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Ultrastructural basis for chloroquine-induced increase in intracellular insulin in adipocytes: Alteration of lysosomal function

(hormone degradation/hormone internalization/lysosome inhibitors/multivesicular bodies/monomeric ferritin-insulin)

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ABSTRACT A quantitative morphological analysis of insulin uptake into adipocytes was undertaken to determine the structural basis for chloroquine-induced increases in intracellular insulin. Adipocytes were incubated with ferritin-labeled insulin in the presence or absence of 50 μM chloroquine at 37°C for 2–90 min and the uptake of the hormone conjugate was determined quantitatively. Quantitative morphometry of cellular organelles also was performed. Chloroquine treatment of adipocytes incubated with 70 nM ferritin-labeled insulin resulted in: (*i*) a 120% increase in the number of lysosomes in the cytoplasm; (*ii*) a 75% increase in the average concentration of ferritin-labeled insulin in a lysosome; and (*iii*) a 25% increase in the percentage of lysosomes containing ferritin-labeled insulin. The cumulative result of these effects was a substantial increase in the amount of intact intracellular hormone within the lysosomes. These morphological data are consistent with biochemical data concerning chloroquine-induced accumulation of ^{125}I-labeled insulin in adipocytes.

The initial interaction between insulin and a cell is the binding of the hormone to specific receptor sites on the cell surface (1), followed by the dissociation, degradation, and internalization of the hormone. Several investigators have shown that adipocytes accumulate insulin, probably through internalization of the hormone–receptor complex (2–5). An increased accumulation of intact insulin in adipocytes has been reported when lysosome function was inhibited by chloroquine (6–8); quantitative biochemical analyses of the insulin uptake process in adipocytes have estimated that about 15–30% of the receptor-bound hormone was internalized (6–8) and approximately 25–50% of the internalized hormone was degraded at a chloroquine-sensitive site (5, 9). In a number of studies chloroquine-sensitive insulin degradation was found to be a small percentage of the total insulin-degradative activity of adipocytes (6–8, 10).

Several laboratories have used morphological techniques to study the binding and uptake of insulin (for a review, see ref. 11). We have found that monomeric ferritin-labeled insulin initially occupied receptor sites on the adipocyte plasma membrane and was endocytosed in pinocytotic invaginations and transported to cytoplasmic vesicles (unpublished data). Approximately 50% of the internalized hormone was incorporated into multivesicular bodies and lysosomes, with the remaining being recycled to the plasma membrane.

The present study investigated the effects of chloroquine on the uptake of insulin into adipocytes. Chloroquine had three major actions on the adipocyte that, in combination, account for the increased accumulation of insulin within the cell. Those effects were: (*i*) a 120% increase in the number of lysosomes in the cytoplasm; (*ii*) a 75% increase in the average concentration of ferritin-labeled insulin within a lysosome; and (*iii*) a 25% increase in the percentage of lysosomes containing ferritin. The first of these effects was possibly due to the ability of chloroquine to affect membrane fusion (12, 13), whereas the latter two occurred as a result of lysosomal protease inhibition (14, 15).

MATERIALS AND METHODS

Cells and Reagents. Adipocytes were prepared from epididymal fat pads of 120-g Sprague–Dawley rats by collagenase digestion as described (16). Collagenase, bovine serum albumin, and chloroquine were obtained from Sigma. Ferritin (6× crystallized) was purchased from Miles. Glutaraldehyde and osmium tetroxide were purchased from Polysciences. Porcine insulin (lot PJ 5682; 23.1 units/mg) was a gift from R. Chance of Eli Lilly. Other materials were obtained from standard sources.

Preparation and Characterization of Monomeric Ferritin-Insulin (Fm-I). The conjugation of insulin to ferritin (16–19) was modified and described in detail (20) recently. Fm-I was subjected to extensive analysis to document its insulin-like properties and its validity as an ultrastructural marker for occupied insulin receptors. Fm-I was found to be fully active biologically, as compared with native insulin, and to specifically bind to the insulin receptor with the same apparent affinity as ^{125}I-labeled insulin (^{125}I-insulin). It was displaced by unlabeled insulin, was stable under a variety of storage and incubation conditions, and contained no unlabeled insulin (20). Fm-I prevented degradation of ^{125}I-insulin by adipocytes identically to unlabeled insulin (unpublished data). The calculated number of occupied insulin receptors per μm^2 of cell surface with comparable concentrations of ^{125}I-insulin or ferritin-labeled insulin was approximately the same (21). Fm-I, like ^{125}I-insulin, will identify only occupied receptor sites and, in the event that the label, the hormone, or the receptor is degraded, the localization of the label relative to the hormone receptor is unknown. Unlike ^{125}I-insulin, when Fm-I is used as an ultrastructural marker the distance between the ferritin particle and the membrane to which it is bound can be used to determine the integrity of the label–hormone–receptor complex (12).

