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THE INHIBITORY EFFECT OF CONJUGATED LINOLEIC ACID (CLA) ON N-6 POLYUNSATURATED FATTY ACID METABOLISM IN A TRANSFORMED YEAST MODEL

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
The Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

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* * * * *

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ABSTRACT

Conjugated linoleic acid (CLA), a mixture of positional and geometric isomers derived from linoleic acid (LA; Δ9,12-18:2), has been shown to modulate immune function and inhibit certain types of cancer in cell culture and animal models. It has been postulated that CLA exerts its beneficial biological effect through suppression of production of n-6 polyunsaturated fatty acids (PUFA), especially arachidonic acid (AA; Δ5, 8,11,14-20:4) and consequently, pro-inflammatory eicosanoids. Since in the PUFA metabolic pathway, Δ6-desaturase, elongase and Δ5-desaturase are directly responsible for synthesis of AA, the hypothesis of this study is that CLA reduces the production of AA by inhibiting the activities of these enzymes. Because the mammalian microsomal system is complex and the purified enzymes are not available, a simple model for testing the hypothesis was needed for this study. Recently, different genes involved in PUFA metabolism have been identified and successfully expressed in baker’s yeast, Saccharomyces cerevisiae. Through the transformed yeast, the activity of Δ6-desaturase, elongase, or Δ5-desaturase could be investigated step-by-step. The objective of this study was to determine whether CLA isomers can affect the Δ6-desaturation which converts...
LA to γ-linolenic acid (GLA; Δ6,9,12-18:3); elongation of LA to EDA (Δ11,14-20:2), elongation of GLA to dihomo-γ-linolenic acid (DGLA; Δ8,11,14-20:3), and Δ5-desaturation of DGLA to AA. Results from this study showed that CLA clearly modulated n-6 PUFA metabolism by inhibiting the two rate-limiting steps of the n-6 PUFA metabolic pathway, Δ6-desaturation of LA and Δ5-desaturation of DGLA. CLA could also inhibit the alternate pathway, i.e. elongation of LA, but not elongation of GLA. The inhibitory effect of CLA on these enzymes is likely due to the competition between substrates and CLA for the enzymes. Among the four CLA isomers studied, c9,t11- and t10,c12-CLA isomers could be elongated to form conjugated EDA (CEDA) isomers by the elongase. These two CLA isomers and the c9,c11-CLA could also be metabolized to form Δ5-desaturation products by the Δ5-desaturase. Since these two CLA isomers also exerted the greatest inhibition, it is likely that these two isomers are the most potent isomers among the CLA mixture as suggested by others.

In conclusion, results in this study show that CLA inhibits the Δ6-desaturation and elongation of LA, and Δ5-desaturation of DGLA. The combined inhibitory effects of CLA on these three steps modulate n-6 PUFA metabolism and hence reduce the production of AA. This, in turn reduces the amount of AA entering the cyclooxygenase/lipoxygenase pathways, resulting in a possible reduction in the biosynthesis of pro-inflammatory eicosanoids in mammalian cells.
Dedicated to the Lord
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PUBLICATIONS

Research Publication

growth induced by expression of HBsAg in the secretion pathway of S. cerevisiae

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**Proceedings and Abstracts**


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CHAPTER 1

INTRODUCTION

1.1 Background

Conjugated linoleic acid (CLA; 18:2) is a collective term for the group of positional and geometric isomers of linoleic acid (LA; Δ9,12-18:2). CLA has been shown to exert many beneficial biological effects in cell culture and animal models. For example, CLA significantly decreases breast cancer cell proliferation and inhibits tumorigenesis in mammary gland, skin, and stomach in experimental animals (Belury et al., 1996; Cunningham et al., 1997; Ha et al., 1990; Ip et al., 1991; Ip et al., 1995). CLA can also decrease the development of atherosclerosis in hamsters and rabbits (Lee et al., 1994; Nicolosi et al., 1997), modulate immune function in rats (Sugano et al., 1998), and modulate body composition and fat distribution in different animals (Ostrowska et al., 1999; Park et al., 1997; Stangl et al., 1999; West et al., 1998).

The mechanisms by which CLA exerts its biological effects are still not fully understood. However, several possible mechanisms have been proposed. CLA has been suggested to function as an antioxidant or to have cytotoxic properties (Ha et
CLA could also modulate the gene expression and/or signal transduction (Belury and Kempa-Steczko, 1997; Lee et al., 1998), manipulate the PUFA metabolism and eicosanoid synthesis (Liu and Belury, 1997; Sugano et al., 1998), and prevent the formation of DNA-adduct (Josyula et al., 1998; Zu and Schut, 1992). Recently, Pariza et al. (Pariza et al., 2000) proposed the concept of multiple biochemical mechanisms to explain various biological effects triggered by CLA. They attribute each of the physiological effects of CLA to the combined result of a number of different biochemical mechanisms. For example, the inhibition of carcinogenesis by CLA could be the result of the collective inhibitory effects of CLA (or its metabolites) on cell differentiation and proliferation, eicosanoid metabolism, and signal transduction (Pariza et al., 2000). Among these mechanisms, the regulatory effects of CLA (or its metabolites) on the n-6 PUFA metabolic pathway, especially arachidonic acid (AA; Δ5,8,11,14-20:4) production and eicosanoid biosynthesis might play the most important role in the “multiple biochemical mechanisms”.

Arachidonic acid is the precursor of the 2- and 4-series of eicosanoids (pro-inflammatory) (Williams and Peck, 1977), a potent activator of peroxisome proliferator-activated receptor alpha (PPARα) and nuclear factor kappa B (NF-κB) (Camandola et al., 1996; Keller et al., 1993), and also a mediator which triggers various signal transduction pathways (Barry et al., 1999; Metzler et al., 1998) (Figure 1.1). In mammalian cells, free cytosolic AA with/without stress signals, including inflammatory cytokines, ultraviolet light, etc. may possibly up-regulate
Figure 1.1: Arachidonic acid (AA) or its metabolites (eicosanoids) can potentially activate multiple pathways through signal transduction. Abbreviation: cAMP, cyclic adenosine monophosphate; COX II, cyclooxygenase; GF, growth factor; 5-LOX, 5-lipoxygenase; NF-κB, nuclear factor kappa B; PPAR, peroxisome proliferator-activated receptor; PLA₂, phospholipase A₂.
and induce the phospholipase A\textsubscript{2} (PLA\textsubscript{2}) to release more AA from membrane phospholipids (Barry et al., 1999; Fujishima et al., 1999; Gudmundsdottir et al., 2001). Cytosolic AA, growth factors and/or stress signals may trigger a series of signal transduction to stimulate the synthesis of eicosanoids by the activation of cyclooxygenase II (COX-II) or 5-lipoxygenase (5-LOX) (Barry et al., 1999; Woo et al., 2000). Later, pro-inflammatory eicosanoids may bind to their respective receptors and activate different downstream cell signaling cascades (Fan and Chapkin, 1998), such as activation of G-protein, adenylate cyclase, etc. and induce the production of several pro-inflammatory factors, e.g. interleukin 2 (IL-2) and tumor necrosis factor (TNF). These cytokines become the stress signals that repeatedly up-regulate PLA\textsubscript{2}, COX-II and 5-LOX, and mediators which cause chronic inflammation. All these findings suggested that AA was the most important factor which activates most pro-inflammatory responses and consequently leads to different diseases. This has led to a hypothesis that the decrease in the production of AA may reduce chronic inflammation.

The argument that the regulatory effect of CLA on the n-6 PUFA metabolic pathway and eicosanoid biosynthesis plays the most important role for CLA exerting its biological effects (Figure 1.2), could be supported by the fact that CLA (or its metabolites) has been shown to decrease the production of arachidonic acid and pro-inflammatory eicosanoids, consequently, modulate the immune function (Liu and Belury, 1997; Sugano et al., 1998). Also the possible regulatory effects of CLA on PUFA metabolic enzymes, including Δ6-desaturase, elongase and Δ5-desaturase
Figure 1.2: Metabolic pathways for conversion of n-6 polyunsaturated fatty acids to pro-inflammatory eicosanoids.
may be responsible for its modulation of the production of AA and pro-inflammatory eicosanoids.

Despite the importance of understanding the effect of CLA on n-6-PUFA metabolism, to date, few studies have been done to examine the effects of CLA on Δ6-desaturase, elongase and Δ5-desaturase. This is because none of these PUFA metabolic enzymes has been successfully purified. Although liver microsomes isolated from the CLA-fed animals were used as a model to study the effects of CLA on those PUFA enzymes (Banni et al., 1995; Sebedio et al., 1997), there is a drawback that, the liver microsomal system contains a variety of endogenous enzymes, including all of the PUFA metabolic enzymes. This complexity makes it difficult to examine how CLA (or its metabolites) affects each step of the n-6 PUFA metabolic pathway.

Recently, utilizing molecular biological techniques, different genes involved in the Δ6-desaturaton, elongation and Δ5-desaturation of long-chain PUFA have been identified and successfully expressed in baker's yeast, *Sacchromyces cerevisiae* (Cho et al., 1999; Huang et al., 1999; Knutzon et al., 1998; Leonard et al., 2000a; Leonard et al., 2000b; Michaelson et al., 1998; Parker-Barnes et al., 2000; Qiu et al., 2001; Watts and Browse, 1999). Since normal baker's yeast does not contain any enzymes involved in PUFA metabolism, yeast introduced with a specific PUFA metabolic enzyme gene converts only the substrate to its specific product, and no further metabolism should be found in this transformed yeast. This distinct
advantage is not observed using the microsomal system. Thus, the transformed yeast provides a unique model for studying the n-6 PUFA metabolism step-by-step.

1.2 Hypotheses and Objectives

The hypothesis of this study is to examine whether CLA reduces the production of AA through its inhibitory effect on n-6 PUFA metabolism, i.e. Δ6-desaturation, elongation and Δ5-desaturation. Since, AA is the precursor of the 2 and 4-series of eicosanoids, including prostaglandins (PGE₂) and leukotrienes (LTB₄) (Williams and Peck, 1977), and a mediator triggering various pro-inflammatory responses (Barry et al., 1999; Camandola et al., 1996; Keller et al., 1993; Metzler et al., 1998), a decrease in production of AA might modulate eicosanoid metabolism, and, in turn, regulate the progress of inflammation and the development of inflammatory diseases.

There are three specific objectives in this study:

(1) The first objective is to examine if CLA itself can be metabolized by the recombinant Δ6-desaturase, and determine whether CLA affects the conversion of LA to GLA by Δ6-desaturase.
(2) In mammalian cells, there is an alternate metabolic pathway for converting LA to PUFA, when Δ6-desaturation of LA is suppressed. Therefore, two elongation steps may be involved in the production of AA from LA. The second objectives are to examine whether CLA inhibits elongation of LA and GLA, and reduce the production of DGLA.

(3) The third objective is to study whether CLA can affect the activity of Δ5-desaturase, and reduce the production of AA.
1.3 **Significance of the Study**

The modulation of n-6 PUFA status by CLA has been documented by different research groups (Banni et al., 1995; Liu and Belury, 1998; Sebedio et al., 1997), but none has specifically examined the interaction between CLA and PUFA metabolic enzymes. By using the unique transformed yeast model, we have demonstrated the inhibitory effect of CLA on these PUFA metabolic enzymes, and provided evidence to support the hypothesis that CLA reduces production of AA by modulating the n-6 PUFA metabolism.
CHAPTER 2

LITERATURE REVIEW

2.1 Conjugated Linoleic Acid

2.1.1 Definition and Chemical Structure

Conjugated linoleic acid (CLA; 18:2) refers to a mixture of conjugated derivatives from linoleic acid (LA; Δ9,12-18:2). Both LA and CLA have 18-carbon chain length and 2 double bonds. The difference in chemical structures between CLA and LA is that the 2 double bonds in CLA are conjugated with no carbon atom between the two double bonds, whereas the two double bonds in LA are interrupted by a methylene (–CH2–) group (Figure 2.1). Because of the presence and location of the two double bonds, CLA is known to have four different geometric isomers (cis-cis, cis-trans, trans-cis and trans-trans) and at least five positional isomers (e.g. 7,9; 8,10; 9,11; 10,12 and 11,13) (Eulitz et al., 1999; Sehat et al., 1998; Yurawecz et al., 1998). Through various combination of these two different types of isomers, a large number (more than 20) of CLA isomers may exist in nature. However, in nature, only c9,t11- and t10,c12- CLA isomers are the most predominant ones, and have long been considered biologically active.
Linoleic Acid, 18:2(c9,c12)

Conjugated Linoleic Acid (CLA), 18:2(9,11)

Conjugated Linoleic Acid (CLA), 18:2(10,12)

Figure 2.1: The chemical structures of linoleic acid (LA; c9,c12-18:2) and conjugated linoleic acid (CLA; t9,t11-, t10,t12-, and c9,t11-18:2).

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CLA is one of the biohydrogenated by-products formed from unsaturated fatty acids by a strain of anaerobic rumen bacteria, *Butyrivibrio fibrisolvens*. Kepler *et al.* (1966) were the first to demonstrate that *B. fibrisolvens* carries out the biohydrogenation of linoleic acid and produces a mixture of geometric and positional isomers of dienoic and monoenoic acids in a two-step process (Kepler *et al.*, 1966). The first step is isomerization – linoleic acid is converted to cis-9,trans-11-octadecadienoate (one of the CLA isomers). In the second step, the conjugated octadecadienoate is reduced by hydrogenation to form predominantly trans-11-octadecenoate. In other rumen bacteria, trans-11-octadecenoate is further converted to stearate (Polan *et al.*, 1964; Shorland *et al.*, 1957; Ward *et al.*, 1964). Although, the role of biohydrogenation in bacteria is still not fully understood, it has been proposed to serve as a mean in depositing the reducing power, a process vital to an anaerobic cells (Lennarz, 1966).

The enzyme responsible for the initial isomerization of LA to cis-9,trans-11-octadecadienoate was isolated from *B. fibrisolvens* and identified as a linoleate cis-12,trans-11-isomerase (Kepler and Tove, 1967). More recently, Chin *et al.* reported that a partially purified isomerase from *B. fibrisolvens* was able to convert LA to
CLA is converted from LA not only by rumen bacteria, but also by rat intestinal flora (Chin et al., 1994).

