

IS UREMIA AN EXAMPLE OF ACQUIRED INHIBITION OF RECEPTOR-MEDIATED ENDOCYTOSIS?

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SUMMARY

• *Many factors that inhibit receptor-mediated endocytosis (RME) at postreceptor level in vitro have been described: (i) high concentration of urea, (ii) lowering of intracellular pH, (iii) hypotonic or hypertonic media, (iv) intracellular potassium depletion, (v) depletion of cellular ATP, (vi) inhibition of the enzyme transglutaminase, (vii) impairment of receptor recycling by rising of the endosomal pH, (viii) disruption of microtubules.*

Nearly all or all of the factors mentioned above are present as disturbances of homeostasis in uremia. The magnitude of the factors inhibiting RME in vitro is considerably greater than the deviations observed in uremia, but in vitro almost complete inhibition of RME is aimed and also in uremia all of the factors act together. The hypothesis that RME is inhibited in uremia is supported by the metabolism of macromolecules known or supposed to be internalized by this process.

Although glucose intolerance in uremia is due to a postreceptor defect in insulin action, impaired RME at postreceptor level, to the best of my knowledge, has not been pointed as a general feature of uremia.

In addition to uremia, there might be other clinical examples of inhibited RME at-postreceptor level as well.

INTRODUCTION

• RME is an important and general process by which cells take up macromolecules from the extracellular environment. It is common to virtually all eukaryotic cells except the mature erythrocyte (1,2). RME begins with clustering of cell surface receptors within specialized invaginations of the plasma membrane, termed clathrin coated pits (CP), prior to internalization. Two significant properties of RME, that distinguish it from fluid phase endocytosis, are the selective uptake of ligands and the considerably higher effectiveness of internalization. CP act as molecular filters concentrating the receptors involved in macromolecules binding and excluding the proteins that are relatively permanent residents of the plasmalemma (3,4,5). The clathrin coat seems to behave like a molecular motor that converts the CP into a coated vesicle (CV) (6). After its formation, the CV quickly sheds its coat and the resultant uncoated vesicle immediately participates in fusion events (7,8). Unclustered or partially clustered ligand-receptor (L-R) complexes may also be taken up by smooth membrane (pinocytic) vesicles (31). Although in broad sense this is also a receptor-mediated uptake, in this article, the term RME is used for designation of internalization via CP/CV.

Clathrin coated pits and vesicles are characteristic structures not only of RME. At the trans-face of the Golgi com-

plex they are also implicated in sorting and transport of newly synthesized lysosomal enzymes and the regulated secretory proteins to their appropriate destinations (9,10).

The cytoplasmic coats of coated pits and vesicles represent lattices consisting of hexagons and pentagons and are built up by clathrin triskelions. Triskelions are stable, flexible, three armed symmetric protein complexes. Each arm of the triskelion is composed of one clathrin heavy chain and one non-covalently bound clathrin light chain. The three heavy chains are also non-covalently connected by their hydrophobic carboxyl terminal regions to form the triskelion vertex (Fig. 1). The arms of different triskelions impose on and connect to each other making the polygons of the clathrin coat (10,11).

Rearrangement of some of the hexagons into pentagons occurs during the CP-CV conversion (12), but there is no evidence that this is the force generating mechanism for invagination (13). The term clathrin means lattice-like. It has been initially introduced for designation of the "major

protein" extracted from CV. In later studies it has been demonstrated that this "major protein" consists of two different types of polypeptides: the clathrin heavy and two clathrin light chains. The triskelion is the functional clathrin unit present in living cells and isolated from CV preparations (11,14). Under the physiologic conditions of pH, ionic strength and low clathrin concentration, the assembly of clathrin coats and their attachment to the underlying membrane are mediated by heterotetrameric protein complexes designated as adaptors (AP). Two such AP have been identified: the AP-2 is restricted to the plasma membrane CP and CV, while the AP-1 is found in the CV of the trans-Golgi network (11,17). AP play a role in receptor clustering within CP. Tyrosine containing motifs within the cytoplasmic domains of receptors taken by RME compete for a common binding site on AP-2. Direct interaction, but probably not via the AP-2 recognizing motif, has been demonstrated between the cytoplasmic tail of mannose-6-phosphate receptor and AP-1 (14,15,16). After clathrin removal, AP-2 remain attached to the underlying membrane and induce aggregation of the formed uncoated vesi-

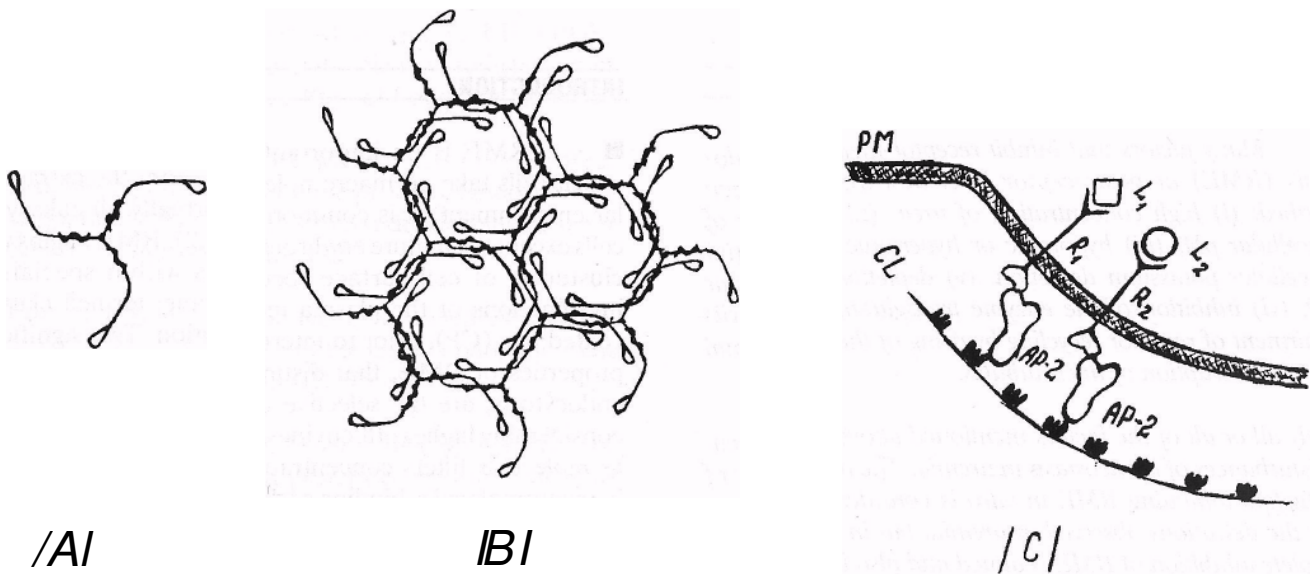




Figure 1.
The triskelion assembly and association.
/A/ clathrin triskelion

 A clathrin heavy chain. The distended end is designated as terminal domain.
 A clathrin light chain

