The relationship of cyclic AMP levels and collagen synthesis in vascular smooth muscle cells following estrogen or beta-adrenergic treatment

George John Dimopoulos
University of Connecticut
The use of estrogens in postmenopausal women has become controversial. Estrogen replacement therapy has been used for many years to slow the progression of atherosclerosis. However, recent clinical studies have shown that menopausal women on hormone replacement therapy have higher incidences of myocardial infarctions and strokes than their counterparts without the therapy. Other studies have demonstrated that a decrease in collagen content in advanced atherosclerotic lesions may weaken the structural integrity of these lesions, resulting in rupture and clot formation. Within lesions, vascular smooth muscle cells (VSMCs) actively synthesize collagen and other types of extracellular matrix proteins during atherogenesis. Estrogen, through activation of the second messenger cAMP, may attenuate collagen synthesis in VSMCs and may promote reduction of collagen content in lesions.

Incubation with Rp-cAMPs, a PKA inhibitor, blocked estrogen's ability to inhibit collagen synthesis in VSMCs. This suggests that estrogen, through stimulation of cAMP, may activate the cAMP-PKA pathway in VSMCs. Treatment of VSMCs with estrogen and phosphodiesterase inhibitors, cilostamide or Ro-20-1724, had an additive effect on increasing cAMP levels and on inhibiting collagen synthesis. VSMCs treated with estrogen and forskolin, an adenylyl cyclase stimulator, also had an additive effect on increasing cAMP levels.
and on attenuating collagen synthesis. When VSMCs were treated with both estrogen and a beta-adrenergic agonist, isoproterenol or terbutaline, there was an additive effect on cellular cAMP levels although the observed decrease in collagen synthetic rates were the same as observed in estrogen treated cells. VSMCs, which were treated with terbutaline, had a significantly higher amount of cAMP located extracellularly compared to estrogen treated cells. The different distribution patterns of cAMP observed in terbutaline and estrogen treated cells may explain why beta agonists may not attenuate collagen synthesis in VSMCs. These results clearly demonstrate that not all agents, which elevate cAMP, may inhibit collagen synthesis in VSMCs. Our data also suggest that the combination of agents, which have an additive effect with estrogen, may have a negative effect on the stability of existing atherosclerotic lesions through the inhibition of collagen synthesis.
THE RELATIONSHIP of CYCLIC AMP LEVELS and COLLAGEN SYNTHESIS in VASCULAR SMOOTH MUSCLE CELLS FOLLOWING ESTROGEN or BETA-ADRENERGIC TREATMENT

George John Dimopoulos, BA
University of New Hampshire, 1994

A Dissertation
Submitted in Partial Fulfillment of the
Requirements for the Degree of
Doctor of Philosophy
At
The University of Connecticut
2003
THE RELATIONSHIP OF CYCLIC AMP LEVELS AND COLLAGEN SYNTHESIS IN VASCULAR SMOOTH MUSCLE CELLS FOLLOWING ESTROGEN OR BETA-ADRENERGIC TREATMENT

Presented by

George John Dimopoulos, B.A.

Major Advisor

Ronald O. Langner

Associate Advisor

Gerard Gianutsos

Associate Advisor

University of Connecticut

2003
ACKNOWLEDGEMENTS

I would like to thank my major advisor, Dr. Ronald 0. Langner, for his mentorship throughout my graduate school career. His encouragement and insight were invaluable. I would also like to thank my associate advisors, Dr. Gerald Gianutsos and Laurine Bow for their support and guidance.

Finally, I would like to thank all the Pharmaceutical Sciences students past and present, especially, Chuan Chen, Dadong Li, Xin Yang, Alex Papanicoulaou, Andreas Goutopoulos, Angie Slitt, Dianna Sahakian, Bakul Butnager, Sid Pattish, Sarah Mowbray, Mike Thibodeau, Ebru Caba, Mark Slein, Peter Symanowicz, Scott Wilkie, Rob Picone, Lee Ayotte, Tony Flores, Jaque Ciarlo, Bindi Doshi, and Gayle Hennig. I would also like to thank all post-doctoral fellows, past and present for their friendship and insight, especially, Spyros Nikas, David Wang, Chara Souli, Sonya Palmer, and Spiro Pavolopoulos (Currently, Professor Pavolopoulos).
DEDICATION

This dissertation is dedicated to my parents, Christos and Evmorfia Dimopoulos, for their patience and understanding.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Approval Page</td>
<td>ii</td>
</tr>
<tr>
<td>Acknowledgement</td>
<td>iii</td>
</tr>
<tr>
<td>Dedication</td>
<td>iv</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>v</td>
</tr>
<tr>
<td>List of Figures</td>
<td>viii</td>
</tr>
</tbody>
</table>

## Introduction

Pathogenesis of Atherosclerosis 1

The Initiation of Atherosclerosis 3

VSMC in Atherosclerosis
  Phenotypic regulation 4
  Inducers of VSMC proliferation and migration during atherogenesis 5
  Other possible Roles of Synthetic VSMCs 6

VSMC and Connective Tissue 8

The Role of Collagen in Atherosclerotic Lesion Development 9

The Role of Collagen in Lesion Stability 11

Factors Regulating Collagen Synthesis 13
Estrogen and Atherosclerosis

Significance of Study

Methods

Animal Protocol

Drugs, Cell Culture Reagents, Isotopes, and Other Chemicals

Isolation of Cells

Characterization of VSMCs

Immunohistochemistry

Experimental Conditions

Determination of Protein Synthetic Rates

Trichloroacetic Acid Extraction Assay

Cyclic AMP Levels
  Principles and protocols of cAMP assays

Protein Assays

Statistical Analysis

Results

Discussion
List of Figures

Fig. 1  An advanced atherosclerotic lesion  1
Fig. 2  Risk factors schematic  2
Fig. 3  Cell types within lesion schematic  14
Fig. 4  Regulators of collagen synthesis in VSMCs  14
Fig. 5  Immunohistochemical identification of alpha-actin in VSMCs  35
Fig. 6  Estrogen-cAMP dose response relationship  36
Fig. 7  Estrogen-collagen dose response relationship  37
Fig. 8  Regression analysis of the relationship between changes in VSMC cAMP levels and rates of collagen synthesis  38
Fig. 9  The effects of Rp-cAMPs on estradiol's inhibition of collagen synthesis in VSMCs  39
Fig. 10  The effects of estradiol and Ro-20-1724 on cAMP levels in VSMCs  40
Fig. 11  The effects of estradiol and Ro-20-1724 on collagen and total protein synthetic rates.  41
Fig. 12  The effects of estradiol and cilostamide on cAMP levels in VSMCs  42
Fig. 13  The effects of estradiol and cilostamide on collagen and total protein synthetic rates in VSMCs  43
Fig. 14  The effects of estradiol and forskolin on cAMP levels in VSMCs  44
Fig. 15  The effects of estradiol and forskolin on collagen and total protein synthetic rates in VSMCs  45
Fig. 16  The effect of estradiol and isoproterenol on cAMP levels in VSMCs  46
Fig. 17  The effect of estradiol and isoproterenol on collagen rates in VSMCs  47
Fig. 18  The effect of estradiol and isoproterenol on total protein synthetic rates in VSMCs  48
Fig. 19  The effect of a prolonged incubation with isoproterenol on collagen, non-collagen, and total protein synthesis in VSMCs  49
Fig. 20  The effect of estradiol and terbutaline on cAMP Levels in VSMCs  50
Fig. 21  The effect of estradiol and terbutaline on collagen And total synthetic rates in VSMCs  51
Fig. 22  The effects of estradiol and terbutaline on Intracellular and extracellular  52
Fig. 23  Cyclic-AMP-adenosine pathway  55
Pathogenesis of Atherosclerosis

Cardiovascular disease, accounting for approximately one million deaths annually, remains as the primary cause of death in the United States (National Institutes of Health, 2002). Most cardiovascular deaths are attributed to myocardial and cerebral infarctions in blood vessels that exhibit atherosclerosis. Atherosclerosis is a disease primarily of the large to medium-sized arteries, which is characterized by raised focal plaques or lesions within the intima and media of the blood vessels. As plaques enlarge, they can impede blood flow to tissue causing tissue necrosis and death. Atherosclerotic plaques can also serve as a focal point for the formation of blood clots, which if large enough can completely stop blood flow to tissues. (Parrish et al., 1967) The fatty streak is thought to be the earliest type of atherosclerotic lesion. It is a lipid-rich lesion, consisting of both lipid-laden macrophages and vascular smooth muscle (VSMCs) (McGill et al., 1968). With increasing age, the fatty streak may progress and become an advanced, complicated plaque, composed of increased amounts of VSMCs, leukocytes, and intracellular and extracellular lipids (Ross et al., 1986). This type of lesion is often surrounded by a dense fibrous cap of VSMCs and collagen (Fig. 1) and is referred to as a fibrous plaque (Ross et al., 1986).

