Insulin Binding Sites Localized to Nerve Terminals in Rat Median Eminence and Arcuate Nucleus

Abstract. *Specific binding sites for blood-borne insulin were determined to be selectively localized on axons and axon terminals in the external median eminence and the hypothalamic arcuate nucleus by means of quantitative fine structural radioautography. This localization suggests that discrete populations of hypothalamic nerve terminals are potential targets for the direct effects of insulin and that insulin may act through synaptic mechanisms to influence hypothalamic circuits regulating energy balance and hypophyseal function.*

Insulin seems to act directly on cells in the medial basal hypothalamus to promote local glucose uptake (*1*) and subsequent metabolism (*2*), to alter the electrical activity of ventral hypothalamic neurons (*3*), and to stimulate the release of hypothalamic norepinephrine (*4*). Insulin also seems to act locally within the ventral hypothalamus to influence feeding behavior (*5*) and to modify the output profile of central autonomic centers participating in the maintenance of peripheral glucose homeostasis (*6*). The key unresolved issue central to the interpretation of these observations, however, is whether these apparently direct hormonal effects result in fact from a receptor-mediated interaction of insulin with a particular cellular component of the ventral hypothalamus.

Recently we have used quantitative light microscopic radioautography to demonstrate the existence of insulin-specific binding sites in the zona externa of the median eminence and in the hypothalamic arcuate nucleus (*7*). We observed that the binding of blood-borne [^{125}I]insulin, as reflected in the intensity of light microscopic radioautographic reactions, was blocked by coinjected unlabeled insulin, insulin analogs, and structurally dissimilar polypeptides in parallel with their ability to competitively inhibit insulin binding to receptors in vitro (*8*). Thus, the in vivo radioautographic assessment of binding specificity, which is analogous to the in vitro competitive binding assay (*9*), demonstrated that insulin binding sites in the ventral hypothalamus had the specificity characteristics of genuine insulin receptors. The biochemical and morphologic specificity of radioautographically identified insulin binding sites in the medial basal hypothalamus is further supported by similar studies revealing a different topographic distribution of specific binding sites for blood-borne lactogen (*10*) and angiotensin II (*11*).

The presence of insulin-specific binding sites in the medial basal hypothalamus implies the existence of local receptive agents capable of directly interacting with insulin. Identifying insulin-receptive elements in the median eminence and arcuate nucleus would possibly elucidate a cellular basis for the mechanism of insulin action in the hypothalamus. Therefore, we have now used quantitative electron microscopic (fine structural) radioautography to determine the exact cellular location of previously defined insulin-specific binding sites in these morphologically heterogeneous regions.

Monoiodinated [^{125}I]insulin (*12*), either alone or in the coinjected presence of a 500-fold excess (830 μg) of unlabeled insulin, was injected into the left cardiac ventricle of anesthetized 200-g male rats (*7*). Five minutes after injection, brains were perfused through an intracardiac cannula, first with lactated Ringer solution to flush out blood and unbound radioactivity, and then with a buffered solution of mixed aldehydes (*13*). Shortly after perfusion and fixation, blocks of medial basal hypothalamus were removed and prepared for electron microscopy (*14*). Coronal sections of the appropriate thickness were then processed for either light microscopic (*15*) or fine structural (*16*) radioautographic visualization of bound radioactivity.

In rats injected with [^{125}I]insulin alone, light microscopic radioautography showed intense reactions over the external median eminence and ventral arcuate nucleus (Fig. 1A), as previously described (*7*). However, quantitative analysis of the cellular distribution of silver grains over fine structural radioautographs of the external median eminence taken from rats injected with [^{125}I]insulin alone showed that 83 percent of all bound [^{125}I]insulin visualized in this region was restricted to neuronal processes (Table 1). Axon terminals were the most frequently labeled (Fig. 2a), accounting for more than 50 percent of all silver grains. Considerable labeling of preterminal axons (30 percent) occurred as well (Fig. 2a), but only one-sixth of all silver grains were localized to glial elements, principally basal tanycyte processes.