/B/ Part of a clathrin lattice
/C/ Part of a coated pit. PM - plasma membrane; Lj, L2 - ligands; R1, R2 - receptors; AP-2 - adaptor type 2; CL - clathrin lattice. Terminal domains of clathrin heavy - chains are protruding inwards.

cles. Since membrane fusion requires close opposition of the two participating bilayers, the AP-2 dependent aggregation may be an important initial step in the fusion of uncoated vesicles and endosome formation (8). A 180kD protein with clathrin assembly activity has been also designated as AP-3. It has uncertain relationship with the other two AP, consists of a single polypeptide, has been detected only in brain and its specific functions in living cells need further elucidation (11,16).

There is another type of vesicles with cytoplasmic protein coats not made by clathrin within the Golgi complex. In contrast to clathrin coated pits and vesicles that mediate selective transport events, the non-clathrin coated vesicles act as non-selective "bulk flow" carriers of the constitutive export through the Golgi complex (18). In my article, if not additionally noted, the term coated refers to clathrin CP and CV.

A process of receptor - dependent uptake of small molecules, termed potocytosis, has been described. Potocytosis embodies certain features of RME, but the L-R uptake is via caveolae covered by delicate filaments instead of CP/CV (19,33).

RME is an ATP dependent process (20). It has been shown that ATP is needed for the initiation of a CP formation and the final budding of a deeply invaginated CP from the plasmalemma (13,21). Clathrin removal from CV requires ATP as well and is mediated by a 70kD heat shock protein (hsp70), designated also as uncoating ATPase (11). The disassembly of the CVs' clathrin shell allows membrane fusion events and delivery of L-R complexes to sorting endosomes (26). There is evidence that a variety of different L-R complexes cluster in one and the same CPs and are subsequently found into common endosomes (22,23). Mixing in the endosomal compartment of ligands taken via CP/CV and molecules internalized by noncoated plasmalemmal invaginations has been also found (41,48).

At the present there is no standard nomenclature for the elements of the endosomal compartment (25,26,37, see Fig. 2) and two models for the intracellular traffic of internalized material have been suggested (27):

A/ The transfer between sorting and late endosomes, and between late endosomes and lysosomes is carried out by transport vesicles - "vesicle shuttle model".

B/ Sorting endosomes are continuously formed de novo. With time the sorting endosome loses its ability to fuse with uncoated vesicles and transforms into late endosome -

"endosome maturation model". Recent investigation of Dunn and Maxfield favour the latter of the proposed models (26, Fig. 2).

Endosomes are acidified by proton pumps residing with-

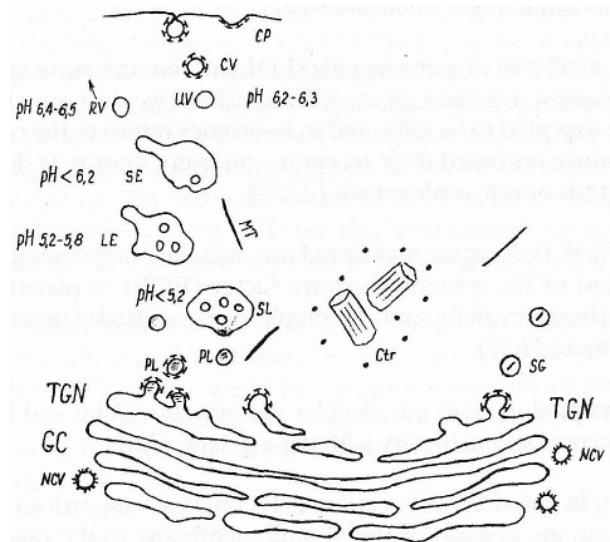


Figure 2.

The "endosome maturation".

CP - clathrin coated pit; CV- clathrin coated vesicle; UV- uncoated vesicle; SE- sorting endosome; RV- recycling vesicle; LE - late endosome; SL - secondary lysosome; PL - primary lysosome; MT - microtubules; Ctr - centrosome; SG- secretory granule; TGN - trans-Golgi network; GC - Golgi complex; NCV - non-clathrin coated vesicle.

in their membranes and have intravesicular pH ranging between 6,3 and 5,2 (see Fig. 2). The acidic nature of the endosomal compartment has emerged as an important factor for the appropriate processing and targeting of ligands and receptors (23,24).

There are several pathways that physiologic L-R complexes may follow once inside a cell:

(i) Ligand and receptor dissociate in the endosomal compartment due to the mildly acidic pH. Receptors return to the plasmalemma and ligands are finally degraded in lysosomes. Ligands as low density lipoproteins (LDL), oc2-macroglobulin, asialoglycoproteins, mannosylated glycoproteins, mannose-6-phosphate glycoproteins (M-6-P), galactose terminal glycoproteins and their relevant receptors follow predominantly this intracellular route (5,22,23).

(ii) L-R complex recycles intact to the cell surface. Transferrin (Tf) has high affinity for its receptor at neutral pH, while apoTf has high affinity at acidic pH and low

affinity at neutral pH. Iron is tightly bound to Tf at neutral pH. Iron is tightly bound to Tf at neutral pH, but dissociates at mildly acidic pH. Tf binds to its receptor at neutral pH at the cell surface, enters the acidic endosomes, donates here its iron atoms, converts to apoTf that remains bound to its receptor and recycles back to the plasmalemma where dissociates (24).

A small part of some ligands (LDL, insulin, mannose and galactose terminal glycoproteins) which would normally be expected to be delivered to lysosomes return to the cell surface on board their receptor - an event known as dia-cytosis or retroendocytosis (23,32).

(iii) Both receptor and ligand are degraded in lysosomes. Most of the epidermal growth factor (EGF) or platelet-derived growth factor L-R complexes are degraded in lysosomes (22,23).