The exact cause of atherosclerosis is unknown, however, factors, such as hypertension, hypercholesterolemia, diabetes, smoking, and bacterial or viral
infections are major risk factors for the development of atherosclerosis (Ross et al., 1976). Work by Ross and others suggest that these risk factors induce injury to the endothelial lining that initiates a variety of responses in the vascular wall, ultimately resulting in the formation of an atherosclerotic plaque (Ross et al., 1993) (Fig.2).
**RISK FACTORS**

<table>
<thead>
<tr>
<th>Smoking</th>
<th>High Blood Pressure</th>
<th>High Serum Lipids</th>
<th>Xenobiotics</th>
<th>Diabetes</th>
</tr>
</thead>
</table>

**Injury to Vascular Wall**

**Response of Arterial Wall**

<table>
<thead>
<tr>
<th>Lipid Accumulation</th>
<th>Cellular Proliferation</th>
<th>Cellular Migration</th>
<th>Calcium Deposition</th>
<th>Connective Tissue Deposition</th>
</tr>
</thead>
</table>

**Vascular Lesion**

**Clinical Manifestations**

Fig. 2 A schematic showing major risk factors causing injury to the arterial wall which results in the pathological manifestations of atherosclerosis

**The Initiation of Atherosclerosis**

Both animal and human studies have suggested that atherosclerotic lesions are characterized by endothelial dysfunction, which is caused by injury to the endothelial lining of blood vessel (Petterson et al., 1993). In response to injury, endothelial cells begin to express adhesion molecules, selectins, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1)). These molecules are believed to aid in the recruitment of monocytes, T- lymphocytes, and other immune cells. (Cybulsky et al., 1991). Additionally, synthetic VSMCs stimulate monocyte and leukocyte recruitment during the early stages of atherogenesis by expressing VCAM-1 and ICAM-1.
The expression of granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF), and monocyte chemotactic protein 1 (MCP-1) by endothelial cells and activated macrophages have been reported to contribute to the early development of atherosclerotic lesions (Rajavashisth et al., 1990 and Boisvert et al., 1998). MCP-1 has been shown to promote chemotaxis and accumulation of additional monocytes to areas of lesion development (Boisvert et al., 1998). GM-CSF and M-CSF have been shown to stimulate differentiation, proliferation, and migration of macrophages and granulocytes, suggesting that these molecules may act as regulatory signals for modulating cell migration and proliferation during the early stages of atherogenesis. (Rajavashisth et al., 1990, Metcalf et al., 1989, Bussolino et al. 1989).

**VSMC in Atherosclerosis**

Migration and proliferation of aortic vascular smooth muscle cells (VSMC) may play an important role in the formation of atherosclerotic lesions. Following injury to the endothelial lining VSMC become phenotypically altered and participate in the repair process of endothelial lining. Ross et al. (1993) have suggested that VSMC involvement during the repair process may become uncontrolled and may contribute to the formation of atherosclerotic lesions.
**Phenotypic Regulation.** VSMCs can present either a contractile or synthetic phenotype. In normal blood vessels, the majority of VSMCs are in a contractile phenotype and express a unique array of contractile proteins, receptors, ion channels, signaling molecules, and other proteins involved with cellular contraction (Owens et al., 1998). Contractile VSMCs proliferate and synthesize extracellular matrix proteins at extremely low rates, but once converted to a synthetic phenotype, as seen in vascular remodeling, these cellular processes are accelerated (Owens et al., 1995). The conversion of VSMCs to the synthetic phenotype is characterized by a reorganization of cytoplasmic microfilaments and organelles. Thyberg et al (1983) has shown that synthetic VSMCs have a reduced number of microfilaments and a larger number of organelles, such as rough and smooth endoplasmic reticulae, golgi, lysosomes, and mitochondria, which are responsible for the biosynthesis and secretion of macromolecules, such as collagen and glycosaminoglycans.

Several studies have demonstrated that, during the atherogenic process, contractile VSMCs are transformed into synthetic VSMCs (Campbell et al 1981). The precise mechanism, by which VSMCs undergo a phenotypic transformation, remains elusive. Studies have shown that certain cytokines and prostaglandins promote the transition of VSMCs from a normal contractile phenotype to a synthetic one (Ross et al., 1981). MCP-1 and eicosanoids, such as, platelet derived 12-hydroxyeicosatetraenoic acid, neutrophil derived leukotriene B4, leukotrienes C4, D4, E4, and prostaglandin E1, all have been shown to be
possible inducers of the secretory phenotype (Sjolund et al., 1984, Palmberg et al., 1989 and Denger et al., 1999).

In vitro studies have shown that culturing VSMC in fetal calf serum promotes conversion to the synthetic phenotype (Hedin et al., 1987). It is believed that growth factors in fetal calf serum induces phenotypic conversion, resulting in proliferation of cultured VSMCs (Hedin et al., 1987). Other studies, examining VSMC growth on various types of matrix proteins, have reported that the extracellular milieu may play a role in phenotypic regulation of VSMCs. Contractile VSMCs cultured on type I collagen or fibronectin displayed conversion to the synthetic phenotype without the addition of exogenous mitogens (Thyberg et al., 1990). On the other hand, VSMCs grown on glycoprotein, laminin, or elastin, exhibited cell attachment, suppression of spreading, and retention of the contractile phenotype for several passages (Thyberg et al., 1990). Yamamoto et al. demonstrated that antibody blockage of the beta-1 integrin receptor in synthetic VSMCs prevented phenotype transition. These data suggest that VSMC integrin receptors are important for matrix-cell interactions, and the attachment to matrix proteins may promote VSMC phenotype modulation during atherogenesis (Yamamoto et al., 1993).

**Inducers of VSMC Proliferation and Migration During Atherogenesis.**

A host of diverse molecules, released from dysfunctional endothelial cells, platelets, synthetic VSMCs and/or various infiltrating immune cells, may alter the activity of synthetic VSMCs (Ross et al., 1976). MCP-1, basic fibroblast growth factor (bFGF), and platelet-derived growth factor B-chain (PDGF-BB) have been shown to stimulate proliferation of synthetic VSMCs (Viedt et al., 2002 and Linder et al., 1995).
Studies with adult rat synthetic VSMCs have shown that VSMCs are able to secrete a PDGF-like mitogen, which promotes cellular proliferation (Sjolund et al., 1988).

Interleukin-1 and tumor necrosis-alpha (TNF-α), released by macrophages, also stimulate VSMC proliferation in addition to inducing VSMC expression of MCP-1 (Cattaruzza et al., 2002). Interleukin-3, which is secreted by activated T lymphocytes and is found in atherosclerotic lesions, may support atherogenesis by promoting proliferation and migration of VSMC within lesions (Brizzi et al., 2001).

A variety of blood products can also stimulate VSMC migration and proliferation. At the site of blood vessel injury, platelets release heparinase and platelet factor 4, promoting the release of bFGF from the extracellular matrix of blood vessels. The release of bFGF has been shown to induce VSMC proliferation (Myler et al., 2002). Thrombin, a known mitogen of VSMCs, can also induce VSMC expression of the urokinase-type plasminogen activator. Urokinase-type plasminogen activator degrades fibrin through the conversion of plasminogen to plasmin, and it is thought that this process promotes VSMC migration (Reuning et al., 1994). Other studies have shown that blood coagulation factors X, Xa, and protein S can also stimulate VSMC proliferation (Gasic et al., 1992).

**Other Possible Roles of Synthetic VSMCs.** In addition to proliferative and migratory roles during atherogenesis, synthetic VSMCs have the
ability to synthesize various types of macromolecules, which are involved in vascular remodeling in response to endothelial injury. (Ross et al., 1976). In response to PDGF treatment, cultured rat arterial VSMCs were shown to secrete MCP-1 (Poon et al., 1996). Poon et al. concluded that MCP-1 expression is a highly regulated process, which may be important in contributing to the inflammatory process during atherogenesis. In vitro studies have shown that MCP-1 induces interleukin-6 release from VSMCs (Viedt et al., 2002). Interleukin-6 promotes differentiation of B lymphocytes into plasma cells that secrete antibody against various components of lesions. These immunomodulators may contribute to continuous inflammation within atherosclerotic lesions (Hansson et al., 1989 and Libby et al., 1995). GM-CSF, which is released from endothelial, can also be secreted by VSMCs, suggesting that both endothelial cells and VSMCs can stimulate proliferative activity in addition to inducing the migration of macrophages and granulocytes at the site of inflammation during the early stages of atherogenesis (Stanford et al., 2002). TNF-α, produced by both immune cells and endothelial cells and increases inflammatory and immune responses, is also released by VSMCs (Newman et al., 1998). The release of TNF-α by VSMCs may cause the infiltration of lymphocytes into lesions and may induce their activation (Newman et al., 1998).

**VSMC and Connective Tissue**

Synthetic VSMCs can synthesize enormous amounts of connective tissue proteins that promote vascular remodeling during atherogenesis (Burk et al., 1979). Numerous types of connective tissue proteins (i.e., collagen, fibronectin, proteoglycans, elastin) are essential in sustaining...
the synthetic VSMC phenotype (Pietila et al., 1983). During atherogenesis, collagen production by VSMCs exceeds the synthesis of all other types of connective tissue proteins, and, thus, comprises the bulk of the lesion (Pietila et al., 1983).

**The Role of Collagen in Atherosclerotic Lesion Development**

It is well known that collagen molecules are structural macromolecules abundantly found in the extracellular matrix (ECM). Collagens are a family of complex proteins, which are comprised of 19 genetically distinct types (Wight et al., 1997). The general structure of collagen consists of a triple helix of polypeptide chains and globular domains. Collagen is unique in that it contains high amounts of the amino acid, hydroxyproline. Besides playing a structural role in vascular tissue, collagen has been shown to be involved with atherosclerotic lesion formation and lesion stability. High levels of collagen within lesions may lead to occlusions in blood vessels, while a deficit in collagen levels may make lesions more susceptible to rupture (Rekhter et al., 1998).

Collagen comprises 60% of total protein within lesions (Smith et al., 1965). Collagen accumulation within lesions contributes to arterial lumen narrowing and may also further lesion development by serving as a depot for macromolecules, such as modified lipoproteins and growth factors (Greilberger et al., 1997,
Studies have reported that collagens can interact with extracellular components in blood vessel walls, which may contribute in lesion development (Wright et al., 1989). Wight et al. observed that advanced atherosclerotic lesions that are abundant in type I collagen are also abundant in glycosaminoglycans (GAGs). In vitro studies have shown that GAGs can strongly link LDL to type 1 collagen, promoting retention of LDL in atherosclerotic plaques (Pentikainen et al., 1997). The mechanism by which GAGs link LDL to collagen is thought to involve both structural and electrostatic associations. The sulfated groups located on GAGs are highly negatively charged, and they can electrostatically bind to positively charged LDL and collagen (Danielson et al., 1997). A study by Hurt, involving isolation of chondroitin 6-sulfate rich proteoglycan from human aortas, demonstrated that GAGs bound to LDL via collagen molecules, caused a 2-4-fold increase in the uptake of cholesterol into human macrophages. Recent studies, employing electron microscopy and computer modeling, reported that the core protein of decorin, a predominant type of GAG macromolecule found in atherosclerotic plaques, has the shape of an arch which can insert itself in the triple helix of type I collagen (Scott et al., 1996 and Weber et al., 1996).

