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Honors Thesis

Localization of Carboxypeptidase O in Madin-Darby Canine Kidney Cells

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## **Abstract**

Carboxypeptidases are enzymes that trim the carboxy-terminal ends of proteins. Carboxypeptidase O (CPO) is a recently discovered enzyme that was proposed to be associated with lipid droplets. Lipid droplets are organelles found in the cytosol of the cell that carry out different roles like storing metabolic energy and protein degradation. To test if CPO and lipid droplets were associated we used immunocytochemistry and lipid droplet staining of Madin-Darby canine kidney (MDCK) cells. Our data shows that lipid droplets can be detected with both BODIPY 493/503 and Nile Blue dye. CPO is associated with lipid droplets, but CPO does not affect the number of lipid droplets. Studies show that lipid droplet proteins contain potential cleavage sites for CPO. This information can possibly help in the advancement of research of metabolic disease.

## **Introduction**

Carboxypeptidases are a specific type of protease enzyme found in various types of organisms. Carboxypeptidases are activated by endoproteases and are involved in hydrolyzing or cleaving peptide bonds found between amino acid residues precisely at the carboxy-terminal end. The first metallo-carboxypeptidase (CP) was found and identified in pancreatic extracts more than 80 years ago and labelled carboxypeptidase A (CPA).

All metallo-carboxypeptidases can be separated into four subfamilies based on domain structure and amino acid sequence similarities. One subfamily referred to as “A/B” includes digestive CPs. All members of this subfamily contain an N-terminal “pro” region. This “pro” region also referred to as a prodomain is made up of approximately 90 amino acids and functions to assist in folding the active CP domain and as a CP inhibitor. There are a total of eight mammalian members of the CPA/B subfamily (Wei et al., 2002).

A recently characterized unique member of the CPA/B subfamily is carboxypeptidase O (CPO). This specific carboxypeptidase is classified as unique because it lacks the N-terminal prodomain which is thought to be necessary for folding. CPO cleaves acidic amino acids from dietary proteins and peptides (Lyons and Fricker, 2011). As a result of CPO being a newly characterized member of the CPA subfamily more information is needed to better understand its role in the cell.

The goal of my project is to answer the question of locality, and identify specifically where in the cell carboxypeptidase O is located. Hypothesized at the start of this research project was the idea that CPO is located in the lipid droplets (LDs) of cells. Lipid droplets are organelles located in the cytosol of the cell that carry out many different roles. Lipid droplets make up the hydrophobic phase in the aqueous environment of the cytosol. Their core stores metabolic energy and they function in protein degradation as well as viral

replication (Walther & Farese, 2012). CPO is highly expressed in intestinal epithelial cells which function to absorb amino acids released from proteins and peptides digested within the intestinal tract (Lyons and Fricker, 2011)

In order to determine whether or not CPO is located in lipid droplets we carried out several trials of immunocytochemistry and lipid droplet staining in the experimentation process. Immunocytochemistry uses antibodies to detect specific antigens, such as those on CPO, in cells and tissues. These bound antibodies can then be detected through immunofluorescence because fluorophores are attached to antibodies. As for the lipid droplets, lipophilic dyes which partition into the nonpolar core of the lipid droplet are used as markers (Listenbeger & Brown, 2007). Madin-Darby kidney cells (MDCK) were used for this experiment because they are epithelial cells. Epithelial cells are found in the intestinal lining of organisms that contain CPO.

## **Experimental Procedures**

### *Cell culture-*

A stock of carboxypeptidase O-expressing MDCK cells and a stock of carboxypeptidase O- non-expressing MDCK cells were thawed from their storage in liquid nitrogen. These cells were grown in separate, CPO-expressing and CPO-non-expressing, flasks with provided Dulbecco's modified Eagle's medium (DMEM) nutrients. The flasks were placed in an incubator at 37°C and 5% CO<sub>2</sub> and allowed to grow until the flask was about 70% confluent with undifferentiated cells. Once the cells reached 70% confluence they were split one to ten. During this process the cold DMEM was aspirated from the cells and they were rinsed once with 5 ml warmed (37°C) phosphate buffered saline (PBS). The PBS was then aspirated and 0.5 ml warmed (37°C) trypsin was applied to the flask. After approximately 3 minutes the cells when the cells were dislodged from the bottom of the flask, 5ml of DMEM was added to the flask. The cells were broken up by pipetting the DMEM up and down a few times then 0.5 ml of these cells and DMEM was pipetted into a new labelled flask that contained 5 ml fresh DMEM. The cells were stored in the new flask and placed in the incubator with a loosely screwed on cap.