The predominant intracellular pathway of insulin and its receptor seems to vary with the cell type (22).

(iv) In polarized cells, certain L-R complexes may traverse from one domain of the plasma membrane to the other where ligand is released intact - transcytosis (2,5,16,22).

(v) After entering within the cell, some ligands or L-R complexes can be delivered to the intracellular sites of their actions. The tyrosine kinase activity of occupied insulin and EGF receptors is retained for a substantial part of their intracellular lifetime and is important for many of the diverse biologic effects of insulin and EGF (32,34,35). Partial prelysosomal proteolysis of some internalized ligands may take place in the endosomal compartment and such degradation may have physiological significance (32,36,38). There is evidence that insulin internalization and processing is required for its late effects as stimulation! of alanine transport, protein synthesis, inhibition of protein degradation (32). Glucose (G1) is transported across the plasma membrane via facilitative carrier proteins. Cell types (endothelial cells, hepatocytes, parenchymal brain cells), in which G1 uptake is not subject to acute regulation, possess G1 transporter isoforms (GLUT1, GLUT2, GLUT3) that are constitutively localized to the plasma membrane. In insulin sensitive tissues (fat and muscle), the augmentation of G1 uptake after insulin stimulation is achieved mainly by translocation of GLUT4 from intracellular tubulovesicular structures to the cell surface. Fat and muscle cells also possess GLUT1, but GLUT4 is present in much greater abundance. GLUT4 is found in CP of the plasmalemma and is more efficiently internalized than GLUT1. Haney et al (95) suggested that the difference in the internalization rate is the cause for the

greater GLUT4 translocation to the cell surface after insulin challenge. In adipocytes GLUT1 and GLUT4 have been found in distinct intracellular vesicles (94,95,96). Insulin internalization seems to be required for the G1 transporters translocation (31,32).

A small amount of several polypeptide hormones and growth factors are transferred to the cell nucleus after internalization, but the mechanism of this delivery is still unclarified (28). It has been demonstrated that insulin, together with its plasmalemmal receptor, is transported to the cell nucleus (29) and there is data suggesting that the EGF nuclear receptors may be identical to that on the cell surface (28). Some experimental results favour the hypothesis that there might be nuclear receptors present a priori and not derived from the plasmalemmal ones (28,30). For some of the best studied ligands as insulin and EGF there is growing evidence that their delivery to the cell nucleus is significant for their late effects: DNA synthesis, RNA transcription and release (28,29,30,32).

Sorting endosomes are the main organelles in which ligand - receptor segregation takes place. The majority of recycling receptors return back to the cell surface as membrane components of recycling vesicles that bud from sorting endosomes (22,26). Receptor recycling from late endosomes is also possible (37). Receptors destined for degradation are removed from the limiting membrane of endosomes by inward vesiculation, a mechanism playing the role of a molecular filter, and late endosomes acquire the appearance of multivesicular bodies (16,37). Primary lysosomes emerged as CV in the Trans-Golgi Network (TGN) containing nascent lysosomal enzymes bound to the M-6-P receptor. After uncoating they fuse late endosomes where lysosomal enzymes dissociate (9,10). CV have not been implicated in receptor recycling from endosomes to the cell surface or in returning of M-6-P receptor from late endosomes back to the TGN (22,39).

The formation of CV seems to be a perpetual process (5,14,49) and there is evidence for delivery of several unoccupied receptors via CP and CV to the endosomal compartment (23). Receptors internalize and recycle in the absence of their ligands, but often at a slower rate (2,24).

The integrity of microtubules is required for the centripetal migration of endosomes to the juxtannuclear area (37,73). It has been shown that movement of lysosomes is directed by microtubules as well (73). Movement of recycling vesicles may also depend on microtubules (40,55).

INHIBITION OF RME IN VITRO

- Urea at concentration of 2M completely dissociates clathrin from isolated CV (3).

Intracellular pH (pH_i) varies in different cell types between 7,0 and 7,3, while extracellular pH (pH_e) is 7,35-7,45 (60). pH_i in most cells follows pH_e to some extent usually lagging by 0,5-1,0 pH unit (42,60). Acidification accentuates the normal tendency of clathrin lattices to curve in vitro, but lowering of pH_i below 6,8 in cultured fibroblasts (42,61) or Hep2 cells (62) inhibits RME by preventing CP budding (pinching off) from the plasma membrane and causing precipitation of free triskelions in microcages. Low cytoplasmic pH also inhibits the rate of intracellular transport from the TGN to the cell surface (61). Samuelson et al have found that $pH_i=6,7$ does not inhibit RME and L-R dissociation in cultured rat hepatocytes, but impaired the ligand transport to lysosomes (63). In macrophages both reduced RME and transport to lysosomes have been observed (43). Clamping of cells (fibroblasts) at neutrality ($pH=7$) with nigericin inhibits RME by creating a preponderance of unusually flat lattices. These results suggest that the physiologic $/H^+$ gradient existing across the plasmalemma (60) is responsible for the constitutive curving of CP and formation of CV.

Hypotonic media inhibit RME by creating unusually flat clathrin coats as well (42).

Hypertonic media and intracellular K^+ depletion inhibit RME in the same way. They induce clathrin polymerization into microcages and cause disappearance of plasmalemmal CP (10,44,45). Clathrin coats in the trans-Golgi region appeared less affected by K^+ depletion (46). Rapid intracellular K^+ depletion by incubation in hypotonic medium and subsequent transfer to isotonic K^+ free buffer inhibits to a greater extent RME than slow K^+ depletion by incubation only in a K^+ free buffer (46,47). K^+ depleted cells by incubation with the Na-K-ATPase inhibitor ouabain show marked reduction of protein and DNA synthesis and fail to respond to EGF and insulin. Biosynthetic functions are restored when intracellular K^+ returns to normal values (46).

Recent investigations have established that RME is sensitive to ATP depletion (discussed before in the text).