In vivo studies, using a cholesterol fed rabbit model, demonstrated that hypercholesterolemia caused the formation of atherosclerotic lesions with a simultaneous increase in both LDL and GAG metabolism uptake and the formation of LDL complexes of GAG in association with collagen (Srinivasan et
al., 1975). Hoover et al., 1996 provided evidence that once LDL undergoes oxidative or metabolism, the resultant oxidized LDL binds to collagen. These data suggest that the internalization of oxidized LDL by macrophages may require the presence of both GAGs and collagen. Studies have also shown that oxidized-LDL may be a stimulant of collagen synthesis in aortic VSMCs. Jimi and colleagues (1995) found that oxidized-LDL increases collagen synthetic rates six-fold, while the native form of LDL had no effect in VSMCs. These studies suggest that oxidized LDL may help to initiate and sustain collagen synthesis in VSMCs (Jimi et al., 1995).

**The Role of Collagen in Lesion Stability**

Vulnerable atherosclerotic lesions can rupture and provide a site for the formation of a vessel-occluding blood clot, culminating in the clinical syndromes associated with many vascular diseases such as myocardial infarction or stroke. (Rabbani et al., 1999). The hallmarks of a vulnerable plaque include a large lipid pool, an abundance of inflammatory cells and mediators, a decreased smooth muscle cell population, reduction in collagen content, and a thin overlying fibrous cap (Rabbani et al., 1999). Rupture can commonly occur at the shoulder regions of lesions where the overlying fibrous cap is thin, necrotic and continuously infiltrated by macrophages (Constantinides et al., 1966). The shoulder regions of plaques are regions, which are exposed to the greatest hemodynamic forces (Cheng et al., 1993). The lipid core within lesions reduces the load-bearing capabilities because of decreased tensile strength (Macisaac et al., 1993). This
promotes higher stress levels in the overlying fibrous cap, which may eventually rupture (Macisaac et al., 1993).

Rises in blood pressure and coronary spasms can cause vulnerable lesions to rupture (Constantinides et al., 1990 and Nobuyoshi et al., 1991). Emotional stress and strenuous physical activity have been strongly associated with acute myocardial infarction (Totter et al., 1990). Both emotional stress and physical activity may cause myocardial infarct by elevating blood pressure and triggering coronary spasm. A study by Constantinides et al., 1966, demonstrated that in animal models, a sudden increase in arterial pressure can promote lesion rupture. After lesion disruption, the highly thrombogenic lipid components and the exposed collagen fragments may promote in the formation of an occlusive thrombus (Falk et al., 1995).

Studies have shown that therapeutic strategies to lower lipid blood levels contributes to lesion stability (Rabbani et al., 1999). Elevated LDL levels have been intricately linked with endothelial dysfunction, and a reduction in LDL levels, using lipid-lowering drugs, has resulted in improved endothelial function (Anderson et al., 1995). Improving endothelial function by lowering LDL levels reduces the level of LDL infiltration within blood vessels, which reduces the amount of LDL that can collect within lesions (Anderson et al., 1995). Decreased LDL pools within lesions resulted in a reduction of expressed matrix degrading enzymes and in an accumulation of interstitial collagen within the intima of atherosclerotic lesions of rabbits (Aikawa et al., 1998). Aikawa el al. suggested that lipid lowering may stabilize vulnerable plaques by decreasing the
degradation of collagen. Additional studies have shown that increases in HDL, which is responsible for lipid removal from tissues, can increase the plaque's relative strength by stimulating collagen synthesis, thus favoring lesion stabilization (Blankenhorn et al., 1989). The conclusion of these findings emphasizes that circulating levels high LDL and low HDL may affect plaque stabilization by promoting a better balance between the amount of lipid in a lesion relative to the amount of collagen in the lesion (Rabbini et al., 1999).

**Factors Regulating Collagen Synthesis**

There are three primary factors, which may control collagen production within lesions. They are: 1) cell type, 2) cell activity, such as migration and proliferation, and 3) chemical and physical factors within atherosclerotic lesions (Rekhter et al., 1999).

**Cell Type**

Synthetic VSMCs, which migrate, proliferate, and produce increased amounts of collagen, are the most important contributors to the development of fibrous atherosclerotic plaques in blood vessels. Some studies have established that endothelial cells can also synthesize collagen during capillary vascularization in thicker plaques (Canfield et al., 1992). Cell types, such as stellate intimal cells and osteoblast-like cells, have also been shown to produce small amounts of type I collagen in human atherosclerotic lesions (Tintut et al., 1998 and Rekhter et al., 1999. (Fig. 3)
Chemical and Physical Factors

In adult rat and rabbit aortic VSMC cultures, there is a strong relationship between the conversion to the synthetic VSMC phenotype and collagen synthesis (Sjolund et al., 1986). Many studies have shown that upon phenotypic conversion to the synthetic from the contractile, VSMCs begin to synthesis and release collagen within the lesion (Thyberg et al., 1996). There are several local chemical factors which can alter collagen production in VSMCs. (Fig. 4).
Studies of VSMC in culture have shown that PDGF induces synthesis of type I and III collagen. (Amento et al., 1991). The mechanism of PDGF stimulation of collagen synthesis in VSMCs is unknown. However, it is believed that PDFG is not a direct stimulator of collagen synthesis but rather a mitogen, which indirectly stimulates collagen synthesis through increase of cell proliferation (Rehkter et al., 1999). Interleukin-I, endothelin-I and angiotensin II have also been shown to stimulate collagen synthesis in SMC cultures (Rehkter et al., 1996). Angiotensin II stimulates collagen production in human arterial SMCs via the AT(1) receptor which results in an increases production of transforming growth factor beta (TGF-β) (Ford et al., 1996).

TGF-β is a potent stimulator of collagen in VSMC cultures (Liau et al., 1989). Nabel et al., has shown that in vivo transfer of the TGF-β gene into VSMCs of a porcine artery induced type I collagen production (Nabel et al., 1993). Studies by Brenner et al. (1994), have shown that there is a TGF-β activation element in the promotor region of the alpha 1 (I) gene, which is responsible for activating transcription of collagen. Connective tissue growth factor (CTGF), a protein, which is thought to be a downstream mediator for TGF-β signaling, has been shown to promote extracellular matrix formation by stimulating collagen synthesis in several cell types, including vascular endothelial cells, epithelial cells, neuronal cells, VSMCs, and fibroblasts. Additionally, CTGF has been shown to promote VSMC proliferation, migration, and adhesion.

CTGF overproduction is proposed to play a major role in pathways that lead to tissue fibrosis, especially in those tissues that are TGF-β dependent.
(Moussad et al., 2000). CTGF, a 38-kDa cysteine-rich protein, is abundantly expressed in atherosclerotic lesions, but only marginally expressed in normal vascular tissues, suggesting that CTGF is one of the factors involved in the development of atherosclerotic lesions (Fan et al., 2002). Recent studies by (Duncan et al., 1999) demonstrated that elevation of intracellular levels of cAMP with either membrane-permeable 8-Br-cAMP or an adenylyl cyclase activator, cholera toxin, inhibited the ability of both TGF-β and CTGF to increase collagen synthetic rates in fibroblasts. Chen et al., 1999, investigated the ability of pentoxifylline to inhibit proliferation and collagen synthesis in rat VSMCs under both basal and PDGF or TGF-β stimulated conditions. Pentoxifylline is a phosphodiesterase inhibitor that increases cellular levels of cAMP by preventing its metabolism. Chen and colleagues showed that pentoxifylline inhibited both VSMC proliferation and collagen synthesis in a dose dependent manner.

Cyclic AMP has been recognized as an inhibitor of collagen synthesis for many years. Studies by Baum et al. (1978) were the first to demonstrate in fibroblasts that elevation of cAMP levels reduced collagen production by as much as 40%. Increasing the levels of cAMP in cultured lung or skin fibroblasts by treatment with either prostaglandins, dibutyryl AMP, or the beta- adrenergic agonist, isoproterenol, resulted in decreased rates of collagen synthesis (Kollros et al., 1987) Clark et al., 1996 reported that PGE2 stimulated intracellular cAMP levels and decreased both lung fibroblast proliferation and collagen production in a dose-dependent manner. Moreland et al., 1998
investigated the mechanism by which the prostaglandin PGE 1 suppresses TGF-β- induced collagen synthesis in human corpus cavernosum smooth muscle cells. These studies demonstrated that PGE 1 inhibited TGF-β- induced collagen synthesis by invoking cAMP synthesis through the activation of adenylyl cyclase activity.

Other studies using intestinal SMC reported that cholera toxin and isobutyl methylxanthine, agents which elevate cAMP levels, also significantly inhibited collagen synthesis. (Perr et al., 1989). The exact mechanism by which increased cAMP suppresses collagen synthesis is not known. However, because a variety of agents, which operate through different mechanisms, elevate cAMP levels and suppress collagen production, it would suggest cAMP plays a central role.

