Insulin Permeability across an *in Vitro* Dynamic Model of Endothelium

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Received September 24, 2001; accepted January 4, 2002

Purpose. Endothelium insulin permeability was investigated using *in vitro*, dynamic culture of endothelial cells.

Methods. Endothelial cells were cultured in a hollow fiber apparatus and continuously exposed to a flow. Transendothelial electrical resistance and permeability to [^{14}C]sucrose and [^{14}C]inulin were used to monitor the integrity of the endothelial monolayer.

Results. Under these experimental conditions, measurements of insulin permeability, investigated at increasing hormone concentrations, suggested that the predominant transendothelial insulin fluxes were attributable to bidirectional convective transport rather than to a saturable transport mechanism, in agreement with *in vivo* experiment results published earlier. Analytical determinations of insulin catabolism demonstrated a low percent of insulin degradation by the endothelium, leading to production of insulin metabolites qualitatively identical to those produced by human monocytes.

Conclusions. The findings of this paper indicated that (a) insulin crosses the endothelial monolayer by paracellular "leak" and endothelial insulin receptors have a minor (if any) role in insulin transport; (b) degradation of the hormone by BAEC is minimal; (c) the *in vitro*, dynamic culture of endothelial cells presented here should represent a valuable transport model system to study permeability mechanisms of insulin and many other drugs.

KEY WORDS: insulin; endothelium; flow; transport; degradation.

INTRODUCTION

Endothelial cells are thought to limit insulin transport from the intravascular to the interstitial space, resulting in attenuated hormonal action at the target sites. Large macromolecules are generally thought to traverse the capillary barrier by passive diffusion (1), vescicular transport (2), or receptor-mediated transcytosis (3–6). King and Johnson (4) claimed that transport of insulin across cultured endothelial monolayers is unidirectional and saturable. This concept, together with the existence of insulin receptors on the endothelial wall (7), supported the hypothesis of receptor-mediated transendothelial insulin transport. Milton and Knutson (8) reported that the insulin receptors expressed in bovine aortic endothelial cells (BAEC) are not involved in insulin transport across the cell monolayer, but that passive diffusion across leaky tight junctions of the cell monolayer accounts for the movement of insulin from the apical to the basolateral compartments. Moreover, *in vivo* studies (9) showed lack of evidence for receptor-operated insulin transport in canine hind limb muscle cells, also suggesting the involvement of a diffusion process. Finally, in rat perfused hearts capillary endothelial cells affect the transcapillary insulin flux by a bidirectional convective transport rather than by a saturable receptor mediated mechanism (10).

Given these conflicting findings, the role of endothelium in transcapillary transport of insulin warrants further investigation. Discrepancies between *in vivo* and *in vitro* studies could be partly due to the static conditions used to perform the experiment with cultured endothelial cells. *In vivo,* such cells are continuously exposed to hemodynamic factors that influence endothelial biology but which are lacking in traditional tissue culture models (11,12). Thus the purpose of the present study was to explore the feasibility of culturing endothelial cells under chronic flow in permeable polypropylene hollow fibers and to take advantage of this dynamic cell culture model to investigate transendothelial insulin permeability.

MATERIALS AND METHODS

Cell Culture

Endothelial cells derived from bovine aorta (BAEC) were obtained from Dr H. Sage's laboratories at the University of Washington (passage 8). Cells were grown to confluence in 75 cm^2 flasks in medium containing: DMEM with 1 g/l glucose (BioWhittaker), 10% fetal bovine serum (FBS) (HyClone), non-essential amino acids (BioWhittaker), 2 mM L-glutamine (BioWhittaker), MEM essential vitamin mixture (BioWhittaker), 1 mM Na pyruvate (BioWhittaker) and PSF consisting of 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone (BioWhittaker). After removal with trypsin, cells were resuspended in DMEM containing 1% FBS and PSF, and seeded into hollow fiber apparatus.