### 2.1.3 CLA in Food

CLA has been found in different foods (Table 2.1), such as ruminant meats and dairy products. Significant amounts of CLA produced by rumen microorganisms are also found in ruminant meats. Among them, lamb contains the highest level (5.6 mg CLA/g fat) and veal contains the lowest (2.7 mg CLA/g fat). In beef, CAL content ranges from 2.9 to 4.3 mg CLA/g fat (Chin et al., 1993).

High levels of CLA are also found in dairy products. It has been reported in two surveys that total CLA content in cow’s milk is about 5.5 mg/g fat and in other dairy products ranges from 3.6 to 8.0 mg/g fat (Chin et al., 1993; Lin et al., 1995).

Generally, the meat from non-ruminant animals contains much less CLA than that from ruminant animals. Pork contains less 0.6 mg/g fat, chicken contains 0.9 mg/g fat, and seafood ranges from 0.3 to 0.6 mg/g fat (Chin et al., 1992; Chin et al., 1993). The exception is turkey, which contains average 2.5 mg CLA/g fat (Chin et al., 1994). The reason why turkey has high CLA level is not clear.

Vegetables and fruits contain none or only traces of CLA. Plant oils also contain extremely low amounts of CLA, ranging from 0.1 mg/g fat (coconut oil) to 0.7 mg/g fat (safflower oil). The presence of CLA in plant oils is likely due to oxidation and/or bleaching effect during processing (Chin et al., 1992).
<table>
<thead>
<tr>
<th>Foods</th>
<th>CLA (mg/g fat)</th>
<th>c9,t11-CLA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lamb</td>
<td>5.6</td>
<td>92</td>
</tr>
<tr>
<td>Group beef</td>
<td>4.3</td>
<td>85</td>
</tr>
<tr>
<td>Veal</td>
<td>2.7</td>
<td>84</td>
</tr>
<tr>
<td>Ground Turkey</td>
<td>2.5</td>
<td>76</td>
</tr>
<tr>
<td>Pork</td>
<td>0.6</td>
<td>82</td>
</tr>
<tr>
<td>Chicken</td>
<td>0.9</td>
<td>84</td>
</tr>
<tr>
<td>Shrimp</td>
<td>0.6</td>
<td>-</td>
</tr>
<tr>
<td>Salmon</td>
<td>0.3</td>
<td>-</td>
</tr>
<tr>
<td>Milk</td>
<td>5.5</td>
<td>92</td>
</tr>
<tr>
<td>Blue cheese</td>
<td>5.7</td>
<td>90</td>
</tr>
<tr>
<td>Cottage cheese</td>
<td>4.5</td>
<td>83</td>
</tr>
<tr>
<td>Cheese whiz</td>
<td>6.4</td>
<td>48</td>
</tr>
<tr>
<td>Butter</td>
<td>4.7</td>
<td>88</td>
</tr>
<tr>
<td>Yogurt</td>
<td>4.8</td>
<td>84</td>
</tr>
<tr>
<td>Beef tallow</td>
<td>2.6</td>
<td>84</td>
</tr>
<tr>
<td>Egg yolk</td>
<td>0.6</td>
<td>82</td>
</tr>
<tr>
<td>Corn oil</td>
<td>0.2</td>
<td>37</td>
</tr>
</tbody>
</table>

Table 2.1: CLA content in various food products (Chin et al., 1992).
2.1.4 CLA Absorption

The absorption and assimilation of dietary CLA by animals and humans have been well described in the literature (Britton et al., 1992; Chin et al., 1994; Huang et al., 1994; Sugano et al., 1997). Generally, dietary CLA is taken up and subsequently incorporated into body lipids. In rats, CLA enters the enterocytes by hydrophobic diffusion, and is then secreted from the intestine in chylomicrons similar to other fatty acids such as linoleic acid (Liu and Belury, 1997; Sugano et al., 1997). However, the lymphatic recovery rate of CLA was slower than that of linoleic acid during a 24-hr period (Sugano et al., 1997). This indicates that CLA exhibits different physical properties as compared to LA, despite the fact that they have similar chemical structures. The slower recovery of CLA could be due to its conjugated double bonds. Banni et al. have proposed that arrangement of these 2 conjugated double bonds makes the backbone of CLA similar to that of oleic acid. Hence, CLA is incorporated into rat liver lipids preferentially into neutral lipids, similar to oleic acid (Banni et al., 1995; Banni et al., 1990).

Once it is absorbed, CLA is incorporated into various tissues in rodents. The amount of CLA incorporated into the tissue is related to the content of neutral lipids in the tissue. For example, tissues with higher contents of neutral lipids, such as adipose and mammary tissues, as compared to liver and plasma, contain higher amounts of CLA (Banni et al., 1999). Dietary CLA consumption also has a dose-dependent effect on CLA incorporation into tissue phospholipids and neutral lipids (Kavanaugh et al., 1999).
Due to their differences in chemical structures, individual CLA isomers are also absorbed and distributed differently (Sugano et al., 1997). More trans-trans CLA isomers than cis-trans and trans-cis isomers were detected in the tissue lipid extracts (Sugano et al., 1997).

2.1.5 CLA Metabolism

Similar to other fatty acids, CLA can be metabolized by the same polyunsaturated fatty acid (PUFA) enzyme system in animal tissues. Banni et al. have reported the appearance of different conjugated diene (CD) fatty acids in animal tissues (Banni et al., 2001; Banni et al., 1996; Banni et al., 1995). In rats fed with CLA, two fatty acids, CD 18:3 and CD 20:3 fatty acids have been identified. In addition, a CD 20:4 has also been found in lamb tissue.

Sébéedio and his colleague (Juaneda and Sebedio, 1999; Sebedio et al., 2001; Sebedio et al., 1997) have also studied the elongation and desaturation of CLA in rats. By utilizing the combined techniques of high-performance liquid chromatography (HPLC) and gas chromatography – mass spectrometry (GC-MS), they have identified three CD metabolites of CLA, Δ8,12,14-20:3, Δ5,8,12,14-20:4 and Δ5,8,11,13-20:4 (Sebedio et al., 1997). Results from their studies also suggested that CLA could be desaturated and elongated in animals without affecting the CD structure.
2.2 Beneficial Effects of CLA

2.2.1 Anti-carcinogenic Effect

In the early 80s, Pariza and his colleagues found factors extracted from grilled ground beef could inhibit carcinogenesis (Ha et al., 1987; Ha et al., 1990; Pariza and Hargraves, 1985). After extraction and isolation, these anti-carcinogenic factors were shown to consist of isomers of conjugated octadecadienoic acid. This finding led to the subsequent discovery of many inhibitory effects of CLA on various types of cancer.

2.2.1.1 Breast Cancer

In recent years, the anti-carcinogenic properties of CLA on the prevention of chemical or biological carcinogen-induced mammary cancer have been vigorously investigated in many rat and mouse models (Banni et al., 1999; Ip et al., 1996; Ip et al., 1991; Ip and Scimeca, 1997; Ip et al., 1995; Ip et al., 1994; Ip et al., 1999; Visonneau et al., 1997; Wong et al., 1997). Results from these studies showed that dietary supplementation of CLA at the 1.0% level significantly inhibited the development of mammary cancer. There were reductions in tumor incidence, total number of mammary adenocarcinomas, and the density of terminal end buds (TEBs), which were the primary sites for the chemical induction of mammary carcinomas in rodents. Also, evidence has shown that as little as 0.1% of CLA in the diet was sufficient to prevent the development of mammary cancer in rats treated with lower doses of the carcinogen, 7,12-dimethylbenz[a]anthracene (DMBA) (Ip et al., 1994).
On the other hand, Wong et al. have carried out a similar study in BALB/c mice induced with a WAZ-2T tumor cell line, and reported that CLA modulated only certain aspects of immune function, but it had no significant effect on the mammary tumor growth (Wong et al., 1997).

The timing of dietary CLA on the prevention of mammary cancer has also been examined. Ip et al. (1995) and Banni et al. (1999) have shown in rats that exposure to CLA during the early post-weaning and pubertal period is critical in reducing the progress of the DMBA or methylnitrosourea-induced (MNU) mammary tumor (Banni et al., 1999; Ip et al., 1995). It is known that TEBs, which develop mainly in the early weaning period (Russo and Russo, 1978), become hyperplastic to form adenocarcinomas by the carcinogenic induction. CLA can either slow or inhibit the development of TEBs and down-regulated mammary epithelial growth during this period. This suggests that the protective effect of CLA on mammary cancer is at the initiation stage of tumorigenesis.

The duration of CLA supplementation also plays an important role in mammary cancer prevention. Evidence has shown that a short-term exposure to CLA after DMBA or MNU administration was not effective in cancer prevention. Significant inhibition was observed only in animals receiving a continuous supplementation of CLA (Ip et al., 1997; Ip et al., 1995). This suggests that some other unknown mechanisms are involved in suppressing the process of neoplastic promotion/progression by CLA.
There were also studies examining the inhibitory effect of CLA on mammary tumor cell lines (Cunningham et al., 1997; Durgam and Fernandes, 1997; Shultz et al., 1992a; Shultz et al., 1992b). Shultz et al. (1992) reported that CLA significantly inhibited the proliferation of human mammary breast cancer cell line (MCF-7), and the inhibitory effect of CLA was dose- and time-dependent (Shultz et al., 1992a). The possible mechanisms underlying this effect might be through its inhibition of lipoxygenase activity (Cunningham et al., 1997) or through its interference with the hormone regulated mitogenic pathway (Durgam and Fernandes, 1997).

2.2.1.2 Skin Cancer

The anti-carcinogenic activity of CLA on skin cancer was first described in the two-stage mouse epidermal carcinogenesis model by Ha et al. (Ha et al., 1987). They showed that CLA, either extracted from fried ground beef or chemically synthesized, consistently reduced the number and incidence of papillomas in mice induced by DMBA. Their finding indicated that the inhibition of CLA on the initiation of skin epidermal tumors was through its inhibitory effect on the activation of DMBA. A later study using a similar mouse model indicated that CLA also inhibited tumor promotion (Belury et al., 1996). These findings suggest that CLA inhibited both the initiation and promotion stages of skin carcinogenesis similar to that observed in mammary carcinogenesis.
2.2.1.3 Forestomach Cancer

There are only a few reports examining the chemopreventive activity of CLA in stomach carcinogenesis. Ha et al. (1990) have shown that CLA inhibited the development of mouse forestomach neoplasia induced by benzo(a)pyrene by significantly reducing the tumor incidence. Ha et al. suggested that c9, t11-CLA might be the most active component in CLA isomers, because this isomer was preferentially incorporated into forestomach membrane phospholipids.

2.2.1.4 Colon Cancer

The inhibitory effect of dietary CLA on colon cancer has also been examined in the aminooimidazoazaarene (such as 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), etc.) induced-rat and mouse models (Liew et al., 1995; Schut et al., 1997; Zu and Schut, 1992). CLA significantly lowered the formation in rat colon of IQ-DNA or PhIP-DNA adducts, which normally lead to a mutation, and subsequently to an initiation of the carcinogenic process (Schut et al., 1997; Zu and Schut, 1992). CLA supplementation could also reduce the number of aberrant crypt foci (ACF), an early preneoplastic biomarker of malignant potential in the process of colon carcinogenesis (Liew et al., 1995). Incubation of human colorectal cell lines (HT-29) with physiological concentrations of CLA also reduced cell proliferation (Shultz et al., 1992b). Results from these studies all showed that CLA modulated the development of colon cancer.
2.2.1.5 Prostate Cancer

Recently, the effect of CLA on the development of prostate cancer in SCID mice induced by human prostatic carcinoma cells (DU-145) has also been examined (Cesano et al., 1998). The results from this study showed that CLA reduced the size of local tumors as compared to the untreated controls, and suggested that dietary CLA might affect the progress of prostate cancer.

2.2.2 Anti-atherosclerotic Effect

In addition to an anti-carcinogenic effect, CLA also exhibited hypocholesterolemic and anti-atherogenic properties in rabbits and hamsters (Lee et al., 1994; Nicolosi et al., 1997). CLA supplementation was found to decrease the levels of total cholesterol, low and very low density lipoprotein (LDL and VLDL) cholesterol and triacylglycerol in experimental animals fed an atherogenic diet. It also lowered the ratio of LDL cholesterol to HDL cholesterol and the ratio of total cholesterol to HDL cholesterol in plasma of CLA-treated rabbits as compared to the control group (Lee et al., 1994). Moreover, CLA reduced the percentage of total aortic surface covered by fatty lesions in CLA-treated animals, although the type of lesions made by accumulation of lipids and matrix fibers were similar in both control and CLA treatment groups. This indicates that CLA inhibited the development of atherosclerosis.

Recently, Munday et al. (1999) studied the effect of CLA on the development of atherosclerosis by using a C57BL/6 mouse atherosclerosis model (Munday et al., 1999).
1999). They found that dietary CLA increased the ratio of HDL cholesterol to total cholesterol and lowered the level of plasma triacylglycerol, and concluded that CLA inhibited the development of atherosclerosis. However, they later observed that CLA significantly increased aortic fatty streak formation in C57BL/6 mice, and suggested that CLA may prevent atherogenic progress in other animals, but not in mice.

2.2.3 Effect on Immune Function

The effect of CLA on immune function has been investigated in both animal and cell culture studies. Generally, clinical symptom of food allergy are induced by the production of inflammatory mediators such as histamine, leukotriene B₄ (LTB₄) and prostaglandin E₂ (PGE₂) triggered by allergen-specific immunoglobulin (Ig)E (Metcalf, 1991). In rats, supplementation with CLA resulted in a decrease in splenic levels of IgE, and an increase in anti-allergic factors, such as IgA and IgG. CLA also significantly reduced the level of LTB₄ in spleen, and levels of PGE₂ in spleen, serum and bone organ culture (Li and Watkins, 1998; Sugano et al., 1998; Sugano et al., 1997).

