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A Study on Morphology and Function

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Abstract

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This study focused on the characterization of the lysosomal and endosomal structures and their function in rat heart muscle cells. Since controversial data has been published on the involvement of lysosomes during myocardial ischemia and necrosis, special emphasis was put on the function of lysosomal and endosomal structures after isoproterenol-induced infarct-like necrosis both in vivo and in vitro.

High-uptake lysosomal enzymes were shown to bind to membrane fractions from rat heart muscle by mannose 6-phosphate recognition. Various parts of the heart muscle showed no apparent differences in their binding efficiency. However, in contrast to the results from other tissues after subcellular fractionation, myocardial sarcolemmal fraction showed a relatively high specific binding of the ligands, suggesting that the endocytic route is important in the delivery of the lysosomal enzymes to lysosomes.

The morphology study showed that, in fact, the bulk of the cation independent mannose 6-phosphate receptor (CI-MPR) is located in large membraneous structures very similar to the prelysosomes recently characterized by Griffiths et al. (1988). Only low amounts of CI-MPR were found in derivatives of the sarcolemma and in the trans-Golgi network (TGN). Cathepsin L and lgp120 were found in CI-MPR-negative structures designated as lysosomes as well as in prelysosomes together with CI-MPR. Prelysosomes were often associated with autophagy.

Infarct-like myocardial necrosis induced by a single injection of a large dose of isoproterenol caused a dramatic change in CI-MPR distribution. During the early course of myocardial cell injury, well before irreversible damage and the inflammatory reaction, the bulk of the CI-MPR, i.e. the late endosomal structures, fragmented and migrated towards the cellular periphery, whereas the distribution of lysosomes was unchanged. Lysosomes seemed, however, to aggregate in larger structures but showed no change in structural latency. Late endosomal fragmentation and migration towards the periphery was also reproduced in vitro by isoproterenol treatment and shown to be microtubule-dependent. These phenomena are most probably due to the lowered cytoplasmic pH during infarct-like myocardial necrosis since after isoproterenol-treatment the myocyte cytoplasm was acidified and similar results were produced by mere acidification of the cytoplasm.

Key words: Isoproterenol; mannose 6-phosphate receptor; prelysosomes; endocytosis.

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List of original publications

This thesis is based on the following original articles, which will be referred to by their Roman numerals:

I. Marjomäki, V. S. & Salminen, A. 1987: Characteristics of lysosomal phosphomannosyl-enzyme receptors in the rat heart. - Basic Res. Cardiol. 82:252-260.

II. Marjomäki, V., Huovila, A., Surkka, M., Jokinen, I., & Salminen, A. 1990: Lysosomal trafficking in rat cardiac myocytes. - J. Histochem. Cytochem. 38 (8): 1155-1164.

III. Marjomäki, V., Ritamäki, V & Gruenberg, J. : Isoproterenol-induced redistribution of endosomes in cardiac myocytes (revised manuscript submitted to Amer. J. Physiol.).

Abbreviations

ADP	adenosine diphosphate
arf	ADP-ribosylation factor
ß-GA	ß-glucosaminidase
BCECF/AM	(acetoxymethyl ester of indicator 2´-7´-bis(carboxyethyl)-5(6)-carboxyfluorescein
cat-L	cathepsin L
CI-MPR	cation independent mannose 6-phosphate receptor
CD-MPR	cation dependent mannose 6-phosphate receptor
CURL	compartment of uncoupling of receptor and ligand
DAMP	3-(2,4-dinintroanilino)-3´-amino-N-methyl-dipropylamine
DMEM	Dulbecco´s modified Eagle medium
ER	endoplasmic reticulum
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
GDI	GDP dissociation inhibitor
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HRP	horseradish peroxidase
IGF-II	insulin-like growth factor II
LAP	lysosomal acid phosphatase
L-enz	lysosomal enzymes
lgp	lysosomal membrane glycoprotein
MPR	mannose 6-phosphate receptor
M6P	mannose 6-phosphate
NSF	N-ethylmaleimide sensitive factor
PBS	phosphate-buffered saline
Rab	rat protein from bovine brain
RER	rough endoplasmic reticulum
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SR	sarcoplasmic reticulum
TGN	trans-Golgi network

Contents

1 INTRODUCTION

In the late 1950s several enzymes with a low pH optimum were found to reside in distinct organelles that were termed lysosomes (de Duve 1959). Later, especially during the last decade, various other organelles along the biosynthetic and endocytic pathways as well as those involved in autophagocytosis were also shown to contain lysosomal enzymes (Griffiths et al. 1988, Gruenberg et al. 1989, Ludwig et al. 1991, Tooze et al. 1990). It was thus gradually realised that lysosomes and lysosomal enzymes could not be studied without considering the complex network of these interconnected pathways. In addition, the receptors responsible for the delivery of lysosomal enzymes to lysosomes were characterized and the prelysosomes containing the bulk of these receptors were discovered (Griffiths et al. 1988, Hoflack & Kornfeld 1985a, Sahagian et al. 1981). More recently, new information has been gained about the routing of lysosomal membrane glycoproteins to lysosomes. The different components of the general vesicle-mediated transport machinery have now also been identified and characterized, including the GTP-binding proteins, the N-ethylmaleimide sensitive factor (NSF) and associated proteins (SNAPs) as well as coat proteins (see review by Pryer et al. 1992).

After the first demonstrations that the lysosomes may leak lysosomal enzymes to the cytoplasm due to various agents that affect the permeability of the lipoprotein membrane, numerous studies followed that suggested lysosome rupture also during cardiac ischemia. These studies led to the development of the "lysosomal hypothesis" according to which the lowered pH in the myocyte cytosol together with the permeability changes of the lysosomal membrane cause increased breakdown of the cellular constituents and contribute to cell death (Wildenthal 1978). Later, the focus was put on the early events before irreversible damage. However, a lot of contradictory data accumulated over the involvement of lysosomes during myocardial ischemia. Technical advances, e.g. the use of cryoelectron microscopy for immunola-

belling and the availability of antibodies and other markers for various cellular compartments as well as the incredible increase of the overall knowledge of the organization of cellular membranes have only very recently also made it possible to study in more detail the lysosomal system and related structures in cardiomyocytes.

This thesis concentrated on characterizing these structures in rat cardiac myocytes both by subcellular fractionation combined with kinetic studies of lysosomal enzyme binding and by immunolabelling lysosomal proteins and their receptor on thin frozen sections. The early events during myocardial cell injury with respect to lysosomes and endosomes were studied after isoproterenol-induced infarct-like necrosis both in vivo and in vitro. The results suggest that the large membranous structures labelling heavily with CI-MPR and significantly with lysosomal membrane proteins and lysosomal enzymes function as prelysosomes in cardiac myocytes. During the early course of cell injury prelysosomes are fragmented and the fragments are shifted towards the periphery in a microtubule-dependent manner due to the lowered cytosolic pH, while the lysosomes remain intact.

2 Review of the literature

2.1 The lysosomal system

Lysosomes were first discovered by de Duve and collaborators in the late 1950s on the basis of their studies of acid phosphatase in rat liver (de Duve & Beaufay 1959). The term lysosome was introduced after the observation that five distinct acid hydrolases with different specificities were associated together within a distinct group of subcellular particles in rat liver (de Duve 1963, de Duve & Beaufay 1959). Since then, more than 50 different acid hydrolases have been discovered that are capable of degrading most biological macromolecules. The early description of lysosomes was that, in addition to containing a set of hydrolases with a low pH optimum, they are spherical in shape, having a mean diameter of 0.4 µm and an average density of 1.15 (de Duve 1959). The structure-linked latency maintained by a lipoprotein membrane was also considered to be one of the fundamental characteristics of lysosomes. If latency was lost due to permeability changes in the limiting membrane, the leaked acid hydrolases might potentially cause cellular injury and lead to cell death ("lysosomal hypothesis " or "suicide-bag" theory).

On the basis of centrifugation studies using acid phosphatase as a marker lysosomes were shown to be an individual group of organelles, distinct from mitochondria and microsomes (Hers et al. 1951, Schneider & Hogeboom 1950). Lysosomes were also defined by cell fractionation in sucrose density gradients, where the lysosomes exhibited unimodal distribution (Beaufay 1969). However, later, when shallow Percoll density gradients were used, lysosomal marker enzymes showed bimodal distribution (Pertoft et al. 1978, Rome et al. 1979a). The low-density peak was localized in the region where endosomes, plasma membrane and Golgi subfractions normally fractionated (Rome et al. 1979a), while the bulk of the enzyme activity fractionated in the high density peak.

In the early morphological studies acid phosphatase activity was often used as a cytochemical marker to localize lysosomes. Hence the term "acid phosphatase positive granules" was widely used (Novikoff 1963). It soon became clear, however, that a simple morphologic definition was too limited due to the variations in the shape of lysosomal structures and their contents. The polymorphistic nature of lysosomes and their involvement in the intracellular digestion of endogenous and exogenous material led to the generation of various terms to describe the different aspects of lysosomes (multivesicular bodies, residual bodies, primary and secondary lysosomes, autophagosomes etc.). It was thus important to define lysosomes by functional criteria in addition to their morphology or physical characteristics.

During the last decade a lot of data has accumulated on the biosynthetic route of lysosomal enzymes, lysosomal membrane proteins and their receptors as well as their close connections to both the endocytic route and autophagocytosis events (Gordon et al. 1992, Gordon & Seglen 1988, Gruenberg & Howell 1989, Kornfeld & Mellman 1989, Tooze et al. 1990). In this complex organization the acid phosphatase-positive dense granules seem rather marginal and inactive. However, as is obvious from the severe nature of various lysosomal storage diseases and from studies with agents that increase the permeability of the lysosomal membrane, functional and latent lysosomes are essential for life (Kornfeld 1985). According to the current view, lysosomes may be defined as terminal digestive compartments in the endocytic pathway containing various mature forms of acid hydrolases.

2.1.1. The biosynthetic route of lysosomal enzymes

Proteins destined for the Golgi, lysosomes, and the nuclear or plasma membrane are synthesized on membrane-bound ribosomes (Walter et al. 1984). The signal sequence first forms a complex with a cytosolic ribonucleoprotein, a signal recognition particle (Walter & Blobel 1982), which then binds to a receptor on the membrane of the rough endoplasmic reticulum (RER) (Meyer et al. 1982). The forming polypeptide is then inserted into the lumen of the RER. A large preformed oligosaccharide (three glucose, nine mannose, and two N-acetylglucosamine residues) is transferred from a lipid-linked intermediate to the nascent polypeptide. Trimming of the oligosaccharides starts already in the RER where three glucoses are excised by glucosidases I (Atkinson & Lee 1984) and II (Hubbard & Robbins 1979) and one of the mannose residues by alpha-mannosidase in the ER (Buendia et al. 1990)

The first feature unique to lysosomal enzymes is revealed shortly after their export from the RER ; namely, a selective recognition marker is added on their oligosaccharide side chains. A recognition marker was first proposed by Hickman and Neufeld in 1972 on the basis of their work on human diseased I-cell fibroblasts (Hickman & Neufeld 1972). Later in 1977, Kaplan et al. showed that the marker is mannose 6-phosphate. Several groups showed later that mannose 6-phosphate marker is synthesized by the sequential action of two Golgi enzymes. Firstly, N-acetylglucosaminyl-phosphotranferase transfers N-

acetylglucosaminyl 1-phosphate to the C-6 hydroxyl position of selected mannose residues in the oligosaccharide to produce a phosphodiester intermediate (Reitman & Kornfeld 1981). This enzyme proved to be deficient in I-cell fibroblasts, which were then unable to synthesize mannose 6-phosphate marker and were secreting lysosomal enzymes to the extracellular medium. After phosphorylation of the mannose residues, N-acetylglucosamine 1-phosphodiester alpha-N-acetylglucosaminidase removes the covering N-acetylglucosamine and exposes the recognition signal generating the active phosphomonoester (Varki & Kornfeld 1981).

Candidates for the initial phosphorylation site are the transitional elements of the ER (Rizzolo & Kornfeld 1988) and the extensive membrane network between the transitional elements and cis side of the Golgi. This structure has been called the intermediate compartment (Saraste & Kuismanen 1984, Schweizer et al. 1990, Tooze et al. 1988), salvage compartment (Warren 1987) and cis-Golgi network (Mellman & Simons 1992). This compartment may be the major site for sorting newly synthesized proteins. It has been suggested that it is the site where the luminal ER proteins are retrieved back to ER by a receptor-mediated mechanism (Pelham 1989, Warren 1987). By attaching the ER retention signal (KDEL) to the lysosomal enzyme cathepsin D, Pelham showed that phosphorylated cathepsin D, with almost all the phosphorylated oligosaccharides being phoshodiesters, accumulated in the ER (Pelham 1988). This, together with the finding that low temperature inhibited the phosphorylation of cathepsin D, strongly suggests that phosphorylation occurs in a pre-Golgi or intermediate compartment (Pelham 1988).

