β - Carotene 15,15’Oxygense-1 (BCO1) Distribution In Parenchymal And Non-Parenchymal Cells In Rat Liver

THESIS

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Abstract

β - Carotene 15,15-oxygenase 1 (BCO1) is a major enzyme catalyzing first step in the synthesis of vitamin A (retinoids) and stellate cells (HSCs) in liver are the major vitamin A storage site in the body. The expression of BCO1 in liver has been confirmed by many studies however only couple of studies have observed the localization of BCO1 \textit{in situ}. A recent study by Dr. Blaner and colleagues shows a relatively high expression of \textit{BCO1} mRNA in non-parenchymal-stellate cell fraction as compared to parenchymal cell fraction. Hepatocytes and stellate cells function in coordination in the metabolism of vitamin A in liver. Hepatocytes primarily take up the chylomicron remnant retinyl esters from the circulation which are transferred to hepatic stellate cells (HSCs) for storage in the form of lipid droplets. In order to gain further insight, we investigated the immunolocalization of BCO1 in normal rat liver section with the help of immunohistchemistry. Antibodies specific to two distinct epitopes of BCO1 were used to study the distribution of BCO1 in liver cells and compare it with desmin, a marker for quiescent hepatic stellate cells and also with carboxylesterase ES-4, a retinyl ester hydrolysing enzyme extensively localized in hepatocytes. It was observed that BCO1 is uniformly distributed in the cytoplasm of the hepatocytes in rat liver. Compared to hepatocytes, a strong staining for BCO1 was observed in non-parenchymal stellate cells...
which colocalizes with the staining for desmin positive hepatic stellate cells. BCO1 also observed to be localized in endothelial cells lining the hepatic artery and portal vein in liver.
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Dedication

Dedicated to my parents and my husband for being a great source of inspiration and motivation.
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Chapter 1: Introduction

1.1 Overview

β-Carotene 15, 15’Oxygenase-1 (BCO1; EC 1.14.99.36) is the sole enzyme that catalyzes the central cleavage of β-carotene to synthesize vitamin A (retinol and its esters) (Goodman and Huang 1965, Olsen and Hayaishi 1965). β-Carotene is an important source of vitamin A in diet, more so for vegetarians. Vitamin A is an essential nutrient for humans as we are incapable of synthesizing retinoids de novo. Vitamin A and its derivatives play an essential role in vision, embryonic development, reproduction and cellular growth and differentiation. All-trans- and 9-cis-retinoic acid serves as ligand for RAR and RXR nuclear hormone receptors that are proposed to modulate the expression of more than 500 genes (Blaner and Blomhoff 2002).

β-Carotene 15, 15’Oxygenase 1(BCO1) and β-carotene 9, 10’monooxygenase (BCO2) are the only two mammalian enzymes known to cleave carotenoids. BCO1 is involved in central cleavage of provitamin A carotenoids which results in retinoids. In contrast to BCO1, BCO2 catalyzes eccentric cleavage of both provitamin and non-provitamin A carotenoids which results in apo-carotenoids (Wyss et al. 2000, Kiefer et al. 2001, Paik et al. 2001). These two enzymes shares 40% sequence homology however the cleavage activity of BCO1 is much higher than BCO2 (Kiefer et al. 2001).
In a well nourished animal, liver stores about 70% of the total vitamin A in the body (Blaner & Olson 1994). Hepatocytes and hepatic stellate cells are the two major cell types that play an important role in the uptake and metabolism of retinoids and carotenoids taken up by liver (Blaner & Olson 1994, Blomhoff et al. 1994). Hepatic stellate cells (HSC) which constitutes 5-8% of the total liver cell population are involved in the storage of 90-95% of the total vitamin A in liver. This is in contrast to hepatocytes which accounts for about 70% of the total liver cells however only 5-10% of the total retinoid stores of liver (Blaner et al. 1985, Blomhoff et al. 1985). Hepatocytes are also a major cellular site for carboxylesterase ES-4, involved in hydrolysis of chylomicron retinyl esters in liver. (Sanghani et al. 2002, Ghosh and Harrison 2009, unpublished work). Due to scant number of stellate cells, researchers use desmin, an intermediate filament protein, in in situ studies to identify stellate cells among other non-parenchymal cells in liver (Yokoi et al. 1984).

*BCO1* mRNA is expressed in both hepatocytes and HSCs with 4 fold high expression in purified HSCs fraction as compared to hepatocytes (Shamarakov et al. 2010). In addition to retinyl esters, β-carotene is also taken into liver which further accumulates in both hepatocytes and HSCs (Lakshman et al. 1989, Shamarakov et al. 2010). Therefore, it is plausible that the serum-borne carotenoids are cleaved in liver by locally available BCO1, which may further metabolize and contribute to extra-intestinal synthesis of retinoids.
1.2 General hypothesis

β-Carotene 15, 15’oxygenase 1 (BCO1) is localized in hepatocytes (parenchyma) and hepatic stellate cells (non-parenchymal) in liver.

1.3 Specific aim

(1). To assess the specificity of BCO1 antibody using western blot and immunofluorescence staining.

(2). To study cell type localization of BCO1 in liver using immunohistochemistry (IHC).
Chapter 2: Literature Review

2.1 Carotenoids

Carotenoids are a large class of natural colored compounds synthesized by plants and some microorganisms. They impart a characteristic yellow to red color in fruits, vegetables and certain photosynthetic organisms. All carotenoids are derived from lycopene, which has acyclic isoprenoid structure of 40-carbon atoms (C\(_{40}H_{56}\)) with 13 conjugated double bonds (During and Harrison, 2006). Carotenoids are synthesized from this parent structure by hydrogenation, dehydrogenation, oxidation or cyclization of either one end (i.e. \(\gamma\)-carotene) or both ends (e.g. \(\beta\)-carotene) of the polyene chain. On the basis of structure, carotenoids can be divided into two major groups: (i) carotenes and (ii) xanthophylls. Carotenes are pure hydrocarbon carotenoids with un-substituted ionone rings and xanthophylls are their oxygenated derivatives (e.g: zeaxanthins) (Moss, 1974).

Of more than 600 carotenoids isolated from nature, only 10% are found in the human diet and only about 3% have been detected in the blood and human tissue (Mangels et al. 1993) Furthermore, only a small percentage of carotenoids provide provitamin A activity where, \(\beta,\beta\)-carotene (\(\beta C\)) is an important and direct precursor of vitamin A.
Vitamin A and its derivatives such as retinal, retinol, all-trans- and 9-cis retinoic acid, are involved in various physiological functions. A crucial role of 11-cis-retinal in visual functions has been known for many years (Saari 1994). Many studies conducted in developing countries have shown the beneficial effects of vitamin A supplementation in combating night-blindness among malnourished children (Sommer 1997, West et al. 1997, Carlier et al. 1993). Recent advances in the field of vitamin A have demonstrated the involvement of retinoic acid in the regulation of more than 500 genes. The isomers of retinoic acid, all-trans and 9-cis- forms act as ligands for retinoic acid receptors (RAR) and retinoid X receptors (RXR), and directly influence the transcription of vitamin A responsive genes. Vitamin A and its analogs also have significant impact on normal growth and differentiation of cells, reproduction, and immune functions (Moore 1957, Goodman 1984, Sporn & Goodman 1994).

2.1.1 Intestinal Digestion of β-carotene and Vitamin A
As mammals are not able to synthesize vitamin A de novo, they must acquire retinoids from the diet either as preformed vitamin A (retinyl esters) or provitamin A carotenoids (Goodman et.al 1965, Goodman, 1984, Blaner and Olson 1994, Vogel et al. 1999). The major dietary form of provitamin A carotenoids is β-carotene and that of preformed vitamin A is retinyl esters (REs) (Plack et al. 1965). In the intestinal lumen, β-carotene is converted by β-Carotene 15, 15’oxygenase-1 (BCO1) into two molecules of retinal and the retinyl esters (REs) are hydrolyzed to free retinol before taken into the enterocytes (van Bennekum et al. 2000, Rigtrup and Ong 1992, Rigtrup et al. 1992). Pancreatic
triglyceride lipase and phospholipase B catalyzes the hydrolysis of REs to free retinol within the lumen and brush border membrane of mucosal cells, respectively (Harrison & Hussain 2001). Retinal formed from β-carotene is converted to retinol by retinal reductase. The free retinol obtained from β-carotene and REs are taken up by the mucosal cells for further processing before their release into the circulation (Dew and Ong 1994).

