Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 459–463, January 1987
Cell Biology

Ultrastructural evidence for the accumulation of insulin in nuclei of intact 3T3-L1 adipocytes by an insulin-receptor mediated process

(endocytosis/electron microscopy/monomeric ferritin-insulin/intracellular hormone processing)

ROBERT M. SMITH AND LEONARD JARETT*

Department of Pathology and Laboratory Medicine, University of Pennsylvania, School of Medicine, Philadelphia, PA 19104

Communicated by Louis B. Flexner, October 3, 1986 (received for review June 4, 1986)

ABSTRACT **Monomeric ferritin-labeled insulin (F_m-Ins), a biologically active, electron-dense marker of occupied insulin receptors, was used to characterize the internalization of insulin in 3T3-L1 adipocytes. F_m-Ins bound specifically to insulin receptors and was internalized in a time- and temperature-dependent manner. F_m-Ins was found in cytoplasmic vesicles within 5–10 min at 37°C and subsequently was observed in multivesicular bodies and lysosomes. In addition, small amounts of F_m-Ins were associated with nuclei after 30 min. The number of F_m-Ins particles observed in nuclei continued to increase in a time-dependent manner until at least 90 min. In the nucleus, several F_m-Ins particles usually were found in the same general location—near nuclear pores, associated with the periphery of the condensed chromatin. Addition of a 250-fold excess of unlabeled insulin or incubation at 15°C reduced the number of F_m-Ins particles found in nuclei after 90 min by 99% or 92%, respectively. Nuclear accumulation of unlabeled ferritin was only 2% of that found with F_m-Ins after 90 min at 37°C. Biochemical experiments utilizing ^{125}I-labeled insulin and subcellular fractionation indicated that intact 3T3-L1 adipocytes internalized insulin rapidly and that ≈3% of the internalized ligand accumulated in nuclei after 1 hr. These data provide biochemical and high-resolution ultrastructural evidence that 3T3-L1 adipocytes accumulate potentially significant amounts of insulin in nuclei by an insulin receptor-mediated process. The transport of insulin or the insulin–receptor complex to nuclei in this cell or in others may be directly involved in the long-term biological effects of insulin—in particular, in the control of DNA and RNA synthesis.**

Most of the effects of insulin are observed within a few seconds after the addition of the hormone to cells. As a result, it is accepted that most of insulin's biological effects are generated as a direct result of binding to specific plasma membrane receptors. However, some insulin-responsive processes are not affected for several minutes or hours (for a review, see ref. 1). The mechanisms by which insulin effects these processes are unknown, although several have been postulated (for a review, see ref. 2), including intracellular insulin processing (3–5). Insulin has been shown by both biochemical and ultrastructural techniques to be internalized by numerous cell types (6–18) and to have effects on isolated nuclei (19, 20), leading to the speculation that internalization, intracellular translocation, and/or processing may be physiologically significant, especially for the long-term effects of insulin.

This laboratory has used high-resolution electron microscopic and biochemical analyses to investigate the binding and subsequent processing of the insulin–receptor complex in various cells (11, 21–25) and has observed cell-specific heterogeneity in receptor organization, distribution, mechanism of internalization, and routes of intracellular processing (for a review, see ref. 26). As part of those studies, insulin–receptor processing in 3T3-L1 adipocytes was investigated (25). A serendipitous observation during the course of those studies was the appearance of monomeric ferritin-insulin (F_m-Ins) particles in the nuclei of these cells after extended incubations. That observation led to this detailed analysis of the accumulation of insulin in nuclei. The results of the present study provide both biochemical and ultrastructural evidence that 3T3-L1 adipocytes transport a small but potentially significant amount of insulin or insulin–receptor complexes to the interior of nuclei, where it is associated with the perimeter of condensed chromatin. These findings, and particularly the association of the insulin with the condensed chromatin, may be related to the long-term biological effects of insulin, since the perimeter of the condensed chromatin has been suggested to be the most active site for gene transcription (27).

