Regulation and intracellular route of the caveolin-mediated endocytosis in rat peritoneal macrophages and HepG2 cells

PhD Thesis

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INRTODUCTION

Caveolae are characterised morphologically as omega-, or flask-shaped membrane invagnations (d = 50-100 nm) (Palade, 1953, Yamada 1955). Biochemically caveolae are specialised highly hydrophobic, cholesterol- and sphingolipid-rich membrane domains (Brown and London 1997, Simons and Ikonen 1997). The major protein components of this lipid rafts are caveolins (caveolin-1, 2 and 3). Caveolin-1 is essential protein of caveolar formation (Parton 1996, Fernandez et al. 2002). Caveolin-2 is present mainly in the Golgi network, but it can also create huge hetero-oligomers with caveolin-1, so it is thought to play an accessory role in caveolae formation (Mora et al. 1999, Scherer et al 1997). Caveolin-3 (M caveolin) is a muscle specific isoform (Song et al. 1996).

Caveolae seem to have multiple functions:

i.) Wide variety of signalling molecules (i.e.: Src family kinases, eNOS) were accumulated in caveolae (Pelkmans and Helenius 2002) via the scaffolding domain of caveolin-1 (Couet et al. 1997). This interaction with caveolin-1 can regulate the activity of these molecules, consequently caveolae are thought to function as pre-assembled signalling complexes, signalling organelles (Smart et al. 1995). Caveolae do not only accumulate lipids in their coat but they play important role in regulation of cellular cholesterol homeostasis as well (Fielding and Fielding 2000).

ii.) For a long time it was debated that caveolae can pinch off from the cell surface, they were described as static, stable components of the plasma membrane. Increasing number of evidence suggest that caveolae are directly involved in the internalisation of membrane components,

extracellular ligands like cholera toxin, folic acid, serum albumin (Anderson et al. 1992, Parton et al. 1994). The binding of a ligand to its receptor triggers phosphorylation of tyrosine residues in proteins associated with caveolar coat (Pelkmans et al. 2002) indicating that caveolar pinching off can be regulated by synchronised function of kinases and phosphatases. The exact mechanism of the regulation, however, is not entirely known.

After caveolar internalization various ligands (SV40 virus, cholera toxin) accumulate in special cellular compartments, called caveosomes. Caveosomes were described as intermediate organelles of caveolae-mediated endocytosis. Until now they are characterized only by their morphology (grape-, or flower-like appearance) and the presence of caveolin-1 at their cholesterol-, and sphingolipid-rich limiting membrane (Pelkmans and Helenius 2002). However, the question remains whether caveosomes are the only destination for internalized caveolin-1 or whether caveolin-1 can travel to the late endosome – lysosome pathway, where it is degraded.

AIM OF OUR WORK

In our work we were interested in caveolin-mediated endocytosis. We studies two types of cells: resident (resting) and elicited (activated) rat peritoneal macrophages and a hepatocellular carcinoma cell line, HepG2 cells.

1. First, we were interested in whether phosphorylation has regulatory role in caveolar internalization. We applied serine/threonine and tyrosine phosphatse inhibitors (ocadaic acid and sodium-orthovanadate), as well as serum albumin to

provoke the pinching off of caveolae in macrophages and HepG2 cells.

2. To investigate the regulatory effect of ocadaic acid, we paid attention to phosphorilation of caveolin isoforms.

3. The intracellular pathway of caveolin-mediated endocytosis is not entirely known. In our work we followed the intracellular route of caveolin-1. We were especially interested in whether communication exists between the clathrin-mediated and caveolin-mediated endocytosis.

4. We focused on caveosomes, that are known as special orgnelles of the caveolin-mediated endocytosis. Their morphology (grape-like group of vesicles) and localization (close to the plasma membrane) suggest that many of them are not individual organelles. Using special surface plasma membrane marker, Ruthenium red, we have done detailed examination about their possible connection with the plasma membrane.

