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Growth of adipose tissue involves both hyperplasia and hypertrophy of adipocytes. In this study, the 3T3-L1 preadipocyte cell line was used as a model to examine the regulation of adipocyte differentiation, with the emphasis on the differentiation-dependent *trans*-acting factors and Ca^{+2}-dependent protein kinases (PKCs).

Depending on the time of treatment, 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD) was effective in inhibiting 3T3-L1 preadipocyte differentiation and mRNA levels of CCAAT/enhancer binding protein (C/EBP) α and peroxisome proliferator activated-receptor (PPAR) γ2, but did not affect mRNA levels of retinoid X receptor (RXR) α and retinoic acid receptor (RAR) α. The time-dependent inhibition of cell differentiation by TCDD was correlated with the levels of aryl hydrocarbon receptor (AhR). TCDD did not alter mRNA or protein levels of C/EBPβ, which is thought to play a role in inducing the C/EBPα and PPARγ2 expression. These results suggest that TCDD inhibited 3T3-L1 preadipocyte differentiation through the AhR pathway, and the change of C/EBPβ mRNA and protein levels was not involved in reducing the mRNA expression of C/EBPα and PPARγ2.

Retinoic acid (RA) inhibits 3T3-L1 preadipocyte differentiation. RA prevented induction of mRNA levels of C/EBPα, PPARγ2, RARα, and RXRα, but not C/EBPβ. The RAR- and RXR-specific ligands were used to determine that the effects of RA were mediated by ligand-bound RARs rather than RXRs.

Four PKC isozymes, PKC α, βI, ε, and ζ were identified in 3T3-L1 adipocytes. The levels of PKC α, βI, and ζ decreased during differentiation, while the amounts of PKC ε increased. Phorbol 12-myristate 13-acetate (PMA), an activating ligand for PKC, inhibited 3T3-L1 preadipocyte differentiation and maintained the basal levels of PKC α, βI, ζ, and ε. Staurosporin, a PKC inhibitor, enhanced 3T3-L1 preadipocyte differentiation. Calpain inhibitor II treatment caused reduction of the 3T3-L1 pre-adipocyte differentiation which was accompanied by sustained PKC levels. Furthermore, TCDD did not affect expression of PKCs, and RA affected only PKCα. Taken together, these results indicate that the calpain-dependent PKC down-regulation and the decrease of PKC levels are associated with 3T3-L1 cell differentiation.

Regulation of 3T3-L1 Preadipocyte Differentiation in Culture

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CONTRIBUTION OF AUTHOR

Dr. Ann E. Brodie was involved in the discussion of chapter II.

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Chapter I

INTRODUCTION

The ultimate goal of animal science research is to increase the efficiency of animal production. In meat animals, concerted efforts are ongoing to enhance the rate and efficiency of meat production by increasing muscle tissue and decreasing adipose tissue. We are interested to study the development of adipose tissue, since understanding of the process of adipose tissue accretion will enable us to design effective approaches to reducing body fat in meat animals.

The fat cells (adipocytes) of adipose tissue are derived from adipocyte precursors (preadipocytes) through the process of cell differentiation. The conversion from preadipocytes into mature adipocytes is induced by extracellular hormones, growth factors, or nutrients. These factors bind to their corresponding receptors in preadipocytes and trigger different signaling pathways, which subsequently result in the activation of specific *trans*-acting factors that initiate the cell differentiation program. An understanding of the regulation of adipocyte differentiation is important and fundamental to the comprehension of adipose tissue development.

In this thesis, the 3T3-L1 preadipocyte cell culture system was used as a model to investigate the roles of protein kinase C signaling pathway in adipocyte differentiation. 2,3,7,8-tetrachlorodibenzo-*p* -dioxin (TCDD) and retinoic acid

(RA), two compounds known to affect adipose development, were applied to study the interactions and expression of the differentiation-dependent *trans*-acting factors during adipocyte differentiation. These studies provided us valuable information for deciphering the mechanisms of adipocyte differentiation.

In this chapter, the general background regarding adipose tissue development and adipocyte differentiation will be addressed.

Adipose tissue development

Adipose tissue has long been considered a type of connective tissue consisting mainly of fat-storing cells, referred to as adipocytes, in association with a rich blood supply. Adipocytes represent between one third and two thirds of the total number of cells in adipose tissue. The remaining cells are various blood cells, endothelial cells, pericytes, fibroblasts, and adipose precursor cells at varying stages of differentiation (Ailhaud *et al.*, 1992).

The storage of triglycerides, the major function of adipose tissue, , is an efficient form of calorie storage since fat has about twice the caloric density of carbohydrate and protein. Thus, when caloric intake exceeds expenditure, metabolic flux is directed into pathways leading to synthesis of triglycerides to be stored in the adipocytes. In contrast, when caloric expenditure exceeds intake, this energy reserve is mobilized to meet the deficiency and provide needed physiological fuel, i.e. free fatty acids, for other cell types. In addition to the storage and mobilization of lipid, adipose tissue also insulates the body to reduce heat loss and protects inner organs from trauma. The recently cloned mouse obesity (*ob*) gene appears to encode an adipose tissue-derived signaling factor for

body weight homeostasis (Zhang *et al.*, 1994). Mice that are monozygous for mutations in this gene exhibit a profound obesity resulted from defects in energy expenditure, food intake, and nutrient partitioning (Bray and York, 1979). Several recent studies have shown that recombinant OB protein (leptin) purified from *Escherechia coli* can correct the obesity related phenotypes in *ob/ob* mice when exogenously administered (Campfield *et al.*, 1995; Pelleymounter *et al.*, 1995; Halaas *et al.*, 1995). The receptor for the leptin (OB-R) has also been cloned from a cDNA expression library prepared from mouse choroid plexus (Tartaglia *et al.*, 1995). Since leptin is an important circulating signal for the regulation of body weight and OB-R mRNA is expressed not only in choroid plexus, but also in several other tissues, including the hypothalamus (Tartaglia *et al.*, 1995), an endocrine function of adipose tissue has been suggested. In addition, adipose tissue appears to have a number of links with the immune system. Several components of the alternative complement pathway are synthesized in adipose tissue. These include complement factors D (adipsin), C_3, and B (Bortell *et al.*, 1992). The presence of tumor necrosis factor (TNF)-α, an immune system regulator, also has been described in adipose tissue. The expression of TNF-α is increased in a number of models of genetic obesity (Hotamisligil *et al.*, 1993). These observations support the interaction of adipose tissue with the immune system.

The growth of adipose tissue is a combination of both hypertrophy and hyperplasia of adipocytes (Hirsch *et al.*, 1989). When lipogenesis exceeds lipolysis, the storage of triglycerides increases in adipocytes to enlarge its size or hypertrophy. Conversely, preadipocytes that are not capable of storing fat become adipocytes through the process of differentiation, which leads to the increase of mature adipocytes in number or hyperplasia. During growth of the adipose tissue mass, well-defined stages could be characterized either by

hyperplastic growth or by hypertrophic growth. In the rat epididymal fat pad, growth of the adipose tissue is hyperplastic from birth until the 4th postnatal week. From the 4th to the 14th week, both hyperplasia and hypertrophy contribute to the enlargement of the adipose tissue mass; from the 14 week until senescence, hypertrophic growth predominates. Therefore, influences early in postnatal life may exert long-lasting effects on the number of fat cells, whereas influences later in life are more likely to change cell size only. Even though adipocyte hypertrophy appears to occur more readily than hyperplasia later in postnatal life (Naslun *et al.*, 1988), severe obesity cannot be accounted for solely by adipocyte hypertrophy since adipocytes containing proportionately large amounts of fat are not found. This implies that hyperplasia must occur as well during periods of excessive fat storage (Hirsch and Batchelor, 1976; Faust *et al.*, 1977; Faust *et al.*, 1984). One study demonstrated that when rats were fed highly palatable diets, they consumed a large excess of calories and their fat cells enlarged to what may be considered maximal size. Once the maximal cell size has been reached, the number of cells begins to increase in the adipose depots. When the overfed rats were returned to a diet of the less palatable laboratory chow, fat cells shrunk back to the more usual sizes, but the number remains elevated (Faust *et al.*, 1977). Clearly, after fat cells are formed, regardless of the stages of development of the animal, they remain throughout its life. Therefore, exerting control over hyperplastic development of adipose tissue may be the most effective way to reduce carcass fat content, improve production efficiency, and provide high quality meat in animal agriculture. Toward this end, complete understanding of the cellular and molecular mechanisms that regulate adipocyte proliferation and differentiation is required.

Adipocyte differentiation

Although no biochemical or molecular marker specific to preadipocytes has been identified, ultrastructural studies have shown the existence of very small fat cells in addition to mature adipocytes (DeMartinis and Francendese, 1982; Francendese and DeMartinis, 1985; Julien *et al.*, 1989). Furthermore, experiments in which [^{3}H]thymidine was administered to rats demonstrated that adipocytes develop by proliferation and differentiation of cells present in the stromal vascular fraction of adipose tissue and that mature, fully differentiated adipocytes have no capacity for cell division *in vivo* (Van, 1985). Subsequently, it became possible to isolate these adipocyte precursor cells and culture them *in vitro* where they proliferate and differentiate into mature adipocytes (Van, 1985). Mature adipocytes, when cultured in *vitro* have a limited capacity for cell division (Sugihara *et al.*, 1987), but it is likely that most hyperplastic capacity of adipose tissue in *vivo* resides in a population of adipocyte precursor cells.

Considerable data on the development of the adipocytes has come from *in vitro* cell culture study. These studies have used either: (i) primary cultures of adipocyte precursor cells, which are prepared by collagenase digestion of adipose tissue and have a limited life span, or (ii) preadipocyte cell lines, which have been isolated and cloned from a number of sources and can grow in culture through many passages. Examples of preadipocyte cell lines that have been isolated are: 3T3-L1 (Green and Kehinde, 1974), 3T3-F442A (Green and Kehinde, 1976), Ob17 (Négrel *et al.*, 1978), TA1 (Chapman *et al.*, 1984), 1246 (Darmon *et al.*, 1981), ST13 (Hiragun *et al.*, 1980), PFC6 (Ailhaud, 1982), and A31T6 (Diamond *et al.*, 1977). The most extensively characterized preadipocyte cell lines are the 3T3-L1, 3T3-F442A, and Ob1771 lines. The 3T3-L1 and 3T3-F442A cell lines were derived from disaggregated mouse embryo cells and were selected for their

propensity to accumulate triacylglycerol lipid droplets. Ob17 cells were derived from the stroma of epididymal fat pads from adult genetically obese mice (*ob/ob*), and several sublines, notably the Ob1771 line, were subsequently selected. There are advantages and disadvantages to using either cell lines or primary cultures of adipocyte precursor cells. The major advantage of cell lines is that they are clonally derived and so are a defined homogeneous population at the same stage of differentiation. In contrast, preparations of the primary adipocyte precursor cells are heterogeneous populations containing other cell types. However, an important advantage of primary cells is that they may more closely resemble those preadipocytes that are present *in vivo*.

The established preadipocyte cell lines have facilitated the study of the molecular details of adipocyte differentiation, mainly because they are homogeneous population and can be passed for many generations. In addition, previous studies have shown that preadipocyte cell lines represent faithful models of preadipocyte differentiation *in vivo*. The most compelling evidence comes from transplantation studies. When 3T3-F442A preadipocytes were injected subcutaneously into Balb-C athymic mice, normal fat pads histologically indistinguishable from white adipose tissue developed at the site of injection within 5 weeks (Green and Kehinde, 1979). Furthermore, fully differentiated adipocytes derived from the preadipocyte cell lines faithfully mimic the metabolism of the adipocytes isolated from adipose tissue. Extensive biochemical analysis has revealed that the accumulation of cytoplasmic triacylglycerol is closely correlated with the coordinate expression of every known enzyme involved in *de novo* fatty acid and triacylglycerol biosynthesis (Cornelius *et al.*, 1994). Finally, detailed electron micrographic studies have verified that mature 3T3-L1 adipocytes possess the ultrastructural features of adipocytes *in situ* (Novikoff *et al.*, 1980).

Prior to differentiation, preadipocytes resemble fibroblasts. Cell/cell contact appears to be a prerequisite for subsequent preadipocyte differentiation, because reversible growth arrest at the G_0/G_1 cell cycle boundary at confluence must occur before preadipocytes can commit to the differentiation program (Spiegelman and Farmer, 1982). Growth factors and other mitogens, therefore, may inhibit adipogenesis indirectly by preventing arrest at the specific stage of the cell cycle (Hayashi *et al.*, 1981; Ignotz and Massague, 1985; Navre and Ringold, 1989). Following cell/cell interaction and growth arrest at confluence, preadipocytes must receive the appropriate combination of mitogenic and adipogenic signals provided by external inducers (See "Regulation of adipocyte differentiation" section below) to proceed through the required clonal expansion and subsequent differentiation. During clonal expansion, preadipocytes undergo several rounds of mitosis. Certain mitogens, e.g. FGF and PDGF, stimulate postconfluence mitosis but do not induce differentiation (MacDougald and Lane, 1995). Thus, DNA replication *per se* appears to be necessary but not sufficient for the induction of differentiation. Presumably, DNA replication and the accompanying changes in chromatin structure increase the accessibility of *cis*-DNA elements to the *trans*-acting factors which activate (or depress) the transcription of the genes that give rise to the adipocyte phenotype (MacDougald and Lane, 1995). As clonal expansion ceases, preadipocytes lose their fibroblastic characteristics, assume a "rounded-up" appearance, and acquire the morphological and biochemical characteristics of adipocytes. The rounding up of the differentiating preadipocytes may be due to changes in the expression of the cytoskeletal and/or extracellular proteins involved in matrix formation (Spiegelman and Farmer, 1982; Bernlohr *et al.*, 1984; Dani *et al.*, 1990). Soon after the induction of differentiation (3-4 days), many cytoplasmic triacylglycerol-containing vacuoles appear and, after an extended period in culture, coalesce to

form unilocular fat droplet, giving rise to the typical "signet-ring" appearance of mature white adipocytes.

Based on two-dimensional electrophoretic analysis of cell extracts before, during , and after adipose conversion, it has been estimated that the expression of at least 100 proteins is altered within 5 hours after the initiation of differentiation (during clonal expansion), and at least 200 additional proteins are differentially expressed by the time terminal differentiation is achieved (Sidhu, 1979; Sadowski *et al.*, 1990). Many of these proteins whose genes are differentially expressed during preadipocyte differentiation have been identified. Proteins which are regulated during differentiation fall into several classes, including those involved in lipogenesis and lipolysis [e.g. fatty acid synthase (Sul and Paulauskis, 1988), hormone sensitive lipase (Kawamura *et al.*, 1981)], hormone action and signaling [e.g. β-adrenergic receptors (Guest *et al.*, 1990), G-proteins (Watkins *et al.*, 1987)], extracellular matrix structure/function [e.g. tubulin (Bernlohr *et al.*, 1984), collagens (Aratani and Kitagawa, 1988)], secretory proteins with extra-adipocyte functions [e.g. angiotensinogen (Saye *et al.*, 1989), adipsin (Spiegelman *et al.*, 1983)], and most importantly, *trans*-acting factors [C/EBP (Cao *et al.*, 1991), mPPARγ2 (Tontonoz *et al.*, 1994b)], which control adipocyte differentiation.

The dramatic changes in cell phenotype that accompany preadipocyte differentiation largely result from the coordinate transcriptional activation of adipocyte-specific genes and the concomitant silencing of preadipocyte genes which are not required for adipocyte functions. A group of candidate *trans*-acting factors that regulate expression of these genes during differentiation have been identified. Presumably, the second messenger systems triggered by the external differentiation inducers (See "Regulation of adipocyte differentiation" section below) activate (or repress) expression of the *trans*-acting factors, which

include the CCAAT/enhancer binding protein (C/EBP) family (Cao *et al.*, 1991), mouse peroxisome proliferator activator receptor γ2 (mPPARγ2, Tontonoz *et al.*, 1994b), fatty acid activated receptor (FAAR, Amri *et al.*, 1995), adipocyte determination and differentiation factor 1 (ADD1, Tontonoz *et al.*, 1993), and HNF3/forkhead (Enerback *et al.*, 1992). Most of these *trans*-acting factors are differentially expressed during the differentiation of preadipocytes into adipocytes, lending credence to their involvement in the process. The time windows during which each of these factors are expressed must be rigidly controlled to facilitate the orderly progression of the differentiation program. The C/EBP family and mPPARγ2 are the two most extensively characterized transcription factors implicated in preadipocyte differentiation.

The C/EBP family includes C/EBPα, β, and δ (Cao *et al.*, 1991) which are sequence-specific DNA binding proteins that bind to CCAAT boxes (Johnson *et al.*, 1987). These proteins have a basic transcriptional activation domain and an adjoining leucine zipper motif that mediates homo- and hetero-dimerization. These transcription factors may play a general role in establishing and maintaining the differentiated, non-proliferative state (Friedman and McKnight, 1990).

The first indication that C/EBPα regulates adipocyte differentiation was its high level of expression in adipose tissue (Birkenmeier *et al.*, 1989) and its increased expression during adipose conversion of the 3T3-L1 preadipocytes (Christy *et al.*, 1989) due to transcriptional activation of the C/EBPα gene (Christy *et al.*, 1991). C/EBPα is virtually undetectable in proliferating preadipocytes and its expression precedes the expression of adipocyte-specific genes during adipocyte differentiation (Cao *et al.*, 1991). A connection to adipocyte-specific genes was established by the discovery that C/EBPα is the differentiation-induced transcription factor that binds specifically to sites within

the promoters of three adipocyte genes [422/aP2, SCD1 (Christy *et al.*, 1989), and GLUT4 (Kaestner *et al.*, 1990)] that are coordinately expressed during differentiation. It was also shown with intact 3T3-L1 preadipocytes (Chrsty *et al.*, 1989; Kaestner *et al.*, 1990) and with a cell-free transcription system (Cheneval *et al.*, 1991) that C/EBPα transactivates reporter gene transcription from the promoters of these genes. Furthermore, Lin and Lane (1992) found that antisense C/EBPα mRNA inhibited the expression of these genes and adipocyte conversion, demonstrating that C/EBPα is required for adipocyte differentiation. The most direct evidence for the involvement of C/EBPα in adipocyte differentiation has come from the recent work of Freytag *et al.* (1994) who used a retroviral expression system to show that ectopic expression of C/EBPα induced adipogenesis in preadipocyte cell lines and fibroblast cell lines which normally do not develop into adipocytes. Thus, it appears that C/EBPα is not only required but sufficient to induce adipocyte differentiation.

C/EBPβ and C/EBPδ, which were cloned from preadipocyte based on sequence similarities to C/EBPα (Cao *et al.*, 1991), are structurally similar to C/EBPα and bind the same recognition DNA sequence. Although expression of both isoforms is high in proliferating preadipocytes (Cao *et al.*, 1991), the levels decline upon growth arrest at confluence, and remain suppressed until differentiation is induced with external inducers. Expression of these isoforms increases immediately, reaching a maximum by 24 hours, and then decreases, with C/EBPδ declining precipitously and C/EBPβ at a much slower rate. The temporal expression of C/EBPβ and C/EBPδ during differentiation, and the presence of a C/EBP binding site within the C/EBPα promoter have led to the hypothesis that C/EBPβ and/or C/EBPδ may, in part, be responsible for activating transcription of the C/EBPα gene and regulating early differentiation (Cao *et al.*, 1991). Yeh *et al.* (1995) reported that retroviral expression of C/EBPβ

promoted adipose differentiation of cultured NIH-3T3 cells. Expression of C/EBPδ, on the other hand, promoted differentiation of established 3T3-L1 preadipocytes, but not of NIH-3T3 cells. These results differ from those of Freytag *et al.* (1994), who observed that retroviral expression of C/EBPβ did not affect adipocyte differentiation.