Perfused Transcapillary Cultures

Cells were cultured in hollow fiber tubes (50 polypropylene artificial capillaries, coated with ProNectin F, each 13 cm long and 330 μm inner fiber diameter) inside a sealed chamber (the 'extraluminal space') with accessible ports (CELLMAX®, Cellco, Germantown, MD, USA), as previously described (13–15). The hollow fiber tubes were connected by gas-permeable tubing to a source of the growth medium. A pulsatile pump forced the medium through the lumen of the artificial tubes, allowing diffusion of nutrients to the extraluminal space (ECS) through the 0.5-μm transcapillary pores, at a controllable rate. Total lumen volume was 135 ml, and ECS volume was 1.5 ml. The entire apparatus resided in a water-jacketed incubator with 5% CO_2 at 37° C. For sterile sampling, it could be moved into a laminar flow hood. BAEC were seeded intraluminally and allowed to establish themselves for 7–15 days. Amount of cells seeded ranged

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ABBREVIATIONS: BAEC, bovine aortic endothelial cells; ECS, extraluminal space; PC, permeability coefficient; TEER, transendothelial electrical resistance.

0724-8741/02/0400-0445/0 © 2002 Plenum Publishing Corporation

from 10×10^6 to 20×10^6. Flow rate was adjusted to 5 ml/min (average estimated endothelial surface shear stress of 4.5 dyne/cm^2) (14).

Cell metabolism inside the apparatus was monitored over time by measuring glucose consumption. Glucose in medium samples taken daily from the reservoir of the culture system was measured using a Glucose Auto & STAT GA 1120 analyzer (DAIICHI, Japan). Growth medium was changed when the glucose concentration approached 50% of starting values.

In order to measure transendothelial electrical resistance (TEER), a significant hardware modification of the existing Cellco system was performed as previously described (15). The parameters measured represent capillary resistance to current flowing perpendicular to the capillary wall, Rm (Ohm/cm^2). Actual endothelial cell resistance was calculated by subtracting the resistance determined in the absence of endothelial cells from that in their presence.

Permeability Measurements

A concentrated bolus of the compound under investigation was dissolved in the medium bottle (100 ml) and perfused intraluminally at a rate of 5 ml/min. Samples were taken from ECS or the lumen as described (15), after the first 10 min, the time period in which the bolus reaches the capillary system. Samples removed from ECS were replaced with equal volumes of medium. 200 μl of [^{14}C]sucrose or [^{14}C]inulin (NEN Life Science) samples were introduced into vials with 4 ml of Ready Protein Beckman scintillation cocktail. Radioactivity was counted using a 1600 scintillation counter (Packard). Highly purified human insulin (Novo, Copenhagen, Denmark) was iodinated as previously described (16). In further experiments [^{125}I]A_{14}-insulin at different concentrations was added to the perfusion system and samples were collected from ECS and the lumen, as previously described (15). [^{125}I]A_{14}-insulin radioactivity was counted with a gamma counter.

The permeability/surface product was calculated by graphical integration of drug concentration in the lumen and in ECS over 40 min, based on previous studies (15). Permeability for a compound x was calculated by integrating the area under the ECS and lumen data points according to the following formula describing the permeability coefficient (PC):

$$PC = \frac{K * [x]_{ECS,final} - [x]_{ECS,t=0}}{\int_{0\rightarrow t}[x]_{lumen} - \int_{0\rightarrow t}[x]_{ECS}}$$

where K is a constant used to normalize rate of efflux flux for the luminal surface and lumen/ECS volume ratios; $[x]_{ECS,final}$ and $[x]_{lumen}$ are the extraluminal space and lumen concentrations of x, as previously reported (15).

[^{125}I]insulin Binding, Internalization, Recycling and Degradation Studies

BAEC were grown to confluence in 100 mm diameter culture dishes and incubated in the fresh growth medium with 0.5 nM [^{125}I]A_{14}-insulin at 37°C in the absence or presence of 17 μM unlabeled insulin for binding, internalization and recycling studies, essentially as previously described (17–20).