5. If caveolin is degraded we were interested in whether the cells use stored or *de novo* synthesis is necessary to replace caveolae at the plasma membrane.

MATHERIALS AND METHODS

Elicitated macrophages were isolated from Charles River rats treated with complete Freund's adjuvant, resident cells were obtained from non-treated rats by washing the peritoneal cavity with phosphate buffered saline. HepG2 cells

from a hepatocellular carcinoma cell line (ATCC: HB8065) was generous gift of Dr Ilona Kovalszky.

We applied a serine/threonine and tyrosine phosphatase inhibitors – ocadaic acid (OA) and sodium orthovanadate, respectively – as well as albumin (a physiological ligand) to provide the pinching off of caveolae from the plasma membrane. To study the possible role of caveolae in endocytosis we followed the flud-phase uptake of horseradish-peroxidase (HRP) in ocadaic acid treated macrophages. In some cases cycloheximide was used for inhibition of protein synthesis.

For morphologycal and biochemical studies the following antibodies were used: rabbit anti-caveolin-1, mouse anti-caveolin-2, mouse anti-syndecan (CD128), mouse anti-CD63 (LIMP-1) and mouse anti-phospho-tyrosine antibodies. For confocal microscopy flourescent stain (Alexa) labeled secondary antibodies were used. Nuclei were stained by DAPI. Bio-Rad Radiance2000 Rainbow confocal microscope was used for confocal images. Cellular fluorescens intensity was determined using Laser Sharp and Confocal Assistant software.

Processing of cells for ultrathin cryosectioning and immuno-labeling according to the protein A-gold method was done as described by Slot et al. 1991 and Liou et al. 1996. Electron microscopic images were taken by Hitachi and Jeol electron microscopes.

Methods:

Morphological methods: fluorescent confocal microscopy, common transmission electron microscopy, surface labelling with Ruthenium-red staining, DAB reaction, ultrathin cryo-sectioning and immuno-gold labeling. We used morphometrical analysis was done according to Weibel

(1966). StatSoft Statistica 6.1. software was used for statistical analysis.
Biochemical techniques: spectrophotometry to measure the amount of internalized HRP, immunoprecipitation, detergent-free membrane purification according to Smart and Conrad (1995) and Western bloting.

RESULTS AND DISCUSSION

To examine the effect of phosphorylation on the internalization of caveolae, we used ocadaic acid (a serine/threonine phosphates inhibitor). First, we studied the changes the number of internalized caveolae after ocadaic (OA) treatment in macrophages. OA resulted in a decrease of surface connected caveolae both in resident and elicited macrophages. The number of clathrin-coated pits also dramatically decreased. When we studied the HRP uptake, surprisingly we found that OA significantly decreased the amount of the internalized HRP. This contradiction may be explained the fact that in rezident macrophages the main endocytotic structures are clathrin-coated pits (Kiss and Kittel 1995), in elicited macrophages mainly caveolae are responsible for the fluid-phase endocytosis (Kiss and Heuse 1997). Since OA decreased the number of both type of vesicles, macrophages do not have enough surface connected vesicles for further uptake of HRP.

To study the effect of the OA on the phosphorylation of different caveolin isoforms, cell lysates were prepared from OA treated macrophages and HepG2 cells. When we immunoprecipitated proteins with anti-caveolin-2 antibody and we used Western blot analysis we found that in macrophages a 29kDa protein was strongly labeled with anti-

phosphotyrosine antibody in a concentration dependent manner. We were not able to detect similar changes in tyrosine phosphorylation of either caveolin-1 or caveolin-2 in HepG2 cells.

Although OA is a serine/threonine phosphatase inhibitor, we found an increased tyrosine phosphorylation. The question arises: how can this possible? It is known that OA has an inhibitory effect on serine/threonine protein phosphatases (PP1 and PP2A), that inhibit Src kinase (Marinessen and Gutkind, 2001). Sequential inhibition activates Src kinase which can phosphorylate caveolin isoforms on tyrosine.