PPARγ was first linked to adipogenic gene expression through the analysis of the fat-specific enhancer from the adipocyte P2 (aP2) gene. The key transcriptional regulator of this gene was initially defined as a novel differentiation-dependent nuclear factor, termed ARF6, that binds to two *cis*-acting DNA elements within the enhancer (Graves *et al.*, 1992). The target sequences for ARF6 in the aP2 enhancer exhibit homology to a direct repeat of hormone-response elements (DR-1) spaced by one nucleotide. DR-1 was previously shown to bind heterodimers of retinoid-X receptor (RXR) and PPAR. The endogenous ARF6 complex has also been purified. Chemical sequencing and mass spectral analysis of tryptic peptides derived from the purified polypeptides definitively identified the ARF6 complex as a heterodimer of RXRα and PPARγ (Tontonoz *et al.*, 1994a). To date, PPARγ is the only transcription factor known to be expressed with adipose specificity (Tontonoz *et al.*, 1994b). Expression of PPARγ is induced very early during differentiation of cultured preadipocyte cell lines, suggesting that it functions early in the differentiation process (Chawla *et al.*, 1994a). It has been recognized for many years that clofibrate and related hypolipidemic drugs could potentiate the differentiation of cultured preadipocyte cell lines such as 3T3-L1 (Brandes *et al.*, 1987) and Ob17 (Gharbi-Chihi *et al.*, 1993). More recently, Chawla and Lazar (1994b) demonstrated that a number of peroxisome proliferators caused differentiation of 3T3-L1 preadipocytes in the absence of other inducing agents. These observations, together with the tissue-specificity of PPARγ expression and the

ability of this factor to activate adipocyte-specific enhancers in non-adipose cells, suggests that PPARγ regulates the process of adipocyte differentiation *per se*. Furthermore, Tontonoz *et al.* (1994c) demonstrated that retroviral expression of PPARγ is sufficient to convert a number of uncommitted fibroblast cell lines into differentiation-competent preadipoyctes. PPAR activators, including naturally occurring polyunsaturated fatty acids, promote the differentiation of PPARγ-expressing cells in a dose-dependent manner. Taken together, these results suggest that PPARγ regulates adipocyte gene expression and differentiation in response to one or more endogenous lipid activators.

The studies reviewed above suggest that both PPARγ and C/EBPα are important regulators of adipocyte gene expression and differentiation. When co-expressed in fibroblasts, PPARγ and C/EBPα act synergistically to induce adipogenesis (Tontonoz *et al.*, 1994c). This suggests that the endogenous differentiation program involves the combined action of both proteins. The temporal expression of these two factors suggests that PPARγ2, which is induced very early, may be important for the activation of certain early genes. C/EBPα, which is induced later in the differentiation process, probably functions to coordinate the expression of late genes and may serve to trigger terminal differentiation and/or lipid accumulation.

Regulators of adipocyte differentiation

External inducers are required for the induction of preadipocyte differentiation, although the specific inducers differ somewhat with different cell lines. The conditions required for preadipocyte differentiation were initially evaluated in serum-containing media using the rate and extent of cytoplasmic

triacylglycerol accumulation as criteria. Most protocols that have been developed allow preadipocyte to proliferate to confluence in media containing calf serum. This process is followed by treatment with one or more of the following inducers: isobutylmethylxanthine (IBMX), dexamethasone (DEX), a high concentration of insulin, and fetal bovine serum (Student *et al.*, 1980). Smith *et al.* (1988) determined that IGF-1, rather than insulin which interacts with the IGF-1 receptor at high concentrations, is the factor required for the induction of differentiation. Interestingly, growth hormone, previously identified as a serum factor required for the induction of adipocyte differentiation (Zezulak and Green, 1986), stimulates the synthesis and secretion of IGF-1 by preadipocytes (Doglio *et al.*, 1987). Thus, growth hormone apparently plays at least a partial role in adipocyte differentiation by initiating an autocrine/paracrine mechanism that leads to the secretion of IGF-1. Recent evidence also suggests that long-chain saturated fatty acids induce preadipocyte differentiation (Amri et al., 1994). In addition, both naturally occurring long-chain polyunsaturated fatty acids, such as arachidonic acids, and synthetic hydrophobic peroxisome proliferating agents, such as clofibrate, activate PPARs and induce preadipocyte differentiation (Tontonoz *et al.*, 1994b). These findings implicate four basic second-messenger signaling pathways for the induction of adipocyte differentiation. These include: 1. the IGF-1-activated tyrosine kinase pathway, 2. the glucocorticoid pathway, 3. the cAMP-dependent protein kinase pathway, and 4. the fatty acid activated receptor pathway. Other agents that enhance preadipocyte differentiation include prostaglandin I_2 (Catalioto *et al.*, 1991) and thyroid hormone (Gharbi-Chihi *et al.*, 1991), which are more effective at promoting Ob1771 cell conversion, and ascorbic acid (Ono *et al.*, 1990) and progesterone (Rondinone *et al.*, 1992), which increase 3T3-L1 preadipocyte differentiation.

A number of negative regulators of adipocyte differentiation also have been identified, including tumor necrosis factor α (TNFα; Cornelius *et al.*, 1988; Pape and Kim, 1988; Gimble *et al.*, 1989), transforming growth factor β (TGFβ; Ignotz and Massague, 1985; Gimble *et al.*, 1989), epidermal growth factor (EGF; Serrero, 1987; Serrero and Mills, 1991), basic fibroblast growth factor (bFGF; Hayashi *et al.*, 1981; Navre and Ringold, 1989), platelet-derived growth factor (PDGF; Hayashi *et al.*, 1981; Navre and Ringold, 1988), retinoic acid (RA; Kuri-Harcuch, 1982; Stone and Bernlohr, 1990), and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; Serrero *et al.*, 1992). Although their mode of action is largely uncharacterized, negative regulators may affect the growth arrest, the cell division that initiated clonal expansion of preadipocytes, or the expression of certain genes.

Many inhibitors of adipocyte differentiation are growth factors, such as bFGF, TGFβ, EGF, and PDGF. Preadipocyte proliferation is stimulated by these factors, indicating that the prevention of preadipocytes from entering growth arrest phase may contribute to the inhibition of cell differentiation by these inhibitors. In addition to inhibition of cell differentiation, bFGF also decreases the expression of adipocyte-specific genes in mature fat cells (Navre and Ringold, 1989). Treatment of rats with EGF reduces the number of adipocytes and increases the number of adipocyte precursors, leading to a reduction in fat pad weight (Serrero and Mills, 1991). This *in vivo* response is consistent with that observed *in vitro* (Serrero, 1987). These observations, coupled with the depressed levels of plasma EGF observed in *ob/ob* obese mice compared with their control littermates, indicate a possible endocrine role for EGF in the regulation of adipocyte development (Serrero *et al.*, 1993).

One of the earliest identified inhibitors of adipocyte differentiation was TNFα. TNFα, secreted by activated macrophages, alters lipid and protein metabolism in adipose and skeletal muscle cells *in vivo* (Fong and Lowry, 1990).

Like bFGF, TNFα also suppresses adipocyte-specific genes in mature adipocytes. It was believed that TNFα altered energy balance during infection and did not have a major role in the normal development of adipose tissue. Recent studies, however, suggest a role for the increased production of TNFα by adipocytes in a number of acquired and genetic models of obesity. Expression of TNFα is increased in adipose tissue in obese strains compared with their lean counterparts (Hotamisligil *et al.*, 1993).

Supraphysiological concentrations of RA inhibit adipocyte differentiation and specifically decrease the expression of certain adipocyte mRNAs in differentiated cells (Antras *et al.*, 1991). RA addition, either prior to or after treatment with differentiation inducers, does not affect differentiation, indicating that RA acts at an early stage of differentiation. The effect of retinoids on adipocyte differentiation appears to be concentration-dependent. For example in Ob1771 cells, supraphysiological concentrations are inhibitory, while concentrations close to the K_d value for the RA receptors (1 pM to 10 nM) accelerate adipose differentiation (Safonova *et al.*, 1994). One proposed hypothesis is that at higher concentrations retinoids may bind other nuclear hormone receptors, thus indirectly influencing transcriptional events (Safonova *et al.*, 1994).

Conclusions

Reduction of fat content of the meat animals is an important issue to the entire livestock industry. Accretion of adipose tissue involves both hypertrophy and hyperplasia of adipocytes. During the growth of adipose tissue, hyperplastic growth precedes hypertrophic growth. Therefore, a means to

regulate adipocyte differentiation to prevent hyperplasia would provide an effective way to repress hypertrophic growth and adipose accretion. Thus, fundamental studies of preadipocyte differentiation become necessary for developing physiological schemes designed to hinder fat deposition.

Considerable data on adipocyte differentiation has been obtained from *in vitro* cell culture studies. Cells are propagated as preadipocytes and induced for adipocyte differentiation after treatment with specific inducers. Differentiation is accompanied by induction of several enzymes involved in fatty acid and triacylglycerol metabolism, as well as increased responsiveness to both lipogenic and lipolytic hormones. This acquisition of the adipocyte phenotype occurs concomitantly with changes in mRNAs coding for specific proteins, indicating that adipogenesis is associated with significant reprogramming of gene expression. A number of *trans*-acting factors that regulate adipocyte differentiation have been cloned. Extensive characterization of C/EBP isoforms, α and β, and PPARγ2 have paved the way for the understanding of the molecular basis of fat cell differentiation, contributing a feasible means to control adipose tissue accretion at the molecular level by regulating the expression of specific *trans*-acting factors.

In addition, the studies on the effect of specific hormones or factors on the regulation of adipocyte differentiation has provided insight into the genetic, endocrine, and autocrine/paracrine aspects of adipocyte development. In particular, compounds that inhibit adipocyte differentiation serve as useful tools to control adipose tissue development. Eventually, the goals to control adipose tissue accretion in meat animals will be fullfilled by the combination of the usage of differentiation inhibitors and the manipulation of the expression of differentiation-dependent *trans*-acting factors mentioned above.

Chapter II

CCAAT/ENHANCER-BINDING PROTEIN β IS NOT AFFECTED BY TCDD INHIBITION OF 3T3-L1 PREADIPOCYTE DIFFERENTIATION

Chu-Liang Chen, Ann E. Brodie, and Ching Yuan Hu

Summary

Differentiation of 3T3-L1 preadipocytes is induced by the coordinate activation of *trans*-acting factors in response to inducers. Depending on the time of treatment, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was effective in inhibiting 3T3-L1 preadipocyte differentiation and the expression of differentiation-dependent *trans*-acting factors. Based on glycerol-3-phosphate dehydrogenase activity, the differentiation of 3T3-L1 cells was decreased 70% in cells treated with TCDD before the induction of differentiation, 25% during induction, and not at all after induction. This time dependent inhibition of cell differentiation by TCDD was correlated with the levels of aryl hydrocarbon receptor (AhR). TCDD treatment decreased the mRNA levels of C/EBPα and PPARγ2, but did not affect the mRNA levels of RXRα and RARα. Furthermore, TCDD did not change the mRNA or protein levels of C/EBPβ, which is thought to play a role in inducing C/EBPα and PPARγ2 expression. These results suggest that TCDD inhibited 3T3-L1 preadipocyte differentiation through the AhR pathway, and the change of C/EBPβ mRNA and protein was not involved in reducing mRNA expression of C/EBPα and PPARγ2.

Introduction

The preadipocyte cell line, 3T3-L1, derived from mouse embryo (Green and Kehinde, 1974), is widely used for the *in vitro* study of adipocyte differentiation. These cells have fibroblast-like morphology and are able to divide indefinitely in culture medium containing 10% fetal bovine serum. However, terminal differentiation can be initiated by the addition of the

differentiation inducers (insulin, dexamethasone [DEX], and isobutylmethylxanthine [IBMX]), to confluent (growth-arrested) cells, causing greater than 90% of the cells to become lipid-accumulating fat cells. Several *trans*-acting factors have been identified as participants in the process of preadipocyte maturation (MacDougald and Lane, 1995; Smas and Sul, 1995). Among them, the CCAAT/enhancer-binding protein (C/EBP) family and peroxisome proliferator activated-receptor γ2 (PPARγ2) are under intense investigation.

The C/EBPα isoform of the C/EBP family has been shown to activate the coordinated expression of many adipocyte-specific genes during 3T3-L1 preadipocyte differentiation (MacDougald and Lane, 1995). In addition, overexpression of C/EBPα is sufficient to trigger differentiation of preadipocytes without exogenous hormonal inducers (Lin and Lane, 1994). Furthermore, in the presence of differentiation inducers, antisense C/EBPα RNA used to block expression of C/EBPα, prevented 3T3-L1 preadipocyte differentiation (Lin and Lane, 1992). However, as the expression of C/EBPα occurs relatively late during differentiation, it is unlikely that the expression of C/EBPα is induced directly by the differentiation inducers. Therefore, two other C/EBP isoforms, C/EBPβ and C/EBPδ, were isolated in an effort to identify new protein factors that might play a regulatory role in the differentiation of adipocytes (Coa *et al.*, 1991). The C/EBPβ and C/EBPδ isoforms are induced by IBMX and DEX, respectively (Coa *et al.*, 1991), and appear to be important for the activation of C/EBPα (Yeh *et al.*, 1995). The C/EBP family is also expressed in a number of other tissues, e.g. liver, intestine, and lung. Thus, it seems unlikely that the expression of C/EBP alone can be responsible for adipocyte-specific differentiation.

One member of the peroxisome proliferator-activated receptor family, PPARγ2, is an adipocyte-specific transcription factor (Tontonoz *et al.*, 1994). In

3T3-L1 preadipocytes, differentiation can be initiated by PPAR activators in the absence of differentiation inducers (Chawla and Lazar, 1994). After activation by a PPAR activator, PPARγ2 forms a heterodimer with the retinoid X receptor α (RXRα) (Tontonoz *et al.*, 1994) and *trans*-activates the expression of aP2 mRNA by binding to the adipocyte-specific enhancer in the 5′ flanking region of the aP2 gene (Tontonoz *et al.*, 1994). The levels of PPARγ2 and RXRα are also increased during 3T3-L1 adipocyte differentiation (Chawla and Lazar, 1994). These observations indicate that PPARγ2 is involved in adipocyte differentiation.

2,3,7,8 tetrachlorodibenzo-*p*-dioxin (TCDD), commonly called dioxin, is a world-wide toxic environmental contaminant (Poland and Knutson, 1982) causing hepatic, gastric, and epidermal lesions, thymic atrophy, and teratogenesis. TCDD enters cells by passive diffusion and primarily exerts its effects on gene expression through the aryl hydrocarbon receptor (AhR), which belongs to the helix-loop-helix family of DNA-binding-proteins (Burbach *et al.*, 1992). This AhR-TCDD complex binds to DNA recognition sites and alters the expression of target genes (Landers and Bunce, 1991). TCDD is a very lipophilic and persistent chemical which accumulates in fat tissue (Kohn *et al.*, 1993). In addition, TCDD treatment results in progressive weight loss with depletion of adipose tissue. These observations suggest that adipose tissue development could be affected by TCDD. Fatty acid synthesis (Lakshman et la., 1989) and glucose uptake (Enan *et al.*, 1992) are decreased in adipose tissue from TCDD-treated animals. Recently, TCDD has been shown to inhibit 3T3-L1 preadipocyte differentiation (Phillips *et al.*, 1995). We propose to utilize this inhibitory compound as a tool to study the fundamental mechanisms underlying normal fat cell differentiation.

In this report we investigated the effect of TCDD, introduced at different developmental stages, on the 3T3-L1 preadipocyte differentiation. As there is

only indirect evidence that AhR is involved with TCDD inhibition in 3T3-L1 differentiation, we examined the temporal presence of AhR in relation to the inhibitory effect of TCDD. We investigated whether the inhibition by TCDD affected the mRNA levels of important differentiation-dependent *trans*-acting factors (C/EBPα, C/EBPβ, and PPARγ2). Our intention was to determine whether any changes of the transcription factors could explain the inhibition of preadipocyte differentiation by TCDD.

Materials and Methods

Cell culture

3T3-L1 preadipocytes were seeded at a density of 4×10^4 cells per well in 6-well plates and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum in a 5% CO_2-humidified atmosphere at 37°C. Cells were first grown to confluence. Two days after cells reached confluence, differentiation was induced by treatment with insulin (10 μg/ml), dexamethasone (1 mM), and isobutylmethylxanthine (0.5 mM) in DMEM with 10% fetal bovine serum (day 0). After 2 days (day 2), cells were changed to culture medium lacking dexamethasone and isobutylmethylxanthine. This medium was replaced every 2 days. TCDD (Cambridge Isotope Lab., MA) was prepared in dimethylsulfoxide (DMSO). Control cells were treated with DMSO alone.

Glycerol-3-phosphate dehydrogenase (GPDH) activity assay

To measure GPDH activity, differentiated cells were harvested in ice-cold lysate buffer (0.25 M sucrose, 1 mM EDTA, 1 mM dithiothreitol, 5 mM Tris, pH

7.4). The cells were ultrasonically disrupted with a sonicator at 40 watts for 10 seconds and centrifuged at 13,000 *x g* for 5 minutes to remove cell debris. Enzyme activity was determined as previously described (Akanbi *et al.*, 1994). The activity was presented as milliunits per mg protein. One unit of GPDH activity represents the oxidation of 1 mmol NADH per minute. Protein concentrations were determined by a dye-binding method (Bradford, 1976).

Oil Red O staining

To stain triacylglycerol droplets, adipocytes were fixed in 10% formalin for 10 minutes and stained with a 0.5% solution of Oil Red O in 100% propylene glycol for 15 minutes. After thorough rinsing, cells were mounted in glycerine jelly (Suryawan and Hu, 1993) and photographed.

RNA isolation and Northern blot analysis

Total RNA was isolated by acid guanidinium thiocyanated-phenol-chloroform extraction (Chomczynski and Sacchi, 1987) and subjected to electrophoresis on formaldehyde-agarose gels. Gels were blotted onto nylon membranes by capillary flow, and fixed by UV cross linking (Sambrook *et al.*, 1989). Membranes were prehybridized in 5X SSPE/50% formamide/0.5% SDS/5X Denhardt's solution, and 100 μg/ml denatured salmon sperm DNA at 42°C for 4 hours. For hybridization, membranes were incubated overnight with ^{32}P-labeled cDNA probes in the prehybridization solution plus 10% dextran sulfate. Membranes were then washed twice in 5X SSPE/0.5% SDS at room temperature, twice in 1X SSPE/0.5% SDS at 37°C, and once in 0.1X SSPE/1% SDS at 50°C. The intensity of radioactivity were measured by phosphorimager (Molecular Dynamics, Inc., CA). The aP2 cDNA probe was from Dr. David A. Bernlohr, University of Minnesota. The C/EBPα and C/EBPβ probes were from

Dr. Steve L. McKnight of Tularik Inc.. The PPARγ2 probe was from Dr. Bruce M. Spiegelman of Dana-Farber Cancer Institute. The RXRα and RARα probes were from Dr. Mark Leid, Oregon State University. The 18S oligo (ACGGGCGGTGTGTG/AC) probe was from Dr. Stephen J. Giovannoni, Oregon State University. Probes were labeled by the random primers method with [α-^{32}P]dCTP (DuPont NEN, DE) using a kit from Boehringer Mannheim Co., IN.

Western blot analysis

Whole cell lysates were subjected to sodium dodecyl sulfate (SDS) 10% polyacrylamide gel electrophoresis according to the method of Laemmli (Laemmli, 1970). The separated proteins were electrophoretically transferred to a nitrocellulose membrane by the method of Towbin *et al.* (1979). Nonspecific sites were blocked by incubation of the membranes with 5% non-fat dry milk buffer in TBST buffer (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.1% Tween-20) for 1 hour at room temperature. Specific antibodies were incubated with the membranes for 2 hours at room temperature. After washing with TBST buffer (3 X 5 min), Horseradish peroxidase-conjugated secondary antibody was added and incubated for 1 hour at room temperature. Following this incubation, the membrane was washed with TBST (5 X 5 min) and specific binding was detected by using ECL detection system (Amersham, IL). Hepa 1c1c7 cells were from Dr. James P. Whitlock, Jr. of Standford University. Ah receptor-specific antibody was a gift from Dr. Gary H. Perdew of Purdue University. C/EBPβ-specific antibody was from Santa Cruz Technology, Inc., CA.

Statistical analysis

Data of experiments were analyzed by the analysis of variance procedure of NCSS (NCSS, 1994), with differences between means tested for significance by Fisher's least significant difference.

Results

The optimal inhibitory concentration of TCDD on 3T3-L1 cell differentiation was determined by treating the cells with TCDD at concentrations varying from 10^{-7} to 10^{-11} M from days 0 to 8. Differentiation was assayed by analyzing GPDH activity from cell extracts prepared on day 8. Cells treated with 10^{-7} M or 10^{-8} M TCDD had significantly lower levels of GPDH activity (data not shown). However, treatments with lower concentrations of TCDD had little effect (10^{-10} M) or no effect (10^{-11} M) on GPDH activity. Hence, experiments were conducted with 5×10^{-8} M TCDD.

To determine the critical stage when introduction of TCDD had an inhibitory effect on preadipocyte differentiation, 3T3-L1 cells were exposed to TCDD from days -4 (4 days before adipocyte differentiation was induced), 0, 2, or 4, to day 8. Cells were then either stained with oil red O (Fig. II.1) or harvested and GPDH activity measured (Fig. II.2: A). TCDD treatment from day -4 to day 8, when oil red O staining was performed, significantly inhibited the accumulation of intracellular triacylglycerol compared to the differentiated control cells. However, treatment on or after day 2 resulted in no difference, compared to control cells, as visualized by oil red O staining and confirmed by the GPDH activity (Fig. II.2: A). Compared to the control cells which were exposed to DMSO, the GPDH activity of TCDD-treated cells was decreased by

Figure II.1: Effect of time of TCDD treatment on 3T3-L1 preadipocyte differentiation: Oil red O staining. 3T3-L1 cells were exposed to 50 nM TCDD or DMSO (control; C) from days -4, 0, 2, or 4 to day 8. Oil red O staining was performed on day 8.