MATERIALS AND METHODS

3T3-L1 preadipocytes obtained from American Type Culture Collection were transformed, and cell suspensions were prepared as described (25). Mono[^{125}I]iodinated insulin was prepared by standard procedures (28) and was >90% [A14-^{125}I]iodoinsulin (^{125}I-insulin). Insulin binding was assessed as described (25). Internalization and nuclear accumulation of ^{125}I-insulin was determined by incubating ≈3–6 × 10^7 cells under the conditions described in Table 2. The cells were incubated for 60 min and diluted 1:9 with 0–4°C Krebs–Ringer Hepes buffer (pH 7.4) with 1% bovine serum albumin (KRH). The cells were collected by centrifugation at 200 × *g* for 10 min and resuspended in KRH buffer. Trypsin (type II, Sigma) was added to a final concentration of 1 mg/ml, and the cells were incubated at 15°C for 15 min to remove plasma membrane insulin receptors. The cells were chilled to 4°C, centrifuged at 200 × *g* for 10 min, and resuspended to their original volume in 5 mM 4-morpholinepropanesulfonic acid (Mops)/1 mM EDTA, pH 7.4. An aliquot of cells was taken for determination of cell-associated ^{125}I-insulin. The remainder of the suspension was left on ice for 5 min. The cell suspension was then forced through a 27-gauge needle and sonicated (30 pulses, 10% duty cycle, setting 5, Branson microtip sonicator). The sonicated cells were immediately diluted with an equal volume of ice-cold 0.5 M sucrose/50 mM Mops/1 mM EDTA, pH 7.4. Cellular debris was removed by centrifugation for 10 min at 150 × *g*. The supernatant was recovered, and the intact nuclei were isolated by centrifugation at 750 × *g* for 20 min. The nuclear pellet was assayed for ^{125}I-insulin.

F_m-Ins was prepared and characterized as described (29). F_m-Ins concentrations expressed in the text, tables, and

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Abbreviation: F_m-Ins; monomeric ferritin-labeled insulin.

*To whom reprint requests should be addressed.

legends to figures are based on the insulin concentration of the conjugate. Unlabeled ferritin, obtained from Miles Laboratories (Naperville, IL), was used at two concentrations to assess the specificity of the F_m-Ins uptake and nuclear accumulation. At the lower concentration (0.25 mg of ferritin per ml), the concentration of unlabeled ferritin was equivalent to the ferritin concentration in the F_m-Ins used in these studies. With this low concentration of unlabeled ferritin, little or no ferritin particles were observed associated with the cell (see *Results*). To observe some "nonspecific" ferritin internalization, the concentration of unlabeled ferritin was increased 20-fold to 5 mg/ml. Incubations and preparation of the cells for electron microscopy were performed as described in the tables or legends to figures and as in previous studies (25). The procedure used for the morphometric analysis of the accumulation of F_m-Ins in nuclei is described in detail in the results.

RESULTS

As previously reported (25), F_m-Ins-occupied receptors in the plasma membrane of 3T3-L1 adipocytes rapidly migrated from the microvilli to the intervillous membrane and microaggregated into small clusters of receptors. F_m-Ins particles were found in pinocytotic invaginations of the plasma membrane and in small vesicles in the cytoplasm within 15 min at 37°C (Fig. 1). Because of the close proximity of the F_m-Ins particle to the inside of the membrane forming the invaginations and cytoplasmic vesicles, it is believed that the F_m-Ins was internalized while it was complexed to the insulin receptor. The observation of F_m-Ins in cytoplasmic vesicles was both time and temperature dependent. The current study showed (Fig. 1) that within 10–15 min, F_m-Ins was observed in larger vesicles resembling multivesicular bodies and lysosomes. Morphometric analysis of the F_m-Ins particle distribution among small vesicles, multivesicular bodies, and lysosomes suggested that the amount of F_m-Ins in these structures reached equilibrium in 15–20 min (data not shown). In the process of quantifying the distribution of F_m-Ins in these intracellular structures, occasional F_m-Ins particles were observed in nuclei.