Ingestion of dietary fats with carotenoids and retinoids promotes their absorption in the intestinal lumen (Noh and Koo 2001, Tso et al. 2001). Of the total provitamin A carotenoids consumed by humans, about half of them are absorbed intact and the remainder are metabolized to retinol (Blomstrand and Werner 1967). But the extent of conversion from provitamin A carotenoids to vitamin A varies widely among individuals. Once inside the mucosal cells, free retinol binds with cellular retinol binding protein II (CRBP-II) which transfers retinol to LRAT (Lecithin: retinol acyltransferase) for re-esterification (MacDonald and Ong 1994, Ong et al. 1987). LRAT esterifies retinol with long chain fatty acids to form retinyl esters (REs) which, along with absorbed β-carotene, are incorporated into chylomicrons (CMs) and released into the lymphatic circulation (Harrison and Hussain 2001, Huang and Goodman 1965). A recent study has localized β-carotene 15, 15’oxygenase 1(BCO1) protein in the microvilli of enterocytes lining the small intestine (Lindqvist & Andersson 2004) which is in agreement with the initial studies regarding extraction and characterization of BCO1 from small intestine (Olson and Hayaishi 1965, Goodman and Huang 1965).
2.1.2 Carotenoid Cleavage Enzymes
Carotenoid cleavage enzymes belong to a family of non-heme iron containing carotenoid oxygenase enzymes (Lindqvist & Andersson 2002). Two types of carotenoid cleavage enzymes are recognized in vertebrates: BCO1 and BCO2, with about 40% sequence homology. Retinal pigment epithelium protein 65 (RPE65) in the eye has also been identified as a carotenoid cleavage enzyme however not much is known about its function (Ho et al. 2007, During & Harrison 2006, von Lintig et al. 2005).

2.1.3 Central cleavage enzyme (BCO1)
β-Carotene 15, 15’Oxygenase 1 (BCO1; EC 1.14.99.36), is the key enzyme involved in the oxidative, central cleavage of β-carotene at the 15,15’ carbon-carbon double bond. Two molecules of all-trans retinal are synthesized in this reaction (Lindquist and Andersson 2002). Carotenoids with polyene chain and at-least one unsubstituted β-ionine ring can serve as substrate for BCO1 enzyme (Wirtz 1998). In humans, β-carotene 15, 15’ oxygenase 1 catalyses the central cleavage of 35% to 90% of all the β-carotene absorbed from the intestine (Parker 1996, Moussa et al. 2008). The symmetrical cleavage of β-carotene by BCO1 is a well defined and widely accepted phenomenon. However, the details of its substrate specificity and reaction mechanism remain to be elucidated.

2.1.4 Eccentric cleavage enzyme (BCO2)
Eccentric cleavage is a newly characterized pathway, which nonetheless was first proposed by Glover in 1954 (Glover and Redfearn 1954). The enzyme catalyzing the
eccentric cleavage is called β-carotene oxygenase 2 (BCO2). BCO2 cleaves β-carotene at
the 9', 10'-carbon double bond (and perhaps others) to produce β-apo-carotenals (Hu et al.
2006, Lindqvist & Anderson 2004 and Keifer et al. 2001). The BCO2 protein is co-
expressed with BCO1 in many tissues although amount of BCO2 protein is less than
BCO1 (Lindqvist and Andersson 2004, Lindqvist et al. 2005). In vivo, the physiological
function of BCO2 has not been completely elucidated, however it has been proposed as
an alternate pathway for the synthesis of vitamin A in case of BCO1 deficient or
knockout state.

2.2 β-Carotene 15, 15'-Oxygenase 1 (BCO1)

2.2.1 Function and Characterization of BCO1

β-Carotene 15,15'-oxygenase 1 cleaves β-carotene at central carbon 15, 15'-double bond
(Olson and Hayaishi 1965) with synthesis of two molecules of retinal (Bloomstrand and
Werner 1967, Goodman et al. 1966). The enzyme activity of BCO1 was first described by
Moore in 1930s (Moore 1930). Later in 1965, Goodman and Olson independently studied
and characterized the β-carotene central cleavage enzyme from cell free extracts of
intestine and liver (Olson and Hayaishi 1965, Goodman and Huang 1965). Both
Goodman and Olson demonstrated the enzymatic cleavage of β-carotene at central 15, 15'
carbon double bond to yield two molecules of retinal. Several subsequent studies have
also demonstrated the central cleavage of dietary β-carotene in intestinal mucosa. The
enzymatic activity of BCO1 was observed to be dependent on molecular oxygen
however, nicotinamide dinucleotide cofactors were not required for catalysis hence the
enzyme was named $\beta,\beta$-carotene 15,15-oxygenase. The pH optimum was in slightly alkaline range and the enzyme activity was inhibited by sulfhydryl alkylating and iron chelating agents. The fact that conversion of $\beta$-carotene to retinaldehyde was not inhibited by cysteine, which is an inhibitor of ferric protoporphyrin, suggested that it is a nonheme iron-containing enzyme (Johannes and Klaus 2004).

Subsequently, the enzyme was characterized in more details. BCO1 is a cytosolic enzyme with molecular weight around 62 kD and $\beta$C is the major substrate for BCO1 for the synthesis of vitamin A in vivo (Olson and Hayaishi 1965, Goodman and Huang 1965, Goodman et al. 1966, Fidge et al. 1969). Although BCO1 is known as cytosolic enzyme however, a study also reported the association of BCO1 with high molecular weight lipid protein fractions. An important characteristic of BCO1 is that only retinal was the identified product when $\beta$C was used as substrate. BCO1 do not cleave carotenoids such as zeaxanthin and lycopene under same conditions as $\beta$-carotene and $\beta$-cryptoxanthin (Singh and Cama 1974). However, carotenoids such as $\beta$-cryptoxanthin (Such et al. 2005) and $\alpha$-carotene (Lakshmann et al. 1968, Lakshmann at al. 1972) can also be used as substrate which supports the hypothesis that carotenoids with at least one un-substituted $\beta$-ring can be accepted as substrate for BCO1 (Wyss and Lintig 2008).

2.2.2 Identification, cloning and homology

Wyss and colleagues in 2001 successfully identified, cloned and sequenced the first vertebrate cDNA for BCO1 from chicken intestine (Wyss et al. 2001). Subsequently,
BCO1 gene was also identified and cloned from rats, mice and humans (reviewed in von Lintig 2010). Human recombinant BCO1 protein was characterized and observed to catalyze the central cleavage of βC and other carotenoids with one un-substituted β-ionone ring (Lindqvist and Andersson 2002). At the amino acid level, the chicken BCO1 shares 81% sequence homology with mouse (Wyss A et al. 2001) and 50% with drosophila. A significantly high sequence homology exists between mouse, chicken and human BCO1 (Ross and Harrison 2007, Lindqvist and Andersson 2002). The chicken BCO1 cDNA also reveals high sequence homology to RPE65 (Hamel 1993, Marlhens 1998) which is a retinal pigment epithelium protein with an unknown function.

2.2.3 Tissue specific expression of BCO1

The tissue specific expression of BCO1 mRNA has been recently studied after the first BCO1 cDNA was purified and cloned from chicken intestine. Several studies have analyzed the expression and distribution of BCO1 mRNA in various tissues using RT-PCR and northern blot experiments. In chicken, BCO1 mRNA was highly expressed in the small intestine and duodenum. However, mouse BCO1 protein and mRNA was mainly expressed in liver followed by testis and marginal levels in kidney (Wyss et al. 2001). Our own work also suggests a high expression of BCO1 protein in liver with comparatively lower levels in small intestine (Paik et al. 2001).

Using recombinant human BCO1, Andersson and Lindqvist observed that BCO1 mRNA was expressed throughout the intestinal tract with highest level in jejunum followed by
ileum and duodenum (Lindqvist and Andersson 2002). Among non-intestinal tissues, human BCO1 mRNA was highly expressed in liver and kidney. A comparatively low BCO1 protein expression was found in testis, ovary, colon, skeletal muscle and prostate tissue (Lindqvist and Andersson 2002). Like human recombinant BCO1, rat BCO1 mRNA was also highly expressed in liver and intestine (Takitani et al. 2006). BCO1 is also expressed in retinal pigment epithelium (RPE) in human eye (Yan et al. 2001). Recently, it has been shown that BCO1 mRNA is expressed in both parenchymal and non-parenchymal cell fraction in liver. However, the relative expression of BCO1 mRNA was 4-fold higher in purified fraction of hepatic stellate cells as compared to hepatocytes (Shamarakov et al. 2010). Thus, it is possible that β-carotene cleared by the liver is cleaved by locally available BCO1 in liver. It is also proposed that in addition to small intestine, vitamin A is also synthesized in the peripheral tissues from plasma-borne provitamin A carotenoids (Lindqvist and Andersson 2002).

2.2.4 Localization of BCO1 in various tissues

BCO1 was characterized in crude intestinal homogenate in mid 1960s (Olson and Hayaishi 1965, Goodman and Huang 1965). It was first purified and cloned in the early 2000s from various species including chicken, rat, mouse and human. Using many experimental approaches BCO1 mRNA expression was analyzed in tissue as described above. However, only a few studies have reported the localization of BCO1 protein in situ. BCO1 protein was first localized in liver and intestine cryosection of chicken (Wyss et al. 2001). Hepatocytes in liver and epithelial cells of small intestine expressed strong
staining for BCO1 protein. The epithelial cells of bronchioles in lungs and glomeruli in kidney also stained for BCO1. In kidney, the endothelium of the blood vessels were also positively labeled with BCO1 (Wyss et al. 2001).

