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dissertation entitled

Cellular Mechanisms for the Adrenomedullin control of Mesangial Growth, Apoptosis and Matrix

presented by

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has been accepted towards fulfillment
of the requirements for

Doctor of Philosophy degree in ~~Physiology~~

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# CELLULAR MECHANISMS FOR THE ADRENOMEDULLIN CONTROL OF MESANGIAL GROWTH, APOPTOSIS AND MATRIX

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Physiology

1999

ABSTRACT

CELLULAR MECHANISMS FOR THE ADRENOMEDULLIN CONTROL OF MESANGIAL GROWTH, APOPTOSIS AND MATRIX

By

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Mesangial cells are structural and functional entities of the renal glomerulus, that play a key role in the pathogenesis of glomerulosclerosis associated with chronic renal failure. Factors affecting the growth of mesangial cells as well as the extracellular matrix production are important in the development of, or guarding against, glomerulosclerosis. Adrenomedullin is a recently described potent vasodilatory peptide that exerts most of its action through an increase in intracellular cyclic AMP via a Gs coupled heterotrimeric G-protein. This thesis examines the role of adrenomedullin in the regulation of mesangial proliferation, apoptosis and matrix production, and the possible involvement of mitogen-activated protein kinase pathways, specifically extracellular signal-regulated kinase (ERK), *jun* amino-terminal kinase (JNK) and P38 mitogen-activated protein kinase (P38 MAPK). We have used rat glomerular mesangial cells in culture as a model system.

Adrenomedullin caused an increase in cyclic AMP in rat mesangial cells, associated with a decrease in proliferation, an increase in apoptosis, and a modest increase in hyaluronic acid. These biological responses were associated with differential regulation of mitogen-activated protein kinase pathways, that is, adrenomedullin decreased ERK activity, but concomitantly increased JNK and P38 MAPK activities. Using selective pharmacological inhibitors of protein kinase A, P38 MAPK and

phosphotidyl inositol-3 kinase (a possible upstream regulator of P38) we examined the importance of the changes in the activities of the above signaling components.

With the exception of JNK activity and hyaluronic acid release, all the other responses, namely the decrease in ERK, increase in P38 MAPK, decrease in proliferation and increase in apoptosis were all dependent on the cAMP-PKA pathway. Although hyaluronic acid release was not PKA-dependent, P38 MAPK inhibitor blocked adrenomedullin-stimulated hyaluronic acid release. Because P38 MAPK activity was also inhibited by PKA inhibitor, the results on hyaluronic acid release also suggests a PKA-independent regulation of P38 MAPK. Furthermore, P38 MAPK inhibitor blocked only P38 MAPK activity without affecting ERK or JNK but inhibited all the three responses of adrenomedullin, namely proliferation, apoptosis and hyaluronic acid release. Wortmannin, a PI3-kinase inhibitor blocked only P38 MAPK activity, suggesting the presence of PI3-kinase upstream of P38 MAPK. Nevertheless, wortmannin inhibited only adrenomedullin-induced apoptosis and hyaluronic acid release, and did not affect adrenomedullin-inhibited proliferation. Because P38 inhibitor blocked all three responses (proliferation, apoptosis and hyaluronic acid), this probably suggests the presence of two P38 MAPK pathways, one sensitive to wortmannin (apoptosis and hyaluronic acid) and the other pathway insensitive to the PI3-kinase inhibitor (proliferation).

The results of the present study indicate that exogenous addition of adrenomedullin to rat mesangial cells in culture regulates mesangial turnover and matrix production. Although most responses are protein kinase A-dependent, it does not preclude the involvement of protein kinase A-independent pathways. This study also suggests an important physiological role for P38 MAPK in the regulation of mesangial proliferation, apoptosis and matrix.

*To my family*

## ACKNOWLEDGEMENTS

I would like to thank my committee members Drs. Kathleen Gallo, Greg Fink, Ponnal Nambi, Harvey Sparks and William Spielman. I would especially like to thank my mentor Dr. Spielman, for the constant encouragement, guidance and support throughout my graduate career. I would also like to thank Dr. Nambi for providing me with all the help that I needed while I was at SmithKline Beecham. In that regard, I would also like to thank Dr. David Brooks for part of the financial support for this project. I would like to thank Dr. Harvey Sparks for the helpful discussions through all the lab meetings, Dr. Gallo for the discussions on cell signaling and Dr. Fink for the help with my statistics questions.

I would also like to take this opportunity to thank my colleagues and friends in the lab specifically Carol Hall, Kwan, and Voytek at MSU, & Mark Pullen, and Charlene Wu at SmithKline Beecham. I would like to sincerely thank the office staff for all the help rendered to me.

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## KEY TO ABBREVIATIONS

| | |
|---|---|
| ADM | Adrenomedullin |
| A-II | Angiotensin-II |
| ANP | Atrial natriuretic peptide |
| AVP | Arginine vasopressin |
| cAMP | Adenosine 3'-5' cyclic monophosphate |
| CGRP | Calcitonin gene-related peptide |
| CRLR | Calcitonin receptor-like receptor |
| dbcAMP | dibutyryl cAMP |
| ERK | Extracellular signal-regulated kinase |
| GPCR | G-protein coupled receptor |
| HA | Hyaluronate or Hyaluronic acid |
| iNOS | Inducible Nitric oxide synthase |
| JNK | *jun*-amino terminal kinase |
| MAPK | Mitogen-activated protein kinase |
| MAPKK | Mitogen-activated protein kinase kinase |
| MAPKKK | Mitogen-activated protein kinase kinase kinase |
| MEK | MAPK/ERK kinase |
| MKK | MAPK kinase |
| MKKK | MAPK kinase kinase |
| NGF | Nerve growth factor |
| P38 MAPK | P38 mitogen-activated protein kinase |
| PDGF | Platelet-derived growth factor |
| PGE2 | Prostaglandin E2 |
| PI3-kinase | Phosphotidyl inositol 3-kinase |
| PKA | Protein kinase A |
| PKC | Protein kinase C |
| PP2a | Protein phosphatase 2a |
| RAMP | Receptor-activity modifying protein |
| TGF | Transforming growth factor |
| TNF | Tumour necrosis factor |
| TPA | Tetradecanoyl phorbol myristoyl acetate |
| VIP | Vasoactive intestinal peptide |

# 1. INTRODUCTION

Proliferation, apoptosis and synthesis of extracellular matrix are fundamental biological processes, which when perturbed individually or together leads to the commonly seen disorders of different systems. In chronic renal failure associated with glomerulonephritis, aberrant mesangial cell proliferation, apoptosis and excess matrix synthesis are keys to the pathogenesis of the disease (Brenner and Stein, 1989; El Nahar et al., 1997; Klahr et al., 1988). Hence, effort in developing different and effective treatment modalities directed at controlling these aberrant processes is necessary to potentially regulate these biological processes.

Adrenomedullin, a recently described vasodilatory peptide, causes a decrease in proliferation, an increase in apoptosis and a slight increase in hyaluronic acid levels in cultured rat mesangial cells (Parameswaran et al., 1999b,e). Understanding and characterizing the intracellular signaling pathways of these biological processes will aid us in targeting these signaling pathways for drug development. This thesis project is primarily focused on the effects of adrenomedullin and their mechanisms thereof, on mesangial growth, apoptosis and matrix synthesis. Accordingly, the major aims of this thesis are: 1. To examine the effects of adrenomedullin on mesangial cell turnover, specifically proliferation and apoptosis. 2. To examine the effects of adrenomedullin on mesangial matrix production, particularly that of hyaluronic acid release. 3. To examine the effects of adrenomedullin on the intracellular signaling molecules, particularly that of adenylate cyclase, ERK, P38 MAPK and JNK. 4. To examine the role of these

intracellular signaling molecules on adrenomedullin-mediated changes in proliferation, apoptosis and hyaluronic acid release, using selective pharmacological inhibitors.

This thesis is essentially divided into 6 chapters. An introduction about this thesis is given in chapter #1. The second chapter deals with the past relevant and current literature that is available on mesangial cells, adrenomedullin, signaling mechanisms in general, and how they can be integrated in understanding the fundamental biological processes in mesangial cells. Chapter #3 is the research project that focuses on the effects of adrenomedullin on mesangial cell proliferation and apoptosis, and also the modulation of mitogen-activated protein kinase pathways. The experiments described in this chapter are designed to test the hypothesis that adrenomedullin-mediated changes in proliferation, apoptosis, ERK, JNK and P38 MAPK activities are cAMP-PKA-dependent. First, in this chapter, the experiments compare the effects of ADM and forskolin (a direct adenylate cyclase activator) on proliferation, apoptosis and MAPK activities. In order to examine the role of cAMP-PKA pathway directly, experiments have also been done in the presence of a potent PKA inhibitor (H89).

Chapter #4 is the research project that deals with the role of adrenomedullin-stimulated P38 MAPK pathway using a selective pharmacological inhibitor, SB203580. The experiments described in this chapter test the following hypothesis: 1. Adrenomedullin-mediated changes in proliferation and apoptosis are P38 MAPK-dependent. 2. Adrenomedullin-mediated changes in P38 and JNK activities are PI3-kinase dependent. (This is done using a selective pharmacological inhibitor of PI3-kinase, namely wortmannin) 3. Adrenomedullin-mediated changes in proliferation and apoptosis are PI3-kinase dependent (using wortmannin as a pharmacological tool)

Chapter #5 deals with the effects and mechanisms of adrenomedullin on hyaluronic acid release. In this project we have examined the role of protein kinase A, P38 MAPK and PI 3-kinase in the regulation of ADM-stimulated hyaluronic acid release using the respective pharmacological inhibitors. The experiments described in this chapter test the following hypotheses: 1. Adrenomedullin-stimulated hyaluronic acid release is cAMP-PKA-dependent. 2. Adrenomedullin-stimulated hyaluronic acid release is PI3-kinase- and P38 MAPK-dependent. Experiments designed in this chapter have also examined the role of forskolin (adenylate cyclase activator) and dibutyryl cAMP (a cell permeable cAMP analog) on rat mesangial cell hyaluronic acid release. Experiments have also been designed to examine the possible role of PKA, P38 MAPK and PI3-kinase on both forskolin- and dbcAMP-stimulated hyaluronic acid release.

The final conclusions and summary of this thesis work including a general discussion on limitations of this study and the positive contributions are provided in chapter 6.

# 2. LITERATURE REVIEW

This chapter of the thesis deals with the current basic concepts on mesangial cells and signaling, and the significance and contribution of these cells to the pathophysiology of glomerulonephritis. Also included in this section is the current literature on adrenomedullin physiology and pharmacology, including some of the signaling aspects from other model systems. Essentially, this chapter is divided into five sections. The first one gives an introduction about the renal glomerulus, the cell types and the main functions of mesangial cells. The second section deals with role of mesangial cell proliferation and matrix synthesis in the progression of glomerulonephritis and chronic renal failure. Discovery of adrenomedullin and its basic pharmacology is provided in section 3. Section 4 deals with the current concepts on adrenomedullin receptor regulation. This section also reviews some important aspects of signaling in general, with emphasis on the mitogen-activated protein kinase pathways. The final section tries to justify why identification of these different pathways may be necessary for drug targeting in general.

## 2.1. THE KIDNEY GLOMERULUS AND MESANGIAL CELLS:

The glomerulus, the filtration unit of the kidney, is a network of capillaries originating in the afferent arteriole. After dividing into four to eight lobules to form the glomerular tuft, the capillaries rejoin to form the efferent arteriole. The one to one-and-a-half million glomeruli present in the human kidney are composed of at least four cell types: (1) endothelial cells, (2) glomerular epithelial cells, (3) mesangial cells, and (4)

parietal epithelial cells. The endothelial cells line the glomerular capillary wall and are characterized by the presence of fenestrae. This layer of endothelium may participate in the restriction of macromolecules from passing across the capillary wall. In certain disease states the fenestrae are replaced by a continuous layer of endothelial cytoplasm. The glomerular epithelial cells participate in the synthesis of glomerular basement membrane that is present in between the endothelium and the epithelium. The basement membrane forms an important component of the filtration unit. The glomerular epithelial cells also participate in the filtration process through pinocytosis of filtered proteins that may have leaked through the glomerular basement membrane. Also, the glomerular epithelial cells contain a cell coat rich in negatively charged sialoglycoproteins and this acts to restrict the passage to anionic molecules across the glomerular basement membrane. In some forms of renal disease associated with proteinuria, the epithelial foot processes are replaced by continuous band of epithelial cytoplasm.

The mesangial cells by virtue of their contractile properties probably influence the rate of plasma flow through glomerular capillaries and thereby the glomerular filtration rate. The other functions of mesangial cells and their role in renal glomerular disease will be dealt in detail later (see below). Finally, the parietal epithelial cells together with the basement membrane material form the outer wall of the Bowman's capsule, which surrounds the glomerular tuft (Valtin and Schaffer, 1995; Klahr *et al.*, 1988; Schlondorff *et al.*, 1996; Pfeilschifter, 1989).

The following filtration model in the glomerulus has been proposed by many workers: The endothelium acts as a gross filter that screens out cells and controls access to the main filter, which is probably the basement membrane. The epithelium may

provide an additional, important barrier, and it can phagocytize macromolecules that have leaked through the basement membrane. The mesangial cells, by contracting and relaxing the capillaries, influence the glomerular filtration rate by affecting the rate of plasma flow. It is important to note that there is no basement membrane between mesangial cells and the capillaries, unlike the epithelium and the capillaries. Thus by abutting the capillary loops directly, the mesangial cells are also thought to recondition and unclog the filter (Valtin and Schaffer, 1995).

Although all the above components are necessary for maintaining the functional integrity of filtration, the mesangial cells are unique in that they act as a "back bone" to the glomerular tuft, that is, mesangial cells provide the structural integrity necessary for maintaining the capillary loops. Immunological models of mesangiolysis and genetic models have provided enough evidence for this role of mesangial cells. Injection of anti-Thy 1.1 antibodies in rats result in profuse mesangiolysis, resulting in the loss of individual capillaries. Upon re-growth of the mesangial cells, the individual capillaries are reestablished (Iversen *et al.*, 1992; Yamamoto *et al.*, 1991). Mice made deficient in PDGF-β or mutated PDGF-β receptor, result in a failure of mesangial cells to grow into the glomerulus during renal development. Although the capillary basement membrane and the glomerular epithelial and endothelial cells develop normally the lack of mesangial cells result in the formation of intra-glomerular vascular sacs, instead of normal capillary network (Soriano, 1994; Leveen *et al.*, 1994).

The following is a summary of the potential roles of mesangial cells:

1. Structural support for the glomerulus, and capillary loops. 2. Generation and turnover of extracellular mesangial matrix. 3. Target site and site of production for various vasoactive agents, inflammatory mediators, growth factors, cytokines, chemokines and adhesion factors. 4. Handling of macromolecules such as lipids, immune complexes and advanced glycation end products (AGE). With enormous responsibility in hand, the mesangial cells carefully balance their turnover and matrix production and degradation, thus avoiding any inflammatory reaction that may culminate in the pathology of glomerulonephritis. In pathologies where mesangial cells are involved, irrespective of the original insult, the end point is usually an excessive mesangial proliferation or aberrant matrix production resulting in glomerulosclerosis. Thus understanding the mechanisms involved in the process of mesangial growth, apoptosis and matrix production will help us target better molecules for drug development in glomerular diseases.

## 2.2. GLOMERULOSCLEROSIS, MESANGIAL CELLS AND CHRONIC RENAL FAILURE:

The progression of chronic renal failure represents one of the biggest challenges in nephrology because it leads to a large number of patients reaching end stage renal insufficiency and requiring long term dialysis replacement therapy (El Nahas *et al.*, 1997). Glomerulosclerosis is known to occur in a wide range of chronic nephropathies regardless of whether the initial insult is glomerular, tubulo-interstitial or vascular. What ever may be the case, the glomerular cells are intrinsically involved in the process of

injury and development of the pathology (Figure 1). Mesangial cells are especially actively involved in the pathogenesis of glomerulosclerosis. As was discussed earlier, mesangial cells are capable of the synthesis and release of various inflammatory mediators, growth factors, vasoactive agents etc. In diseased conditions, these agents can also act on the mesangial cells in an autocrine fashion resulting in aberrant proliferation and matrix production. Not only are autocrine factors involved, paracrine factors released from epithelial and endothelial cells in the glomeruli also affect mesangial cell and matrix turnover (Klahr *et al.*, 1988; Brenner and Stein, 1989).

One of the key features of various human glomerular diseases including IgA nephropathy, membranoproliferative glomerulonephritis, variants of idiopathic focal sclerosis, lupus nephritis and possibly diabetic nephropathy, is the proliferation of intrinsic glomerular mesangial cells (Floege *et al.*, 1993). It is believed that a number of conditions such as hyperlipidemia, hemodynamic abnormalities, increased glomerular metabolism, local hypercoagulopathy, mesangial macromolecular deposition etc. lead to glomerular injury and activation of the glomerular growth promoters that lead to an increase in glomerular hypertrophy including mesangial expansion and matrix synthesis (Couchman *et al.*, 1994; Rupprecht *et al.*, 1996). Thus, as mentioned before, there is a remarkable resemblance in the histopathology of kidney specimens of end stage kidney disease obtained from a wide variety of primary disease conditions, leading to a possible hypothesis that there is a common intermediary stage in the pathogenesis of such chronic renal diseases (El Nahas *et al.*, 1997).

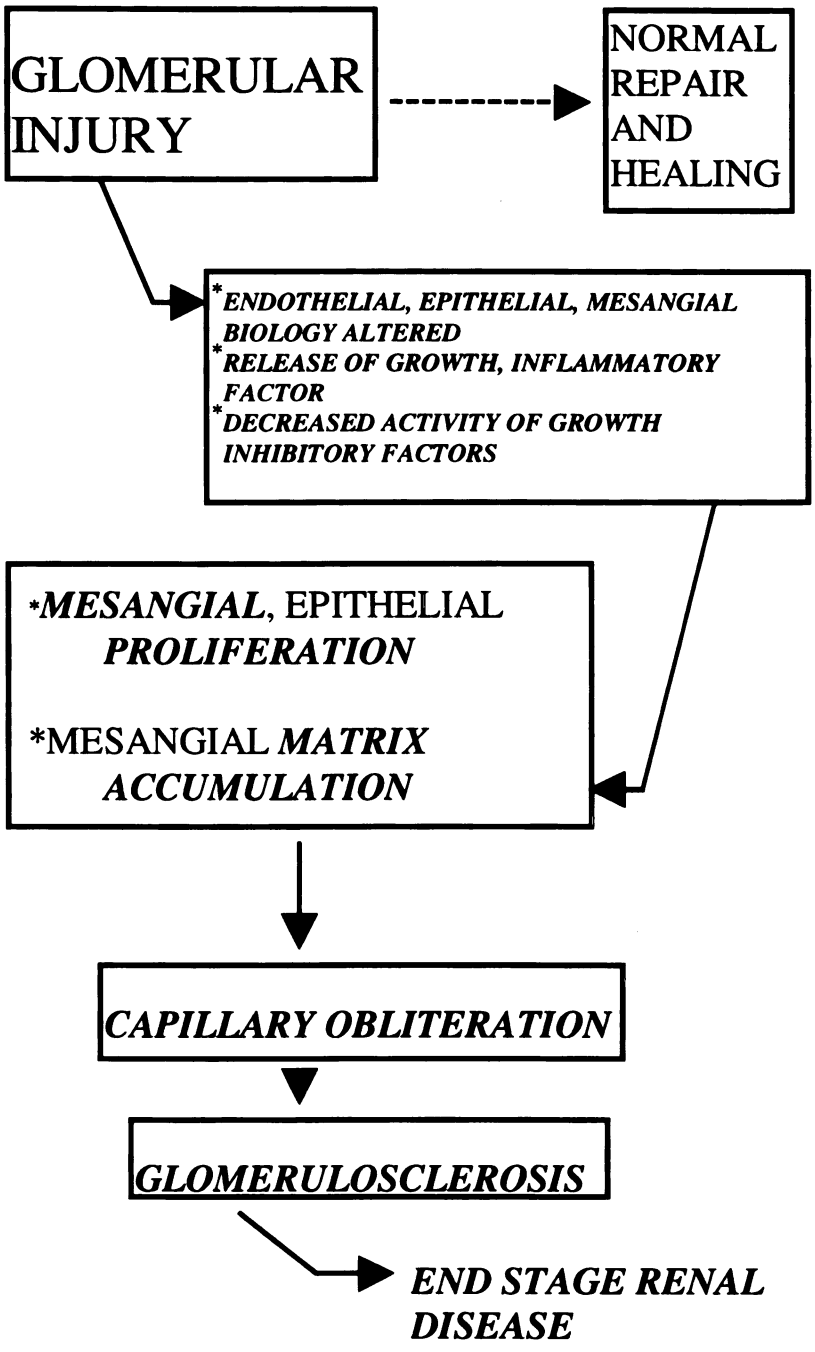



Fig 1: Possible mechanisms of development of glomerulosclerosis and end-stage renal disease.

Because mesangial growth and matrix turnover are important in the pathogenesis of glomerulonephritis and chronic renal failure, understanding the mechanisms of mesangial proliferation and matrix production may be helpful in the long-term goal of drug development for such renal diseases. Accordingly, in this thesis we have examined the mechanisms of adrenomedullin-mediated decrease in mesangial proliferation and increase in apoptosis and matrix (hyaluronic acid), with the idea of possibly identifying some targets for drug development related to cell turnover and matrix production.

## 2.3. ADRENOMEDULLIN-

## DISCOVERY, PHYSIOLOGY AND PHARMACOLOGY:

Adrenomedullin is a multifunctional peptide hormone with a potent vasodilatory activity. It was discovered using a platelet adenylate cyclase bioassay system by Kitamura *et al.* (1993a) and subsequently cloned from extracts of a pheochromocytoma. The human adrenomedullin gene encodes a 185 amino acid preprohormone (Kitamura *et al.*, 1993b). The genetic sequence is well preserved among species and the level of peptide homology is extensive. Rat adrenomedullin differs from the human in only six positions and is 50 amino acids in length (52 amino acids in human) (Sakata ., 1993). Post-translational processing of human preproadrenomedullin results in the production of at least two biologically active peptides, adrenomedullin (ADM) and proadrenomedullin N-terminal 20 peptide (PAMP). PAMP is also a vasodilator and 20 amino acids in length (Kitamura *et al.*, 1994). Although adrenomedullin has been shown to interact with calcitonin gene-related peptide (CGRP) receptors, PAMP interacts with a unique yet

uncharacterized receptor. Current understanding of the adrenomedullin receptors will be described later (please see section 2.4.).

**Sites of adrenomedullin production:**

In addition to being synthesised and released by pheochromocytomas and normal adrenal glands, transcription of the adrenomedullin gene occurs in a variety of tissues throughout the body. Adrenomedullin-like immunoreactivity has also been identified in multiple tissues including adrenal medulla, blood vessels, kidney, anterior pituitary, hypothalamus, heart, lung, thyroid, submandibular gland, liver, pancreas, stomach, intestine, testis, and choroid plexus (Ichiki *et al.*, 1994; Jougasaki *et al.*, 1995; Satoh *et al.*, 1995; Satoh *et al.*, 1996; Washimini *et al.*, 1995). Circulating levels of adrenomedullin in humans and experimental animals have been reported to be in the low pg/ml range (Kitamura *et al.*, 1994; Sato *et al.*, 1995; Washimini *et al.*, 1995). Plasma levels of adrenomedullin are significantly elevated in a number of human pathologies including essential hypertension, pulmonary hypertension, heart failure, liver diseases, renal failure, thyrotoxicosis, acute asthma, and type I and II diabetes mellitus (Cheung *et al.*, 1997; Ishimitsu *et al.*, 1994; Jougasaki *et al.*, 1995; Kitamura *et al.*, 1994; Kohno *et al.*, 1996a; Kohno *et al.*, 1996b; Shimokubo *et al.*, 1995; Taniyama *et al.*, 1997; Washimine *et al.*, 1995; Nakamura *et al.*, 1998; Garcia-Unzueta *et al.*, 1998). It is still not known whether these elevations are as a result of tissue damage or as a physiologically relevant compensatory mechanism. In septic shock, adrenomedullin levels are elevated leading to profound hypotension. No information is available on transgenic overexpression or knockout of ADM gene in animal models.

**Regulation of adrenomedullin production and secretion:**

In a recent comprehensive study by Isumi *et al.* (1998), the effect of 43 different substances on adrenomedullin secretion was tested from cultured rat endothelial cells. They also demonstrated that the endothelial cells secreted adrenomedullin at a rate 5.8 times higher than vascular smooth muscle cells. Out of 43 substances tested 26 were found to significantly affect adrenomedullin secretion. Tumor necrosis factor, interleukin-1, and lipopolysaccharide augmented adrenomedullin secretion from endothelial cells, suggesting that adrenomedullin secreted by endothelial cells may participate in the induction of hypotension in septic shock. The same effects were also observed in another study in vascular smooth muscle cells (Sugo *et al.*, 1995). While bovine serum albumin was found to be a potent stimulator, fetal calf serum significantly inhibited adrenomedullin secretion in endothelial cells but stimulated in vascular smooth muscle cells. Transforming growth factor β1 was found to be the most potent inhibitor of adrenomedullin secretion both in endothelial and vascular smooth muscle cells. Basic FGF and EGF showed weak but significant inhibitory effects on adrenomedullin secretion in endothelial cells. Among the three interferons, only interferon-γ showed a weak inhibitory effect on adrenomedullin secretion in both endothelial cells and smooth muscle cells. Thrombin potently stimulated adrenomedullin secretion in endothelial cells. All the six steroid hormones that were tested (glucocorticoids, mineralocorticoids, and sex steroids) also increased adrenomedullin secretion modestly in these cultured cells. In vascular smooth muscle cells, retinoic acid, mineralo- and glucocorticoids and thyroid hormones weakly stimulated adrenomedullin gene transcription. While isoproterenol and

norepinephrine slightly but significantly increased adrenomedullin secretion, phenylephrine and epinephrine did not alter adrenomedullin secretion. Among the different vasoactive peptides tested, CGRP and endothelin-1 decreased adrenomedullin secretion. In vascular smooth muscle cells, angiotensin-II, endothelin-1, bradykinin, substance-P and adrenaline stimulated adrenomedullin production while VIP inhibited its (Sugo *et al.*, 1995). Neither the nitric oxide synthase inhibitor nor the nitric oxide generator altered adrenomedullin secretion. Oxidized LDL also stimulated adrenomedullin secretion to a small but significant level.

Among the various substances that modulate second messenger systems, only TPA was shown to have a stimulatory effect on adrenomedullin secretion. Forskolin and 8-Br-cAMP did not show any effect even up to very high concentrations in endothelial cells. 8-Br-cGMP also did not alter adrenomedullin levels in endothelial cells (Isumi *et al.*, 1998). On the other hand, in vascular smooth muscle cells cAMP was shown to be a potent inhibitor of adrenomedullin gene transcription (Sugo *et al.*, 1995).

**Actions of adrenomedullin:**

Adrenomedullin has been shown to have a number of effects in different systems including the heart, lung, kidney, adrenal gland, vasculature, pituitary and brain. In general, most effects of ADM can be explained in terms of an increase in intracellular cAMP levels although a few exceptions have been encountered. Most important effects of ADM are summarized in Table 1 (Modified from Samson, 1998).

