Chloroquine Extends the Lifetime of the Activated Insulin Receptor Complex in Endosomes*

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Insulin signal transduction, initiated by binding of insulin to its receptor at the plasma membrane, activates the intrinsic receptor tyrosine kinase and leads to internalization of the activated ligand-receptor complex into endosomes. This study addresses the role played by the activated insulin receptor within hepatic endosomes and provides evidence for its central role in insulin-stimulated events in vivo. Rats were treated with chloroquine, an acidotrophic agent that has been shown previously to inhibit endosomal insulin degradation, and then with insulin. Livers were removed and fractionated by density gradient centrifugation to obtain endosomal and plasma membrane preparations. Chloroquine treatment increased the amount of receptor-bound insulin in endosomes at 2 min after insulin injection by 93% as determined by exclusion from G-50 bound insulin in endosomes at 2 min after insulin injection and by 90% as determined by polyethylene glycol precipitation (p < 0.02). Chloroquine treatment also increased the insulin receptor content of endosomes after insulin injection (integrated over 0–45 min) by 31% when compared with controls (p < 0.05). Similarly, chloroquine increased both insulin receptor phosphotyrosine content and its exogenous tyrosine kinase activity after insulin injection (64%; t < 0.01 and 90% and p < 0.001, respectively). In vivo chloroquine treatment was without any observable effect on insulin binding to plasma membrane insulin receptors, nor did it augment insulin-stimulated receptor autophosphorylation or kinase activity in the plasma membrane. Concomitant with its effects on endosomal insulin receptors, chloroquine treatment augmented insulin-stimulated incorporation of glucose into glycogen in diaphragm (p < 0.001). These observations are consistent with the hypothesis that chloroquine-dependent inhibition of endosomal insulin receptor dissociation and subsequent degradation prolongs the half-life of the active endosomal receptor and potentiates insulin signaling from this compartment.

Insulin signal transduction is initiated by binding of insulin to its receptor at the plasma membrane, which in turn leads to the rapid autophosphorylation of multiple tyrosine residues on the intracellular portion of the β-subunit and the activation of the receptor tyrosine kinase toward exogenous substrates (1, 2). Following autophosphorylation, the activated ligand-receptor complex is internalized into endosomes in liver (3–6) and low density membranes in adipocytes (7, 8) and muscle (9). Endocytosis of activated receptors has the twin effects of concentrating receptors within endosomes and allowing the insulin receptor tyrosine kinase to phosphorylate substrates that are spatio-temporally distinct from the plasma membrane (Ref. 10; reviewed in Ref. 11). Subsequent termination of signal transduction is achieved by endosomal insulin degradation (12–16) following dissociation of insulin from its receptor (14, 17) as the intraluminal environment of the endosome acidifies (18). This loss of the ligand-receptor complex attenuates any further ligand-driven receptor re-phosphorylation, and the receptor is dephosphorylated by extraluminal endosomally associated phosphotyrosine phosphatase(s) (5, 6, 19).

The anti-malarial drug chloroquine has been shown to elicit a number of effects on insulin metabolism. Thus, in rats, chloroquine treatment leads to hepatic retention of intact insulin within endosomes (12, 20) due to inhibition of endosomal insulin degradation (13, 21, 22). This effect does not rely solely on the acidotrophic nature of chloroquine (whereby it is accumulated within acidic vesicles neutralizing the pH; Ref. 23), as chloroquine’s inhibition of insulin degradation persists in detergent disrupted endosomes (13, 21). Kinetic analysis of the rates of endosomal insulin degradation in the absence of chloroquine show it to be a bi-exponential process where the values of the rate constants are very similar to those for the dissociation of insulin from its receptor (21), suggesting that dissociation from the receptor is the rate-limiting step in degradation. Chloroquine results in a 2-fold decrease in the rate constant of the slow process, together with an increase in the proportion of degradation proceeding via the slow process (21, 24). Additionally, chloroquine has been shown to increase insulin binding in cultured hepatoma cells (25, 26) and IM9 lymphocytes (27). Modeling of insulin binding to purified plasma membranes in the presence of chloroquine demonstrated that the augmentation of binding was due to a decrease in the rate constant for insulin dissociation (28). Clinical manifestations of chloroquine action have also been observed. Thus, in non-insulin-dependent diabetes mellitus, chloroquine improves glucose tolerance (29), increases peripheral glucose disposal, and decreases the metabolic clearance rate of insulin (30). In insulin-dependent diabetes mellitus, chloroquine has been shown to reduce insulin resistance (31).