**Estrogen and Atherosclerosis**

Post-menopausal women in their 50’s and 60’s experience an acceleration in coronary heart disease and usually match their male counterparts in the number of incidences of cardiovascular complications (Witteeman et al., 1989). Estrogen replacement therapy has been used for many years to slow the progression of atherosclerosis. Recent epidemiological studies have strongly questioned this use of estrogen since it has shown that post-menopausal women on estrogen replacement therapy may experience an increased incidence of cardiovascular disease (Farquhar et al., 2002 and Lemaittre et al., 2002). Prior to these publications it was generally believed that the administration of estrogen to post-menopausal women decreased the risk of cardiovascular disease (for review see Bittner et al., 2000). The exact
mechanism of estrogen's perceived antiatherogenic effects has not been established. Studies suggested that estrogen had both lipid and non-lipid effects. The lipid effects include estrogen's ability to decrease both total cholesterol and LDL levels and to increase HDL levels (Bittner et al., 2000 and Lemay et al., 2002). Some of the non-lipid mechanisms of estrogen action include decreasing insulin resistance, serum fibrinogen, factor VII, and plasminogen activator inhibitor-1 (Weidmann et al., 1985 and Kauser et al., 1994). Estrogens were also reported to maintain endothelial cell integrity, decrease expression of adhesion molecules, decrease platelet aggregation, inhibit VSMC proliferation, and exert antioxidant activity (for review, see Mendelsohn et al., 2002). Several studies have demonstrated that estrogen has a direct effect on inhibiting collagen synthesis in VSMC (Beldekas et al., 1981). During the early stages of atherosclerosis, the over production of collagen is an important component in the formation of complex fibrous atherosclerotic lesions (Okada et al., 1993). The natural presence or the administration of estrogens may aid in retarding the progression of plaques via inhibiting collagen synthetic rates in VSMCs.

In vivo experiments, involving estrogen treatment of cholesterol-fed rabbits and estrogen treatment of renal hypertensive rats have shown that the administration of estrogen resulted in a reduction of aortic collagen synthesis in both the rat and the rabbit (Fischer et al., 1981). Ovariectomy of rabbits fed a high cholesterol diet resulted in an increased formation of atherosclerotic lesions (Fischer et al., 1981). Too, in cholesterol-fed rabbits an increase in the rate of aortic collagen synthesis was also evident (Fischer et al., 1981). Estrogen treatment of ovariectomized cholesterol-fed
rabbits resulted in fewer atherosclerotic lesions with reduced levels of aortic collagen (Fischer et al., 1985). These studies suggested to Fischer et al. (1985) that estrogen's ability to inhibit atherosclerotic lesion formation was a result of estrogen's ability to reduce the rate of aortic collagen synthesis. These in-vivo observations were consistent with the initial observations of Beldekas et al., (1981) who found that treatment of VSMC in vitro resulted in a decreased production of collagen. The mechanism by which estrogen treatment reduces collagen synthesis is not completely understood but is probably related to estrogens ability to increase cell levels of cAMP. Studies by Dubey et al.,(2000) have shown that estrogen treatment of VSMCs results in a dose dependent increase in cellular levels of cAMP with corresponding decreases in collagen synthetic rates. Although the exact mechanism by which estrogen increases cAMP levels is unknown, studies have shown that stimulation of cAMP by estrogen in aortic VSMCs occurs via a calcium dependent process that does not involve nuclear transcription, suggesting that estrogen's effects on cAMP levels are mediated by direct interaction of the hormone with specific membrane binding sites (Farhat et al., 1996 and Dubey et al., 2000).

**Significance of Study**

The relationship of estrogen-induced changes in cAMP levels and collagen synthesis in the presence of other agents is not known. Tissue levels of cAMP are a product of its rate of synthesis minus its rate of metabolism. Cyclic AMP is synthesized from ATP by adenylyl cyclase and is regulated by many G-protein coupled receptor types. Cyclic AMP is eliminated from the cell by a
combination of hydrolysis, which is catalyzed by cyclic nucleotide phosphodiesterases, and efflux by several plasma membrane transport proteins. In most instances cAMP produces its effects by activating cAMP-dependent protein kinases, which in turn phosphorylate a variety of molecules, such as metabolic enzymes, transport proteins, and numerous regulatory proteins (Chin et al., 2002). There are several clinically used agents, which have the potential to significantly alter cell cAMP levels by either altering its rate of synthesis or elimination. Since changes in cAMP levels could have a significant effect on collagen synthesis in VSMC, it is important that the relationship of estrogen, cAMP and collagen synthesis should be investigated. The hypothesis, which was investigated in this study, is that agents which elevate cAMP, when used in combination with estrogen, will have an additive effect in inhibiting collagen synthesis in VSMCs.
Methods

Animals

Male Sprague-Dawley rats (Charles River Laboratories), weighing between 190-220 grams, were used for the purpose of isolating vascular smooth muscle cells (VSMC). Rats were housed three per cage at 27 °C with a 12-hour light and dark cycle. Commercial Purina Rat chow and water were provided ad libitum. All rats were fasted overnight prior to VSMC isolation. All procedures were approved by the University of Connecticut Animal Care Committee.

Drugs, Cell Culture Reagents, Isotopes, and other Chemicals

1713 Estradiol, isoproterenol, propranolol, terbutaline, forskolin, pentobarbital, elastase, collagenase, and trichloroacetic acid were purchased from Sigma Chemical (St. Louis, MO). Cilostamide and Ro-24-1720 were purchased from Calbiochem-Novabiochem (San Diego, CA). Hanks Balanced Salt Solution, Medium 199, fetal bovine serum, penicillin-streptomycin, kanamycin, and amphotericin B were purchased from BRL Gibco Life Technologies (Rockville, MD). $^{[14]}$C L-proline was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Optiflour scintillation fluid was purchased from Perkin Elmer Life Sciences (Beltsville, MD).

Isolation of Cells

Rats were killed with a lethal injection of pentobarbital at dose of 120 mg/kg and the thoracic cavity was opened immediately. The aorta, from the aortic arch to the femoral bifurcation, was quickly removed and cleaned under cold running tap water. During this cleaning process the adventitia from each
isolated aorta was removed by blunt dissection and the remaining aorta was placed in sterile Hanks Balanced Salt Solution (HBSS) pH 7.5. HBSS contained 2.7uM amphotericin B, 250units/ml penicillin-streptomycin and 170uM kanamycin. The intima from each aorta was removed by scraping with a rubber policeman, and the remaining medias were minced into small cuboidal pieces in HBSS. The minced medias were digested in plain sterile Medium-199, pH 7.5, containing 2mg/ml collagenase and 0.4 mg/ml elastase for 3 hours at 37 °C in a shaking water bath. After digestion, cells were initially plated in 25 cm flasks and were left in the flask for eight days. Studies have reported that all freshly isolated VSMCs should be in the synthetic phenotype by the eighth day in culture (Thyberg et al., 1983). Following the eighth day, VSMCs were divided and replated into two separate flasks, and the cells were allowed to grow to confluency. This step was repeated eight times to propagate adequate numbers of cells for experiments.

**Characterization of VSMCs**

Two methods were used to characterize VSMCs following isolation from aorta. The first method consisted of immunohistochemistry of a specific isoform of the alpha-actin cytoskeletal protein exclusively found in VSMCs. The second method included morphological studies of VSMCs in confluent cultures. Confluent VSMCs have a characteristic "hill and valley" pattern that can be identified in cultures.
To assess the identity of VSMCs after isolation, detection of VSMC actin levels was conducted with the Ultra Streptavidin Detection System (Signet Laboratories, Dedham, MA). The primary antibody used in this study is specific for the actin that is only found in VSMCs (Owens et al., 1986). Isolated cells were grown on cover slips for 24 hours and then were fixed with 100% acetone for four minutes. Samples were rinsed with phosphate buffered saline (PBS) for three minutes and then were treated for five minutes with normal serum to block nonspecific binding. Primary antibody, specific for VSMC actin, was incubated with samples overnight at 4°C. Primary antibody serial dilutions of 1:40, 1:30, and 1:20 were used on test samples to ascertain correct dilution for adequate staining of VSMCs. This step demonstrated that 1:30 dilution was optimal for staining VSMCs. After the primary antibody wash step, cytology samples were incubated with biotinylated anti-immunoglobulin serum linking reagent (secondary antibody) for 20 minutes at room temperature, and the peroxidase labeled ultra-streptavidin was incubated with samples for an additional 20 minutes at room temperature. The first 20-minute incubation allowed the binding of secondary antibody to the primary antibody, while the second 20-minute incubation allowed the peroxidase to bind the secondary antibody. Lastly, the substrate chromogen, 3-amino-9-ethylcarbazole (AEC), was incubated with samples for five minutes at room temperature to allow the chromogen to react with the peroxidase. The counterstain used in these samples was Mayer's hematoxylin.
Experimental Conditions

Each experiment consisted of incubating VSMCs with various experimental agents for one hour. VSMCs were plated with M-199 medium and 15% bovine serum in six well plates with a diameter of 35mm, and these cells were then grown to 70-80% confluency. Before experimentation, each well was washed twice with plain M-199 medium, and the appropriate agent(s) was/were prepared in M-199 medium and dispensed in duplicate or triplicate into designated wells. Separate studies were conducted for ascertaining cAMP levels and protein synthetic rates. To reduce variability, the values obtained from two-three separate wells were averaged and were reported as a single experimental value (N=1) for all studies.

Determination of Protein Synthetic Rates

$[1^4C] - L-Proline Incorporation$ $[14^C]-L$-proline incorporation was utilized to assess the rate of collagen, and non-collagen, protein synthesis in VSMCs. Treated cells were incubated with isotope for one hour at 37°C. Preliminary studies were conducted to determine the proper conditions for incorporation of $^{14}$C-proline. The results of these studies suggested that a 1-hour incorporation would allow for accurate determination of either inhibition or stimulation of protein synthetic rates. In all experiments VSMCs were incubated with experimental agents and isotope for 1 hour. Upon completion of the 1-hour incubation period, controls and treated samples were placed on ice. Cell medium was aspirated from each well and placed in an equal volume of ice cold 10% TCA. The cells in
each well were loosened by scrapping and were then suspended in 100mM EDTA. VSMCs were then sonicated 3 times for 30 seconds, and 100ul of the resulting homogenate was set aside for assessing the total protein concentration for normalizing the collagen and non-collagen synthetic rates (e.g., cpms/mg/ml protein). The remaining protein in the cell homogenate was precipitated in 5% TCA by adding an appropriate volume of 10% trichloroacetic acid (TCA) to each sample. Both cell medium and homogenates were then combined and centrifuged at low speeds (800xg) for 5 minutes. Supernatant, containing unincorporated [1\(^4\)C]-L-proline, was discarded, and the pellets were resuspended with 4ml of 5% TCA and centrifuged at 800xg for five minutes. This wash step was repeated 6 times to ensure that all unincorporated [1\(^4\)C]-L-proline is removed.