Degradation studies were carried out in BAEC medium samples taken from the lumen of the perfused capillary system, denoted as "lumen medium", and in BAEC medium samples taken from the culture on Petri dishes, denoted as "cell medium". Intact [^{125}I]A_{14}-insulin and its degradation products in the luminal and cell media were characterized by high-performance liquid chromatography (HPLC) after the radioactivity extraction procedure (21). Media were centrifuged at 14,000 g for 2 min, at 4°C. Radioactivity in the supernatants was extracted using a Sep-Pak C18 cartridge (Millipore, Bedford, MA) (16). HPLC analysis was performed using an isocratic HPLC system (Waters Associates, Milford, MA) and a C18 μBondapak (average particle size 10 μm) column (300 × 3.9 mm I.D.) (Waters Associates) and, as mobile phase, 0.01 M sodium phosphate buffer-isopropanol-acetonitrile (68:11:21; vol./vol./vol.), containing 0.15 M ammonium acetate (11.56 g/L of eluent) and adjusted to pH 3 with hydrochloric acid (16).

Finally, degradation products in the incubation media of human monocytes were characterized by HPLC, as previously described (16).

Results of HPLC elution are expressed as capacity values ($K = (V_1\text{-}V_0)/V_0$, where V_1 is the retention volume of the compound and V_0 the void volume).

Statistical Methods

Results are expressed as means ± SEM. Statistical significance was tested by one-way analysis of variance (ANOVA). *P* values of 0.05 or less were considered to indicate statistically significant differences.

RESULTS

Endothelial Cell Monolayer in the Hollow Fiber Apparatus

Endothelial cells grown intraluminally for 7 days in the hollow fiber apparatus developed a TEER value of 39 ± 15 Ω/cm^2, and no significant change in TEER was observed over a period of 4 weeks. The measurement of TEER across the endothelial cell monolayer is indicative of barrier function, as previously reported (22,23). In addition, assessment of the barrier function was carried out by permeability coefficient (PC) measurement of selected molecules such as inulin and sucrose (22–24). These molecules were chosen as paracellular markers for their inability to cross cell membranes (8).

[^{14}C]Inulin and [^{14}C]sucrose transport was measured as passage of substances from the luminal side of the cell monolayer to the extraluminal space (ECS). The permeability coefficient (PC) across the endothelium was $6.36 \pm 0.14 \times 10^{-6}$ cm/sec for [^{14}C]inulin, and $14.3 \pm 1.19 \times 10^{-6}$ cm/sec for [^{14}C]sucrose, calculated from the initial (40 min) time period of the time-transport profiles (Fig. 1). Hollow fiber apparatus without endothelial cells showed that these fibers did not *per se* represent a barrier to the movement of inulin and sucrose (Fig. 2).

Insulin Transport across Endothelial Cell Monolayer in Hollow Fiber Apparatus

Figure 3A represents the profile of the insulin concentrations inside the lumen and ECS compartments vs. time. The PC value across the endothelium calculated from the initial 40 min period was $5.03 \pm 1.09 \times 10^{-6}$ cm/s, a value not

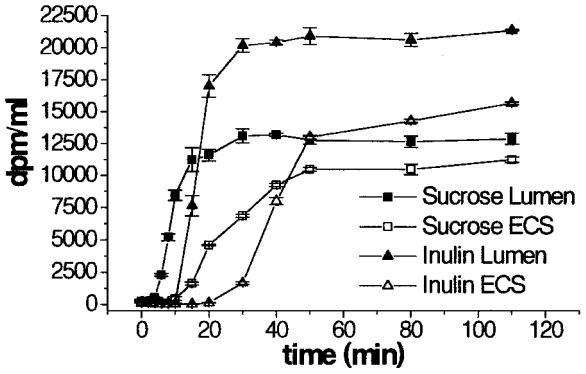

Fig. 1. Profile of [^{14}C]sucrose and [^{14}C]inulin concentration in the lumen (solid symbols) and extraluminal space (open symbols) compartments vs. time. Each point represents the mean ± SEM (n ≥ 3).

significantly different compared to PC values calculated for inulin.

The permeability coefficient of insulin across BAEC monolayers increased significantly with increasing insulin concentrations from 3 to 600 pM, corresponding to 0.5–100 μU/ml (Fig. 3C).

We found no evidence that transendothelial transport of insulin involved a saturable (unidirectional) mechanism, because its PC value did not reach a plateau with increasing insulin concentrations. Furthermore, to obtain a saturation of insulin receptors, the monolayer was preloaded with 1 μM unlabeled insulin, which is 50,000 times more concentrated than labeled insulin, the latter being injected 2h later to measure transendothelial transport. Under these conditions, ECS [^{125}I]A_{14}-insulin concentration increased significantly, as shown in Fig 3D.