This augmentation of insulin-receptor interaction observed with chloroquine treatment prompted the present study to ascertain whether in vivo chloroquine treatment in the rat: 1) increases the amount of insulin bound to endosomal insulin receptors, 2) inhibits dissociation and hence degradation of insulin within endosomes, 3) augments and/or prolongs the

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autophosphorylation state and activity of the endosomal insulin receptor (tyrosine kinase, 4) affects the temporal flux of insulin receptors through the endosome compartment, and 5) augments insulin-stimulated metabolic events.

EXPERIMENTAL PROCEDURES

Animals—Male Sprague-Dawley rats 1 (180–200 g, body weight) were fed ad libitum on a standard chow diet and housed at 22 ± 1 °C with 12-h light cycles.

Materials—Porcine insulin suspension was purchased from Calbiochem (Notts, UK). Chloroquine (7-chloro-4-(4-diethylamino-1-methyl)butylamino)quinoline), RIA-grade BSA and most other chemicals were purchased from Sigma (Poole, Dorset, UK). Carrier-free Na[125I] and γ[32P]ATP (9 TBq/μmol) were from Amer sham International (Bucks., UK). Metafane anesthetic was from C-VET Ltd. (Bury St. Edmonds, Suffolk, UK), and Sagital (sodium pentabarbitone) was from May and Baker Ltd. (Dagenham, UK). Polyethylene glycol 6000 (PEG-6000; molecular weight 6000–7500) was from BDH (Poole, Dorset, UK). The synthetic peptide FYF (RDIFEADYFRK) was synthesized commercially (Lake Placid, NY). A monoclonal antibody (CT-3) raised to, and reacting with, the last 43 amino acids of the insulin receptor β-subunit was used for immunocapture assays. CT-3 was prepared as using as a GST-fusion protein containing the terminal 100 amino acids of the human insulin receptor (36) and purified as described previously (37).

Preparation of 125I-[A14]Insulin—Monocomponent porcine insulin was prepared from insulin zinc suspension (British Pharmaceuticals) as outlined by Christensen et al. (33) and was iodinated with lactoperoxidase (34). The 125I-[A14]insulin isomer was separated by HPLC as described previously (35).

Antibodies—A monoclonal phosphotyrosine antibody 4G10 (1G10) used for immunocapture assays was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). A monoclonal antibody (CT-3) raised to, and reacting with, the last 43 amino acids of the insulin receptor β-subunit was used for immunocapture assays. CT-3 was prepared as using as a GST-fusion protein containing the terminal 100 amino acids of the human insulin receptor (36) and purified as described previously (37).

Preparation of Liver Subcellular Fractions—Following sacrifice, livers were minced with scissors in ice-cold homogenization buffer. Plasma membranes were prepared as described by Bevan et al. (10) and endosomes prepared as described below. Livers were minced with scissors in ice-cold homogenization buffer (10 mM HEPES buffer, pH 7.4, containing 0.25 mM sucrose, 1 mM MgCl₂, 0.1 mM phenylmethanesulfonic fluoride, 0.2 mM AEBSSF, 0.02 mM EDTA, 0.02 mM pepstatin A, 2 mM NaF, and 2 mM sodium orthovanadate). All preparative procedures were performed at 4 °C in the presence of the same concentration of phosphatase/protease inhibitors and buffer with only the sucrose concentration changing as indicated. The livers were homogenized (5 ml of homogenization buffer/g of liver) in a Potter-Elvehjem homogenizer with five passes of a motorized Teflon pestle to achieve a 20% homogenate and then filtered through a 50-μm nylon mesh to remove fibrous and undigested tissue. A 10-ml aliquot of homogenate was layered onto a discontinuous gradient of 11 ml ρ = 1.09 g cm⁻³ (0.67 mM) and 12 ml ρ = 1.14 g cm⁻³ (1.05 mM) sucrose in 33 ml centrifuge tubes and centrifuged in a Sorvall AH-629 swing-out rotor (Du Pont) at 110,000 × gₑₑₑ (29,000 rpm, 75 min, 4 °C) in a Sorvall OT75B centrifuge (Du Pont). Endosomes were collected at the interface of the 1.09 and 1.14 g cm⁻³ sucrose solutions.

