Innovative Methodology

**Insulin uptake across the luminal membrane of the rat proximal tubule in vivo and in vitro**

*Pavel Kolman*\(^1,6\), *Angelo Pica*\(^2,4\), *Nicolas Carvou*\(^5\), Alan Boyde\(^3,6\), Shamshad Cockcroft\(^5\), Andrew Loesch\(^6\), Arnold Pizzey\(^7\), Mariadelina Simeoni\(^2,4\), Giovambattista Capasso\(^2\) and Robert J. Unwin\(^4,5\)

\(^1\)Institute of Physical Engineering, Brno University of Technology, Brno, Czech Republic; \(^2\)Department of Internal Medicine, Second University of Naples, Naples, Italy; \(^3\)Barts and The London School of Medicine and Dentistry; \(^4\)Centre for Nephrology; \(^5\)Department of Physiology; \(^6\)Department of Anatomy and Developmental Biology; \(^7\)Department of Haematology, University College London, London, UK

**Running Title**: Proximal tubule insulin uptake in vivo and in vitro

**Keywords**: intravital confocal microscopy, fluorescence, kidney, proximal tubule, insulin

*Joint first authors

Correspondence to:
Robert Unwin
Centre for Nephrology (London Epithelial Group)
University College London
Royal Free Campus
Rowland Hill Street
London NW3 2PK
UK

Copyright © 2009 by the American Physiological Society.
Innovative Methodology

Abstract

We visualized insulin uptake \textit{in vivo} across the apical membrane of the rat proximal tubule (PT) by confocal microscopy; we compared it with \textit{in vitro} findings in a rat PT cell line (WKPT) using fluorescence microscopy and flow cytometry. Surface tubules were observed \textit{in vivo} with a 633 nm single laser-illuminated real-time video-rate confocal scanning microscope in upright configuration for optical sectioning below the renal capsule. Fields were selected containing proximal and distal tubules; Cy5-labelled insulin was injected twice (the second time after \textasciitilde 140 min) into the right jugular vein and the fluorescence signal (at 650-670 nm) recorded. Fluorescence was detected almost immediately at the brush border (BBM) of PT cells only, moving inside cells within 30-40 min. As a measure of insulin uptake, the ratio of the fluorescence signal after the second injection to the first doubled (ratio 2.11±0.26, \(n=10\); mean±SEM), indicating a ‘priming’, or stimulating, effect of insulin on its uptake mechanism at the BBM. This effect did not occur after pre-treatment with intravenous lysine (ratio 1.03±0.07, \(n=6\); \(P<0.01\)). Cy2 or Cy3 labeled insulin uptake in a PT cell line \textit{in vitro} was monitored by 488 nm excitation fluorescence microscopy using an inverted microscope. Insulin localized toward the apical membrane of these cells. Semi-quantitative analysis of insulin uptake by flow cytometry also demonstrated a priming effect (up-regulation) on insulin internalization in the presence of increasing amounts of insulin, as was observed \textit{in vivo}; moreover, this effect was not seen with, or affected by, the similarly endocytosed ligand \(\beta\)2-glycoprotein.
Innovative Methodology

**Introduction**

Renal handling of insulin has been described previously using radio-labeled insulin combined with histochemical studies after timed injections *in vivo* (2), or in isolated proximal tubule (PT) perfusion studies *in vivo* (10) and *in vitro* (21, 20, 17), and in cultured cells (24). Insulin uptake in rabbit PT has also been monitored by electron microscopy after perfusion of gold-labeled insulin and subsequent cross-linking to its receptor with disuccinimidylsuberate (DSS) (18). These studies have shown that insulin binding and uptake occurs predominantly at the brush border membrane (BBM) of proximal tubule (PT) cells (2, 22), where it is then translocated to endocytic vesicles and vacuoles, and eventually degraded in lysosomes; a small portion (~0.5%) is directly transcytosed to the basolateral membrane (19). However, unlike many other filtered peptides (34), insulin seems not to be degraded by brush border enzymes (11). The receptors for insulin binding are recycled to the plasma membrane via dense apical tubules, either from small vesicles or from larger endocytic vacuoles, although the early mechanisms involved in insulin binding and internalization in the PT are still unclear.

Higher levels of insulin receptors are expressed on the basolateral membrane of PT cells than on the apical membrane (9, 29), and it is the basolateral receptors - present at other nephron sites (4) - that are believed to mediate insulin’s biological effects (32). Thus, it was originally proposed that non-specific charge interactions were responsible for the initial binding of insulin to the BBM (28). However, later studies reported a high capacity, low affinity receptor for insulin in the PT (15), which was eventually thought to be the endocytic receptor megalin. Orlando et al (23), using chemical cross-linking of $^{125}$I-insulin to a renal microvillar membrane preparation, identified megalin as the binding protein, which required a 1000-fold excess of unlabeled insulin to compete for binding; it was also competed with by glucagon. In contrast, RAP (Receptor-Associated Protein), which inhibits binding of other
Innovative Methodology
endocytosed ligands to megalin, such as retinol binding protein (RBP) (13), transthyretin (27) and β2-glycoprotein (β2gpI) (16), did not inhibit binding. In the same study (23), uptake of FITC-labeled insulin in L2 yolk sac-derived cells (which are rich in megalin) was inhibited by a 50-fold excess of unlabeled insulin and by anti-megalin IgG, but again not by RAP.