Most amines are weak bases and principally penetrate through the plasmalemma in their uncharged form. From the cytosol amines accumulate in acidic organelles as endosomes, lysosomes and elements of the trans-Golgi. Intracellularly, uncharged amines accept H^+ and rise the

pH both of cytosol and acidic vesicular structures. In vitro addition of amines diminish the difference between pH_i and pH_e (24,52,60). Elevation of the endosomal pH inhibits RME by preventing L-R dissociation and the subsequent receptor recycling. The transfer of internalized ligands or L-R complexes from endosomes to lysosomes, partial endosomal proteolysis and lysosomal degradation may be also inhibited by amines (23,24,52). Rising of the endosomal pH suppresses the recycling of receptors that have been internalized unoccupied as well (23,24) and prevents the penetration of internalized enveloped viruses and certain protein toxins through the endosomal membrane into the cytosol (24). Reduced uptake of Tf (52) or unchanged uptake and recycling of Tf but inhibited intracellular iron delivery caused by amines have been reported (23,53).

A correlation between the ability of primary amines to prevent clustering and uptake of several ligands and to inhibit transglutaminase led to the suggestion that this enzyme was essential in the internalization process (50,51). However, other investigations failed to confirm this suggestion (52).

Although the influences of acidophilic agents on the exocytic pathway have been less extensively studied, there are examples showing that the transport of membrane and secretory proteins is altered by amines (24,97).

Ostlund et al found that the microtubule disrupting agent colchicine significantly reduced the receptor-mediated uptake and degradation of LDL (54). The experimental results of Sandvig and vanDeurs showed that colchicine did not affect the initial internalization of Tf, but its receptor recycling might be inhibited (55).

All of the factors discussed above reversibly inhibit RME and the process is restored when the suppressing agent(s) is removed.

Although the experimental results are not equivocal, Hansen et al (56) concluded that hypertonic media, K^+ depletion or acidification of the cytosol, which efficiently block RME, reduce the uptake of fluid phase markers or ricin, that is able to enter cells both from CP and other membrane areas, only to some extent. The sensitivity of fluid phase endocytosis to these factors also seems to depend on the cell type (42,44,45,56). Microtubule disrupting agents(55) and amines (52) may decrease the rate of fluid phase endocytosis as well.

A PARALLEL BETWEEN THE FACTORS INHIBITING RME IN VITRO AND DEVIATIONS OF HOMEOSTASIS IN UREMIA

- The concentration of urea is much higher than that of all the other potential uremic toxins combined. Urea is best known to chemists as a global protein-unfolding agent (denaturant) at high concentrations ($\sim 1M$), but the nuclear magnetic resonance spectroscopy data have shown that important effects also occur at the lower concentrations seen in uremia and are thus likely to be important in the pathophysiology of this condition (58). This suggests that although the urea levels in uremia are considerably lower than the concentrations used in vitro for complete clathrin removal, they may have effect on clathrin, especially in combination with other factors disturbing the normal intracellular clathrin behavior.

Hyperchloremic metabolic acidosis can develop early in the course of the renal disease but the acidosis transforms to an increased anion-gap form when the glomerular filtration rate falls below 20ml/min (59). In uremia $/pH_j/$ was found to be normal (66,67) or decreased (68,69,71). Normal $/pH_j/$, but prolonged slight intracellular acidosis after a mild exercise was reported in skeletal myocytes of uremic patients when compared to controls (70).

Metabolic acidosis in uremia should be associated with efflux of K^+ from intracellular to extracellular fluids (74).

Decreased Na-K-ATPase activity has been established in erythrocytes, brain, leukocytes, intestine and skeletal myocytes of uremic subjects (64,65,83). Normal (83) or reduced (65) Na-K-ATPase activity has been found in myocardium. As Na^+ influx is not diminished, intracellular Na^+ concentration rises. Increased Na^+ concentration will restore the product of the rate constant and Na concentration to that observed prior to enzyme inhibition. A new steady state will be reached in which Na^+ efflux equals Na^+ influx, but at higher concentration of intracellular Na^+ . As intracellular Na^+ rises the intracellular concentration of K^+ falls. Since the gain of Na^+ exceeds the loss of K^+ , cell osmolality rises, i.e., the extracellular fluid becomes hypotonic. This leads to an increase in cell water (65). It has been found that erythrocytes, leukocytes and muscle cells from uremic patients have higher Na^+ , lower K^+ and increased intracellular water contents than normal (64,65). Hemodialysis partially restores Na-K-ATPase activity (65).

As a result of the impaired renal function and inappropriate diet or superimposed aggravating conditions hyper- or hyponatremia may occur as well (74,82).

Erythrocytes and brain cells from uremic subjects have elevated ATP, while in leukocytes ATP content is low (65). No difference has been found in skeletal muscle ATP concentration in normal and uremic subjects, but in the latter glycolysis dominates over oxidative phosphorylation as a source of ATP. During exercise phosphocreatine breakdown, the rate of intracellular acidification and lactic acid accumulation is increased in uremia. The half-time of phosphocreatine recovery is also longer, suggesting diminished mitochondrial function (84). In uremia energy utilization tends to be decreased, especially in severe untreated cases (72). Perhaps disturbances in ATP production and utilization play only a minor, if any, additive role in the inhibition of RME, since in vitro almost complete depletion is needed for suppression of this process (20,21). Direct effects of uremic toxins on clathrin uncoating ATPase, by analogy with Na-K-ATPase, have not been investigated.

Amines, including primary ones, are considered to be uremic toxins and their concentrations are increased in end stage renal disease (65,75,76). In uremia amines may inhibit RME by the mechanisms observed in vitro. Increased levels of amines may play a role in the cases when normal $/pH_j/$ and metabolic acidosis is present. Probably amines are also the reason or one of the reasons for the absent initial cytoplasmic acidification and enhanced subsequent alkalization, observed in neutrophils isolated from uremic subjects or in normal neutrophils preincubated in effluent peritoneal dialysate, after stimulation with the chemotactic peptide FMLP (66).

Lee et al (58) have speculated that some methylamines may offset the effects of urea on proteins, but this suggestion needs further investigation.

Braguer et al (77) have reported that a fraction of uremic middle molecules inhibits microtubule formation and may also disrupt preformed microtubules.

