ABSTRACT

Title of Document: THE POTENTIAL ROLE OF BUTYROPHILIN AND XANTHINE OXIDOREDUCTASE IN CONTROLLING THE AMOUNT AND SIZE OF MILK-FAT DROPLETS

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The secretion of fat droplets from mammary epithelial cells requires the expression of two major proteins, butyrophilin1A1 (BTN) and xanthine oxidoreductase (XOR). Ablation of the BTN or XOR gene in mice results in the accumulation of large fat droplets suggesting a reciprocal relationship between BTN/XOR concentration and milk-fat droplet size. We tested this hypothesis by correlating BTN/XOR concentrations in cow and mouse with their fat droplet size. The amount of BTN in mouse was 75 times less than in bovine samples. The size of fat droplets in mice was larger than in cow, but no correlation was found between fat-droplet size and the amount of BTN/XOR. Experimental reduction in fat-droplet size in mice did not change the concentration of BTN. We propose that a low amount of BTN is sufficient to mediate its role in milk-fat secretion and that it may have additional functions to its potential role as a structural protein.

THE POTENTIAL ROLE OF BUTYROPHILIN AND XANTHINE OXIDOREDUCTASE IN CONTROLLING THE AMOUNT AND SIZE OF MILK-FAT DROPLETS

By

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List of Abbreviations

BCA	bicinchoninic acid
BTN	butyrophilin 1a1
BTNL2	butyrophilin-like 2 protein
BSA	bovine serum albumin
CLA	conjugated linoleic acid
CLD	cytoplasmic lipid droplet
2D	two dimensional
ER	endoplasmic reticulum
MFD	milk-fat depression
MFGM	milk-fat globule membrane
MLD	microlipid droplet
MOG	myelin oligodendrocyte glycoprotein
PMSF	phenylmethyl sulfonyl fluoride
SDS	sodium dodecyl sulfate
SDS/PAGE	SDS/ polyacrylamide gel electrophoresis
TAG	triacylglycerol
TEMED	tetramethylethylenediamine
TLCK	Na-*p*-tosyl-L-lysine chloromethyl ketone
TPCK	Na-*p*-tosyl-L-phenylalanine chloromethyl ketone
XOR	xanthine oxidoreductase
XO	xanthine oxidase
XDH	xanthine dehydrogenase

Introduction

Milk is composed of a plethora of nutrients that are necessary for the nourishment and growth of the newborn. Milk contains proteins, lipids, carbohydrates, minerals and vitamins at a defined percentage in any given species, but this composition changes with stages of lactation, dietary factors, species, breed, genetics and environmental conditions (Cerbulis and Farrell, 1975; Torres-Hernandez and Hohenboken, 1979; Gonzalo, *et al.,* 1994). The whole milk can be separated into skim milk and cream by low speed centrifugation. Skim milk contains the majority of the soluble proteins, casein micelles, carbohydrates and water, while the cream portion contains most of the lipids. On centrifugation of skim milk at high speed, casein micelles sediment as a pellet, leaving a soluble fraction called whey.

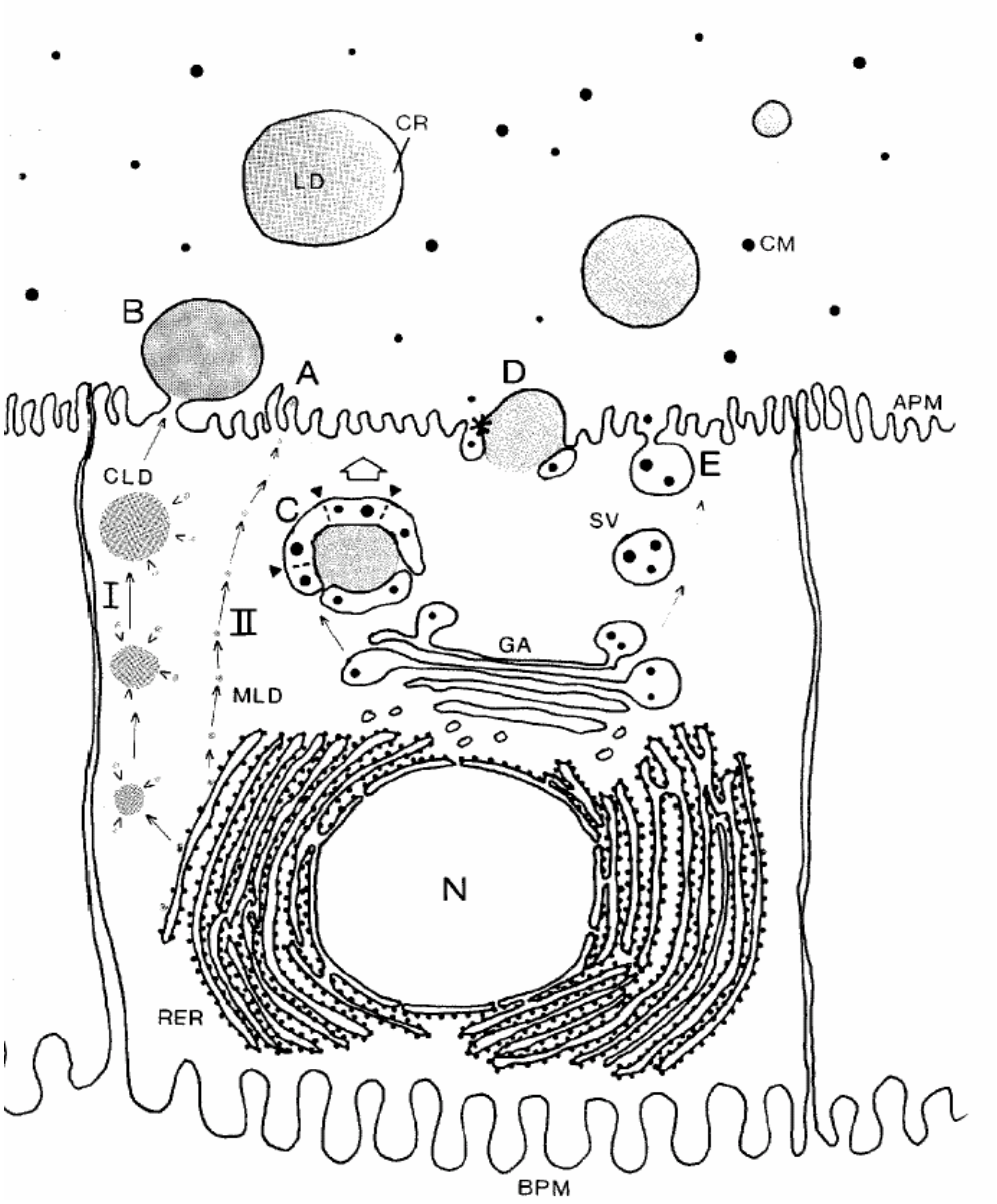
Of the different components of milk, lipids are very important in many species as they comprise the major source of energy. The fat content of milk varies with species (reviewed by Jenness, 1974 and Oftedal, 1993). In cattle, the amount of fat in milk, on average, is 4g per 100g of milk (Jenness, 1974) while in mouse it is 15g (Linzell and Fleet, 1969) and in blue whales (Oftedal, 1993) and seals (Jenness, 1974) it is as high as 50 g. The high fat percentage in seal's milk provides large amounts of energy which is deposited as adipose stores and therefore helps to keep the pup's body temperature constant in an extreme cold climate.

Triacylglycerols (TAGs) are the major lipids found in milk comprising about 98% of the total (Patton and Keenan, 1975). TAGs are esters of fatty acids and glycerol. Milk TAGs are synthesized in the rough endoplasmic reticulum (ER) of the alveolar epithelial cells of mammary gland (Zaczek and Keenan, 1990) by the action of acyl Co-A: diacyl

glycerol acyl transferase enzymes, inactivation of which results in defective milk-fat secretion (Smith *et al.*, 2000). The fatty acids, derived either from blood or synthesized *de novo* in mammary gland epithelium (reviewed by Bauman and Davis, 1974), are esterified to form TAGs. Besides TAGs, other lipids including phospholipids and cholesterol are mostly associated with the milk-fat-globule membrane (MFGM) or skim milk membrane and they comprise only a small amount of lipid in milk (Patton and Jensen, 1975).

The different components of the milk are secreted into the alveoli by different pathways. While proteins and lactose are secreted into the lumen of alveoli by exocytosis from Golgi-derived secretory vesicles, milk-fat secretion occurs by an entirely different mechanism. TAGs may be synthesized on the surface of the rough ER (Stein and Stein, 1967; Dylewski *et al*., 1984) or between the leaflets of the ER membrane (Zaczek and Keenan, 1990; Murphy and Vance, 1999), and the mature lipid droplets then bud from the ER membrane and move into the cytoplasm (Fig.1). During this process they carry a monolayer of phospholipids and lipid-droplet associated proteins (Murphy and Vance, 1999; Martin and Parton, 2006). These droplets may then coalesce together to form small lipid droplets or micro lipid droplets (MLDs) of sizes less than 1 μm in diameter. MLDs either fuse together to form larger droplets called cytoplasmic lipid droplets (CLDs) or they may be secreted directly from cells (Valivullah *et al.*, 1988; Deeney *et al.*, 1985) (Fig. 1). Thus the sizes of lipid droplets in milk can vary from small droplets less than 1 μm to large droplets greater than 10 μm. It is not clear whether the fusion of small droplets to form larger ones is a controlled or a random process (Mather and Keenan, 1998). The lipid droplets are surrounded by a coating of proteins and polar lipids. This

Fig. 1 Proposed pathways of milk-lipid droplet transit and secretion from mammary epithelial cells. The components of milk are secreted into the lumen of alveoli by different pathways. Whereas proteins and lactose are secreted by the classical secretory pathway (E), fat is secreted by a unique mechanism (A and B). Small fat droplets that bud from the ER travel through the cytoplasm to reach the apical cell membrane. These small fat droplets or MLDs may be secreted out as such or may combine with pre-formed lipid droplets called CLDs to form larger fat droplets before they are secreted out. While budding from the apical membrane, the fat droplets are surrounded by a thin layer of membrane known as the MFGM. MFGM contains proteins derived from the ER, the cytoplasm and the apical cell membrane. Sometimes, secretory vesicles may surround CLD's and form intracytoplasmic vesicles before they are secreted out (C). A combination of both B and C can also occur (D). Apical plasma membrane (APM); basal plasma membrane (BPM); cytoplasmic crescent (CR); rough endoplasmic reticulum (RER); lipid droplet (LD); Golgi apparatus (GA); cytoplasmic lipid droplet (CLD); microlipid droplet (MLD); nucleus (N); secretory vesicle (SV). Figure and modified legend reproduced from Mather and Keenan, (1998) with permission.


CR
LD
CM
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A
D
APM
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CLD
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SV
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II
GA
MLD
N
RER
BPM

may help to prevent coalition with other cellular lipids but at the same time helps in the fusion of milk-lipid droplets. Endogenous gangliosides may have an important role in fusion of the droplets as gangliosides were detected on MLDs isolated from mammary tissue and exogenously supplied gangliosides promoted fusion of droplets (Valivullah *et al.*, 1988). As lipid droplets fuse and become larger they also migrate towards the cell surface and finally interact with the apical cell membrane. The mechanisms involved in the migration of lipid droplets towards the cell membrane are not clear. Microtubules and cytoskeletal elements may be involved in this process of transit (discussed in Mather and Keenan, 1998). Even though microtubules and actin containing microfilaments are abundant in milk-secreting cells (Nickerson and Keenan, 1979; Franke *et al.*, 1976; Franke *et al.*, 1980), morphological evidence to show specific interaction between lipid droplets and cytoskeletal elements are lacking. Similarly, treatment with agents that interfere with microtubule assembly have yielded contradictory results (Patton *et al.*, 1974; Nickerson *et al.*, 1980; discussed in Mather and Keenan 1998). Hence the specific roles of microtubules and microfilaments, if any, are still not known. As per the widely accepted scheme, the fat droplets approach the apical surface, interact with proteins on the apical plasma membrane and are gradually coated with the plasma membrane and bud from the cell. The lipid droplets therefore become surrounded by a surface membrane together with the initial phospholipid monolayer and protein derived from the rER and cytoplasm. The entire coat structure is collectively called the MFGM (Bargmann and Knoop, 1959). Formation of the MFGM is essential for maintaining the stability of fat droplets in milk (Ogg *et al.*, 2004).

Previously, several investigators have studied the milk-fat globule size distribution in several species, especially in cow. The size distribution of milk-fat globules changes with species, breed, stages of lactation, etc. Fat globules in bovine milk range in size from less than 0.2 μm to 20 μm in diameter or more (Walstra *et al.*, 1969). The average size of fat droplets of Holstein and Jersey cows were 3.4 and 4.5 μm in diameter respectively (Walstra *et al.*, 1969). Milk-fat droplets less than 1 μm in diameter represent more than 80% of the total number, but they account for less than 10% of the total lipid volume. Droplets larger than 1 μm in diameter account for more than 90% of the total lipid volume, even though their total number is small. Attaie and Richter (2000) reported that 90% of fat droplets in bovine and caprine milk were less than 5.21 μm and 6.42 μm respectively. They found that the mean size of fat globules was larger in bovine milk (3.51 μm) than in goat's milk (2.76 μm). Tedman (1982) reported that the mean size of milk-fat globules of Weddell seal was 6.4 μm in diameter at 4 days post-partum, which is much higher than in bovine milk-fat samples. He also found a higher frequency of smaller droplets at 23 and 45 days post-partum than at 4 days post-partum indicating changes in the size of globules depending upon the stage of lactation. The average size of human milk-fat droplets were much smaller (Simonin *et al.*, 1984) and ranged from 2.2 μm at the beginning of lactation to 2.7 μm after 40 days of lactation.

Previously, Wiking *et al.* (2004) reported that milk fat globule size is affected by fat production in dairy cows. A correlation was found between average diameter of fat droplets and fat yield of cows. Furthermore, among the three subpopulations of milk fat globules i.e. small, medium and large, it was the medium sized globules that increased in

size. It was assumed that the increase in the size of fat droplets with higher fat synthesis was due to a limitation in the production of MFGM.

Major proteins found in the MFGM are butyrophilin1A1 (BTN), xanthine oxidoreductase (XOR), Mucin 1 (MUC-1), adipophilin, PAS VI/VII (MFG-E8) and CD-36 (Fig. 2). BTN is the most abundant protein in bovine MFGM, accounting for nearly 40% of total proteins on a weight basis (Mather *et al.*1980). It is a type 1 membrane protein belonging to the immunoglobulin superfamily. Human BTN genes comprise three subfamilies - BTN1A1, BTN2A1-3 and BTN3A1-3 (Ruddy *et al.*, 1997; Tazi-Ahnini *et al.*, 1997). While BTN subfamily 2 and 3 members are widely expressed in many tissues (Ruddy *et al.* 1997; Rhodes *et al.*, 2001), BTN is solely expressed in lactating mammary gland potentially indicating a role in the process of milk-fat secretion or some other functions in lactation (Franke *et al.*, 1981; Ogg *et al.*, 1996). BTN has an N-terminal exoplasmic domain comprising two immunoglobulin domains, a short transmembrane anchor and a long C-terminal cytoplasmic domain. The cytoplasmic domain also contains a so-called B30.2 domain, which may be self-adhesive and function in interactions with other proteins (Woo *et al*., 2006; Vernet *et al.*, 1993; Henry *et al.*, 1998).

