

## INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

**The quality of this reproduction is dependent upon the quality of the copy submitted.** Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

# UMI

A Bell & Howell Information Company  
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA  
313/761-4700 800/521-0600





Université d'Ottawa • University of Ottawa



# **A Paracrine/Autocrine Role of Prostaglandins in Adipose Tissue Development**

**Veena A. Kumar**

A thesis submitted to the Department of Biochemistry in partial fulfillment of the requirements  
for the degree of Master of Science

University of Ottawa  
Ottawa, Ontario, Canada

May 1997

© Veena A. Kumar, Ottawa, Ontario, Canada



National Library  
of Canada

Acquisitions and  
Bibliographic Services

395 Wellington Street  
Ottawa ON K1A 0N4  
Canada

Bibliothèque nationale  
du Canada

Acquisitions et  
services bibliographiques

395, rue Wellington  
Ottawa ON K1A 0N4  
Canada

*Your file / Votre référence*

*Our file / Notre référence*

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-28431-X

**Canada**

# ABSTRACT

Paracrine interactions between constituent cells of adipose tissue (preadipocytes, adipocytes and endothelial cells) may play an important role in regulating the process of adipose tissue development. The present study investigated the paracrine/autocrine role of prostaglandins in the development of the adipocyte phenotype. To determine the paracrine role of adipocyte-derived prostaglandins on differentiation of preadipocytes, test preadipocytes were cultured with medium conditioned by isolated mature adipocytes (ACM). ACM has been shown by our laboratory to contain factors released by mature adipocytes which induce differentiation. Addition of indomethacin, an inhibitor of prostaglandin synthesis, to the culture of adipocytes (ACM+I) significantly augmented differentiation induced by ACM. ( $p \leq 0.05$ ). In contrast, addition of indomethacin to preadipocytes (ACM+Ia) resulted in significant inhibition of differentiation ( $p \leq 0.05$ ) induced by ACM and standard differentiation mixture (MIX). These findings suggested that adipocytes secrete prostaglandins that may act as negative paracrine regulators of preadipocyte differentiation, whereas, preadipocytes may secrete prostaglandins that stimulate their differentiation in an autocrine manner. Cultured adipocytes and preadipocytes released PGI<sub>2</sub> and PGE<sub>2</sub> into medium. However, the amount of PGI<sub>2</sub> and PGE<sub>2</sub> released by preadipocytes was 1.4- and 11-fold greater than those released by adipocytes. When the effect of exogenous prostaglandins on preadipocyte differentiation was examined, PGI<sub>2</sub> and PGE<sub>2</sub> stimulated preadipocyte differentiation, but the effective concentration ( $\geq 10^{-7}$  M) was much higher than the concentration found to be released in the culture medium of adipocytes. PGF<sub>2 $\alpha$</sub> , in contrast, inhibited differentiation significantly ( $p \leq 0.05$ ) at a lower concentration ( $10^{-8}$  M). These findings suggested that adipocytes may release PGE<sub>2</sub> and PGI<sub>2</sub> in amounts that are insufficient to stimulate

preadipocyte differentiation. Hence the differentiating promoting activity of ACM cannot be due to the presence of PGI<sub>2</sub> or PGE<sub>2</sub>. A significant increase in differentiation observed in the presence of indomethacin may be due to the presence of inhibitory prostaglandins such as PGF<sub>2α</sub> in adipocytes. A considerably larger secretion of PGE<sub>2</sub> and PGI<sub>2</sub> by preadipocytes may be sufficient to stimulate their differentiation in a paracrine/autocrine manner.

The study also points to a potential role for the products of lipoxygenase pathway such as HETEs and leukotrienes in adipose-cell regulation. Nordihydroguaiaretic acid (NDGA), an inhibitor of lipoxygenase, inhibited preadipocyte differentiation induced by MIX. The addition of indomethacin (100 μM) significantly augmented MIX-induced differentiation which was also reduced significantly by NDGA ( $p \leq 0.05$ ). The addition of NDGA to ACM, however, stimulated differentiation induced by ACM. NDGA also failed to inhibit the increased differentiation induced by ACM in the presence of indomethacin. These results suggest that differentiation induced by MIX may involve stimulatory HETEs or leukotrienes, whereas that induced by ACM may involve inhibitory lipoxygenase products.

In summary, the present data suggest that arachidonic acid metabolites produced by cyclooxygenase and lipoxygenase pathways may play key roles in regulating adipose tissue mass via paracrine/autocrine mechanism(s).

# **DEDICATION**

This thesis is dedicated to the ones I love the most, my husband, Ashok Kumar and my children, Ritesh and Aditi whose love, support and inspirations have made this work a success.

# ACKNOWLEDGEMENTS

I would like to first thank my supervisor, Dr. D. C. W. Lau for giving me the opportunity to work in his laboratory. I extend my heart-felt gratitude to Dr. Lau for his able guidance, superior knowledge and constructive criticism in conducting this investigation successfully.

I equally express my indebtedness to Dr. Gillian Shillabeer, my co-supervisor, for introducing me to research, for her untiring help, indispensable advice and for creating the spirit of independent thinking, during the entire course of this investigation. Her critical review and valuable suggestions while going through the manuscript are greatly acknowledged.

I thank Garry Hatch, our ex-technician, for his help with the cell culture techniques and G3PDH assay. I would also like to thank Emma Tibbo and Claudia Lampercht, our present technicians, for boosting my morale from time to time.

I thank Dr. Odette Lanueville, Dr. Mary Allen Harper and Dr. Gillian Shillabeer, for their helpful suggestions as members on my committee. Dr. Lanueville's help in performing TLC analysis is greatly appreciated. The encouragement and help received from my friend Dheerja Pardasani is also acknowledged gratefully.

Last but not least, my deep sense of gratitude goes to my beloved husband Ashok Kumar and my loving children Ritesh Kumar and Aditi Kumar without whose constant love, support and inspirations, this work would not have seen the light of the day.

# LIST OF TABLES

<b>Table#</b>	<b>Title</b>	<b>Page</b>
1	PGE <sub>2</sub> and 6-keto PGF <sub>1α</sub> production in rat and human adipocytes under basal conditions.....	17
2	Measurement of PGE <sub>2</sub> and PGI <sub>2</sub> released by preadipocytes and adipocytes using RIA.....	50
3	Inhibition of PGI <sub>2</sub> production (measured as 6-keto PGF <sub>1α</sub> ) by indomethacin in rat adipocytes.....	51
4	Inhibition of PGE <sub>2</sub> production in rat adipocytes by indomethacin..	52

## LIST OF FIGURES

<b>Figure #</b>	<b>Title</b>	<b>Page</b>
1	Pathway for the formation of eicosanoids.....	14
2	Photomicrographs of preadipocytes showing lipid inclusions....	37
3	Effect of ACM on rat preadipocyte differentiation as determined by measuring G3PDH activity.....	38
4	Phase contrast photomicrographs of rat preadipocytes showing increased lipid droplets in response to indomethacin (100 $\mu$ M)....	40
5	Effect of indomethacin (100 $\mu$ M) on G3PDH activity of differentiating preadipocytes.....	41
6	Dose-response curve of indomethacin in ACM-induced preadipocyte differentiation.....	43
7	Dose-response curve of indomethacin in MIX-induced preadipocyte differentiation.....	44
8	Effect of indomethacin (0.01 $\mu$ M) on G3PDH activity of differentiating preadipocytes.....	46
9	TLC autoradiogram of prostaglandins after incubation of preadipocytes with [1- <sup>14</sup> C] arachidonic acid.....	48
10	Phase contrast photomicrographs illustrating the cytoplasmic lipid inclusions in rat preadipocytes upon exposure to increasing concentrations of exogenous cPGI <sub>2</sub> (a stable analogue of PGI <sub>2</sub> )..	54
11	Effect of increasing concentrations of exogenous prostaglandins (cPGI <sub>2</sub> , PGE <sub>2</sub> , 15-d PGJ <sub>2</sub> and PGF <sub>2<math>\alpha</math></sub> ) on adipose conversion of rat preadipocytes in primary culture as measured by G3PDH specific activity.....	55

12	Phase contrast photomicrographs of rat preadipocytes exposed to increasing concentrations of $\text{PGF}_{2\alpha}$ .....	57
13	Effect of NDGA on G3PDH activity of differentiating preadipocytes.....	58
14	Effect of NDGA on ACM-induced preadipocyte differentiation.....	61

## LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
AA	Arachidonic acid
ACM	Adipocyte-derived conditioned medium
ACM + I	ACM collected in the presence of indomethacin
ACM + Ia	Indomethacin added to ACM after its collection
cAMP	Cyclic Adenosine monophosphate
BAT	Brown adipose tissue
BMI	Body mass index
BSA	Bovine serum albumin
COX	Cyclooxygenase
DMEM-F12	Dulbecco's Modified Eagle Medium and Ham's F12
FAS	Fatty acid synthase
FBS	Fetal bovine serum
FBS + I	FBS + indomethacin
G3PDH	Glycerol-3 phosphate dehydrogenase
HDL	High density lipoprotein
HETE	Hydroxyeicosatetraenoic acid
HPETE	Hydroperoxyeicosatetraenoic acid
IBMX	3 isobutyl-1-methyl xanthine
ITT	DMEM-F12 supplemented with insulin, transferrin and triiodothyronine
LPL	Lipoprotein lipase
LOX	Lipoxygenase
$\alpha$ MEM	Alpha Minimum Essential Medium

MIX	Standard differentiation cocktail
NADH	$\beta$ -nicotinamide adenine dinucleotide
NDGA	Nordihydroguaiaretic acid
NIDDM	Non-insulin dependent diabetes mellitus
NSAID	Non-steroid anti-inflammatory drugs
PBS	Phosphate buffered saline
PG	Prostaglandin
cPGI <sub>2</sub>	Carbaprostacyclin
PLA <sub>2</sub>	Phospholipase A <sub>2</sub>
RIA	Radioimmunoassay
TG	Triacylglycerols
TGF- $\beta$	Transforming growth factor- $\beta$
TLC	Thin Layer Chromatography
TNF $\alpha$	Tumor necrosis factor- $\alpha$
UCP	Uncoupling protein
WAT	White adipose tissue

# TABLE OF CONTENTS

	<b>Page #</b>
ABSTRACT	i
DEDICATION	iii
ACKNOWLEDGEMENTS	iv
LIST OF TABLES	v
LIST OF FIGURES	vi
LIST OF ABBREVIATIONS	viii
1. INTRODUCTION.....	1
1.1    Prevalence and Definition of Obesity.....	2
1.2    Adipose Tissue Cell Biology.....	3
1.3    Adipocytes as Secretory / Endocrine Cells.....	4
1.4    Control of Adipose Tissue Growth and Development.....	5
1.5    Cell Culture Models for the Study of Adipocyte Development.....	5
1.5.1    Preadipocyte Cell Lines.....	5
1.5.2    Primary Preadipose Cells.....	7
1.6    Adipose Cell Differentiation.....	8
1.6.1    Sequence of Events.....	8
1.7    Exogenous Modulators of Preadipocyte Differentiation.....	9
1.8    Paracrine Interaction in Adipose Tissue Development and Growth	10
1.9    Prostaglandins in Adipose Tissue.....	12
1.9.1    Prostaglandin Biochemistry.....	12
1.9.2    Synthesis of Lipoxygenase Products.....	13
1.9.3    Mechanism of Eicosanoids Actions: Eicosanoids as Local Hormones.....	15

1.9.4	Prostaglandin Biosynthesis in Adipocytes.....	15
1.9.5	Inhibition of Prostaglandin Biosynthesis.....	18
1.9.6	Role of Prostaglandins as Paracrine Regulators of Adipocyte Development.....	19
<b>2. HYPOTHESIS AND RESEARCH OBJECTIVES</b>		
2.1	Hypothesis.....	22
2.2	Objectives.....	22
2.3	Rationale.....	22
<b>3. MATERIALS</b>		
3.1	Chemicals.....	24
3.2	Sera and Cell Culture Medium.....	24
3.3	Tissue Culture Plastic Ware.....	25
<b>4. METHODS</b>		
4.1	Preadipocyte Primary Culture.....	26
4.2	Collection of Adipocyte-derived Conditioned Medium.....	27
4.3	Assessment of Differentiation.....	27
4.4	Preparation of Celi Extract for G3PDH and Protein assay.....	28
4.5	Measurement of G3PDH activity.....	28
4.6	Protein determination.....	29
4.7	Determination of Paracrine Effect of Prostaglandins on Preadipocyte Differentiation.....	29
4.8	Determination of Prostaglandin Release.....	31
4.8.1	Thin Layer Chromatography.....	31
4.8.2	Radioimmunoassay.....	32
4.9	Study of Direct Effects of Prostaglandins on Preadipocyte Differentiation.....	33

4.10	Effects of Lipoxygenase (LOX) Products on Preadipocyte Differentiation.....	34
<b>5. RESULTS</b>		
5.1	Induction of Preadipocyte Differentiation by ACM.....	36
5.2	The effect of Indomethacin (100 $\mu$ M) on Preadipocyte Differentiation.....	39
5.3	Dose-response Curve of Indomethacin.....	42
5.4	The Effect of Indomethacin (0.01 $\mu$ M) on Preadipocyte Differentiation.....	45
5.5	Measurement of Prostaglandins released by preadipocytes and Adipocytes.....	47
5.6	Effect of Exogenous Prostaglandins on Preadipocyte Differentiation.....	53
5.7.1	Effect of increasing Concentrations of cPGI <sub>2</sub> , PGE <sub>2</sub> and 15d-PGJ <sub>2</sub> on Preadipocyte differentiation.....	53
5.7.2	Effect of Increasing Concentration of PGF <sub>2<math>\alpha</math></sub> on Preadipocyte Differentiation.....	56
5.8	Effect of Lipoxygenase (LOX) Products on Preadipocyte Differentiation.....	59
<b>6. DISCUSSION.....</b>		<b>62</b>
<b>7. SUMMARY AND CONCLUSIONS.....</b>		<b>71</b>
<b>8. FUTURE DIRECTIONS.....</b>		<b>75</b>
<b>REFERENCES.....</b>		<b>77</b>

# 1. INTRODUCTION

The critical association of obesity with such metabolic complications as high blood pressure, cardiovascular diseases, and non-insulin dependent diabetes mellitus (NIDDM) (Krotkiewski *et al.*, 1983; Kissebah *et al.*, 1987) makes it important to study the mechanism(s) that control the process of adipose tissue growth and development. The last several years have produced some breakthroughs in obesity-related areas of investigation, such as identification of genetic loci at which mutations cause obesity in rodents (Zang *et al.*, 1994) and the identification of transcription factors leading to the development of the adipocyte phenotype (Cao *et al.*, 1991; Kaestner *et al.*, 1990; Freytag and Geddes, 1992; Tontonoz *et al.*, 1994). However, the mechanism(s) regulating the process of adipogenesis despite extensive research, remains unclear. The treatment of this disorder, which is generally aimed at correcting positive energy balance by caloric restriction and exercise, will remain empirical until we have a better understanding of the normal and pathophysiologic states of adipose tissue development and growth.

New fat cells are formed as a result of replication and differentiation of their precursor cells. A number of yet unidentified factors are known to influence adipose differentiation. Elucidation of these factors may provide new insight into the pathogenesis and ultimately the treatment of obesity. The present study is aimed at studying paracrine factors specifically prostaglandins that might contribute to the regulation of adipose tissue development. The following literature review will provide an up-to-date perspective on advances in adipocyte development. A review on the role of prostaglandins as effectors of adipocyte development is also presented.

## 1. 1. Prevalence and Definition of Obesity

Obesity, the phenotypic expression of excessive body fat, results from an imbalance between energy intake and energy expenditure. The condition has reached an epidemic proportion in North America affecting more than 30% of the population (Olefsky, 1995). Many factors influence the development and maintenance of obesity such as diet, physical activity, age, sex, education and genetic susceptibility (McGinnis and Ballard-Barbash, 1991). Whatever may be the cause, obesity is expressed by an increase in adipose tissue mass. The prevalence of overweight or obesity depends upon the criteria used. One of the measures commonly used in obesity clinics and medical practice is weight and height (Bray and Gray, 1988). Although this measure can give reasonable estimates of relative body fatness in most cases, it can not separate lean from obese (Gray, 1989). The body mass index (BMI), defined as weight in kilograms/ height<sup>2</sup> in meters, appears to be a better index of fatness as shown in epidemiological studies (Bray and Gray, 1988). It is independent of body frame and thus, identical reference values can be used for all adults, men and women. Overweight or mild obesity is defined as a body weight exceeding 120% of reference values or a BMI greater than 27 kg/ m<sup>2</sup>. Moderate obesity is defined as a BMI greater than 30 kg/ m<sup>2</sup>, and morbid obesity is defined as a BMI greater than 37 kg/ m<sup>2</sup> or exceeding 170% of the reference values (Gray, 1989).

Recently, body fat distribution has been suggested as a better predictor of cardiovascular risk, lipid disorders, hypertension and type 2 diabetes than the degree of obesity. A high ratio of visceral (intra-abdominal) to subcutaneous fat, or upper body obesity as assessed by waist to hip circumference ratio, has been positively correlated with insulin resistance and NIDDM (Kissebah *et al.* 1987; Ferland *et al.* 1991).

## **1. 2. Adipose Tissue Cell Biology:**

Adipose tissue occurs as discrete depots and within other tissues such as muscles. The functional unit of adipose tissue consists of an adipocyte and a precursor cell (preadipocyte) in contact with at least one capillary (Markman, 1989). Mature adipocytes are usually large, > 100µm in diameter, and are about 95% triacylglycerol by weight.

Two types of adipose tissues have been identified: white adipose tissue (WAT) and brown adipose tissue (BAT). White adipose tissue is composed of cells with a single large central lipid droplet surrounded by a very thin rim of cytoplasm and accounts for the bulk of body fat, whereas BAT is made up of cells containing multilocular cytoplasmic lipid droplets and abundant mitochondria (Markman, 1989; Nechad, 1986). The brown pigment of BAT is attributable to the high content of cytochrome in the mitochondria. WAT and BAT play different roles in energy metabolism. While the main function of WAT is to store energy in the form of triacylglycerols (TG) in periods of nutritional abundance and release unesterified fatty acids when they are needed, BAT functions to utilize TG to produce heat (Trayhum, 1990). Heat production by these cells is dependent upon mitochondrial uncoupling protein (UCP). This UCP, the so-called thermogenin dissociates substrate oxidation from the production of adenosine triphosphate resulting in heat production. This thermoregulatory function is important in small mammals, neonates and in hibernation. In human adults, BAT is present only in small amounts.

### 1. 3. Adipocytes as Secretary/ Endocrine Cells

Traditionally, the adipocyte has been viewed as a passive organ for the storage and release of excessive energy. Recent knowledge of adipose cell biology, however, suggests the adipocyte as an endocrine/secretory cell with an ability to communicate with other cells through the production of a number of secreted molecules. Some of these secreted molecules have been found to be implicated in the regulation of its tissue mass and lipid metabolism (Flier, 1995). For example, adipocytes synthesize and secrete lipoprotein lipase and as shown more recently, cholesterol ester transfer protein, both of which are major regulators of TG and high density lipoprotein (HDL) metabolism (Quinet *et al.*, 1993). Acylation stimulating protein, a component of the alternative complement pathway secreted by adipocytes has been suggested to play a role in lipoprotein metabolism (Baldo *et al.*, 1993). Adipocytes also secrete angiotensinogen and monobutylin, vasodilators thought to play a role in regulating blood flow in the microvasculature (McGehee *et al.*, 1993; Wilkison *et al.*, 1991). More recently, leptin, the product of the obese gene secreted and released by adipocytes, has been shown to influence food intake and energy balance (Campfield *et al.*, 1995; Halaas *et al.*, 1995; Pelleymounter *et al.*, 1995). Tumor necrosis factor  $\alpha$  (TNF  $\alpha$ ) is another molecule found to be secreted by adipose tissue (Hotamisligil *et al.*, 1993) and to have significant effects on lipid metabolism such as lipolysis and inhibition of adipose differentiation (Beutler and Cerami, 1987).

Our own laboratory has adduced evidence for the release of yet unidentified factor(s) by adipocytes that induce preadipocyte differentiation (Shillabeer *et al.*, 1989). Taken together, the adipose cell is capable of secreting a large number of molecules that could act via a paracrine/autocrine mode of communication to influence adipose tissue

growth and development. Elucidation of these components/factor(s) may help us understand the mechanism(s) that control adipose tissue development.

#### **1. 4. Control of Adipose Tissue Growth and Development**

As mentioned earlier, adipose tissue is composed mainly of mature adipocytes, preadipocytes and microvascular endothelial cells. Preadipocytes are fibroblast-like precursor fat cells which are capable of undergoing replication. When induced to differentiate, these precursor cells accumulate lipid and convert into mature adipocytes (Roncari, 1984). Adipose tissue mass is a result of an increase in number (hyperplastic growth) or size (hypertrophic growth) of mature adipocytes or both (Rosche, 1981). Previously, it was believed that there was a fixed number of mature fat cells during adult life (Hirsch & Knittle, 1970; Faust *et al.*, 1978). Hence, weight gain in adults could occur only by hypertrophic deposition. We now know that adipocyte precursors are present throughout life and change in number and size of adipocytes can occur any time during life. A dynamic equilibrium between slow preadipocyte replication and differentiation leads to a stable body fat content in normal adults. An increase in the rate of preadipocyte replication and differentiation could lead to the development of obesity.

#### **1. 5. Cell-culture Models for the study of Adipocyte Development**

##### **1. 5. 1. Propagating Preadipocyte Cell Cultures**

The study of adipocyte development *in vitro* has been facilitated to a great extent by the availability of propagating cell cultures. Cultured preadipocytes when appropriately stimulated differentiate into cells possessing the biochemical and

morphological phenotypes of adipocytes, indicating they can be considered as true fat cell precursors *in vitro* (Van & Roncari, 1982). These cell lines can be derived from multipotent stem cell lines or preadipocyte cell lines. Cell-lines exhibiting stem cell characteristics include 10T1/2, CHEF/18, Balb/c 3T3 and 1246. Following treatment with 5-azacytidine, an inhibitor of DNA methylation (Taylor and Jones, 1979), these cells can generate several cell types, each of which can terminally differentiate into a specific lineage such as, adipocytes, myocytes, or chondrocyte lineage (Konieczny and Emerson, 1984). 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 (Green and Kehinde, 1974; Green & Kehinde, 1975; Green & Kehinde, 1976). Preadipocyte cell lines represent a late stage of adipose development in which cells have already undergone commitment to the adipocyte lineage and can further differentiate only into adipocytes. The most extensively characterized preadipocyte cell lines are the 1246 and Ob 1771 lines. Ob 17 cells were derived from the stroma of epididymal fat pads from adult Ob/Ob genetically obese mouse (Negrel *et al.*, 1978) and several sublines such as Ob 1771 lines were selected. Preadipocyte cell lines such as TA1 and 30A5 are also determined preadipocytes derived from 10T1/2 multipotent stem cells following treatment with 5-azacytidine.

### 1. 5. 2. Primary Preadipose Cells

Primary cultures of stromal-vascular cells from adipose tissue have also been used extensively (Deslex *et al.*, 1986; Hauner *et al.*, 1988; Shillabeer *et al.*, 1989), to delineate the physiological effectors of adipose differentiation *in vitro*. Although clonal preadipocytes exhibit many similar characteristics and have been very useful for the study of adipocyte development, they suffer a disadvantage because they are aneuploid and therefore, possess characteristics that differ from those of tissue preadipocytes. Monolayer confluence, which is considered to be a prerequisite for differentiation in 3T3-L1 cells (Green and Meuth, 1974; Green and Kehinde, 1975; Eckel *et al.*, 1977) was not required by rat preadipocytes in primary culture (Shillabeer *et al.*, 1989). Some of the reports also suggest that the response of clonal preadipocytes to agents that induce/repress differentiation also differ significantly from cultured rat and human preadipocytes. For example, cat serum did not induce differentiation in 3T3-L1 cells but it induced significant adipose conversion in rat preadipocytes in primary culture (Djian *et al.*, 1985). Epidermal growth factor that has been reported to inhibit adipose conversion in rat preadipocytes in culture by many investigators, including our laboratory, stimulated differentiation of 3T3-L1 cells (Li *et al.*, 1993). Hence, data drawn on studies using aneuploid cells should be interpreted with caution and, whenever possible, verified by using diploid preadipose cells in primary culture (Cornelius *et al.*, 1994).

## **1. 6. Adipose Cell Differentiation**

### **1. 6. 1. Sequence of Events**

Differentiation of preadipocytes is a complex process. It takes place in an orderly sequence. Preadipocytes replicate until cell-cell contact at confluence density inhibits mitosis (Bernlohr *et al.*, 1985). The cell-cell contact at confluent density is thought to be a prerequisite for adipocyte differentiation in 3T3-L1 cells (Green and Meuth, 1974; Green and Kehinde, 1975; Eckel *et al.*, 1977), however, adipose conversion has been shown to occur in low density primary cultures containing high concentrations of human plasma (Krawisz and Scott, 1982; Shillabeer *et al.*, 1989). Preadipocytes enter a unique growth-arrested stage at the  $G_0/G_1$  cell cycle boundary which is permissive for subsequent differentiation. This stage was referred to as  $G_D$  by Scott and colleagues (Scott *et al.*, 1982). At this stage, preadipocytes begin to express the early markers of adipocyte differentiation including lipoprotein lipase. After having ceased to divide at confluence, preadipocytes can be induced to differentiate with appropriate adipogenic stimuli.