Iacopetta et al (127) observed in vitro that elevated intracellular Ca^{2+} had little or no effect on the internalization of insulin and transferrin, but in other investigations inhibition of RME of EGF by the same impact was found. Also increased Ca^{2+} burden of cells is associated with impaired mitochondrial oxidation and ATP production (128). Elevated intracellular Ca^{2+} , as Ca^{2+} -calmodulin complexes, can depolymerize microtubules (129,130). Hyperparathyroidism of chronic renal failure enhances Ca^{2+} entry and causes increased basal levels of Ca^{2+} in a lot of cell types (128). Many hormones and growth factors transiently elevate intracellular Ca^{2+} (131) and if their stay at the plasma membrane is prolonged due to inhibited RME, this may contribute for further increase of intra-

cellular Ca^{++} . CV seem to be Ca^{++} sequestering organelles as well (132). Chervu et al (128) established reduced Fc-receptor mediated phagocytosis by the polymorphonuclear leukocytes from uremic rats and provided evidence that the suppression is caused by the increased intracellular Ca^{++} due to secondary hyperparathyroidism.

Although there is no doubt in the lifesaving effect of hemodialysis treatment, there are factors that commonly occur and may temporarily contribute to the inhibition of RME during the hemodialysis session: (a) in standard hemodialysis (Na^{+} concentration of dialysate \approx 140 mmol/L) there is a movement of water from the extracellular to the intracellular fluid, i.e., the extracellular fluid becomes hypotonic compared to the cytosol (78,79,80), (b) if dialysates with increased osmolality are used the water flux is just the opposite (78,98), (c) in the beginning of acetate hemodialysis there is initial lowering of both pH_e and pH_i (69,80,81).

The data about the parallel made between inhibition of RME in vitro and disturbances of homeostasis in uremia are summarized in Table 1.

The factors inhibiting RME in vitro are of considerably greater magnitude than the observed deviations of homeostasis, but in uremia all factors act together, not separately. Also in vitro nearly complete inhibition of RME is aimed.

Since lowering of the pH of caveolae during potocytosis is considered to be important for L-R dissociation and ligand entering within the cell (19,33), potocytosis may also be inhibited in uremia as a result of increased concentrations of amines. It has not been investigated if plasma membrane changes observed in uremia (99) influence potocytosis.

METABOLISM OF MACROMOLECULES KNOWN OR PRESUMED TO BE INTERNALIZED VIA CP/CV IN UREMIA

- The carbohydrate metabolism in uremia is characterized with fasting euglycemia, diminished glucose (Gl) uptake by peripheral tissues and elevated insulin levels. Early insulin response following Gl loading may be normal, increased or decreased, but persistent hyperinsulinemia is always observed during the final part of the Gl tolerance test, regardless of the insulin secretion (65,88,90). Insulin receptor tyrosine kinase activity, necessary for many of insulin effects and considered to be important for receptor-mediated insulin internalization (32), has been found not decreased in uremia both in situ and in vitro

Table 1

Parallel between the inhibition of RME in vitro and the metabolic disturbances in uremia

Factors inhibiting RME in vitro	Uremia
Urea	Concentration observed in uremia may have effect on clathrin conformation
Intracellular acidification or reduced pH_e - pH_i difference	Metabolic acidosis, decreased or normal pH_i . Increased intracellular acidification during and after exercise.
Hypotonic or hypertonic media	Hypotonic extracellular fluid, rarely hypernatremia
Unracellular K^{+} depletion	Decreased intracellular K^{+}
Cellular ATP depletion	Decreased ATP levels established only in leukocytes. Impaired ATP production and utilization.
Amines a) inhibition of transglutaminase by primary amines b) rising of endosomal pH and inhibition of receptor recycling	Increased concentrations of amines including primary ones.
Disruption of microtubules	Uremic middle molecules inhibiting microtubules assembly and probably disrupting preformed microtubules as well.
Increased intracellular Ca^{++}	Established in many cell types.

(89,93). Uremia induces insulin resistance in peripheral tissues causing a postreceptor defect in insulin action. Even pharmacological concentrations of insulin, that should overcome a possible binding defect do not normalize Gl uptake (88,89). Jacobs et al (93) established decreased concentration of GLUT, without determining their type, in adipocytes isolated from uremic rats. In plasma membranes obtained from these cells the concentration of GLUT was decreased by 16% and 30% in basal and insulin treated cells, respectively. Concomitantly, microsomal membranes prepared from uremic cells treated in the absence and presence of insulin had a 28% and 15% decrease in the number of GLUT, respectively. These results suggest that in addition to the diminished total amount of GLUT, there is a reduced translocation of GLUT from the intracellular vesicular structures to the cell surface. As discussed before in the text, insulin internal-

ization is necessary for stimulation of GI uptake (movement of GLUT4 to the plasmalemma) in insulin sensitive tissues. Effective internalization of GLUT4 via CP/CV and the subsequent intracellular sequestration are also important for the transfer of GLUT4 to the cell surface after insulin challenge (95,96).

Like the inhibition of RME by the factors discussed before, GI intolerance in uremia is reversible. Hexose transport in adipocytes from uremic rats is restored after 20 hours of incubation in physiologic medium and is reduced in normal adipose tissue preincubated for 3 hours with uremic serum (87). GI metabolism also improves after the beginning of hemodialysis treatment (100).

Effects of insulin as activation of amino acid transport, stimulation of protein synthesis and inhibition of protein breakdown, which depend on insulin internalization and its appropriate intracellular processing (32), are all depressed in uremia (65). In addition to disturbing insulin action (100), acidosis may also directly enhance protein degradation (101).

In uremia the half-life of insulin is markedly prolonged. After ten weeks of dialysis insulin clearance returns towards normal and increase in insulin degradation by the liver and skeletal muscle has been observed (88,100). Obviously, during this time the renal function does not change. In my opinion, inhibited receptor-mediated uptake via CP/CV play a role in the delayed insulin clearance, but its contribution is difficult to be quantitatively defined for the following reasons: (i) in extrarenal tissues insulin is degraded both at the cell surface and intracellularly (for review see ref. 32, (ii) insulin can enter within the cell both via CP and non-coated plasmalemmal invaginations (31,102, 103), and (iii) nonreduced insulin binding to a variety of cell types, obtained from uremic subjects, has been found (85,86,87,88,89). In other studies, an uremic peptide inhibiting insulin binding to erythrocytes has been isolated (105). Insulin receptors from different tissues are heterogeneous (105) and insulin binding is not uniformly influenced by certain molecules *in vitro* (91). This results suggest that the uremic peptide inhibiting insulin binding to erythrocytes may have weaker effect on other tissues. Experimentally induced acidosis reduces insulin-receptor binding, tissue insulin extraction and causes GI intolerance as the hyperglycemia might be due either to binding or/and postbinding defects (92,100). There is a view that uremia without acidosis does not reduce insulin-receptor binding (100). In uremia metabolic acidosis aggravate, but is not the only reason for the impaired GI metabolism (104). If cells from uremic subjects are incubated in media different from the uremic plasma, prior to the measurement of

insulin binding, then factors as the uremic peptide or acidosis will be abolished. If such incubation is long enough, restoration of non-coated and probably to a lesser extent of CP/CV endocytosis may occur. It seems that this is the reason for the results of Kauffman and Caro (85), who established in hepatocytes from uremic rats increased number of insulin receptors, unimpaired insulin binding and internalization. They also found decreased insulin degradation both at the cell surface and intracellularly. In another experiment, Maloff et al (87) observed not reduced insulin binding and degradation by liver membranes and adipocytes from uremic rats during the 1 hour long insulin binding assays.