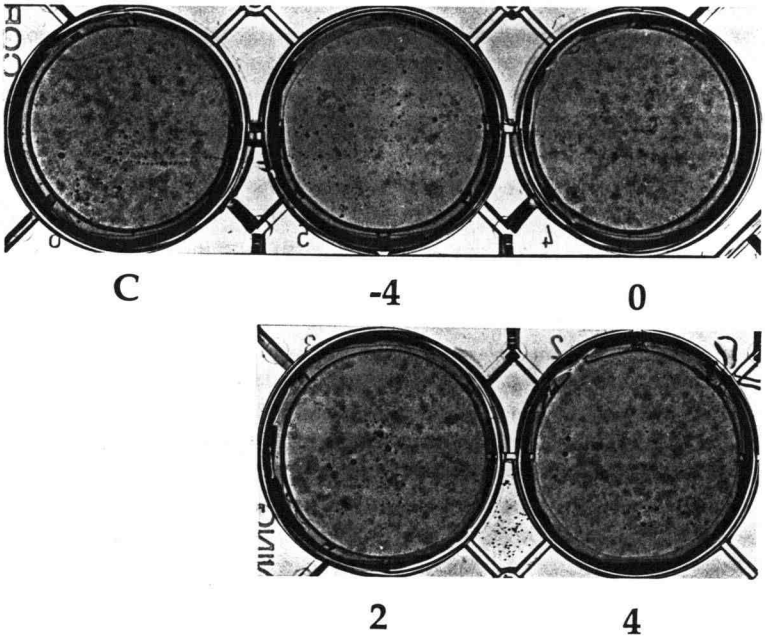
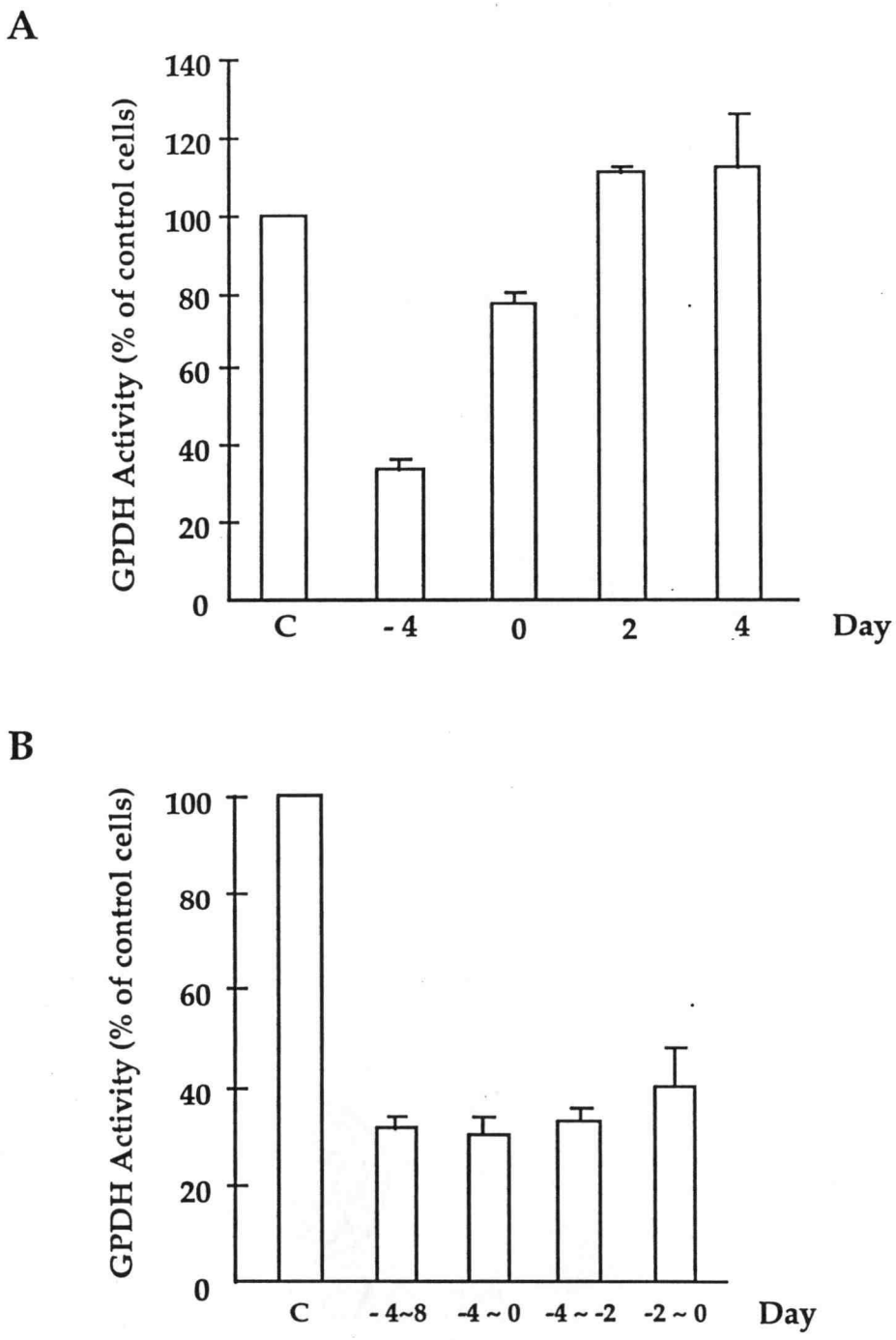


Figure II.2: Effect of time of TCDD treatment on 3T3-L1 preadipocyte differentiation: GPDH activity. 3T3-L1 cells were exposed to 50 nM TCDD or DMSO (control; C) from days -4, 0, 2, or 4 to day 8 (A) or from day -4 to 0, -4 to -2, -2 to 0, or -4 to 8, respectively (B). Cells were harvested and GPDH activity measured on day 8.



70% in cells treated on day -4, and by 25% if treated on day 0. For cells treated with TCDD on or after day 2, GPDH activity was not altered.

Similar studies were performed to specify the length of TCDD treatment required to inhibit preadipocyte differentiation. 3T3-L1 cells were treated with TCDD from day -4 to 0, day -4 to -2, day -2 to 0, or day -4 to 8. TCDD inhibition of GPDH activity was similar with each treatment (Fig. II.2: B). These results indicated that a two-day exposure to TCDD before the onset of differentiation was sufficient to reduce GPDH activity by 70%.

As the AhR mediates the action of TCDD, we determined its expression during cell differentiation using western blot analysis. In a typical example (Figure II.3), AhR was present in 3T3-L1 preadipocytes before the induction of differentiation (i.e. days -4, -2, and 0). The addition of differentiation inducers on day 0 dramatically decreased the expression of AhR to an undetectable level by day 2, and it remained absent through day 8. Surprisingly, in cells treated two days with TCDD, from day -4 to -2, the AhR was significantly down-regulated, remaining so to day 0. The addition of differentiation inducers on day 0 further decreased the expression of AhR to an undetectable level (Fig. II.3).

To further confirm that TCDD inhibited the differentiation of 3T3-L1 preadipocytes, we examined the expression of an adipocyte-specific marker, adipose P2 (aP2) mRNA. Northern blot analysis indicated that TCDD-treated cells expressed a lower level of aP2 mRNA than control cells (Fig. II.4).

The effect of TCDD on the expression of adipocyte differentiation-dependent transcription factors was subsequently investigated. A typical example, as shown in Figure II.4, demonstrated that the mRNA levels of C/EBPα and PPARγ2 in TCDD-treated cells were significantly lower than those in control cells. However, the mRNA levels of C/EBPβ, RXRα, and retinoic acid receptor α (RARα) were not altered by TCDD treatment.

Figure II.3: AhR expression during 3T3-L1 preadipocyte differentiation. 3T3-L1 cells were treated (+TCDD) or not treated (-TCDD) with 50 nM TCDD from day -4 to day -2. Whole cell lysates obtained on days -4, -2, 0, 2, 5, and 8 were analyzed by western blot with the antibody to AhR. Cell extracts prepared from Hepa cells (+) were used as a positive control for the AhR protein.

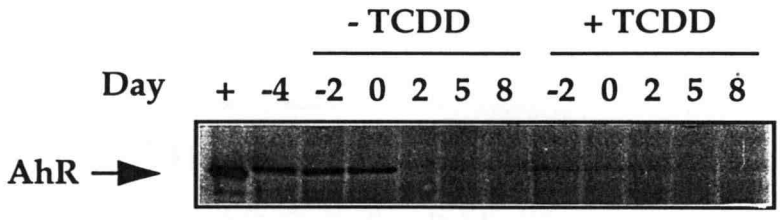
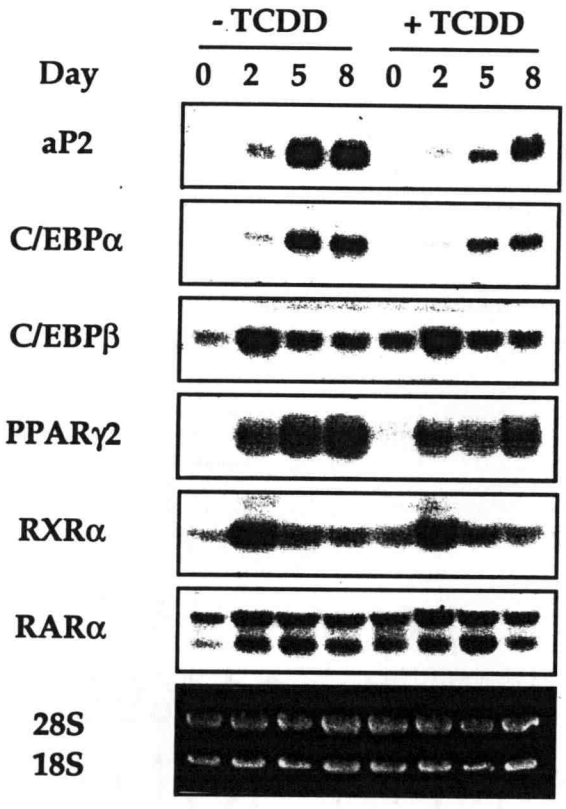


Figure II.4: Effect of TCDD on the mRNA levels of differentiation-dependent markers during 3T3-L1 preadipocyte differentiation. 3T3-L1 cells were treated with 50 nM TCDD (+TCDD) or DMSO (-TCDD) from day -4 to day -2. Total RNA isolated on days 0, 2, 5, and 8 was analyzed by northern blot with the indicated probes. Equal amounts of RNA were loaded per lane as judged by ethidium bromide staining of 18S and 28S.



The mRNA expression of C/EBPβ induced by IBMX alone also was not affected by TCDD treatment (Fig. II.5). Both control and TCDD-treated confluent 3T3-L1 cells were treated with IBMX alone or with the complete set of differentiation inducers as a positive control. After four hours, total RNA was isolated and subjected to northern blot analysis. The mRNA level of C/EBPβ was increased substantially after the addition of the complete set of differentiation inducers and increased somewhat less by treatment with IBMX alone. However, there was no difference in mRNA level of C/EBPβ between TCDD-treated and control cells, induced by either IBMX alone or the complete set of differentiation inducers.

It has been suggested that the C/EBPβ isoform induced by IBMX activates the expression of C/EBPα gene (Cao et al., 1991, Yeh *et al.*, 1995). As shown above, TCDD treatment inhibited the expression of C/EBPα mRNA, but the expression of C/EBPβ mRNA was not affected. Although the mRNA of C/EBPβ was unaffected, the C/EBPβ protein could be altered by the TCDD treatment. However, consistent with the pattern of mRNA expression, the protein level of C/EBPβ, as measured by western blot, was similar in TCDD-treated and control cells during differentiation (Fig. II.6).

Discussion

The maximal inhibitory effect of TCDD on 3T3-L1 preadipocyte differentiation was observed when TCDD was applied before induction of differentiation (i. e. before day 0). Cell differentiation was only slightly inhibited when TCDD treatment occurred during exposure to differentiation inducers (day 0 to day 2) and was not affected when applied after the treatment with

Figure II.5: Effect of TCDD on the IBMX induced expression of C/EBPβ mRNA. 3T3-L1 cells were treated with DMSO (lanes 1-3) or 50 nM TCDD (lanes 4-6) from day -4 to day -2. On day 0, cells were exposed to media alone (-; lanes 1 and 4), media plus IBMX (I; lanes 3 and 6), or the complete set of differentiation inducers (+; lanes 2 and 5). Total RNA was isolated and analyzed by northern blot with C/EBPβ probe. The blot was re-probed with labeled 18S oligo probe to serve as an internal control.

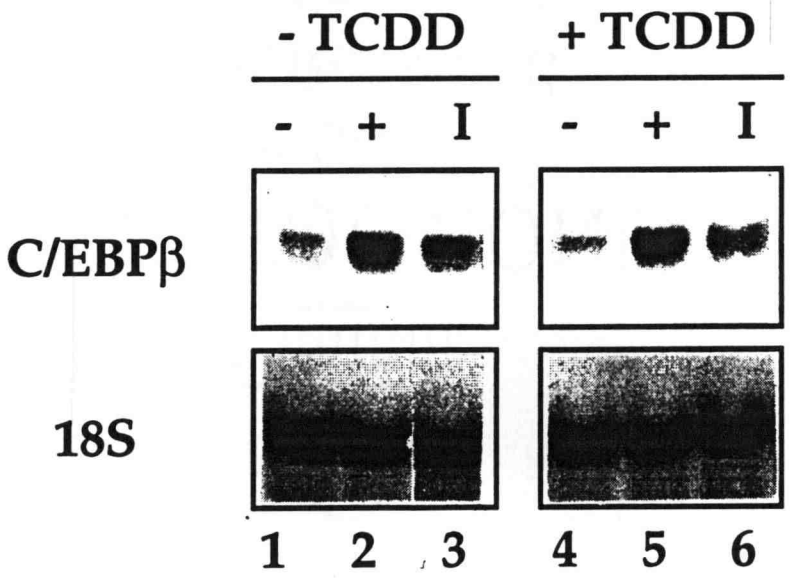
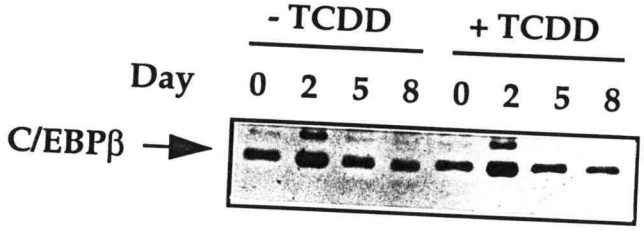


Figure II.6: Effect of TCDD on the protein levels of C/EBPβ during 3T3-L1 preadipocyte differentiation. 3T3-L1 cells were treated with 50 nM TCDD (+TCDD) or DMSO (-TCDD) from day -4 to day -2. Whole cell lysates obtained on days 0, 2, 5, and 8 were analyzed by western blot with the antibody to C/EBPβ.



differentiation inducers. This distinct pattern of TCDD inhibition on 3T3-L1 cells at different stages of development indicates different levels of sensitivity of the cells during development. One explanation for this pattern of sensitivity could be the change in the presence of the AhR.

AhR was expressed in 3T3-L1 preadipocytes before the induction of differentiation. With the addition of differentiation inducers to the cells for two days, the level of AhR decreased to an undetectable level, where it stayed throughout the remaining culture period. There are other examples of the cellular level of AhR changing during growth and differentiation. In HaCaT-keratinocytes, for instance, the mRNA level of the AhR was shown to increase during development from proliferating basal cells to cells that have entered the differentiation pathway after withdrawal from the cell cycle (Wanner *et al.*, 1995). It is important to note that the degree and time of inhibition of differentiation by TCDD treatment of 3T3-L1 cells was corresponded to the pattern of the presence of AhR during cell development. Even though a second pathway not involving AhR, as proposed by Enan and Matsumura (1995), might mediate TCDD inhibition on 3T3-L1 cell differentiation, this seems unlikely. Not only is the inhibition and AhR level correlated, but a TCDD antagonist, which competes with TCDD for binding to the AhR, is able to block the TCDD-induced inhibition of 3T3-L1 cell differentiation (Phillips *et al.*, 1995). Our result suggests that the level of the AhR accounts for the distinct periods of response of the 3T3-L1 cells to TCDD inhibition.

In addition, we observed that the AhR was down-regulated by TCDD treatment in 3T3-L1 cells (Fig. 3). Ligand-dependent downregulation of the AhR was also demonstrated in Hepa 1c1c9 cells (Giannone *et al.*, 1995). Giannone *et al.* (Giannone *et al.*, 1995) suggested that the downregulation of AhR by TCDD was

the result of protein degradation by a short-lived protease; however, the actual mechanism remains unknown.

TCDD decreased the mRNA levels of C/EBPα and PPARγ2, the most characterized differentiation-dependent transcription factors in 3T3-L1 cells. PPARγ2 forms a heterodimer with the RXRα upon activation by a PPAR activator (Tontonoz *et al.*, 1994). The RARα also heterodimerizes with RXRα after activation by retinoic acids, which have been shown to prevent 3T3-L1 preadipocyte differentiation (Stone and Bernlohr, 1990). Our results indicated that TCDD did not affect the levels of RARα or RXRα mRNA. This suggests that TCDD decreases formation of the PPARγ2/RXRα complex by decreasing PPARγ2 expression and that the retinoic acid signaling pathway might not be involved in the action of TCDD.

It has been suggested that C/EBPβ plays a role in inducing the expression of C/EBPα (Yeh *et al.*, 1995) and PPARγ2 (Wu *et al.*, 1995). Therefore, we would expect to see the C/EBPβ level reduced by TCDD treatment. However, our results showed that both the mRNA and protein levels of C/EBPβ did not change in the cells exposed to TCDD, compared to control cells. This observation indicates that unknown factors, which can regulate the expression of C/EBPα or PPARγ2, may be affected by TCDD treatment of the 3T3-L1 cells. As an example, post-translational modification of C/EBPβ by TCDD may cause the decreased expression of C/EBPα and PPARγ2. TCDD has been shown to decrease the formation of estrogen receptor/estrogen response element complex without significantly affecting the steady state mRNA levels of estrogen receptor in MCF-7 human breast cancer cells (Wang *et al.*, 1993). Therefore, it is possible that TCDD altered the binding of C/EBPβ to its DNA response element to cause the reduction of cell differentiation. Since TCDD was only applied to 3T3-L1 preadipocytes before the induction of differentiation, but had a profound effect

on cell differentiation which occurred much later, TCDD appears to be a good tool for identifying and defining the interactions of various *trans*-acting factors in the early stages of 3T3-L1 adipocyte differentiation.

In summary, AhR plays an important role in the TCDD inhibition of 3T3-L1 preadipocyte differentiation. Although C/EBPβ has been identified as an important factor for the activation of preadipocyte differentiation, it seems that the TCDD effect does not involve a change in C/EBPβ mRNA or protein.

Chapter III

EXPRESSION OF PROTEIN KINASE C ISOZYMES DURING 3T3-L1 PREADIPOCYTE DIFFERENTIATION

Chu-Liang Chen and Ching Yuan Hu

Summary

Protein kinase C (PKC) has been shown to be important for the regulation of adipocyte differentiation. In this report, we examined the expression of PKC isozymes during the differentiation of an established adipocyte cell line, 3T3-L1. Four PKC isozymes, PKC α, βI, ε, and ζ were identified in the 3T3-L1 adipocytes using western blot analysis. Treating the cells with Phorbol 12-myristate 13-acetate (PMA), an activating ligand for PKC, caused all of these isozymes to be translocated from the cytosol to membrane and to be subsequently down-regulated. The levels of PKC α, βI, and ζ decreased during adipocyte differentiation, while the amount of PKC ε increased. PMA inhibited 3T3-L1 preadipocyte differentiation as well as maintaining basal levels of PKC α, βI, ζ, and ε. Staurosporin, a PKC inhibitor, enhanced 3T3-L1 preadipocyte differentiation. The patterns of the protein levels of PKC isozymes were similar in the presence or absence of staurosporin. These results indicate the involvement of PKC isozymes in adipocyte differentiation.