**Trichloroacetic Acid Extraction Assay**

The hot TCA extraction procedure was utilized to separate collagen from other cell proteins. The amount of collagen in tissues can be estimated by measuring tissue levels of hydroxyproline, an amino acid which is primarily found only in collagen. Hydroxyproline in collagen is formed by the post-translational hydroxylation of one of the prolines in the repeating amino sequence of G-P-P, which is common to collagen. Studies have reported that treatment of collagen with TCA at 90°C solubilizes collagen by hydrolysis of the peptide bond between proline and hydroxyproline (Last et al., 1975). In our studies, after samples were treated with 90°C TCA the supernatant was collected and was used to assess
collagen synthesis, and the remaining pellet was used to determine non-collagen protein synthesis.

Homogenate samples were hydrolyzed in 5% TCA by incubating the samples in a 90 °C water bath for 60 minutes. The hydrolyzed samples were then centrifuged at 800xg for 10 minutes. Supernatant samples, containing collagen, were collected in separate tubes and the pellets, consisting of non-collagen protein, were resuspended with 0.5N NaOH. A 0.5 ml aliquot of each sample (collagen and non-collagen) was placed in 5ml of scintillation cocktail, and the radioactivity of incorporated \(^{14}\text{C}\) L-proline was determined by liquid scintillation counting. The average of two 10-minute counts were used in all experiments to obtain a statistically significant number of counts. All data were expressed as counts per minute (CPMs).

**Cyclic AMP Levels**

Cyclic AMP levels were assessed by either the \(^3\text{H}\) cAMP detection system (Amersham Pharmacia Biotech, Piscataway, NJ) or the cAMP enzyme immunoassay system (EIA) (Amersham Pharmacia Biotech, Piscataway, NJ). The preparation steps for both cAMP detection systems were identical. After experimentation, VSMCs were immediately placed on ice. The incubation medium from VSMCs was removed and placed into separate tubes. VSMCs were then resuspended in 100mM EDTA and were then sonicated twice for 30 seconds. A 100 µl aliquot from each sample was placed into separate tubes for determining total protein concentrations. Total protein concentrations were used for normalizing cellular cAMP levels. The medium and cell homogenate from
each sample were combined and boiled for seven minutes to coagulate protein. Samples were then centrifuged at 800xg for 10 minutes, and the resulting supernatant was assayed for cAMP content.

**Principles and Protocols of cAMP Assays**

The [$^3$H] cAMP detection system is based on competition between unlabeled cAMP (sample) and a fixed quantity of tritium labelled cAMP for binding to a protein which has a high affinity for cAMP (Gilman et al., 1970). The amount of labelled cAMP-protein complex formed is inversely related to the amount of unlabeled cyclic AMP present in the assay. The concentration of cAMP in the unknown is assessed by comparison with a standard curve. (2pmol - 32pmol).

100ul of sample or standard were added to 1.5ml microcentrifuge tubes. A 50ul aliquot of labeled cAMP was added to each of the samples, which was followed by the addition of 100ul of binding protein. After a 2-hour incubation period at 4°C, separation of the protein bound cAMP from the unbound cAMP (labeled/unlabeled) was accomplished by adsorption of free cAMP on charcoal, followed by microcentrifugation (800xg) (Brown et al., 1971). A 200ul aliquot of the supernatant was removed for liquid scintillation counting as described above.

The cAMP enzyme immunoassay system (EIA) is based on competition between unlabeled cAMP (sample) and a fixed quantity of peroxidase-labelled cAMP, for a limited number of binding sites on a cAMP specific antibody. The peroxidase-labeled cAMP conjugate is immobilized onto pre-coated microtiter plates which is then analyzed on a microplate reader. The amount of cAMP in
the unknown sample is inversely related to the amount of peroxidase-labelled cAMP, which is bound to the cAMP specific antibody. The amount of unknown cAMP in each sample was determined by comparison with a linear standard curve which was determined for each experiment using a 3200 fmol/ml cAMP standard stock solution.

Beginning with the standards and ending with the unknowns, a volume of 100µl of each sample was added to the specified well plate. Next, a volume of 100µl of cAMP antibody was added to the sample wells, and these samples were incubated for two hours at 4°C. Immediately following the 2-hour incubation, 50µl of cAMP-peroxidase conjugate was added into the wells, and the samples were incubated for 1 hour at room temperature. Following a wash step to remove excess antibody, samples were treated with 150µl of tetramethylbenzidine (TMB), which reacts with the bound cAMP-peroxidase conjugate, for one hour at room temperature. Upon completion of the enzyme substrate incubation, 100µl of 1.0M sulfuric acid was added into the well to stop the reaction, and each well was read by a fluorimeter (Dynex Corporation) at an optical density of 450nm within 30 minutes. Blank and nonspecific binding wells were included along with the sample wells.

**Protein Assays**

The Bicinchoninic Acid Protein Assay Kit (BSA) (Pierce, Rockville, IL) or the Coomassie Plus Protein Assay Kit (Pierce, Rockford, IL) was used to determine the protein concentrations of each sample. Total protein concentrations were used to normalize cAMP content and collagen, non-
collagen, and total protein synthetic rates. A Linear standard curve 25-240 g/ml (BSA) or 1-25 µg/ml (Coomassie,) was used for extrapolating protein concentration values.

**Statistical Analysis**

The statistical significance of the data from each study was analyzed by use of a one-way ANOVA to test for the overall treatment effect. If a significant difference was indicated, the data was further analyzed using Tukey’s procedure. Data which had a P value of <0.05 were statistically significant in all experiments.
RESULTS

Immunohistochemical and histological procedures were used to characterize isolated vascular smooth muscle cells (VSMC). Immunohistochemistry was utilized to identify a specific isoform of the α-actin cytoskeletal protein, which is unique to SMCs. As shown in Fig. 5A, the reddish-brownish staining of α-actin cytoskeletal filaments, suggests that the isolated cells were VSMCs. Histologically, the distinct spindle-shape morphology and the hill-valley pattern of these cells in culture is also a recognized a unique characteristic of VSMCs. To ensure that the isolated cells were not fibroblasts, mouse fibroblasts were immunochemically stained for α-actin. As seen in Fig. 5D, the mouse fibroblasts exhibited an absence of staining for α-actin, which was expected since fibroblasts lack α-actin.

The ability of estrogen to stimulate the production of cAMP in VSMCs is shown in Fig. 6. VSMCs, incubated with increasing concentrations (10^{-6} to 10^{4} M) of estradiol for 1 hour, resulted in a corresponding increase in cAMP from control levels of approximately 10 pmoles/mg protein to 40 pmoles/mg protein. The effect of increasing concentrations of estradiol on collagen and non-collagen protein synthesis is shown in Fig. 7. When cells were incubated for 1 hour with concentrations of estradiol ranging from 10^{-6} to 10^{4} M there was a corresponding decrease in the rate of collagen and non-collagen protein synthesis in VSMCs. The potential correlation between increasing amounts of cAMP and decreasing rates of collagen synthesis was investigated.
and is shown in Fig. 8. In Fig 4, the percent increase in cell cAMP levels, invoked by increasing concentrations of estradiol, was plotted against the percent inhibition of collagen synthesis. Analysis of the data showed a correlation coefficient of 0.88, which was statistically significant at P <0.1.

The purpose of this study was to examine the relationship of cAMP-protein kinase and estrogen's ability to inhibit collagen synthesis. Several studies have shown that cAMP activates the cAMP-protein kinase A pathway to elicit various cellular processes (Chin et al., 2002). As shown in Fig. 9, treatment of VSMCs with the protein kinase inhibitor, Rp-cAMP (10^-5 M) for 1 hour completely inhibited estradiol's (10^-5 M) inhibition of collagen synthesis in VSMCs.

The ability of inhibitors of cAMP metabolism to alter cAMP levels and collagen synthesis, when used alone or in combination with estradiol, was investigated with phosphodiesterase inhibitors. Treatment of VSMCs with Ro-20-1724 (10^-6 M), an inhibitor of phosphodiesterase type 4, resulted in a significant increase in cAMP levels. Cyclic AMP levels were approximately 5-fold greater than the amounts observed with just estradiol alone. When estradiol and Ro-20-1724 were combined there was a significant increase in cAMP levels compared to just estradiol treated cells (Fig 10). Treatment of VSMCs with either estradiol (10^-5M) or Ro-20-1724 (10^-6 M) resulted in a 50% inhibition of both collagen and total protein synthesis (Fig 11). The combination of estradiol and Ro-20-1724 resulted in a reduction in collagen and total protein synthesis,
which was significantly greater than the reduction observed when either agent used alone (Fig 11). Treatment of VSMCs with cilostamide (10\(^{-6}\) M), an inhibitor of phosphodiesterase type 3, also resulted in a significant increase in cAMP levels (Fig. 12). Cyclic AMP levels were the same as observed with just estradiol alone. Co-treatment of estradiol and cilostamide resulted in cAMP levels that were significantly greater than those observed in VSMCs treated with estrogen alone (Fig 12). Treatment of VSMCs with either estradiol (10\(^{-5}\)M) or cilostamide (10\(^{-6}\) M) resulted in a 50% inhibition of both collagen and total protein synthesis (Fig. 13). The combination of estradiol and cilostamide reduced both collagen and total protein synthesis, which was significantly greater than the reduction observed when either agent was used alone (Fig 13) but was similar to the effects observed when cells were treated with Ro-20-1724 (Fig. 11).

