INFORMATION TO USERS

While the most advanced technology has been used to photograph and reproduce this manuscript, the quality of the reproduction is heavily dependent upon the quality of the material submitted. For example:

- Manuscript pages may have indistinct print. In such cases, the best available copy has been filmed.

- Manuscripts may not always be complete. In such cases, a note will indicate that it is not possible to obtain missing pages.

- Copyrighted material may have been removed from the manuscript. In such cases, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, and charts) are photographed by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each oversize page is also filmed as one exposure and is available, for an additional charge, as a standard 35mm slide or as a 17"x 23" black and white photographic print.

Most photographs reproduce acceptably on positive microfilm or microfiche but lack the clarity on xerographic copies made from the microfilm. For an additional charge, 35mm slides of 6"x 9" black and white photographic prints are available for any photographs or illustrations that cannot be reproduced satisfactorily by xerography.
Prostanoid and arachidonic acid metabolism in cultured cells. Studies with cyclosporine A, bacterial lipopolysaccharide and human low density lipoproteins

Zhang, Hanfang, Ph.D.
The Ohio State University, 1987
PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy. Problems encountered with this document have been identified here with a check mark ✓.

1. Glossy photographs or pages ✓
2. Colored illustrations, paper or print
3. Photographs with dark background ✓
4. Illustrations are poor copy
5. Pages with black marks, not original copy
6. Print shows through as there is text on both sides of page
7. Indistinct, broken or small print on several pages ✓
8. Print exceeds margin requirements
9. Tightly bound copy with print lost in spine
10. Computer printout pages with indistinct print
11. Page(s) lacking when material received, and not available from school or author.
12. Page(s) seem to be missing in numbering only as text follows.
13. Two pages numbered. Text follows.
14. Curling and wrinkled pages
15. Dissertation contains pages with print at a slant, filmed as received ✓
16. Other

__________________________________________________________

__________________________________________________________

University Microfilms International
PROSTANOID AND ARACHIDONIC ACID METABOLISM IN CULTURED CELLS.
STUDIES WITH CYCLOSPORINE A, BACTERIAL LIPOPOLYSACCHARIDE AND HUMAN
LOW DENSITY LIPOPROTEINS

DISSERTATION

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

By

Hanfang Zhang, Diploma (M.D. equivalent), M.S.

* * * * *

The Ohio State University
1987

Dissertation Committee:
Dr. David G. Cornwell
Dr. Gerald P. Brierley
Dr. J. David Johnson
Dr. Rao V. Panganamala

Approved by

Advisor
Department of Physiological Chemistry
ACKNOWLEDGMENTS

I express my sincere appreciation to Dr. David G. Cornwell for his encouragement, guidance and insight throughout this research. I thank Drs. Gerald P. Brierly, J. David Johnson and Rao V. Panganamala for their participation and thoughtful suggestions as the members of the reading and examination committee. I am particularly grateful to Drs. W. Bruce Davis, Kenneth H. Jones, Arthur Struch, and Ronald Whisler for their ideas, techniques and generosity with resources. Mrs. Ruth Thinguldstad provided able secretarial assistance.
VITA

November 19, 1952

Born - Huangpi, Hubei Province, The People's Republic of China

1974-1977

Diploma (M.D. equivalent), Tongji Medical University, Wuhan, Hubei Province, The People's Republic of China

1977-1979

Special Training Class, Tongji Medical University, Wuhan, Hubei Province, The People's Republic of China

1979-1982

Assistant (Instructor equivalent), Department of Biochemistry, Tongji Medical University, Wuhan, Hubei Province, The People's Republic of China

1982-1984

M.S., Department of Physiological Chemistry, The Ohio State University, Columbus, Ohio, The United States of America

1984-1985

Teaching Assistant, Department of Physiological Chemistry, The Ohio State University, Columbus, Ohio, The United States of America

1985-1987

Research Assistant, Department of Physiological Chemistry, The Ohio State University, Columbus, Ohio, The United States of America


TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>11</td>
</tr>
<tr>
<td>VITA</td>
<td>111</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>x</td>
</tr>
<tr>
<td>LIST OF PLATES</td>
<td>xii</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xiii</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>REVIEW OF LITERATURE</td>
<td>3</td>
</tr>
<tr>
<td><strong>BIOSYNTHESIS OF PROSTAGLANDINS AND ARACHIDONIC ACID METABOLISM</strong></td>
<td>3</td>
</tr>
<tr>
<td>Biologically Active Lipids and Arachidonic Acid</td>
<td>3</td>
</tr>
<tr>
<td>Polyunsaturated Fatty Acids and Prostaglandins</td>
<td>4</td>
</tr>
<tr>
<td>Phospholipase Activity, Arachidonic Acid</td>
<td>7</td>
</tr>
<tr>
<td>Release and Prostaglandin Synthesis</td>
<td>11</td>
</tr>
<tr>
<td>Prostaglandin Endoperoxide Synthase Activity</td>
<td>11</td>
</tr>
<tr>
<td>in Prostaglandin Synthesis</td>
<td>15</td>
</tr>
<tr>
<td>Peroxidation of Arachidonic Acid</td>
<td></td>
</tr>
<tr>
<td><strong>EFFECTS OF CYCLOSPORINE A ON MONOCYTE/MACROPHAGE PROSTAGLANDIN SYNTHESIS IN IMMUNITY</strong></td>
<td>19</td>
</tr>
<tr>
<td>Polyunsaturated Fatty Acids and Cell-Mediated Immunity</td>
<td>19</td>
</tr>
<tr>
<td>Prostaglandin E and MØ in Cell-Mediated Immunity</td>
<td>20</td>
</tr>
<tr>
<td>Cyclosporine A and MØ in Immunity</td>
<td>24</td>
</tr>
<tr>
<td>Mechanism of Cyclosporine A Effects on Arachidonic Acid Metabolism</td>
<td>25</td>
</tr>
<tr>
<td><strong>EFFECTS OF BACTERIAL LIPOPOLYSACCHARIDE ON PROSTAGLANDIN SYNTHESIS IN ENDOTOXIC SHOCK</strong></td>
<td>26</td>
</tr>
<tr>
<td>Enhanced Prostaglandins in Endotoxic Shock</td>
<td>26</td>
</tr>
<tr>
<td>Topic</td>
<td>Page</td>
</tr>
<tr>
<td>----------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Uptake of $[^{14}C]$-AA by SMC in Culture</td>
<td>64</td>
</tr>
<tr>
<td>Separation of Phospholipids by TLC</td>
<td>67</td>
</tr>
<tr>
<td>Distribution of $[^{14}C]$-AA in Major Lipids of SMC</td>
<td>67</td>
</tr>
<tr>
<td>Release of $[^{14}C]$-AA Metabolites from Labeled SMC with CsA, LPS and A23187</td>
<td>67</td>
</tr>
<tr>
<td>Identification of $[^{14}C]$-Metabolites by HPLC</td>
<td>73</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>85</td>
</tr>
<tr>
<td>EFFECTS OF OXIDIZED-LDL ON CELL VIABILITY AND PROSTANOID METABOLISM IN SMC</td>
<td>92</td>
</tr>
<tr>
<td>RESULTS</td>
<td>92</td>
</tr>
<tr>
<td>Preparation of LDL Solutions</td>
<td>92</td>
</tr>
<tr>
<td>Immunoreactive Products of LDL Preparations</td>
<td>95</td>
</tr>
<tr>
<td>Effects of LDL on Morphology and Viability of SMC</td>
<td>97</td>
</tr>
<tr>
<td>Effects of BHT-LDL on Prostanoid Synthesis in SMC</td>
<td>105</td>
</tr>
<tr>
<td>Effects of Oxidized-LDL on Prostanoid Synthesis in SMC</td>
<td>105</td>
</tr>
<tr>
<td>Release of $[^{14}C]$-AA Metabolites from Labeled SMC with Oxidized-LDL</td>
<td>112</td>
</tr>
<tr>
<td>Identification of $[^{14}C]$-Metabolites by HPLC</td>
<td>112</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>121</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>127</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Effect of CsA or LPS on prostanoid synthesis in SMC</td>
<td>59</td>
</tr>
<tr>
<td>2. Effect of CsA or LPS on prostanoid synthesis in MØ and inhibitory effects with dexamethasone</td>
<td>60</td>
</tr>
<tr>
<td>3. Effect of exogenous AA on the stimulation of prostanoid synthesis in SMC with CsA or LPS</td>
<td>62</td>
</tr>
<tr>
<td>4. Effect of exogenous AA on the stimulation of PGE₂ synthesis in MØ with CsA or LPS</td>
<td>63</td>
</tr>
<tr>
<td>5. Effect of pre-incubation with CsA or A23187 on the Synthesis of 6-Keto-PGF₁α in SMC stimulated with LPS or TPA</td>
<td>65</td>
</tr>
<tr>
<td>6. Distribution of [¹⁴C]-AA in SMC lipids</td>
<td>72</td>
</tr>
<tr>
<td>7. Radioactivity recovered from media when SMC were labeled with [U-¹⁴C]-AA and then incubated with IM, CsA, LPS or A23187</td>
<td>74</td>
</tr>
<tr>
<td>8. Radioactivity recovered from media when SMC were labeled with [¹-¹⁴C]-AA and then incubated with IM, CsA, LPS or A23187</td>
<td>75</td>
</tr>
<tr>
<td>9. The ratio of 6-keto-PGF₁α to PGE₂ in SMC with CsA, A₂₃₁₈₇ or LPS</td>
<td>79</td>
</tr>
<tr>
<td>10. The Summary of data with CsA, A₂₃₁₈₇ or LPS</td>
<td>87</td>
</tr>
<tr>
<td>11. TBAR material in oxidized-LDL and BHT-LDL preparations</td>
<td>93</td>
</tr>
<tr>
<td>12. Prostanoid cross-reactivities with antibodies in LDL preparations with different levels of TBAR material</td>
<td>96</td>
</tr>
</tbody>
</table>
13. Effect of BHT-LDL and IM on prostanoid synthesis in SMC................................................................. 106

14. Radioactivity recovered from media when SMC were labeled with [U-14C]-AA and then incubated with oxidized-LDL preparations containing different TBAR material content................................................. 113
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. The metabolic pathways of n-6 and n-3 fatty acids...</td>
<td>6</td>
</tr>
<tr>
<td>2. Arachidonic acid cascade.................................</td>
<td>12</td>
</tr>
<tr>
<td>3. Autoxidation leading to cyclic peroxide compounds...</td>
<td>17</td>
</tr>
<tr>
<td>4. Scheme for possible modulation of cell-mediated immune response by PGE2..........................</td>
<td>23</td>
</tr>
<tr>
<td>5. Time course for the uptake of AA by SMC challenged with 0.625 μM [14C]-AA.................................</td>
<td>66</td>
</tr>
<tr>
<td>6. TLC of major phospholipids...............................</td>
<td>68</td>
</tr>
<tr>
<td>7. The separation of cellular lipids by TLC.............</td>
<td>70</td>
</tr>
<tr>
<td>8. The separation of standard prostanoids and AA by HPLC.....................................................</td>
<td>76</td>
</tr>
<tr>
<td>9. HPLC analysis of prostanoids and free AA released from SMC by CsA or A23187..........................</td>
<td>77</td>
</tr>
<tr>
<td>10. HPLC analysis of labeled prostanoids and free AA released from SMC by LPS...............................</td>
<td>80</td>
</tr>
<tr>
<td>11. HPLC analysis of labeled prostanoids and free AA released from SMC by CsA in the presence or in the absence of IM........................................</td>
<td>81</td>
</tr>
<tr>
<td>12. HPLC analysis of labeled prostanoids and free AA released from SMC by A23187 or LPS in the presence or in the absence of IM.............................</td>
<td>82</td>
</tr>
<tr>
<td>13. Schematic diagram showing the points where CsA and LPS affect prostanoid synthesis..................</td>
<td>90</td>
</tr>
<tr>
<td>14. LDL from individual sera is oxidized in the absence of BHT at different rates.......................</td>
<td>94</td>
</tr>
</tbody>
</table>
15. Time course of production of PGE₂ immunoactivity in oxidized-LDL preparations ................................................. 98
16. Prostanoid cross-reactivity in LDL preparations .................. 100
17. Effect of oxidized-LDL on morphology and viability of SMC ................................................................. 102
18. Effect of oxidized-LDL with the lowest content of TBAR material on PGE₂ synthesis in SMC ......................... 107
19. Effect of oxidized-LDL with the intermediate content of TBAR material on PGE₂ synthesis in SMC ................. 108
20. Effect of oxidized-LDL with the highest content of TBAR material on PGE₂ synthesis in SMC ................................................. 109
21. Effect of oxidized-LDL with different TBAR content on 6-Keto-PGF₁α synthesis in SMC ................................................. 111
22. HPLC Analysis of labeled prostanoids and free AA released from SMC ................................................................. 114
23. HPLC analysis of labeled prostanoids and free AA released from SMC by oxidized-LDL that had an intermediate TBAR material content ................................................. 117
24. HPLC analysis of labeled prostanoids and free AA released from SMC with 800 μg/ml cholesterol LDL containing different TBAR material content ................................................. 119
<table>
<thead>
<tr>
<th>PLATE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td>56</td>
</tr>
</tbody>
</table>

Reactivity of confluent SMC to fluorescent antibodies prepared against human umbilical artery F-actin.
LIST OF ABBREVIATIONS

AA  Arachidonic acid
ACAT Acyl CoA: cholesterol acyltransferase
BHT Butylated hydroxytoluene
CsA Cyclosporine A
DDA Dideoxy adenosine
DM Dexamethasone
EC Endothelial cell
EDGF Endothelial cell-derived growth factor
EDTA Ethylenediamine tetraacetic acid
EFA Essential fatty acid
FBS Fetal bovine serum
FDA-PI Fluorescein diacetate-propidium iodide
GC/MS Gas-liquid chromatograph/mass spectrometer
HDL High density lipoprotein
HHT Hydroxy-heptadecatrienoic acid
15-HPETE 15-Hydroperoxy-eicosatetraenoic acid
HPLC High performance liquid chromatography
IL-1(2) Interleukin-1 (2)
LDL Low density lipoprotein
LPC Lysophosphatidylcholine

xiii
LPS  Lipopolysaccharide
MDA  Malondialdehyde
MDGF Macrophage-derived growth factor
MHC  Major histocompatibility complex
M-LDL Modified-LDL
MØ  Monocyte/macrophage
NCEH Neutral cytosolic cholesteryl ester hydrolase
NL  Neutral lipid
NSAI Non-steroid anti-inflammatory
PA  Phosphatidic acid
PBMC Peripheral blood mononuclear cell
PC  Phosphatidylcholine
PDGF Platelet-derived growth factor
PE  Phosphatidylethanolamine
PG  Prostaglandin
PHA Phytohemagglutinin
PI  Phosphatidylinositol
PMNL Polymorphonuclear leukocyte
PS  Phosphatidylserine
PUFA Polyunsaturated fatty acid
RIA Radioimmunoassay
SMC Smooth Muscle cell
Sph Sphingomyelin
SOD Superoxide dismutase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBAR</td>
<td>Thiobarbituric acid-reacting</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TNS</td>
<td>6-p-toluidino-2-naphthalenesulfonic acid</td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-tetradecanoylphorbole-13-acetate</td>
</tr>
<tr>
<td>TXA₂</td>
<td>Thromboxane A₂</td>
</tr>
<tr>
<td>(β-)VLDL</td>
<td>(β-) Very low density lipoprotein</td>
</tr>
</tbody>
</table>
INTRODUCTION

Prostanoid derivatives of arachidonic acid (AA) modulate a number of physiological and pathophysiological processes involved in inflammation and repair such as cell-mediated immunity, endotoxic shock and atherosclerosis. The accessory cell function of the monocyte/macrophage (MØ) in the immune system involves the expression of I$_a$ antigens and the human "I$_a$-like" HLA-DR antigens. Cellular immunity is modulated by cyclosporine A (CsA) and prostaglandin E$_2$ (PGE$_2$), and it is interesting that CsA stimulates prostanoid synthesis in both MØ and somatic cells. These correlations suggest that prostanoids mediate the suppression of MØ function by CsA. Dramatically enhanced prostanoid synthesis is involved in the pathophysiological process in endotoxic shock, and agents in this process such as lipopolysaccharides (LPS) enhance prostanoid synthesis in MØ from many animal sources. Atherosclerosis is a disease characterized by MØ accumulation, smooth muscle cell (SMC) proliferation, lipid accumulation and connective tissue deposition. Prostanoids may affect growth factors which are responsible for SMC proliferation. Prostanoids also influence lipid metabolism in aortic cells. A number of studies have shown that lipoproteins such as high density lipoprotein (HDL) and low density lipoprotein (LDL) have both positive and negative effects on prostanoid synthesis and these lipoproteins are important agents in the
pathogenesis of atherosclerosis. Thus the regulation of prostanoid synthesis by CsA, LPS and lipoproteins may be important in understanding CsA effects on the immune response, LPS in the pathophysiology of endotoxic shock and lipoproteins in the development of atherosclerosis.

Modulating agents act at several points in the AA metabolic cascade which leads to prostaglandin synthesis. Agents such as zymosan and A23187 promote the release of AA from phospholipids. The preliminary observation that CsA has little effect in the presence of exogenous AA suggests that this agent also acts on AA release. Lipid X, a biologically active moiety of LPS, releases AA. LPS itself enhances prostanoid synthesis in the presence of exogenous AA suggesting that LPS acts at a different point from lipid X in the AA cascade by stimulating prostaglandin synthase. Lipid hydroperoxides both activate (low concentrations) and inhibit (high concentrations) cyclooxygenase. LDL contains esterified AA that is readily susceptible to lipid peroxidation. LDL with different levels of lipid peroxidation may have either positive or negative effects on prostanoid synthesis.

CsA, LPS and LDL have not been studied in sufficient detail to establish the exact points at which they affect the AA cascade. Mechanisms for the effects of CsA, LPS and LDL on prostanoid synthesis are the subject of this dissertation. These studies are restricted to two prostanoids, PGE2 and PGI2, and two cells, the human Mø and the SMC from guinea pig aorta. These studies are designed to approach the fundamental role of prostanoids in inflammation and repair.
Biologically Active Lipids and Arachidonic Acid

A finding that a strip of human uterus could either contract or relax in diluted fresh human seminal fluid was reported in 1930 by the gynecologists Kurzrok and Lieb (1930). A few years later, Goldblatt (1933, 1935) in England and U.S. von Euler (1934, 1935a) in Sweden independently investigated the effects of human seminal plasma on the stimulation of smooth muscle. Euler (1936) found that the active substance from semen was dialysable and soluble in water, alcohol, acetone, ether and chloroform. The compound was liable at high temperatures, more so in alkali than in acid, and the active material which was soluble in lipid solvents when it was in the acid state had acidic properties. Euler (1935b, 1936) announced the discovery of a biologically active fatty acid which he named "prostaglandin" (PG).

Addition of permanganate to the active compound showed that complete inactivation occurred suggesting that the active compound was unsaturated (Euler, 1939). Bergström (1949) later found that the biologically active material appeared to be associated with unsaturated hydroxy acids. Two pure prostaglandins - PGE₁ and PGF₁α - were isolated in crystalline form by Bergström, Krabisch, and Sjövall (1960).
and Bergström and Sjövall (1957, 1960a, 1960b). The structures of the primary prostaglandins (\(E_1\), \(E_2\), \(E_3\), \(F_1\alpha\), \(F_2\alpha\) and \(F_3\alpha\)) were quickly elucidated (Bergström, Dressler, et al., 1962a, 1962b; Bergström, Ryhage, et al., 1962, 1963; Nugteren, Dorp, et al., 1966; Samuelsson, 1963a, 1963b, 1963c, 1964; Hamberg and Samuelsson, 1965, 1966). The locations of cis-double bounds in prostaglandins suggested that prostaglandins might have something to do with the essential unsaturated fatty acids. Dorp et al. (1964a) and Bergström, Danielsson and Samuelsson (1964) simultaneously reported that the incubation of \(^3H\)-labelled arachidonic acid (AA) with homogenized sheep glands produced a mixture of \(E_2\) and \(F_2\alpha\) prostaglandins.