Incubation Conditions. Binding was determined by incubating 10^6 adipocytes per ml in Krebs–Ringer phosphate buffer (pH 7.4), with 3% bovine serum albumin and 0.2% dextrose, at 37°C for 2–90 min in the presence or absence of 50 μM chloroquine with 7 or 70 nM Fm-I. Specific details of each experiment are described in the legend to the appropriate figure or table. All experiments contained controls to monitor nonspe-

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Abbreviation: Fm-I, monomeric ferritin-insulin.

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cific binding of Fm-I, including the addition of 4 μM native insulin along with the Fm-I or an amount of unlabeled ferritin equivalent to that in the Fm-I added to a separate set of cells. Native insulin prevented binding to or uptake of Fm-I by viable cells. Unlabeled ferritin did not bind to or appear to be internalized in intact adipocytes at the concentrations used in these studies.

In some experiments an aliquot of cells was prefixed for 5 min at 37°C with 0.1% glutaraldehyde in Krebs–Ringer phosphate buffer (pH 7.4). The fixed cells were washed and incubated for 15 min at 37°C with a Krebs–Ringer solution buffered with 50 mM Tris·HCl (pH 7.4) to eliminate nonspecific binding of ferritin; they then were washed and resuspended in fresh incubation buffer. Fm-I was added and the cells were incubated for 30 min. The same types of controls for nonspecific binding were performed with results identical to the fresh adipocytes.

Preparation of Adipocytes for Electron Microscopy. After the incubations with Fm-I, the cell suspensions were diluted with a 10-fold excess of Krebs–Ringer phosphate buffer (pH 7.4) at 4°C and were centrifuged rapidly at 1,000 × g. The isolated cells were prepared for electron microscopy as described (18).

Quantitative Analysis of Electron Micrographs. Morphometry of the adipocytes and analysis of the distribution of Fm-I receptor sites were performed on micrographs taken along the perimeter of adipocytes at magnifications appropriate to visualize the entire depth of the cytoplasm as well as identify the desired cellular structures and ferritin particles. The measurements made included: (*i*) the length of plasma membrane; (*ii*) the number and circumference of pinocytotic invaginations; (*iii*) the number and size of coated pits; (*iv*) the number and diameter of cytoplasmic vesicles; (*v*) the number and diameter of multivesicular bodies; (*vi*) the number and diameter of dense bodies; (*vii*) the depth of the cytoplasm; (*viii*) the number and size of the mitochondria; and (*ix*) the number and distribution of Fm-I particles associated with any of the above cellular structures.

The definition of a group of receptors was based on criteria established earlier by using computer-assisted analysis of receptor site distribution on adipocytes (22). Certain structural definitions were adhered to in the analysis. Pinocytotic invaginations were flask shaped and clearly connected to the plasma membrane. Where multiple invaginations were connected in a rosette-like structure (23) and a clear connection with the plasma membrane was observed, the structures were considered to be invaginations. Cytoplasmic vesicles were smooth surfaced and not connected to the plasma membrane, regardless of the distance between the vesicle and the membrane. This definition might result in some invaginations being classified as vesicles because the section failed to reveal the plasma membrane connection. Results presented suggest that the error was small and did not affect the analysis. Lysosome-like structures were divided into two major types—i.e., multivesicular and dense bodies—depending on the appearance of the contents of their lumen. Acid phosphatase cytochemistry (24) suggested that few, if any, multivesicular bodies contained acid phosphatase activity, whereas approximately 50% of the dense bodies contained reaction product (data not shown). Coated pits were identified by their characteristic electron-dense, bristle-like, submembrane layer and were readily identified without specialized staining.