In studies where PEG precipitation, HPLC, and gel permeation chromatography were used, experiments were performed at 4 °C in the presence of the same concentration of phosphatase/protease inhibitors and buffer with only the sucrose concentration changing as indicated. The livers were homogenized (5 ml of homogenization buffer/g of liver) in a Potter-Elvehjem homogenizer with five passes of a motorized Teflon pestle to achieve a 20% homogenate and then filtered through a 50-μm nylon mesh to remove fibrous and undigested tissue. A 10-ml aliquot of homogenate was layered onto a discontinuous gradient of 11 ml ρ = 1.09 g cm⁻³ (0.67 mM) and 12 ml ρ = 1.14 g cm⁻³ (1.05 mM) sucrose in 33 ml centrifuge tubes and centrifuged in a Sorvall AH-629 swing-out rotor (Du Pont) at 110,000 × gₑₑₑ, (29,000 rpm, 75 min, 4 °C) in a Sorvall OT75B centrifuge (Du Pont). Endosomes were collected at the interface of the 1.09 and 1.14 g cm⁻³ sucrose solutions.

In studies where PEG precipitation, HPLC, and gel permeation chromatography were under investigation, endosomes were prepared in homogenization and gradient buffers containing 10 mM HEPES buffer, pH 7.4, 2.5 mM N-ethylmaleimide (NEM), 1 mM 1,10-phenanthroline, and 1 mM chloroquine. The homogenizer buffer to prepare samples for HPLC analysis additionally contained 60 unit/ml bacitracin.

Solubilization Procedure for Immunocapture and Tyrosine Kinase Assay—Plasma membrane and endosomal fractions (0.5 ml) were solubilized in 0.5 ml of freshly prepared 2 × solubilization buffer (50 mM HEPES buffer, pH 7.4, containing 1% Triton X-100, 150 mM NaCl, 10 mM EDTA, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 30 mM NaF, 0.5 mM phenylmethanesulfonic fluoride, 2.5 mM benzamidine, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1 μg/ml antipain; final concentration). After incubation for 10 min at 4 °C, the samples were centrifuged at 13,000 × gₑₑₑ (12,000 rpm, 5 min, 4 °C).

Tyrosine Kinase Assay—Tyrosine kinase activity was assessed using a modified microtitre plate immunocapture method as described previously (38, 39). Fifty microliters of CT-3 (20 μl NaHCO₃, pH 9.6, at a concentration of 0.5 μg/ml) was incubated for 16 h at 4 °C in 96-well F16 Maxisorb loose Nunc-immuno microwell module plates. Any unattached antibody was removed with three applications (100 μl) of wash buffer (50 mM HEPES buffer, pH 7.4, 150 mM NaCl, 0.1% BSA, 0.1% Triton X-100, and 0.1% Tween 20). Duplicate solubilized plasma membrane or endosome samples (200 μl) were added to wells and incubated for 16 h at 4 °C. The samples were then aspirated and plates washed three times with ice-cold wash buffer (100 μl) and 20 μl of kinase reaction mixture applied (130 mM HEPES, pH 7.4, 100 μM MgCl₂ BSA, 1 mM EGTA, 12 mM MgCl₂, 0.2 mM FYY, and 74 KBq [γ-32P]ATP). The incubation was terminated after 20 min at room temperature by the addition of 5 μl of 2 M HCl. 32P-Labeled peptide substrate was captured onto phosphocellulose columns (Sephacel™, Pierce, Chester, UK) by centrifugation in an Eppendorf centrifuge at 13,000 × gₑₑₑ (12,000 rpm, 0.5 min, 22 °C). The filter was washed twice with 75 mspHosphoric acid (500 μl) and centrifuged as before. Finally, the filter cartridge was removed and counted in 10 ml of scintillation mixture in a Canberra Packard 1500 Tri-Carb liquid scintillation analyzer.