Introduction

Cell differentiation is controlled by the modulation of different signaling pathways in response to extracellular factors. The activation of protein kinase C (PKC), a serine/threonine kinase, has been demonstrated to play an important role in the signal transduction pathways through which hormones, growth factors, and other extracellular stimuli activate or inhibit differentiation of target cells (Clemens *et al.*, 1992, Asaoka *et al.*, 1992). For

instance, following exposure to phorbol ester (Phorbol 12-myristate 13-acetate, PMA), a substance that activates PKC, the HL-60 promyelocytic leukemia cell line undergoes differentiation (Vandenbark *et al.*, 1984). And conversely, the induction of neuroblastoma cell differentiation is observed when PKC activity is inhibited by PKC inhibitors (Felipo *et al.*, 1990) or PKC-specific antibodies (Leli *et al.*, 1992).

The 3T3-L1 preadipocyte cell line established by Green and Kehinde (1974) has been used as a model for the study of adipocyte differentiation. In the past two decades, an outline of the process of adipocyte differentiation has been established through the characterization of hormonal regulators and signaling pathways involved in the expression of genes that regulate 3T3-L1 preadipocyte differentiation. Three inducers are required for induction of 3T3-L1 preadipocyte differentiation in serum-containing culture system: insulin-like growth factor-1 (IGF-1), glucocorticoid, and isobutyl-methyl-xanthine (IBMX). IGF-1 triggers the IGF-1-activated tyrosine kinase pathway, glucocorticoid binds to its receptors and activates a receptor/transcription factor pathway, and IBMX elevates the concentration of cyclic-AMP (cAMP) and initiates the cAMP-dependent protein kinase pathway. In addition, fatty acids have been shown to increase adipocyte differentiation by the fatty acid-activated receptor pathway (MacDougald and Lane, 1995).

PKC is activated by Ca^{+2}, phospholipids, and diacylglycerol (DAG), which are produced from the receptor-mediated hydrolysis of membrane phospholipids (Asaoka *et al.*, 1992). Activation of PKC is usually associated with their translocation from the cytoplasm to the plasma membrane, and is often followed by down-regulation by proteolytic degradation (Nishizuka, 1992). The tyrosine kinase pathway is thought to activate phospholipase C and produce DAG, which activates the PKC signaling pathway (Nishizuka,

1992). PKC has been shown to be associated with insulin action in mature rat adipocytes (Arnold *et al.*, 1993). In rat primary preadipocyte serum-free culture, PKC inhibitors enhance adipocyte differentiation (Shinohara *et al.*, 1994). In 3T3-L1 preadipocytes, both PKC α and β activities are decreased when cells differentiate in culture, and the inactivation of PKC by staurosporin enhances differentiation (Ueda *et al.* 1991). In addition, chronic hypoxia impairs 3T3-L1 preadipocyte differentiation, which was shown to be associated with the sustained activation of PKC (Sahai *et al.* 1994). Therefore, it is very likely that the PKC pathway is involved in the process of adipocyte differentiation.

In this study, we intended to learn whether expression of PKC is correlated with 3T3-L1 preadipocyte differentiation. We identified the isozymes of PKC in 3T3-L1 adipocytes using western blot analysis. PMA, a DAG analog, causes translocation, activation, and subsequent down-regulation of PKC (Kikkawa *et al.*, 1989) and has been a useful tool for studying signal transduction pathways involving PKC. Therefore, the responses of PKC to PMA treatment in 3T3-L1 preadipocytes were characterized. We then performed a time-course study to examine whether the protein levels of PKC changed during differentiation. Furthermore, the effects of PMA or staurosporin on 3T3-L1 preadipocyte differentiation were examined by measuring glycerol-3-phosphate dehydrogenase (GPDH) activity. Finally, we determined whether the modulation of cell differentiation by PMA or staurosporin treatments was associated with alteration of the protein levels of PKC during differentiation.

Materials and Methods

Cell culture

3T3-L1 preadipocytes (American Type Culture Collection, MD) were seeded at a density of 4 X 10^4 cells per well in 6-well plates and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone, UT) in a 5% CO_2-humidified atmosphere at 37°C. Cells were first grown to confluence. Two days after cells reached confluence, differentiation was induced by treatment with insulin (10 μg/ml), dexamethasone (1 mM), and isobutylmethylxanthine (0.5 mM) in DMEM with 10% fetal bovine serum (day 0). After 2 days (day 2), cells were changed to culture medium lacking dexamethasone and isobutylmethylxanthine. This medium was replaced every 2 days. Phorbol 12-myristate 13-acetate (PMA) and staurosporin (Sigma, MO) were prepared in dimethylsulfoxide (DMSO). Control cells were treated with DMSO alone.

Glycerol-3-phosphate dehydrogenase (GPDH) activity assay

To measure GPDH activity, differentiated cells were harvested in ice-cold lysate buffer (0.25 M sucrose, 1 mM EDTA, 1 mM dithiothreitol, 5 mM Tris, pH 7.4). The cells were ultrasonically disrupted with a sonicator at 40 watts for 10 seconds and centrifuged at 13,000 *x g* for 5 minutes to remove cell debris. Enzyme activity was determined as previously described (Akanbi *et al.*, 1994). The activity was presented as milliunits per mg protein. One unit of GPDH activity represents the oxidation of 1 mmol NADH per minute. Protein concentrations were determined by a dye-binding method (Bradford, 1976).

Oil red O staining

To stain triacylglycerol droplets, adipocytes were fixed in 10% formalin for 10 minutes and stained with a 0.5% solution of Oil Red O in 100% propylene glycol for 15 minutes. After thorough rinsing, cells were mounted in glycerine jelly (Suryawan and Hu, 1993) and photographed.

Preparation of cell extracts

Cells were recovered in ice-cold homogenizing buffer (20 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 50 mM β-mercaptoethanol, 1 mM PMSF, 200 μg/ml of leupeptin, 5 mM EDTA, 2 mM EGTA, 10 mM benzamidine) from culture plates after washing with PBS. These cells were homogenized using a Dounce homogenizer and centrifuged at 100,000 *x g* for 20 minutes at 4 °C. Supernatants were used as the cytosolic fractions. To obtain membrane fractions, cell pellets were extracted with homogenizing buffer containing 1% Triton X-100. After one hour incubation at 4 °C, the samples were centrifuged at 100,000 *x g* for 20 minutes and the supernatants were collected as solubilized membrane fraction. Protein concentrations of individual samples were determined by the method of Bradford (1976) using BSA as a standard.

Western blot analysis

Protein samples were subjected to sodium dodecyl sulfate (SDS)10% polyacrylamide gel electrophoresis according to the method of Laemmli (1970). The separated proteins were electrophoretically transferred to a nitrocellulose membrane by the method of Towbin *et al.* (1979). Nonspecific sites were blocked by incubation of nitrocellulose membranes with either 5% non-fat dry milk or 3% BSA in TBST buffer (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.1% Tween-20) for 1 hour at room temperature. Anti-PKC isozyme

specific antibodies were incubated with the membranes for 2 hours at room temperature. After washing with TBST buffer (3 X 5 min), horseradish peroxidase (HRP) conjugated secondary antibody (Sigma Chemical Co., MO) was added and incubated for 1 hour at room temperature. Following incubation, the membrane was washed with TBST (5 X 5 min) and the specific binding of anti-PKC antibodies was assayed using the ECL detection system (Amersham, IL). Anti-PKC α, γ, δ, and ε isozyme-specific antibodies were from GIBCO (NY). Anti-PKC βI and ζ isozyme-specific antibodies were from Santa Cruz Technology, Inc. (CA).

Statistical analysis

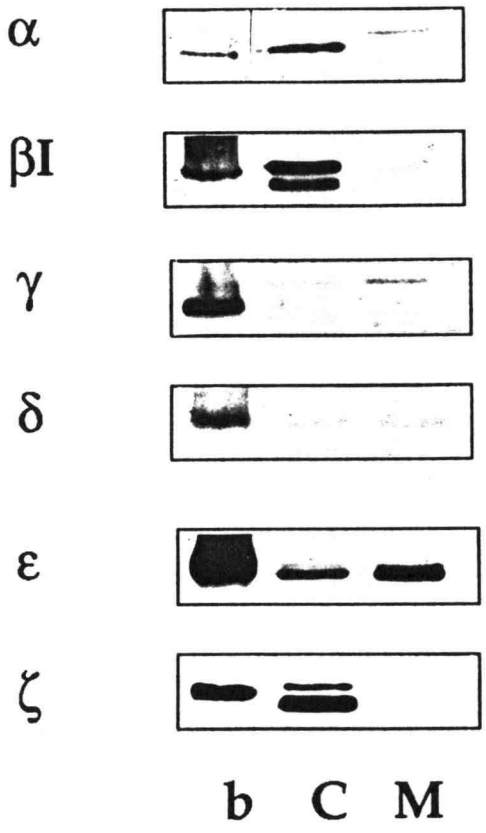
Data of experiments were analyzed by the analysis of variance procedure of NCSS (18), with differences between means tested for significance by Fisher's least significant difference.

Results

Identification of PKC isozymes in 3T3-L1 adipocytes

PKC isozymes in 3T3-L1 adipocyte cytosolic and membrane fractions were tested from adipocytes 8 days after differentiation inducers were added to confluent cells. The cell extract from rat brain was used as a positive control for the PKC polypeptides. As shown in Figure III.1, PKC isozyme-specific antibodies detected PKC α, βI, ε, and ζ, but not isozymes γ and δ. The binding of antibodies to PKC on the blots was blocked by competitive peptides provided by the manufacturer (data not shown).

Figure III.1: Identification of PKC isozymes in 3T3-L1 adipocytes. Cytosolic (C) and membrane (M) proteins were isolated from adipocytes 8 days after differentiation inducers were added to confluent cells. Different PKC isozymes were identified by PKC α, βI, γ, δ, ε, or ζ isozyme-specific antibodies using western blot analysis on SDS-10% polyacrylamide gels. Cell extracts prepared from rat brain (b) were used as a positive control for the PKC polypeptides.



Translocation and down-regulation of PKC isozymes of 3T3-L1 preadipocytes in response to PMA treatment

PMA was administered to confluent cells for different times to examine whether PKC α, βI, ε, and ζ in 3T3-L1 preadipocytes responded similarly. As shown in Figure III.2, in cells that were not treated with PMA, the PKC α was detected predominantly in the cytosolic fractions, PKC ε, in the membranes, and PKC βI and ζ equally in both fractions. Shortly after the addition of PMA, these PKC isozymes were detected in the membrane fractions. All four PKC isozymes tested were depleted from the cytosolic fraction and translocated to the membrane fraction within 10 minutes. Then the amounts of both PKC α and βI in the membrane fractions gradually decreased to the original levels within 6 hours after PMA treatment. However, PKC ε and ζ appeared to be less sensitive to PMA, because trace quantities of these enzymes were still detectable after 21 hours of treatment.

Effects of PMA and staurosporin on 3T3-L1 preadipocyte differentiation

Cells were treated with five different concentrations of PMA (0, 10 nM , 100 nM, 1 mM, or 10 mM) or staurosporin (0, 1 nM, 10 nM, 100 nM, or 1 mM) from 1 day before the induction of differentiation to day 8. At day 8, cell extracts were prepared and GPDH activity, an indicator of adipocyte differentiation, measured. The results indicated that at the concentration as low as 10 nM, PMA was able to inhibit GPDH activity by almost 80%. The GPDH activity was further inhibited by higher doses of PMA (Fig. III.3). On the contrary, the inclusion of staurosporin at the concentration of 1 nM increased the GPDH activity by 180 % (Fig. III.3). However, when 10 nM or higher concentrations of staurosporin were applied, cytotoxic effects were observed by visual analysis under microscope.

Figure III.2: Translocation and down-regulation of PKC isozymes of 3T3-L1 preadipocytes in response to PMA treatment. 3T3-L1 preadipocytes were grown to confluence and 500 nM PMA was administered for 0, 10 minutes, 0.5 , 1, 3, 6, or 21 hours. Cytosolic and membrane fractions were subsequently prepared. The proteins were resolved in SDS-10% polyacrylamide gels and analyzed by western blot analysis using PKC α, βI, ε, or ζ isozyme-specific antibodies. Cell extracts prepared from rat brain were used as a positive control for the PKC polypeptides (lane b).

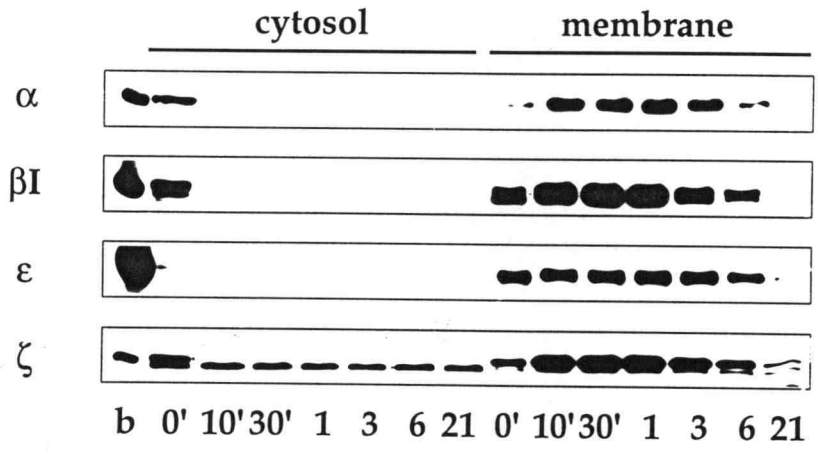
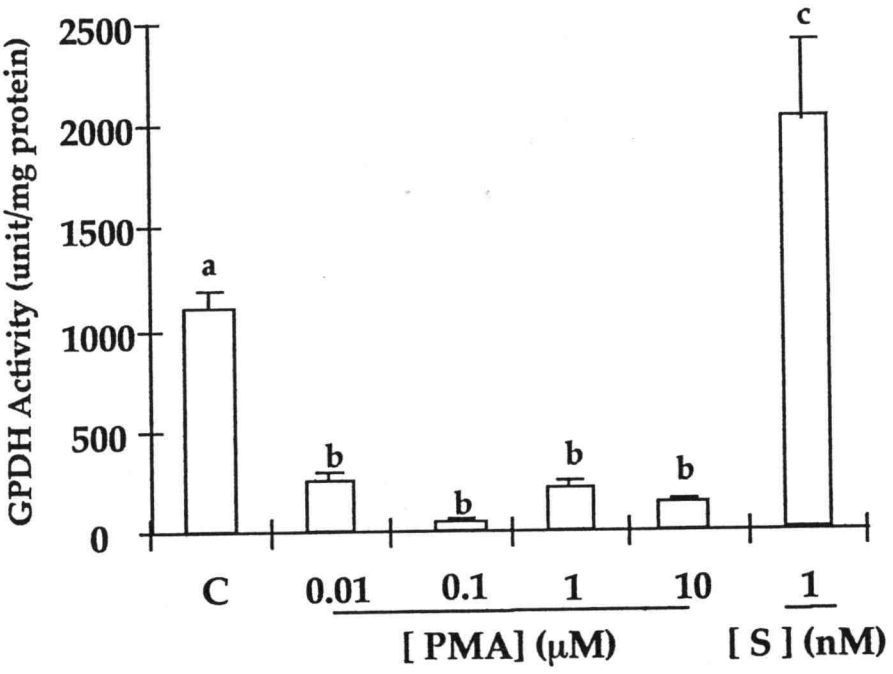


Figure III.3: Effects of PMA and staurosporin on 3T3-L1 preadipocyte differentiation. Confluent 3T3-L1 preadipocytes were treated with DMSO (control; C), PMA (10 nM, 100 nM, 1 mM, or 10 mM) or staurosporin (S) (1 nM) from 1 day before the induction of differentiation to day 8 when cells were harvested. Cell extracts were prepared and GPDH activity measured. Means (bars) with different superscripts are different ($p<0.05$).



In PMA-treated cells, which were shown above to exhibit substantially reduced levels of adipocyte differentiation, cell morphology remained fibroblastic and accumulation of intracellular triacylglycerol was inhibited as visualized by oil red O staining (Fig. III.4). However, staurosporin enhanced the accumulation of triacylglycerol droplets significantly compared to the control differentiated cells.

Expression of PKC isozymes during 3T3-L1 preadipocyte differentiation

Confluent cells were stimulated to differentiate under normal conditions. In undifferentiated cells at day 0, PKC α, βI, and ζ were found in both cytosolic and membrane fractions (Fig. III.5). Expression of cytosolic isozymes decreased during differentiation. The levels of plasma membrane-associated PKC α, βI, and ζ declined dramatically after two day's exposure to differentiation inducers. On the other hand, the majority of PKC ε was detected in the membrane fraction at day 0 and its expression increased slightly in the plasma membrane fraction and dramatically in the cytosolic fraction during differentiation (Fig. III.5). A higher molecular weight band found in the blot of PKC α at day 5 and 8 (Fig. III.5) was a non-specific binding of antibodies because the binding of antibodies to PKC was not blocked by competitive peptides provided by the manufacturer (data not shown).

Expression of PKC isozymes during differentiation of 3T3-L1 preadipocytes treated with PMA or staurosporin

Because both PMA and staurosporin are capable of affecting the PKC signaling pathway, we investigated whether the effects of PMA or staurosporin on cell differentiation were correlated with alteration of the profile of PKC levels. Confluent 3T3-L1 preadipocytes were treated with

Figure III.4: Fat accumulation of 3T3-L1 adipocytes after treatment with PMA or staurosporin. Confluent 3T3-L1 preadipocytes were treated with solvent alone (A), PMA (100 nM) (B), or staurosporin (1 nM) (C) from one day before the induction of differentiation to day 8. On day 8, cells were fixed in 10% formalin. Triacylglycerol droplets in adipocytes were visualized by oil-red O staining. Confluent cells without induction of differentiation were used as a negative control (D).

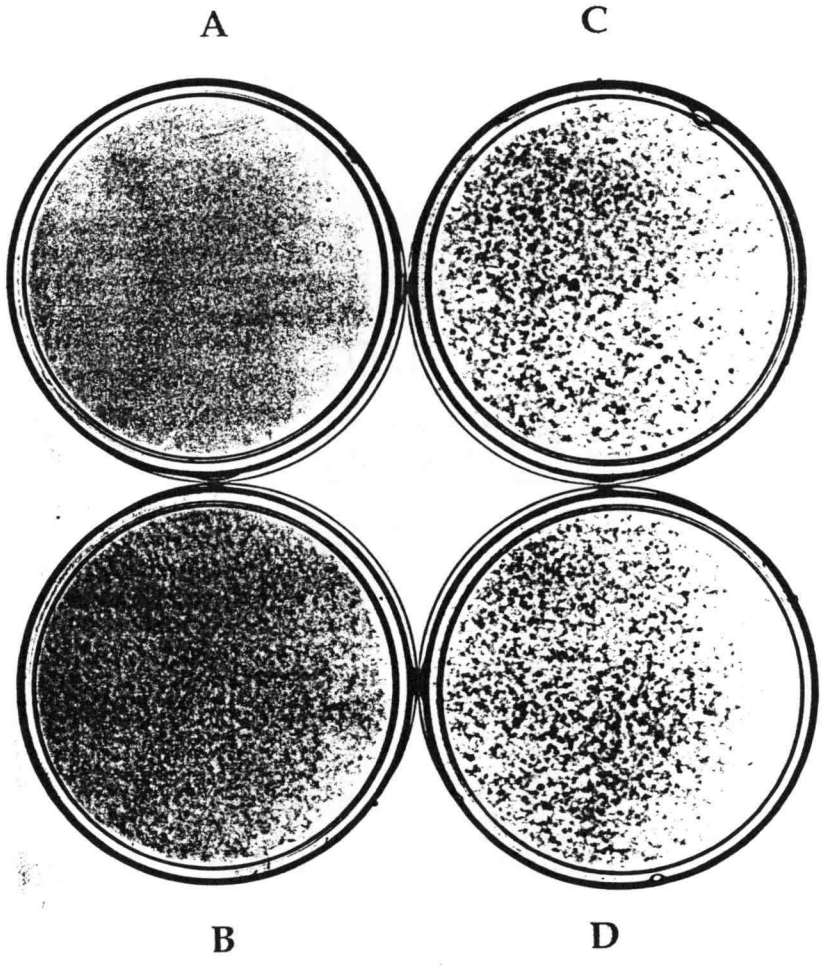
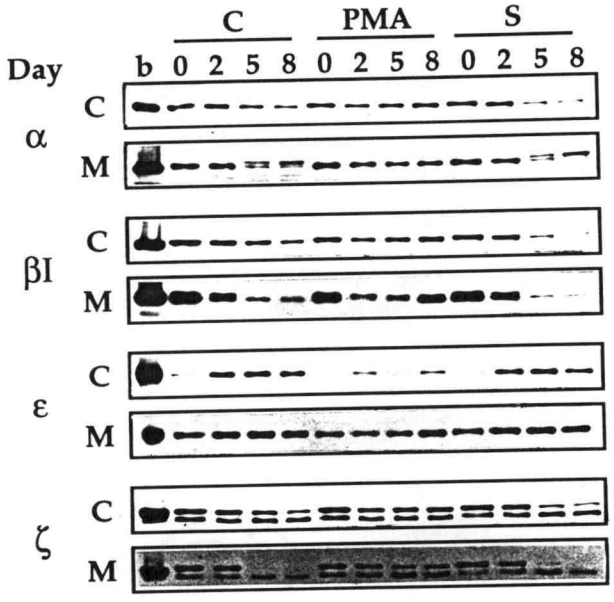


Figure III.5: Expression of PKC isozymes during the differentiation of normal, PMA-treated, or staurosporin-treated 3T3-L1 cells. Confluent 3T3-L1 preadipocytes were treated with DMSO (control), 100 nM PMA, or 1 nM staurosporin (S) from 1 day before the induction of differentiation to day 0, 2, 5, or 8 when cells were harvested. Western blot analyses using PKC α, β1, ε, or ζ isozyme-specific antibodies were carried out following the preparation of the cytosolic (C) and plasma membrane (M) fractions. Cell extracts prepared from rat brain were used as a positive control for the PKC polypeptides (+).



either PMA or staurosporin from 1 day (day -1) before the induction of differentiation to days 0, 2, 5, or 8 when cells were harvested. Compared to control cells, there were relatively low levels of PKC α, βI, and ζ in the membrane fractions of PMA-treated cells at day 0 (Fig. III.5), consistent with the down-regulation by PMA on PKC. However, the levels of PKC α, βI, and ζ were stabilized in the PMA-treated cells and did not decline on day 5 and 8 as those observed in control cells. In PMA-treated cells, the increase of PKC ε was inhibited in both membrane and cytosolic fraction. There was no significant difference in the pattern of PKC levels between control cells and staurosporin-treated cells. However, compared to control cells, the expression of PKC α, βI, and ζ was slightly lower in the staurosporin-treated cells (Fig. III.5).