BTN proteins share structural similarities with B7 family members, which are regulators of T-cell activation and tolerance. Recently it was reported that a BTN family member, butyrophilin-like 2 (BTNL2) can regulate T-cell activation (Nguyen *et al.*, 2006), which indicates that BTN proteins may be involved in some immune functions. Similarly, closely related family members of BTN such as BTN3A1 can interact with T-cells (Nguyen *et al.*, 2006; Compte *et al.*, 2004) whereas BTN2A1 can interact with the lectin, DC-SIGN (Malcherek *et al.*, 2007), a receptor expressed on immature monocyte-

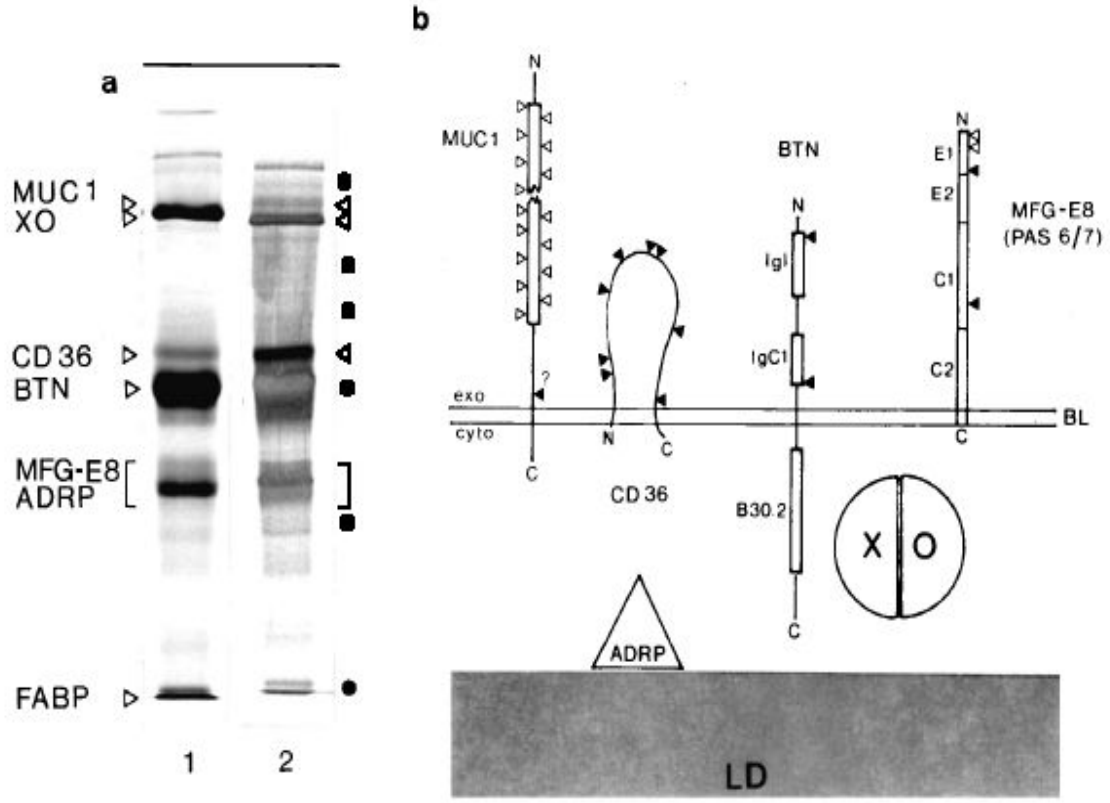


Fig. 2 Major proteins of bovine milk-fat globular membrane and their topology.

(a). Bovine MFGM proteins separated by SDS/PAGE followed by staining with Coomassie blue (lane 1) and with silver (lane 2). (b) Schematic diagram of the topology of major MFGM proteins. BTN is a transmembrane protein with an exoplasmic domain containing two immunoglobulin-like folds, a transmembrane domain and a cytoplasmic tail containing a B30.2 domain. Xanthine oxidoreductase (XO) is a cytoplasmic protein that may bind to the cytoplasmic tail of BTN, thus stabilizing the fat droplets. Adipocyte differentiation related protein (ADRP), also known as adipophilin, is proposed to be on the surface of lipid droplets that may bind to XO (Mather and Keenan, 1998).

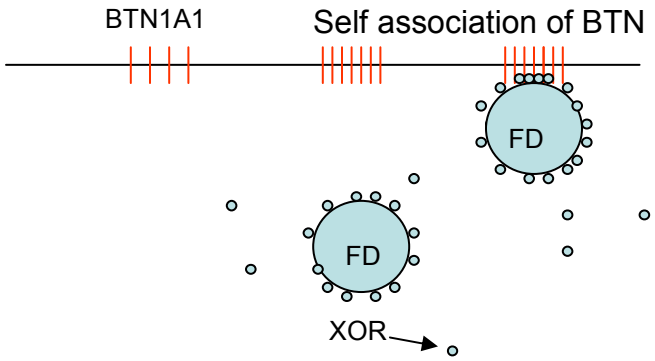
derived dendritic cells. Moreover, recent demonstration of molecular mimicry between BTN and myelin oligodendrocyte glycoprotein (MOG), one of the proteins involved in the etiology of multiple sclerosis, an autoimmune disease affecting the structural integrity of myelin (Guggenmos *et al.*, 2004), indicates that BTN, apart from the milk-fat secretion process may play pathophysiological roles.

XOR is expressed in most cell types and is involved in the catabolism of purines (Hille and Nishino, 1995). In mammals, the enzyme can exist as one of two interconvertible forms, an oxidase (XO) form and a dehydrogenase (XDH) form (Waud and Rajagopalan, 1976). In tissues, the enzyme is present mainly as XDH whereas in milk it is present as the XO (Waud and Rajagopalan, 1976; Hille and Nishino, 1995). XDH uses NAD+ as the preferred electron acceptor whereas XO uses molecular O_2 as the electron acceptor (Hille and Nishino, 1995) and both forms, but especially XO, contribute to the synthesis of many reactive oxygen species (reviewed by Vorbach *et al.*, 2003). The XOR gene is expressed at very high levels in lactating mammary epithelium, indicating other roles for XOR during lactation, other than purine metabolism (Kurosaki *et al.*, 1996; McManaman *et al.*, 2002). XOR is a cytoplasmic protein, and some studies suggest that it can interact with membrane proteins, especially BTN (Ishii *et al.,* 1995; McManaman *et al.*, 2002; Vorbach *et al.*, 2002; A. R. Rao, J. K. Jeong and I. H. Mather unpublished observations). Milk contains high levels of XOR (Hille and Nishino, 1995) which is concentrated in the MFGM.

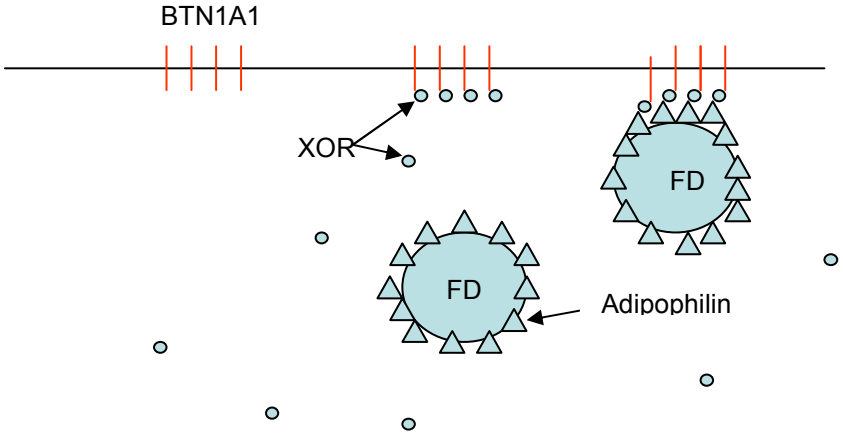
Several models have been proposed to explain the process of milk-fat secretion. In one model (Fig. 3A), the fat droplets are coated with XOR as they transit through the cytoplasm and when the fat droplets reach the cell surface, XOR interacts with the C-

Fig. 3 Postulated models for the secretion of milk-fat droplets. A. BTN in the apical membrane self associates to form tetrameric or pentameric aggregates as per the model proposed by Mather and Keenan (1998). XOR, a cytoplasmic protein, interacts with lipid droplets as they traverse the cytoplasm. As the lipid droplets reach the plasma membrane, XOR interacts with the cytoplasmic domain of BTN. The fat droplets are pushed against the membrane and finally pinch off coated with MFGM containing BTN and XOR on the surface of the droplets; B. Mcmanaman *et al.* (2002) proposed that XOR is diffusely distributed in the cytoplasm during pregnancy, but during lactation it moves to the apical membrane and interacts with BTN. Adipophilin, a lipid-droplet associated protein then interacts with the BTN-XOR complex when the fat droplets reach the apical membrane. Once the complex is formed, the droplets pinch off forming the MFGM; C. Robenek *et al.* (2006) proposed that BTN, associates with lipid droplets in the cytoplasm and then interacts with BTN in the apical membrane thus forming a complex in which BTN is more abundant in the fat droplet than in the bilayer. In this model, neither XOR nor adipophilin are important for milk-lipid secretion, even though they are found in the MFGM. XOR is present in the cytoplasm and also in the fat droplet derived from the ER, but it is diffusely distributed on the surface of the fat droplets. Milk secretion is dependent only on BTN-BTN interactions. BTN1A1, Butyrophilin subfamily1 member A1; FD, fat droplet; XOR, xanthine oxidoreductase.

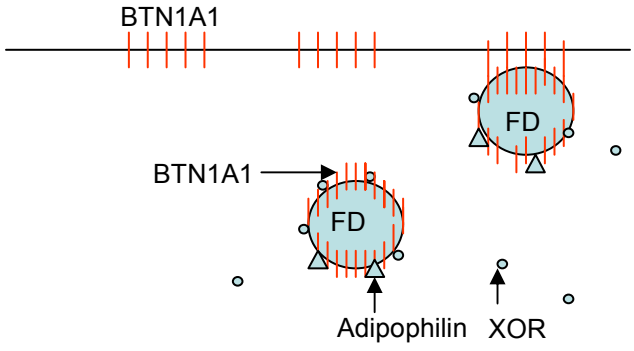
A.


BTN1A1
Self association of BTN
FD
FD
XOR

B.


BTN1A1
XOR
FD
FD
Adipophilin

C.


BTN1A1
FD
BTN1A1
FD
Adipophilin
XOR

terminal domain of BTN in the apical plasma membrane (Mather and Keenan, 1998). BTN may act as a transmembrane scaffold which stabilizes the membrane-fat droplet interactions. It has also been proposed that BTN may act as a signaling receptor which interacts with XOR, stimulating the production of reactive oxygen species which regulates expression of the components of the milk-fat secretion process (Ogg *et al.*, 2004). Once these proteins interact, the fat droplet gradually pushes against the apical plasma membrane of the mammary epithelium and pinches off from the surface of the cell carrying a bilayer membrane around its outer surface. Another model was proposed by McManaman *et al.* (2002) and Vorbach *et al.* (2002) in which, during lactation, XOR binds to the cytoplasmic C-terminal domain of BTN in the apical membrane. Adipophilin, which is a lipid-droplet associated protein then binds to the BTN-XOR complex at the apical plasma membrane (Fig. 3B). These two models propose that XOR is important for the milk-fat secretion process and are consistent with the findings of Mondy and Keenan, (1993), who found that BTN and XOR occur in a constant molar proportion in the MFGM of Holstein and Jersey cows. However, using freeze-fracture immunocytochemistry, Robenek *et al.*, (2006) proposed a different model in which milk secretion is solely controlled by interactions between BTN in the plasma membrane and BTN associated with lipid droplets in the cytoplasm (Fig. 3C). According to this model, BTN does not form a complex with XOR, and milk-lipid secretion is controlled by BTN-BTN interactions alone.

Ogg *et al.* (2004) tested the importance of BTN in lipid secretion by generating homozygous $Btn^{-/-}$ mice. Regulated secretion of milk lipid was severely affected in such BTN null mice. Large pools of TAGs accumulated in the cytoplasm of secretory cells and

many luminal spaces were filled with aggregated lipid-droplets (Fig. 4A). The lipid droplets were two or three times larger than those from the wild type mice (Fig.4 B) and some of them appeared to escape into the alveolar lumen by breaking through the apical cell membrane. Many of the secreted droplets were misshapen and had an unstable MFGM. The importance of BTN and that of the MFGM in milk secretion was emphasized by the observation that half of the pups died in the first 20 days and that the surviving pups were only 60-80% of the weight of wild-type mice. The pups were unable to get sufficient amounts of milk as the lumen of the alveoli were filled with unstable fat which presumably clogged the ducts making the milk unavailable to the pups. BTN is therefore necessary for maintaining the stability of the lipid droplets.

The importance of XOR in lipid secretion was studied by McManaman *et al.* (2002). By using RT-PCR, XOR mRNA was found to be expressed in the mammary gland during pregnancy and lactation. The levels of XOR mRNA increase in mid-pregnancy, reach peak levels during lactation and finally decrease during involution. In comparison, a significant change in the levels of XOR mRNA was not noticed in liver during these periods suggesting that XOR is important in lactation. During the stages of pregnancy, XOR was found diffusely distributed in the cytoplasm, but during lactation, it co-localized with two other proteins, BTN and adipophilin (McManaman *et al.*, 2003). During involution, XOR moved away from the apical membrane and was found again diffusely distributed in the cytoplasm, but BTN was still associated with the apical membrane. McManaman *et al.* suggested that XOR helps with the interaction of the lipid droplets with the apical cell membrane. The importance of XOR in lipid secretion was

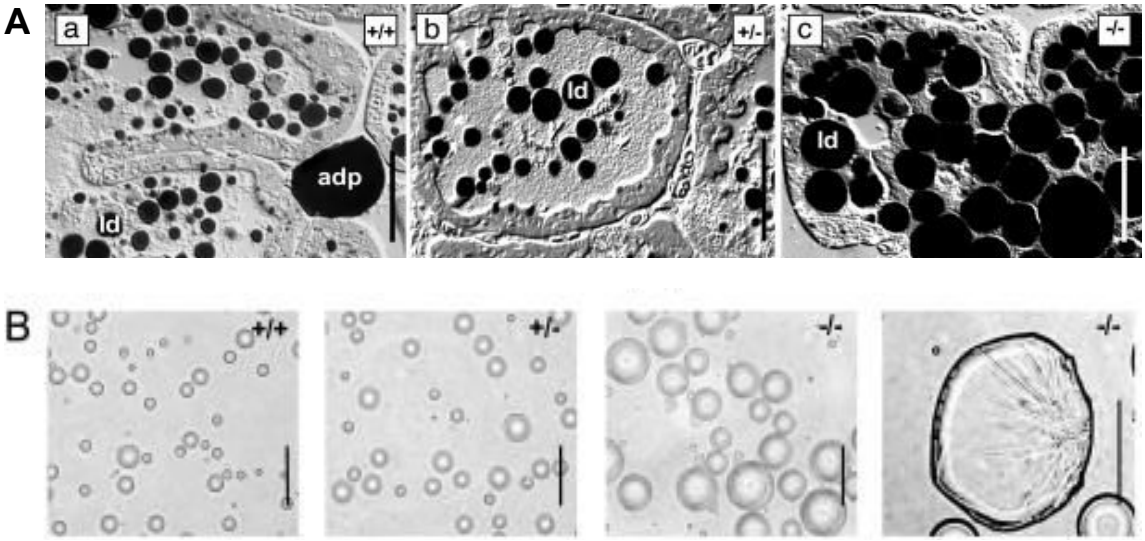


Fig. 4 Effect of ablation of BTN gene on milk-fat secretion in mice. A. Differential interference contrast micrographs of lactating mammary tissue from wild type ($Btn^{+/+}$), heterozygous ($Btn^{+/-}$) and knock-out ($Btn^{-/-}$) mice. Lipid droplets (ld) and adipocytes (adp) are stained black by osmium tetroxide (Bars: 50 μm). B. Phase contrast micrographs of lipid droplets in milk from $Btn^{+/+}$, $Btn^{+/-}$ and $Btn^{-/-}$ mice (Ogg *et al.*, 2004) (Bars: 25 μm).

confirmed by Vorbach *et al.* (2002) who showed that heterogenous XOR mutant mice ($Xdh^{+/-}$) were unable to maintain lactation, resulting in death of the pups. Mammary epithelial cells showed accumulation of large milk-fat droplets in the cytoplasm (Fig 5). Transmission electron microscopy showed severely deformed fat droplets enveloped by abnormal membrane. These mice also underwent premature involution of the mammary gland resulting from rupture of the mammary epithelium.