In the present study, we describe a method we have developed for quantitative assessment of the disposition of fluorescently labeled substances filtered at the kidney glomerulus using conventional confocal, rather than multiphoton (8), microscopy in vivo. We chose to investigate insulin, because of the available literature for comparison, and as a prototypical filtered peptide hormone. We found that insulin’s visible uptake in vivo, and in real-time, is confined to the PT, that it occurs apically; and we made the novel observation that insulin uptake is self-priming and up-regulated. This finding was also confirmed in vitro in cultured PT cells and contrasted with the similarly endocytosed ligand β2gpI.
Innovative Methodology

**Methods**

**Insulin labeling**

Insulin was purchased from Sigma and β2gpI was provided by SCIPAC (Kent, UK). For the *in vitro* cell culture experiments, insulin was prepared and labeled with Cy2 or Cy3, as previously described (6). For the *in vivo* study, Cy5 was chosen as a suitable fluorophor, because we were using a 633 nm HeNe laser to curtail mitochondrial autofluorescence (3) (Cy5 is a far red fluorescent dye), and to improve reflection mode imaging. Insulin was labeled with Cy5 according to the manufacturer’s instructions, as follows: 1 mg insulin was mixed with 75 μg Cy5 and the labeled insulin separated by size exclusion using a Sephadex G-15 column and eluted with PBS. Labeling efficiency was estimated to be ~30%.

**Confocal microscopy in vivo**

Male Sprague-Dawley rats (n = 4) weighing ~200 g were maintained on a standard laboratory diet and handled according to Institutional and UK Home Office license regulations. They were anesthetized with sodium thiopentone (100 mg/kg ip; Link Pharmaceuticals, Horsham, Sussex, UK) and prepared surgically as for micropuncture of the left kidney (5). Rats were infused intravenously via a right jugular venous catheter with a 0.154 M NaCl solution (‘normal saline’) at a rate of 2 ml/h. During each experiment, the rat was placed beneath the objective of a confocal microscope (Noran Odyssey video-rate laser scanning confocal unit with a 633 nm laser on a Nikon Optiphot upright microscope) on a specially adapted and thermo-regulated stage. We used a 40/1.0 oil immersion objective lens with glass coverslips sandwiched to a thickness of 425 μm to fill the space between the kidney surface and the objective lens; this also stabilized the kidney surface and minimized transmitted movement.
Innovative Methodology

from respiration and arterial pulsation. The confocal slit for the fluorescence signal was set at 100 µm.

In those experiments in which the rats were pre-infused with lysine, a 10% solution in 0.154 M NaCl was started ~30 min before the first insulin injection and continued throughout the experiment at dose of 200 mg/kg body weight, according to the protocol of Tucker et al. (33).

*Image recording and analysis*

Insulin uptake was rapid and suitable for imaging renal tubules at video-rate. The Noran Odyssey video-rate laser scanning confocal unit has standard video outputs, one for reflection mode, and another for fluorescence, and one for the mixed signal (not used in this study). We were not able to record both channels simultaneously. The signal was recorded using a Sony digital camcorder. We used a micro-switch controlled by the left knee of the operator to select the reflection or fluorescence imaging modes. The time delay between the captured reflection and fluorescence images was 1 or 2/25 sec. The image proved stable enough to treat the two frames as coming from the same place.

Cy5-insulin was injected into the jugular vein catheter (0.1 mg/kg body weight) in a volume of 0.1 ml (although we also did some experiments using a femoral artery cannula in the aorta and placed just above the left renal artery). Two consecutive injections were given at an interval of ~140 min. The reflection and fluorescence images (Figures 1a and 1c, respectively) were ‘grabbed’ from the short video sequences using Pinnacle Studio software (Pinnacle Systems, Avid Technology Inc., California, USA). The average time interval between repeated recording sequences was ~10 min; the format of the grabbed image frames was 640 x 480 pixels, 8 bit/pixel. Recordings were made from several tubules (in different fields) during each experiment. Once we had captured the images, we used JASC Paint Shop Pro 5 (Corel Corporation, Ontario, Canada) - with its freehand smart edge selection tool - to
Innovative Methodology

select from the reflection image (Figure 1a) the tubule segment to be analyzed; filling in the cells, including their white reflective (negative black in Figures 1a-d) brush border, to create a binary mask. (The images shown in Figures 1a-d are cropped free of artifacts.)

For further analysis, we wrote a procedure in MATLAB software (The MathWorks Inc., Massachusetts, USA). The fluorescence intensity image (Figures 1c) was transformed to an insulin pseudo-concentration map using a calibration function determined from calibration measurements. The mask was then divided into equal width strips of ~0.85 µm (Figure 1b); it was applied to monitor the spatial distribution of insulin in the tubular cells of the selected PT segment by calculating the averaged concentration in each strip separately. The concentration values were corrected using another calibration function to compensate for the effect of photo-bleaching.