RESULTS

The effect of increasing concentrations of Fm-I on the distribution of occupied insulin receptors was determined because experiments in this study were performed with various concentrations of Fm-I (Table 1). The distribution pattern of receptor sites in groups of various sizes was found to be virtually identical at all concentrations of Fm-I. These data suggest that occupied receptor sites seen at 1.8 nM Fm-I are representative of receptor site distribution. Total receptor occupancy levels, as reflected by the concentration of receptors per μm^2 of cell surface, were found to increase with increasing concentrations of Fm-I, as expected from biochemical studies of ^{125}I-insulin binding. At higher Fm-I concentrations, receptor sites were close to full saturation; therefore, little increase in receptor number was observed between 7 and 70 nM Fm-I.

Table 1. Distribution and concentration of occupied Fm-I receptors determined with various concentrations of Fm-I

Concentration		Receptor sites per group, %*					
Fm-I, nM	Receptors, no. per μm^2	1	2	3	4	5	6
1.8	8.3	37.3	22.7	17.9	13.5	6.6	2.0
3.5	17.4	36.8	25.7	22.9	10.1	3.0	1.5
7	21.6	35.3	23.6	18.9	11.0	7.0	4.2
70	25.7	35.1	27.2	22.6	11.2	2.4	1.5

Adipocytes were incubated at 37°C for 60 min with various concentrations of Fm-I, washed, and prepared for electron microscopy as described. Cell surface receptors were analyzed to determine the distribution of the receptors into groups (1–6) of various sizes as described (22). A minimum of 500 Fm-I molecules was observed in each experimental condition.

* Data are expressed as percentage of total occupied receptor sites.

Fig. 1 illustrates that when intact isolated adipocytes were incubated with 70 nM Fm-I, the concentration of occupied receptors on the unspecialized region of the plasma membrane remained constant from 2 to 90 min and it was the same as on the prefixed cells. Chloroquine had no effect on Fm-I binding to the plasma membrane. The concentration (mean ± SEM) of Fm-I receptors in coated pits from 2 to 90 min was 1.3 ± 0.1 receptors per μm^2, or about 5% of the concentration on the rest of the plasma membrane, and did not differ from that found on

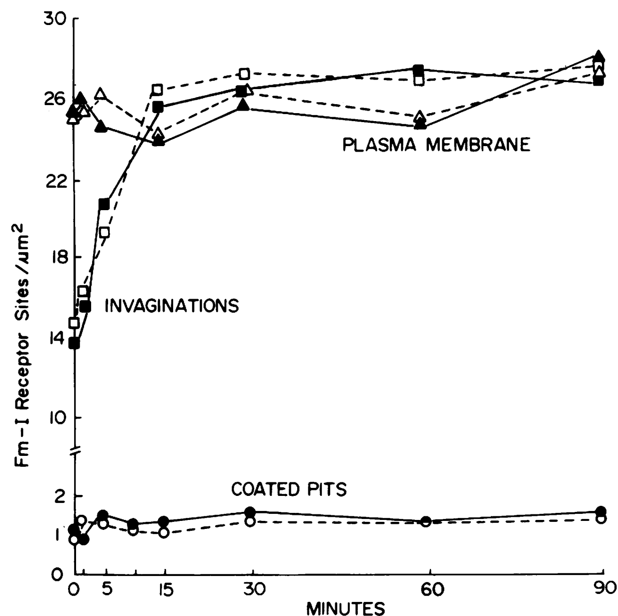

FIG. 1. Quantitative analysis of the effect of chloroquine on the concentration of Fm-I receptors on adipocyte plasma membranes, pinocytotic-like invaginations, and coated pits. Fixed adipocytes were used as a 0 time value. Fresh adipocytes were incubated for 2–90 min at 37°C with 70 nM Fm-I in the absence (closed symbols) or presence (open symbols) of 50 μM chloroquine. Analysis of the concentrations of receptor sites per μm^2 of plasma membrane (▲, △), invaginations (■, □), and coated pits (●, ○) was performed as described.

the prefixed cells. The data from the longest incubation times indicated that insulin receptors were not being concentrated into the coated pits. Chloroquine had no effect on the concentration of receptor sites in the coated pits. A time-dependent increase in the concentration of insulin receptor sites in pinocytotic invaginations was observed during the first 10 min of incubation, although chloroquine had no effect on the rate of increase or final concentration of receptor sites observed. The steady-state level achieved in invaginations was approximately equal to the concentration seen on the unspecialized plasma membrane.