Normal (89) or even increased number (85) of surface insulin receptors along with elevated insulin concentration indicate impaired insulin receptor down-regulation in uremia. Occupancy of receptors by insulin enhances their association with CP and subsequent internalization. Cellular entry is necessary for ligand-induced receptor down-regulation (106).

The clinical significance of impaired RME, due to inherited defects in low-density lipoprotein (LDL) receptor, for accelerated development of atherosclerosis is well known (57,109). In renal failure, dyslipoproteinemia can be detected when glomerular filtration rate is reduced below 40-50 ml/min and becomes more accentuated with deterioration of renal function (107,108). Although decreased activities of lipoprotein lipase, hepatic triglyceride lipase, lecithin/cholesterol acyltransferase, as well as increased concentration of apolipoprotein CIII, have been reported, the complete mechanism of altered lipoprotein metabolism in uremia has not been established (99,107,108,110). Delayed removal of very-low-density lipoproteins (VLDL) and chylomicrons, due to diminished lipoprotein lipase activity, is considered to be the main cause for the hypertriglyceridemia in uremia (86,108,109). Many experimental results indicate that impaired RME, including at a postreceptor level, can be the missing factor in the pathogenesis of dyslipoproteinemia of end-stage renal disease:

(i) The synthesis of lipoprotein lipase is controlled by insulin (111) and impaired RME and effect on protein synthesis of the hormone may be one of the causes for the diminished concentration of lipoprotein lipase in uremia (65,112).

(ii) The concentration of intermediate-density lipoproteins (IDL) is increased in uremia (110,112), although their production should be decreased as a result of reduced lipoprotein lipase activity (108,109). IDL are internalized by the hepatic LDL receptor or are further transformed to LDL

by the lipoprotein lipase (109,113). LDL are usually not elevated in uremia (107,108), although cases of type-IIa and type-IIb Fredrickson patterns of hyperlipidemia in dialysis patients have been described (121). Also Tsukamoto et al (122) have found elevated LDL concentration in hypertriglyceridemic undialysed uremic patients.

(iii) Weintraub et al. (114) have established a severe defect in the clearance of postprandial chylomicron remnants in dialysis patients with and without fasting hypertriglyceridemia. Chylomicron remnants are taken up by the liver via the LDL receptor or via specific chylomicron remnant receptor (109,113). Portman et al (115) have established an acquired defect in LDL receptor function in peripheral blood mononuclear cells from uremic patients. They have proposed that the defect is due to decreased LDL receptor expression because of the reduction of LDL receptor mRNA. Diminished LDL receptor expression is probably a result of decreased reductive capacity of uremic serum and creation of oxysterols, some of which are 100 times more potent than LDL in inhibiting LDL receptor biosynthesis (115). By this mechanism is difficult to explain the delayed clearance of chylomicron remnants, since the chylomicron remnant receptor is unaffected by intracellular accumulation of cholesterol (57). The LDL receptor-related protein is thought to represent the chylomicron remnant receptor and the promoter region of its gene does not contain a sterol regulatory element of the kind associated with the LDL receptor gene (116).

(iv) Reduced high-density-lipoprotein (HDL) cholesterol especially in HDL2 subfraction, increase in HDL triglyceride, decreased ratio of HDL2 cholesterol to HDL3 cholesterol and impaired conversion of HDL2 to HDLg are characteristic for uremic dyslipoproteinemia. Approximately only 50% of the deviations in HDL cholesterol content can be explained by changes in VLDL triglycerides or changes in the activities of lipoprotein lipase, hepatic triglyceride lipase and lecithin/cholesterol acyltransferase (112). Inhibition of RME may influence several steps in HDL metabolism (a) macrophages possess specific receptors for HDLg without apolipoprotein E (apo-E). After internalization these HDL3 are converted to larger apo-E containing, cholesterol rich HDL2-like particles. HDL2 with apo-E are exocytosed by macrophages and hence the excessive cholesterol is removed from the cells (117) (b) one of the mechanisms for cholesteryl esters removal from HDL is the uptake of HDL containing apo-E by the hepatic LDL receptors (113,123). HDL without apo-E can also be endocytosed by hepatocytes, but, to the best of my knowledge, the role of CP/CV in this internalization is not defined (123,124).

Modified, including oxidized, LDL are recognized and taken up by the macrophage scavenger receptors and accelerate the formation of foam cells in atherosclerotic lesions. Endocytosis of scavenger receptors is presumably via CP/CV (118). It is interesting if in uremia the formation of foam cells is delayed due to the possible impairment of RME. The atherosclerotic plaque bears many similarities to chronic inflammatory conditions (119). In uremia the disturbed lipoprotein metabolism should promote and the suppressed immune response (126) should retard the development of atherosclerosis. Probably this is the reason for the variable results obtained in cross sectional and prospective studies about atherosclerosis in uremic subjects (107).

Apo-E containing lipid complexes and LDL receptors are involved in peripheral nerve regeneration and remyelination (113). Uremic peripheral neuropathy, characterized by primary axonal degeneration and secondary segmental demyelination (125), may be due, at least in part, to reduced RME. Also unimpaired RME of nerve growth factor is required for the full expression of its activity (120). Specific binding sites for nerve growth factor have been detected in the nuclei of certain cells (28).