Methods for the identification of the structure of prostaglandins contributed to the study of the occurrence of prostaglandins in animal tissues. Prostaglandins have been demonstrated in a number of different tissues, such as seminal plasma, lung, brain, kidney and iris (reviewed by Euler and Eliasson, 1967; Bergström, Carlson and Weeks, 1968).

**Polyunsaturated Fatty Acids and Prostaglandins**

Prostaglandins are cyclopentanoic acids which are derived from all-cis-\(C_{20}\) polyunsaturated fatty acids (PUFA). The conversion of AA into \(\text{PGE}_2\) and \(\text{PGF}_{2\alpha}\), demonstrated by Dorp et al. and Bergström et al., was the first true insight into a function for the essential fatty acids. These investigators studied the enzymatic conversions of all-cis-\(C_{20}\) PUFA into primary prostaglandins (Dorp et al., 1964b;
Bergström, Danielsson, Klenberg, et al., 1964). They showed that the PG₂ family was obtained from AA and that two other families, PG₁ and PG₃ were obtained from all-cis - 8,11,14 eicosa - trienoic acid (r-homo-linolenic acid) and all-cis 5,8,11,14,17 eicosa pentaenoic acid (EPA), respectively.

AA [20:4 (n-6)]* and linoleic acid [18:2 (n-6)], the n-6 family of fatty acids, are designated "essential fatty acids" (EFA) (Burr and Burr, 1929, 1930) since they can not be synthesized de novo in animals. EPA [20:5 (n-3)] and linolenic acid [18:3 (n-3)] are members of the n-3 family of fatty acids. The n-6 family of fatty acids is mainly obtained from plants, seeds and leaves. Linolenic acid is found in some seeds, but the n-3 family of fatty acids is mainly obtained from the phytoplankton — fish oil chain.

When n-6 and n-3 fatty acids are taken up by cells, they can undergo three metabolic reactions. These reactions are: 1) desaturation; 2) chain elongation; and 3) retroconversion (Sprecher, 1982; E.L. Smith et al., 1983). Examples of the first 2 reactions are shown in biosynthetic pathways outlined in Fig. 1. AA accumulates in most animal cells leading to the biosynthesis of PG₂ family in preference to the PG₁ family. The PG₃ family is synthesized in animals supplied in a diet containing marine fish oils.

*Fatty acids are designated by the number of carbon atoms; number of double bonds, and the position of the first double bond from the methyl terminus of the acyl chain is noted in parentheses; 18:2 (n-6), 9,12-octadecadienoic acid; 18:3 (n-3), 9,12,15-octadecatrienoic acid; 20:3 (n-6), 8,11,14-eicosatrienoic acid; 20:4 (n-6), 5,8,11,14-eicosatetraenoic acid; 20:5 (n-3), 5,8,11,14,17-eicosapentaenoic acid. Fatty acid families are designated by the position of the first double bond from the methyl terminus.
Fig. 1. The metabolic pathways of n-6 and n-3 fatty acids. Modified from Sprecher (1982) and E.L. Smith et al. (1983).
Phospholipase Activity, Arachidonic Acid Release and Prostaglandin Synthesis

The release of AA from the phospholipids of cell membranes is important in prostaglandin biosynthesis. Derksen and Cohen (1975) reported that AA amounted to 32 percent of the total fatty acids in human platelet membranes and 99 percent of the AA was esterified to phospholipids. When labeled AA was incubated with a variety of cultured cells or tissue slices, the distribution of labeled material was found predominantly in phospholipids (Flower and Blackwell, 1975; Levine and Alam, 1982; Spector et al., 1983). In tissue perfusion experiments over 90 percent of labeled AA was also recovered in phospholipids (Sickle et al., 1986; Hsueh et al., 1977). AA was selectively incorporated into phospholipids (Isakson, Raz, et al., 1976). However, Lands and Samuelsson (1968) and Vonkeman and Dorp (1968) demonstrated that prostaglandins were not formed from the fatty acids which were still attached to the glycerol moiety of phospholipids. Only free AA was the precursor for prostaglandin synthesis.

Prostaglandins are not stored in cells. Their release is preceded by synthesis (Vogt, 1978). Tissue perfusion with phospholipase A2 enhanced prostaglandin release (Vogt et al., 1969; Bartels et al., 1970), and the secretion of prostaglandins and other related products was accompanied by the loss of AA from phospholipids in a variety of cells and tissues (Flower and Blackwell, 1975; Isakson, Raz, Hsueh, et al., 1978; Bills et al., 1977; Blackwell, Duncombe, et al., 1977; Hong and Levine, 1976; Flower, 1981). These data showed
that the first regulatory step in prostaglandin biosynthesis was the release of AA from phospholipids by phospholipase A$_2$.

The activity of phospholipase A$_2$ can be regulated both by specific non-enzymatic proteins and by the availability of Ca$^{++}$ ions. Following the discovery that nonsteroid anti-inflammatory agents such as aspirin and indomethacin (see next Section) inhibited prostaglandin synthesis (Vane, 1971), it was postulated that all anti-inflammatory compounds could exert their effect by suppression of prostaglandin synthesis. Surprisingly, it was found that anti-inflammatory steroids did not affect the synthesis of prostaglandins in tissue homogenates or partially purified enzymes. The inhibitory effect of steroids on the biosynthesis of prostaglandins could only be demonstrated in whole animals, organs or tissues (reviewed by Salmon, 1981). Since the inhibitory effects of steroids but not indomethacin were abolished by exogenous AA, Gryglewski, Panczenko, et al. (1975) concluded that steroids inhibited the release of AA. This conclusion was also supported by other studies in different systems (Kantrowitz et al., 1975; Tashjian et al., 1975; Hong and Levine, 1976; Tam et al., 1976). A number of experiments have demonstrated that steroids are taken up by the cells, bind to a cytoplasmic receptor and induce the synthesis of a protein through the induction of RNA. This induced protein inhibits phospholipase A$_2$ by combining with the active enzyme to form an inactive complex (Flower and Blackwell, 1979; Danon and Assouline, 1978; Russo-Marie et al., 1979; DiRosa and Persico, 1979; Hirata et al., 1980; Tsurufuji et al., 1979). The steroid-induced inhibitory protein has been purified and called "lipomodulin" or

Calcium is required for the activity of phospholipase A₂. Kunze et al., 1974; Frei and Zahler, 1979; Garcia et al., 1975) showed that the addition of Ca²⁺ enhanced the release of labeled fatty acids in the assay of phospholipase A₂ activity when bovine seminal vesical homogenate was incubated with 1-acyl-2-[1-¹⁴C] linoleoyl-sn-glycero-3-phosphoryl ethanolamine. Ethylenediamine tetracetic acid (EDTA) inhibited phospholipase A₂ activity. Similar results were also obtained with human platelet membrane phospholipase A₂ in the presence of Ca²⁺ or EDTA (Derksen and Cohen, 1975).

The Ca²⁺-ionophore A₂³₁₈⁷ facilitates Ca²⁺ movement across membranes or mobilizes the intracellular store of Ca²⁺ and therefore, A₂³₁₈⁷ increases the intracellular level of Ca²⁺. This effect in turn enhances the release of AA by the activation of phospholipase A₂ (Rittenhouse-Simmons and Deykin, 1978; Pickett et al., 1977; Feinman and Detwiler 1974). The sequence, A₂³₁₈⁷ — high intracellular level of Ca²⁺ — phospholipase A₂ activity — release of AA, is demonstrated by a number of studies. First, the A₂³₁₈⁷-induced release of AA and stimulation of prostaglandin synthesis are Ca²⁺-dependent (Förstermann and Hertting, 1979; Rittenhouse-Simmons and Deykin, 1978) although it should be noted that other investigators have reported Ca²⁺-independent AA release by A₂³₁₈⁷ (Pickett et al., 1977). The addition of Ca²⁺ alone did not enhance labeled AA release from phospholipids in human platelets, but the addition of both A₂³₁₈⁷ and Ca²⁺ had a synergistic effect on AA release. The A₂³₁₈⁷ effect was abolished when Ca²⁺ was
replaced by Mg$^{++}$ (Rittenhouse-Simmons and Deykin, 1978). Secondly the phospholipase A$_2$ activation induced by A$_{23187}$ is inhibited by a phospholipase A$_2$ inhibitor. The released AA follows either the cyclooxygenase pathway leading to the synthesis of prostaglandins or the lipoxygenase pathway (see next Section). The production of reactive oxygen species from AA, probably from the lipoxygenase pathway, was monitored by chemiluminescence in rat peritoneal MØ challenged with A$_{23187}$ (Lim et al., 1983). The chemiluminescence induced by A$_{23187}$ was diminished either by the phospholipase inhibitor bromophenacyl bromide or by the antioxidant nordihydroguaiaretic acid (NDGA). The cyclooxygenase inhibitor, indomethacin, had no effect on chemiluminescence suggesting that reactive oxygen compounds in the lipoxygenase pathway originated from AA released by phospholipase A$_2$ which was activated by A$_{23187}$.

Phospholipase A$_2$ is important in the regulation of AA release, and alternatively, AA is also released in platelets by the combined action of phospholipase C and a diglyceride lipase (Lagarde et al., 1981; Chap, et al., 1979). Phospholipase C catalyzes the formation of diacylglycerol from phosphatidylinostol (PI). PI contains stearic and arachidonic acids esterified to the first and second carbon atoms of glycerol, respectively (Rittenhouse-Simmons, 1979; Bell et al., 1980). This diacylglycerol can be subsequently cleaved by diglyceride lipase to release AA (Bell et al., 1979, 1980; Lagarde et al., 1981; Prescott and Majerus, 1983). Alternatively the diacylglycerol can be used by a kinase to form phosphatidic acid which may activate phospholipase A$_2$. (Lapetina and Cautrecasas, 1979; Billah et al., 1979).
Prostaglandin Endoperoxide Synthase Activity in Prostaglandin Synthesis

The arachidonic acid cascade describes the several pathways by which this fatty acid is enzymatically converted into different biologically active products (Fig. 2). These biosynthetic sequences can be classified according to the type of enzyme catalyzing the primary oxygen addition reaction. One class initiated by prostaglandin endoperoxide synthase leads through bisdioxgenation to the endoperoxides, PGG\(_2\) and PGH\(_2\). The other class comprises a group of lipoxygenases that add a molecule of oxygen to various positions on AA forming hydroperoxides, one of which can be transformed into leukotrienes (LTs). Only endoperoxide synthase will be discussed in this review.

Prostaglandin endoperoxide synthase, also called cyclooxygenase or prostaglandin H synthase, adds a molecule of dissolved oxygen into AA to produce an endoperoxide bridge between C\(_9\) and C\(_{11}\). Subsequent stereospecific rearrangement leads to a bicyclic structure with a bond between C\(_9\) and C\(_{12}\). Another molecule of oxygen is incorporated generating a hydroperoxide (PGG\(_2\)) (Samuelsson et al., 1975; Smith and Lands, 1972). The peroxidase component of the same bifunctional enzyme (Ohki et al., 1979) reduces PGG\(_2\) to PGH\(_2\), a key intermediate for all prostaglandins (Nugteren and Hazelhof, 1973; Hamberg and Samuelsson, 1973). PGH\(_2\) can be isomerized to thromboxane A\(_2\) (TXA\(_2\)) by thromboxane synthase (Hamberg et al., 1975) and PGH\(_2\) can be isomerized to PGI\(_2\) by PGI\(_2\) synthase (Moncada et al., 1976). These two compounds are rapidly and non-enzymatically deactivated by hydration to the stable compounds, TXB\(_2\) and 6-Keto-PGF\(_{1\alpha}\). PGH\(_2\) can be converted into PGE\(_2\) (Ogino et al.,
MEMBRANE PHOSPHOLIPIDS

Phospholipase A₂

AA → HPETE → LTs

Lipoxygenase

Cyclooxygenase

PGG₂ → PGH₂

(Endoperoxides)

PGI₂ → 6-keto-PGF₁α

PGE₂ → PGF₂α

PGD₂

TXA₂

HHT + MDA

TXB₂

Fig. 2. Arachidonic acid cascade.
1977), PGD₂ (Christ-Hazelhof and Nugteren, 1979; Shimizu et al., 1979) and PGF₂α (Lee and Levine, 1974). PGF₂α can also be formed from PGE₂ by prostaglandin E 9-ketoreductase (Lee and Levine, 1974). Finally, PGH₂ can be cleaved into malondialdehyde (MDA) and hydroxy-heptadecatrienoic acid (HHT) either chemically or enzymatically by thromboxane synthase. Not every cell is able to synthesize all prostaglandins. For example, TXA₂ is the major product from blood platelets; whereas PGI₂ is mainly made by arterial endothelial cells (Hamberg et al., 1975; Johnson et al., 1976; Korbut and Moncada, 1978; MacIntyre et al., 1978).

The enzymes in the AA cascade can be regulated by various agents. The most important enzyme is cyclooxygenase which is regulated by lipid peroxides and non-steroid anti-inflammatory drugs. The lipid peroxides play a role in both activating and inactivating cyclooxygenase. The addition of H₂O₂ and long-chain lipid hydroperoxides at low concentration stimulates cyclooxygenase activity (Hemler and Lands, 1980; Lands and Byrnes, 1982). Panganamala et al. (1974) showed that the addition of catalase inhibited prostaglandin synthesis in the microsomal incubation with labeled 20:3 (n-6) since catalase broke down H₂O₂ or hydroperoxyl intermediates. The activity of cyclooxygenase can be inhibited by glutathione peroxidase which can remove peroxides (Lands, Lee, et al., 1971; Cook and Lands, 1976). Inhibition occurred when glutathione peroxidase was added as AA metabolism progressed suggesting that peroxides acted as activators and were continuously required for cyclooxygenase activity (Lands, Kulmacz, et al., 1984). However, when the levels of peroxides increased above
the need for activation, deactivation occurred (Gale and Egan, 1984; Lands, Kulmacz, et al., 1984). Cyclooxygenase activity can be monitored by the consumption of oxygen. Gale and Egan (1984) showed that oxygen uptake during prostaglandin synthesis stopped before all AA or all oxygen was used. Although a second addition of AA did not cause increased oxygen consumption, the addition of fresh enzyme induced further oxygen uptake and these data precluded substrate depletion. Inhibition was evidently caused by the high peroxide levels existing in the system. Inhibition could also occur by the pre-incubation of the enzyme with a variety of hydroperoxides (O'Brien and Rahimtula, 1976; Egan, Paxton, et al., 1976; Egan, Gale, et al., 1981). The inhibition was irreversible and was not due to classic product inhibition. Egan, Paxton, et al. (1976) and Gale and Egan (1984) proposed that peroxidase action on the hydroperoxides released oxidizing equivalents which deactivated the enzyme.

Another functional component of cyclooxygenase is its peroxidase activity which leads to the formation of PGH₂ from PGG₂ (Ouderaa et al., 1979; Ohki et al., 1979). It is paradoxical that prostaglandin synthase has both oxygenase and peroxidase activity. The peroxide level needed for the activation of cyclooxygenase is much lower than the Km value of peroxidase so that cyclooxygenase activity is enhanced by peroxide levels that are too low for removal by peroxidase. Thus, cyclooxygenase is regulated by the peroxide levels. Lower concentrations activate the enzyme and higher concentrations irreversibly deactivate the enzyme (Lands, Kulmacz, et al., 1984).
In 1971, Vane reported that the aspirin-like drugs inhibited the biosynthesis of prostaglandin in homogenates of guinea pig lung (Vane, 1971). This drug had a similar effect in human platelets (Smith and Willis, 1971) and perfused dog spleen (Ferreira et al., 1971). This discovery provided a plausible explanation for the therapeutic effects of the aspirin-like drugs which have been used for many years. This discovery also provided evidence that prostaglandins participated in the pathogenesis of inflammation, fever and pain. The mechanisms by which aspirin-like drugs exert their inhibitory effects vary. Aspirin itself acts by acetylating a serine residue at the active site of the cyclooxygenase (Roth and Majerus, 1975; Roth et al., 1975). The inhibition by aspirin is irreversible. Other drugs have quite different mechanisms of action. It is understood that aspirin-like drugs only inhibit the oxygenase activity of cyclooxygenase and these drugs have no significant effect on peroxidase activity (Gale and Egan, 1984; Salmon, 1981). Commonly used non-steroid anti-inflammatory drugs include aspirin, indomethacin, phenylbutazone, meclofenamic acid, ibuprofen and naproxen (Nickander et al., 1979).

Peroxidation of Arachidonic Acid

Lipid peroxidation is a complex process in which molecular oxygen and lipid react in a free radical chain sequence. Methylene-interrupted PUFA such as linoleic acid [18:2 (n-6)] or AA [20:4 (n-6)] undergo oxidation more readily than monounsaturated fatty acids such as oleic acid (Porter, 1986). The oxidation of lipids generally consists of initiation (eq. 1), propagation (eq. 2a, 2b) and termination (eq. 3a,
The oxidation of PUFA is usually estimated by measuring the yield of MDA which can react with thiobarbituric acid (TBA) to give a pink color with maximal absorbance at 532 nm (Wilbur et al., 1949). However, TBA test is not specific for MDA, but it still can show good relationship to the extent of lipid peroxidation (Bird and Draper, 1984) although other biological compounds react with TBA to give the same color (Slater, 1972). So TBA test is also called TBA-reacting (TBAR) material assay.

\[
\begin{align*}
\text{RH} &\rightarrow \text{R} \cdot + \text{IH} & \quad (1) \\
\text{R} \cdot + \text{O}_2 &\rightarrow \text{ROO} \cdot & \quad (2a) \\
\text{ROO} \cdot + \text{RH} &\rightarrow \text{ROOH} + \text{R} \cdot & \quad (2b) \\
2\text{R} \cdot &\rightarrow \text{R} - \text{R} & \quad (3a) \\
\text{R} \cdot + \text{ROO} \cdot &\rightarrow \text{ROOR} & \quad (3b) \\
2\text{ROO} \cdot &\rightarrow \text{ROOR} + \text{O}_2 & \quad (3c)
\end{align*}
\]

When the peroxyl radical (ROO·) is formed, trans, cis-conjugated dienes of hydroperoxide are normally produced in autoxidation. Only a small amount of trans, trans-products are detected during AA autoxidation (Porter, 1986). If the autoxidation temperatures are raised, more trans, trans-products will be formed. Peroxyl radicals derived from AA can undergo cyclization reactions that lead to cyclic peroxide compounds (Fig. 3) in addition to the formation of non-cyclic peroxides.
Fig. 3. Autoxidation leading to cyclic peroxide compounds.

Modified from Porter (1986).
Phenolic antioxidants such as 2R, 4'R, 8'R-alpha tocopherol (also called d-alpha-tocopherol, or natural vitamin E) not only stop or slow autoxidation but also direct isomer formation. The antioxidant (ArOH) inhibits autoxidation by trapping intermediate peroxyl radicals (ROO·) in two ways. First, peroxyl radicals are trapped by H-atom transfer forming hydroperoxide and aryloxy radicals (eq. 4). The rate of H-atom transfer from vitamin E is about four orders of magnitude faster than the rate of hydrogen transfer from hydrocarbons to peroxyl radicals.

\[
ROO· + ArOH \rightarrow ROOH + ArO· \quad (4)
\]

\[
ROO· + ArO· \rightarrow ROO-ArO \quad (5)
\]

Secondly, aryloxy radicals react by radical-radical coupling to form peroxide products (eq. 5). The extent of lipid peroxidation is reduced in biological systems with sufficient levels of protective vitamin E. Furthermore fatty acid or ester hydroperoxides formed in the presence of antioxidants only have the trans,cis-conjugated diene structure (Porter, 1986). Trans, trans-conjugated dienes are formed in the absence of antioxidants.
Polyunsaturated Fatty Acids and Cell-Mediated Immunity

The regulatory actions of PUFA on cell-mediated immunity and the protective effects of PUFA on the prevention of allograft rejection have been investigated either in vitro with lymphocyte culture systems or in whole animals. Essential fatty acid-induced suppression in cultures of mitogen-or antigen-stimulated human peripheral lymphocyte transformations were studied by a number of investigators (Mertin and Hughes, 1975; Weyman et al., 1977; Mihas et al., 1975). Both linoleic acid and AA at concentrations comparable to those normally found in serum inhibit lymphocyte activation induced either by the T cell mitogen phytohemagglutinin (PHA) or by a purified protein derivative of tubercle bacillus (Mertin and Hughes, 1975).