In the Golgi the next step is the binding of phosphorylated lysosomal enzymes to the receptors that recognize the mannose 6-phosphate residues on their oligosaccharide side chains. The binding requires that the mannose 6-phosphate residues are uncovered, which means that the most proximal site for the binding is the mid-Golgi where the uncovering enzyme is localized (Varki & Kornfeld 1981). In addition to the phosphorylated high-mannose units, several of the oligosaccharides in lysosomal enzymes are processed further, in a manner similar to that of the secretory and membrane glycoproteins, building up galactose and sialic acid residues in the hybrid and complex type oligosaccharides (Vladutiu 1983). Lysosomal enzymes are therefore thought to pass the trans-Golgi cisternae en route to lysosomes (Fedde & Sly 1985). Goldberg and Kornfeld showed that bulk of the receptor-bound phosphorylated oligosaccharides could be fractionated in the light Golgi subfractions close to the galactosyltransferase activity (Goldberg & Kornfeld 1983). This together with immunocytochemical data on double labellings with CI-MPR and lysosomal enzymes suggests that most lysosomal enzymes traverse the entire Golgi to the TGN en route to lysosomes (Fedde & Sly 1985, Geuze et al. 1984, Goldberg & Kornfeld 1983, Griffiths et al. 1988, Griffiths & Simons 1986)

The segregation of lysosomal enzymes from other soluble proteins to be secreted starts after their binding to the receptors. Double and triple immunolabellings in electron microscopy have suggested that the segregation occurs in the trans-Golgi network (Griffiths et al. 1988). However, Geuze and coworkers showed that albumin colocalized with CI-MPR and lysosomal enzymes still in the coated buds of the trans-Golgi reticulum (Geuze et al. 1985). Theoretically

the most distal part for segregation is the plasma membrane.

From the trans-Golgi network, packaged lysosomal enzyme-receptor complexes are delivered to the prelysosomal compartment (late endosomes) via clathrin-coated vesicles (Fig. 1). These vesicles contain clathrin-associated proteins that are distinct from those present in the plasma membrane-derived clathrin-coated vesicles (see review by (Robinson 1992). Once in the prelysosomes the receptor-enzyme complexes dissociate due to the low luminal pH. A small proportion of the lysosomal enzymes, usually 5-20%, is secreted before their delivery to lysosomes (Willingham et al. 1981). These enzymes may bind to MPRs on the cell surface and be internalized and delivered to the lysosomes. Very recently Ludvig et al. (Ludwig et al. 1991) showed that, in fact, a significant portion of the phosphorylated lysosomal enzymes is found in the early endosome. These enzymes most probably come directly from the Golgi but leave the early endosome with the bulk flow or as bound ligands to the late endosomes.

2.1.2. Mannose 6-phosphate receptors

So far, two distinct mannose 6-phosphate receptors, that selectively recognize mannose 6-phosphate residues on oligosaccharide side chains have been identified (Hoflack & Kornfeld 1985a, Sahagian et al. 1981). Both receptors have their strongest affinities for extended oligosaccharide structures that include the Man α 1,2 Man sequence. However, these receptors differ both immunologically, in their dependence on divalent cations, and in their pH optimum for lysosomal enzyme binding (Hoflack et al. 1987, Hoflack & Kornfeld 1985b, Sahagian, et al. 1981). The cation-independent mannose 6-phosphate receptor has been extensively characterized (see review by Kornfeld 1992). It is a 270 kDa integral membrane glycoprotein that was first purified by Sahagian et al. from bovine liver (Sahagian et al. 1981). The 46 kDa mannose 6-phosphate receptor is also an integral membrane protein but it needs the presence of divalent cations for the binding of lysosomal enzymes, hence the name cation-dependent mannose 6-phosphate receptor (CD-MPR) (Hoflack & Kornfeld 1985b). The CD-MPR was first purified from mouse $P388D_1$ macrophage-like cells that do not synthesize the CI-MPR (Hoflack & Kornfeld 1985b).
Recently cDNAs for the CI-MPR and CD-MPR have been cloned from bovine tissue and later also from other sources (Dahms et al. 1987, Lobel et al. 1987). The structures of the receptors are now relatively well known (Dahms et al. 1987, Lobel et al. 1987, Lobel et al. 1989). The structural similarity of the two MPRs implies that the two receptors are derived from a common ancestor. The CD-MPR exists as a monomer, dimer or tetramer depending on the ambient pH, receptor concentration and temperature, while the CI-MPR exists as a monomer or oligomer (Hoflack & Kornfeld 1985b, Kornfeld 1992). It seems that the CD-MPR can undergo these changes while cycling between different cellular compartments.

The intracellular distribution of the CI-MPR is now well established (Table 1). Early biochemical studies on the binding of lysosomal enzymes

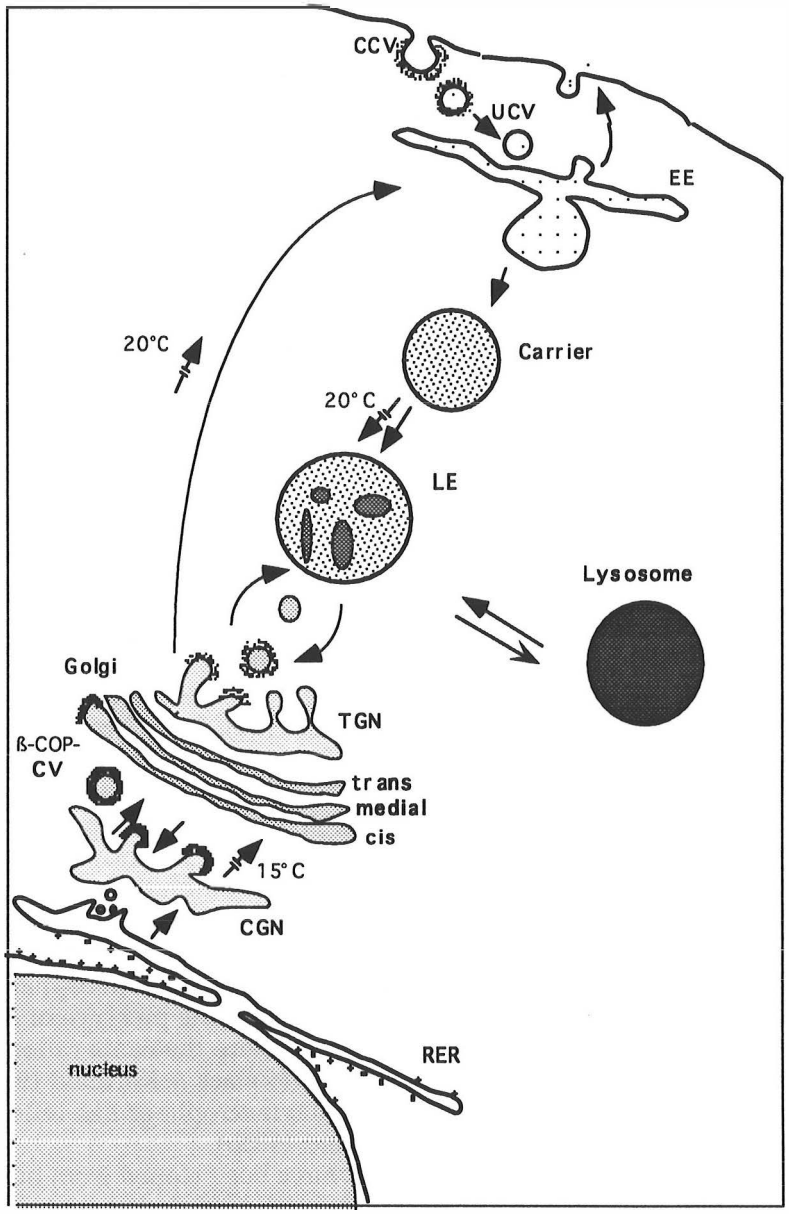

Fig. 1 A section of a non-polarized cell showing structures along the biosynthetic and endocytic pathways.
ß-cop CV = ß-cop coated vesicle ; CCV = clathrin coated vesicle ; CGN = cis-Golgi network ; EE = early endosome ; LE = late endosome ; PLC = prelysosomal compartment ; RER = rough endoplasmic reticulum ; TGN = trans-Golgi network ; UCV = uncoated vesicle

Table 1. Characteristics of lysosomes and endosomes

	morphology [1]	pH [2]	characteristic proteins [3]
early endosomes	tubulo-vesicular	6 - 6.3	rab4,5 CI-MPR + lgp´s + L-enz +
late endosomes	high content of internal membranes	≈ 5.5	rab7,9 CI-MPR +++ lgp´s ++ L-enz ++
lysosomes	spherical, electron dense	≈ 4.5	lgp´s +++ L-enz +++

[1] (Gruenberg & Howell 1989)

[2] (Mellman et al. 1986)

[3] (Griffiths et al. 1988, 1990 ; Ludvig et al. 1991 ; Gruenberg & Clague 1993)

proposed that the bulk of the MPR is localized in intracellular membranes (Fischer et al. 1980b). Various immunocytochemical studies have since shown later that about 10% of the CI-MPR is present on the plasma membrane (Bleekemolen et al. 1988, Geuze et al. 1988, Griffiths et al. 1990, Klumperman et al. 1993). From the intracellular pool the bulk of the CI-MPR is localized to endosomes, especially late endosomes (Geuze et al. 1988, Griffiths et al. 1988, Griffiths et al. 1990, Klumperman, et al. 1993). The CI-MPR is present in low amounts in the Golgi , predominantly the TGN, and in coated vesicles (Geuze et al. 1984, 1988; Griffiths et al. 1988, 1990) , whereas it was shown to be absent from lysosomes (Brown et al. 1986, Griffiths, et al. 1988, Sahagian & Neufeld 1983, Willingham et al. 1983) . Morphological studies on the CD-MPR have not been as extensive, but it seems that both receptors exhibit a similar distribution (Bleekemolen et al. 1988, Klumperman et al. 1993).

There appears to be a single pool of CI-MPR molecules that participate both in lysosomal enzyme sorting in the Golgi and in endocytosis at the cell surface. Cell-surface CI-MPR labeled with [^{3}H]galactose at 4°C were shown to reach the Golgi since the MPRs gained sialic acid residues after the cells were warmed to 37°C (Duncan & Kornfeld 1988). Early biochemical studies showed that the MPR has to dissociate from its ligand and participate in many rounds of transport in order to internalize the high amount of ligands detected (Gonzalez-Noriega et al. 1980, Rome et al. 1979b). Receptor-ligand complexes were shown to dissociate below pH 5.7 (Gonzalez-Noriega et al. 1980, Sahagian et al. 1981). Weak bases, e.g. ammonium chloride, and ionophores, e.g. monensin, that raise the pH of acidic organelles above 6, could indeed block the sorting of endogenous lysosomal enzymes and the endocytosis of exogenous lysosomal enzymes (Gonzalez-Noriega et al. 1980, Hasilik & Neufeld 1980). Since immunocytochemical studies have shown that the CI-MPR is localized mainly in endosomes and not in lysosomes, and furthermore that the pH for the dissociation of the receptor-enzyme complex is 5.7 or lower, the dissocia-

tion most likely occurs in a late endosomal compartment of pH 5.5 or less (Schmid et al. 1989). After dissociation of these complexes the CI-MPRs are then free to recycle back to the Golgi or plasma membrane.

The early studies that suggested the involvement of a receptor in lysosomal enzyme targeting were done with diseased cells from patients suffering from a lysosomal storage disorder. Later endocytosis of exogenous ligands was, however, shown to comprise only about 5-20% of the enzyme targeting, while the bulk of the CI-MPR is involved in intracellular trafficking (Vladutiu & Rattazzi 1979, Willingham et al. 1981). Recently, it was shown that the CD-MPR does not bind its ligands at the neutral pH of the extracellular medium (Hoflack et al. 1987, Stein et al. 1987) . Hence, the CD-MPR may not be involved in the endocytosis of exogenous phosphorylated ligands but might function solely in intracellular traffic (Stein, et al. 1987). It is likely that the phosphorylated hydrolases bound to this receptor dissociate upon their arrival at the plasma membrane and are then released to the extracellular medium. It was shown recently that overexpression of the CD-MPR could induce increased secretion of lysosomal enzymes (Chao et al. 1990). The possible regulation of the secretion of lysosomal enzymes could explain why two mannose 6-phosphate receptors with different binding properties are found in mammalian cells.