## Table 1: Actions of Adrenomedullin

| Tissue | Effect of Adrenomedullin |
|---|---|
| Vasculature | Hypotension, antiproliferation, survival factor |
| Heart | Positive chronotropism and inotropism, increased coronary blood flow, increased ANP gene transcription, antimitogenesis, potentiation of iNOS expression in cytokine stimulated cultured myocytes. |
| Lung | Vasodilation, bronchodilation, anti-inflammatory |
| Adrenal gland | Inhibition of K+ and A-II-stimulated aldosterone secretion |
| Kidney | Increased renal blood flow, diuresis, natriuresis, inhibition of mesangial cell proliferation, stimulation of renin release, stimulation of hyaluronic acid release from mesangial cells. |
| Pituitary gland | Inhibition of ACTH secretion |
| Brain | Inhibition of thirst (water drinking) and salt appetite*, inhibition of gastric emptying, stimulation of sympathetic outflow (hypotension), inhibition of AVP secretion, increased cerebral blood flow. |

*ADM antisense oligonucleotide treatment significantly lowers the peptide content in the hypothalamo-paraventricular nucleus and exaggerates sodium consumption.

In addition to the above effects, adrenomedullin has also been shown to affect insulin secretion and blood glucose metabolism. In isolated rat islets, adrenomedullin inhibited insulin secretion in a dose-dependent manner (Martinez *et al.*, 1996). Monoclonal antibody against bio-active adrenomedullin was able to increase insulin release by about five fold and this effect was reversed by the addition of synthetic adrenomedullin. Furthermore intravenous injection of adrenomedullin reduced insulin levels in the blood with a concomitant increase in circulating glucose in rats.

**Growth effects of adrenomedullin:**

Because this thesis deals with the mechanisms of adrenomedullin-mediated growth, this section exclusively deals with the current knowledge on the growth effects of ADM. Depending on the cell type, ADM has been shown to have a positive or a negative influence on cell proliferation. In a variety of human tumor cell lines ADM acts as an autocrine growth promoter (Miller *et al.*, 1996). This autocrine effect was abolished by the addition of monoclonal antibodies against ADM. But in smooth muscle cell types, including vascular smooth muscle cells and mesangial cells, ADM acts as a negative regulator of cell growth (Chini *et al.*, 1995; Chini *et al.*, 1997). It not only inhibits basal proliferation in these cell types but also suppresses mitogen-stimulated proliferation. Although the mechanisms of these effects are not currently known, most of these actions can be related to its effects on intracellular cAMP levels. In VSMC and mesangial cells, cAMP elevation decreases proliferation and so does ADM. Moreover protein kinase-A inhibitors effectively reverse adrenomedullin's effect on proliferation in these cell types (Chini *et al.*, 1997; Parameswaran *et al.*, 1999b).

A recent study using differential display PCR protocol reported that a specific downregulation of adrenomedullin gene by overexpression of MC29 v-Myc oncoprotein in C3H10T1/2 mouse fibroblast. Furthermore, Wang *et al.* (1999) identified the transcript of the ADM gene as an mRNA that is specifically downregulated in v-Myc overexpressing C3H10T1/2 cell line as well as in Rat 1a cell line inducible for c-Myc. Results from these studies suggest that in these cell lines ADM gene expression in incompatible with deregulated growth. The authors propose a model in which repression of ADM gene expression by Myc is important to the role of this oncoprotein as a potentiator of cellular transformation in C3H10T1/2 cell line.

In addition to its effects on proliferation, ADM also has significant effect on apoptosis of cells. In endothelial cells ADM protects the cells from apoptosis through a protein kinase A-independent mechanism (Kato *et al.*, 1997). On the other hand, in mesangial cells it induces apoptosis through a protein kinase-A-dependent mechanism (Parameswaran *et al.*, 1999b). This thesis is focussed primarily on the mechanisms of how the changes in proliferation and apoptosis are brought about in mesangial cells. In addition to an increase in proliferation, an aberrant apoptotic machinery is also thought to contribute to the development of proliferative glomerulonephritis involving mesangial cells. We believe if the mechanisms for these biological processes are characterized, it will help us target better molecules for drug development for glomerular diseases.

As mentioned in previous section(s) not only an aberrant growth leads to glomerulosclerosis, an increase in matrix components also contributes to the disease process. Although to our knowledge there are no reports so far on the effects of ADM on matrix components, results from our laboratory suggest that ADM might regulate

hyaluronic acid release, an important matrix component in mesangial cells (Parameswaran *et al.*, 1999). Because Chapter #5 deals with the mechanisms of hyaluronic acid release in mesangial cells, the importance of hyaluronic acid and its role in mesangial cell homeostasis will be discussed in that chapter.

**Adrenomedullin in diseased states:**

As mentioned previously, plasma adrenomedullin levels are elevated in a variety of disorders. Only a few of those studies are cited here. In a study by Kubo *et al.* (1998), 37 patients with chronic glomerulonephritis and 39 healthy volunteers were enrolled. Plasma ADM concentrations were higher and urinary ADM levels were lower in patients with chronic glomerulonephritis than in healthy volunteers. Furthermore the plasma ADM levels positively correlated with the degree of proteinuria while urinary ADM levels negatively correlated with proteinuria. In another study involving patients with non-insulin-dependent diabetes mellitus Nakamura *et al.* (1998), found that the plasma ADM level increased depending on the severity of the diabetic nephropathy and retinopathy. In a small scale human study involving 58 male subjects (eight with essential hypertension, 12 with heart failure, 10 with ascites due to cirrhosis, 12 with chronic renal failure, four with hypoxia due to chronic obstructive pulmonary disease and 12 control subjects) plasma levels of ADM were significantly increased in patients with essential hypertension, congestive heart failure and renal failure compared to the controls. Plasma levels of ADM were also elevated in patients with ascites due to liver cirrhosis and chronic pulmonary disease with hypoxia (Cheung and Leung, 1997).

## 2.4. ADRENOMEDULLIN RECEPTOR, SIGNAL TRANSDUCTION AND MITOGEN-ACTIVATED PROTEIN KINASE PATHWAYS:

Adrenomedullin shares significant structural homology with calcitonin gene-related peptide (CGRP) and in several systems has been shown to act through a receptor that is blocked by the CGRP receptor antagonist CGRP(8-37) (Samson, 1998). This is especially evident in the vascular beds where the vasorelaxant effect of ADM is blocked by the CGRP receptor antagonist. However, the blood pressure lowering effect of intravenous infusion of ADM is not blocked by CGRP(8-37) and the increased renal blood flow observed in isolated kidney or in situ tissue preparation is also not blocked by the CGRP receptor antagonist (reviewed in Samson, 1998). Adrenomedullin-(22-52) the peptide fragment from proadrenomedullin was recently shown to antagonize adrenomedullin's action in endothelial cells (Eguchi *et al.*, 1994). In mesangial cells the adrenomedullin receptor antagonist inhibits cAMP response to both ADM and CGRP. Furthermore CGRP receptor antagonist [CGRP98-37)] also blocks both ADM- and CGRP-stimulated cAMP response in the same cells (Osajima *et al.*, 1996).

Recently Kapas *et al.*, (1995) identified a cDNA clone to be adrenomedullin receptor. This was originally cloned as an orphan receptor. When expressed in COS-7 cells this receptor was shown to have specific binding affinity [Kd of 8.2x10(-9)M] for adrenomedullin and increased cAMP levels significantly. Also, Hanze *et al.*, (1997) reported the cloning of a novel human receptor gene with homology to rat adrenomedullin receptor with high expression in heart and immune system. In order to confirm the above two findings Kennedy *et al.*, (1998) transfected the putative rat and human adrenomedullin receptors in COS-7 cells. Although they found specific binding

for adrenomedullin in the putative adrenomedullin receptor transfected cells, they also found a similar binding in vector transfected cells. Moreover they found that adrenomedullin failed to evoke any cAMP response in these transfected cells. These authors claim that the cloned receptors may not be authentic adrenomedullin receptors.

The calcitonin family of peptides includes calcitonin, amylin, two CGRPs (CGRP 1&2) and adrenomedullin. Genes encoding the calcitonin, CGRP and adrenomedullin receptors have been cloned (Lin *et al.*, 1991; Kapas *et al.*, 1995; Aiyar *et al.*, 1996) but that still has not solved the problem of why ADM would act through a CGRP receptor in some systems and not others. Moreover it was found that it was not possible to express the cloned CGRP receptor in any cell line except a few.

A recent report by McLatchie *et al.* (1998) might suggest some explanation for these effects. McLatchie's group reported a novel mechanism of receptor regulation for the receptors of calcitonin related peptides. These researchers cloned a 148 amino acid protein called receptor-activity modifying protein (RAMP). Initially they cloned RAMP1 and subsequently cloned RAMP 2 and 3 from SKNMC cells (these cells are derived from human neuroblastoma and have endogenous CGRP receptor). Surprisingly they found that overexpression of RAMP1 in xenopus oocytes (which have endogenous CGRP receptor) led to a large dose-dependent response to CGRP than without RAMP1. These RAMP1 expressing oocytes showed no significant response to ADM, calcitonin and amylin. Over expression of RAMP1 also did not affect other endogenous receptors namely adenosine, VIP and beta-adrenergic receptor. Hence they concluded that RAMP1 was specific for CGRP-mediated responses. On the other hand, overexpression of RAMP1 in mammalian cells not expressing an endogenous CGRP receptor did not affect

cAMP response to CGRP. When HEK 293 cells were transfected with calcitonin-receptor like receptor (CRLR) and RAMP1, then the cells responded to CGRP significantly. (The members of the calcitonin family of peptides except CGRP were originally thought to be candidate ligands for CRLR).

In further experiments the authors showed evidence that the mechanism of RAMP1-mediated increase in CGRP response was by targeting the CRLR to the cell membrane. Transfection of both RAMP1 and CRLR led to a dramatic increase in the CRLR in the cell membrane. Thus RAMP was concluded to be a membrane targeting protein of the receptor. By cloning and identifying RAMP 2 and 3, the authors showed that RAMP 2 and 3 did not affect the response to CGRP. On the other hand they dramatically increased the response to ADM. Both the ADM receptor antagonist [ADM(22-52)] and the CGRP receptor antagonist [CGRP(8-37)} blocked the responses to ADM in the cells expressing RAMP2 and CRLR. Thus the authors showed evidence that the CRLR can induce a signal to either ADM or CGRP based on the presence of the type of RAMP. That is, CRLR has two alternative pharmacological profiles that are conferred by the accessory proteins RAMP 1 (producing CGRP receptor) and RAMP 2 (producing ADM receptor). They proposed that RAMPs probably control the transport and glycosylation of the CRLR and the difference in pharmacology could be due to the differential glycosylation and/or the presence of a particular RAMP.

Thus, the existence of RAMPs indicates a new and novel mechanism of receptor regulation. The regulatory events that change the responsiveness of tissues to different neuropeptides need to be determined in the context of RAMP. In mesangial cells, the cAMP responses are blocked by both ADM and CGRP receptor antagonists to both ADM

and CGRP. Further studies are necessary in this area to identify the type of RAMP that is expressed and the mechanisms of how they are regulated in both health and disease.

**Mitogen-activated protein kinase pathways and Signal transduction:**

Stimulation of any receptor that leads to a biological event, has many intermediary biochemical pathways that are orderly, defined and specific. In that regard, more than a decade ago, a module of kinase pathways was defined and named the mitogen-activated protein kinase pathways. A generic model of such pathway includes a MAPKKK (MKKK), that phosphorylates Ser/Thr residues and activates a MAPKK (MKK). Activated MKK is a dual phosphorylating kinase that phosphorylates a MAPK at threonine and tyrosine residues. The activated MAPK has a number of cellular targets including the transcription factors. The transcription factors then lead to changes in gene expression and the possible biological event. Although most receptor activation leads to this generic event, the pathways are specific because of the presence of different isoforms and types of kinases within the module. Currently there have been 14 MKKK, 7 MKK and 12 MAPK identified in mammalian cells. The mammalian MAPK can be divided into five parallel MAPK pathways. They are the extracellular signal-regulated kinase-1/2, *jun*-amino terminal kinase, P38 mitogen-activated protein kinase pathways, extracellular signal-regulated kinase-3/4 and the extracellular signal-regulated kinase-5. Of these only the first three, namely the ERK1/2, JNK and the P38 pathways, are well characterized (Denhardt, 1996; Neary, 1997; Robinson and Cobb, 1997; Widmann *et al.*, 1999). The schematic in figure 2 gives some idea on how these pathways are organized.

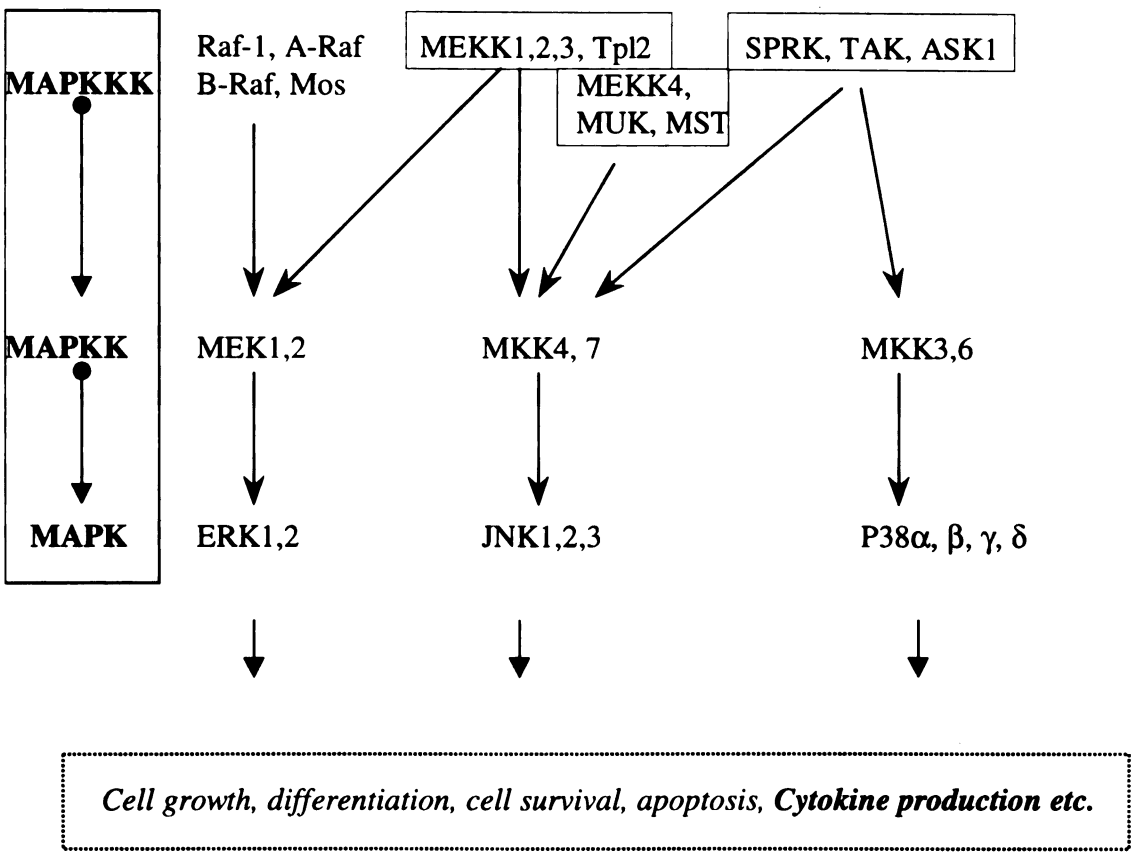



Figure 2: A schematic of the MAPK pathway. The small G proteins such as ras, cdc42, rab, rac are also involved in the activation of MAPKKK. These are usually activated via growth factor, GPCR, cytokine receptors, although it depends on the ligand and cell type. The second messenger systems of the receptors can also integrate into the MAPK pathway by mechanisms that are not yet well defined.

A brief description of each of the three pathways is given below. There is a vast number of cell culture literature available on these pathways and is increasing every day. Only the ones that are relevant to the present study are cited here.

**The ERK pathway**: Activation of the ERK1/2 pathway results in the activation or inhibition of many substrates with diverse functions in cells. There is a good correlation between activation of ERK and proliferation of cells. Growth factors such as PDGF and EGF, and G-protein coupled receptor agonists such as endothelin and thrombin, that stimulate cell proliferation including mesangial cells cause an increase in ERK activity (Denhardt, 1996; Widmann *et al.*, 1999). Suppression of ERK activity [either by an inhibitor of MEK (Dudley *et al.*, 1995; Pang *et al.*, 1995) (an immediate upstream activator of ERK-Please see figure 2) or dominant negative mutants of the pathway especially of Raf (Pages *et al.*, 1993)] has been shown to result in the inhibition of proliferation induced by these mitogens. However, activation of ERK may not always be required for proliferation. Interleukin-4 stimulation of B cells does not significantly activate ERK signaling but still induces proliferation (Wang *et al.*, 1992). In smooth muscle cells activation of ERK leads to PGE2 synthesis that actually inhibits proliferation. But in cells lacking this pathway of ERK-mediated stimulation of PGE2 synthesis, ERK activation stimulates proliferation. Activation of ERK may also provide protection against apoptosis in some cell types (Bornfeldt *et al.*, 1997). In PC-12 cells, NGF withdrawal inhibits ERK activity and induces cell death. But constitutive activation of ERK protects the cells during NGF withdrawal (Xia *et al.*, 1995). Support of the hypothesis on the involvement of ERK pathway in survival signal comes from studies on

B-Raf knockout mice. These mice die of vascular defects during mid-gestation (Wojnowski *et al.*, 1997). In mesangial cells it appears that the activation of the ERK pathway leads to an increase in proliferation. In fact, in proliferative glomerulonephritis increase in ERK activity in the mesangium has been reported (Bokemeyer *et al.*, 1997).

The ERK pathway can be modulated by a number of agonists including growth factors, G-protein-coupled receptors, cytokine receptors etc. Even if the final common pathway for these ligands is the activation or inhibition of the MAPK module of the ERK pathway, it does so by different mechanisms. For example, the growth factor receptors when activated, increase their intrinsic tyrosine kinase activity, that leads to the binding of adapter proteins (Shc, Grb2, Sos) causing the small G-protein ras to be activated by exchange of GTP in the membrane. Activation of ras leads to the activation of raf1 or other MKKKs in the ERK module leading to the activation of ERK pathway. For receptors lacking an intrinsic tyrosine kinase activity, tyrosine phosphorylation of the receptor and/or the adapter proteins (Shc, Grb2, Sos) is accomplished by recruitment and activation of Src family of tyrosine kinases including Lck, Lyn, Fyn etc. The recruitment of adapter proteins is followed by the activation of the ERK module as described for the growth factor receptors (Widmann *et al.*, 1999; Denhardt, 1996). The mechanism of how G-protein coupled receptors lead to the activation of ERK has been an intense area of research over the past 5-8 years. The consensus is that in case of Gi-coupled receptors the release of $G\beta\gamma$ on receptor activation results in the activation of phosphotidyl inositol 3-kinase$\gamma$. PI3-kinase then recruits the adapter proteins through a tyrosine kinase, possibly Src, and this results in the activation of ERK module (Gutkind, 1998).

A recent report from Lefkowitz's laboratory, provided evidence for the mechanism of MAPK activation through β-adrenergic receptors in cultured cells. β-adrenergic receptor is usually coupled to Gs and increases cAMP and protein kinase A activity upon activation. In HEK293 cells activation of PKA by β-AR results in the phosphorylation of the receptor, leading to the uncoupling of the receptor from Gs and coupling of the β-AR to Gi. The Gβγ subunit of the now Gi coupled receptor activates the ERK pathway (Daaka *et al.*, 1997). This may be specific for the β-AR because results from our laboratory indicate that in the same cultured cells activation of CGRP receptor that results in the PKA activation does not lead to uncoupling of the receptor from Gs and coupling to Gi. CGRP still activates ERK pathway in these cells (Parameswaran *et al.*, 1999a). Thus different ligands may activate ERK by different pathways.

A novel report by Luttrell *et al.* (1999) recently has increased the complexity of the mechanism of ERK activation by G-protein coupled receptors to another level. They showed that activation of β-AR leads to the recruitment of Src, a tyrosine kinase that then results in the recruitment and activation of the adapter proteins and the ERK module. In that study they showed evidence for how Src is recruited. As in any other G-protein coupled receptor, activation of the receptor leads to its desensitization through involvement of G-protein coupled receptor kinases (GRKs) and proteins called arrestins. Arrestin binding to the phosphorylated receptor (receptor being phosphorylated by the GRKs) results in the arresting of the catalytic activity of the receptor. They showed that arrestin not only binds the phosphorylated receptor but also the tyrosine kinase Src, thus recruiting it to the membrane. The receptor-arrestin-Src complex was then shown to be targeted to clathrin coated pits. The arrestin-mediated Src kinase recruitment and receptor

targeting to clathrin-coated pits were required for the β-AR-mediated activation of ERK pathway. Thus, activation of β-AR results in the activation of an effector like adenylate cyclase. At the same time proteins required for termination of this signal is recruited to the membrane leading to the targeting of the receptor to the clathrin-coated pits. This initiates a second wave of signal leading to the activation of the ERK pathway. The desensitization phenomenon thought to be the one that turns off a signal from the receptor, may actually be a phenomenon that initiates a second signaling pathway.

The activities of these ERKs can also be decreased by receptor-mediated events. For example, activation of protein kinase-A phosphorylates and inhibits raf-1 activity that then inhibits the ERK module (Denhardt, 1996). In fact in mesangial cells, adrenomedullin decreases raf-1 activity (Parameswaran *et al.*, 1997). A decrease in the activity of these kinases can also be brought about by activation of phosphatases for example MAPK phosphatase-1 (MKP-1) and protein phosphatase 2a (PP2a). In mesangial cells Togawa *et al.* (1997) showed that adrenomedullin and other cAMP activators could induce the expression of MKP-1 which is associated with a decrease in ERK activity and proliferation. Results from our laboratory have provided evidence that adrenomedullin can stimulate protein phosphatase-2a. Okadaic acid, a selective inhibitor of PP2a, reverses the effect of adrenomedullin on ERK pathway without affecting other MAPK pathways. This was also associated with a reversal of the effects of adrenomedullin on proliferation and apoptosis (Parameswaran *et al.*, 1999c). Use of this inhibitor also provides indirect evidence that in mesangial cells inhibition of the ERK pathway by adrenomedullin can lead to the inhibition of proliferation and the induction of

apoptosis. Direct evidence for this can be obtained only when a specific activator of the ERK pathway is available.

**The JNK pathway:** The JNK pathway was biochemically identified in 1991 (Pulverer *et al.*, 1991). This pathway, similar to the ERK module, has MKKs and MKKKs that sequentially activate the JNK. Also like ERK, JNK has many substrates but unlike ERK, these are exclusively transcription factors. (ERK has substrates outside the nucleus also.) The mechanism of activation of the JNK pathway is not as well characterized as that of the ERK pathway. JNKs have been shown to be activated through cell surface receptors from a variety of families including TNF, GPCR, tyrosine kinase receptors and cytokine receptors. In addition, stress inducers like UV light and hydrogen peroxide can also stimulate the JNK pathway, although the mechanisms are not well characterized. Similar to the ERK pathway, activation of the JNK module also involves the recruitment of small G-proteins like Rac and Cdc42 (Widmann *et al.*, 1999; Denhardt, 1996; Fanger *et al.*, 1997).

JNK activity has been implicated in response to cell stress, specifically apoptosis. Inhibition of JNK signaling by introduction of dominant inhibitory mutants of JNK, or its downstream target (c-Jun), or its upstream activator (MKK4), show that JNK is necessary for apoptosis in response to growth factor withdrawal, stress, DNA damage, and ligation of the Fas on the cell surface (Widmann *et al.*, 1999). In JNK3 deficient mice, administration of an epileptogenic dose of kainic acid does not cause seizures that are as severe as those compared to the wild type mice (Yang *et al.*, 1997). The onset of seizures coincides with the cell death in an area of the hippocampus. In the JNK3 knock out mice

no cell death is observed in that area providing evidence for the role of JNK3 in apoptosis in these cell types. In spite of these results, JNK in some cell lines also appears to mediate cell growth or survival response (Widmann *et al.*, 1999). JNK activity has been shown to be upregulated in proliferative glomerulonephritis in mesangial cells (Bokemeyer *et al.*, 1997). The relevance of this increase is not known. A commercial inhibitor for JNK is not currently available. Hence the role of JNK in mesangial cells is not quite clear. There is some indirect evidence that supports a role for JNK in mesangial cell apoptosis. This can be resolved when a selective inhibitor becomes available.

**The P38 MAPK pathway**: P38 a kinase involved in the third parallel pathway, was recently cloned and characterized. It has the greatest homology to the yeast Hog1, which is activated by hyperosmotic shock. In mammalian cells, activation of P38 can also be achieved by cellular stress (UV irradiation, osmotic shock, heat shock, lipopolysaccharides, protein synthesis inhibitors), certain cytokines and G-protein coupled receptors. Similar to the JNK pathway, the P38 pathway is also stimulated via small G proteins like Cdc42 and Rac1. Because P38 can phosphorylate several substrates, it is thought that P38 can also have different biological effects (Widmann *et al.*, 1999; Denhardt, 1996). This enigma was solved to an extent by the discovery of a selective inhibitor of P38 (Lee *et al.*, 1994). Use of this inhibitor has suggested several functions for this pathway including cytokine production, cytokine-stimulated cell proliferation, apoptosis, cardiomyocyte hypertrophy etc. Involvement of P38 in apoptosis may be specific to the type of induction of the apoptosis and the cell type used. It is likely that involvement of both JNK and P38 pathways in survival versus death responses is

dependent on the integrated sum of all the biochemical pathways (Widmann *et al.*, 1999; Neary, 1997).

There is some evidence in the literature which suggests that some ligands can have dual effects, activating one MAPK pathway and at the same time inhibiting another pathway. In addition to the one described in this thesis by adrenomedullin, glia maturation factor activates the P38 pathway and at the same time inhibits the ERK pathway. Glia maturation factor is a 17-kDa brain protein which when phosphorylated by PKA, specifically enhances the P38 activity but concomitantly inhibits ERK activity (Lim *et al.*, 1996; Zaheer and Lim, 1996). Thus, the PKA-dependent pathway is able to regulate the different MAPK pathways differentially similar to that in mesangial cells. Whether there is a protein similar to glia maturation factor in mesangial cells is not known. Because of the different effects of these kinases in different systems and the differential regulation of these pathways by different ligands, it becomes necessary to characterize each of these so that a specific function can be associated with each of these ligands and the biochemical pathways modulated by them. Characterizing these pathways will increase our knowledge with respect to the mechanisms of the biological functions, and will also help us initiate some drug development strategies for manipulating these responses for the prevention or control of diseases. The next section will briefly summarize why understanding and integrating these biochemical pathways may be necessary, and how useful it has been so far, especially in the cancer research field. The same principles can be applied to other diseases such as chronic renal failure associated with aberrant mesangial proliferation.

## 2.5. SIGNAL TRANSDUCTION IN DISEASE AND DRUG DEVELOPMENT:

Over the past one to two decades, considerable advances have been made in understanding the cellular and molecular basis for intracellular and intercellular communication. It has also been observed that maintenance of normality relies on the fidelity of these signaling pathways. In spite of the redundancy of these biochemical pathways, a small change in a single regulatory component in the signaling pathway has been found to result in the malfunctioning of the pathway, leading to diseases like cancer, atherosclerosis, rheumatoid arthritis, multiple sclerosis and tissue rejection to name a few(Levitzki, 1996). Hence, identification of these signaling components becomes critical for the development of drugs related to such diseases where one or more of the components are malfunctioning. Inhibition and/or activation of signal transduction pathways can be achieved by a variety of agents like small molecules, antibodies, DNA encoding dominant-negative proteins, anti-sense RNA and target-specific RNA ribozymes. This strategy of drug development based on the signal transduction pathways has already produced a couple of promising new leads. For example, antibodies against the HER-2/neu receptor that is overexpressed in a number of breast, ovary and lung cancers has been found quite useful. Moreover the use of EGF receptor antibody in cancers overexpressing EGF receptor has also been found to be very useful (Levitzki, 1996; Levitzki, 1994; Powis, 1994).