In the arcuate nucleus of rats injected with [^{125}I]insulin alone, axons were the most frequently labeled structures (Fig. 2b and Table 1). Surprisingly, synaptic terminals were also labeled (Fig. 2, c and

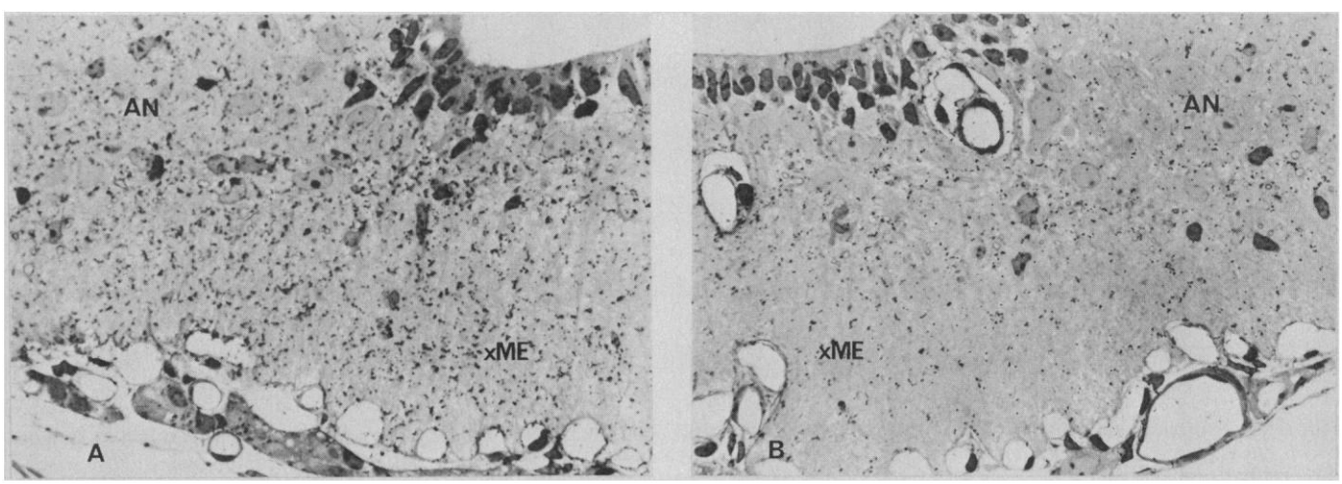

Fig. 1. Comparison of light microscopic radioautographic reaction intensities over sections 1 μm thick including the external median eminence (*xME*) and the hypothalamic arcuate nucleus (*AN*) 5 minutes after injection of [^{125}I]insulin alone (A) or in the coinjected presence of a 500-fold excess of unlabeled insulin (B) (*17*).

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Table 1. Cellular distribution of silver grams (percentage of the total) (24) in five structural radioautographs of the external median eminence and the hypothalamic arcuate nucleus 5 minutes after systemic injection of [^{125}I]insulin alone or in the presence of a 500-fold excess of unlabeled insulin.

Treatment	External median eminence					Arcuate nucleus						
	Grains scored (No.)	Neuronal		Glial	Other (extracellular space)	Grains scored (No.)	Neuronal				Glial	Other [unidentified (25)]
		Terminal	Preterminal axon				Synapse	Preterminal axon	Dendrite	Soma		
Labeled insulin	470	52.5*	30.3	15.3	1.9	452	12.4	57.1	3.5	2.4	19.5	5.1
Labeled + unlabeled insulin	339	8.3	8.5	81.4	1.8	372	2.4	12.3	6.3	1.3	74.5	3.2

*Approximately 8 percent of all terminals (labeled and unlabeled).

d). Labeled synaptic terminals commonly contained clear vesicles and a few dense-core vesicles; they were usually observed contacting dendritic spines and shafts. Only one-fifth of all arcuate grains were localized to glia.