Insulin Degradation Studies

In order to obtain insight into the pattern of insulin metabolism in our experimental model, lumen medium HPLC was performed after 15 min of [^{125}I]A_{14}-insulin perfusion (Fig. 4A), as reported in the Methods. Eight main peaks of radioactivity were found: peak 1, which co-eluted with standard [^{125}I]A_{14}-tyrosine, was considered as a terminal degradation product. The peak denoted as [^{125}I]A_{14}-insulin co-eluted with [^{125}I]A_{14}-insulin, and represented intact undegraded insulin (about 90% of luminal radioactivity). Six additional peaks were found; five of these (2, 3, 4, 5 and 6) eluted at an intermediate position between peaks 1 and [^{125}I]A_{14}-insulin, and one (7) eluted after the insulin peak. These represent intermediate products of insulin degradation. In parallel, HPLC analysis showed that neither fresh medium in the cell-free capillary system, nor BAEC conditioned medium induced insulin degradation.

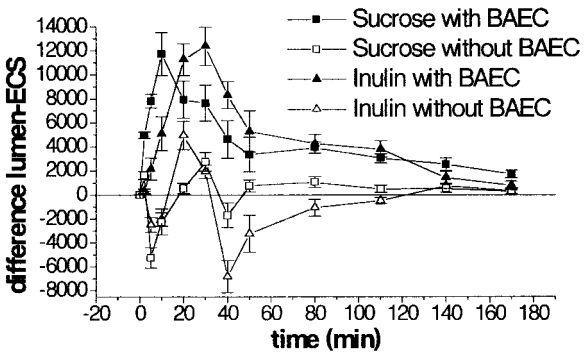

Fig. 2. Difference between lumen and extraluminal space (ECS) [^{14}C]sucrose and [^{14}C]inulin concentrations at various times, in the hollow fiber apparatus without (open symbols) and with (solid symbols) the endothelial cell monolayer. Each point represents the mean ± SEM (n ≥ 3).

There is considerable evidence to support the idea that insulin degradation is a cell-mediated process dependent on insulin receptor complex formation, so that bound insulin alone represents the substrate for the degradation process (25,17). In this perspective, specific insulin receptors were assessed on the endothelial cells used in this study. After cells were grown on Petri dishes to confluence, [^{125}I]A_{14}-insulin was incubated at 37° for different periods of time. Maximum insulin binding (1.1 ± 0.2%/mg protein) and internalization (0.8 ± 0.1%/mg protein) were found after 10 min [^{125}I]A_{14}-insulin incubation. HPLC analysis of 15 min incubation cell medium showed a pattern of degradation products superimposable to that observed when luminal radioactivity was studied.

The time course of the appearance of insulin degradation products (sum of all peaks, excluding the [^{125}I]A_{14}-insulin peak, i.e., intact insulin) in both cell medium and lumen medium is shown in Fig. 5. Insulin degradation, evaluated after exposing [^{125}I]A_{14}-insulin to BAEC at the indicated time intervals, was minimal (10% HPLC detected at 2 h), with a rapid increase after 15 min, in agreement with the fact that most of the insulin bound was internalized in this period.

Finally, the pattern of luminal endothelial insulin degradation was compared to that produced by freshly isolated human monocytes, incubated with [^{125}I]A_{14}-insulin at 37°for 15 min. As shown in Fig. 4B, the incubation medium of human monocytes proved to contain products of insulin degradation with an HPLC elution pattern similar to that recorded for luminal endothelial radioactivity.

DISCUSSION

The model system used for these studies results from a modification of traditional static bidimensional "Petri dish" culturing conditions. Cell culture on hollow fibers was first described by Knazek *et al.* (13) and has since then been extensively exploited (12,14,15). Studies of flow-mediated effects on endothelial cell growth suggest that culture under these conditions promotes endothelial cell differentiation (12,14).

Cell growth was monitored daily by measuring the amount of glucose consumed by the cells, since the inherent structural characteristic of the hollow fiber apparatus does not allow direct visualization of cell growth. Interestingly, the pattern of endothelial cell growth in the intraluminal portion of the hollow fibers was characterized by an initial rapid expansion followed by quasi static growth (data not shown).