Discussion

Based on the primary structure of the polypeptides, at least twelve PKC isozymes (α, βI, βII, γ, δ, ε, ζ, η, θ, ι, λ, and μ) have been identified in different species, tissues and cell lines (Dekker and Parker, 1994). They can be divided into two major groups: the Ca^{+2}-dependent or conventional PKC, such as PKC α, βI, βII, and γ, and the Ca^{+2}-independent or novel PKC, such as PKC δ, ε, ζ, η, and λ. In this study, we demonstrated the presence of PKC α, βI, ε, and ζ in 3T3-L1 adipocytes. However, PKC γ and δ, which were detectable in rat adipocytes (Farese *et al.*, 1992), were not found in our study. Part of our findings confirm a previous study which reported PKC α and β, but not PKC γ, in 3T3-L1 cells (Ueda *et al.* 1991).

A feature of the regulation of PKC is its translocation to membranes and subsequent down-regulation following interaction with phorbol ester (Kikkawa et la., 1989; Borner *et al.*, 1992). To further characterize PKC α, βI, ε, and ζ in 3T3-L1 preadipocytes, we investigated the response of these PKC isozymes to PMA treatment. All four expressed PKC isozymes reacted similarly with respect to PMA-induced redistribution from the cytosol to membrane fraction in the 3T3-L1 adipocytes, in spite of the fact that the relative distribution of each individual PKC isozyme was quite different before PMA treatment (Figure 2). As to down-regulation of PKC, PKC ε and ζ appeared to be less responsive to PMA treatment than PKC α and βI. This may reflect a difference in the factors that PKC α and βI require for activation from those required by PKC ε and ζ (Nishizuke, 1992). Standaert *et al* (1993) reported that in rat adipocytes chronic treatment of PMA had little effect on the PKC ε level. In addition, it has previously been suggested that PKC ζ does not bind to DAG/phorbol esters and, therefore, is not activated and down-regulated by PMA treatment (Ono *et al.*, 1989). However, our findings, and other studies which reported PKC ζ responses to PMA treatment in human platelets (Crabos *et al.*, 1991) and rat-6 (R6) fibroblasts (Borner *et al.*, 1992), suggested cell-specific responses of PKC ζ to PMA treatment. What distinguishes these types of cells with respect to PKC ζ translocation remains to be determined. It is of interest that in rat adipocytes, acute PMA treatment provokes little or no change in PKC ζ expression, however, with prolonged (20 h) PMA treatment, there is a substantial loss of PKC ζ (Farese *et al.*, 1992). As more cell types and species are tested for the effects of PMA on PKC redistribution and down-regulation, it appears that the response of the PKC to PMA may not be an intrinsic property of the isozymes.

Long term changes in the activity of the PKC pathway achieved by modulating the levels of PKC isozymes, as exemplified by the action of growth factors, may regulate cell differentiation (Clemens *et al.*, 1992). In human promyelocytic leukemia (HL-60) cells, expression of PKC α, β, and γ increases during differentiation (Markowske *et al.*, 1988). An elevation of PKC α during differentiation was also observed in murine hemopoietic cells (Mischak *et al.*, 1991) and rat aortic vascular smooth muscle cells (Haller *et al.*, 1994). In contrast, a decrease of PKC ε seems to accompany the differentiation of mouse erythroleukemia cells (Powell *et al.*, 1992), and the differentiation of a neuroblastoma cell line (SH-SY5Y) is promoted by an inactivation of PKC α and ε (Leli *et al.*, 1992). Therefore, the role of PKC in cell differentiation may depend on the cell types. Recently, a study conducted in F9 teratocarcinoma cells showed that the mRNA and protein levels of PKC α increased, whereas those of PKC β and PKC γ become undetectable during F9 cell differentiation (Kindregon *et al.*, 1994). In our study, increased PKC ε protein level was observed during 3T3-L1 cell differentiation, primarily in the cytosol fraction (Figure 5). On the other hand, in the membrane fraction PKC α, βI, and ζ protein levels decreased during differentiation, becoming nearly undetectable in the late stage (day 5 and 8) of cell differentiation. These results are consistent with the suggestion that there are cell-specific patterns of expression of PKC and that the amount, number, and distribution of PKC isozymes also vary in a given cell during cell differentiation.

In 3T3-L1 cells, PMA treatment has been demonstrated to inhibit cell differentiation, as indicated by a decreased percentage of differentiated adipocytes (Shimizu *et al.*, 1983). A similar inhibitory effect of PMA on cell differentiation was observed in another adipogenic cell line, TA1, which also did not stimulate cell growth (Navre and Ringold, 1988). Our results from

GPDH activity agree with these observations (Figure 3). PMA also down-regulated all four PKC isozymes and the levels of PKC isozymes were stabilized toward the end of culture, which may reflect decreased responsiveness of PKC to relevant stimulants after the prolonged PMA treatment. Alternatively, PMA may sequentially activate different signaling pathways to preserve the levels of PKC. As PKC appeared to be completely depleted one day after cells were treated with 500 nM PMA (Fig. 2), but was detected with a lower concentration of PMA (100 nM) (Fig. 5), the degree of PKC down-regulation appeared to be dose related. Previous observations in other cell types, such as C6 glioma cells (Chen *et al.*, 1995) and Swiss 3T3 cells (Olivier and Parker, 1992) demonstrated similar dose responses.

Staurosporin has been widely used as a PKC inhibitor for the study of signal transduction (Tamaoki *et al.*, 1986). It has been shown that PKC inactivation by staurosporin enhanced adipocyte differentiation of rat adipose precursor cells in serum-free culture (Shinohara *et al.*, 1994) and 3T3-L1 preadipocytes (Ueda *et al.*, 1991). Staurosporin had no significant effect on the patterns of PKC protein levels during differentiation in this study (Figure 5), but, in the cytosol fraction, less PKC α, βI, and ζ were found at late stages (day 5 and day 8) of cell differentiation.

In conclusion, it is generally accepted that the multiple, similar PKC isozymes are responsible for different specialized physiological processes. Because all four PKC isozymes identified in 3T3-L1 cells were responsive to PMA and staurosporin treatments, the observed inhibitory effect of chronic PMA treatment or stimulatory effect of staurosporin on adipocyte differentiation could be mediated by any of the PKC isozymes or a combination. It is difficult to determine which PKC isozyme is most critical during differentiation. In a leukemia cell line, HL-60, the levels of both PKC

α and β increased with differentiation. However, only PKC β was demonstrated to have a specific role in the process of differentiation (Gamard *et al.*, 1994). Ueda *et al.* (1993) observed that one 3T3-L1 cell line formed stratified layers and did not differentiate after the treatment with differentiation inducers. Only PKC α was found in this 3T3-L1 cell line, indicating that PKC β is more important for the differentiation of inducible 3T3-L1 cells. In order to determine the role of PKC isozymes in cell differentiation, further experiments should evaluate whether the different isozymes elicit distinct responses and how these contribute to cell differentiation. This could be achieved through selective knockout or selective activation of individual isozymes.

Chapter IV

SUSTAINED PROTEIN KINASE C LEVELS BY CALPAIN INHIBITOR II IMPAIR 3T3-L1 PREADIPOCYTE DIFFERENTIATION

Chu-Liang Chen and Ching Yuan Hu

Summary

Regulation of protein kinase C (PKC) levels has an important role in mediating cell differentiation. In 3T3-L1 cells, PKC levels and activities decrease during differentiation. Because calpain has been identified as the intracellular protease that catalyzes the down-regulation of PKC, we hypothesized that inhibition of calpain activity may lead to sustained PKC levels and consequently, inhibition of 3T3-L1 cell differentiation. Calpain inhibitor II treatment caused a reduction of 3T3-L1 preadipocyte differentiation by 50% as indicated by glycerolphosphate dehydrogenase (GPDH) activity, and that this was accompanied by the sustained PKC levels. However, m-calpain was unresponsive to phorbol ester (PMA) treatment with respect to translocation and its proteolytic activity on PKC down-regulation. These results indicate that calpain plays a role in regulating the PKC levels and the decreased PKC levels are associated with 3T3-L1 cell differentiation.

Introduction

Cell differentiation is controlled by the modulation of different signaling pathways in response to extracellular factors. The protein kinase C (PKC) family plays an important role in the signal transduction pathways through which hormones, growth factors, and other extracellular stimuli promote or inhibit differentiation of target cells (Clemens *et al.*, 1992). PKC is activated by Ca^{+2}, phospholipids, and diacylglycerol (DAG), which are produced from the receptor-mediated hydrolysis of membrane phospholipids (Asaoka *et al.*, 1992). Activation of PKC is usually associated with their translocation from the

cytoplasm to the plasma membrane, and is often followed by their down-regulation by proteolytic degradation (Nishizuka, 1992). Activated or membrane-associated PKC has been shown to be a proteolytic target for calpain, a calcium-dependent protease (Kishimoto *et al.*, 1983; Ohno *et al.*, 1990; Suzuki *et al.*, 1990). This limited proteolysis by calpain initiates the degradation of PKC (Kikkawa *et al.*, 1989). Calpain exists as an inactive cytosolic proenzyme, and is translocated and activated at the membrane under conditions quite similar to those for PKC (Mellgren , 1987; Suzuki *et al.*, 1987). Two ubiquitous isozymes, μ-calpain and m-calpain, with distinct calcium sensitivity *in vitro* are known (Suzuki *et al.*, 1992); and PKC is a preferred substrate for m-calpain (Cressman *et al.*, 1995). The role of calpain in the regulation of the cellular responses mediated through the PKC pathway has been demonstrated by blocking calpain activity using calpain inhibitors in a number of cell types. For example, granule exocytosis is reduced in the human neutrophils treated with calpain inhibitors, which inactive calpain activity and accordingly decrease PKC degradation (Pontremoli *et al.*, 1990).

Sustained changes in the PKC level have been observed to participate in the regulation of cell differentiation. For example, expression of PKC β increases during human promyelocytic leukemia (HL-60) cell differentiation (Markowske *et al.*, 1988). Antisense oligonucleotides used to attenuate up-regulation of PKC β level decreases HL-60 cell differentiation (Gamard *et al.*,1994). A sequential degradation of PKC α and ζ is also found during differentiation of hexamethylenebisacetamide-induced murine erythroleukemia (MEL) cells. This degradation of PKC is promoted by highly controlled activation and activity of calpain (Melloni *et al.*, 1989). Use of an anti-calpain antibody in MEL cells to inhibit the protease catalytic activity decreases the rate of down-regulation of PKC, and a delay in the onset of differentiation (Sparatore *et al.*, 1994). In 3T3-L1

preadipocyte cells, we observed a decrease in the protein levels of PKC α, βI, and ζ during differentiation induced by the medium containing insulin, dexamethasone, and isobutyl-methyl-xanthine. It has been shown that inactivation of PKC by staurosporin, a PKC inhibitor, enhances the differentiation of 3T3-L1 preadipocytes (Ueda *et al.*, 1991) and rat adipose precursor cells (Shinohara *et al.*, 1994). Moreover, while chronic hypoxia impairs the differentiation of 3T3-L1 cells, a sustained PKC activation and expression is observed (Sahai *et al.*, 1994). Taken together, these studies suggest that a degradation/inactivation of PKC is associated with the differentiation of 3T3-L1 preadipocytes.

In this study, we explored the possible role of calpain in the degradation of PKC during 3T3-L1 preadipocyte differentiation. Because the protein levels of PKC α, βI, and ζ started decreasing when 3T3-L1 cells became differentiated, we applied calpain inhibitor II to reduce calpain activity and examined whether this would sustain PKC protein levels and ultimately modulate differentiation. Phorbol 12-myristate 13-acetate (PMA), mimicking the action of DAG, causes translocation, activation, and subsequent degradation of PKC (Kikkawa *et al.*, 1989). PMA also translocates calpains to membranes and facilitates PMA-induced PKC degradation. This PKC degradation is reduced in cells exposed to calpain inhibitors along with PMA, such as muscle cells (Hong *et al.*, 1995), platelets (Patel *et al.*, 1994), and neuroblastoma cells (Shea et la., 1995). Therefore, we also investigated whether translocation of m-calpain was promoted by PMA treatment, and whether calpain inhibitor II could hinder PMA-induced PKC degradation.

Materials and Methods

Cell culture

3T3-L1 preadipocytes (American Type Culture Collection, MD) were seeded at a density of 4×10^4 cells per well in 6-well plates and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone, UT) in a 5% CO_2-humidified atmosphere at 37°C. Cells were first grown to confluence. Two days after cells reached confluence, differentiation was induced by treatment with insulin (10 mg/ml), dexamethasone (1 mM), and isobutylmethylxanthine (0.5 mM) in DMEM with 10% fetal bovine serum (day 0). After 2 days (day 2), cells were changed to culture medium lacking dexamethasone and isobutylmethylxanthine. This medium was replaced every 2 days. Calpain inhibitor II, *N*-acetyl-leucyl-leucyl-methioninal (Boehringer Mannheim Co., IN), and Phorbol 12-myristate 13-acetate (PMA; Sigma, MO) were prepared in dimethylsulfoxide (DMSO). Control cells were treated with DMSO alone.

Glycerolphosphate dehydrogenase (GPDH) activity assay

To measure GPDH activity, differentiated cells were harvested in ice-cold lysate buffer (0.25 M sucrose, 1 mM EDTA, 1 mM dithiothreitol, 5 mM Tris, pH 7.4). The cells were ultrasonically disrupted with a sonicator at 40 watts for 10 seconds and centrifuged at 13,000 *x g* for 5 minutes to remove cell debris. Enzyme activity was determined as previously described (Akanbi *et al.*, 1994). The activity was presented as milliunits per mg protein. One unit of GPDH activity represents the oxidation of 1 mmol NADH per minute. Protein concentrations were determined by a dye-binding method (Bradford, 1976).

Preparation of cell extracts

Cells were recovered in ice-cold homogenizing buffer (20 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 50 mM β-mercaptoethanol, 1 mM PMSF, 200 μg/ml of leupeptin, 5 mM EDTA, 2 mM EGTA, 10 mM benzamidine) from culture plate after washing with PBS. These cells were homogenized using a Dounce homogenizer and centrifuged at 100,000 *x g* for 20 minutes at 4 °C. Supernatants were used as the cytosolic fractions. To obtain membrane fractions, cell pellets were extracted with homogenizing buffer containing 1% Triton X-100. After one hour incubation at 4 °C, the samples were centrifuged at 100,000 *x g* for 20 minutes and the supernatants were collected as solubilized membrane fraction. Protein concentrations of individual samples were determined by the method of Bradford (1976) using BSA as a standard.

Western blot analysis

Protein samples were subjected to sodium dodecyl sulfate (SDS)10% polyacrylamide gel electrophoresis according to the method of Laemmli (1970). The separated proteins were electrophoretically transferred to a nitrocellulose membrane by the method of Towbin *et al.* (1979). Nonspecific sites were blocked by incubation of nitrocellulose membranes with either 5% non-fat dry milk or 3% BSA in TBST buffer (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.1% Tween-20) for 1 hour at room temperature. Anti-PKC isozyme specific antibodies were incubated with the membranes for 2 hours at room temperature. After washing with TBST buffer (3 X 5 min), horseradish peroxidase (HRP) conjugated secondary antibody (Sigma Chemical Co. MO) was added and incubated for 1 hour at room temperature. Following this incubation, the membrane was washed with TBST (5 X 5 min) and detected by using ECL detection system (Amersham, IL) for the specific binding of anti-PKC antibodies. The antibody

specific to m-calpain was from Dr. Neil E. Forsberg of Oregon State University (Hong *et al.*, 1995). Anti-PKC α isozyme-specific antibody was from GIBCO (NY). Anti-PKC βI and ζ isozyme-specific antibodies were from Santa Cruz Technology, Inc. (CA).

Statistical analysis

Data were analyzed by the analysis of variance procedure of NCSS (NCSS, 1984), with differences between means tested for significance by Fisher's least significant difference.

Results

To investigate if calpain plays a role in the process of 3T3-L1 preadipocyte differentiation, we examined whether elimination of the calpain activity by calpain inhibitor II treatment had any effects on differentiation. Confluent 3T3-L1 cells were exposed to selected concentrations of calpain inhibitor II beginning 1 day before the induction of differentiation to day 8. At day 8, cell extracts were prepared and GPDH activity, an indicator of adipocyte differentiation, measured. The result indicated that cell differentiation was decreased by more than 50% in cells exposed to 60 μM of calpain inhibitor II compared to control cells which were exposed to DMSO alone (Fig. IV.1). Treatment with concentrations lower than 10 μM had no effects on GPDH activity, whereas concentrations higher than 75 μM had cytotoxic effects on cells (data not shown).

Experiments to determine whether the inhibition of differentiation was correlated with the alteration of the protein levels of PKC were conducted using western blot analysis. Figure IV.2 shows that calpain inhibitor II-treated cells

Figure IV.1: Effects of calpain inhibitor II on 3T3-L1 preadipocyte differentiation. Cells were treated with 0 (control cells), 15, 30, or 60 μM of calpain inhibitor II from one day before the induction of differentiation to day 8. At day 8, cell extracts were prepared and GPDH activity, an indicator of adipocyte differentiation, measured. Means (bars) with different superscripts are different ($p<0.05$).