Figure 2 illustrates the output of the MATLAB program in a representative control and lysine experiment. Figures 2A[i] and 2B[i] show the temporo-spatial concentration of insulin inside proximal tubular cells. The origin of the spatial axis corresponds to the inner border of the tubule (the apical brush border). Figures 2A[ii-iii] and 2B[ii-iii] show the linear uptake and rate of uptake, respectively, after the first and second injections of labeled insulin; insulin values are in pseudo-concentration units (see calibration below).

Calibration of the fluorescence signal

We found that the intensity distribution in fluorescence mode across the field of view was not constant and that a calibration procedure had to be performed to correct for this, again using MATLAB. Calibration of insulin labeled-Cy5 concentration was performed in vitro with Cy5. We used a special bacteriological chamber with a depth of 10 µm (Weber Scientific, New Jersey, USA). A series of solutions with different Cy5-insulin concentrations was then measured. The image data taken as a short video sequence were averaged using MATLAB software, and the resulting image was smoothed in Paint Shop Pro 5 using a Gaussian blur.
Innovative Methodology

filter, radius 3. However, we found that the intensity of fluorescence for calibration measurements of high concentrations (~0.26 mg/ml) of Cy5-insulin varied by as much as 50%, probably because of significant evaporation from a small sample volume on a relatively large surface area. Therefore, it was not possible to calibrate and transform the intensity values recorded into real concentration values for insulin. The intensity values were in the range of 0 to 255, with a value of 16 for the blank (water). Thus, pseudo-concentrations were derived assuming a linear relationship between a given mean fluorescence intensity in the image ‘I’ and Cy5-insulin concentration \( c = \frac{1}{255}(I-16) \).

**Correction for bleaching**

Following each Cy5-insulin injection tubules were imaged approximately every 10 min and the duration of irradiation in every measurement was ~10 sec. This gave a total of ~5 min of irradiation in any one region, so that the effect of photo-bleaching could not be ignored. We determined experimentally how the fluorescence intensity dropped on bleaching the labeled insulin in renal tubular cells. The concentration values calculated from the fluorescence images were corrected using these data (see Figure 3).

**Insulin localization by electron microscopy (EM) in kidney tissue at ~1 min and ~45 min after insulin injection**

**Tissue preparation**

For ultrastructural study, kidneys were perfused through the renal artery at 1 min and 30-45 min after the insulin-injection with 5 ml of normal saline, followed by 10 ml of fixative, consisting of 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The kidneys were then dissected out and immersed in the same fixative for 5 h (at 4°C), followed by washing with phosphate buffer and 0.1 M TRIS-buffered saline (TBS, pH 7.6).
Innovative Methodology

Sixty-70 μm thick sections were cut through the kidney on a vibratome and collected in TBS. These were then processed with insulin antibody, using a pre-embedding immunogold-silver labeling technique for electron microscopy (see below).

**Immunogold-silver labeling**

To improve immunoreactivity, the vibratome sections were infiltrated for 45 min with cryoprotectant, consisting of 25% sucrose and 10% glycerol in phosphate buffer, before immersion in liquid nitrogen cooled isopentane, then in liquid nitrogen for ~10 sec, and then thawed in cryoprotectant, before being washed in TBS and placed for 1 h at room temperature in 10% non-immune normal goat serum (British BioCell Int., Cardiff, UK) diluted in TBS containing 0.1% sodium azide. Sections were then (i) exposed for 20 h at 4°C to a mouse monoclonal insulin antibody (see below for details) at a concentration 0.3 μg/ml of TBS with 0.1% sodium azide; (ii) washed 3 x 10 min in TBS; (iii) incubated for 20 h at 4°C with a goat anti-mouse IgG:1nm gold conjugate (GAM1, British BioCell Int) diluted 1:100 in TBS with sodium azide; (iv) washed in TBS and then in distilled water; (v) fixed for 10 min with 1% glutaraldehyde; (vi) washed 10 x 5 min with de-ionized distilled water, and (vii) subjected to augmented gold labeling for 10 min with a silver enhancement kit (British Biocell Int.). The specimens were then washed with distilled water, sodium cacodylate buffer (0.1 M), treated with 1% osmium tetroxide for 10 min, dehydrated in progressively higher concentrations of ethanol, followed by propylene oxide. The specimens were embedded flat in Araldite. Ultrathin sections were stained with uranyl acetate and lead citrate, and subsequently examined with a JEM-1010 transmission electron microscope (TEM).

**Immunocytochemical controls**

The insulin antibody (2D11-H5) used in this study was a mouse monoclonal IgG1 antibody corresponding to amino acids 1-84, representing full-length porcine insulin (Santa Cruz...
Innovative Methodology

Biotechnology, Inc., California, USA). According to the supplier, this antibody reacts with insulin of human, porcine and bovine origin by immunohistochemistry, immunoprecipitation and ELISA. In the present study, the specificity of immunolabeling was investigated routinely by omission of the insulin antibody and IgG steps, as well as by replacement of primary antibody with non-immune normal goat serum.