Analysis of the distribution of Fm-I occupied receptors into groups of various sizes was performed on the entire plasma membrane—including invaginations and coated pits—to further investigate the possibility of insulin receptor aggregation. It was shown previously that insulin receptors on adipocytes exist both as single molecules, about 35% of the total receptors, and in small groups of two to six receptors (refs. 16, 19, 22; Table 1). Prolonged incubations at 37°C did not result in any perceptible aggregation or formation of larger groups of receptors (Table 2). Chloroquine had no effect on the distribution of receptor sites.

Experiments designed to quantitatively determine both the time course and the amount of Fm-I taken up, relative to the amount bound to the membrane receptors, were performed with 70 nM Fm-I (Fig. 2). The invaginations on prefixed (0 time point) cells contained about 10% as much Fm-I as the membranes. On control cells the amount of Fm-I rapidly increased in the invaginations, until at 10 min a steady state was reached with the invaginations having 17–18% as much Fm-I as the plasma membrane (Fig. 2*A*). This was consistent with the data in Fig. 1, demonstrating a time-dependent increase in the Fm-I concentration in the invaginations. The cytoplasmic vesicles of control cells showed appreciable accumulation of Fm-I within 2 min but lacked any substantial Fm-I in prefixed cells (Fig. 2*B*). The lack of Fm-I in the vesicles of prefixed cells suggested that the possible error in incorrectly identifying vesicles and invaginations was small. At steady state, vesicles contained about 11% as much Fm-I as the plasma membrane. The combination of multivesicular and dense bodies accumulated Fm-I until a steady state was reached at 15 min, with an amount of Fm-I equivalent to approximately 6% of plasma membrane Fm-I. Chloroquine at 50 μM had no effect on the amount or time course of Fm-I accumulation in the invaginations or vesicles (Fig. 2 *A* and *B*, respectively). There was a marked effect of

Table 2. Effect of increasing time of incubation and chloroquine on the distribution of occupied Fm-I receptor sites on adipocytes into groups of various sizes

Time	Chloroquine	Receptor sites per group, %*		
		1	2	3–6
Prefixed	−	38.6	27.1	34.3
	+	34.2	28.4	37.4
2 min	−	35.2	29.8	35.0
	+	38.1	27.6	34.3
30 min	−	36.5	29.1	34.4
	+	36.2	27.0	36.8
90 min	−	34.6	29.6	35.8
	+	36.5	29.1	34.4

Prefixed or fresh adipocytes were incubated with 70 nM Fm-I at 37°C for the indicated times in the presence (+) or absence (−) of 50 μM chloroquine. The cells were washed and prepared for electron microscopy as described. Analysis of occupied receptor sites on the plasma membrane was performed on a minimum of 500 Fm-I molecules at each time point.

* Data are expressed as percentage of total occupied receptor sites.