Coinjection of [^{125}I]insulin with a 500-fold excess of unlabeled insulin resulted in a 77 percent reduction in light microscopic reaction intensity over the external median eminence and a 69 percent reduction over the arcuate nucleus (Fig. 1B) (*17*). This residual reaction reflects the presence of bound radioactivity not competitively blocked by the 500-fold excess of unlabeled insulin. In fine structural radioautographs of the external median eminence and arcuate nucleus from coinjected rats, the density of silver grains was similarly reduced, as predicted by light microscopic radioautography (Fig. 1B). However, in coinjected rats, quantitative analysis of the cellular distribution of silver grains representing bound radioactivity resistant to competitive binding inhibition showed that most residual grains were restricted to glia (Table 1). Thus, in both regions of the medial basal hypothalamus, the 500-fold excess of unlabeled insulin profoundly reduced [^{125}I]insulin binding to axons and axon terminals, but had no obvious blocking effect on [^{125}I]insulin binding to neighboring glia. The selective neuronal blockade of [^{125}I]insulin binding by unlabeled insulin indicates the previously defined specific binding sites for blood-borne insulin in the external median eminence and arcuate nucleus (*7*) are in fact associated with axons and axon terminals.

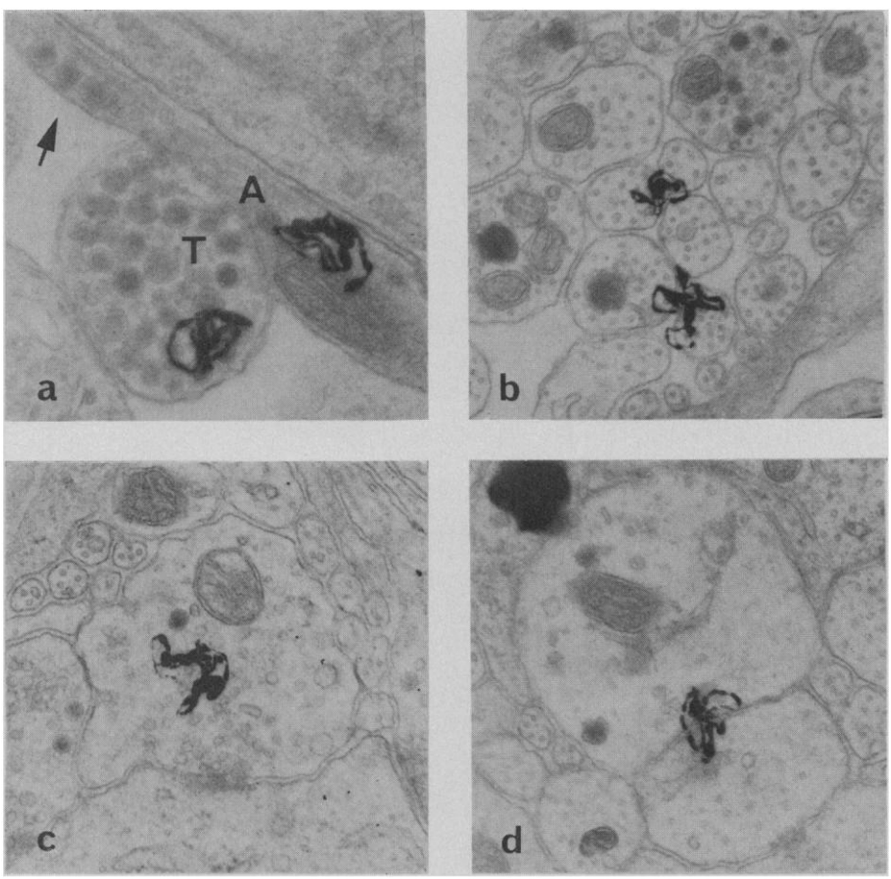

Fig. 2. Fine structural radioautographs illustrating the localization of [^{125}I]insulin binding sites (silver grains) to axons and axon terminals in median eminence and arcuate nucleus of the rat. (a) External median eminence. Silver grains are located over a free nerve ending (*T*) and a preterminal axon (*A*) containing dense-core vesicles (arrow). (b) Arcuate nucleus. Silver grains are located over axons. (c and d) Arcuate nucleus. Silver grains are located over vesicles within a synaptic terminal (c) and over the synaptic cleft (d).