Andersson and colleagues in 2004 performed the first and only immunohistochemical study on the localization of β-carotene 15, 15-oxygenase 1 (BCO1) in human tissues. In agreement with BCO1 mRNA expression in small intestine, BCO1 protein was also expressed in the microvilli of enterocytes and epithelium of the mucosal lining in small intestine. BCO1 labeling was not observed in either lamina propria or submucosa of stomach and colon. In extra-intestinal tissues, BCO1 protein was immunolocalized in steroidogenic cells in testis, ovary and adrenal gland. Epithelium of skin was also positive for BCO1. In pancreas, the exocrine gland forming cells were positive for BCO1 however, the islets of langerhans, which are also a form of epithelial cells in pancreas, did not express BCO1. The skeletal muscle cells and the glandular cells in endometrium, prostate and mammary tissues exhibited BCO1 positive staining. In human liver, BCO1 was shown to be expressed in hepatocytes with no observations reported for non-parenchymal cells (Lindqvist and Andersson 2004). The immunolocalization of BCO1 in various tissues suggest an alternate pathway to meet the retinoid requirement in the extra-intestinal tissues.

2.2.5 Tissue distribution of β-carotene
Several studies have demonstrated the distribution of β-carotene (βC) in animal tissues. βC has been found in spleen, liver, adrenals and ovaries of rat, mice and guinea pigs (Mathews-Roth et al. 1977). Among different tissues, liver accumulates majority of the ingested amount of dietary βC (Shapiro et al. 1984). Increased amounts of dietary β-carotene increases the vitamin A levels in liver although the plasma retinol levels remain unchanged (Mayne and Parker 1986) which proposes a homeostasis mechanism for retinoid concentrations in plasma.

Absorption and storage of βC in liver has been reported earlier in literature. βC accumulates in rat liver in a dose dependent manner (Shapiro et al. 1984). Significantly a high level (80%) of intravenously administered βC is accumulated in non-parenchymal hepatic stellate cells as compared to parenchymal cells. However on feeding dietary βC, majority of the βC is recovered from parenchymal cells (Lakshman et al. 1989). A recent study reported a significant accumulation of βC in both hepatocytes and HSCs in BCO1 deficient animal (Shamarakov et al. 2010). Thus it is plausible that both parenchymal and non-parenchymal cells are an important site for the uptake, accumulation and metabolism of dietary βC in liver.

Expression of BCO1 overlaps with many βC storing tissues such as small intestine and liver. In small intestine, BCO1 is expressed in enterocytes (Lindqvist and Andersson 2004) where dietary βC is cleaved to retinoids. These retinoids are released into
lymphatic circulation for further storage and utilization in liver and other peripheral tissues. βC has been found in the mitochondrial fraction of the liver (Mayne and Parker 1986) and primarily localized in cytosolic fraction of liver cells (Lakshman et al. 1989).

2.2.6 BCO1 Deficiency
Animal studies with BCO1 deficiency are important in understanding the physiological role of BCO1 in body. βC is a potential pre-retinoid carotenoid in human diet. BCO1 deficiency increases the accumulation of βC in liver (Shmarakov et al. 2010). In addition to βC, hepatic triglyceride levels and gene expression of peroxisomal proliferator activator protein-γ (PPAR γ) also increases tremendously in BCO1 deficient state (Shmarakov et al. 2010). Thus, it is probable that BCO1 plays a role in regulation of fat in liver and the diseases related to it like hepatic steatosis. BCO2 mRNA expression also increases significantly in the face of BCO1 deficiency (Shmarakov et al. 2010) Hence, it is conceivable that eccentric cleavage by BCO2 contributes to synthesis of vitamin A and thus its homeostasis in absence of BCO1.

2.3 LIVER
2.3.1 Structure and organization of liver
Liver is the largest glandular organ in the body. It weighs approximately 2.5% of the total body weight in adults. It is located in abdominal cavity enclosed in fibrous connective tissue and well protected by the ribcage. Anatomically, the human liver can be divided into four lobes; two large (right and left) and two small lobes (quadrate and caudate).
Liver is also the major vitamin A storing organ in the body. In a healthy individual, liver stores about 80-90% of the total vitamin A present in the body. After intestinal absorption, liver is the next major organ involved in the uptake, metabolism and storage of retinoids.

2.3.2 Classic Liver Lobule
The basic functional units of liver are called hepatic lobules, as shown in figure 2.1. Each classic lobule has hexagonal appearance with portal areas at the periphery and hepatic vein at the center of each lobule.
Figure 2.1: Diagram of classic liver lobule. Diagram is showing hexagonal structure of classic liver lobule. Hepatic vein is situated at the centre and branches of hepatic artery, portal vein and bile duct at the periphery of each lobule. Arrangement of hepatocytes is shown with sinusoids running between hepatic plates.

The liver is supplied by arterial and venous blood, which flows from the portal areas towards the hepatic/central vein. Portal area consists of small branches of portal vein, hepatic artery and bile duct along with lymph vessel (Figure 2.1, 2.2). Portal triads are surrounded by connective tissue stroma (Rappaport et al. 1958).

Blood from hepatic artery and portal vein reaches liver cells via sinusoidal capillaries (sinusoids) running between the cords of hepatocytes and empties into the central vein (Figure 2.2, D). The hepatic portal vein is the source of most of the (75%) blood supply which carries venous blood deficient in oxygen. The blood supplied by the portal vein
(PV) comes directly from the digestive tract hence contains nutrients and toxic materials largely absorbed in the intestine. Hepatic artery (HA) supplies oxygenated blood from heart and provides 25% of the total blood supply to the liver. The bile duct (BD) is composed of cuboidal epithelial cells and is involved in the exocrine function of the liver by being the vessel involved in secreting bile produced by the liver. Bile flows opposite to the flow of blood, towards the portal areas (Figure 2.2 C).

Based upon the supply of blood to liver cells, liver lobule can be divided into three zones. Zone 1 is composed of the region around portal triads and majority of peripheral region of the lobules. This zone has the richest supply of oxygenated blood from the portal triads. It is also called perilobular zone. Zone 2 is the middle zone of the hepatic lobule with reduced metabolic activity as compared to zone 1. Zone 2 is also known as intermediate zone. And Zone 3 is closest to the central vein and receives the least amount of the oxygenated blood. It is also called centrolobular zone (Figure 2.2, B).

### 2.3.3 Liver Cell Types

Liver cells can be categorized into parenchymal and non-parenchymal cells. Parenchymal cells mainly consist of hepatocytes and non-parenchymal cells consist of four major cell types: kupffer cells, stellate cells, pits cells and endothelial cells (Bouwens et al. 1992) (as shown in Figure 2.3). Hepatocytes are separated by sinusoids which are the blood carrying capillaries that also bathe hepatocytes. Many non-parenchymal cell types like kupffer and pit cells are located in the sinusoids (Figure 2.3). Hepatic plates and sinusoids
are separated by the space of Disse also called perisinusoidal space. This perisinusoidal space is lined with a thin sheet of endothelial cells. The hepatic stellate cells are located within these perisinusoidal spaces.

2.3.3.1 Hepatocytes

Hepatocytes are parenchymal cells in liver and comprise about 70-80% of the total liver cells. They are arranged in cords with sinusoidal capillaries running between them. Hepatic cords radiate from the central vein and extend towards the portal triads. Hepatocytes are well recognized by their cuboidal shape with spherical nucleus at the center (Figure 2.3) (Bioulac-Sage et al. 1991). The basolateral surface of the hepatocytes faces the sinusoids and the apical surface of two adjacent hepatocytes forms bile canaliculi (Figure 2.3). Bile canaliculi carry the bile, produced in hepatocytes, and drains into the bile ducts in portal areas (Figure 2.2, C) (Phillips 1987).
Figure 2.2: H&E stain of normal rat liver used in this study. A: Portal triad in parenchyma. B: Bright field image showing profiles of central vein (CV), portal area (PT) and zones of liver lobule. C: Portal triad at higher magnification showing portal vein (PV), hepatic artery (HA) and bile duct (BD) embedded in connective tissue (CT) stroma. D: Central vein (CV) with large sinusoidal capillaries. Hepatocytes with spherical nucleus are arranged in cords with sinusoids.

2.3.3.2 Kupffer cells

Kupffer cells are the macrophages of liver, located in the sinusoidal spaces. They comprise 15% of the cells in liver. The distribution of Kupffer cells is not homogenous in liver. A large number of Kupffer cells are localized in periportal areas than in pericentral regions in rat liver (Figure 2.2, D) (Wisse et al. 1996).
2.3.3.3 *Endothelial cells*

Endothelial cells play significant roles in liver by lining the sinusoidal capillaries. Endothelial cells lack a basement membrane which gives them a “sieve plate” form. Endothelial lining in sinusoids form a small space of Disse between hepatocytes and endothelial cells (Figure 2.3). Free movement of small molecules and exchange of nutrients takes place in the space of Disse (Wisse et al. 1996).

2.3.3.4 *Stellate cells*

Hepatic stellate cells (HSCs) are also known as fat storing cells in normal liver and are located in the space of Disse in close contact with endothelial cells and hepatocytes. HSCs comprise about 5-8% of the cells in rat liver (Reviewed in Friedman 2008). They are specifically recognized by the presence of intracellular lipid droplets and cytoplasmic filament proteins such as desmin, vimentin and many more.

2.3.4 *Hepatic Stellate Cells (HSCs)*

2.3.4.1 *Morphological Features*

Hepatic stellate cells (HSCs), also known as Ito cells, lipocytes, and perisinusoidal fat storing cells, are the major vitamin A storing cells in liver (reviewed in Blaner et al. 2009). Hepatic stellate cells along with Kupffer cells and endothelial cells constitute the non-parenchymal cell population in liver. They constitute 5-10% of the total liver cell population and one-third of the non-parenchymal cells (Reviewed in Friedman 2008). Due to their stellate like structure, they were first named as “Sternzellen” by Kupffer in
1876 (Reviewed in Friedman 2008). Now they are widely accepted by the name “hepatic stellate cells” (reviewed in Blaner et al. 2009).