The immunosuppressive effects of PUFA in vivo were demonstrated by measuring the survival time of allografts transplanted from mice of one strain to mice of another strain. Mice maintained under normal dietary conditions and treated with orally or subcutaneously administered EFA showed a significant prolongation of allograft survival time (Mertin, 1976; Mertin and Hunt, 1976). The prolongation of kidney allograft survival was also improved by PUFA (Mertin et al., 1973). In addition, PUFA deficient diets caused acceleration of graft rejection (Mertin and Hunt, 1976). Studies using different unsaturated fatty acids demonstrated that only EFA of the n-6 family prolonged skin and kidney allograft survival whereas oleate had no effect (Mertin, 1976).
The prolongation of survival time was greater with increasing chain length and number of double bonds in fatty acids. For example, arachidonic acid was more potent than linoleic acid (Mertin, 1976).

There is also clinical evidence that EFA shows immunosuppressive effects in vivo. Uldall et al. (1974) and McHugh et al. (1977) reported that in human kidney transplants, treatment with PUFA in addition to conventional immunosuppressive drugs, prolonged graft survival and function when compared to human transplants treated with drugs alone.

Prostaglandin E and MØ in Cell-Mediated Immunity

The preventive effects of PUFA on tissue rejection may be explained by the modulation of cell-mediated immunity with prostaglandin E synthesized from PUFA. It is necessary to briefly review fundamental cellular events in allograft rejection in order to explore prostaglandin modulation. Allograft rejection is generally considered as a cell-mediated immune response which results from the maturation and proliferation of cytotoxic T-cells. The initiation of T cell proliferation requires interactions with accessory cells, mainly MØ. MØ provide two essential functions. First, MØ process and present antigens in conjunction with major histocompatibility complex (MHC) Class II gene products, for example Ia antigens, in a way suitable for recognition and binding by T cells. Secondly, MØ can synthesize and secrete interleukin-1 (IL-1), also known as lymphocyte activating factor, for full T cell function (Unanue and Allen, 1987). Following initiation, some T cells release a second interleukin (IL-2)
which drives antigen activated T cells into proliferation (Unanue, 1981; Stobo, 1982). Prostaglandin E and other EFA metabolites (Foegh et al., 1986) are involved in the modulation of the MØ activation process. The inhibitory effects of PGE on the immune response occur by several mechanisms. First, PGE suppresses human T cell mitogenesis and inhibits PHA stimulation of human lymphocytes (Goodwin et al., 1977). The addition of indomethacin and PHA led a much higher increase in 3H-thymidine incorporation in T-cell than PHA alone (Goodwin and Ceuppens, 1985). Secondly, PGE₂ appears to inhibit IL-2 production, while cyclooxygenase inhibitors raise IL-2 production above normal levels (Walker et al., 1983; Gordon et al., 1976; Rappaport and Dodge, 1982). These studies suggest that exogenous and endogenous prostaglandins have immunosuppressive effects. PGE modulation of T cell proliferation may occur by inhibiting both IL-2 production and action (Shaw, 1985). Thirdly, PGE₂ may decrease the ability of MØ to express Ia antigens (Synder et al., 1982). These Ia antigens and presenting antigens also formed by MØ provide a signal for the recognition and binding of T cells and MØ. The amount of Ia antigens expressed by MØ is crucial for recognition and binding (Stobo, 1982). Finally, PGE₂ also stimulates suppressor T cells which inhibit T cell-mediated immune response (Fulton and Levy, 1981; Fisher et al., 1985; Webb and Nowowiejski, 1978; Rogers, Campbell, et al., 1982; Rogers, Nowowiejski, et al., 1980).

The protective effects of PGE₂ on the rejection of transplanted organs were also investigated in animal studies. The administration of PGE prolonged the survival of mouse skin allografts (Anderson et al.,
1976, 1977) and cardiac hamster-rat xenographs (Kakita et al., 1975). The administration of indomethacin decreased allograft survival in mice, and the injection of PGE\textsubscript{2} analogue completely blocked this effect (Anderson et al., 1977).

PGE\textsubscript{2}, which is synthesized by M\textsubscript{0}, and may act in a negative feedback inhibition of the immune response (Schultz et al., 1978). A number of studies have shown that prostaglandins are synthesized and released by isolated M\textsubscript{0} from different species (Gordon et al., 1976; Goodwin et al., 1977; Humes et al., 1977; Gemsa et al., 1978; Kurland and Bockman, 1978; Hsueh and Kuhn, 1979; Whisler et al., 1984). The prostaglandins in these studies were measured by radioimmunoassays, analyzed in the cultures of M\textsubscript{0} labeled with radioactive AA or measured in a gas-liquid chromatograph/mass spectrometer assay (GC/MS). M\textsubscript{0} from many species of animals synthesize PGE, but the isolation of other AA metabolites depends on the animal species and the tissue source of the M\textsubscript{0} (Goldyne and Stobo, 1981). The major prostaglandin isolated from human peripheral blood M\textsubscript{0} is PGE\textsubscript{2}, but TXB\textsubscript{2}, PGF\textsubscript{2}\alpha and other products were also reported (Goldyne and Stobo, 1981). Human peripheral blood M\textsubscript{0} do not appear to synthesize PGI\textsubscript{2} as measured by quantitative GC/MS (Goldyne and Stobo, 1979; Morley et al., 1979) and by radio-thin layer chromatography (TLC) (Kennedy et al., 1980). However, small amounts of 6-keto-PGF\textsubscript{1\alpha} were produced by human monocytes as determined by high performance liquid chromatography (HPLC) (Pawlowski et al., 1983). A study by Gordon et al. (1976) suggested that there was a possible feedback loop involving M\textsubscript{0} - T cells $\rightarrow$ lymphokines $\rightarrow$ M\textsubscript{0} $\rightarrow$ prostaglandines $\rightarrow$ lymphocytes (Unanue, 1981). Figure 4 shows some of
Fig. 4. Scheme for possible modulation of cell-mediated immune response by PGE$_2$. Modified from Shaw (1985).
the mechanisms by which PGE may be involved in the modulation of the rejection of organ transplants. This scheme emphasizes the inhibitory effects of PGE on the immune response, but the scheme does not include the evidence (Mertin and Stackpoole, 1981) that small amounts of PGE may be necessary for the induction of the immune response.

Cyclosporine A and MØ in Immunity

Cyclosporine A (CsA) which is a cyclic undecapeptide improves allograft survival and suppresses certain T-cell-mediated immune responses. The immunosuppressive mechanisms of CsA have been extensively studied. It is generally considered that CsA inhibits an early phase of the immune response instead of impairing the activity of functionally competent T cells (Homan et al., 1980a, 1980b). Some investigators examined the effects of CsA on IL-2 mediated events and reported that CsA either decreased IL-2 receptors (Larsson, 1980; Dos Reis and Shevack, 1982) or IL-2 production, or both (Bunjes et al., 1981; Hess et al., 1982; Andrus and Lafferty, 1982). Palacios and Moller (1981) showed that pretreatment of T cells with CsA blocked T cell receptors for the recognition of HLA-DR antigens, and as a consequence pretreatment inhibited the proliferation of T cells. Other investigators focused on the effects of CsA on macrophages which provide positive and negative signals for the control T cell activation. MØ facilitatory functions for T cell activation are the secretion of IL-1 and processing and presenting antigen in conjunction with Ia antigen expression. Both functions may be inhibited by CsA. Andrus and Lafferty (1982) showed that IL-1 secretion by the macrophage
cell line PU5-1R was decreased by CsA, and Wagner (1983) demonstrated that secretion of IL-1 in the presence of T cells was impaired by the addition of CsA. Also a decrease in the expression of HLA-DR (MHC Class II gene) by MØ was reported when MØ were incubated with CsA (Whisler et al., 1985). PGE2 expression is enhanced by MØ and this process regulates T cell activation. Whisler et al. (1984) showed that CsA increased PGE2 production by peripheral blood monocytes, and the PGE2 levels obtained with CsA were equivalent to the levels previously shown to suppress immune function. The enhancing effect of CsA on prostaglandin synthesis was also reported in somatic cells (Lindsey et al., 1983). Thus CsA induction of PGE formation in MØ could profoundly alter multiple events crucial for the initial activation of T cells. T cell activation is important for the proliferation of functional T cell. CsA also has direct effects on T cells.

**Mechanism of Cyclosporine A Effects on Arachidonic Acid Metabolism**

The mechanism by which CsA increases the production of prostaglandins has not been investigated extensively. Whisler et al. (1984) and Lindsey et al. (1983) showed that exogenous AA and CsA did not have a synergistic effect on prostaglandin synthesis in cultured cells. They suggested that CsA stimulated prostaglandin synthesis through endogenous AA release rather than the conversion of free AA to prostaglandins.
ENHANCED PROSTAGLANDINS IN ENDOXIC SHOCK

Endotoxins are lipopolysaccharides (LPS) that are derived from the cell wall of gram-negative microorganisms. LPS exhibit a wide spectrum of biological activities. Prostaglandin participation in endotoxic shock, endotoxemia, and sepsis has been studied extensively. Enhanced prostaglandin levels are apparently involved in the pathophysiology of endotoxic shock. First, increased endogenous prostaglandin synthesis is observed in both bolus and septic models of endotoxic shock, in vivo, in all species studied including man (reviewed by Flynn, 1985). Shortly after the injection of endotoxin, plasma prostaglandin concentrations increase in mixed venous and arterial blood (Harris et al., 1980; Fletcher et al., 1976). TXA₂ and PGI₂ levels have received the most attention, although PGE and PGF were measured in early studies (Harris et al., 1980; reviewed by Fletcher, 1985 and by Flynn, 1985). Secondly, non-steroid anti-inflammatory (NSAI) drugs improve circulatory function and increase survival in endotoxin shock. The pre-treatment with NSAI drugs of animals subjected to gram-negative peritonitis (without antibiotics) increases survival (Short et al., 1981). The effects of post-treatment with NSAI drugs vary depending on the lethality of the model or species. Fletcher and Ramwell (1980) reported that one or two doses of indomethacin improved survival in a LD₇₃ baboon endotoxin model after shock occurred. There are several opinions about the effects of
NSAI drugs in shock since NSAI drugs inhibit serotonin release, stabilize lysosomal enzymes, inhibit the platelet release reaction and decrease vascular permeability, and these are all factors that may be involved in shock (Flynn, 1985; Fletcher and Ramwell, 1980). Thirdly, EFA deficient animals are more resistant to endotoxin-induced shock than normal animals (Cook et al., 1981). If esterified AA was supplied to EFA deficient animals two days prior to a challenge with LPS, the animals supplied with AA showed a 100 percent mortality compared to 24 percent mortality in EFA deficient animals. These investigations suggested that EFA or their metabolites were involved in the pathophysiologic changes of endotoxic shock. Finally, other reports have shown that enhanced prostanoid levels in endotoxic shock were not due to decreased catabolism or clearance of prostaglandins in endotoxic shock (Harris et al., 1980; Flynn and Lefer, 1977).

It is well established that prostaglandins participate in the pathophysiology of endotoxic shock. However, detailed mechanisms and target tissues have not been established for the enhanced levels of each prostanoid that responds to LPS. One should realize that the involvement of the AA cascade in endotoxic shock is extremely complex. Elevated AA metabolites modify the sensitivity or responsiveness to homeostatic mechanisms. A specific prostaglandin may exert a beneficial action in one phase and a harmful effect in another phase in endotoxic shock.
Mechanism of Lipopolysaccharide Effects on Arachidonic Acid Metabolism

Little information exists about the cellular involvement of bacterial LPS in the metabolism of AA, even though prostaglandin biosynthesis is enhanced by LPS both in vivo and in vitro. A number of studies have shown that prostaglandins are increased in monocytes, macrophages and platelets from different animal sources challenged with LPS (reviewed by Flynn, 1985). Kurland and Bockman (1978) showed that there was a linear relationship between PGE production and LPS concentrations (0.1-20 ug/ml range) in both human blood and mouse peritoneal MØ. The study by Feuerstein et al. (1981) demonstrated that LPS dramatically increased prostaglandin synthesis in rat peritoneal MØ. Although they did not mention the fact, their data showed that the 6-keto-PGF\(_1\alpha\)/PGE\(_2\) ratio decreased from 1.63 in the control to 0.97 in the LPS treated group. They found that both the cyclooxygenase inhibitor indomethacin and the phospholipase A\(_2\) inhibitor quinacrine suppressed the LPS stimulatory effect on prostaglandin synthesis.

Wightman and Raetz (1984) reported that lipid X, a biologically active moiety of LPS, activated protein kinase C in a macrophage-like cell line and released arachidonic acid. Prostaglandin synthesis was not measured in this study. Aderem et al (1986) showed that LPS "primed" macrophages for a second trigger such as A23187 or zymosan to increase the secretion of AA from either lipoxygenase or cyclooxygenase pathways in mouse peritoneal macrophages. In contrast to other investigators (Kurland and Bockman, 1978; Feuerstein et al., 1981; Whisler et al., 1985), Aderem et al. (1986) could not show any enhancement of prostanoids by LPS alone. They suggested that LPS
primed MØ by some unknown mechanisms that potentiated phospholipase activity but not cyclooxygenase or lipoxygenase activity. Similar experiments carried out in human polymorphonuclear leukocytes (PMNL) by Salari et al. (1982) indicated that the pre-incubation of LPS for various time intervals prior to the addition of A23187 had different effects on the stimulation of lipoxygenase metabolites by A23187. The pre-incubation with LPS for short time intervals (5-30 min) increased the sensitivity of PMNL to A23187, whereas the pre-incubation of LPS for long time intervals (60-120 min) inhibited the sensitivity of PMNL to A23187. A short time pre-incubation with LPS had no effect on the A23187 response in the presence of exogenous AA, but a long time pre-incubation with LPS significantly inhibited the response of cells to A23187 in the presence of exogenous AA. These data suggested that LPS had a non specific inhibitory effect on lipoxygenases rather than on phospholipases (Salari, 1982).

EFFECTS OF LOW DENSITY LIPOPROTEINS ON PROSTANOID SYNTHESIS AND THE CONSEQUENCES OF THESE EFFECTS IN ATHEROSCLEROSIS

Cell Proliferation and Lipid Accumulation in Atherosclerosis

Atherosclerosis, a disease causing half of all deaths in America (Brown and Goldstein, 1984), is characterized by MØ accumulation, SMC proliferation, extra- and intracellular lipid accumulation, and connective tissue deposition (Brown and Goldstein, 1983; Ross, 1979b). The exact mechanisms of atherosclerosis are not yet known, although the disease has been studied for over 100 years (Ross, 1981). Elevated
plasma cholesterol levels, high blood pressure, cigarette smoking, diabetes mellitus and platelet hyperaggregability are considered as risk factors (Steinberg, 1983; Willis et al., 1986).

There are three popular hypotheses used to explain the pathogenesis of atherosclerosis. The "endothelial injury hypothesis," or "response to injury hypothesis," (Ross and Glomset, 1976) proposes that damage to the integrity of the endothelial lining results in platelet adherence and aggregation and as a consequence the release of the platelet-derived growth factor (PDGF). PDGF stimulates SMC migration and proliferation and SMC secrete the connective tissue matrix elements collagen, elastic fibers and proteoglycans (Ross, 1981; 1979a, 1979b; Ross et al., 1974). The "lipid infiltration hypothesis" derived from Virchow in 1856 (reviewed by Adams, 1981) proposes that high levels of low density lipoprotein (LDL) result in LDL infiltration and the increased uptake of LDL by endothelial cells (EC), SMC and MØ. Cholesterol accumulates in these cells as a consequence of LDL uptake. Steinberg (1981, 1983) has suggested a unified hypothesis in which the two previous hypotheses are combined together by many interacting factors. Lipid accumulation and cell proliferation are very important events in all hypotheses proposed to explain atherosclerosis.

SMC proliferation may be induced by the release of growth factors from platelets, EC and MØ. When EC are injured or damaged by mechanical means or by increased LDL concentration (Henriksen et al., 1979; Ross and Glomset, 1976; Ross and Harker, 1976), platelets adhere to the injured surface and release substances such as serotonin and TXA2. Platelets also release PDGF (Ross et al., 1974; Antoniades,
which stimulate SMC migration and growth. Proliferation does not occur experimentally in animals in the absence of platelets (Friedman et al., 1977; Spaet et al., 1975) or by the administration of drugs that inhibited platelet reactions (Harker et al., 1976). When EC are activated (for example, in susceptible areas of vessels where there is increased blood turbulence), EC release endothelial cell-derived growth factor (EDGF) (Gajdusek et al., 1980; DiCorleto and Bowen-Pope, 1983). MØ which infiltrate the early lesion release a growth factor called MØ-derived growth factor (MDGF) (Leibovitch and Ross, 1975; Glenn and Ross, 1981). All these growth factors are cellular mitogens which stimulate SMC proliferation. Finally, LDL, especially from hypercholesterolemic animals, enhance the growth of SMC (Fischer-Dzoga et al., 1976).

Lipid accumulation refers mainly to the extra and intracellular deposition of cholesteryl esters (Ross, 1979b). Foam cells are formed from SMC and MØ by the accumulation of cholesteryl esters (Fagglotto et al., 1984; Fagglotto and Ross, 1984; Gerrity, 1981a, 1981b; Jonasson et al., 1986; Schaffner et al., 1980; Brown and Goldstein, 1983). LDL, the most abundant cholesterol carrier in human plasma (Cornwell et al., 1961a, 1961b; Edelstein, 1986), have long been considered atherogenic components responsible for the lipid accumulation in atherosclerosis (Brown and Goldstein, 1984; Goldstein and Brown, 1977; Rudel et al., 1986; Steinberg, 1983). According to Brown and Goldstein (1983, 1984, 1986), Goldstein and Brown (1977) and Steinberg (1981, 1983), SMC take up cholesterol via either the LDL receptor-dependent pathway or
the LDL receptor-independent pathway. Only the receptor-dependent pathway is subject to feedback regulation (or down regulation). The receptor-independent pathway is regulated by the LDL concentration (Goldstein and Brown, 1977; Steinberg, 1981). After LDL bind to the receptors, rapid internalization occurs forming a vesicle which then fuses with the lysosome. The lysosomal cholesteryl ester hydrolase hydrolyzes cholesteryl ester into cholesterol which is then released into the cytoplasm. Free cholesterol suppresses LDL receptor synthesis by decreasing receptor messenger RNA, suppresses the transcription of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase, a key enzyme in cholesterol synthesis), and increases acyl CoA:cholesterol acyltransferase (ACAT, a cholesteryl-esterifying enzyme) activity. The sequence is called feedback regulation (Brown and Goldstein, 1984; Goldstein and Brown, 1977). LDL are taken up through LDL receptor-independent pathway and cholesterol accumulates in SMC forming foam cells when the LDL concentration is elevated. The mechanism of lipid uptake by MØ differs from lipid uptake by SMC since MØ do not have the native LDL receptor. A number of studies have shown that MØ have, in addition to a β-very low density lipoprotein (β-VLDL) receptor, a receptor called the scavenger or acetyl LDL receptor (Brown and Goldstein, 1983, 1985; Goldstein et al., 1979; Fogelman et al., 1980). Scavenger receptors of MØ only mediate the uptake of chemically modified LDL (M-LDL). Chemical modification diminishes the number of positive lysine residues, increases the net negative charge of the LDL, and converts the LDL into M-LDL for scavenger receptors (Brown and Goldstein, 1983). M-LDL include acetyl-LDL, maleylated LDL,
succinylated LDL (Goldstein et al., 1979), acetoacetylated LDL (Mahley et al., 1979) and malondialdehyde-LDL (Fogelman et al., 1980). The scavenger receptor pathway in MØ is not subject to feedback regulation when the cholesterol content of MØ is increased (Goldstein et al., 1979).