In this thesis work effort has been made to identify the signaling pathways that are activated by adrenomedullin, and also the potential role of these signaling components in the mesangial cell and matrix turnover. Understanding these pathways will help us target

drug development towards these molecules. Because aberrant mesangial cell and matrix turnover form the pathophysiological basis for diseases like proliferative mesangiopathies and other forms of glomerulonephritis associated with chronic renal failure, development of drugs targeting these components might help in the prevention and/or control of these renal diseases.

## 3. Regulation Of Glomerular Mesangial Cell Proliferation and Apoptosis In Culture By Adrenomedullin

### 3.1. Introduction:

Adrenomedullin, a derivative of proadrenomedullin, is a potent vasodilator and natriuretic factor. Discovered in 1993, it is thought to belong to the Calcitonin Gene Related Peptide (CGRP) superfamily (Kitamura *et al.*, 1993; Sakata *et al.*, 1993). Since its initial discovery, a number of reports have appeared describing the actions of adrenomedullin, both in animal and cell culture models (Ebara *et al.*, 1994; Gardiner *et al.*, 1995; Haynes and Cooper, 1995; Jougasaki *et al.*, 1995). Adrenomedullin has been shown to decrease proliferation or thymidine incorporation in mesangial cells (Chini *et al.*, 1995; Segawn *et al.*, 1996). In most systems including mesangial cells, adrenomedullin activates adenylate cyclase, with a subsequent increase in cyclic-AMP accumulation and protein kinase-A activation (Chini *et al.*, 1995; Kohno *et al.*, 1995; Osajima *et al.*, 1996). In endothelial cells, in addition to stimulation of cyclic-AMP, an increase in intracellular calcium release and phosphatidyl-inositol hydrolysis in response to adrenomedullin was reported (Shimekake *et al.*, 1995). The mechanisms of adrenomedullin-mediated responses, however, have not been completely elucidated.

The major aim of the present study was to evaluate the effect of adrenomedullin on mesangial cell proliferation (using [$^3$H]thymidine incorporation as an index), apoptosis (using nucleosome-associated DNA fragmentation as an index) and mitogen-activated protein kinase (MAPK) pathways, specifically that of extracellular signal regulated kinase (ERK), *jun*-amino terminal kinase (JNK) and P38 mitogen-activated protein kinase (P38 MAPK) activities. We have also designed experiments to test the

hypothesis that adrenomedullin-mediated changes in proliferation, apoptosis, and MAPK pathways are cAMP-PKA-dependent. For that, we have compared adrenomedullin-mediated responses to that of forskolin, another cAMP elevating agent and a direct activator of adenylate cyclase. We have also done experiments in the presence of a potent protein kinase A inhibitor, H89 to directly test the hypothesis that adrenomedullin-mediated responses are cAMP-PKA-dependent. Our data indicates that protein kinase-A-dependent and –independent pathways are modulated by adrenomedullin in rat mesangial cells.

## 3.2. Materials and Methods:

### 3.2.1. Materials:

Adrenomedullin and adrenomedullin-(22-52) were purchased from Phoenix Pharmaceuticals (Belmont, California), myelin basic protein (MBP), from Sigma (St. Louis). Polyclonal anti-ERK2, anti-P38 MAPK and anti-JNK1 antibodies were purchased from Santa Cruz laboratories (Santa Cruz, California). Glutathione-S-transferase conjugated cJUN (GST-cJUN) was purchased from Alexis Biochemicals (San Deigo, California). RPMI-1640, fetal bovine serum, penicillin and streptomycin were from Gibco (Grand Island, NY). All other reagents were of high quality available.

### 3.2.2. Cell culture:

Rat mesangial cells were obtained from the glomeruli of kidney cortex isolated from Sprague Dawley rats as described before (Albrightson *et al.*, 1992), and were grown in RPMI-1640 with 15% fetal bovine serum. Passages between 15 and 30 were used for the experiments.

### 3.2.3. Cyclic nucleotides:

CyclicAMP measurements were performed as described before, with slight modifications (Haneda *et al.*, 1996). Cells were plated in 24 well plates (50,000cells/well) and grown for 2 days and serum starved overnight. Cells were preincubated with 0.5 mM isobutyl methyl xanthine (IBMX) for 10 min and then agonist solutions (prepared in phosphate buffered saline containing 0.2% bovine serum albumin, 0.2% magnesium chloride and 0.1% glucose) were added to the wells and incubated for an additional 5 min at 37 C. Reactions were stopped by adding 50 µl of 100% trichloroacetic acid. The cells in trichloroacetic acid were collected in separate tubes and centrifuged. The supernatants were collected after brief centrifugation and ether extracted three times with water-saturated ether. After overnight evaporation of the ether, a portion of the sample was used for measurement of cAMP levels, using a radio-immunoassay (RIA) kit from PerSeptive Biosystems (Framingham, MA). Each experiment was done in triplicate and repeated 5 times.

### 3.2.4. [$^3$H]thymidine incorporation:

Cells were plated in 24 well plates (30000 cells/well) and grown for 2 days, and then serum starved for 48 h. Cells were then treated with the compounds for a period of 16 h and pulsed with [$^3$H]thymidine (1 µCi/ml) for 4 h. The radioactivity was counted in Beckman LS counter, after washing the cells and stopping the reaction with 5% trichloro acetic acid and solubilising the cells in 0.5 ml of 0.25 N sodium hydroxide. Each experiment was done in quadruplicates and was repeated 4 times.

### 3.2.5. Kinase assays:

Cells were plated in p100 plates and were serum starved overnight on reaching 80% confluency. The agonist solutions were prepared in the growth media without serum. Cells were treated with the agonists for 30 minutes. Our preliminary time course experiments suggested that the changes in all the kinase activities were maximal after 30 minutes of adrenomedullin treatment. Hence that time point was chosen for further experiments. The cell lysates were prepared as described (Bogoyevitch, *et al.*, 1995; Li *et al.*, 1995). In the meantime, specific antibodies (10 µg/reaction) were incubated with protein A agarose (Gibco) for 30 min at room temperature. After normalizing for protein concentration, the cell lysates were incubated with the specific antibody agarose conjugate for 2 h at 4 C with constant shaking. The kinase assays were done after washing the immunoprecipitates three times with HNTG (20 mM HEPES pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol) buffer and two times with kinase buffer (50 mM Tris-HCl, 100 mM NaCl, 10 mM MnCl2 and 0.1 mM sodium ortho vanadate). The functional assay was done in the presence of 50 µM ATP, 5 µCi 32P-ATP, 10 µg of specific substrate (MBP-for ERK2 and P38 MAPK, and GST-cJUN- for JNK1), and the immunoprecipitate. The reactions were performed at 30 C for 15 min and then stopped with Sodium-dodecyl sulfate (SDS) buffer. The samples were electrophoresed in 12% polyacrylamide gel with proper molecular weight standards. The gels were dried and subjected to autoradiography or phosphoimager plates. The intensity of the bands in the autoradiogram was visualized using an ARCUS high-resolution optical scanner and quantitated using NIH image software or quantitated using imagequant program (for the

gels exposed to phosphoimager plates). Results are expressed as percent change from the basal of the relative densitometric units or phosphoimager units.

**3.2.6. Western blots:**

Western blot analysis was done as described before (Guo *et al.*, 1998). Briefly, equal concentration of protein samples were subjected to sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) along with molecular weight standards and transferred to nitrocellulose membranes. The membranes were then blocked with 5% non-fat dry milk in Tris buffered saline containing 0.05% Tween-20 and incubated with primary antibodies followed by horse radish peroxidase-conjugated secondary antibodies according to manufacturer's instructions. The blots were then visualized by an enhanced chemiluminescence (ECL) kit obtained from Pierce.

**3.2.7. ELISA for apoptosis:**

The ELISA kit was obtained from Boehringer Mannheim (Indianapolis, IN). The principle of the kit is based on the fact that on induction of apoptosis cells undergo DNA fragmentation, leading to oligonucleosomal DNA fragments in the cytoplasm of the cells. For the experiments, the cells were plated in 96 well plates for overnight, after which they were labeled with bromodeoxyuridine for 8 h and then treated with different agonists. At the end of the incubation period, the cells were lysed, centrifuged to separate the cytoplasmic DNA fragments from the nuclear DNA and then an ELISA was done to quantitate the cytoplasmic fragmented DNA, and the absorbance was read at 490 nm using a plate reader (Biorad, model550). Each experiment was done in quadruplicates and the experiment was repeated at least 3 times. The experiments were also repeated using a different ELISA kit (Boehringer Mannheim, Indianapolis, IN), which specifically detects

the cytoplasmic nucleosome-associated DNA fragments. For that, cells were plated in 48 well plates and after 24 hours were serum starved overnight. Different agonists (prepared in the media) were added to the cells and the incubation continued for another 20 h. The cells were lysed with the lysis buffer and centrifuged to separate cytoplasmic and nuclear fractions. The cytoplasmic fraction was then tested for DNA still attached to nucleosomes using the ELISA protocol from Boehringer Mannheim, Indianapolis, IN. The assay was done in triplicates or quadruplicates and repeated at least 3-5 times. The results that are presented here are from the second ELISA method, which detects the nucleosome-associated DNA fragments in the cytoplasm. Similar results were also obtained with the first ELISA method.

**3.2.8. Caspase activity assays**: Cultured rat mesangial cells in P100 plates were treated with vehicle or test compounds for 18 hours. Cell lyasates were prepared as described (Yue *et al.*, 1998). Briefly, the cells were washed with ice-cold phosphate buffered saline without calcium and magnesium, harvested and suspended in buffer containing 25 mM HEPES, pH 7.5, 10% sucrose, 0.1% CHAPS, 2 mM DDT, 5 mM EDTA, 1 mM PMSF, and 1 μM pepstatin A and allowed to swell for 20 min on ice. The suspension was forced through a 25 gauge needle to break the cells. The homogenate was centrifuged at 4 C for 1 hour in a Beckman Airfuge at 90,000 rpm. The cleared lysates were stored at -70 C until used for assays.

**Enzyme assays**: The assay buffer and the substrates for caspase-3 like activity were used for the peptide-pNA hydrolysis assays as described before (Yue *et al.*, 1998). The composition of the assay buffer was as follows: HEPES (pH 7.5), CHAPS (0.1%), DTT (1 mM), 50 mM KCl. Ac-DEVD-pNA was used as substrate. Cell extracts containing 20-

30 μg of protein were diluted into the assay buffer and preincubated for 10 min for 30 C prior to the addition of the substrate. Levels of released pNA were measured with a plate reader colorimetrically at 405 nm for 30 min.

### 3.2.9. Data analysis:

Results are expressed as mean±S.E. Analysis of Variance (ANOVA) was used to compare 3 or more treatments, followed by Bonferoni's multiple comparison between treatments, and student's t test for 2 treatment comparisons. A P value of less than 0.05 was considered significant.

## 3.3. Results:

### 3.3.1. Cyclic AMP:

Adrenomedullin increased cAMP levels significantly above basal (Figure 3) Forskolin, a direct activator of adenylate cyclase also increased cAMP levels (2666±578% change from basal).

### 3.3.2. [$^3$H]Thymidine incorporation (Proliferation):

Adrenomedullin decreased [$^3$H]thymidine incorporation in a concentration-dependent manner (Figure 4). In addition, forskolin also decreased [$^3$H]thymidine incorporation in mesangial cells significantly below basal levels (Figure 5). Furthermore, H89, a potent protein kinase-A inhibitor completely reversed the adrenomedullin-mediated proliferation response at a concentration as low as 20 nM (Figure 6). At both 20 and 200nM H89 by itself did not cause any statistically significant change in proliferation of rat mesangial cells. But at 2μM it caused approximately a 2-fold increase in mesangial cell proliferation.

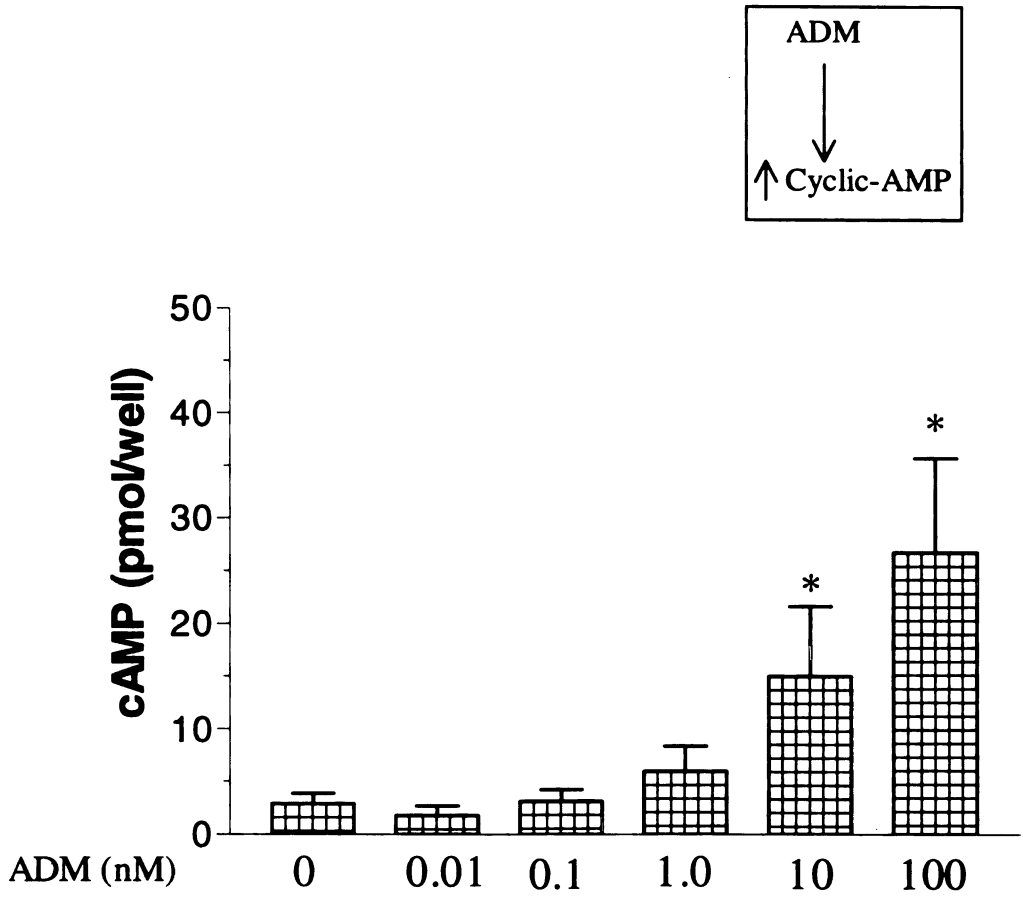



Figure 3. Effect of adrenomedullin (ADM) on cAMP levels in rat mesangial cells. Each experiment was done in triplicates and repeated 5 times. ANOVA P value<0.05. (* P<0.05 compared to basal). ADM significantly increased cAMP levels in rat mesangial cells.

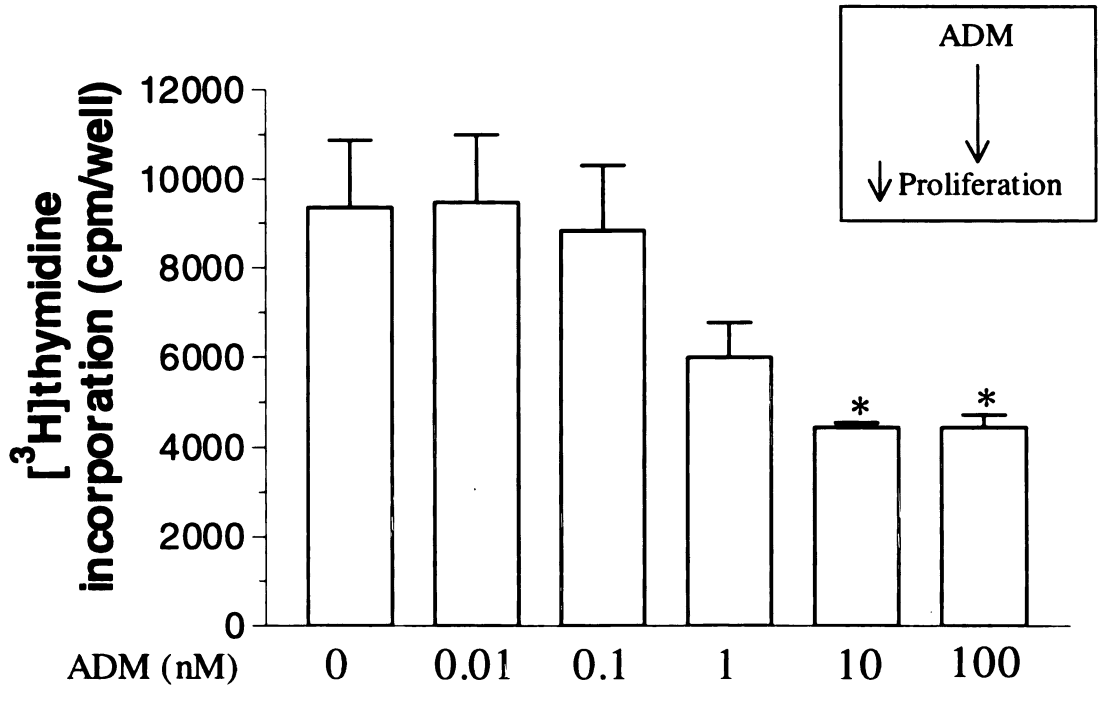



Figure 4. Effect of adrenomedullin (ADM) on [$^3$H]thymidine incorporation (an index of proliferation) in rat mesangial cells. Each experiment was done in quadruplicates and repeated four times. ANOVA P value<0.05. (* P value<0.05 compared to basal). ADM decreased rat mesangial cell proliferation significantly.

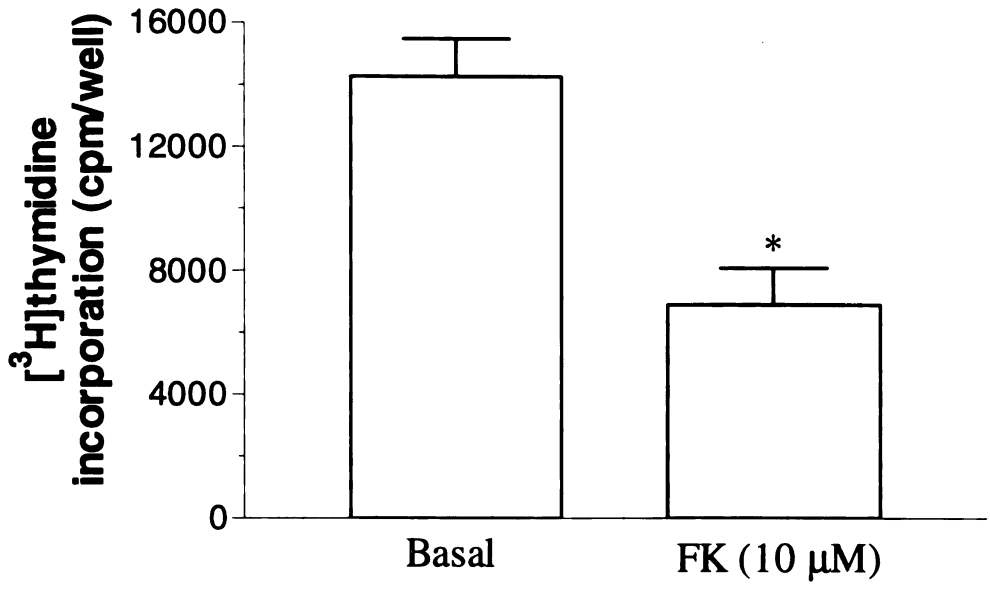



Figure 5. Effect of Forskolin (FK) on [$^3$H]thymidine incorporation in rat mesangial cells. n=3 ($P<0.01$) Forskolin is a direct activator of adenylate cyclase and increases intracellular cAMP. Because FK decreases rat mesangial cell proliferation, an elevation of intracellular cAMP levels probably decreases proliferation in mesangial cells.

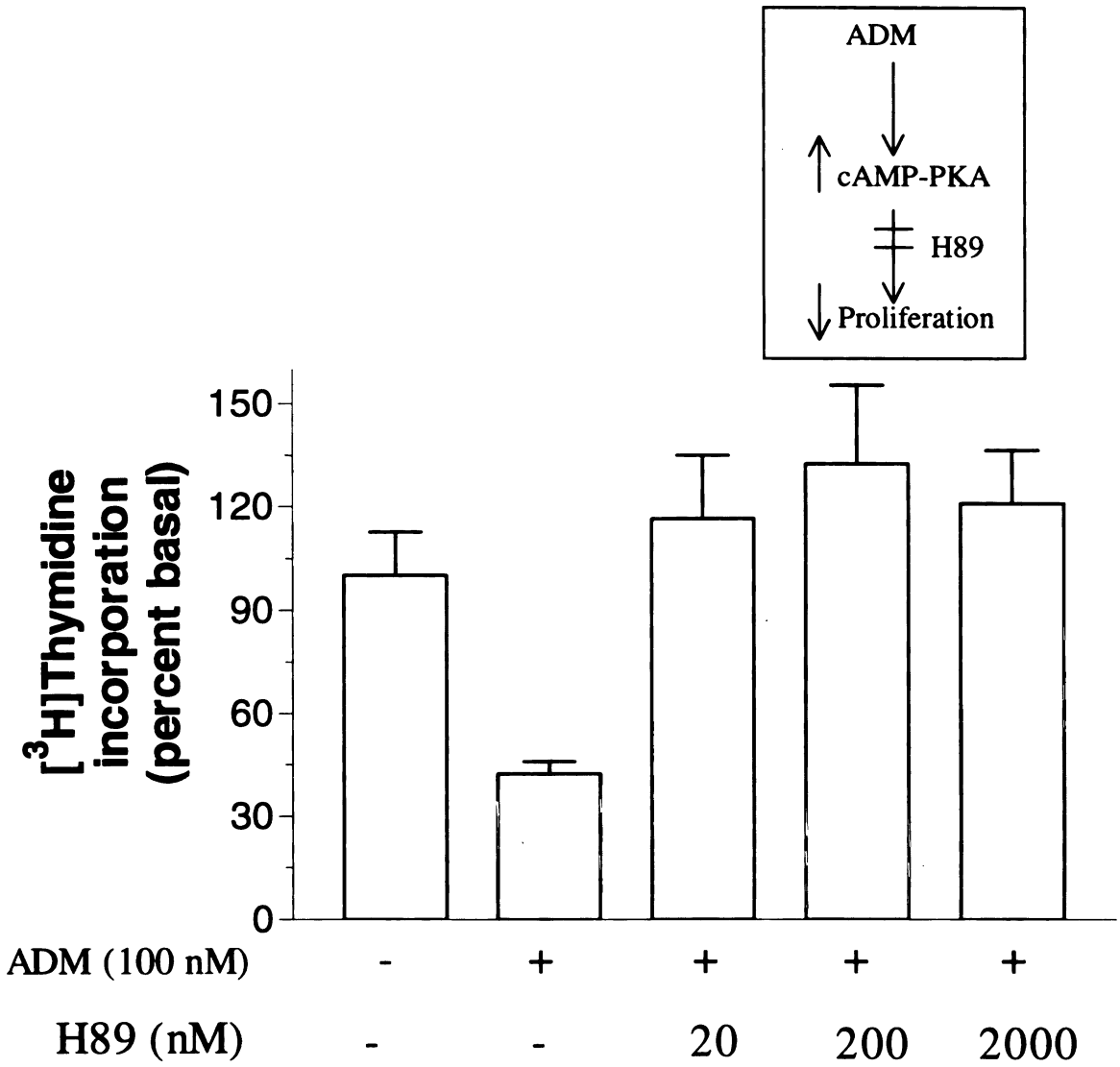



Figure 6. Effect of H89, protein kinase-A inhibitor on adrenomedullin (ADM)-mediated response on [$^3$H]thymidine incorporation in rat mesangial cells. n=3. The raw values were analyzed using ANOVA ($P<0.05$). H89 by itself stimulated proliferation of mesangial cells significantly only at 2μM. PKA inhibitor significantly reversed ADM-inhibited proliferation.

### 3.3.3. Nucleosome-associated DNA fragmentation (Apoptosis):

Associated with a decrease in [$^3$H]thymidine incorporation, adrenomedullin also caused an increase in cytoplasmic nucleosome-associated DNA fragmentation (an index of apoptosis), in a concentration-dependent manner (Figure 7). Furthermore, forskolin also increased mesangial cell apoptosis (Figure 9). In addition, H89, a potent protein kinase-A inhibitor significantly inhibited adrenomedullin-stimulated apoptosis (Figure 10), although at that concentration of H89, there was no significant change in the basal DNA fragmentation/apoptosis.

**3.3.4. Caspase-3-like activity**: To confirm the induction of apoptosis biochemically, we examined the effect of adrenomedullin on caspase-3-like (CPP 32 or apopain) activity in rat mesangial cells. Eighteen hour treatment with ADM caused a significant induction of caspase-3 activity confirming biochemically, the induction of apoptosis by ADM in rat mesangial cells (Figure 8).

**3.3.5. MAPK activities**:

**Extra-cellular signal regulated Kinase (ERK):** Associated with a decrease in proliferation and an increase in apoptosis, adrenomedullin and forskolin also caused a decrease in ERK2 activity (Figure 11, 12). Also, H89 a protein kinase A inhibitor completely reversed the ERK inhibition caused by adrenomedullin (Figure 13, 14). By itself H89 did not affect the basal ERK activity.

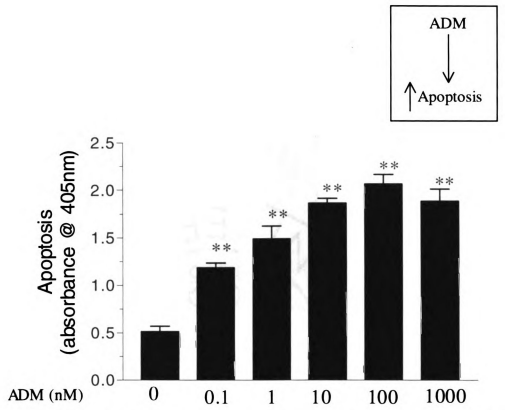



Figure 7. Effect of adrenomedullin (ADM) on nucleosome-associated DNA fragmentation (apoptosis), in rat mesangial cells. Each experiment was done in triplicates and repeated 3 times. ANOVA P value< 0.01. (**P<0.01 compared to basal). ADM induced rat mesangial cell apoptosis significantly, in culture.

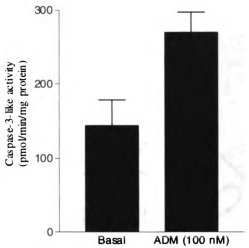



Figure 8. Effect of adrenomedullin on caspase-3-like activity in rat mesangial cells ($P<0.01$) n=3. Activation of caspase-3, a protease activated during apoptosis, confirms the biochemical activation of the apoptosis cascade by adrenomedullin in rat mesangial cells in culture.