Figure 2.3: Liver cell types. Parenchymal cells-hepatocytes are arranged as single cell thick plates. Basolateral membrane of hepatocytes faces space of Disse. Non-parenchymal-hepatic stellate cells are located in the space of Disse in contact with endothelial cells and hepatocytes. Kupffer cells are anchored to endothelial cells within the sinusoids.

In a normal liver, HSCs are well recognized by their characteristic retinoid containing lipid droplets in the cytoplasm (reviewed in Blaner et al. 2009). These cytoplasmic lipid
droplets give stellate cells a unique in morphology as compared to other non-parenchymal cells. These lipid droplets also exhibit blue-green fluorescence when excited with light at 328nm. Hepatic stellate cells have cytoplasmic processes that extend around sinusoids and hence are implicated in the regulation of blood flow through the capillaries (reviewed in Blaner et al. 2009).

2.3.4.2 Distribution of Hepatic Stellate cells in liver

It is well established that the stellate cells are located in the “space of Disse” between hepatocytes and endothelial cells lining the sinusoids. HSCs are uniformly distributed in liver lobules under normal condition. Some studies suggested an increased number of cells around central vein as compared to periportal regions (reviewed in Rockey and Friedman 1992). However, others observed more frequent distribution in peri-portal regions than in peri-central locations with desmin staining (Yokoi et al. 1984).

2.3.4.2 Cytoskeletal Phenotype

The cytoplasm of the stellate cells is rich in certain intermediate filaments and microtubules (Rockey and Friedman 1992). Several studies have established that desmin, an intermediate filament protein, is identified in the cytoplasm of the HSCs (Yokoi et al. 1984). Immunocytochemical studies have also identified desmin in the muscle cells of hepatic artery and portal vein (reviewed in Rockey and Friedman 1992). The expression
of desmin has been extensively studies in stellate cells and hence proposed to be a reliable marker of quiescent stellate cells in rat liver.

2.4 Vitamin A metabolism in liver

2.4.1 Overview

Liver stores approximately 80-90% of the total body vitamin A under vitamin A sufficient conditions (reviewed in Geerts 2001). Of the total vitamin A stored in liver, the majority (75%) are stored in the lipid droplets of stellate cells in the form of retinyl esters (RE) and predominantly as retinyl palmitate (Reviewed in Friedman 2008). Hepatocytes also store about 20% of the total hepatic retinoid and a small amount is distributed among other non-parenchymal cells (reviewed in Geerts 2001). In the enterocytes, dietary retinol is esterified with long-chain fatty acids to form retinyl esters (REs). These REs are incorporated into chylomicrons and released into the circulation (Geerts 2001) where chylomicrons undergo lyposis and loose some of their triglycerides and the resulting particles are called chylomicron remnants (Green and Glickman 1981). These chylomicron remnants which contain all of the esterified retinol are cleared by the liver via specific receptor mediated endocytosis (Goodman et al. 1965, Blomhoff et al. 1984). In the vitamin A sufficient state, chylomicron retinyl esters are initially taken up by hepatocytes (Blomhoff et al. 1982) and then transferred to hepatic stellate cells (HSCs) for storage. However during vitamin A deficiency, retinoids are directly transferred from hepatocytes to extra-hepatic tissue in the form of retinol binding protein bound retinol.
βC circulating in the blood in association with low density lipoproteins is also taken up by liver via receptor mediated endocytosis. Once inside the liver, it is transferred to the lysosomes for further processing (Krinsky et al. 1958, Mathews-Roth and Gulbrandsen 1974). Hence, liver cells are an important site for the metabolism of retinoids as well as β-carotene under physiological conditions.

2.4.2 Role of parenchymal cells in vitamin A metabolism
Retinyl esters (RE) taken up by hepatocytes are hydrolyzed to retinol by retinyl ester hydrolases (REHs) (Harrison and Gad 1989). Hydrolysis either takes place in plasma membrane or in early endosomes. Free retinol thus formed is transferred to endoplasmic reticulum (ER). In ER, retinol binds with retinol-binding protein (RBP) (Blomhoff et al. 1988) and secreted from the hepatocytes and enters the circulation. From the circulation retinol binding protein bound retinol (retinol-RBP) is either transferred to peripheral tissues for further processing and utilization or reabsorbed by hepatocytes and hepatic stellate cells (Rask et al. 1983, Geerts 2001, Friedman 2008). Free retinol may also bind to CRBP I and is further processed in hepatocytes before being transferred to hepatic stellate cells (Geerts 2001). The transfer of retinol from hepatocytes to the stellate cells has not been completely elucidated; however retinol binding protein (RBP) is proposed to play a significant role in the transfer mechanism (reviewed in Geerts 2001).

2.4.4 Role of hepatic stellate cells in retinoid metabolism
Under normal conditions, hepatic stellate cells (HSCs) store 50-80% of the total body vitamin A in the form of retinyl esters (Blomhoff et al. 1985). Lecithin: retinol acyltransferase (LRAT) and acyl coenzyme A: retinol acyltransferase (ARAT) are two enzymes involved in the esterification of retinol in hepatic stellate cells. ARAT specifically uses free retinol or retinol bound to serum RBP and LRAT has high specificity for CRBP-I bound retinol for esterification. In vitro studies reveal a preferential esterification of RBP-retinol before storage (Troen et al. 1994). Esterified retinol along with triglycerides and phospholipids is stored in cytoplasmic lipid droplets of HCSs. Stellate-like cells have also been described in extrahepatic tissues, implying that tissues other than intestine and liver are also involved in retinoid metabolism (Yamada and Hirosawa 1976). However, the storage ability for vitamin A is highest in HSCs as compared to other cell types in the body.

2.4.5 Hepatic stellate cell lipid droplets

It has been proposed by many researchers that HSCs contain two types of lipid. The type I lipid droplets are smaller in size and usually bound by a unit membrane. The type II droplets are usually large in size and not bound by any membrane (Wake 1980, Wake 1974, Yamamoto & Ogawa 1983, Geerts et al. 1990). It is well established that the lipid droplets of HSCs are the major storage site for vitamin A, where retinoids are stored as retinyl esters (REs). The dietary intake has been observed to affect the composition number and size of the lipid droplets (Friedman 2008). Studies have also shown that along with retinoids, provitamin A carotenoids also get deposited in the hepatic stellate
cells in animals (Lakshman et al. 1989, Mayne and Parker 1986). Hence, hepatic stellate cells are significantly involved in the metabolism of retinoids and also the provitamin A carotenoids taken up from circulation or from other liver cell types.

2.5 Enzymes involved in the metabolism of vitamin A in liver

2.5.1 Retinyl Ester Hydrolases (REH)

In liver, retinyl esters are the storage form of retinoids in HSCs in the normal state. During inadequate dietary vitamin intake, retinoid stores are mobilized and retinyl esters are hydrolyzed by retinyl ester hydrolases (REH) (Silveira & Moreno 1998). Retinyl esters can be hydrolyzed by acid or/and neutral retinyl ester hydrolases (Troen et al. 1994). After hydrolysis, free retinol is bound to retinol-binding protein (RBP) and secreted into circulation as retinol-RBP complex. Several research groups have identified the neutral and acid bile salt – independent REH activity from rat liver homogenates (Harrison & Gad 1989, Harrison and Napoli 1990, Napoli et al. 1989, Gad and Harrison 1991, Boerman and Napoli 1991). Carboxylesterases ES-2 and ES-10 (pI 6.0/6.1 esterase) were purified from rat liver microsomes and shown to have neutral REH activity (Sun et al. 1997). Later, three major neutral rat liver carboxylesterases (ES-10, ES-4, and ES-3) were purified exhibiting retinyl palmitate hydrolase activity (Sanghani et al. 2002). Hepatocytes are the primary liver cells involved in hydrolysis of chylomicrom remnant retinyl esters in an acidic environment in endosomal compartments (Hagen et al. 1999). Hydrolysis of retinyl esters, stored in hepatic stellate cell lipid droplets are also proposed to involve acidic REH activity (Harrison et al. 1995). More recently,
carboxylesterase ES-10 was purified from rat liver and shown to have both neutral and acid REH activity. On comparing mRNA levels of major carboxylesterases proposed to be involved in retinoid metabolism ES-10 protein had highest expression followed by ES-3 and ES-4 and ES-2 in rat liver (Linke et al. 2005). A recent immunohistochemical study reveals that ES-4 enzyme is highly expressed in hepatocytes and more specifically in the endoplasmic reticulum in rat liver (Ghosh and Harrison 2009, unpublished work).