The localization of F_m-Ins in nuclei is shown in Fig. 2. F_m-Ins particles were most frequently observed near the periphery of the nucleus, almost always near apparent nuclear pores, and in or at the periphery of the condensed chromatin. In most instances, multiple F_m-Ins particles were observed in the same general area as illustrated. F_m-Ins was never found in the cytoplasm outside the nuclear pores or associated with the cytoplasmic side of the nuclear membrane.

To demonstrate whether the observed F_m-Ins particles were the result of receptor-mediated internalization and intracellular transport, cells were incubated with F_m-Ins under various conditions (Table 1). The number of nuclear-associated ferritin particles was determined in 100 randomly selected thin-sectioned nuclei in intact cells from five different embedded samples of each experimental condition. Coded specimen grids were randomly observed, so that the microscopists were not aware of the incubation condition of the specimen. Since most of the F_m-Ins particles had been found at the perimeter of the nucleus and in order to standardize the volume of nuclei examined, only the perimeter of the entire nucleus, including an area 0.5 μm on both sides of the nuclear membrane, was observed. All ferritin particles were counted and photographed. The result of this analysis is shown in Table 1. As can be seen, cells incubated with 100 ng of F_m-Ins per ml at 37°C had detectable levels of F_m-Ins by 15 min and continued to accumulate the ligand for 90 min. In the specimens incubated for 90 min, F_m-Ins particles, or groups of particles as illustrated in Fig. 2, were

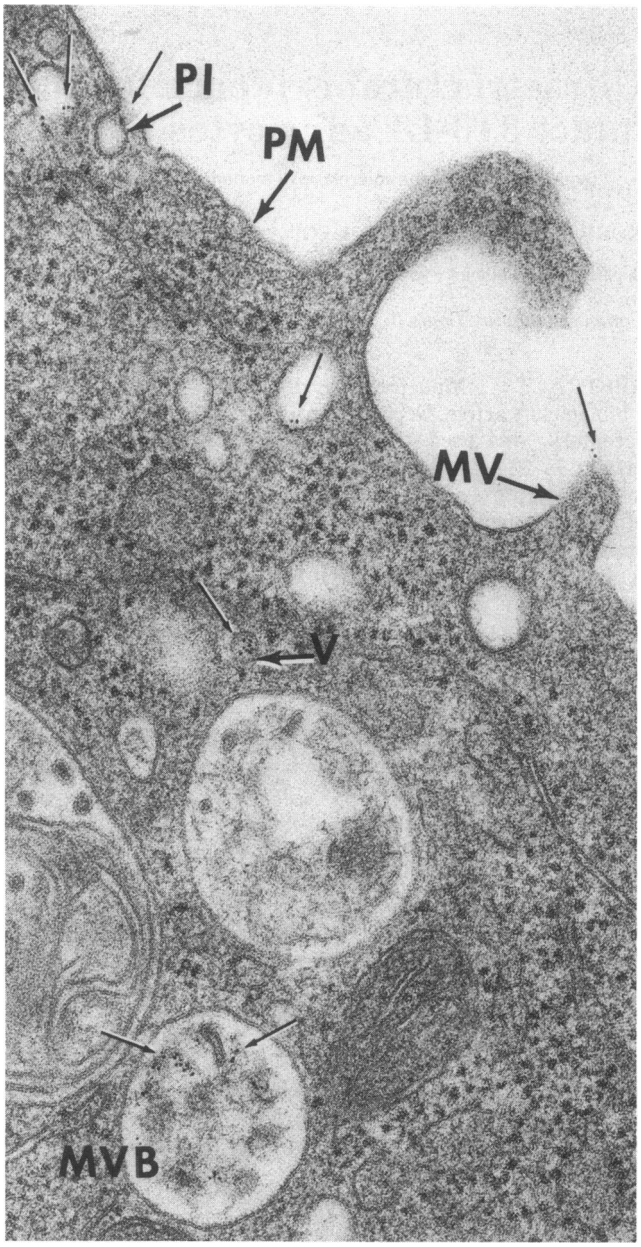

FIG. 1. 3T3-L1 adipocytes were incubated 15 min at 37°C with 20 ng of F_m-Ins per ml and were processed for electron microscopy as described (30). This electron micrograph shows that F_m-Ins particles (thin arrows) were found on the microvilli (MV), on intervillous plasma membrane (PM), in pinocytotic invaginations of the plasma membrane (PI), in cytoplasmic vesicles (V), and in multivesicular bodies (MVB). (×74,250.)