### *Immunocytochemistry-*

MDCK cells, all CPO expressing, were cultured on polylysine coated slides in a 6-well plate. Cells were rinsed twice with cold DMEM and once with PBS, then fixed in 4% paraformaldehyde (PFA) in PBS for 10 minutes. Following the fixation the cells were rinsed 5 times with PBS. The cells were permeabilized with 0.1% Triton (Tx)-100 in PBS for 10 minutes, and then rinsed 3 times with PBS. The cells were then blocked in 5% bovine serum albumin (BSA) in PBS (warmed at 37°C) for 45 minutes at room temperature. After blocking the cells were immunostained for 1 hour at room temperature with the primary antibody

diluted 1:1000 in 5% BSA. The cells were then washed 4 times for 5 minutes in PBS on the rocker. After the wash the secondary antibody, which was diluted 1:1000 in 5% BSA, was administered for 1 hour at room temperature. The cells were washed as before. The staining of the lipid droplets, if performed, followed these washes.

*Staining of lipid droplets-*

All the liquid from the above wash was aspirated and BODIPY 493/503, diluted 1:1000 in PBS, was added to each well and incubated in the dark for 10 minutes at room temperature. The cells were then washed 3 times in PBS for 5 minutes each on a rocker and protected from ambient light. All the liquid was the aspirated, coverslips were removed from the wells, and cells were mounted using buffered glycerol with anti-fade on slides. Coverslips were secured with nail varnish at the corners and left to dry for 10 minutes (in the dark). The slides were kept in a slide box and stored at 4°C.

*Microscopy-*

Images were taken on a Leitz Laborlux D Fluorescence Microscope.

## **Results**

In order to determine if CPO is associated with lipid droplets, we needed to visualize lipid droplets with a lipophilic fluorophore. Literature reports that BODIPY 493/503 and Nile Red are good lipophilic dyes as well as Nile Blue. In our lab we had access to Nile Blue which is a modified form of Nile Red, and typically has a small percentage of Nile Red present. Our results indicate that both Nile Blue and BODIPY 493/503 can identify lipid droplets (Fig. 1).

However, BODIPY 493/503 was the preferred dye to use to identify lipid droplets in conjunction with immunocytochemistry because this dye only fluoresced in the green channel. Since BODIPY 493/503 only fluoresced in the green channel this eliminated any confusion of the lipid droplets in the red channel where CPO would fluoresce.

Our hypothesis that CPO is associated with lipid droplets was supported. Immunocytochemistry was performed along with lipid droplet staining and fluorescence imaging performed with a Leitz Laborlux D Fluorescence Microscope (Fig 2). Merging both the immunofluorescent (red) and lipid droplet stain (green) often showed CPO to be in contact with lipid droplets. Sometimes, however, CPO staining was not associated with lipid droplets, suggesting that there may be another pool of CPO. To determine whether or not CPO had an effect on the number of lipid droplets, lipid droplets were quantified in CPO-expressing and CPO-non-expressing MDCK cells (Fig. 3). This quantification displays there is no significant difference between the number of lipid droplets in CPO expressing and non-expressing MDCK cells.

Previous studies (Brasaemle, D.L. *et al.*, 2004; Sato, S. *et al.* 2006; Zhang, H. *et al.*, 2007) indicated that some lipid droplet proteins contain potential cleavage sites for CPO. In Table 1 a select few lipid droplet-associated proteins were chosen to display the C-terminus

where CPO would cleave at glutamates and aspartates. Heat shock proteins usually function to fold and assemble glycoproteins in the endoplasmic reticulum as well as bind calcium found in the endoplasmic reticulum.