After differentiation has been initiated, cells can dedifferentiate and reenter mitosis only for a period of time. Dedifferentiation can be initiated by disrupting cell/cell contact or by exposing cells to certain agents, for example, retinoic acid (Hoerl *et al.*, 1984) and tumor necrosis factor  $\alpha$  (Ron *et al.*, 1992; Petruschke and Hauner, 1993). Experiments with Balb/c 3T3 cells suggest that once cells have gone beyond a specific stage, they can no longer undergo dedifferentiation but rather are committed to terminal differentiation (Wier and Scott, 1986; Filipak *et al.*, 1988; Wang and Scott, 1993). A serum protein referred to as a proliferin and purified by Wier and Scott (Wier and Scott,

1986) was found to be capable of inducing terminal differentiation. Once differentiated terminally, mature adipocytes are believed to lose their ability to replicate.

Differentiation of preadipocytes is characterized by morphological and biochemical changes. Morphologically, cells round up and accumulate cytoplasmic lipid thereby losing their fibroblastic appearance and acquire the characteristic signet-ring appearance of mature adipocytes (Green and Meuth, 1974). The biochemical conversion involves an increase in TG content and in the activity of the enzymes that are involved in the synthesis of fatty acids and TG, such as acetyl CoA carboxylase, fatty acid synthase (FAS), and glycerol-3 phosphate dehydrogenase (G3PDH). These lipogenic enzymes, as well as lipoprotein lipase (LPL), are expressed in a temporal sequence, with LPL being an early marker (0-1 day post confluence), whereas G3PDH is a late marker of differentiation. The differentiated cells also acquire the proteins necessary for the release of TG such as hormone sensitive lipase (Roncari *et al.*, 1980). Consistent with the transition from fibroblast morphology, a concomitant decrease occurs in the cellular content of the cytoskeletal proteins, actin and tubulin (Bernlohr *et al.*, 1985; Cook *et al.*, 1985).

### **1. 7. Exogenous Modulators of Preadipocyte Differentiation**

Normally preadipocytes, upon reaching monolayer confluence, undergo differentiation in culture only with enrichment of culture conditions. A number of agents have been studied using embryonic fibroblasts and preadipocytes in primary culture that induce differentiation *in vitro*. The most widely used hormonal cocktail includes glucocorticoids, high levels of insulin (which may act through insulin-like growth factor-1 receptor) and 3-isobutyl-1-methylxanthine (IBMX, a cAMP phosphodiesterase

inhibitor) (Student *et al.*, 1980). The agents most frequently used to differentiate 3T3-F442A cells are insulin and calf serum (Green and Kehinde, 1975). Differentiation of 3T3-L1 and 30A5 cells can be induced with dexamethasone and insulin in media enriched with fetal bovine serum (Student *et al.*, 1980). Several other agents have been reported to either induce or inhibit adipocyte differentiation. Those that induce differentiation include thyroid hormones (Gharbi-chihi *et al.*, 1991), sodium butyrate (Toscani *et al.*, 1990), ascorbic acid (Ono *et al.*, 1990), prostacyclin (Vassaux *et al.*, 1992), aldosterone (Rondinone *et al.*, 1993) and arachidonic acid (Gaillard *et al.*, 1989) etc. Some of the investigated inhibitory agents include TNF  $\alpha$  (Beutler and Cerami, 1987), transforming growth factor  $\beta$  (Ignotz & Maasague, 1985), phorbol ester (Diamond *et al.*, 1977), retinoic acid (Stone and Bernlohr, 1990) and prostaglandin F<sub>2</sub> $\alpha$  (Serrero *et al.*, 1992).

Conditions have been developed for achieving adipocyte differentiation in the absence of serum using clonal cell lines and primary stromal preadipocytes (Guller *et al.*, 1988; Hauner, 1990; Schmidt *et al.*, 1990; Catalioto *et al.*, 1992). The serum free medium contains insulin, triiodothyronine, growth hormone, transferrin and agents which increase cAMP concentration. This advancement has made it possible to characterize some of the extracellular and intracellular signals that are involved in the control of adipose conversion.

### **1. 8. Paracrine Interactions in Adipose Tissue Development and Growth.**

As described earlier, the adipocyte is a metabolically very active cell. A number of products are synthesized and secreted in white adipose tissue that may influence appetite and food intake regulation (Campfield *et al.*, 1995; Halaas *et al.*, 1995).

lipoprotein and cholesterol metabolism as well as preadipocyte replication and differentiation (Flier *et al.*, 1987; Pykalisto *et al.*, 1975; Lau *et al.*, 1987a; Lau *et al.*, 1987b). While the molecular mechanism of these molecules is under investigation, there is evidence suggesting that these molecules may influence some of these processes via a paracrine or autocrine mode of action or both. Regional differences in the growth of adipose tissue *in vivo* (Faust *et al.*, 1978) and *in vitro* (Djian *et al.*, 1983; Lau *et al.*, 1987a; Lau *et al.*, 1987b; Shillabeer *et al.*, 1990) suggest the role of local factors in the control of adipose tissue development. Preadipocytes from retroperitoneal fat pads were shown to replicate faster in response to high dietary fat as compared to preadipocytes from epididymal or subcutaneous pads (Faust *et al.*, 1978). A paracrine mode of action was further supported by the studies from our laboratory using constituent cells of rat adipose tissue. Replication of rat preadipocytes was found to occur due to factors released by proliferating preadipocytes from massively obese humans (Lau *et al.*, 1987a). Extracellular matrix components secreted by adipose tissue-derived microvascular endothelial cells stimulated preadipocyte differentiation *in vitro* (Varzaneh *et al.*, 1994). Our laboratory has also adduced evidence for paracrine signals originating from mature rat adipocytes that regulated preadipocyte differentiation (Shillabeer *et al.*, 1989). Medium conditioned by mature adipocytes induced differentiation of its precursor cells grown in primary culture. This adipogenic effect of the medium was not due to the release of TGs from broken fat cells, but rather secondary to some other unidentified factor(s). One objective of the present study was focused on the elucidation of positive regulator(s) of adipocyte differentiation originating from adipose tissue's own microenvironment.

## 1. 9. Prostaglandins (PG) in Adipose Tissue

### 1. 9. 1. PG Biochemistry

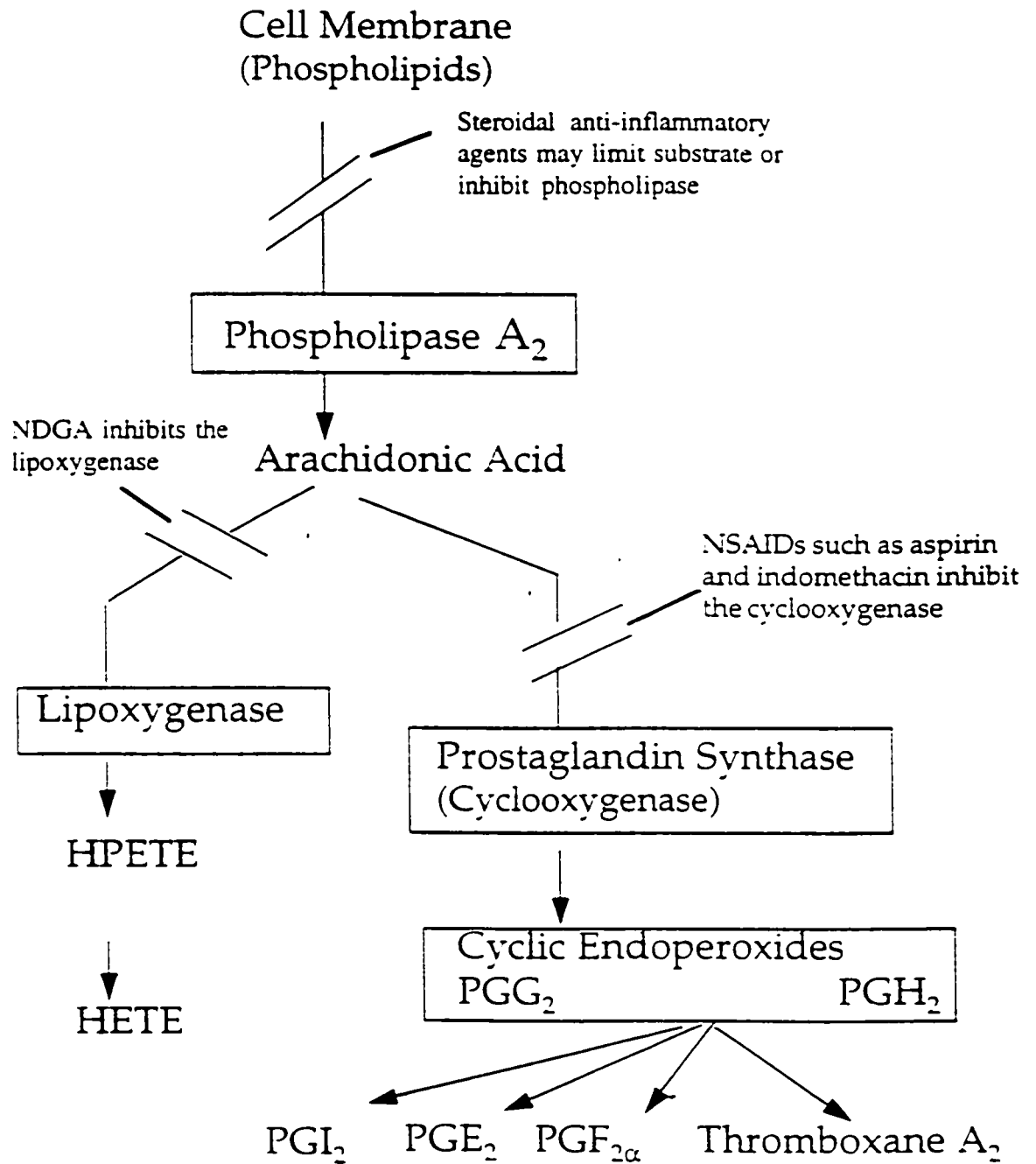
Prostaglandins are unsaturated hydroxy fatty acids with one cyclopentane ring and two side chains for a total of 20 carbon atoms (Samuelsson *et al.*, 1978; Moncada & Vane, 1979; Corey *et al.*, 1980). They are formed from polyunsaturated fatty acids through oxygenation and cyclization under the control of the enzyme cyclooxygenase. Three essential fatty acids, dihomono- $\gamma$ -linolenic acid (20:3  $\omega$ -6), arachidonic acid (20:4  $\omega$ -6) and eicosapentaenoic acid (20:5  $\omega$ -3), serve as substrates for the synthesis of prostaglandins of the '1', '2' and '3' series, respectively. These fatty acids are contained in meat and fish constituents of the diet and in some plant foods (Willis and Stone, 1976). Arachidonic acid (AA), the precursor of dienoic prostaglandins, is the most abundant of these three fatty acids present in membrane phospholipids mainly bound at the 2-position of the glycerol moiety in most species (Van den Bosch, 1980; Irvine, 1982) and can be obtained directly from the diet or by desaturation and chain elongation from dietary linoleic acid (18:2  $\omega$ -6). This explains why prostaglandins of the '2' series are commonly detected. Arachidonic acid can be released from cell membranes by the action of phospholipases which thus becomes available for PG synthesis. The correlation of AA release with prostaglandin production suggests 'AA release' as an important control point. Synthesis of prostaglandins takes place in three stages (Smith, 1986): (a) release of AA from phospholipids, (b) oxygenation and cyclization of free AA to PGG<sub>2</sub> and PGH<sub>2</sub> endoperoxides by cyclooxygenase (COX) and (c) metabolism of PGH<sub>2</sub> to specific biologically active PG or thromboxanes. PGG<sub>2</sub> and PGH<sub>2</sub> endoperoxides are unstable in aqueous solution (half-life approximately 5 min at 37°C) and decompose quickly to the stable metabolites, PGE<sub>2</sub>, D<sub>2</sub> and F<sub>2 $\alpha$</sub>  and prostacyclin (PGI<sub>2</sub>). PGI<sub>2</sub> is also unstable and

metabolizes to its stable metabolite 6-keto PGF<sub>1α</sub>. The various prostaglandins differ from each other by the degree of unsaturation and saturation in the cyclopentane ring or aliphatic side chains. Prostaglandins are designated A through I depending on the exact structure of the cyclopentane ring. The subscript numbers (1, 2 and 3) denote the number of double bonds in the side chains.

### **1. 9. 2. Synthesis of Lipoxygenase Products**

Catabolism of arachidonic acid by lipoxygenases (LOX) rather than COX generates another class of biologically active compounds which includes leukotrienes and a variety of hydroxy and hydroperoxy fatty acids. Three different mammalian lipoxygenases catalyse the insertion of molecular oxygen into arachidonic acid at positions 5, 12 or 15. The initial product formed by each of these reactions is a hydroperoxyeicosatetraenoic acid (5-, 12-, or 15 HPETE). HPETE is unstable and is reduced to the corresponding stable hydroxyeicosatetraenoic acid (5-, 12-, or 15 HETE) (Samuelsson *et al.*, 1987). HETEs have been known to be involved in a number of biological processes including blood pressure regulation and renal functions. Very recently, 8(s) HETE, a relatively less studied HETE, was found to be adipogenic to 3T3-L1 cells by binding to a nuclear receptor, peroxisome proliferator activating receptor. (Yu *et al.*, 1995). Together, the various oxygenated arachidonic acid metabolites such as prostaglandins, leukotrienes, and lipoxins are called eicosanoids (Moncada & Vane 1979; Corey *et al.*, 1980). The pathway for the formation of eicosanoids is presented in Figure 1.

**Fig. 1. Pathway For The Formation of Eicosanoids**



### **1.9. 3. Mechanism of Eicosanoid Actions: Eicosanoids as Local Hormones**

Ferreira and Vane (1967) demonstrated in their studies that infused PGE and PGF $\alpha$  derivatives failed to survive a single pass through the circulation suggesting that prostaglandins act in close proximity to their site of synthesis. Two observations support this suggestion: first, that plasma concentrations of eicosanoids in physiological conditions are less than  $10^{-9}$  M (Granstorm and Samuelsson, 1978; Dunn et al, 1978) and second, that eicosanoid synthesis occurs in large variety of tissues rather than a central endocrine organ (Smith, 1986; Borgeat, 1987). The action of locally produced PG *in vivo* is rapidly terminated by local metabolism resulting in products that normally have little or no biological activity (Moncada & Vane, 1979). Prostaglandins are not stored within cells (Piper & Vane, 1971; Bills *et al.*, 1977), but quickly exit the cell. So, eicosanoids are considered to be extracellular mediators eliciting their biological effects by binding to specific membrane receptors (MacIntyre, 1985), but it seems possible that some of them could exert their effects via nuclear receptors (Forman *et al.* 1995; Kliewer *et al.* 1995).

### **1. 9. 4. Prostaglandin Biosynthesis in Adipocytes**

It has been known for some time that adipose tissue is a site of prostaglandin production (Shaw and Ramwell, 1968). As discussed above, in most cell types the precursor of prostaglandin formation is mainly arachidonic acid present in the phospholipids of cell membranes, which can be released for PG synthesis by the action of a specific phospholipase A<sub>2</sub> (or by other phospholipases) (Van den Bosch, 1980; Irvine, 1982). In adipocytes, however, arachidonic acid may not only be esterified in the phospholipids but may also be stored in neutral lipids (triacylglycerol) (Christ & Nugteren, 1970; Dalton & Hope, 1974; Negrel & Ailhaud, 1981) and may become

available for prostaglandin synthesis during triacylglycerol lipolysis. The release of PG from adipose tissue of various species in response to lipolytic agents has been demonstrated in a number of studies (Lewis and Mathews, 1970; Dalton and Hope, 1974; Axelrod and Levine, 1981; Richelsen, 1987). Some of the vaso-active agents such as bradykinin and angiotensin were also able to stimulate prostaglandin formation in rat adipocytes (Axelrod *et al.*, 1985; Darimont *et al.*, 1994). These agents liberate arachidonic acid stored in phospholipids without affecting lipolysis.

Prostaglandins are metabolized very rapidly *in vivo*. However, in isolated rat adipocytes it has been shown that more than 90% of added PGE<sub>2</sub> could be recovered from the adipocyte suspension after incubation for 60-120 min at 37<sup>0</sup>C (Axelrod *et al.*, 1985; Richelsen and Pedersen, 1987). PGI<sub>2</sub> is known to decompose rapidly into a chemically stable, but biologically inactive derivative, 6-keto-PGF<sub>1α</sub>. Studies of this reaction *in vitro* established that PGI<sub>2</sub> has a half life of about 3 minutes (Cho and Allen, 1978). Due to this spontaneous hydrolysis of PGI<sub>2</sub>, the quantitation of 6-keto-PGF<sub>1α</sub> is generally accepted by many researchers as a measure of PGI<sub>2</sub> formation (Fitzpatrick *et al.*, 1979). Currently, various radioimmunological assays (RIA) have been used to determine prostaglandin production. These assays are highly sensitive and have the advantage of accomodating large number of samples compared to bioassay and chromatographic assays. Table 1 summarizes the production of PG in adipocytes. When measured by RIA, the release of PGI<sub>2</sub> (measured as 6-keto-PGF<sub>1α</sub>) from isolated rat adipocytes under basal conditions was reported to be in excess of PGE<sub>2</sub>.

**Table 1. PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> production in rat and human adipocytes under basal conditions.** PG were extracted from the cell-free medium and measured by RIA.

Source	PGE <sub>2</sub>	6-keto-PGF <sub>1α</sub>	Reference
	ng/10 <sup>6</sup> cells		
Rat	0.25	0.51	Richelsen, 1987
	0.56	1.23	Axelrod and Levine, 1981 Axelrod et al, 1985
Human	0.12	0.59	Richelsen, 1987

*In situ* microdialysis of rat adipose tissue further confirmed the production of PGI<sub>2</sub> and PGE<sub>2</sub> in this tissue (Darimont *et al.*, 1994). The basal extracellular concentrations of 6-keto-PGF<sub>1α</sub> and PGE<sub>2</sub> determined by this method in rat adipose tissue were estimated to be 1 and 0.3 nM respectively. Thus PGE<sub>2</sub> and PGI<sub>2</sub> have been detected in considerable amounts in adipose tissue in different studies, whereas PGF<sub>2α</sub> has been found in smaller quantities (Dalton and Hope, 1974; Axelrod and Levine, 1981) with little or no PGE<sub>1</sub> (Christ & Nugteren, 1970; Dalton & Hope, 1974). The formation of PG has also been demonstrated in human subcutaneous adipocytes (Chang *et al.*, 1977; Brain & Lewis, 1981; Richelsen, 1987). In agreement with the findings in rat adipocytes, the main PG were found to be PGI<sub>2</sub> and PGE<sub>2</sub> (Richelsen, 1987; Table 1). However, if adipocyte size and surface area are taken into account, the formation of both prostaglandins is considerably smaller in human than in rat adipocytes (5-10 times less) (Richelsen, 1987).

Adipogenic cell lines such as ob17 and 3T3-L1 have also been reported to produce prostaglandins (Negrel & Ailhaud, 1981; Hyman *et al.*, 1982). In the

preadipocyte stage of 3T3-L1 cells, however, PGE<sub>2</sub> was the major prostaglandin produced (783 ± 100 pmol/ mg protein) and 6-keto-PGF<sub>1α</sub> was found only at low levels (269 ± 65 pmol/ mg protein) (Hyman *et al.*, 1982). The rate of PGE<sub>2</sub> and 6-keto-PGF<sub>1α</sub> synthesis in clonal preadipocytes was maximal during the growth phase and decreased dramatically after confluence (Negrel and Ailhaud, 1981; Hyman *et al.*, 1982). In summary, it is now established that prostaglandins are produced in both rat and human adipocytes.

### **1. 9. 5. Inhibition of Prostaglandin Synthesis**

As in other cell systems, prostaglandin formation in adipocytes can be inhibited by non-steroidal antiinflammatory drugs (NSAID), such as aspirin and indomethacin. The enzyme COX, which generates and releases prostaglandins in response to various stimuli, is ubiquitous in mammalian tissues and is sensitive to NSAID. The inhibition of PG synthesis by NSAID has been demonstrated in adipose tissue both *in vivo* and *in vitro*. Indomethacin and aspirin inhibited PGE<sub>2</sub> and 6-oxo-PGF<sub>1α</sub> production in rat and human adipocytes (Richelsen, 1987). For that reason, these drugs are widely used as a tool to evaluate the physiological role of prostaglandins. Upon exposure to aspirin, the enzyme is acetylated that leads to irreversible COX inhibition (Smith and Lands, 1971) and thus new enzyme synthesis is required before more prostaglandins can be produced. Indomethacin, meclofenamate and flurbiprofen also cause irreversible inactivation of the COX activity but without covalent modification of the enzyme (Rome and Lands, 1975; Pace-Asciak and Smith, 1983). NSAID inhibit specifically the COX and not the LOX activity (Flower and Vane, 1973; VanderOuderaa *et al.*, 1980). Nordihydroguaiaretic acid (NDGA) is reported to be inhibitor of LOX (Higgs and Vane, 1983).

Anti-inflammatory steroids such as glucocorticoids also inhibit the release of prostaglandins from various tissues including adipose tissue (Lewis and Piper, 1978; Mitchell *et al.*, 1983), but these drugs do not inhibit the COX enzyme (Lewis and Piper, 1978; Flower and Vane, 1974). They inhibit the activity of phospholipase A<sub>2</sub> and hence reduce the release of arachidonic acid. However, the involvement of glucocorticoid in the release of arachidonic acid in many tissues including adipose tissue is controversial. Treatment of Ob1771 preadipocytes with corticosterone elicited enhanced arachidonic acid release and increased synthesis of PGI<sub>2</sub> (Gaillard *et al.*, 1991). Dexamethasone also stimulated arachidonic acid conversion to PGE<sub>2</sub> in human amnion cells (Zakar and Olson, 1989). In conclusion, anti-inflammatory steroids influence the prostaglandin system by mechanism which differs from that of NSAID.

#### **1. 9. 6. Role of Prostaglandins as Paracrine Regulators of Adipocyte Development**

Prostaglandins play an important role in cellular processes. In adipose tissues, prostaglandins have been shown to influence adipocyte functions, such as lipolysis (Christ and Nugteren, 1970; Richelsen and Pedersen, 1987). Recent evidence suggests that they may also be important paracrine regulators of preadipocyte development. Vassaux *et al.* (1992) suggested PGI<sub>2</sub> as a local hormone playing the role of a hyperplastic agent in the development of adipose tissue. However, the effect of prostaglandins on adipose differentiation has yielded variable results. Prostaglandins have been shown to both accelerate and inhibit adipocyte differentiation depending on the cell line and culture conditions tested. Prostacyclin (PGI<sub>2</sub>) stimulated preadipocyte differentiation in Ob 1771 cell lines and rat and human preadipocytes in the presence of serum free medium. (Negrel *et al.*, 1989). PGF<sub>2α</sub> potentiated the effect of cPGI<sub>2</sub> in adipose conversion in ob1771 cells (Catalioto *et al.*, 1991). PGF<sub>2α</sub> also stimulated differentiation of 3T3-L1 cells (Hopkins

& Gorman, 1981). More recently, 15-d PGJ<sub>2</sub>, a metabolite of PGD<sub>2</sub> has been reported to be adipogenic in C3H10T1/2 fibroblasts (Kliwer et al, 1995).

In contrast to stimulation observed in Ob1771 cells, rat and human preadipocytes, PGI<sub>2</sub> inhibited the differentiation of 3T3-L1 (Hopkins and Gorman, 1981). PGF<sub>2α</sub> also inhibited differentiation of adipocyte precursors in primary cultures (Serrero et al, 1992a) and of cell line 1246 (Serrero *et al*, 1992b). Very recently, Casimir and his coworkers have reported inhibition of differentiation in 3T3-L1 preadipocytes by PGF<sub>2α</sub>, PGE<sub>2</sub> and PGD<sub>2</sub> (Casimir *et al*, 1996). Cyclooxygenase catalyzes the initial step in the breakdown of arachidonic acid to prostaglandins, and the cyclooxygenase inhibitor, indomethacin, promoted differentiation in 3T3-L1 (Williams & Polakis, 1977) and TA1 cells (Knight, *et al*, 1987), consistent with the reports suggesting PG as negative regulators of preadipocyte development. However, the concentration of indomethacin (100 μM) required for a significant stimulation of differentiation in TA1 cells was much greater than that needed for complete inhibition of cyclooxygenase and may have actions independent of inhibition of prostaglandin synthesis.

In one study, the PLA<sub>2</sub> activity, a rate limiting enzyme in the production of prostaglandins, was found to be low in undifferentiated 1246 preadipocytes (Gao and Serrero, 1990). However, the activity increased significantly during differentiation, with a concomitant increase in the level of PGF<sub>2α</sub>, whereas the PGE<sub>2</sub> level remained unchanged. This also provides evidence for the production of prostaglandins, particularly PGF<sub>2α</sub> by mature adipocytes and for a mechanism by which differentiated cells might regulate the differentiation of precursor cells. The role of prostaglandins in the regulation of adipose tissue development is, however, controversial. The effective concentration of prostaglandins in most of these studies was much higher than the concentration actually

found in adipose tissue (Darimont *et al.*, 1994). Much more work is needed to elucidate the role of prostaglandins as paracrine/autocrine regulators of adipocyte development at the physiological level.

## **2. HYPOTHESIS AND RESEARCH OBJECTIVES**

The overall objective for studying adipose tissue development was to identify the factors that control the process of differentiation and to determine how these factors act *in vivo*. The proposed work examined the following hypothesis with 3 specific objectives.