The estimated [^{14}C]inulin and [^{14}C]sucrose PCs and TEER values obtained in our study reflected those reported from previous studies (8,24), suggesting integrity of the endothelial monolayer structure. The main advantage of using this dynamic *in vitro* endothelium model was the possibility of maintaining barrier features unchanged for many weeks. Therefore, each experiment was carried out on the same cell

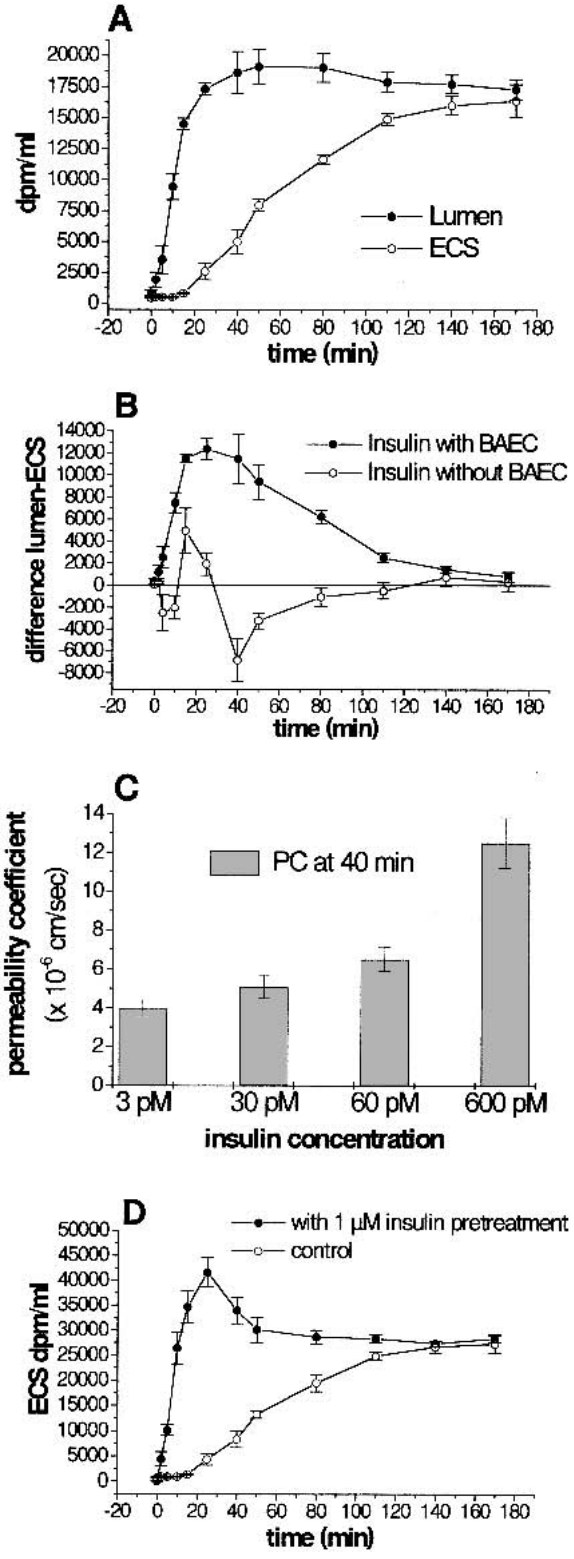

Fig 3. A: Profile of $[^{125}I]A_{14}$-insulin concentration in the lumen and extraluminal space (ECS) compartments vs. time. Each point represents the mean ± SEM B: Difference between lumen and extraluminal space $[^{125}I]A_{14}$-insulin concentration at various times, in the hollow fiber apparatus without (open circles) and with (solid circles) the endothelial cell monolayer. Each point represents the mean ± SEM C: Concentration dependence of insulin permeation at 37°C across the bovine aortic endothelial cell monolayer in the dynamic capillary system. Data are means ± SEM ($n \geq 3$). D: Profile of $[^{125}I]A_{14}$-insulin concentration in the extraluminal space as a function of time. Prior to injection of $[^{125}I]A_{14}$-insulin, the monolayer was pretreated with (solid symbol) 1 μM unlabeled insulin for 2 hours. The control was not pretreated (open symbol). Each point represents the mean ± SEM ($n = 3$).