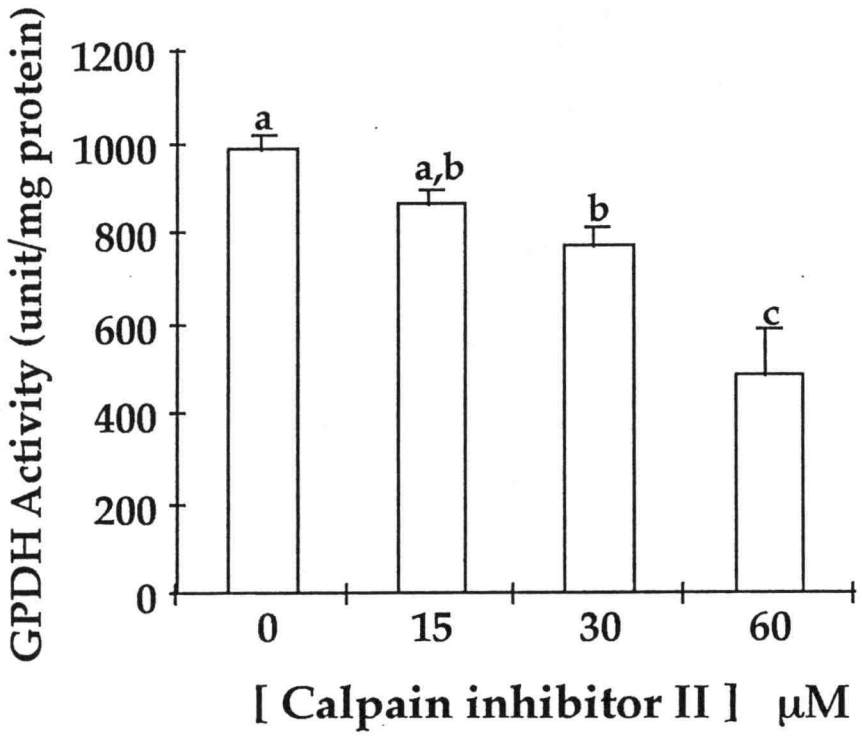
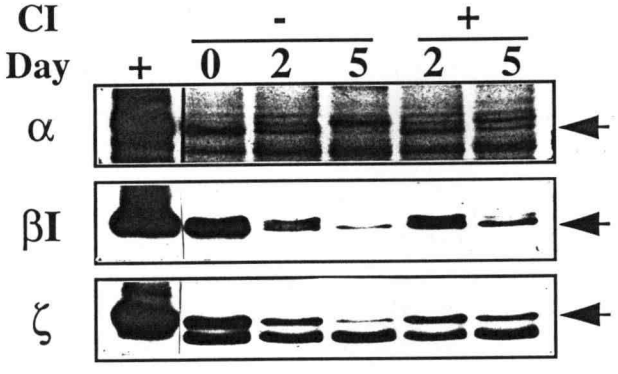


Figure IV.2: Effects of calpain inhibitor II (CI) on PKC α, βI, and ζ levels during 3T3-L1 preadipocyte differentiation. Cells were treated with 60 μM of calpain inhibitor II from 1 day before the induction of differentiation to days 0, 2, or 5 when cells were harvested. Membrane proteins were isolated, resolved in SDS-10% polyacrylamide gels and analyzed by western blot analysis using PKC α, βI, or ζ isozyme-specific antibodies. Cell extracts prepared from rat brain were used as a positive control for the PKC polypeptides (+).



exhibited higher protein levels of PKC α, βI, and ζ than DMSO-treated cells at day 2, and even more significantly at day 5. The protein levels of PKC decreased dramatically with differentiation in control cells, and this decrease of PKC expression was partially blocked by calpain inhibitor II treatment (Fig. IV.2). A higher molecular weight band found at day 5 in the blot of PKC α was a non-specific binding of antibodies because the binding of antibodies to PKC was not blocked by competitive peptides provided by the manufacturer (data not shown).

The role of calpain on the PMA-dependent PKC down-regulation was examined next. Confluent 3T3-L1 preadipocytes were treated with 500 nM PMA in the absence or presence of 60 μM calpain inhibitor II for 0, 7, or 21 hours. Cytosolic PKC α, βI, and ζ were translocated to membrane in response to PMA, and the subsequent PKC down-regulation was not affected by calpain inhibitor II treatment (Fig. IV.3). In addition, PMA-dependent m-calpain translocation to membranes was not found in 3T3-L1 preadipocytes (Fig. IV.4). Cytosolic and membrane proteins were isolated from cells exposed to PMA for various periods of time, and tested for the expression of m-calpain using western blot analysis. Both cytosolic and membrane-associated m-calpains were found in 3T3-L1 preadipocytes, and this cellular distribution profile was not changed by PMA treatment (Fig. IV.4).

Discussion

Calpains have been implicated in differentiation of a number of cell types, such as T-lymphocytes (Murachi *et al.*, 1990), chondrocytes (Yasuda *et al.*, 1995), erythroleukaemia cells (Sparatore *et al.*, 1994), neuroblastoma cells (Saito *et al.*,

Figure IV.3: Effects of calpain inhibitor II (CI) on the translocation and down-regulation of PKC isozymes of 3T3-L1 preadipocytes in response to PMA treatment. Confluent 3T3-L1 preadipocytes were treated with 100 nM PMA in the absence or presence of 60 μM calpain inhibitor II for 0, 7, or 21 hours. The cytosolic and membrane fractions were prepared for western blot analysis. The proteins were resolved in SDS-10% polyacrylamide gels and analyzed using PKC α, βI, or ζ isozyme-specific antibodies. Cell extracts prepared from rat brain were used as a positive control for the PKC polypeptides (+).

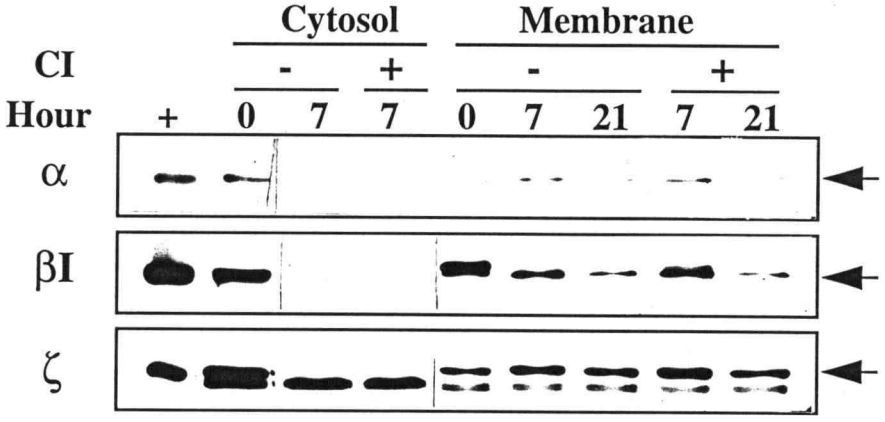
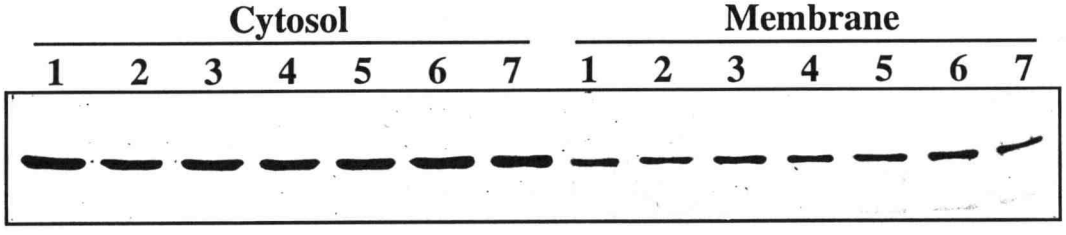


Figure IV.4: Effects of PMA on the translocation of cytosolic m-calpain to the membrane. Confluent 3T3-L1 preadipocytes were treated with 500 nM PMA for 0 (lane 1), 10 minutes (lane 2), 0.5 (lane 3), 1 (lane 4), 3 (lane 5), 6 (lane 6), or 21 hours (lane 7). The cytosolic and membrane fractions were prepared for western blot analysis. The proteins were resolved in SDS-10% polyacrylamide gels and analyzed using m-calpain specific antibodies.



1994), muscle cells (Kumar *et al.*, 1992; Cottin *et al.*, 1994), and intestinal cells (Ibrahim *et al.*, 1994). One direct evidence is provided by the use of calpain inhibitors to block calpain activities, which results in the modulation of cell differentiation (Kumar *et al.*, 1992; Saito *et al.*, 1994; Sparatore *et al.*, 1994; Yasuda *et al.*, 1995). However, there has been no report on the role of calpains in preadipocyte differentiation. We previously observed that PKC α, βI, and ζ protein levels decreased with differentiation, and this decrease was most predominant in membrane fractions, where PKC α, βI, and ζ proteins were nearly undetectable during late development. In the present study we demonstrated that 3T3-L1 cell differentiation was inhibited by calpain inhibitor II treatment (Fig. 1), and that this is associated with the sustained expression of PKC (Fig. 2). Initially, calpain-mediated proteolysis was thought to be part of the activation mechanism for PKC (Tapley and Murray, 1985; Kishimoto *et al.*, 1983), but more recent studies suggest that it is an important down-regulation mechanism (Kishimoto *et al.*, 1989). Our results suggest the possibility of calpain-dependent down-regulation of PKC in 3T3-L1 preadipocyte differentiation.

A feature of PKC regulation is its translocation to membranes and subsequent down-regulation following interaction with phorbol ester (Kikkawa *et al.*, 1989, Borner *et al.*, 1992). In 3T3-L1 preadipocyte culture, PKC α, βI, and ζ were depleted from the cytosolic fraction and translocated to the membrane fraction within 10 minutes of PMA treatment (data not shown). Membrane-associated PKC isozymes then gradually degraded, though PKC ζ appeared to be less sensitive to PMA-induced down-regulation than PKC α and βI. Calpain is important for the PMA-dependent down-regulation, because inhibition of calpain by addition of calpain inhibitor II prevents the disappearance of PKC in L8 muscle cells (Hong *et al.*, 1995). However, this PMA-dependent down-

regulation of PKC was not prevented by calpain inhibitor II treatment in 3T3-L1 cells (Fig. 3). In addition, PMA-dependent m-calpain translocation was not found in 3T3-L1 cells (Fig. 4), suggesting that m-calpain may not be involved in the PMA-dependent PKC down-regulation in 3T3-L1 cells. Junco *et al.* (1994) also proposed that m-calpain was not responsible for PMA-dependent PKC α down-regulation. These authors produced mutant PKC α proteins resistant to m-calpain, and observed that these mutant proteins were still down-regulated in COS-1 cells on exposure to PMA. They suggested that a more general process or multiple proteases, including proteasome and lysosomal protease, may be involved in PMA-dependent PKC down-regulation. It is likely that separate pathways mediating PKC down-regulation in response to extracellular ligands exist in 3T3-L1 cells.

Calpain inhibitor II, a competitive inhibitor for calpain, also inhibits proteasome and other cysteine proteases including the lysosomal cathepsins (Sasaki *et al.*, 1990). It is very unlikely that calpain inhibitor II reduced PKC degradation by inhibiting the lysosomal cathepsins, since lysosomes typically do not degrade membrane-associated proteins. It is possible that the prevention of PKC degradation by this treatment may be not contributed by inhibition of calpain activity alone. Nevertheless, sustained PKC levels were correlated with inhibition of cell differentiation after calpain inhibitor II treatment of 3T3-L1 cells. PKC also plays a key role in mediating hypoxia induced impairment of 3T3-L1 cell differentiation (Sahai *et al.*,1994). These results indicate that modulation of PKC levels by inducers in 3T3-L1 cells is critical during differentiation.

In summary, calpain-dependent degradation of PKC was linked to 3T3-L1 cell differentiation. Inhibition of calpain by calpain inhibitor II resulted in preservation of PKC expression, and subsequently decline of cell differentiation.

However, m-calpain was unresponsive to PMA treatment with respect to translocation and its proteolytic activity on PKC down-regulation. These results suggest that down-regulation of PKC is regulated by other pathways in response to different extracellular factors, and the expression of PKC is an important factor during 3T3-L1 cell differentiation.

Chapter V

RETINOIC ACID INHIBITION OF 3T3-L1 PREADIPOCYTE DIFFERENTIATION

Chu-Liang Chen and Ching Yuan Hu

Summary

Retinoic acid (RA) has been shown to inhibit the differentiation of 3T3-L1 preadipocytes. In the present study, we showed that RA prevented induction of the adipogenic factors C/EBPα, PPARγ2, and aP2, but not C/EBPβ. We determined that the effects of RA were mediated by liganded RA receptors (RARs) rather than retinoid X receptors (RXRs) using receptor-specific synthetic retinoids. RA treatment also inhibited the expression of RXRα and RARα. These results suggested that the decreased expression of RXRα and PPARγ2 was part of the mechanism of RA inhibition of adipocyte differentiation, and C/EBPβ : C/EBPα cascade regulation may not be involved during RA inhibition of adipocyte differentiation at early stages.

Introduction

Vitamin A and its natural and synthetic derivatives (the retinoids) are required for several essential life processes, including vision, reproduction, metabolism, differentiation, hematopoiesis, bone development, and pattern formation during embryogenesis (Sporn *et al.*, 1984). These small hydrophobic ligands are passively transported across biological membranes and control cell functions by using specific intracellular receptors as signal transducers. These intracellular receptors, located in the nucleus, function as ligand-activated transcription factors that modulate gene expression after binding specific DNA sequences located in the regulatory regions of target genes (Evans, 1988). The complexity of the biologic responses to vitamin A suggested that several metabolites might function as ligands. By the mid-1980s, however, an

abundance of evidence led researchers to believe that most of these effects were due to one specific metabolite, retinoic acid (RA), and more specifically all-*trans* RA (at-RA; Sporn *et al.*, 1984).

RA regulates cellular functions by binding to intracellular RA receptors, which are members of the steroid and thyroid receptor superfamily (Green and Champon, 1988). Two families of RA receptors, each consisting of three receptor types, α, β, and γ, have been identified: the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs) (Leid *et al.*, 1992; Mangelsdorf *et al.*, 1994). Distinct patterns of tissue distribution and developmental stage specificity in the expression of the RARs/RXRs have been demonstrated. For instance, RARα and RARβ are found in many tissues, while RARγ occurs predominantly in skin and lung (Zelent *et al.*, 1989). RXRγ is found mainly in liver, lung, and muscle (Mangelsdorf *et al.*, 1990). The physiological significance of multiple receptor isoform expression in tissues has not been explained. at-RA binds to RAR to form RAR-RXR heterodimer that acts as a *trans*-acting factor and controls the transcription initiated from the promoters of target genes by interacting with the *cis*-acting DNA response elements. In addition to at-RA, 9c-RA, a stereoisomer of at-RA, is generated by intracellular isomerization of at-RA (Levin *et al.*, 1992; Heyman *et al.*, 1992), and binds to both RARs and RXRs (Mangelsdorf *et al.*, 1994), even though there is low sequence homology between RARs and RXRs. The binding of 9c-RA to RAR triggers the RAR-RXR signaling pathway, while the binding of 9c-RA to RXR forms RXR homodimers and triggers a retinoid signaling pathway distinct from the RAR-RXR pathway (Fig. V.1).

Although RA induces differentiation in a number of biological systems (Sporn and Roberts, 1980), it inhibits adipocyte differentiation during early development (Sato *et al.*, 1980; Kuri-Harcuch, 1982; Stone and Bernlohr, 1990; Xue *et al.*, 1996). The adipocyte differentiation program is regulated by

transcriptional regulators, which coordinate the expression of genes involved in initiating and maintaining the adipocyte phenotype (MacDougald and Lane, 1995). Among these transcription factors, C/EBPα, C/EBPβ, and PPARγ are the most characterized ones, and ectopic expression of each of them is sufficient for the induction of adipocyte differentiation (Freytag *et al.*, 1994; Lin and Lane, 1994; Wu *et al.*, 1995; Tontonoz *et al.*, 1995). Adipocyte differentiation, induced either by a standard hormone cocktail or by PPAR activators, is prevented by the addition of RA only when the RA is added within 24 to 48 hours of the exposure of preadipocytes to differentiating conditions (Kuri-Harcuch, 1982; Stone and Bernlohr, 1990; Xue *et al.*, 1996).

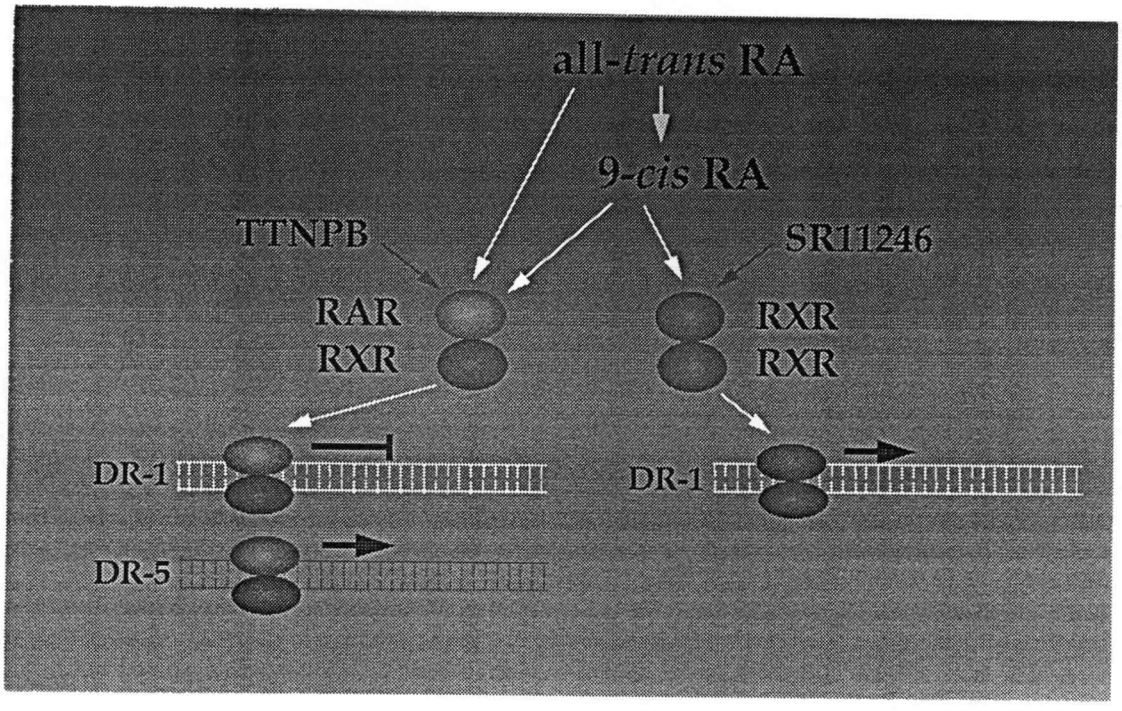
The discovery of synthetic retinoids that discriminate between the RARs and RXRs has helped understand the roles of these receptors in governing the biological effects of RA. In this study, we applied a RAR-specific agonist, (E)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)propenyl]benzoic acid (TTNPB), and a RXR-specific agonist, 4-[1-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)cyclo-propyl]benzoic acid (SR11246) to determine the retinoid receptors which mediate the RA signal (Fig. V.1). Furthermore, in order to investigate the mechanism of RA inhibition of adipocyte differentiation, we examined the effect of RA on the expression of RARs, RXRs, and adipogenic factors including C/EBPα, C/EBPβ, and PPARγ.

Materials and Methods

Cell culture

3T3-L1 preadipocytes were seeded at a density of 4×10^4 cells per well in 6-well plates and cultured in Dulbecco's modified Eagle's medium (DMEM)

Figure V.1: Retinoic acid receptor (RAR) and retinoid X receptor (RXR) signaling pathway. TTNPB: a RAR specific ligand. SR11246: a RXR specific ligand. DR-1: DNA binding element direct repeat-1. DR-5: DNA binding element direct repeat-5.



supplemented with 10% fetal bovine serum in a 5% CO_2-humidified atmosphere at 37°C. Cells were first grown to confluence. Two days after cells reached confluence, differentiation was induced by treatment with insulin (10 µg/ml), dexamethasone (1 mM), and isobutylmethylxanthine (0.5 mM) in DMEM with 10% fetal bovine serum (day 0). After 2 days (day 2), cells were changed to culture medium lacking dexamethasone and isobutylmethylxanthine. This medium was replaced every 2 days. Cells were treated with all-*trans* RA, 9-*cis* RA, TTNPB ((E)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)propenyl]benzoic acid), or SR11246 (4-[1-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)cyclo-propyl]benzoic acid) prepared in dimethylsulfoxide (DMSO). Control cells were treated with DMSO alone.

Glycerol-3-phosphate dehydrogenase (GPDH) activity assay

To measure GPDH activity, differentiated cells were harvested in ice-cold lysate buffer (0.25 M sucrose, 1 mM EDTA, 1 mM dithiothreitol, 5 mM Tris, pH 7.4). The cells were ultrasonically disrupted with a sonicator at 40 watts for 10 seconds and centrifuged at 13,000 *x g* for 5 minutes to remove cell debris. Enzyme activity was determined as previously described (Akanbi *et al.*, 1994). The activity was presented as milliunits per mg protein. One unit of GPDH activity represents the oxidation of 1 mmol NADH per minute. Protein concentrations were determined by a dye-binding method (Bradford, 1976). This study was repeated three time, three replicates for each treatment.