Oxidized LDL and Its Cytotoxicity

M-LDL are taken up by MØ, but few of the chemically modified forms are generated biologically (Brown and Goldstein, 1983; Steinberg, 1983). Recent investigations indicate that some mechanisms by which M-LDL are produced occur in biological reactions (Heinecke, Baker et al., 1986; Heinecke, Rosen, et al., 1984; Henriksen, Mahoney and Steinberg, 1981, 1982, 1983; Hiramatsu et al., 1987; Morel et al., 1983, 1984; Parthasarathy, Printz, et al., 1986; Parthasarathy, Steinbrecher, et al., 1985; Steinbrecher, Parthasarathy et al., 1984). These mechanisms involve LDL lipid peroxidation with EC, MØ or SMC to produce oxidized-LDL. Henriksen et al. (1981) first reported that LDL conditioned by incubation with a rabbit aortic EC line (EC-modified LDL) was taken up and degraded by mouse peritoneal MØ and by a tumor line of mouse MØ (J774 cells) 3 to 4 times more rapidly than native LDL. The uptake and degradation of EC-modified LDL were inhibited by acetyl LDL suggesting that the uptake and degradation of EC-modified LDL was a scavenger receptor-mediated process. The properties of EC-modified LDL and the mechanisms of modification were investigated by Steinbrecher, Parthasarathy, et al. (1984) and Henriksen et al. (1982, 1983). They reported that the electrophoretic mobility and density of
EC-modified LDL were increased (Henriksen et al., 1982). Steinbrecher, Parthasarathy, et al. (1984) showed that the TBA-reacting (TBAR) material increased as modification occurred and antioxidants, such as vitamin E and butylated hydroxytoluene (BHT), totally blocked the modification of LDL by EC. These data indicated that modification involved lipid peroxidation. Similar modifications occurred in the presence of Cu\(^{2+}\) and in the absence of EC. Phospholipase A\(_2\) activity was enhanced during modification by EC resulting in the hydrolysis of as much as 40 percent of the LDL phosphatidyl-choline (PC) to lysophosphatidylcholine (LPC) (Steinberecher, Parthasarathy, et al., 1984; Parthasarathy, Steinbrecher, et al., 1985). The phospholipase A\(_2\) inhibitor P-bromophenacyl bromide blocked the hydrolysis of LDL PC, and diminished MØ degradation of EC-conditioned LDL suggesting that phospholipase A\(_2\) was associated with purified LDL. Parthasarathy, Steinbrecher, et al. (1985) postulated that phospholipase A\(_2\) released peroxidized free fatty acids, and these fatty acids propagated lipid oxidation by freely interacting with other fatty acids in LDL. They suggested that the peroxidized free fatty acid interacted with the apoprotein B of the LDL to modify its structure (Schuh et al., 1978). It was reported that decrease in reactive amino groups occurred during LDL oxidation (Steinbrecher, Witztum, et al., 1987). Antioxidants and a phospholipase A\(_2\) inhibitor both inhibited the modification processes. Similar modifications were also reported for EC by other investigators (Morel et al., 1984; Masana et al., 1986). Proubcol, a drug used in the treatment of hypercholesterolemia, blocked the oxidative modification of LDL by EC (Parthasarathy, Young, et al., 1986).
SMC (Morel et al., 1984; Heinecke et al., 1984, 1986) and MØ (Parthasarathy, Young, et al., 1986; Hiramatsu et al., 1987) also modify the LDL by oxidation. The oxidation of LDL by SMC and MØ involved superoxide, since superoxide dismutase (SOD) inhibited the modification reaction (Heinecke et al., 1986; Hiramatsu et al., 1987). Hiramatsu et al. (1987) showed that the early addition of SOD blocked oxidation but the later addition of SOD had a very small inhibitory effect on oxidation suggesting that the superoxide mainly initiated oxidation. BHT also could block the modification of LDL by SMC and MØ. The inhibitory effect of catalase varied in different cells, but mannitol (a hydroxyl radical scavenger) was without effect (Hiramatsu et al., 1987; Heinecke et al., 1986). LDL modified by oxidation has already been isolated from human plasma (Bon et al., in press). The properties of oxidized LDL from human plasma were very similar to the properties of LDL modified by oxidation with EC, SMC, and MØ in tissue cultures. The amount of oxidized LDL in atherosclerotic patients was higher than in normal subjects. Zhang et al. (in press) showed that LDL isolated from individual human sera varied in their susceptibilities to lipid peroxidation when the LDL were incubated in 0.15 M NaCl at 37°C in the absence of cells or transitional metal ions. These data suggested that differences in oxidation rate explain why lipid peroxide levels are significantly different in individual sera (Yagi, 1982; Lee, 1980; Szczeklik and Gryglewski, 1980; Szczeklik et al., 1981).

Oxidized LDL are cytotoxic. Henriksen et al. (1979) and Hessler, Robertson, et al. (1979) reported that LDL injured cultures of human EC, SMC and skin fibroblast when LDL were incubated with these cells
for 2 or 3 days. Cellular injury led to $^{51}$Cr release from cells and reduction in cell numbers. These effects were LDL dose-dependent and they were inhibited by high density lipoproteins (HDL). The phenomenon of cytotoxicity stimulated these investigators to study LDL modified by incubation with EC. It is now well known that the cytotoxicity of LDL is caused by lipid peroxidation, (Morel et al., 1983; Hessler, Morel, et al., 1983; Zhang et al., in press) since a study by Hessler, Morel, et al. (1983) indicated that toxic material in LDL was found in the chloroform-methanol extractable fraction. Morel et al. (1983) showed that the presence of antioxidants or EDTA in the LDL preparation prevented the formation of cytotoxic LDL, and that SOD and catalase partially blocked its formation. However, the addition of BHT, EDTA, SOD or catalase could not prevent the cytotoxicity of oxidized LDL (Morel et al., 1983). In a later study, Zhang et al. (in press) showed that the addition of BHT to the SMC cultures prevented the cytotoxic effects of oxidized LDL.

Effects of Prostaglandins on Atherosclerosis

Sinclair (1956) and Cornwell and Panganamala (1982) suggested that atherosclerosis might be mediated to a deficiency of EFA. Huttner, Gwebu, et al. (1977) and Cornwell et al. (1979, 1984) showed that relatively high PGE and PUFA levels inhibited proliferation in cultures of guinea pig aortic SMC. A number of recent investigators have continued to study prostaglandins and atherosclerosis. These studies have focused on platelet aggregation, cell proliferation and lipid metabolism.
The balance between PGI$_2$ and TXA$_2$ is very important in platelet aggregation. TXA$_2$ is an important platelet metabolite which is a powerful vasoconstrictor and aggregating agent, while PGI$_2$ is a major product of endothelial cells and is a powerful vasodilator and antiaggregatory agent. Gryglewski (1980a, 1980b) has postulated that atherosclerosis is a disease due to a deficiency of PGI$_2$ since platelet hyperaggregability is one of the major risk factors in atherosclerosis.

Studies by Huttner, Gwebu, et al. (1977), Cornwell et al. (1979), Cornwell and Panganamala (1982), Mahmud et al. (1984), D.L. Smith et al. (1984) and Wiley et al. (1983) showed that PGE and other prostaglandins also inhibited aortic SMC proliferation and other cell growth. PGI$_2$ did not have an inhibitory effect on cell proliferation (Willis et al., 1986). However, PGI$_2$ inhibited the release of mitogenic materials derived from platelets, EC and MØ (Willis et al., 1986). This concept is particularly important since PDGF, EDGF and MDGF all are involved in the stimulation of SMC proliferation in atherosclerosis.

Prostaglandins are also involved in the modulation of cholesteryl ester metabolism. After cholesterol is taken up by cells, some free cholesterol is excreted, and the rest is re-esterfied by cytoplasmic ACAT. When cholesteryl ester accumulates in cells, protective mechanisms function to remove the excessive cholesterol. The cytosolic cholesteryl ester hydrolases (or called neutral cholesteryl ester hydrolase, NCEH) convert the ester into fatty acid, and free cholesterol which is released depending on the availability of acceptors such HDL and red blood cells. Cholesteryl esters undergo a
cycle of hydrolysis and re-esterification, and this is called the cholesteryl ester cycle (Brown and Goldstein, 1983).

Prostaglandins influence the deposition of cholesterol by their effects on ACAT and NCEH in the cholesteryl ester cycle. Hajjar et al. (1982) reported that cultures of SMC challenged for two weeks with PGI$_2$ significantly decreased their total cholesterol content by 35 percent compared to untreated control. Hajjar, (1984) indicated that PGI$_2$ stimulated both lysosomal and cytosolic cholesteryl ester hydrolases, but not ACAT. It was suggested that the effects of PGI$_2$ on the hydrolases was mediated by the enhancement of intracellular cAMP. Thus the pre-incubation of cells with the adenylate cyclase inhibitor dideoxyl adenosine (DDA) diminished the effects of PGI$_2$ (Hajjar et al., 1982).

PGE$_2$ also decreased cholesteryl ester accumulation. Morisaki et al. (1986) showed that exogenous PGE$_2$ inhibited cholesteryl ester accumulation in MØ challenged with β-VLDL in both a dose and a time-dependent manner. They suggested that PGE$_2$ may enhance the hydrolysis of cholesteryl ester or PGE$_2$ may increase the secretion of free cholesterol. Other investigators (Berberian et al., 1977; Hajjar 1985; Subbiah and Dicke, 1977; Hajjar and Weksler, 1983) reported that PGE$_2$ decreases the activity of ACAT. The presence or absence of DDA had no influence on the inhibitory effect of PGE$_2$ on ACAT suggesting that the mechanism of PGE$_2$ action on ACAT is not mediated by cAMP (Hajjar, 1984). It is clear that the inhibition of ACAT by PGE$_2$ and activation of cholesteryl ester hydrolase by PGI$_2$ decrease cholesteryl ester and increase free cholesterol which escapes from cells and is transported by acceptors.
Prostaglandin Synthesis and LDL in Atherosclerosis

Studies on prostaglandin synthesis in atherosclerosis and the effects of LDL on prostaglandin synthesis have attracted the investigators for some time. Gryglewski et al. (1978) reported that the production of PGI$_2$ by slices of mesenteric arteries and aortas was inhibited in animals maintained for 1-3 months on atherogenic diets, but the production of PGI$_2$ returned to normal after 5 months on the diets. Sinzinger et al. (1979) confirmed Gryglewski's work by investigating atherosclerotic human arteries in which the production of PGI$_2$ was less than the production of PGI$_2$ in morphologically normal arteries. In Sinzinger's animal study (1979), enhanced PGI$_2$ synthesis was found in arteries from animals fed an atherogenic diet for only 4 weeks. Mehta et al. (1986) showed that the synthesis of PGI$_2$ and TXA$_2$ was increased as measured by RIA in arterial segments from cholesterol-fed rabbits. These authors suggested that the formation of PGI$_2$ may be a compensatory response to atherogenesis. The aortas from Watanabe heritable hyperlipidemic rabbits also produced more 6-Keto-PGF$_{1a}$ than the aortas from New Zealand White rabbits (Pfister et al., 1986). Rolland et al. (1984) investigated prostaglandin synthesis by aortic microsomes from normal and atherosclerotic humans. In the presence of exogenous AA, reduced glutathione and epinephrine, increased PGE$_2$ and decreased PGI$_2$ measured by RIA were found in atherosclerotic aortas. However, increased PGI$_2$ production has also been reported in atherosclerotic patients (Fitzgerald et al., 1984).

The overall metabolism of prostaglandin in atherosclerosis is not clear, and similar problems are encountered in studies on the
effects of LDL on prostaglandin synthesis. Szczeklik and Gryglewski (1980) showed that LDL which functioned as a carrier for lipid peroxides was responsible for the inhibitory effect of LDL on PGI$_2$ synthesis. Later, Szczeklik et al. (1981) found that the lipid peroxides in LDL were produced mainly during LDL isolation, although lipid peroxides were higher in LDL from patients with coronary heart disease and hyperlipoproteinemia than LDL from normal people. BHT (0.02 percent) blocked peroxide formation during the preparation of LDL, but LDL from 25 percent of the patients still inhibited PGI$_2$ synthesis. These investigators suggested that inhibitory effects may have been caused by small amount of pre-existing lipid peroxides because very small amount of 15-hydroperoxy-eicosatetraenoic acid (15-HPETE) was a powerful inhibitor for PGI$_2$ synthase (Gryglewski et al., 1976). Beltz and Förster (1980) reported that low concentration of LDL (0.5 mg cholesterol/ml) increased PGI$_2$ synthesis, in microsomal fraction in the presence of PGH$_2$, but high concentrations of LDL decreased PGI$_2$ synthesis in a dose-dependent manner. Beltz et al. (1983) found that BHT (0.02 percent) did not block lipid peroxidation during the preparation of LDL and HDL from the plasma of control subjects or patients with hyperlipoproteinemia. LDL with low amounts of lipid peroxides inhibited PGI$_2$ synthesis, and HDL with higher amounts of lipid peroxides enhanced PGI$_2$ synthesis suggesting that the inhibitory effects of LDL were not dependent on lipid peroxides. Beltz et al. (1983) postulated that the fatty acid pattern and lipid class composition determined the effect of LDL on PGI$_2$ synthesis.
LDL also influences PGE$_2$ metabolism. Yokode et al. (1985) reported that oxidized-LDL obtained by Cu$^{2+}$-induced oxidation stimulated PGE$_2$ release as measured by RIA and GC/MS in mouse peritoneal MØ. A stimulatory effect did not occur with native LDL, acetyl LDL or free lipid peroxides. Pomerantz and Hajjar (1987) showed that aortic SMC, cultured with cationized LDL, had increased intracellular free and esterified cholesterol (principally cholesterol-linoleate). These cholesterol-rich SMC produced much less 6-Keto-PGF$_{1\alpha}$ and/or PGE$_2$ than control cells in response to A$_{23187}$ or exogenous AA. The uptake of $[^{14}C]$-AA was the same in cholesterol-rich and control SMC. The possible mechanisms by which the synthesis of prostanoids were decreased were 1) inhibition of cyclooxygenase or PGI$_2$ synthase or 2) inhibition of AA uptake into phospholipid.

Pomerantz et al. (1984) showed that either HDL or LDL significantly enhanced both PGI$_2$ and PGE$_2$ syntheses by SMC as measured by RIA. The enhancement of prostaglandin synthesis by HDL and LDL was time and dose dependent. The stimulatory effect of HDL on the synthesis of prostaglandin was greater than the effect of LDL. In these experiments, LDL lipid peroxidation was not measured after the incubation of SMC with LDL. It is interesting that lipid peroxidation in LDL occurred when LDL were incubated with SMC (Henriksen et al., 1982; Heinecke et al., 1984, 1986). Thus oxidized-LDL may have influenced prostanoid metabolism.
Mechanisms of LDL Effects on Arachidonic Acid Metabolism

Gryglewski (1980a, 1980b) postulated that lipid peroxides selectively inhibit PGI₂ synthetase, but not cyclooxygenase in arterial walls. The inhibition of PGI₂ may cause more PGH₂ to be converted to PGE₂. A number of studies have shown consistently that atherosclerotic aortas from either humans or animals contain lipid peroxides (Glavind et al., 1952; Fukuzumi, 1969; Filipovic et al., 1976; Henriksson et al., 1985) and that lipid peroxides are measurable in human plasma (Demopoulos, 1973; Tsuchida et al., 1985; Warso and Lands, 1985; Yagi, 1982). LDL is very highly susceptible to lipid peroxidation in vitro (Ray et al., 1954; Szczeklik and Gryglewski, 1980; Zhang et al., in press) or in biological system (Henriksen et al., 1981, 1982; 1983; Parthasarathy et al., 1986; Morel et al., 1984; Heinecke et al., 1984, 1986), and the oxidized-LDL may be carriers for lipid peroxides (Szczeklik and Gryglewski, 1980).

An alternative mechanism demonstrated by Pomerantz et al. (1984) was that lipoproteins provided substrate for the synthesis of prostaglandin. In their experiments, both LDL and HDL stimulated prostaglandin synthesis in the same manner in which the stimulation of PGE₂ synthesis was bigger than the stimulation of 6-Keto-PGF₁α. The effect of HDL was greater than LDL. HDL was chosen for the study of mechanisms. The stimulatory effect of HDL was not caused by apoproteins since the delipidated apoproteins of HDL had no effect on Prostaglandin synthesis. Labeled prostaglandin syntheses were enhanced by A23187 after SMC were pre-incubated with recombinant HDL-containing cholesteryl [1-¹⁴C]-AA. In contrast, HDL themselves
did not have an effect on the release of labeled prostaglandin from SMC pre-labeled with [1-14C]-AA. The data from Pomerantz et al. (1984) strongly suggested that lipoproteins provided AA for the synthesis of prostaglandins. Zhang et al. (in press) showed that oxidized-LDL affected PGE_2 and PGI_2 synthesis in the same manner. The effects of oxidized-LDL on prostaglandin synthesis depended both on the degree of LDL oxidation and on the LDL concentration. LDL containing small amounts of lipid peroxides stimulate both PGE_2 and 6-keto-PGF_1α synthesis as measured by RIA. Highly oxidized-LDL inhibited the synthesis of 6-keto-PGF_1α and PGE_2. Highly oxidized LDL apparently contains material which cross-reacts with the PGE_2 antibody in the RIA of PGE_2 since the cultures of SMC contained similar amounts of PGE_2 after incubation with oxidized-LDL in the presence or absence of indomethacin.
MATERIALS AND METHODS

MATERIALS

Arachidonic acid [20:4 (n-6)] was purchased from Nu-Check (Elysian, MN.), purified by elution from a Unisil column with hexane-ether (9:1 v/v), and used only when thin-layer chromatography showed that lipid peroxides were absent (Huttner et al., 1977). Other reagents were obtained from the following sources: \[1^{-14}\text{C}] - 20:4 (n-6) (54.5 mCi/mmol), \[U^{-14}\text{C}] - 20:4 (n-6) (1.0 Ci/mmol), [5,6,8,11,12,14,15 -\text{3H}(N)] - \text{PGE}_2 (200.0 Ci/mmol) and 6 - [5,8,9,11,12,13,15 -\text{3H}(N)] - \text{keto-PGF}_{1\alpha} (163.5 Ci/mmol) (New England Nuclear, Boston, MA); phosphatidylinositol (PI) and phosphatidylserine (PS) (Avanti Polar-Lipids, Inc., Birmingham, AL); indomethacin (IM), phosphatidic acid (PA), lysophosphatidylcholine (LPC), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (Sigma Chemical Company, St. Louis, MO); sphingomyelin (Sph) (Serdary Research Lab., London, Ontario, Canada); trilinolenin (Nu-Check, Elysian, MN); BHT and 1,1,3,3-tetramethoxypropane (Aldrich, Milwaukee, WI); calcium ionophore A23187 (Calbiochem-Behring, Corp., La Jolla, CA); Escherichia coli WE027:B8 LPS (Difco Laboratories, Detroit, MI).
METHODS

Tissue Culture of SMC

Primary cultures of SMC were established from the dissected medial layer of guinea pig aorta from prepubertal males (Huttner, Cornwell et al., 1977; Gavino et al., 1981a, 1981b). The medium for growing cells to confluency (Growth Medium) was prepared from 1X Eagle's minimum essential medium containing Hank's salts and 25mM HEPES buffer (GIBCO, Grand Island, NY) supplemented with 50 mg/ml of gentamycin sulfate (Schering, Kenilworth, NJ), 2 mM glutamine, 1X non-essential amino acids (Microbiological Associates, Walkersville, MD), 1 mM sodium pyruvate, and 1.3 mg/ml of sodium bicarbonate. This medium was supplemented with 10 percent fetal bovine serum (FBS) (Sterile Systems, Logan, UT: Hyclone lots 100348 and 1111574). Subcultures were split in a 1 to 2 ratio after cell detachment by trypsin. Each split grown to confluency was counted as one passage (Schaeffer, 1979). SMC were identified in the cultures by their reactivity to antibodies prepared from smooth gizzard muscle (Morisaki et al., 1982a, 1982b) and antibodies against human umbilical artery F-actin which was shown to react specifically with muscle actin isoforms (Strauch et al., 1984). SMC were used for experiments at passage level 3 or 4. The medium in experimental studies with confluent cells (Experimental Medium) consisted of Growth Medium supplemented with 20 percent FBS, 1X essential amino acids, and 1X essential vitamins.
Morphology and Viability of SMC

For morphologic studies, SMC were fixed in 3.7 percent phosphate buffered formalin and stained with filtered Giemsa. For viability studies, unfixed cells were stained simultaneously with fluorescein diacetate (FDA) and propidium iodide (PI) (Sigma, St. Louis, MO) (Jones and Senft, 1985). Stains were rinsed off after 5 min and the cells were allowed to dry. Cultures were then examined with epifluorescence illumination. Live cells fluoresced green since fluorescein is released from FDA by esterases and trapped in the live cell. Non-living cells fluoresced red because PI, which is excluded from the live cell, enters dead cells and intercalates with nucleic acids forming a red fluorescent complex.