Erythroid cells obtain iron by RME of transferrin (Tf). The acidic endosomal pH allows Fe^{3+} dissociation from Tf and the formed apotransferrin, remaining bound to the Tf receptor, recycles back to the cell surface. Released Fe^{3+} is reduced to Fe^{2+} by a transendosomal membrane reductase and is then translocated across the endosomal membrane (133). About 80% of the iron leaving the plasma is incorporated into hemoglobin. Since erythrocyte precursors possess Tf receptors (TfR) to the reticulocyte stage, the iron uptake will depend greatly on the rate of erythropoiesis (137). In reticulocytes, TfR are sequestered into the membranes of endosomal intraluminal vesicles and are subsequently exocytosed (23). By enzyme-linked immunosorbent assay it has been established that the plasma TfR protein (TfRP) levels have a constant relationship to tissue TfR and in most instances reflect the rate of erythropoiesis (137). The erythron Tf uptake (ETU) is derived from hematocrit, plasma iron, plasma iron half-life and Tf saturation (for the exact formulas see ref. 134). The possible influences of endosomal pH on Fe^{3+} release from Tf are not taken into consideration. Also direct measurements of TfRP or cell TfR do not participate in the calculations. Since TfRP levels are proportional to the erythroid cells TfR, the ETU/TfRP ratio may serve as an indicator for the effective endocytosis and recycling of TfR. In healthy individuals, after several applications of erythropoietin there is an increase in both TfRP and ETU, but the ETU/TfRP ratio remains relatively constant (see table III).

The anemia of end-stage renal disease is normocytic, normochronic, hypoproliferative and is due to a combination of several factors: (i) relative erythropoietin deficiency, (ii) decreased bone marrow response, (iii) decreased red cell survival and (iv) external blood losses (138,139). In hemotransfusion dependent uremic patients (group A, Table 2) increased plasma iron, Tf saturation and prolonged plasma iron half-life together with the reduced radioiron red-cell utilization (RCU) and ETU indicate diminished iron uptake. In the same time the reticulocyte index and the corrected reticulocyte count suggest reticulocyte production about the normal rate. In uremic patients that do not require hemotransfusions (group B, Table 2) the indices for iron utilization are about normal values, but the retic-

ulocyte index and the corrected reticulocyte count show accelerated, although insufficient for the degree of anemia, erythropoiesis, i.e., increased number of erythroid cells possessing TfR. The facts about ferrokinetics and erythropoiesis in uremic patients, summarized in Table 2, imply reduced rate of iron utilization per erythrocyte precursor cell in end stage renal disease. After four doses of erythropoietin TfRP and ETU increase in uremic patients (135), but the ETU/TfRP ratio changes as well (see Table 3). Since there is evidence that RME, receptor recycling and exocytosis are inhibited in uremia (discussed before), it is reasonable to suggest that the exocytosis of TfR by reticulocytes is diminished. In my opinion, the values of ETU/TfRP ratio before and after erythropoietin in uremic

Table 2.

	Hct (%)	PI µg/dL	Tf sat. (%)	TIBC µg/dL	T _{1/2} min.	TfRP µg/ml	Ferr. ng/ml	RCU (%)	PIT mg/dL/d	ETU µmol/L/d	Ret. (%)	CRC (%)	RI	Reference
Normal subjects (reference values)	♂ 46-62 ♀ 42-54	75-175	20-55	250-410			20-200				0.5-1.5			140
Normal subjects	42-+4	112-+43	35-+11		80-+15			85-+4	0.71-+0.17	60-+12			~1	
Renal Failure (A)	23-+3	206-+38	80-+15		232-+55			26-+10	0.73-+0.16	35-+11			1.2-+0.6	134
Renal Failure (B)	26-+5	79-+24	28-+6		81-+22			71-+13	0.77-+0.18	73-+21			1.5-+0.7	
Normal subjects	42.4-+4.5 40.9-+5.1	95-+35 96-+30	30-+11 30-+9	299-+38 322-+32		4.8-+1.5 5.5-+1.2	80-+75 58-+38		0.69-+0.16 0.78-+0.20	62-+16 73-+17		1.6-+0.1 1.4-+0.1		modified from 135*
Renal Failure	20.3-+1.7 20.5-+2.5 20.4-+3.4	144-+90 143-+71 113-+68	56-+37 57-+33 47-+29	269-+14 269-+53 255-+50		3.7-+1.4 2.96-+0.73 2.8-+1.5	2022-+1315 890-+818 508-+451		0.65-+0.19 0.48-+0.14 0.52-+0.11	41-+14 22-+9 33-+15		1.4-+0.4 0.7-+0.3 1.4-+1.0		
Normal subjects	47-+1	110-+6	41-+3		85-+5			82-+1	0.75-+0.02		0.6-+0.01			modified from
Renal Failure (A)	23-+2	259-+14	86-+2		221-+27			23-+3	0.98-+0.12		1.8-+0.05	0.8-+0.4		
Renal Failure (B)	29-+2	88-+8	35-+5		78-+14			80-+4	0.92-+0.12		2.9-+0.6	1.8-+1.3		136**
Normal subjects	47-+5					7.772-+1.029				60-+13				137
Renal Failure	21-+3					4.973-+2.276				33-+15				

Ferrokinetics and erythropoiesis in uremic patients.

Hct - hematocrit; PI - plasma iron; Tf - transferrin; TIBC - total iron binding capacity; T_{1/2} - plasma radioiron half-life; TfRP - plasma transferrin-receptor protein; Ferr. - ferritin; RCU - radioiron red cell utilization; PIT - plasma iron turnover; ETU - erythron transferrin uptake; Ret. - reticulocytes; CRC - corrected reticulocyte count; RI - reticulocyte index;

(A) - hemotransfusion dependent group; (B) - group not requiring hemotransfusions

*-for normal subjects without hemochromatosis and uremic patients

** - the hematocrit of normal subjects is accepted for basal

patients (Table 3) support such a presumption. It is quite unlikely that TfR are used more efficiently in uremia, since in addition to the inhibited CP/CV internalization the Fe⁺ release from Tf should also be impaired by the elevated endosomal pH due to increased concentrations of amines. Therefore, the number of cell TfR may be higher than the predicted from TfRP. The capacity for Tf uptake by erythrocyte precursors is considerable as implied by the ability of normal bone marrow to increase erythropoiesis five to sevenfold after a maximal stimulation (141). Probably this is the reason for the normocytic, normochromic anemia of end-stage renal disease, where the rate of erythropoiesis is about normal or slightly increased. The same speculations can be made for vitB⁻, which is also internalized via CP/CV (2). If maturation of erythrocyte precursors is accelerated in uremic patients by erythropoietin treatment (152), iron deficiency easily develops, particularly in patients whose anemia has been severe (153).