Cell culture

The WKPT 9302 Cl.2 cell line was a gift from Professor F. Thevenod, Germany and cultured as described previously (31). This epithelial cell line is derived from the early segment (S1) of the proximal tubule of normotensive Wistar-Kyoto rats and transduced with the oncogene SV40 large T-antigen (35); it forms confluent, electrically resistive polarized monolayers expressing apical microvilli, tight junctions, and convolutions of the basolateral plasma membrane. Cells were maintained in Dulbecco’s Modified Eagle’s medium (DMEM)/Ham’s nutrient Mixture F-12 (1:1) (Sigma) supplemented with 10% bovine calf serum, 1.2 mg/ml NaHCO₃, 5 μg/ml insulin, 5 μg/ml apo-transferrin, 4 μg/ml dexamethasone, 10 ng/ml EGF, 100 units/ml penicillin and streptomycin. Cells were grown in 75 cm² flasks at 37°C with 5.0 % CO₂ and split 1/10 or 1/20 three times a week.
Innovative Methodology

**In vitro endocytosis assay**

WKPT cells were plated onto 13 mm diameter glass coverslips placed in 16 mm diameter wells (24 well plate) and washed twice in warm HEPES buffer (20 mM Hepes/137 mM NaCl/3.0 mM KCl/2.0 mM MgCl₂/1.0 mM CaCl₂/1 mg/ml glucose, 37°C). The-Cy labeled ligand (1-5 μM) was added to the cells (250 μL/well) and incubated for 2 h at 37°C. The cells were incubated in 0.2 M acetic acid, 0.5 M NaCl for 5 min at room temperature to remove any residual extracellular fluorescence, washed x 4 with PBS and either fixed in 4.0% formaldehyde for 10 min at room temperature for analysis by confocal microscopy, or detached in trypsin-EDTA for analysis by flow cytometry. In competition experiments, the cells were pre-incubated with unlabeled insulin or β2gpI for 10 min at 37°C, before adding the Cy2-labeled ligand. In priming experiments, the cells were incubated at 37°C for 60 min with unlabeled insulin or unlabeled β2gpI and washed x 4 with HEPES buffer prior to incubation with labeled ligand.

**Confocal microscopy in vitro**

After washing, the fixed cells x 3 in PBS, the coverslips were mounted onto a 10 μL drop of Mowiol overnight at 4°C. Cell fluorescence was recorded by excitation at 488 nm with an Omnichrome series 43 (PerkinElmer, Milan, Italy) argon ion laser system using an Olympus IX70 microscope fitted with a x100 oil immersion objective, unless stated otherwise. Images were acquired with a charged coupled device camera (PerkinElmer) cooled to -35°C, and controlled with the Ultraview 4.0 software (PerkinElmer).
Innovative Methodology

**Analysis by Flow Cytometry**

After 2 h incubation with fluorescent ligand, the WKPT cells were washed x 4 with ice cold HEPES buffer supplemented with 1mM EDTA. Cells were then incubated on ice with HEPES buffer + 10 mM EDTA for 10 min, and detached with Trypsin-EDTA at 37°C for 5-15 min. The trypsin was then quenched by addition of WKPT culture medium and the cells were pelleted by centrifugation at 1,300 RPM for 5 min. The cell pellets were re-suspended in ice-cold HEPES buffer + 1 mM EDTA, and kept on ice until ready for analysis by flow cytometry. At least 10,000 cells per data point were analyzed for fluorescence on an EPICS Elite Flow cytometer (Beckman-Coulter, High Wycombe, UK). A gating strategy was employed that excluded cell aggregates and high-scatter cell events judged to be non-viable cells. The mean cell fluorescence was recorded for each data point. The data were then processed with Summit v3.1 software (Cytomation Inc., Colorado, USA). Histogram charts were produced for each sample and their mean fluorescence resolved.

**Statistics**

All statistical comparisons were made using non-parametric Kruskal-Wallis 1-way ANOVA and/or unpaired Mann-Whitney tests; P<0.05 was considered significant.
Innovative Methodology

Results

*In vivo confocal microscopy*

*Descriptive analysis*

As reported by us before, using the Noran Odyssey confocal microscope, the reflection mode image showed details of capillary blood flow, identifiable and distinguishable proximal and distal tubules: proximal tubules were recognized by their intensely bright (light scattering) brush border, especially early proximal tubule segments (3) (Figure 1a - see supplementary video); erythrocytes were strongly reflective, and white cells recognizable by their slower motion, adhering to capillary walls and some even marginating (3; 26). After the injection of free Cy5, fluorescence was evident in the lumen of distal tubules within 1 min, but no uptake of fluorescence was seen in either the proximal or distal tubules (not shown).

After injection of Cy5-insulin, fluorescence appeared in peritubular capillaries within 0.5 min; soon after fluorescence was seen at the proximal tubule brush border, although fluorescence intensity and distribution were variable (see supplementary video). By ~20-30 min there was an increase in intracellular fluorescence with a punctate pattern distributed toward the basolateral membrane (Figures 1c and 1d - see supplementary video). No fluorescence was detectable in the distal tubules (Figures 1c and 1d - see supplementary video). In each recording in fluorescence mode, before injection of Cy5-insulin, there was no tubular autofluorescence; after Cy-5 insulin there were no changes in the reflection mode images.