2.5.2 Retinol esterifying enzyme (LRAT)

As mentioned earlier, hepatic stellate cells (HSC) store more than 90% of the total vitamin A in the body and retinyl ester is the major storage form of vitamin A in the lipid droplets of stellate cells. The formation of retinyl esters in HSCs is catalyzed by lecithin retinol acyltransferase (LRAT), a 25kD protein (Matsuura 1997). CRBP bound retinol acts as substrate for LRAT that esterifies retinol to all-trans-retinyl esters. The LRAT activity is observed to be higher in non-parenchymal cell fraction as compared to parenchymal cells in normal liver (Matsuura et al. 1997, Nagatsuma et al. 2009). It is proposed that HSCs have high specific activity and protein expression of LRAT as compared to hepatocytes (Matsuura 1997).

The LRAT activity and mRNA expression has been observed to be highly regulated in liver. In vitamin A deficient state, LRAT activity in rat liver was observed to be negligible and induced on repletion with vitamin A (Randolph & Ross 1991, Matsuura & Ross 1993). This suggests that enzymes that are involved in vitamin A metabolism are
regulated by liver to conserve the plasma retinol concentrations under conditions of dietary insufficiency of vitamin A.

LRAT activity was also expressed in lungs, testis, intestine, keratinocytes and retinal pigment epithelial cells in eye (Yostt et al. 1988, Matsuura et al. 1997, Saari et al. 1993, Kurlandsky et al. 1996, Zolfaghari & Ross 2002, Shingleton et al. 1989, Ong et al. 1991). According to a recent immunolocalization study, LRAT protein distribution has been observed among hepatic stellate cells in the space of Disse and the endothelial cells lining the sinusoids in normal human and rat liver. It was proposed that LRAT is involved in an unknown function in endothelial cells and these sinusoidal lining cells might be involved in the metabolism of retinoids in liver (Nagatsuma et al. 2009).

2.5.3 Transporters of Retinoids: Extracellular Retinol Binding Protein
In the cytoplasm, numerous carrier proteins are involved in the metabolism of retinoids. Plasma retinol binding protein (RBP) assists in the transport of all-trans-retinol in plasma (Newcomer & Ong 2000). Retinol bound to RBP is released into circulation from where it reaches extra-hepatic tissues for further processing and utilization (Smith et al. 1975). The liver parenchymal cells express RBP mRNA and within cell, the endoplasmic reticulum is the major site for the synthesis of RBP (Soprano & Blaner 1994).

2.5.4 Intracellular retinol binding proteins (CRBP-I)
Cellular retinol-binding protein-1 (CRBP-I) helps in transport of hydrophobic retinoids in the aqueous environment in cell (reviewed in Blaner et al. 2009). It also mediates esterification of retinol to REs and oxidation of retinol to the biologically active form of retinoids such as retinal and retinoic acid. CRBP-I is expressed in parenchymal and non-parenchymal stellate cells in liver. Approximately 90% of the total liver CRBP-I is expressed in hepatocytes however in HSCs its concentration is 20 times higher than hepatocytes in respect to per unit protein. (Blomhoff & Wake 1991) and proposed to be a good marker for stellate cells in liver (Lepreux et al. 2004).

The physiological significance of CRBP-I was investigated in overexpressing and knockout model of CRBP-I in mice (Ghyselinck et al. 1999, Troen et al. 1999). There was no visible phenotypic change in CRBP-I overexpressing model. However, the lipid deposition increased with increased retinol esterification in CRBP-I transfected stellate cells (O’Byrne & Blaner 2005, reviewed in Geerts 2001). Induction of CRBP-I deficiency reduced the accumulation of retinyl esters in HSCs by almost 50% (Nilsson et al. 1997), implicating its significance in storage of retinoids in HSCs.

The expression of CRBP-I protein was studied in quiescent hepatic stellate cells using immunohistochemistry on in formalin fixed paraffin embedded human liver sections (Lepreux et al. 2004, Rossen et al. 2009). CRBP-I expression was observed in the cytoplasmic processes of hepatic stellate cells (Lepreux et al. 2004). CRBP-I was also
marginally expressed in post-injury activated stellate cells (Rossen et al. 2009). Along with hepatic stellate cells, CRBP-I was also demonstrable in epithelial cells of bile duct in the portal triads (Lepreux et al. 2004).
Chapter 3: Materials and Methods

3.1 Cell Lines

3.1.1 Hepatic cell lines

The present study used two rat liver cell lines: HSC-T6 cells and McArdle-RH777 cells. HSC-T6 is a rat liver stellate cell line (Vogel et al. 2000) maintained in Waymouth MB 752/1 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 10% horse serum (HS), 1% penicillin-streptomycin (P/S) and 0.2% fungizone (Invitrogen). These cell lines were gifts from Dr. William Blaner, Columbia University, NY.

McA (McArdle-RH777) is a rat hepatoma cell line (American Type Culture Collection, Manassas, VA, USA, #CRL-1601), maintained in Dulbecco’s modified Eagle’s medium (DMEM) enriched with glucose and L-glutamine (Invitrogen, Carlsbad, CA, USA), supplemented with 10% FBS, 10% HS (Sigma-Aldrich, Inc., St. Louis, MO, USA), 1% penicillin-streptomycin and 0.2% fungizone.

3.1.2 BCO1 transfected cell line (NY CHO)

Chinese hamster ovary (CHO) cells stably transfected with mouse BCO1 cDNA, referred as NY CHO cell in this study, were a generous gift from Dr. Blaner, New York
(Paik et al. 2001). NY CHO cell line was used as positive control in assessing the specificity of the antibodies for BCO1 protein. They were grown in Minimum Essential Medium (MEMα, Invitrogen) supplemented with 10% FBS, 1% P/S and 0.2% fungizone.

3.1.3 Chinese hamster ovary cells (ATCC CHO)

Chinese hamster ovary cell line was obtained from American Type Culture Collection (ATCC, # CRL-10154) and is referred to as ATCC CHO. These cells were used as a negative control in this study. Cells were grown in T-75 flask at 37°C in a basic medium, MEMα, with 10% FBS, 1% penicillin-streptomycin, 0.2% fungizone and 0.1% methotrexate (MTX) in an incubator with 5% CO2 level and 95% humidity.

3.2 Cell culture

Cells (NY CHO, ATCC CHO, HSC and McA) were allowed to grow in their respective growth media until 70-80% confluency was obtained before passaging them into T-75cm² flasks. Medium and other solutions were preheated to 37°C before use. Spent medium was aspirated and the cells were washed with PBS. Thereafter, cells were trypsinized by adding 2 ml 0.25% Trypsin/EDTA (Invitrogen, Carlsbad, CA, USA) in calcium and magnesium-free phosphate buffered saline (PBS), and incubated for 2-3 minutes in the incubator. The cells were observed under the microscope (Olympus-CKX41) to ensure that all the cells were detached and floating. The trypsinized cell solution was inactivated by adding 5ml of fresh medium and flushed gently to ensure that all the cells are detached. The cell suspension was centrifuged at 1000 rpm for 5 minutes
at 24°C. The supernatant was discarded and cell pellet was resuspended in 2 ml fresh medium. The cells were counted with hematocytometer. Approximately, 1x10^6 cells were re-suspended in fresh medium and transferred to new sterilized T-75 flask. All cell lines were maintained in humidified environment at 37°C with 5% CO₂ for approximately 72 hours.

3.3 Antibodies

Primary antibodies were: rabbit polyclonal antibody against mouse BCO1 (IgG) referred to as “rabbit anti-BCO1”, a generous gift from Dr. Blaner, Columbia University, NY; chicken polyclonal antibody against human recombinant BCO1 (IgY), referred as “chicken anti-BCO1” (Genway biotech, San Diego, CA); chicken anti-ES-4 (Genway biotech, San Diego, CA) and rabbit antibody against human desmin (Novus biological, Littleton, CO). Secondary antibodies used were Biotin-Streptavidin-conjugated donkey anti-rabbit (IgG) and anti-chicken (IgY) for Immunohistochemistry (IHC) (Jackson Immunoresearch, West Grove, PA). For western blotting, secondary antibodies used were Infrared dye (IRDye) 800 conjugated donkey anti-chicken and goat anti-rabbit (LI-COR biosciences, Lincoln, NE). For immunofluorescence staining secondary antibody used was Alexa Fluor 488 – coupled donkey anti-rabbit (Invitrogen, Carlsbad, CA, USA). Peroxidase conjugated mouse anti-biotin (IgG) (Jackson Immunoresearch) was used as tertiary antibody in IHC. Summary of antibodies used in the study with respective dilutions is shown in table 3.1.
<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Source</th>
<th>Dilution</th>
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<td>1:300 (IHC)</td>
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<td></td>
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<td>1:1000 (IF)</td>
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<td></td>
<td>1:100 (IHC)</td>
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<tr>
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<td></td>
<td></td>
<td>1:500 (IHC)</td>
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Abbreviations: BCO1, β-carotene oxygenase-1; ES-4, carboxylesterase ES-4; SP, Streptavidin; IR, Infrared; WB, western Blot; IHC, Immunohistochemistry; IF, Immunofluorescence.

**Table 3.1: Antibodies used in this study**
3.4 Western Blotting

3.4.1 Preparation of cell lysates and liver homogenate

Cells were seeded at a density of $1.5 \times 10^5$ cells/well in 6 well plates for 48 hrs. Fresh medium was added after 24 hrs. On attaining 70-80% confluency, cells were washed with PBS then lysed with 1x lysis buffer prepared with Radio-Immunoprecipitation Assay (RIPA, Thermo Scientific, Rockford, IL) and protease inhibitor cocktail (PI, Roche Diagnostics, Indianapolis, IN). Lysed cells were then collected in 1.5 ml eppendorf tubes and centrifuged at 14000 rpm at 4°C for 4 minutes. Supernatant was collected and analyzed for protein concentration using the BCA Assay.