### **2.1. Hypothesis**

Prostaglandins released by adipocytes or preadipocytes act via paracrine/autocrine mechanism(s) to modulate preadipocyte differentiation.

### **2.2. Objectives**

1. To determine if mature adipocytes or preadipocytes release prostaglandins that influence preadipocyte differentiation.
2. To measure the release of prostaglandins by adipocytes and preadipocytes.
3. To study the physiological effect of prostaglandins on adipocyte development.

### **2.3. Rationale**

Adipose cell size and number are carefully regulated by yet unknown mechanisms in each adipose tissue depot. The regional variations in body fat suggest the role of paracrine/autocrine factors in the control of adipose tissue growth. Previous work from our laboratory has shown that adipocytes secrete some factor(s) in culture medium that

play an important role in the differentiation of preadipocytes. (Shillabeer *et al.* 1989). However, the precise identity of these factors has not been elucidated. Prostaglandins are produced and released by adipose tissue (in the extracellular matrix) and have been suggested to play a role in adipose cell differentiation. The role of prostaglandins in adipose cell differentiation, however, remains controversial. In one study, PGE<sub>2</sub>, PGI<sub>2</sub> and PGF<sub>2α</sub> (main PG of adipose tissue) all markedly inhibited differentiation, while the same prostaglandins were shown to stimulate differentiation in another study. Moreover, the effective concentration of prostaglandins in most of the studies was many fold greater than the physiological amount secreted in the adipose tissue microenvironment. Much of the prostaglandin literature has focused on the pharmacological concentrations of prostaglandins on adipocyte differentiation.

The present study was aimed at determining the paracrine/autocrine effect of prostaglandins derived directly from primary rat adipocytes/preadipocytes on the differentiation of primary preadipocytes. An understanding of the paracrine role of prostaglandins in adipose differentiation at physiological level may provide a new insight into the mechanisms that control the process of adipocyte development.

## **3. MATERIALS**

### **3. 1. Chemicals**

Collagenase type II, bovine insulin, bovine serum albumin, corticosterone, 3-isobutyl-1-methyl xanthine, adenosine, hepes, triiodothyronine, holo-transferrin, dihydroxyacetone phosphate,  $\beta$ -nicotinamide adenine dinucleotide (reduced form,  $\beta$ -NADH), triethanolamine, 2, 2, 4- trimethyl pentane, diethyl ether, Tris-HCl, trypsin, indomethacin and nordihydroguaiaretic acid were purchased from Sigma Chemical Co. (St. Louis, Mo). All prostaglandins were obtained from either Cayman Chemicals (Ann Arbor, MI) or Sigma and their specified purities were always > 99%. [1- $^{14}$ C] arachidonic acid, PGE<sub>2</sub> and 6-keto PGF<sub>1 $\alpha$</sub>  radioimmunoassay kits were purchased from Amersham (Oakville, ON). 2-Mercaptoethanol was obtained from Aldrich (Milwaukee, WI). Isotone II was from Coulter Diagnostics (Burlington, ON). All other reagents were bought from either Fisher Scientific Ltd. (Nepean, ON) or BDH Inc. (Toronto, ON).

### **3. 2. Sera and Cell Culture Media**

Alpha-minimum essential medium ( $\alpha$ MEM), Dulbecco's modified Eagle's medium with Ham's F12 (DMEM/F12), fetal bovine serum (FBS), calf serum (CS), penicillin and streptomycin were all purchased from Gibco (Burlington, ON).

### 3.3. Tissue Culture Plastic Ware

Tissue Culture plastic ware (6- and 24- well plates, and 25- and 75-cm<sup>2</sup> flasks) was obtained from Becton- Dickinson (Oxnard, CA). Filters (0.22 µm) used to filter all cell culture solutions and media were purchased from Millipore (Bedford, MA) or Nalgene (Rochester, NY).

## 4. METHODS

All studies were conducted on rat preadipocytes propagated in primary culture. Primary rat adipocytes were used to collect the conditioned medium or were used as test conditions. Epididymal and retroperitoneal fat depots of adult male Sprague-Dawley rats (Charles River Breeding Laboratories Inc., Montreal, Quebec) weighing about 300-400 g were the source of both preadipocytes and adipocytes. The rats were housed two per cage for a minimum of 7 days before sacrifice. The temperature of the animal room was 22°C with a 12-hr light/dark cycle. The rats were provided with standard rat chow and tap water *ad libitum*. Rats were killed by cervical dislocation under light halothane anesthesia and immediately submerged in phenomyicide solution, a germicidal detergent, for at least five minutes. Following a midline incision and retraction of skin, fat pads were resected under sterile conditions, pooled and processed to isolate preadipocytes and adipocytes according to a previously reported method (Shillabeer *et al.*, 1989).

#### 4.1. Preadipocyte Primary Culture

After excision, the fat pads were minced and digested with 1.5 mg/ml type II collagenase in Krebs-Ringer-Hepes buffer, pH 7.4, containing 2 mM glucose, 200 nM adenosine and 1% bovine serum albumin (BSA), with gentle shaking in a rotatory water bath at 37<sup>0</sup> C for 1 h. Following digestion, the cell suspension was filtered through a 150 µm nitex mesh filter. The floating layer which consisted of mature adipocytes was processed separately to collect the conditioned medium, whereas the infranatant was used to isolate preadipocytes. The infranatant was centrifuged at 100 x g for 10 min at room temperature. The pellet was resuspended in αMEM and filtered through a 25 µm mesh filter. The cells thus obtained consisted mainly of preadipocytes. Aliquots of preadipocyte suspension were counted electronically with a multisizer Coulter Counter II (Coulter Electronics Inc., Hialeah, FL) and cells were seeded at a density of 4 x 10<sup>4</sup> cells/cm<sup>2</sup> in 24-well plates (unless specified otherwise) in αMEM supplemented with 10% FBS, penicillin (63 mg/L), streptomycin (134 mg/L) and NaHCO<sub>3</sub> (2.2 g/L). Cells were propagated in a cell culture chamber maintained at 37<sup>0</sup>C in a humidified atmosphere of 95% air - 5% CO<sub>2</sub>.

After 24 h, the adherent cells were washed thoroughly with fresh αMEM to remove blood cells and unattached cells and maintained in αMEM supplemented with 10% FBS till they became confluent. Preadipocytes reached monolayer confluence approximately four days after seeding and were used as test cells in all the experiments.

## **4.2. Collection of Adipocyte-Derived Conditioned Medium (ACM)**

The floating layer of adipocytes resulting from the filtration of the digested cell suspension through 150 micron nylon mesh was washed thrice with fresh Krebs-Ringer-Hepes buffer. Washed adipocytes were resuspended in  $\alpha$ MEM supplemented with 10% FBS and 4% BSA and centrifuged at 50 x g for 5 min at room temperature. The medium below adipocyte layer was aspirated and mature adipocytes were resuspended in fresh medium corresponding to 1/4 (vol/vol) of the adipocyte layer. After enumerating with a hemacytometer (Hausser Scientific, Horsham, PA), adipocytes were seeded at a density of  $4 \times 10^5$  cells into a precoated 25 cm<sup>2</sup> flask. The flasks were precoated with a small volume of  $\alpha$ MEM supplemented with 10% FBS and 4% BSA to promote cell adhesion. Counting of adipocytes was done once and the same estimated volume was used for all other times. After seeding, the flasks were inverted quickly to allow cells to adhere to the surface of the flasks and incubated at 37<sup>0</sup>C for 60-90 min. After cells had attached to the surface, flasks were returned to their original positions and 5 ml of  $\alpha$ MEM supplemented with 10% FBS was added gently making sure that adipocytes did not detach. The conditioned medium was collected after 48 h. A second collection was also made after the next 48 h.

## **4.3. Assessment of Differentiation**

Biochemical measurement of glycerol-3-phosphate dehydrogenase (G3PDH) activity, a lipogenic enzyme that appears late during the course of adipocyte maturation, was used as an index of preadipocyte differentiation. Morphological changes and lipid accretion were observed by looking at cells by phase contrast microscopy.

#### **4.4. Preparation of Cell Extract for G3PDH and Protein Assay**

Following preadipocyte culture, on day 10 after seeding, except in serum-free conditions in which cells were cultured for longer time, differentiated cells were washed thrice in phosphate buffered saline (PBS), scraped with a rubber policeman into 25 mM Tris-HCl, pH 7.4, containing 1 mM EDTA and 1 mM 2-mercaptoethanol and stored at  $-80^{\circ}\text{C}$  till used. On the day of assay, the cell suspension was sonicated at  $4^{\circ}\text{C}$  at 30 W for 5-10 sec (Fisher Sonic Dismembrator, Model 300, Ottawa, ON) and centrifuged at  $95,000 \times g$  for 10 min at  $4^{\circ}\text{C}$ . The supernatant fraction was separated and assayed for G3PDH activity and protein determination, to be described as follows.

#### **4.5. Measurement of G3PDH Activity**

Assay of G3PDH activity was performed using the method of Kozak and Jensen (Kozak and Jensen, 1974). G3PDH activity was measured in the aliquots of supernatant section of the cell homogenate spectrophotometrically (Beckman Model DU-50 recording spectrophotometer). A known volume of the sample was mixed with the reaction mixture (60  $\mu\text{l}$ ) in a final volume of 200  $\mu\text{l}$ . The reaction mixture contained 1M triethanolamine, pH 7.4, 5M 2-mercaptoethanol, 25 mM EDTA and 0.2 mM  $\beta$ -NADH. The reaction was started by adding 4 mM dihydroxyacetone phosphate. The activity was determined under zero order kinetics with an interval time of 15 sec. and total time of 2 min. The initial rate of oxidation of NADH was measured at room temperature by a change in the absorbance at 340 nm. The values of the enzyme activity were calculated as follows and expressed in units/min/mg protein. One unit of enzyme activity corresponded to 1 nmole of NADH oxidized.

Calculations:

$[\text{Absorbance at time 0} - \text{absorbance at 2 min.} / 2 \text{ min.}] \times 32.154 / \text{volume of sample} \times 1000 = \text{unit/ml/min.}$

#### **4.6. Protein Determination**

The cellular protein content was determined by the Coomassie Blue method using BSA as the standard (Spector, 1978). The measurement of the protein concentration was based on the colorimetric reaction between Coomassie Blue G and the protein in the sample. The complex was formed in about 2 minutes and was stable for 1 h. The absorbance was recorded at 595 nm in a Beckman model DU-50 recording spectrophotometer. A standard curve was generated using dilution of BSA in a concentration range of 0 mg/ml to 21 mg/ml.

#### **4.7. Effect of Prostaglandins on Preadipocyte Differentiation**

To study the effect of prostaglandins on adipose differentiation, confluent primary preadipocytes were exposed to indomethacin, an inhibitor of prostaglandin synthesis, in the presence of  $\alpha$ MEM with 10% FBS, the standard differentiation mixture and ACM. Preadipocytes exposed to indomethacin in the presence of  $\alpha$ MEM with 10% FBS and standard differentiation mixture served as negative and positive controls, respectively. The standard differentiation mixture, also designated as MIX consisted of  $\alpha$ MEM supplemented with 10% calf serum,  $10^{-8}$  M insulin,  $10^{-7}$  M corticosterone and 0.5 mM 3-isobutyl-1-methylxanthine for the first 48 h after which the last was replaced with 0.5% (v/v) lipid emulsion and the cells were maintained in this medium till harvested for G3PDH assay. To inhibit prostaglandins released by adipocytes, indomethacin (100 $\mu$ M)

was added to  $\alpha$ MEM containing 10% FBS which was then added to the adherent adipocytes, as described earlier, in the preparation of adipocyte-conditioned medium (ACM). ACM thus containing indomethacin at the time of collection (ACM+I) was collected after 48 h. When exposed to test preadipocytes, the presence of indomethacin in this medium was presumed to inhibit prostaglandins released by the test preadipocytes as well. In a separate group, test preadipocytes were exposed to ACM to which indomethacin was added after its collection (ACM+Ia). The purpose of this group was to inhibit prostaglandins released by the test preadipocytes only.

Media were changed after every 2 days. Each experimental condition was analyzed in triplicate. Photomicrographs of cells were taken prior to harvesting. Cells were harvested in Tris-HCl and stored at  $-80^{\circ}\text{C}$  until assayed for G3PDH activity. The above experiment was repeated at least four times. The following summarizes the culture conditions to which confluent preadipocytes were exposed to study the effect of prostaglandins:

Adipocyte-derived conditioned medium (ACM)

ACM collected in the presence of indomethacin (ACM+I)

ACM+indomethacin added after collection (ACM+ Ia)

**Controls (Basal and Positive)**

$\alpha$ MEM supplemented with 10% FBS (FBS)

FBS + indomethacin (FBS+I)

Standard differentiation cocktail (MIX)

MIX + indomethacin (MIX+I)

#### **4. 8. Determination of Prostaglandins Release**

The production of prostaglandins by preadipocytes and adipocytes was determined by measuring their release into culture medium. Due to very short half life of PGI<sub>2</sub> (Cho and Allen, 1978), the production of this PG was measured by the release of its stable degradation product 6-keto-PGF<sub>1α</sub> (Fitzpatrick *et al* 1979). The release of prostaglandins by adipocytes and preadipocytes was examined under basal conditions (αMEM containing 10% FBS) by the following methods.

#### **4. 8. 1. Thin Layer Chromatography**

Isolated preadipocytes were seeded in 100 mm culture dishes (10<sup>4</sup> cells/cm<sup>2</sup>) in αMEM containing 10% FBS. On day 4, growing cells were washed with PBS at least three times and harvested with a rubber policeman. The pooled cell pellet was resuspended in 0.5 ml serum-free medium (αMEM). For assay of prostaglandin synthesis from arachidonic acid, cells were incubated with 10 μM [1-<sup>14</sup>C] AA (0.4 μCi) for 30 min at 37<sup>0</sup>C in a total volume of 0.5 ml. Cell-free medium (0.5 ml) incubated with [1-<sup>14</sup>C] arachidonic acid served as control. Radioactive prostaglandins released into the medium were extracted twice with 3 volumes of diethyl ether, methanol and 0.2 M citric acid (30:4:1, v/v/v). The cell suspension was immediately vortexed and centrifuged at 200 x g for 5 min. The organic layer was removed and evaporated to dryness under a stream of N<sub>2</sub> and redissolved in 50 μl of solvent system (see below) used to develop thin layer chromatography (TLC) plates. The samples and standard prostaglandins {6-keto PGF<sub>1α</sub>, PGE<sub>2</sub>, PGF<sub>2α</sub>, PGD<sub>2</sub> and 15-deoxy Δ<sup>12, 14</sup> PGJ<sub>2</sub> (15-d PGJ<sub>2</sub>)} were applied to silica gel TLC plates (Merck, F-254, 20x20 cm) and developed in an organic phase of ethyl acetate/ 2,2,4-trimethyl pentane/ acetic acid/ water (100:50:20:100, v/v/v/v) solvent system. The radioactive bands were visualized by autoradiography. The locations of standard

prostaglandins were spotted with iodine vapor, and the radioactive zones were defined. The identified labeled prostaglandins were scraped from the glass plates, added to 5 ml scintillation liquid and the radioactivity was determined in a liquid scintillation counter (Beckman LS 3801). The protein content of the cells was determined by the method of Spector, 1978 as described earlier in this chapter.

#### **4. 8. 2. Radioimmunoassay**

Duplicate samples of 24 h culture media conditioned by preadipocytes or adipocytes were also analyzed for prostaglandin concentrations by radioimmunoassay using commercial kits (Amersham). Preadipocytes were isolated and plated at a density of  $4 \times 10^4$  cells/cm<sup>2</sup> in 24 well plates in duplicates in  $\alpha$ MEM supplemented with 10% FBS. After 24 h, a period during which almost no cell replication occurs (Djian *et al.*, 1983), adherent cells were washed extensively to get rid of blood cells and maintained in 1 ml of  $\alpha$ MEM containing 10% FBS/ well. Culture medium was collected the following day in 2 ml of ethanol and stored at -20<sup>0</sup>C till measured. Preadipocytes were detached with 1 ml trypsin solution (50 mg/100 ml, 0.25 mM EDTA) and counted using the Coulter Counter. The release of PGE<sub>2</sub> and 6-keto PGF<sub>1 $\alpha$</sub>  by adipocytes was measured in ACM. ACM with or without indomethacin supplementation was collected after 24 h as described in section 4.2. The medium was collected in 2 volumes of ethanol and stored at -20<sup>0</sup>C until assayed for prostaglandin measurement.

Extractions of prostaglandins were performed using ethyl bonded silica minicolumns supplied with the kit. Samples were acidified with 0.25 ml 1M HCl before applying to columns. The prostaglandins were extracted with 5 ml of methyl formate.

The eluates were dried under nitrogen and reconstituted to 0.1 ml with assay buffer (phosphate-buffered saline with gelatin).

The assay is based on the competition between unlabeled prostaglandin and a fixed quantity of  $^3\text{[H]}$  PG for a limited number of binding sites on the prostaglandin specific antibody. In the standard reaction, 0.1 ml of antiserum was added to the samples and vortexed. An aliquot of 0.1 ml of  $^3\text{[H]}$  PG was added to each tube and the resultant mixture was incubated at  $4^{\circ}\text{C}$  for overnight. Separation of unbound ligand from the protein bound was achieved by adsorption of the free ligand on to 1.0 ml of dextran-coated charcoal, followed by centrifugation at  $100 \times g$  for 10 min at  $4^{\circ}\text{C}$ . Radioactivity in the supernatant liquid was quantitated in the scintillation counter (Beckman LS 3801). The concentration of unlabeled prostaglandins in the sample was determined from a linear standard curve. The detection limit for  $\text{PGE}_2$  and 6-keto  $\text{PGF}_{1\alpha}$  by radioimmunoassay was 0.2 pg and 2.5 pg per 100  $\mu\text{l}$ , respectively.

#### **4. 9. Study of Direct Effect of Prostaglandins on Preadipocyte Differentiation**

To investigate if the concentration of prostaglandins released by adipocytes and preadipocytes was sufficient to induce the effect observed, confluent rat preadipocytes were exposed to exogenous prostaglandins. Serum-free conditions were used to study the specific effect of individual prostaglandin on preadipocyte differentiation. Exogenous prostaglandins ( $\text{PGI}_2$ ,  $\text{PGE}_2$ ,  $\text{PGF}_{2\alpha}$  and 15-d  $\text{PGJ}_2$ ) were added to preadipocytes in concentrations ranging from  $10^{-10}$  M to  $10^{-6}$  M. Owing to very short half life of  $\text{PGI}_2$ , a stable and active analogue, carboprostacyclin (c $\text{PGI}_2$ ), was used (Negrel *et al.*, 1989). Isolated preadipocytes were propagated in  $\alpha\text{MEM}$  containing 10% FBS until confluent. On day 4 when cells reached monolayer confluency, serum supplemented medium was

replaced with hormone supplemented serum-free ITT. ITT refers to Dulbecco's modified Eagle's medium- Ham's F-12 (DME-F12) medium (1:1 mixture) supplemented with insulin (100 nM), transferrin (10 nM) and triiodothyronine (200 pM). Prostaglandins were added on day 4 in the presence of ITT and maintained in the culture medium throughout the experiment. Stock solutions of prostaglandins were prepared in ethanol and stored at -20°C. The final concentrations were made by dilutions with culture media. The final concentration of ethanol in culture media were < 0.1%. Media were changed after every two days and fresh prostaglandins were added at that time. Cells were harvested on day 14 after seeding and assayed for differentiation by measuring G3PDH activity.

#### **4. 10. Effect of Lipooxygenase (LOX) Products on Preadipocyte Differentiation**

To study the effect of lipoxygenase products on preadipocyte differentiation, rat preadipocytes were treated with nordihydroguaiaretic acid (NDGA), an inhibitor of LOX (Higgs and Vane, 1983), in the presence of MIX and ACM. Preadipocytes from retroperitoneal and epididymal fat pads of male rats were isolated and seeded at a density of  $4 \times 10^4$  cells/cm<sup>2</sup> in 24- well plates. Cells were grown till monolayer confluence (approximately four days post inoculation) in  $\alpha$ MEM containing 10% FBS. The medium was then changed to one of the following conditions:

- a) ACM
- b) ACM with NDGA (10  $\mu$ M)
- c) ACM with indomethacin (100  $\mu$ M)
- d) ACM with indomethacin (100  $\mu$ M) plus NDGA (10  $\mu$ M)

## **Controls**

- a) Standard hormonal mixture (MIX)
- b) MIX with NDGA (10  $\mu$ M)
- c) MIX with indomethacin (100  $\mu$ M)
- d) MIX with indomethacin (100  $\mu$ M) plus NDGA (10  $\mu$ M)

The culture medium was changed every three days. Cells were extracted for assessment of differentiation on day 10 after seeding. The experiment was repeated at least five times using triplicate dishes per condition.

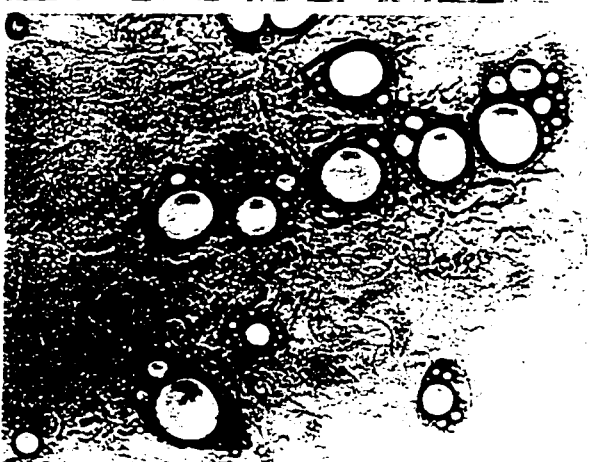
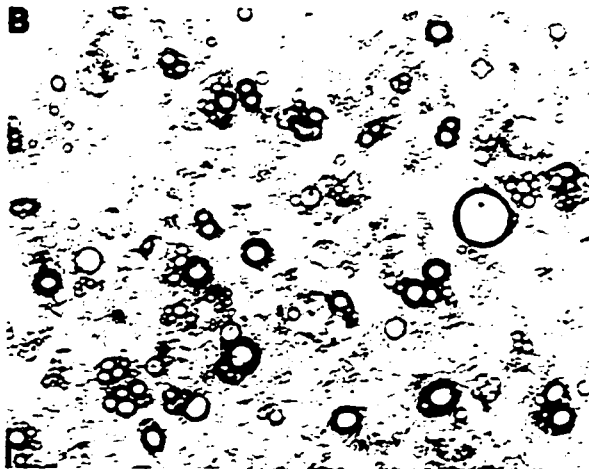
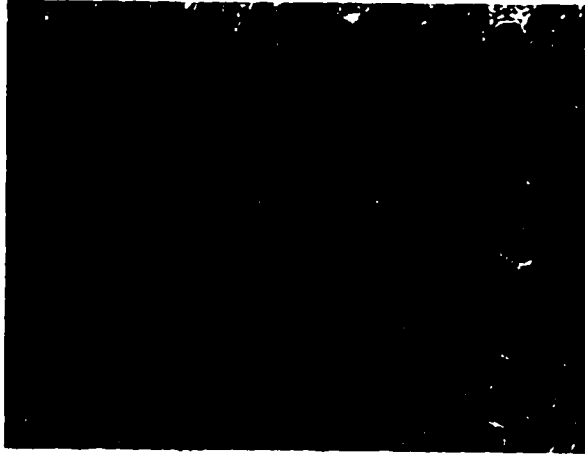
## 5. RESULTS

### 5. 1. Induction of Preadipocyte Differentiation by ACM

In the present study, adipocyte-derived conditioned medium (ACM) was used as a tool to study the paracrine mechanism. To demonstrate the use of ACM, the differentiation activity of ACM was tested. Confluent rat preadipocytes were induced to differentiate with ACM and the differentiation activity was compared with the control medium ( $\alpha$ MEM containing 10% FBS) and a standard differentiation cocktail (MIX). Rat preadipocytes showed no differentiation in the presence of  $\alpha$ MEM supplemented with 10% FBS (10% FBS), as the cells maintained fibroblastic appearance (Figure 2, panel A). However, in the presence of ACM, preadipocytes differentiated very rapidly. The cytoplasmic lipid droplets were visible from day 2 of differentiation initiation when examined under phase contrast microscopy and the number and size of differentiating cells increased progressively until day 10 (Figure 2, panel B). Preadipocytes treated with MIX also showed increased differentiation. The proportion of preadipocytes containing lipid droplets when induced to differentiate with MIX, was not different from those cultured with ACM. However, the size of lipid droplets in MIX conditions was much larger than ACM (Figure 2, panel C).