observed in 27 of the 100 nuclear thin sections. In 9 sections, two groups of particles were observed at different locations in the nuclei. Incubation in the presence of excess unlabeled insulin decreased nuclear accumulation by 99% at 90 min. Incubations at 15°C reduced the nuclear association of F_m-Ins to 8% of that found at 37°C. Unlabeled ferritin added at the same concentration as the ferritin in 100 ng of F_m-Ins per ml (0.25 mg of ferritin per ml) did not bind to the plasma membrane and was not detected inside nuclei or in other intracellular structures. When the concentration of unlabeled ferritin was increased 20-fold, ferritin was observed bound to the plasma membrane and, in cytoplasmic organelles, in an amount approximately equivalent to that found with F_m-Ins. However, nuclear accumulation of unlabeled ferritin was <2% of F_m-Ins accumulation at 60 and 90 min. Ferritin was

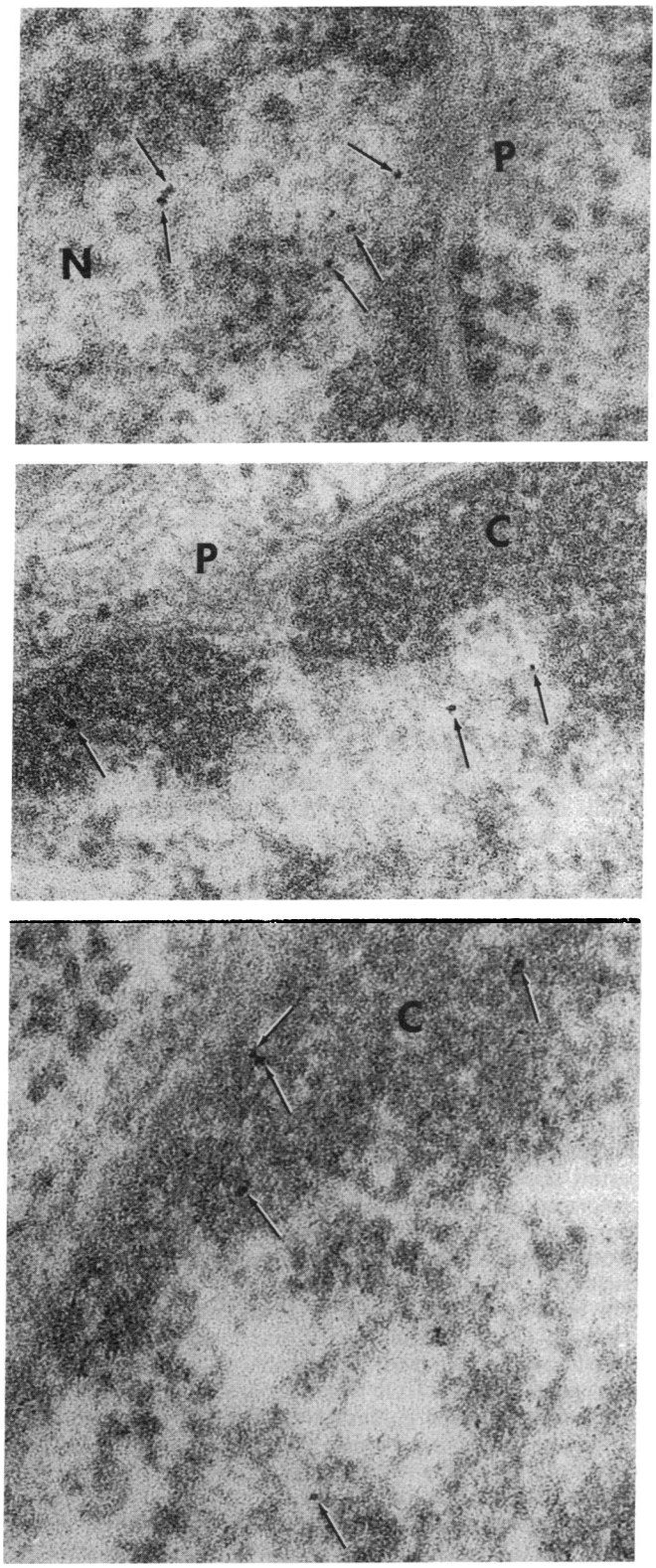

FIG. 2. 3T3-L1 adipocytes were incubated for 60 min at 37°C with 20 ng of F_m-Ins per ml. These three electron micrographs demonstrate F_m-Ins particles (arrows) inside the nucleus (N). The particles were frequently found near a nuclear pore (P), and there usually were multiple F_m-Ins particles in the same general area. Most of the nuclear-associated F_m-Ins particles were found in or at the periphery of the condensed chromatin (C). (×175,000.)

not observed in the cytoplasm outside the nuclear membrane under any incubation condition.

To provide biochemical support for the ultrastructural observations and to quantify the amount of insulin accumulated by nuclei, purified nuclei were prepared from intact 3T3-L1 adipocytes after incubation of the intact cells with ^{125}I-insulin at 20 ng/ml under the conditions described in Table 2. Electron microscopy revealed that the isolated nuclei were intact and the fraction contained <5% membrane contamination (data not shown). However, ^{125}I-insulin bound to this amount of plasma membrane contamination would