*Quantitative analysis*

Quantitative analysis of the intracellular fluorescence signal after Cy5-insulin injection (Figure 2) showed an increase in intracellular fluorescence intensity up to ~20 min, when it almost reached a steady state. However, after the second injection of insulin, uptake of
Innovative Methodology

insulin seemed to be enhanced in magnitude (Figure 2A[ii]) and rate (Figure 2A[iii]). The mean ratio for the change in concentration, as a measure of insulin uptake, after the second injection (second/first) was 2.11±0.26 (mean±SEM). After pre-treatment with lysine, this enhancement, or priming effect, was abolished with a ratio of 1.03±0.07 (Figures 2B[ii] and 2B[iii]). These ratio were significantly different when compared using the nonparametric Mann-Whitney test (two-tailed exact test, P<0.01) with median values of 2.03 versus 0.98, respectively.

Electron microscopy (EM) of insulin immuno-labeling

EM examination of rat kidney tissue after injecting insulin showed localization of insulin at +1 and +45 min. Insulin immuno-labeling was evident in proximal tubule cells: 1 min after insulin injection, label was detected predominantly along microvilli of the brush border of proximal tubular cells (Figure 4A); after 45 min, there was mainly intracellular localization (Figure 4B). Label was present throughout the cytoplasm, and also in endocytic vesicles and or multivesicular bodies (Figure 4B). No immuno-labeling was detected in control sections (Figure 4C), when no insulin was injected or insulin was injected without incubation with anti-insulin antibody, or when the primary antibody step was omitted in sections from insulin injected animals.

In vitro cell line experiments

We have shown previously that WKPT cells endocytose insulin and β2gpl, and that uptake is temperature-dependent and that these two proteins do not co-localize (6). To confirm that insulin and β2gpl do not compete for uptake, Cy2-β2gpl was incubated in the presence of excess unlabeled insulin or β2gpl (Figure 5A [a-c]). A 10 molar excess of β2gpl blocked Cy2-β2gpl endocytosis (Figure 5A [b]), while the same concentration of insulin did not affect Cy2-β2gpl uptake (Figure 5A [c]). In the reciprocal experiment, 10 molar excess of β2gpl
Innovative Methodology

did not block Cy2-insulin uptake (Figure 5B [d vs. e]). A 10 molar excess of insulin did not block uptake of Cy2-insulin, but appeared to enhance it (Figure 5B [d vs. f]). These results indicate that insulin and β2gpI are endocytosed independently and bind to different sites on the surface of WKPT cells. In separate experiments, we had examined whether their endocytic compartments could be differentiated based on staining with EEA1, a marker for early endosomes. We found that EEA1 partially co-localizes with Cy2-β2gpI, but not with Cy2-insulin (6).

Analysis by flow cytometry was used to quantify the uptake of 5 μM Cy2-insulin in the presence of increasing amounts of unlabeled insulin (Figure 6). The mean fluorescence of the sorted cells increased in a linear manner and nearly doubled from 23.8 AU ± 1.19 SEM when no unlabeled insulin was added, to 51.0 AU ± 2.44 SEM with 100 μM unlabeled insulin, before reaching a plateau with 200 μM unlabeled insulin (50.5 AU ± 0.93 SEM). The opposite trend was observed with β2gpI (Figure 7): in cells exposed to 1 μM Cy2-β2gpI for 2 h the mean fluorescence decreased from 13.3 AU ± 0.03 SEM to the control value (4.6 AU ± 0.47 SEM) in the presence of 10 μM β2gpI.

L-lysine did not seem to affect insulin uptake in vivo (although it did prevent the priming effect), so we tested its effect in vitro (Figure 8). Again, addition of 40 μM unlabelled insulin significantly enhanced Cy2-insulin uptake compared to the positive control; however, neither 40 μM β2gpI (as expected) nor 40 μM L-Lysine affected insulin uptake (although we were unable to test the priming effect in the same way did in vivo).

In another set of experiments WKPT cells were primed with increasing amounts of insulin for 1 h, washed and then incubated with 5 μM Cy2-insulin. After 2 h incubation, insulin uptake was measured in the insulin-primed cells: Cy2-insulin uptake was enhanced (Figure 9). Together, these results suggest that endocytosis of insulin is regulated by a
Innovative Methodology

positive feedback mechanism, possibly involving the up-regulation of its endocytic receptor. This is in contrast to β2gpI uptake, where excess β2gpI competes effectively with labeled β2gpI, indicating that the uptake system is saturable.

In L2 cells, which express high levels of megalin, β2gpI binds to megalin and RAP is known to inhibit its uptake (16). Insulin internalization is also mediated by megalin, but RAP has no effect on insulin uptake in L2 cells (13). To study the effect of RAP on insulin and β2gpI endocytosis in WKPT cells, the uptake of Cy2-insulin or Cy2-β2gpI was monitored in the presence of various concentrations of RAP (Figure 10). RAP integrity was tested by SDS-PAGE (Figure 10A) and because BSA was present in the RAP buffer, a BSA control was used in the uptake assay (Figure 10B and 10C, column 3). RAP did not inhibit insulin uptake (Figure 10B) or β2gpI internalization, which is at odds with findings in L2 cells, and suggests that in WKPT cells β2gpI internalization is not mediated by megalin.
Innovative Methodology

Discussion

In vivo experiments

An important aim of our study was to devise a method for quantifying labeled insulin uptake in renal tubules in vivo using conventional single laser confocal microscopy, and to see if we could inhibit it and/or detect any change in uptake. Video rate, long wavelength confocal microscopy is a powerful technique for this purpose, because of its optical sectioning property at increased depth within the intact functioning kidney in both reflection and fluorescence modes. Renal tubule morphology is readily discriminated in reflection mode and insulin labeled with a fluorescent dye is observable in fluorescence mode. When capturing a set of images and analyzing them by computer, we can get a spatio-temporal distribution of insulin molecules in renal tubular cells.