CLA is also effective in preventing the growth depression induced by immune stimulation (lipopolysaccharide injection, LPS) in chicks, rats and mice (Cook et al., 1993). The effect on the mitogen-induced weight loss was suggested to be through modulation of immune responses. Further studies showed that CLA significantly reduced the mitogen-induced levels of interleukin-2 (IL-2) in pig
macrophage (Chew et al., 1997), interleukin-6 (IL-6) in rat macrophages (Turek et al., 1998), and lowered the chemical-induced PGE$_2$ production in rat macrophage, mouse epidermis and murine keratinocytes (Kavanaugh et al., 1999; Liu and Belury, 1998; Turek et al., 1998).

2.2.4 Effect on Body Composition and Fat Distribution

Besides the preventive role of CLA in certain diseases, the effect of dietary CLA on body composition has also been investigated. Results from studies with mice, rats and pigs show that CLA treatment significantly decreased whole body fat and increased whole lean body mass (whole body water, body protein, and body ashes) (DeLany et al., 1999; Ostrowska et al., 1999; Park et al., 1997; Park et al., 1999a; Park et al., 1999c; Stangl et al., 1999; West et al., 1998). The mechanisms for this effect are not clear. It has been suggested that a reduction of fat accumulation might be the result of inhibition of adipocyte proliferation (Evans et al., 2000; Satory and Smith, 1999).

More recent studies showed that the reduced adipose tissue mass by dietary CLA was due to a smaller size rather than a reduced number of adipocytes (Azain et al., 2000; Evans et al., 2000). Nevertheless, CLA reduces lipoprotein lipase activity in 3T3-L1 adipocyte cell lines, subsequently reduces the levels of intracellular triacylglycerol and glycerol, and enhances the release of glycerol into the medium (Evans et al., 2000; Park et al., 1999c). There is also evidence that dietary CLA not only significantly reduced the fat deposition, but also increased the metabolic rate in
mice (DeLany et al., 1999; West et al., 1998). CLA is also known to enhance fatty acid oxidation through an increase in CLA-induced carnitine palmitoyltransferase activity in fat pads and skeletal muscle (Park et al., 1997), causing a significant decrease in fat mass.

In humans, CLA unlike the results found in experimental animals, has no significant effect on changes in lean mass, fat mass and percentage body fat, or body weight. CLA has also no significant effects on fatty acid or glycerol metabolism, body composition, energy expenditure, fat oxidation, or respiratory exchange ratio (Zambell et al., 2001; Zambell et al., 2000).

2.3 Possible Mechanisms for Overall Beneficial Effects of CLA

The biological effects of CLA on prevention of diseases and modulation of immune function have been well established. However, the mechanism(s) involved in these functions is still not fully understood. To date, several mechanisms have been proposed to explain the many beneficial functions of CLA. These mechanisms include antioxidant activity (Ha et al., 1990; Ip et al., 1991), prooxidant cytotoxicity (Shultz et al., 1992a), modulation on n-6 fatty acid metabolism and eicosanoid synthesis (Liu and Belury, 1998; Sugano et al., 1998; Sugano et al., 1997), inhibition of DNA adduct formation (Josyula et al., 1998; Josyula and Schut, 1998; Zu and Schut, 1992), and modulation of gene expression and/or signal transduction (Belury and Kempa-Steczko, 1997; Lee et al., 1998).
2.3.1 Antioxidant Activity

Ample evidence has indicated that free radicals and radical-mediated oxidation processes play important roles in the development of certain cancers and atherosclerosis. As mentioned previously, CLA with two conjugated double bonds exhibited anti-carcinogenic and anti-atherogenic properties in various animals. Since these 2 conjugated double bonds can function as active scavenger of free radicals, it is possible that biological effects of CLA may be due to its antioxidant properties.

CLA was shown to be an excellent antioxidant tested in an *in vitro* study. Ha *et al.* (1990) found that CLA was as effective as butylated hydroxytoluene (BHT) in suppressing peroxide formation from unsaturated fatty acids, and was much more effective than α-tocopherol (Ha *et al.*, 1990). The study of Ip (1991) showed that lipid peroxidation was decreased in mammary glands when mice were fed CLA (Ip *et al.*, 1991). This evidence suggested that CLA might serve as an *in situ* defense system in the membrane against the attack of free radicals.

However, this point of view was not supported by Chen *et al.* (1997) and van den Berg *et al.* (1995). Results from their studies suggest that CLA is neither an antioxidant in fats and oils (Chen *et al.*, 1997) nor an efficient radical scavenger comparable to vitamin E or BHT (van den Berg *et al.*, 1995). In fact, the oxidative susceptibility of CLA was as high as that of arachidonic acid, and even higher than that of linoleic acid (van den Berg *et al.*, 1995). These observations suggest that CLA is more prone to oxidation, and is unlikely to exhibit its biological effects through antioxidant properties (Banni *et al.*, 1998).
2.3.2 Prooxidant cytotoxicity

Previously, studies have shown that two naturally occurring 18-carbon conjugated fatty acids such as cis-parinaric acid (c9,t11,t13,c15-18:4) containing four conjugated double bonds, and conjugated trienoic acid (mainly α-eleostearic acid; c9,t11,t13-18:3) containing three conjugated double bonds, are toxic to various human tumor cell lines, but not to normal cells (Cornelius et al., 1991; Igarashi and Miyazawa, 2000). Similarly, CLA containing two conjugated double bonds has also been reported to reduce the proliferation of human tumor cell lines, i.e., malignant M21-HPB melanoma, HT-29 colorectal, and MCF-7 breast cells (Shultz et al., 1992a; Shultz et al., 1992b). However, unlike parinaric acid, CLA does not exert the selective cytotoxicity on normal and tumor cell lines.

The cytotoxicity of parinaric acid seems to involve lipid peroxidation, because this toxic action can be blocked by the addition of BHT (Cornelius et al., 1991). Results from recent studies also showed that CLA increased lipid peroxidation in different tumor cell lines (O'Shea et al., 1999; Schonberg and Krokan, 1995). Although the antioxidant enzyme systems (superoxide dismutase, catalase, and glutathione peroxidase) in cell lines were induced, they did not protect these tumor cells from the cytotoxic peroxidation of CLA (O'Shea et al., 1999). In hepatocytes from rats fed CLA, the level of cellular lipid peroxidation was not changed (Banni et al., 1995; Cantwell et al., 1999), but the cellular antioxidant defense system was significantly decreased (Cantwell et al., 1999; Cantwell et al.,
The latter finding suggests that dietary CLA actually compromises the endogenous antioxidant defense system in normal cells.

2.3.3 Modulation of n-6 Fatty Acid Metabolism and Eicosanoid Synthesis

CLA has also been proposed to be involved in the modulation of n-6 PUFA metabolism and eicosanoid biosynthesis. The beneficial effect of CLA is exerted through its competition with other fatty acids for incorporation into membrane phospholipids (Ha et al., 1990; Ip et al., 1991).

Since CLA is a conjugated isomer of LA, the similarity in chemical structure suggested that CLA and/or its metabolites may compete with n-6 PUFA for the endogenous enzyme system (Figure 2.2). Several conjugated fatty acids, such as conjugated γ-linolenic acid (CD18:3), conjugated dihomo-γ-linolenic acid (CD20:3), and conjugated arachidonic acid (CD20:4) were found in the phospholipids of rat and lamb liver (Banni et al., 1996; Banni et al., 1995). Later, by using combined chromatography techniques, Sebedio and his co-workers identified the 20-carbon conjugated fatty acids as c8,t12,c14-20:3; c8,c11,t13-20:3; c5,c8,t12,c14-20:4; and c5,c8,c11,t13-20:4, respectively (Juaneda and Sebedio, 1999; Sebedio et al., 2001; Sebedio et al., 1997; Sebedio et al., 1999). They have suggested that these conjugated diene fatty acids were formed by desaturation and elongation of the c9,t11- and t10,c12c-18:2 (CLA). All of these findings demonstrated that CLA could be metabolized, and that itself and its metabolites could compete with n-6 PUFA for the enzyme system, and modulate the production of n-6 PUFA.
Figure 2.2: Metabolic pathways for conversion of n-6 polyunsaturated fatty acids to 2- and 4-series of eicosanoids, and possible metabolic pathway of CLA.
CLA has been shown to decrease the Δ6-desaturation of LA significantly (Bretillon et al., 1999), and reduce the production of LA metabolites (including 18:3-GLA, 20:3-DGLA and 20:4-AA) in animal and cell culture studies (Banni et al., 1999; Liu and Belury, 1998). A decrease in production of AA by CLA (Banni et al., 1999; Liu and Belury, 1998), would result in a decrease in synthesis of 2- and 4-series eicosanoids (including PGE\(_2\), LTB\(_4\) and LTC\(_4\)) (Liu and Belury, 1998; Miller et al., 2001; Sugano et al., 1998; Sugano et al., 1997). Since these 2- and 4-eicosanoids, which are oxygenated metabolites of PUFA via cyclooxygenase and lipoxygenase pathways, are potent pro-inflammatory mediators (Williams and Peck, 1977), it has been hypothesized that manipulating PUFA and eicosanoid metabolism could modulate the process of inflammation and the development of cancers.

### 2.3.4 Inhibition of DNA Adduct Formation

IQ and PhIP, like other carcinogenic heterocyclic amines, can react with DNA to form carcinogen-DNA adducts after being activated by liver microsomes through N-hydroxylation and subsequent esterification, such as O-acetylation (Aoyama et al., 1990; Battula et al., 1991; Kato, 1986; Synderwine et al., 1988). These DNA-adducts have been related to the increased susceptibility of DNA to chemical-induced mutation, which leads to initiation of carcinogenesis (Schut and Castonguay, 1984). Thus, inhibition of carcinogen activation might be the key factor to prevent the initiation of carcinogenesis (Aoyama et al., 1990; Battula et al., 1991; Synderwine et al., 1988).
Indeed, CLA has been shown to lower the formation of IQ- and PhIP-DNA adducts significantly in rodents (Josyula et al., 1998; Josyula and Schut, 1998; Zu and Schut, 1992). Schut et al. (1997) have proposed that CLA might modulate the removal of PhIP- and IQ-DNA adducts but provided no evidence to support their hypothesis (Schut et al., 1997). Recently, Josyula et al. (1998a and 1998b) have proposed that the protective effect of CLA may be exerted through an unknown mechanism other than the inhibition of either the N-hydroxylation or O-acetylation of PhIP or IQ (Josyula and Schut, 1998).

2.3.5 Modulation of Gene Expression and/or Signal Transduction

Modulation of gene expression is another possible mechanism through which CLA exerts its biological effect.

CLA, especially the t10,c12- isomer, has been shown to significantly decrease hepatic stearoyl-CoA desaturase activity in mouse liver and mouse liver cell lines (H2.35) by repressing mRNA expression of stearoyl-CoA desaturase gene (SCD1) (Lee et al., 1998). Stearoyl-CoA desaturase catalyzes the rate-limiting step in the synthesis of monounsaturated fatty acids, mainly oleate (Δ9-18:1) and palmitoleate (Δ9-16:1), which are the major monounsaturated fatty acids of membrane phospholipids, cholesteryl esters, and triglycerides. A similar finding has also been reported in cultured adipocytes. Choi et al. (2000) reported that treating differentiating 3T3-L1 preadipocytes with t10,c12-CLA isomer resulted in a dose-dependent decrease in SCD1 mRNA expression, protein level and enzyme activity.
(Choi et al., 2000). These findings suggested that the suppressive role of CLA in SCD1 gene expression may contribute to the mechanisms through which CLA reduces body fat in mice.

In addition, CLA modulates signal transduction triggered by various transcription factors, such as peroxisome proliferator-activated receptors (PPAR), which may also account for the biological effects of CLA.

PPARα is predominantly expressed in liver, kidney, and heart, and is involved in the regulation of inducible (peroxisomal β-oxidation) and constitutive (mitochondrial β-oxidation) lipid metabolizing enzymes, such as acyl-CoA oxidase (ACO), liver fatty acid-binding protein (L-FABP), palmitoyl-CoA synthase, microsomal CYP4A, etc. (Aoyama et al., 1998; Belury and Kempa-Steczko, 1997; Moya-Camarena et al., 1999). CLA has been demonstrated to have high binding affinity for PPARα (Moya-Camarena et al., 1999), and may activate this transcription factor to enter the nucleus. Once inside the nucleus, activated PPARα may interact with specific DNA response elements (Schoonjans et al., 1996), PPAR-responsive elements (PPRE) which are located at upstream of the responsive gene, and thus modulate gene expression of the enzymes and proteins involved in lipid homeostasis (Vanden Heuvel, 1999).

Belury and her co-workers showed that CLA isomers, especially c9,t11-CLA, activated PPARα and also increased levels of ACO, L-FABP and CYP4A1 mRNA in rat hepatoma cell lines and in mice (Belury and Kempa-Steczko, 1997; Moya-
Camarena et al., 1999). Thus, CLA regulates lipid metabolism through binding and activation of PPARα.

2.4 Summary

Conjugated linoleic acid, a mixture of positional and geometric isomers derived from linoleic acid, has been shown to decrease the development of atherosclerosis, affect body weight composition and fat distribution, modulate immune function and inhibit certain types of cancer in cell culture and animal models. Although the mechanism(s) involved in these functions is still not fully understood, several mechanisms have been proposed to explain many beneficial functions of CLA to date. These mechanisms include antioxidant activity, prooxidant cytotoxicity, modulation on n-6 fatty acid metabolism and eicosanoid synthesis, inhibition of DNA adduct formation, and modulation of gene expression and/or signal transduction. However, each proposed mechanism could only partially explain biological effects. Recently, the concept of “multiple biochemical mechanism” suggested by Pariza et al. may provide a better way to illustrate how CLA exerts its biological effects (Pariza et al., 2000). More studies needed to elucidate the exact mechanisms involved in these biological effects.