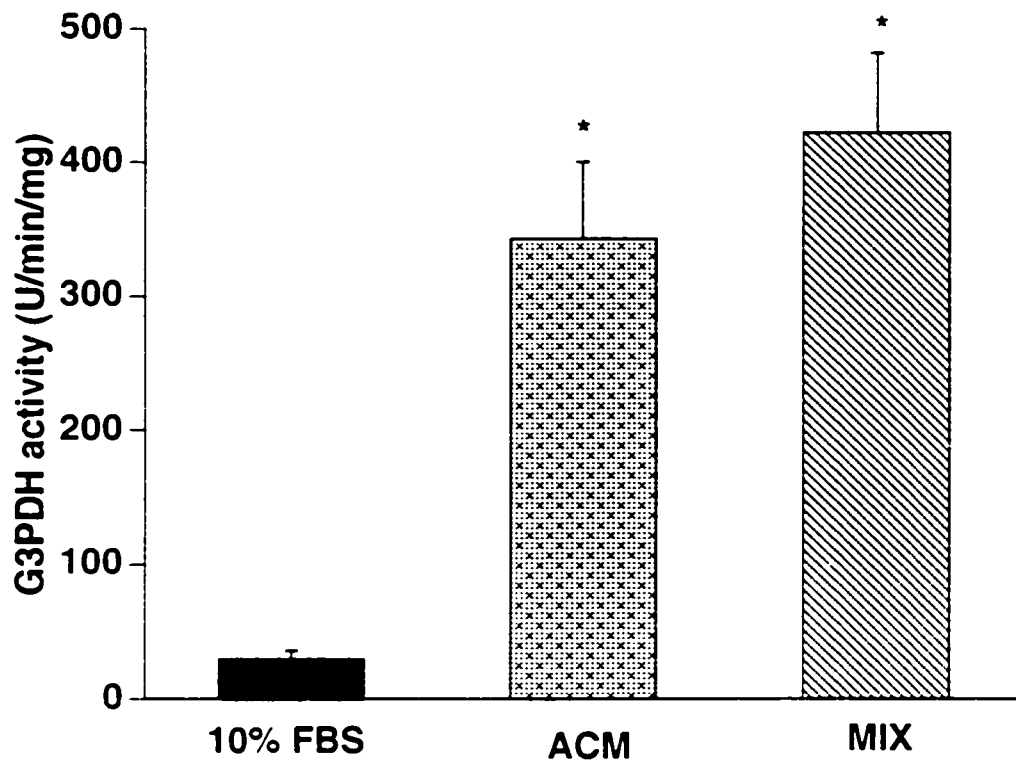
The morphological changes were accompanied with the biochemical evidence of differentiation as determined by G3PDH measurement. As shown in Figure 3, ACM induced an 11- fold increase in the G3PDH activity of differentiated preadipocytes compared to those cultured in control medium (10% FBS) (ACM:  $342 \pm 58$  vs  $30 \pm 6$  nmoles NADH/min/mg for controls). The ACM-induced G3PDH activity was

**Figure 2. Photomicrographs of preadipocytes with cytoplasmic lipid inclusions** in response to (A)  $\alpha$ MEM supplemented with 10% FBS (10% FBS), (B) ACM, (C) standard differentiation mixture (MIX). Preadipocytes were isolated from retroperitoneal and epididymal fat pads of male rats and propagated in primary culture as described in Methods. Cells were grown in 10% FBS until monolayer confluence. The media were then changed to the above culture conditions and cells were maintained in those conditions for an additional 6 days. Original magnification x 320.



**Figure 3. Effect of ACM on rat preadipocyte differentiation as determined by measuring G3PDH activity.**

Confluent test preadipocytes were induced to differentiate with (a) 10% FBS (control), (b) ACM and (c) MIX, as described in the legend of Figure 2. Media were changed after every 2 days. Cells were harvested on day 10 after seeding and assessed for G3PDH activity. One unit of enzyme activity corresponds to 1 nmole of NADH oxidized. The data were analyzed by one-way analysis of variance (ANOVA) and Tukey's test. Values are means  $\pm$  SEM of triplicate determinants from five different experiments. \* denotes values that are significantly different from controls,  $p \leq 0.05$



comparable to that induced by MIX (MIX:  $422 \pm 60$  vs  $342 \pm 58$  U for ACM). The larger size of lipid droplets in MIX conditions did not make a difference in the G3PDH activity. This comparable activity of ACM and MIX on differentiation induction confirmed our previous report that ACM can serve as a suitable tool to study adipose cell growth and differentiation (Shillabeer *et al.*, 1996).

## **5. 2. The Effect of Indomethacin (100 $\mu$ M) on Preadipocyte Differentiation**

To delineate the role of prostaglandins in preadipocyte differentiation, the effect of indomethacin, a commonly used inhibitor of prostaglandin synthesis, was examined under various test culture conditions as described in section 4.6 of Methods. To determine the autocrine role of prostaglandins, test preadipocytes were exposed to indomethacin in the presence of ACM (ACM+Ia), the standard differentiation mixture (MIX+I) and the control medium, 10% FBS (FBS+I). Addition of indomethacin (100  $\mu$ M) to confluent test preadipocytes stimulated differentiation in a dramatic fashion in all three conditions, including the control conditions (FBS+I). By 3rd day of indomethacin addition, almost all cells in MIX+I and ACM+I conditions exhibited adipose cell morphology and had accumulated lipid droplets (Figure 4A to 4G). These morphological changes were associated with a parallel increase in the G3PDH activity. An increase of 3-, 4-, and 11-fold in the level of G3PDH activity was observed in MIX, ACM, and control conditions, respectively as illustrated in Figure 5. These results suggested that preadipocyte differentiation was probably mediated by a mechanism independent of inhibition of PG synthesis.

To determine the paracrine role of adipocyte-derived prostaglandins on preadipocyte differentiation, ACM was prepared from rat primary adipocytes cultured in the absence or presence of indomethacin (ACM+I), as mentioned in section 4.2. of

**Figure 4. Phase contrast photomicrographs of rat preadipocytes showing increased lipid droplets in response to indomethacin (100  $\mu$ M).** Original magnification x 320. Preadipocytes were grown till confluence in  $\alpha$ MEM supplemented with 10% FBS (10% FBS) and induced to differentiate with:

(A) 10% FBS (FBS)

(B) 10% FBS + indomethacin (FBS+I)

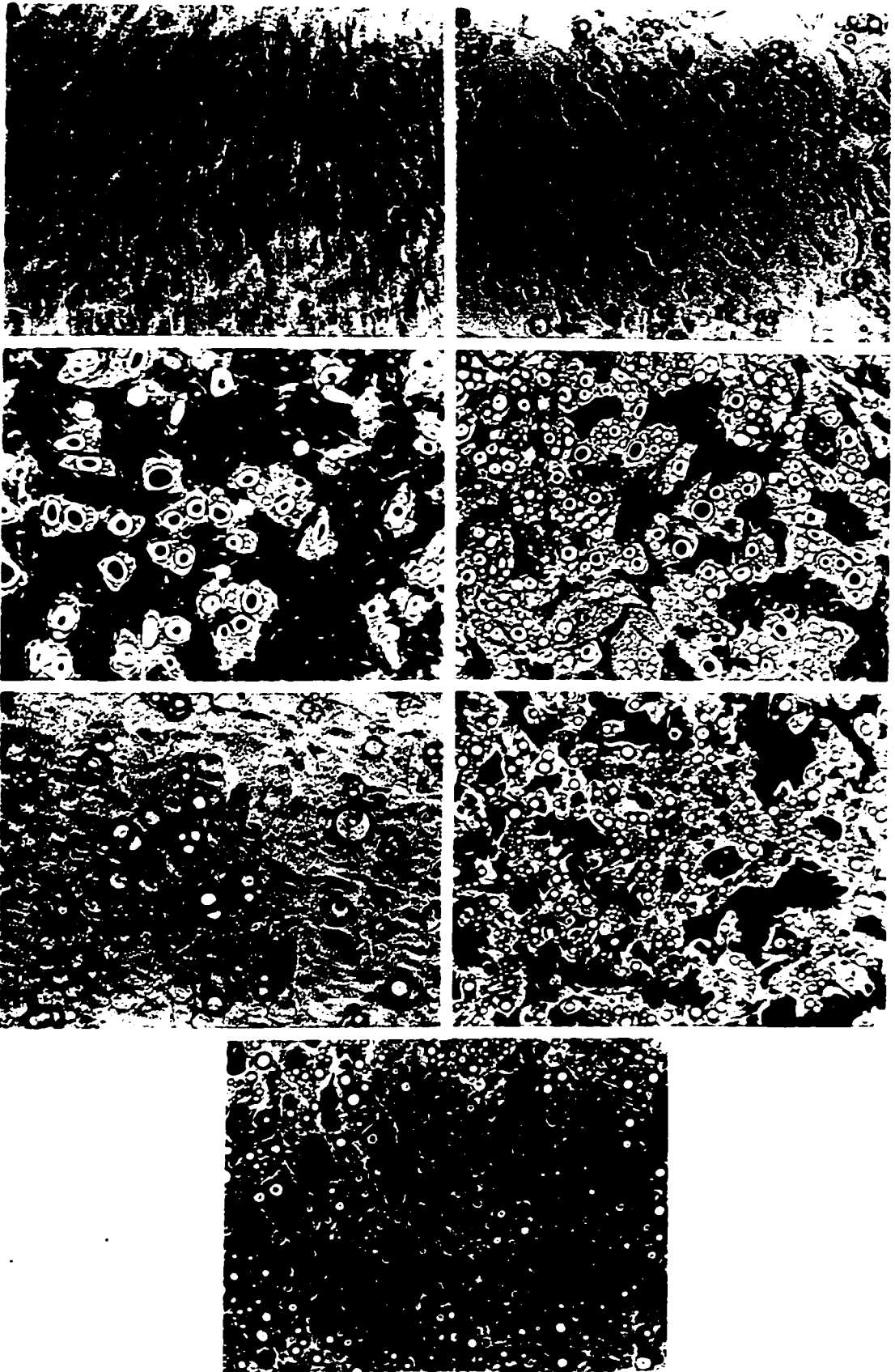
(C) (MIX)

(D) MIX + indomethacin (MIX+I)

(E) ACM

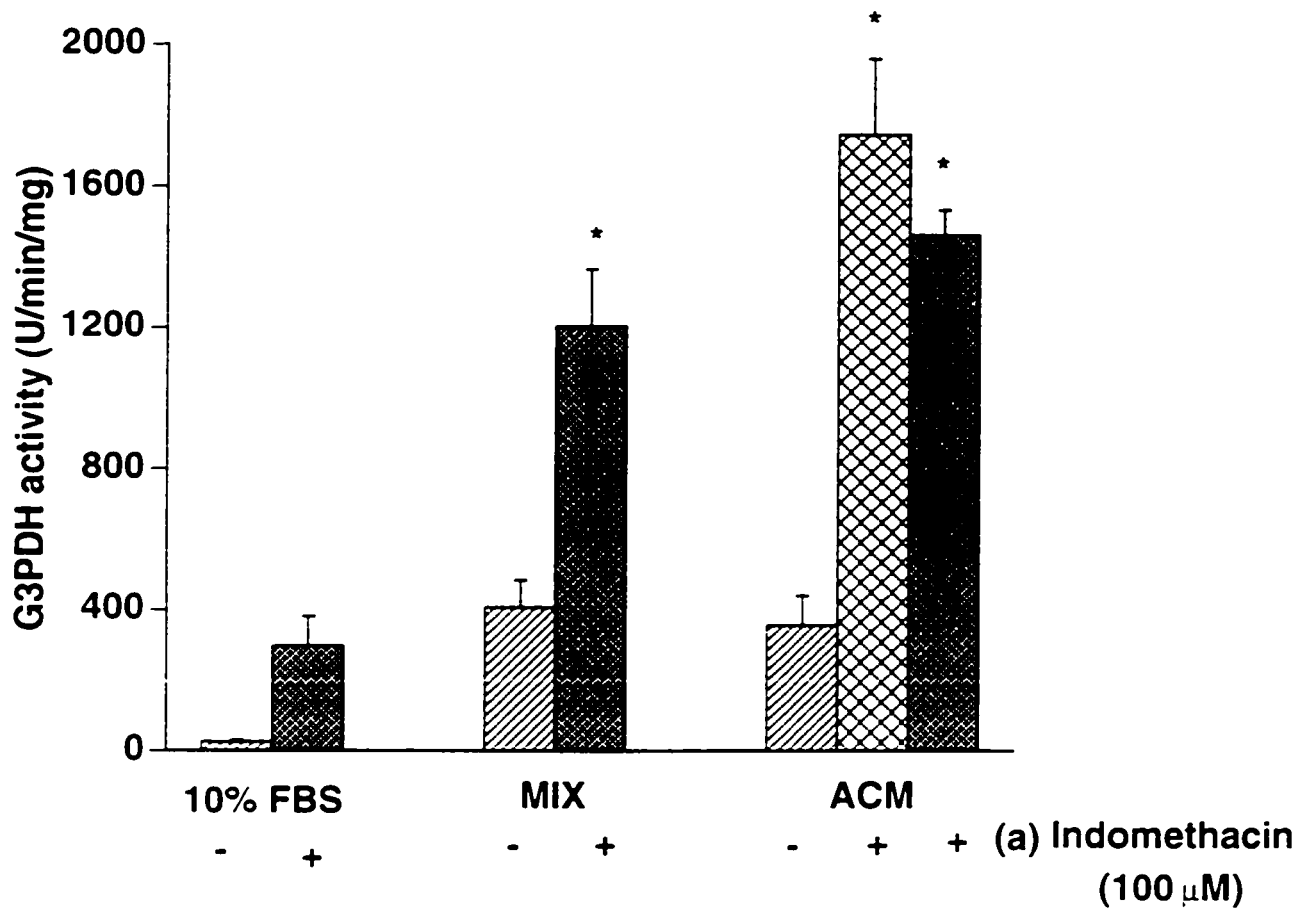
(F) ACM + indomethacin added at the time of collection (ACM+I)

(G) ACM + indomethacin added after collecting medium (ACM+Ia)



**Figure 5. Effect of indomethacin (100  $\mu$ M) on G3PDH activity of differentiating preadipocytes.**

Confluent test preadipocytes were induced to differentiate with same culture conditions as described in the legend of Figure 4. Cells were extracted for G3PDH activity on day 10 after seeding. One unit of the enzyme activity corresponds to the oxidation of 1 nmole NADH. The data were analyzed by ANOVA and Tukey's test. Values are means  $\pm$  SEM of five experiments. \* denotes values that are significantly different from their respective controls ( $P \leq 0.05$ ).



Materials and Methods. Confluent test preadipocytes were induced to differentiate with ACM and ACM+I culture conditions. Cultures of test preadipocytes with ACM+I also showed significantly increased G3PDH activity (5-fold) compared to those cultured with ACM alone (Figure 5).

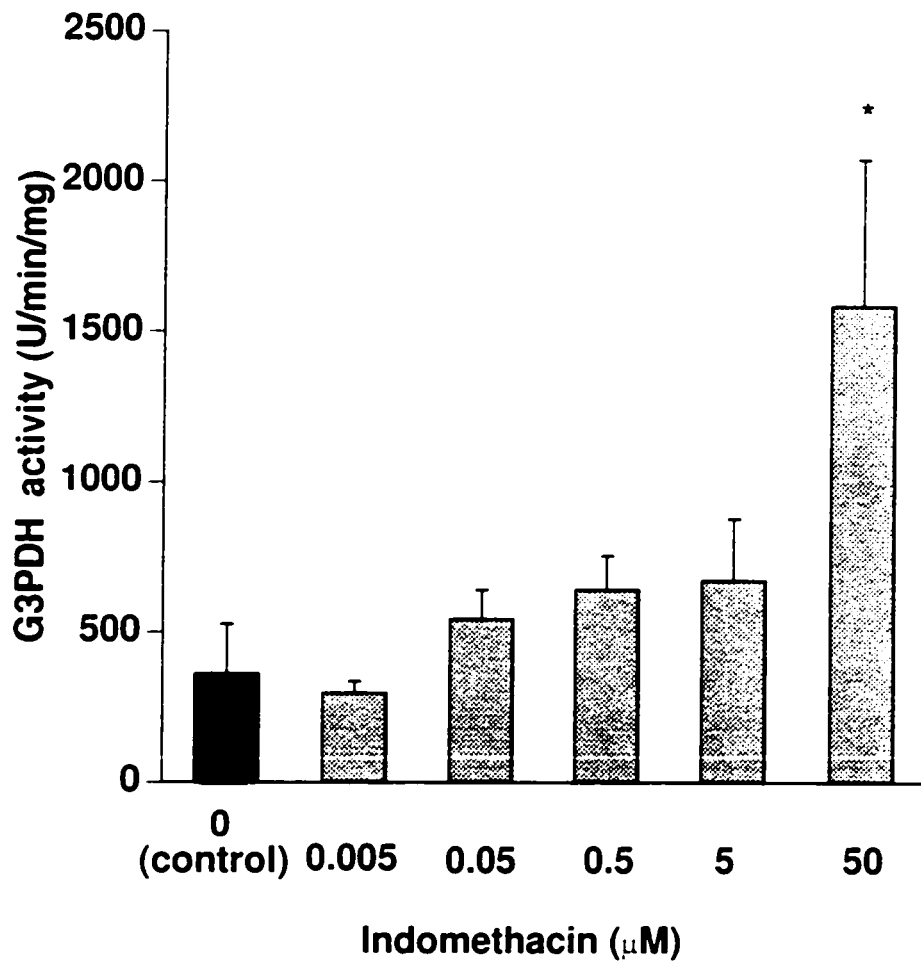
The G3PDH activity induced by ACM+I condition was greater (about 20 %) than that induced by ACM+Ia condition (Figure 5). Although, the difference in the stimulation of differentiation between these two groups did not reach the level of significance, the trend of greater differentiation in ACM+I than ACM+Ia was found in all five repeats of the experiment. These results suggest that in the absence of indomethacin, adipocytes may release prostaglandins that are inhibitory for preadipocyte differentiation, but again the effect of blocking inhibitory prostaglandins might have been masked by some other mechanism of indomethacin unrelated to prostaglandin synthesis inhibition.

### **5. 3. Dose-dependent Effect of Indomethacin on Preadipocyte Differentiation**

Confluent preadipocytes were exposed to indomethacin in concentrations ranging from 0.005  $\mu\text{M}$  to 50  $\mu\text{M}$  in the presence of ACM or MIX. As shown in Figure 6, at lower doses, a moderate differentiation promoting effect of indomethacin was observed on ACM-induced preadipocyte differentiation, for example, ACM:  $362 \pm 165$  vs ACM+I(5 $\mu\text{M}$ ):  $672 \pm 208$  U, G3PDH. Whereas, at higher concentration (50  $\mu\text{M}$ ), the addition of indomethacin resulted in a dramatic increase of preadipocyte differentiation. Cytoplasmic lipid accumulation was visible within 2 days of indomethacin addition and by day 9, almost every cell had accumulated lipid droplets. The G3PDH activity was also increased significantly by 5-fold from  $362 \pm 164$  U for control (ACM) to  $1586 \pm 844$  U,  $p \leq 0.05$  (Figure 6). Similar results were observed with the increasing concentrations of

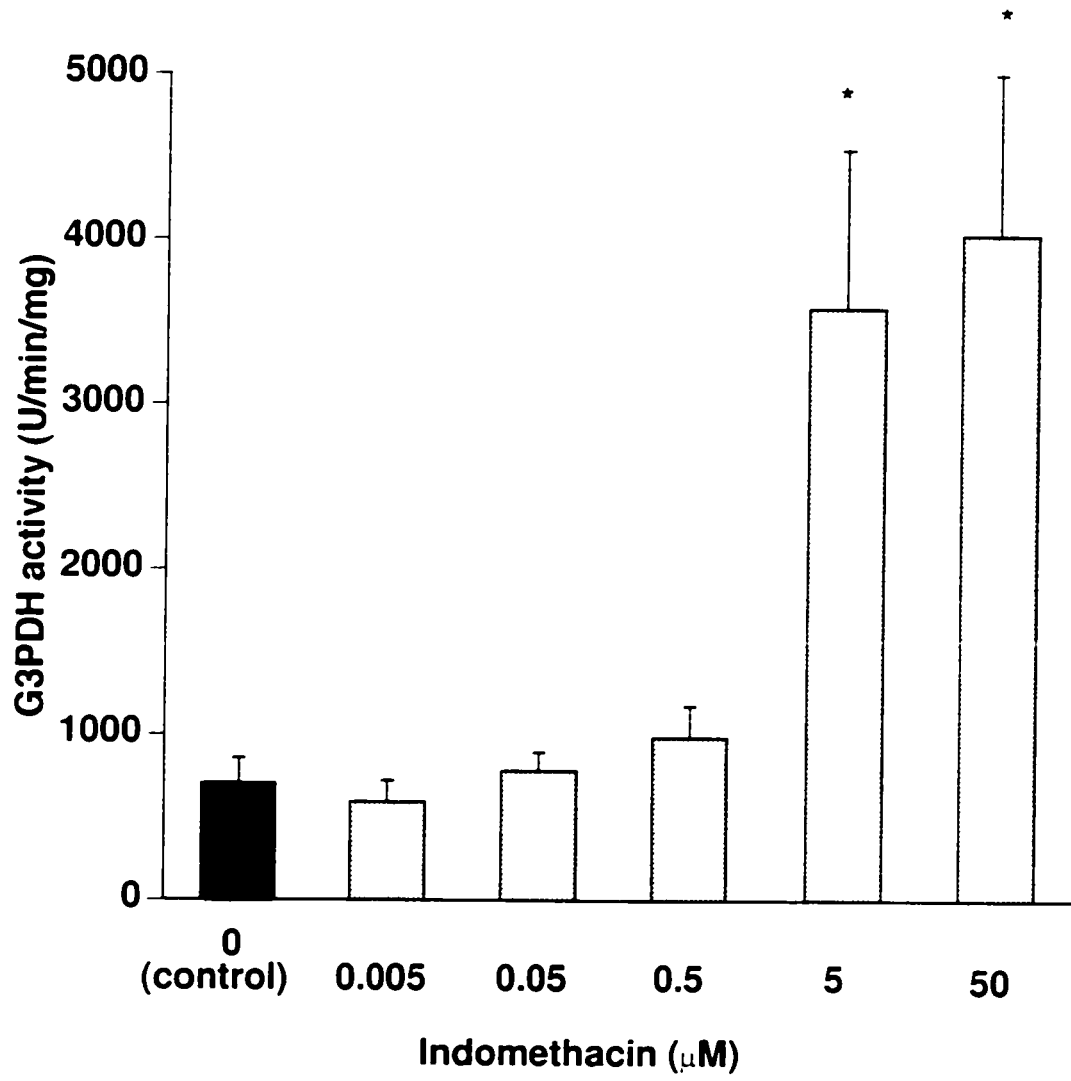
**Figure 6. Dose-response curve of indomethacin in ACM-induced preadipocyte differentiation.**

Isolated rat preadipocytes were cultured at a high density of  $4 \times 10^4$  cells/cm<sup>2</sup> in 24-well plates and allowed to grow in  $\alpha$ MEM containing 10% FBS. On day 4 when cells were confluent, the media were changed to ACM (control) and ACM with increasing concentrations of indomethacin (0.005  $\mu$ M to 50  $\mu$ M). On day 10 after seeding, cells were extracted for G3PDH assay as described in Methods. Enzyme activity was expressed as nmole NADH oxidized/min/mg protein. The data were analyzed by ANOVA and Tukey's test. The values represent means  $\pm$  SEM of 3 experiments. \* denotes values that are significantly different from control ( $P \leq 0.05$ ).



**Figure 7. Dose response curve of indomethacin in MIX-induced preadipocyte differentiation.**

Confluent test preadipocytes were exposed to MIX (control) and MIX with increasing concentrations of indomethacin (0.005  $\mu$ M to 50  $\mu$ M). Cells were harvested on day 10 after seeding and assessed for G3PDH activity. One unit of enzyme activity corresponds to 1 nmole of NADH oxidized. The data were analyzed by ANOVA and Tukey's test for multiple comparisons. The values are means  $\pm$  SEM of 3 experiments. \* denotes values that are significantly different from control ( $P \leq 0.05$ ).



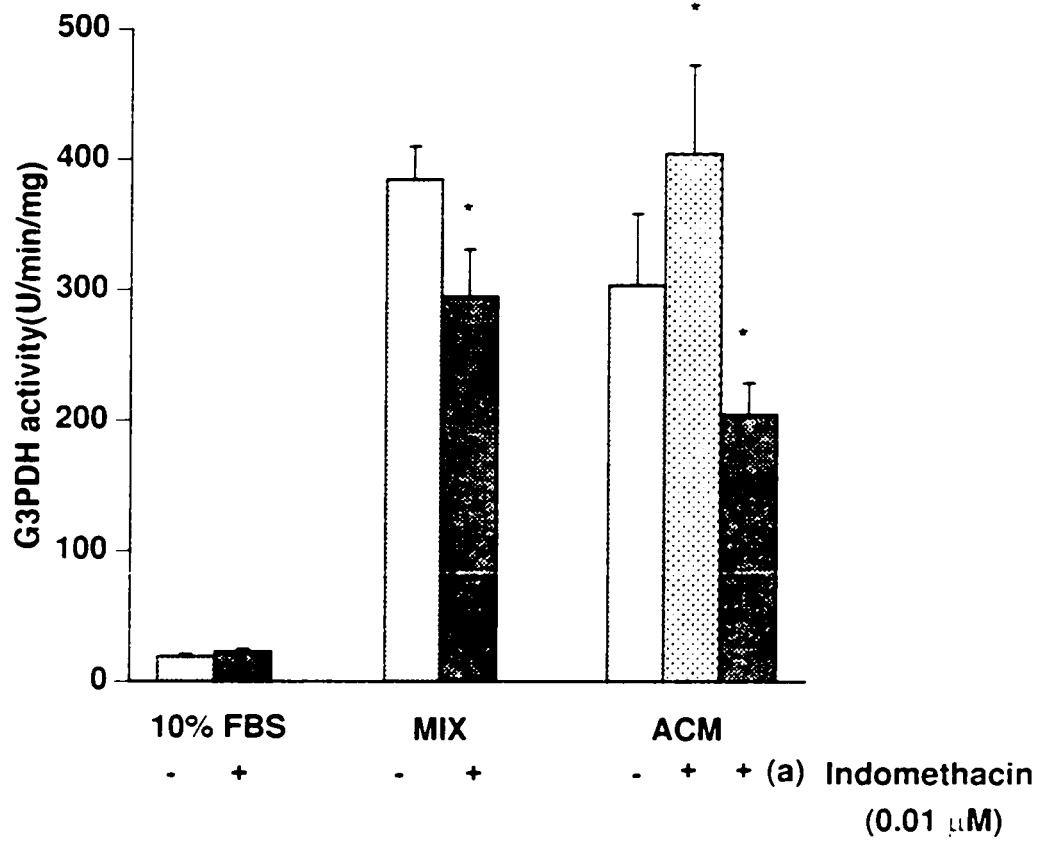
indomethacin (0.005  $\mu\text{M}$  to 50  $\mu\text{M}$ ) when differentiation was induced with MIX (Figure 7). Up to a concentration of 0.5  $\mu\text{M}$ , indomethacin in MIX showed a slight increase in the G3PDH activity ( $702 \pm 154$  U vs  $984 \pm 194$  U). However, the addition of indomethacin at higher concentrations (5 and 50  $\mu\text{M}$ ) resulted in a 5-fold increase in the G3PDH activity of the differentiating preadipocytes ( $702 \pm 154$  U, MIX vs  $3583 \pm 966$  and  $4033 \pm 980$  U with 5 and 50  $\mu\text{M}$  indomethacin, respectively).