RNA isolation and Northern blot analysis

Total RNA was isolated by acid guanidinium thiocyanated-phenol-chloroform extraction (Chomczynski and Sacchi, 1987) and subjected to electrophoresis on formaldehyde-agarose gels. Gels were blotted onto nylon

membranes by capillary flow, and fixed by UV cross linking (Sambrook *et al.*, 1989). Membranes were prehybridized in 5X SSPE/50% formamide/0.5% SDS/5X Denhardt's solution, and 100 μg/ml denatured salmon sperm DNA at 42°C for 4 hours. For hybridization, membranes were incubated overnight with ^{32}P-labeled cDNA probes in the prehybridization solution plus 10% dextran sulfate. Membranes were washed twice in 5X SSPE/0.5% SDS at room temperature, twice in 1X SSPE/0.5% SDS at 37°C, and once in 0.1X SSPE/1% SDS at 50°C. The intensity of radioactivity were measured by phosphorimager (Molecular Dynamics, Inc., CA). The aP2 cDNA probe was from Dr. David A. Bernlohr, University of Minnesota. The C/EBPα and C/EBPβ probes were from Dr. Steve L. McKnight of Tularik Inc.. The PPARγ2 probe was from Dr. Bruce M. Spiegelman of Dana-Farber Cancer Institute. The RXRα and RARα probes were from Dr. Mark Leid, Oregon State University. Probes were labeled by the random primers method with [α-^{32}P]dCTP (DuPont NEN, DE) using a kit from Boehringer Mannheim Co., IN.

Statistical analysis

Data were analyzed by the analysis of variance procedure of NCSS (NCSS, 1984), with differences between means tested for significance by Fisher's least significant difference.

Results

The concentrations of at-RA, 9c-RA, TTNPB, and SR11246 that inhibited 3T3-L1 cell differentiation were determined by treating cells from days 0 to 2 with doses that ranged from 10^{-6} to 10^{-10} M. Differentiation was assayed by

analyzing GPDH activity in cell extracts prepared on day 8 (Fig. V.2). Cells treated with 10^{-6} M at-RA or 9c-RA had significantly lower levels of GPDH activity (60 and 70% reductions, respectively). Treatments with lower concentrations had little (10^{-8} M) or no effect (10^{-9} M) on GPDH activity. The GPDH activity of TTNPB-treated cells was decreased by 30% at 10^{-9} M, and by 90% at 10^{-6} M. SR11246 treatment had no effect on GPDH activity compared to control cells exposed to DMSO (Fig. V.2).

To confirm that at-RA, 9c-RA, and TTNPB inhibited the differentiation of 3T3-L1 preadipocytes, we examined the expression of an adipocyte-specific marker, adipose P2 (aP2) mRNA. Northern blot analysis indicated that treated cells expressed lower levels of aP2 mRNA than control cells (Fig. V.3; Fig. V.4).

The effect of RA on the expression of adipocyte differentiation-dependent transcription factors was subsequently investigated. Figure V.3 shows that the mRNA levels of C/EBPα, PPARγ2, and RXRα in RA-treated cells were significantly lower than those in control cells. The mRNA level of RARα was decreased slightly on day 2 but was not different from that in control cells on day 5. The mRNA levels of C/EBPβ were not altered by RA treatment. The same results were observed in the TTNPB treated cells (Fig. V.4).

Discussion

The RARs and RXRs exhibit differential *trans*-activational activity on several naturally occurring and synthetic retinoic acid-responsive promoters (Nagpal *et al.*, 1992). This suggests that the responses of these two receptor systems to RA have evolved through distinct pathways. Because both RARs and RXRs can be activated by either at-RA or 9c-RA, RAR- or RXR-specific retinoids

Figure V.2: Effect of at-RA, 9c-RA, TTNPB, or SR11246 treatment on 3T3-L1 preadipocyte differentiation: GPDH activity. 3T3-L1 cells were exposed to at-RA, 9c-RA, TTNPB, or SR11246 at different concentrations as indicated in figure or DMSO (0) from days 0 to 2. Cells were harvested and GPDH activity measured on day 8.

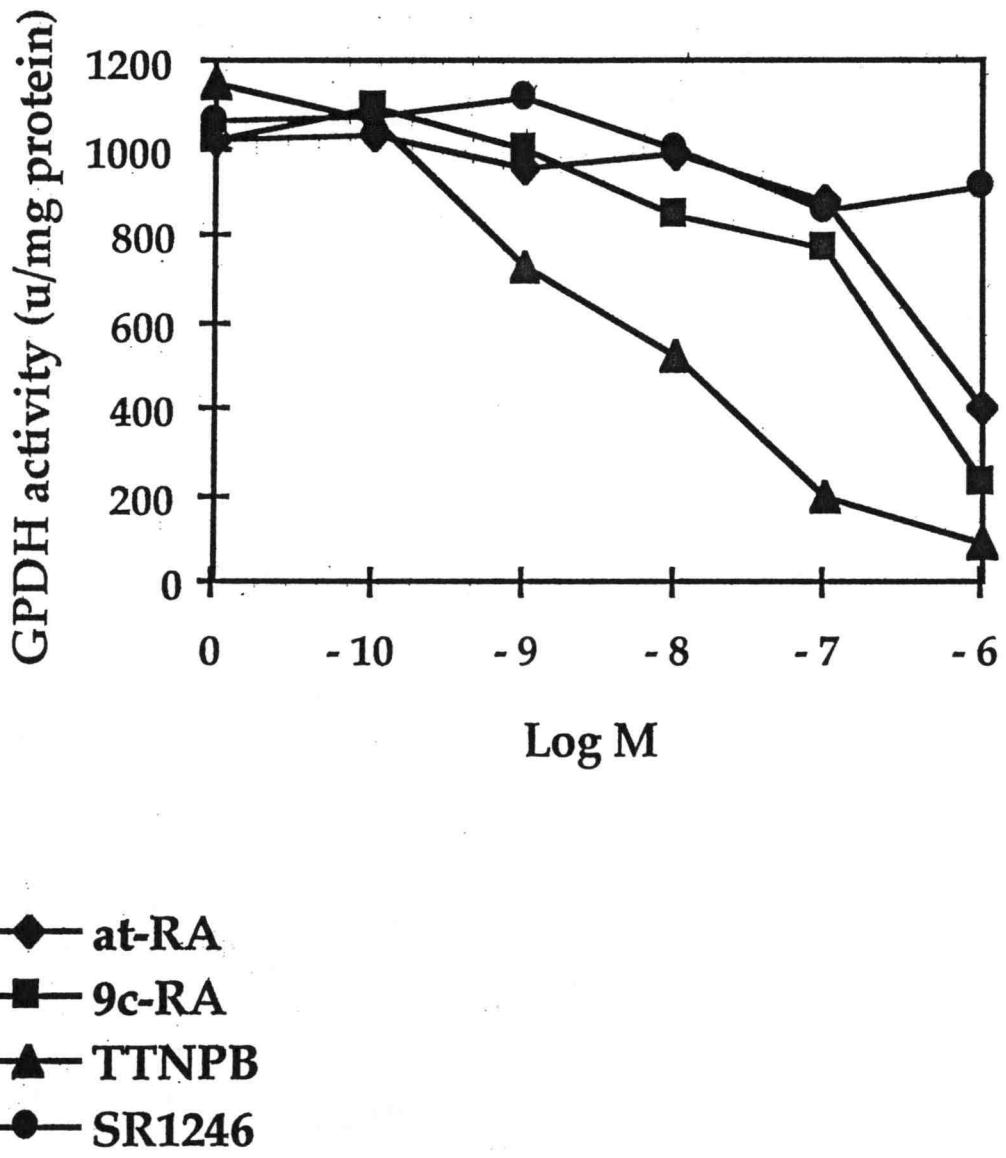


Figure V.3: Effect of at-RA or 9c-RA treatment on the mRNA levels of differentiation-dependent markers during 3T3-L1 preadipocyte differentiation. 3T3-L1 cells were treated with 10^{-6} M at-RA, 10^{-6} M 9c-RA or DMSO (-) from day 0 to day 2. Total RNA isolated on days 0, 1, 2, and 5 was analyzed by northern blot with the indicated probes. Equal amounts of RNA were loaded per lane as judged by ethidium bromide staining of 18S and 28S. This is a representative blot of two independent experiments.

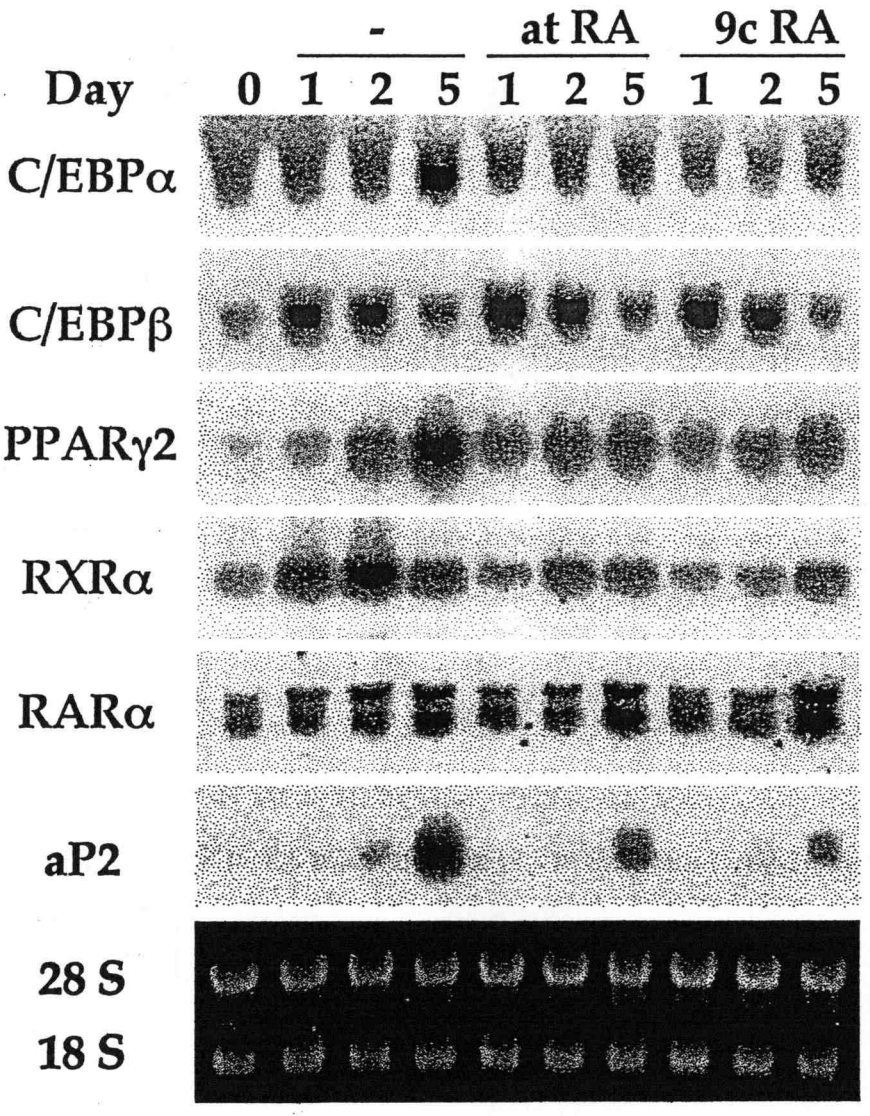
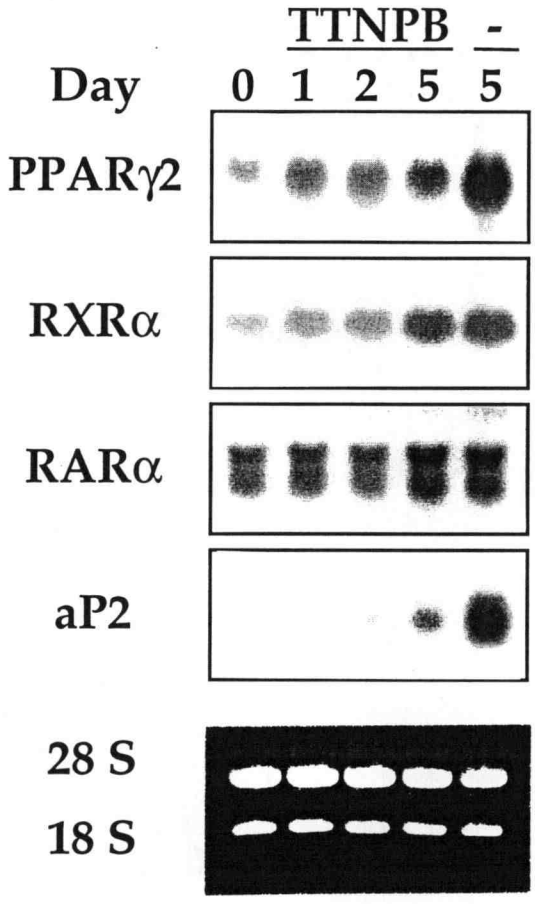


Figure V.4: Effect of TTNPB treatment on the mRNA levels of differentiation-dependent markers during 3T3-L1 preadipocyte differentiation. 3T3-L1 cells were treated with 10^{-5} M TTNPB or DMSO (-) from day 0 to day 2. Total RNA isolated on days 0, 1, 2, and 5 was analyzed by northern blot with the indicated probes. Equal amounts of RNA were loaded per lane as judged by ethidium bromide staining of 18S and 28S. This is a representative blot of two independent experiments.



are required to identify which retinoid receptor pathway mediates the RA inhibition of 3T3-L1 preadipocyte differentiation. The RAR-specific retinoid TTNPB was more effective at inhibiting adipocyte differentiation than at-RA or 9c-RA, as assayed by GPDH activity (Fig. V.2). In contrast, the RXR-specific retinoid SR11246 (Dawson *et al.*, 1995) was ineffective at inhibiting adipocyte differentiation. Therefore, the inhibitory effects of RA on adipocyte differentiation were likely mediated by RARs. These results indicate that genes with RAR-responsive promoters are candidates for the RA-mediated inhibition of adipocyte differentiation.

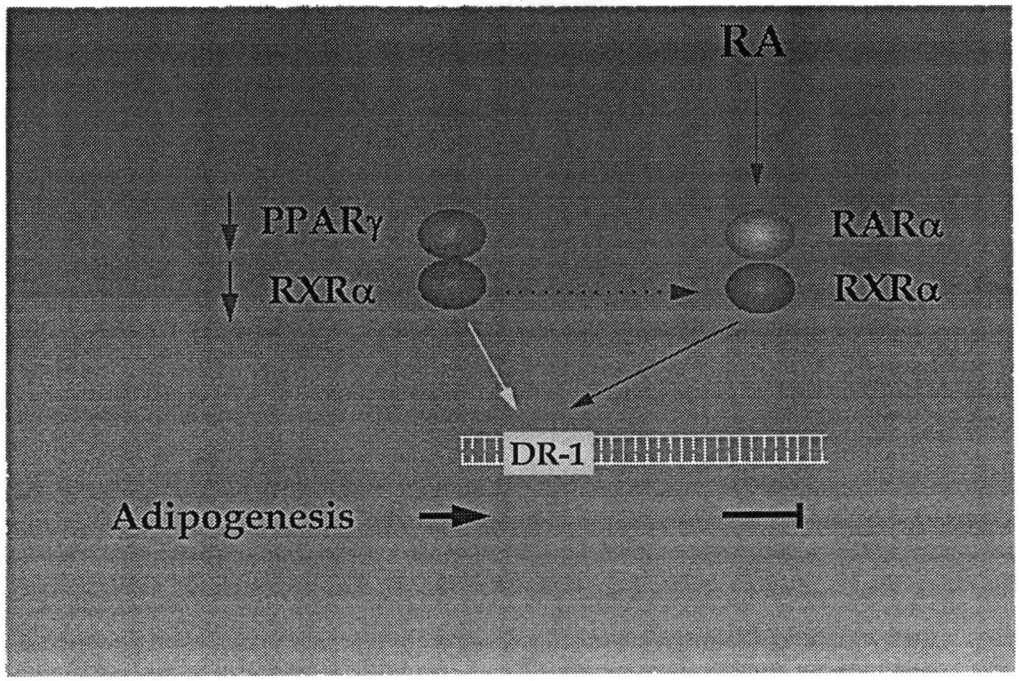
The process of adipocyte differentiation involves the regulation of a large number of genes and transcription factors (Sadowski *et al.*, 1990). The regulation of individual genes at specific times after the induction of differentiation suggests the existence of a regulatory hierarchy or cascade of events (Cao *et al.*, 1991; Yeh *et al.*, 1995). The fact that inhibition of C/EBPα expression prevents differentiation (Lin and Lane, 1992) and ectopic expression of C/EBPα induces adipogenesis (Freytag *et al.*, 1994; Lin and Lane, 1994; Yeh *et al.*, 1995) underscores the importance of C/EBPα in this process. However, C/EBPα is not expressed until 2 to 3 days after induction of differentiation, likely in response to transcription factors produced earlier in adipocyte differentiation, including C/EBPβ (Yeh *et al.*, 1995) or PPARγ (Chawla *et al.*, 1994a; Tontonoz *et al.*, 1994b). The cascade model of adipocyte differentiation is further supported by the observation that RA is effective at much earlier times. This suggests that while inhibition of C/EBPα expression by RA is important for inhibiting adipogenesis, it likely is an indirect effect. Furthermore, the fact that the mRNA level of C/EBPβ was not affected by RA treatment (Fig. V.3), suggests that the C/EBPβ : C/EBPα cascade regulation (Yeh *et al.*, 1995) is not involved in RA inhibition of adipocyte differentiation at early stages.

RARα and RARγ are strongly expressed in adipose tissue and 3T3-L1 cells (Kamei *et al.*, 1993), and the prevention of adipose differentiation of 3T3-L1 cells by RA is elicited through RARα (Kamei *et al.*, 1994). The expression of RARα mRNA was down-regulated slightly during day 0 to 2 when cells were exposed to RA (Fig. V.3). PPARγ induces adipocyte differentiation by forming heterodimers with RXRα and interacting with DNA response elements in the promoter of target genes. The expression of both PPARγ and RXRα mRNA was inhibited by RA treatment (Fig. V.3). These results suggest that decreased expression of RXRα, PPARγ, and possibly RARα, was involved in the RA inhibition of adipocyte differentiation.

PPARγ/RXRα heterodimers bind the DNA response elements (DR-1) and activate the expression of target gene. RAR/RXR heterodimers also bind DR-1 after RA treatment. However, the binding of RAR/RXR to DR-1 supresses transcription (Kurokawa *et al.*, 1994; Mangelsdorf *et al.*, 1994). It is possible that the binding of RAR/RXR to DR-1 after RA treatment prevents the binding of PPARγ/RXRα to the same DNA response element, and consequently inhibits the adipocyte differentiation initiated by PPARγ. In addition, RAR may compete with PPARγ for RXRα and decrease the binding of PPARγ/RXRα to DR-1 site (Fig. V.5). The relative binding of PPARγ/RXRα and RAR/RXR to DR-1 following RA treatment of 3T3-L1 cells could be measured using a gel mobility shift assay.

In summary, we presented evidence that RA inhibition of 3T3-L1 preadipocyte differentiation was mediated by the RAR pathway using receptor-specific retinoids. The expression of PPARγ, RXRα, and RARα, but not of C/EBPα and C/EBPβ was involved in RA-mediated effects on adipogenesis at early stages. Decreased expression of PPARγ, RXRα, and RARα also play a role in the RA inhibition of adipocyte differentiation.

Figure V.5: Mechanism of retinoic acid inhibition of 3T3-L1 preadipocyte differentiation. PPARγ: peroxisome proliferator activated-receptor γ. RA: retinoic acid. RARα: retinoic acid receptor α. RXRα: retinoid X receptor α. DR-1: DNA binding element direct repeat-1.



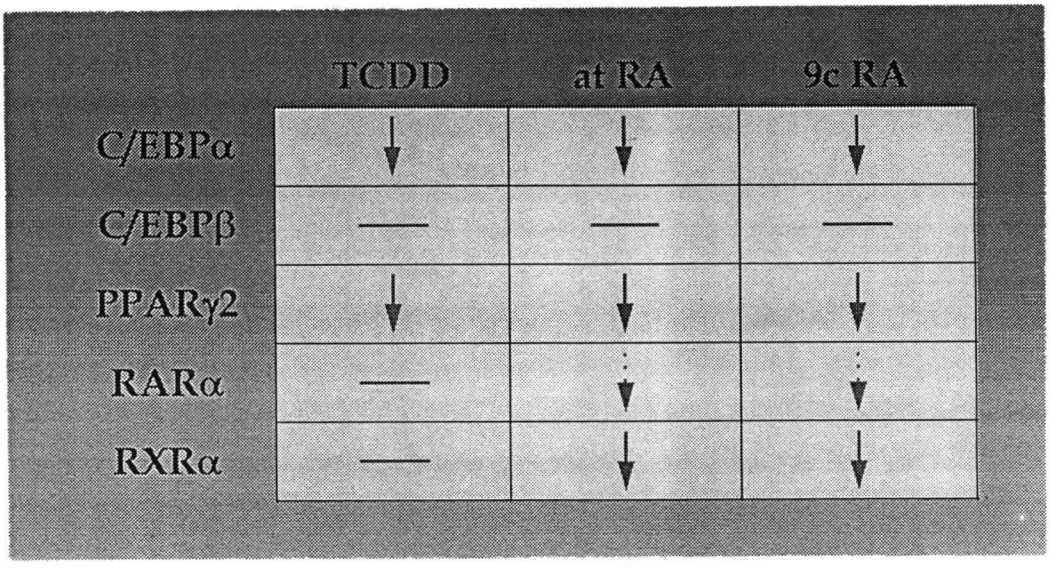
Chapter VI

CONCLUSIONS

Adipose tissue development involves both hypertrophy and hyperplasia of adipocytes in response to external signals. These signals can be nutrients, hormones, growth factors, or factors secreted by adjacent cells that act in a paracrine or autocrine manner. The accumulation of triacylglycerol in adipocytes through lipogenesis contributes to the increase of adipocyte size. On the other hand, the increase in number of adipocytes is hypothesized to originate from a group of fibroblast-like adipose precursor cells through differentiation. During the growth of adipose tissue, hyperplastic growth precedes hypertrophic growth. Therefore, a means to reglulate adipocyte differentiation to prevent hyperplasia would provide an effective way to repress hypertrophic growth and adipose accretion. In this sense, interruption of adipocyte differentiation at the molecular level may be the most direct way to reduce fat content in meat animals.