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CHAPTER 3

MATERIALS AND METHODS

3.1 Sources of Materials

Tritridecanoin (a synthetic triacylglycerol containing 3 molecules of tridecanoic acid 13:0), triheptadecanoin (containing 3 molecules of heptadecanoic acid, 17:0), linoleic acid (LA; Δ6,9-18:2), γ-linolenic acid (GLA; Δ6,9,12-18:3), eicosadienoic acid (EDA), dihomo-γ-linolenic acid (DGLA; Δ8,11,14-20:3), arachidonic acid (AA; Δ5,8,11,14-20:4) and a mixture of conjugated linoleic acid (CLA) isomers (free fatty acid form, containing 41% of c9,t11 isomer, 44% of t10,c12 isomer, and others) were purchased from Nu-Chek-Prep, Inc. (Elysian, MN). Four individual isomers of CLA (c9,t11-; t9,t11-; c9,c11- and t10,c12-) and a mixture of conjugated eicosadienoic acid (CEDA) isomers (free fatty acid form, containing 53% of c11,t13 isomer, 29% of c11,c13 isomer, and others) were obtained from Matreya Inc. (Pleasant Gap, PA). The fluorescent dye, 2,7-dichlorofluorescein was from Sigma Chemical Co. (St. Louis, MO). Dropout base (DOB) medium, dropout base with galactose (DOB w/GAL), complete supplement
mixture minus leucine (CSM-LEU) and complete supplement mixture minus uracil (CSM-URA) were from Bio 101, Inc. (Vista, CA). The YPD medium which contained yeast extract, peptone and dextrose was from Difco Laboratories (Detroit, MI). Hexane, chloroform and methanol were from Burdick and Jackson (Muskegon, MI). Dimethyl sulfoxide (DMSO), anhydrous sodium sulfate, sulfuric acid, acetic acid, sodium chloride and formaldehyde were from Mallinckrot Baker, Inc (Paris, KY). Hexane was UV grade and other solvents were distilled-in-glass quality. Phenol, Nylon membrane and DIG High Prime Random Labeling kit were from Roche (Indianapolis, IN). Agarose gel was from FMC BioProduct (Rockland, ME). Thin layer chromatography (TLC) plates were from Whatman (Clifton, NJ). The Omegawax™ 320 Fused Silica Capillary Column (30 m x 0.32 mm i.d. x 0.25 μm film thickness) was from Supelco (Bellefonte, PA).

3.2 Transformed Yeast

3.2.1 Plasmids and Yeast Strains

All transformed yeast strains were gifts from Ross Product Division, Abbott Laboratories (Columbus, OH). Three plasmids pYES2 (vector only), pCGR5 (Δ6-desaturase gene from Mortierella alpina) and pCGR4 (Δ5-desaturase gene from M. alpina) were constructed and transformed into a host strain of baker's yeast (Saccharomyces cerevisiae), SC334, respectively (Huang et al., 1999; Knutzon et al., 1998). Plasmids pYX242 (vector only), pRAE58 (human elongase cDNA) and
pRAE89 (human Δ6-desaturase cDNA) were also constructed and transformed into SC334 (Cho et al., 1999; Leonard et al., 2000a). The transformation protocol and growth conditions followed the procedures described previously (Knutzon et al., 1998; Leonard et al., 2000a).

3.2.2 Incubation conditions

Colonies of transformed yeasts were allowed to grow overnight in YPD medium at 30°C [Appendix A]. Cultures (1×10⁸ cells) of the transformed yeast were then inoculated into 50 ml of Yeast minimal medium (YMM) [Appendix A]. Cell numbers were maintained at the same level in all studies. Culture conditions for transformed yeast strains have previously been shown to be optimal for expression of all recombinant enzymes (Huang et al., 1999; Knutzon et al., 1998; Leonard et al., 2000a). Cells were harvested by centrifugation, and cell pellets washed once with sterile distilled-deionized water.

3.3 General Methods

3.3.1 Analysis of Yeast Lipids

Fatty acid profiles in total lipids of the yeasts and individual fractions (cholesterol ester, triacylglycerol, free fatty acid, diacylglycerol and phospholipid) of the total lipids were determined. The total CLA level and CLA isomers in yeast lipids were determined using the same method. Because no purified PUFA metabolic enzymes were available for testing, and the “index” of enzyme activity

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was used in this study to reflect the enzyme activity. The index of enzyme activity is defined by the conversion (i.e. Δ6-desaturation, elongation or Δ5-desaturation) of substrates to products, based on the ratio of [product] / [products + substrate] ×100%. The levels of substrate and its metabolic products taken up by the yeast were calculated, based on the absolute amounts of fatty acids in total yeast lipids.

3.3.1.1 Total Lipid Extraction

The method for yeast lipid extraction followed the procedure described previously (Chuang et al., 2001a; Folch et al., 1957). Briefly, the rinsed cell pellet was extracted with 20 ml of chloroform/methanol (2:1, v/v) at 4°C for overnight. The extracted lipids in the chloroform phase were separated from the aqueous phase by adding 4 ml of saline solution. The lipid extract in chloroform was collected and dried with anhydrous sodium sulfate [Appendix B].

3.3.1.2 TLC Separation of Lipid Fractions

The total lipid extract was further fractionated into cholesteryl ester, triacylglycerol, free fatty acid, diacylglycerol and phospholipid by TLC.

The fractionation was performed using a 20 × 10 cm Whatman HTLC (layer thickness, 200 μM, 60A) LHPK silica gel plates. Aliquots of total yeast lipids in chloroform were applied onto the plates, and they were developed with a solvent mixture of hexane:diethyl ether:acetic acid (70:30:1; v/v/v). The bands
corresponding to different lipid fractions were visualized by spraying the plate with
2.7-dichlorofluorescein/ethanol solution. The bands of interest were marked with a
pencil under UV light (Mineralight Lamp, Model UVGL-58, UVP, Inc., San Gabriel,
CA) and scraped off individually from the plates. Lipids in bands were methylated
as description on the following section.

3.3.1.3 Fatty Acid Methyl Ester Preparation

The extracted yeast lipids were treated with 1% of sulfuric acid in methanol
and 0.5 ml DMSO for 20 min at 95°C to prepare fatty acid methyl esters (Yamasaki
et al., 1999). The fatty acid methyl esters were then extracted by hexane and used
for Gas Chromatography (GC) analysis [Appendix B].

3.3.1.4 Gas Chromatography Analysis of Fatty Acid Profile

The methyl esters of CLA and other fatty acids were quantified using a gas
chromatograph (GC) method (Chuang et al., 2001b). A Hewlett-Packard 5890 gas
chromatograph equipped with an on-column automatic injector, a flame ionization
detector, and a Hewlett-Packard Chemstation (Avondale, PA) were used. The
operation conditions were set as following: methyl esters of fatty acids were
separated by using a Omegawax™ 320 Fused Silica Capillary Column (30 m × 0.32
mm i.d. × 0.25 μm film thickness). Helium was used as the carrier gas.
Temperatures of injector was set at 250°C, and detector at 280°C. The temperature
of oven was initially at 120°C, raised to 205°C at 4°C/min and held for 25 min, then
raised to 245°C at 50°C/min and held for 10 min. The peaks of CLA and other fatty acids were identified by comparing their retention times to those of known standard materials. When necessary, GC-Mass Spectrum (GC-MS) was also used to help identify or confirm the identity of unknown peaks. In this case, a Hewlett Packard mass selective detector (model 5972) was used by operating at an ionization voltage of 70 eV with a scan range of 20-500 Da. The mass spectrum of an unknown peak was compared with that of a standard material in the database NBS75K.L (National Bureau of Standards). The quantification was determined by using the technique of internal standardization.

3.3.2 Isolation and Analysis of RNA

To examine the mRNA level, total RNAs were isolated by hot phenol-chloroform extraction as described previously (Vijayraghavan et al., 1989). Equal amounts of total RNA were separated on a 1% formaldehyde-agarose gel electrophoresis, transferred to a nylon membrane, and auto cross-linked to the membrane with an UV Stratalinker 1800 (Strategene, La Jolla, CA). The DNA probes used for hybridization overnight at 50°C were synthesized using the DIG High Prime Random Labeling kit (Roche, Indianapolis, IN). The *M. alpina* \( \Delta6 \)-desaturase gene from pCGR5 was used for the desaturase mRNA detection (Huang et al., 1999), and the 2.2 kb *HindIII* fragment of cryI (Chang et al., 1997) was used to show that equal amounts of RNA had been loaded. Following hybridization, detection of the signal was performed with reagents from Roche following the
manufacturer's instructions. The hybridization signals were quantitated by a densitometry (IS-1000 system, Alpha Innoteck, San Leandro, CA).

### 3.3.3 Statistical Analysis

Data were analyzed by analysis of variance (ANOVA) and least significant difference (LSD) to determine differences between means of the cellular content and between means of the conversion rates. Means differences were considered significant at the $P \leq 0.05$ level.
4.1 Objective

It has been reported by Banni and Sebedio that CLA can be metabolized by Δ6-desaturase to form conjugated ω-linolenic acid (conjugated-18:3) (Banni et al., 1995; Sebedio et al., 1997). They suggested that by competing with LA for Δ6-desaturase, CLA inhibits the conversion of LA to GLA. However, to this date there is no direct evidence to support suggestion. In order to verify this theory, the objectives of this study were 1) to determine if CLA could indeed be metabolized by the enzyme, Δ6-desaturase, and 2) to examine whether CLA affects the conversion of LA to GLA by Δ6-desaturase.
4.2 Experimental Design and Results

4.2.1 Enzyme Activities of Recombinant Δ6-desaturases

Recently, the genes for Δ6-desaturase from the fungus of Mortierella alpina and from human have been identified and successfully expressed in yeasts (Cho et al., 1999; Huang et al., 1999). The yeasts transformed with either fungal or human Δ6-desaturase gene were used as a model for the present study. To test the validity of using the transformed yeasts with Δ6-desaturase as a suitable model for this study, the enzyme activity of Δ6-desaturase (the first step of n-6 PUFA metabolism) needed to be determined. Yeasts transformed with fungal or human Δ6-desaturase gene were incubated at 25°C for 48 h in the media containing 25 μM of LA as the exogenous substrate. The activity of recombinant Δ6-desaturase was expressed by the conversion of LA to GLA.

Figure 4.1B showed a significant level of GLA (Δ6,9,12-18:3) was converted from LA (Δ9,12-18:2) in the yeast transformed with fungal Δ6-desaturase [334(pCGR5)] (Figure 4.1B). A low level of Δ6,9-16:2 (converted from Δ9-16:1) was also observed. None of these fatty acids was found in the yeast transformed with vector only [334(pYES2)] (Control) (Figure 4.1A). Similar findings were also observed in yeast transformed with human Δ6-desaturase [334(pRAE89)] incubated with 25 μM LA for 48 h (Figure 4.2). Results from both experiments indicated that recombinant Δ6-desaturases were expressed successfully in the transformed yeasts.
Figure 4.1: Gas chromatographic analysis of fatty acid methyl esters of total lipids from baker's yeast, *Saccharomyces cerevisiae*, transformed with (A) only the vector [334(pYES2)] or (B) the fungal Δ6-desaturase [334(pCGR5)].

All yeast cells were incubated in the medium containing 25 μM linoleic acid (LA). Solid arrows indicate the appearance of Δ6,9-16:2, the Δ6-desaturation product of Δ9-16:1, and γ-linolenic acid (GLA), the Δ6-desaturation product of LA.
Figure 4.2: Gas chromatographic analysis of fatty acid methyl esters of total lipids from transformed yeast with (A) only the vector [334(pYX242)] or (B) the human Δ6-desaturase [334(pRAE89)] incubated with LA. All yeast cells were incubated in the medium containing 25 μM LA. Solid arrows indicate the appearance of γ-linolenic acid (GLA), the Δ6-desaturation product of LA.
4.2.2 Characterization of Transformed Yeast with \( \Delta 6 \)-desaturase Gene

To characterize the enzyme activities in the transformed yeast, the effect of environmental factors, such as incubation time, substrate dose response, etc. have been studied.

4.2.2.1 Incubation Time

The effect of incubation time on the conversion of LA to GLA was examined in the strains of yeasts transformed with fungal or human \( \Delta 6 \)-desaturase genes. They were incubated with the specific media containing 25 \( \mu \)M of LA at 25°C for a period of 24 or 48 h. The absolute amount of LA incorporated by the yeasts and that of GLA formed in the yeasts were determined.

Figure 4.3A show that the level of GLA in the yeast transformed with fungal \( \Delta 6 \)-desaturase [334(pCGR5)] increased as incubation time increased. The level of LA was rapidly increased during the first 24 h, but decreased afterward. The decrease in the level of LA was due to its increased conversion to GLA (from 18% to 29%) (Figure 4.3B).

Similar results were also observed in the yeast transformed with human \( \Delta 6 \)-desaturase [334(pRAE89)] (Figure 4.4). However, the level of LA taken up and the rate of conversion of LA to GLA (from 6% to 11%) by the yeast transformed with human \( \Delta 6 \)-desaturase were significantly lower than those in the yeast transformed with fungal \( \Delta 6 \)-desaturase.
Figure 4.3: The effect of incubation time on (A) cellular uptake of LA or GLA, and (B) Δ6-desaturation of LA to GLA by yeast transformed with fungal Δ6-desaturase gene.

The yeast cells were cultured in 25 μM LA for 24 or 48 h. The cellular uptake of LA or GLA was calculated based on the absolute weights of LA and GLA in yeast lipids. Δ6-desaturation of LA to GLA was calculated as [product] / ([product] + [substrate]) × 100%. All results are mean ± SE of three incubations.
Figure 4.4: The effect of incubation time on (A) cellular uptake of LA or GLA, and (B) Δ6-desaturation of LA to GLA by yeast transformed with human Δ6-desaturase gene.

The yeast cells were cultured in 25 μM LA for 24 or 48 h. The cellular uptake of LA or GLA, and Δ6-desaturation of LA to GLA were defined as the same as the legend of Figure 4.3. All results are mean ± SE of three incubations.
4.2.2.2 Substrate Concentration

The effect of the levels of LA on the activity of both recombinant Δ6-desaturases was also studied. The transformed yeasts were incubated at 25°C for 48 h in media containing four different levels of LA (10, 25, 50 or 100 μM).

Figure 4.5A showed that LA was rapidly incorporated into yeast cells, and a portion of LA was converted to GLA. The levels of LA in the yeasts transformed with fungal Δ6-desaturase were increased when exogenous LA into medium increased. However, the levels of GLA in the transformed yeast were not increased. When the conversion rate of LA to GLA was calculated, there was a progressive decrease in the conversion of LA to GLA (from 35% to 15%) in the yeast transformed with fungal Δ6-desaturase (Figure 4.5B).

In contrast, both LA and GLA levels in the yeast transformed with human Δ6-desaturase were progressively increased as the concentration of LA in the medium increased (Figure 4.6B). The rate of conversion of LA to GLA in yeast transformed with human Δ6-desaturase stayed constant (Figure 4.6B).