## **Discussion**

Carboxypeptidases are well established as protease enzymes that cleave peptide bonds at the carboxy-terminal ends of proteins. CPO is found to function in the intestinal epithelial cells in the digestive systems of humans (Lyons and Fricker, 2011). CPO has been recognized as a digestive carboxypeptidase due to its role in cleaving at glutamate and aspartate in proteins (Lyons and Fricker, 2011). Studies show that lipid droplet proteomes contain potential cleavage sites for CPO which is noteworthy because lipid droplets are the main component of adipose tissue.

Adipose tissue or 'fat' has a main role in storing energy in the form of lipids; it also cushions and insulates the body. Medical conditions like obesity arise when adipose tissue is found in excess and produces adverse effects on the health. Carboxypeptidases that play a role in metabolic regulations such as Prolyl carboxypeptidase (PRCP), a protease expressed in the hypothalamus, lead me to believe carboxypeptidases are a worthy subject of study in the effort to combat obesity. PRCP is responsible for the degradation of  $\alpha$ -melanocyte-stimulating hormone which plays a central part in regulating energy uptake and expenditure (Jeong *et al.* 2012).

Since CPO has potential cleavage sites in the proteome of lipid droplets this can possibly hint to a role in metabolic regulation. Heat shock proteins, which function in protein folding, are proteins found in lipid droplets where CPO substrates are located. Exercise increases the levels of heat shock proteins through the mechanical deformation of tissues. Exercise-induced transient increases of heat shock protein inhibit the generation of inflammatory mediators and vascular inflammation ((Noble and Shen, 2012). This plays part in metabolic disorders associated with type 1 and 2 diabetes as well as atherosclerosis.

Evidence suggests that the activation of heat shock protein induced by exercise or metabolic disorders may play dual role in inflammation (Noble and Shen, 2012). As more studies are performed in the area of CPO, other carboxypeptidases, lipid droplets, and metabolic disease more information will be acquired which may lead to advancement in treating numerous metabolic diseases.

The next step to the research project I completed might be to find information on the function of CPO within lipid droplets. As more experiments involving carboxypeptidase O are completed more question will arise, which will provide further knowledge into this unique enzyme.

## Literature Cited

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**Table 1****Lipid droplet proteomes contain potential CPO substrates.**

<b>Protein Name</b>	<b>Accession #</b>	<b>C-terminus</b>
BiP protein	14916999	EEDTAEKDEL
Calnexin precursor	160333216	SPRNRKPRRE
Calsequestrin-1	227430322	DDDDDDDDDDDD
CGI-58	13385690	KVKEICHTVD
Heat shock 70 kDa protein 1-like	124339838	ATGPTIEEVD
Heat shock cognate 71 kDa protein	31981690	SSGPTIEEVD
Heat shock protein HSP 90-beta	40556608	EDASRMEEVD
Heat shock-related 70 kDa protein 2	31560686	SSGPTIEEVD
HSP 70	31981690	SSGPTIEEVD
Mannose 6 phosphate receptor binding protein 1	20127486	ITEKAPEEKK

Carboxypeptidase O cleaves at C-terminal glutamates (E) and aspartates (D). The table shows some of the proteins found in lipid droplets that have potential CPO cleavage sites (Brasaemle, D.L. et al., 2004; Sato, S. et al. 2006; Zhang, H. et al., 2007). Some C-terminal lysines (K) and leucines (L) are likely to be cleaved prior to CPO cleavage.

BODIPY 493/503

Nile Blue

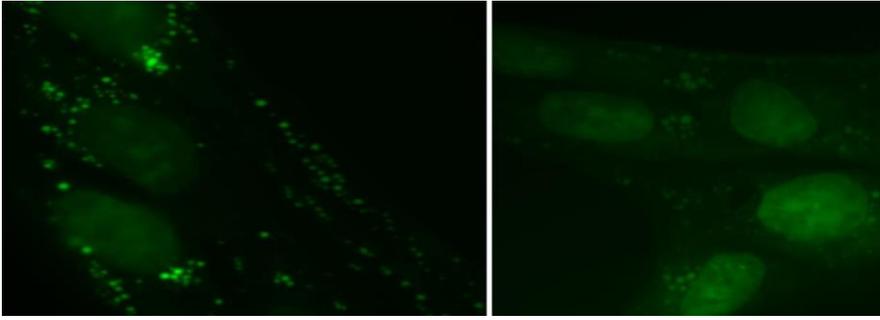


Figure 1. **Lipid droplets were stained with BODIPY 493/503 and Nile Blue.** BODIPY493/503 was the preferred dye over Nile Blue for dyeing the lipid droplets. Nile Blue fluoresced in both the red (not shown) and green channels, which made it unsuitable for subsequent immunocytochemistry, whereas BODIPY 493/503 only fluoresced in the green channel.