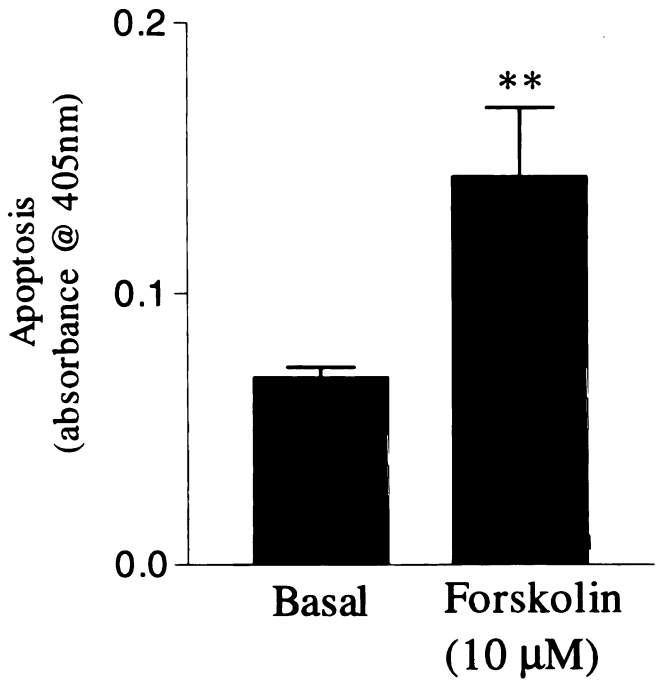



Figure 9. Effect of forskolin (FK) on nucleosome-associated DNA fragmentation (apoptosis) in rat mesangial cells. n=3 (*P<0.01). Induction of apoptosis by forskolin indicates that elevation of cAMP levels in mesangial cells probably induces apoptosis.

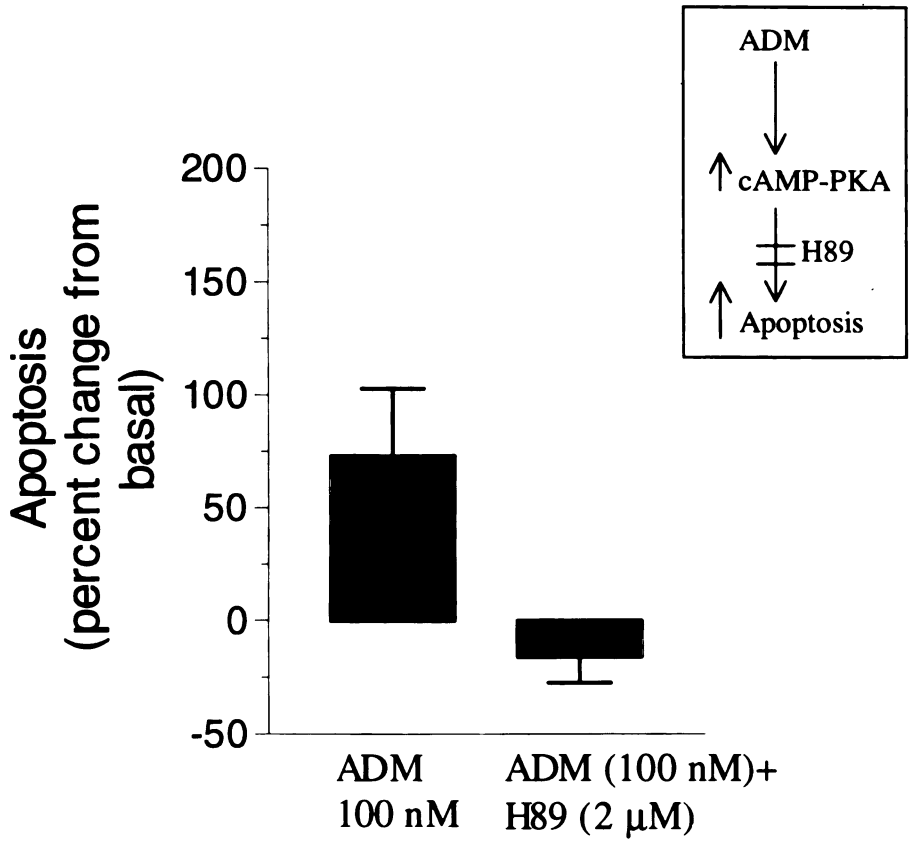



Figure 10. Effect of inhibition of protein kinase-A, by H89, on adrenomedullin-induced nucleosome-associated DNA fragmentation (apoptosis) in rat mesangial cells. Analysis of raw values for stimulation of apoptosis by adrenomedullin indicated a significant increase in apoptosis ($P<0.05$). Pretreatment with H89 completely blocked adrenomedullin-induced apoptosis. H89 by itself did not affect the basal DNA fragmentation. ( n=3)

***Jun*-amino terminal kinase (JNK):** Adrenomedullin increased JNK1 activity significantly above basal levels (Figure 11, 12). But forskolin, a direct activator of adenylate cyclase did not cause any consistent change in JNK1 activity. Furthermore, H89 did not have any consistent effect on adrenomedullin-stimulated JNK1 activity (Figure 13, 14), indicating a possible protein kinase-A -independent activation. By itself H89 did not affect the basal JNK activity.

**P38 mitogen-activated protein kinase (P38 MAPK):** Adrenomedullin and forskolin significantly increased P38 activity (Figure 11, 12). In addition, H89 almost completely inhibited adrenomedullin-stimulated P38 MAPK activity (Figure 13, 14).

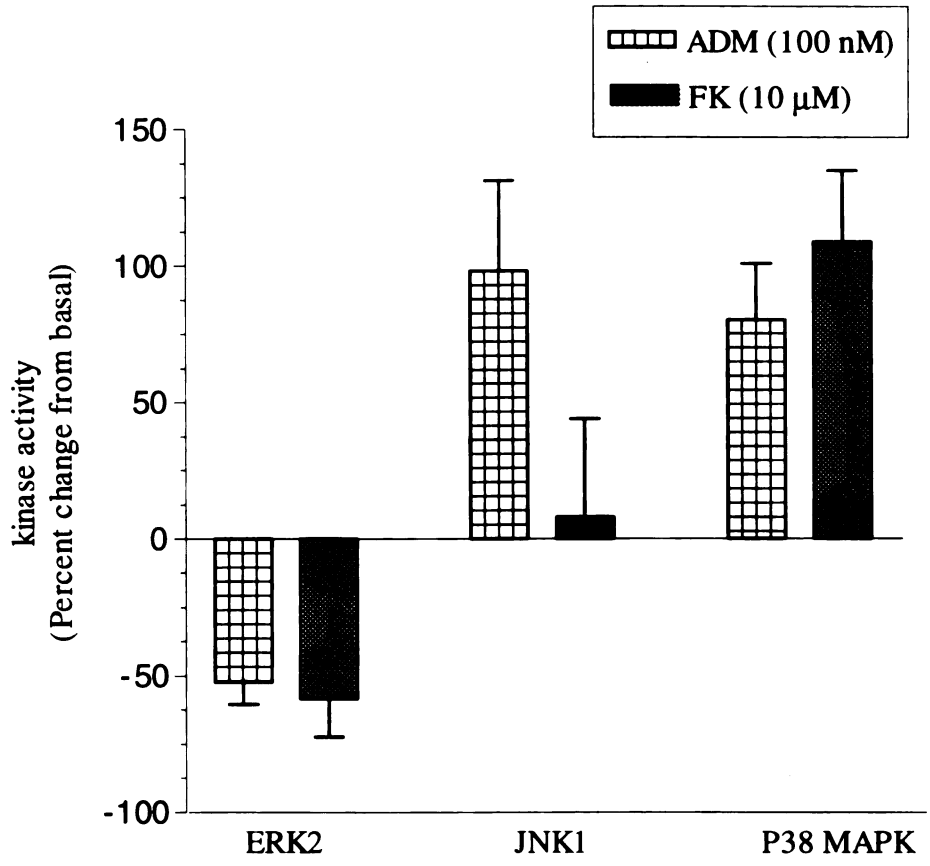
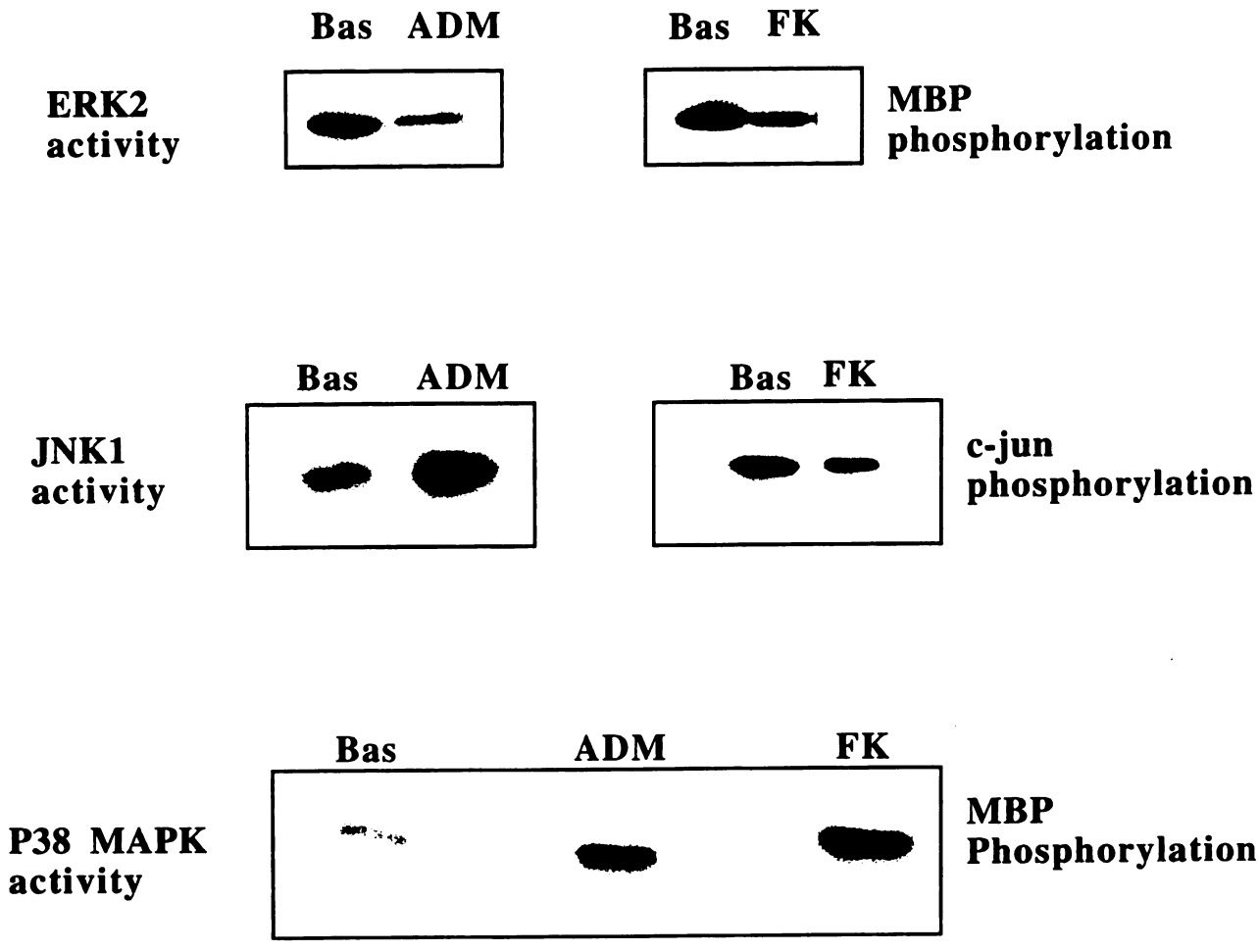



Figure 11. Effect of adrenomedullin (ADM) and forskolin (FK) treatment (for 30 min) on ERK2, JNK1 and P38 MAPK activities in rat mesangial cells. (n: ADM=6, forskolin=3). Our preliminary time course experiments indicated that the activities were maximal at 30 minutes after treatment.

Figure 12. Representative autoradiograms showing the effect of adrenomedullin (ADM) and forskolin (FK) on ERK2, JNK1 and P38 MAPK activities. ERK2 activity was determined by specific immuno-complex assay with MBP as the substrate as described in methods. The degree of phosphorylation of MBP by the immunoprecipitated ERK2 indicates the activity of the enzyme. JNK1 activity was determined by specific immuno-complex assay with c-jun as the substrate. The degree of phosphorylation of c-jun by the immunoprecipitated JNK1 indicates the activity of the enzyme. P38 MAPK activity was determined by specific immuno-complex assay with MBP as the substrate. The degree of phosphorylation of MBP by the immunoprecipitated P38 MAPK indicates the activity of the enzyme.


Bas ADM
Bas FK
ERK2 activity
MBP phosphorylation
Bas ADM
Bas FK
JNK1 activity
c-jun phosphorylation
Bas
ADM
FK
P38 MAPK activity
MBP Phosphorylation

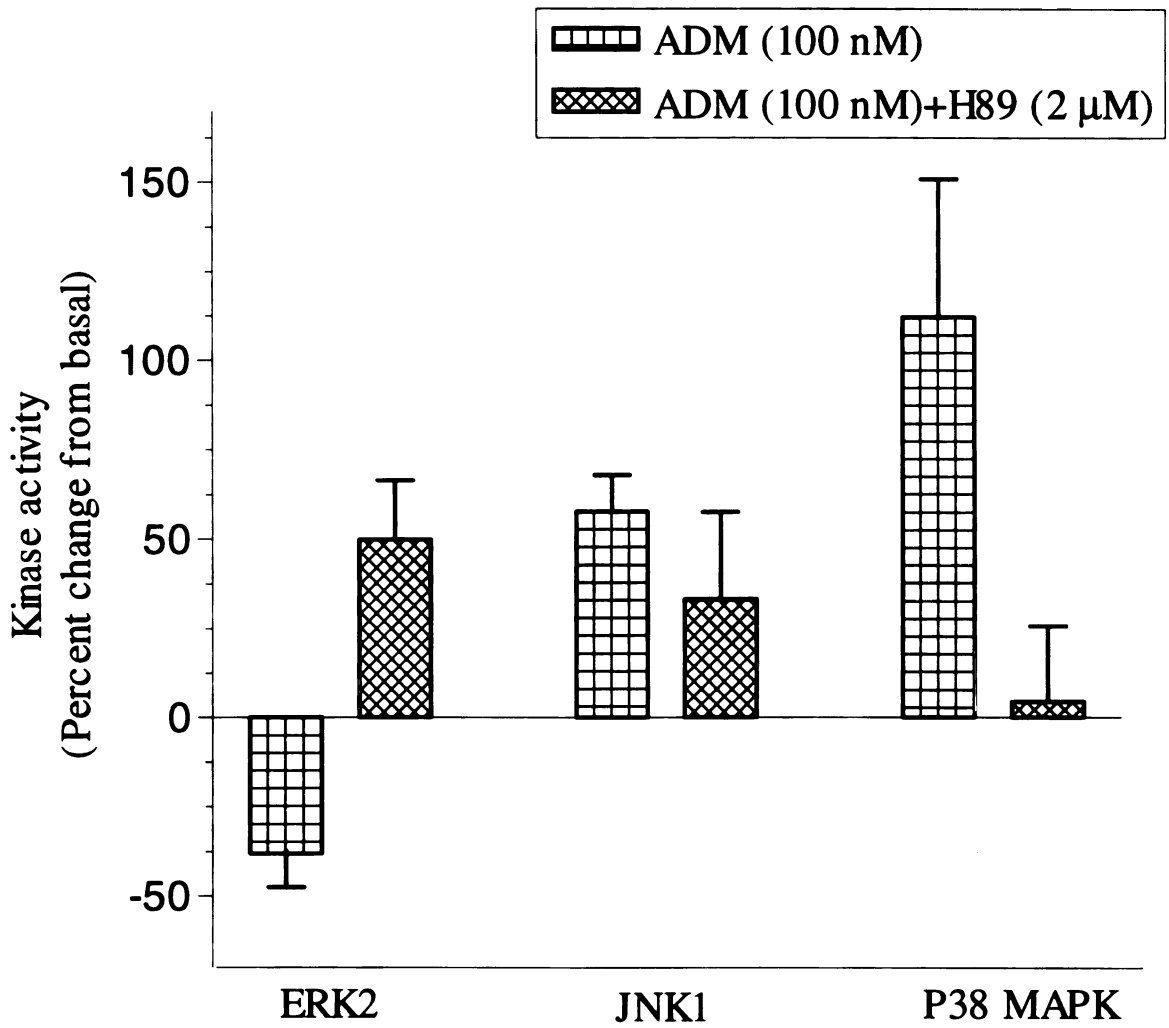



Figure 13. Effect of H89, a protein kinase A inhibitor on ERK2, JNK1 and P38 MAPK activities modulated by adrenomedullin treatment (for 30 min) in rat mesangial cells. H89 did not affect the basal kinase activities significantly (n=4). H89 only reversed ADM-modulated ERK2 and P38 activities and did not consistently affect JNK activity. ($P<0.05$ between ADM and ADM+H89 for ERK2 and P38 MAPK. Between ADM and ADM+H89 for JNK-not statistically significant)

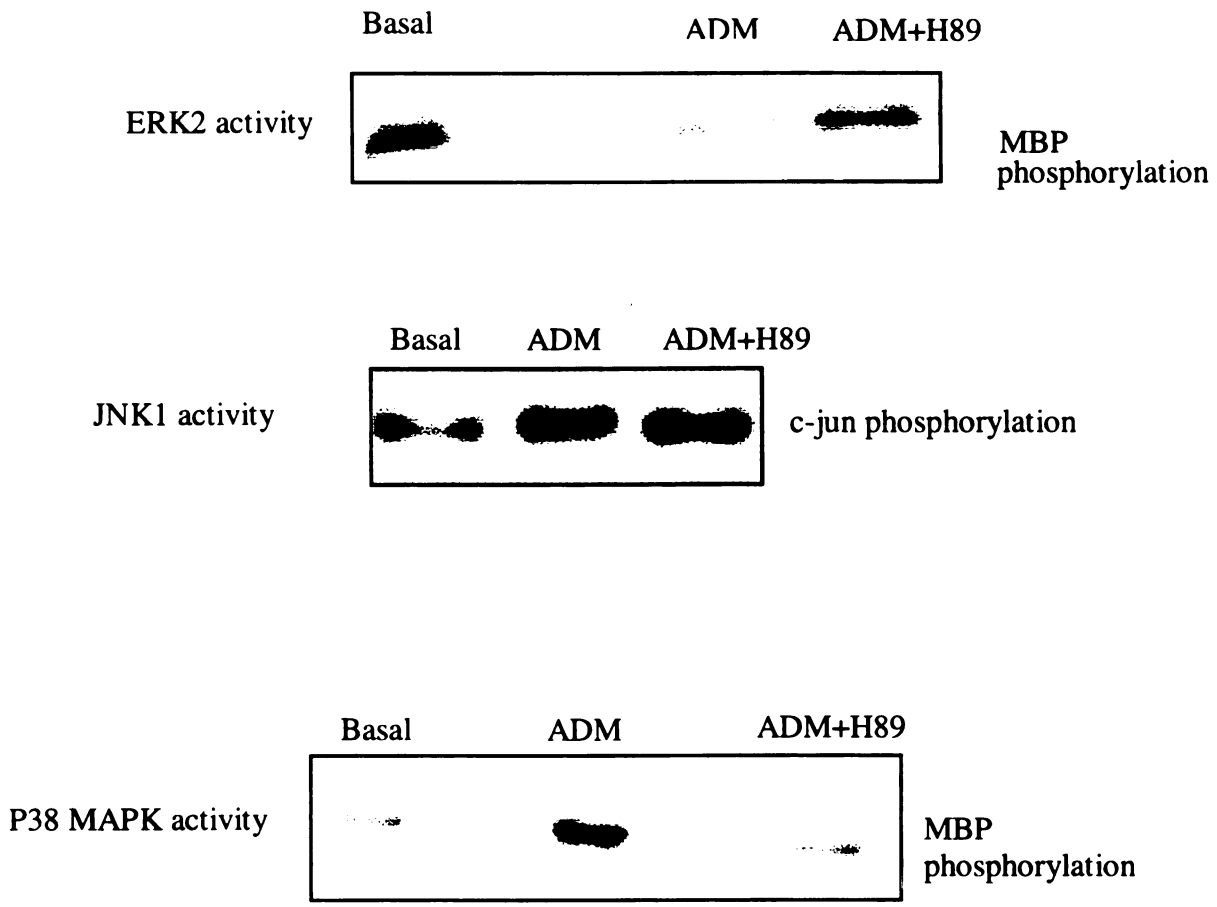



Figure 14. Representative autoradiograms showing the effect of adrenomedullin and H89 on ERK2, JNK1 and P38 MAPK activities. Experiment was done as described in methods section and in Figure 12.

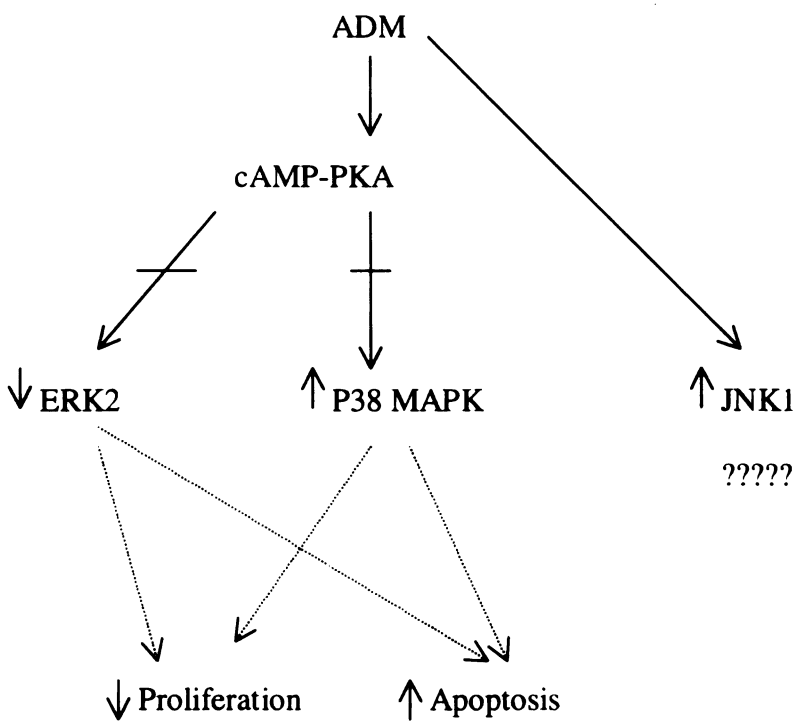



Figure 15. A schematic model based on the results presented in chapter 3, indicating the possible pathways of adrenomedullin-mediated effects in rat mesangial cells in culture

### 3.4. Discussion:

An increase in cAMP resulting in the activation of protein kinase-A is followed by a multitude of changes in the signaling systems in a cell, such as changes in the MAPK pathways, leading to biological responses such as proliferation, apoptosis and matrix production. These responses have important implications in the initiation and progression of diseases such as glomerulonephritis. A common finding in proliferative glomerulonephritis is the aberrant proliferation of mesangial cells. Several hormones, growth factors and inflammatory cytokines have been shown to modulate mesangial cell turnover in culture, indicating a possible role in nephropathies associated with aberrant proliferation (Brenner and Stein, 1989; El Nahar *et al.*, 1997; Klahr *et al.*, 1988).

Several studies have shown that adrenomedullin causes an increase in cAMP in mesangial cells, resulting in the activation of protein kinase-A (Chini *et al.*, 1995; Chini *et al.*, 1997). An increase in cAMP causes changes in proliferation depending on the cell type (Dumont *et al.*, 1989; Withers *et al.*, 1996). In mesangial cells, cAMP causes a decrease in proliferation (Floege *et al.*, 1993). Our results using a protein kinase-A inhibitor indicate that adrenomedullin causes a decrease in proliferation of rat mesangial cells through activation of protein kinase-A.

In addition to an aberrant proliferation, an altered apoptotic machinery may be an important mechanism in the progression of proliferative diseases. Although in some cases, resolution of renal disease or proliferative glomerulonephritis is seen, the mechanisms involved in these processes are not understood (Baker *et al.*, 1994; Sugiyama *et al.*, 1996). Mesangial cell apoptosis has recently been proposed to be one of the important mechanisms of resolution of hypercellularity in some of the experimental

models of proliferative mesangiopathies (Baker *et al.*, 1994). Accordingly, it is thought that alterations of the apoptotic machinery in the glomerulus which leads to the survival of excess mesangial cells, leads to further complications of the disease. We report here for the first time that adrenomedullin causes an increase in nucleosome-associated DNA fragmentation, an index of apoptosis (Kroemer *et al.*, 1995; Martin *et al.*, 1994). Our findings confirm a recent report by Muhl *et al.*, (1996) who showed that elevation of cAMP levels in mesangial cells was associated with an increase in apoptosis. We have also demonstrated that inhibition of protein kinase-A by H89 can inhibit adrenomedullin-induced apoptosis. These results are in contrast to the effect of adrenomedullin on endothelial cells, where it protects the cells from apoptosis through a cAMP-independent mechanism (Kato *et al.*, 1997).

Recently, numerous reports have suggested a role for MAPKs, specifically of ERK, JNK and P38 MAPK in the regulation of proliferation and apoptosis (Denhardt, 1996; Neary, 1997; Robinson and Cobb, 1997). To understand the mechanism of adrenomedullin-induced apoptosis and adrenomedullin-mediated decrease in proliferation, we measured kinase activities of ERK2, JNK1 and P38 MAPK in response to adrenomedullin and forskolin. While both these agents caused a decrease in ERK2 activity and an increase in P38 MAPK, only adrenomedullin caused an increase in JNK1 activity. Previous reports have demonstrated that the activity of a myelin basic protein phosphorylating kinase decreases in response to adrenomedullin in the total cell lysate (Chini *et al.*, 1995; Chini *et al.*, 1997; Haneda *et al.*, 1996). Our study shows for the first time that adrenomedullin causes specifically an increase in P38 MAPK and JNK1 and a decrease in ERK2 activities. Xia *et al.*, (1995) recently showed that in PC12 cells, nerve

growth factor withdrawal induces apoptosis through a mechanism that involves all of these three kinases; specifically, a decrease in ERK2 and a simultaneous increase in P38 MAPK and JNK1 were obligatory for the induction of apoptosis. It remains to be determined if the same prerequisite is necessary for adrenomedullin-induced apoptosis in mesangial cells. Forskolin-induced apoptosis, however, does not require JNK activity because it increases only P38 MAPK (in addition to a decrease in ERK2). The same could be true for adrenomedullin-induced apoptosis because inhibition of protein kinase-A with H89, inhibits adrenomedullin-stimulated apoptosis, although it does not consistently affect adrenomedullin-induced JNK activity. We have also found recently that SB203580, a P38 inhibitor completely inhibits both adrenomedullin-stimulated apoptosis and adrenomedullin-inhibited proliferation (N. Parameswaran *et al.*, 1999d), suggesting that there might be redundant pathways stimulated by adrenomedullin, which may or may not result in the same biological response. The role of adrenomedullin-stimulated JNK in mesangial cell function remains to be elucidated.

Based on our results using H89, adrenomedullin-mediated decrease in ERK2 and increase in P38MAPK activities are possibly mediated through protein kinase-A pathway, while JNK1 activity is probably protein kinase-A -independent. Several laboratories studying G-protein coupled receptors in the recent years have shown that the $\beta\gamma$ subunit of the G-protein, can activate several effectors including ERK, P38 MAPK and JNK through a signaling cascade (Gutkind, 1998; Lopez-Ilasaca, 1998; Yamauchi *et al.*, 1997). In view of the fact that the only second messenger system for adrenomedullin so far identified in mesangial cells is cAMP, and that the adrenomedullin receptor is G-protein coupled, we postulate that the increase in JNK activity by adrenomedullin may be

through βγ subunit. Further experiments with specific inhibitor of βγ subunit would be necessary to prove that.

It is quite surprising that even though forskolin elevated cAMP levels higher than adrenomedullin (about 3 fold), most of the other responses of forskolin are comparable with that of adrenomedullin or only slightly better than that of adrenomedullin. In fact, adrenomedullin-stimulated apoptotic response is even slightly higher than that of forskolin. Furthermore, in our studies on hyaluronic acid production in mesangial cells, forskolin-stimulated hyaluronic acid production is much higher than adrenomedullin and also, H89 does not inhibit adrenomedullin-stimulated hyaluronic acid secretion, while it inhibits forskolin-induced hyaluronic acid production (N. Parameswaran *et al.*, 1999e).