Studies with cDNAs for the CI-MPR mutated in the cytoplasmic domain have shown that the cytoplasmic domain contains different signals for rapid endocytosis and efficient lysosomal enzyme sorting (Canfield et al. 1991, Johnson & Kornfeld 1992, Lobel et al. 1989). Moreover, the tyrosine residues at positions 24 and 26 are needed for endocytosis analogous to the findings concerning the LDL receptor (Davis et al. 1987) and influenza virus hemagglutinin (Lazarovits & Roth 1988). Studies on coat proteins named adaptins have suggested that tyrosine residues are needed for the association with plasma membrane derived HA-II adaptors thus allowing rapid endocytosis (Glickman et al. 1989). Golgi-associated HA-I adaptors bind to the cytoplasmic tail of the CI-MPR independent of the tyrosine residues, thus allowing diversion to the prelysosomal sorting compartment (Glickman et al. 1989).

In 1987 Morgan et al. (1987) reported that the sequence of the human insulin-like growth factor II (IGF-II) receptor bears 80% identity to that of the bovine CI-MPR. The CI-MPR, but not the CD-MPR, bears distinct binding sites for mannose 6-phosphate and IGF-II (a nonglycosylated polypeptide hormone) (Braulke et al. 1988, Tong et al. 1988). Murayama et al. showed that the addition of IGF-II to human CI-MPR stimulates a putative trimeric G protein coupled with GTPase activity, and similar results were observed with rat CI-MPR (Murayama et al. 1990, Okamoto et al. 1990). This activation is negatively controlled by lysosomal proteins. However, the coupling of the CI-MPR with the G_i does not affect lysosomal protein binding and endocytosis (Murayama et al. 1990). It still remains to be seen whether G proteins are really activated in vivo and what the putative downstream effectors are.

2.1.3 Lysosomal membrane proteins

The limiting membrane of lysosomes is enriched in heavily glycosylated and sialylated integral membrane proteins (see reviews by Kornfeld & Mellman 1989 and Fukuda 1991). These glycoproteins are also found in lower amounts in endocytic vesicles and in the plasma membrane (Geuze et al. 1988, Griffiths et al. 1988, Lippincott-Schwartz & Fambrough 1986, 1987). Two distinct groups of proteins have been discovered that comprise about 50 % of the total integral membrane proteins in lysosomes, namely lgp-A and lgp-B (see review by Kornfeld & Mellman 1989 ; Marsh et al. 1987). These groups are 65-70% similar and contain only species-specific versions of the same protein (Howe et al. 1988). The lpg-A group contains rat and mouse lgp120, mouse LAMP-1, human lamp-1 and chicken LEP-100, whereas the lgp-B group consists of rat and mouse lgp110 and human lamp-2. All the lgps have a single hydrophobic membrane spanning domain and short cytoplasmic tails of 10-11 residues in length. The cytoplasmic tails of the group lgp-A are identical with each other but only about 50% identical to group lgp-B. The lgps do not contain the mannose 6-phosphate residue and must therefore be targeted to lysosomes by a different mechanism (D'Souza & August 1986, Lippincott-Schwartz & Fambrough 1986). Although the mature lysosomal acid phosphatase (LAP) is a soluble lysosomal enzyme it is also synthesized as an integral membrane protein precursor and transported to lysosomes independent of the mannose 6-phosphate signal (Waheed et al. 1988). LAP does not have any sequence similarity with lgps in the cytoplasmic tail but shows a conserved glycine-tyrosine in about the same relative position.

The route by which the lysosomal membrane proteins reach lysosomes is currently under debate in the literature. Whether lysosomal membrane proteins are delivered to lysosomes via endocytosis from the cell surface or directly from the Golgi remains controversial. The cytoplasmic domain of several lysosomal membrane proteins was shown to contain the information for delivery to lysosomes (Peters et al. 1990, Williams & Fukuda 1990). There is also some evidence that LAP and some other lysosomal membrane proteins are first delivered to the plasma membrane and then directed to lysosomes (Lippincott-Schwartz & Fambrough 1986, Nabi et al. 1991, Peters et al. 1990). On the basis of studies using LEP100 deletion mutants and chimeras of LEP100 and VSV-G protein, Mathews et al. (1992) proposed that the pathway via the cell surface is the major pathway for lysosomal membrane proteins. However, the recent work by Harter and Mellman suggests that rat lgp-A is preferentially delivered to lysosomes intracellularly without moving through the cell surface (Harter & Mellman 1992). Letourneur and Klausner proposed, according to their study on chimeras containing extracellular and transmembrane domains of Tac antigen fused to different cytoplasmic domains, that, in fact, two interchangeable distinct domains exist, namely a di-leucine and a tyrosine motif (Letourneur & Klausner 1992). They propose that the existence of both motifs makes it more probable that the protein is directly sorted into lysosomes.

2.2 The endocytic route

Endosomes are a heterogenous population of endocytic vacuoles through which fluid components, receptors, ligands and their complexes pass en route to lysosomes. The first observation that internalized proteins pass through prelysosomal structures before entering lysosomes was made by Werner Strous in 1964 (Strous 1964). He localized lysosomes with acid phosphatase and simultaneously stained internalized peroxidase in rat kidney epithelium. He discovered that at early time points peroxidase and acid phosphatase remained in separate structures, peroxidase in the peripheral cytoplasm and acid phosphatase in the perinuclear location. Later both peroxidase and acid phosphatase were colocalized in perinuclear vacuoles.

Classically endocytosis has been divided into two types: phagocytosis for large particles and pinocytosis for small soluble particles that enter the cells by virtue of their presence in the fluid (fluid phase pinocytosis) or bind to the membrane first and are then internalized (adsorptive pinocytosis; specific i.e. receptor-mediated or unspecific). Receptor-mediated endocytosis has been intensively studied since the demonstration of the receptor-mediate uptake of low density liporoteins by Goldstein et al. (1979). Endosomes have been visualised using different fluid phase markers, e.g. horseradish proxidase and non-specifically adsorbed markers, e.g. cationized ferritin or physiological ligands, for receptor-mediated endocytosis coupled with suitable markers for light or electron microscopy. The low temperature block (16-20°C) has been instrumental in the dissection of the endocytic pathway between endosomes and lysosomes (Dunn et al. 1980, Marsh et al. 1983). Originally, the difficulty was that there were no obvious unique morphological characteristics for endosomes. However, it is becoming clear that early endosomes, late endosomes and lysosomes exhibit specific characteristics.

Several techniques have now made it possible to separate these structures. Endocytosed material was first observed in the lower density than the bulk of the lysosomal enzymes in self-forming Percoll-gradients, and thus endosomes were found to be lighter than mature lysosomes (Pertoft, et al. 1978). In addition, early and late endosomes can be separated from each other using density gradient centrifugation (Storrie et al. 1984), immuno-isolation (Gruenberg et al. 1989) and free-flow electrophoresis (Schmid et al. 1988).

Endosomes were first observed to contain a low internal pH by Tycko and Maxfield (1982) and Van Renswoude et al. (1982) using intact cells and fluorescein-labelled endocytic markers. The low pH is generated by a proton-ATPase in the endosomal membrane. Recently, a recycling Na^+,K^+ -ATPase has also been proposed to participate in controlling the pH in early endosomes by opposing the action of proton-ATPase (Cain et al. 1989, Fuchs et al. 1989).

Endocytic tracers, like HRP, cationized ferritin and alpha2-macroglobulin are observed within 1 to 2 minutes in uncoated vesicles, i.e. vesicles that have just lost their clathrin coats and are derived from clathrin coated pits (Fig. 1). These vesicles have a neutral pH and do not contain lgp120, whereas within 2 - 5 min the tracers are detected in more acidic peripheral early endosomes, where also small amounts of lysosomal enzymes, lgp120 and MPRs are de-

tected (Geuze et al. 1988, Griffiths et al. 1988, 1990 ; Gruenberg et al. 1989). Early endosomes have been also referred to as CURL (compartment of uncoupling of receptor and ligand, (Geuze et al. 1983) and sorting endosomes (Salzman & Maxfield 1989). After 15 - 30 min of exposure endocytic tracers are found in the perinuclear location in late endosomes where the bulk of the cellular MPRs and high amounts of lysosomal enzymes and lysosomal membrane glycoproteins are found (Geuze et al. 1988, Griffiths et al. 1990)

Early endosomes have an internal pH of 6 - 6.3 (Schmid et al. 1989) that is sufficiently acidic for some receptor-ligand complexes to dissociate, allowing receptors to recycle back to the plasma membrane (LDL-receptor, α_2-macroglobulin receptor). By contrast, in the internal pH of 5.5 or less in late endosomes, lysosomal enzymes are able to dissociate from their receptors. In addition to the differences in morphology (see review by Gruenberg & Howell 1989) and ambient pH (see review by Mellman et al. 1986) early and late endosomes also differ in their protein composition (Table I). Late endosomes contain rab7 and high amounts of CI-MPR in addition to lysosomal enzymes and lgps (Chavrier et al. 1990, Griffiths et al. 1988). Later structures that are more acidic than late endosomes (pH about 4.5), devoid of CI-MPR, but label heavily with lysosomal enzymes, endocytic tracers after longer incubations and lgps have been designated as lysosomes (Geuze et al. 1988, Griffiths et al. 1988, Mellman et al. 1986).

The transport vesicles that leave the TGN carrying MPRs and newly synthesized lysosomal enzymes most likely fuse with late endosomes, but it is possible that a fraction of those vesicles fuse first with early endosomes and are then delivered to late endosomes (Klumperman et al. 1993, Ludwig et al. 1991).There is not much data on how lysosomal enzymes finally reach lysosomes due to the difficulties of specifically inhibiting this step.

Nonetheless, two theories exist which differ in their view of endosomes as transient (the maturation model) or stationary organelles (the vesicle shuttle or stable compartment model). According to the maturation model, clathrin coated vesicles from the plasma membrane quickly lose their clathrin coat and fuse together to form an early endosome (see review by Helenius et al. 1983), This endosome then gradually acidifies and matures into a late endosome which fuses with Golgi-derived vesicles bearing newly-synthesized lysosomal enzymes (so called primary lysosomes) forming a functional lysosome or secondary lysosome as was proposed by de Duve and colleagues in the late fifties (de Duve 1959). The difference between early and late endosomes would then be merely the difference in their location and the fact that early endosomes would more readily fuse with peripheral structures and late endosomes with, e.g. Golgi-derived perinuclear structures. However, some biochemical differences between early and late endosomes have been proposed since, being transient organelles, different molecules should be introduced transiently into endosomes and then recycled back to the plasma membrane, Golgi, etc.

According to the stable compartment model, early and late endosomes are pre-existing organelles, located in peripheral and perinuclear regions respectively, and are connected to each other via recycling vesicles (see review by Griffiths & Gruenberg 1991). This model was originally proposed by Palade in 1975 (Palade 1975). In favor of the stable compartment model early and late en-

dosomes have been shown to bear characteristic structures (reviewed by Gruenberg & Howell 1989)). In addition, transport from early to late endosomes is discontinuous and mediated by multivesicular-type carrier vesicles (Gruenberg et al. 1989). Griffiths et al. (1989) showed that HRP endocytosed by BHK cells was detected already within 2 minutes in early endosomes where the concentration increased with no change in the size of the compartment. After 15-20 min of continuous internalization, HRP was observed in perinuclear late endosomes where the concentration of HRP increased with time. Two-dimensional gel-analysis of subcellular fractions have shown differences in polypeptide compositions between coated pits, endosomes and lysosomes as well as between early endosomes and later stages (Beaumelle et al. 1990, Schmid et al. 1988). In addition, the in vitro fusion assays as well as localization studies of small ras-like GTP-binding proteins, that are involved in the regulation of membrane traffic, have shown distinct differences between these endosomal compartments (see review by Pryer et al. 1992).

Recently, based on the observations of fluorescently labeled macropinosomes, a combination of these two theories has been suggested to occur in macrophages (Racoosin & Swanson 1993). The early steps of macropinosome endocytosis showed features of maturation gaining gradually rab7 or lgp-A label, whereas later the vesicles fused with a fluorescently labeled pre-existing tubular lysosome compartment.

2.3 Molecular mechanisms of vesicle-mediated transport

Secretory proteins and proteins destined for cellular organelles are tranported from their site of synthesis on the RER by transfer vesicles that shuttle between organelles. Vesicles bud from a donor compartment leaving the resident proteins behind, identify the target compartment and fuse with it in a very specific manner. Different components of the transport machinery have now been identified and characterized, including GTP-binding proteins, the N-ethylmaleimide sensitive factor (NSF) and associated proteins (SNAPs) as well as coat proteins.

Two types of coated vesicles of distinct coat compositions have been characterized, namely clathrin-coated and COP-coated vesicles (Kreis 1992, Robinson 1992) . Clathrin-coated vesicles that bud from the plasma membrane and from the TGN differ in their associated adaptins that are thought to provide the molecular basis for protein sorting (Robinson 1992). The adaptins form adaptor complexes that contain either α - and β - adaptins (HAII) or β- and γ -adaptins (HAI), being specific for plasma membrane and TGN-derived clathrin-coated vesicles, respectively. Adaptors have been shown to bind to the cytoplasmic tails of LDL and CI-MPR receptors suggesting that they mediate the selectivity of coated vesicles for certain membrane proteins (Glickman et al. 1989). While clathrin coats are involved in steps that require selection of the cargo, the COP-coated vesicles (containing α,β,γ and δ -cop's) seem to mediate transport between the Golgi compartments by a bulk flow.

