes and 250% for smooth muscle cells (average of four experiments). Both cell types also showed significant stimulation at insulin concentrations of 10 ng/ml ( $1.7 \times 10^{-9}$  M).

Stimulation of [<sup>3</sup>H]thymidine incorporation into DNA. Endothelial cells from retinal capillary and aorta exhibited different responses to insulin with respect to growth-promoting effect (Fig. 4A). Insulin was able to increase [<sup>3</sup>H]thymidine incorporation by five-to sixfold in retinal endothelial cells starting at 10 ng/ml ( $1.7 \times 10^{-9}$  M) and the maximal response was observed at  $10 \,\mu$ g/ml. Aortic endothelial cells and human endothelial cells (Table I and Fig. 4) were only minimally responsive (<10%) to insulin. However, all the cells tested responded well to 10% serum. In addition, endothelial cells growth factor was very effective in human endothelial cells.

For the vascular support cells, in both retinal pericytes and aortic smooth muscle cells, insulin was able to increase DNA synthesis, although the dose-response curves for the two cells are different (Fig. 4B). With the pericytes, insulin stimulated [<sup>3</sup>H]thymidine incorporation by 10-fold starting at 10 ng/ml with maximal response around 1  $\mu$ g/ml. In aortic smooth muscle cells, higher concentrations were required and a lower maximal response was observed (Fig. 4).

#### DISCUSSION

In this study we have compared insulin receptors and actions between vascular cells from the capillaries, namely retinal endothelial cells and pericytes, and from bovine aorta and human umbilical vein, namely endothelial cells and smooth muscle cells. High affinity



INSULIN CONCENTRATION (ng/ml)

FIGURE 3 Effect of insulin on the stimulation of  $[^{14}C]$ glucose incorporation into glycogen of endothelium on the left (A, aortic; B, retinal capillary) and vascular supporting cells on the right (C, aortic smooth muscle cells; D, retinal pericytes). Experiments were carried out on monolayered cells grown on gelatin plates and incubated with insulin and  $[^{14}C]$ glucose for 60 min at 37°C. The details of the procedure are described in Methods.



FIGURE 4 Effect of insulin on the stimulation of  $[^{3}H]$ thymidine incorporation into DNA of endothelium (A) and vascular supporting cells (B). Confluent cultures on gelatin-coated plates were incubated with insulin for 18 h and pulsed with  $[^{3}H]$ thymidine for 30 min in PBS at pH 7.4. The details of the study are described in Methods.

receptors for insulin were present on all the cells tested, although aortic smooth muscle cells have a smaller number of receptors. In measuring the incorporation of [<sup>14</sup>C]glucose into glycogen as an example of a metabolic effect, retinal endothelial cells, retinal pericytes, and aortic smooth muscle cells were responsive to insulin at low and physiological concentrations, whereas aortic and umbilical vein endothelial cells were not sensitive to insulin. Likewise for growth-promoting activity, i.e., stimulation of thymidine incorporation into DNA, insulin was rather ineffective on endothelial cells from large vessels, in contrast with its good activity on retinal endothelium and pericytes, and aortic smooth muscle cells.

 TABLE I

 Comparison of Insulin's Growth-Promoting Effect

 on Various Types of Endothelial Cells

Type of endothelium	( <sup>8</sup> H)Thymidine incorporation into DNA*			
	Basal	Insulin (10 µg/ml)	Serum (10%)	ECGF
	cpm/mg protein			
Bovine				
Retinal capillaries	1,180	8,108	15,300	NOT DONE
Aorta	3,394	3,916	15,280	NOT DONE
Human				
Umbilical Vein	1,560	1,780	7,594	9,840

• Data were calculated from one typical experiment. Each point is the average of three wells.

ECGF, endothelial cell growth factor, partially purified by method of Maciag et al. (15).

Several conclusions can be drawn from these findings. First, endothelial cells from the retinal capillary are very different from aortic and human umbilical vein endothelium as characterized by their responsiveness to insulin in both metabolic and growth-promoting effects. The vascular support cells, pericytes, and aortic smooth muscles, on the other hand, are similar in their response to insulin, but they differ strikingly in the number of insulin receptors. Secondly, insulin is able to stimulate metabolic and growth effects in retinal capillary cells at physiological concentrations. This suggests that insulin may be important for the metabolism and growth of retinal capillaries. In disease states such as diabetes, the presence of insulin deficiency or insulin excess (as a result of treatment) may have an etiological role for the development of diabetic retinopathy. The latter point may be especially important in view of the recent finding that many patients treated with continuous subcutaneous infusion have higher than normal insulin levels (21).

These findings suggest that endothelial cells derived from large vessels and capillaries are different in their response to insulin. However, endothelial cells from capillaries and large vessels from other animal and tissue sources have to be checked in order to determine whether our data can be applied in general. In addition, our report provided biochemical evidence that insulin may have a metabolic role in the vascular cells.

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