Adrenomedullin, through its antiproliferative and apoptotic effect on mesangial cells, may play a major role in the normal turnover of mesangial cells. It is known that the plasma levels of adrenomedullin are increased in hypertension and renal failure (Cheung *et al.*, 1997; Ishimitsu *et al.*, 1994). Tissue-specific expression of adrenomedullin under these pathophysiological conditions might provide insight into the role of adrenomedullin in mesangial cell turnover in such abnormal conditions.

In summary, adrenomedullin decreases proliferation and increases apoptosis possibly through a protein kinase-A pathway. In addition adrenomedullin decreases ERK and increases JNK and P38 MAPK activities in rat mesangial cells. Only adrenomedullin-modulated ERK and P38 are sensitive to H89, a protein kinase-A inhibitor, while JNK activity is not consistently sensitive to H89 (Figure 15). These results suggest that while adrenomedullin-stimulated protein kinase-A pathway is critical

for most responses, it does not preclude the involvement of adrenomedullin-stimulated protein kinase-A -independent pathways.

# 4. A P38 MAPK Inhibitor Reverses Adrenomedullin's Effect On Proliferation And Apoptosis In Cultured Mesangial Cells

## 4.1. Introduction:

Adrenomedullin, a derivative of proadrenomedullin, is a 52 amino acid peptide and is a potent vasodilator and natriuretic factor. Discovered in 1993, it is thought to belong to the Calcitonin Gene-related Peptide (CGRP) superfamily (Kitamura *et al.*, 1993; Sakata *et al.*, 1993). Since its initial discovery, a number of reports have appeared describing the physiological and pharmacological actions of adrenomedullin, both in animal and cell culture models (Chini *et al*, 1997; Ebara, *et al.* 1994, Gardiner *et al.*, 1995; Haynes and Cooper, 1995). In all systems studied so far, adrenomedullin has been shown to activate adenylate cyclase resulting in the accumulation of cAMP (Chini *et al*, 1997; Kohno *et al.*, 1995; Osajima *et al*, 1996). In some cellular systems, adrenomedullin receptor is also coupled to phosphatidyl inositol hydrolysis (Shimekake *et al.*, 1995). In glomerular mesangial cells adrenomedullin activates adenylate cyclase without any increase in phosphotidyl inositol hydrolysis (Osajima *et al.*, 1996).

Mesangial cell proliferation is a common feature in chronic renal diseases such as glomerulonephritis (El Nahas *et al.*, 1997; Floege *et al.*, 1993; Klahr *et al.*, 1988) and, therefore, understanding the mechanism of mesangial cell turnover is critical for our understanding of the role of mesangial cells in the development of chronic renal disease. Although a number of agents have been shown to have proliferative and antiproliferative effects, the mechanisms of action of these agents, however, have not been completely characterized. Adrenomedullin is one such peptide that has been shown recently to have an anti-proliferative effect in mesangial cells (Chini *et al.*, 1997). We have also reported an apoptotic effect of adrenomedullin in rat mesangial cells (Parameswaran *et al.*, 1999). The purpose of the present study was to delineate the mechanisms of these two responses.

Stimulation of a receptor leading to the activation of second messenger sytems have been shown to be coupled to divergent intracellular signaling pathways including the MAPK pathway. Three parallel MAPK pathways are currently known. They are the extracellular signal-regulated kinase (ERK), cJun N-terminal kinase (JNK) and P38 mitogen-activated protein kinase (P38 MAPK) pathways. Although these MAPKs are stimulated through a kinase cascade, the exact nature of the proximal signaling event depends on the cell system as well as the ligand (Denhardt, 1996; Gutkind, 1998). In mesangial cells, adrenomedullin receptor is coupled to all the three pathways differently, that is, adrenomedullin causes a decrease in ERK and an increase in JNK and P38 MAPK activities. Only the decrease in ERK and the increase in P38 MAPK are protein kinase-A mediated (Parameswaran *et al.*, 1999). The aims of the present study were (1) to study the involvement of the MAPK pathways modulated by adrenomedullin specifically, P38 MAPK, in adrenomedullin-mediated inhibition of proliferation (using [$^3$H]thymidine incorporation as an index) and stimulation of apoptosis (using nucleosome-associated cytoplasmic DNA fragmentation as an index) and (2) to characterize the proximal signaling pathways, using pharmacological inhibitors of specific signaling molecules, specifically the role of PI3-kinase. In order to understand the role of MAPKs as well as phosphotidyl inositol-3-kinase in adrenomedullin-mediated apoptosis and inhibition of proliferation, we used two inhibitors of signaling molecules, SB203580 {[4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole}, a P38 MAPK inhibitor (Lee at al., 1994) and wortmannin {[1S-(1α, 6bα, 9aβ, 11α, 11bβ)]-11-(Acetyloxy)-1, 6b, 7, 8, 9a, 10, 11, 11b-octahydro-1-(methoxymethyl)-9a, 11b-dimethyl-3H-furo[4, 3, 2-de]indeno[4, 5-h]-2-benzopyran-3, 6, 9-trione}, a phosphotidyl inositol 3-kinase inhibitor (Ui *et al.*, 1995). SB203580 (IC50 600nM-1μM) preferentially inhibits P38 MAPK without affecting other kinases significantly (Lee at al., 1994; Young *et al.*, 1997). Wortmannin is a potent, selective, cell permeable and irreversible inhibitor of phosphotidyl inositol-3-kinase (IC50 1-10nM). At 100 fold higher concentrations, it has

also been shown to inhibit phosphotidyl inositol-4 kinase, myosin light chain kinase (MLCK) and phospholipase-D (Bonser *et al.*, 1991; Nakanishi *et al*, 1992; Ui *et al.*, 1995). Wortmannin was also shown to inhibit a novel isoform of phosphotidyl inositol-3-kinase with a lower potency (IC50 of around 200-300 nM) (Stephens *et al*, 1994).

Using these two inhibitors the following hypotheses were tested: 1. Adrenomedullin-mediated changes in proliferation and apoptosis are mediated through P38 MAPK. 2. Adrenomedullin-induced p38 and JNK activities are mediated through PI3-kinase activation. 3. Adrenomedullin-mediated changes in proliferation and apoptosis are dependent on PI3-kinase activation. The results of this study indicate a role for P38 MAPK in adrenomedullin-mediated mesangial cell turnover. Furthermore we also show the possible presence of a wortmannin-sensitive kinase upstream of a P38 MAPK but not of ERK or JNK. Wortmannin affects only ADM-stimulated apoptosis and not ADM-inhibited proliferation. These data indicate differential signaling pathways controlling adrenomedullin-mediated changes on mesangial cell proliferation and apoptosis.

## 4.2. Materials and Methods:

**4.2.1. Materials:** Adrenomedullin was purchased from Phoenix Pharmaceuticals (Belmont, California), myelin basic protein, from Sigma (St. Louis). Polyclonal anti-ERK2, anti-P38 MAPK and anti-JNK1 antibodies were purchased from Santa Cruz laboratories (Santa Cruz, California). GST-cJUN was purchased from Alexis Biochemicals (San Diego, California). RPMI-1640, fetal bovine serum, penicillin and streptomycin were from Gibco (Grand Island, NY). SB203580 was a kind gift from Dr. John Lee, SmithKline Beecham Pharmaceuticals. Wortmannin was from Calbiochem. All other reagents were of high quality available.

**4.2.2. Cell culture**: Rat mesangial cells were obtained from the glomeruli of kidney cortex isolated from Sprague Dawley rats as described before (Albrightson *et al.*, 1992), and were grown in RPMI-1640 with 15% fetal bovine serum. Passages between 15 and 30 were used for the experiments.

**4.2.3. [$^3$H]-Thymidine incorporation (Proliferation):** Cells were plated in 24 well plates at 30000 cells/well and grown for 2 days, after which they were serum starved for 48 h. Cells were then treated with the test compounds for a period of 16 h and pulsed with [$^3$H]thymidine (1 μCi/ml) for 4 h. The radioactivity was counted in Beckman LS counter, after washing the cells and stopping the reaction with 5% trichloro acetic acid and solubilising the cells in 0.5 ml of 0.25 N sodium hydroxide. Each experiment was done in quadruplicates and was repeated at least 3 times.

**4.2.4. Kinase assays**: Cells were plated in p100 plates and were serum-starved overnight on reaching about 80% confluency. The agonist solutions were prepared in the growth media without serum. Cells were treated with the agonists for 30 minutes. In experiments where inhibitors were used, the cells were pretreated with the inhibitor for a period of 30 minutes before adrenomedullin treatment. The time points and experimental protocols were similar to that described before (Parameswaran *et al.*, 1999). The cell lysates were prepared as described (Bogoyevitch *et al.*, 1995; Li *et al.*, 1995). In the meantime specific antibodies (10 μg/reaction) were incubated with protein A agarose (Gibco) for 30 min at room temperature. After normalizing for protein concentration, the cell lysates were incubated with the specific antibody agarose conjugate for 2 h at 4°C with constant shaking. The kinase assays were done after washing the immunoprecipitate three times with HNTG (20 mM HEPES pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol) buffer and two times with kinase buffer (50 mM Tris-HCl, 100 mM NaCl, 10 mM $MnCl_2$ and 0.1 mM sodium ortho vanadate). The functional assay was done in the presence of 50 μM ATP, 5 μCi [$^{32}$P]ATP, 10 μg of specific substrate (myelin basic protein (MBP)-for ERK2 and P38 MAPK, and glutathione-S-transferase-c-Jun (GST-cJun) for JNK1), and

the immunoprecipitate. The reactions were performed at 30°C for 15 minutes and then stopped with sodium dodecyl sulphate buffer. The samples were electrophoresed on 12% polyacrylamide gel with appropriate molecular weight standards. The gels were dried and subjected to autoradiography or phosphoimager plates. The intensity of the bands in the autoradiogram was visualized using an ARCUS high-resolution optical scanner and quantitated using NIH image software or quantitated using imagequant program (for the gels exposed to phosphoimager plates).

**4.2.5. Enzyme linked immunosorbant assay (ELISA) for apoptosis**: The ELISA kit was obtained from Boehringer Mannheim (Indianapolis, IN), which specifically detects the cytoplasmic nucleosomal DNA. Cells were plated in 48 well plates and after 24 hours were serum starved for 24 h. Different agonists (prepared in the media) were added to the cells and incubated for another 20 h. The cells were lysed with the lysis buffer and centrifuged to separate cytoplasmic and nuclear fractions. The cytoplasmic fraction was then tested for DNA still attached to nucleosomes using the ELISA protocol from Boehringer Mannheim, Indianapolis, IN. The assay was done in triplicates or quadruplicates and repeated 3-5 times.

**4.2.6. Data analysis**: Results are expressed as mean±S.E. Analysis of Variance (ANOVA) was used to compare 3 or more treatments, followed by Bonferoni's multiple comparison between treatments, and student's t test for 2 treatment comparisons. A P value of less than 0.05 was considered significant.

**4.3. Results**:

**3.1. [$^3$H]Thymidine incorporation (Proliferation):**

Exposure of rat mesangial cells to adrenomedullin resulted in a significant decrease in basal [$^3$H]thymidine incorporation (-38.13±12.5%) (Figure 16). SB203580 completely reversed adrenomedullin-mediated decrease in proliferation (Figure 16). At higher concentrations (10 μM), SB203580 by itself caused a significant increase (275±106%) in [$^3$H]thymidine incorporation (Figure 16). However, at 1 μM, SB203580

by itself did not increase proliferation consistently (33±50%); but it completley reversed adrenomedullin-mediated decrease in [$^3$H]thymidine incorporation (34±4.7%).

Wortmannin, at concentrations known to be effective for inhibition of phosphotidyl inositol-3-Kinase activity (100-200 nM), did not affect adrenomedullin-mediated inhibition of [$^3$H]thymidine incorporation (Figure 17). At 200 nM, wortmannin by itself had no consistent effect on thymidine incorporation, but at higher concentrations (2 μM), it inhibited basal [$^3$H]thymidine incorporation in mesangial cells significantly (-57±8.5%) (Figure 17). In addition, at these concentrations, wortmannin also inhibited nucleosome-associated cytoplasmic DNA fragmentation (see below).

**4.3.2. Cytoplasmic nucleosome-associated DNA fragmentation (Apoptosis):**

Adrenomedullin caused a significant increase in nucleosome-associated cytoplasmic DNA fragmentation. Unlike [$^3$H]thymidine incorporation, adrenomedullin-mediated increase in cytoplasmic DNA fragmentation was inhibited by both inhibitors (SB203580 as well as wortmannin). SB203580 inhibited adrenomedullin-mediated increase in cytoplasmic DNA fragmentation significantly (even at 0.1 μM) with no significant effect on its own (Figure 18). Wortmannin also inhibited adrenomedullin-mediated increase in cytoplasmic DNA fragmentation (Figure 20). Although wortmannin by itself had no effect on basal apoptosis at 200 nM, it had a significant effect on basal apoptosis at 2 μM (-62±3%) (Figure 20).

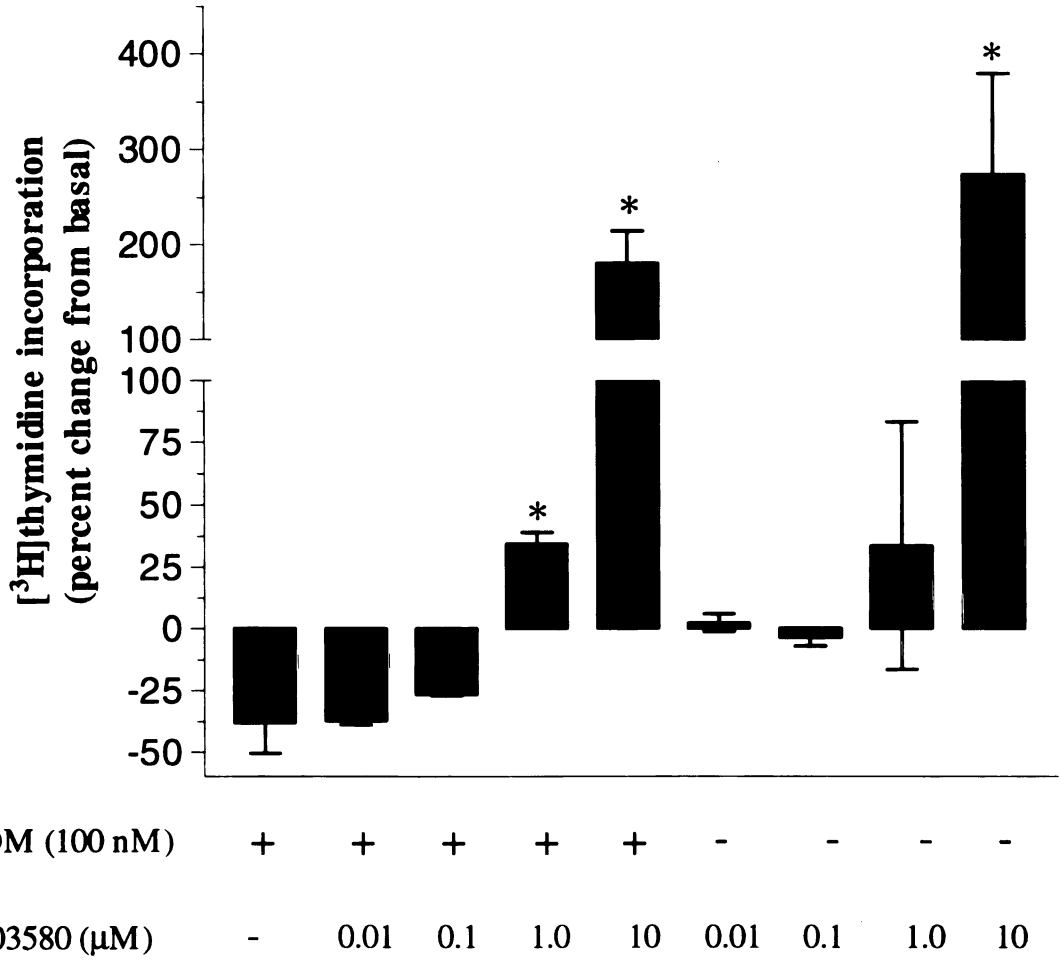



Figure 16. Effect of adrenomedullin (ADM) and SB203580 (P38 MAPK inhibitor) on [$^3$H]thymidine incorporation in rat mesangial cells. Experiment was done as described in the methods part. Cells were pretreated with the inhibitor for a period of 30 minutes before the addition of adrenomedullin. ADM caused a significant decrease in [$^3$H]thymidine incorporation [n=3 except basal and ADM(=6)]. Analysis of raw values indicated significant decrease in [$^3$H]thymidine incorporation by ADM alone compared to basal. * $P<0.01$ compared to ADM.

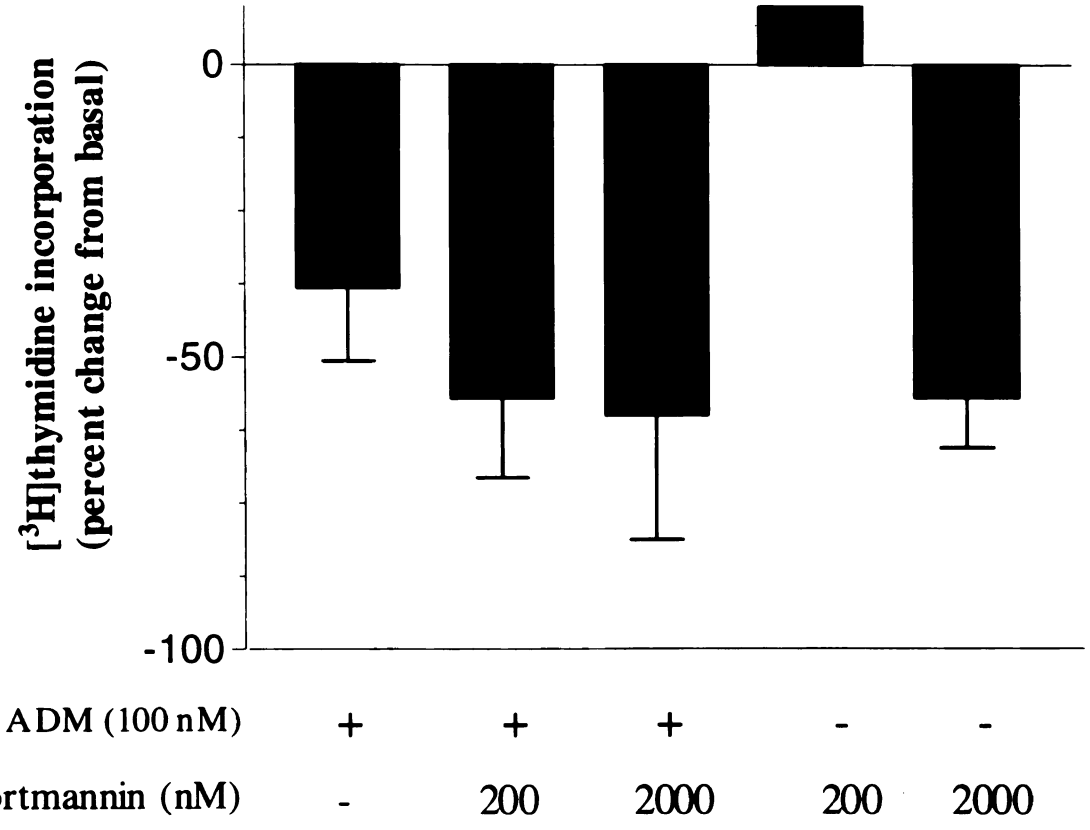



Figure 17. Effect of adrenomedullin (ADM) and wortmannin (PI-3 Kinase inhibitor) on [$^3$H]thymidine incorporation in rat mesangial cells. Experiment was done as described in the methods part. Cells were pretreated with the inhibitor for a period of 30 minutes before the addition of adrenomedullin. (ADM caused a significant decrease in [$^3$H]thymidine incorporation). Effect of wortmannin in the presence of ADM was not significantly different from ADM treatment (n=3).

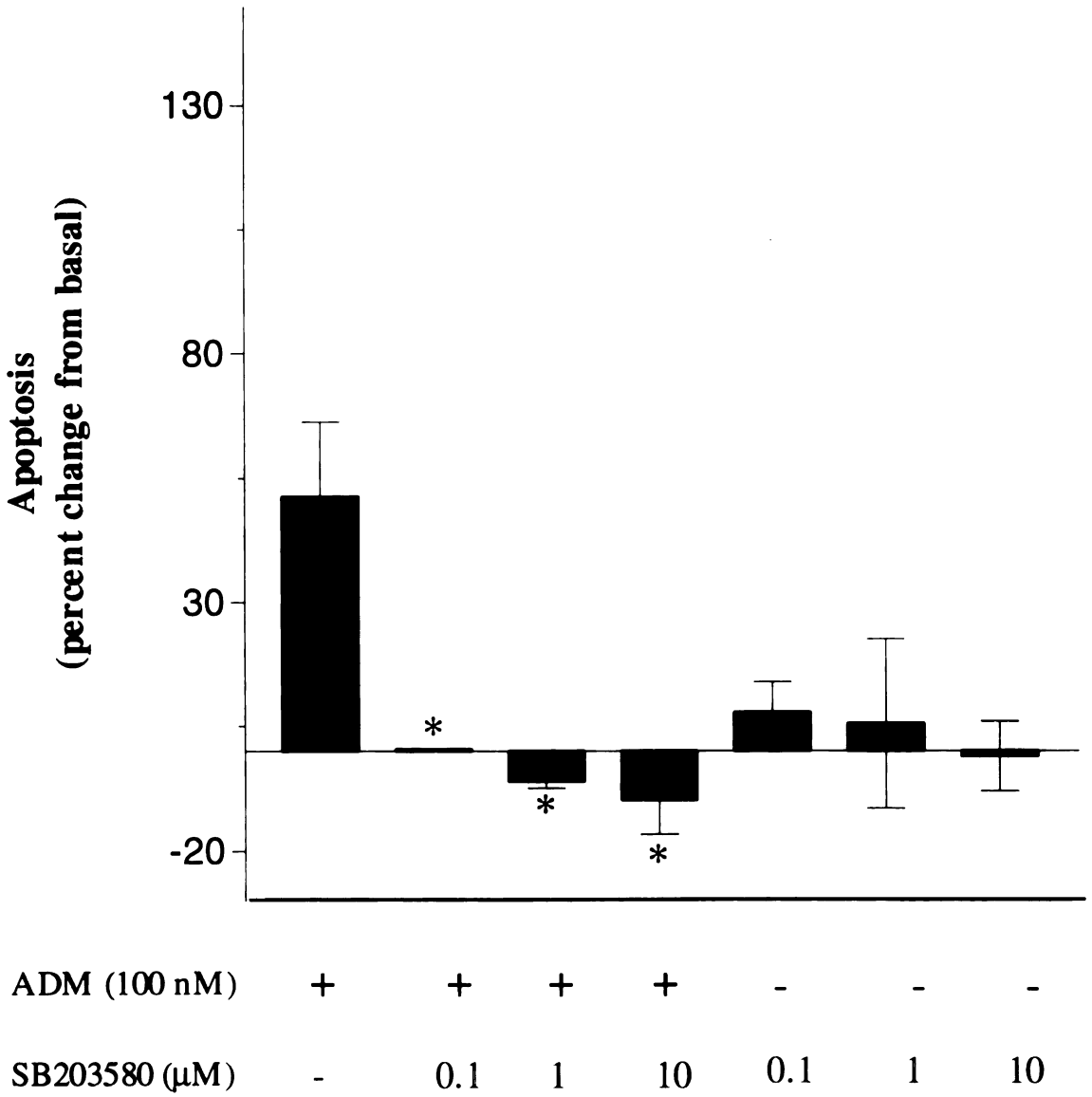



Figure 18. Effect of adrenomedullin (ADM) and SB203580 (P38 MAPK inhibitor) on cytoplasmic nucleosome-associated DNA fragmentation (an index of apoptosis) in rat mesangial cells. Cells were pretreated with the inhibitor for a period of 30 minutes before the addition of adrenomedullin. ADM caused a significant increase in DNA fragmentation. SB203580 by itself did not affect DNA fragmentation at any of the concentrations tested [n=3 except basal and ADM (n=6)]. *P<0.01 compared to ADM.

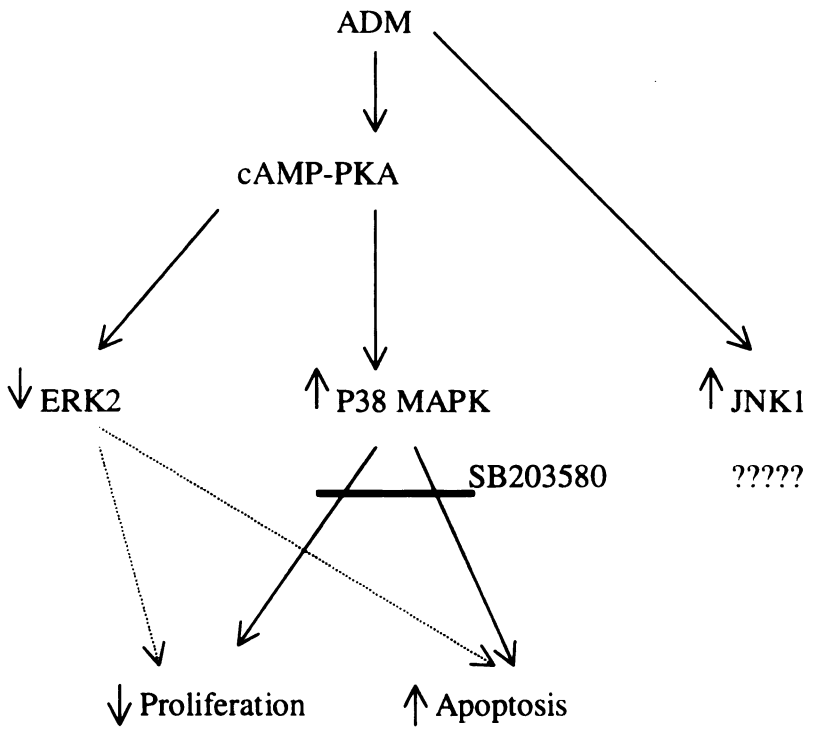



Figure 19. A schematic pathway based on the results from chapter 3, and Figures 16 and 18 of chapter 4. P38 MAPK inhibitor (SB203580) completely reversed ADM-mediated changes in proliferation and apoptosis in rat mesangial cells. It is assumed here that SB203580 does not affect ERK or JNK activities.