NSF and the soluble NSF attachment proteins (SNAPs) have been proposed to be the components of the fusion machinery common to all eucaryotic cells (Rothman & Orci 1992). It seems likely that NSF, SNAPs and the receptor, SNARE, on the membrane form a fusion particle which regulates the fusion by controlled assembly and disassembly (Rothman & Orci 1992). The fact that SNAREs were purified both on the vesicles and on the target membrane suggest that NSF/SNAPs mediate targeting (Sollner et al. 1993)

The involvement of small GTP-binding proteins in vesicular transport was first proposed by Novick et al. who studied the yeast SEC4 gene product (Salminen & Novick 1987). They observed that temperature-sensitive SEC4 mutations showed an accumulation of invertase-containing secretory vesicles at the restrictive temperature. Later, the SEC4 gene product was shown to localize on the surface of the secretory vesicle (Goud et al. 1988) and bind and hydrolase GTP (Kabcenell et al. 1990). Another GTP-binding yeast gene product, YPT1, was shown to be important in ER to Golgi transport (Schmitt et al. 1988, Segev et al. 1988). Studies with SEC4 and YPT1 strongly suggested that they function in vesicle targeting and fusion events, which led to the search of mammalian counterparts for these proteins.

So far, more than twenty different SEC4 and YPT1-related gene products have been discovered (see reviews by Bourne et al. 1991 and Pryer et al. 1992) and have been termed a rab-proteins (Ras-like proteins from rat brain). Rab-proteins are 21-25 kDa and about 30% identical to the proto-oncogene product Ras. Another class of small GTP binding proteins, Arf proteins (ADP-ribosylation factor) also function in intracellular transport (Taylor & Melançon 1991). Different rab proteins have been localized to distinct organelles along the biosynthetic and endocytic pathways which also suggests a role for them in vesicular transport (see review by Pryer et al. 1992). The organelle-specificity of rab-proteins was shown by Chavrier and coworkers to depend on the 34 most C-terminal residues (Chavrier et al. 1991). Another line of evidence comes from the in vitro assays that reconstitute the series of steps in intracellular transport and show that GTP is essential for those transport steps and that yeast mutant fractions are defective in reconstituting the steps see review by (Pryer et al. 1992).

GTP-binding rab and arf proteins are cytosolic proteins that need post-translational modifications for their functional activity and association with intracellular organelles. Rab proteins are modified by a geranylgeranyl(C_{20})-prenyl moiety on C-terminal cysteine residues, whereas arf proteins acquire N-terminal myristate. Rab proteins are extracted from the membranes by a Rab GDI that was originally purified as a GDP dissociation inhibitor for rab3a (Sasaki et al. 1990). Since then, GDI has been shown to interact with Sec4p as well as many other rab proteins (Ullrich et al. 1993). This association appears to be regulated by GDI phosphorylation (Steele-Mortimer et al. submitted). The current view is that GDI functions as an escort protein during rab protein recycling back to the donor membrane.

In addition to vesicle targeting and fusion vesicle formation was also shown to involve small GTP-binding proteins, and recently also the action of the large heterotrimeric G proteins (Melançon et al. 1987 ; see also review by Barr et al. 1992) which are known mediators in signal transduction at the

plasma membrane (Gilman 1987). These pertussis-toxin sensitive G-proteins were detected in intracellular membranes, e.g. the Golgi complex (Ercolani et al. 1990). Recent evidence suggests that trimeric G-proteins are also involved in endosome fusion events (Colombo et al. 1992).

2.4 Cardiac myocytes

2.4.1 The lysosomal system in cardiac myocytes

Cardiac muscle tissue was shown to contain lysosomes and biochemically detectable lysosomal enzyme activity fairly late. The low interest previously shown in studying lysosomes in cardiac muscle was mostly due to the notion that myocytes contained few lysosomes and that non-myocytic cells contributed to most of the lysosomal enzyme activity in heart muscle tissue (Borgers et al. 1971). The earliest anatomical evidence of lysosomal structures in heart muscle cells was based on lysosomal enzyme histochemistry localizing e.g. aryl sulphatase as well as ß-glucuronidase and acid phosphatase activity in perinuclear ovoid granules (Abraham et al. 1967, Hibbs et al. 1965b, Romeo et al. 1966).

Later, Topping and Travis (1974) suggested, on the basis of their ultrastructural study, that two distinct morphological types of primary lysosomes exist in myocardial cells: those with granular matrices and those with membranous matrices. The cytochemical study showed activity for acid phosphatase, aryl sulphatase and cathepsin B either amidst the cone-shaped cluster of mitochondria at the juxtanulear zone or in the mitochondrial rows among the myofilament bundles. They proposed that the structures far from the nucleus would evolve directly from the smooth ER, whereas those in the perinuclear area would come from the Golgi.

Some alterations were reported for lysosomal structures in diseased states, e.g. the increased number and size of lysosomes, and increased number of apparent autophagic vesicles (Ferrans et al. 1972, Hibbs et al. 1965a, Van Noorden et al. 1971, Wheat 1965). Lipofuscin granules were also reported to accumulate during normal aging and in several disease states (Fawcett & Mcnutt 1969, Hibbs et al. 1965a). However, it was difficult to link certain changes to particular diseases and show a correlation between such changes and the severity of a particular disease. Thus, it was widely thought that changes in lysosomal structures show only a nonspecific response to cardiac stress or injury.

Technical advances such as the production of specific antibodies against lysosomal enzymes as well as an improved method for isolating cardiac lysosomes and the ability to identify separate populations of lysosome-related structures have provided better tools to studying the lysosomal structures in the heart (Franson et al. 1972, Poole et al. 1972, Smith & Bird 1975). Also, progress in understanding the lysosomal organization in general and the presence of markers

specific to the different cellular compartments has only very recently facilitated the study of lysosomal behaviour in various pathological conditions.

2.4.2 Myocardial ischemia and necrosis

Ischemia results from an imbalance between the myocardial demand for, and the vascular supply of, coronary blood (see review by Hearse 1980). Ischemia creates a deficit of oxygen, substrates and energy in the tissue but it also results in a reduced capacity to remove potentially toxic metabolites such as lactate, carbon dioxide and protons. Ischemia may also arise under normal coronary flow conditions during an increased work or energy demand on the heart.

Myocardial ischemia has classically been studied using mechanical occlusion of the main coronary artery in the dog (Jennings 1969), but other methods, such as isolated perfused rat heart under reduced oxygen tension or catecholamine administration in vivo (Ferrans et al. 1969), have been used. The method using foetal mouse hearts in culture has the advantage that the experimental conditions are easily controlled (Ingwall et al. 1975).

The acute effects of myocardial ischemia first accompany reversible alterations which may lead to irreversible damage if the ischemic period is extended over the "point-of-no-recovery". The severity of experimental ischemia and the time course of the appearance of irreversible alterations depends to some extent on the method used. The occlusion of the coronary artery causes irreversible injury to a few cells after only 20-30 min. There is a gradual shift from reversible to irreversible ischemic damage as more and more cells pass the point-of-no-recovery. Even after 45 min of coronary occlusion 35-66% of the cells remain viable (Jennings 1969). After catecholamine administration the reversible alterations seem to occur as rapidly as after coronary ligation whereas necrosis seems to develop more slowly (Ferrans et al. 1969).

Soon after the onset of ischemia, supplies of glycogen and high energy phosphates are depleted from the tissue (Jennings 1969). As as result of anaerobic glycolysis, increased concentrations of hydrogen ions and lactate accumulate in the tissue and eventually leak from the cells. Electron microscopy of reversibly injured cells shows clumping of the nucleoplasm and the swelling of mitochondria (Brachfeld 1969).

The irreversible damage is most easily seen in mitochondria, which show disorganized cristae and amorphous densities, most probably due to excessive calcium accumulation (Jennings & Ganote 1974). Breaks in the sarcolemma lead eventually to the leakage of macromolecules, e.g. creatine kinase, which is one the biochemical markers used for ischemic damage (Hearse 1980). Restoration of coronary blood flow during reversible damage leads to increased autophagocytosis of damaged cellular constituents, whereas reperfusion during irreversible damage does not prevent cell death (Ingwall et al. 1975, Ridout et al. 1986b). During 12 to 24 hours of ischemia, the histological evidence of necrosis appears concomitantly with the beginning of a massive infiltration of inflammatory cells into the tissue (Jennings 1969). Later, phagocytosis and repair of the damage takes place.

Recently, free radicals and active oxygen species have been proposed to be the key elements contributing to ischemic injury (Mc Cord 1988). Several metabolic pathways may lead to an increase in the production of oxygen-free radicals during ischemia, including an increase in xanthine and xanthine oxidase, and a decrease in oxygen free radical scavengers, such as superoxide dismutase and glutathione proxidase (Jennings & Reimer 1981). Free radicals exert their cytotoxic effect by causing lipid peroxidation of membrane phospholipids, which can result in membrane permeability changes (Mc Cord 1988). Kalra et al. (1989) observed in vitro some release of lysosomal enzymes from lysosomal fractions using exogenous oxygen free radicals (Kalra et al. 1989). The release of the enzymes could be prevented by superoxide dismutase

Circulating levels of natural catecholamines like noradrenaline and adrenaline are known to increase markedly during stress and to cause myocardial hypertrophy and myocardial cell damage as well as cardiomyopathy (Rona 1985, Rona et al. 1959). It is also known that during myocardial ischemia high amounts of natural catecholamines are released from sympathetic nerve terminals into the extracellular space of the ischemic area reaching micromolar concentrations capable of producing myocardial necrosis even in the nonischemic areas in the heart (Schömig 1988). Catecholamine-induced myocardial necrosis appears to be multifactorial (see review by Rona 1985). In stressful situations catecholamines act primarily through ß-adrenergic receptors. Beta-adrenergic stimulation dilates blood capillaries, activates contraction of the myocytes through excessive calcium influx and increases oxygen consumption, which finally leads to a relative hypoxia and anaerobic metabolism. Low levels of catecholamines increase cAMP production by activating the ß-adrenoceptor -linked adenylate cyclase system (Akimoto et al. 1990). High levels of catecholamines and their accumulation in cardiac myocytes, however, cause desensitization of the adenylate cyclase system and have been shown to increase lactate production and thus exert glycolysis (see Akimoto et al. 1990 and also references therein).

Isoproterenol, a synthetic counterpart of natural catecholamines has been widely used to study infarct-like myocardial necrosis since its cardiotoxicity was first proposed in 1959 by Rona et al. It is heart-specific at doses under 85 mg/kg in rats. Its effects are also dose dependent and reproducible, which makes it ideal for studying experimental myocardial necrosis in vivo. Large doses of isoproterenol induce a fully developed necrosis in the heart apex, the papillary muscles and in the subendocardial portion of the left ventricle (Ciplea & Bock 1976, Ferrans et al. 1964, Rona 1985). This induced myocardial damage is very similar to that caused by coronary artery ligation in rats (Rona et al. 1959). It also closely resembles human myocardial infarction (Ferrans et al. 1964, Rona et al. 1959). Light and electron microscopic observations of cardiac myocytes show the typical changes observed after coronary artery occlusion in the rat, e.g. swelling of mitochondria 30 min after the onset of ischemia, and more severe changes e.g. loss of cristae as well as swollen sarcoplasmic reticulum (SR) after 2 hours (Rona et al. 1959). The cytotoxicity caused by isoproterenol has been shown to be mediated primarily by phospholipase activation through increased calcium influx affecting sarcolemmal membrane permeability and leading to irreversible necrosis (Kondo et al. 1987, Okumura et al. 1983,

Persoon-Rothert et al. 1989, Takasu et al. 1988).