4.2.3 Metabolism and Distribution of CLA in Transformed Yeast

4.2.3.1 Metabolism of CLA in Transformed Yeast

It was suggested by others that the beneficial effect of CLA was exerted through its competition with LA for the same metabolic enzyme, Δ6-desaturase, and
Figure 4.5: The effect of different levels of LA on (A) cellular uptake of LA or GLA, and (B) Δ6-desaturation of LA to GLA by yeast transformed with fungal Δ6-desaturase gene.

The yeast cells were cultured in 10, 25, 50 or 100 μM of LA for 48 h. All results are mean ± SE of three incubations. The cellular uptake of LA or GLA, and Δ6-desaturation of LA to GLA were defined as the same as the legend of Figure 4.3.
Figure 4.6: The effect of different levels of LA on (A) cellular uptake of LA or GLA, and (B) \( \Delta 6 \)-desaturation of LA to GLA by yeast transformed with human \( \Delta 6 \)-desaturase gene.

The yeast cells were cultured in 10, 25, 50 or 100 \( \mu \text{M} \) of LA. All results are mean \( \pm \) SE of three incubations. The cellular uptake of LA or GLA, and \( \Delta 6 \)-desaturation of LA to GLA were defined as the same as the legend of Figure 4.3.
CLA could be converted to form conjugated GLA (18:3) (Banni et al., 1995; Sebedio et al., 1997). To confirm this hypothesis, both control yeast and transformed yeast were incubated in media containing 25 μM of CLA at 25°C for 48 h.

Results from Figure 4.7 and 4.8 demonstrated that there was no Δ6-desaturation metabolites of CLA formed by both transformed yeasts, 334(pCGR5) or 334(pRAE89). Thus, under the current condition described in this study CLA could not be metabolized by the fungal and human Δ6-desaturases.

4.2.3.2 CLA Distribution in Transformed Yeast

To determine if the inability to convert CLA to conjugated GLA was due to the failure of yeast cells to take up CLA, the level of CLA intake and the distribution of CLA in transformed yeast were further examined. Yeasts transformed with either fungal or human Δ6-desaturase were incubated with various levels of CLA (10, 25, 50 or 100 μM) for 48 h.

Results in Figure 4.9 show that CLA was taken up and incorporated mostly into the phospholipid and free fatty acid fractions of the transformed yeast. The amount of CLA in these two fractions particularly, the free fatty acid fraction increased when the levels of CLA supplemented into medium increased.
Figure 4.7: Gas chromatographic analysis of fatty acid methyl esters of total lipids from transformed yeast with (A) only the vector [334(pYES2)] or (B) the fungal Δ6-desaturase [334(pCGR5)] incubated with conjugated linoleic acid (CLA).

All yeast cells were incubated in the medium containing 25 μM CLA. Solid arrows indicate the appearance of Δ6,9-16:2 and the Δ6-desaturation product of CLA.
Figure 4.8: Gas chromatographic analysis of fatty acid methyl esters of total lipids from transformed yeast with (A) only the vector [334(pYX242)] or (B) the human Δ6-desaturase [334(pRAE89)] incubated with CLA. All yeast cells were incubated in the medium containing 25 µM CLA. A solid arrow indicates the appearance of the Δ6-desaturation product of CLA.
Figure 4.9: CLA distribution in transformed yeast cells.

Yeast transformed with fungal Δ6-desaturase gene was cultured in 10, 25, 50 or 100 μM of CLA. The distribution of CLA in different fractions are calculated based on the cellular uptake of CLA.
4.2.4 Inhibitory Effect of CLA on Fungal Δ6-desaturase Activity

The following study was designed to examine if CLA affects the conversion of LA to GLA by fungal Δ6-desaturase. The transformed yeast was incubated with both LA and CLA (in combination or individual isomers) at the concentration of 25 μM.

Figure 4.10 shows that addition of CLA mixture into the growth medium significantly inhibited the conversion of LA to GLA by 33%. When individual CLA isomers were added to the medium, the extent of inhibition varied. The c9, t11-CLA exerted the greatest inhibition (40%). The other three CLA isomers, t10, c12-, c9, c11-, and t9, r11-CLA also significantly inhibited the conversion of LA to GLA, but to a lesser extent. The extent of inhibition was not significantly different among them. A separate study examined the inhibitory effect of four individual CLA isomers supplemented in media at different levels (10, 25 and 50 μM) on the enzyme activity of fungal Δ6-desaturase in the transformed yeasts. Results in Figure 4.11 show the inhibitory effects were dose-dependent (Figure 4.11).

The rate of uptake of individual CLA isomers by the transformed yeasts is shown in Figure 4.12. Generally, the level of uptake was in a direct proportion to the concentration of CLA isomers added in the medium. However, the rate of uptake varied significantly among the four CLA isomers. The levels of t9, t11- and c9, c11-CLA isomers taken up by the yeasts were greater than those of c9, t11- and t10, c12-.
Figure 4.10: Effect of CLA isomers on the activity of fungal Δ6-desaturase.
Transformed yeast with fungal Δ6-desaturase were incubated with both LA and CLA (in combination or individual isomers) at the concentration of 25 μM. Transformed yeast incubated with only 25 μM of LA was designed as the control (100%). All results are mean ± SE of three incubations. Values with different letters are significantly different from each other at $P < 0.05$. The Δ6-desaturase activity was defined as the same as Δ6-desaturation of LA to GLA (Figure 4.3)
Figure 4.11: Effect of different levels of CLA isomers on the activity of fungal Δ6-desaturase.

Transformed yeast with fungal Δ6-desaturase was incubated with 25 μM of LA and different concentrations (10, 25 and 50 μM) of CLA individual isomers. Transformed yeast incubated with 25 μM of LA was designed as the control (100%). All results are means of three incubations. Values with different letters are significantly different from each other at \( P < 0.05 \). The Δ6-desaturase activity was defined as the same as the legend of Figure 4.10. c9,t11-CLA (♦), c10,c12-CLA (■), c9,c11-CLA (▲) and r9,t11-CLA (●).
Figure 4.12: Uptake of different CLA isomers by yeast transformed with fungal Δ6-desaturase.

The uptake of CLA isomers is indicated by the presence of CLA isomers (% total lipids) in yeast. All results are means of three incubations. Values with different letters are significantly different from each other at $P < 0.05$. $c_9,t_{11}$-CLA (●), $t_{10},c_{12}$-CLA (■), $c_9,c_{11}$-CLA (▲) and $t_9,t_{11}$-CLA (●).
4.2.5 **Inhibitory Effect of CLA on Human Δ6-desaturase Activity**

A similar study examining the inhibitory effect of CLA on the activity of human Δ6-desaturase was also carried out. The yeasts transformed with human Δ6-desaturase gene were incubated with media supplemented with both LA and CLA (in a mixture or individual isomers) at the concentration of 25 μM for 48 h.

Results in Figure 4.13 show that addition of the CLA mixture inhibited conversion of LA to GLA (38%) in the transformed yeast. Among four individual isomers, only τ10, ω12-CLA substantially inhibited the conversion of LA to GLA (76%), followed by c9, τ11- (28%) and τ9, τ11- (10%). The c9, ω11-CLA isomer exhibited no inhibitory effect. The rate of different CLA isomers taken up by the yeast transformed with human Δ6-desaturase is shown in Figure 4.14. The rate of uptake varied significantly among the four CLA isomers: τ9, ω11-CLA were greatest (48%), followed by c9, ω11- (19%), τ10, ω12- (6%) and c9, τ11- (5%).

The inhibitory effect of different levels (5, 10, 25, 50, and 100 μM) of τ10, ω12-CLA isomer on the activity of human Δ6-desaturase was also examined. Figure 4.15 shows that the inhibitory effect increased from 44% to 89%, when the concentrations of the isomer in the medium increased. The levels of τ10, ω12-CLA isomer taken up by the transformed yeasts were in proportion to the concentration of CLA isomers added in the medium (Figure 4.16).
Figure 4.13: Effect of CLA isomers on the activity of human Δ6-desaturase.
Transformed yeast with human Δ6-desaturase were incubated with both LA and CLA (in combination or individual isomers) at the concentration of 25 μM. Transformed yeast incubated with only 25 μM of LA was designed as the control (100%). All results are mean ± SE of three incubations. Values with different letters are significantly different from each other at \( P < 0.05 \). The Δ6-desaturase activity was defined as the same as the legend of Figure 4.10.
Figure 4.14: Uptake of different CLA isomers by yeast transformed with human Δ6-desaturase.
The uptake of CLA isomers is indicated by the presence of CLA isomers (% total lipids) in yeast. All results are mean ± SE of three incubations. Values with different letters are significantly different from each other at $P < 0.05$. 

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Figure 4.15: Effect of different levels of r10,c12-CLA isomer on the activity of human Δ6-desaturase.
Transformed yeast with human Δ6-desaturase were incubated with 25 μM of LA and different levels (5, 10, 25, 50 and 100 μM) of r10,c12-CLA isomer. Transformed yeast incubated with only 25 μM of LA was designed as the control (100%) All results are mean ± SE of three incubations. Values with different letters are significantly different from each other at $P < 0.05$. The Δ6-desaturase activity was defined as the same as the legend of Figure 4.10.
Figure 4.16: Uptake of different levels of t10,c12-CLA isomer by yeast transformed with humanΔ6-desaturase.

The uptake of t10,c12-CLA isomer is indicated by the presence of CLA isomers (% total lipids) in yeast. All results are mean ± SE of three incubations. Values with different letters are significantly different from each other at $P < 0.05$. 

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4.2.6 Modulation of Δ6-desaturase Gene Expression by CLA

Results from previous experiments clearly demonstrated that CLA inhibits the activity of the recombinant Δ6-desaturases. To determine whether or not the inhibitory effect of CLA on Δ6-desaturase was exerted through its regulation on gene expression, Δ6-desaturase mRNA was isolated and quantified by using the technique of Northern hybridization. The levels of Δ6-desaturase mRNA in the yeasts transformed with fungal Δ6-desaturase gene incubated with 50 μM of LA (Control) and those incubated with both 50 μM of CLA and 50 μM of LA were compared. Results in Figure 4.17 shows that there were no significant difference in the relative abundance of Δ6-desaturase mRNA between the transformed yeasts treated with or without CLA.
Figure 4.17: Effect of CLA on fungal Δ6-desaturase mRNA expression in transformed yeast [334(pCGR5)]. Data are expressed as the ratio of Δ6-desaturase/CryI mRNA. Similar results were observed in three separate experiments.

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CHAPTER 5

INHIBITORY EFFECT OF CLA ON ENZYME ACTIVITY OF ELONGASE

5.1 Objective

The Δ6-desaturation of LA to form γ-linolenic acid (GLA) in mammalian cells, is considered to be the rate-limiting step of the formation of n-6 long chain polyunsaturated fatty acid. When this step is suppressed, an alternate metabolic pathway for LA to form n-6 PUFA could also take place. In this instance, LA could be elongated to form eicosadienoic acid (EDA; Δ11,14-20:2), followed by Δ8-desaturation to form dihomo-γ-linolenic acid (DGLA; Δ8,11,14-20:3). DGLA is then converted to arachidonic acid (AA; Δ5,8,11,14-20:4) by the action of Δ5-desaturase. Since results in the previous study showed that CLA could significantly inhibit Δ6-desaturation of LA, it would be of interest to find whether CLA affected the elongation of LA to EDA. Moreover, the same elongation enzyme has also been shown to elongate GLA to form DGLA. Thus, the objectives of this experiment were to examine whether CLA could affect the elongation of LA to EDA and GLA to DGLA, and modulate the production of DGLA.
5.2 Experimental Design and Results

5.2.1 Enzyme activity of Recombinant Elongase

Recently, several genes involved in the elongation of long-chain PUFA have been identified from human and fungal sources, and these genes have been successfully expressed in baker's yeast, *Saccharomyces cerevisiae* (Leonard et al., 2000a; Parker-Barnes et al., 2000). For the present experiment, the yeasts transformed with human elongase [334(pRAE58)] were used as the study model to examine the effect of CLA on elongation of PUFA.

Prior to the study, the validity of using the transformed yeast as a suitable study model needed to be established. The transformed yeasts were incubated at 25°C for 48 h in the mediums with 100 μM of exogenous GLA or LA, and the enzyme activity of elongase (elongation of GLA to its metabolites) examined.

When GLA was added as the substrate, a significant portion (51.0%) of GLA was elongated to form dihomo-gamma-linolenic acid (DGLA; Δ8,11,14-20:3) and a substantial portion (15.7%) of newly synthesized DGLA was subsequently elongated...
to form docosatrienoic acid (n-6 DTrA; Δ10,13,16-22:3) (Figure 5.1B). Small amounts of Δ11-18:1, the elongation product of palmitoleic acid (Δ9-16:1), and Δ13-20:1, the elongation product of Δ11-18:1 were also observed. None of these fatty acids was found in yeast transformed with vector only [334(pYX242)] (Figure 5.1A).

The elongation of LA to EDA in yeasts transformed with human elongase has also been studied. Results in Figure 5.2B show in yeasts transformed with human elongase that a substantial portion (13%) of LA was elongated to form EDA. Small levels of elongation products (Δ11-18:1 and Δ13-20:1) were also observed.

Results from both experiments demonstrated that recombinant human elongase has been successfully expressed in the yeast. This transformed yeast was subsequently used for studying the effect of CLA on elongation of LA or GLA.

5.2.2 Characterization of Recombinant Human Elongase

The following studies were designed to characterize the elongase activity in the transformed yeast, and the effect of incubation time, substrate dose, etc.

5.2.2.1 Incubation Time

In this study, to examine the effect of incubation time on elongation of GLA to its metabolites (DGLA and n-6 DTrA), the transformed yeast was incubated in the medium containing 25 μM of GLA at 25°C for a period of 24 or 48 h. The amounts of GLA taken up and incorporated in the cells and the levels of two
Figure 5.1: Gas chromatographic analysis of fatty acid methyl esters of total lipids from yeast transformed with (A) only the vector [334(pYX242)] or (B) the human elongase [334(pRAE58)].