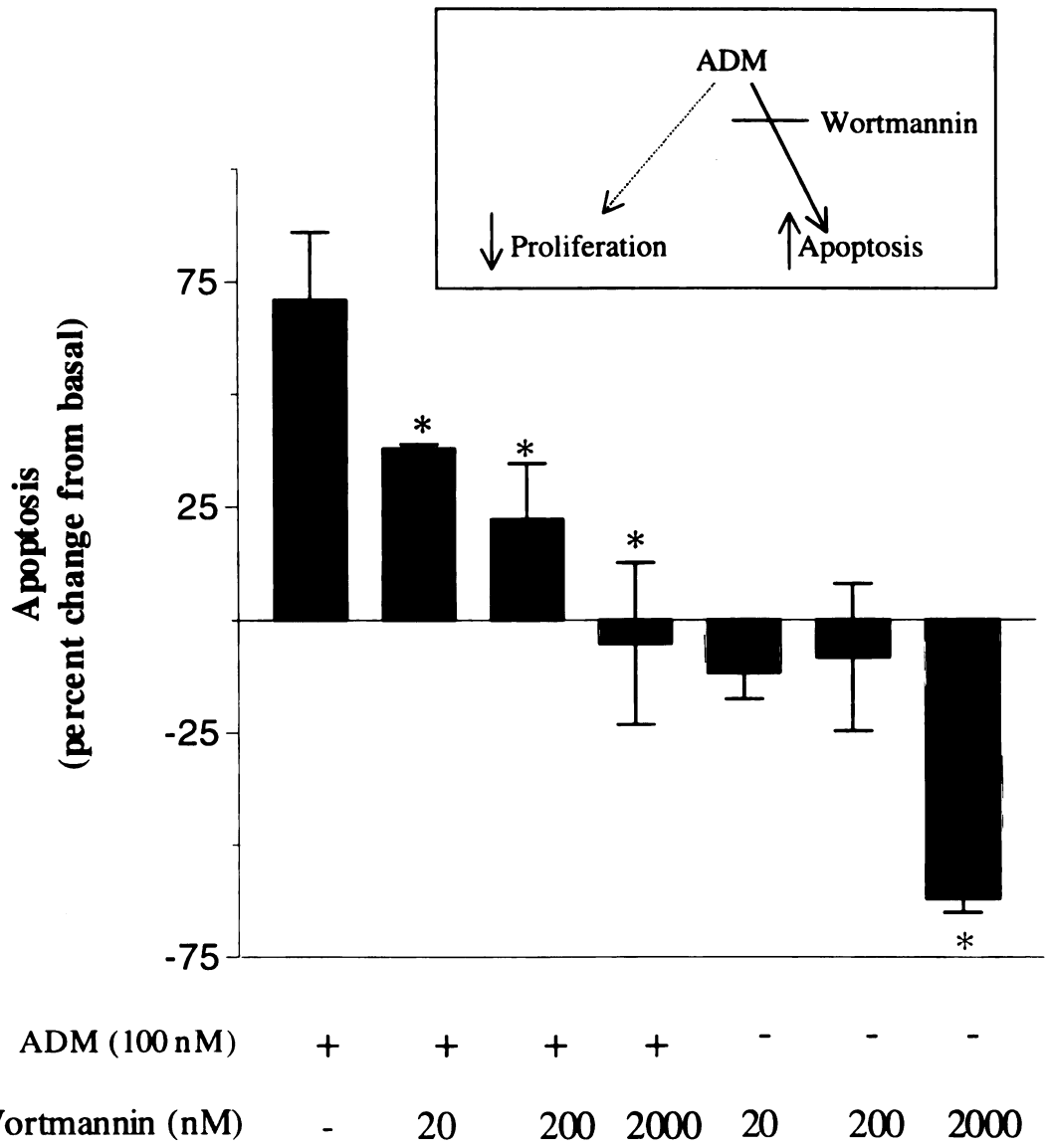



Figure 20. Effect of adrenomedullin (ADM) and wortmannin (PI3 kinase inhibitor) on cytoplasmic nucleosome-associated DNA fragmentation (an index of apoptosis) in rat mesangial cells. Cells were pretreated with the inhibitor for a period of 30 minutes before the addition of adrenomedullin. ADM caused a significant increase in DNA fragmentation (n=4). *P<0.05 compared to ADM.

**4.3.3. MAPK pathway**:

Because of the differential effects observed with SB203580 and wortmannin on adrenomedullin-mediated proliferation and apoptosis, it was of interest to test the activities of various mitogen-activated protein kinases. The kinase assays were done after 30 minutes treatment with adrenomedullin. In experiments where inhibitors were used, the cells were pretreated with the inhibitors for a period of 30 minutes before adrenomedullin treatment.

**4.3.3.1. ERK2**: Adrenomedullin decreased ERK2 activity significantly below basal levels as measured by immuno-complex assay with specific anti-ERK2 antibody using MBP as substrate. The effect of adrenomedullin on ERK2 activity was not affected by SB203580 or wortmannin (Figure 21, 22).

**4.3.3.2. JNK1**: Adrenomedullin increased JNK activity significantly above basal levels as measured by immuno-complex assay using specific anti-JNK1 antibody and GST-cJun as substrate. Neither SB203580 nor wortmannin had any consistent effect on adrenomedullin-stimulated JNK1 activity (Figure 23, 24).

**4.3.3.3. P38 MAPK**: Adrenomedullin increased P38 MAPK activity significantly above basal levels as measured by immuno-complex assay using specific anti-P38 MAPK antibody and MBP as substrate (Figure 25). Only P38-MAPK activity was sensitive to SB203580. Similar to SB203580, wortmannin inhibited adrenomedullin-stimulated P38 MAPK activity (Figure 27).

The inhibitors by themselves did not cause any significant change in the activities of these kinases within the time frame tested.

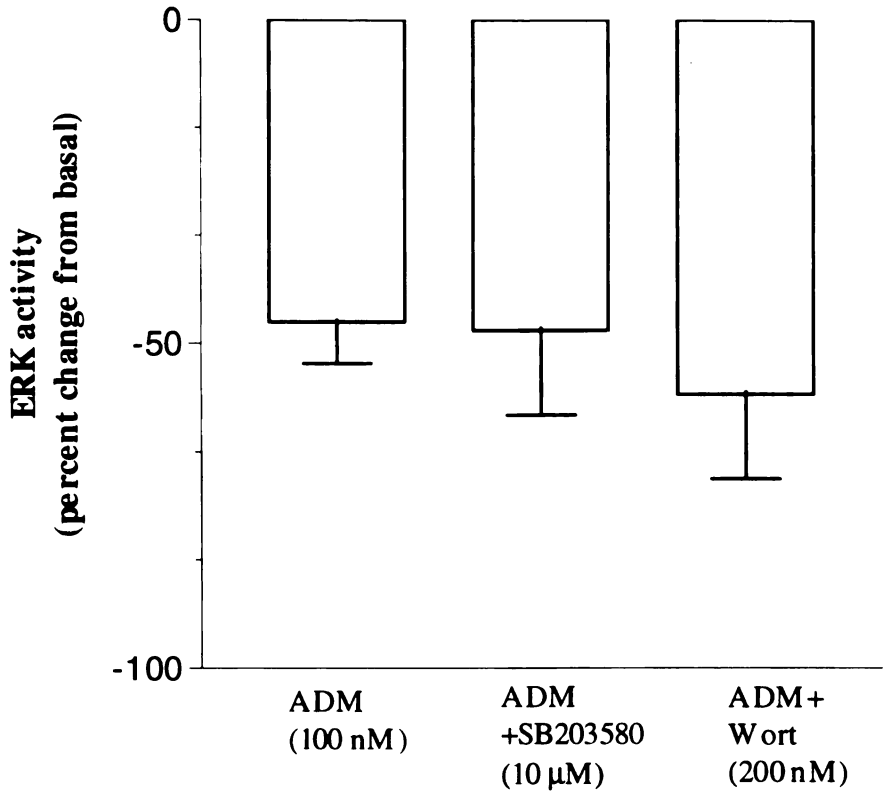



Figure 21. Effect of ADM, SB203580 and wortmannin on ERK2 activity in rat mesangial cells (n=3). SB203580 or wortmannin alone did not affect ERK2 activity. Adrenomedullin decreased ERK2 activity significantly below basal levels as measured by immuno-complex assay with specific anti-ERK2 antibody using MBP as substrate.

The kinase assays were done after 30 minutes treatment with adrenomedullin (ADM). In experiments where inhibitors were used, the cells were pretreated for a period of 30 minutes before adrenomedullin treatment.

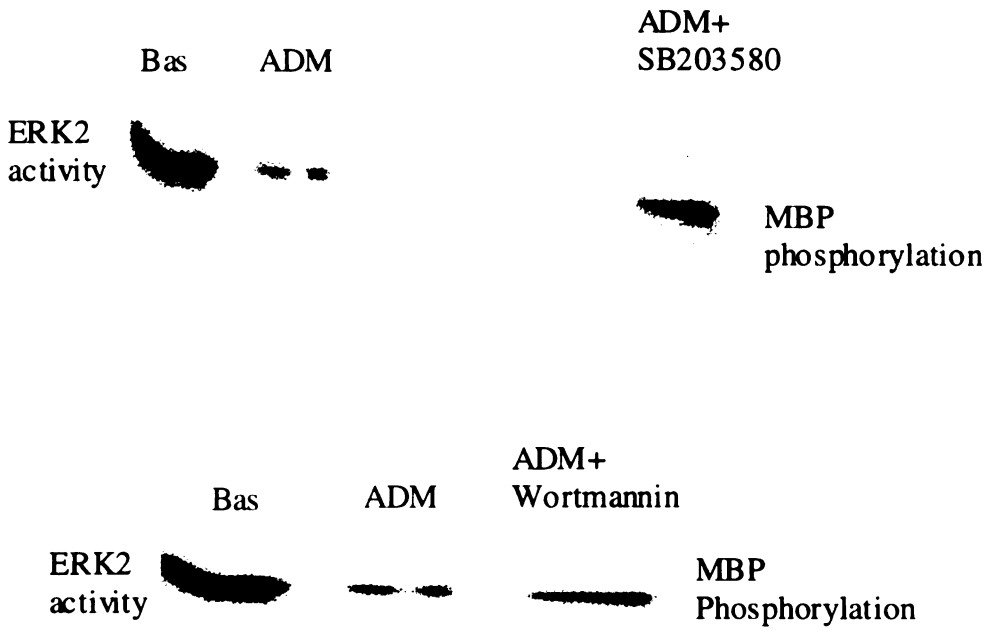



Figure 22. A representative autoradiogram showing the effect of ADM and SB203580 (top), and ADM and wortmannin (bottom) on ERK2 activity in rat mesangial cells.

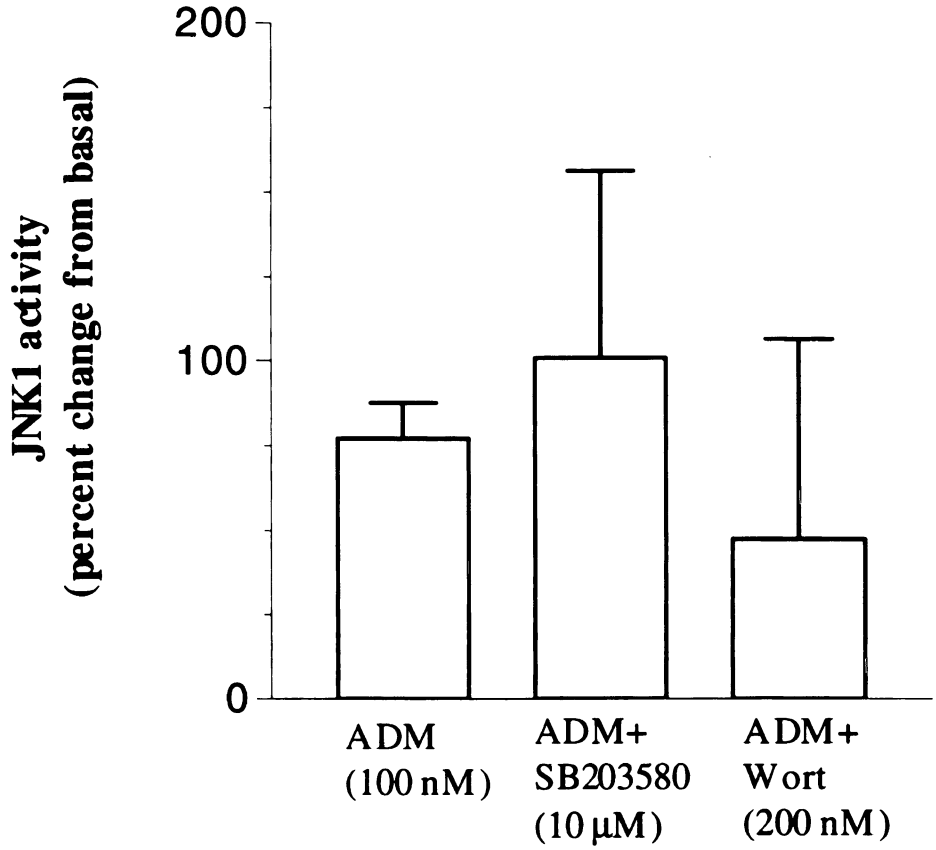



Figure 23. Effect of ADM, SB203580 and wortmannin on JNK1 activity in rat mesangial cells (n=3). Neither of the inhibitors consistently affected ADM-stimulated JNK activity. SB203580 or wortmannin alone did not affect JNK1 activity. Adrenomedullin increased JNK activity significantly above basal levels as measured by immuno-complex assay using specific anti-JNK1 antibody and GST-cJun as substrate

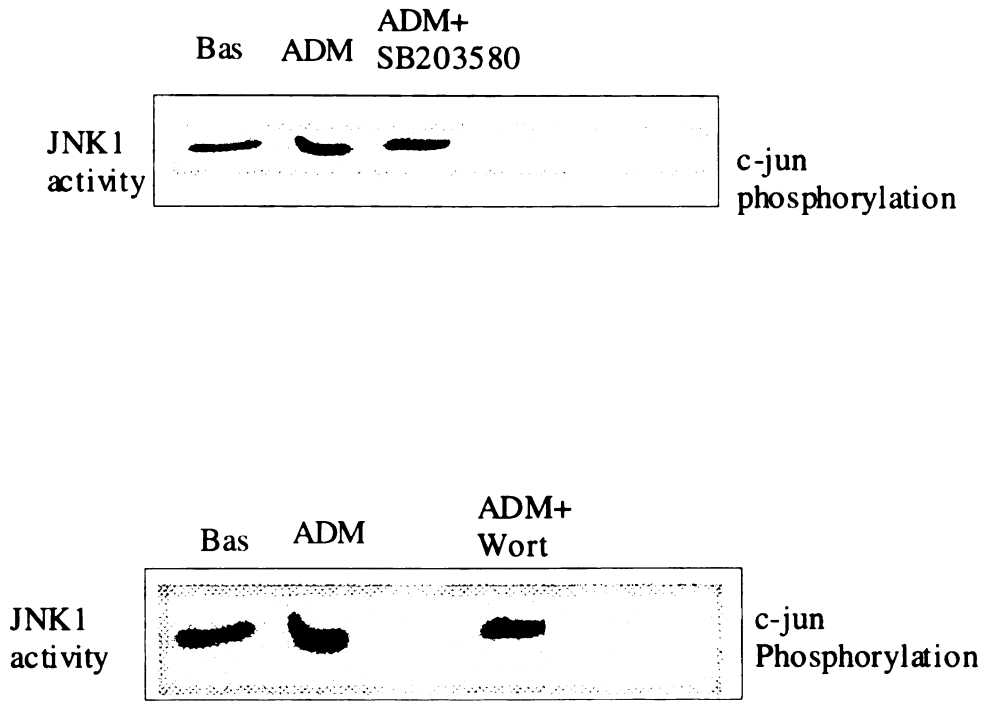



Figure 24. A representative autoradiogram showing the effect of ADM and SB203580 (top), and ADM and wortmannin (bottom) on JNK1 activity in rat mesangial cells.

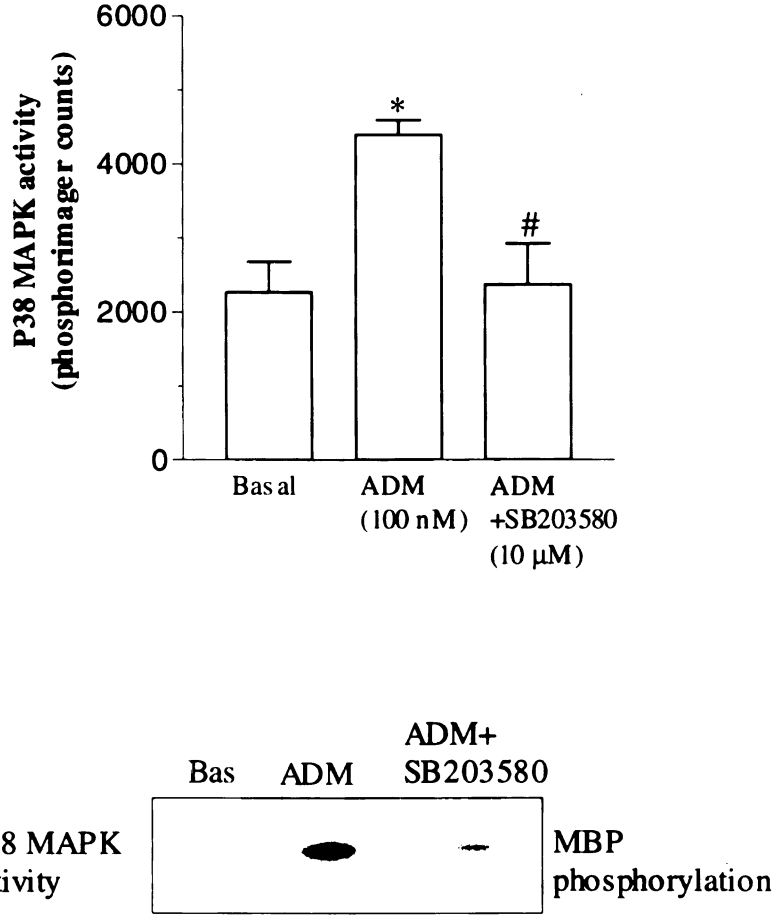



Figure 25. Effect of ADM and SB203580 on P38 MAPK activity in rat mesangial cells. (n=4) *P<0.01 compared to basal and #P<0.01 compared to ADM. Adrenomedullin increased P38 MAPK activity significantly above basal levels as measured by immuno-complex assay using specific anti-P38 MAPK antibody and MBP as substrate. A representative autoradiogram (bottom panel) showing the effect of ADM and SB203580 on P38 MAPK activity in rat mesangial cells.

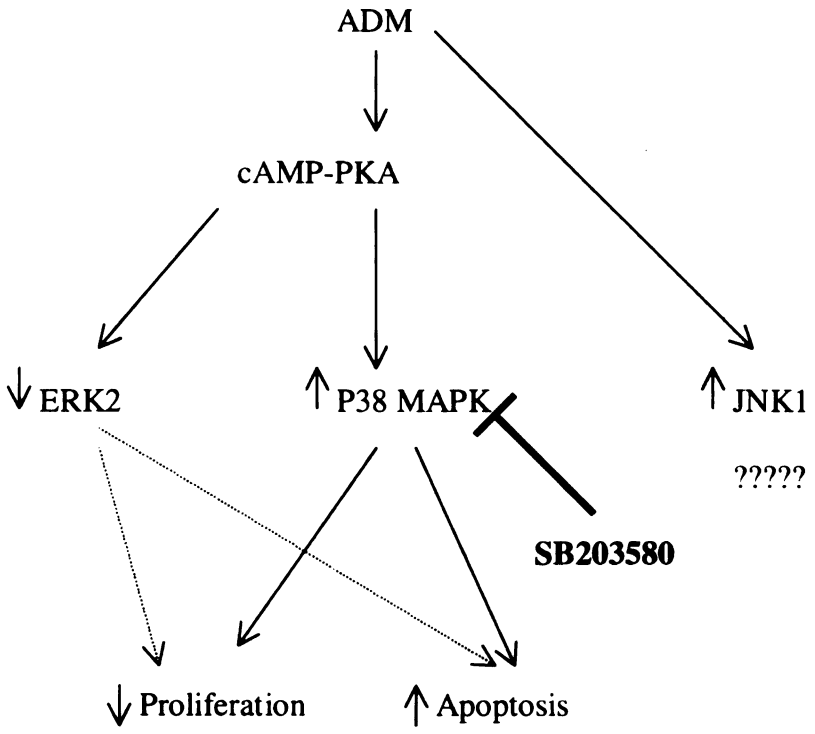



Figure 26. A schematic signaling pathway modulated by ADM based on the results from chapter 3 and figures 16, 18, 21-25 of chapter 4. It was assumed in the scheme in figure 19 that SB203580 inhibits only P38 activity. Results from Figures 21-25 indicate the same. Thus, ADM through cAMP-PKA pathway stimulates P38 MAPK activity, that induces apoptosis and decreases proliferation in cultured rat mesangial cells.

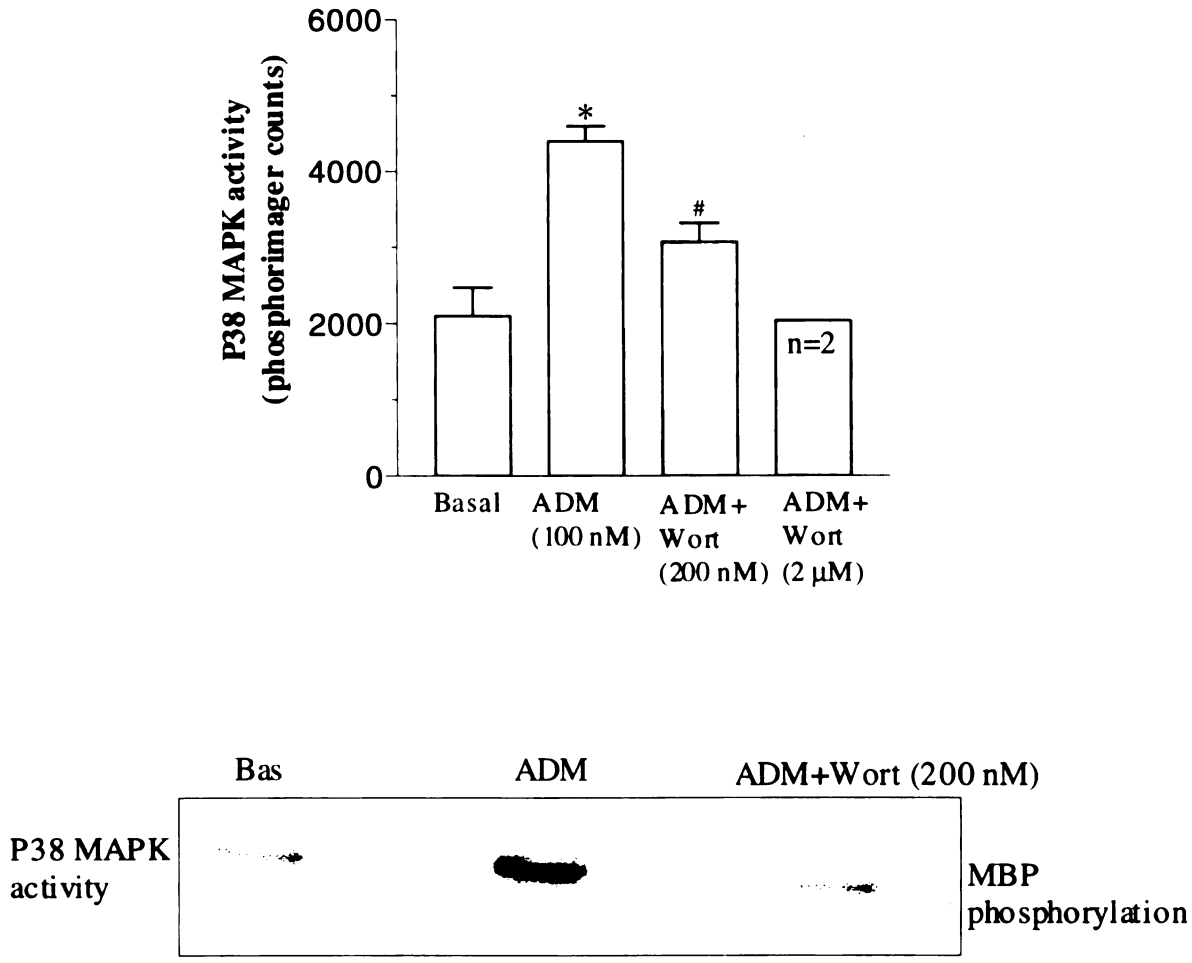



Figure 27. Effect of ADM and wortmannin on P38 MAPK activity in rat mesangial cells. (n=3) *P<0.01 compared to basal and #P<0.01 compared to ADM. A representative autoradiogram (bottom panel) showing the effect of ADM and wortmannin (200 nM) on P38 MAPK activity in rat mesangial cells.

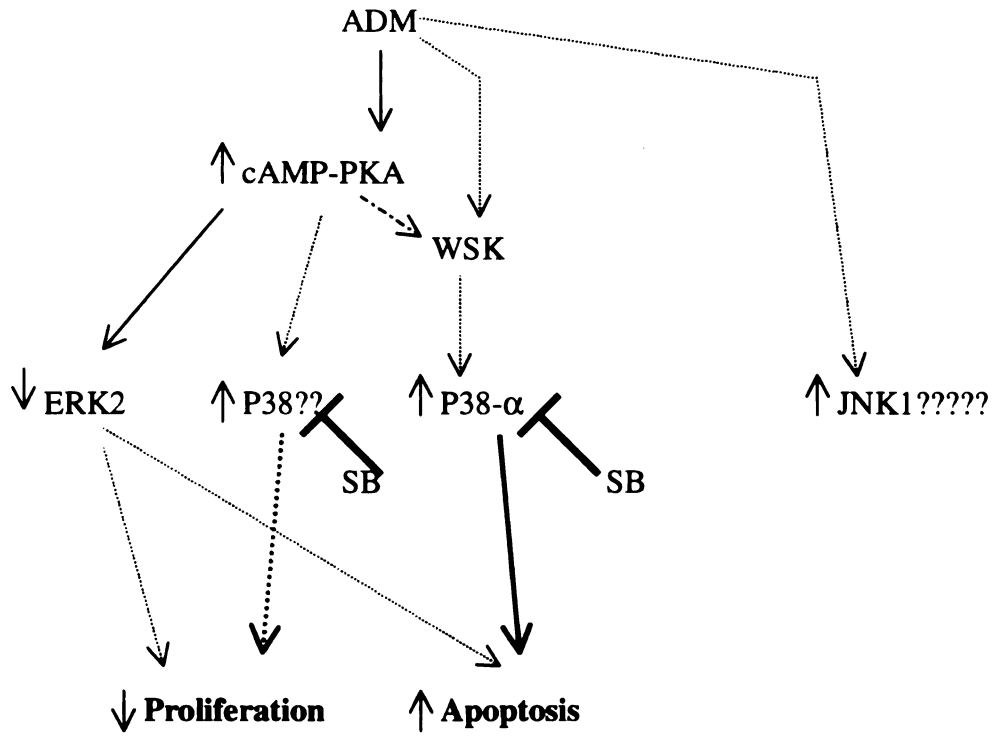



Figure 28. A proposed signaling pathway based on the results from chapters 3 and 4. Wortmannin inhibited ADM-stimulated apoptosis but did not affect ADM-inhibited proliferation pathway. Wortmannin also inhibited ADM-stimulated P38 MAPK activity. But inhibition of P38 activity with SB203580 reversed both proliferation and apoptosis responses of ADM, thus suggesting two p38 pathways, one sensitive to wortmannin (the apoptosis pathway) and the other insensitive to wortmannin (the proliferation pathway). [WSK-wortmannin sensitive kinase, SB-SB203580 (P38 MAPK inhibitor)]

4.4. Discussion:

In mesangial cells, adrenomedullin caused a significant decrease in [$^3$H]thymidine incorporation (an index of proliferation) and an increase in cytoplasmic nucleosome-associated DNA fragmentation (an index of apoptosis). In addition, treatment of mesangial cells with adrenomedullin resulted in a decrease in ERK2 and an increase in JNK1 and P38 MAPK activities. SB203580, a P38 MAPK inhibitor completely reversed the effect of adrenomedullin on both proliferation and apoptosis, suggesting that adrenomedullin-stimulated P38 MAPK may be involved in adrenomedullin-mediated decrease in proliferation and increase in apoptosis (Figure 26). The fact that SB203580 did not affect either ERK2 or JNK1 activity, further indicates that the effect of this inhibitor is likely through inhibition of P38 MAPK. Recent reports using other cell lines have indicated a role for P38 MAPK in apoptosis (Wang *et al.*, 1998). Although P38 MAPK has been shown to be involved in other biological responses such as cardiac myocyte hypertrophy, and cytokine secretion (Lee *et al.*, 1994; Wang *et al.*, 1998), this is the first demonstration of the involvement of P38 MAPK in adrenomedullin-mediated effects on mesangial cell proliferation. The fact that SB203580 by itself at high concentrations stimulated proliferation, suggests that a P38 MAPK is active at basal state that controls the normal turnover. This may have important implications in progressive renal diseases where aberrant proliferation of mesangial cells is a characterisitc feature. It remains to be seen whether P38 MAPK activity is altered in the progression of such diseases. Using 5/6$^{th}$ nephrectomized rats as chronic renal failure models we found P38 and JNK activities to be upregulated at specific time points in the kidney. Although the implications of these findings are not readily apparent, it is quite possible that these are involved in the pathogenesis of the disease expression in these rats (N. Parameswaran *et al.*, 1999, Manuscript in preparation).