#### **5. 4. The Effect of Indomethacin (0.01 $\mu\text{M}$ ) on Preadipocyte Differentiation**

To eliminate the adipogenic effect of indomethacin on preadipocyte differentiation, the paracrine/autocrine role of prostaglandins in differentiation was reinvestigated using a concentration of indomethacin (0.01  $\mu\text{M}$ ) that specifically inhibited prostaglandin formation in adipocytes by more than 60-70% (data shown in later section of this chapter). In contrast to significant stimulation observed under all conditions with high concentration of indomethacin (Figure 5), low concentration (0.01  $\mu\text{M}$ ) did not influence preadipocyte differentiation when added to control (10% FBS) (Figure 8). Moreover, preadipocytes exposed to indomethacin under MIX (MIX+I) and ACM (ACM+Ia) conditions at this concentration revealed a significantly ( $p \leq 0.05$ ) reduced differentiation when compared to their respective controls (Figure 8). These results indicate that indomethacin inhibited prostaglandin mediated differentiation in preadipocytes induced by MIX and ACM. In the absence of indomethacin, stimuli in MIX and ACM may induce the release of prostaglandins by preadipocytes and stimulate differentiation in an autocrine fashion.

**Figure 8. Effect of indomethacin (0.01  $\mu$ M) on G3PDH activity of differentiating preadipocytes.**

Confluent test preadipocytes were induced to differentiate with the same culture conditions as described in the legend of Figure 5. Cells were harvested on day 10 after seeding and analyzed for G3PDH activity. Values are means  $\pm$  SEM of six experiments except for MIX conditions where n = 5. \* denotes values that are significantly different ( $P \leq 0.05$ ) from their respective controls.

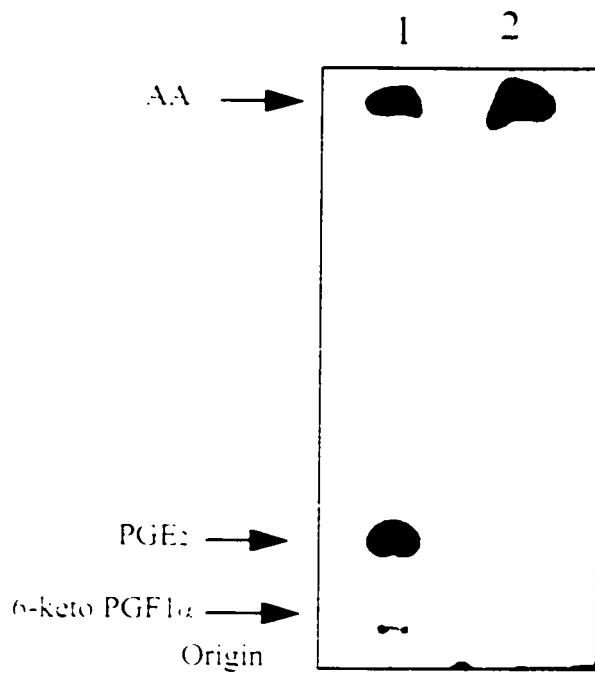


To determine whether indomethacin at this concentration (0.01  $\mu$ M) affected the prostaglandins synthesized by adipocytes and subsequent differentiation, test preadipocytes were cultured with ACM and ACM collected in the presence of indomethacin (ACM+I) as described before. As shown in Figure 8, preadipocytes cultured in the presence of ACM+I showed greater differentiation as compared to those cultured with ACM alone (ACM:  $304 \pm 55$  U vs ACM+I:  $405 \pm 68$  U, n=6). The G3PDH activity induced by ACM+I condition was also significantly greater than that induced by ACM+Ia condition (ACM+I:  $405 \pm 68$  vs ACM+Ia:  $205 \pm 24$  U,  $p < 0.05$ ). These results suggested that increased differentiation observed under ACM+I culture conditions compared to ACM alone and ACM+Ia conditions, may be due to the inhibition of inhibitory prostaglandins such as PGE<sub>2</sub> and PGF<sub>2 $\alpha$</sub>  in adipocytes by indomethacin. These inhibitory prostaglandins may remain active in ACM harvested in the absence of indomethacin.

### **5. 5. Measurement of Prostaglandins Released by Preadipocytes and Adipocytes**

The production of radioactive prostaglandins from [1-<sup>14</sup>C] arachidonic acid by preadipocytes and adipocytes was examined using thin layer chromatography (TLC) as described in section 4.7 of Methods. Figure 9 shows the autoradiogram of the TLC plates developed after an exposure of 96 h. Preadipocytes converted exogenous arachidonic acid into prostaglandins that comigrated with standard PGE<sub>2</sub> and 6-keto PGF<sub>1 $\alpha$</sub> , a stable metabolite of PGI<sub>2</sub>. PGE<sub>2</sub> accounted for 48% of the total radioactive metabolites, whereas 6-keto PGF<sub>1 $\alpha$</sub>  accounted for 33% of the labeled prostaglandins. No

**Figure 9. TLC autoradiogram of Prostaglandins after incubation of preadipocytes with [1-<sup>14</sup>C] AA.** Preadipocytes were seeded at a density of 10<sup>4</sup> cells/cm<sup>2</sup> in 100 mm culture dishes in  $\alpha$ MEM containing 10% FBS. Metabolism of exogenous [1-<sup>14</sup>C] arachidonic acid by these cells was analyzed on day 4, as described in Methods. Lane 1, preadipocytes; Lane 2, Preadipocytes plus indomethacin (10  $\mu$ M). Arrows indicate the locations of standard arachidonic acid and prostaglandins. The data are representative of four experiments.



PGF<sub>2α</sub>, PGD<sub>2</sub> and 15d-PGJ<sub>2</sub> were detected in the culture medium of growing preadipocytes. The effect of indomethacin (10 μM) on synthesis of these prostaglandins was also examined. Indomethacin blocked ≥ 90% of conversion of arachidonic acid into products that comigrated with PGE<sub>2</sub> and PGI<sub>2</sub> (Figure 9).

In order to assess the actual amount of PGE<sub>2</sub> and PGI<sub>2</sub> formed, radioimmunoassays (RIA) for these prostaglandins were utilized. As shown in Table 2, the amount of PGI<sub>2</sub> (measured as 6-keto PGF<sub>1α</sub>) released by preadipocytes was 4.8 ± 0.6 ng/10<sup>6</sup> cells corresponding to a medium concentration of 1.3 x 10<sup>-9</sup> M, while the amount of secreted PGE<sub>2</sub> was 5.2 ± 0.8 ng/10<sup>6</sup> cells and this corresponded to 1.4 x 10<sup>-9</sup> M.

Rat adipocytes were also found to release PGI<sub>2</sub> and PGE<sub>2</sub> into the culture medium conditioned by these cells for 24 h (ACM) when measured by RIA. The amount of 6-keto PGF<sub>1α</sub> released by adipocytes was 0.64 ± 0.2 ng/10<sup>6</sup> cells, whereas the amount of released PGE<sub>2</sub> was 0.4 ± 0.3 ng/10<sup>6</sup> cells. The amount of PGI<sub>2</sub> released by adipocytes corresponded to a medium concentration of 9 x 10<sup>-10</sup> M and that of PGE<sub>2</sub> corresponded to the concentration of 1.25 x 10<sup>-10</sup> M. The synthesis of PGI<sub>2</sub> and PGE<sub>2</sub> by adipocytes in culture was greatly decreased in the presence of indomethacin in a dose dependent manner (Table 3 and 4). At the concentration of 0.05 μM, indomethacin was able to inhibit ≥ 60% of PGI<sub>2</sub> (Table 3) and ≥ 70% PGE<sub>2</sub> (Table 4) production in adipocytes. The presence of prostaglandins in adipocytes could not be detected by TLC due to some technical problems. A very high content of triacylglycerols(TG) in adipocytes seemed to take up the radioactive arachidonic acid and the prostaglandins formed could not be separated from TG by the solvent system used in this TLC assay.

**Table 2. Measurement of PGE<sub>2</sub> and PGI<sub>2</sub> released by preadipocytes and adipocytes using RIA.**

Type of cell	PGE <sub>2</sub>	6-keto-PGF <sub>1α</sub> *
	(ng/10 <sup>6</sup> cells)	
Preadipocytes	5.2 ± 0.8 (1.4 x 10 <sup>-9</sup> M)	4.8 ± 0.6 (1.3 x 10 <sup>-9</sup> M)
Adipocytes	0.4 ± 0.3 (1.25 x 10 <sup>-10</sup> M)	0.6 ± 0.2 (9 x 10 <sup>-10</sup> M)

After isolation, mature rat adipocytes were incubated with  $\alpha$ MEM containing 10% FBS for 24 h. The ACM was collected the following day for assay of PG released. To quantitate amount of PG released by preadipocytes, cells were seeded at a density of 4 x 10<sup>4</sup> cells/cm<sup>2</sup> in 24 well plates in  $\alpha$ MEM containing 10 % FBS. After 24 h, cells were washed extensively with  $\alpha$ MEM and maintained in  $\alpha$ MEM supplemented with 10% FBS for the next 24 h. The medium was collected after 24 h. Preadipocytes were trypsinized and counted as described in Methods. The amount of PG released was measured in pg/ml and calculated in terms of ng/10<sup>6</sup> cells. Results are mean  $\pm$  S. D. (n=2)

\* PGI<sub>2</sub> was measured as its stable metabolite, 6-keto-PGF<sub>1α</sub>

**Table 3. Inhibition of PGI<sub>2</sub> production (measured as 6-keto-PGF<sub>1α</sub>) by indomethacin in rat adipocytes.**

Indomethacin (μM)	PGI <sub>2</sub> (pg/ml)	Inhibition of PGI <sub>2</sub> (%)
None	320 ± 113	0
0.005	130 ± 28	≥60
0.05	128 ± 24	≥60
0.5	105 ± 21	≥68
5.0	91 ± 14	≥70

Isolated rat adipocytes were seeded at a density of approximately  $5 \times 10^4$  cells in 25 cm<sup>2</sup> flask and incubated with αMEM supplemented with 10% FBS with or without further supplementation of indomethacin. ACM was collected after 24 hr. 6-keto PGF<sub>1α</sub> was determined by radioimmunoassay. Results were expressed as mean ± S. D. (n=2).

**Table 4. Inhibition of PGE<sub>2</sub> production in rat adipocytes by indomethacin.**

Indomethacin ( $\mu$ M)	PGE <sub>2</sub> (pg/ml)	Inhibition of PGE <sub>2</sub> (%)
None	240 $\pm$ 28	0
0.005	92 $\pm$ 2	$\geq$ 60
0.05	56 $\pm$ 18	$\geq$ 70
0.5	42 $\pm$ 3	$\geq$ 80
5.0	29 $\pm$ 13	$\geq$ 90

The experimental protocol was same as for Table 3. Results were expressed as mean  $\pm$  S.D. (n=2).

## **5. 6. Effect of Exogenous Prostaglandins on Preadipocyte Differentiation**

To investigate if the concentration of prostaglandins released by adipocytes and preadipocytes was sufficient to induce differentiation, confluent rat preadipocytes were exposed to exogenous prostaglandins (cPGI<sub>2</sub>, PGE<sub>2</sub>, PGF<sub>2α</sub> and 15d-PGJ<sub>2</sub>) in concentrations between physiological and pharmacological ranges (10<sup>-10</sup> M to 10<sup>-6</sup> M) in a defined medium (ITT). To inhibit the endogenous production of prostaglandins by test cells, indomethacin (0.01 μM) was added to preadipocytes that were exposed to a physiological concentration of prostaglandins (10<sup>-10</sup> M).

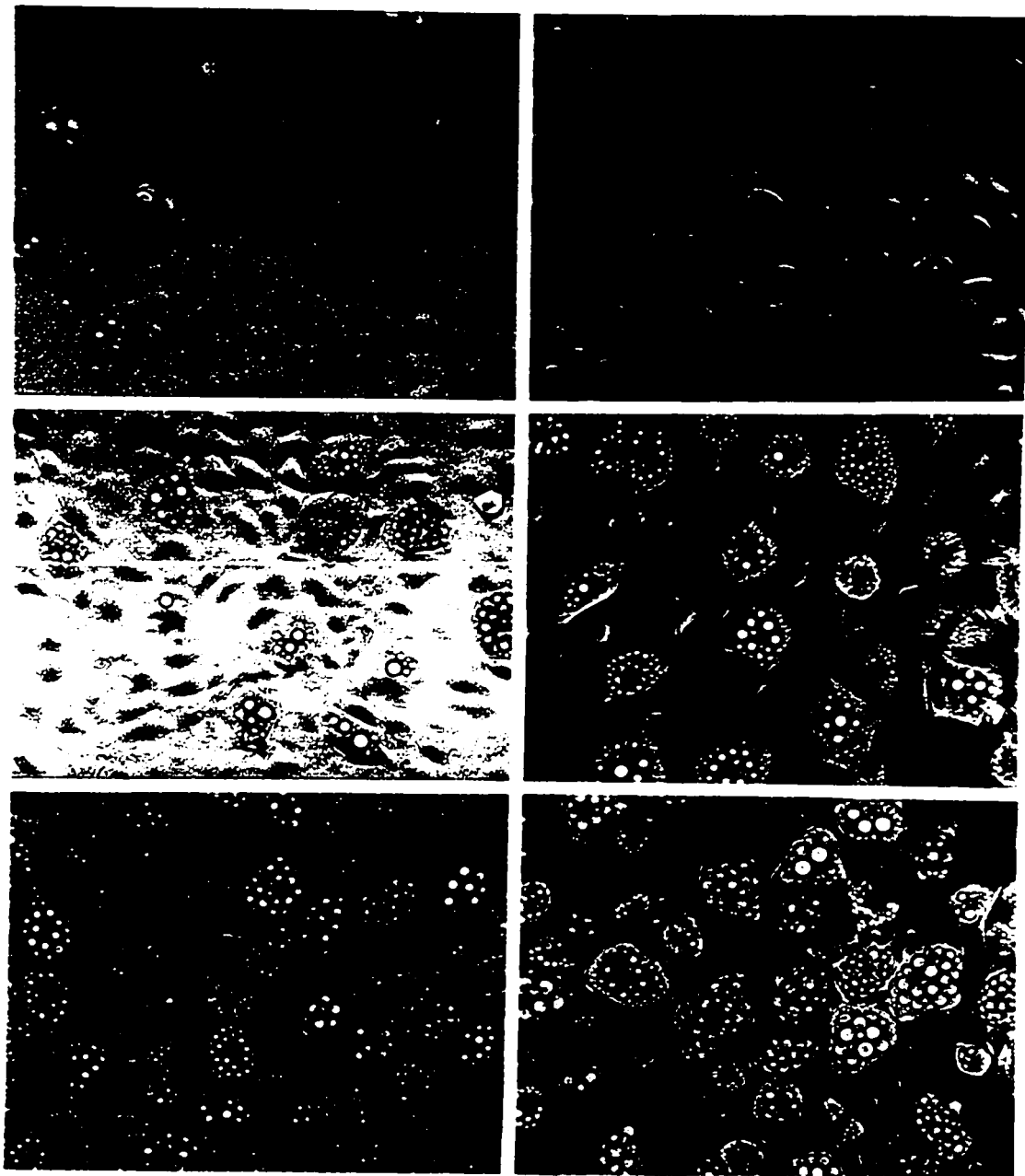
### **5. 6. 1. Effect of Increasing Concentrations of cPGI<sub>2</sub>, PGE<sub>2</sub> and 15d-PGJ<sub>2</sub> on Preadipocyte Differentiation**

cPGI<sub>2</sub> stimulated differentiation of adipocyte precursors in serum free medium only when added at concentrations > 10<sup>-8</sup> M. At concentrations below this, no increase in cytoplasmic lipid accretion was noticeable compared to control (ITT), when examined under phase contrast microscopy (Figure 10, panel A to D). However, lipid accumulation was rapid at concentration 10<sup>-7</sup> and 10<sup>-6</sup> M (Figure 10, panel E and F). Cytoplasmic lipid droplets were noticeable on day 2 after PGI<sub>2</sub> addition at these concentrations, as compared to control. The number of differentiated cells increased progressively and by day 14 almost every cell was lipid laden as illustrated in the photomicrographs.

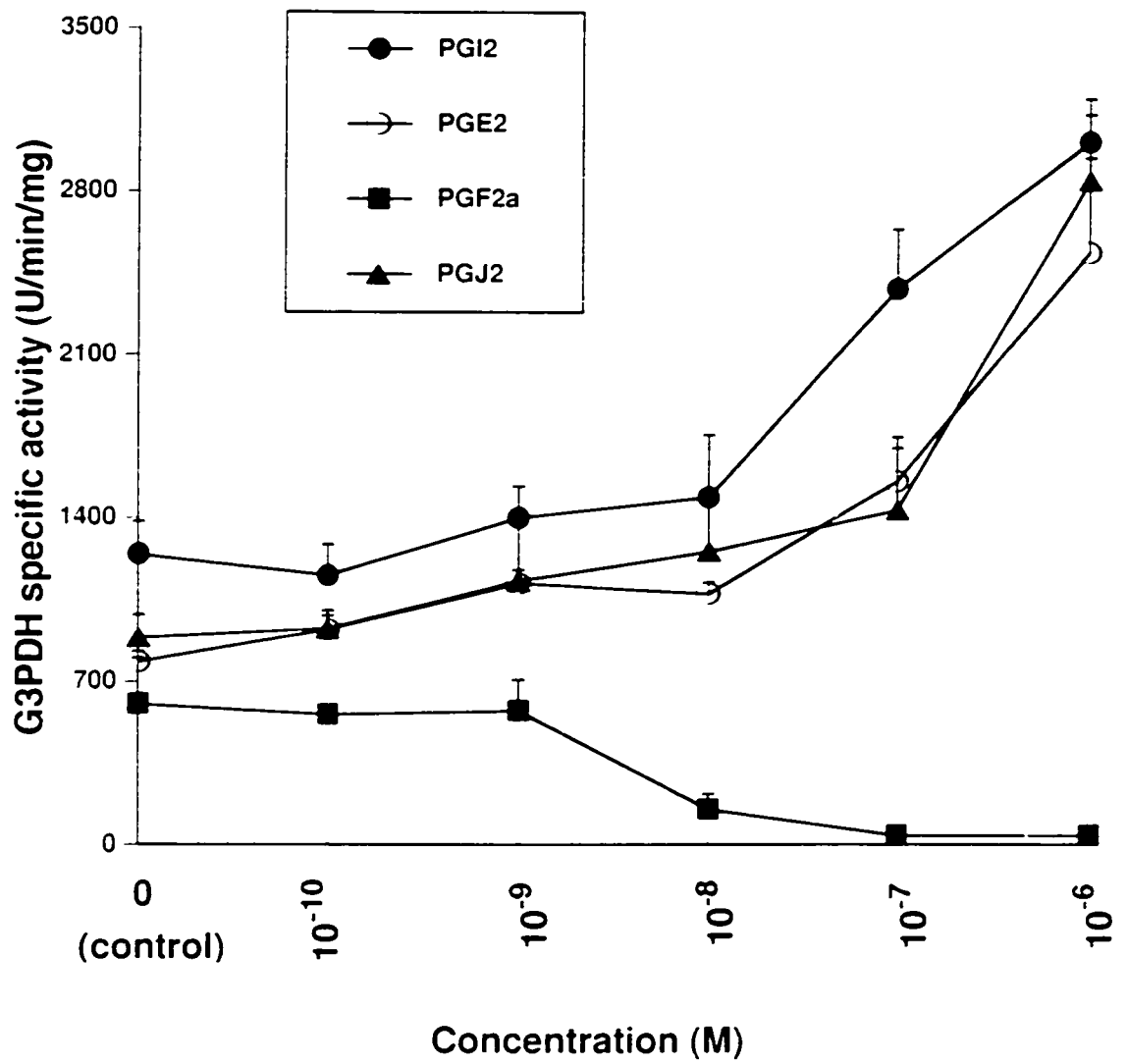
Biochemical assessment as measured by G3PDH activity showed similar results (Figure 11). A significant 2-3 fold increase ( $p \leq 0.05$ ) in G3PDH values was observed only at concentrations 10<sup>-7</sup> and 10<sup>-6</sup> M (2385 ± 255 and 3016 ± 185 U, respectively, vs 1244 ± 139 U for control). Concentrations lower than 10<sup>-7</sup> M did not lead to an increase

**Figure 10. Phase contrast photomicrographs illustrating the cytoplasmic lipid inclusion in rat preadipocytes upon exposure to increasing concentrations of exogenous cPGI<sub>2</sub> (a stable analogue of PGI<sub>2</sub>).** Confluent primary rat preadipocytes grown in  $\alpha$ MEM containing 10% FBS were shifted (day 5) to serum free, hormone supplemented medium, ITT, supplemented further or not with cPGI<sub>2</sub>. The concentration of cPGI<sub>2</sub> ranged from 10<sup>-10</sup> M to 10<sup>-6</sup> M. Medium was changed after every 2 days and fresh cPGI<sub>2</sub> was added at that time. Experiment was terminated on day 14 after seeding. Original magnification x 320.

Panel (A) control (ITT), (B) ITT + 10<sup>-10</sup> M cPGI<sub>2</sub>, (C) ITT + 10<sup>-9</sup> M cPGI<sub>2</sub>, (D) ITT + 10<sup>-8</sup> M cPGI<sub>2</sub>, (E) ITT + 10<sup>-7</sup> M cPGI<sub>2</sub>, (F) ITT + 10<sup>-6</sup> M cPGI<sub>2</sub>.



**Figure 11. Effect of increasing concentrations of exogenous PG (cPGI<sub>2</sub>, PGE<sub>2</sub>, 15d-PGJ<sub>2</sub> and PGF<sub>2α</sub>) on adipose conversion of rat adipocyte precursors in primary culture as measured by G3PDH specific activity.** Confluent rat preadipocytes were exposed to exogenous PG under same conditions as described in the legend of Figure 10. On day 14, cells were harvested to measure G3PDH activity. One unit of enzyme activity corresponds to 1 nmole of NADH oxidized. The data were analyzed by ANOVA and Tukey's test for multiple comparisons. The values are means ± SEM of triplicate determinations obtained in 3 separate experiments except for PGF<sub>2α</sub> where n=4.



in G3PDH activity when compared to controls (Figure 11).