Thus, a common feature of BTN knockout mice and $Xdh^{+/-}$ mice is the presence of abnormally large fat droplets in the epithelial cells and lumina and an unstable MFGM which results in improper secretion of milk-lipid. Therefore, we hypothesize that both BTN and XOR are necessary for fat secretion and that a reduction in BTN or XOR concentrations results in the accumulation of fat droplets in the cytoplasm which fuse together to form larger droplets. This suggests that the amounts of BTN and XOR expressed during lactation may regulate the size of lipid droplets. If this is the case, the size of milk-lipid droplets should be inversely related to the amount of BTN and XOR in the MFGM across species. In addition, experimental manipulation of the size of milk fat droplets within species should alter the amounts of BTN and XOR associated with the MFGM, i.e. decreased droplet size should correlate with increased amounts of BTN and XOR in the MFGM.

One method for manipulating the amount and size of lipid droplets in milk is by dietary means. One such example is milk-fat depression (MFD) noticed in animals fed on a high carbohydrate, low roughage diet (Gaynor *et al.*, 1995), and also in animals fed with oil supplements rich in polyunsaturated fatty acids (Bauman *et al.*, 2000). Conjugated linoleic acids (CLAs) are a group of positional and geometric isomers of the

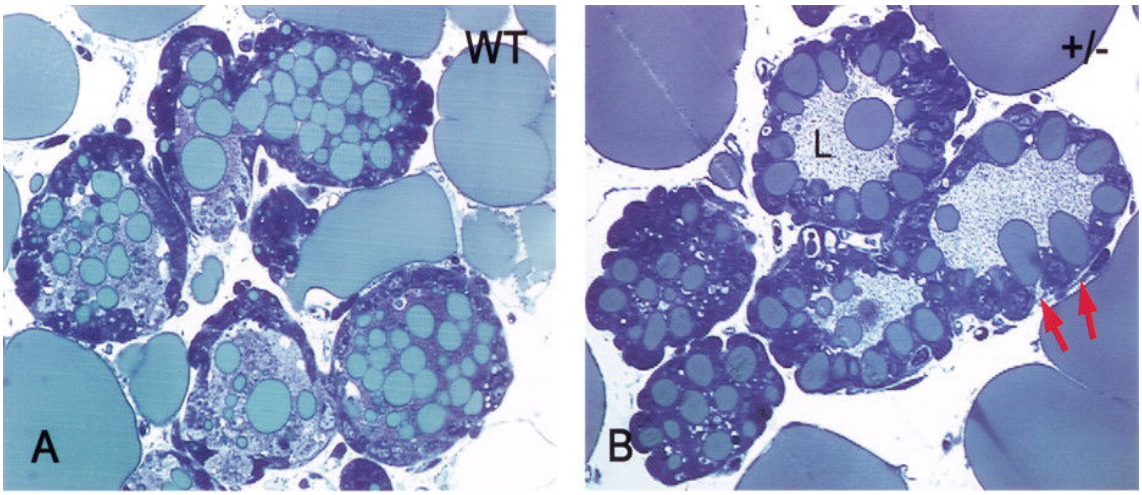


Fig. 5. Effect of milk-fat secretion in xanthine oxidoreductase heterozygous mice. Histological analysis of mammary glands of wild type mice (A) and xanthine oxidoreductase heterozygous ($Xdh^{+/-}$) mice (B) at lactation day 1. In Xdh+/- mice, large fat droplets that accumulated in the cell bulge from the apical membrane (indicated by red arrows). L, lumen of alveoli. (Vorbach *et al.*, 2002).

unsaturated long chain fatty acid, linoleic acid. Depending on the position of the double bond, several CLA isomers are possible. In ruminants, unsaturated fatty acids undergo incomplete biodehydrogenation in the rumen and some of the intermediates escape the rumen and reach body fat and milk (Bauman *et al.*, 2000). The main source of dietary CLAs in non-ruminants is the meat and dairy products of ruminants. CLAs reduce the amount of fat in milk dramatically in both ruminants and non-ruminants.

Even though CLAs have a profound influence on milk fat depression, it is not known whether they have any influence on the size of milk-fat droplets or whether they affect the expression of proteins involved in milk secretion such as BTN or XOR.

In this thesis research, a quantitative assay for BTN and XOR was devised in species having significantly different milk fat percentages and droplet sizes including cows, seals and different strains of mice. The absolute amounts of BTN and relative amounts of XOR in milk-fat samples were then correlated with the size of fat droplets in their milk. The amounts of BTN and XOR were also determined after feeding CLA to mice to influence milk-fat percentage and potentially the milk-fat droplet size.

Materials and methods

Materials:

Tris hydroxymethyl amino methane (Tris), sodium dodecyl sulphate (SDS), ammonium persulphate, N,N,N',N' tetramethylethylenediamine (TEMED), goat anti-(rabbit-IgG) conjugated to horse-radish peroxidase, nitrocellulose, urea, immobilized pH gradient (IPG) strips, mineral oil, ampholines, bromophenol blue, Coomassie brilliant blue R-250, acrylamide, N,N' methylenebisacrylamide, and Triton X-100 were obtained from Bio-Rad Laboratories (Hercules, CA). N-p-tosyl-L-phenylalanine chloromethyl ketone (TPCK), Nα-p-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), DL-dithiothreitol (DTT), aprotinin and glycerol were obtained from Sigma-Aldrich Inc. (St.Louis, MO). Bicinchoninic acid (BCA) protein assay reagents were from Pierce Chemical Company (Rockford, IL). ECL™ Western blotting detection reagents and Hyperfilm™ were obtained from Amersham Life Sciences (Buckinghamshire, England). Bovine serum albumin (BSA) and phenylmethyl sulfonyl fluoride (PMSF) were from Calbiochem (CA). CLAs were from Nu-Chek Prep, Inc. (Elysian, MN). All other chemicals and reagents were from Fisher (Pittsburg, PA).

Methods

Collection and processing of milk samples

Mice: Milk from seven strains of mice (five animals each), which were in their first lactation was collected at day 10 of lactation. The strains of mice used were C57, CD-1, C3H, Fvb, Swiss Webster, Balb/c and 129S. Mice were separated from their litters for 4 h prior to milking. They were injected intraperitoneally with avertin (125-250 mg/kg body weight) and 0.2 I.U. of oxytocin in physiological saline. Milk was collected using a hand-held vacuum device and collected into capillary tubes (Teter *et al.*, 1990). Milk (1 µl) was diluted with 24 µl of PBS and spotted onto microscope slides and sealed under 18 X 18 mm coverslips with nail polish to examine the fat droplets by microscopy. The capillary tubes were then centrifuged at 15,000 g for 15 min using a hematocrit centrifuge to separate fat from skim milk (Teter *et al.*, 1990). The percentage of milk-fat was determined by "creamatocrit" (Lucas *et al*., 1978). The capillary tube was broken at the skim-milk/fat interface and then the two pieces stored separately at -20^{o} C.

Cows: Milk from five Holstein cows from the dairy herd at USDA, Beltsville was collected at 6-8 weeks of lactation. 250 ml of milk was then centrifuged at 10,000 rpm in a Sorvall RC-5 superspeed centrifuge using a GSA rotor. The floating layer of fat was washed once with PBS (250 ml) and stored at -20^{o} C.

Seals: Milk samples from Weddell seal (*Leptonychotes weddelli*) were provided by Dr. Olav Oftedal (National Zoological Park, Smithsonian Institution, Washington, D. C.) as part of a project in Antartica. Milk was collected by a manual expressor from lactating

seals after an injection of oxytocin subcutaneously at a dose of 0.1 to 0.25 units/ kg body weight. Due to restricted facilities in Antartica, the whole milk could not be separated into skim milk and cream and hence it was kept frozen as whole milk at -80^{o} C until analyzed (Tedman *et al.*, 1982).

Preparation of BTN and XOR standards

Bovine BTN was isolated from bovine MFGM by preparative SDS-PAGE to use as a protein standard (Rao, 2003). Bovine XOR was purified from the MFGM of bovine milk as described by Sullivan et al. (1982). Mouse BTN was expressed and purified using the Yeast Expression and purification system in which Glutathione-S-transferase (GST)-mouse Btn-cyto fusion protein was expressed in *Saccharomyces pombe* and purified by affinity chromatography on glutathione agarose (Rao, 2003).

SDS-Polyacrylamide Gel Electrophoresis and Western Blot

Protein samples stored with protease inhibitors (0.25 mM TLCK, 0.25 mM TPCK, 1.25 mM PMSF, 0.2 mM ε-amino-n-caproic acid and 0.15 units of aprotinin) were prepared for electrophoresis by heating with SDS-PAGE Laemmli buffer (50 mM Tris- HCl, pH 6.8, 10% glycerol, 2% SDS, 0.05% bromophenol blue) containing 150 mM DTT for 3 min at 95^{o}C. Proteins were separated by SDS-PAGE in 8 or 10% (w/v) polyacrylamide gels as described by Laemmli, (1970). Samples were separated at a constant current of 30mA/slab gel for 5-6 h.

For Western blot, the separated proteins were transferred onto nitrocellulose membrane using the method of Towbin *et al.* (1979). The membranes were blocked

overnight using 5% non-fat dry milk. After washing with high-salt TBS (20 mM Tris and 0.5M NaCl, pH 7.4), the membranes were incubated with primary antibodies to BTN or XOR in 2.5% non-fat dry milk for 2 h. The membranes were washed three times in high salt TBS for 15 min each and then incubated with goat-anti-rabbit IgG conjugated to horse radish-peroxidase in 2.5% non-fat dry milk for 2 h. The membranes were then washed three times as above. The bound peroxidase was detected using the Amersham Western blotting detection kit. The blots were then quantified using the computer software program, Quantity One (Biorad). Protein bands were detected by Coomassie blue staining and the molecular weights of proteins were estimated by comparing their electrophoretic mobilities with the mobilities of protein standards (Kaleidoscope standards- BioRad).

Two dimensional (2D) gel electrophoresis

Bovine and mouse MFGM were prepared by alternate thawing and freezing of milk-fat samples followed by centrifugation. Fat and supernatants were removed and the pellets resuspended in a small volume to get a high concentration of MFGM proteins. The protein concentration was then determined by the BCA assay (Smith et al., 1985).

BioRad immobilized pH gradient (IPG) strips were rehydrated using rehydration buffer containing 9.8M urea, 2% NP-40, 20 mM DTT, 0.2% Bio-Lyte ampholytes (3-10) and 0.001% bromophenol blue to make a final volume of 125 µl. The IPG strips (7 cm) were rehydrated according to manufacturer's instructions. Each of the IPG strips was overlaid by 2 ml of mineral oil to prevent evaporation. The rehydration tray was covered with a plastic lid and left on a level bench overnight for rehydration at room temperature.

Following overnight rehydration, the mineral oil from the IPG strips was drained and the strips were transferred to the corresponding channel in an isoelectric focusing (IEF) tray maintaining the gel side down. A paper wick was placed at each end of the channel covering the wire electrode to remove salts and non-amphoteric constituents of the samples. 50 µg of total milk-fat sample protein along with 10 µl of Biorad 2D gel electrophoresis markers were mixed in rehydration buffer to make a final volume of 25 µl. The protein sample was then added to the tray along the channel. Each IPG strip was again overlaid with 2 ml of fresh mineral oil and the plastic lid was covered as before.

The PROTEAN IEF cell (BioRad) was programmed using a default cell temperature of 20^0C and a maximum current of 50µA/IPG strip. Electrofocusing was done by gradually increasing the voltage in steps from 150 to 300, 600, 1000 and 2000V for 1h each, followed by 4000V for 2.5h, for a total of 14,000 V-h. IPG strips were drained of mineral oil and used directly for second dimension SDS-PAGE or stored in the rehydration tray with gel side up at -80^0C for future use.

IPG strips stored at -80^0C were thawed for 10-15 min and briefly dipped into a graduated cylinder containing SDS PAGE running buffer and then pushed using a forceps onto the top of an SDS-polyacrylamide slab gel and overlaid with agarose as per manufacturer's instructions. SDS-PAGE followed by Western blotting was done as described previously.

Size distribution of milk-fat globules

Five separate images of the milk-fat globules of each sample were recorded by phase contrast microscopy using a Leica DMIRE2 epifluorescence/differential

interference contrast microscope and saved as Microsoft office document imaging files. The diameter and area of individual globules were determined using the software, Image-pro Express (Media Cybernetics, Bethesda, MD). A histogram was constructed based on the size distribution of the fat droplets.

Dietary treatment of mice with CLA

Twelve C57/BL6 mice of first lactation were split into two groups of six each. One group was fed with a standard mouse diet (Table 1) and the other group with the same diet supplemented with CLAs as the test group. The test group was fed with trans-10, cis-12 CLA isomers for six days from day 4 to day 9 of lactation as per Table 1.

Milk was collected on day 10 of lactation, the percentage of milk-fat determined by creamatocrit and fat samples separated and stored as described above. The concentrations of BTN and XOR in milk-fat samples and the size distribution of fat droplets were determined as above.

Protein determination

Protein concentration was determined by the BCA method according to manufacturer's instructions, using BSA as a standard (Smith *et al.*, 1985).

Statistical analysis

Differences in BTN and XOR amounts, BTN/XOR ratio and fat droplet sizes among the seven strains of mice were analyzed using ANOVA test. Similarly the differences in these

Table 1. Composition of mouse diet

Ingredient	g/kg of diet (dry weight)
Sucrose	590
Vitamin free casein	200
Alphacel	50
Fat:	
Cocoa butter	14.3
Corn oil	30.9
Olive oil	34.8
Oleic acid[#]	20.0
AIN 76 mineral mix	40
AIN 76 vitamin mix	15
DL-methionine	3
Choline bitartrate	2

[#] In the test group, 6.7 g of oleic acid/kg was replaced with an equal weight of trans-10, cis-12 CLA.

parameters between cow and mouse and those between control and CLA-treated groups of mice were analyzed using ANOVA. A *P*-value <0.05 was regarded as significant. Duncan's multiple range tests was used as post hoc test to find which groups were significantly different from each other. Relationship between fat droplet size and amount of BTN, XOR and BTN/XOR ratio was studied using correlation statistics. The statistical analyses were performed using the Statistical Analysis System (SAS).

Results

In the current three models of milk-fat secretion, it is assumed that the process of secretion is the same in all mammals and that BTN and XOR are major proteins in the MFGM, irrespective of species. This assumption is based on analysis of the protein composition of the MFGMs of a number of species including cow, goat, sheep, human, pig, rat and guinea pig (Heid *et al.* 1983) In order to expand the analysis, we devised a quantitative assay for BTN and XOR in cow, mouse and seal, three species which differ significantly in milk-fat percentage. Since BTN null mice produce unusually large fat droplets (Ogg *et al* 2004) and BTN heterozygous mice produce fat droplets of intermediate size when compared to normal mice, a reciprocal relationship between BTN concentration and milk-fat droplet size was predicted. Also, a similar role for XOR in regulating fat droplet size was predicted since XOR heterozygous mice produce larger fat droplets than wild type mice (Vorbach *et al.*, 2002), and BTN and XOR bind to each other in *in vivo* and *in vitro* assays. Potential correlations between milk fat percentages and BTN/XOR concentration and fat droplet size were also tested.