Wortmannin, on the other hand, did not affect [$^3$H]thymidine incorporation that was inhibited by adrenomedullin but inhibited adrenomedullin-stimulated apoptosis at concentrations that are known to be selective for phosphotidyl inositol-3-kinase inhibition. The rationale for testing wortmannin was to identify the proximal signaling mechanisms of JNK and P38 MAPK activation. A number of reports have suggested recently that both these kinase pathways could be regulated by phosphotidyl inositol-3 kinase especially through the $\beta\gamma$ subunit of G- protein involved in receptor activation (Gutkind, 1998; Lopez-Ilasaca *et al.*, 1998; Yamauchi *et al.*, 1997). In HL60-granulocytes, wortmannin was found to inhibit ERK and P38 MAPK activities but not JNK activity stimulated by formyl peptide receptor (Rane *et al.*, 1997). Moreover, we have also found that adrenomedullin-stimulated hyaluronic acid secretion can be inhibited significantly by wortmannin (N. Parameswaran *et al.*, 1999e). Our present results indicate that adrenomedullin-stimulated P38 MAPK may be dependent on a wortmannin-sensitive kinase. We found JNK activity to be very inconsistent with regard to the inhibitors including wortmannin.

Wortmannin inhibited adrenomedullin-stimulated P38 MAPK activity. This suggests that the kinase sensitive to wortmannin is present upstream of P38 MAPK. Wortmannin affected only adrenomedullin-induced apoptosis and not the proliferation response while SB203580, a P38 MAPK inhibitor affected both. These findings lead us to suggest that, there are probably two different pathways that are P38/SB203580 sensitive and only one is possibly sensitive to wortmannin (Figure 28). This might indicate that the effect of SB203580 could be a combination of P38 MAPK-dependent and -independent mechanisms or alternatively, that the wortmannin-sensitive pathway might be activating an isoform of P38 MAPK that affects apoptosis but not proliferation. The fact that SB203580 did not affect either ERK or JNK activity (the most closely related kinases to P38 MAPK), possibly argues against the former. Different isoforms of P38 have been shown to cause different effects in myocardial cells (Wang *et al.*, 1998).

The fact that 10 μM SB203580 caused mesangial cell proliferation while, did not affect basal apoptosis suggests that the enzymes regulating these two processes are active differently, in quiescent cells.

The differential effects of wortmannin could also be unrelated to P38. That is, wortmannin has been shown to decrease receptor-dependent regulation of calcium-entry in human platelets, without affecting intracellular calcium stores (Von Appen *et al.*, 1997). Since late apoptosis requires an increase in calcium, it is possible that wortmannin might be inhibiting apoptosis by inhibiting the calcium entry. Obviously extensive studies are necessary to test this hypothesis.

Other factors have been shown to induce mesangial cell apoptosis. Of particular interest are the fas and ceramide-mediated apoptosis (Coroneos *et al.*, 1996; Gonzalez-cuadrado *et al.*, 1997). It is not quite clear what MAPKs are required for the apoptosis induced by these agents. But Fas-induced apoptosis in T cells was shown to be independent of P38, even though Fas increased P38 activity (Salmon *et al.*, 1997) . Further studies examining the regulation of apoptosis by these factors and ADM will be quite useful in understanding the pathophysiology of proliferative glomerulonephritis.

Our results demonstrate for the first time that the adrenomedullin-mediated decrease in proliferation and increase in apoptosis are sensitive to SB203580 and hence possibly mediated through P38 MAPK pathway. Our data also suggests the presence of wortmannin-sensitive and -insensitive p38 MAPK/SB203580 sensitive pathways, the former possibly regulating apoptosis and the latter proliferation. Taken together, these results indicate that P38 MAPK may play an important role in mesangial cell turnover mediated by adrenomedullin.

# 5. Mechanism Of Adrenomedullin-Stimulated Hyaluronic Acid release In Rat Mesangial Cells

## 5.1. Introduction:

Hyaluronic acid, a non-sulfated glycosaminoglycan, is widely distributed in the extracellular space in animals. It is an important extracellualr matrix component and also a potential mitogen secreted by mesangial cells (Couchman *et al.*, 1994; Mahadevan *et al.*, 1996). Serum hyaluronic acid levels are increased during renal insufficiency and chronic renal failure (Hallgreen *et al.*, 1987). Hyaluronic acid release by the glomerulus is also increased in experimental diabetes and postulated to be a significant factor in glomerular hypercellularity (Mahadevan *et al.*, 1996). In addition, the expression of hyaluronic acid receptor is significantly increased in experimental proliferative glomerulonephritis (Nikolic-Paterson *et al.*, 1996). Although certain factors have been shown to modulate hyaluronic acid release in different cell systems, there is little information on the molecular mechanisms of regulation of hyaluronic acid release, especially the role of mitogen-activated protein kinase (MAPK) pathways (Heldin *et al.*, 1992; Heldin *et al*, 1989; Honda *et al.*, 1993; Suzuki *et al.*, 1995).

Adrenomedullin, discovered in 1993, increases cAMP in a variety of systems including rat mesangial cells (Chini *et al.*, 1995; Kohno *et al.*, 1995). Recently, we reported that in rat glomerular mesangial cells adrenomedullin causes a decrease in extracellular signal-regulated kinase-2 (ERK2) activity and an increase in *jun*-amino terminal kinase-1 (JNK1) and P38 mitogen-activated protein kinase (P38 MAPK) activities with an associated induction of apoptosis and decrease in proliferation (Parameswaran *et al.*, 1999b). The major aim of this study was to identify the effects and molecular mechanisms of adrenomedullin, forskolin, and cAMP on hyaluronic acid release in rat mesangial cells. We have designed experiments to test the hypothesis that

adrenomedullin-mediated increase in hyaluronic acid levels is through activation of protein kinase-A, P38 MAPK and PI3-kinase. We have used selective pharmacological inhibitors to test this hypothesis. We have also compared all these effects in response to forskolin, an adenylate cyclase activator and dibutyryl cAMP, a cell permeable cAMP analog. We demonstrate here that adrenomedullin, forskolin and dibutyryl-cAMP can stimulate hyaluronic acid release in mesangial cells, and that it is dependent on P38 MAPK as well as wortmannin-sensitive kinase pathways. While forskolin- and dbcAMP-stimulated HA release is dependent on PKA, that of ADM is not. These findings indicate a novel pathway of hyaluronic acid release in rat mesangial cells by adrenomedullin and cAMP elevating agents.

## 5.2. Materials and Methods:

### 5.2.1. Materials:

Adrenomedullin was purchased from Phoenix Pharmaceuticals (Belmont, California), RPMI-1640, fetal bovine serum, penicillin and streptomycin were from Gibco (Grand Island, NY). SB203580 was a kind gift from Dr. John Lee (SmithKline Beecham pharmaceuticals, King of Prussia, PA). H89, forskolin, and wortmannin were from Calbiochem. Dibutyryl cyclic AMP was from Sigma. All other reagents were of the highest quality available.

### 5.2.2. Cell culture:

Rat mesangial cells were obtained from the glomeruli of kidney cortex isolated from Sprague Dawley rats as described before (Albrightson *et al.*, 1992), and were grown in RPMI-1640 with 15% fetal bovine serum. Passages between 15 and 30 were used for the experiments.

**5.2.3. Radiometric assay for hyaluronic acid**:

Measurement of hyaluronic acid was done using a radiometric assay kit obtained from Pharmacia and Upjohn Diagnostics Division, (Kalamazoo, MI). The principle of the radiometric assay is as follows:

The hyaluronic acid released in the cell culture media reacts with $^{125}$I-HABP (Hyaluronic acid binding protein) in solution. The unbound $I^{125}$-HABP is then quantitated by incubating with hyaluronic acid covalently coupled to sepharose particles of small size and low density. Separation is performed by centrifugation followed by decanting. The radioactivity bound to the particles is measured by a gamma counter and the counts are inversely proportional to the concentration of hyaluronic acid in the sample. For our experiments, cells were plated in 24 well plates at 50,000 cells/well and serum starved overnight, after 48 h of plating. Cells were then treated with different agents in triplicates or quadruplicates for a period of 18 h and then the media were collected and assayed for hyaluronic acid released. Results are expressed as mean±S.E. of hyaluronic acid in ng/ml of media.

**5.2.4. Data analysis:** Results are expressed as mean±S.E. Analysis of Variance (ANOVA) was used to compare 3 or more treatments, followed by Bonferonni's multiple comparison between treatments, and student's t test for 2 treatment comparisons. A P value of less than 0.05 was considered significant.

**5.3. Results:**

Exposure of mesangial cells to adrenomedullin resulted in a concentration-dependent increase in hyaluronic acid release (Figure 29). In addition to adrenomedullin, forskolin and db-cAMP also increased hyaluronic acid release significantly above basal levels (Figure 30 and 31).

Adrenomedullin-stimulated hyaluronic acid release was not significantly inhibited by H89, a potent protein kinase A inhibitor (Figure 32), whereas forskolin- and dbcAMP-

mediated hyaluronic acid release was inhibited by H89 (Figure 35, 36). Both wortmannin (a phosphatidyl inositol-3 Kinase inhibitor) and SB203580 (a P38-MAPK inhibitor) significantly blocked adrenomedullin-, forskolin-, and dbcAMP-stimulated hyaluronic acid release (Figure 33, 34, 35, 36).

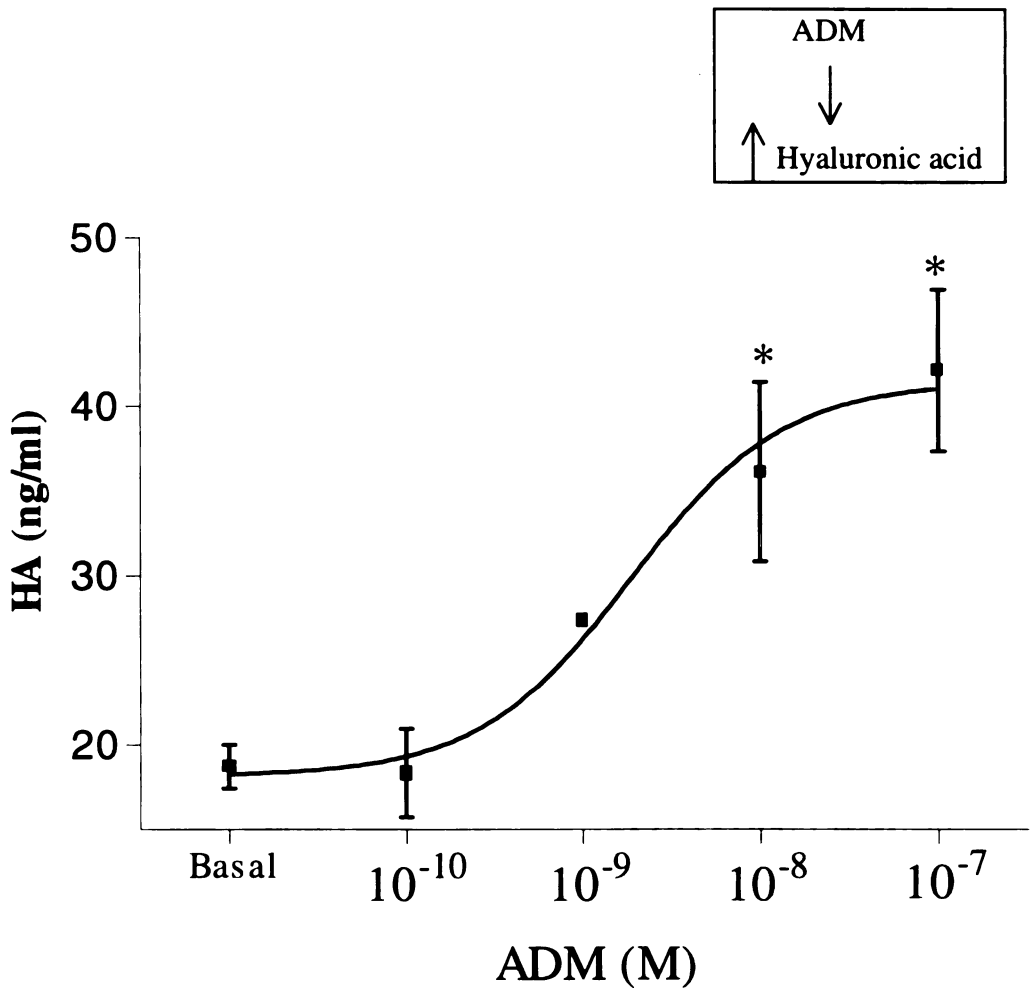



Figure 29. Adrenomedullin (ADM) activation of hyaluronic acid (HA) release from rat mesangial cells. Adrenomedullin caused a concentration-dependent increase in hyaluronic acid release. *P<0.05 compared to basal. Experiments were done in triplicates and repeated three times.

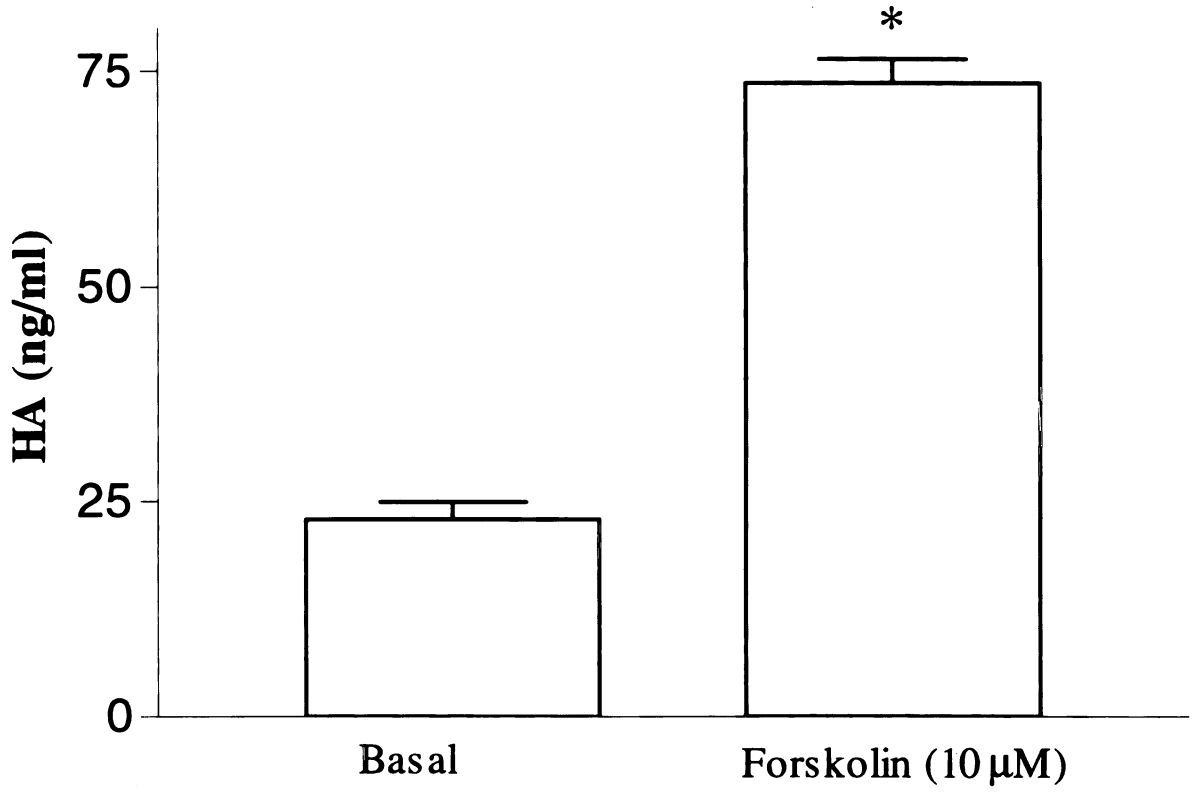



Figure 30. Effect of forskolin (adenylate cyclase activator) on HA release in rat mesangial cells. Forskolin caused a significant increase in hyaluronic acid release. *P<0.01 compared to basal. n=3.

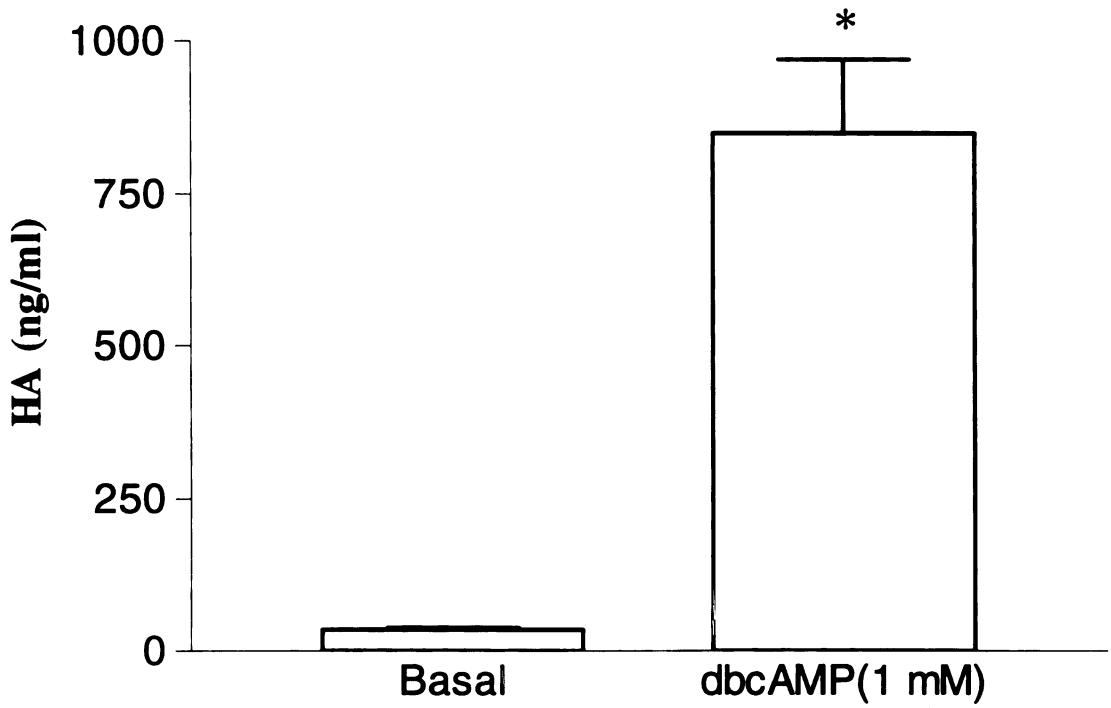



Figure 31. Effect of dibutyryl cAMP (cell permeable cAMP analog) on HA release in rat mesangial cells. Dibutyryl cAMP caused a significant increase in hyaluronic acid release. *P<0.01 compared to basal. n=3.

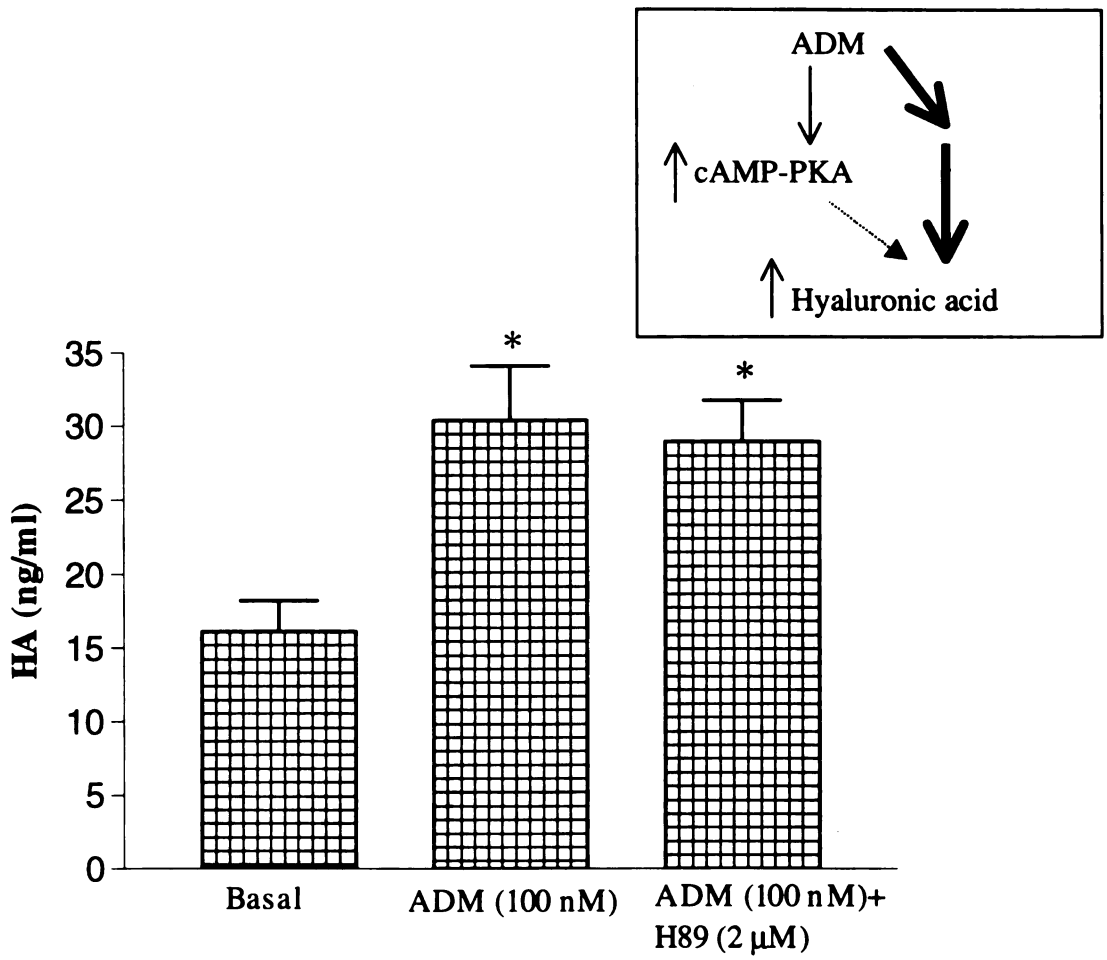



Figure 32. Effect of adrenomedullin and H89 (protein kinase A inhibitor) on hyaluronic acid release from rat mesangial cells. H89 did not significantly affect adrenomedullin stimulated hyaluronic acid release. Experiments were done in triplicates and repeated four times. H89 by itself did not affect the basal hyaluronic acid release significantly. *P<0.05 compared to basal. The insert on the top shows the proposed pathway. ADM appears to use a cAMP-independent pathway to stimulate HA release, although forskolin and dbcAMP significantly increased HA release, suggesting that an increase in cAMP can increase HA release in mesangial cells.

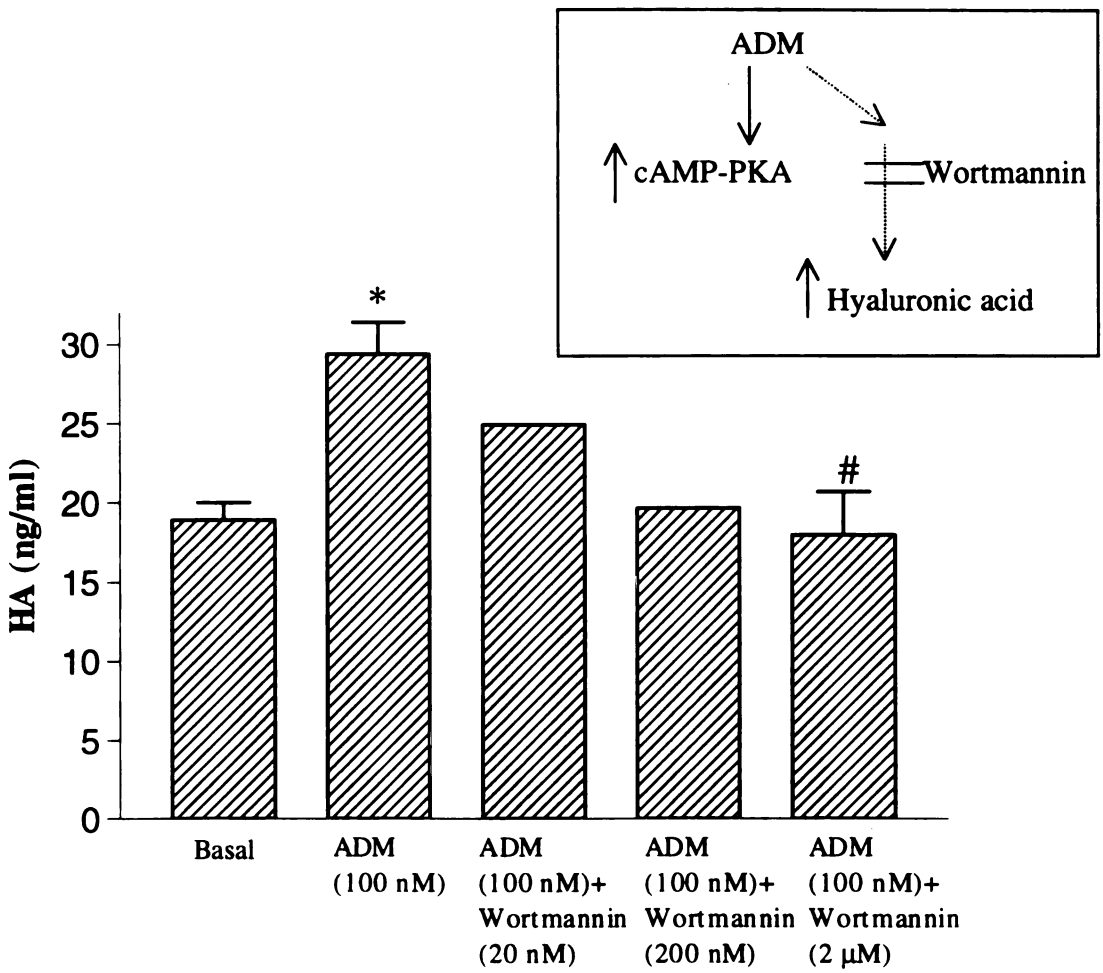



Figure 33. Effect of adrenomedullin and wortmannin (phosphotidyl inositol 3-kinase inhibitor) on hyaluronic acid release from rat mesangial cells. Wortmannin significantly inhibited adrenomedullin-stimulated hyaluronic acid release. *P<0.01 compared to basal, #P<0.05 compared to ADM. Experiments were done in triplicates and repeated three times. Wortmannin by itself did not affect basal hyaluronic acid release

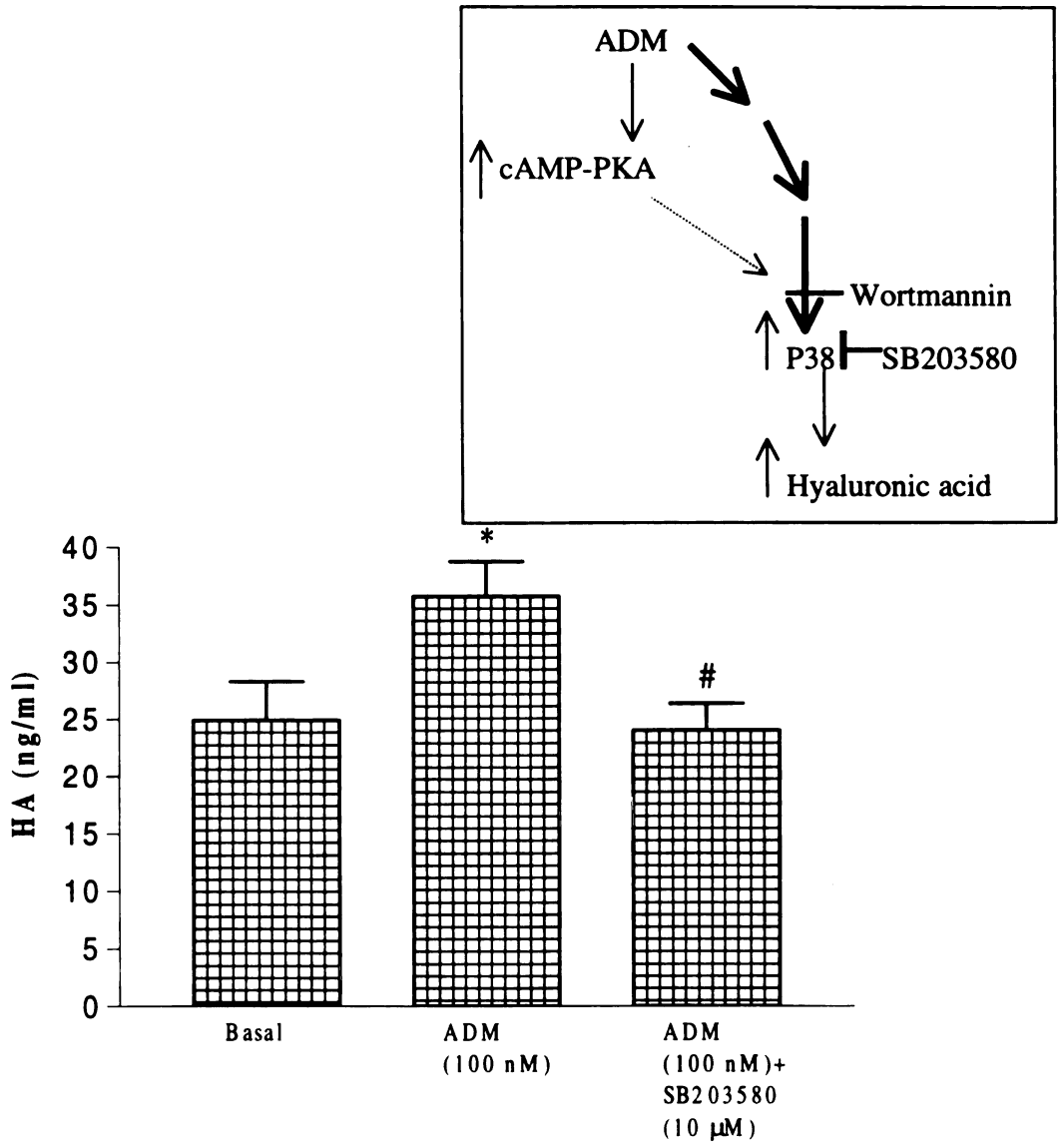



Figure 34. Effect of ADM and SB203580 (P38 MAPK inhibitor) on hyaluronic acid release from rat mesangial cells. SB203580 significantly inhibited ADM-stimulated hyaluronic acid release. *P<0.05 compared to basal, #P<0.05 compared to ADM. SB203580 by itself did not affect basal hyaluronic acid release. Experiments were done in triplicates and repeated four times.