Fresh rat liver (1 gm) was homogenized in 3 ml lysis buffer (1x RIPA and phosphatase inhibitor) with Omni tissue homogenizer (Thomas Scientific Swedesboro, NJ). The homogenate was further diluted with deionized distilled water in 1:10 (v/v). The protein content of cells and tissue homogenate was determined using bicinchoninic acid assay (BCA, Thermo Scientific, Rockford, IL). BCA was added to a CuSO$_4$ pentahydrde solution at a ratio of 1:50 and 100 μl of this reagent was added to 1 μl of cell lysate in 99 ul water and incubated at room temperature for 45 min. The absorbance was measured at 562 nm using the spectrophotometer. Bovine serum albumin (BSA) was used as standard.

3.4.2 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed with cell lysates and rat liver homogenate. Samples were dissolved with SDS-Laemml reducing buffer (SDS, glycerol, Tris-HCL, bromophenol,
β-mercaptoethanol). 50-60µg protein per sample was then loaded onto 10% acrylamide gel overlaid with 5% acrylamide stacking gel. IR Dye two color molecular weight ladder (LI-COR biosciences, Lincoln, NE) was used to determine the protein molecular mass. Gels were run in running buffer (0.25M Tris-glycine, SDS, water, pH 8.3) using the Mini-Protean tetra-Electrophoresis system (Bio-rad, Hercules, CA) at 120V until the dye front reached the bottom of the gel.

3.4.3 Western Blotting

Immunoblotting was performed with nitrocellulose membranes (Millipore, Bedford, MA). Resolved proteins were transferred to membrane in transfer buffer (pH 8.3, Tris-glycine) in tank transfer unit overnight at 0.05 Amps at 4°C. Successful transfer of protein from gel to membrane was confirmed by Ponceau staining. Unspecific binding sites were blocked by incubating in blocking buffer (LI-COR biosciences, Lincoln, NE) for an hour at room temperature. Following this, the blots were incubated in one of the following primary antibodies; diluted in blocking buffer with 0.01% Tween-20, rabbit anti-BCO1 (1:5,000); chicken anti-BCO1 (1:10,000); goat anti-desmin (1:5000); chicken anti-ES-4 (1:10,000). Incubation was performed overnight in a cold room (4°C) on a rocker. Four washes of five min each were made in PBS-Tween (0.1%). Membranes were incubated in IRDye 800 conjugated secondary antibodies, goat anti-rabbit and donkey anti-chicken for 30 min at room temperature and washed 5 times in PBS-Tween (0.1%) thereafter. PBS alone was used in last two washes to remove the tween from blot. Blot
was protected from direct light after secondary antibody incubation and observed with
ODYSSEY–LICOR-Infra-red Imaging System (LI-COR biosciences, Lincoln, NE).

3.5 Immunofluorescence staining

For further characterization of antibodies for BCO1, fluorescence immunostaining was
used to visualize the expression of BCO1 in NY CHO cells. NY and ATCC CHO cells
were seeded at a density of 1.5 x 10⁵ cells/well on the coverslips in Falcon 6-well plate.
After incubating for 42 hours, cells were washed in PBS and fixed with 4% (w/v)
paraformaldehyde and permeabilized with 0.3% Triton X 100 (Fisher Scientific, Fair
Lawn, NJ). After permeabilization, cells were washed and blocked with 10mg/ml bovine
serum albumin (BSA, Fischer Scientific) and 5% normal donkey serum (NDS, Jackson
Immunoresearch) in PBS for 30 minutes to inhibit non-specific binding of primary and
secondary antibodies. The primary antibody solution was applied, which comprised of
rabbit polyclonal anti-mouse BCO1 antibody (1:1,000) diluted in 10mg/ml bovine serum
albumin and 5% normal donkey serum in PBS. Cells were incubated overnight at 4°C.
After incubating with primary IgG, cells were washed three times with PBS and
incubated with Alexa Fluor 488 conjugated donkey anti-rabbit (1:5,000) which fluoresces
green color when excited at 499nm. Prolong Gold antifade (Invitrogen, Carlsbad, CA,
USA) mounting medium was applied and cells were visualized using Olympus IX71
epiphotofluorescence microscope and Olympus cell sense software. Controls were performed
for both NY and ATCC CHO cells, by incubation in secondary IgG without the primary
IgG.
3.6 Immunohistochemistry

Immunohistochemical staining was performed to demonstrate the presence and localization of BCO1 protein in rat liver. Formalin fixed paraffin embedded rat liver sections were prepared by Veterinary Histology Laboratory Services, The Ohio State University (Columbus, OH, USA). Before proceeding with staining, paraffin sections were first deparaffinized and rehydrated by placing in xylene followed by 100%, 95% and 70% ethanol and distilled water. The slides were then placed in 3% hydrogen peroxide in PBS (pH 7.4) to block endogenous peroxidase activity in the liver section. Following this, slides were rinsed with PBS and blocked with 3% bovine serum albumin (BSA) + 1.28% normal donkey serum (NDS) in PBS, (pH 7.4) in humidifying chamber. Blocking will inhibit any unspecific binding of primary or secondary antibodies. After blocking for 1 hr at room temperature, sections were incubated with primary antibodies: rabbit anti-mouse BCO1 (1:300); chicken anti-BCO1 (1:1500); rabbit anti-desmin (1:100); chicken anti-Esterase-4 (1:500). Incubation with primary antibody was carried out overnight at 4°C in humidifying chamber.

After overnight incubation with primary antibody, tissue sections were washed with PBS to remove unbound primary antibodies from the surface of the tissue. Sections were next incubated with the appropriate secondary antibodies diluted in 3% BSA + 1.28% NDS in PBS. Secondary antibody concentrations were as follows: donkey anti-rabbit in 1:500 dilution for detection of BCO rabbit antibody and desmin; donkey anti-chicken IgY in 1:500 dilution for detecting ES-4 and 1:2000 for BCO1 chicken antibody. After
subsequent washes in PBS, sections were incubated with horseradish peroxidase (HRP) conjugated tertiary antibody followed by diaminobenzidine (DAB, Dako, Carpinteria, CA) and counterstained with Gill’s Hematoxylin (Fisher Scientific, Fair Lawn, NJ). Controls were performed by removing the primary antibody from the protocol. Sections of rat liver tissue were examined using Olympus BX51 light microscope with 10x, 20x, 40x and 60x objective. DP Manager Software was used to digitally record images.
Chapter 4: RESULTS

4.1. Western Blotting - Specificity of the BCO1 antibodies

4.1.1 Specificity of the rabbit- and chicken anti-BCO1 antibodies

Antibodies that recognize distinct epitopes of BCO1, referred as rabbit anti-BCO1 and chicken anti-BCO1, as described in “Methods”, were used for the analysis of BCO1 protein in rat liver sections. The two antibodies were tested for the specificity towards BCO1 protein using western blot.

Chinese hamster ovary cells stably transfected with BCO1 cDNA (NY-CHO) were used as positive control and a non-transfected cell line (ATCC-CHO) was used as negative control. Both, rabbit anti-BCO1 and chicken anti-BCO1 reacted with a ~62 kD protein in the NY CHO cells (Figure 4.1, NY), whereas no BCO1 protein band was observed in ATCC CHO cells (Figure 4.1, ATCC), demonstrating that both antibodies were specific for BCO1 enzyme. In the absence of BCO1 specific antibodies no labeling was observed in either NY CHO (Figure 4.2, NY) or ATCC CHO cell (Figure 4.2, ATCC).
Figure 4.1: Detection of BCO1 by immunoblotting. The specificity of the two antibodies used in the IHC experiments, were compared using lysates from NY-CHO cells (Lane NY) and ATCC-CHO cells (Lane ATCC). Both rabbit anti-BCO1 and chicken anti-BCO1 antibodies show protein band at 62kD (arrow).

Figure 4.2: Control for rabbit and chicken anti-BCO1 antibodies. Cell lysates from NY CHO and ATCC CHO cells do not react with any protein in the absence of primary antibodies (rabbit anti-BCO, chicken anti-BCO1)
4.1.2 Expression of BCO1 in hepatic cell lines

To examine whether BCO1 protein is expressed in parenchymal and non-parenchymal cells, western blot was performed using transformed cell lines derived from hepatocytes and hepatic stellate cells. Rat liver homogenate was used to observe the expression of BCO1 protein in whole liver. A comparison of BCO1 expression was also made between whole liver homogenate and BCO1 transfected cell line.

![Rabbit anti-BCO1](image)

Figure 4.3: BCO1 expression in hepatic cell lines and rat liver. Cell lines derived from hepatocytes (McA-RH777), hepatic stellate cells (HSC-T6) and rat liver homogenate (Liver) show a protein band at ~62 kD with rabbit anti-BCO1 antibody (Right). (Left) BCO1 expression in NY and ATCC CHO cells is compared with rat liver homogenate. Arrows mark the position of the respective BCO1 protein bands. The molecular weight markers are shown in figure above.