In previous work exploiting the advantages of real-time video rate confocal scanning light microscopy, we have used a Noran Odyssey scanner coupled to an upright Nikon microscope to study sub-capsular layers in the rat kidney (3, 26). The preparation of the animal for kidney imaging is as for renal micropuncture experiments. In the original configuration (3) using an argon ion laser and the dominant 488 nm line, we identified proximal and distal tubule segments by their morphology, and we imaged capillary flow in reflection mode and mitochondrial autofluorescence in the yellow-orange spectral range. A number of fluorescent substances were used to visualize capillary plasma, highlighting red cells in negative contrast, pre-labeled white cells, and passage into and transit through the tubular lumen (3). The strong mitochondrial autofluorescence in the yellow-orange spectral range limited the utility of the fluorescence approach with dyes excited by the 488 nm laser line (3). Therefore, we modified the instrument by fitting a 633 nm HeNe laser with the expectation that the reduction in light scattering at the longer wavelength would give a useful improvement in reflection mode imaging, which we used to study diuretic-induced changes in renal tubular
Innovative Methodology

diameter (26). A similar in vivo approach has also been described, though using dual-photon microscopy (8), which has some advantages (particularly the ability to use more than one fluorophor), although it is more costly and less widely available. This technique has also been largely descriptive (25), and usually without parallel or complementary in vitro studies, although attempts have been made to make more quantitative analyses of glomerular filtration and permeability (12, 36).

In the present study, we have shown that we can visualize native Cy5 and Cy5-labeled substances introduced into the circulation, and monitor their immediate binding, rapid uptake and passage through the renal tubule in real-time in vivo. Indeed, this pattern in the proximal tubule was confirmed on immuno-EM of kidney sections taken at early and late time points after insulin injection. However, we were faced with the challenge of quantifying the fluorescence signal to measure uptake of fluorescent label. Difficulties arose because of: (a) the non-uniformity of fluorescence excitation within the field of view, which we solved by calibrating every pixel address separately; and (b) a longer-term bleaching effect for bound Cy5 label, solved by knowing the irradiation time for each field and an empirically determined bleaching rate, and by applying an appropriate correction. Based on our solutions, we have been able to show that the method can be used for relative quantification of physiological measurement of fluorescent label uptake in vivo.

Previous studies have shown that L-lysine can inhibit tubular reabsorption of filtered peptides (14), including albumin (33); lysine has also been shown recently to alter endocytic receptor trafficking (30). We tested lysine’s effect on insulin uptake by infusing L-lysine intravenously for 90 min, followed immediately by a first injection of labeled insulin; the second injection followed after 140 min. In control experiments we found that the first injection of insulin stimulated its uptake following a second injection - a priming effect - and that after lysine pre-treatment this effect was blunted.
Innovative Methodology

In one experiment we did try to combine proximal tubule micropuncture with confocal microscopy to visualize the uptake of insulin injected directly into the tubule lumen. While this approach allowed more accurate dosage, as well as localized delivery of insulin (or any other fluorescently labeled substance), it proved technically demanding, because of the short working distance of the high aperture objective lenses needed for confocal microscopy, which made insertion of the micropipette under the objective difficult (see supplementary video).

In vitro experiments

We have also confirmed in vitro that insulin and β2gpI (which was not tested in vivo) are endocytosed by two pathways in WKPT cells: insulin and β2gpI do not compete for binding and do not co-localize in the same intracellular compartment after uptake; β2gpI endocytosis is constitutive and can be blocked with excess β2gpI, whereas insulin uptake is again up-regulated (primed) in the presence of increasing amounts of insulin.

We have shown recently that the uptake of both insulin and β2gpI is mediated by clathrin and that insulin internalization occurs via the adaptor complex AP-2, while AP-2 expression is not required for β2gpI uptake; moreover, uptake of β2gpI is widespread and insulin uptake is predominantly apical (6). The lack of interference of insulin endocytosis by insulin was previously noted in 1980 when ¹²⁵I-labeled insulin was injected in the whole animal: quantitative autoradiography showed extensive labeling over proximal convoluted tubules, which was not depressed by co-injection of unlabeled insulin (1). Our finding of the effect of insulin appears to relate to the increase in its binding sites: once cells have been sensitized to insulin, insulin can be removed and the cells still show enhanced uptake. This suggests that
insulin can stimulate signal transducing pathways that can mobilize sequestered proteins from the interior of the cell to the plasma membrane for the enhanced uptake of insulin.