Table 3.
The calculations made after the data in reference 135 for normal subjects (without hemochromatosis) and uremic patients.

	ETU/TfRP			
		Erythropoietin		
		15 U/kg	50 U/kg	150U/kg
Normal subjects	12.85±4.24	13.76±5.2	12.17±5.54	12.38±2.32
	(N=16)	(N=6)	(N=4)	(N=6)
Chronic Renal Failure	11.55±5.7	17.98±9.36	18.8±6.58	21.41±17.34
	(N=23)	(N=4)	(N=5)	(N=14)

Erythropoietin (Epo) is a glycoprotein. It is metabolized by the kidney, bone marrow and via the liver asialoglycoprotein receptors after its desialylation. The first two ways have only little contribution to the Epo clearance (142). The plasma half-life of human Epo injected in uremic animals is significantly prolonged, considerably more than would be expected solely from cessation of the urinary excretion of the hormone (143,144). The serum Epo half-life was found to be not markedly different in patients with end-stage renal disease compared to those with normal renal function (142,154). Kindler et al (145) found no significant difference in the recombinant human Epo half-life in patients various degrees of renal failure. Shannon et al (146) demonstrated increased glycosidase activity in the serum of patients with chronic renal failure. Therefore, the cleavage of the carbohydrate moiety from Epo should be enhanced. Accelerated submission of Epo to the hepatic

asialoglycoprotein receptor system, possessing a great capacity for ligand uptake, can compensate the probable inhibition of RME and may be the reason for the similar clearance rates of Epo in patients with renal failure and normal individuals.

Burst forming unit erythroid cells (BFU-E), colony forming unit erythroid cells (CFU-E), proerythroblasts and erythroblasts possess a small number of specific Epo receptors. The number of Epo receptors and sensitivity to Epo of erythroblasts show a steady decline during maturation (154,156). After binding, Epo is internalized by RME (142,154). In addition to Epo, several other growth factors as interleukin-3, granulocyte-macrophage colony stimulating factor and insulin-like growth factor I (IGF-I) modulate erythropoiesis (154,155,158).

There are different opinions about the importance of uremic inhibitions of erythropoiesis (135,139). Several facts show decreased bone marrow response in chronic renal failure: (i) In end-stage renal disease Epo levels are low for the degree of anemia, but as absolute values are normal or slightly elevated (135,138,157). In healthy subjects, after blood losses such as one-unit blood-bank donation, the red-cell mass slowly returns to its steady-state level without significant changes in the rate of erythropoiesis and Epo production. In normal individuals banking multiple units of blood, although Epo levels are significantly increased, they are not significantly above the upper limit of normal for the assay (154). Therefore, if the uremic bone marrow response is normal, the shortened erythrocyte lifespan and external blood losses may be overcome by the existing levels of Epo, (ii) It has been observed that serum Epo increases with the progression of renal insufficiency when the hematocrit decreases, but after the partial correction of the uremic syndrome by dialysis the serum Epo declines, together with the improvement of anemia. Whereas the relationship between Epo and hematocrit is inverse in pre-dialysis patients, this relationship is either lost or becomes positive after dialysis has been started. Absence and existence of correlation between IGF-I levels and hematocrit have been found in pre-dialysis and dialysis patients, respectively (148,158), and (iii) Kushner et al (159) observed that uremic sera inhibited erythroid colony (CFU-E) formation to a significantly greater degree than granulocyte-macrophage colony formation. They also established a noncompetitive inhibition of the Epo bioactivity on CFU-E by polyamines.

The low number of Epo receptors on erythrocyte precursors, the evidences for decreased bone marrow response and the high doses of recombinant Epo used for the improvement of anemia of end-stage renal disease (161)

suggest a post-binding defect in the Epo action.

In addition to the decreased renal clearance, the increased levels of growth hormone, parathyroid hormone, glucagon, prolactin and luteinizing hormone in uremia may be also due to impaired RME in extrarenal tissues, predominantly the liver. Normal or elevated concentrations of insulin-like growth factors, adrenocorticotropin and follicle-stimulating hormone have been observed (65,147,148,160).

It is interesting if in uremia post-binding or post-receptor defects are common feature of ligands requiring unimpaired internalization for the full expression of their activity.

Inhibited RME should result in prolonged stay of ligands at the cell surface and may enhance some of the effects carried out at the plasmalemma. Impaired RME will also depress the ligand induced receptor down-regulation. For example, the glucose output and cyclic AMP formation by hepatocytes is increased in uremia after glucagon challenge. Like insulin receptors, the hepatic glucagon receptors are also not efficiently down-regulated in end-stage renal disease.

Although increased concentrations of growth hormone and glucagon can induce hyperglycemia, they are not the primary and the main reason for the GI intolerance in uremia (see ref. 65,88).

Increased concentrations of tumor-necrosis factor, interleukin-1 and interleukin-6 are found in pre-dialysis and dialysis patients (149). Inhibited RME may contribute to the elevation of these cytokines in uremia.

Significant increase in serum α_2 -macroglobulin has been observed at the end and early post-dialysis periods regardless of the membrane used (150). Increased levels of insulin after dialysis have been found as well (151). Impairment of RME during the hemodialysis session (discussed before) may be one of the reasons for these elevations of α_2 -macroglobulin and insulin. Tissue sensitivity to insulin increases after dialysis (100). Compared to the pre-dialysis period, after ten weeks of dialysis treatment considerable improvement of insulin clearance has been reported (88). Similar levels of growth hormone and glucagon have been observed before and after dialysis. Normalization of the glycemic reaction to glucagon after dialysis has been also found (65,88). It seems that the time of measurements influences the concentrations of the studied ligands.

CONCLUSION

- The variety of factors inhibiting RME in vitro suggests that there might be other clinical examples of acquired inhibition of RME, but there is no other case, except uremia, lasting for a such long period of time and in which the factors inhibiting RME in vitro are so fully presented. The hypothesis presented herein may give a key not only to the pathogenesis of uremic syndrome, but may help in finding answers to some general questions concerning RME. It may also contribute to further improvement of the treatment of patients with end-stage renal disease.

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Received for publication 25 June 1993

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