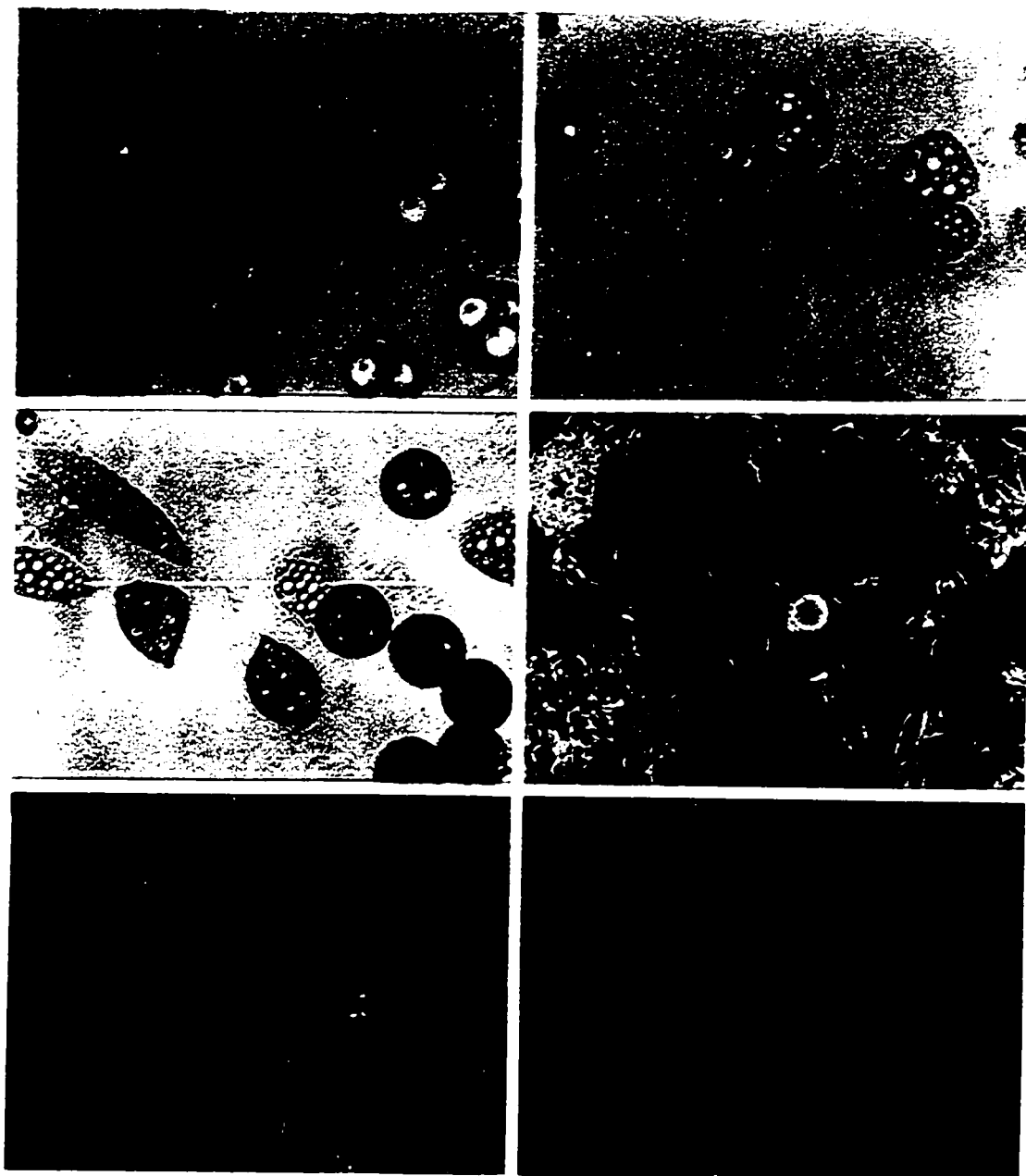
Similar results were obtained in a reproducible manner for PGE<sub>2</sub> and 15d-PGJ<sub>2</sub> with respect to morphological as well as biochemical evidence of differentiation (Figure 11). After a 10-day exposure of PGE<sub>2</sub>, the increase in G3PDH activity reached values close to 2-fold compared to controls, but only at 10<sup>-6</sup> M concentration (ITT: 792 ± 47 U vs ITT + PGF<sub>2α</sub> (10<sup>-7</sup> M): 1559 ± 191 U). There was a slight dose-relationship between G3PDH activity and the concentration of 15d-PGJ<sub>2</sub>. An increase in 15d-PGJ<sub>2</sub> concentration stimulated G3PDH activity, with 3-fold increase observed at the concentration of 10<sup>-6</sup> M (control: 888 ± 99 U vs 10<sup>-6</sup> M PGJ<sub>2</sub>: 2850 ± 284 U).

#### **5. 6. 2. Effects of Increasing Concentrations of PGF<sub>2α</sub> on Preadipocyte Differentiation**

The addition of exogenous PGF<sub>2α</sub> to confluent test preadipocytes in a concentration range of 10<sup>-10</sup> M to 10<sup>-6</sup> M resulted in a dose dependent inhibition of preadipocyte differentiation (Figure 11). About 75% inhibition was observed with 10<sup>-8</sup>M PGF<sub>2α</sub> (151 ± 68 vs 603 ± 198 U G3PDH for control) with maximum inhibition occurring at 10<sup>-7</sup>M (38 ± 6 vs 603 ± 198 U for control). In addition to having lower levels of G3PDH activity, the cells cultivated in the presence of PGF<sub>2α</sub> remained fibroblastic, whereas the cells maintained in ITT alone (control) rounded up and accumulated lipid in their cytoplasm (Figure 12).

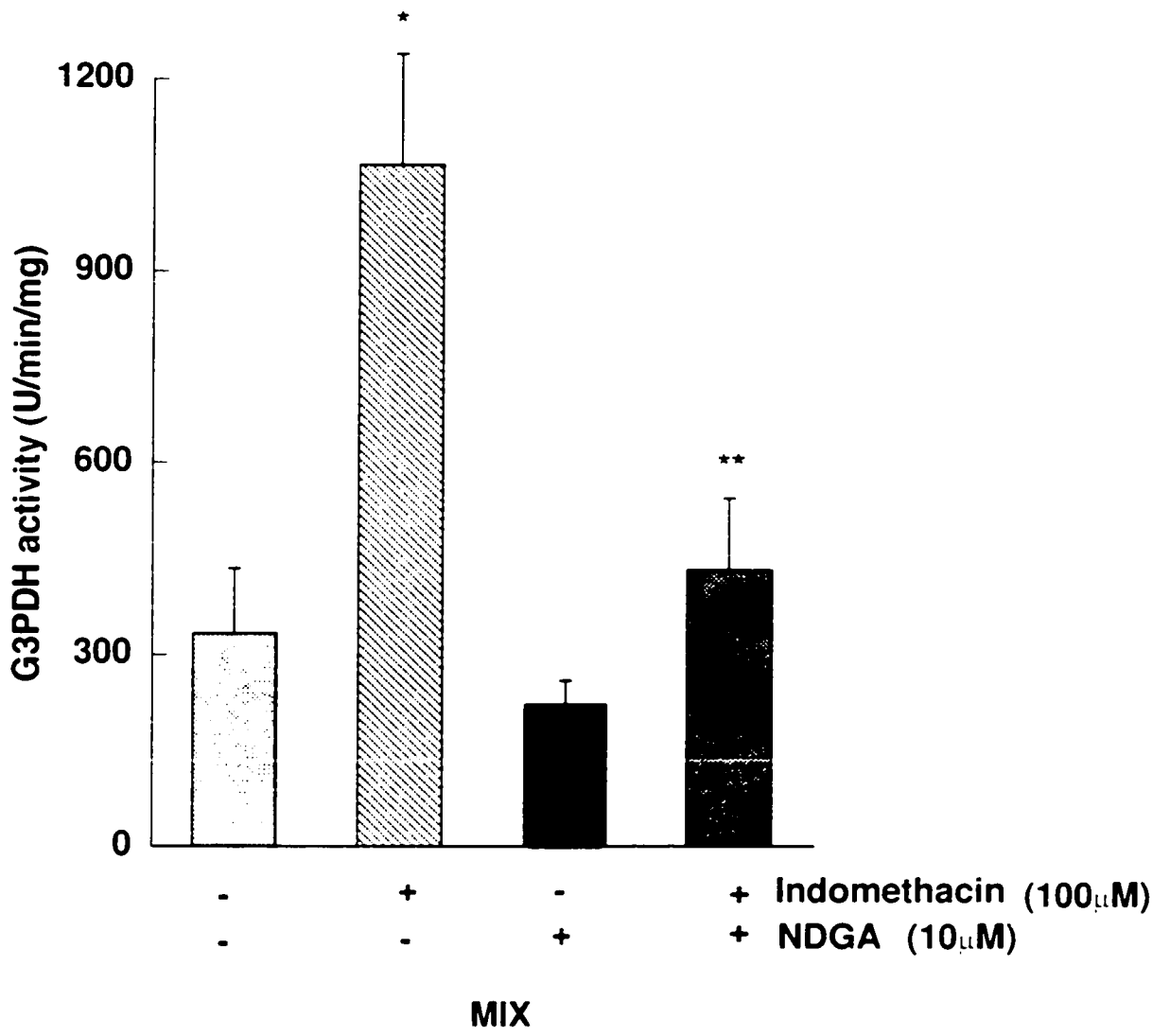
**Figure 12. Phase contrast photomicrographs of rat preadipocytes exposed to increasing concentrations of PGF<sub>2α</sub>.** Rat preadipocytes were seeded at a density of  $4 \times 10^4$  cells/ cm<sup>2</sup> in 24 well plates in  $\alpha$ MEM containing 10% FBS. On day 5 when cells reached monolayer confluency, medium was changed to serum free medium (ITT) supplemented or not with increasing concentrations of PGF<sub>2α</sub>. Fresh PGF<sub>2α</sub> was added after every 2 days when medium was changed. The experiment was terminated on day 14 after seeding. Original magnification x 320.

Panel A: control (ITT), B: +  $10^{-10}$  M PGF<sub>2α</sub>, C: +  $10^{-9}$  M PGF<sub>2α</sub>, D: +  $10^{-8}$  M PGF<sub>2α</sub>, E: +  $10^{-7}$  M PGF<sub>2α</sub>, F: +  $10^{-6}$  M PGF<sub>2α</sub>.



**Figure 13. Effect of NDGA on G3PDH activity of differentiating preadipocytes.**

Confluent test preadipocytes were induced to differentiate with a) MIX (control), b) MIX plus NDGA, c) MIX plus indomethacin, d) MIX plus indomethacin plus NDGA, as described in Methods, section 3.9. The experiment was terminated on day 10 after seeding. Cells were extracted to measure G3PDH activity. One unit of enzyme activity corresponds to 1 nmole NADH oxidized. The values represent means  $\pm$  SEM of triplicate determinations of 7 sets of experiments. \* denotes values that are significantly different from control ( $P \leq 0.05$ ); \*\* significantly different from \* ( $P \leq 0.05$ ).



### 5.7. Effect of Lipoxygenase (LOX) Products on Rat Preadipocyte Differentiation

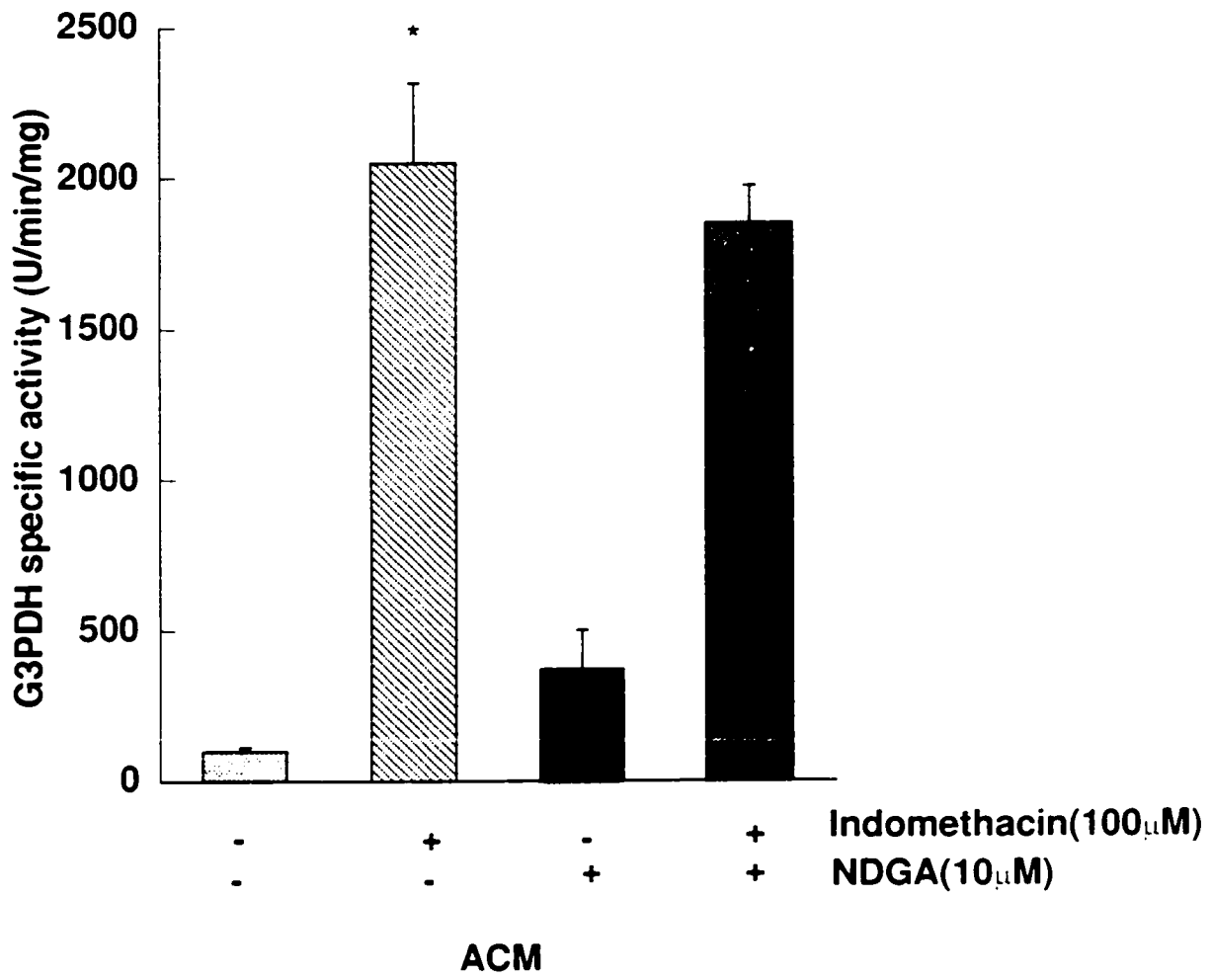
To test the involvement of LOX products in preadipocyte differentiation, confluent preadipocytes were induced to differentiate with MIX and ACM with or without the addition of NDGA, an inhibitor of LOX (Higgs and Vane, 1983). The differentiation of rat preadipocytes induced by MIX was inhibited in the presence of NDGA (10  $\mu$ M). MIX-treated preadipocytes revealed no appreciable amount of cytoplasmic lipid accumulation in the presence of NDGA compared to preadipocytes treated with MIX alone. When assessed biochemically, a decrease in the G3PDH activity was observed in NDGA treated cells (MIX:  $333 \pm 102$ ; MIX  $\pm$  NDGA:  $222 \pm 37$  U, Figure 13). Although the difference in the G3PDH values was not statistically significant, the trend of lower G3PDH values in NDGA treated cells was found in all 7 repeats of the experiment. As expected, the addition of indomethacin (100  $\mu$ M) to MIX treated cells stimulated a 3-fold increase in G3PDH activity (MIX:  $333 \pm 102$  vs MIX+Ind:  $1066 \pm 102$  U, Figure 13). This indomethacin-stimulated differentiation of MIX treated preadipocytes was also significantly ( $P \leq 0.05$ ) reversed by NDGA (Figure 13), suggesting that LOX products may be a positive regulators of rat preadipocyte differentiation.

In contrast to inhibition observed in MIX conditions, NDGA stimulated preadipocyte differentiation in the presence of ACM. The number of lipid accumulating preadipocytes increased progressively in the presence of NDGA than in ACM alone, with a simultaneous increase in the G3PDH activity (ACM:  $99 \pm 13$ ; ACM+NDGA:  $369 \pm 139$  U, Figure 14). Again the difference in the differentiation level of these two groups was not statistically significant, but the trend of higher G3PDH activity in NDGA treated cells was observed in all 5 repeats of the experiment. Furthermore, NDGA failed to inhibit

differentiation augmented by high concentration of indomethacin (Figure 14).

**Figure 14. Effect of NDGA on ACM-induced preadipocyte differentiation.**

The experimental protocol was same as described in the legend of Figure 13, except that ACM was used as a control to induce preadipocyte differentiation. G3PDH activity was measured on day 10 after seeding. The values are representative of means  $\pm$  SEM of 5 separate sets of experiments. \* denotes values that are significantly different compared to control (ACM alone),  $P \leq 0.05$ .



## 6. DISCUSSION

Adipose cell differentiation is regulated by many yet unknown mechanisms. Considering the close relationship of obesity with a broad spectrum of metabolic disorders, elucidation of mechanisms underlying adipose tissue development would be of tremendous benefit in developing the therapeutic strategies. With the emerging image of adipocyte as a secretory cell, it is reasonable to think that adipocyte may regulate its own environment. Evidence generated from our laboratory as well as from other investigators for the paracrine interactions between the constituent cells of adipose tissues prompted us to investigate the role of potential paracrine/autocrine factors in the regulation of adipose tissue mass. Prostaglandins are one of many molecules known to be produced in adipose tissue and are shown to influence adipose cell differentiation. The role of prostaglandins in the past has been addressed by using pharmacological prostaglandins. In this study, we demonstrate for the first time that endogenously released prostaglandins in an adipose tissue may function in both positive and negative manner in the development of adipocyte lineage. We, herein, present evidence that in a given adipose tissue microenvironment, prostaglandins produced and released by adipocytes may act as negative paracrine modulators of precursor cell differentiation, whereas those synthesized and released by the precursor cells (preadipocytes) may play a positive role in adipocyte development by means of an autocrine mechanism.

Since the overall objective of this study was to understand the physiological regulation of preadipocyte differentiation *in vivo*, the primary culture system was used. Primary culture being close to *in vivo* conditions, offers the benefit of studying physiological regulators of adipose development *in vitro*. However, when using

preadipocytes in primary culture. an important question is whether the isolated preadipocytes are a homogenous population without being contaminated with endothelial cells. The homogeneity of preadipocytes in our culture is justified with the observation that over 90% of the cells developed into adipocytes in the presence of high concentrations of indomethacin and prostaglandins such as PGI<sub>2</sub>, PGE<sub>2</sub> and PGJ<sub>2</sub>. Furthermore, the cells seemed to be homogenous without contamination with endothelial cells when examined under microscope.

The paracrine effect was studied by culturing preadipocytes with medium conditioned by adipocytes (ACM). ACM is shown by our laboratory to contain differentiation-promoting factors released by adipocytes (Shillabeer *et al.* 1989). Our finding that prostaglandins released by adipocytes *in vitro* may act to inhibit preadipocyte differentiation in a paracrine fashion was supported by the following observations: 1) suppression of prostaglandin synthesis in adipocytes with cyclooxygenase inhibitor, indomethacin, resulted in a significant increase in preadipocyte differentiation suggesting that in the absence of indomethacin, adipocytes may release prostaglandins that are inhibitory for differentiation. 2) Adipocytes secreted PGI<sub>2</sub> and PGE<sub>2</sub> into ACM at concentrations of  $9 \times 10^{-9}$  M and  $1.25 \times 10^{-10}$  M, respectively. These prostaglandins were found to be stimulatory for differentiation, but the effective concentration ( $10^{-7}$  M) was much higher than that released by adipocytes in the culture medium (Figure 11). It is likely that the concentration of PGE<sub>2</sub> and PGI<sub>2</sub> detected in ACM may be lower than the actual concentration released by adipocytes *in vivo*, as adipocytes may utilize these prostaglandins for other cell functions. However, a difference of more than 100-fold between the concentrations released and those required for differentiation clearly indicates that at physiological concentrations these prostaglandins cannot act as positive modulators of adipocyte differentiation. 3) PGF<sub>2α</sub>, in contrast, inhibited preadipocyte

differentiation significantly ( $p \leq 0.05$ ) at lower concentration ( $10^{-8}\text{M}$ ), suggesting that at physiological level, the negative paracrine regulation may be due to the presence of  $\text{PGF}_{2\alpha}$  in adipocytes. Although a direct release of  $\text{PGF}_{2\alpha}$  was not measured in this study, the production of this prostaglandin in adipocytes has been previously reported (Negrel and Ailhaud, 1981; Hopkin and Gorman, 1981; Mitchell *et al.*, 1983). The presence of inhibitory prostaglandins in rat adipocytes such as  $\text{PGF}_{2\alpha}$ , and the paracrine negative control of precursor cell differentiation by these prostaglandins may represent a physiological signal involved in maintaining cells as preadipocytes. A feed-back mechanism in which prostaglandins synthesised by adipocytes inhibit an excessive continuing increase of adipocyte numbers in adipose tissue may well be a physiological phenomenon.

The anti-inflammatory drug indomethacin acts as a potent inhibitor of prostaglandin synthesis in a wide variety of tissues (Smith and Lands, 1971; Richelsen, 1987) and for that reason has been used as a tool to evaluate the physiological role of prostaglandins. However, in our experiment, indomethacin at high concentration ( $100\ \mu\text{M}$ ) stimulated preadipocyte differentiation considerably even under control conditions (10% FBS). These results indicated that significant stimulation observed in differentiation with indomethacin under MIX and ACM conditions may not be solely due to the inhibition of inhibitory prostaglandin synthesis. While this dissertation was in preparation, a study demonstrated indomethacin ( $100\ \mu\text{M}$ ) as a ligand for  $\text{PPAR}\gamma_2$ , a transcription factor involved in adipogenesis (Lehmann *et al.*, 1997). Therefore, it was quite possible that such an adipogenic activity of indomethacin had masked the effect of blocking inhibitory prostaglandins. However, the effect of indomethacin on preadipocyte differentiation did not appear to result from its adipogenic activity, when optimal concentration ( $0.01\ \mu\text{M}$ ) was used to inhibit prostaglandin synthesis (Figure 8).

Radioimmunoassays, in this study, revealed the production of PGI<sub>2</sub> (measured as 6-keto PGF<sub>1α</sub>) and PGE<sub>2</sub> by adipocytes as measured by their release into ACM. The ratio of 6-keto PGF<sub>1α</sub> formed versus PGE<sub>2</sub> (1.5 : 1) in ACM was in agreement with the previous findings (Axelrod and Levine, 1981; Richelsen, 1987). Whether the experimental manipulation of preadipocyte/adipocytes such as collagenase treatment, induces any non-specific stimulation of prostaglandin formation in adipocytes is presently unknown. However, the quantity of prostaglandin produced in our experiment was comparable with the data from fat tissue (Christ and Nugteren, 1970), suggesting that collagenase treatment might not have had an effect on prostaglandin synthesis. Furthermore, inhibition by indomethacin of about 90% of the prostaglandins produced eliminates the possibility of non-specific prostaglandin formation.

When we examined the direct effect of prostaglandins on differentiation, cPGI<sub>2</sub> proved to be a stimulator of rat preadipocyte differentiation. However, higher concentrations of PGI<sub>2</sub> ( $\geq 10^{-7}$  M) were necessary to observe significant stimulation in rat preadipocyte differentiation. These observations are in well agreement with previous studies (Catalioto *et al.*, 1991; Vassaux *et al.*, 1994). The concentration required to obtain a significant effect upon G3PDH expression for cPGI<sub>2</sub> in these studies was  $10^{-5}$  M.

Similarly, PGE<sub>2</sub> in this study stimulated rat preadipocyte differentiation, but again the effective concentration for differentiation ( $\geq 10^{-7}$  M) was many fold greater than that released by rat adipocytes in ACM (Figure 11). The effect of PGE<sub>2</sub> on adipose differentiation has been variously reported. Some of the previous studies have observed an inhibitory effect of PGE<sub>2</sub> on clonal preadipocytes (Williams and Polakis, 1977; Casimir *et al.*, 1996), whereas Serrero's group was unable to demonstrate any effect of

PGE<sub>2</sub> (10<sup>-7</sup> M) on differentiation of rat primary preadipocytes (Serrero et al, 1992a) and 1246 clonal preadipocytes (Serrero *et al.*, 1992b). PGE<sub>2</sub>, in other cell types, is able to operate through two different receptors, a stimulatory receptor coupled to G<sub>s</sub> which is involved in the activation of adenylate cyclase, and an inhibitory receptor coupled to G<sub>i</sub> that is involved in the inhibition of adenylate cyclase (Smith, 1989). In rat preadipocytes, only G<sub>i</sub> proteins have been reported to be present (Lu *et al.*, 1988). The stimulatory response of PGE<sub>2</sub> observed in our experiment, however, suggests the presence of PGE<sub>2</sub> receptors coupled to G<sub>s</sub> proteins as well in preadipocytes. Moreover, PGE<sub>1</sub> has been shown to bind with PGI<sub>2</sub> receptors in preadipocytes (Vassaux *et al.*, 1992). So, it is quite possible that in our case PGE<sub>2</sub> exerted its adipogenic effect via PGI<sub>2</sub> receptors. However, the mechanism behind the apparent stimulatory effect of PGE<sub>2</sub> remains to be elucidated.

In the present study, addition of increasing concentrations of PGF<sub>2α</sub> to the culture of rat adipocyte precursors resulted in inhibition of differentiation in a dose-dependent manner. Similar effect of PGF<sub>2α</sub> has also been reported by Serrero's and Casimir's groups (Serrero *et al.*, 1992a; Serrero *et al.*, 1992b; Casimir *et al.*, 1996). PGF<sub>2α</sub> in these studies (Serrero *et al.*, 1992a and Casimir *et al.*, 1996) inhibited differentiation of rat preadipocytes and 3T3 L1 preadipocytes by 50% at subnanomolar concentrations (3 x 10<sup>-9</sup>M and 0.4 nM, respectively). Some investigators, however, using Ob1771 preadipocytes have reported the reverse (Negrel *et al.*, 1989; Catalioto *et al.*, 1991). The nature of this different effect of PGF<sub>2α</sub> in Ob1771 cells could be due either to a difference in the cell line or to the culture conditions used. Moreover, these studies did not examine the direct effect of PGF<sub>2α</sub> on adipocyte differentiation as PGF<sub>2α</sub> was always added in the presence of cPGI<sub>2</sub>. Although PGF<sub>2α</sub> was found to be a positive modulator

of Ob1771 cell differentiation, it was previously reported to inhibit differentiation of the cell line Ob17, from which Ob1771 cells are derived (Irvine, 1982).