To devise a quantitative assay for BTN and XOR in species differing significantly in milk-fat percentages and between different strains of mice, the milk collected from all species was separated into cream and skim milk. The amount of BTN and XOR in bovine cream was determined by comparing the intensity of BTN and XOR bands on Western blots with known amounts of BTN and XOR standards respectively (Fig. 6A, Fig. 7). Using this procedure, the amounts of BTN and XOR in bovine milk samples were determined to be 372.9 ± 32.2 ng (Fig. 6B) and 203.8 ± 11.3 ng/µg of total protein, respectively (Fig. 7). On a percentage basis, BTN and XOR comprise 37.3 ± 3.2% and

20.4 ± 1.1% of total protein respectively which is consistent with earlier findings calculated as the percentage of total Coomassie-blue stained protein (Mather *et al.*, 1980; Mondy and Keenan 1993). Therefore, these results confirm earlier findings and validate this quantitative immunoassay.

Using the same procedure, the amount of BTN in mouse samples was determined to be, on an average, 5 ng/μg of total protein (Fig. 8, Table 2) which is 75 times lower than in the bovine samples. Similarly, the amount of XOR in mouse cream was very low when compared to bovine samples (Fig. 9), although an absolute amount of XOR could not be determined due to the unavailability of standards. Significant differences in BTN and XOR levels were measured among different strains of mice. However, the BTN/XOR ratio showed no significant difference between the different strains of mice (Table 2).

To confirm that the amounts of BTN and XOR are substantially lower in mouse MFGM compared to cow, cream samples were separated and analyzed by 2D electrophoresis and immunoblot (Fig. 10). Even though a large difference was noticeable in BTN amount between bovine and mouse samples after Coomassie staining, the fold reduction in the mouse samples appeared less than estimated from the immunoassays. Western blot also showed a significant difference in BTN amount between the two species.

The milk fat percentage varied greatly among the three species. While bovine milk is relatively low in fat (3.5-4%), seal's milk contains approximately 50% fat. The average milk fat percentage in seven strains of mice was 18.2±0.9 (Fig. 11).

Fat droplet sizes were estimated by phase contrast or differential interference contrast microscopy in the same samples analyzed for BTN and XOR protein. The size of

fat droplets differed significantly between bovine and mouse samples (Fig. 12). The mean diameter of mouse milk-fat droplets was 6.21±0.24 μm, which is double the size of bovine fat droplets (2.97±0.26 μm) (Table 3). Also, the distribution of fat droplet sizes differs between the two species. In cows, most of the fat droplets were in the range of 1-4 μm in diameter (Fig. 13A), with relatively few fat droplets of larger size. None of the samples contained fat droplets larger than 10 μm. However, in mice, the size of the fat droplets ranged from 3-10 μm in diameter (Fig.13B-H) and fat droplets larger than 15 μm were also detected, although in smaller numbers.

A quantitative analysis of BTN and XOR in seal's milk could not be conducted due to the unavailability of purified BTN and XOR standards. However, the presence of BTN and XOR in the seal's milk samples was confirmed using rabbit antipeptide antibodies to the C-terminal 21-amino acid peptide of mouse BTN (Ogg *et al.* 2004) and the C-terminal 21 aminoacid peptide of mouse XOR (Rao, 2003), respectively (Fig. 14). Rabbit antibodies to bovine BTN and XOR did not cross-react with seal BTN or XOR (data not shown). The protein profile of the seal milk samples showed significant differences from cow and mouse milk-fat samples (Fig. 15). This difference may be due to the high fat content in seal's milk which may require expression of a different set of proteins, or may be a consequence of prolonged handling of the samples after collection in Antartica and shipment to the U.S. Also, these samples are highly contaminated with skim-milk proteins as the cream was not separated and washed after collection.

Potential correlation between BTN and XOR levels and the size of fat droplets was investigated based on the above data using SAS. No correlation was detected between BTN or XOR levels and fat droplet size either in cows or in mice. A positive

correlation was detected between BTN/XOR ratio and fat droplet size in cows, but not in mouse (Table 2).

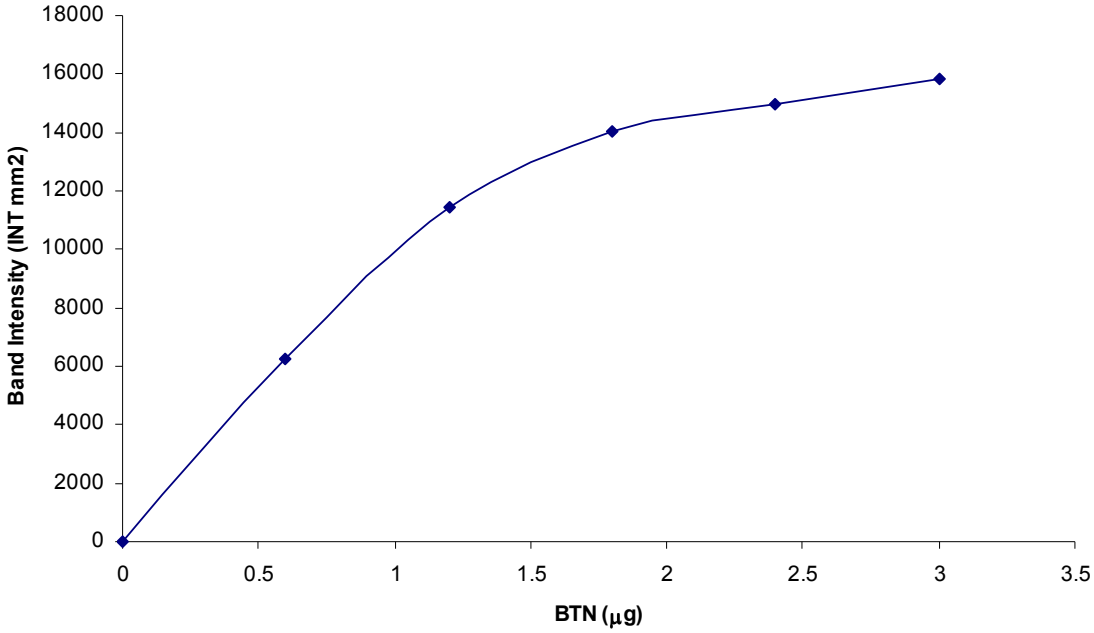
To test whether the expression levels of BTN or XOR can be changed by changing the percentage of fat in the milk, mice were fed with a diet rich in CLAs, known to reduce milk-fat percentage. In comparison to the control group, in which the milk-fat percentage was 25.4%, a significant drop to 13.3% was recorded in mice fed with CLAs, thus reducing the milk-fat percentage by about 50% (Fig. 16). The amount of BTN did not differ significantly between the two groups (Fig. 17); but, a significant difference in XOR levels was noticed in CLA treated mice (Fig. 18). The BTN/XOR ratio did not differ significantly between the two groups (Table 2).

The milk-fat droplet size was reduced significantly in the CLA treated group (Fig. 19). The mean fat droplet size in the CLA group was half the size of the control group (Table 4). The distribution of fat droplet size also varied between the two groups. The majority of the fat droplets in the control group were 3-8 μm in diameter, whereas in the CLA treated group, the majority of the droplets were 1-4 μm in diameter (Fig. 20). No fat droplets above the size of 7 μm diameter could be seen in any of the CLA-treated samples.

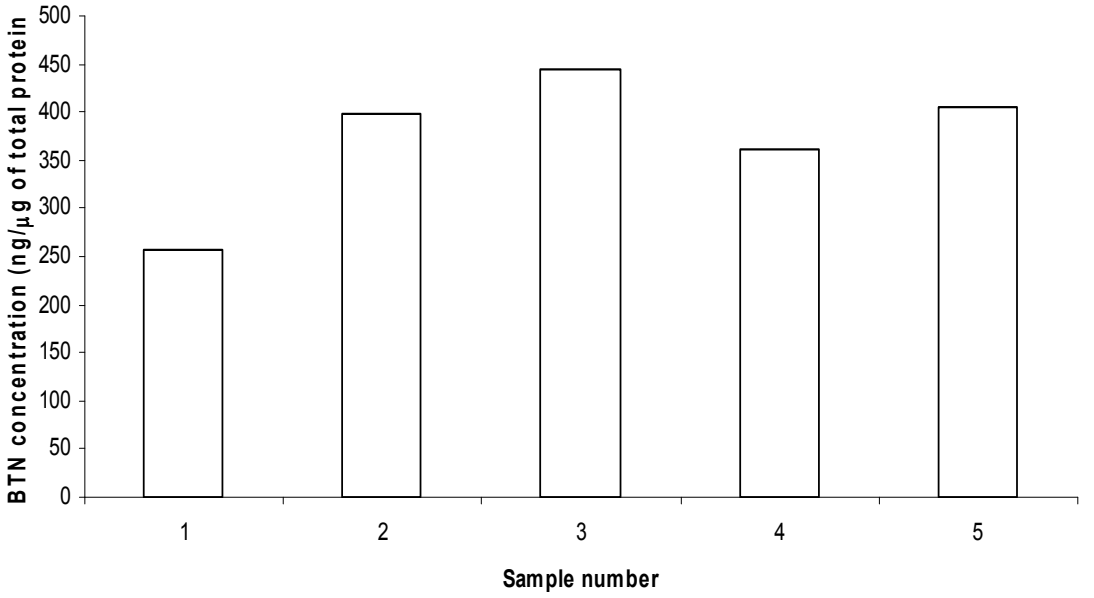
No significant correlation was found between BTN, XOR or BTN/XOR ratio and fat droplet size between the above two groups (Table 2).

Fig. 6 Validation of BTN immunoassay. Milk samples (approx. 200 ml) from five Holstein cows were collected at the regular morning milking and separated into cream and skim milk fractions by centrifugation. Total cream samples (30 μg of total protein/well) were separated by SDS-PAGE in 10% polyacrylamide gels and electrophoretically transferred to nitrocellulose together with known amounts of purified bovine BTN. Bovine BTN on the paper was detected using rabbit antibody (1 in 2,500 fold dilution) to BTN protein purified from bovine milk followed by goat anti-(rabbit IgG) conjugated to horse-radish peroxidase (1 in 10,000 dilution) as described in Materials and Methods. A. Standard curve for BTN (μg) against intensity of band in Western blot. B. BTN concentration in cream from five cows.

A


18000
16000
14000
12000
10000
8000
6000
4000
2000
0
Band Intensity (INT mm2)
0
0.5
1
1.5
2
2.5
3
3.5
BTN (µg)

B


500
450
400
350
300
250
200
150
100
50
0
BTN concentration (ng/µg of total protein
1
2
3
4
5
Sample number

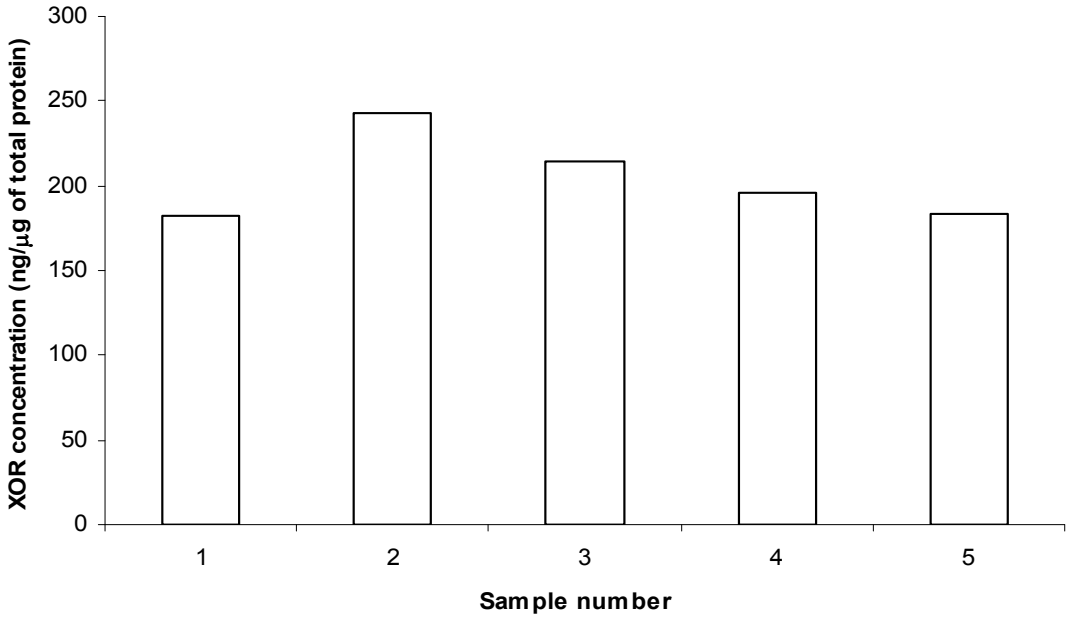
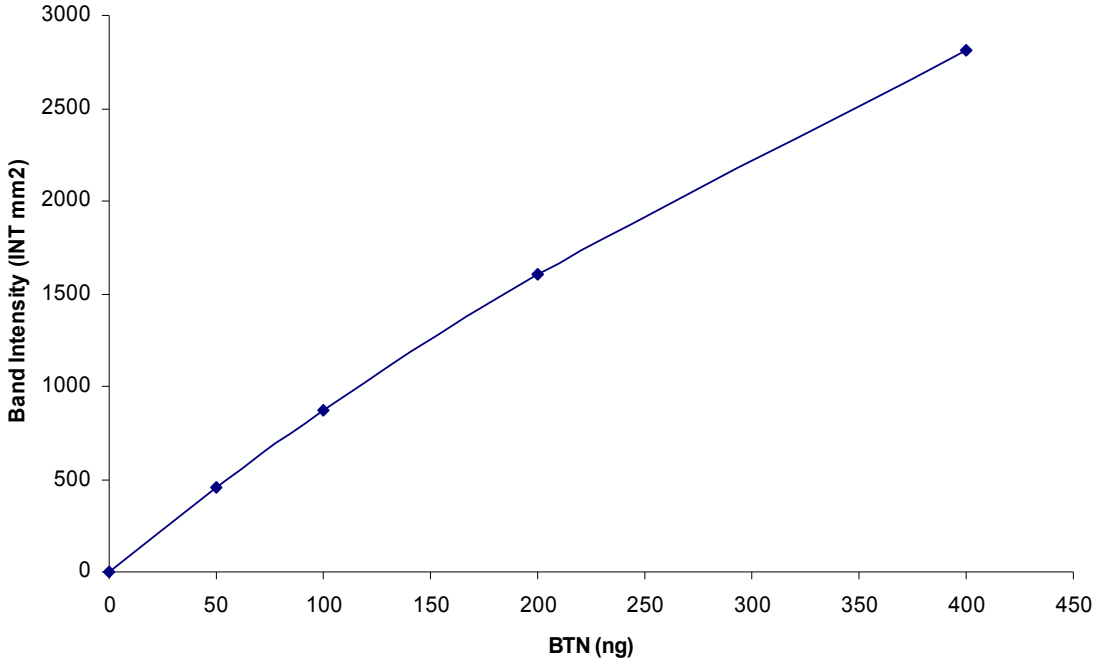


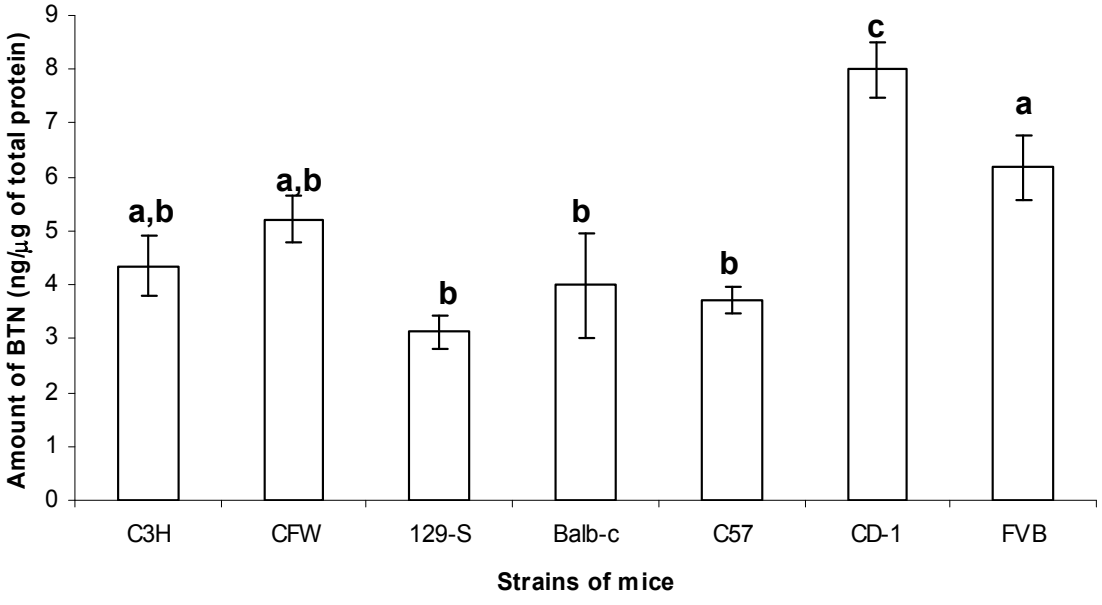
Fig. 7 Amount of XOR in bovine milk-fat samples. Samples were collected and prepared as described in the legend of Fig. 7, except that bovine XOR on the nitrocellulose paper was detected using rabbit antibody (1 in 5,000 dilution) to XOR protein purified from bovine milk.