Quantitation of Insulin Receptor and Its Phosphotyrosine Content by Immunocapture Assay—Solubilized plasma membrane and endosomes were incubated with microserit plates as described above in the kinase assay up to the point of kinase reaction mixture addition, except that in the case of phosphotyrosine content estimation, the wells were plated with phosphotyrosine antibody 4G10 at a concentration of 1 μg/ml instead of CT-3 and the sample volume was 100 μl. Following aspiration of the sample and three washes with wash buffer, the bound antibody was incubated for 16 h at 4 °C with 50 μl of binding buffer (100 mM HEPES buffer, pH 7.4, 120 mM NaCl, 1 mM EDTA, 15 mM sodium acetate, 1.2 mM MgSO₄, 10 mM glucose, and 1% BSA) containing 333 Bq of [125I]-[A14]insulin. The wells were then aspirated and washed three times with 100 μl of PBS, pH 7.4, and the bound insulin released by the addition of 100 μl of 0.03% SDS in water at 20 min room temperature. The 100 μl was removed from the wells and counted for radioactivity.

![FIG. 1. Determination of the amount of receptor-bound insulin present in freshly isolated endosomes. Anesthetized rats were injected with approximately 1 MBq of [125I]-[A14]insulin via the hepatic portal vein over 30 s. The liver was removed after an additional 2 min and endosomes prepared as described under “Experimental Procedures.” Inhibitors of insulin degradation were omitted from the sucrase solutions. Endosomes were disrupted and extracted into 0.1% Triton X-100 and applied to a G-50 Sepharose column in the presence of 2.5 mM NEM, 1 mM 1,10-phenanthroline, and 1 mM chloroquine. Fractions (1 ml) were collected and counted for radioactivity. The figure is representative of two experiments from two separate animals.](image341x607to531x729)
Identification of $^{125}$I-[A14]Insulin and Degradation Products within Endosomes—Following hepatic portal vein injection of $^{125}$I-[A14]insulin (1 MBq in 0.5 ml of PBS, 5% RIA-grade BSA), endosomes were prepared by discontinuous sucrose gradient centrifugation as described above. The isolated endosomal population was subsequently washed free of cytosol by a 10-fold dilution in 10 mM HEPES buffer, pH 7.4 (containing 0.1 M KCl, 2.5 mM NEM), and sedimented by centrifugation in a Sorvall T-865.1 rotor at 100,000 x g (37,000 rpm, 30 min, 4 °C). The endosomes were solubilized with a final concentration of 0.1% Triton X-100 at 4 °C for 10 min and then clarified by centrifugation in a Beckman TLA 100.2 rotor at 120,000 x g (60,000 rpm, 10 min, 4 °C). HPLC separation of extracted endosomal contents was performed at a flow rate of 1 ml/min, in 0.1 M ammonium acetate buffer, pH 5.5, with an acetonitrile gradient. The precise changes in acetonitrile concentration required to effect the separation of degradation intermediates were as follows: 1) 0–5 min at 0%, 2) 5–20-min gradient rising to 8.45%, 3) instantaneous rise to 28.5%, 4) 20–40-min gradient rising to 32.5%. Detection of $^{125}$I was achieved by an on-line counter attached to a reverse phase HPLC Hypersil-BDS, 5 μm, C-18 column, 250 x 4.6 mm, fitted with an additional 10-mm guard cartridge of the same material and purchased from Shandon HPLC (Runcorn, Cheshire, UK).

PEG Precipitation of Receptor-Bound Insulin—Endosomes containing $^{125}$I-[A14]insulin were prepared as described above from animals that had received an injection of $^{125}$I-[A14]insulin (1 MBq in 0.5 ml of PBS, 5% RIA-grade BSA) via the hepatic portal vein. The endosomes were solubilized with a final concentration of 0.1% Triton X-100 at 4 °C for 10 min and then clarified by centrifugation in a Beckman TLA 100.2 rotor at 120,000 x g (60,000 rpm, 10 min, 4 °C). A 0.25-ml aliquot of the supernatant was mixed with 0.5 ml of bovine γ-globulin (1.25 mg/ml PBS, pH 7.4) and 0.5 ml of PEG-6000 (25% in water). The mixture was left for 20 min at 4 °C and then centrifuged in a Jouan KR 422 centrifuge at 3000 x g (3200 rpm, 25 min, 4 °C). The pellet was then counted for radioactivity using an LKB 1282 γ-counter.