2.4.3 Lysosomal involvement in myocardial cell injury: cause or consequence?

The first observation by de Duve and collaborators that during liver ischemia the decrease in the proportion of particle-bound lysosomal enzyme activity could be measured aroused interest in studying similar phenomena in ischemic heart tissue (de Duve & Beaufay 1959). This original observation was followed by several other similar observations which then led to the general "lysosomal hypothesis", suggesting that in the liver acidotic and hypoxic conditions lead to the loss of lysosome latency, i.e. the labilization of lysosomal membranes and the release of lysosomal enzymes to the cytosol, and hence increased degradation of macromolecules under acidotic milleu (Slater & Greenbaum 1965, Van Lancker & Holtzer 1959 ; see also reviews by Wildenthal 1975, 1978). Cardiologists studying myocardial infarction were immediately attracted to this idea since, if proven true, lysosomal stabilization would provide a valuable method of treatment during early coronary occlusion. The early work done on myocardial ischemia had provided evidence that in some conditions lysosomal enzyme distribution changed from particulate to supernatant fraction (Brachfeld 1969, Brachfeld & Gemba 1965, Leighty et al. 1967). Ricciutti suggested further that the low level of cellular injury and the increase in supernatant fraction of lysosomal enzymes before irreversible necrosis is a proof of the lysosomal hypothesis (Ricciutti 1972a, b). Moreover, some ultrastructural studies showed evidence of membrane rupture and loss of enzyme activity from lysosomes (Hoffstein et al. 1974). Much data has since accumulated on the fact that changes in lysosomal enzyme redistribution to the unsedimentable fraction occur at some point during myocardial infarction (Decker et al. 1977, Decker & Wildenthal 1978, Hoffstein et al. 1975a, 1976, Ravens & Gudbjarnason 1969).

However, some studies found no apparent changes in the distribution of lysosomal enzymes from the particulate to the supernatant fraction even after 2 hours of ischemia (Akagami et al. 1976, Ingwall et al. 1975). The difficult homogenization of cardiac muscle tissue as well as the various different fractionation procedures used has made it difficult to directly compare the results of different studies. In taking a biochemical approach to study lysosome latency it is also difficult to rule out the contribution of non-myocyte cells and changes due to their migration to the heart muscle. The timing of these events is another critical question. If the lysosomal hypothesis was to be proven true, the sequence of events well before the stage of irreversible damage would need to be evaluated. In fact, ultrastructural studies were often unable to show any changes in the appearance of lysosomes early during ischemia (Hoffstein et al. 1975b, Kloner et al. 1974). In some other experimental conditions accompanying necrosis lysosomal rupture has been shown to occur only after the onset of irreversible necrosis (Friedman et al. 1969, Lesch 1977). Instead, lysosomes seem to participate actively in the repair process in reversibly injured myocytes

(Ingwall et al. 1975, Ridout et al. 1986a, b).

As yet there has been no definitive answer to the problem of the role of lysosomes during myocardial cell injury. Combined morphological and biochemical approaches together with additional data to determine the extent of cellular damage are needed in order to study lysosomal involvement in progressive injury.

3 AIM OF THE STUDY

The aim of this thesis was to characterize lysosomal and endosomal structures in cardiac myocytes. The focus was both on the morphology of lysosomes and endosomes and on the function of these structures in normal conditions and during infarct-like myocardial cell injury. The specific issues addressed were as follows:

1. Biochemical and morphological characterization of lysosomal and endosomal structures in cardiac myocytes with special emphasis on the mannose 6-phosphate receptor function and distribution.

2. Structural and functional changes in lysosomes and endosomes during infarct-like myocardial necrosis in vivo.

3. In vitro-study of isoproterenol-induced changes in lysosomes and endosomes. Comparison with artificial acidification of the myocyte cytoplasm.

4 SUMMARY OF MATERIALS AND METHODS

4.1 Experimental animals

Hearts from adult male Sprague-Dawley rats were used for subcellular fractionation (I) and for immunocytochemical studies (II). The hearts were perfused free of blood using the Langendorff perfusion apparatus (I, II). Hearts for immunocytochemistry were either directly fixed by perfusion (II) or adult myocytes were first isolated by combined collagenase and hyaluronidase perfusion (II).

Adult male Wistar rats were used for the isoproterenol administration in vivo (III). A single dose of (-)-Isoproterenol -HCl (Sigma I-6504) was injected subcutaneously (n= 29). Saline was injected in the control animals (n= 11). The animals were killed either 30 minutes or 2, 24 or 48 hours after the injection.

4.2 Cultured cells

Primary monolayer cultures of 3 to 6 day old neonatal rat cardiac myocytes were isolated using cycles of combined collagenase and hyaluronidase digestion and plated in DMEM (II,III). Myocytes were separated from non-muscle cells using differential attachment to the culture plates. The majority of non-muscle cells adhered to the tissue culture plates during the first hour after plating, whereas most of the myocytes remained in the supernatant. Monolayers were used on the third to the sixth day after plating, when the cultures were 70-80% confluent.

Isoproterenol was added to the subconfluent cultures at a concentration of 1 mM in DMEM at pH 7.4.

Experimental acidification of the cytoplasm was induced in the subconfluent cultures with 70 mM acetate in Dulbecco's modified Eagle medium (DMEM) buffered to pH 6.5.

4.3 Antibodies used for immunocytochemistry and biochemical experiments

Antibodies against CI-MPR were produced in female white rabbits using 300-500 µg CI-MPR purified from bovine liver (II). Antisera were further purified by salt-fractionation (II), and protein A-purified IgG fraction (II) or affinity-purified antibodies (III) were used for most of the experiments.

Antibodies against the lysosomal glycoprotein lgp120 (II) were the generous gift of Dr. Ira Mellman (Yale University, New Haven, CT).

The antiserum against the luminal lysosomal enzyme, cathepsin L (produced against the purified MEP from KNIH-cell secretion) was kindly provided by Dr. Michael Gottesman (National Cancer Institute, Bethesda, MD).

The monoclonal antibody 4A1 (Dr. Jean Gruenberg, EMBL, Heidelberg) detects an acidic glycoprotein of 120 Kd that distributes in BHK cells (F. Aniento, N. Emans, G. Griffiths and J. Gruenberg, manuscript in preparation) essentially identically to lgp120 in NRK cells (Griffiths et al. 1988).

The monoclonal antibody E7 (Developmental Studies Hybridoma Bank, Iowa City, IA) recognises ß-tubulin.

The antiserum against dinitrophenol (61-006, ICN ImmunoBiologicals, Lisle, Illinois IL) was used to detect DAMP (3-(2,4 dintiroanilino)-3'-amino-N-methyl-dipropylamine) after aldehyde fixation as first described by Anderson (Anderson et al. 1984). DAMP was kindly provided by Dr. Anderson (Southwestern Medical School, Dallas, TX).

4.4 Microscopical methods

4.4.1 Immunofluorescence

Indirect immunofluoresence labeling was perfOrmed on subconfluent cultured neonatal rat cardiac myocytes using either 4% PFA or cold methanol as a fixative (II,III). Cells were permeabilized with 0.2% Triton X-100, reacted with antibodies diluted in 1% BSA/PBS and revealed with either anti-rabbit IgG-FITC or anti-mouse IgG-TRITC conjugates. Immunolabeling for cryostat sections was perfOrmed either on unfixed frozen sections or on fixed tissue treated with increasing concentrations of sucrose (III).

4.4.2 Cathepsin B staining

Cathepsin B activity was revealed on unfixed cryostat sections using N-CBZ-ala-arg-arg-4-methoxy-2-naftylamide (Bachem, Bubendorf, Switzerland) as a substrate according to the method described by van Noorden et al. (Van Noorden et al. 1989). The enzymatic reaction was performed at 22°C for 20 min and photographed immediately on fluorescence microscope.

4.4.3 Cryosectioning and immunoelectron microscopy

Fixed adult isolated cardiac myoytes (II) as well as tissue blocks fixed either by perfusion (II) or by immersion (III) were used for cryosectioning and subsequent immunolabeling. After fixation with 8% PFA, samples were infused with 2.1M sucrose and prepared for cryosectioning as described by Griffiths et al. (Griffiths et al. 1984). Thin frozen sections were cut using glass knives and a Reichert 4D ultramicrotome. Single and double immunolabeling was performed as described by Griffiths et al. (Griffiths et al. 1984) using either commercial protein A -gold particles of 5 and 10 nm in diameter (Janssen; Beerse,Belgium) or self-made particles of 6 and 9 nm in diameter prepared as described by Slot and Geuze (Slot & Geuze 1985).

4.4.4 Measurement of the cytoplasmic pH

Cytoplasmic pH was measured using the fluorescent pH indicator BCECF/AM (acetoxymethyl ester of indicator 2´-7´-bis(carboxyethyl)-5(6)-carboxyfluorescein ; Molecular Probes Inc., Eugene, OR, USA) as described by Gradenpiesser et al. (1989, III). The myocytes were loaded with 1 µm BCECF for 30 min at 37 C. For the isoproterenol treatment, myocytes were pretreated for 90 min with 1 mM isoproterenol and continued incubating with BCECF for a further 30 min. For the low pH -treatment, myocytes were loaded with BCECF in Krebs-Ringer buffer of pH 6.5 containing 70 mM acetate. The myocytes were washed well before measuring the fluorescence ratio F_{495} /F_{440}. This ratio value was converted to an intracellular pH value with a calibration curve, which was obtained by exposing the myocytes to the 140 mM KCl solution containing 10 µM nigericin at various pH values. The measured intensity ratios were divided by the ratio at pH 7.0 and a calibration curve $R(pH_i)/R(pH\ 7.0) = f(pH_i)$ was plotted.

4.5 Biochemical methods

4.5.1 Enzymatic measurements

ß-GA enzyme activity was measured from homogenate samples as described by Barrett (1972) and HRP enzymatic activity from Percoll fractions as described by Gruenberg et al. (1989). Lactate and creatine kinase activities were measured from the serum samples taken directly from the rat hearts (Lactate and Creatine kinase Monotest kits ; Boehringer Mannheim). Lactate dehydrogenase activity was measured from the culture media of cultured myocytes (LDH monotest kit ; Boehringer Mannheim).

4.5.2 Lysosome latency

Lysosome latency is considered as the particle-bound lysosomal enzyme activity that has not leaked through the lysosome membrane (de Duve 1959). Latency was determined by the distribution of the marker enzyme ß-GA in the pellet (sedimentable activity) and the supernatant (free activity) after heavy sentrifugation (180 000g for 30 min, I ; 250 000 g for 20 min, III) of the homogenate (I) or the post-nuclear supernatant (III). 0.5% saponin was used to release the latent free activity (releasable). The activity associated with pelletable membranes was considered as endogenous receptor-bound activity as also suggested by Fischer et al. (Fischer et al. 1980b).

4.5.3 Subcellular fractionation

Sarcolemmal, sarcoplasmic reticulum and mitochondrial fractions were separated from post-nuclear-supernatants prepared from adult rat hearts using discontinuous sucrose gradient as described by Jaqua-Stewart et al. (Jaqua-Stewart et al. 1979). The purity of the various fractions were analyzed by electron microscopy and by specific enzyme activity for those fractions.

In the endocytosis experiments cultured neonatal cardiac myocytes were fractionated in self-forming Percoll gradients. After the pulse with HRP and treatments with either isoproterenol or low pH the cells were scraped off the dish and homogenized by pipetting. Post-nuclear-supernatants were produced and 2 - 3 mg protein was mixed with 90% Percoll, homogenization buffer and BSA to create 20% Percoll mixture. Fractions from the gradient were analyzed for their respective HRP- and ß-GA activities.

4.5.4 MPR binding activity

Cardiac membrane fraction of 1000 - 35 000 g, a similar fraction obtained from different parts of the heart muscle (atria, right ventricle and the subendo- and subepicardial layers of the left ventricle), and sarcolemmal, sarcoplasmic reticulum and mitochondrial fractions from the left ventricle were used in the lysosomal enzyme binding studies as described by Fischer et al. (1980b). The membrane fractions were treated with saponin to release the latent free lysosomal enzyme activity and with alkaline buffer to remove the endogenous lysosomal enzyme activity.

High-uptake lysosomal enzymes were concentrated from fibroblast secretions as described (Fischer et al. 1980b). In the standard binding assay 200 µl of membrane fraction (150 - 200 µg membrane protein), 400 µl of enzyme preparation and 100 µl of homogenate buffer with or without 50 mM mannose 6-phosphate (final concentration) were incubated at 20 °C for 60 min. Incubation was stopped by adding ice-cold homogenate buffer and by centrifugation at 35 000g. ß-GA enzyme activity was measured as described by Barrett (Barrett 1972) and protein by Peterson (Peterson 1977).

4.5.5 Immunoblotting

Proteins were separated using the standard procedure for SDS-polyacrylamide-gel-electrophoresis described by Laemmli (Laemmli 1970). Immunoblotting was performed as described by Towbin et al. (Towbin et al. 1979). CI-MPR was visualized in the blot using an alkaline phosphatase- conjugated second antibody (II).

4.5.6 Metabolic labelling and immunoprecipitation

Subconfluent myocyte cultures were labeled with a 30-min or 2-hour pulse of [^{35}S] -methionine and either homogenized directly or chased for 4 hours with an excess of non-radioactive L-methionine (III). After lysis of the cells the extracts were treated with antibodies against CI-MPR and cathepsin L for 60 minutes. Antibody-antigen complexes were then collected using protein A-Sepharose and washed extensively before SDS-PAGE. To reduce non-specific binding the cellular extracts were treated with protein A-Sepharose before adding antibodies, and the protein A-Sepharose was then treated with non-radioactive cellular extract. After running 10% SDS-PAGE for cathepsin L and 7.5% for CI-MPR the gels were treated with enhancers, dried and processed for autoradiography.