Fig. 8 Amount of BTN in mouse milk-fat samples determined by immunoassay. Milk samples from seven strains of mice (five samples each) were collected on day 10 of lactation and separated into cream and skim milk fractions by centrifugation. Total cream samples (30 µg of total protein/well) were separated by SDS-PAGE in 10% polyacrylamide gels and electrophoretically transferred to nitrocellulose together with known amounts of the purified mouse cytoplasmic portion of BTN fused to GST (GST-BTN cyto) as a standard. Mouse BTN on nitrocellulose paper was detected using rabbit antibody (1 in 5,000 dilution) to a 21 amino acid peptide in the C-terminus of mouse BTN (Ogg *et al.* 2004) and goat anti-rabbit (IgG) conjugated to horse-radish peroxidase (1 in 10,000 dilution), as described in Materials and Methods. A. Standard curve for BTN (ng) against intensity of band in Western blot. B. BTN concentration in cream from seven strains of mice. Similar letters denote no statistically significant difference, $p>0.05$.

A


3000
2500
2000
1500
1000
500
0
Band Intensity (INT mm2)
0
50
100
150
200
250
300
350
400
450
BTN (ng)

B


9
8
7
6
5
4
3
2
1
0
Amount of BTN (ng/μg of total protein)
a,b
a,b
b
b
b
c
a
C3H
CFW
129-S
Balb-c
C57
CD-1
FVB
Strains of mice

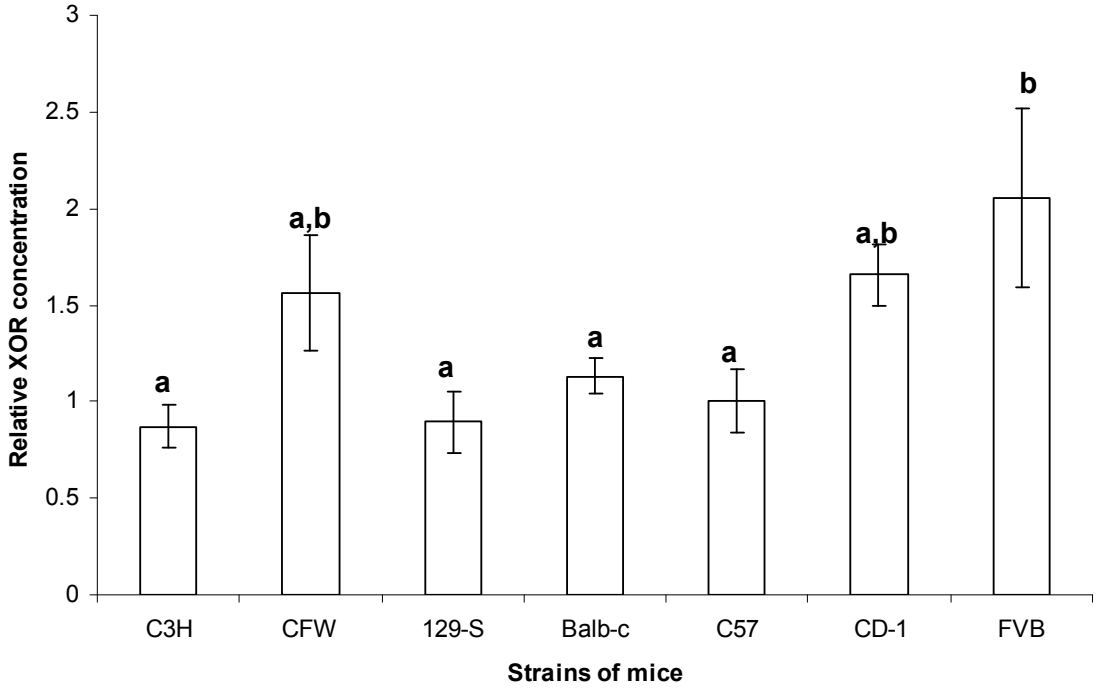
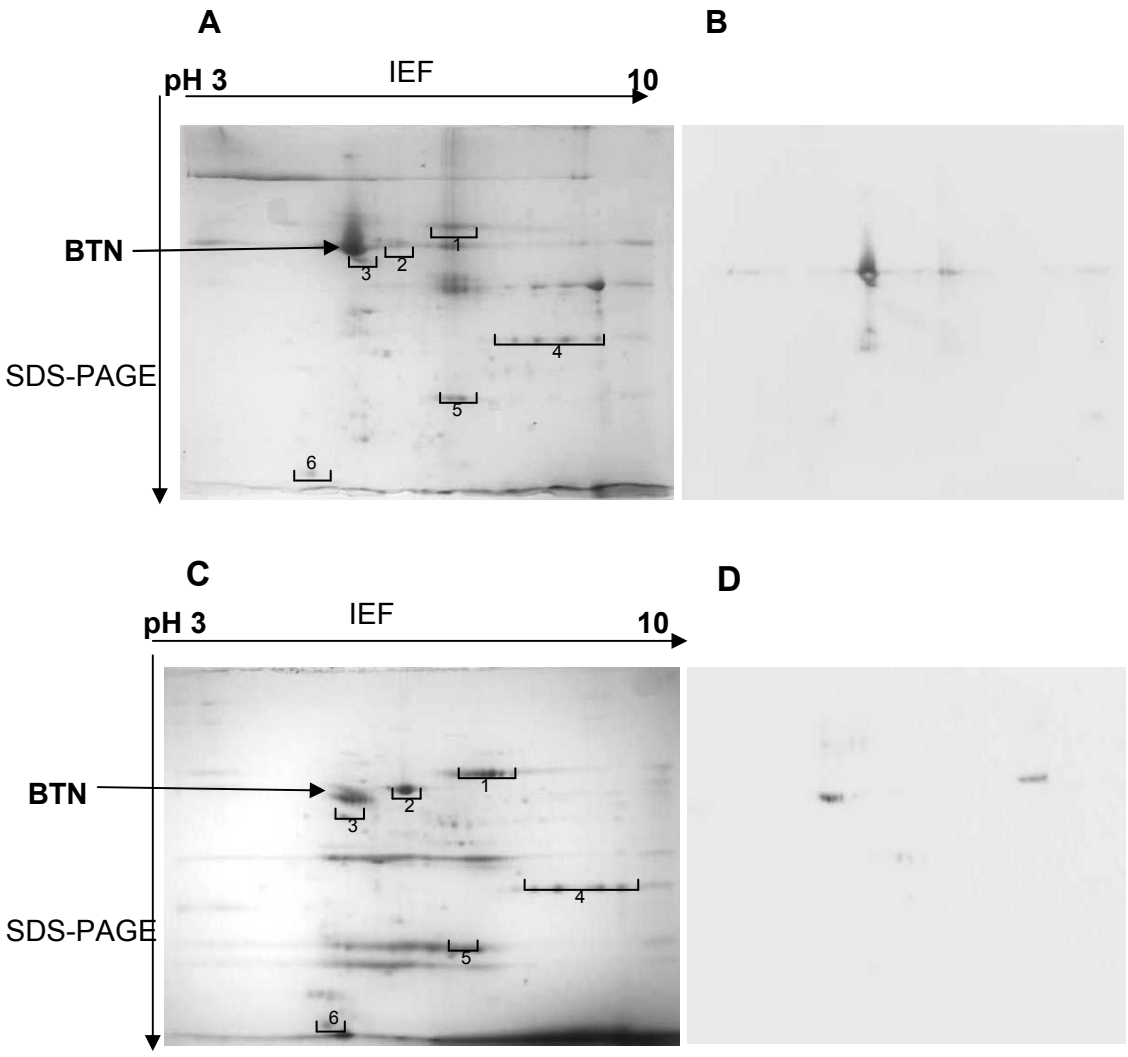


Fig. 9 Relative amount of XOR in mouse milk-fat samples. Milk samples from seven strains of mice (five each) were collected on day 10 of lactation and separated into cream and skim milk fractions by centrifugation. Due to unavailability of standards, an absolute amount of XOR could not be determined. Amount of XOR in the different strains of mice was compared relative to the amount of XOR in C57 mice, set arbitrarily to 1.0. Total cream samples (30 μg of total protein/well) were separated by SDS-PAGE in 10% polyacrylamide gels and electrophoretically transferred to nitrocellulose. Mouse XOR on nitrocellulose paper was detected using rabbit antibody (1 in 2,500 dilution) to purified mouse XOR and goat anti-(rabbit IgG) conjugated to horse-radish peroxidase (1 in 10,000 dilution) as described in Materials and Methods. Similar letters denote no statistically significant difference, $p>0.05$.

Fig. 10 Detection of BTN in bovine and mouse milk-fat samples using 2D gel electrophoresis and Western blot. 50 μg of bovine and mouse milk-fat samples and 10 μl of BioRad 2D gel electrophoresis markers were separated by 2D gel electrophoresis as described in Materials and Methods and detected by Western blot as described previously. A. Coomassie staining after 2D gel electrophoresis of bovine milk-fat samples. B. Western blot of bovine BTN. C. Coomassie staining after 2D gel electrophoresis of mouse milk-fat samples. D. Western blot of mouse BTN. 1, Hen egg white conalbumin M_r 76,000, pI 6.0-6.6; 2, BSA, M_r 66,200, pI 5.4-5.6; 3, Bovine muscle actin, M_r 43,000, pI 5.0-5.1; 4, Rabbit muscle GAPDH, M_r 36,000, pI 8.3-8.5; 5, Bovine carbonic anhydrase, M_r 31,000, pI 5.9-6.0; 6, Soybean trypsin inhibitor, M_r 21,500, pI 4.5.


A
B
pH 3
IEF
10
BTN
SDS-PAGE
1
2
3
4
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6
C
D
pH 3
IEF
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BTN
SDS-PAGE
1
2
3
4
5
6

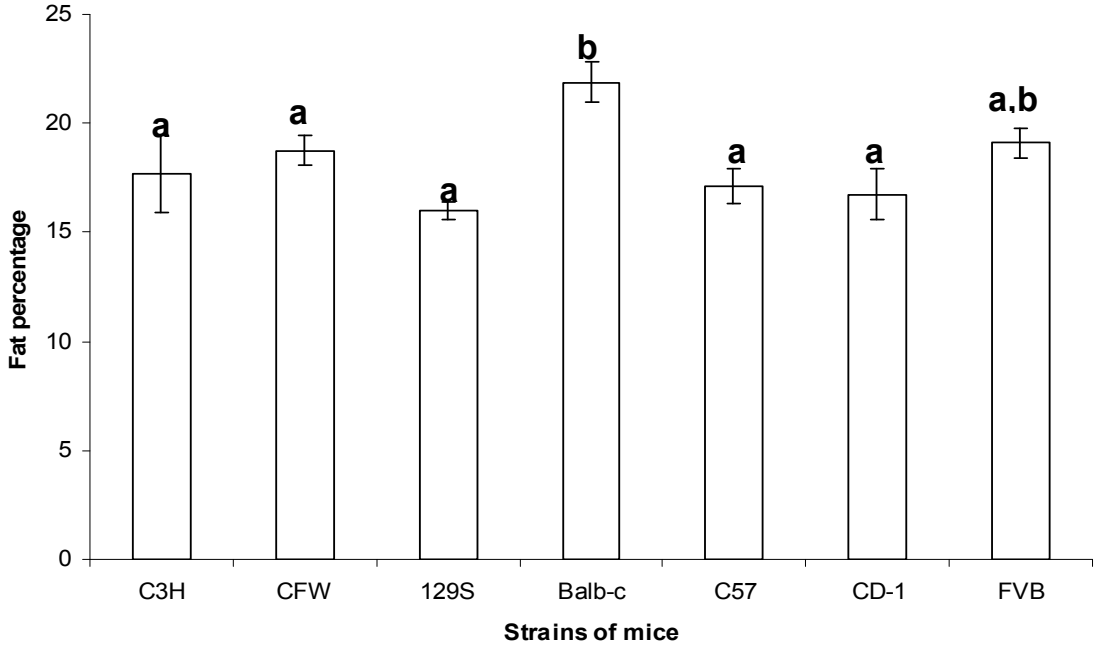


Fig. 11 Concentration of milk lipid (%) in seven strains of mice. Milk was collected from five mice of each strain on day 10 of lactation and separated into cream and skim milk samples, and fat percentage determined by creamatocrit, as described in the text. Similar letters denote no statistically significant difference, $p>0.05$.