Gel Permeation Chromatography—A 0.5-ml aliquot of solubilized endosomes containing $^{125}$I-[A14]insulin prepared as above was applied to a Sephadex G-50 column (40 x 1 cm) equilibrated with 50 mM HEPES buffer, pH 8.0, containing 17 mM NaCl, 2.5 mM NEM, 1.0 mM l,10-phenanthroline, 1 mM chloroquine, 0.5% BSA. Elution was achieved with the same buffer, and 1-ml fractions were counted for radioactivity using LKB 1282 γ-counter.

Trichloroacetic Acid Precipitation of Intact Insulin—Aliquots (250 μl) derived from endosomes containing $^{125}$I-[A14]insulin were added to 500 μl of ice-cold 20% trichloroacetic acid and incubated on ice for 10 min prior to centrifugation in an Eppendorf centrifuge at 13,000 x g. 

### Table I

| Treatment      | Trichloroacetic acid precipitability (%) | PEG precipitability (%) | Sepharose G-50 chromatography (%) |
|---------------|----------------------------------------|-------------------------|----------------------------------|
| (-) Inhibitors | 86.3 ± 5.5                             | 19.2 ± 2.7              | 18.5                             |
| (-) Chloroquine|                                        |                         |                                  |
| (+) Inhibitors | 97.5 ± 0.4                             | 27.7 ± 1.6              | 29.7                             |
| (+) Chloroquine|                                        |                         |                                  |

FIG. 2. Effect of chloroquine on endosomal insulin degradation and receptor-binding in vitro. Anesthetized rats were injected with approximately 1 MBq of $^{125}$I-[A14]insulin via the hepatic portal vein over 30 s. The liver was removed after another 2 min and endosomes prepared as described above with controls receiving PBS. The liver was removed after another 2 min and endosomes prepared as described under “Experimental Procedures.” Endosomes were incubated with the indicated concentration of chloroquine for 1 h and then either subjected to trichloroacetic acid precipitation (A) or extracted into 0.1% Triton X-100 and PEG-precipitated (B). Trichloroacetic acid precipitability indicates intact insulin, while PEG precipitability indicates receptor-bound insulin. Values represent the mean and S.E. from three to five separate animals. *, p < 0.05; **, p < 0.01.

FIG. 3. Determination of the amount of receptor-bound insulin present in freshly isolated endosomes following chloroquine treatment in vivo. Anesthetized rats were injected with approximately 1 MBq of $^{125}$I-[A14]insulin via the hepatic portal vein over 30 s, having first received an intraperitoneal injection of either PBS or 20 μmol/100 g body weight chloroquine 2 and 1 h prior to insulin administration. The liver was removed after another 2 min and endosomes prepared as described under “Experimental Procedures.” Endosomes were disrupted and extracted into 0.1% Triton X-100 and applied to a G-50 Sepharose column in the presence of 2.5 mM NEM, 1 mM l,10-phenanthroline, and 1 mM chloroquine. Fractions (1 ml) were collected and counted for radioactivity. The figure is representative of two experiments from two separate animals.
RESULTS

Determination of the Amount of Receptor-bound Insulin in Freshly Isolated Hepatic Endosomes and the Effect of Insulin Dissociation on Subsequent Endosomal Degradation—It has been shown previously that hepatic insulin degradation in vivo occurs within endosomes (12), with dissociation of insulin from the receptor being a prerequisite for this process to occur (14, 17). To determine the percentage of insulin in endosomes that is receptor-bound, rats were administered $^{125}$I-[A14]insulin via the hepatic portal vein and hepatic endosomes freshly isolated. Fig. 1 shows the elution profile from a G-50 gel permeation column of material generated from solubilized hepatic endosomes. The first peak represents receptor-bound insulin, which is size-excluded from the column and elutes in the void volume. This comprised 18.5% of the total. The amount of receptor-bound insulin was confirmed by an independent method of assessment, namely PEG precipitation of the insulin-receptor complex (19.2%; Table I). The remaining 80% of the insulin eluted in the second peak from the G-50 column and comprised dissociated insulin, of which 86.3% remains intact as assessed by trichloroacetic acid precipitation (Table I). After insulin injection, 2 min of in vivo processing time, and endosome preparation time, 80% of endocytosed insulin has already dissociated from its receptor and is accessible for intra-endosomal degradation.