Levels of cAMP and protein synthesis were estimated in VSMCs following forskolin (10\(^{-6}\)M) induction of adenyl cyclase. Treatment of VSMCs for 1 hour with forskolin resulted in a significant increase in cAMP levels when compared to either control or estradiol treated cells (Fig. 14). The combination of VSMCs with estradiol and forskolin appeared resulted in an increase in cAMP levels (Fig 15). Although treatment of cells with forskolin resulted in a reduction in collagen and total protein synthesis, this reduction was similar to the inhibitory effect seen with estradiol treatment even though forskolin treatment resulted in a much greater increase in cAMP levels than the increase in cAMP levels produced
by estradiol. The combination of estradiol with forskolin resulted in a further reduction in collagen and total protein synthesis (Fig. 15).

Changes in levels of cAMP and collagen and total protein synthesis were investigated in VSMCs following stimulation or inhibition of autonomic beta-adrenergic receptors. Treatment of VSMCs with isoproterenol, a beta agonist, for 1 hour significantly increased cAMP levels compared to control or estradiol treated cells (Fig. 16). The combination of estradiol plus isoproterenol resulted in an additional increase in cAMP levels compared to treatment of VSMCS with isoproterenol alone. Treatment of cells with propranolol, a beta antagonist, for 1 hour resulted in no significant changes in cAMP levels. The combined treatment of cells with propranolol plus isoproterenol significantly reduced cAMP levels compared to cells treated with just isoproterenol. The administration of propranolol to estrogen treated cells had no significant effect on cAMP levels.

Treatment of VSMCs with isoproterenol, for 1 hour had no significant effect on collagen or total protein synthesis (Figs 17, 18). This was true whether the cells were incubated with isoproterenol for 1 hour (Figs.17, 18) or 6 hours (Fig. 19). Treatment of cells with propranolol had no effect on collagen or total protein synthesis. The combination of either isoproterenol or propranolol with estradiol resulted in a decrease in collagen and total protein synthesis, which was similar to treatment with only estradiol (Fig. 17, 18).

To further evaluate the effects of beta stimulation on VSMC cAMP levels and collagen and total protein synthesis, cells were treated with terbutaline (10\(^{-6}\) M), a selective $\beta_2$ adrenergic agonist. Treatment of cells with terbutaline for 1
hour resulted in a significant increase in cAMP levels, which was similar to levels observed in VSMCS with just estradiol alone. When cells were treated with estradiol plus terbutaline, cAMP levels were significantly greater than the levels seen in cells treated with just estradiol (Fig. 20). Incubation of VSMCs with terbutaline had no effect on collagen or total protein synthesis (Fig. 21). When terbutaline was co-administered with estradiol, the rate of collagen and total protein synthesis was the same as observed when cells were treated with estradiol alone (Fig. 21). The ratio of cellular to extracellular location of cAMP following either estradiol or terbutaline are shown in Fig 22. Following treatment with estradiol approximately, 40% of cAMP was located intracellularly, while 60% was located extracellularly. This distribution of cAMP was also observed in non-estradiol treated control VSMCs. In VSMCs treated with terbutaline, only 10% of cAMP was located intracellularly and 90% was located extracellularly. This relative distribution of cAMP was significantly different from the distribution of cAMP seen in control or estradiol treated VSMCs.
Fig. 5 Immunohistochemical identification of alpha-actin in isolated cells. (SA) isolated cells without alpha-actin antibody. (5B) Isolated eels with alpha-actin staining; note the reddish-brownish staining of cells, confirming the presence of alpha-actin. (SC & D) shows alpha-actin antibody staining of fibroblasts. (SC) fibroblasts, without alpha-actin antibody. (5D) Fibroblasts with alpha-actin staining. Note no staining in both the control and in fibroblasts incubated with alpha-actin antibody.
**Fig. 6. The effect of varying concentrations of estrogen on cAMP levels in VSMCs.**

VSMCs were treated with estradiol for one hour and were then assayed for cAMP as described in methods. In this figure a dose response relationship demonstrates that increasing estradiol concentrations ($10^{-6} - 10^{-4} M$) results in increasing cAMP levels (pmoles/mg protein) in VSMCs. Each point represents the mean of three observations which is plotted as mean +/- S.E.
Fig. 7. The effect of varying concentration of estradiol on collagen and noncollagen synthetic rates in VSMCs. VSMCs were treated with estradiol and $^{14}$C-L-proline for one hour, and were assayed for collagen and noncollagen synthesis as described in methods. In this figure a dose response relationship demonstrates that increasing estradiol concentrations (10$^{-6}$ M - 10$^{-4}$ M) can result in decreasing levels of collagen and non-collagen in VSMCs. Each point represents the mean of three observations which is plotted as mean+/-S.E.
Fig. 8. The relationship of estradiol induced changes in cellular levels of cAMP and collagen synthesis in VSMCs. A regression analysis of the data in Figures 2 and 3 was conducted to determine the relationship between changes in VSMC cAMP levels and rates of collagen synthesis. An R value of 0.8787 was statistically significant at P<0.1
Fig. 9. The effects of Rp-cAMPs on estradiol's inhibition of protein synthesis in VSMCs. VSMCs were incubated with agents and $^{14}$C-L-proline for one hour and were assayed for protein synthetic levels as described in methods. Estradiol $10^{-5}$ M reduced collagen synthesis. Rp-cAMPs $10^{-5}$ M had no effect on collagen synthesis. Estradiol $10^{-5}$ M + Rp-cAMPs $10^{-5}$ M treatment had no effect on collagen synthesis. Each value represents the mean±SE of five observations. * p< 0.05 compared to control cells.
Fig. 10. The effects of estradiol and Ro-20-1724 on cAMP levels in VSMCs. VSMCs were incubated with agents for one hour and were assayed for cAMP as described in methods. Estradiol $10^{-5}\text{M}$ significantly elevated cAMP two fold, while Ro-20-1724 $10^{-6}\text{M}$ elevated cAMP approximately 10 fold. Treatment of VSMC with estradiol $10^{-5}\text{M} + \text{Ro-20-1724} 10^{-6}\text{M}$ had an additive effect on cAMP levels. Each value represents the mean± SE of three observations. * $p<0.05$ compared to control cells. ** $p<0.05$ compared to estradiol treated cells.
Fig. 11. The effects of estradiol and Ro-20-1724 on collagen and total protein synthetic rates in VSMCs. VSMCs were incubated with agents and $^{14}$C-L-proline for one hour and were assayed for collagen and total protein synthetic rates as described in methods. Estradiol $10^{-5}$ M and Ro-20-1724 (Ro) $10^{-6}$ M reduced both collagen and total protein synthesis by 50%. Treatment of VSMCs with estradiol $10^{-5}$ M + Ro-20-1724 $10^{-6}$ M significantly reduced collagen and total protein synthesis when compared to treatment of cells with estrogen or Ro-20-1724 alone. Each value represents the mean ± SE of six observations. * $p<0.05$ compared to control cells. ** $p<0.05$ compared to estradiol or Ro-20-1724 treated cells.
Fig. 12. The effects of estradiol and cilostamide on cAMP levels in VSMCs VSMCs were incubated with agents for one hour and were assayed for cAMP as described in methods. Estradiol (Estra) 10-SM and cilostamide (Cilo) 10 M significantly increased cAMP levels in VSMCs. The combination of estradiol 10^{-1} M + cilostamide 10 M resulted in a significantly increase in cAMP levels when compared to estrogen treated cells. Each value represents the mean ± SE of seven to ten observations. * p< 0.05 compared to control cells ** p< 0.05 compared to estradiol treated cells
Fig. 13. The effects of estradiol and cilostamide on collagen and total synthetic rates in VSMCs. VSMCs were incubated with agents and 50µCi ¹⁴C-L-proline for one hour and were assayed for collagen synthetic levels. Treatment of VSMCs with either Estradiol (Estra) 10⁻¹⁰ M or Cilostamide (Cilo) 10⁻⁸M significantly reduced collagen and total protein synthetic rates. Treatment of cells with estradiol 10⁻⁸M + cilostamide 10⁻⁶M had additive effect on both collagen and total protein synthetic rates. Each value represents the mean ± SE of five to ten observations. * p< 0.05 compared to control cells. ** p< 0.05 compared to estradiol treated cells.
Fig. 14. The effects of estradiol and forskolin on cAMP levels in VSMCs. VSMCs were incubated with for one hour and were assayed for cAMP as described in methods. Estradiol (Estra) 10-SM significantly increased cAMP levels. Forskolin (Fors) 10-sM significantly increased cAMP levels compared to estrogen treated cells. Estradiol 10-SM + forskolin 10-sM appeared to have an additive effect on cAMP levels compared to the cells treated with just forskolin. Each value represents the mean ± SE of three observations. * p< 0.05 compared to control cells. ** p< 0.05 compared to estradiol treated cells.
Fig. 15. The effects of estradiol and forskolin on collagen and total protein synthetic rates in VSMCs. VSMCs were incubated with agents and 2.5μCi ¹⁴C-L-proline for one hour and were assayed for collagen and total protein synthetic levels. Estradiol (Estra) 10⁻⁵M and forskolin (Fors) 10⁻⁶M both significantly reduced collagen and total protein synthesis. Treatment of VSMCs with estradiol 10⁻⁵M+forskolin 10⁻⁶M significantly reduced collagen and total protein synthesis compared to estradiol treated cells. Each value represents the mean ± SE of six observations. * p< 0.05 compared to control cells ** p< 0.05 compared to estradiol treated cells
Fig. 16. The effect of estradiol and isoproterenol on cAMP levels in VSMCs. VSMCs were incubated with agents for one hour and assayed for cAMP. Estradiol (Estra) $10^{-5}$M, significantly increased cAMP levels 2 fold, while isoproterenol (Iso) $10^{-6}$M significantly increased cAMP levels 5 fold. The combination of isoproterenol + estrogen appeared to have an additive effect on cAMP levels. Propranolol (Pro) $10^{-6}$M alone had no effect on cAMP levels, but significantly decreased isoproterenol's stimulation of cAMP levels. Propranolol had no effect on estrogens ability to increase cAMP levels. Each value represents the mean ± SE of at least four observations. * $p<0.05$ compared to control cells.
Fig. 17. The effect of estradiol and isoproterenol on collagen synthetic rates in VSMCs. VSMCs were incubated with agents and $^{14}$C-L-proline for one hour, and were assayed for collagen synthetic activity as described in methods. Estradiol (Estra) significantly reduced collagen synthesis by 50%, while isoproterenol (Iso) and propranolol (Pro) had no effect on collagen synthetic activity. The combination of either isoproterenol or propranolol with estrogen had no effect on estrogen's ability to inhibit collagen synthesis. Each value represents the mean ± SE of five observations. * p< 0.05 compared to control cells.
Fig. 18. The effect of estradiol and isoproterenol on total protein synthetic rates in VSMCs. VSMCs were incubated with agents and $^{14}$C-L-proline for one hour, and were assayed for total protein synthetic activity as described in methods. Estradiol (Estra) 10^{-5} M significantly reduced total protein synthesis by 50%, while isoproterenol (Iso) and propranolol (Pro) had no effect on total protein synthetic activity. The combination of either isoproterenol or propranolol with estrogen had no effect on estrogen's ability to inhibit total protein synthesis. Each value represents the mean ± SE of five observations * p< 0.05 compared to control cells.
Fig. 19. The effect of a prolonged incubation with isoproterenol on collagen, non-collagen, and total protein synthetic rates in VSMCs VSMCs were incubated with agent and $^{14}$C-L-proline for six hours and were assayed for collagen, noncollagen, and total protein synthetic levels as described in methods. Isoproterenol 10-6M had no effect on collagen, non-collagen, or total protein synthetic rates. Each value represents the mean ± SE of nine observations. Significance (p< 0.05) compared to control cells.
Fig. 20. The effect of estradiol and terbutaline on cAMP levels in VSMCs. VSMCs were incubated with agents for one hour and were assayed as described in methods. Estradiol (Estra) $10^{-5}$ M and terbutaline (Terb) $10^{-6}$ M significantly increased cAMP levels 2-fold. Treatment of VSMCs with estradiol $10^{-5}$ M + terbutaline $10^{-6}$ M had an additive, elevating cAMP levels three fold. Each value represents the mean ± SE of four to five observations. * $p< 0.05$ compared to control cells. ** $p< 0.05$ compared with estradiol treated cells.
Fig. 21. The effect of estradiol and terbutaline on collagen and total synthetic rates in VSMCs. VSMCs were incubated with agents and 2.5μCi of \(^{14}\)C-L-proline for one hour and were assayed for collagen and total synthetic levels as described in methods. Estradiol (Estra) 10\(^{-5}\) M significantly reduced collagen and total synthesis. Treatment of VSMCs with estradiol 10-SM + terbutaline 1o-oM also significantly reduced both collagen and total protein synthesis. Terbutaline 10-0 Malone had no effect on collagen or total protein synthesis. Each value represents the mean ± SE of three observations. * p<0.05 compared to control cells
Fig. 22. The effects of estradiol and terbutaline on intracellular and extracellular cAMP levels in VSMCs. VSMCs were incubated with agents for one hour, and the medium and cellular extracts were assayed for cAMP as described in methods. Treatment of VSMCs with Terbutaline (Terb) $10^{-6}$M resulted in 90% of total cAMP in the extracellular environment. Control and estradiol (Estra) 10.sM treated cells had significantly less cAMP in the extracellular milieu and significantly more cAMP intracellularly compared to the terbutaline treated cells. Each value represents the mean ± SE of eight observations. * p< 0.05 compared to control cells.
Discussion