Preparation of MØ

Peripheral blood mononuclear cells (PBMC) were obtained by the centrifugation of blood from normal donors over a Ficoll-Hypaque gradient (Boyum, 1968). Adherent MØ were prepared from PBMC by surface adherence and gently harvested with a rubber policeman. Over 90 percent of the cells were MØ as demonstrated by esterase staining (Yam, 1971). MØ were suspended in RPMI 1640 (Microbiological Associates, Walkersville, MD) containing 10 percent FBS.

Prostanoid Synthesis in SMC and MØ

SMC were seeded at 1.3 x 10⁴ cell/cm² in Corning T-25 flasks or 35 mm plates (indicated in specific experiments) containing experimental
medium. The cells were allowed to grow to confluency (4-5 days) at
37° C in 4 percent CO2 and 96 percent air, and the treatments were
initiated with fresh experimental media (4 ml in T-25 flask, 1.3 ml in
35 mm plate). MØ were adjusted to 2 x 10^6/ml in 10 percent FBS RPMI
media with different treatments and incubated in multiwell tissue
culture plates. Reagents such as CsA, A23187, LPS, AA and LDL
solutions were added directly to the fresh media. A23187, IM, AA and
CsA were added in ethanol. LPS was added in incomplete media and LDL
was added in 0.15 M NaCl. The final concentration of ethanol in any
experiment was less than 0.3 percent. The same volume of solvents was
added to each control.

RIA for Prostanoids

PGE2 and 6-Keto-PGF1α in culture media were measured by a
standard RIA procedure (Levine et al., 1971). The supernants were
diluted with Tris-albumin buffer (pH 7.5). Each sample was incubated
for 16 hours at 40° C with [3H]-PGE2 or [3H]-6-Keto-PGF1α and a
predetermined optimal dilution of rabbit antiserum directed against
PGE2 or 6-keto-PGF1α in a total volume of 600 μl. At the end of the
incubation period each sample received 500 μl of 0.05 percent dextran
and 0.5 percent charcoal in Tris-albumin buffer to absorb the free
[3H]-prostanoids. The sample was mixed and centrifuged at 3000 rpm for
20 min at 40°C. Supernatants were removed and 5.5 ml of ACS II
scintillation medium was added. Radioactivity was measured with a
Beckman LS 8100 counter. Antiserum were kindly supplied by Dr.
Lawrence Levine (Brandeis University, Waltham, MA). The cross-
reactivity of PGE antibody was: 6-keto-PGF$_{1\alpha}$, 0.4 percent; PGE$_1$, 0.76 percent; PGF$_{2\alpha}$, 0.31 percent; PGD$_2$, 0.051 percent; AA, 0.00045 percent. The cross-reactivity of 6-keto-PGF$_{1\alpha}$ antibody was: PGE$_2$, 0.15 percent; PGD$_2$, 0.02 percent; PGF$_{2\alpha}$, 0.10 percent; AA, 0.005 percent. The amounts of prostanoid in the culture medium were calculated from the standard curve generated with known concentrations of each prostanoid.

**TBAR Material Assay**

Lipid peroxides in lipoproteins were assayed by a modified TBA procedure (Gavino et al., 1981b). Lipoprotein solutions were adjusted to a concentration of 700 μg LDL cholesterol in a 0.4 ml volume. Two ml of 0.1 N HCl containing 0.375 percent TBA and 15 percent trichloroacetic acid was added and then followed with 10 μl of 5 mM Fe$^{3+}$ as a catalyst to decompose lipid peroxides. The mixture was incubated at 97°C for 20 min, cooled and centrifuged. The absorbance of clear supernatants was measured at 532 nm and converted to nmols of MDA from a standard curve generated with 1,1,3,3-tetramethoxypropane.

The lipid peroxide content of LDL in experimental media was also measured by TBA assay. LDL at a concentration of 800 μg LDL cholesterol/ml in experimental media was incubated for different time intervals. A 0.4 ml aliquot was taken for the TBA assay, and the data were reported as absorbance at 532 nm (A$_{532}$).
**LDL Preparations**

Human LDL (density, 1.019-1.063 g/ml) were isolated by a sequential ultracentrifugation procedure (Cornwell et al., 1961a; Havel et al., 1955). Individual units of freshly drawn human plasma were obtained from the Red Cross and converted to serum by the addition of CaCl\(_2\) (0.18 percent final concentration of CaCl\(_2\)). Serum was adjusted to a density of 1.019 and centrifuged at 40,000 rpm in a Beckman preparative ultracentrifuge in a Type 40 rotor for 18 h at 17°C. The top fraction containing VLDL was separated with a tube cutter. The bottom fraction was readjusted to a density of 1.063 and centrifuged in a Type 40 rotor at 40,000 rpm for 18 hr at 17°C. The orange-yellow LDL band at the top of the centrifuge tube was separated with a tube cutter and collected. The LDL solution was dialyzed at 4°C for 16 h against 400 volumes of 0.15 M NaCl and then sterilized by passage through a 0.45 μm Millipore filter.

Oxidized-LDL preparations with different TBAR contents were prepared by incubating sterilized LDL (around 4 mg/ml LDL cholesterol) for various time intervals. The oxidation reaction was closely monitored by the disappearance of the yellow carotenoid color and by the TBA assay. Unoxidized LDL (BHT-LDL) were prepared by the immediate addition of 100 μM BHT to a second portion of the fresh LDL preparation before dialysis. This mixture was dialyzed at 4°C for 16 h against 400 volumes of 0.15 M NaCl saturated with BHT and sterilized by filtration. BHT-LDL was incubated at 37°C for the same number of hours as oxidized-LDL. LDL preparations were characterized by electrophoresis in an agarose gel (Noble, 1968). Total cholesterol was measured by an
established procedure (Witte et al., 1974). All LDL solutions were used immediately after their preparation.

**Labeling of Cellular AA**

AA labeling was initiated in SMC cultures that were subconfluent. The cells were seeded at $1.3 \times 10^4$ cell/cm$^2$ in Corning 35 mm plates. After cells were grown for 3 or 4 days, SMC conditioned medium was obtained by collecting medium from some of the plates and centrifuging the medium at 2000 rpm for 3-5 min to remove any debris. $[^{14}\text{C}]$-AA in ethanol was evaporated to dryness and re-dissolved in SMC conditioned media. Two hundred μl of the labeled AA solution was added to subconfluent SMC. The AA concentration in the media was 0.625 μM. Media were collected at specific time intervals and the rate of AA uptake was measured. Each plate was rinsed twice with the same volume of experimental medium. In some experiments, the cells were seeded at $1.3 \times 10^4$ cell/cm$^2$ in Corning 25 T-flasks. After cells were grown for 3 or 4 days, media was aspirated, 1μM or 8 μM of $[^{14}\text{C}]$-AA added and the incubation continued for another 24 h. At the end of incubation period, media was collected, and each flask was rinsed twice with the same volume of experimental media. A 0.5 ml aliquot was taken from the media and each wash, mixed with scintillation solution and counted. The quenching factor was around 4 percent with 0.5 ml of medium. Counts were not corrected by this quenching factor. The radioactivity that disappeared from the media was assumed to be taken up by the cells. This radioactivity was divided by the amount of label AA originally added to the culture and AA uptake was then expressed as percent.
Cellular Lipid Extraction

Cellular lipids were extracted by a modification of several published procedures (Pong et al., 1977). After an incubation of 16 h, media were collected and the plate was washed twice with experimental medium. Washing removed any free fatty acid that remained attached to the cells. The cells were quick-frozen over dry ice. One and a half ml of 70 percent methanol in water was added. The cells were detached with a rubber policeman and transferred to a flask. The plate was rinsed three times with 1.0 ml of methanol, and the rinses were added to the cell suspension. Nine ml of chloroform was added to the flask. The mixture was extracted with periodic shaking at 4°C for approximately 3 h. The suspension was then passed through a glass wool plug. The extract was evaporated to a small volume in a Buchi evaporator. The wall of the flask was washed with 10 ml of methanol and the extract was evaporated to dryness. The residue was dissolved in chloroform, and this solution was filtered through a 0.20 µm membrane filter. The flask was washed 5 times with about 2 ml portions of chloroform and all wash solutions were filtered and collected. Chloroform was evaporated with N₂ and the residue was dissolved in 3.0 ml of chloroform. An aliquot of the solution was counted. The extraction efficiency was expressed as the total cpm in the cell extract relative to the cpm taken up by the cells during the incubation period. The extraction efficiency was 90.8 ± 3.3 percent (mean ± SEM, n=8).
Separation of Phospholipids by TLC

Phospholipid classes were separated by thin layer chromatography (TLC) on Whatman LK 5D plates (Whatman Inc., Clifton, NJ). The developing solvent consisted of chloroform-methanol-40 percent methylamine (60: 20: 5, v/v) (Hajlagapiou and Spector, 1987). Phospholipid standards PI, LPC, Sph, PC, PE were well separated. PS was not separated from PA. Neutral lipids moved near the solvent front in this system. Standards were also added to some labeled cell extracts, and lipids were visualized under u.v. light after spraying with 1 mM 6-P-toluidino-2-naphthalenesulfonic acid (TNS) in buffer solution (Johns, 1982). Labeled phospholipids extracted from cells were detected by a radioscan. The peaks in the radiochromatogram coincided with bands visualized under u.v. light. Bands were scraped and counted. The distribution of labeled AA in the different lipid fractions was calculated as percent of total cpm recovered from the whole TLC plate.

Assay of Total Labeled Metabolite Release

Confluent SMC prelabeled with $[^{14}\text{C}]$ AA were washed twice with experimental media and refed with fresh experimental media containing different agents. The cells were incubated at 37°C for 24 h. At the end of the incubation period, the media were withdrawn and the plate or flask was rinsed once with an equal amount of saline. The media and saline wash were centrifuged at 2000 rpm for 3-5 min and transferred to separate tubes. Aliquots, 0.5 ml, of media and saline wash were counted. The total radioactivity in media and wash was divided by the
total label in the cells and expressed as percent of AA metabolites released from the cells.

**Extraction of \([^{14}\text{C}]\)-Metabolites from Media**

The \([^{14}\text{C}]\)-AA metabolites in the culture media were extracted by a method modified from Unger et al. (1971) and Morisaki et al. (1984). One part containing equal volumes of media and rinse was mixed with two parts of ethanol. The mixture was acidified to pH 3-4 with 88 percent (wt/wt) formic acid. Three parts of H$_2$O or 0.15 M NaCl were added. This mixture was extracted three times with 2 volumes of ethylacetate. The upper phases were combined and evaporated in a Buchi evaporator. Five or ten ml of methanol was added to wash the flask and this solution was evaporated to dryness. The residue was suspended in 0.2-0.4 ml of a mixture containing equal volumes of ice cold acetonitrile and H$_2$O: methanol (100:5 v/v) and filtered through a 0.20 \(\mu\)m membrane filter. This procedure was repeated 5 times and the filtrates combined. Ten ml of methanol was added and the mixture was evaporated to dryness, re-dissolved in 1 ml of methanol and filtered. The recoveries of radioactive 6-keto-PGF$_{1\alpha}$, PGE$_2$ and AA were consistently greater than 90 percent when each compound was added to culture media.

**High Performance Liquid Chromatography**

High performance liquid chromatography (HPLC) was used to isolate and to identify prostanoids and AA. The HPLC procedure was modified from Peters et al. (1983), Rollins et al. (1982) and Morisaki et al.
(1984) using a Beckman model 334 chromatograph which consisted of dual Beckman Model 110A pumps (A and B), Altex 210A injector with a 20 μl loop, Beckman 421 controller, Beckman 164 variable wavelength detector, and an Altex Model C-RIA integrator. HPLC was performed at room temperature on a 250 x 4.6 mm Ultrasphere-ODS (5μm particles) reversed-phase column preceded by a 35 x 4.6 mm guard column packed with ODS (5μm) (Beckman, Irvine, CA).

The elution solvents for HPLC consisted of various mixtures of acetonitrile-aqueous phosphoric acid (pH 2). Solvent compositions were: Solvent A, HPLC grade water pH = 2 adjusted by phosphoric acid; Solvent B₁; acetonitrile; and solvent B₂, 3 percent isopropanol in acetonitrile. The flow rate of elution solvents was 1.0 ml/min. The elution steps were as follows: time 0 to 14 min, 36.7 percent solvent B₁; time 14 to 41 min, 60 percent solvent B₁; time 41 min to the end, 90 percent solvent B₂. At 34 min, solvent B₁ was changed to solvent B₂. Fractions were collected at 0.5 min or 1.0 min intervals, and the radioactivity of each fraction was counted and graphed. Retention times decreased with extensive column usage.

Statistics

Data are reported as mean ± SEM. The significance of differences in a treatment series was determined by a one-way analysis of variance (F-ratio). Individual treatments were compared with the control by the Tukey-HSD test (sample sizes equal), the Scheffe test (sample sizes unequal) or the Student t test when only one treatment group was involved.
RESULTS

Characteristics of SMC

The typical SMC at confluence were spindle-shaped with centrally located nuclei. They had large nuclei, 2 to 6 prominent nucleoli and a granular area. Electron microscopy showed that SMC contained myofilaments (Miller et al., 1980). FDA-PI stain showed that confluent SMC cultures contained only viable cells. SMC were identified in these cultures by their reactivity to antibodies against human umbilical artery F-actin which was shown to react specifically with muscle actin isoforms (Stauch et al., 1984). A typical experiment (Plate I) showed that almost all cells in confluent cultures were SMC.

Effects of CsA and LPS on Prostanoid Synthesis in Confluent SMC

Confluent cultures of SMC were used to test the effects of CsA and LPS on prostanoid synthesis. Cultures were treated with various amounts of CsA ranging from 10 to 100 μM and LPS ranging from 1 to 50 μg/ml. After a 24 h incubation period media were removed and the amounts of PG12 (measured as 6-keto-PGF1α) and PGE2 were determined by RIA. Both CsA and LPS significantly stimulated prostanoid synthesis in
Plate I

Reactivity of confluent SMC to fluorescent antibodies prepared against human umbilical artery F-actin.
a dose dependent manner. The results are shown in Table 1. Maximal stimulation with CsA occurred with 50 μM CsA. Decreased prostanoid synthesis was observed with 100 μM CsA. Detached and floating SMC indicated that the high concentration of CsA was cytotoxic.

Stimulation with LPS reached a plateau at 25 μg/ml. LPS did not have a cytotoxic effect on SMC throughout the concentration range tested. LPS had a greater stimulatory effect than CsA on prostanoid synthesis (Table 1).

**Effects of CsA and LPS on Prostanoid Synthesis in MØ and Inhibitory Effects with Dexamethasone**

Freshly isolated MØ were incubated at 2 x 10⁶ cells/ml with either CsA or LPS. At the end of a 24 h incubation period, media were centrifuged to remove any cells. Prostanoid concentrations in the media were determined by RIA. Very little 6-keto-PGF₁α was found in the MØ. Both CsA and LPS significantly stimulated PGE₂ synthesis in MØ (Table 2).

Dexamethasone (DM), a steroid that inhibits phospholipase A₂ through the induction of an inhibitory protein, diminished the stimulatory effects of both CsA and LPS. The inhibitory effect of DM was greater with CsA than LPS (Table 2).
Table 1.
Effect of CsA or LPS on Prostanoid Synthesis in SMC\textsuperscript{a}

<table>
<thead>
<tr>
<th>Treatment</th>
<th>6-keto-PGF\textsubscript{1\alpha} (nmoles/culture)\textsuperscript{b}</th>
<th>PGE\textsubscript{2} (nmoles/culture)\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>CsA (\mu M)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.83 ± 0.054 (19)</td>
<td>0.62 ± 0.149 (14)</td>
</tr>
<tr>
<td>10</td>
<td>1.68 ± 0.098 (18)\textsuperscript{c}</td>
<td>0.83 ± 0.146 (14)</td>
</tr>
<tr>
<td>50</td>
<td>2.41 ± 0.072 (6)\textsuperscript{c}</td>
<td>1.98 ± 0.317 (4)\textsuperscript{c}</td>
</tr>
<tr>
<td>100</td>
<td>1.56 ± 0.220 (4)\textsuperscript{c}</td>
<td>1.03 ± 0.168 (4)</td>
</tr>
<tr>
<td>F ratio</td>
<td>42.309</td>
<td>6.646</td>
</tr>
<tr>
<td>P</td>
<td>0.0001</td>
<td>0.0013</td>
</tr>
</tbody>
</table>

LPS (\mu g/ml)

| 0 | 0.79 ± 0.049 (3) | 0.30 ± 0.035 (3) |
| 1 | 2.83 ± 0.117 (3)\textsuperscript{c} | 1.37 ± 0.032 (3)\textsuperscript{c} |
| 10 | 5.02 ± 0.489 (3)\textsuperscript{c} | 3.57 ± 0.154 (3)\textsuperscript{c} |
| 25 | 5.36 ± 0.070 (3)\textsuperscript{c} | 4.26 ± 0.205 (3)\textsuperscript{c} |
| 50 | 5.40 ± 0.351 (3)\textsuperscript{c} | 4.59 ± 0.256 (3)\textsuperscript{c} |
| F ratio | 53.638 | 133.227 |
| P | 0.0001 | 0.0001 |

\textsuperscript{a} Confluent cultures of SMC in 25 T-flask were incubated for 24 h with increasing concentrations of CsA or LPS. Prostanoids were estimated by RIA.
\textsuperscript{b} Mean ± SEM; number of experiments in parentheses.
\textsuperscript{c} Treatment groups differed significantly from media alone (Tukey HSD test and Scheffe test).
Table 2.
Effect of CsA or LPS on Prostanoid Synthesis in H0
and Inhibitory Effects with Dexamathasone^a

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PGE2 (ng/2 x 10^6 H0)b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CSA (μM)</strong></td>
<td><strong>DM (μg/ml)</strong></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0.01</td>
</tr>
<tr>
<td>5</td>
<td>0.05</td>
</tr>
<tr>
<td>5</td>
<td>0.10</td>
</tr>
<tr>
<td>5</td>
<td>0.30</td>
</tr>
<tr>
<td>5</td>
<td>1.20</td>
</tr>
<tr>
<td><strong>LPS (μg/ml)</strong></td>
<td><strong>DM (μg/ml)</strong></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>0.30</td>
</tr>
<tr>
<td>10</td>
<td>1.20</td>
</tr>
<tr>
<td>10</td>
<td>2.40</td>
</tr>
<tr>
<td>10</td>
<td>3.60</td>
</tr>
</tbody>
</table>

F ratio 124.445
P 0.0001

^a Suspension cultures of H0 (2 x 10^6 cells/ml) were incubated for 24 h with CsA and LPS and increasing concentrations of DM. PGE2 was estimated by RIA.
^b Mean ± SEM; number of experiments in parentheses.
^c Treatment groups differed significantly from media alone (Tukey HSD test).
^d Treatment groups differed significantly from LPS alone (Tukey HSD test).
Effects of Exogenous AA on the Stimulation of Prostanoid Synthesis in SMC and MØ with CsA and LPS