A previous study (28) had shown chloroquine capable of enhancing the association of insulin to its receptor. The effect of chloroquine in vitro was examined on insulin-receptor dissociation and degradation. Hepatic endosomes, containing $^{125}$I-[A14]insulin internalized over a 2-min time period, were incubated for 1 h at 25 °C with concentrations of chloroquine ranging from 0.5 to 5 mM. In the absence of chloroquine, a 1-h incubation resulted in 18.5% of the insulin remaining receptor-associated (Fig. 2B) and 56.5% degraded (Fig. 2A). Inclusion of chloroquine in the incubation buffer augmented the amount of receptor-associated insulin to a maximum of 28% (p < 0.05) in the presence of 5 mM chloroquine with only 16.3% now being in a degraded state (p < 0.01). Thus, this in vitro experiment confirmed the need for insulin to dissociate from its receptor for subsequent degradation, and the inhibitory role played by chloroquine in this process.
insulin administration and sacrificed between 0 and 45 min later. Following preparation of hepatic endosomes the amount of insulin receptor, its phosphotyrosine content and tyrosine kinase activity were established using microtiter plate capture assays.

The flux of insulin receptors through the endosomal compartment following insulin administration in vivo, as determined by immunocapture, is shown in Fig. 5. The data are normalized so that the basal value of the control group is set to 100%. In the absence of chloroquine, the control group showed a rapid doubling of endosomal insulin receptor content by 5 min and return to basal values by 18 min. Following chloroquine treatment, the integrated response for endosomal receptor content was increased throughout the time course by 31% (p < 0.05; **, p < 0.01). Even at 45 min after insulin administration, the endosomal insulin receptor content had not returned to control basal levels, indicative of chloroquine-sensitive inhibition of receptor recycling. Receptor phosphotyrosine content (Fig. 6) demonstrated a similar time course, rapidly increasing by 5 min after insulin injection and remaining elevated at 10 min until return to basal level at 18 min. Chloroquine treatment augmented the phosphotyrosine content, leading to a 64% increase in the integrated response-time curve (p < 0.01). Finally, the most striking effect of chloroquine was observed with the insulin receptor tyrosine kinase activity toward an exogenous substrate (Fig. 7). Tyrosine kinase activity reached a rapid peak of 300% over basal values at 5 min after insulin injection, remained elevated at 10 min, and returned to base line by 18 min. Chloroquine treatment substantially augmented the tyrosine kinase activity throughout the time course, giving a 96% increase in the integrated response (p < 0.001). Thus, chloroquine potentiated the normal insulin-in-

**Fig. 6.** Effect of chloroquine administration on endosomal insulin receptor phosphotyrosine content following administration of insulin in vivo. Animals were treated and endosomes prepared as in Fig. 5. Endosomes were then solubilized and insulin receptors immunocaptured onto microtiter plates, precoated with an antibody to phosphotyrosine (4G10), and quantified by 125I-[A14]insulin binding analysis as described under “Experimental Procedures.” Each point represents the mean and S.E. from five to six separate animals. *, p < 0.05. The inset shows the integrated response over the time course and the effect of chloroquine was tested by ANOVA. ***, p < 0.001.

**Fig. 7.** Effect of chloroquine administration on endosomal insulin receptor tyrosine kinase activity following administration of insulin in vivo. Animals were treated and endosomes prepared as in Fig. 5. Endosomes were then solubilized and insulin receptors immuno-captured onto microtiter plates, precoated with an antibody to the insulin receptor (CT-3). Insulin receptor tyrosine kinase activity was assayed as described under “Experimental Procedures.” Each point represents the mean and S.E. from five to six separate animals. *, p < 0.05; **, p < 0.01; ***, p < 0.001. The inset shows the integrated responses over the time course, and the effect of chloroquine treatment was tested by ANOVA. ***, p < 0.001.
Chloroquine Increases Endosomal Insulin Receptor Signaling

Anesthetized rats were injected with 1.5 μg/100 g body weight insulin via the jugular vein, having already received an intraperitoneal injection of chloroquine or PBS 2 and 1 h prior to insulin injection. Livers were removed 30 s later and plasma membranes prepared. Tyrosine kinase activity and tyrosine phosphorylation of the insulin receptor were determined as described under “Experimental Procedures.” Values represent the mean and S.E. from three separate animals.