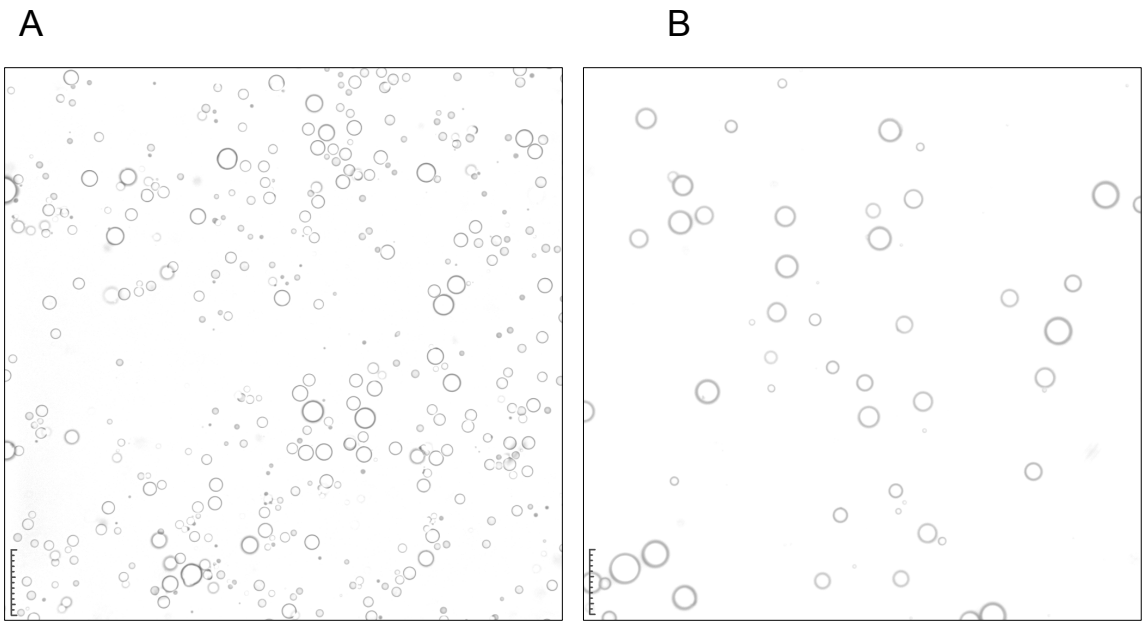
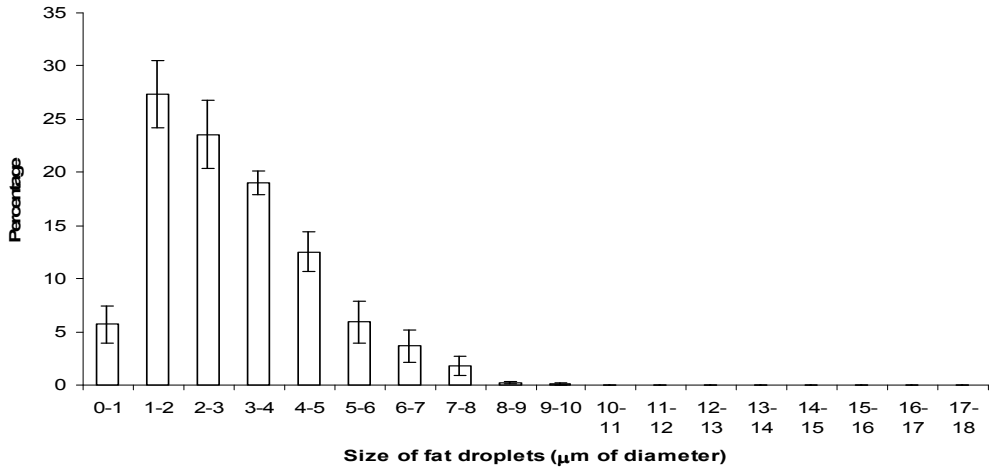


Fig. 12 Comparison of fat droplet size of mouse and cow. Milk (1 µl) was diluted with 24 µl of PBS and spotted onto microscope slides, covered with a cover slip which was sealed with nail polish as described in Materials and Methods. Images of the milk-fat globules were recorded by phase contrast microscopy using a Leica DMIRE2 epifluorescence/differential interference contrast microscope and saved as Microsoft office document imaging files. A. Bovine milk-fat droplets; B. Mouse milk-fat droplets (Bars: 20µm).

A


Percentage
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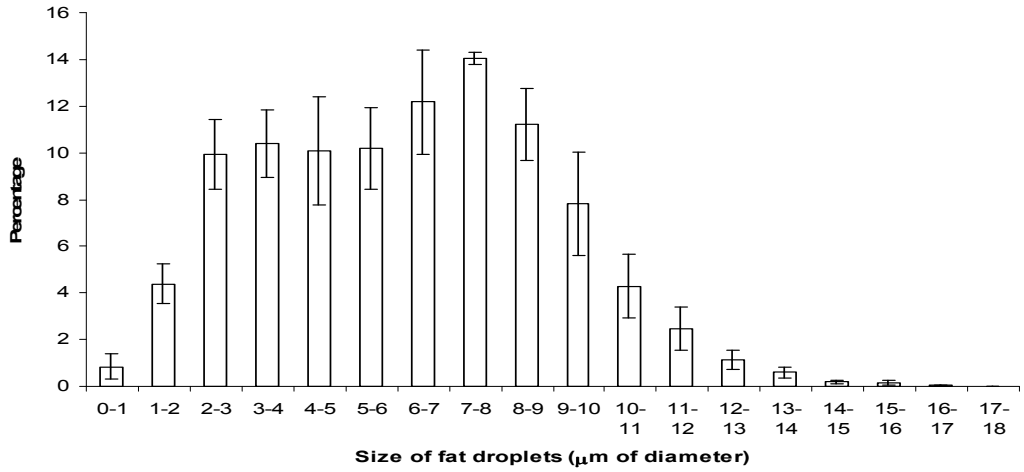
30
25
20
15
10
5
0
0-1
1-2
2-3
3-4
4-5
5-6
6-7
7-8
8-9
9-10
10-11
11-12
12-13
13-14
14-15
15-16
16-17
17-18
Size of fat droplets (μm of diameter)

B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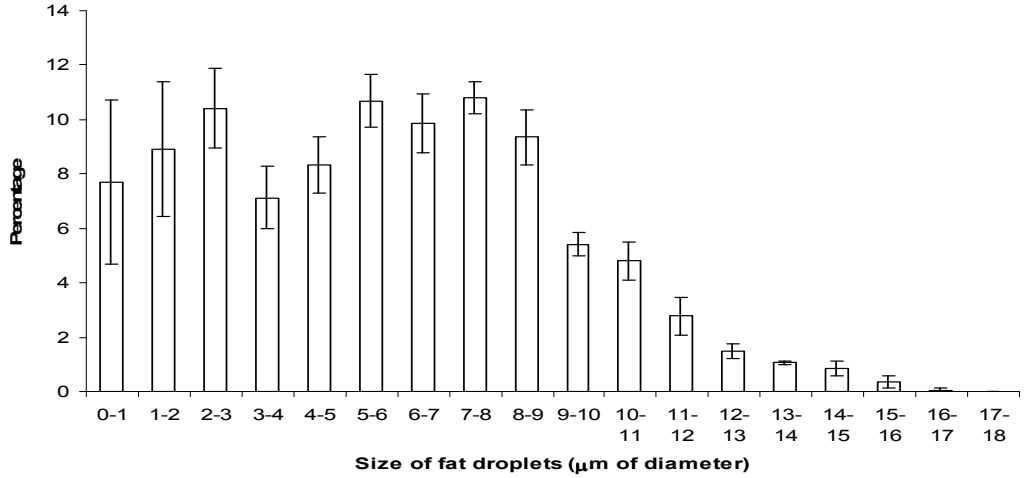
17-18
Size of fat droplets (μm of diameter)

C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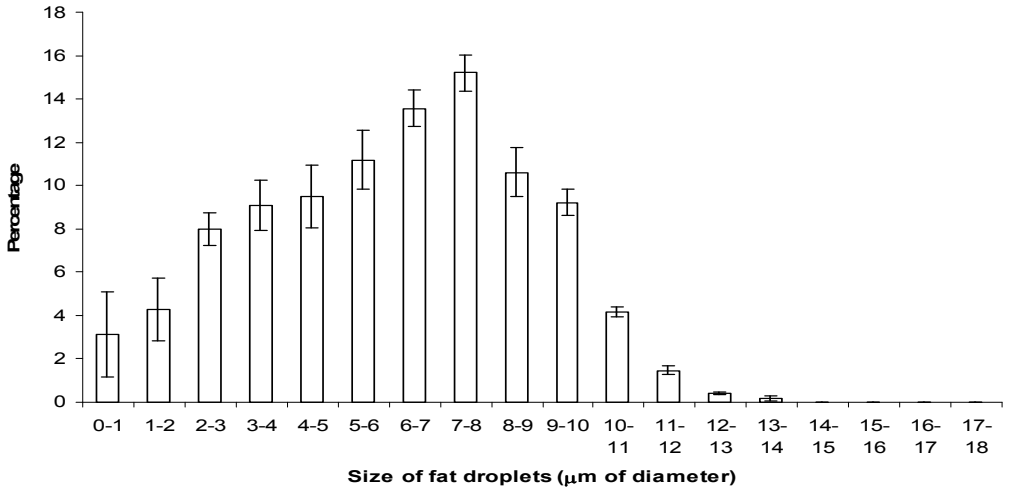
17-18
Size of fat droplets (μm of diameter)

D


Percentage
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6-7
7-8
8-9
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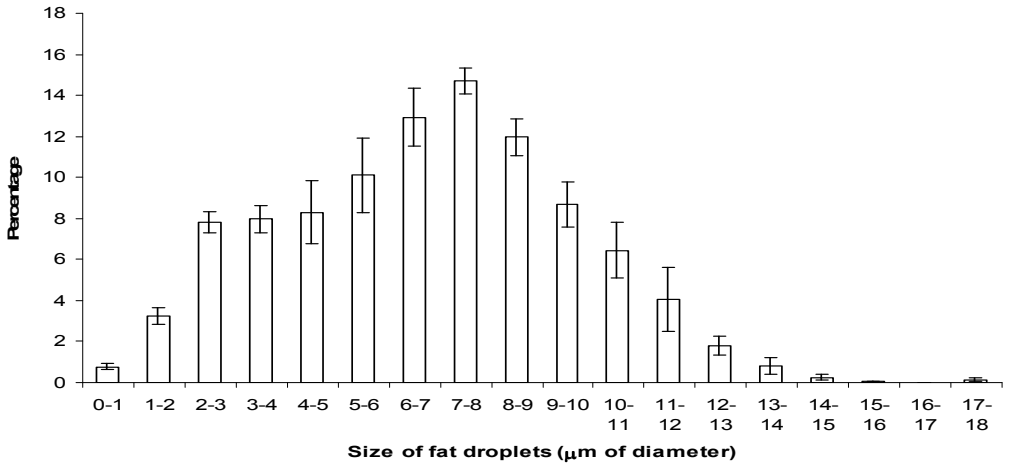
16-17
17-18
Size of fat droplets (μm of diameter)

E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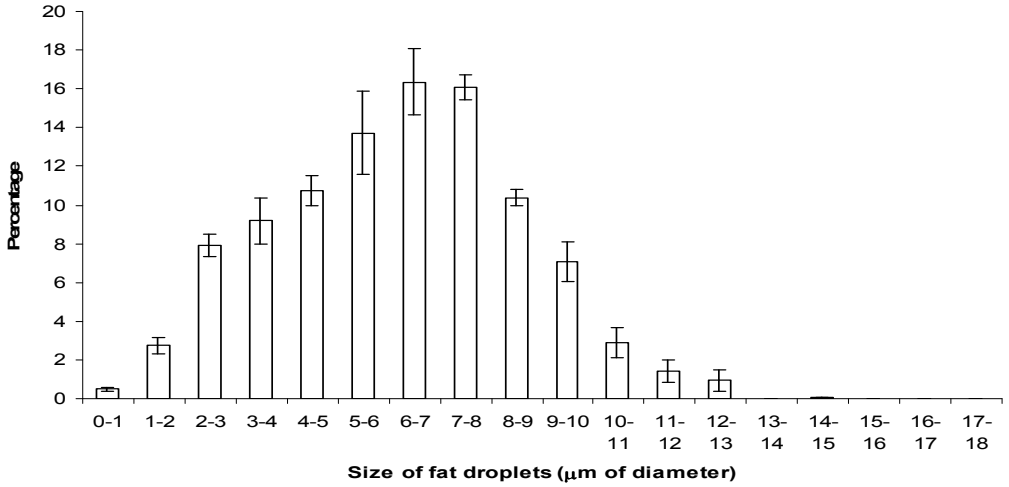
17-18
Size of fat droplets (μm of diameter)

F


Percentage
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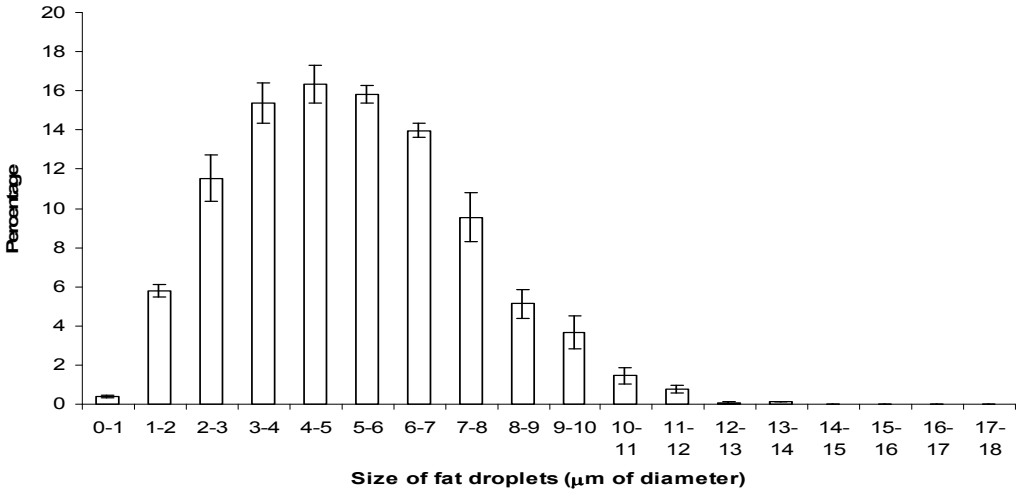
17-18
Size of fat droplets (μm of diameter)

G



H

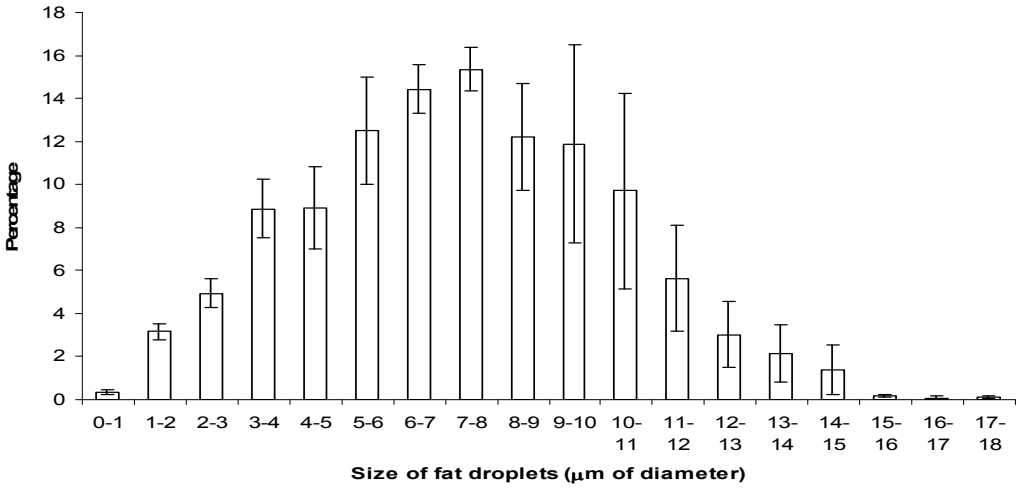


Fig. 13 Size distribution of milk-fat droplets in cows and in different strains of mice. The size of individual fat droplets was determined using the software program, Image Pro Express as described in Table 2. A, cow; B, C3H; C, CFW; D, 129S; E, Balbc, F, C57; G, CD-1; H, FVB.