5 Review of the results

5.1 Lysosomal enzyme binding of cardiac membranes (I)

5.1.1 Specificity for mannose 6-phospate recognition

The binding of high-uptake lysosomal enzymes to cardiac membranes was saturated by increasing the enzyme concentrations, whereas a linear increase in binding occurred when the amount of membrane protein was increased. Mannose 6-phosphate inhibited the lysosomal enzyme binding on both occasions, whereas glucose 6-phosphate and mannose 1-phosphate had a less dramatic effect. Alkaline phosphatase treatment of lysosomal enzymes converted the high-uptake forms to low-uptake forms, which decreased the lysosomal enzyme binding by 58%.

The integrity of receptor-ligand complexes under various pH values ranging from pH 5 to 9 was tested. In contrast to the studies by other groups, the receptor-ligand complexes were relatively stable still at pH 5.5, whereas they were effectively dissociated under alkaline conditions.

Triton X-100 (1%) and trypsin removed 62.1% and 45.8% of the membrane protein respectively. Under these conditions the content of endogenously-bound lysosomal enzymes and lysosomal enzyme binding decreased by values close to those mentioned above (56.1 and 39.1 respectively).

5.1.2 Binding to various cardiac fractions

The membrane fractions derived from different areas of the rat heart muscle

(atria, right ventricle and subendo- and subepicardial areas from the left ventricle) showed no statistical differences in the amount of bound endogenous lysosomal enzymes which varied between 40 and 55% of the total activity. Neither was any difference detected between the various parts in binding capacity of specific lysosomal enzymes. Instead, clear differences were detected between the subcellular fractions. The sarcolemmal membranes showed the highest specific binding capacity, as well as the highest amount of endogenous activity, about two-fold compared to the SR fraction, the mitochodrial membranes showing the lowest value for specific activity.

5.2 Distribution of lysosomal and endosomal markers in cardiac myocytes

Immunofluorescent labeling of CI-MPR in neonatal rat cardiac myocytes showed perinuclear labeling (II, III), as has been shown in other cell types (Griffiths et al. 1988). Lgp120, on the other hand, showed a punctate labeling of small vesicles throughout the cytoplasm (II). 4A1, which distributes in a manner essentially similar to lgp120 in NRK cells, showed a fairly similar distribution, being more tubular or reticular in nature (III). The antibodies against the luminal lysosomal enzyme, cathepsin L, showed the typical punctate labeling of small round vesicles throughout the cytoplasm (II, III).

Immunoelectron microscopy on thin frozen sections showed that the bulk of the CI-MPR label localized in large membranous structures in perinuclear areas, but sometimes also in more peripheral areas near the sarcolemma (II). These structures labeled heavily with CI-MPR and contained tightly packed membranes. Double-labeling studies of CI-MPR with lgp120 or cathepsin L showed that both CI-MPR and cathepsin L were mostly associated with inner membranes, whereas lgp120 was preferentially associated with the limiting membrane (II). Apart from the large membranous structures the tubular structures adjacent to the Golgi, presumably TGN, also showed some CI-MPR. CI-MPR was also found in low amounts on the sarcolemma and in the tubular extensions of the sarcolemma, the T-tubules inside the cells.

Frequently both CI-MPR and cathepsin L were observed to be associated with mitochondria undergoing degradation. (II) Membranous structures labeling heavily with CI-MPR and cathepsin L were often seen adjacent to mitochondrial structures that showed no apparent signs of degradation, whereas when degradation was apparent they were seen surrounded by both CI-MPR and cathepsin L label.

Structures that were devoid of CI-MPR and only labeled with cathepsin L were only occasionally observed in the thin frozen sections.

5.3 Myocardial cell injury in vivo

5.3.1 Infarct-like myocardial cell injury

Within 30 min after a single subcutaneous injection of isoproterenol the animals showed fatigue and exhaustion. Lactate measured from the rat sera showed a distinct increase 30 min after the injection. (III: Table 1) Typically the serum creatine kinase showed an increase 24 hours after the injection (III: Table 1). Light microscopic succinate dehydrogenase staining showed intense compensatory staining in addition to a changed fibrillar staining pattern 2 hours after the injection (Fig. 2). Haematoxylin-eosin staining showed also that 2 hours after the injection the first signs of tissue injury could be seen (not shown). At later time points tissue edema became apparent and the infiltration of inflammatory cells was observed.

The thin frozen sections showed irregularities in the contracting filaments in the form of the thickening of Z-bands and "contraction bands" as well as the disintegration of filamental structures already 2 hours after administration of the drug (III: Fig. 1). Signs of irreversible damage, e.g. precipitates in mitochondria and distinct irregularities in the structure of the cristae in mitochondria, were observed more intensively at later time points.

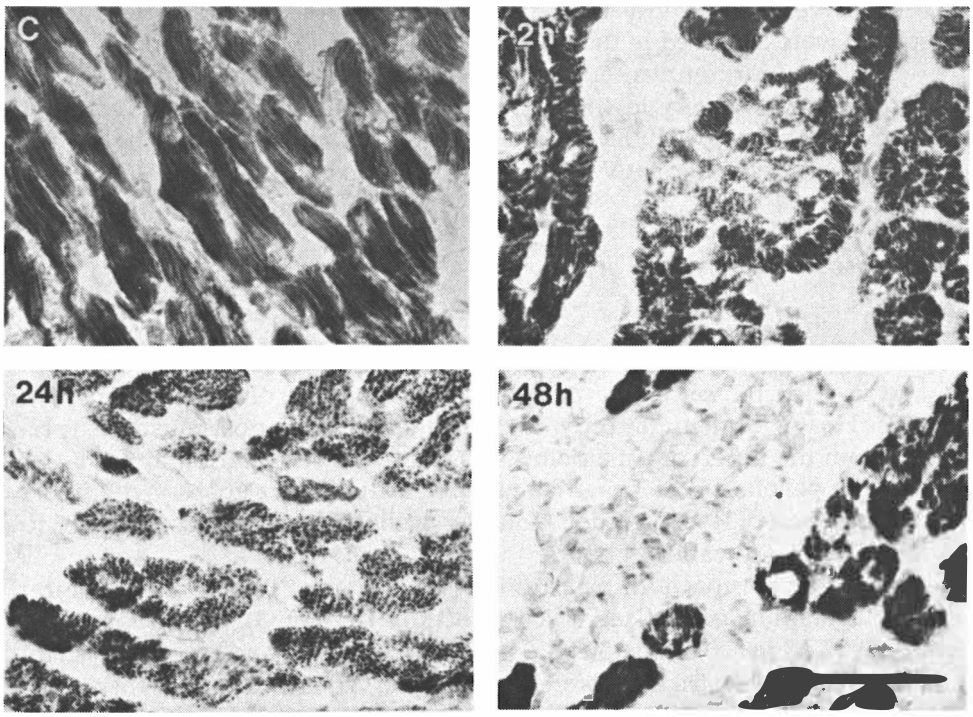

Fig. 2 Succinate dehydrogenase staining shows a dramatic change in the staining pattern already 2 hours after the isoproterenol injection. Original magnification = 25x. Bar = 100 μm.

5.3.2 Changes in the levels of lysosomal enzymes and CI-MPR

Measurement of the specific activity of ß-GA from the heart homogenates showed a distinct increase 2 hours after the injection (III: Table 2), whereas the amount of cathepsin L and CI-MPR remained unchanged (data not shown). After 24 hours the specific activity of ß-GA was, however, normalized. After 48 hours the activity was again increased as well as the amounts of cathepsin L and CI-MPR (not shown) most probably in response to the inflammatory reaction and the vast amount of inflammatory cells infiltrating the tissue.

5.3.3 CI-MPR and cathepsin L distribution

Immunofluorescent labeling of CI-MPR and cathepsin L showed striking differences in their distribution soon after the onset of ischemia (III: Fig. 4). The perinuclear labeling of CI-MPR showing relatively large vesicles around the nuclei shifted to the periphery and showed small vacuolar label already 2 hours after the injury(III: Fig. 4 the left panel). In contrast, cathepsin L accumulated in larger structures losing some small vacuolar label in the cytoplasm and showed no apparent shift to the periphery (III: Fig. 4, the right panel). Immunoelectron microscopy on thin frozen sections from the same time point showed that, indeed, small vesicles heavily labeled for CI-MPR and to a lesser extent for cathepsin L were observed in the periphery (III: Fig. 5).

Twenty-four hours after the onset of ischemia the labeling for CI-MPR and cathepsin L was very low both in the immunofluorescence study and in the electron microscopy (III: Fig. 4 and 6c). After 48 hours, however, the surviving muscle cells showed relatively similar labeling to the control cells.

5.3.4 Lysosome latency

The classical latency measurement was performed using the marker enzyme ß-GA and its distribution to the pelletable and the supernatant fractions (III: Table 2). Heavy centrifugation was performed for the post-nuclear-supernatants from the different time points. No change in the distribution of ß-GA between the pellet and the supernatant ("free activity") was observed 2 hours after the injection of isoproterenol. However, a slight decrease was observed in the pelletable fraction after 24 hours.

Another approach for measuring lysosome latency was a cytochemical cathepsin B staining on unfixed cryostat sections (III: Fig. 3). Incubation of the sections at 22 °C for 20 min showed a fairly low amount of cathepsin B activity in the control cells. A striking difference was observed after 2 hours when some cells showed punctate vesicles throughout the cytoplasm and highly increased activity. However, no diffuse appearance or overall cytoplasmic stain was observed. The cytochemical reaction was lower after 24 hours and the diffuse cy-

toplasmic cathepsin B stain was more apparent after 48 hours.

5.4 Isoproterenol treatment in vitro

5.4.1 Myocardial cell injury in vitro

Isoproterenol was added to the subconfluent cultures at 1 mM concentration in DMEM at pH 7.4. To confirm that 1 mM isoproterenol was an appropriate concentration to use in vitro, the lactate dehydrogenase activity was measured from the culture media. One millimolar concentration of isoproterenol caused a leakage of LDH from cardiac myocytes after 12 and 24 hours ($p < 0.001$) but not during early incubation (30 minutes, 2 or 4 hours ; Fig. 3). Similarly, LDH activity measured in the sera of rats treated in vivo, peaked after 24 hours (data not shown), as did creatine kinase (III: Table 1). 250 µM or 500 µM isoproterenol did not cause any leakage of LDH even after 24 hours in vitro. Thus 1 mM isoproterenol caused cell death on a very similar time scale compared to the in vivo administration of isoproterenol but showed no cellular leakage 2 hours after the isoproterenol treatment.

The lactate measurement from the culture media showed that 1 mM isoproterenol caused a leakage of lactate already after 30 minutes similar to that in the in vivo experiment (III, Fig. 8). No depletion in the intracellular ATP content was, however, noticed during this period. The intracellular pH measurement using the fluorescent indicator BCECF/AM showed that the cytosolic pH was lowered from 7.25 to 7.0 after isoproterenol treatment for 2 hours (III, Table 3). The distribution of acidic vesicles in the myocyte cytoplasm was also changed as studied using the fluorescent DAMP probe (III, Fig. 7). More acidic vesicles were observed in the periphery and at the cell leading edge after 1 to 2 hours of isoproterenol treatment and vesicles were often observed to be aligned in rows.

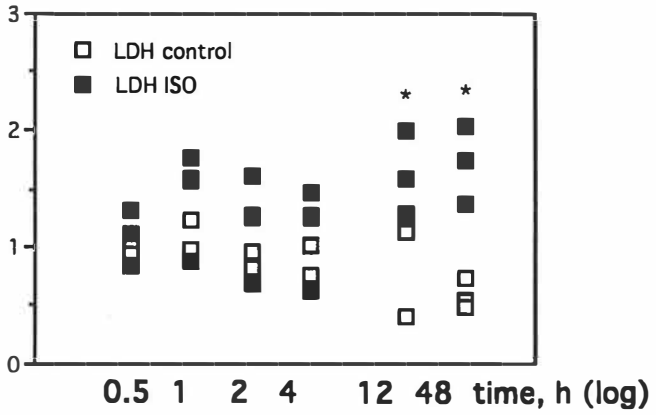

Fig. 3. Lactate dehydrogenase released from myocyte cultures during 1 mM isoproterenol (ISO) treatment. LDH is expressed as relative activity (0 time = 1). Individual values from 3 control and 3 isoproterenol-treated dishes are shown.; * : $p < 0.05$ (Kruskal-Wallis -test).