| Treatment       | Tyrosine phosphorylation | Tyrosine kinase activity |
|-----------------|--------------------------|--------------------------|
|                 | Receptor content         | -Fold increase over basal| Receptor content         | -Fold increase over basal|
| PBS + insulin   | 0.61 ± 0.08              | 36.39 ± 6.06             | 60.91 ± 10.66            |
| Chloroquine + insulin | 0.67 ± 0.01        | 41.43 ± 4.49             | 54.64 ± 0.92             |

TABLE III
Effect of chloroquine on insulin-stimulated endosomal insulin receptor tyrosine phosphorylation and kinase activation expressed per unit receptor in vivo

Animals were treated and endosomes prepared as in Fig. 5. Endosomes were then solubilized and insulin receptors immunoprecipitated onto microtiter plates. Quantitation of insulin receptor and its phosphotyrosine content was achieved as described in the legend to Figs. 5 and 6. Quantitation of insulin receptor tyrosine kinase activity was assayed as described in the legend to Fig. 7. Ratios of phosphotyrosine content and tyrosine kinase activity per insulin receptor content were taken and normalized to the basal control which was set at 100%. Each point represents the mean and S.E. from five to six separate animals. The integrated responses in the absence and presence of chloroquine were compared by ANOVA. *, p < 0.05.

| Activity/unit receptor | Time (min) | Treatment       | PBS + insulin | Chloroquine + insulin |
|------------------------|------------|-----------------|---------------|-----------------------|
| Tyrosine phosphorylation| 0          | 100 ± 0         | 89 ± 17       |
|                        | 5          | 156 ± 34        | 194 ± 39      |
|                        | 10         | 111 ± 38        | 147 ± 55      |
|                        | 15         | 106 ± 30        | 110 ± 36      |
|                        | 45         | 20 ± 10         | 64 ± 15       |
| Tyrosine kinase*       | 0          | 100 ± 0         | 131 ± 22      |
|                        | 5          | 140 ± 50        | 266 ± 63      |
|                        | 10         | 134 ± 23        | 182 ± 42      |
|                        | 18         | 127 ± 32        | 205 ± 69      |
|                        | 45         | 106 ± 31        | 111 ± 27      |

Discussion

Previous studies have shown that subsequent to insulin administration in vivo, hepatic insulin receptors bind insulin at the plasma membrane, followed by very rapid internalization of receptor (3–5) and bound insulin (43, 44) into endosomes where the number of receptors increases approximately 5-fold (5, 6). It has been demonstrated that the endosomal insulin receptor tyrosine kinase remains tyrosine-phosphorylated and is even more active than its receptor located within the plasma membrane (4–6). The selective activation of the endosomal insulin receptor kinase, by the phosphotyrosine phosphatase inhibitor bisperoxovanadium phenanthroline, in the absence of insulin, has been shown to lead to phosphorylation of IRS-1 and to elicit a hypoglycemic response in vivo (10, 11). Comparable studies of the hepatic epidermal growth factor and its receptor have revealed a similar phenomenon (45–47) where endosomes containing activated ligand-bound receptors constitute the major locus for ScH tyrosine phosphorylation (48, 49). Together, these findings have prompted the suggestion that endosomes play a pivotal role in signal transduction, giving the receptor tyrosine kinase access to substrates distal to and topographically distinct from the plasma membrane (11). In this study, we have demonstrated that in vivo administration of chloroquine, a compound that inhibits both dissociation of insulin from its receptor (28) and subsequent endosomal degradation of insulin (12), leads to the prolongation of the activated insulin-receptor complex and augments insulin-stimulated responses. A key observation is that the in vivo locus for both the inhibition of insulin dissociation and degradation by chloroquine is restricted almost entirely to the endosomal compartment (Figs. 1–4). These findings are in agreement with previous in vitro studies, where it was observed that chloroquine augmented the binding of insulin to its receptor in purified rat liver plasma membrane preparations (28) and inhibited endosomal insulin degradation in isolated vesicles (12).