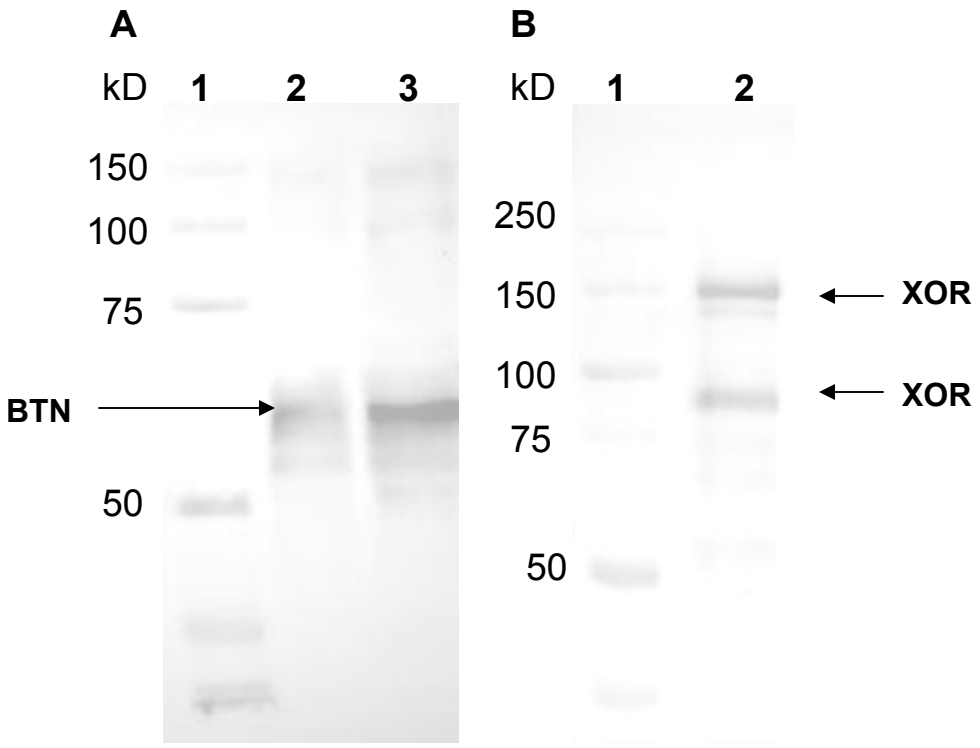


Fig. 14 Cross reactivity of seal BTN and XOR with antibodies to mouse BTN and XOR. Milk proteins were separated by SDS-PAGE in 10% (for BTN) or 8% (for XOR) polyacrylamide gels and electrophoretically transferred to nitrocellulose paper. Seal BTN (A) and XOR (B) were detected using antipeptide antibodies to mouse BTN (1 in 2,500 dilution) or XOR (1 in 2,000 dilution), respectively, followed by goat anti-(rabbit IgG) conjugated to horse-radish peroxidase (1 in 10,000 dilution) as described in Materials and Methods. A. Lane 1, molecular weight markers; lane 2, milk-fat sample of seal (10 μg); lane 3, milk-fat sample of seal (30 μg) B. Lane 1, molecular weight markers; lane 2, milk-fat sample of seal (20 μg).

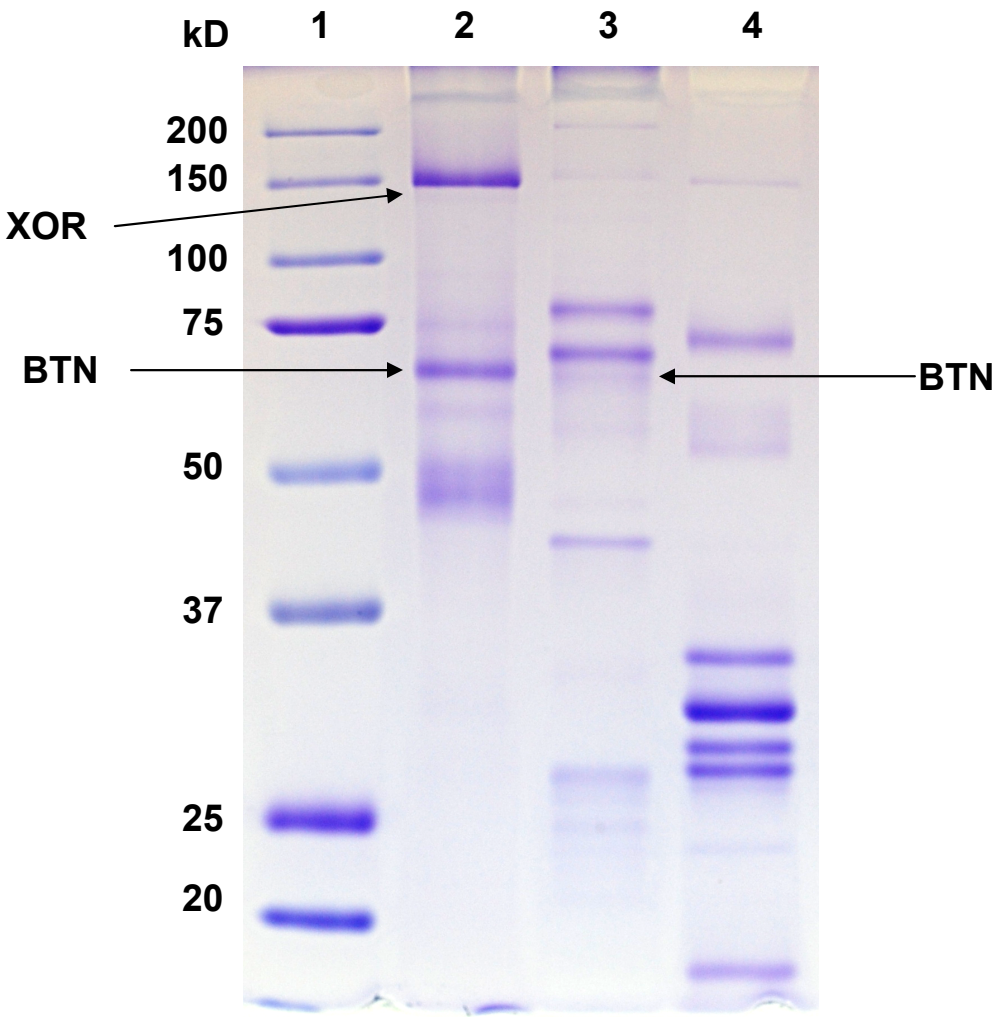


Fig. 15 Comparative protein profiles of milk-fat samples of cow, mouse and seal. Proteins were separated by SDS-PAGE in a 10% polyacrylamide gel and stained with Coomassie Blue. Lane 1, molecular weight molecular weight markers; lane 2, cow (8 µg of cream); lane 3, mouse (8µg of cream); lane 4, seal (20µg of cream).

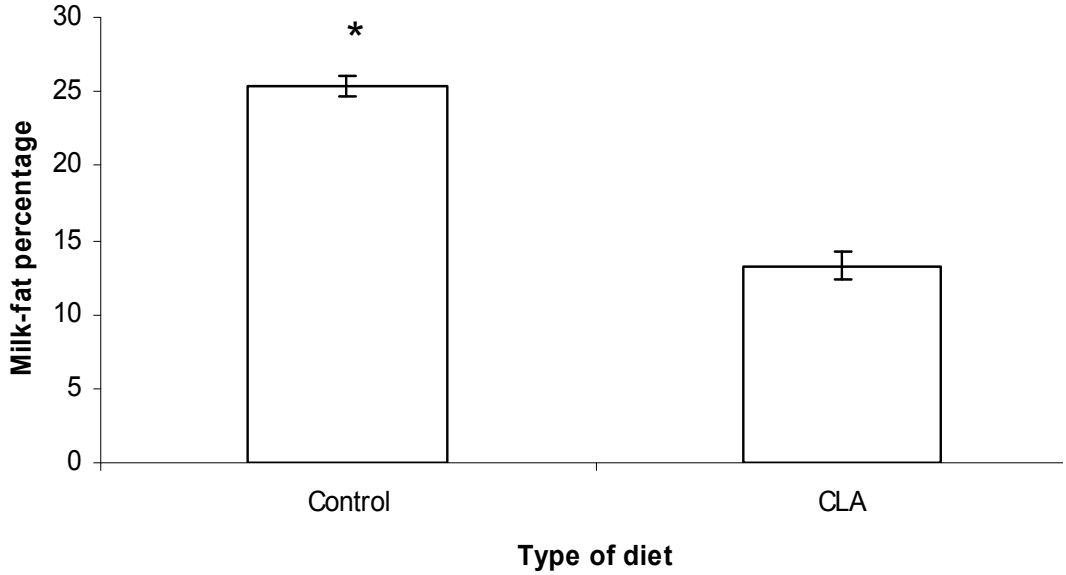


Fig. 16 Milk-fat percentages in control and CLA treated C57 mice. Milk was collected on day 10 of lactation from two groups of C57 mice (5 each), one group fed on a control diet and the other on a CLA diet, and separated into cream and skim milk samples. Fat percentage were determined by creamatocrit, as described in Materials and Methods.

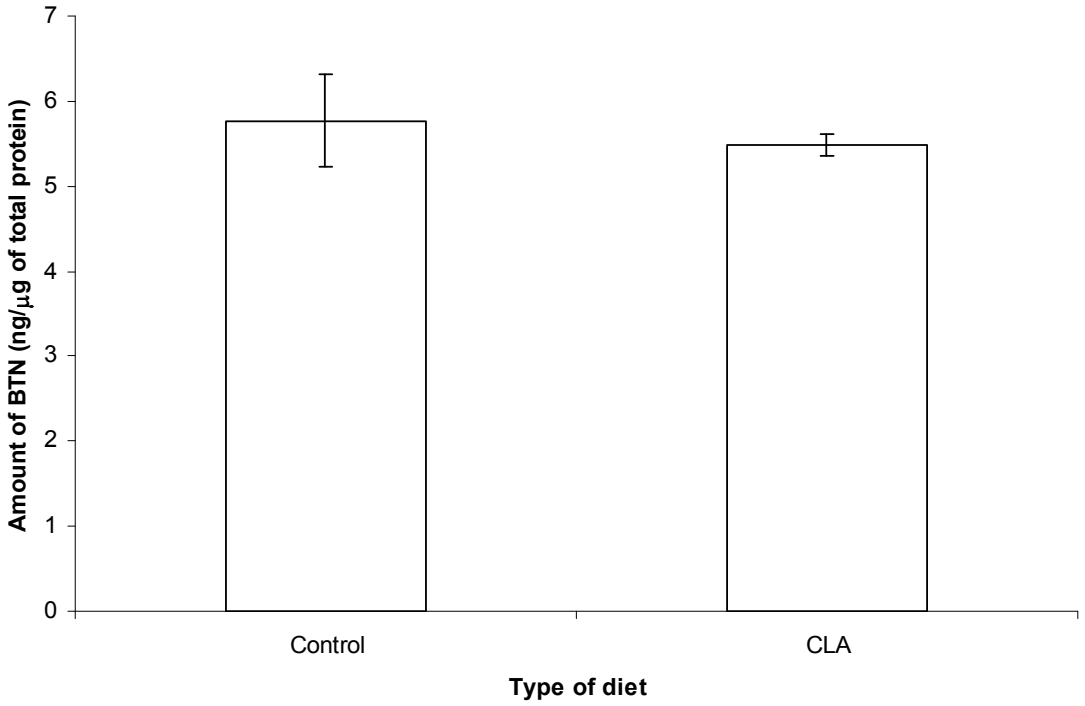


Fig. 17 Amount of BTN in control and CLA-treated mice. The amount of BTN in the fat samples of two groups of C57 mice (5 each), fed on control or CLA diets was determined by Western blot and quantitative densitometry as described in the legend to Fig. 9.

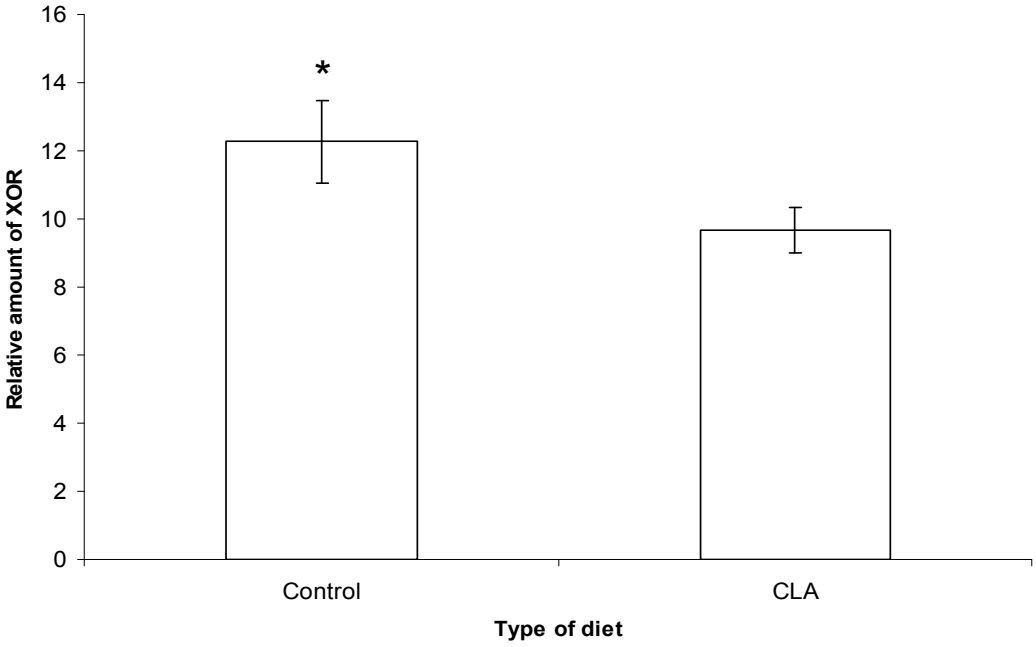


Fig. 18 Relative amount of XOR in control and CLA-treated mice. The relative amount of XOR in the fat samples of two groups of C57 mice (5 each), fed on control or CLA diets, was determined by Western blot and quantitative densitometry as described in the legend to Fig. 10.