5.4.2 Distribution of CI-MPR, 4A1 and cathepsin L

DAMP labeling of cells suggested that acidic compartments in perinuclear positions and scattered throughout the cytoplasm acquired, at least to some extent, a peripheral localization after 1 to 2 hours of isoproterenol treatment. To characterize these vesicles further, double immunofluorescence was performed using antibodies against CI-MPR, cathepsin L and 4A1, an acidic glycoprotein of 120 kD. In the control myocytes the bulk of CI-MPR label was found in late endosomes located around the nuclei (III, Fig. 9). Isoproterenol treatment caused vesicularization of this perinuclear labelling, a decrease in CI-MPR labeling in the nuclear vicinity and the appearance of small CI-MPR-positive vesicles in the periphery. By contrast, isoproterenol had less effect on the overall 4A1 labelling pattern (III, Figs. 9 and 10).

In the control myocytes 4A1 exhibited a vesicular/tubular labelling pattern throughout the cytoplasm but also colocalised with CI-MPR close to nuclei, consistent with its presence in both late endosomes and lysosomes. According to earlier studies (II), the luminal lysosomal enzyme cathepsin L colocalizes with CI-MPR in late endosomes but also labels lysosomes throughout the cytoplasm . In this study cathepsin L not only colocalised with 4A1 in the perinuclear location but also more peripherally (III, Fig. 10). After isoproterenol treatment cathepsin L accumulated in slightly larger vesicles but showed no apparent shift towards the periphery (III, Fig. 10).

5.4.3 Involvement of microtubules

As judged by immunofluorescent tubulin-labeling, microtubules were intact after treatment with 1 mM isoproterenol for 4 hours in vitro (data not shown). Treatment with 33 µM nocodazole caused dispersal of CI-MPR- and 4A1-labelled structures in agreement with the fact that the distribution of both late endosomes and lysosomes is microtubule-dependent [(Matteoni & Kreis 1987) ; III: Fig. 11]. By contrast, treatments with isoproterenol did not further modify the distribution of these compartments (III, Fig. 11).

When myocytes were treated with isoproterenol for 2 hours and chased for an additional 10 minutes with normal culture medium (pH 7.4) CI-MPR -labeled vesicles sometimes seemed to align in rows resembling a tubular labeling pattern (III Fig. 11). Occasionally this incubation resulted in punctate CI-MPR and 4A1 labeling throughout the cytoplasm (III Fig. 11), and caused the disappearance of vesicular labeling from the leading edge of the cells.

5.4.4 Physico-chemical changes in late endosomes

Next, it was investigated whether redistribution and fragmentation would ac-

company the changes in the physical properties of the corresponding vesicles. In these experiments, 20 % Percoll gradients were used because minor differences in densities can be measured due to the shallow slope of the gradient. HRP was endocytosed for 5 minutes at 37°C and chased for 25 minutes into late endosomes and lysosomes. A PNS was prepared and fractionated on a 20% Percoll gradient. Late endosomes and lysosomes were found in the dense fractions of the 20% Percoll gradient, judging from the colocalization of the bulk of the lysosomal enzymes, and HRP endocytosed for 30 minutes (III, Fig. 12). Early endosomes labeled with HRP endocytosed for 5 minutes were found in the light fractions of the gradient well separated from the later structures. When chasing was carried out in the presence of 1 mM isoproterenol in the medium for an additional 60 minutes, no change in the distribution of HRP was observed.

5.4.5 Synthesis and processing of cathepsin L and CI-MPR

Since late endosomal structures redistributed to the periphery with little or no changes in the distribution of lysosomes, the fate of newly synthesized CI-MPR and cathepsin L during the isoproterenol treatment was investigated. It was also interesting to see whether vesicle redistribution to the periphery was coupled with the secretion of lysosomal enzymes. Cells were pulsed with [^{35} S]-methionine for 30 min and then chased with or without isoproterenol for 4 hours (III, Fig. 13a). Isoproterenol reduced the extent of cathepsin L processing, since the amount of 39 kD precursor form after isoproterenol treatment was increased 2.5 -fold when compared to the control. Immunoprecipitation from the chase culture medium showed a 47 % decrease in the secretion of cathepsin L, the 39 kD form being the only secreted form. Since the study in vivo showed increased specific activity of ß-GA and cathepsin B already 2 hours after the injection of isoproterenol, the possible changes in the synthesis of CI-MPR and cathepsin L were investigated in vitro. A two hour pulse with [^{35} S]-methionine showed no difference in the synthesis of CI-MPR and cathepsin L (III, Fig. 13b).

5.4.6 Artificial acidification in vitro

To directly demonstrate that late endosomes are sensitive to low cytoplasmic pH, the cytosol was artificially acidified with a culture medium of pH 6.5 containing 70 mM acetate, which was shown earlier to induce redistribution of late endosomes and lysosomes in other cell types (Heuser 1989, Parton et al. 1991). The low pH -medium lowered the cytoplasmic pH to 6.8 as judged from the fluorescence BCECF/AM measurement (III, Table 3). Cytosolic acidification caused redistribution of CI-MPR-labelled vesicles, and to some extent 4a1-positive elements, to the periphery, very similar to that caused by the isoproterenol treatment (III, Fig. 8, low pH). The redistribution was evident 30 minutes after cytosolic acidification, whereas isoproterenol induced a similar distribution af-

ter 2 hours.

Microtubules were intact during cytosolic acidification and acidification after microtubule depolymerization did not further modify the distribution of late endosomes as has been shown earlier in other cell types (Heuser 1989, Parton et al. 1991). As with the isoproterenol treatment in vitro, late endosomes showed no density change after cytosolic acidification.

6 Discussion

6.1 Receptor-mediated binding of lysosomal enzymes in cardiac tissue

The saturation of macromolecule binding to membrane proteins as well as competitive inhibition with molecules that show a high steric resemblance to the natural ligand are characteristic of receptor recognition. This study showed that phosphorylated "high-uptake"-enzymes bind to cardiac subcellular membranes in a saturable manner (I). Recognition was based on mannose 6-phosphate side chains on lysosomal enzymes since mannose 6-phosphate inhibited the binding of the enzymes and also because removal of the phosphate group from the lysosomal enzymes decreased binding.

Mannose 6-phosphate recognition was first shown by Kaplan et al. (1977). Mannose 6-phosphate receptor was then shown to function in lysosomal enzyme binding in various cells and tissues, e.g. fibroblasts and hepatocytes also including rat heart muscle (Fischer et al. 1980b). The enzyme binding was shown to be pH dependent (Fischer et al. 1980a, b), so that the dissociation of the bound enzyme could be accelerated by lowering the pH to levels comparable to the intralysosomal pH level. Interestingly, in this study, receptor-ligand complexes were not easily dissociated even at a rather low pH of 5.5, suggesting the possibility of a different mechanism for dissociation in vivo (I).

This study further characterized lysosomal enzyme binding to membrane fractions of rat heart muscle. No apparent differences were observed in enzyme binding to the different parts of the rat heart muscle (I). However, the subcellular fractions showed considerable differences in specific binding capacity. The fractionation method used in the preparation of the endoplasmic reticulum, plasmalemmal and mitochondrial membranes was specifically meant for heart muscle . Heart muscle tissue contains a large network of plas-

mamembrane, i.e. the plasmalemma and sarcoplasmic reticulum, throughout the cytoplasm and these comprise the bulk of the membranous mass in myocytes. In fibroblasts and hepatocytes about 90% of the enzyme binding was shown to occur in intracellular membranes (Fischer et al. 1980a). However, in the heart the lysosomal enzyme binding to the plasmalemma was about two-fold and, furthermore, the total binding capacity was about four-fold compared to the sarcoplasmic reticulum fraction. The difference in protein contents between these organelles in myocytes and hepatocytes partly explains the difference in enzyme binding capacity: the protein content of the endoplasmic reticulum is seven-fold compared with that of the plasma membrane, whereas in myocytes the amount of the plasmalemma is more than double the amount of the sarcoplasmic reticulum (Page & McAllister 1973, Jaqua-Stewart et al. 1979).

These results originally proposed that the pinocytosis route for lysosomal enzymes might be more important in myocytes compared to hepatocytes and fibroblasts, and that interstitial cells showing high amounts of lysosomal enzyme activity could be an important source of enzymes for cardiac myocytes. However, in the light of present knowledge of the endocytic and biosynthetic pathways in other cell types, it seems more likely that the intracellular routing of lysosomal enzymes to lysosomes occurs to a large extent via sarcolemma and endocytic structures. This idea is supported by the fact that the sarcolemma comprises a huge network in cardiac myocytes extending to every sarcomere and forming close complexes with sarcoplasmic reticulum.

The membraneous fractions gained with the purification procedure most probably contain fragments of TGN and late endosomes, which have been recently shown to contain high amounts of CI-MPR. The mitochondrial fraction especially, which showed relatively high specific binding activity, might contain late endosomes since they are large and heavy organelles and in electron microscopic images are often associated with mitochondria.

6.2 Organization of lysosomal and endosomal structures in cardiac myocytes

The immunofluorescence studies of both cultured cells and cryostat sections as well as the immunoelectron microscopy studies showed that, in fact, the bulk of the receptor is found in large membranous structures in myocytes and only to a lesser extent in the plasmalemma and sarcoplasmic reticulum (II, III). The tubular structures close to the Golgi, most probably representing the TGN, showed moderate labelling for CI-MPR (II). The large structure that labeled heavily for CI-MPR bore a close resemblence to the structure that was only recently characterized in NRK cells and named prelysosomes by Griffiths et al. (1988, 1990). Similar structures were referred to as endosomes by Geuze et al. (1984, 1988) . These large membranous structures in myocytes also labelled for lgp120 and cathepsin L (II). Since lysosomes were earlier shown to be devoid of CI-MPR, these large membranous structures that contain the bulk of the receptor and colocalize with lysosomal enzymes and lysosomal glycoproteins most likely

represent the meeting point between the biosynthetic and endocytic routes (Geuze et al. 1984, Griffiths et al. 1988, Willingham et al. 1983).

In cardiac myocytes these large prelysosomes were usually found among clusters of mitochondria and often associated with mitochondria under degradation (II). This association and possible cofractionation with mitochondria may explain the high amount of lysosomal enzyme binding activity measured in the enzyme binding assays (I). Other vesicles showing lower amounts of CI-MPR and some lgp120 most probably represent endosomes that are earlier structures than prelysosomes in the endocytic pathway (II).

Both lgp120 and cathepsin L showed typical lysosomal localization distinct from CI-MPR (II, III). For some reason, lysosomes were easily detected in the immunofluorescence study, but upon immunoelectron microscopy, probably due to their low amount in myocytes, pure lysosomes were less frequently found. For immunoelectron microscopy an antibody against a mixture of lysosomal enzymes would be more appropriate for localising mature lysosomes.

6.3 Lysosome latency is not changed early during infarct-like myocardial cell injury

A single subcutanous injection of a large dose of isoproterenol showed the typical characteristics of myocardial infarction and necrosis in the rat heart (III). Lactate accumulation and changes in the oxidative mitochondrial enzymes were the earliest detectable responses, followed by irreversible cell injury and inflammatory reaction significantly later. This study concentrated on the events occurring early after the administration of isoproterenol during the period when most cells were still undergoing reversible injury.

During this early period no apparent change in lysosome latency was observed. The classical latency study based on centrifugation of heart homogenates showed no increase in the unsedimentable fraction (free activity) of lysosomal enzymes (III). A slight increase was observed after 24 hours concomitantly with the inflammatory reactions and massive cell death. This measurement was performed using centrifugation at a high speed, exceeding that reported in many studies. The original procedure described by de Duve suggested 30-min centrifugation at 100 000g (de Duve 1959). However, a procedure using centrifugal forces of 20 000g and 22 000g for 20 min has been widely used, probably in order to obtain only the dense lysosomes. Recently, lysosomal enzymes have been localized, however, to the TGN, endosomes and plasma membrane in various tissues and, in addition, to the sarcoplasmic reticulum in heart muscle tissue (Geuze et al. 1988; Griffiths et al. 1988, 1990; Hoffstein et al. 1975b). This versatile localization also in lighter structures that are difficult to sediment at low g forces may have partly caused the controversial results. Furthermore, if fragmentation of some of these structures were to occur during ischemia or myocardial necrosis it would be detected as an increase in supernatant activity after light centrifugation. Indeed, it was shown that prolonged starvation causes a major increase in nonsedimentable cathepsin

D activity but, however, no leakage of the enzyme activity to the cytoplasm, whereas an increased distribution into the sarcoplasmic reticulum was observed (Decker et al. 1980, Wildenthal et al. 1975). Kennett and Weglicki (1978) measured myocardial blood flow during experimental ischemia using microspheres, and showed that, after 2 hours, contrary to severely ischemic areas (blood flow 9% of control), no change in lysosomal enzyme distribution occured in moderately ischemic areas (blood flow 53% of control).