In the present study, chloroquine was without apparent effect on insulin receptors present in the plasma membrane (Table II). However, differences in conditions between the studies may explain the apparent discrepancy. The concentration of chloroquine required to demonstrate the phenomenon was in the order of 1 mM, whereas in this study the blood concentration of chloroquine was in the low micromolar range (data not shown) and thus could not elicit an effect on the receptors in the plasma membrane. However, since chloroquine is an acidotrophic agent and accumulates in acidic environ-
ments such as insulin-containing endosomes (18) and lysosomes (50), high millimolar concentrations are rapidly achieved within these organelles (23) allowing the effect to occur within endosomes. In addition, since insulin’s interaction with the plasma membrane receptor is very transient (<30 s) before entry into endosomes, its window of opportunity for an effect is very limited. Thus, the effect of chloroquine on insulin dissociation and degradation in vivo is restricted to endosomes.

The findings that in vivo chloroquine treatment inhibited insulin dissociation from its receptor and reduced insulin degradation led us to investigate whether in vivo chloroquine treatment could augment the insulin signaling cascade from this compartment as hypothesized previously (28). The flux of insulin receptors through the endosomal compartment of the liver following insulin administration was significantly increased in amount and duration in the presence of chloroquine (Fig. 5). This increased accumulation suggested either that chloroquine was inhibiting recycling of receptors back to the plasma membrane following their insulin-induced endocytosis or that insulin receptors were endocytosing more rapidly in the presence of chloroquine. The retention of receptors within the endosome might result from sustained receptor tyrosine phosphorylation. Dephosphorylation has been suggested as a possible signal required before return of receptors to the plasma membrane can be effected (51, 52), and endosomal insulin receptors have been shown to be specifically dephosphorylated by phosphotyrosine phosphatase(s) prior to their return to the plasma membrane (6, 19). Such a mechanism would require sustained endosomal insulin receptor tyrosine phosphorylation following chloroquine treatment. This was demonstrated by the data in Fig. 6, where insulin-induced receptor tyrosine phosphorylation was shown to be augmented by chloroquine throughout the time course. In a similar manner, the endosomal insulin receptor exogenous tyrosine kinase activity was also increased (Fig. 7). Therefore, the chloroquine-augmented insulin-receptor complex formation within endosomes not only prolonged the residency of the insulin receptor within the endosome.
dosomal compartment but enhanced its activation in both duration and amount. Enhanced interaction of insulin with its receptor leading to sustained receptor tyrosine phosphorylation and signaling has also been observed using analogues of insulin whose binding characteristics are greatly augmented (53).

Consequential to the sustained endosomal activation of the insulin receptor, assessment of chloroquine’s effect on an insulin-dependent event, in vivo incorporation of glucose into glycogen in rat diaphragm (40, 42) resulted in an augmentation of glycogen synthesis (Fig. 8). This response was determined in diaphragm, since it is not easily shown in rat liver due to the glucose paradox (54). Additionally, in another study, chloroquine resulted in a pronounced improvement in glycemic control following an oral glucose challenge in rats. These observations correlated well with effects in human non-insulin-dependent diabetes mellitus patients treated with chloroquine. Here, improvement in glucose tolerance (29), increased peripheral glucose disposal, and decreased metabolic clearance rates of insulin (30) were observed. In insulin-dependent diabetics mellitus patients, chloroquine has been shown to reduce insulin resistance (31). These augmentations of insulin-induced metabolic effects all appear consequential to a prolongation of the effective half-life of insulin in endosomes as described in this study. Indeed, in an investigation of rat adipocytes where the endosomal insulin receptor tyrosine kinase is known to be active (7, 8), IRS-1, the distribution of which was 20% endosomal and 80% cytosolic, demonstrated that its tyrosine phosphorylation paralleled that of the receptor tyrosine kinase in the endosome (8). Consequently, a strong case for a role of the activated endosomal insulin receptor has been made in the recruitment of the insulin-sensitive GLUT 4-containing vesicles from their intracellular depot to the plasma membrane following insulin stimulation in adipocytes (8, 55).

In summary, these observations are consistent with a chloroquine-dependent inhibition of endosomal insulin-receptor dissociation and subsequent degradation, sustaining the half-life of the active endosomal receptor and potentiating insulin signaling from this compartment as depicted in Fig. 9. Additionally, it demonstrates the usefulness of chloroquine as research tool to help elucidate the role endosomes in signal transduction.

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