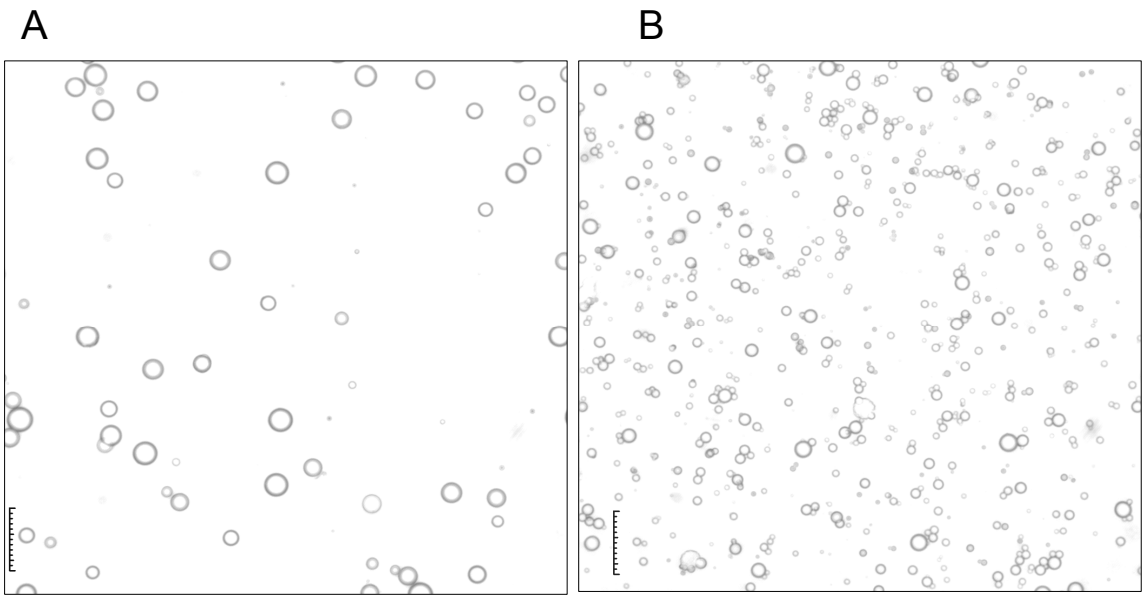
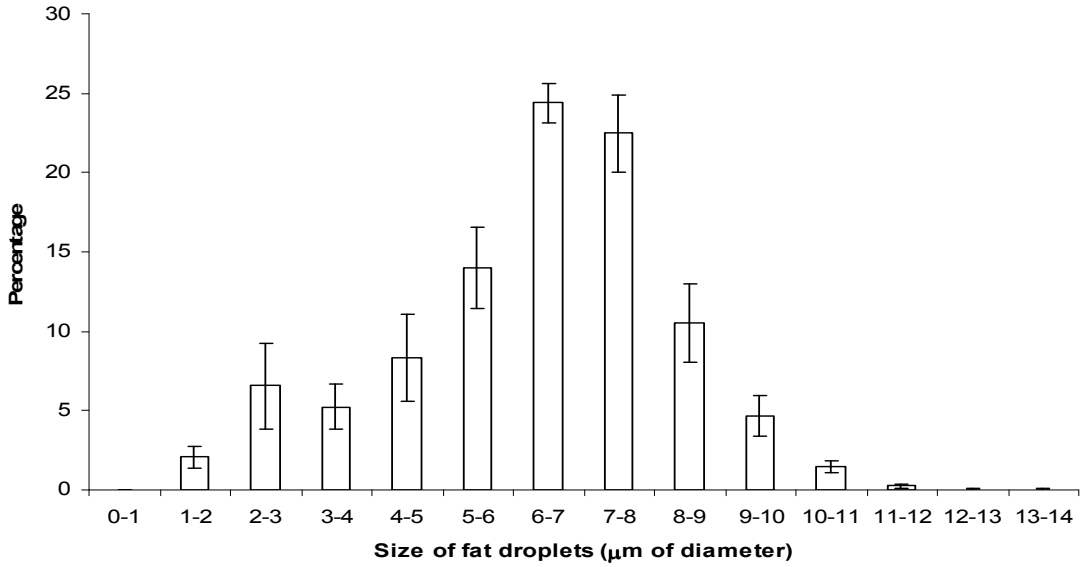


Fig. 19 Comparison of milk-fat droplet size in control and CLA treated mice. Milk (1 µl) from C57 mice fed on a control diet and a CLA diet was diluted with 24 µl of PBS and spotted onto microscope slides, covered with a cover slip which was sealed with nail polish as described in the Materials and Methods section. Images of the milk-fat globules were recorded by phase contrast microscopy using a Leica DMIRE2 epifluorescence/differential interference contrast microscope and saved as Microsoft office document imaging files. A. Control; B. CLA treated mice (Bars: 20 µm)

A



B

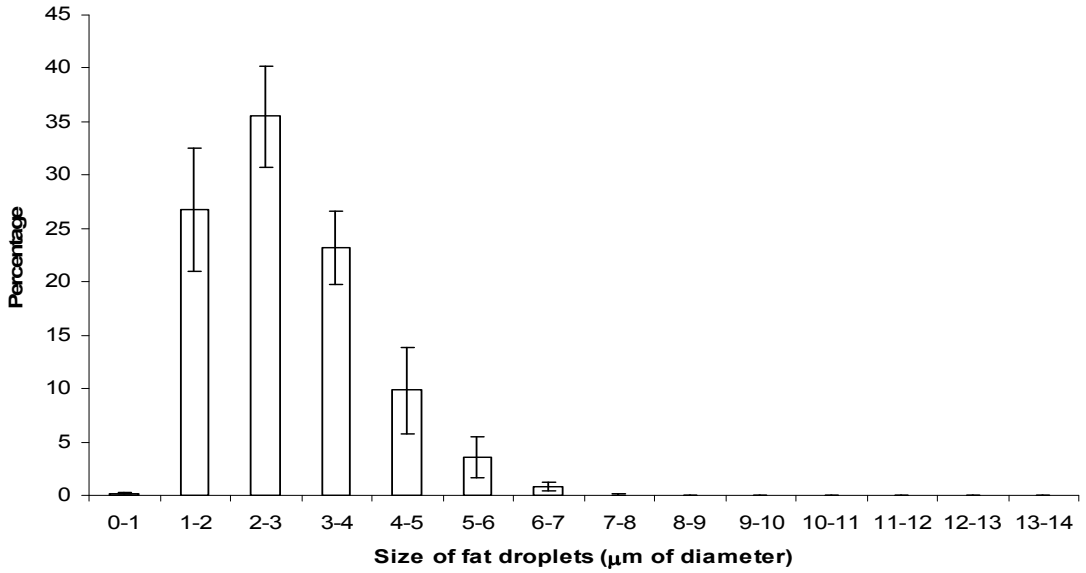


Fig. 20 Distribution of milk-fat droplets of control and CLA-treated groups based on size. A, control; B, CLA. The size of individual fat droplets was determined using the software program, Image Pro Express as described in the legend of Table 2.

Table 2. Statistical analysis of different parameters in cow and mouse

A

Parameters	Between mice strains (P value)	Between cow and mouse (P value)	Between control and CLA-treated mice (P value)
Average size of fat droplets	0.0076	0.0001	0.0004
BTN amount	0.0001	>0.0001	0.5387
XOR amount	0.009	N.A.	0.0238
BTN/XOR ratio	0.4801	0.0022	0.113
Size distribution of fat droplets	N.A.	All sizes except 4-5 μm of diameter are statistically different	All sizes except 4-5 μm of diameter are statistically different

B

	Cow		Mouse	
Parameters	Pr>F	R^2 value	Pr>F	R^2 value
BTN and fat droplet size	0.2367	0.4203	0.2482	0.0332
XOR and fat droplet size	0.7704	0.0329	0.195	0.0416
BTN/XOR ratio and fat droplet size	0.034	0.8212	0.0015	0.2253

A. Statistical analysis of different parameters in cow and mouse. $P<0.05$ is considered statistically different. B. Table showing correlation between BTN, XOR and BTN/XOR ratio to fat droplet size in cow and mouse. $P<0.05$ is considered statistically different. N.A., not applicable.

Table 3. Average size of milk-fat droplets in cows and seven strains of mice

Animal	Mean diameter of milk-fat droplets (μm) ± s.e.m.
Cow	2.97±0.26
Mouse:	
C3H	6.30±0.32
CFW	5.76±0.39
129S	6.14±0.18
Balb-c	6.78±0.26
C57	6.21±0.12
CD-1	5.20±0.18
FVB	7.10±0.54
Mean size in all mouse strains	6.21±0.24

Milk (1 μl) was diluted with 24 μl of PBS and spotted onto microscope slides, covered with a cover slip which was sealed with nail polish as described in the Materials and Methods section. Five separate images of the milk-fat globules of each sample were recorded by phase contrast microscopy using a Leica DMIRE2 epifluorescence/differential interference contrast microscope and saved as Microsoft office document imaging files. The size of individual fat droplets was determined using the software program, Image Pro Express.

Table 4. Average size of milk-fat droplets in control and CLA treated groups of C57 mice

Sample Number	Mean diameter of milk-fat droplets (µm)	
	Control group	CLA group
1	4.64	2.55
2	5.51	3.75
3	6.02	2.57
4	4.51	2.63
5	5.97	2.49
Mean	5.27±0.32	2.74±0.24

Milk (1 µl) was diluted with 24 µl of PBS and spotted onto microscope slides, covered with a cover slip which was sealed with nail polish as described in the Materials and Methods section. Five separate images of the milk-fat globules of each sample were recorded by phase contrast microscopy using a Leica DMIRE2 epifluorescence/differential interference contrast microscope and saved as Microsoft office document imaging files. The size of individual fat droplets was determined using the software program, Image Pro Express.

Discussion

BTN is an integral membrane protein specifically expressed on the apical membrane of lactating mammary gland epithelial cells. It has important roles in milk-fat secretion since ablation of the BTN gene in mice results in defective milk-fat secretion (Ogg *et al.*, 2004). Other roles of BTN, if any, are not clear. However, closely related family members of BTN such as BTNL2 and BTN2A1 have immunological functions (Nguyen *et al.*, 2006; Compte *et al.*, 2004; Malcherek *et al.*, 2007). BTNL2 inhibits T-cell activation (Nguyen *et al.*, 2006; Compte *et al.*, 2004), whereas BTN2A1 can interact with DC-SIGN (Malcherek *et al.,* 2007), a receptor expressed on immature monocyte-derived dendritic cells (MoDC). Because of structural similarities between family members, it is possible that BTN may also have immunological functions apart from its role in milk-fat secretion.

XOR is expressed in almost all cells and is important in purine metabolism. However, in mammary gland epithelial cells, the expression of XOR is highly increased during the later stages of pregnancy and throughout lactation suggesting that it may play some role in milk secretion, especially milk-fat secretion (Kurosaki *et al.*, 1996; McManaman *et al.*, 2002). Moreover, XOR heterozygous mice show many similar phenotypes to BTN knock-out mice such as defective milk-fat secretion, unusually large fat droplets in the mammary epithelium and death and weight loss of the pups (Vorbach *et al.*, 2002), indicating that XOR may work together with BTN in milk-fat secretion. Model 1 and 2 of milk-fat secretion (Introduction, Fig. 3) suggest that the C-terminal domain of BTN interacts with XOR and that this interaction helps to stabilize the fat droplets. This explains why lactation is defective in BTN knock-out mice (Ogg *et*

al.,2004) and XOR heterozygous mice (Vorbach *et al.*, 2002). The third model suggesting that BTN interacts with itself in the secretion process (Robenek *et al.*, 2006) does not explain the defective milk-fat secretion observed in XOR heterozygous mice.

All current models of milk-fat secretion assume that the mechanism of secretion is the same in all species. Similarly, all three models of milk-fat secretion are based on the assumption that the amount of BTN is high enough to play a significant structural role in the secretion process. On a percentage basis, BTN and XOR alone comprise approximately 60% of the total proteins in bovine MFGM (Mather *et al.*, 1980; Mondy and Keenan 1993). However, as shown in this thesis, quantitative assay of BTN and XOR shows that the amount of BTN and XOR varies significantly between at least two species- cow and mouse (Fig. 6, 7, 8 and 9; Table 2). A 75-fold lower BTN level in mice compared to cows suggests that a very low level of BTN is sufficient for mediating its role in milk-fat secretion in mice.

Based on freeze fracture immunocytochemistry of bovine and human milk fat globules, Robenek *et al.* (2006) suggested that milk-fat secretion is mediated by interaction between BTN on the apical plasma membrane and BTN on the surface of intracellular lipid droplets. They found that BTN is more abundant on the monolayer of the CLD surface than in the bilayer of the MFGM and that it occurs in a well defined network which mediates physical apposition between bilayer and monolayer. This model suggests that a network of BTN containing sites links both the layers indicating that a high amount of BTN is necessary for milk-fat secretion. However, a very low level of BTN in mouse MFGM questions the validity of this model. In addition, this model suggests that XOR is not involved in the secretion process. However, the fact that milk-

fat secretion is defective in XOR heterozygous mice (Vorbach *et al.*, 2002) implies that XOR plays a specific role in the secretion process. XOR is also most likely to play a role in milk-fat secretion in cows because Mondy and Keenan (1993) reported that in both Holstein and Jersey cows, BTN and XOR occur in constant molar proportions of about 4:1 throughout lactation, thus suggesting some common functions for XOR and BTN. Similarly, the fact that XOR can directly interact with membrane proteins, especially BTN (Ishii *et al.,* 1995; McManaman *et al.*, 2002; Vorbach *et al.*, 2002; A. R. Rao, J. K. Jeong and I. H. Mather unpublished observations), also indicates its role in milk-fat secretion.

Coomassie staining of SDS-PAGE gels (Fig. 15) showed a relatively thick band for bovine BTN but a very faint band for mouse BTN. To confirm that the amount of BTN was significantly lower in mouse milk-fat samples, the bovine and mouse MFGM samples were analyzed by 2D-gel electrophoresis followed by Western blot. 2D gel electrophoresis and Western blot (Fig. 10) using specific antibodies to bovine and mouse BTN revealed a large difference in BTN amount between the two species. However the amount of BTN in mouse MFGM did not qualitatively appear to be 75 fold lower than bovine MFGM. This apparent discrepancy may reflect differences in methodology. SDS-PAGE analyzes essentially all proteins in a mixture, whereas it is difficult to solubilize all proteins in membrane fractions for 2-D electrophoresis because ionic detergents such as SDS cannot be used. Therefore, only a variable portion of the total proteins can be analyzed by 2D electrophoresis.

The low levels of BTN and XOR in mouse MFGM imply that these proteins may not play a structural role at all but function in some other capacity in milk-lipid secretion.

Earlier, it was suggested that BTN could function as a signaling molecule as it has the canonical structure of a receptor (Taylor *et al.* 1996; Linsley *et al.*, 1994; Ogg *et al.*, 2004). XOR, in this case, may act as an intermediate molecule in the signaling pathway, which, after interaction with BTN triggers downstream pathways via the generation of reactive oxygen species. However, neither the ligand for BTN nor the downstream signaling pathways for XOR are known.

Based on the previous studies with BTN knock-out mice in which unusually large fat droplets were seen in epithelial cells as well as in the lumen (Ogg *et al.* 2004), a reciprocal relationship between BTN or XOR and fat droplet size was predicted. Even though no correlation could be found between BTN or XOR amount and fat droplet size in this work (Table 2), there was a high positive correlation between BTN/XOR ratio and fat droplet size in cows (R^2 value of 0.82), but not in mouse (R^2 value of 0.22). The reason for these differences is not clear.

If BTN or XOR levels were the determinants of fat droplet size, a change in BTN or XOR concentration would be expected with lower fat droplet size noticed in CLA-treated mice. However, no correlation was found between BTN concentration and fat droplet size. Similarly, there was no correlation between BTN/XOR ratio and fat droplet size after CLA treatment. However, it is known that CLA changes the fatty acid composition of milk (Chouinard *et al.* 1999) with a pronounced decrease in short and medium chain fatty acids which represent the portion synthesized de novo by mammary gland epithelial cells. Reduction in the size of fat droplets may be a consequence of the change in fatty acid composition. If this is the case, one cannot expect a change in BTN concentration and hence cannot conclude that BTN does not influence the fat droplet size.

The size of milk-fat droplets may be affected by a number of factors. Previous studies showed a positive correlation between fat droplet size and milk-fat yields in cows (Wiking *et al.*, 2004). Among the three species discussed in this thesis (cow, mouse and seal), the size of fat droplets is lowest for cow, intermediate for mouse (Table 3) and slightly larger than mouse in seal (Tedman 1982). It can be argued that, because of physiological constraints, there must be a limit for fat droplet size, which may explain why fat droplets in seal are not proportionally larger than in mice, even though the fat percentage is two and a half times more. No correlation was observed between fat-droplet sizes among different strains of mice, presumably because lipid droplets are at the physiological limit in this species. Interestingly, when mice were treated with CLA, a highly positive correlation with milk-fat droplet size was noticed. Reduction in fat percentage correlated well with reduction in the size of the fat droplets.

In conclusion, the size of fat droplets may be influenced by different factors depending on the species. BTN alone does not influence the size, at least in mouse. Even though BTN knock-out mice exhibit defective milk-fat secretion, indicating its specific role in secretion, it is possible that BTN may have other roles in lactation. A very low level of BTN in mouse suggests that it may not function as a structural protein, but rather may function as a signaling molecule with XOR acting as an intermediate in this signaling pathway.

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