Since the present study was also focused on determining the differentiation promoting factor (adipogenic factor) released by rat adipocytes into ACM (Shillabeer *et al.*, 1989), it was postulated that PGI<sub>2</sub> may be the potential paracrine factor involved in the development of adipocyte phenotype and hence contributing towards the adipogenic activity of ACM. PGI<sub>2</sub> has been shown to be secreted in the interstitial fluid of rat adipose tissue (Darimont *et al.*, 1994) and to stimulate differentiation of rat preadipocytes in primary culture (Vassaux *et al.*, 1994) and Ob1771 cells (Catalioto *et al.*, 1991). However, in light of our results, the differentiation-promoting activity of ACM can not be ascribed to the presence of PGI<sub>2</sub>. ACM may contain both stimulatory (adipogenic factor) as well as inhibitory factors, such as PGF<sub>2α</sub> and its ability to promote differentiation may depend upon a balance between these two factors. Further studies are required to characterize the adipogenic factor present in ACM.

In support of second part of my hypothesis, I have demonstrated that prostaglandins produced by precursor cells (preadipocytes) act as positive modulators of differentiation in an autocrine mode of action. The following observations account for this finding. 1) Inhibition of prostaglandin synthesis in preadipocytes using optimal concentration of indomethacin (0.01 μM) resulted in significant inhibition of differentiation induced by both MIX and ACM, suggesting that in the absence of indomethacin, preadipocytes may release prostaglandins that are stimulatory for differentiation (Figure 8). 2) Preadipocytes released PGE<sub>2</sub> and PGI<sub>2</sub> (prostaglandins stimulatory for differentiation) in considerably greater amount into culture medium, compared to adipocytes. The amount of PGE<sub>2</sub> and PGI<sub>2</sub> released by preadipocytes was

11- and 1.4-fold greater, respectively than those released by adipocytes. This suggests that the collective production of these prostaglandins at physiological level may be sufficient to increase the number of adipocytes from dormant preadipocytes by an autocrine mechanism. Furthermore,  $\text{PGF}_{2\alpha}$  (prostaglandin found inhibitory for differentiation) could not be detected in the culture medium of preadipocytes (Figure 9).

To our knowledge, this is the first study to report  $\text{PGE}_2$  and  $\text{PGI}_2$  release by rat primary preadipocytes *in vitro*. Clonal preadipocytes such as Ob1771 (Negrel and Ailhaud, 1981) and 3T3-L1 (Hyman *et al.*, 1982) have been reported to produce and secrete these prostaglandins in amounts comparable to our study. *In vivo* measurement of prostaglandins in interstitial fluid of rat adipose tissue (Darimont *et al.*, 1994) was unable to identify the cellular source of these prostaglandins (preadipocytes, mature adipocytes or endothelial cells). Chatzipanteli and coworkers (Chatzipanteli *et al.*, 1992) demonstrated that  $\text{PGE}_2$  and  $\text{PGI}_2$  production by rat adipose tissue required the cooperation of adipocytes and endothelial cells, but did not measure prostaglandins released by preadipocytes. Taken together, the synthesis of  $\text{PGE}_2$  and  $\text{PGI}_2$  by preadipocytes in culture in considerably greater amounts compared to adipocytes indicates that at physiological level these prostaglandins may act in concert to modulate preadipocyte differentiation positively in an autocrine manner.

The present study also demonstrated the adipogenic effect of 15-deoxy- $\Delta^{12,14}$ -prostaglandin  $\text{J}_2$  (15d-PG $\text{J}_2$ ) on primary rat preadipocyte differentiation. 15d-PG $\text{J}_2$ , which is a terminal metabolite of  $\text{PGD}_2$  (Fitzpatrick and Wynalda, 1983; Kikawa *et al.*, 1984), was recently shown to stimulate adipose conversion in stem cells through its interaction with  $\text{PPAR}\gamma$  (Forman *et al.*, 1995; Kliewer *et al.*, 1995). Although

physiological concentrations of 15-d PGJ<sub>2</sub> have not been established, our results point to a possible role for this metabolite of PGD<sub>2</sub> in adipose tissue development.

As discussed earlier, indomethacin at high concentration (100 μM) in this study induced differentiation dramatically. The inhibition of cyclooxygenase pathway by aspirin or indomethacin increased 12-HETE production in human platelets indicating that arachidonate metabolized for prostaglandin production can be routed to the alternate lipoxygenase pathway (Hamberg *et al.*, 1974). We, thus examined the effect of lipoxygenase metabolites on adipose cell differentiation. Our results by blocking lipoxygenase pathway using either NDGA (inhibitor of LOX) alone, or in the presence of indomethacin (100 μM) raise the possibility that lipoxygenase metabolites such as leukotrienes or HETEs may be important paracrine regulators of adipose cell differentiation. Recently, 8(s) HETE was shown to induce differentiation in 3T3-L1 fibroblasts (Yu *et al.*, 1995). It will be of interest to consider that HETEs or leukotrienes may play a crucial role in adipose cell regulation, particularly when inhibition of enzyme cyclooxygenase provides an alternative pathway for eicosanoid biosynthesis.

In contrast to MIX conditions, the presence of NDGA in ACM conditions stimulated ACM-induced preadipocyte differentiation. This contradictory effect of NDGA observed in the presence of ACM, suggests that ACM induces differentiation by a mechanism that may not involve these metabolites. It is also likely that different types of HETES or leukotrienes are activated in the presence of ACM. Moreover, the presence of agents such as IBMX, insulin and glucocorticoids at pharmacological concentrations in MIX conditions may also differentially affect the synthesis of eicosanoids (Lewis and Piper, 1978; Mitchell *et al.*, 1983; Gaillard *et al.*, 1991) and hence may lead to a

mechanism that acts differently than ACM. Further work will be needed to study the direct effect of these less studied arachidonate metabolites on paracrine regulation of adipose tissue in both normal and pathophysiological conditions.

It would be relevant to take some considerations concerning the use of indomethacin in detecting an effect of endogenous prostaglandins. At lower concentrations, indomethacin blocks cyclooxygenase activity. However, at higher concentrations, it may not only inhibit cyclooxygenase activity but also promote PPAR-activated adipocyte differentiation (Lehmann *et al.*, 1977). In addition, indomethacin at higher concentrations may shift the arachidonic acid metabolism from the cyclooxygenase pathway to lipoxygenase pathway resulting in the production of leukotrienes and HETEs.

In summary, the study has provided new insight into the proposed paracrine/autocrine mechanism(s) of regulating adipose cell growth. The positive autocrine regulation of adipose tissue by PGE<sub>2</sub> and PGI<sub>2</sub> may be important during the growth and development of adipose tissue, whereas the paracrine negative control of differentiation by inhibitory prostaglandins, such as PGF<sub>2α</sub> may be important to maintain the pool of undifferentiated precursor cells. Whether this negative regulation can be turned off when the need for differentiation arises and vice versa, would be the next question of investigation.

## SUMMARY AND CONCLUSION

The study of factors and mechanisms involved in the regulation of adipose tissue is important for the development of preventive and therapeutic strategies for obesity. Many mechanisms have been proposed which may act alone or in concert to influence adipose tissue development. Beyond the simple understanding that energy intake must exceed energy expenditure, the precise mechanisms underlying the pathogenesis of obesity remains unresolved. Studies from our laboratory and other investigators have presented evidence for the role of paracrine factors in adipose tissue development. The purpose of this study has been to extend our understanding of autocrine/paracrine mechanisms involved in adipose tissue development. Adipocytes, in addition to serving a site for the storage and release of triacylglycerols, are known to secrete a number of molecules that may be implicated in regulating adipose tissue. We investigated the paracrine/autocrine role of prostaglandins, one of the molecules produced and released by adipocytes/preadipocytes into the extracellular milieu, on precursor cell (preadipocyte) differentiation.

The first objective of the study was to determine if prostaglandins released by adipocytes and preadipocytes influence preadipocyte differentiation in a paracrine/autocrine manner. Cells derived from the epididymal and retroperitoneal adipose tissue of male Sprague-Dawley rats were used to study adipose cell differentiation. To determine the paracrine role of prostaglandins released by adipocytes in the differentiation of preadipocytes, test preadipocytes were cultured with medium conditioned by adipocytes (ACM) collected in the presence of indomethacin, an inhibitor of cyclooxygenase (ACM+I). To determine the autocrine role of prostaglandins, test

preadipocytes were exposed to indomethacin in the presence of standard differentiation mixture: MIX (MIX+I) and ACM (ACM+Ia). Preadipocytes exposed to ACM containing indomethacin at the time of collection revealed significantly greater differentiation ( $p < 0.05$ ) compared to ACM and ACM to which indomethacin was added after its collection, suggesting that adipocytes may release prostaglandins that negatively modulate the extent of differentiation. In contrast, preadipocytes induced to differentiate under ACM+Ia and MIX+I conditions showed significant reduction in differentiation ( $p < 0.05$ ) compared to their respective controls. These results suggested that in the absence of prostaglandin synthesis inhibitor, preadipocytes may release prostaglandins that stimulate differentiation in an autocrine mode of action.

The second objective was to determine the production and release of prostaglandins by adipocytes and preadipocytes. Preadipocytes and adipocytes were both found to release  $PGE_2$  and  $PGI_2$  into culture medium. However, Preadipocytes released considerably greater amount of  $PGE_2$  (11-fold) and  $PGI_2$  (1.4-fold) in culture medium compared to adipocytes.

The third objective was to determine if the amount of released prostaglandins was sufficient to induce the effect observed in the presence of indomethacin. Test preadipocytes were exposed to exogenous prostaglandins ( $PGE_2$ ,  $PGI_2$ , 15-d  $PGJ_2$  and  $PGF_{2\alpha}$ ) in concentrations between physiological ( $10^{-10}$  M) and pharmacological ( $10^{-6}$  M) ranges. Although exogenous  $PGE_2$  and  $PGI_2$  stimulated preadipocyte differentiation, the effective concentration ( $\geq 10^{-7}$  M) was much higher than the concentration found in the culture medium of adipocytes. The concentration of  $PGI_2$  and  $PGE_2$  in culture medium of adipocytes was found to be  $9 \times 10^{-9}$  M and  $1.25 \times 10^{-9}$  M, respectively. At concentrations below  $10^{-7}$  M,  $PGE_2$  and  $PGI_2$  did not influence preadipocyte

differentiation.  $\text{PGF}_{2\alpha}$ , in contrast, inhibited preadipocyte differentiation significantly ( $p < 0.05$ ) at a lower concentration ( $10^{-8}$  M). These results suggested that in physiological conditions, adipocytes may release insufficient amounts of  $\text{PGE}_2$  and  $\text{PGI}_2$  to stimulate preadipocyte differentiation. The negative paracrine regulation observed in the presence of indomethacin may be due to the presence of  $\text{PGF}_{2\alpha}$  in adipocytes. A considerably larger secretion of  $\text{PGE}_2$  and  $\text{PGI}_2$  by preadipocytes may be sufficient to stimulate differentiation in an autocrine manner.

The present study also investigated the involvement of arachidonic acid metabolites produced via the lipoxygenase pathway. Treatment of preadipocytes with a lipoxygenase inhibitor, nordihydroguaiaretic acid (NDGA), inhibited adipose conversion induced by MIX. Addition of indomethacin ( $100 \mu\text{M}$ ) significantly augmented MIX-induced differentiation which was also inhibited by NDGA. In contrast, addition of NDGA to ACM stimulated ACM-induced differentiation. NDGA also failed to inhibit the increased differentiation induced by ACM in the presence of indomethacin. These findings suggested the involvement of stimulatory lipoxygenase products in MIX-induced differentiation and inhibitory lipoxygenase products in ACM-induced differentiation.

In conclusion, this work extended our understanding of the cellular mechanisms which may play important roles in the development of adipose tissue, specifically:

1. Prostaglandins produced and released by adipocytes in the adipose tissue microenvironment may act to inhibit precursor cell differentiation. This negative paracrine control of differentiation by inhibitory prostaglandins of adipocytes, such as  $\text{PGF}_{2\alpha}$ , may be important to maintain the pool of undifferentiated

precursor cells in normal and pathophysiological conditions of adipose tissue growth and development.

2. Precursor cells may release sufficient amounts of PGE<sub>2</sub> and PGI<sub>2</sub> into the extracellular matrix to modulate their differentiation positively by an autocrine mode of action. This positive autocrine regulation may be important during times requiring the growth and development of adipose tissue.
3. Arachidonate metabolites oxidized via the lipoxygenase pathway may contribute towards the growth and development of adipose tissue. The action and mechanisms underlying the function of these products, however, remain to be seen.

## FUTURE DIRECTIONS

Although this study attempted to address many questions, many more have arisen. Further studies described below should provide further insight into mechanisms underlying adipose tissue growth and development.

By using exogenous 15-deoxy  $\Delta^{12,15}$  PGJ<sub>2</sub> (15-d PGJ<sub>2</sub>) we have shown that this prostaglandin at micromolar concentrations may play a positive role in adipose conversion. But is this prostaglandin present in sufficient concentration *in vivo* to serve as a stimulator of precursor cell differentiation? The production or presence of this prostaglandin in adipocytes or preadipocytes, at present, is unknown. Once the production and release of this prostaglandin in adipose tissue is determined, the physiological relevance of its action in adipose tissue growth and development could be established.

Most prostaglandins are known to mediate their response through interaction with cell-surface receptors. However, 15-d PGJ<sub>2</sub> is shown to stimulate preadipocyte differentiation by binding to a nuclear receptor (Kliwer *et al.* 1995; Forman *et al.* 1995). The transfer system of PGJ<sub>2</sub> from cytosol to nucleus in L-1210 murine leukemia cells has been reported (Narumiya *et al.* 1986). Understanding the conditions in which PGJ<sub>2</sub> taken up by cells is transferred to cell nuclei in adipose-cell system may reveal a novel mechanism of adipose conversion by this prostaglandin.

Strong evidence for the role of arachidonate metabolites through the lipoxygenase pathway in adipose-cell regulation can be obtained from studies of the direct effect of

these products on adipocyte growth and development. Once their role in differentiation/proliferation is determined, understanding the mechanism of their action could be the next possible direction in delineating the mechanisms underlying adipose tissue growth and development.

## REFERENCES

- Axelrod, L and Levine, L. (1981) Prostacyclin production by isolated rat adipocytes. Diabetes 30, 163-167.
- Axelrod, L., Minnich, A. K. and Ryan, C. A. (1985) Stimulation of prostacyclin production in isolated rat adipocytes by angiotensin II, vasopressin and bradykinin: evidences for two separate mechanisms of prostaglandin synthesis. Endocrinology 116, 2548-2553.
- Baldo, A., Sniderman, A. D., St.-Luce, S., Kohen, R., Maslowka, M., Hoang, B., Monge, J. C., Bell, A., Muley, S. and Cianflone, K. (1993) The adipsin-acylation stimulating protein system and the regulation of intracellular triglyceride synthesis. J. Clin. Invest 92, 1543-1558.
- Bernlohr, D. A., Bolanowski, M. A., Kelly, T. J. and Lane, M. D. (1985) Evidence for an increase in transcription of specific mRNAs during differentiation of 3T3-L1 preadipocytes. J. Biol. Chem. 260, 5563-5567.
- Beutler, B. and Cerami, A. (1987) Cachectin: more than a tumor necrosis factor. New Engl. J. Med. 316, 379-385.
- Bills, T. K., Smith, J. B. and Silver, M. J. (1977) Selective release of arachidonic acid from the phospholipids of human platelets in response to thrombin. J. Clin. Invest. 60, 1-6.
- Borgeat, P. (1987) In: Handbook of Eicosanoids, Prostaglandins and Related Lipids Ed.: A. L. Willis (Boca Raton, FL) pp 193-211.
- Brain, S. D. and Lewis, G. P. (1981) Can endogeneous prostaglandins exert a negative feed back control of lipolysis? Br. J. Pharmacol. 785.
- Bray, G. A. and Gray, D. S. (1988) In: Anthropometric Standardization Manual Ed.: T. G. Lohman, A. Roch, R. Martorell, I. L. Champaign (Human Kinetics) pp 131-136.
- Campfield, L. A., Smith, F. J., Guisez, Y., Devos, R. and Burn, P. (1995) Recombinant mouse Ob protein: evidence for a peripheral signal linking adiposity and central neural networks. Science 269, 546-549.
- Cao, Z., Umek, R. M. and Mcknight, S. L. (1991) Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. Genes Develop. 5, 1538-1552.
- Casimir, D. A., Miller, C. W. and Ntambi, J. M. (1996) Preadipocyte differentiation blocked by prostaglandin stimulation of prostanoid FP2 receptor in murine 3T3-L1 cells. Differentiation 60, 203-210.
- Catalioto, R. M., Gaillard, D., Maclouf, J., Ailhaud, G. and Negrel, R. (1991) Autocrine control of adipose cell differentiation by prostacyclin and PGF<sub>2α</sub>. Biochim. Biophys. Acta 1091, 364-369.

- Catalioto, R. M., Gaillard, D., Ailhaud, G. and Negrel, R. (1992) Terminal differentiation of mouse preadipocyte cells: The mitogenic-adipogenic role of growth hormone is mediated by the protein kinase C signalling pathway. Growth Factors 6, 255-264.
- Chang, J., Lewis, G. P. and Piper, P. J. (1977) The effect of inflammatory drugs on the prostaglandin system in human subcutaneous adipose tissue. Br. J. Pharmacol. 61, 446-447.
- Chatzipanteli, K., Rudolph, S. and Axelrod, L. (1992) Coordinate control of lipolysis by prostaglandin E<sub>2</sub> and prostacyclin in rat adipose tissue. Diabetes 41, 927-935.
- Cho, M. J. and Allen, M. A. (1978) Chemical stability of prostacyclin (PGI<sub>2</sub>) in aqueous solution. Prostaglandins 15, 943-954.
- Christ, E. J. and Nugteren, D. H. (1970) The biosynthesis and possible function of prostaglandins in adipose tissue. Biochim. Biophys. Acta 218, 296-307.
- Cook, K. S., Hunt, C. R., Spiegelman, B. M. (1985) Developmentally regulated mRNAs in 3T3-adipocytes: analysis of transcriptional control. J. Cell Biol. 100, 514-520.
- Corey, E. J., Niwa, H., Falck, J. R., Mioskowski, C., Arai, Y. and Marfat, A. (1980) Recent studies on the chemical synthesis of eicosanoids. Prostaglandin Thromboxane Res. 6, 19-26.
- Cornelius, P., Enerback, S., Bjursell, G., Olivecrona, T. and Pekala, P. H. (1988). Regulation of lipoprotein mRNA content in 3T3-L1 cells by tumor necrosis factor. Biochem. J. 249, 765-769.
- Cornelius, P., MacDougald, O. A. and Lane, M. D. (1994) Regulation of preadipocyte development. Ann. Rev. Nutr. 14, 99-129.
- Dalton, C. and Hope, H. R. (1974) Cyclic AMP regulation of prostaglandin biosynthesis in fat cells. Prostaglandins 6, 227-242.
- Darimont, C., Vassaux, G., Gaillard, D., Ailhaud, G. and Negrel, R. (1994) *In situ* microdialysis of prostaglandins in adipose tissue: stimulation of prostacyclin release by angiotensin II. Int. J. Obesity 18, 783-788.
- Deslex, S., Negrel, R., Vannier, C., Etienne, J. and Ailhaud, G. (1986) Differentiation of human adipocyte precursors in a chemically defined serum-free medium. Int. J. Obesity 10, 19-27.
- Diamond, L., O'Brien, T. G. and Rovera, G. (1977) Inhibition of adipose conversion of 3T3 fibroblasts by tumor promoters. Nature 269, 247-249.
- Djian, P., Roncari, D. A. K., and Hollenberg, C. H. (1983) Influence of anatomic site and age on replication and differentiation of rat precursors in culture. J. Clin. Invest. 72, 1200-1208.
- Djian, P., Roncari, D. A. K. and Hollenberg, C. H. (1985) Adipocyte precursor clones vary in capacity for differentiation. Metabolism 34, 880-883.

- Dunn, M. J., Liard, J. F. and Dray, F. (1978) Basal and stimulated rates of renal secretion of prostaglandin E<sub>2</sub>, F<sub>2α</sub>, and 13, 14, dihydro 15-keto-Fα in dog. Kidney Int 13, 136-143.
- Eckel, R. H., Fujimoto, W. Y. and Brunzell, J. D. (1977) Development of lipoprotein lipase in cultured 3T3-L1 cells. Biochem. Biophys. Res. Commun. 78, 288-293.
- Faust, I. M., Johnson, P. R., Stern, J. S. and Hirsch, J. (1978) Diet induced adipocyte number increase in adult rats: a new model of obesity. Am. J. Physiol. 235, E279-E286.
- Ferland, M., Despres, J. P., Nadeau, A., Moorjani, S., Trmbley, A., Lupien, P. J., Theriault, G. and Bouchard, C. (1991) Contribution of glucose tolerance and plasma insulin levels to the relationships between body fat distribution and plasma lipoprotein levels in women. Int. J. Obest. 15, 677-688.
- Ferreira, S. H. and Vane, J. R. (1967) Prostaglandins: their disappearance from and release into the circulation. Nature 216, 868-873.
- Filipak, M., Sparks, R. L., Tzen, C-Y, Scott, R. E. (1988) Tumor necrosis factor inhibits the terminal event in mesenchymal stem cell differentiation. J. Cell Physiol. 137, 367-373.
- Fitzpatrick, F. A., Stringfellow, D. A., Maclouf, J. and Rigaud, M. (1979) In: Prostacyclin Eds.: J. R. Vane and S. Bergstrom (Raven Press, New York) pp 55.
- Fitzpatrick, F. A. and Wynalda, M. A. (1983) Albumin-catalyzed metabolism of prostaglandin D<sub>2</sub>. Identification of products formed *in vitro*. J. Biol. Chem. 258, 11713-11718.
- Flier, J. S., Cook, K. S., Usher, P., and Spiegelman, B. M. (1987) Severely impaired adipin expression in genetic and acquired obesity. Science 237, 402-404.
- Flier, J. S. (1995) The adipocyte: storage depot or node on the energy information highway? Cell 80, 15-18.
- Flower, R. J., and Vane, J. R. (1973) In: Prostaglandins Synthetase Inhibitors Ed.: H. J. Robinson and J. R. Vane (Raven Press, New York) pp 9-18.
- Flower, R. J. and Vane, J. R. (1974) Inhibition of prostaglandin biosynthesis. Biochem. Pharmacol. 23, 1439-1450.
- Forman, B. M., Tontonoz, P., Chen, J., Brun, R. P. and Spiegelman, B. M. (1995) 15-Deoxy-Δ<sup>12,14</sup>-prostaglandin J<sub>2</sub> is a ligand for the adipocyte determination factor PPARγ. Cell 83, 803-812.
- Fredholm, B. B. and Hamberg, M. (1976) Metabolism and effect of prostaglandin H<sub>2</sub> in adipose tissue. Prostaglandins 11, 506-518.
- Freytag, S. O. and Geddes, T. J. (1992) Reciprocal regulation of adipogenesis by Myc and C/EBP. Science 256, 379-382.

- Gaillard, D., Negrel, R., Lagarde, M. and Ailhaud, G. (1989) Requirement and role of arachidonic acid in the differentiation of pre-adipose cells. Biochem. J. 257, 389-397.
- Gaillard, D., Wabitch, M., Pipy, B. and Negrel, R. (1991) Control of terminal differentiation of adipose precursor cells by glucocorticoids. J. Lipid Res. 32, 569-579.
- Gao, G. and Serrero, G. (1990) Phospholipases A<sub>2</sub> is a differentiation dependent enzymatic activity for adipogenic cell line and adipocyte precursors in primary culture. J. Biol. Chem. 265, 2431-2434.
- Gharbi-Chihi, J., Facchinetti, T., Berge-LeFranc, J. L., Bonne, J. and Toressani, J. (1991) Triiodothyronine control of ATP-citrate lyase and malic enzyme during differentiation of a murine preadipocyte cell line. Horm. Metab. Res. 23, 423-427.
- Granstorm, E. and Samuelsson, B. (1978) Quantitative measurement of prostaglandins and thromboxanes: general considerations. Adv. Prostaglandin Thromb. Res. 5, 1-13.
- Gray, D. S. (1989). Diagnosis and prevalence of obesity. Med. Clin. North Am. 73, 1-11.
- Green, H. and Meuth, M. (1974) An established preadipose cell-line and its differentiation in culture. Cell 3, 127-133.
- Green, H. and Kehinde, O. (1974) Sublines of mouse 3T3 cells that accumulate lipid. Cell 1, 113-116.
- Green, H. and Kehinde, O. (1975) An established adipose cell line and its differentiation in culture II. Factors affecting the adipose conversion. Cell 5, 19-27.
- Green, H. and Kehinde, O. (1976) Spontaneous heritable changes leading to increased adipose conversion in 3T3 cells. Cell 7, 105-113.
- Guller, S., Corin, R. E., Mynarcik, D. C., London, B. M. and Sonenberg, M. (1988) Role of insulin in growth hormone-stimulated 3T3 cell adipogenesis. Endocrinology 122, 2084-2089.
- Halaas, J. L., Gajiwala, K. S., Maffei, M., Cohen, S. L., Chait, B. T., Rabinowitz, D., Lollone, R. L., Burley, S. K. and Friedman, J. M. (1995) Weight-reducing effects of the plasma protein encoded by the obese gene. Science 269, 543-546.
- Hamberg, M., Svensson, J. and Samuelsson, B. (1974) Prostaglandin endoperoxides: a new concept concerning the mode of action and release of prostaglandins. Proc. Natl. Acad. Sci. 71, 3824-3828.
- Haunner, H., Wabitch, M. and Pfeiffer, E. F. (1988) Differentiation of adipocyte precursor cells from obese and non-obese adult women and from different adipose tissue sites. Horm. metab. Res. 19, 35-39.
- Hauner, H. (1990) Complete adipose differentiation of 3T3-L1 cells in a chemically defined medium: comparison to serum-containing culture conditions. Endocrinology 127, 865-872.