Two mechanisms may account for the preferential concentration of blood-borne insulin by axonal processes in the arcuate nucleus: (i) The arcuate neuropil borders closely on the permeable capillary plexus of the median eminence, such that there appears to be no effective barrier to prevent blood-borne polypeptides from diffusing into this region (*18*). Consequently, blood-borne insulin may be retained in the arcuate nucleus in part by directly binding to receptive axons and synaptic terminals. (ii) Insulin may be bound initially to receptive terminals located in the external median eminence, internalized, and then transported by retrograde flow through connecting axons into synaptic terminals in the arcuate nucleus. Collaterals of tuberoinfundibular axons giving origin to terminals innervating both the median eminence and arcuate nucleus (*19*) may provide this pathway. Preferential concentration of insulin by internalization and retrograde axonal transport may explain the presence of inordinately large amounts of insulin in the rat hypothalamus (*20*).

In either case, blood-borne insulin rapidly translocates in vivo to specific binding sites in association with axonal elements in the medial basal hypothalamus. The specific interaction of insulin with axon terminals in this region raises the possibility that insulin may directly affect the release of hypophysiotropic substances. To our knowledge a direct effect of insulin on hypophysiotropic function has not been demonstrated. However, of interest in this regard is the report that, in hyperinsulinemic-hyperphagic rats, catecholamine turnover in the median

eminence is selectively increased (*21*). The existence of specific binding sites in the external median eminence for other polypeptide hormones, namely, lactogen (*10*), angiotensin II (*11*), and calcitonin (*22*), indicates that nerve terminals in this region have receptors for a variety of polypeptide hormones in addition to insulin. This broad hormone-recognition capacity suggests that the median eminence plays an important but unappreciated receptive role in hormone-to-brain communication.

Axonal or synaptic insulin receptors in the arcuate nucleus, on the other hand, seem ideally situated to influence synaptic transmission, and thereby to alter hypothalamic electrical activity, such as that following either systemic insulin administration (*23*) or microinjection of insulin into the ventral hypothalamus (*3*). In view of this possibility, a direct action of insulin on insulin-receptive axons and synaptic terminals in the arcuate nucleus may underlie a fundamental mechanism whereby fluctuations in blood-borne insulin can rapidly modulate the activities of hypothalamic circuits programming feeding behavior, body weight, and glucose homeostasis.