In this study, the 3T3-L1 cell line was used as a model to study the regulation of adipocyte differentiation with emphasis on the expression of specific differentiation-dependent *tran*-acting factors (C/EBPα, C/EBPβ, and PPARγ2) and Ca^{+2}-dependent protein kinases (PKC). TCDD and RA were applied to test their effects on adipocytes differentiation. Adipocyte differentiation was inhibited by TCDD or RA treatment at early stages. The effects of TCDD, at-RA, and 9c-RA on the levels of specific differentiation-dependent *trans*-acting factors are summarized in Figure VI.1. These two agents prevented the induction of C/EBPα but not C/EBPβ. Since C/EBPα was

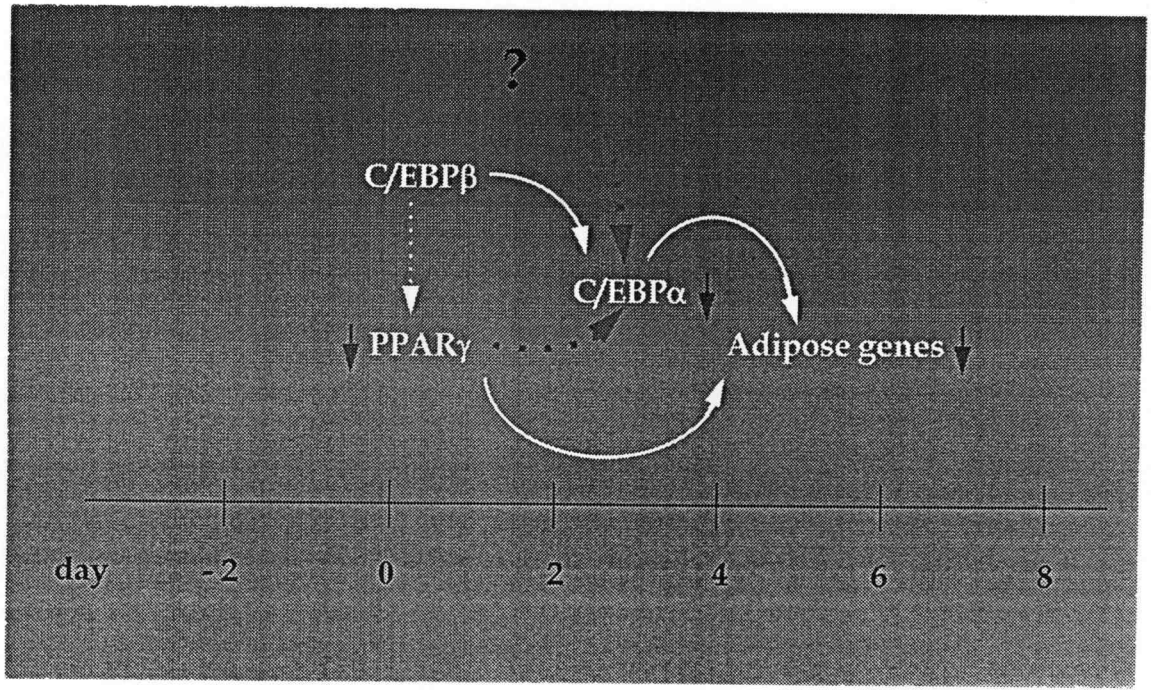
Figure VI.1: Effects of TCDD and retinoic acids on the mRNA levels of differentiation-dependent markers during 3T3-L1 preadipocyte differentiation.



considered a later marker during differentiation, it is unlikely that the TCDD or RA interfered with the C/EBPβ : C/EBPα cascade regulation at early stages. The expression of PPARγ2 occurred early during differentiation and was inhibited by TCDD or RA treatment, indicating that PPARγ2 is one of the target genes for the TCDD or RA action. In addition, these results suggest that the possible existence of unidentified factors that regulate C/EBPα expression and the possible role of PPARγ2 in the induction of C/EBPα during adipocye differentiation (Fig. VI.2). We also demonstrated that RA inhibition of adipocyte differentiation was mediated by the RAR pathway. RA treatment inhibited RXRα expression, suggesting that expression of RXRα and PPARγ2 is partly responsible for regulation of adipocyte differentiation. On the other hand, RXRα expression was not affected by TCDD treatment in preadipocytes. The expression of AhR appeared to be abolished within 2 days after the induction of differentiation. Whether the down-regulation of AhR is related to the differentiation program remains to be examined. The endogenous ligand for AhR also awaits identification.

Four PKC isozymes, PKC α, βI, ε, and ζ were identified in the 3T3-L1 adipocytes. Immunoblotting analyses showed that the levels of PKC α, βI, and ζ decreased during adipocyte differentiation, while the amounts of PKC ε increased. In the presence of phorbol 12-myristate 13-acetate (PMA), an activating ligand for PKC, the 3T3-L1 preadipocyte differentiation was inhibited while the basal levels of PKC α, βI, ζ, and ε were maintained. The 3T3-L1 preadipocyte differentiation was enhanced by staurosporin, a PKC inhibitor. Moreover, the down-regulation of PKC is believed to be partly mediated by the intracellular protease calpain. Calpain inhibitor II treatment led to the apparent sustenance of the PKC levels concomitant with a reduction of 3T3-L1 preadipocyte differentiation. Taken together, these results indicate the calpain-

Figure VI.2: Proposed mechanism of TCDD and retinoic acids inhibition of 3T3-L1 preadipocyte differentiation.



dependent PKC down-regulation and the decrease of PKC levels are associated with 3T3-L1 cell differentiation. The sustained expression of PKCs in the PMA- or calpain inhibitor II-treated cells was not a consequence but a cause of differentiation inhibition. Furthermore, the temporal expression pattern of PKCs was not affected by the TCDD treatment (Appendix II), indicating that the TCDD inhibition of adipocyte differentiation did not involve the change of PKC expression. On the other hand, RA treatment affected only the expression of PKCα (Appendix III), suggesting a possible role for PKCα in the process of adipocyte differentiation.

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APPENDICES

Appendix I: Nucleotide sequence of rat adipocyte tissue hsp 60 cDNA

In prokaryotic and eukaryotic cells, proper protein folding and assembly require a class of proteins referred to as "chaperonins" or "heat shock proteins (Hsps)". Within eukaryotic cell mitochondria, a protein analogous to Hsp60 in yeast (GroES in *Escherichia Coli* or P1 in mammalian cells) facilitates folding of proteins imported from the cytoplasm (Craig, 1993).

In order to identify rat inguinal adipose tissue-specific proteins, a monoclonal antibody (RI-1) was developed which reacted with a 25 kDa protein present in inguinal adipose tissue but not in epidydimal- or perirenal adipose tissues (Chen and Hu, unpublished data). In the process of screening for the cDNA encoding the 25-kDa inguinal adipose-specific protein using the RI-1 monoclonal antibody, the rat adipose hsp60 cDNA sequence was obtained and determined.

Poly $(A)^+$-containing RNA was prepared from rat inguinal adipose tissue. The construction and antibody screening of a λgt11 expression cDNA library were carried out as described in the protocols provided by the Stratagene ZAP-cDNA synthesis kit (La Jolla, CA). One positive clone was obtained and subjected to dideoxynucleotide DNA sequencing. Results of DNA sequencing revealed that the insert contained the entire Hsp60 cDNA fragment which is 2247 nucleotides long with a 24-nucleotide poly-A tail (Figure 1). The coding sequence encodes for a polypeptide of 1722 residues (M_r 60.95).

Although extensive sequence identity between the rat adipose hsp60 and the published rat liver hsp60 (Peralta et al., 1990), or rat kidney () hsp60 cDNA sequences was revealed by sequence alignment, several unique features were found in the adipose Hsp60 sequence. First, compared to liver

hsp60 sequence, adipose hsp60 cDNA has an additional 47 and 25 nucleotides in the 5′ and 3′ untranslated regions, respectively. Likewise, kidney hsp60 cDNA is 131- and 28-nucleotides shorter in the 5′ and 3′ regions, respectively. Second, three nucleotide substitutions between the adipose and liver cDNA sequence were found. The substitutions result in three amino acid substitutions, two of which were conserved. At the third position (537), instead of a proline residue, a serine residue was found in the adipose Hsp60 protein. Similarly, there are four nucleotide replacements which distinguish the adipose from the kidney cDNA sequence. At the amino acid sequence level, two silent as well as two conserved mutations were detected. Studies of both human and Chinese hamster genomic libraries suggested that mammalian cells contain only one functional hsp60 gene per haploid genome, despite of the presence of multiple copies of hsp60 gene in the genome (Venner et al., 1990). Therefore, it is unlikely that the sequence differences among the hsp60 cDNA from three different tissues of rat are derived from different hsp60 gene copies. Taken together, the results suggested that the liver and kidney hsp60 cDNA sequences available in the databank were probably not complete at both the 5′ and 3′ ends. Due to the missing 131 nucleotides at the 5′ end of the kidney hsp60 cDNA, the predicted amino acid sequence is 39 amino acid residues shorter than that of the adipose Hsp60 polypeptide. Alternatively, it is possible that the differences in the 5′ and 3′ regions of the mRNA are introduced by tissue-specific RNA processing.

Figure 1. nucleotide sequence of rat adipose hsp60 cDNA.

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1    GCAGGAATTCGGCACGAGCTCGCCTCGTGCTCTTCCGCCGCCGCCCCGCAGAAATGCTTCGACTACCCACAGTCCTTCGCCAGATGAGACCA
                                                        M  L  R  L  P  T  V  L  R  Q  M  R  P

93   GTGTCTCGGGCACTGGCTCCTCATCTCACTCGGGCCTATGCCAAAGATGTAAAATTTGGTGCGGATGCTCGAGCCTTAATGCTTCAAGGT
     V  S  R  A  L  A  P  H  L  T  R  A  Y  A  K  D  V  K  F  G  A  D  A  R  A  L  M  L  Q  G

183  GTAGACCTTTTAGCCGATGCTGTAGCTGTTACAATGGGGCCAAAGGGAAGAACAGTGATTATTGAACAGAGTTGGGGAAGTCCCAAAGTA
     V  D  L  L  A  D  A  V  A  V  T  M  G  P  K  G  R  T  V  I  I  E  Q  S  W  G  S  P  K  V

273  ACAAAAGATGGGGTCACTGTTGCAAAGTCAATTGATTTAAAGGATAAATACAAAAATATCGGAGCTAAGCTTGTTCAGGATGTTGCCAAT
     T  K  D  G  V  T  V  A  K  S  I  D  L  K  D  K  Y  K  N  I  G  A  K  L  V  Q  D  V  A  N

363  AACACAAATGAAGAGGCTGGGGATGGCACCACCACTGCCACTGTTCTGGCACGGTCTATTGCCAAGGAGGGCTTTGAGAAGATCAGCAAA
     N  T  N  E  E  A  G  D  G  T  T  T  A  T  V  L  A  R  S  I  A  K  E  G  F  E  K  I  S  K

453  GGGGCTAATCCAGTGGAAATCCGGAGAGGTGTGATGTTGGCTGTTGATGCTGTAATTGCTGAACTTAAGAAACAATCTAAACCTGTGACA
     G  A  N  P  V  E  I  R  R  G  V  M  L  A  V  D  A  V  I  A  E  L  K  K  Q  S  K  P  V  T

543  ACCCCTGAAGAAATTGCTCAGGTTGCTACAATTTCTGCAAACGGAGACAAAGACATTGGGAACATCATTTCTGATGCAATGAAGAAGGTT
     T  P  E  E  I  A  Q  V  A  T  I  S  A  N  G  D  K  D  I  G  N  I  I  S  D  A  M  K  K  V

633  GGAAGAAAGGGTGTCATCACAGTGAAGGATGGAAAAACCCTGAATGATGAGCTAGAAATTATTGAAGGCATGAAGTTTGATAGAGGATAT
     G  R  K  G  V  I  T  V  K  D  G  K  T  L  N  D  E  L  E  I  I  E  G  M  K  F  D  R  G  Y

723  ATTTCCCCATATTTTATTAACACATCAAAAGGTCAAAAATGTGAATACCAAGATGCCTATGTTTTGTTGAGTGAAAAGAAAATTTCTAGT
     I  S  P  Y  F  I  N  T  S  K  G  Q  K  C  E  Y  Q  D  A  Y  V  L  L  S  E  K  K  I  S  S

813  GTTCAGTCCATTGTACCTGCTCTTGAAATTGCCAATGCTCACCGGAAGCCCTTGGTCATAATTGCTGAAGATGTTGATGGAGAAGCTCTT
     V  Q  S  I  V  P  A  L  E  I  A  N  A  H  R  K  P  L  V  I  I  A  E  D  V  D  G  E  A  L

903  AGCACACTGGTTTTGAACAGGCTAAAAGTTGGTCTTCAGGTTGTAGCAGTCAAAGCTCCAGGGTTTGGGGACAACAGGAAGAACCAGCTT
     S  T  L  V  L  N  R  L  K  V  G  L  Q  V  V  A  V  K  A  P  G  F  G  D  N  R  K  N  Q  L

993  AAAGATATGGCTATCGCTACTGGTGGTGCGGTGTTTGGAGAAGAGGGTTTGAATCTAAATCTTGAAGATGTTCAAGCTCATGATTTAGGG
     K  D  M  A  I  A  T  G  G  A  V  F  G  E  E  G  L  N  L  N  L  E  D  V  Q  A  H  D  L  G

1083 AAAGTTGGAGAGGTCATCGTCACCAAAGATGATGCCATGCTTTTGAAAGGAAAAGGTGACAAAGCTCACATTGAAAAACGTATTCAAGAA
     K  V  G  E  V  I  V  T  K  D  D  A  M  L  L  K  G  K  G  D  K  A  H  I  E  K  R  I  Q  E

1173 ATCACTGAGCAGCTAGACATCACCACTAGTGAATATGAGAAGGAAAAGCTGAACGAGCGACTTGCTAAACTCTCAGATGGAGTAGCCGTG
     I  T  E  Q  L  D  I  T  T  S  E  Y  E  K  E  K  L  N  E  R  L  A  K  L  S  D  G  V  A  V

1263 TTGAAGGTTGGAGGGACAAGTGATGTTGAAGTGAATGAGAAGAAAGACAGAGTTACAGATGCTCTCAATGCTACAAGAGCAGCTGTTGAA
     L  K  V  G  G  T  S  D  V  E  V  N  E  K  K  D  R  V  T  D  A  L  N  A  T  R  A  A  V  E

1353 GAAGGCATTGTTCTAGGAGGGGGCTGTGCTCTACTTCGGTGCATCCCAGCCTTGGATTCATTAAAGCCTGCTAATGAAGATCAGAAGATA
     E  G  I  V  L  G  G  G  C  A  L  L  R  C  I  P  A  L  D  S  L  K  P  A  N  E  D  Q  K  I

1443 GGTATAGAAATTATTAAAAGAGCACTTAAAATTCCTGCAATGACGATTGCTAAGAATGCAGGTGTTGAAGGATCTTTGATAGTTGAAAAA
     G  I  E  I  I  K  R  A  L  K  I  P  A  M  T  I  A  K  N  A  G  V  E  G  S  L  I  V  E  K

1533 ATTCTGCAGAGTTCCTCAGAGGTTGGCTATGATGCCATGCTTGGAGATTTTGTGAACATGGTGGAAAAGGGAATCATTGATCCAACAAAG
     I  L  Q  S  S  S  E  V  G  Y  D  A  M  L  G  D  F  V  N  M  V  E  K  G  I  I  D  P  T  K

1623 GTTGTAAGAACTGCTTTACTGGATGCTGCTGGGGTGGCCTCCCTGCTAACTACAGCCGAAGCTGTAGTGACAGAAATTCCTAAAGAAGAG
     V  V  R  T  A  L  L  D  A  A  G  V  A  S  L  L  T  T  A  E  A  V  V  T  E  I  P  K  E  E

1713 AAGGACCCTGGAATGGGTGCAATGGGTGGAATGGGAGGGGGTATGGGAGGTGGCATTTTCTAACTCCTAGAATAGTGCTTTGCCCTTATC
     K  D  P  G  M  G  A  M  G  G  M  G  G  G  M  G  G  G  I  F  *

1803 AATGAACTGTGGCAGGAAGCTCAAGGCAGGTTCCTCACCAATAACTTCAGAGAAGTCACCTGAAGAAAATGACTGAAGAGAAGGCTGGCT
1893 GATCACTGTAACCATCAGTTACTGGTTTCCTTTGACAATACATAATGGTTTACTGCTGTCATTGTCCATGCCTACAGATAATTTATTTTG
1983 TATTTTTGAATAAAGACATTTGTACATTCCTGATGCTGGGTGCAAGAGCCAGTGTCCTGCTTTCAACTTAAATCACTGAGGCATCTCTAC
2073 TGTTCTGTTAGCATCAGGACTGTAGCGCTGTGTCACCACATGAGAAGTTCAGAAGCAGCCTTTCTGTGGAGGGTGAGAATGATTGTGTAC
2163 AGAGTAGAGAAGTATCCAATTATGTGACAACCTTTGTGTAATAAAATTTTGTTTAAAGTTCAAAAAAAAAAAAAAAAAAAAAAAAA
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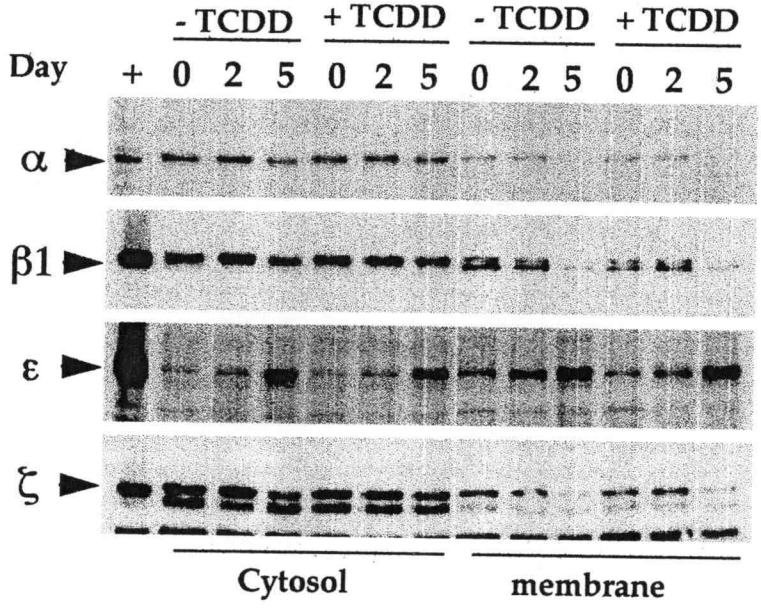
Appendix II: Effects of TCDD on the expression of PKC isozymes during 3T3-L1 preadipocyte differentiation

3T3-L1 cells were treated (+TCDD) or not treated (-TCDD) with 50 nM TCDD from day -4 to day -2. Western blot analyses using PKC α, β1, ε, or ζ isozyme-specific antibodies were carried out following the preparation of the cytosolic and plasma membrane fractions. Cell extracts prepared from rat brain were used as a positive control for the PKC polypeptides (+).



Appendix III: Effects of retinoic acid (RA) on the expression of PKC isozymes during 3T3-L1 preadipocyte differentiation

3T3-L1 cells were treated with 1 μM DMSO (-), all-*trans* RA (at RA) or 9-*cis* RA (9c RA) from day 0 to day 2. Western blot analyses using PKC α, β1, ε, or ζ isozyme-specific antibodies were carried out following the preparation of the cytosolic and plasma membrane fractions. Cell extracts prepared from rat brain were used as a positive control for the PKC polypeptides (+).