- Higgs, G. A. and Vane, J. R. (1983) Inhibition of cyclooxygenase and lipoxygenase. Br. Med. Bull. 39, 265-270.
- Hirsch, J. and Knittle, J. L. (1970) Cellularity of obese and non obese human adipose tissue. Federation Proc. 29, 1516-1521.
- Hoerl, B. J., Wier, M. L. and Scott, R. E. (1984) Biological mechanisms for the loss of the differentiated phenotype by non terminally differentiated adipocytes. Exp. Cell Res. 155, 422-434.
- Hopkins, N. K. and Gorman, R. R. (1981) Regulation of 3T3-L1 fibroblasts differentiation by prostacyclin (prostaglandin I<sub>2</sub>). Biochim. Biophys. Acta 663, 457-466.
- Hotamisligil, G. S., Shargil, N. S. and Spiegelman, B. M. (1993) Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science 259, 87-91.
- Hyman, B. T., Stoll, L. L. and Spector, A. A. (1982) Prostaglandin production by 3T3-L1 cells in culture. Biochim. Biophys. Acta. 713, 375-385.
- Ignatz, R. A. and Massague, J. (1985) Transforming growth factor controls the adipogenic differentiation of 3T3 fibroblasts. Proc. Natl. Acad. Sci USA 82, 8530-8534.
- Irvine, R. F. (1982) How is the level of free arachidonic acid controlled in mammalian cells? Biochem J. 204, 3-16.
- Kaestner, K. H., Christy, R. J. and Lane, M. D. (1990) Mouse insulin-responsive glucose transporter gene: characterization of the gene and trans-activation by the CCAAT/enhancer binding protein. Proc. Natl. Acad. Sci. USA 87, 251-255.
- Kikawa, Y., Narumiya, S., Fukushima, M., Wakatsuka, H. and Hayaishi, O. (1984) 9-deoxy- $\Delta^9$ ,  $\Delta^{12-13}$ , 14 dihydroprostaglandin D<sub>2</sub>, a metabolite of prostaglandin D<sub>2</sub> formed in human plasma. Proc. Natl. Acad. Sci. USA 81, 1317-1321.
- Kissebah, A.H., Peiris, A. and Evans, D. J. (1987) In: Recent Advances in Obesity Research Eds.: E.M. Berry, H. E. Blondheim and E. Shafir (London, U. K.) pp 54-59.
- Kliwer, S. A., Lenhard, J. M., Wilson, T. M., Patel, I., Morris, D. C. and Lehmann, J. M. (1995) A prostaglandin J<sub>2</sub> metabolite binds peroxisome proliferator-activated receptor  $\gamma$  and promotes adipocyte differentiation. Cell 83, 813-819.
- Knight, D. M., Chapman, A. B., Navre, M., Drinkwater, L., Bruno, J. J. and Ringold, G. M. (1987) Requirements for triggering of adipocyte differentiation by glucocorticoids and indomethacin. Mol. Endocrinology 1, 36-43.
- Konieczny, S. F. and Emerson, C. P. (1984) 5-Azacytadine induction of stable mesodermal stem cell lineages from 10T1/2 cells: evidence for regulatory genes controlling determination. Cell 38, 791-800.

- Kozak, L. P. and Jensen, J. T. (1974) Genetic and developmental control of multiple forms of L-glycerol-3-phosphate dehydrogenase. J. Biol. Chem. 249, 7775-7781.
- Krawisz, B. R. and Scott, R. E. (1982) Coupling of preadipocyte growth arrest and differentiation. I. by heparanized medium containing human plasma. J. Cell. Biol. 94, 394-399.
- Krotkiewski, M., Bjorntorp, P., Sjostrom, L. and Smith U. (1983) Impact of obesity on metabolism in men and women: importance of regional tissue distribution. J. Clin. Invest. 72, 1150-1162.
- <sup>a</sup>Lau, D. C. W., Roncari, D. A. K. and Hollenberg, C. H. (1987) Release of mitogenic factors by cultured preadipocytes from massively obese human subjects. J. Clin. Invest. 79, 632-636.
- <sup>b</sup>Lau, D. C. W., Wong, K. L., and Tough, S. C. (1987) Regional differences in the replication rate of cultured rat microvascular endothelium from retroperitoneal and epididymal fat pads. Clin. Exp. Metab. 36, 631-636.
- Lehmann, J. M., Lenhard, J. M., Oliver, B. B., Ringold, G. M. and Kliewer, S. A. (1997) Peroxisome proliferator-activated receptors  $\alpha$  and  $\gamma$  are activated by indomethacin and other non-steroidal anti-inflammatory drugs. J. Biol. Chem. 272, 3406-3410.
- Lewis, G. P. and Mathews, J. (1970) The mechanism of functional vasodilatation in rabbit epigastric adipose tissue. J. Physiol. 207, 15-30.
- Lewis, G. P. and Piper, P. J. (1978) Interaction of anti-inflammatory steroids with PG system in adipose tissue. Biochem. Pharmacol. 27, 1409-1412.
- Li, Z. H., Sun, M. H., and Lau, D. C. W. (1993) The inhibitory effect of EGF on preadipocyte differentiation is independent of PKC pathway. Clin. Invest. Med. 16(B), 244.
- Lu, Z., Pineyro, M. A., Kirkland, J. L., Li, Z. H. and Gregerman, R. I. (1988) Prostaglandin-sensitive adenylyl cyclase of cultured preadipocytes and mature adipocytes of the rat: Probable role of  $G_i$  in determination of stimulatory or inhibitory action. J. Cell Physiol. 136, 1-12.
- MacIntyre, D. E. (1985) In: Biochemistry of arachidonic acid metabolism. Ed.: W. E. M. Lands ( Martinus Nijhoff, Boston) pp 243-269.
- Markman, B. (1989) Anatomy and physiology of adipose tissue. Clin. Plast. Surg. 16, 235-244.
- McGehee, R. E., Ron, D., Braiser, A. R. and Habener, J. F. (1993) Differentiation-specific element: a cis-acting developmental switch required for the sustained transcriptional expression of the angiotensinogen gene during hormonal-induced differentiation of 3T3-L1 fibroblasts to adipocytes. Mol. Endocrinol. 7, 551-560.

- McGinnis, J. M. and Ballard-Barbash, R. M. (1991) Obesity in minority population: policy implications of research. Am. J. Clin. Nutr. 53, 1512s-1514s.
- Mitchell, M. D., Cleland, W. H., Smith, M. E., Simpson, E. R. and Mendelson, C. R. (1983) Inhibition of prostaglandin biosynthesis in human adipose tissue by glucocorticoids. J. Clin. Endocrinol. Metab. 57, 771-776.
- Moncada, S. and Vane, J. R. (1979) Pharmacology and endogenous roles of prostaglandin endoperoxides, thromboxane A<sub>2</sub>, and prostacyclin. Pharmacol. rev. 30, 293-331.
- Narumiya, S., Ohno, K., Fujiwara, M. and Fukushima, M. (1986) Site and mechanism of growth inhibition by prostaglandins. II. Temperature-dependent transfer of a cyclopentenone prostaglandin to nuclei. J. Pharmacol. Exp. Therapeut. 239, 506-511.
- Nechad, M. (1986) In: Brown adipose tissue. Ed.: P. Trayhurn and D. G. Nicolls (Arnold, London) pp 1-30.
- Negrel, R., Grimaldi, P. and Ailhaud, G. (1978) Establishment of preadipocyte clonal line from epididymal fat pad of ob/ob mouse that responds to insulin and lipolytic hormones. Proc. Natl. Acad. Sci. USA 75, 6054-6058.
- Negrel, R. and Ailhaud, G. (1981) Metabolism of arachidonic acid and prostaglandin synthesis in the preadipocyte clonal line OB 17. Biochem. Biophys. Res. Comm. 98, 768-777.
- Negrel, R., Gaillard, D. and Ailhaud, G. (1989) Prostacyclin as a potent effector of adipose cell differentiation. Biochem. J. 257, 399-405.
- Olefsky, J. M. (1995) Weighing in on the lean genes. J. Clin. Invest. 95, 2427-2428.
- Ono, M., Aratani, Y., Kitagawa, I. and Kitagawa, Y. (1990) Ascorbic acid phosphate stimulates Type IV collagen synthesis and accelerates adipose conversion of 3T3-L1 cells. Exp. Cell Res. 187, 309-314.
- Pace-Asciak, C. R. and Smith, W. L. (1983) In: The Enzymes. Ed.: P. D. Boyer (Academic Press, New York) pp 543-603.
- Pelleymounter, M. A., Cullen, M. J., Baker, M. B., Hecht, R., Winters, D., Boone, T. and Collins, F. (1995) Effects of the Ob gene products on body weight regulation in Ob/Ob mice. Science 269, 540-543.
- Petruschke, Th. and Hauner, H. (1993) Tumor necrosis factor- $\alpha$  prevents the differentiation of human adipocyte precursor cells and causes delipidation of newly developed fat cells. J. Clin. Endocrinol. Metab. 76, 742-747.
- Piper, P. J. and Vane, J. R. (1971) The release of prostaglandins from lung and other tissues. Ann. N. Y. Acad. Sci. 180, 363-385.
- Pykalisto, O. J., Smith, P. H., and Brunzell, J. D. (1975) Determinants of human adipose tissue lipoprotein lipase: effect of diabetes and obesity on basal- and diet-induced activity. J. Clin. Invest. 56, 1108-1117.

- Quinet, E. M., Huerta, P., Nancoo, D., Tall, A. R., Marcell, Y. L., and Mcpherson, R. (1993) Adipose tissue cholesteryl ester transfer mRNA in response to probucol: cholesterol and species dependent. J. Lipid Res. 34, 347-446.
- Richelsen, B. (1987) Factors regulating the production of PGE<sub>2</sub> and prostacyclin (PGI<sub>2</sub>) in rat and human adipocytes. Biochem. J. 247, 289-394.
- Richelsen, B. and Pedersen, S. B. (1987) Antilipolytic effect of prostaglandin E<sub>2</sub> in perfused rat adipocytes. Endocrinology 121, 1221-1226.
- Roche, A. F. (1981) The adipocyte number hypothesis. Child Dev. 52, 31-43.
- Rome, L. H., and Lands, W. E. M. (1975) Structural requirements for time-dependent inhibition of prostaglandins biosynthesis by anti-inflammatory drugs. Proc. Natl. Acad. Sci. USA. 72, 4863-4867.
- Ron, D., Brasier, A. R., McGehee, R. E. Jr. and Habener, J. F. (1992) Tumor necrosis factor-induced reversal of adipocyte phenotype of 3T3-L1 cells is preceded by a loss of nuclear CCAAT/enhancer binding protein (C/EBP). J. Clin. Invest. 89, 223-233.
- Roncari, D. A. K., Wang, H. and Desai, K. S. (1980) Cyclic AMP metabolism and lipolysis in cultured human adipocyte precursors. Can. J. Biochem. 58, 201-205.
- Roncari, D. A. K. (1984) Preadipose cell replication and differentiation. TIBS 9, 486-489.
- Rondinone, C. M., Rodbard, D. and Baker, M. E. (1993) Aldosterone stimulated differentiation of mouse 3T3-L1 cells into adipocytes. Endocrinology 132, 2421-2426.
- Samuelsson, B., Goldyne, M., Granstorm, E., Hamberg, M., Hammarstorm, S. and Maimsten, C. (1978) Prostaglandins and thromboxanes. Ann. Res. Biochem. 47, 997-1029.
- Samuelsson, B., Dahlen, S. E., Lindgren, J. A., Rouzer, C. A. and Serhan, C. H. (1987) Leukotrienes and lipoxins: structures, biosynthesis and biological effects. Science wash. DC 237, 1171-1176.
- Schmidt, W., Poll-Jordan, G., Loeffler, G. (1990) Adipose conversion of 3T3-L1 cells in a serum free culture system depends on epidermal growth factor I, corticosterone, and cAMP. J. Biol. Chem. 265, 15489-15495.
- Scott, R. E., Florine, D. L., Wille, J.J. and Yum, K. (1982) Coupling of growth arrest and differentiation at a distinct state in G<sub>1</sub> phase of the cell cycle: G<sub>D</sub>. Proc. Natl. Acad. Sci. USA 79, 845-849.
- <sup>a</sup>Serrero, G., Lepak, N. M. and Goodrich, S. P. (1992) Prostaglandin F<sub>2α</sub> inhibits the differentiation of adipocyte precursors in primary culture. Biochem. Biophys. Res. Commun. 183, 438-442.

- <sup>b</sup>Serrero, G., Lepak, N. M. and Goodrich, S. P. (1992) Paracrine regulation of adipose differentiation by arachidonate metabolites: Prostaglandin F<sub>2α</sub> inhibits early and late markers of differentiation in the adipogenic cell line 1246. Endocrinology 131, 2545-2551
- Shaw, J. E. and Ramwell, P. W. (1968) Release of prostaglandins from epididymal fat pad on nervous and hormonal stimulation. J. Biol. Chem. 243, 1498.
- Shillabeer, G., Forden, J. M. and Lau, D. C. W. (1989) Induction of preadipocyte differentiation by mature fat cells in the rat. J. Clin. Invest. 84, 381-387.
- Shillabeer, G., Forden, J. M., Russell, J. C. and Lau, D. C. W. (1990) Paradoxically slow preadipocyte replication and differentiation in corpulent rats. Am. J. Physiol. 258, E368-E376.
- Shillabeer, G., Li Z-H, Hatch, G., Kumar, V. and Lau D. C. W. (1996) A novel method for preadipocyte differentiation *in vitro*. Int. J. Obesity. 20 (suppl. 3): S77-S83.
- Smith, W. L. and Lands, W. E. M. (1971) Stimulation and blockade of prostaglandin biosynthesis. J. Biol. Chem. 246, 6700-6703.
- Smith, W. L. (1986) Prostaglandin synthesis and its compartmentation in vascular smooth muscle and endothelial cells. Ann. Rev. Physiol. 48, 251-262.
- Smith, W. L. (1989) The eicosanoids and their biochemical mechanisms of action. Biochem. J. 259, 315-324.
- Spector, T. (1978) Refinement of Coomassie Blue method of protein quantitation. Anal. Biochem. 86, 142-146.
- Stone, R. L. and Bernlohr, D. A. (1990) The molecular basis for inhibition of adipose conversion of murine 3T3-L1 cells by retinoic acid. Differentiation 45, 119-127.
- Student, A. K., Hsu, R. Y. and Lane, M. D. (1980) Induction of fatty acid synthetase synthesis in 3T3-L1 preadipocytes. J. Biol. Chem. 255, 4745-4750.
- Taylor, S. M. and Jones, P. A. (1979) Multiple new phenotypes included in 10T1/2 and 3T3 cells treated with 5-azacytadine. Cell 17, 771-779.
- Toscani, A., Soprano, D. R. and Soprano, K. J. (1990) Sodium butyrate in combination with insulin or dexamethasone can terminally differentiate actively proliferating swiss 3T3 cells into adipocytes. J. Biol. Chem. 265, 5722-5730.
- Tontonoz, P., Hu, E. and Spiegelman, B. (1994) Stimulation of adipogenesis in fibroblasts by PPAR $\gamma$ 2, a lipid-activated transcription factor. Cell 79, 1147-1156.
- Trayhurn, P. (1990) Energy expenditure and thermogenesis: animal studies on brown adipose tissue. Int. J. Obesity. 14, 17-29.

- Van, R. L. R. and Roncari, D. A. K. (1982) Complete differentiation *in vivo* of implanted cultured adipocyte precursors from adult rats. Cell Tiss. Res. 225, 557-566.
- Van den Bosch, H. (1980) Intracellular phospholipases A. Biochim. Biophys. Acta 604, 191-246.
- VanderOuderaa, F. J., Buytenhek, M., Nugteren, D. H. and Van Drop, D. A. (1980) Acetylation of prostaglandin endoperoxide synthetase with acetylsalicylic acid. Eur. J. Biochem. 109, 1-8.
- Varzaneh, E. F., Shillabeer, G., Wong, K. L. and Lau, D. C. W. (1994) Extracellular matrix components secreted by microvascular endothelial cells stimulate preadipocyte differentiation *in vitro*. Metabolism 43, 906-912.
- Vassaux, G., Gaillard, D., Ailhaud, G and Negrel, R. (1992) Prostacyclin is a specific effector of adipose cell differentiation. Its dual role as a cAMP and Ca elevating agent. J. Biol. Chem. 267, 11092-11097.
- Vassaux, G., Negrel, R., Ailhaud, G. and Gaillard, D. (1994) Proliferation and differentiation of rat adipose precursor cells in chemically defined medium: differential action of anti-adipogenic agents. J. Cell. Physiol. 16, 249-256.
- Verrando, P., Negrel, R., Grimaldi, P., Murphy, M. and Ailhaud, G (1980) Differentiation of Ob 17 preadipocytes to adipocytes. Triggereing effects of clofenapate and indomethacin. Biochim. Biophys. Acta 663, 255-265.
- Wang, H and Scott, R. E. (1993) Inhibition of distinct steps in the adipocyte differentiation pathway in 3T3 T mesenchymal stem cells by dimethyl sulphoxide (DMSO). Cell Prolif. 26, 55-66.
- Wier, M. L., and Scott, R. E. (1986) Regulation of the terminal event in cellular differentiation: biological mechanism of the loss of proliferative potential. J. Cell Biol. 102, 1955-1964.
- Wilkison, W. O., Choy, L. and Spiegelman, B. M. (1991) Biosynthetic regulation of monobutyryn, an adipocyte-secreted lipid with angiogenic activity. J. Biol. Chem. 264, 1811-1815.
- Williams, I. H. and Polakis, S. E. (1977) Differentiation of 3T3-L1 fibroblasts to adipocytes. The effect of indomethacin, prostaglandin E1 and cAMP on the process of differentiation. Biochem. Biophys. Res. Comm. 77, 175-186.
- Willis, A. L. and Stone, K. J. (1976) In: Handbook of Biochemistry and Molecular Biology. Eds.: G. D. Fasman (CRC Press, Cleveland) pp 312-423.
- Yu, K., Bayona, W., Kallen, C. B., Harding, H. P., Ravera, C. P., McMahon, G., Brown, M. and Lazar, M. A. (1995) Differential activation of peroxisome proliferator-activated receptors by eicosanoids. J. Biol. Chem. 270, 23975-23983.
- Zakar, T. and Olson, D. M. (1989) Dexamethasone stimulates arachidonic acid conversion to prostaglandin E<sub>2</sub> in human amnion cells. J. Dev. Physiol. 12, 269-272.

Zang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L. and Friedman, J. M. (1994) Positional cloning of the mouse obese gene and its human analogue. Nature 372, 425-432.

## CURRICULUM VITAE

**Name** Veena Kumar

**Address** 1557 Blohm Drive  
Ottawa, Ontario, Canada  
K1G 4R7

**Telephone** 613-736-1882

### Education

Sep.1994-May 1997 M. Sc., Biochemistry  
University of Ottawa  
Ottawa, Ontario, Canada

1991-1992 Completed supplementary courses for the program  
in Dietetics (accredited by American Dietetic  
Association). University of Rhode Island, RI, USA.

1978-1981 M.Sc. (Foods and Nutrition)  
Minor, Biochemistry  
Haryana Agricultural University  
Hisar, Haryana, India

1973-1977 B.Sc.  
Haryana Agricultural university  
Hisar, Haryana, India

### Abstracts/Presentations

1. **Kumar V, G** Shillabeer and D. C. W. Lau. 1995. Indomethacin augments differentiation of preadipocytes induced by adipocyte-derived conditioned medium. Presented as a poster at the 64th annual meeting of The Royal College of Physicians and Surgeons of Canada in Montreal Sep. 13-17, 1995.
2. **Kumar V, G**, Shillabeer and D. C. W. Lau. 1996 The paracrine role of prostaglandins on preadipocyte development. Presented as a poster at the 65th annual meeting of The Royal College of Physicians and Surgeons of Canada in Halifax: Sep. 25-29, 1996.
3. **Kumar V, G** Shillabeer and D. C. W. Lau. 1996. The paracrine/autocrine role of prostaglandins in preadipocyte differentiation. Paper presented in meeting of National Association of Studies in Obesity. Oct. 12-15, 1996.

4. **Kumar V**, E Tibbo, D.C.W. Lau and G Shillabeer. 1997. Products of the lipoxygenase pathway may promote adipogenesis. Paper presented in 66th annual meeting of The Royal College of Physicians and Surgeons of Canada. Sep 25-29, 1997.

### **Publications**

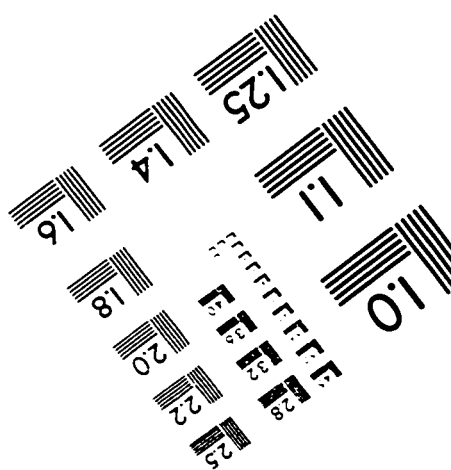
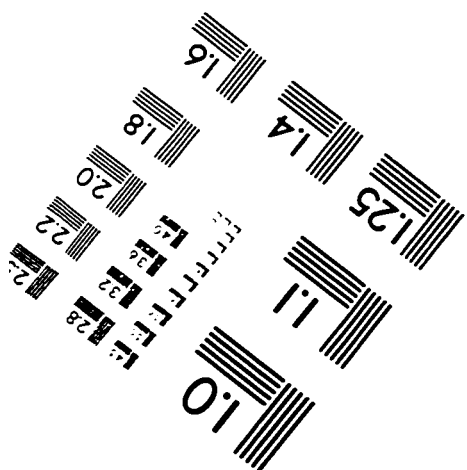
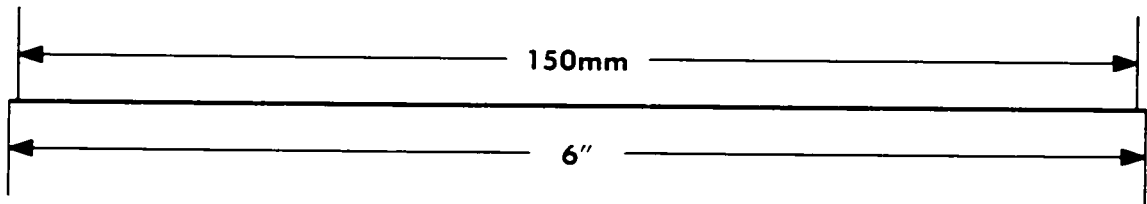
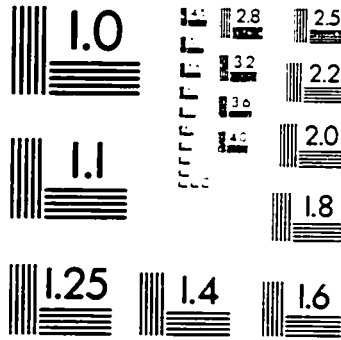
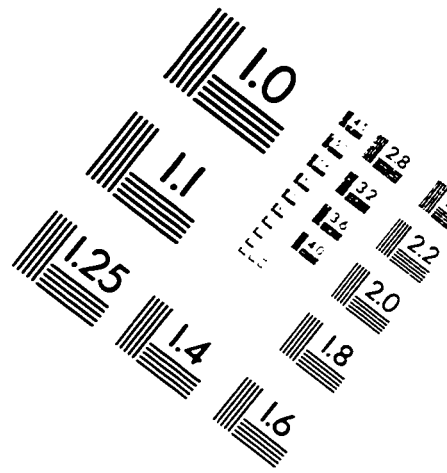
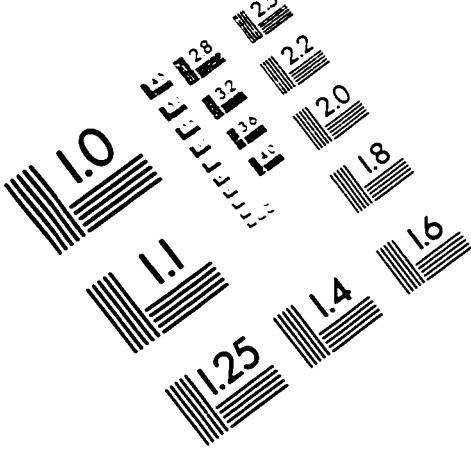
1. Shillabeer G, Li Z-H, Hatch G, **Kumar V** and Lau D. C. W. 1996. A novel method for preadipocyte differentiation *in vitro*. Int. J. Obesity. 20 (suppl. 3): S77-S83.

2. Shillabeer, G, **Kumar V**, Tibbo, E. and D. C. W. Lau. 1997. Arachidonic acid metabolites of the lipoxygenase as well as the cyclooxygenase pathway may be involved in regulating preadipocyte differentiation. (Metabolism, in press).

3. **Kumar V** and A. C. Kapoor. 1983. Availability of Zinc as affected by phosphates. Nutrition Reports International. 28:1.

4. **Kumar V** and A. C. Kapoor. 1984. Trace mineral composition of different varieties of cereals and pulses. Ind. J. Nutr. Dietet. 21:137.

# TEST TARGET (QA-3)



**APPLIED IMAGE . Inc**  
1653 East Main Street  
Rochester, NY 14609 USA  
Phone: 716/482-0300  
Fax: 716/288-5989

© 1993, Applied Image, Inc., All Rights Reserved