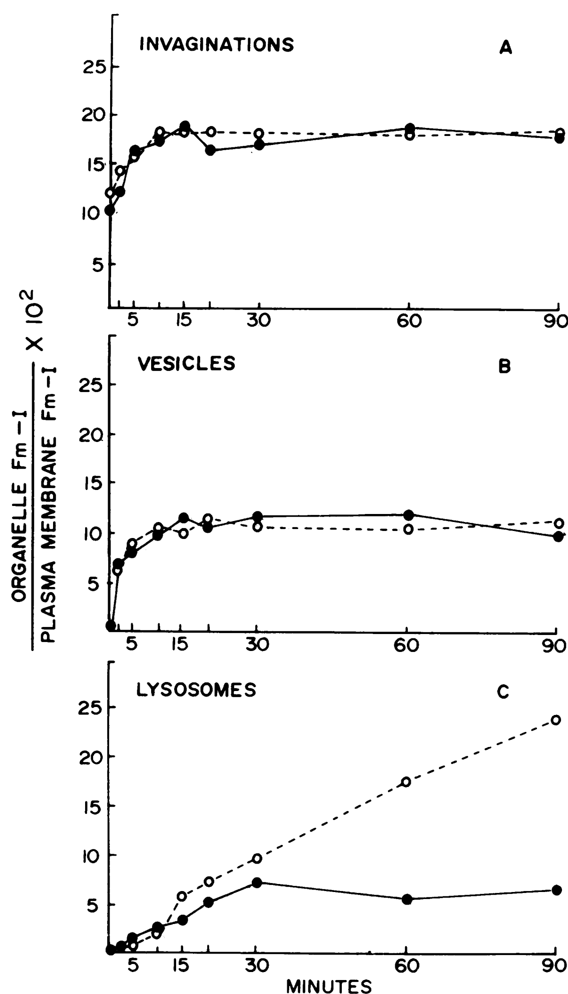

FIG. 2. Quantitative analysis of Fm-I uptake in adipocytes and the effect of chloroquine. Fixed adipocytes were used as a 0 time value. Fresh adipocytes were incubated for 2–90 min at 37°C with 70 nM Fm-I in the absence (●) or presence (○) of 50 μM chloroquine. Analysis of the amount of Fm-I on the plasma membrane and associated with invaginations (*A*), cytoplasmic vesicles (*B*), and lysosomes (*C*) was performed as described. The results are expressed as the ratio of the number of ferritin insulin particles associated with each organelle to the number of ferritin insulin particles on the plasma membrane.

chloroquine on the amount of Fm-I found in the multivesicular and dense bodies that became apparent after 15 min of incubation. Chloroquine prevented the structures from attaining a steady-state level and by 90 min the amount of Fm-I was about 24% as much as found on the plasma membrane. This represented a 4-fold increase in the amount of Fm-I contained in these structures as compared with control cells.

Morphometric analysis performed on control and chloroquine-treated cells showed chloroquine had no effect on the size of either the cells or various intracellular organelles (data not shown). However, as shown in Table 3, chloroquine increased the number of multivesicular bodies by 50% ($P < 0.05$) and the number of dense, lysosome-like structures by 120% ($P < 0.001$). There was no detectable effect of chloroquine on the number of cytoplasmic vesicles, mitochondria, or plasma membrane-associated structures.

It was necessary to determine if the increase in total intracellular Fm-I in lysosomal structures (Fig. 2*C*) caused by chloroquine was a result of an increase in the number of those structures (Table 3) or the amount of Fm-I within the structures, or both. After 60 min of incubation, chloroquine caused a marginal

Table 3. Effect of 50 μM chloroquine on adipocyte cellular structures

Structure	Structures per 10-μm cell surface	
	Control cells	Chloroquine-treated cells
Pinocytotic invaginations	57.0 ± 1.8	53.0 ± 1.8
Coated pits	1.6 ± 0.4	2.0 ± 0.3
Cytoplasmic vesicles	190.5 ± 7.6	184.5 ± 6.8
Multivesicular bodies	2.6 ± 0.7	3.8 ± 0.5*
Dense bodies	5.4 ± 0.5	11.7 ± 0.6†
Mitochondria	45.0 ± 2.8	44.5 ± 3.1

At least 50 randomly selected micrographs of both control and chloroquine-treated cells in each of six experiments were analyzed. The length of plasma membrane, the width of the cytoplasm, and the number of organelles present were recorded. The average depth of the cytoplasm (plasma membrane to central lipid droplet) (±SEM) was found to be 1.6 ± 0.3 μm and was not significantly different between the control and chloroquine-treated cells. The only significant differences between the control and chloroquine-treated cells were found in the number of multivesicular bodies and dense bodies. Values are expressed as the mean number of organelles per 10 μm of linear cell surface (±SEM) in six experiments. Corrections were made for the magnification of the micrograph.

* Significantly different from control; $P < 0.05$.

† Significantly different from control; $P < 0.001$.

increase of 7% in the percentage of multivesicular bodies containing Fm-I (Table 4). The percentage of dense bodies with Fm-I was increased by 25%. When Fm-I concentrations were calculated on the basis of the total number of structures observed, whether they contained Fm-I or not, chloroquine increased the average number of Fm-I per structure in a thin section only in the dense bodies (Table 4). If the increases in the number of lysosome-like structures and in the concentration of Fm-I per structure are taken into account, chloroquine would have a marked effect on the total amount of Fm-I contained in these structures.