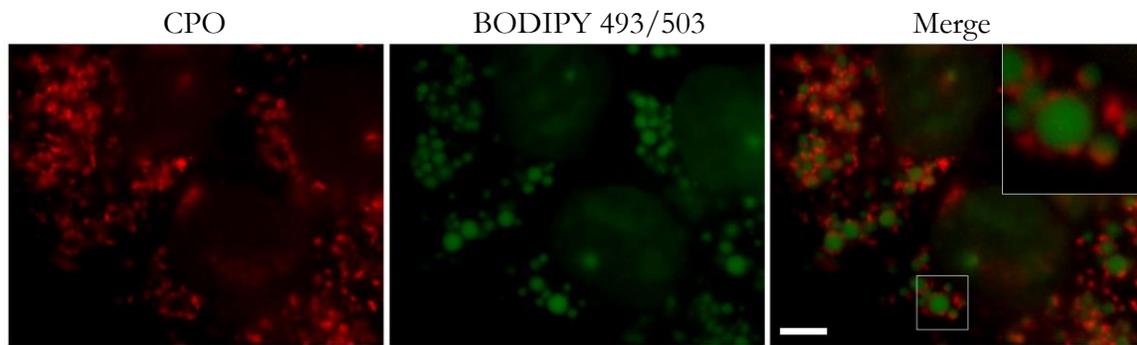


Figure 2. **CPO associates with lipid droplets in MDCK cells.** CPO was identified by immunocytochemistry and lipid droplets were stained with BODIPY 493/503. The scale bar picture indicates 5 micrometers. The insert shows a higher magnification, indicating close association of CPO (red) with lipid droplets (green).

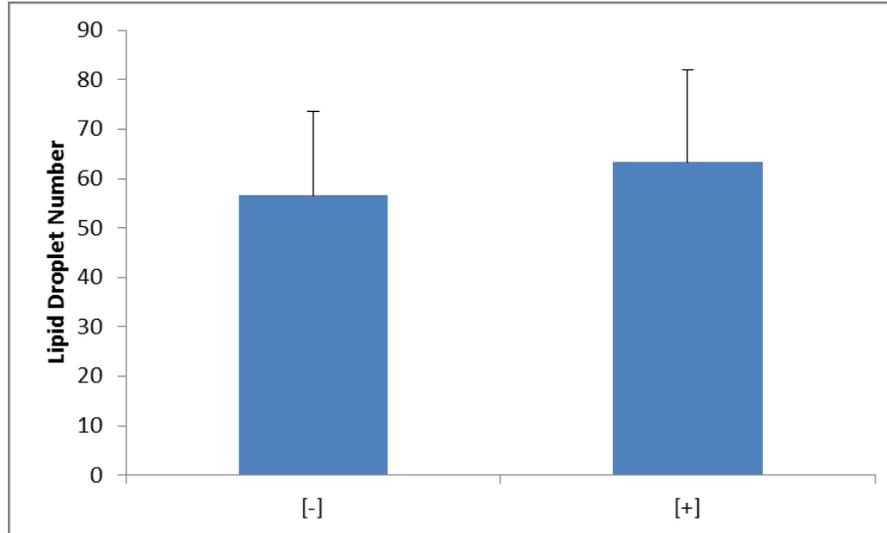


Figure 3. **CPO expression does not affect the amount of lipid droplets in MDCK cells.** Lipid droplets were stained using BOPIDY 493/503 and counted by eye using fluorescence microscopy. Average number of lipid droplets per cell are shown; the (-) indicates CPO-non-expressing cells and (+) indicates CPO-expressing cells. Error bars indicate standard deviation. n=20