Immunoblotting of the cell lines with rabbit anti-BCO1 antibody revealed a band of ~62 kD in both McA-RH777 (derived from hepatocytes) (Figure 4.3, McA) and HSC-T6 (hepatic stellate cell line) (Figure 4.3, HSC). As compared to whole liver homogenate
(Figure 4.3, Liver), the expression of BCO1 was noticeably high in NY CHO cells (Figure 4.3, NY). As expected, ATCC CHO cells do not express BCO1 protein (Figure 4.3, ATCC). Thus, BCO1 rabbit antibody recognizes BCO1 enzyme in liver and liver cells.

4.1.3 Specificity of rabbit anti-BCO1 antibody using Immunofluorescence staining

Immunofluorescence staining was performed for NY CHO cells, transfected with BCO1 cDNA and ATCC CHO, a non-transfected cell line, using rabbit anti-BCO1 antibody. NY CHO cells were positively stained by the antibody to BCO1 (Figure 4.4, Image A). ATCC CHO shows no specific fluorescence labeling in the cells (Figure 4.4, Image B). In the absence of BCO1 antibody no labeling was observed in either BCO1 transfected cells (Figure 4.5, A) or non-transfected cells (Figure 4.5, B) thus confirming the specificity of the antibody for BCO1.

4.1.4 Specificity of ES-4 and Desmin antibodies

The expression of carboxylesterase ES-4, a marker for hepatocytes, and desmin, a marker for hepatic stellate cells, was investigated by immunoblotting. Expression of ES-4 was detected at about predicted molecular weight of 64 kD (arrow) in liver; no labeling observed in either NY CHO or ATCC CHO cell lysates (Figure 4.6, blot A). Similarly, blotting with desmin antibody labeled a protein at appropriate molecular weight of ~ 52 kD with no reactivity in NY and ATCC CHO cell lysates (Figure 4.6, blot B).
Figure 4.4: The specificity of the rabbit anti-BCO1 antibody verified by immunofluorescence staining. NY CHO and ATCC CHO cells were incubated with primary (rabbit anti-BCO1) antibody. Specific staining was observed with secondary antibody conjugated to Alexa Fluor 488 fluorophore. Image A: Specific staining in NY CHO cells. Image B: ATCC CHO cells show green background without specific staining in cells. 100x.

Figure 4.5: Control for rabbit anti-BCO1 antibody. ATCC and NY CHO cell lines incubated with Alexa Fluor 488 conjugated secondary antibody in the absence of primary (rabbit anti-BCO1) antibody. Image A: NY CHO cells stably transfected with BCO1, image B: ATCC CHO non-transfected cell line (100x).
Thus ES-4 and desmin antibodies recognized appropriate sized protein bands in rat liver lysate.

Figure 4.6: Western blotting for anti-ES-4 and anti-desmin antibodies. Detection of ES-4 and desmin was assessed in rat liver using chicken anti-ES4 and rabbit anti-desmin antibodies. The protein bands of expected molecular weight for ES-4 and desmin are shown with the arrow. No protein band observed in NY and ATCC CHO cells as expected. Molecular weight markers are shown on the left of each blot.
4.2 Immunohistochemical localization of BCO1 in liver

To determine the cell type localization of BCO1 protein, immunohistochemistry (IHC) was performed using antibodies recognizing two distinct epitopes of BCO1 protein in rat liver. Rat liver paraffin sections were immunostained with both BCO1 antibodies, referred as rabbit anti-BCO1 and chicken anti-BCO1.

4.2.1 Localization of BCO1 in liver cells

The comparison of BCO1 labeling with both chicken (top image – chicken anti-BCO1) and rabbit (image – rabbit-anti-BCO1) antibodies is shown in Figure 4.7. Both antibodies moderately stained the hepatocytes with relatively strong staining in non-parenchymal stellate-like cells. At a higher magnification, as shown in the insets, chicken anti-BCO1 strongly stained hepatic stellate like cell (HSC) in the perisinusoidal spaces of liver lobules (Figure 4.7; chicken anti-BCO1) and rabbit anti-BCO1 antibody recognizes lipid containing stellate cell (Figure 4.7; rabbit anti-BCO1, inset). Both BCO1 antibodies, albeit with difference in epitope recognition, displayed immunostaining of perisinusoidal hepatic stellate cells (HSC).
Figure 4.7: Immunostaining of rat liver paraffin section with BCO1 antibodies. Perisinusoidal hepatic stellate cells (inset, chicken anti-BCO1) are positive with chicken anti-BCO1 antibody and hepatic stellate cells with lipid droplets (inset, rabbit anti-BCO1) shows positive staining for rabbit anti-BCO1 antibody. Uniform positive staining was observed in hepatocytes for both chicken and rabbit anti-BCO1 (star). Scale bars 200µm. HSC; Hepatic stellate cell. (Arrows indicating HSCs)
4.2.2 Co-localization of BCO1 and Hepatic Stellate Cell marker (desmin)

4.2.2.1 Co-localization of BCO1 and desmin in hepatic stellate cells using rabbit anti-BCO1 antibody

Desmin is an accepted histochemical marker for rodent HSCs (Yokoi et al. 1984). The distribution of BCO1 positive cell types in rat liver section was compared with desmin in the serial sections of a rat liver parenchymal region (Figure 4.8). The hepatocytes exhibit uniform positive staining for BCO1 (Figure 4.8A, C) and expectedly show no expression for desmin (Fig 4.8B, D). Some non-parenchymal stellate like cells distributed between the hepatocytes were positive for BCO1 (←; Figure 4.8, A, C) corresponding with the staining of HSCs for desmin (←; Figure 4.8, B, D). A similar distribution of desmin positive HSCs are observed in the serial section (Figure 4.8, B and D). Each inset in Figure 4.8 shows HSCs between cords of hepatocytes, at a higher magnification.
Figure 4.8: Immunostaining of rat liver serial sections for rabbit anti-BCO1 and rabbit anti-desmin. Panel A & C showing anti-BCO1 and panel B & D is showing anti-desmin staining in parenchyma. A & C) Rabbit anti-BCO1 antibody shows positive staining in hepatocytes and non-parenchymal stellate cell (arrows). B & D) Desmin shows positively stained hepatic stellate cells (inset) and negative staining in hepatocytes. Each arrow shows non-parenchymal perisinusoidal cells. Each inset shows HSCs. Magnification 600x A, B and 400x (C, D).
In the portal triads, strong staining was observed in endothelial cells lining the hepatic artery “tunica intima” (Figure 4.9A, arrow) with BCO1 antibody. As expected, desmin moderately stained the smooth muscle layer of hepatic artery (Figure 4.9B arrow) and portal vein (Figure 4.9B) as it is a smooth muscle protein. The expression of BCO1 and desmin were clearly distinct in portal vessels. As a smooth muscle intermediate filament
protein, the expression of desmin in smooth muscle cells was in agreement with other histochemical studies (Yokoi et al. 1984).

Figure 4.10: Immunostaining for BCO1 and desmin in the endothelial cells lining the portal vein in serial sections of rat liver. A) Endothelial cells lining the portal vein showing positive staining for BCO1 (arrows). Magnified images endothelial cells shown by arrows in section A & B are shown below the respective images. B) Endothelial cells lining the portal vein are negative for desmin (arrows). PV, portal vein; EC, endothelial cells, 600x A, B.

Furthermore, Figure 4.10 shows the difference in the staining for BCO1 and desmin in the endothelial cells of portal vein. BCO1 antibody strongly stains the oval shaped the endothelial cells lining the portal vein (Figure 4.10A, lower panel, arrows). Desmin
staining is negative in the endothelial cell lining the portal vein; however it shows positive staining in the thin wall of portal vein (Figure 4.10B, lower panel, arrows).

BCO1 and desmin labeling in the central vein region is shown in Figure 4.11. Staining for BCO1, in contrast to desmin, was localized in hepatocytes in a uniform manner (Figure 4.11, A). Hepatocytes are negative for desmin (Figure 4.11, B). Endothelial cells lining the central vein are negative for both BCO1 (Figure 4.11A, arrows) and desmin (Figure 4.11B, arrow).

4.2.2.2 Co-localization of BCO1 and desmin in hepatic stellate cells using chicken anti-BCO1 antibody

Immunostaining with chicken anti-BCO1 (A, C) and anti-desmin (B, D) in the serial sections of rat liver is shown in Figure 4.12. Consistent with rabbit BCO1 labeling, a uniformly diffused staining was observed in hepatocytes with chicken BCO1 antibody (Figure 4.12, A & C).
Figure 4.11: Rat liver serial sections immunostained with rabbit BCO1 and anti-desmin antibody. A) Uniform positive labeling for BCO1 is shown in hepatocytes. Endothelial cells lining the central vein show negative staining for BCO1 (A; arrows). B) Endothelial cells lining the central vein (B; arrow) and hepatocytes are negative for desmin. Each arrow shows endothelial cell in central vein (←). EC, endothelial cells; CV, central vein, magnification 600x.
Figure 4.12: Serial sections of rat liver immunostained with chicken anti-BCO1 and anti-desmin antibodies. Each inset shows a magnified view of hepatic stellate cells. A) Chicken anti-BCO1 antibody shows diffused positive staining in hepatocytes and strong positive staining in sinusoids. Strongly positive perisinusoidal hepatic stellate cell (arrow) is shown in inset of A & C. B) Desmin positive hepatic stellate cells (arrow) are shown in insets of B & D. HSC; hepatic stellate cells, BD; bile duct, HA; hepatic artery, PV; portal vein. Scale bar 200μm.