The stimulatory effect of CsA and LPS may be caused either by the enhanced conversion of endogenous AA to prostanoids or by an increase in the release of endogenous AA from phospholipids. Prostanoid synthesis in cultures challenged with either CsA or LPS and exogenous AA were compared with cultures challenged with CsA, LPS or AA alone to distinguish between these mechanisms. The results with SMC and MØ cultures are summarized in Tables 3 and 4. Exogenous AA alone increased prostanoid synthesis in both SMC and MØ (compare AA alone with baseline data in Tables 1 and 2). CsA in cultures challenged with AA failed to enhance further the synthesis of prostanoids in either SMC or MØ. However, LPS in cultures challenged with AA acted synergistically and increased the synthesis of prostanoids in both SMC and MØ. These data indicated that CsA was unable to potentiate the conversion of exogenous AA to prostanoids, and these data suggested that the mechanism responsible for increased prostanoid synthesis with CsA may be the increased release of endogenous AA from phospholipids by CsA. These data indicated that LPS was able to potentiate the conversion of exogenous AA to prostanoids, and these data suggested that the mechanisms responsible for increased prostanoid synthesis may be via increased cyclooxygenase activity.
Table 3.
Effect of Exogenous AA on the Stimulation of Prostanoid Synthesis in SMC with CsA or LPSa

<table>
<thead>
<tr>
<th>Treatment</th>
<th>6-keto-PGF1α (nmoles/culture)b</th>
<th>PGE2 (nmoles/culture)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>CsA (µM)</td>
<td>AA (µM)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>120</td>
<td>4.20 ± 0.232 (8)</td>
</tr>
<tr>
<td>10</td>
<td>120</td>
<td>4.39 ± 0.306 (8)</td>
</tr>
<tr>
<td>50</td>
<td>120</td>
<td>4.85 ± 0.413 (6)</td>
</tr>
<tr>
<td>100</td>
<td>120</td>
<td>4.21 ± 0.538 (4)</td>
</tr>
<tr>
<td>F ratio</td>
<td></td>
<td>0.730</td>
</tr>
<tr>
<td>P</td>
<td>N.S.</td>
<td></td>
</tr>
<tr>
<td>LPS (µg/ml)</td>
<td>AA (µM)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>120</td>
<td>4.70 ± 0.235 (3)</td>
</tr>
<tr>
<td>1</td>
<td>120</td>
<td>5.62 ± 0.245 (3)</td>
</tr>
<tr>
<td>10</td>
<td>120</td>
<td>9.33 ± 0.684 (3)c</td>
</tr>
<tr>
<td>25</td>
<td>120</td>
<td>9.96 ± 0.487 (3)c</td>
</tr>
<tr>
<td>50</td>
<td>120</td>
<td>11.13 ± 1.030 (3)c</td>
</tr>
<tr>
<td>F ratio</td>
<td></td>
<td>21.161</td>
</tr>
<tr>
<td>P</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

a Confluent cultures of SMC in 25 T-flask were incubated for 24 h with AA and increasing concentrations of CsA or LPS. Prostanoids were estimated by RIA.
b Mean ± SEM; number of experiments in parentheses.
c Treatment groups differed significantly from media (Tukey HSD test).
d Treatment groups differed significantly from 1 µg/ml LPS (Tukey HSD test).
Table 4.
Effect of Exogenous AA on the Stimulation of PGE₂ Synthesis in MØ with CsA or LPS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PGE₂ (ng/2 x 10⁶ MØ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CsA (µM)</td>
<td>AA (µM)</td>
</tr>
<tr>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td>0.5</td>
<td>18</td>
</tr>
<tr>
<td>2.5</td>
<td>18</td>
</tr>
<tr>
<td>5.0</td>
<td>18</td>
</tr>
<tr>
<td>15.0</td>
<td>18</td>
</tr>
<tr>
<td>0</td>
<td>45</td>
</tr>
<tr>
<td>0.5</td>
<td>45</td>
</tr>
<tr>
<td>2.5</td>
<td>45</td>
</tr>
<tr>
<td>5.0</td>
<td>45</td>
</tr>
<tr>
<td>15.0</td>
<td>45</td>
</tr>
<tr>
<td>LPS (µg/ml)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>P</td>
<td>&lt; 0.025c</td>
</tr>
<tr>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>P</td>
<td>&lt; 0.025d</td>
</tr>
</tbody>
</table>

a Suspension cultures of MØ (2 x 10⁶ cell/ml) were incubated for 24 h with AA and increasing concentrations of CsA or LPS. PGE₂ was estimated by RIA.
b Mean ± SEM; number of experiments in parenthesis.
c Student t test for unequal variances. t = 3.97, df = 3.11, p < 0.025.
d Student t test for unequal variances. t = 4.07, df = 3.00, p < 0.025.
Effects of Pre-Incubation with CsA or A23187 on Synthesis of Prostanoids in SMC Stimulated with LPS or TPA

A23187 is a well known ionophore that increases phospholipase A2 activity by enhancing the concentration of intracellular Ca++. 12-o-tetradecanoylphorbol-13-acetate (TPA) enhances AA release from phospholipids (Levine et al., 1984) and stimulates both cyclooxygenase and lipoxygenase pathways (Kato et al., 1984; Morisakei et al., 1985). If the endogenous AA pool is mobilized and depleted by phospholipase A2 activity, the subsequent synthesis of prostanoids with stimulatory agents will be decreased. CsA had no effect on prostanoid synthesis when cultures were incubated simultaneously with CsA and either LPS or TPA (Table 5). SMC cultures were pre-incubated for 24 h with either CsA or A23187, then treated with fresh media containing either CsA, LPS or TPA. The results in Table 5 demonstrated that pre-treatment with either CsA or A23187 diminished the subsequent stimulatory effect of LPS, TPA or CsA on prostanoid synthesis. Cells pre-treated with A23187 showed, like CsA, decreased prostanoid synthesis during subsequent treatment with TPA.

Uptake of $[^{14}C]$-AA by SMC in Culture

The foregoing studies suggested mechanisms by which CsA and LPS stimulated prostanoid synthesis in SMC and MØ. SMC labeled with small amounts of $[^{14}C]$-AA provided another model to study these mechanisms. Labeled AA at a final concentration of 0.625 μM was added to sub-confluent SMC without a media change and incubated for specific time intervals. The time course for AA uptake is shown in Fig. 5. AA uptake
**Table S.**

<table>
<thead>
<tr>
<th>Treatment I</th>
<th>6-keto-PGF$_1$α (molecules/culture)</th>
<th>Treatment II</th>
<th>6-keto-PGF$_1$α (molecules/culture)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CsA (μM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>$1.70 \pm 0.16$ (5)</td>
<td>10</td>
<td>$2.78 \pm 0.08$ (4)$^b$</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPS (μg/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>10</td>
<td>9.28 (2)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>11.98 (2)</td>
<td></td>
</tr>
<tr>
<td>TPA (μM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.01</td>
<td>8.35 (2)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.01</td>
<td>10.88 (2)</td>
<td></td>
</tr>
<tr>
<td>CsA (μM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1.50 (2)</td>
<td>0</td>
<td>2.62 (2)</td>
</tr>
<tr>
<td>10</td>
<td>2.67 (2)</td>
<td>10</td>
<td>1.89 (2)</td>
</tr>
<tr>
<td>LPS (μg/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1.67 (2)</td>
<td>10</td>
<td>10.39 (2)</td>
</tr>
<tr>
<td>10</td>
<td>2.77 (2)</td>
<td>10</td>
<td>10.80 (2)</td>
</tr>
<tr>
<td>TPA (μM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1.47 (2)</td>
<td>0.01</td>
<td>12.23 (2)</td>
</tr>
<tr>
<td>10</td>
<td>3.00 (2)</td>
<td>0.01</td>
<td>4.93 (2)</td>
</tr>
<tr>
<td>A23187 (μg/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.53 (2)</td>
<td>0.01</td>
<td>8.52 (2)</td>
</tr>
<tr>
<td>0.5</td>
<td>1.67 (2)</td>
<td>0.01</td>
<td>5.73 (2)</td>
</tr>
<tr>
<td>1.0</td>
<td>1.99 (2)</td>
<td>0.01</td>
<td>3.91 (2)</td>
</tr>
</tbody>
</table>

$^a$ Confuent cultures of SMC ($1.1 \times 10^6$ cells/culture) were incubated for 24 h with the specified agent(s) and the prostanoïd was measured by RIA. In Treatment II, cultures from Treatment I were incubated for an additional 24 h in fresh media with the specified agent(s) and the prostanoïd was measured by RIA.

$^b$ CsA differed significantly (one-tailed t test).
Fig. 5. Time course for the uptake of AA by SMC challenged with 0.625 μM [14C]-AA. Data are expressed as mean ± SEM.
was rapid during the initial 6 h period and reached a plateau at 16 h. The addition of unlabeled AA at the same concentration, 0.625 μM, had little effect on the basal synthesis of PGE₂ by SMC as measured by RIA [control, 0.038 ± 0.008 nmol/plate (n = 6); control + 0.625 μM AA, 0.046 ± 0.006 nmole/plate (n = 8); mean ± SEM].

Separation of Phospholipids by TLC

Figure 6 showed that standards of major phospholipids were separated by TLC. The separation of major phospholipid classes by TLC is shown in Fig. 6. PS was not separated from PA in this system. The neutral lipid (NL) trilinolenin moved near the solvent front.

Distribution of [¹⁴C]-AA in Major Lipids of SMC

The distribution of [¹⁴C]-AA in different lipid classes is shown in Figure 7 and Table 6. Subconfluent SMC were incubated with 0.625 μM [¹⁴C]-AA for 16 h and cellular lipids were extracted and separated by TLC. Most of the radioactivity was found in phospholipids, especially in PI, PC and PE. Only 7.1 percent of the labeled AA was incorporated into neutral lipids. The data are consistent with previous studies (Flower and Blackwell, 1975; Spector et al., 1983; Levine and Alam, 1981).

Release of [¹⁴C]-AA Metabolites from labeled SMC with CsA, LPS and A₂₃₁₈₇

The release of [¹⁴C]-AA and its metabolites from the cellular lipids of SMC challenged with CsA, LPS and A₂₃₁₈₇ in the presence or in
TLC of major phospholipids. Lanes 1 to 6 and 8 contained individual phospholipids (Lane 2 contained PS and Lane 3 contained PA). Lane 7 contained PE, PC and Sph. Lane 9 was a mixture of Trilinolenin, PE, PC, Sph, LPC, PS and PI. Each standard compound was spotted on the Whatman LK5D plate. The plate was developed in a solvent consisting of CHCH₃ : CH₃OH : 40 percent CH₃-NH₂ (60 : 20 : 5, v/v). The bands were visualized under u.v. light and marked after spraying with TNS.
Fig. 6
Fig. 7

The separation of cellular lipids by TLC. Subconfluent SMC were incubated with 0.625 \( \mu \text{M} \) \(^{14}\text{C}\)-AA for 16 h. After cellular lipids were extracted, the sample and standards were loaded on the plate and separated by TLC (TLC system used as in Fig. 6). A shows the TLC plate scanned for radioactivity, and B shows bands visualized by u.v. after spraying with TNS.
Fig. 7
Table 6.

Distribution of $^{14}$C-AA in SMC Lipids$^a$

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Radioactivity (%)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Origin</td>
<td>$1.9 \pm 0.4 (6)$</td>
</tr>
<tr>
<td>PI</td>
<td>$15.2 \pm 1.1 (6)$</td>
</tr>
<tr>
<td>PS + PA</td>
<td>$2.0 \pm 0.1 (6)$</td>
</tr>
<tr>
<td>LPC</td>
<td>$1.5 \pm 0.1 (6)$</td>
</tr>
<tr>
<td>Sph</td>
<td>$1.2 \pm 0.2 (6)$</td>
</tr>
<tr>
<td>PC</td>
<td>$23.1 \pm 0.7 (6)$</td>
</tr>
<tr>
<td>PE</td>
<td>$44.9 \pm 1.1 (6)$</td>
</tr>
<tr>
<td>NL</td>
<td>$7.1 \pm 0.4 (6)$</td>
</tr>
</tbody>
</table>

$^a$ Subconfluent cultures of SMC were incubated with 0.625 $\mu$M $^{14}$C-AA for 16 h. Cellular lipids were extracted and separated by TLC. Bands were scraped and counted. The distribution of labeled AA was calculated as the percent of total label recovered on the TLC plate.

$^b$ Mean $\pm$ SEM; number of experiments in parentheses.
the absence of the cyclooxygenase inhibitor IM is reported in Table 7 and 8. CsA, LPS, and A23187 all stimulated the release of radioactivity from the cells during a 24 h incubation period.

The cyclooxygenase inhibitor IM had no effect on the release of radioactivity during incubations with CsA and A23187. However, IM diminished the release of radioactivity during incubations with LPS. These data support the data obtained by RIA which suggest that CsA and A23187 act by stimulating fatty acid release and LPS acts by stimulating cyclooxygenase activity.

Identification of [14C]-Metabolites by HPLC

Labeled AA metabolites released into the media by SMC (summarized in Table 7) were extracted and identified by HPLC. The separation of reference compounds is shown in Fig. 8. Control cultures of SMC released labeled 6-keto-PGF1α, PGE2 and AA into the media (Fig. 9-A). The cultures of SMC synthesized more 6-keto-PGF1α than PGE2, and the ratio of 6-keto-PGF1α to PGE2 was 2.4 (Table 9). CsA and A23187 stimulated prostanoid synthesis and did not affect the ratio of 6-keto-PGF1α to PGE2 (Fig. 9 and Table 9). The stimulatory effect of LPS was greater than either CsA or A23187, and equivalent amounts of 6-keto-PGF1α and PGE2 were formed in the presence of LPS (Fig. 10 and Table 9). More free [14C]-AA was released by CsA and A23187 than by LPS (Fig. 9 and 10). IM totally blocked the prostanoid synthesis (Fig. 11 and 12). The release of free [14C]-AA was almost doubled, and increased amounts of identified peaks with retention time between 30
Table 7.
Radioactivity Recovered from Media when SMC were Labeled with [U-\(^{14}\)C]-AA and then Incubated with IM, CsA, LPS or A\(_{23187}\)\(^a\)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Radioactivity in media(^b) (% of cellular label)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IM (µM)</td>
</tr>
<tr>
<td>0</td>
<td>4.0 ± 0.3 (4)</td>
</tr>
<tr>
<td>10</td>
<td>2.0 (2)</td>
</tr>
<tr>
<td>CsA (µM)</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>9.7 ± 0.8 (4)</td>
</tr>
<tr>
<td>50</td>
<td>6.0 (2)</td>
</tr>
<tr>
<td>A(_{23187}) (µg/ml)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>21.0 ± 3.7 (3)</td>
</tr>
<tr>
<td>1</td>
<td>30.2 (1)</td>
</tr>
<tr>
<td>LPS (µg/ml)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>17.4 ± 0.6 (4)</td>
</tr>
<tr>
<td>10</td>
<td>2.7 (2)</td>
</tr>
</tbody>
</table>

\(\^{a}\) [U-\(^{14}\)C]-AA (0.625 µM) labeled SMC in 35 mm plates were incubated for 24 h with fresh media containing the specified agent(s). Radioactivity in the media was measured and reported as percent of the initial cellular radioactivity.

\(\^{b}\) Mean ± SEM; number of experiments in parenthesis.
Table 8.
Radioactivity Recovered from Media when SMC were Labeled with [1-\textsuperscript{14}C]-AA and then Incubated with IM, CsA, or LPS or A\textsubscript{23187}\textsuperscript{a}

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Radioactivity in media\textsuperscript{b} (% of cellular label)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IM (\textmu M)</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>CsA (\textmu M)</td>
</tr>
<tr>
<td></td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>A\textsubscript{23187} (\textmu g/ml)</td>
</tr>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>LPS (\textmu g/ml)</td>
</tr>
<tr>
<td></td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>10</td>
</tr>
<tr>
<td>F ratio</td>
<td>45.096</td>
</tr>
<tr>
<td>P</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

\textsuperscript{a} [1-\textsuperscript{14}C] AA (1 \textmu M) labeled SMC in 25 T-flasks were incubated for 24 h with fresh media containing the specified agent(s). Radioactivity in the media was measured and reported as percent of the initial cellular radioactivity.

\textsuperscript{b} Mean ± SEM; number of experiments in parentheses.

\textsuperscript{c} Treatment groups differed significantly from media alone (Scheffe test for unequal n).

\textsuperscript{d} IM differed significantly (Scheffe test for unequal n).
Fig. 8. The separation of standard prostanoids and AA by HPLC. A mixture containing each compound was applied to the column. The separation procedure was described in the Methods.
HPLC analysis of $[^{14}C]$ labeled prostanoids and free AA released from SMC by CsA or A$_{23187}$. SMC were labeled by incubation with 0.625 μM [U-$^{14}C$]-AA for 16 h and were then incubated for an additional 24 h with fresh media containing CsA or A$_{23187}$. Labeled materials in media were extracted and separated by HPLC. HPLC fractions were collected, counted and graphed.
Fig. 9
Table 9.
The Ratio of 6-keto-PGF₁α to PGE₂ in SMC with CsA, A₂₃₁₈₇ or LPS.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ratio of 6-Keto-PGF₁α/PGE₂&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.4 (n = 3)</td>
</tr>
<tr>
<td>CsA (50μM)</td>
<td>3.0 (n = 2)</td>
</tr>
<tr>
<td>A₂₃₁₈₇ (1μg/ml)</td>
<td>2.6 (n = 2)</td>
</tr>
<tr>
<td>LPS (10μg/ml)</td>
<td>1.0 (n = 2)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data were calculated from cpm of the peaks separated by HPLC. See legend in Fig. 9 for the procedure. Number of HPLC experiments in parenthesis.
Fig. 10. HPLC analysis of labeled prostanoids and free AA released from SMC by LPS. See legend in Fig. 9 for the procedure.
Fig. 11. HPLC analysis of labeled prostanoids and free AA released from SMC by CsA in the presence or the absence of IM. See legend in Fig. 9 for the procedure.
Fig. 12.

HPLC analysis of labeled prostanoids and free AA released from SMC by A23187 or LPS in the presence or in the absence of IM. See legend in Fig. 9 for the procedure.
Fig. 12

Retention Time (min)

Radioactivity (cpm)

C: 10 mg/ml LPS

With 10xIM

Without IM

Radioactivity (cpm)

B: 1 mg/ml LPS

Radioactivity (cpm)

A: Control

With 10xIM

Without IM

G: 10 mg/ml LPS
and 40 min were found with CsA and A_{23187} in the presence of IM (Fig. 11 and 12). The release of free [\textsuperscript{14}C]-AA was also increased in the presence of LPS and IM, but the unidentified peaks with retention time between 20 and 30 min found with LPS were completely blocked by IM (Fig. 12). HPLC data again demonstrated that CsA behaved like A_{23187} in stimulating release of AA and increasing prostanoid synthesis. LPS, unlike CsA and A_{23187}, had little effect on the release of free AA, but LPS had a dramatic effect on cyclooxygenase as shown by the very large enhancement in prostanoid synthesis and by the change in the ratio of 6-keto-PGF_{1\alpha} to PGE_{2}. 
DISCUSSION

Since prostanoids are not stored in cells (Piper and Vane, 1971; Vogt, 1978), their release is preceded by synthesis. Stimulatory agents act at different points in the AA cascade leading to increased prostanoid synthesis. Cellular AA is found predominantly in the phospholipids of cell membranes (Derken and Cohen, 1975), and cyclooxygenase does not catalyze the synthesis of prostanoids from esterified AA (Lands and Samuelsson, 1968; Vonkeman and Dorp, 1968). Free AA release from phospholipids controls prostanoid synthesis through substrate availability. Prostanoid synthesis is also regulated by cyclooxygenase activity, but the effects at this control point generally involve the inhibitory regulation with enzyme inhibitors such as aspirin and indomethacin (Vane, 1971).