clone at the same passage, i.e. the same endothelial monolayer. This implies that the differences found in this study cannot be accounted for by difference of time in culture or differences in passage number. Moreover, the presence of a fluid flowing through the system made it possible to mimic the physiological conditions. Physiological levels of venous and arterial shear stresses range between 1 and 5 dyne/cm^2 and 6 and 40 dyne/cm^2, respectively (26). We used a flow rate producing a shear stress of 4.5 dyne/cm^2, a value that is in the physiological range.

Using the described dynamic tridimensional endothelium model we found that insulin, in the concentration range used in the experiments, crossed the endothelium in a nonsaturable manner, suggesting an undetectable contribution of the receptor-mediated process. The high values of ECS $[^{125}I]A_{14}$-

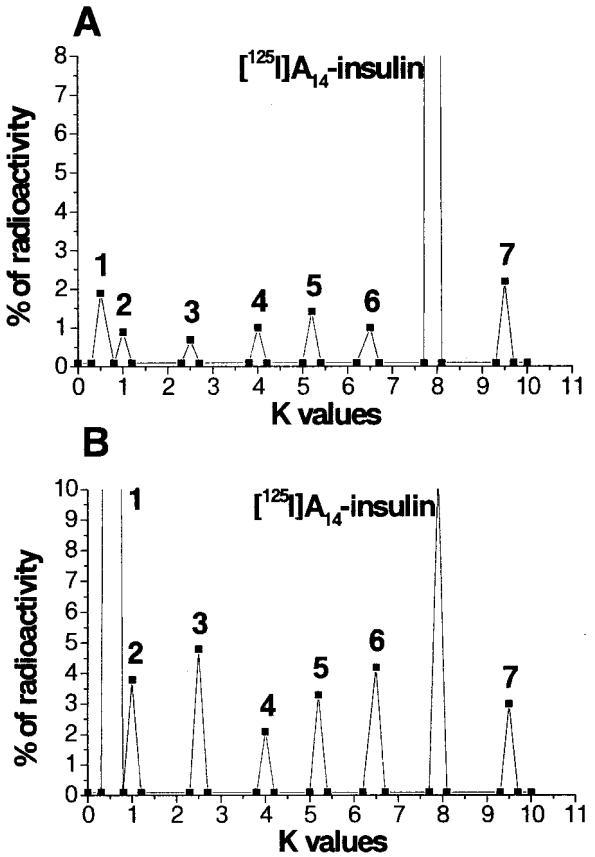

Fig. 4. Representative elution profiles from HPLC of bovine aortic endothelial cell luminal medium (A), and monocyte medium (B) radioactivity. Figures show endothelial cell and human monocyte medium radioactivity after 15 min of $[^{125}I]A_{14}$-insulin incubation at 37° C; at this time the peak heights of intermediate degradation products (numbered 1-7) were higher. The $[^{125}I]A_{14}$-insulin peak represents intact insulin. Results of HPLC elution are expressed as capacity values (K values) as described in the Methods. The experiment was performed three times with similar results.

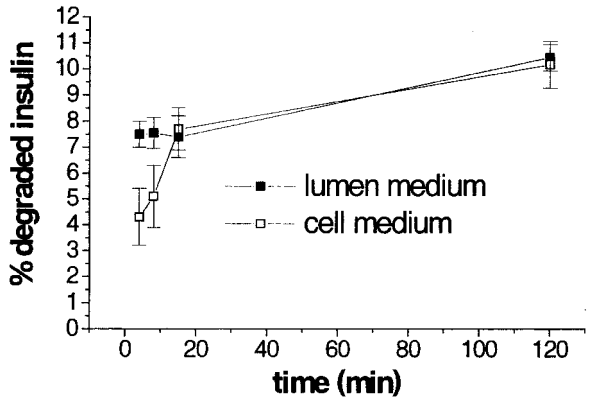

Fig. 5. Percentage of degraded insulin (calculated from the sum of HPLC-detected radioactivity associated at all peaks excluding the [^{125}I]A_{14}-insulin peak, intact insulin) as a function of time, in cell medium (from dish-cultured cells) (open symbols) and in lumen medium (from capillary-cultured cells) (solid symbols). Cells were incubated with [^{125}I]A_{14}-insulin for the times indicated, and HPLC was then performed as described in the Methods. Data are means ± SEM (n = 3).