All yeast cells were incubated in the medium containing 25 µM gamma-linolenic acid (GLA). Solid arrows indicate the appearance of Δ13-20:1, the elongation product of Δ11-18:1, and dihomo-γ-linolenic (DGLA) and docosatrienoic acid (DTrA), the elongation products from GLA.
Figure 5.2: Gas chromatographic analysis of fatty acid methyl esters of total lipids from transformed yeast with (A) only the vector [334(pYX242)] or (B) the human elongase [334(pRAE58)] incubated with LA. All yeast cells were incubated in the medium containing 25 μM LA. Solid arrows indicate the appearance of Δ13-1:20:1, the elongation product of Δ11-18:1, and eicosadienoic acid (EDA), the elongation product of LA.
metabolites (DGLA and n-6 DTrA) formed in yeast were determined.

Results in Figure 5.3A showed that the levels of DGLA and n-6 DTrA in transformed yeast varied significantly when incubation time was increased from 24 to 48 h. The level of GLA in the cells increased rapidly in the first 24 h, but decreased somewhat between 24 and 48 h of incubation. The decrease of GLA was due to its increased elongation to its metabolites as shown by the increase of both DGLA and DTrA, and the rate of conversion of GLA to its metabolites (Figure 5.3B). A significant increase in elongation of GLA to its metabolites (from 63% to 90%) was also observed in yeast transformed with human elongase when incubation time was increased from 24 to 48 h (Figure 5.3B).

5.2.2.2 Substrate Concentration

The effect of varying substrate levels of GLA or LA on the activity of human elongase has also been studied. The transformed yeast were incubated at 25°C for 48 h with four different levels (10, 25, 50 or 100 μM) of GLA or LA added in the medium.

Figure 5.4A showed that GLA was rapidly incorporated into yeast cells, and a significant portion of GLA was elongated to DGLA, and subsequently to n-6 DTrA. As levels of exogenous GLA increased, the levels of GLA, DGLA and n-6 DTrA in yeast cells were also increased. However, the rate of elongation of GLA to its metabolites stayed constant (Figure 5.4B).

Similarly, when four different levels of LA (10, 25, 50 or 100 μM) were
Figure 5.3: The effect of incubation time on (A) cellular uptake of GLA and its metabolites, and (B) elongation of GLA to DGLA and DTrA by yeast transformed with human elongase gene.

The yeast cells were cultured in 25 μM GLA for 24 or 48 h. The cellular uptake of GLA or its metabolites were calculated based on the absolute weight of GLA, DGLA or DTrA in yeast lipids. All results are mean ± SE of three incubations. Elongation of GLA to its metabolites was calculated as [product] / ([product] + [substrate]) x 100%.
Figure 5.4: The effect of different levels of GLA on (A) cellular uptake of GLA or its metabolites, and (B) elongation of GLA to its metabolites by yeast transformed with human elongase gene.

The yeast cells were cultured in 10, 25, 50 or 100 μM of GLA. All results are mean ± SE of three incubations. The cellular uptake of GLA and elongation of GLA were defined as the same as the legend of Figure 5.3.
supplemented into the medium and incubated for 48 h, the levels of LA and EDA in
the transformed yeast cells were increased as the level of exogenous LA increased
(Figure 5.5A). However, when the rate of conversion of LA to EDA in transformed
yeast was calculated, a decrease (from 22% to 12%) in elongation of LA to EDA was
observed (Figure 5.5B)

5.2.3 **Inhibitory Effect of CLA on Elongation of LA**

The following study was designed to examine the effect of varying levels of
CLA on elongation of LA to EDA in yeasts transformed with human elongase.

The transformed yeasts were incubated in the medium containing 25 μM of
LA supplemented with four different levels (10, 25, 50 or 100 μM) of CLA isomers
(in mixture or individual). Results in Figure 5.6A show that addition of the CLA
mixture at 10 μM inhibited the elongation of LA to EDA in the transformed yeast.
At equal concentration (25 μM), CLA significantly inhibited elongation of LA to
EDA by 50%. However, no additional inhibitory effect was observed when the
concentration of CLA exceeded 25 μM.

The inhibitory effect of individual CLA isomers on elongation of LA to EDA
was also examined. Figure 5.6B show that only c9,t11- and t10,c12-CLA isomers
could exert the inhibition on elongation of LA to EDA, whereas the other two
isomers (c9,c11- and t9,t11-CLA) had no such effect. The extent of inhibition of
c9,t11- and t10,c12-CLA isomers was also related to the level of CLA isomers added
Figure 5.5: The effect of different levels of LA on (A) cellular uptake of LA and eicosadienoic acid (EDA), and (B) elongation of LA to EDA by yeast transformed with human elongase gene. The yeast cells were cultured in 10, 25, 50 or 100 µM of LA for 48 h. All results are mean ± SE of three incubations. The cellular uptake of LA and elongation of LA to EDA was defined as the same as the legend of Figure 5.3.
Figure 5.6: Effect of different concentrations of CLA isomers in a mixture (A) or individually (B) on elongation of LA to EDA in yeast transformed with human elongase gene.

Transformed yeast with elongase was incubated with 25 μM of LA and different levels (10, 25, 50 and 100 μM) of CLA. All the results are mean ± SE of three incubations. Elongation of LA to EDA was defined as the same as the legend of Figure 5.3. Values with different letters and signs are significantly different from each other at $P < 0.05$. $c_9,c_{11}$-CLA ($\bullet$), $r_9,t_{11}$-CLA ($\bigcirc$), $c_9,t_{11}$-CLA ($\square$) and $r_{10},c_{12}$-CLA ($\bigtriangleup$).
in the medium.

5.2.4 Possible Mechanisms as to How CLA Inhibits Elongation of LA

5.2.4.1 Elongation of CLA isomers

Results from the previous experiment showed that only \( c_9,\omega_{11} \)- and \( \omega_{10},c_{12}- \) CLA isomers inhibited the elongation of LA to EDA. There are several explanations on how CLA isomers exerted the inhibitory effect. One of the possibilities is that CLA could itself serve as the substrate, be elongated to form conjugated EDA (CEDA) by human elongase. The competition with LA for elongase may result in inhibition of elongation of LA to EDA. To test this hypothesis, yeasts transformed with human elongase were incubated with 100 µM CLA mixture at 25°C for 48 hours.

The GC chromatogram in Figure 5.7 shows that two additional peaks (Peak A and B) were detected in yeasts transformed with human elongase [334(pRAE58)], but not in the control yeast [334(pYX242)]. In comparison with authentic standards, results in Figure 5.8 show that the retention time (24.99 min) of peak A was identical to that of the authentic \( c_{11},\omega_{13}- \) CEDA standard. Further analysis by GC-MS shows that the mass peak (\( m/z = 322 \)) and fragmentation pattern of peak A were identical to those of the authentic \( c_{11},\omega_{13}- \) CEDA standard (Figure 5.9). Thus, peak A was identified as \( c_{11},\omega_{13}- \) CEDA, an elongation product of \( c_9,\omega_{11}- \) CLA. The peak B as compared to peak A also had the same mass peak (\( m/z = 322 \)) and a similar fragmentation pattern but different in intensity (Figure 5.10), and different retention
Figure 5.7: Gas chromatographic analysis of fatty acid methyl esters of total lipids from transformed yeast with (A) only the vector [334(pYX242)] or (B) the human elongase [334(pRAE58)] incubated with CLA mixture. All yeast cells were incubated in the medium containing 25 μM CLA mixture. Solid arrows indicate the appearance of Δ13-1:20:1, the elongation product of Δ11-18:1, and two additional peaks.
Figure 5.8: Comparison of fatty acid methyl esters of conjugated EDA (CEDA) standard and total lipids from transformed yeast [334(pRAE58)] incubated with CLA mixture.
Solid arrows indicate the appearance of two extra peaks (Peak A and Peak B).
Figure 5.9: Comparison of mass spectrums between the CEDA standard and the peak A extracted from transformed yeast.
No 12,14-eicosadienoic acid, methyl ester standard MS spectrum available

Figure 5.10: Mass spectrum of the peak B extracted from transformed yeast.
time (25.33 min) (Figure 5.8). Although at the time of analysis, no authentic standard was available for comparison, evidence obtained from the following study suggests that peak B was t12,c14-CEDA.

The yeasts transformed with human elongase were also incubated with four individual isomers. However, only two isomers, c9,t11- and t10,c12-CLA were elongated to form c11,t13-20:2 and t12,c14-20:2 (Figures 5.11A and B). No elongation metabolites were detected in the transformed yeast incubated with either c9,c11- or t9,t11-CLA isomers (Figures 5.12A and B).

To examine whether the differential inhibitory effect was due to the level of individual CLA isomers taken up by the yeast, the uptake of the individual CLA isomers and the elongation of individual CLA isomers were also examined in the transformed yeasts. Results in Figure 5.13 show that approximate 25% c9,t11- and t10,c12-CLA were taken up by the transformed yeast, and significant amounts of these isomers were elongated to their respective CEDA. On the other hand, significant amounts of c9,c11-CLA (40%) and t9,t11-CLA (70%) isomers were taken up, but no elongation products were detected. These findings demonstrated that the inhibitory effect of c9,t11- and t10,c12-CLA on elongation of LA to EDA was due to its competition with LA as substrate for human elongase. The failure of both c9,c11- and t9,t11-CLA isomers to inhibit the elongation activity was not due to the lower intake.

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Figure 5.11: Gas chromatographic analysis of free fatty acid methyl ester from the total lipids of transformed yeast with only the vector (Control) or the elongase gene incubated with c9,t11- or t10,c12-CLA isomers. Yeast cells were incubated in their respective medium containing c9,t11-CLA (A) or t10,c12-CLA (B). Arrows indicate the appearance of Δ13-20:1 and CEDA.
Figure 5.12: Gas chromatographic analysis of free fatty acid methyl ester from the total lipids of transformed yeast with only the vector (Control) or the elongase gene incubated with c9,c11- or t9,t11-CLA isomers. Yeast cells were incubated in their respective media containing c9,c11-CLA (A) or t9,t11-CLA (B). Arrows indicate the appearance of Δ13-20:1 and CEDA.

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Figure 5.13: Cellular uptake of CLA (A) and elongation of CLA to CEDA (B) in yeasts transformed with human elongase gene. The yeast cells were cultured in 100 μM of four individual isomers, respectively. The cellular uptake of CLA isomers was calculated based on the sum of CLA and CEDA isomers (% total lipids) in yeast lipids. Elongation of CLA to CEDA was defined as the same as the legend of Figure 5.3. All results are mean ± SE of three incubations. Values with different letters and symbols are significantly different from each other at \( P < 0.05 \).
5.2.4.2 Substrate Inhibition in Elongation of LA

The above described results clearly showed that only c9,t11- and t10,c12-CLA could be elongated and only these two isomers inhibited the elongation of LA to EDA. To further verify the theory that substrate inhibition was the cause of the inhibition, transformed yeast was incubated with 50 μM of LA and the two CLA isomers (c9,t11- and t10,c12-CLA). The elongation rate and cellular uptake of each substrate were then determined.

Figure 5.14 shows that the levels of two CLA isomer taken up by the yeast cells were lower than LA. However, the elongation rates of the two CLA isomers were significantly greater than that of LA. Approximate 12% of LA was elongated to EDA, but greater than 25% of the CLA isomers were elongated to CEDA.

5.2.4.3 Feedback Inhibition in Elongation of LA

The other possible mechanism by which CLA isomers might inhibit elongation of LA to EDA is through the feedback inhibition. To examine whether the elongation product of CLA (CEDA) could affect LA elongation, 25 μM of CLA and CEDA were supplemented separately to the medium containing 25 μM of LA. Results in Figure 5.15 show that CEDA was as effective as CLA on inhibiting elongation of LA to EDA.
Figure 5.14: Comparison of (A) cellular uptake of LA and CLA isomers, and (B) elongation of LA to EDA and CLA to CEDA in yeasts transformed with human elognase gene.

The concentration of LA and CLA in the medium was 50 μM. Cellular uptake and elongation of substrates were defined as the same as Figure 5.3. All results are mean ± SE of three incubations. Values with different letters and symbols are significantly different from each other at \( P < 0.05 \).
Figure 5.15: Effect of CEDA on elongation of LA to EDA in yeast transformed with human elongase gene.

Transformed yeast was incubated with media containing 25 μM of LA and 25 μM of CLA or CEDA. Yeast incubated with only 25 μM LA was designed as the control (100%). Elongation of LA to EDA was defined as the same as the legend of Figure 5.3. All the results are mean ± SE of three incubations. Values with different letters are significantly different from each other at \( P < 0.05 \).
5.2.5 Inhibitory Effect of CLA on Elongation of GLA

Evidence has also shown that the human elongase can elongate GLA to DGLA. The following study was designed to examine if CLA has effects on the elongation of GLA to DGLA by human elongase. When an equal level (25 µM) of GLA and \( c_{9,11} \)-CLA was added separately into transformed yeast culture, the elongation rates of GLA and CLA were found to be 68% and 33%, respectively. When equal levels (25 µM) of both GLA and \( c_{9,11} \)-CLA were added together in the medium, the elongation rate of GLA was not affected, but elongation of CLA was significantly decreased by the presence of GLA (Figure 5.16).

The transformed yeast was incubated with 25 µM of GLA and different levels of CLA (0, 10, 25, 50 or 100 µM). Results in Figure 5.17 show that addition of CLA at 10 and 25 µM had no inhibitory effect on the elongation of GLA. However, slight inhibitory effect could be observed when CLA concentration was greater than 50 µM.
Figure 5.16: Effect of CLA on elongation of GLA to DGLA in yeast transformed with human elongase gene.
Transformed yeast was incubated with medium containing 25 μM of GLA, CLA, or the combination at the same level (25 μM). The elongation of substrates to products was defined as the same as the legend of Figure 5.3. All results are mean ± SE of three incubations. Values with different letters or symbols significantly differ from each other at $P < 0.05$. 

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Figure 5.17: Effect of different concentrations of CLA mixture on elongation of GLA to its metabolites in yeast transformed with human elongase gene. Transformed yeast was incubated with 25 μM of GLA and different levels (10, 25, 50 and 100 μM) of CLA mixture. Yeast incubated with medium containing only 25 μM GLA were designed as the control (100%). Elongation of GLA to DGLA was defined as the same as the legend of Figure 5.3. All the results are mean ± SE of three incubations. Values with different letters are significantly different from each other at $P < 0.05$. 