have prevented estimation of specific nuclear accumulation of insulin. Therefore, the plasma membrane-associated insulin was removed from the intact cells with trypsin before isolated nuclei were prepared. In cells incubated at 4°C, 9% of the total cell-associated insulin remained on the cells after treatment with trypsin (Table 2). Approximately 5% of the trypsin-insensitive radioactive material was recovered subsequently in the nuclear pellet. Since internalization of ^{125}I-insulin is unlikely at 4°C, this radioactivity most probably represents ^{125}I-insulin remaining on the plasma membrane after the trypsin treatment and plasma membrane contamination of the nuclear pellet, respectively. These data at 4°C were used to estimate the radioactivity attributable to plasma membrane occupied receptors after trypsin treatment and the radioactivity contributed by plasma membrane contamination of the nuclei for the incubations at 15°C and 37°C. The theoretical radioactivity remaining on the plasma membrane at 15°C or 37°C, based on 9% of the total radioactivity bound, was overestimated because 7% and 30% of the total cell-associated insulin was actually internalized at 15°C and 37°C, respectively. Consequently, the theoretical plasma membrane-associated radioactivity in the nuclear pellet was also overestimated, and the amount of ^{125}I-insulin associated with the nuclei was a conservative estimate. With these theoretical calculations, little specific association of insulin could be demonstrated at 15°C, consistent with the data in Table 1. At least 3% of the intracellular insulin (calculated by subtracting theoretical plasma membrane cpm after trypsin from observed cpm bound after trypsin) and >1% of the total cell-associated hormone was recovered with the nuclei of cells incubated at 37°C. Virtually identical results were obtained in five different experiments at 37°C, with a mean (± SEM) of 3.1 ± 0.4% of the internalized hormone specifically associated with the nuclear fraction.