Our results demonstrated that prostanoid synthesis in both SMC and MØ increases when cells are challenged with LPS, CsA and A23187 (Tables 1 and 2). SMC incubated with media alone (control cultures) release both 6-keto-PGF_1α and PGE_2. MØ in control cultures mainly release PGE_2, and the amount of 6-keto-PGF_1α is not detectable by RIA.

Enhanced prostanoid synthesis in SMC is dose dependent in cells challenged with both CsA and LPS. However, high concentrations of CsA fail to increase prostanoid synthesis since CsA is cytotoxic as shown by the detachment of adherent cells from the cultures. CsA like A23187 increases the synthesis of both 6-keto-PGF_1α and PGE_2. LPS unlike CsA and A23187 preferentially increases PGE_2 synthesis (Table 9). The stimulatory effect obtained with LPS is much greater than the stimulatory effects obtained with CsA or A23187. The studies described above
suggest that LPS acts at a different control point than either CsA or A23187 in the AA cascade. The data supporting these mechanisms are summarized in Table 10.

Studies with exogenous AA help to elucidate the different mechanisms by which CsA and LPS increase prostanoid synthesis. The failure of CsA to increase the conversion of exogenous AA to prostanoids in either SMC or MØ suggests that CsA effect is focused on the release of endogenous AA from phospholipids. The increased availability of free AA from either an endogenous or exogenous source apparently increases prostanoid synthesis. Exogenous AA saturates the cyclooxygenase complex and additional free AA released through the action of CsA has no effect on prostanoid synthesis (Table 10). The synergy between exogenous AA and LPS (Table 10) suggests that LPS effect is focused on the stimulation of cyclooxygenas activity. Thus activated enzymes lead to dramatically enhanced prostanoid synthesis in the presence of exogenous AA.

Differences between the mechanisms by which CsA and LPS stimulate prostanoid synthesis are also distinguished by studies in which SMC are labeled with radioactive AA. Media from the incubation of cultures with labeled SMC contains free AA and a number of AA metabolites including prostanoids. Agents which enhance AA release from cellular phospholipid may increase the total radioactivity in the media by increasing the level of free AA and its metabolites. Agents which enhance cyclooxygenase activity may increase the total radioactivity in the media mainly by stimulating the formation of prostanoids and related metabolites.
Table 10

The Summary of Data with CsA, A23187 or LPS

<table>
<thead>
<tr>
<th></th>
<th>CsA</th>
<th>A23187</th>
<th>LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG Synthesis</td>
<td>+a</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Exogenous AA</td>
<td>NE</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>DM</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>$[^{14}C]$-Release (no IM)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>$[^{14}C]$-Release (+IM)</td>
<td>+</td>
<td>+</td>
<td>NE</td>
</tr>
<tr>
<td>6-keto-PGF$_{1a}$/PGE$_2$</td>
<td>3.0</td>
<td>2.6</td>
<td>1.0</td>
</tr>
<tr>
<td>Free $[^{14}C]$-AA (+IM)</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>

* +, increase effect; -, decrease effect; NE, no effect.*
Total radioactivity in the media is increased when cells are challenged with CsA, A23187 and LPS (Tables 7 and 8). However, total radioactivity is unaffected when IM is added to cells challenged with CsA and A23187, and on the other hand total radioactivity is greatly diminished when IM is added to cells challenged with LPS (Tables 7 and 8). These data are consistent with a mechanism involving the action of CsA and A23187 on AA release from cellular phospholipids and a second mechanism involving the action of LPS on the cyclooxygenase and related enzyme systems.

Lipid X, an acidic phospholipid-like derivative of LPS, stimulates the release of labeled AA and PGE$_2$ from a macrophage-like tumor cell pre-labeled with tritiated AA (Wightman and Raetz, 1984). In these studies Lipid X and A23187 have similar properties. However, Lipid X is an acidic phospholipid-like material that binds to Ca$^{++}$, and it is not unexpected that this moiety of LPS behaves like A23187. Intact LPS and A23187 have very different effects on pre-labeled SMC with [$^{14}$C]-AA (see above).

Further evidence for differences between the effects of CsA and LPS on AA metabolism is provided by experiments in which labeled metabolites in the media are identified by HPLC. In these experiments, SMC incubated in media alone have a small amount of labeled free AA and large amounts of labeled prostanoids in media isolated at the end of the incubation period (Fig. 9-A). The 6-keto-PGF$_{1\alpha}$ to PGE$_2$ ratio is 2.4 in these studies (Table 9). Increased amounts of labeled AA, 6-keto-PGF$_{1\alpha}$, PGE$_2$ and a number of unidentified labeled AA metabolites are found when the cells are challenged with CsA and A23187 (Fig. 9).
Furthermore, the 6-keto-PGF₁α to PGE₂ ratio is similar to the control. IM blocks the formation of 6-keto-PGF₁α and PGE₂, but the amounts of labeled AA and other AA metabolites are increased (Figs. 11 and 12). Two peaks with retention time between 30 and 40 min are characteristic of cells challenged with CsA and A₂₃₁₈₇, and these peaks are enhanced with IM. These data are consistent with an increase in total radioactivity due to the release of AA and its subsequent metabolism to a number of labeled products.

Cells challenged with LPS behave in a very different manner from cells challenged with CsA and A₂₃₁₈₇. Media from cells with LPS contain very large and equal amounts of labeled 6-keto-PGF₁α and PGE₂ (Figs. 10 and 12). Furthermore, media from these cells contain new metabolites with peaks that have retention time between 20 and 30 min (Fig. 10 and 12). The synthesis of these metabolites is inhibited by IM (Fig. 12). Finally, media from cells challenged with LPS do not contain the two characteristic IM insensitive peaks previously noted in media from cells challenged with CsA and A₂₃₁₈₇ (Figs. 11 and 12). The LPS data are consistent with an increase in total radioactivity due to enhanced activity of the cyclooxygenase complex.

Differences between LPS and both CsA and A₂₃₁₈₇ are summarized in the schematic diagram (Fig. 13). This diagram shows the points where CsA, A₂₃₁₈₇ and LPS can affect AA metabolism in ways that are consistent with the data obtained in this study. The mechanisms described in this diagram are consistent with other preliminary studies from this laboratory on enhanced PGE₂ synthesis in Mφ treated with CsA and LPS (Lindsey et al., 1983; Whisler et al., 1984).
Fig. 13. Schematic diagram showing the points where CsA and LPS affect prostanoid synthesis.
CsA not only increases PGE\(_2\) synthesis in MØ, but also diminishes HLA-DR expression by these cells (Whisler et al., 1984, 1985). These effects are causally related since IM diminishes PGE\(_2\) synthesis in MØ challenged with CsA and these cells continue to express HLA-DR. Thus enhanced PGE\(_2\) synthesis in MØ treated with CsA may help to explain the immuno-suppressive effects of CsA (see section of cell-mediated immunity in the Review of Literature).

The data that LPS increases MØ and SMC prostanoid synthesis support many previous reports with MØ and platelets (Kurland and Bockman, 1978; Feuerstein et al., 1981; Whisler et al., 1985) although in one study Aderem et al. (1986) showed that LPS alone did not increase prostanoid synthesis in mouse peritoneal macrophages. The data from Feuerstein et al. (1981) showed that the 6-keto-PGF\(_1\alpha\) to PGE\(_2\) ratio decreased from 1.63 in control to 0.97 in LPS treated group in rat peritoneal MØ although they did not calculate this ratio in their studies. Our observations are consistent with the data from Feuerstein et al. (1981).

LPS acts predominantly through enhanced cyclooxygenase activity in promoting prostanoid synthesis. The overall increase in radioactivity evidently occurs because labeled AA is shunted into prostanoid metabolites and not re-esterified into cellular phospholipids. Thus CsA, A\(_{23187}\) and LPS all increase the total radioactivity of the media but they act through different mechanisms that generate different metabolite profiles.
EFFECTS OF OXIDIZED LDL ON CELL VIABILITY AND PROSTINOID METABOLISM IN SMC

RESULTS

Preparation of LDL Solutions

LDL solutions were prepared with different levels of autoxidized lipid. LDL preparations, isolated from individual sera and dialyzed at 4°C against 0.15 M NaCl, contained 0.7 ± 0.1 nmol MDA/200 µg LDL cholesterol (mean ± SEM). These LDL preparations were further oxidized at different rates when they were incubated in 0.15 M NaCl at 37°C to yield oxidized-LDL. However, the same degree of lipid peroxidation was achieved, 1.9 ± 0.1 nmol MDA/200 µg LDL cholesterol, when oxidation was continued only until the disappearance of the yellow carotenoid color in the oxidized-LDL preparation (Table 11 and Fig. 14). Oxidation was not affected by IM. Since LDL contains 30.8 percent cholesterol and 22.4 percent protein (Oncley et al., 1957), the MDA content of oxidized-LDL with 1.9 ± 0.1 mmole MDA/200 µg cholesterol was 2.6 ± 0.1 nmol MDA/200 µg protein. Thus MDA values for this oxidized-LDL, measured by a sensitive TBA assay that employs a Fe³⁺ catalyst (Morisak, et al., 1984; Gavino et al., 1981b), are comparable to MDA values that have been reported by other investigators for control LDL preparations that were not oxidized with metal ions or cells (Masana et
<table>
<thead>
<tr>
<th>Serum Number</th>
<th>Oxidation time in h</th>
<th>MDA Oxidized-LDL</th>
<th>MDA BHT-LDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>13</td>
<td>2.0</td>
<td>0.2</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>1.6</td>
<td>0.3</td>
</tr>
<tr>
<td>3</td>
<td>24</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>1.6</td>
<td>0.1</td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>1.9</td>
<td>0.2</td>
</tr>
<tr>
<td>6</td>
<td>12</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>14</td>
<td>1.9</td>
<td>0.2</td>
</tr>
<tr>
<td>8</td>
<td>28</td>
<td>1.6</td>
<td>0.1</td>
</tr>
</tbody>
</table>

a LDL, isolated from individual sera, was either dialyzed in the presence of BHT (BHT-LDL) or dialyzed in the absence of BHT (oxidized-LDL). Both preparations were incubated at 37°C until the carotenoid color disappeared in the preparation that did not contain BHT. TBAR materials were measured and are reported as nmol MDA/200 μg LDL cholesterol.
Fig. 14. LDL from individual sera is oxidized in the absence of BHT at different rates. Three LDL preparations (A, B, and C) isolated at same time were dialyzed in the absence of BHT and then incubated at 37°C. TBAR materials were measured at incubation times of 5, 10, and 15 h and are reported as nmol MDA/200 µg LDL cholesterol.
Mild oxidation was confirmed by electrophoresis. The relative electrophoresis mobility was 1.1 for oxidized-LDL with 1.9 nmol MDA/200 μg cholesterol compared to freshly isolated LDL with 0.7 nmol MDA/200 μg cholesterol. Highly oxidized-LDL preparations have much greater electrophoretic mobilities (Masana et al., 1986; Heinecke et al., 1986; Morel et al., 1984; Parthasarathy et al., 1986; Steinbrecher et al., 1985). BHT-LDL was prepared by the addition of 100 μM BHT to an aliquot of the LDL preparation at the end of the second ultracentrifugation step in the isolation procedure. This sample was dialyzed against 0.15 M NaCl saturated with BHT and then incubated at 37°C for the same time as another aliquot of the LDL preparation that did not contain BHT. Oxidation data for the BHT-LDL preparations are reported in Table 11. BHT-LDL which contained 0.2 ± 0.03 nmol MDA/200 μg cholesterol was similar in MDA content to other BHT-LDL preparations reported in the literature (Morel et al., 1984; Steinbrecher et al., 1984). The relative electrophoretic mobility was 1.0 for BHT-LDL compared to freshly isolated LDL.

**Immunoreactive Products of LDL Preparations**

Since prostanoids are synthesized through cyclic endoperoxides and lipid autoxidation forms a number of acyclic and cyclic peroxides (Pryor et al., 1976; Porter, 1986), the cross-reactivity of autoxidation products with antibodies to PGE₂ and 6-keto-PGF₁α was measured in different LDL preparations (Table 12). LDL preparations
Table 12
Prostanoid Cross-Reactivities with Antibodies in LDL Preparations with Different Levels of TBAR Material

<table>
<thead>
<tr>
<th>LDL preparations</th>
<th>MDA (nmol/plate)\textsuperscript{b}</th>
<th>PGE\textsubscript{2} (nmole/plate)\textsuperscript{b}</th>
<th>6-keto-PGF\textsubscript{1\alpha} (nmole/plate)\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidized-LDL</td>
<td>4.3 ± 0.466 (6)</td>
<td>0.007 ± 0.001 (16)</td>
<td>0.002 ± 0.0001 (6)</td>
</tr>
<tr>
<td>After further oxidation in saline at 37°C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BHT-LDL</td>
<td>1.7 ± 0.748 (4)</td>
<td>0.003 ± 0.001 (15)</td>
<td>0.003 ± 0.0012 (3)</td>
</tr>
<tr>
<td>Oxidized-LDL</td>
<td>8.3 ± 0.483 (5)</td>
<td>0.037 ± 0.002 (18)</td>
<td>0.005 ± 0.0006 (14)</td>
</tr>
<tr>
<td>Oxidized-LDL (+IM)</td>
<td>7.7 (2)</td>
<td>0.032 ± 0.001 (8)</td>
<td>0.006 (2)</td>
</tr>
</tbody>
</table>

\textsuperscript{a} LDL concentration was adjusted to 800 \( \mu \)g/ml cholesterol, and immunoreactivity was measured immediately after the preparation or further oxidation of each LDL preparation.

\textsuperscript{b} Mean ± SEM nmol/plate (1.3 ml/plate); number of experiments in parenthesis.
all showed a low level of cross-reactivity to the 6-keto-PGF$_{1\alpha}$ antibody, which was not related to the MDA content of the LDL preparation and was not sensitive to 10 \( \mu \)M IM (Table 12). LDL preparations showed very high cross-reactivity to the PGE$_2$ antibody and this cross-reactivity varied with the MDA content of the LDL preparation (Table 12).

The amount of material cross-reacting with the PGE$_2$ antibody increased linearly, but the MDA contents remained unchanged (Fig. 15) when oxidized-LDL was added to experimental media at 37°C and incubated without cells for different time intervals. The total amount and rate of formation of cross-reacting material varied directly with the MDA content of the LDL preparations (Fig. 15). Data are shown in Figure 16 for a number of LDL preparations incubated without cells for 24 h. IM failed to block the formation of cross-reacting material during the 24 h incubation, and these data showed that the cross-reacting material was not formed from PGE$_2$ biosynthesis (Fig. 16). It is important to note that material with cross-reactivity to 6-keto-PGF$_{1\alpha}$ was not formed during the incubation period (Fig. 16).

**Effects of LDL on Morphology and Viability of SMC**

There is morphologic evidence of a dose-associated effect of oxidized-LDL on cell viability and proliferation. SMC in control cultures (no LDL) were spindle-shaped, polygonal or elongated with centrally located nuclei (Fig. 17-A). The majority of cells appeared to contain nuclei of similar size and staining characteristics. A
Fig. 15

Time course of production of PGE$_2$ immunoreactivity in oxidized-LDL preparations. The LDL concentration was adjusted to 800 μg/ml cholesterol and the LDL was incubated without cells for 0, 24, 48 and 72 h at 37°C. At the end of the incubation period, MDA and PGE$_2$ were measured. The data describe oxidized-LDL with a low initial MDA concentration (△–△) and oxidized-LDL with a high initial MDA concentration (●–○).
Fig. 15
Fig. 16

Prostanoid cross-reactivity in LDL preparations. LDL preparations with different MDA content were prepared, adjusted to 800 μg/ml cholesterol and incubated without cells for 24 h at 37°C in the presence or in the absence of 10 μM IM. RIA was measured at the end of incubation period.
Fig. 16

PROSTANOID (n mole/plate) vs MDA (n mole/plate)

-IM  +IM

6-keto-PGF$_{1\alpha}$  △  ▲
PGE$_2$  ○  ●

Fig. 16
Effect of oxidized-LDL on morphology and viability of SMC. SMC were incubated without oxidized-LDL (A and B), and with oxidized-LDL (8.3 ± 0.5 nmoles MDA/plate, 800 µg/ml cholesterol) in the absence (C and D) or in the presence (E and F) of 40 µM BHT. SMC were incubated for 24 h at 37°C. A, C and E are Giemsa stain and B, D and F are FDA-PI stain.
small proportion of the cells had very large nuclei suggesting polyploidy. Mitotic figures (indicated by arrows in Fig. 17-A) were evident and the mitotic index was estimated at 3 to 4 percent. Unfixed cells were stained with FDA-PI in order to discriminate between viable and non-viable cells. All cells in control cultures accumulated fluorescein as the result of FDA hydrolysis (Jones and Senft, 1985) and these cells gave the green fluorescence characteristic of viable cells (Fig. 17-B). SMC were also incubated with 200, 400 and 800 BHT-LDL cholesterol/ml and these lipoprotein preparations, which did not contribute (TBA-reactive materials, also had no effect on morphology, viability or the mitotic index. Oxidized-LDL with low MDA (4.33 ± 0.47 nmol/plate at 800 μg/ml) also had no effect on morphology and viability in SMC.

SMC cultures were also incubated for 24 h with 200, 400, and 800 μg/ml cholesterol of highly oxidized-LDL (8.27 ± 0.48 nmol MDA/plate at 800 μg/ml). Cultures incubated with 200 μg appeared similar to control cultures, whereas cultures incubated with 400 μg were similar to control cultures but did not show mitotic figures. Almost all cells in the 200 μg and 400 μg oxidized-LDL were viable (FDA-PI). Cultures incubated with 800μg oxidized-LDL were altered dramatically by the treatment. Cells retained a cellular outline, but their cytoplasm was shrunken and their refractive indices changed (Fig. 17-C). Almost all nuclei in these cultures were pyknotic (Fig. 17-C). These cells gave the red fluorescence (Fig. 17-D) that is characteristic of non-viable cells where PI accumulates and intercalates with nucleic acids to form a red fluorescent complex (Jones and Senft, 1985).
SMC cultures were also incubated with 400 μg/ml highly oxidized-LDL in the presence of 10, 20 and 40 μM BHT. 40 μM BHT maintained the mitotic figures in these cultures (data not shown). Lower concentrations of BHT, 10 and 20 μM had no effect on cultures incubated with 800 μg/ml oxidized-LDL. However, when the 800 μg/ml highly oxidized-LDL cultures were incubated with 40 μM BHT (Fig. 17-E), the cells appeared similar morphologically to control cultures but no mitotic figures were seen. The cells in these cultures also gave the green fluorescence characteristic of viable cells (Fig. 17-F). It is evident that 40 μM BHT diminished the level of TBAR materials sufficiently to maintain viability without restoring the mitotic index.

**Effect of BHT-LDL on Prostanoid Synthesis in SMC**

Confluent cultures of SMC were incubated for 24 h with increasing amounts of BHT-LDL and the release of immunoreactive prostanoids was measured in the media. BHT-LDL, a preparation which did not contain large amounts of MDA or immunoreactive PGE$_2$ materials and did not affect morphology and viability, had no effect on prostanoid levels in these cultures (Table 13).