The use of estrogens in postmenopausal women has recently become controversial. Estrogen replacement therapy has been used for many years to slow the progression of atherosclerosis. Recent studies have suggested that estrogen replacement therapy may inflict harmful effects, such as myocardial infarcts and strokes than beneficial effects. Estrogens have been shown to inhibit collagen synthesis (Dubey et al. 1998). Collagen is known to have an important role in atherosclerotic lesions, and its accumulation within atherosclerotic lesions is known to promote narrowing of the arterial lumen and may serve as a depot for macromolecules (i.e., lipoproteins and growth factors). Other studies have suggested that collagen may add to lesion stability (Rekhter et al., 1999) and that a decrease in collagen can make atherosclerotic lesions vulnerable to rupture, which may promote the formation of vessel-occluding blood clots. These data suggest that either too much or too little collagen within atherosclerotic lesions could have a negative effect on blood flow in diseased blood vessels. The mechanism by which estrogen treatment reduces collagen synthesis is not completely understood but is probably related to estrogens ability to increase cell levels of cAMP. The hypothesis, which was investigated in this study, was the following: agents, which elevate cAMP in VSMC, when used in combination with estrogen, will have an additive effect in inhibiting collagen synthesis in VSMC.
There are many studies that show estrogen eliciting a fast-acting biological response through nongenomic processes in a variety of cell types (Dos Santos et al., 2002, Cambiasso et al., 2001, and Farhat et al., 1996). Our results demonstrate that increasing concentrations of estrogen (10^{-6}M, 10^{-5}M, 10^{-4}M) resulted in elevated levels of cAMP in VSMCs and that estrogen at a dose of 10^{-5}M can increase cAMP levels 2-fold within 1 hour. (Fig. 6). These results agree with recent study data by Farhat et al., (1996) and Dubey et al., (2000), which demonstrated that estrogen may induce cAMP within 5-30 minutes in VSMCs. Farhat et al. further demonstrated that estrogen increases cAMP levels by stimulating adenylate cyclase activity through a calcium-dependent process. Estrogen is an inhibitor of collagen synthesis in VSMCs; however, the exact mechanism is not known. One of the first studies investigating estrogen effects on collagen synthesis showed that estrogen inhibited total collagen production and reduced procollagen type 1 and type 2 fractions in bovine aortic smooth muscle cells (Beldekas et al., 1981 and Dubey et al. 2000), demonstrated that estrogen, increased cAMP levels, which resulted in inhibition of both, total protein and collagen synthesis. The results from our studies agree with the findings of Dubey et al., In our study, VSMCs treated with increasing concentrations of estrogen displayed decreasing rates in both collagen and noncollagen protein synthesis in addition to increasing levels of cAMP, suggesting a dose response relationship (Fig. 7). When collagen synthetic rates in VSMCs were compared to cellular levels of cAMP, there was an apparent inverse correlation, indicating that as cellular levels of cAMP increased,
a related reduction in collagen synthesis followed. (Fig. 8). The mechanism by which increased cAMP levels reduces rates of collagen synthesis is unknown. Dubey and colleagues suggested that estrogen inhibits protein synthesis in VSMCs by activating a cAMP-adenosine pathway (Dubey et al., 2000). In this pathway, extracellular adenosine levels are elevated by the transport of cAMP to the extracellular space where the cAMP is converted to AMP by ecto-phosphodiesterase (ECTO-PDE) and subsequently to adenosine (ADO) by ecto-5'-nucleotidase (ECTO-5'-NT) (Fig 23).

![Smooth Muscle Cell Diagram](image)

**Fig. 23** A schematic showing the cAMP-adenosine pathway in VSMCs

Dubey et al., 2000. Hypertension 35:262-6

The newly formed adenosine binds the adenosine, subtype 2 receptor, (A2), which has an inhibitory effect on collagen and total protein synthesis. These data suggested to Dubey et al. that the inhibitory effects of estrogen are
mediated through the cAMP-adenosine pathway and not by activation of intracellular protein kinase A (PKA) activity.

The results of these studies, using the phosphodiesterase inhibitor, pentoxifylline, showed that by preventing breakdown of cAMP, the cAMP-protein kinase A (PKA) pathway may be activated, and this resulted in inhibition of collagen synthesis in VSMCs (Chen et al., 1999). Co-incubation of pentoxifylline with H-89, a selective inhibitor of PKA, prevented the pentoxifylline induced inhibition of collagen synthesis. Since estrogen treatment increases cAMP levels, the role of the cAMP-PKA pathway in suppressing collagen and total protein synthesis in estrogen- treated VSMC tissue culture model was explored. Co-treatment of VSMCs with estrogen and Rp-cAMP, a PKA inhibitor, significantly reduced estrogen’s ability to inhibit collagen and total protein synthesis in VSMCs (Fig.9). These data suggest that in our model, estrogen attenuates protein synthesis by activating the cAMP-PKA effector pathway. In support of our data, other studies by Walsh et al., (1994), have found that PKA can halt translation of proteins by phosphorylating the initiation factor eIF-2. Possibly, estrogen may impair collagen synthesis by activating a cascade of kinases, which are switched on by the PKA pathway.

To determine if the inhibition of cAMP breakdown to AMP by PDE can block the collagen/protein reduction effects of estrogen as suggested by the studies of Dubey et al., VSMCs were treated with two different PDE inhibitors. Treatment of VSMCs with cilostamide, a phosphodiesterase type III inhibitor or Ro-20-1724, a phosphodiesterase type IV inhibitor, resulted in
increased cAMP levels (Fig. 10 and 12) and decreased rates of collagen and total protein synthesis (Fig. 11 and 13). Since cilostamide appears to be less potent than Ro-20-1724 in elevating cell cAMP levels, it is tempting to speculate that inhibition of PDE type IV is more effective than PDE type III in elevating cAMP levels. Additional dose response studies using both agents would be necessary to confirm this.