DISCUSSION

Adipocytes internalize membrane-associated insulin and this internalization results in the degradation of the hormone (2–8) or the receptor (25, 26). The degradation occurs at a chloroquine-sensitive location (6–8, 25, 26) and therefore, it has been presumed that adipocyte lysosomes accumulate and degrade

Table 4. Effect of chloroquine on Fm-I content in intracellular multivesicular and dense bodies

Structure	% with Fm-I	Fm-I per structure
Multivesicular bodies		
Control	24.3	1.8
Chloroquine	26.0	2.0
Dense bodies		
Control	23.9	2.0
Chloroquine	29.8	3.5

Isolated adipocytes were incubated with 70 nM Fm-I for 60 min at 37°C and the cells were washed and prepared for electron microscopy as described. The total number of multivesicular and dense bodies seen in the electron micrographs was determined along with the number of those structures containing Fm-I. The percentage of the observed structures with Fm-I was calculated. The total number of Fm-I particles was determined and the average number of Fm-I per structure was calculated on the basis of the total number of structures observed. This average number is indicative only of the number of particles seen in thin section and it underestimates the true concentration of Fm-I in the lysosomes by at least an order of magnitude.

insulin by a receptor-mediated endocytotic process. This study documents ultrastructurally the effects of chloroquine on insulin uptake in adipocytes and supplies morphological confirmation of the biochemical studies.

We have examined the endocytosis of insulin in adipocytes and found that insulin bound to small groups of receptors on the plasma membrane and that 10–20% of the occupied receptors were internalized in pinocytotic invaginations by a receptor-mediated process that did not involve the concentration of insulin receptors into coated pits on the plasma membrane (unpublished data). Cytoplasmic vesicles transported about 50% of the internalized insulin to lysosomes where the receptor and the hormone could potentially be degraded. The other 50% was apparently recycled to the plasma membrane in a system of vesicles and exocytotic invaginations. The lack of involvement of coated pits in the uptake process in adipocytes is in contrast with observations made with other techniques and cell types, particularly fibroblasts (27, 28). In other studies, epidermal growth factor, a polypeptide similar to insulin, was endocytosed in coated pits in fibroblasts (29) but not in A431 carcinoma cells (12, 30). It also has been reported that ϕ and H63, two surface antigens, were excluded from coated pits on fibroblast plasma membranes (31). These variations suggest that different types of cells probably possess different mechanisms for endocytosis of specifically bound molecules.

This study showed that chloroquine had no effects on the initial binding of insulin to its receptor, on the distribution of insulin receptors on the cell surface, or on the initial phases of the uptake process. Chloroquine had three demonstrable effects on the processing of internalized insulin. First, the total number of lysosomes was increased, with the greatest increase occurring in the number of dense bodies. This suggested that chloroquine prevented normal lysosomal processing, therefore causing a buildup of these structures within the cell. The mechanism by which chloroquine affects lysosome processing in various cell types has been attributed to various processes (12, 32, 33). It is unclear whether the apparent discrepancies in these observations are due to tissue-specific differences in the actions of chloroquine, the result of differences in morphometric methodology, or semantic differences in the definition or recognition of "lysosomes." The second effect of chloroquine was to increase the amount of Fm-I observed in the dense bodies, as demonstrated by the increase in the number of Fm-I per dense body. This would be consistent with the ability of chloroquine to inhibit lysosomal proteases (14, 15). The third observation was that the percentage of dense bodies in which Fm-I was observed was increased in the chloroquine-treated cells. This also is probably due to the inhibition of proteases. It is the combination of these effects on adipocyte lysosomes that results in the marked increase in the amount of intracellular Fm-I and, by inference, insulin in chloroquine-treated cells. The quantitative estimates of Fm-I uptake in control and chloroquine-treated cells determined morphologically in this study are similar to those reported in adipocytes when using ^{125}I-insulin (2-8).

Differences in the distribution of insulin receptors on the cell surface have been shown in various tissues (34, 35) along with apparent differences in the insulin uptake processes of various cells (refs. 11, 36, 37; unpublished data). When the apparently variable effects of chloroquine on intracellular structures (12, 13, 32, 33) are considered along with these differences in insulin uptake processes, it is entirely possible that the effects of chloroquine on insulin accumulation may be different in adipocytes than in some other cells. Chloroquine has been reported to increase cell-associated insulin by altering receptor affinity in IM9 lymphocytes (38) and to lead to an accumulation of insulin in Golgi vesicles in liver (39). A specific and high resolution marker

for insulin receptors, such as Fm-I, should provide the means for documenting the mechanism(s) by which chloroquine affects the metabolism of insulin in various cells.

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