The immunofluorescent labelling of isoproterenol-treated heart samples in vivo showed that cathepsin L accumulated in larger structures but showed no leakage to the sarcoplasm (III, Fig. 4). The bright fluorescence due to the larger structures and hence the "halo" around these structures might have led other groups to suggest a change in latency. Accumulation of lysosomal label during myocardial ischemia has also been observed in electron microscopy studies by other groups (Decker & Wildenthal 1978, Samuelson et al. 1987).

The cathepsin B staining in the injured myocardium was used to gain more relevant information about precisely where active lysosomal enzyme is localized (III, Fig. 3). A large fraction of the myocytes were clearly affected 2 hours after drug administration, showing increased activity in various locations. Leaked enzyme activity should have given diffuse staining in the sarcoplasm, as was detected after 48 hours but, after 2 hours, cathepsin B was localised in distinct vesicles. An increased in specific activity of ß-GA was also observed in biochemical measurements of heart homogenates (III, Table 2). The measurement of the amounts of CI-MPR and cathepsin L from the protein A-[^{125}I] -blot showed, however, no apparent differences in protein contents after 2 hours, suggesting that during the early course of myocardial infarction specific lysosomal enzyme activity is indeed increased without any change in the synthesis of the enzymes.

Interestingly, the staining patterns of the cytochemical enzymatic activity of cathepsin B and the immunofluorescent localization of cathepsin L were different (III: Figs. 3 and 4). Cathepsin L-positive structures appeared less numerous and larger than cathepsin B-positive structures. It seems likely that in the injured myocytes lysosomal enzymes could, due to the lowered intracellular pH, also be activated in structures that normally contain a higher pH than the optimal pH for their activity. Recently, cathepsin B as well as acid phosphatase were shown to hydrolase their substrates to some extent already in early endosomes where the ambient pH is known to be around 6 - 6.3 (Bowser & Murphy 1990, Roederer et al. 1987). In cardiac myocytes the localization of vesicles stained by cathepsin B largely resemble the localization of the sarcoplasmic reticulum as well as the Golgi structures labeled with ß-cop and mannosidase II, suggesting that activation of this enzyme could occur at some point along its biosynthetic route. Indeed, some groups have reported increased lysosomal enzyme activity very early after the onset of ischemia, and some evidence that activation may occur in the sarcoplasmic reticulum during ischemia has been acquired (Katagiri et al. 1983, Nakamura et al. 1983, Sasai et al. 1982, Toba et al. 1978). The fact that the degree of immunolabelling of cathepsin L was less prominent than the cytochemical detection of cathepsin B could then be due to the inability of the cathepsin L antibody to detect premature forms of the enzyme, as is often the case in immunocytochemistry.

6.4 Isoproterenol produces fragmentation and redistribution of late endosomes in vivo and in vitro

Immunofluorescense study of the lysosomal markers in the ischemic myocardium showed clearly that the distribution of CI-MPR i.e. the late endosomes, shifted from the perinuclear location to the periphery close to the surrounding sarcolemma, whereas the lysosomes, i.e. cathepsin L label, showed no apparent change in their distribution (III: Fig. 4). The late endosomal structures were significantly smaller in size whereas the lysosomes seemed to aggregate and form fewer larger structures. Aggregation of lysosomes has been observed earlier in ischemic tissue and also after artificial acidification of the cytoplasm (Decker & Wildenthal 1978, Samuelson et al. 1987). This fragmentation and peripheral movement occurred 2 hours after the isoproterenol administration during reversible ischemic injury, whereas later, during the inflammatory reaction and cell necrosis, both markers were almost depleted from the cardiac muscle cells. This immunofluorescence observation was verified by electron microscopic labeling of thin frozen sections, which showed that large amounts of CI-MPR was indeed found in small structures close to the periphery (III, Fig. 5). Such high local concentrations of CI-MPR have not so far been observed in other elements of the endocytic or the biosynthetic pathway (Geuze et al. 1984, 1988 ; Griffiths et al. 1988, 1990).

The treatment of cultured neonatal heart muscle cells with a high amount of isoproterenol caused myocardial cell necrosis on a very similar time scale. This was proven by lactate dehydrogenase secretion from damaged cells, which showed an increase after 12 hours but not yet after 4 hours (Fig. 3). Also an increase in lactate secretion was observed very similarly to the in vivo experiment (III, Fig. 8). Isoproterenol also caused the fragmentation of late endosomes and their movement towards the periphery during 1 to 2 hours following treatment (III, Fig. 9). Similarly to the in vivo study, the distribution of cathepsin L as well as 4A1, a lysosomal membrane protein, was not affected, but instead, cathepsin L showed some aggregation (III, Fig. 10). Labeling of the Golgi and pre-Golgi with antibodies against mannosidase II and ß-cop showed no change in their distribution, suggesting that the Golgi is not affected during isoproterenol treatment (data not shown).

The metabolic labelling of cathepsin L showed that its processing from the 39 kDa precursor to the active 21 kDa form was slowed down (III, Fig. 13). At the same time, however, the amount of synthesis of cathepsin L during the first two hours was unaffected, suggesting that the fragmentation and redistribution of late endosomes also affects the receptor recycling and routing of lysosomal enzymes to lysosomes. Indeed, Chua et al. (1979) have shown that myocardial ischemia inhibits protein degradation.

6.5 Redistribution and fragmentation of late endosomes is microtubule-dependent and caused by low pH

The first evidence that low pH may fragment late endosomal/lysosomal structures and that the detaching small vesicles travel to the cell periphery near the plasma membrane came from the study by Heuser on cultured macrophages and fibroblasts (Heuser 1989). Later, Parton et al. (1991) showed that the low cytoplasmic pH fragments late endosomes to the basal membrane in polarized epithelial cells and, in the case of neuronal cells, that they move out along the axons and dendrites. The outward movement of the fragmented vesicles during acidification was shown to be microtubule-dependent and could be reversed at least partially by alkalinization of the cytoplasm. The movement of the late endosomal fragments during isoproterenol treatment also depended on microtubules since the depolymerization of microtubules with nocodazole inhibited redistribution (III, Fig. 11). Isoproterenol did not affect the physico-chemical properties of the late endosomes as proven by the unaltered distribution of HRP-labeled late endosomes in shallow Percoll-gradients (III, Fig. 12). This observation also confirms that the structures observed at the cell periphery are of late endosomal origin and do not reflect a redistribution of the markers towards peripheral early endosomes.

Heuser (1989) referred to lysosomes when he studied the distribution of HRP -loaded structures during acidification. Earlier work on macrophages also suggested that lysosomes occasionally acquire a more tubular conformation along microtubules (Swanson et al. 1987). Parton et al. (1991) showed, however, that low pH principally affected the late endosomes and to a lesser extent lysosomes. Recently, Rabinovitz et al. (1992) described extended tubular structures in macrophages that were kinetically and structurally similar to the tubular lysosomes described by Swanson et al. (1987). These tubular structures contained, in addition to lysosomal glycoproteins LAMP 1 and 2, significant levels of CI-MPR and rab7, a late endosome specific GTP-binding protein, demonstrating that these structures have characteristics of late endosomes.

Cytoplasmic pH is known to fall rapidly in cardiac ischemia as a result of anaerobic glycolysis. Nuclear magnetic resonance measurements show that intracellular pH in ischemic myocytes during global ischemia (i.e. no flow through the myocardium) may drop as low as 5.7 - 6.6 (Garclick et al. 1979, Jacobus & Taylor 1977). High doses of isoproterenol are known to exert glycolysis and increase lactate production. Lactate release detected in the sera soon after isoproterenol administration in vivo suggests that cytoplasmic pH is lowered in cardiac myocytes due to the intracellular accumulation of lactate (III, Table 1) Also in vitro, myocytes secreted lactate to the culture medium with no apparent loss of intracellular ATP content during isoproterenol treatment. Indeed, the intracellular pH measurement showed that the cytoplasmic pH was significantly lowered in myocytes after isoproterenol administration (III, Table 3). The artificial acidification of the pH of the cytoplasm to 6.8 caused a peripheral movement of late endosomal elements similar to that induced by the isoproterenol treatment (III, Fig. 6). These results suggest that low pH induces this peripheral movement and fragmentation of late endosomes during isopro-

terenol treatment in vitro, which is also a reproduction of the similar phenomenon during isoproterenol-induced myocardial necrosis in vivo. In line with these observations Chua et al. (1979) have shown that protein degradation is inhibited by mere lactate or hydrogen ion addition to the perfused myocardial tissue, thus suggesting that they may interfere with the routing of lysosomal enzymes to lysosomes. In addition, some morphological alterations that also occur during ischemia have been reproduced by mere lactate treatment of aerobic hearts in vivo and during perfusion in vitro (Armiger et al. 1975, 1977).

Recently kinesin was shown to be responsible for the anterograde movement of organelles along microtubules (Vale et al. 1985) and dynein for the retrograde movement (Schroer et al. 1989). It is possible that these motor proteins are affected by the cytoplasmic pH. Low pH was, however, shown to affect only late endosomes and not other organelles in this study as well as in other studies by other groups, which suggests that a direct effect on motor proteins is unlikely.

Conclusions

This study focused on the characterization of both the morphology and function of lysosomal and endosomal structures in rat cardiac myocytes in vivo and in vitro. The main conclusions are:

1. Lysosomal enzymes are targeted to lysosomes by mannose 6-phosphate receptors in rat heart. The relatively high amount of receptors in the light sarcolemmal fraction suggests that the endocytic route is involved in the delivery of lysosomal enzymes to lysosomes in cardiac myocytes.

2. The bulk of the CI-MPR resides in large membranous prelysosomes colocalizing with lysosomal enzymes (cathepsin L) and lysosomal membrane glycoproteins (lgp120 and 4A1). Prelysosomes are mainly perinuclear and often associated with autophagy.

3. During isoproterenol-induced myocardial necrosis fragmentation of prelysosomes and movement of the fragments from the perinuclear position to the periphery occurs in cardiac myocytes.

4. Myocardial cell injury is not accompanied by the release of lysosomal enzymes to the cytoplasm during its early phase.

5. Redistribution and fragmentation of prelysosomes disturbs lysosomal enzyme delivery to lysosomes.

6. Redistribution and fragmentation of prelysosomes is microtubule-dependent and caused by low pH.

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Yhteenveto

Lysosomit ja endosomit sydänlihassoluissa

Tämä väitöskirja pyrki karakterisoimaan lysosomaalisia ja endosomaalisia rakenteita ja niiden toimintaa rotan sydänlihassoluissa in vivo ja in vitro. Koska lysosomien roolista sydänsoluiskemian ja nekroosin kehittymisen aikana on julkaistu paljon ristiriitaisia tuloksia, tässä työssä selvitettiin myös lysosomaalisten ja endosomaalisten rakenteiden toimintaa kokeellisesti isoproterenolilla aiheutetun infarktia muistuttavan sydänsoluvaurion aikana in vivo ja in vitro.

Lysosomaalisten entsyymien osoitettiin sitoutuvan solusisäisiin kalvofraktioihin mannoosi-6-fosfaatti -tunnistuksen avulla. Solunsisäiset kalvofraktiot osoittivat suuria eroja kalvoihin sitoutuvien entsyymien määrässä. Solukalvofraktion suhteellisesti suuri sitomiskapasiteetti antoi viitteitä endosytoosireitin tärkeydestä lysosomaalisten entsyymien kuljetuksessa lysosomeihin.

Immunosytokemiallinen tutkimus osoitti, että suurin osa mannoosi-6-fosfaattireseptoreista sijaitsee suurissa kalvorakenteita sisältävissä vesikkeleissä, jotka muistuttavat suuresti Griffithsin ym. (1988) prelysosomeiksi nimittämää rakennetta. Reseptoria löytyi pienempiä määriä myös solukalvolta ja sen jatkeista solun sisällä (T-tubuluksista) sekä trans-Golgi verkostolta.

Isoproterenolilla rotan sydämeen in vivo aiheutetun sydänsoluvaurion varhaisvaiheen aikana reseptorilla leimautuva myöhäinen endosomi fragmentoitui ja fragmentit siirtyivät solukalvon läheisyyteen. Samanaikaisesti lysosomit aggregoituivat, mutta pysyivät rakenteellisesti ehjinä. Myöhäiset endosomit fragmentoituivat myös neonataalien sydänsolujen viljelmissä isoproterenolin vaikutuksesta, ja näiden endosomifragmenttien siirtyminen oli riippuvaista ehjistä mikrotubuluksista. Fragmentoituminen ja fragmenttien liikkuminen johtui todennäköisimmin sytoplasman happamoitumisesta isoproterenolikäsittelyn aikana, sillä pelkkä sytoplasman keinotekoinen happamoittaminen sai aikaan samat ilmiöt.

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ORIGINAL PAPERS

I

Characteristics of lysosomal phosphomannosyl-enzyme receptors in the rat heart

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II

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III

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