Treatment of VSMCs with estrogen and cilostazol or Ro-20-1724 had an additive effect on cAMP levels (Fig. 10 and 12) and on inhibition of collagen and total protein synthetic rates (Fig. 11 and 13). Contrary to the studies by Dubey et al., these findings suggest that the breakdown of cAMP to AMP and subsequently to adenosine may not be a necessary process for inhibiting protein synthesis in VSMCs. The results of our studies using a PKA inhibitor and phosphodiesterase inhibitors are consistent with the hypothesis that estrogen-induced changes in cAMP activates the cAMP-PKA effector pathway to attenuate protein synthesis in VSMCs.

The phosphodiesterase inhibitor data (cilostamide and Ro-20-1724) appear not to support Dubey's hypothesis that estrogen utilizes the cAMP-adenosine pathway to inhibit collagen synthesis. Instead, our data suggest that estrogen-induced cAMP is not required to be converted to adenosine to inhibit collagen synthesis. The co-incubation of cilostamide or Ro-20-1724 phosphodiesterase inhibitor with estrogen produced an additive effect on cAMP levels and collagen inhibition. Furthermore, our beta-adrenergic agonist data bolsters our phosphodiesterase inhibitor data because it demonstrates that the beta-adrenergic receptor is not involved with collagen inhibition since
more of its cAMP is exported to the extracellular compartment.

As shown in figures 11 and 13, the level of protein synthesis inhibition is approximately the same in VSMCs treated with cilostamide or Ro-20-1724 even though Ro-20-1724 caused a greater increase in cAMP compared to cilostamide treated cells. A possible explanation for this observation is that not all the cAMP generated by blocking the PDE was available to inhibit protein synthesis. Studies have shown that cAMP can be compartmentalized within the cell and used for activating separate pathways to elicit distinct biological responses. For example, compartmentation studies in the perfused heart have compared effects of forskolin and isoproterenol on cAMP accumulation and cardiac tension. England et al., (1987) found that, at approximately equal cardiac tensions, the levels of cAMP elicited by isoproterenol were generally smaller than those levels elicited by forskolin. When increased concentrations of isoproterenol were used to elevate cAMP to similar levels of forskolin treatment, the cardiac tensions were larger with isoproterenol than those observed with forskolin. England et al. concluded that only a portion of the total cAMP, induced by forskolin, may be responsible for the measured cardiac effects and that the remaining cAMP may be collected within intracellular compartments involved in different cellular functions. Similar observations were made in studies of the relationship of cAMP and the modulation of Ca\(^{2+}\) movement through cell membrane calcium channels of isolated canine ventricular myocytes (Hohl et al., 1991).
Ca²⁺ correlated best with amount of cAMP in the particulate fraction. As a result, treatment of myocytes with isoproterenol resulted in a greater proportion of total cAMP in the particulate fraction than with forskolin treatment. Since isoproterenol had a greater effect on calcium movement than forskolin, Hohl & Li hypothesized that, in myocytes, differences in responses to agents that increased cAMP may be explained in part by compartmentalization of cAMP.

In our experiments, forskolin treatment of VSMCs elevated cAMP levels (Fig 14) and suppressed collagen and total protein synthesis (Fig. 15). This finding agrees with Dubey et al. (2000) who reported that forskolin treatment has an inhibitory effect on collagen synthesis in VSMCs. Our studies showed that co-incubation of VSMCs with estrogen and forskolin produced an additive effect on cAMP production (Fig. 15) and an inhibitory effect on collagen and total protein synthesis (Fig. 16). Although the levels of cAMP induced by forskolin (Fig. 15) exceeded those induced by estrogen, collagen and total protein inhibition (Fig. 16) for all these agents was similar (~50% of controls). Based upon the studies by England et al., (1987) and Hohl (1991), it can be hypothesized that the bulk of the cAMP induced by forskolin may be compartmentalized for alternative functions not related to protein synthesis. Studies in human fetal lung fibroblasts have shown that treatment of cells with isoproterenol, a beta adrenergic, agonist, significantly elevated cAMP content and inhibited collagen synthesis (Baum et al., 1981). In our studies, treatment of VSMCs with isoproterenol significantly increased cAMP levels in VSMCs (Fig.
As expected, the addition of propranolol, a beta- adrenergic antagonist, to isoproterenol treated cells significantly reduced the level of cAMP, which was observed in cells treated with isoproterenol alone. Propranolol had no effect on estrogen's ability to inhibit cAMP which was not surprising since estrogen has been reported to increase cAMP levels by directly stimulating adenylyl cyclase activity. (Farhat et al., 1996).

Surprisingly, isoproterenol had no inhibitory effects on collagen or total protein synthetic rates even though isoproterenol treatment of VSMCs significantly elevated cell cAMP levels (Fig. 16, 17 and 18). Studies by Crystal et al. (1981) reported that a 6-hour incubation of lung fibroblasts with isoproterenol resulted in a decrease in collagen synthetic rates. Since our VSMCs were incubated with isoproterenol for only 1 hour, a 6-hour incubation study was conducted. As seen in figure 19 a 6-hour incubation did not result in any significant alteration in collagen or total protein synthetic rates, suggesting that there may be a fundamental physiological difference between lung fibroblasts and VSMCs. Treatment of VSMCs with isoproterenol and estrogen had an additive effect on cAMP levels (Fig. 16) but had no additive effect on collagen and total protein synthesis (Fig 17 and 18). The level of inhibition of cellular protein synthesis by this type of combination treatment was similar to the level of inhibition with estrogen treatment alone (Fig. 17 and 18).

VSMCs primarily have beta<sub>2</sub> adrenergic receptors that regulate vasodilation under normal physiological conditions (Vatner et al., 1986). Since isoproterenol is a nonspecific beta agonist, terbutaline, a selective beta<sub>2</sub>-
adrenergic receptor agonist, was utilized in our studies to further characterize the relationship of beta stimulated increases in cAMP and protein synthetic rates. Treatment of VSMCs with terbutaline resulted in a significant increase in cAMP levels, which was similar to estrogen's effects but was much less than the cAMP changes induced by isoproterenol (Fig. 20). As seen with isoproterenol, terbutaline also had no effect on protein synthesis in VSMC when administered to VSMCs either alone or in combination with estrogen (Fig. 21).

Numerous studies have shown that activation of the beta-adrenergic receptor can mediate transport of cAMP to the extracellular compartment, where it can serve an essential cellular function (Rosenberg et al., 1995). For example, in cortical cultures, which are co-cultures of neurons and astrocytes, upon isoproterenol stimulation of the beta-adrenergic receptor, cAMP is exported out from astrocytes to the extracellular compartment where it is hydrolyzed to adenosine (Rosenberg et al., 1995). These data suggest that the extracellular transport of cAMP is essential in replenishing adenosine levels, which are necessary for neuromodulation in the cerebral cortex (Rosenberg et al. 1995). In our studies, estrogen and the beta agonists, isoproterenol and terbutaline, had induced cAMP, but only estrogen inhibited protein synthesis in VSMCs, indicating that estrogen and beta agonist stimulation of cAMP levels result in some form of compartmentalization of cAMP within VSMCs. To further examine this hypothesis VSMCs were treated with either estrogen or terbutaline, and the distribution patterns of cAMP were measured. In VSMCs, treated with terbutaline, the distribution pattern of cAMP was 90%
extracellular and 10% intracellular, while in VSMCs, treated with estrogen, the cAMP distribution pattern was 70% extracellular and 30% intracellular. The distribution of cAMP following estrogen treatment was similar to the distribution of cAMP seen in untreated VSMCs. (Fig. 22). These data suggest that the transport of cAMP to the extracellular compartment following treatment with a beta agonist may explain why beta-adrenergic stimulation of cAMP in VSMCs does not result in inhibition of protein synthesis in VSMCs. (Fig.22). Since beta-adrenergic stimulation results in increased levels of cAMP in the extracellular compartment, it further questions the significance of the adenosine pathway as described by Dubey et al. (2000) in regulating protein synthesis in VSMCs.

Conclusion

The hypothesis, which was investigated in this study, was that agents which elevate cAMP in VSMC when used in combination with estrogen will have an additive effect in inhibiting collagen synthesis in VSMC. Our studies found that phosphodiesterase III inhibitor, cilostamide, phosphodiesterase IV inhibitor, Ro-24-1724, and forskolin in combination with estrogen had additive effects on estrogen induced cAMP levels and on estrogen reduced collagen synthetic rates, while beta-adrenergic agonists, isoproterenol or terbutaline, when used in combination with estrogen were able to elicit an additive effect on cAMP levels but had no effect on collagen synthetic rates. Furthermore, our studies show that once the beta-adrenergic receptor stimulation induces cAMP, it is actively transported to the extracellular milieu. These data suggest that the loss of most cAMP due to its transport out of the cell may be the reason why
beta-adrenergic receptor stimulation is unable to inhibit collagen synthesis in VSMCs.

Our study has shown that estrogen and other cAMP elevating agents, such as cilostamide, Ro-20-1724, and forskolin, have an inhibitory effect on collagen synthesis in VSMCs. It is well established that during the later stages of lesion development reducing collagen content in lesions may weaken lesions and cause them to rupture. The clinical significance of the data is that estrogen replacement therapy and medications which elevate cAMP may reduce collagen levels in lesions, causing them to eventually rupture. Furthermore, our data also suggest that the combination of these agents, which have an additive effect on estrogen, may have a negative effect on the stability of existing atherosclerotic lesions by inhibiting collagen synthesis.
References


Kauser K., Rubamyi J., 1994 17 estradiol attenuates endothelial dysfunction in male spontaneously hypertensive rats. J. Am Col. Cardiol. 295A


Rosenberg PA, Li Y. 1995. Adenylyl cyclase activation underlies intracellular cyclic AMP accumulation, cyclic AMP transport, and extracellular adenosine accumulation evoked by beta-adrenergic receptor stimulation in mixed cultures of neurons and astrocytes dcerived from rat cerebral cortex. Brain Res. 692(1-2):227-32