Two previous studies in yolk sac L2 cells (which express high levels of the endocytic receptor megalin) reported that β2gpl and insulin were endocytosed by binding to megalin. However, whilst β2gpl uptake was Ca\(^{2+}\)-dependent and completely blocked by RAP (16), insulin binding to megalin did not require Ca\(^{2+}\) and insulin uptake was not inhibited by the megalin binding protein RAP (23). In the WKPT cell line the expression level of megalin is low and similar to the LLCPK cell line. RAP did not inhibit endocytosis of insulin or β2gpl, which suggests that uptake of β2gpl and insulin, does not depend on megalin. The observation that megalin-dependent uptake is mainly apical, whereas we observed that β2gpl uptake is more widespread, suggests an alternative receptor. Although β2gpl uptake is clathrin-mediated, the adaptor is unlikely to be AP-2, since depletion of the μ2-subunit of AP2, AP-50, had no effect on endocytosis; in contrast, insulin uptake is apical and is inhibited by depletion of AP-50 protein (6).

Retinol-binding protein (RBP) uptake \textit{in vivo} is also mediated almost entirely by megalin, as suggested by the absence of RBP-containing granules in the proximal tubule of megalin-knockout mice (7). In immortalized rat renal proximal tubule (IRPT) cells, RBP transcytosis is also mediated by megalin, but RBP transport to the lysosome and subsequent degradation (55% of internalized RBP) is independent of megalin (13). Therefore, in the IRPT model other megalin-independent mechanisms of endocytosis must account for RBP degradation: other non-megalin receptors or fluid phase pinocytosis have been suggested.

Finally, we have shown that it is possible to not only image the distribution of filtered fluorescently-labeled substances in real-time, but also that it is feasible to develop methods for relative quantification of the fluorescence signal \textit{in vivo}; moreover, in the case of insulin,
Innovative Methodology

there is a novel priming effect on its uptake in the early proximal tubule following initial exposure, although the mechanism of this phenomenon is not apparent from our experiments.

Several questions will need to be addressed in future studies, for example: What is the mechanism underlying the priming effect observed with insulin in vivo and in vitro, and does it have any functional significance for the effects of insulin? Does it extend to other filtered proteins or peptides? Does it have any implication for protein handling by the proximal tubule?
Innovative Methodology

Acknowledgements

We thank Frank Thevenod for providing the WKPT cell-line and the NKRF and St Peter’s Trust for funding support. The Odyssey confocal microscope was funded by the Wellcome Trust and its upgrade to red laser operation by the Royal Society of London. β-2 Glycoprotein-I was a generous gift from Scipac Ltd., Sittingbourne, Kent, UK. We thank Harvey McMahon, George Banting and Mark Marsh for reagents. The real-time confocal Cy5 fluorescence studies were conducted in the Department of Anatomy and Developmental Biology, UCL.
Innovative Methodology

Figure legends

Figure 1

Real-time reflection and fluorescence microscopy images of rat kidney *in vivo*

The images are presented in negative (inverted) contrast and a black border has been added to make the comparison between images more reliable. **a)** Reflection mode, a single video frame used to define the area of interest for analysis - a distal tubule is seen in the left hand side of the field of view (without the more intense black brush border); **b)** Contour map derived from the binary mask that defines the linear strips within which fluorescence was quantified to estimate the change in mean fluorescence signal intensity within and across (apical to basolateral) PT cells; **c)** Fluorescence mode, a single video frame from which intensity is calculated; **d)** Fluorescence mode, an average of 5 video frames to show morphology more clearly.

Figure 2

Insulin uptake *in vivo*: A. Control; B. Lysine

[i] Insulin pseudo-concentration as a function of time and position within proximal tubule cells in the rat kidney *in vivo* estimated from the fluorescence of Cy5-insulin. [ii] Uptake curve showing mean insulin pseudo-concentration vs. time after injection, comparing the first (●) and second (×) insulin injections. [iii] Logarithmic plot of pseudo-insulin concentration against time after injection - the slope of each curve is a measure of the rate of insulin uptake after the first (●) and second (×) injections. The colored inserts in the top left hand corner of [i] are planar views of the 3D color maps of pseudo-concentration.
Innovative Methodology

**Figure 3**

**Derivation of the bleaching correction**

The curve shows the pseudo-concentration of Cy5-labelled insulin as a function of time under constant conditions of laser irradiation (see text for details).

**Figure 4**

**Electron microscopy of insulin binding and uptake into cells of the proximal tubule detected by an immunogold-silver labeling method at 1-2 min (A) and 30-45 min (B) after insulin injection; (C) control**

A) insulin immuno-gold-silver grains (arrows) localize to the brush border; B) insulin immuno-gold-silver grains now localize in the cytoplasm (arrows) and in an apical vacuole/multivesicular body (mvb) (at ~45 min post-injection); C) no insulin immuno-gold-silver grains are seen when the primary antibody step is omitted or in control tissue (not shown). N is the cell nucleus, Mv are brush border microvilli, and Cap is a peritubular capillary. Bar = 1 μm.

**Figure 5**

**Absence of competition between insulin and β2gp1 uptake**

WKPT cells were incubated with 1 μM Cy2-labeled β2gp1 (A, panels a-c) or Cy2-labeled insulin (B, panels d-f) for 2 hours at 37°C in the presence of either 10 μM unlabeled β2gp1 (b and e) or 10 μM unlabeled insulin (c and f). For the control (a and d) no unlabeled ligand was added. Uptake of insulin and β2gp1 was assessed by confocal microscopy.
Innovative Methodology

Figure 6

Analysis of insulin uptake by flow cytometry

WKPT cells were incubated with 5 µM Cy2-labeled insulin for 2 hours at 37°C in the presence of 0, 20, 50, 100, or 200 µM unlabeled insulin. A) Flow cytometry results: The grey area shows the distribution for the no-ligand negative control and the hatched area shows the distribution for the sample. B) The mean fluorescence of the cells analyzed by flow cytometry. Mean±SEM (n = 3).