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CHAPTER 6

INHIBITORY EFFECT OF CLA ON Δ5-DESATURASE

6.1 Objective

Results from studies in Chapters 4 and 5 showed that CLA significantly modulated n-6 PUFA metabolism by suppressing Δ6-desaturation and elongation of LA. The objective of this study was to further examine if CLA could also affect the Δ5-desaturation which converts dihomo-γ-linolenic acid (DGLA) to arachidonic acid (AA).

\[
\begin{align*}
\Delta6,9-18:2 & \rightarrow \Delta6,9,12-18:3 \\
LA & \rightarrow \text{GLA} \\
\Delta8,11,14-20:3 & \rightarrow \Delta5,8,11,14-20:4 \\
DGLA & \rightarrow \text{AA} \\
18:2 \text{ CLA} &
\end{align*}
\]

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6.2 Experimental Design and Results

6.2.1 Enzyme Activities of Recombinant Δ5-desaturase

Recently, Δ5-desaturase genes from different organisms have been identified and expressed in yeast and mammalian cell cultures (Knutzon et al., 1998; Leonard et al., 2000b; Watts and Browse, 1999). The yeasts transformed with the Δ5-desaturase from the fungus, *M. alpina*, was used as a model for this study (Knutzon et al., 1998). In order to prove the validity of using the yeast transformed with the Δ5-desaturase gene as a model, the activity of Δ5-desaturase (conversion of DGLA to AA) was determined. Yeasts transformed with the Δ5-desaturase gene were incubated at 25°C for 48 h in the medium containing 25 μM of DGLA as the exogenous substrate. The activity of recombinant Δ5-desaturase was determined by measuring the amount of DGLA being converted to AA.

Figure 6.1B showed that a significant level of AA (Δ5,8,11,14-20:4) in the yeast transformed with fungal Δ5-desaturase [334(pCGR4)]. A small level of Δ5,9-18:2 fatty acid (converted from Δ9-18:1) was also found. None of these fatty acids was found in the yeast transformed with vector only [334(pYES2)] (control) (Figure 6.1A). Results from this experiment indicated that the recombinant Δ5-desaturase was expressed successfully in the transformed yeast. The transformed yeast was thus proven to be a valid model for studying the effect of CLA on Δ5-desaturation of n-6 PUFA.

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Figure 6.1: Gas chromatographic analysis of fatty acid methyl esters of total lipids from yeast transformed with (A) only the vector [334(pYES2)] or (B) the fungal Δ5-desaturase [334(pCGR4)]. All yeast cells were incubated in the medium containing 25 μM dihomo-γ-linolenic acid (DGLA). Solid arrows indicate the appearance of Δ5,9-18:2, the Δ5-desaturation product of Δ9-18:1, and arachidonic acid (AA), the D5-desaturation product of DGLA.
6.2.2 Characterization of Transformed Yeast with Δ5-desaturase Gene

To characterize the activity of Δ5-desaturase in the transformed yeast, the effect of incubation time and substrate dose response on the enzyme activity were studied.

6.2.2.1 Incubation Time

The effect of incubation time on conversion of DGLA to AA was examined in the yeast transformed with fungal Δ5-desaturase. The transformed yeast was incubated with a medium containing 25 µM of DGLA at 25°C for 24 or 48 h. The absolute amount of DGLA taken up by the yeast and that of GLA formed in the yeast were determined.

Results in Figure 6.2A show that the levels of DGLA and AA in the transformed yeast increased with incubation time. The highest levels of DGLA and AA were found at the end of 48 h. The increase in DGLA was parallel to that of AA after 24 h. The conversion of DGLA to AA increased from 31% at 24 h to 40% at 48 h (Figure 6.2B).

6.2.2.2 Substrate Concentration

The effect of different levels of DGLA on the activity of Δ5-desaturase has also been studied. The transformed yeast was incubated in the medium containing four different levels of DGLA (10, 25, 50 or 100 µM) at 25°C for 48 h.
Figure 6.2: The effect of incubation time on (A) cellular uptake of DGLA and AA, and (B) Δ5-desaturation DGLA to AA by yeast transformed with fungal Δ5-desaturase gene.

The yeast cells were cultured in 25 μM DGLA for 24 or 48 h. The cellular uptake of DGLA or AA were calculated based on the absolute weights of DGLA or AA in yeast lipids. Δ5-desaturation of DGLA to AA was calculated as \[ \frac{[\text{product}]}{([\text{product}] + [\text{substrate}])} \times 100\% \]. All results are mean ± SE of three incubations.
Figure 6.3A show that the incorporation of DGLA into yeast cells was proportional to the level of DGLA in the medium. The amount of AA in the transformed yeast increased to approximately 20 mg. However, it remained at that level despite that the concentration of DGLA was increased in the medium, and the yeast cells. When the conversion rate of DGLA to AA was calculated, there was a progressive decrease in conversion of DGLA to AA (from 54% to 20%) in the transformed yeast when the levels of DGLA in medium increased (Figure 6.3B).

6.2.3 Inhibitory Effect of CLA on Δ5-desaturase Activity

The following study was designed to examine if CLA affected the conversion of DGLA to AA in the yeast transformed with fungal Δ5-desaturase gene. The transformed yeast was incubated with both DGLA and CLA (in combination or individual isomers) at the concentration of 25 μM.

Figure 6.4 shows that the addition of CLA mixture to the medium reduced the conversion of DGLA to AA by 30%. Among four CLA isomers, the extent of inhibition of Δ5-desaturase activity varied. The c9,c11-CLA had the most potent (38%) inhibition, followed by c9,t11- (33%), t9,t11- (26%) and t10,c12-CLA (17%). The rate of CLA (in a mixture or four individual isomers) taken up by yeasts transformed with the Δ5-desaturase gene is shown in Figure 6.5. The uptakes of CLA (in a mixture or individual isomers) by transformed yeast were similar with the exception of the t9,t11-CLA isomer, which is significantly higher.
Figure 6.3: The effect of different levels of DGLA on (A) cellular uptake of DGLA and AA, and (B) Δ5-desaturation of DGLA to AA by yeast transformed with fungal Δ5-desaturase gene.

The yeast cells were cultured in 10, 25, 50 or 100 μM of DGLA for 48 h. All results are mean ± SE of three incubations. The cellular uptake of DGLA or AA, and Δ5-desaturation of DGLA to AA were defined as the same as the legend of Figure 6.2.
Figure 6.4: Effect of CLA isomers on the activity of fungal Δ5-desaturase.
Transformed yeast with fungal Δ5-desaturase were incubated with both DGLA and CLA (in combination or individual isomers) at the concentration of 25 μM. Transformed yeast incubated with only 25 μM of DGLA was designed as the control (100%). All results are mean ± SE of three incubations. Values with different letters are significantly different from each other at $P < 0.05$. The Δ5-desaturase activity was defined as the same as Δ5-desaturation of DGLA to AA (See the legend of Figure 6.2)
Figure 6.5: Uptake of different CLA isomers by yeast transformed with fungal Δ5-desaturase.

The uptake of CLA isomers is indicated by the presence of CLA isomers (% total lipids) in yeast. All results are mean ± SE of three incubations. Values with different letters are significantly different from each other at $P < 0.05$. 

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A separate study examining the inhibitory effect of four individual isomers supplemented in medium at different levels (10, 25, 50 and 100 μM) on the activity of Δ5-desaturase has also been carried out in the transformed yeast. Results in Figure 6.6 show the inhibition of the four individual CLA isomers on the conversion of DGLA to AA. The inhibition was dose-dependent from 10 to 100 μM. The exception was the t9,t11-CLA isomer which exerted inhibition at 10 μM, and no further inhibition when its concentration exceeded 10 μM (Figure 6.6).

6.2.4 A Possible Mechanism as to how CLA Isomers Inhibit Δ5-desaturation of DGLA to AA

6.2.4.1 Δ5-desaturation of CLA Isomers

Results from the above experiment clearly demonstrated that CLA inhibited the Δ5-desaturation of DGLA to AA. Since Knutzon et al. (1998) have previously shown that LA could be converted to pinolenic acid (PNA; Δ5,9,12-18:3) by yeasts transformed with fungal Δ5-desaturase (Knutzon et al., 1998), it is possible that CLA could be converted to Δ5-conjugated 18:3 fatty acid by the recombinant Δ5-desaturase, and at the same time inhibit Δ5-desaturation of DGLA to AA. This possibility was tested in this study by looking into whether CLA could be metabolized by the recombinant Δ5-desaturase. The transformed yeast was incubated with a medium containing 25 μM of individual CLA isomers for 48 h.
Figure 6.6: Effect of different concentrations of CLA isomers on Δ5-desaturation of DGLA to AA in yeast transformed with fungal Δ5-desaturase gene. Transformed yeast with Δ5-desaturase was incubated with 25 μM of DGLA and different levels (10, 25, 50 and 100 μM) of CLA individual isomers. Yeast incubated with medium containing only 25 μM DGLA were designed as the control (100%). All the results are mean ± SE of three incubations. Δ5-desaturation of DGLA to AA was defined as the same as the legend of Figure 6.2. Values with different letters and signs are significantly different from each other at \( P < 0.05 \).
The GC chromatograms in Figure 6.7A, 6.8A and 6.9A show the appearance of an unknown peak (possible Δ5-desaturation product of CLA) which was eluted at a later time than CLA in the transformed yeasts incubated with c9,t11-, t10,c12- or c9,c11-CLA isomer, respectively. But no such peak was found in the chromatogram when the yeast was incubated with t9,t11-CLA isomer (Figure 6.10). Since there are no commercially available authentic standard of these CLA metabolites to help the identification, these unknown peaks were further examined by GC-MS. Results in Figure 6.7B, 6.8B and 6.9B show that these three unknown peaks share similar fragmentation patterns with different intensities, and have identical mass peaks (m/z = 292). These mass peaks reduced from m/z = 294 down to m/z = 292 which matched the mass peak of Δ5-desaturation product of CLA. Therefore, these peaks were tentatively identified as Δ5,c9,t11-18:3, Δ5,c9,t11-18:3, Δ5,c9,t11-18:3, respectively.

6.2.4.2 Substrate Inhibition in Δ5-desaturation of DGLA to AA

From the previous results, it is evident that CLA significantly inhibited the conversion of DGLA to AA, and three of four CLA isomers may be converted to their respective Δ5-conjugated 18:3 isomers. Since the inhibition of Δ5-desaturation of DGLA to AA by CLA could be due to substrate competition between CLA and DGLA, it is important to determine whether this competition indeed exists. To examine this possibility, yeasts transformed with the fungal Δ5-desaturase gene were
Figure 6.7: Gas chromatographic and gas chromatographic-mass spectrum analyses of fatty acid methyl esters of total lipids from transformed yeast incubated with c9,t11-CLA isomer.
Figure 6.8: Gas chromatographic and gas chromatographic-mass spectrum analyses of fatty acid methyl esters of total lipids from transformed yeast incubated with t10,c12-CLA isomer.
Figure 6.9: Gas chromatographic and gas chromatographic-mass spectrum analyses of fatty acid methyl esters of total lipids from transformed yeast incubated with c9,c11-CLA isomer.
Figure 6.10: Gas chromatographic analysis of fatty acid methyl esters of total lipids from transformed yeast incubated with c9,c11-CLA isomer.
incubated with a medium containing 25 μM of individual CLA isomer (c9,t11-,
t10,c12- or c9,c11-) alone, or in a combination with 25 μM of DGLA. Results in
Figure 6.11 show that Δ5-desaturation of three individual CLA isomers to their
metabolites was suppressed (c9,t11-, from 45% to 34%; t10,c12-, from 20% to 10%
and c9,c11-, from 20% to 17%), when DGLA was present in the medium.
Figure 6.11: Inhibitory effect of DGLA on Δ5-desaturation of CLA to Δ5-desaturation product of CLA in yeast transformed with Δ5-desaturase gene.
Transformed yeast was incubated with medium containing 25 μM of three CLA isomers individually, or the combination of one of CLA isomers and DGLA at the same level (25 μM). Δ5-desaturation of substrates to products was defined as the same as the legend of Figure 6.2. All the results are mean ± SE of three incubations. Values with different letters and symbols are significantly different from each other at P < 0.05.
CHAPTER 7

DISCUSSION AND CONCLUSION

7.1 Metabolism of n-6 PUFA

By using baker’s yeast transformed with PUFA-specific enzyme genes, this study demonstrated that CLA suppressed the n-6 PUFA metabolism through competitive inhibition of Δ6-desaturation and elongation of LA, and Δ5-desaturation of DGLA. These findings suggested that the suppressive effect of CLA on AA production might lead to a reduction in eicosanoid synthesis, thus modulating the immune function and other biological effects.

7.1.1 Rate-limiting Steps of n-6 PUFA Metabolic Pathway

The n-6 PUFA metabolic pathway has been well established in studies using liver microsomes. It is generally agreed that both Δ6- and Δ5-desaturation are the rate-limiting steps for the synthesis of n-6 PUFA (Brenner, 1981; Sprecher, 1981). However, due to the complexity of the microsomal system and unavailability of methodologies for purifying the Δ6- and Δ5-desaturases, direct evidence supporting
the observation has not yet been reported. In this study, by using baker’s yeast transformed with fungal and human Δ6-, and Δ5-desaturase genes, the specific interaction between CLA and different metabolic enzymes could be assessed.

7.1.2 Inhibitory Effect of CLA on Δ6-desaturation

Results from this study indicate that CLA modulated the first rate-limiting step of n-6 PUFA metabolism, i.e. reduced conversion of LA to GLA, by significantly inhibiting the activities of both fungal and human Δ6-desaturases. This finding confirmed the earlier observation reported by Bretillon et al. (1999) in which hepatic microsomes were used as the source of Δ6-desaturase (Bretillon et al., 1999). The result in this report shows that CLA could not be metabolized to form conjugated GLA, despite its similarity to LA in chemical structure. This inability to convert CLA to conjugated GLA is not caused by the failure of yeast cells to take up the CLA isomers, but possibly due to the enzyme specificity. These findings of this study differ from previous studies which found 18:3-CLA metabolites (conjugated-GLA) in CLA-fed animals (Banni et al., 1995; Sebedio et al., 1997).