Co-localization for chicken BCO1 and desmin was observed in HSCs of periportal areas as shown in insets of Figure 4.12. BCO1 positive diffused labeling was observed in endothelial cells lining the sinusoids (inset Figure 4.12A). However, the following can be observed in the figure: (1) In contrast to rabbit anti-BCO1, endothelial cells in portal vessels (artery and vein) were negative for chicken anti-BCO1 antibody. (2) In rat liver,
immunostaining with chicken anti-BCO1 shows low expression in hepatocytes. (3) Chicken anti-BCO1 highlighted the HSCs and sinusoidal lining throughout the lobule. (Inset, Figure 4.12A). (4) Interestingly, some desmin positive HSCs were negative for BCO1 and some BCO1 positive non-parenchymal cells were negative for desmin.

Consistent with earlier results, desmin showed positive staining in smooth muscle cells of hepatic artery and portal vein (Figure 4.12, arrows). Hepatic stellate cells with lipid droplets did express desmin (inset in Figure 4.12, B & D). Desmin positivity was localized in the perinuclear cytoplasm of the HSCs. However, BCO1 was strongly expressed in HSCs with lipid droplets (inset). Both morphology and location of the positive staining identified them as HSCs.

**4.2.3 BCO1 control**

In formalin fixed paraffin embedded rat liver sections, BCO1 staining was revealed in hepatocytes (Figure 4.13, A & C) and non-parenchymal stellate cells in figure 4.13C inset. In the absence of BCO1 primary antibody, no staining was observed in any of the cell types which were stained for BCO1 (Figure 4.13, B and D).
Figure 4.13: BCO1 control. Rat liver serial sections immunostained with antibody against BCO1 and its serial section is stained without primary antibody. A) Rat liver section stained for BCO1 shows uniform positive staining in hepatocytes and non-parenchymal cells (arrow). B) Control for BCO1 is negative in hepatocytes and non-parenchymal cells (arrow). C) Inset in this section showing positively stained stellate cell in sinusoid. D) Inset in control section showing negative labeling for stellate cell shown by arrow. Inset and arrow (→) in section A & C are showing BCO1 positive hepatic stellate cell in sinusoids. Inset and arrow (→) in D&B are showing no staining in stellate cells with control. Magnification 600x A and B, 400x C and D.

4.2.4 Co-localization of BCO1 and carboxylesterase ES-4

In the serial sections of rat liver, staining for BCO1 is similar to ES-4 with consistent uniform expression in hepatocytes (Figure 4.14, A&B and C&D). However, perinuclear cytoplasm in hepatocytes was strongly stained by ES-4 as compared to BCO1 protein.
(Figure 4.14, B and D). Also the endothelial cells lining the central vein are negative for both ES-4 (arrow, Figure 4.14, B) and BCO1 protein (arrow, Figure 4.14, A).

Figure 4.14 also shows the ES-4 and BCO1 localization in portal areas of rat liver. In comparison to BCO1, ES-4 positive staining in hepatocytes is accentuated in the perinuclear cytoplasm (Figure 4.14, D). As with BCO1, uniform moderate positive staining was seen in cytoplasm of hepatocytes (Figure 4.14, C). Differences in ES-4 and BCO1 staining were observed in bile duct epithelial cells [Figure 4.14D-(BD)]. ES-4 is strongly expressed in bile duct epithelial cells whereas negative for BCO1 [Figure 4.14, C-(BD)]. Table 4.1 summarizes the staining for BCO1, ES4, and desmin in rat liver cells and tissue components.
Figure 4.14: BCO1 and ES-4 immunostaining in serial sections of rat liver. A) Rat liver sections stained for BCO1 shows moderate positive staining in hepatocytes. Endothelial cells lining the central vein (arrows) shows negative staining for BCO1. B) Strong positive staining in hepatocytes for ES-4 and negative staining in EC (arrow). C) In portal area, bile duct epithelial cells were negative for BCO1 and endothelial cells (arrow) lining portal vein shows moderate positive staining for BCO1 protein. D) Portal areas show strong positive staining in hepatocytes and moderate positive staining in the endothelial wall of portal vein (arrow). ES-4 shows strong positive staining in the bile duct epithelial cells. BD; bile duct, CV; central vein, EC; endothelial cells, 600x (A, B) and 400x (C, D).
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Abbreviations: HSC, hepatic stellate cells; HA, hepatic artery; PV, portal triad; PT, portal triad; CV, central vein.

Immunostaining grades as: strong (+ + +), moderate (+ +), weak (+), negative (-), not determined (ND)

Table 4.1: Summary of immunohistochemical detection of BCO1 in liver cell types and tissue components in rat liver.
Chapter 5: Discussion

The present study offers new insight regarding the localization of BCO1 protein in liver cell types and tissue components. In this study we demonstrated the immunolocalization of BCO1 protein in parenchymal and non-parenchymal cells in rat liver paraffin sections. Previous studies have reported the presence of BCO1 mRNA in liver tissue (Ref). In 2004, Lindqvist and Andersson reported that BCO1 is localization is limited to hepatocytes in liver. However, a recent study by Blaner and colleagues, suggested a relatively high expression of BCO1 mRNA in purified fractions of stellate cells as compared to a lower expression in hepatocytes (Shamarakov et al.2010). In agreement with the results of the Blaner study, we found significantly strong staining for BCO1 protein in non-parenchymal stellate like cells with lipid droplets and a diffused uniform staining in cytoplasm of the hepatocytes.

For confirmation, we compared the staining pattern for BCO1 with desmin and carboxylesterase ES-4. Desmin is a widely accepted histochemical marker for HSCs and ES-4 is a retinyl ester hydrolase enzyme localized in hepatocytes, specifically in endoplasmic reticulum in rat liver (Ghosh and Harrison 2009, unpublished work).
Negative controls were performed for BCO1 antibody. These controls did not show any staining in the cells which were positive for BCO1 expression.

The present immunohistochemical study revealed that BCO1 protein was present in the non-parenchymal stellate like cells that co-localized with HSCs stained for desmin in the serial sections of rat liver. The staining for BCO1 and desmin differentiated in portal triad and parenchymal cells. Hepatocytes were moderately stained for BCO1 whereas a strong reactivity was observed in the endothelial cells in portal vein and hepatic artery in the portal areas. An interesting observation was that endothelial cells were positively stained for BCO1 in the portal vein while they were negative in the central vein. As expected, hepatocytes were devoid of desmin staining and a moderate staining was observed in smooth muscle cells of hepatic artery and portal vein, as desmin is a smooth muscle protein (Yokoi et al. 1984).

Co-localization of BCO1 and carboxylesterase ES-4 was observed in hepatocytes with moderate staining for BCO1 protein, in contrast to a significantly high expression for ES4. Both ES-4 and BCO1 were localized in perinuclear cytoplasm of the hepatocytes. Differences in the localization of BCO1 and ES-4 were observed in the portal areas. In contrast to BCO1, which strongly reacted with the tunica intima of hepatic arteries, ES-4 was absent in hepatic arteries. We also found ES-4 expression in bile duct epithelial cells that were negative for BCO1 expression. The expression of ES-4 in bile ducts in our
study co-localizes with CRBP-I protein (Lepreux et al. 2004) which may suggest an unknown function of retinoid metabolizing enzymes in bile secretion.

Interestingly, endothelial cells of hepatic artery and portal vein were stained by antibody to BCO1 and sinusoidal endothelial lining was also positive for antibody to BCO1. Although these observations were unexpected, there is indirect evidence that endothelial cells and stellate cells in liver are functionally and structurally related. The cytoplasmic processes of stellate cells are proposed to control the blood flow through sinusoidal capillaries in coordination with fenestrated endothelial cells lining the sinusoids (Shibuya 2000, Wake 1999). Additionally, among other non-intestinal tissues, kidney also expresses BCO1 in the endothelium of its blood vessels (Wyss et al. 2001).

In this study, CHO cells transfected with BCO1 were used to demonstrate the specificity of antibodies to BCO1 protein. In addition to the immunolocalization of BCO1 protein in hepatocytes and hepatic stellate cells in rat liver paraffin section, we also reported the expression of BCO1 in rat liver homogenate and hepatic cell lines: McArdle-RH777 (McA) and HSC-T6 that corresponds to hepatocytes and hepatic stellate cell.

β-Carotene is the major dietary source of vitamin A in mammals as its cleavage by BCO1 synthesizes two molecules of retinoids. There is increased evidence regarding overlapping expression of BCO1 with βC accumulating tissues. A recent study reported a
significant accumulation of dietary \( \beta \)C in hepatocytes and hepatic stellate cells in liver (Shamarakov et al. 2010). Thus, observation of the present study that BCO1 is localized in parenchymal and non-parenchymal cells indicates that liver is also an important site in the metabolism of dietary \( \beta \)-carotene and subsequent synthesis of vitamin A in liver. Or Liver is an important extra-intestinal site in the synthesis of vitamin A. The precise function of BCO1 in hepatic stellate cells and endothelial cells remains to be elucidated.
References


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