**Effects of Oxidized-LDL on Prostanoid Synthesis in SMC**

Confluent cultures of SMC were incubated with increasing amounts of three oxidized-LDL preparations which contained different levels of MDA. Cultures were incubated in the presence or in the absence of 10 μM IM. Immunoreactive PGE$_2$ material was measured in the media after a 24 h incubation period. The results are shown in Figs. 18, 19 and 20.
Table 13.
Effect of BHT-LDL and IM on Prostanoid Synthesis in SMC\(^a\)

<table>
<thead>
<tr>
<th>BHT-LDL (µg/ml cholesterol)</th>
<th>6-keto-PGF(_{1α}) (nmol/plate)</th>
<th>PGE(_2) (nmol/plate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMC (No IM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.084 (2)</td>
<td>0.038 (2)</td>
</tr>
<tr>
<td>200</td>
<td>0.081 (2)</td>
<td>0.036 (2)</td>
</tr>
<tr>
<td>400</td>
<td>0.103 (1)</td>
<td>0.040 (2)</td>
</tr>
<tr>
<td>800</td>
<td>0.109 (2)</td>
<td>0.044 (2)</td>
</tr>
<tr>
<td>SMC (10 µM IM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.011 (2)</td>
<td>0.003 (2)</td>
</tr>
<tr>
<td>200</td>
<td>0.011 (2)</td>
<td>0.003 (2)</td>
</tr>
<tr>
<td>400</td>
<td>0.009 (2)</td>
<td>0.003 (2)</td>
</tr>
<tr>
<td>800</td>
<td>0.012 (2)</td>
<td>0.004 (2)</td>
</tr>
</tbody>
</table>

\(^a\) Increasing concentrations of BHT-LDL (0.88 mmol MDA/plate at 800 µg/ml cholesterol) were incubated for 24 h with confluent SMC cultures in 35 mm plates. Media were analyzed for 6-keto-PGF\(_{1α}\) and PGE\(_2\) by RIA.
Fig. 18. Effect of oxidized-LDL with the lowest content of TBAR material on PGE₂ synthesis in SMC. Confluent SMC were incubated with increasing amounts of oxidized-LDL for 24 h in the presence or in the absence of 10 μM IM. PGE₂-reactive material in the media were measured by RIA. PGEₜ, in the absence of IM; PGEIM in the presence of IM; PGE, the difference between PGEₜ and PGEIM.
Fig. 19. Effect of oxidized-LDL with the intermediate content of TBAR material on PGE\textsubscript{2} synthesis in SMC. See legend in Fig. 18 for details.
Fig. 20. Effect of oxidized-LDL with the highest content of TBAR material on PGE₂ synthesis in SMC. See legend in Fig. 18 for details.
Immunoreactivity toward the PGE\(_2\) antibody in the absence of IM is designated as PGE\(_T\). Immunoreactivity toward the PGE\(_2\) antibody in the presence of IM is designated as PGE\(_{IM}\). The difference between PGE\(_T\) and PGE\(_{IM}\) represents net biosynthesis of PGE\(_2\) from SMC. At the lowest MDA level (Fig. 18), PGE\(_T\) and PGE\(_{IM}\) both increased in SMC cultures incubated with increasing concentrations of oxidized-LDL. However, the difference between PGE\(_T\) and PGE\(_{IM}\) showed that a net increase in PGE\(_2\) biosynthesis occurred in these cultures.

Different results were obtained when cultures were incubated with LDL that had an intermediate MDA level (Fig. 19). PGE\(_T\) and PGE\(_{IM}\) again increased with increasing LDL concentration but the relative increase was greater in PGE\(_{IM}\) than PGE\(_T\). The data showed that PGE\(_2\) biosynthesis was unchanged in these cultures.

Cytotoxicity occurred when cells were incubated with LDL that had the highest MDA level. PGE\(_T\) and PGE\(_{IM}\) were nearly equal in these cultures (Fig. 20). These data showed that PGE\(_2\) biosynthesis was almost completely suppressed in cultures incubated with the most highly oxidized-LDL.

Oxidized-LDL affect PGI\(_2\) biosynthesis in the same way as they affect PGE\(_2\) biosynthesis. PGI\(_2\) data are more readily obtained than PGE\(_2\) data because oxidized lipids in LDL do not cross-react with 6-keto-PGF\(_{1\alpha}\) (Fig. 16 and Table 12). Data showing the effect of oxidized-LDL on PGI\(_2\) synthesis are summarized in Fig. 21. Oxidized-LDL with the lowest level of MDA increased 6-keto-PGF\(_{1\alpha}\) biosynthesis. Oxidized-LDL that had an intermediate MDA level had no effect on 6-keto-PGF\(_{1\alpha}\) biosynthesis. Oxidized-LDL with the highest MDA level decreased 6-keto-PGF\(_{1\alpha}\)
Fig. 21. Effect of oxidized-LDL with different TBAR content on 6-keto-PGF\(_{1\alpha}\) synthesis in SMC. Confluent SMC were incubated with increasing amounts of oxidized-LDL for 24 h. 6-Keto-PGF\(_{1\alpha}\) was measured by RIA.
biosynthesis. These data indicated that oxidized-LDL had both stimulatory (low MDA level) and inhibitory (high MDA level) effects on PGI\textsubscript{2} biosynthesis. The prostanoid level depended both on the degree of LDL oxidation and on the LDL concentration.

**Release of [\textsuperscript{14}C]-AA Metabolites from labeled SMC with Oxidized-LDL**

The release of [\textsuperscript{14}C]-AA and its metabolites from the cellular lipids of SMC challenged with oxidized-LDL containing different levels of MDA is reported in Table 14. Preparation of oxidized-LDL that did not affect cell morphology and viability (3.5 or 5.9 nmol MDA/plate) had no effect on metabolite release. Oxidized-LDL with the highest MDA content was cytotoxic and this LDL preparation enhanced the release of radioactive metabolites, an increase that may be attributed to cell death.

**Identification of [\textsuperscript{14}C]-Metabolites by HPLC**

Labeled AA metabolites released into the media by SMC were extracted and identified by HPLC. The separation of standard [U-\textsuperscript{14}C]-AA as a single peak is shown in Fig. 22-A. Control cultures of SMC released 6-keto-PGF\textsubscript{1\alpha}, PGE\textsubscript{2} and a small amount of AA (Fig. 22-B). Unlabeled exogenous AA, 30 \(\mu\)M, decreased the amount of labeled prostanoids released from SMC (Fig. 22-C). Unlabeled exogenous AA evidently competed with labeled endogenous AA as a substrate for prostanoid synthesis.
Table 14
Radioactivity Recovered from Media when SMC were Labeled with $[^{14}\text{C}]$-AA and then Incubated with Oxidized-LDL Preparations Containing Different TBAR Material Content $^a$

<table>
<thead>
<tr>
<th>Oxidized-LDL ($\mu$g/ml cholesterol)</th>
<th>MDA (nmole/plate)</th>
<th>Radioactivity in media (% of cellular label)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>3.8 ± 0.2 (7)</td>
</tr>
<tr>
<td>800</td>
<td>3.5 ± 0.4 (4)</td>
<td>4.9 ± 0.5 (5)</td>
</tr>
<tr>
<td>400</td>
<td>5.9</td>
<td>5.4 ± 1.3 (3)</td>
</tr>
<tr>
<td>800</td>
<td>11.9 ± 2.1 (4)</td>
<td>63.9 ± 45 (5)</td>
</tr>
</tbody>
</table>

$^a$ SMC in 35 mm plates were labeled with 0.625 $\mu$M $[^{14}\text{C}]$-AA for 16 h. $[^{14}\text{C}]$-AA labeled SMC were then incubated for an additional 24 h with fresh media containing LDL solutions with different amounts of TBAR material. Radioactivity in the media was measured and reported as percent of the initial cellular radioactivity.

$^b$ Mean ± SEM; number of experiments in parentheses.
Fig. 22

HPLC analysis of labeled prostanoids and free AA released from SMC. SMC labeled with 0.625 μM [U-\(^{14}\)C]-AA for 16 h were incubated for an additional 24 h with and without 30 μM exogenous cold AA. Labeled materials from media were extracted and separated by HPLC. HPLC fractions were collected, counted and graphed. A, \(^{14}\)C (U)-AA standard; B, SMC incubated without exogenous AA; C, SMC incubated with 30 μM AA.
Fig. 22
Labeled metabolites from cultures incubated without LDL (Fig. 23-A) were compared with labeled metabolites from cultures incubated with oxidized-LDL containing an intermediate level of MDA (Fig. 23-B). Decreased amounts of labeled prostanoids and free AA were obtained suggesting that oxidized-LDL, like exogenous AA (Fig. 22), supplied unlabeled AA as a substrate for prostanoid synthesis in these cultures.

SMC cultures released a number of unidentified metabolites and these metabolites increased in amount when cultures were incubated with oxidized-LDL (Fig. 23). These labeled metabolites were not products of prostanoid metabolism since IM which blocked the formation of labeled prostanoids had little effect on these metabolites. In fact, some metabolites and free AA actually increased when IM was added to the incubation media (Fig. 23).

Labeled metabolites from cultures incubated with high concentrations of two oxidized-LDL preparations were compared in Fig. 24. Oxidized-LDL with low amount of MDA increased prostanoid synthesis, and this LDL preparation provided substrate like exogenous and decreased the amount of labeled prostanoids (Fig. 24-A compared to Fig. 22-B). Highly oxidized-LDL was cytotoxic and this preparation showed an unusual metabolite profile (Fig. 24-B). Very small amounts of prostanoids and a large amount of labeled free AA were obtained. An unusual number of unidentified peaks were obtained with the highly oxidized-LDL preparation. These peaks will be identified in subsequent studies.
HPLC analysis of labeled prostanoids and free AA released from SMC by oxidized-LDL that had an intermediate TBAR material content. SMC labeled with 8 μM [1-14C]-AA for 24 h were incubated for an additional 24 h with 400 μg cholesterol/ml of oxidized-LDL. See legend in Fig. 22 for details.
Fig. 23
Fig. 24

HPLC analysis of labeled prostanoids and free AA released from SMC with 800 μg/ml cholesterol LDL containing different TBAR material content. SMC labeled with 0.625 μM [U-14C]-AA for 16 h were incubated for an additional 24 h with 800 μg/ml LDL cholesterol containing different TBAR material content. See legend in Fig. 22 for details.
Fig. 24

**A. 800 µg LDL/ml**
- 6-keto-PGF1\(\alpha\)
- MDA 4.18 nmoles/plate

**B. 800 µg LDL/ml**
- MDA 10.70 nmoles/plate
DISCUSSION

A number of studies have shown that LDL is highly susceptible to lipid peroxidation and LDL is readily oxidized during its preparation (Ray et al., 1954; Oncley et al., 1957; Szczeklik and Gryglewski, 1980) or subsequent incubation either with transition metal ions (Parthasarathy et al., 1984; Yokode et al., 1985; Heinecke et al., 1986) or cell cultures (Parthasarathy et al., 1984; Heinecke et al., 1984, 1986; Henriksen et al., 1981, 1982, 1983; Steinbrecker et al., 1984; Parthasarathy et al., 1986; Morel et al., 1984; Hiramatsu et al., 1986). The present data on the MDA content of oxidized-LDL are consistent with previous reports, but oxidized-LDL in the present study were obtained by LDL oxidation during incubation at 37°C in the absence of cells or transition metal ions. This oxidation procedure is much easier to control than oxidation in the presence of transition metal ions. It is important to control oxidation since different LDL preparations vary in their susceptibility to lipid peroxidation and purified LDL from the sera of different subjects are oxidized at very different rates (Table 11 and Fig. 14).

Prostanoids are synthesized through cyclic endoperoxides, and lipid autoxidation of LDL produces a number of acyclic and cyclic peroxides (Porter, 1986). These cyclic peroxides apparently cross-react with antibodies against prostanoids. The structures of cyclic peroxides are evidently closer to the structure of PGE₂ than 6-keto-PGF₁α since cyclic peroxides only cross-react with the PGE₂ antibody (Table 12 and Fig. 16).
Figure 15 shows that the increase in PGE\(_2\) cross-reactivity is time dependent. In this study, LDL was incubated in the absence of cells with experimental media which contained 20 percent FBS. The cross-reactivity with the PGE\(_2\) antibody depended on the initial TBAR content of the LDL (Fig. 16), and the formation of PGE\(_2\) reactive material was not sensitive to IM. These data suggested that the production of material with a cross-reactivity to the PGE\(_2\) antibody did not involve cyclooxygenase activity.

The interactions between different forms of LDL and cells in culture are complex. Unoxidized-LDL (BHT-LDL) is obtained when BHT is added to fresh LDL preparation before dialysis against 0.15M NaCl. BHT-LDL contain very small amounts of MDA (Table 11). BHT-LDL and oxidized-LDL with a low TBAR material content (4.3 nmol MDA/plate at 800 \(\mu\)g/ml cholesterol) are not cytotoxic to SMC at all concentrations. Oxidized-LDL with an intermediate TBAR material content (8.4 nmol MDA/plate at 800 \(\mu\)g/ml cholesterol) is cytotoxic at a LDL concentration of 800 \(\mu\)g/ml cholesterol and leads to cell death. BHT at a 40\(\mu\)M concentration blocks the cytotoxic effect of the oxidized-LDL, and cells maintain their viability. Other investigators (Morel et al., 1983) have reported that BHT and other antioxidants did not prevent the cytotoxicity of oxidized-LDL. The present studies show that the protective effect of BHT is concentration dependent and cytotoxicity is abolished when the BHT level is sufficiently elevated.

The effects of LDL on prostanoid synthesis depend on lipoprotein concentration and the lipid peroxide content of the LDL preparations. BHT-LDL, a preparation with a very small amount of MDA and without
cytotoxicity in SMC, has no effect on either 6-keto-PGF\(_{1\alpha}\) or PGE\(_2\) synthesis (Table 13). Oxidized-LDL with a low lipid peroxide content (4.3 nmol MDA/plate at 800 \(\mu\)g/ml cholesterol) stimulates both 6-keto-PGF\(_{1\alpha}\) and PGE\(_2\) synthesis in SMC, whereas highly oxidized-LDL (8.4 or 15.8 nmol MDA/plate at 800 \(\mu\)g/ml cholesterol) inhibits prostanoid synthesis.

Enhanced prostanoid levels may occur by stimulating different points in the AA cascade. \(\text{A}_{23187}\) and CsA increase endogenous AA release which provides substrate for cyclooxygenase, while LPS stimulates cyclooxygenase. Oxidized-LDL with a low MDA content evidently provides unlabeled AA as a substrate for prostanoid synthesis. This oxidized-LDL preparation enhances total prostanoid levels measured by RIA (Fig. 18) but has no effect on the release of labeled metabolites from cells pre-labeled with AA (Table 14). These data are explained if the AA substrate is provided both from a labeled endogenous source (cellular phospholipids) and an unlabeled exogenous source (oxidized-LDL).

HPLC data also show that oxidized-LDL with a low MDA level like exogenous AA lowers the synthesis of radioactive prostanoids in SMC pre-labeled with \([^{14}\text{C}]\)-AA (Fig. 24-A). RIA, radioactive metabolite release, and HPLC data all suggest that oxidized-LDL is a source of exogenous AA for increased prostanoid synthesis.

As lipid peroxidation increases, oxidized-LDL with a high amount of TBAR material inhibits cyclooxygenase, and this observation is consistent with the concept that high lipid peroxide levels inactivate cyclooxygenase (Gale and Egan, 1984; Lands et al., 1984). Oxidized-LDL
provide free AA and additional free AA is released during cell death (Fig. 24-B), but high lipid peroxide levels inhibit cyclooxygenase activity. The balance between these two effects explains why net prostanoid biosynthesis is unchanged with oxidized-LDL containing an intermediate TBAR material level (Fig. 19). Complete inhibition of cyclooxygenase activity explains why little prostanoid biosynthesis occurs with oxidized-LDL containing a high TBAR material level (Fig. 20).

The present studies support the suggestion of Pomerantz et al. (1984) that lipoproteins supply AA to a phospholipase sensitive pool accessible to cyclooxygenase. Pomerantz et al. (1984) did not evaluate lipid peroxidation in their lipoprotein preparations, but several observations in their studies are consistent with lipid peroxidation. First, they found that lipoproteins particularly LDL caused morphologic changes in SMC. Oxidized-LDL have similar morphologic effect. Secondly, they found that PGE₂ increased more than 6-keto-PGF₁α when these prostanoids were assayed by RIA. This observation could be explained by the cross-reactivity of PGE₂ and oxidized lipids in LDL. Finally, they found that 6-keto-PGF₁α production was lower when cells were plated at high densities allowed to grow for 5 days and then incubated with lipoprotein. Lipoproteins are oxidized by SMC (Morel et al., 1984; Heinecke et al, 1984, 1986) and then more high oxidized lipoprotein formed in the presence of larger number of SMC could have diminished prostanoid synthesis.

Differences in the extent of lipoprotein oxidation and lipoprotein concentration may explain why different investigators find
either an increase or a decrease in prostanoid synthesis with lipoproteins. Szczeklik and Gryglewski (1980) reported that isolated LDL with a high content of MDA inhibited 6-keto-PGF₁α synthesis in both rat aortic slices and superfused bovine coronary arteries. Beitz and Forster (1980) showed that LDL (500 µg/ml cholesterol) increased PGII₂ synthesis from PGH₂ in microsomal fraction. But high concentration of LDL (2000 µg/ml cholesterol) decreased PGII₂ synthesis. In their studies the MDA content in lipoprotein was not measured and they did not evaluate whether the production of PGII₂ was totally from the conversion of PGH₂ or from both the conversion of PGH₂ and the addition of the AA substrate from lipoproteins. The inhibitory effect of a high concentration of LDL may have been caused by lipid peroxidation since LDL is susceptible to oxidation even during isolation (Ray et al., 1954; Szczeklik and Gryglewski, 1980; Zhang et al., in press).

The effects of LDL on the prostanoid synthesis may explain a paradox that seems to occur in studies with vitamin E. Neither the omission of vitamin E nor the addition of vitamin E in high concentration has a significant effect on prostanoid synthesis by microsomes (Panganamala and Cornwell, 1982; Panganamala et al., 1977; and Carpenter, 1982) and cells in culture (Cornwell et al., 1979; Morisaki et al., 1984). These observations indicate that vitamin E has no effect on prostanoid synthesis. Paradoxically, prostanoids such as PGE₂ and PGF₂α are elevated in the serum of animals with vitamin E deficiencies (Hope et al., 1975) and prostanoid levels return to normal when vitamin E is restored to the diet. LDL may play a role in the effect of vitamin E on prostanoid synthesis. Since vitamin E blocks
lipid peroxidation and LDL with an appropriate level of lipid peroxidation stimulates prostanoid synthesis, the absence of oxidized-LDL rather than vitamin E itself may explain the decrease in serum prostanoid levels when vitamin E is added to the diet. This mechanism would not obtain in microsomes and cell cultures where prostanoid synthesis is studied in the absence of LDL.
REFERENCES


Gryglewski, R.J., Bunting, S., Moncada, S., Flower, R.J., and Vane, J.R. (1976). Arterial walls are protected against deposition of platelet thrombi by a substance/prostaglandin X which they make from prostaglandin endoperoxides. Prostaglandins, 12, 685-713.


Johnson, R.A., Morton, D.R., Kinner, J.H., Gorman, R.R., McGuire, J.C.,
Sun, F.F., Whittaker, N., Bunting, S., Salmon, J., Moncada, S.,
and Vane, J.R. (1976). The chemical structure of prostaglandin
X (prostacyclin). Prostaglandins, 12, 915-928.

Jonasson, L., Holm, J., Skaland, O., Bondjers, G., and Hansson, G.K.
(1986). Regional accumulation of T cells, macrophages and smooth
muscle cells in the human atherosclerotic plaque. Arteriosclerosis,
6, 131-138.

cell viability by simultaneous staining with fluorescein
diacetate-propidium iodide. J. Histochem. Cytochem., 33, 77-79.

prostaglandin E hydrochloride in prolonging the survival and
procarbazine of vascularized cardiac hamster-rat xenografts.
Transplantation, 20, 439-442.

Corticosteroids inhibit prostaglandin production by rheumatoid

lipoxygenase products of arachidonic acid in tumor-promoting
activity of TPA. In H. Thaler-Dao, A.C. de Paulet, and R.
Paoletti (Eds.), Icosanoids and Cancer (pp. 101-113). New York:
Raven Press.

synthesis of prostaglandins and related lipids by populations of
human peripheral blood mononuclear cells. Prostaglandins, 20,
135-145.

Korbut, R., and Moncada, S. (1978). Prostacyclin (PGI2) and thromboxane
A2 interaction in vivo. Regulation by aspirin and relationship

anaesthetics on prostaglandin biosynthesis in vitro. Biochim.

human blood monocytes and mouse peritoneal macrophages. J. Exp.
Med., 147, 952-957.