We would have liked to know more about the intracellular route of caveolin-mediated endocytosis. After OA, sodium-orthovanadate and albumin treatments we found grape-like multicaveloar clusters in the cytoplasm near the plasma membrane. According to their morphology and caveolin-1 content of thier membrane they could be caveosomes. Electron microscopic images showed that some of them were obviously connected to the plasma membrane via a narrow tubular membrane channel. Ruthenium red stained these organeles suggesting that many of them had connection with the plasma membrane. These data clearly show that most of them are not individual intracellular organelles.

We were also interested in the intracellular pathway of caveolin-1. Since caveolar internalization is a slow process we used long term (1 and 3 hours) albumin incubation. The presence of albumin increased the number of surface-connected caveolae both after 1 and 3 hours. On the same time the number of organelles containing caveolain-1 were also increased. These organelles were either grape-like

caveolae-clusters or late endosomes/multivesicular bodies (MVBs).

Late endosomes or multivesicular bodies (MVB) can be identified by numerous intra-luminal vesicles and the presence of CD63 (LIMP-1) antigen (Eskelinen et al 2003). In HepG2 cells many CD63 positive MVBs are present in the cytoplasm. In non-treated cells only few of these MVBs stained with caveolin-1. After albumin incubation the number of caveolin-1 containing CD63-labeled endosomes significantly increased. These data indicate that after prolonged incubation with albumin caveolin-1 enters to the organelles of the degradative pathway: MVBs and lysosomes. Using a detergent-free method of Conrad and Smart we isolated plasma membrane and intracellular membranes of HepG2 cells. The caveolin-1 content of these membranes was analyzed by Western blot. We found increased amount of caveolin-1 in intracellular membranes after long term albumin incubation. These data are in good agreement with our morphological analysis and suggest degradation of caveolin-1. After OA treatment however we could not detect significant changes of the number of double labeled multivesicular bodies. These data clearly show that OA arrests the intracellular trafficing of caveolin-1.

One would expect that an increased uptake and degradation of caveolae would lead to a depletion of caveolin-1 at the plasma membrane. Instead we observe an increase, suggesting that degradation of caveolin-1 is accompanied by de novo formation of caveolae. To address the question whether the increase in plasma membrane-associated caveolin-1 represents newly synthesized protein, we added cycloheximide to our assays to block protein synthesis. 3 hours simultaneous incubation with albumin and

cycloheximide results in a significant decrease in the amount of caveolin-1 present in both the plasma membrane, and the intracellular membrane fractions. These data indicate that the increase of caveolae and increased level of caveolin-1 at the plasma membrane requires *de novo* synthesis.

DISCUSSION AND CONCLUSION

1. We found thet phosphatese inhibitors (OA and sodium-orthovanadate) and albumin stimulate the internalization of caveolae in macrophages and HepG2 cells. We worked out possible explanation how ocadaic acid can result in tyrosine phosphorylation of caveolin isoforms.

2. We immunoprecipitated a 29kDa molecular weight protein in macrophages, which was tyrosine phosphorylated after OA treatment. We think that this isoform is responsible for the regulation of caveolar pinching off of macrophages.

3. Following the intracellular pathway of caveolin-1 after long term albumin incubation, numerous CD63 (LIMP-1) positive late endosomes/multivesicular bodies were found to contain caveolin-1, suggesting that upon albumin incubation caveolin-1 is endocytosed and enters the degradative pathway.

4. We proved that many of the grape-like structures, which are described as caveosomes are not independent structures. Their Ruthenium-red labeling clearly showed that they have connection with the plasma membrane.

5. Using cycloheximide we found out that replenished caveolae at the plasma membrane require *de novo* synthesis of caveolar components.

LIST OF PUBLICATIONS

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IF: 2,786

Cumulative IF: 14,245
Original publications IF: 9,922

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