DISCUSSION

This study used high-resolution ultrastructural and cell-fractionation techniques to demonstrate specific receptor-mediated accumulation of insulin in nuclei of intact 3T3-L1 adipocytes. Previous studies have shown that the insulin–receptor complex was internalized; in some cases, the ligand and/or receptor was recycled to the plasma membrane (8, 11–14) and accumulated in cytoplasmic vesicles or lysosomes (10, 11, 14) or Golgi (10, 15–18). Many studies (6–18) have not been able to detect significant amounts of insulin associated with nuclei. However, Goldfine and others have demonstrated specific insulin binding to isolated nuclei (32, 33) and nuclear membranes (33, 34) and the uptake and nuclear association of ^{125}I-insulin in IM-9 lymphocytes (35), rat hepatocytes (36), and pancreatic exocrine cells (37). Insulin binding has been reported in most cases to be associated with the nuclear membrane or envelope (32–34), although ultrastructural analysis of the localization of the hormone based on autoradiography has been hindered by the low resolution of that technique. Two recent autoradiographic studies of insulin accumulation in nuclei suggested that most of the hormone was associated with nucleoplasm of pancreatic exocrine cells (37) and the condensed chromatin of hepatocytes (D. A. Podelecki, R.M.S., M. Kao, P. Tsai, T. Huecksteadt, D. Brandenburg, R. S. Lasher, L.J., and J. M.

Table 1. Specificity, time dependence, and temperature dependence of F_m-Ins accumulation in 3T3-L1 adipocyte nuclei

Incubation conditions		F_m-Ins particles per 100 thin-sectioned nuclei with increasing incubation time				
Additions	°C	5*	15*	30*	60*	90*
F_m-Ins (100 ng/ml)	37	0	4	33	142	206
+ Ins (25 μg/ml)	37	0	0	0	2	5
Unlabeled ferritin (0.25 μg/ml)	37	0	0	0	0	0
(5 mg/ml)	37	—	—	4	2	4
F_m-Ins (100 ng/ml)	15	0	0	5	9	16

Intact 3T3-L1 adipocytes were incubated 5–90 min at 37°C with 100 ng of F_m-Ins per ml [based on insulin (Ins) concentration] in the presence or absence 25 μg of excess insulin or with unlabeled ferritin at the same concentration as the ferritin in F_m-Ins (0.25 mg of ferritin per ml) or at a concentration giving approximately the same amount of intracellular ferritin particles as the F_m-Ins (5 mg of ferritin per ml). Some cells also were incubated with F_m-Ins at 15°C. The cells were washed and prepared for electron microscopy as described (25).
*Minutes of incubation.

Olefsky, unpublished data). Although insulin has been shown to affect nuclear processes (20) and incubation of isolated nuclei with insulin affects biologic processes (38, 39), previous studies have often failed to provide convincing evidence that a significant amount of internalized insulin can accumulate in the nuclei of intact cells. The current study provides that evidence.

The primary advantage of F_m-Ins over autoradiography is the ability to localize the occupied insulin receptor within a 10- to 15-nm radius of the electron-dense marker. Therefore, particles seen within the nuclei, on the nuclear membranes, or within vesicles outside nuclei, can be correctly attributed to each respective organelle. This obviates the need for theoretical, computer or statistical models often utilized with autoradiography. The present study showed that in the nuclei containing F_m-Ins, several F_m-Ins molecules were found, usually near nuclear pores. The F_m-Ins was found predominantly associated with the periphery of condensed chromatin. This latter finding may be related to long-term biological effects of insulin, since the perimeter of the condensed chromatin has been suggested to be the most active site for gene transcription (27), and this localization is similar to that found for occupied progesterone receptors (40).

Table 2. Nuclear accumulation of ^{125}I-insulin in intact 3T3-L1 adipocytes

Incubation temperature	^{125}I-insulin bound, cpm per 10^6 cells		
	Observed	Theoretical PM*	Minimum specific nuclear†
4°C			
Total binding	16,443		
After trypsin	1,463	1,463	
Nuclear fraction	73	73	
15°C			
Total binding	37,567		
After trypsin	5,649	3,342	
Nuclear fraction	187	166	21
37°C			
Total binding	62,828		
After trypsin	23,874	5,590	
Nuclear fraction	835	278	557

3T3-L1 adipocytes were incubated with ^{125}I-insulin at 20 ng/ml for 60 min at the temperatures shown. Cells were treated with trypsin at 1 mg/ml, and the nuclear fractions obtained under each incubation condition were isolated as described in the methods. ^{125}I-insulin was determined in triplicate.