Figure 7

Analysis by flow cytometry of β2gpI uptake

WKPT cells were incubated with 1µM Cy2-labeled β2gpI for 2 hours at 37°C in presence of 0, 4, 10 or 20 µM unlabeled β2gpI. A) FACS results. The grey area shows the distribution for the no-ligand negative control and the hatched area shows the distribution for the sample. B) Mean fluorescence of the cells analyzed by FACS. Mean±SEM (n = 3).

Figure 8

L-Lysine does not affect insulin uptake in vitro

Confluent WKPT cells were incubated with 5 µM Cy2-insulin for 2 hours at 37°C in presence of 40 µM unlabelled insulin, 40 µM β2gpI or 40 µM L-Lysine. Uptake of Cy2-insulin was assessed by confocal microscopy. A) Confocal visualization of endocytosed Cy2-insulin (x40 objective). B) Histogram showing calculated mean fluorescence for five random fields. Mean±SEM (n = 5).
Innovative Methodology

**Figure 9**

**Priming of insulin uptake in vitro**

Confluent WKPT cells were incubated with unlabeled insulin (0-100 μM) for 1 h at 37°C. The cells were then washed 4 times and incubated with 5 μM Cy2-labeled insulin for 2 hours at 37°C. **A)** Confocal visualization of endocytosed Cy2-insulin. **B)** Histogram showing the calculated mean fluorescence for ten random fields. Mean±SEM (n = 10); *P<0.05, **P<0.01 versus (0) no insulin (Kruskall-Wallis 1-way ANOVA, followed by unpaired Mann-Whitney test).

**Figure 10**

**RAP does not inhibit uptake of either insulin or β2gpI**

**A)** Analysis of recombinant RAP (0.5, 1.0 and 2.0 μg) by SDS-PAGE. BSA samples (0.5, 1.0, 2.0 and 3.0 µg) were loaded as a control since BSA was present in the RAP. RAP (40 kDa.) is indicated on the gel.

**B)** and **C)** Confluent WKPT cells were pre-incubated with 0.2, 0.5 or 1.0 μM RAP for 1 h at 37°C. 5 μM Cy2-β2gpI (**B**) or Cy2-insulin (**C**) was subsequently added to the cells, and incubated for 2 h at 37°C. The fluorescence of the cells was measured by confocal microscopy. For each condition the mean fluorescence for 10 random fields was quantified. Mean±SEM (n =10).
Innovative Methodology

Legend to supplementary video sequences

The video clip shows *in vivo* confocal microscopy (50 μm field width, Zeiss 40/1.0 oil immersion objective lens with 2 glass coverslips sandwiched to a thickness of 425 μm) recordings of a rat kidney. The first few frames show reflection images of a distal tubule (centre of field) among proximal tubules (distinguished by reflective – white – brush borders) and surrounding peritubular capillary blood flow. The next sequence (taken with a 488 nm Argon laser) shows the fluorescence signal (switching from fluorescence to reflection modes, and combined) immediately after injection of FITC-labeled insulin, and zooming in on a proximal tubule (with reflective brush border). This is followed by images of fluorescent proximal tubules approximately 40 min after insulin injection, showing more intense fluorescence of early compared with late proximal tubules (field of 3 proximal tubules - a brighter early proximal tubule flanked by 2 later proximal tubules). There is clearly variation in fluorescence intensity among tubules, but this heterogeneity is not unlike the findings *in vivo* renal tubule micropuncture experiments. The final sequence (taken with a 633 nm HeNe laser - see main text) shows fluorescence after injection of Cy5-labeled insulin directly into a proximal tubule via a micropipette (tip visible top right).
References


Figure 2

A

B
Regression curve \[ c = 0.12 \cdot e^{-\frac{t}{5.25}} \]

Increment

Uncorrected value (from the fluorescence image)

Duration of irradiation

Figure 3
Figure 4
Figure 5

A
1μM Cy2-β2gpl

Control 10μM β2gpl 10μM Insulin

B
1μM Cy2-Insulin

d e f
Figure 6

Insulin concentration (µM) -ve Ctrl 0 20 50 100 200

Mean fluorescence (arbitrary units, n=3)

Cy2 fluorescence Count

Figure 6
Figure 7

A

0 µM β2gpl

4 µM β2gpl

10 µM β2gpl

20 µM β2gpl

Cy2 fluorescence

B

Mean fluorescence (arbitrary units, n = 3)

β2gpl concentration (µM)

ctrl 0 4 10 20

0 2 4 6 8 10 12 14
Cy2 Insulin endocytosis

-ve CTRL +ve CTRL 40 uM Insulin 40uM B2GP1 40uM L-lysine

Mean integrated fluorescence (arbitrary units, n=5)

Figure 8
Insulin priming concentration (μM) -ve Ctrl 0 10 50 100

0.0 0.5 1.0 1.5 2.0

Mean fluorescence (arbitrary units X 10^6, n=10)

Figure 9