The CLA mixture inhibited the activity of Δ6-desaturase (Figure 4.10) by 33%. However, in yeasts transformed with fungal Δ6-desaturase, the c9,t11-CLA isomer when compared with other isomers was not taken up as much by the yeast cells (Figure 4.12), but it exerted the greatest inhibition on enzyme activity (40% at 25 μM) (Figure 4.10). The inhibitory effect of CLA mixture could be attributed to
the presence of high percentage (41%) of $c_{9,11}-/t_{9,11}$-CLA isomers in the mixture. This finding suggests that $c_{9,11}$-CLA is the most active CLA isomer. This is in agreement with the results found in the *in vivo* feeding studies by Ha *et al.* (Ha *et al.*, 1990) and Ip *et al.* (Ip *et al.*, 1991). They have suggested that $c_{9,11}$-CLA was the active form of CLA.

CLA mixture also exhibited significant inhibition (38%) on the activity of human $\Delta_6$-desaturase (Figure 4.13). However, the $t_{10,c12}$-CLA isomer, instead of $c_{9,t11}$-, demonstrated the most significant inhibition on the activity of $\Delta_6$-desaturase (76% vs. 28%). At the concentration as low as 5 $\mu$M, this isomer still exhibited a substantial level of inhibition (44%) (Figure 4.15). Thus, in this instance, the $t_{10,c12}$-CLA isomer was the active isomer. This is in agreement with findings reported by Yotsmoto (Yotsmoto *et al.*, 1999), Kang (Kang and Pariza, 2001) and Baumgard (Baumgard *et al.*, 2001).

Results from studies using fungal and human $\Delta_6$-desaturase genes indicated that CLA significantly inhibited the $\Delta_6$-desaturation of LA. However, the most active isomers exerting the inhibition of fungal or human $\Delta_6$-desaturase were different. The reason for this difference is not clear at the present time. It is possible that fungal and human $\Delta_6$-desatureases have different properties in binding site where only a substrate with a specific configuration is favorably incorporated.

Previously, Lee *et al.* (Lee *et al.*, 1998) have shown that CLA reduced stearoyl-CoA desaturase activity by decreasing the level of mRNA of stearoyl-CoA
desaturase gene in mouse liver and H2.35 cells. Similarly, the inhibitory effect of
CLA on Δ6-desaturase activity could also be due to its ability to modulate cellular
signal transduction by up-regulating gene expression and protein production.
However, results in the present study showed that there were no changes in the level
of Δ6-desaturase mRNA in the transformed yeast cells treated with CLA (Figure
4.17). This indicates that the inhibitory effect of CLA on Δ6-desaturase activity was
not exerted through the suppression of Δ6-desaturase gene transcription. The
alternative explanation for CLA inhibition on Δ6-desaturation is that CLA due to
similarity in their chemical structures might compete with LA for binding to the
catalytic sites of Δ6-desaturase. Further studies are needed to verify this hypothesis.

7.1.3 Inhibitory Effect of CLA on Chain Elongation

Using yeasts transformed with a human PUFA specific elongase gene, we
have examined the effect of CLA on two elongation steps: from LA to EDA and
from GLA to DGLA.

7.1.3.1 Effect of CLA on Elongation of LA to EDA

Results in Figure 5.6A demonstrated that CLA significantly inhibited the
alternate pathway of LA metabolism, i.e. elongation of LA to EDA (Figure 5.6A).
Among the four available isomers, only c9,t11- and t10,c12-CLA isomers, could
inhibit LA elongation (Figure 5.6B). And only the same two isomers could be
elongated to form CEDA (Figure 5.11). The other two isomers (c9,c11- and r9,t11-) (Figure 5.12) could not be elongated as shown in Figure 5.12, despite the fact that they were more readily into the yeasts transformed with human elongase than the two active CLA isomers (Figure 5.13A). Thus, the differential elongation of CLA isomers was the result of substrate specificity of the recombinant elongase. It is possible that the recombinant elongase recognizes only the cis-,trans- and trans-,cis-configurations, but not the cis-,cis- and trans-,trans-configurations of CLA. These findings again suggested that c9,t11- and t10,c12-CLA isomers are the two biologically active CLA isomers as suggested by the previous Δ6-desaturase study in this report and in earlier literatures (Ha et al., 1990; Park et al., 1999b; Park et al., 1999c; Yotsmoto et al., 1999).

The inhibitory effect of CLA (only c9,t11- and t10,c12-CLA isomers) on the elongation of LA can be attributed to substrate competition. This was further demonstrated in the following studies. Figure 5.14 shows that the elongation rates of the two CLA isomers to CEDA (>25%) are greater than that of LA to EDA (12%). When both CLA and LA were present, the effect of the co-presence of LA on the elongation of CLA was significantly less than that of CLA on the elongation of LA. These findings suggest that c9,t11- and t10,c12-CLA isomers are the preferred substrates of the recombinant elongase rather than LA, and the inhibitory effect of these two CLA isomers on LA elongation was a result of substrate competition for the recombinant elongase in the transformed yeasts.
In addition to substrate competition, the inhibition of CLA on elongation of LA to EDA could also be due to the formation of CEDA, the CLA elongation product. To test this hypothesis, yeasts transformed with elongase were incubated with 25 µM of CEDA mixture (containing approximate 82% of CEDA isomers). Results in Figure 5.15 show that the inhibitory effect of CEDA (25 µM) on elongation of LA was comparable to that of CLA (25 µM). This finding indicates that CEDA is not likely to play a significant role in the inhibition.

7.1.3.2 Effect of CLA on Elongation of GLA to DGLA

Results in Figure 5.16 show that CLA has no significant effect on elongation of GLA to DGLA. The elongation rate of GLA was higher than that of CLA (68% vs. 33%), when they were supplemented to the medium separately. When CLA and GLA were both present in the medium, the elongation of GLA to DGLA was not affected, but the elongation of CLA to CEDA was significantly inhibited (Figure 5.17). These results suggest that the failure of CLA to inhibit the elongation of GLA to DGLA, was due to the fact that GLA is the preferred substrate of the elognase rather than CLA.

7.1.4 Inhibitory effect of CLA on Δ5-desaturation

In this study, by using the yeast transformed with fungal Δ5-desaturase gene, the effect of CLA on the second rate-limiting step of n-6 PUFA metabolism, i.e.
conversion of DGLA to AA was demonstrated. Results in Figure 6.4 show that CLA significantly inhibited the activity of δ5-desaturases.

Three of the four CLA isomers (c9,t11-, t10,c12- and c9,c11-) were metabolized by the recombinant δ5-desaturase to form the δ5-desaturation products of CLA (Δ5,c9,t11-, Δ5,t10,c12- and Δ5,c9,c11-18:3), respectively. Only t9,t11-CLA was not metabolized. Previously, it has been shown that this δ5-desaturase, although it preferred DGLA as the substrate, could also act on various PUFAs. For example, LA was converted to form pinolenic acid (PNA; Δ5,9,12-18:3) by the same δ5-desaturase according to Knutzon (Knutzon et al., 1998). Since t9,t11-CLA was readily taken by yeast cells, the inability to convert t9,t11-CLA to Δ5,t9,t11-18:3 was likely due to the substrate specificity of δ5-desaturase.

Results in the above study clearly show that CLA inhibited the conversion of DGLA to AA, and both CLA (three of four isomers) and DGLA could be metabolized. Could the inhibitory effect of CLA on δ5-desaturation of DGLA to AA be attributed to substrate competition? Results in Figure 6.4 and 6.11 show that when DGLA and CLA were both present in the medium the rate of δ5-desaturation of DGLA to AA and that of CLA isomers (c9,t11-, t10,c12- and c9,c11-CLA) to their δ5-desaturation products were decreased. This finding suggests that the competition between DGLA and CLA may play a major role in the inhibition of δ5-desaturation of DGLA to AA. Interestingly, t9,t11-CLA, the only CLA isomer which could not be metabolized by the δ5-desaturase (Figure 6.10), exerted the same
inhibitory effect on the conversion of DGLA to AA (Figure 6.4). However, the inhibition of this isomer on \( \Delta 5 \)-desaturase activity reached 30% when its level was at 10 \( \mu \)M in the medium (Figure 6.6), and maintained the same level of inhibition even when its concentration exceeded 10 \( \mu \)M. It is possible that \( \tau 9,\tau 11 \)-CLA may interfere with the binding of DGLA to the catalytic site of \( \Delta 5 \)-desaturase by simply occupying the same site due to their similarities in chemical structures. More studies are needed to verify this hypothesis.

7.2 Conclusion

It has been suggested that CLA and its metabolites may play competitive roles in the regulation of PUFA metabolism (Banni et al., 1995; Liu and Belury, 1998; Sebedio et al., 1997; Sugano et al., 1998). The present study, by using a simple and unique transformed yeast model, demonstrated that CLA significantly inhibited the \( \Delta 6 \)-desaturation and elongation of LA, and the \( \Delta 5 \)-desaturation of DGLA. The inhibition was due to the competition between the substrates and CLA for the enzymes.

The present study also demonstrated that CLA was not converted by \( \Delta 6 \)-desaturase to form conjugated 18:3. Hence, the finding from this study does not support the findings of Banni et al. and Sebedio et al. which stated that CLA was metabolized by the enzyme to form conjugated 18:3 (conjugated-GLA) (Banni et al., 1995; Sebedio et al., 1997). On the other hand, both \( \tau 9,\tau 11 \)- and \( \tau 10,\tau 12 \)-CLA
isomers were elongated to form CEDA isomers by the elongase, and metabolized to form Δ5-desaturation products by the Δ5-desaturase, and these two isomers exerted a significant inhibition on the Δ6-desaturation and elongation of LA and the Δ5-desaturation of DGLA. This study supports the argument that these two isomers are the most potent isomers among the four CLA isomers as suggested by Ha (Ha et al., 1990), Ip (Ip et al., 1991), Park (Park et al., 1999b; Park et al., 1999c) and Yotsmoto (Yotsmoto et al., 1999).

In conclusion, there is no doubt that CLA modulates n-6 PUFA metabolism by inhibiting the two rate-limiting steps of the n-6 PUFA metabolic pathway, i.e. Δ6-desaturation of LA and Δ5-desaturation of DGLA, and also by inhibiting the elongation of LA when going through the alternate pathway. A combination of all these inhibitory effects would enable CLA to modulate n-6 PUFA metabolism and thus reduce the production of AA. A reduction in the amount of AA entering the cyclooxygenase/lipoxygenase pathways, would lead to a decreased biosynthesis of pro-inflammatory eicosanoids in mammalian cells.

7.3 Future Studies and Implications

This study using a transformed yeast model demonstrates the effect of CLA on n-6 PUFA metabolism in three steps, including the Δ6-desaturation and elongation of LA, and the Δ5-desaturation of DGLA. But, it remains to be confirmed whether a decrease in the production of AA would result in a decrease in
synthesis of pro-inflammatory eicosanoids in cell culture and animal models. In addition, recent evidence has suggested that CLA affects signal transduction through transcription factors, such as PPAR or NF-κB. Further studies are needed to answer these questions. Nevertheless, the present study provides evidence that CLA modulates n-6 PUFA metabolism and reduces the formation of AA, the precursor of the pro-inflammatory eicosanoids. This beneficial effect enables CLA to be considered as useful means in nutraceutical prevention and pharmaceutical treatment. It goes without saying that more detailed studies on the mechanisms of its function and on its beneficial effects in various clinical studies are required.


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APPENDIX A

YEAST CULTURE MEDIUM

I. YPD Medium – for overnight inoculation

The ingredients of per liter Difco YPD Broth contains:

Yeast Extract 10 g
Peptone Extract 20 g
Dextrose Extract 20 g

1. Dissolve 50 g of YPD broth in 1 L distilled-deionized water
2. Autoclave at 121°C for 15 min

II. Yeast Minimal Medium (YMM)

a. YMM (DOB-URA) – for 334(pYES2), 334(pCGR4) and 334(pCGR5) transformed yeast strains

The ingredients of per liter YMM (DOB) contains:

Yeast Nitrogen Base 1.7 g
Dextrose 20 g
Ammonium Sulfate 5 g
1. Dissolve 26.7 g of DOB in 1 L distilled-deionized water.

2. Add 0.77 g of Complete Supplement mixture minus uracil (CSM-URA) into DOB.

3. Autoclave at 121°C for 15 min.

b. YMM (DOB-LEU) – for 334(pYX242), 334(pRAE89) and 334(pRAE58) transformed yeast strains

The ingredients of per liter YMM (DOB w/GAL) contains:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast Nitrogen Base</td>
<td>1.7 g</td>
</tr>
<tr>
<td>Galactose</td>
<td>20 g</td>
</tr>
<tr>
<td>Ammonium Sulfate</td>
<td>5 g</td>
</tr>
</tbody>
</table>

1. Dissolve 26.7 g of DOB w/GAL in 1 L distilled-deionized water.

2. Add 0.69 g of complete supplement mixture minus leucine (CSM-LEU) into DOB w/ GAL.

3. Autoclave at 121°C for 15 min.
APPENDIX B

FATTY ACID ANALYSIS

I. Method of Lipid Extraction
1. Add 20 ml chloroform/methanol (2:1, v/v) to the tube, mix well, then allow to set for at least 1 hour at room temperature (or overnight at 4°C).
2. Add 4 ml saline to extract, vortex for 30 seconds and set to settle.
3. Transfer the bottom phase (chloroform phase) to a new tube.
4. Evaporate the solvent with Nitrogen by Zymark Turbo Vap LV Nitrogen Evaporator (Hopkinton, MA).

II. Method of Methylation
1. Add 2 ml methanol with 1% sulfuric acid and 0.5 ml DMSO to the test tube with extracted lipid.
2. Flush with nitrogen and cap the tube with Teflon-lined cap.
3. Boil gently at 95°C for 20 minutes and then cool down to room temperature.
4. Add 2 ml saline solution and 4 ml hexane to extract fatty acid methyl esters.
5. Transfer the upper phase (hexane phase) to a new test tube.

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6. Evaporate hexane with nitrogen by Turbo Vap.

7. Re-dissolve fatty acid methyl ester with 1 ml hexane.