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12. Crystalline porcine zinc insulin (24.4 U/mg) was a gift from Connaught Research Laboratories, Willowdale, Ontario. Insulin was moniodinated to a specific activity of 134.4 μCi/μg by the chloramine-T method [B. I. Posner, *Diabetes* **23**, 209 (1974)]; 1.97×10^7 count/min was delivered in a volume of 0.25 ml with 2.5 percent bovine serum albumin in 25 m*M* tris HCl, *p*H 7.4).
13. The mixture contained 3 percent glutaraldehyde and 1 percent formaldehyde [generated from paraformaldehyde (W. D. Belt)] buffered to *p*H 7.4 with 0.1*M* sodium cacodylate.
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15. Semithin sections (1 μm thick) were stained with iron-hematoxylin and coated with emulsion (Kodak NTB2) [B. M. Kopriwa and C. P. Leblond, *J. Histochem. Cytochem.* **10**, 269 (1962)]. After being exposed for 3 weeks, radioautographs were developed (Kodak D-170). The quantitation of radioautographic reactions was performed with the aid of a photoscope (Zeiss) and an ocular grid (11 by 11 mm), which provided a unit area of 6959 μm^2 at the magnification used for grain counting (×1600). In the external median eminence and in the arcuate nucleus, grains encompassed by a total area of 83,508 μm^2 (12 unit areas) were scored for each injection group, and the average number of silver grains per unit area was calculated. The difference in regional radioautographic reaction intensity between injection groups (that is, [^{125}I]insulin alone versus [^{125}I]insulin along with the 500-fold excess of unlabeled insulin) was thereby estimated as the percentage of reduction in average reaction per unit area. Unstained sections confirmed the absence of chemographic artifacts, and stained sections from nonradioactive brains were used to determine background fog.
16. Thin sections (pale gold interference color) were covered with a monolayer of emulsion (Ilford L-4) [B. M. Kopriwa, *Histochemie* **37**, 1 (1973)] and exposed for 4 months before development in fresh Kodak D-19B [B. M. Kopriwa, *ibid.* **44**, 201 (1975)]. Radioautographs were then stained in uranyl acetate and lead citrate and examined in an electron microscope (Hitachi HS-7S). Grains were photographed wherever they occurred (×8000) and enlarged (×22,000). The relative content of label in neuronal and glial structures was analyzed by allocating and tabulating grains according to the underlying structure (direct scoring method). An additional 205 grains were analyzed by both direct scoring and the statistical method of N. J. Nadler [*J. Cell Biol.* **49**, 877 (1971)] through the use of two different resolution circles (50 percent and 95 percent probability of enclosing the source of radioactivity). Computerized analysis of these results (N. J. Nadler and G. Levine, *Biol. Cellulaire*, in press) showed no statistical difference in the distribution obtained by direct scoring compared with resolution circles. This exercise demonstrates in our study that the simplified technique of direct scoring is as accurate in localizing the structural source of bound radioactivity as the more complicated methods available for quantitative fine structural radioautography, and that locating silver grains by direct scoring can be taken with maximum confidence to designate the location of [^{125}I]insulin binding sites.
17. In rats injected with [^{125}I]insulin alone, the average reaction per 6959 μm^2 of external median eminence was 145 ± 9.5 grains [mean ± standard error of the mean (S.E.M.)], and 119 ± 7.2 grains over the arcuate nucleus. In coinjected rats, the average reaction was 33 ± 2.6 grains and 37 ± 3.1 grains, respectively. Background fog was 7.2 ± 0.8 grains per 6959 μm^2. [The reaction reductions obtained from our analysis of radioautographs of sections (1 μm thick) of Epon-embedded hypothalamus fixed with aldehydes are in close agreement with our previous results obtained from radioautographs of sections (4 μm thick) of paraffin-embedded brain fixed with Bouin's solution (*7*), in which competitive binding sites for insulin in the ventral hypothalamus had the recognition specificity properties of biologically important insulin receptors.]
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24. The arbitrarily chosen number of grains analyzed in fine structural radioautographs from each region does not reflect the striking differences in grain density between injection groups (Fig. 1). Thus, in order to score large numbers of silver grains in fine structual radioautographs, from coinjected rats (Fig. 1B), many more sections were examined than were required for rats injected with [^{125}I]insulin alone (Fig. 1A).
25. Many unidentified structures resembled the "growth-cone-like" processes previously described in this region [M. van Houten and J. R. Brawer, *J. Comp. Neurol.* **179**, 719 (1978)].
26. We thank Y. Clermont and C. P. Leblond for their support of these studies, J. J. M. Bergeron and K. Ruf for critical comments, and B. Patel for technical assistance. The support and encouragement of S. O. Freedman is gratefully acknowledged. A preliminary report of these findings was made at the 18th Annual Meeting of the American Society for Clinical Investigation, Washington, D.C., 7 May 1979. This work was aided by grants from the Canadian Diabetic Association, the Medical Research Council (MRC) of Canada (MT-4182 and MA-5948), the U.S. Public Health Service (1R01, AM1953-01), and by the QRC Establishment grant 291-96 and MRC scholarship 24783.

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Local Effect of the Blastocyst on Estrogen and Progesterone Receptors in the Rat Endometrium

Abstract. *Nuclear receptors for both estradiol and progesterone were present in twofold higher concentrations in implantation sites than in nonimplantation regions of the endometrium of 6-day pregnant rats. Decidualization in the absence of an embryo was not accompanied by a similar increase in the concentration of nuclear receptors. Moreover, this difference in receptor distribution between the implantation and nonimplantation areas persisted when a major part of the maternal supply of sex steroids was suppressed by ovariectomy on day 5 of pregnancy. These results support the hypothesis that steroids originating from the embryo affect the endometrial implantation site.*

The hormonal mechanism that triggers implantation of the ovum is poorly understood. Administration of estrogen or progesterone, or both, depending on the species, is necessary for implantation to occur in ovariectomized females (*1*). It has been proposed that steroids synthesized or accumulated by the blastocyst play an important role in implantation through a local action on the adjacent endometrium (*2*). Various histological and biological modifications have been shown to occur at the implantation sites, and the presence and metabolism of steroids in the blastocyst have been demonstrated. Thus the hypothesis according to which these local changes are brought about by hormones originating from the

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