*cpm recovered from 10^6 cells theoretically associated with the plasma membrane (PM) after trypsin treatment or the PM contamination of the nuclear fraction. These theoretical values were calculated based on the finding that 9% of the total cpm bound at 4°C remained associated with the cells after trypsin treatment. Therefore, no more than 9% of the total cpm at 15°C or 37°C would be expected to remain on the PM after trypsin treatment.

†At 4°C, 5% of the cpm attributed to the PM after the trypsin treatment were recovered in the nuclear pellet. Therefore, a similar percentage of the theoretical PM cpm should be recovered at 15°C and 37°C in the nuclear pellet. Because of the internalization of insulin that occurs at 15°C and 37°C but not at 4°C, the theoretical calculations overestimate the amount of non-nuclear-associated insulin, leading to a conservative calculation of the amount of specific nuclear-associated ^{125}I-insulin. Data presented are from a representative experiment.

This study has not determined whether only insulin or the insulin-receptor complex, or both, were transported to the nucleus. A recent report suggested that nuclei may have specific receptors for the insulin receptor molecule (41). In addition, Podelecki and colleagues (unpublished data) have demonstrated, using biochemical and autoradiographic techniques, that photoreactive insulin crosslinked to the insulin receptor was accumulated in nuclei of intact hepatocytes. The results of that study suggested that the insulin–receptor complex was transported to the nuclei. Since the F_m-Ins particle may represent either insulin or the insulin–receptor complex, the observations of these two studies are compatible.

The amount of F_m-Ins found in nuclei was too small to permit accurate morphometric quantitative analysis to be performed on randomly taken electron micrographs. Therefore, it was impractical to attempt to quantify, even relative to other intracellular structures, the amount of insulin in the nuclei. F_m-Ins particles were observed in 27% of the nuclear thin sections after 90 min at 37°C, but, because of the relatively small volume of each nuclei actually observed in the thin sections, it was not possible to estimate either the percentage of all nuclei in which F_m-Ins accumulated nor the absolute amount of F_m-Ins accumulated by each nucleus. Studies were performed with ^{125}I-insulin that provided both corroboration of the ultrastructural observations and quantification of the amount of insulin associated with nuclei. These measurements demonstrated that a small but potentially physiologically relevant amount of the internalized insulin was found specifically associated with the nuclei.

The mechanism by which F_m-Ins was transferred to the nucleus was not observed in the present study. It is believed that vesicular transport of the hormone–receptor complex and vesicle fusion with the nucleus is the most plausible explanation. The ultrastructural study of Locke and Huie (42) has shown nuclear membrane invaginations that may serve to translocate molecules from the cytoplasm or cytoplasmic organelles to the nucleus and vice versa. The inability to detect F_m-Ins-containing vesicles fusing with the nuclear membrane in the present study may be due to both the low

number of occurrences and the presumed rapidity of the event. In fact, despite the large amounts of F_m-Ins found in multivesicular bodies in 3T3-L1 adipocytes and other cell types (26), the fusion of F_m-Ins-containing vesicles with multivesicular bodies has been visualized infrequently.

This study provided high-resolution ultrastructural and biochemical evidence of a time- and temperature-dependent, receptor-mediated accumulation of insulin or of the insulin–receptor complex in nuclei of intact 3T3-L1 adipocytes. The amount of labeled hormone found in the nuclei and its location in the condensed chromatin suggests that this process represents a mechanism by which insulin affects a number of long-term effects, including insulin-sensitive gene transcription and DNA, RNA, and protein synthesis.

The authors thank Dr. N. Gonatas for his review and comments concerning the manuscript and to Ms. N. Laudenslager, N. Shah, and B. Wire for their technical assistance. These studies were supported by grants from the National Institutes of Health (AM28143 and AM19525) and by the R. J. Reynolds Corp.

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