insulin concentration obtained after pretreatment with 1 μM unlabeled insulin could be due to an influence of the elevated hormone concentration on vascular function. In this perspective, the influence of insulin on liver cell ion permeability has been described in a recent study (27).

Finally, [^{125}I]A_{14}-insulin binding studies performed to assess the presence and the characteristics of insulin receptors on bovine aortic endothelial cells used in this study showed low values of insulin binding and internalization, in agreement with previous studies (18,19).

Results in the present study are both in conflict and accordance with other *in vitro* studies. In the past King and Johnson (4) showed that insulin transport across cultured BAEC is receptor mediated, saturable, and unidirectional. On the basis of this evidence, the authors argued that a specific transendothelial transport of insulin contributes to the hormone's metabolic effects, while also pointing out that their endothelial system differs from physiological conditions in some important respects, including the absence of pressure differences and flow dynamics. On the other hand, Milton and Knutson (8) showed that insulin transport across bovine aortic endothelial cells does not significantly differ from inulin diffusion. In a recent *in vivo* clamp study, Steil *et al.* (9) found no evidence for receptor-operated insulin transport in the hind limb of dog, and although they were unable to rule out the existence of a small receptor-mediated insulin transport pathway, they indicated diffusion as the dominant process *in vivo*. Moreover, Brunner and Wascher (10) concluded that insulin is transported by bidirectional convective transport rather than by a saturable receptor-mediated mechanism in rat isolated perfused hearts, although capillary endothelial cells limit insulin transport from the vascular to the interstitial space, resulting in attenuated hormonal action at target sites.

For all molecules tested in the dynamic endothelium model, permeability was comparable to that found in *in vivo* and *ex vivo* studies, particularly as reported by Steil *et al.* (9), and Paaske and Sejrsen (28) for inulin and insulin and by Crone (29) for sucrose.

Therefore, in this *in vitro* dynamic endothelium model a functional parameter such as insulin degradation by endothelial cells was also monitored. Insulin degradation results show that luminal metabolites were derived from the endothelial cell degradation pathway, and that the amount of degraded insulin in the medium was low. Moreover, endothelial production of insulin metabolites was qualitatively identical to metabolite production by human monocytes. Comparison between BAEC and human circulating monocytes took into account that the latter has been extensively used to assess insulin degradation, and the degradation products have been characterized. In addition, strong evidence exists to support the identity between monocyte insulin-degrading activity and that of various human tissues, including liver, muscle, kidney, and brain (30).

In conclusion, our results provide measurements of solute permeability in an *in vitro* perfused endothelium model. Detection of a substantial permeability barrier to macromolecules under control conditions suggests that the model will be valuable for future studies on permeability mechanisms. The novelty of this model as compared with previously described studies (4,8) opens up the possibility of conducting experiments using long term *in vitro* culture of endothelial cells in microvessel-like structures in the presence of flow. Thereby, giving a basis of comparison with the physiological process of endothelial transport. Furthermore, data on insulin transport and processing by endothelium demonstrated that 1) the hormone crosses the BAEC monolayer by paracellular "leak" and that, if a receptor-mediated component does exist, it is not quantitatively significant for insulin transport; 2) degradation of insulin by BAEC is minimal; 3) insulin degrading activity proved to be qualitatively identical in bovine endothelial cells and human cells (monocytes). The latter result suggests that insulin degradation "machinery" is strongly preserved through different species, indicating a strategic biologic relevance of this mechanism.

These findings raise the possibility that the precise stimuli and biochemical mechanisms involved in transendothelial transport of insulin could be further defined by using this dynamic, *in vitro* model.

ACKNOWLEDGMENTS

The authors thank Dr. A. Bertelli and Dr. F. Borromeo for their technical support. This work was supported by a grant from the University of Pisa.

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