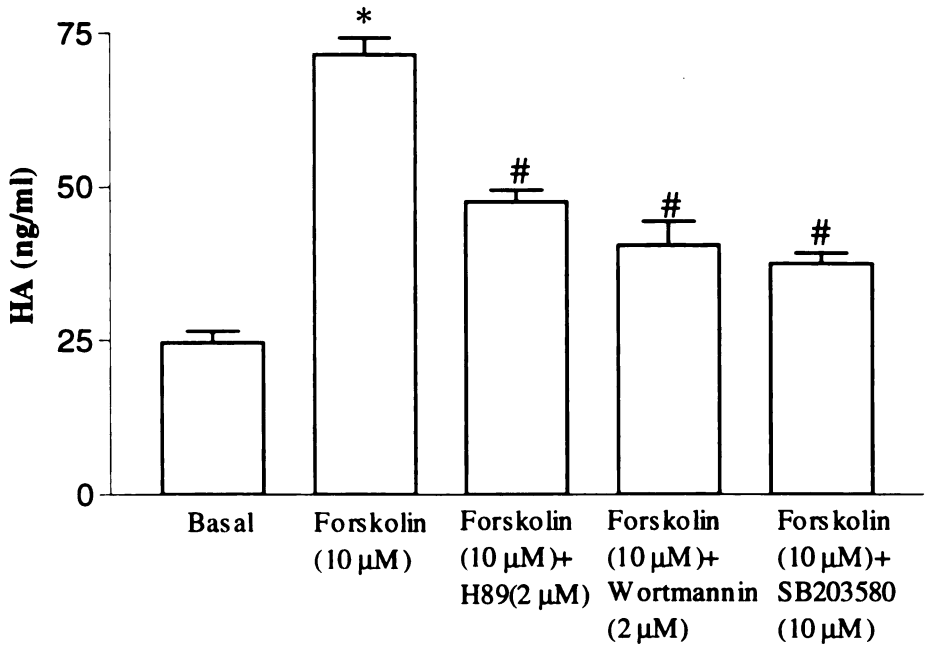



Figure 35. Effect of forskolin (adenylate cyclase activator), H89 (protein kinase A inhibitor), wortmannin (phosphotidyl inositol 3-kinase inhibitor), and SB203580 (P38 MAPK inhibitor) on hyaluronic acid (HA) release from rat mesangial cells. All the inhibitors significantly inhibited forskolin-stimulated hyaluronic acid release from mesangial cells. *P<0.01 compared to basal, #P<0.01 compared to forskolin. n=3.

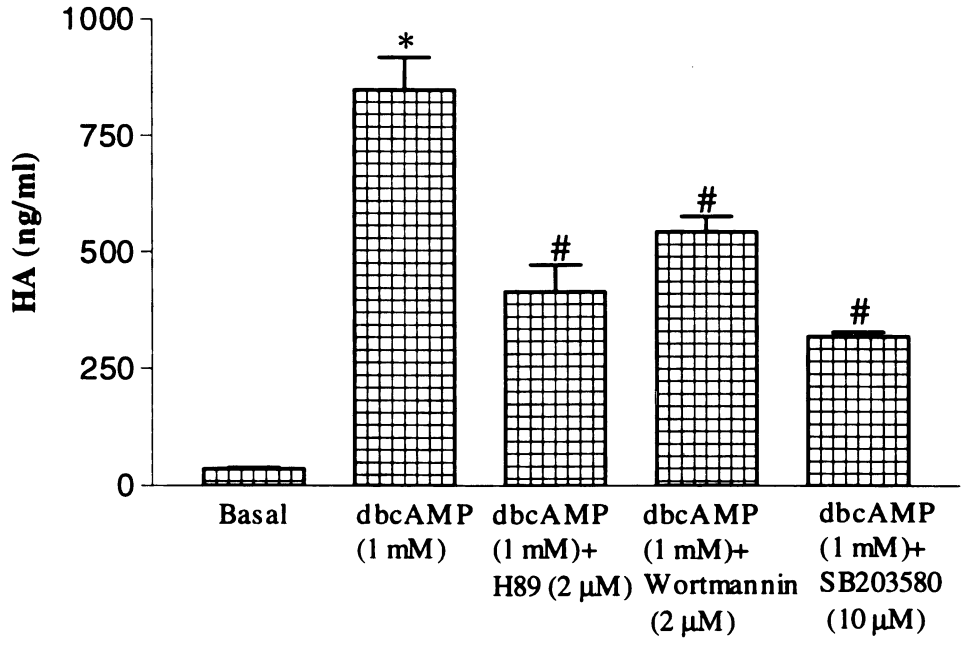



Figure 36. Effect of dibutyryl cAMP (cell permeable cAMP analog), H89 (protein kinase A inhibitor), wortmannin (phosphotidyl inositol 3-kinase inhibitor), and SB203580 (P38 MAPK inhibitor) on hyaluronic acid (HA) release from rat mesangial cells. All the inhibitors significantly inhibited dbcAMP-stimulated hyaluronic acid release from mesangial cells. *P<0.01 compared to basal, #P<0.01 compared to dbcAMP. n=3.

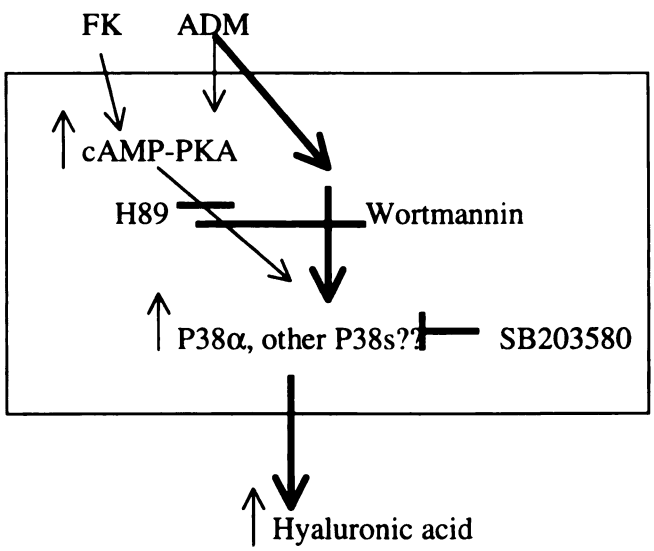



Figure 37. A proposed signaling pathway for ADM-stimulated hyaluronic acid release in rat mesangial cells. Both ADM and forskolin (and dbcAMP) are dependent on a wortmannin- and SB203580-sensitive pathways for HA release in rat mesangial cells, but only forskolin (and dbcAMP) is cAMP-PKA-dependent, while ADM is not. H89 and wortmannin are depicted upstream of P38 based on the kinase activity data from chapters 3 and4.

## 5.4. Discussion:

The present study was initiated to evaluate the role of adrenomedullin and cAMP in mesangial cell matrix production, particularly that of hyaluronic acid. Because glomerular extracellular matrix content plays a key role in the pathophysiology of glomerulonephritis, understanding the mechanisms of the extracellular matrix production is critical for therapeutic intervention. Moreover, understanding the regulation of hyaluronic acid release is important because of its possible role in mesangial cell proliferation in experimental diabetes (Mahadevan *et al.*, 1996). The major aim of this study was to delineate the possible pathways especially, the role of MAPKs in adrenomedullin-, forskolin- and dbcAMP-induced hyaluronic acid release.

Mitogen-activated protein kinase (MAPK) pathway typically consists of a small G-protein like Ras, activating a kinase cascade that ultimately leads to the activation of a MAPK. Three parallel MAPK pathways have been well characterized until now, namely the ERK, JNK and P38 MAPK pathways. In a previous study, we reported that adrenomedullin and forskolin produced a decrease in ERK and an increase in P38 activities, whereas, only adrenomedullin increased JNK activity. We also found that only adrenomedullin-modulated ERK and P38 MAPK activities could be inhibited by H89, a protein kinase A inhibitor. Forskolin, which stimulates cAMP through activation of adenylate cyclase did not stimulate JNK activity; also, H89 (protein kinase A inhibitor) did not consistently inhibit adrenomedullin-stimulated JNK activity indicating a cAMP-independent pathway regulating JNK activity (Parameswaran *et al.*, 1999b). In the present study our results using forskolin and dbcAMP clearly show that an increase in

cAMP levels can induce hyaluronic acid release in rat mesangial cells, and that the inhibition of cAMP-PKA pathway with H89 clearly inhibits FK- and bdcAMP-stimulated HA release. Although adrenomedullin stimulates cAMP levels in rat mesangial cells and causes an increase in protein kinase A activation, H89, at the same concentration that inhibited forskolin- and dbcAMP-stimulated hyaluronic acid release, did not have any effect on adrenomedullin-mediated increase in hyaluronic acid release. We have found that all the other responses (proliferation, apoptosis) mediated by adrenomedullin through cAMP can be completely inhibited by 2 μM H89 (Parameswaran *et al.*, 1999b). In a recent study in rat vascular smooth muscle cells, adrenomedullin was found to elevate MAPK activity and increase proliferation through a cAMP-independent mechanism, although cAMP is the only second messenger so far identified in vascular smooth muscle cells (Iwasaki *et al.*, 1998). Our results indicate that the mechanism of adrenomedullin-stimulated hyaluronic acid release is likely to be predominantly dependent on a cAMP-independent pathway; for example, the pathway stimulated by ßγ-subunit of the G protein or the JNK pathway. Since adrenomedullin receptor is G-protein coupled, activation of adrenomedullin receptor will lead to the release of αs and ßγ-subunit of the Gs protein. Recent studies have shown that the release of ßγ-subunit can lead to a variety of downstream effects like the activation of different MAPK pathways, such as ERK, JNK and P38 in different cell systems (Gutkind, 1998). We postulate that ADM-stimulated HA-release is predominantly dependent on βγ subunit.

There appears to be a cell type specific regulation of hyaluronic acid release. In fibroblast cultures phorbol myritoyl acetate (PMA), platelet-derived growth factor-BB (PDGF-BB) and transforming growth factor-β1 (TGF-β1) stimulated hyaluronic acid production whereas forskolin did not have any effect on hyaluronic acid release (Suzuki

*et al.*, 1995). But in rabbit pericardial cells, prostaglandin $E_2$, forskolin and dbcAMP all stimulated hyaluronic acid release and hyaluronic acid synthase activity and also the prostaglandin $E_2$-mediated effects were shown to be mediated through the activation of protein kinase A (Honda *et al.*, 1993).

SB203580, a selective inhibitor of P38 MAPK, has been shown to be powerful tool for evaluating the role of P38 MAPK in a number of systems (Lee *et al.*, 1994). For example, P38 MAPK has been shown to play a key role in apoptosis and cardiac hypertrophy (Wang *et al.*, 1998). Our results indicate that the adrenomedullin- and cAMP-stimulated hyaluronic acid release in mesangial cells are sensitive to SB203580. The concentration of SB203580 we have used inhibits only P38 MAPK and does not inhibit JNK or ERK (Parameswaran *et al.*, 1999d). To our knowledge this is the first demonstration on the role of P38 MAPK on hyaluronic acid release in rat mesangial cells.

In a previous study, we found that adrenomedullin-stimulated P38 MAPK activity can be inhibited by wortmannin, a selective inhibitor of phosphatidyl inositol 3-kinase (Parameswaran *et al.*, 1999d). It was a surprising finding that- wortmannin not only inhibited adrenomedullin-stimulated hyaluronic acid production, but also that of forskolin and dbcAMP. It remains to be determined if phosphatidyl inositol 3-kinase activity can be stimulated by protein kinase A either directly or indirectly in rat mesangial cells. It is tempting to hypothesise that the stimulation of a wortmannin-sensitive kinase in response to adrenomedullin, forskolin and dbcAMP causes downstream activation of P38 MAPK, which then leads to an increase in hyaluronic acid release (Figure 37). In fibroblast cultures, growth factor-stimulated hyaluronic acid release was not affected by wortmannin (Heldin *et al.*, 1992). This is again probably because of the differences in cell system and hence cell-specific regulation of hyaluronic acid production.

In glomerular cores, Dunlop *et al.* (1996) have found that fibronectin and PDGF can increase hyaluronic acid release from both diabetic and non-diabetic models. They also showed that both were protein kinase C-dependent mechanisms. While fibronectin-stimulated hyaluronic acid release was dependent on prostaglandin production, that of PDGF was not. Also in fibroblast cultures, stimulation of hyaluronic acid release by PDGF and TGFβ1 were dependent on protein kinase C pathway. Whether all the above findings were MAPK pathway-dependent or not, is not known.

In summary, adrenomedullin, forskolin and dbcAMP can stimulate hyaluronic acid release in mesangial cells, and that, the stimulation elicited by these three agents is dependent on P38 MAPK pathway and a wortmannin-sensitive kinase. While ADM-stimulated HA release is not PKA-dependent, FK- and dbcAMP-stimulated HA release is PKA-dependent. These results have significant implications with regard to our understanding of the pathophysiology of mesangial cell proliferation and matrix production. Further studies are necessary to identify the role of JNK or G protein βγ subunit in adrenomedullin-mediated hyaluronic acid production. These can be performed when specific inhibitors are available.

## 6. SUMMARY AND CONCLUSIONS

The major aims of this thesis were to examine the biological effects of adrenomedullin in rat mesangial cells and the mechanisms of these effects. Accordingly, the following hypotheses were tested:

1. a. Adrenomedullin-mediated changes in proliferation, apoptosis and hyaluronic acid release are protein kinase A-dependent.

   b. Adrenomedullin-mediated changes in mitogen-activated protein kinase pathways specifically, ERK2, JNK1 and P38 MAPK pathways are protein kinase-A-dependent.

2. Adrenomedullin-mediated changes in proliferation, apoptosis and hyaluronic acid release are MAPK-dependent. Because of the availability of only a P38 inhibitor, only the role of P38 was tested here.

3. a. Adrenomedullin-mediated changes in proliferation, apoptosis and hyaluronic acid release are PI3-kinase-dependent.

   b. Adrenomedullin-mediated changes in JNK and P38 activities are PI3-kinase-dependent, while that of ERK2 is not.

Following results were obtained when the above hypotheses were tested (Also see Figure 38):

1. Exposure of rat mesangial cells in culture to adrenomedullin increased intracellular cAMP levels. The elevation in cAMP levels in response to ADM was associated with a decrease in ERK2 activity and an increase in P38 and JNK1 activities. ADM-mediated changes in the above intracellular signaling mediators

were accompanied by a decrease in proliferation, an increase in apoptosis and hyaluronic acid release.

a. The decrease in proliferation and increase in apoptosis mediated by exogenous administration of adrenomedullin in rat mesangial cells in culture was dependent on cAMP-PKA pathway, while that of hyaluronic acid release was not PKA-dependent.

b. ADM-mediated decrease in ERK2 and increase in P38 activities were PKA-dependent. ADM-mediated increase in JNK was not consistently dependent on PKA pathway.

2. All the biological responses of ADM namely, changes in proliferation, apoptosis and hyaluronic acid release were mediated through P38 MAPK.

3. a. ADM-mediated decrease in proliferation was not mediated through PI3-kinase/wortmannin-sensitive kinase. ADM-mediated increase in apoptosis and hyaluronic acid release were both PI3-kinase/wortmannin sensitive kinase-dependent.

b. ADM-mediated increase in P38 MAPK activity was dependent on a PI3 kinase/wortmannin-sensitive kinase while that of ERK or JNK was not.

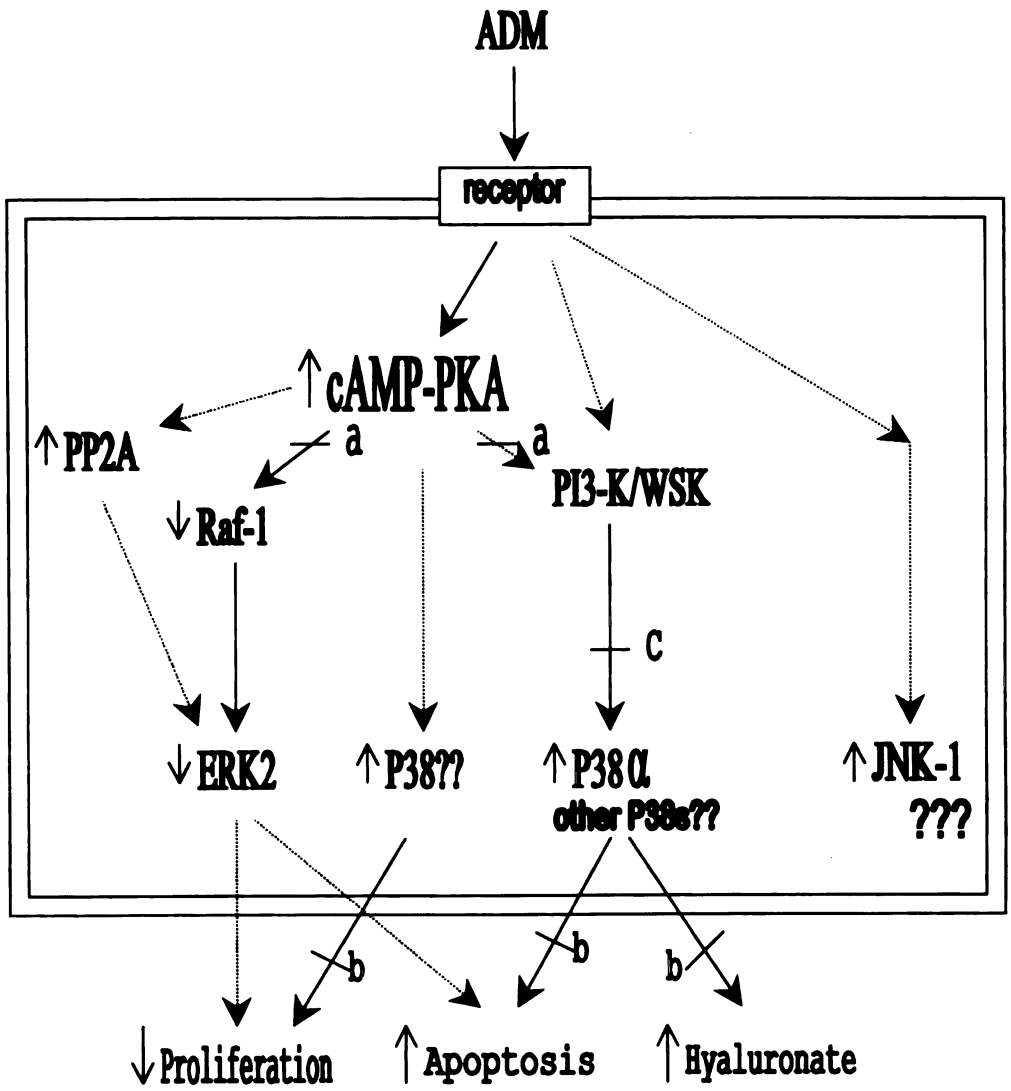
Wortmannin, a PI3-kinase inhibitor, has also helped us delineate the pathways for ADM-mediated proliferation and apoptosis. Because P38 MAPK pathway regulates all biological responses whereas PI3-kinase/wortmannin-sensitive kinase regulates only apoptosis and hyaluronic acid release mediated by ADM, it is possible that there are two P38 MAPK pathways, one sensitive to PI3-kinase/wortmannin-sensitive kinase and the other insensitive to wortmannin.

Figure 38. Proposed model for adrenomedullin mediated signal transduction in cultured rat mesangial cells.

a- H89, a protein kinase A inhibitor, prevented or abolished ADM's effect on ERK and P38α activities. H89 also prevented or abolished ADM's effect on proliferation and apoptosis but not on hyaluronic acid levels.

b- SB203580, a P38-MAPK inhibitor, inhibited ADM's effect on proliferation, apoptosis and hyaluronic acid release. Because of the fact that ADM-stimulated hyaluronic acid release was not inhibited by a protein kinase A inhibitor, it is proposed in this model that ADM might be preferentially acting through a cAMP-independent pathway to stimulate hyaluronic acid release.

c- Wortmannin, a PI3-kinase inhibitor blocked ADM-stimulated P38α activity and also inhibited ADM-induced apoptosis and hyaluronic acid release.


ADM
receptor
↑cAMP-PKA
↑PP2A
a
a
PI3-K/WSK
↓Raf-1
c
↓ERK2
↑P38??
↑P38α
other P38s??
↑JNK-1
???
b
b
b
↓Proliferation
↑Apoptosis
↑Hyaluronate

Although our experiments were primarily designed to examine the effects of ADM, they have also shed some light into the mechanisms of PKA-regulated pathways, particularly to that of hyaluronic acid release. The results suggest that although the PKA pathway may be beneficial in terms of cell turnover, it might also have negative effects with regard to the matrix production, particularly to that of HA release.

ADM modulated cAMP-dependent protein kinase, ERK, JNK and P38 activities. However, only the role of PKA and P38 were examined in this study. The effect of decrease in ERK activity was not directly examined for lack of a direct activator of ERK. Nevertheless, experiments from our laboratory, using indirect means have suggested a role for the decrease in ERK activity. That is, as indicated in the literature review section, a decrease in ERK activity could be mediated through either activation of PP2A or MKP-1. Okadaic acid at low nanomolar concentrations is a very selective inhibitor of PP2A activity. In fact we have shown that, 1.25 nM okadaic acid completely reversed ADM-inhibited ERK activity. Associated with that, okadaic acid also reversed ADM-mediated decrease in proliferation and increase in apoptosis in rat mesangial cells (Parameswaran et al., 1999c). These results suggest that a decrease in ERK activity is also important for proliferation and apoptosis mediated by ADM, in addition to an increase in P38 activity. The role of JNK could not be examined for want of a commercially available inhibitor. It would not at all be surprising if JNK pathway also regulated some or all of these biological responses. Redundancy in signaling systems is a physiologically important phenomenon. In spite of this redundancy, we encounter such diseases like cancer and others, wherein

aberrant proliferation or apoptosis is a key phenomenon because of the activation or inhibition of one crucial signaling molecule. Present research has focussed itself in identifying these critical signaling molecules in order to understand the initiation and progression of diseases like cancer. Not only in cancer, but also in other proliferative diseases like chronic renal disease, activation/inhibition of a signaling molecule may be the cause for the disease at the cellular level. In fact, in our experiments in 5/6 nephrectomized rats, we found P38 and JNK activities to be upregulated at specific time periods before the onset of a morphological change in the kidney (Parameswaran et al., 1999f). Other laboratory groups have shown the activation of one or more of these pathways in proliferative glomerulonephritis and in acute renal failure. Extensive work is necessary in order to understand the importance of the changes in these signaling molecules. But based on our knowledge in cell culture on the role of ERK, P38 and JNK, it wouldn't be surprising to expect an aberrant apoptotic/proliferative/matrix synthesis machinery operative during the time points associated with the changes in these signaling molecules.

**Major Limitations of this study:**

1. The primary limitation of this study is that it is a cell culture study and not in a whole animal model. For practical reasons, a cell culture model was used in this study.
2. Cell culture derived from rat may not necessarily reflect that in human cells. Again for practical reasons in obtaining and maintaining human mesangial cells, both in terms of cost and labor, rat mesangial cell was chosen as a model system.

3. Exogenous administration of ADM brought about all the changes in the responses in rat mesangial cells. Furthermore, the concentrations used may not be reflective of what is actually present physiologically/pathologically in the immediate vicinity of mesangial cells. Until a definite concentration is available both in health and disease in the vicinity of mesangial cells, no argument can be made regarding this.
4. The role of signaling pathways were examined using pharmacological inhibitors which are limited by their specificity or lack there of. Because of this one should be cautious in interpreting the results. Nevertheless, it should also be noted that the concentrations that were used in this study have been used by others and claimed to be specific within that range.

**Positive outcomes of this thesis work:**

The results presented in this thesis work is the first demonstration of a direct link between PKA pathway and cell turnover and matrix production, particularly to that of apoptosis and hyaluronic acid release. Because ADM was able to decrease proliferation and increase apoptosis, it might be beneficial to develop an ADM agonist to test in diseases such as proliferative glomerulonephritis. Obviously, one should also keep in mind the negative effects on HA release, that is, the increase in HA production. This is also the first demonstration of a link between P38 MAPK pathway and cell turnover and matrix production. Future research should focus on the identification of the different P38 MAPKs that selectively regulate the different pathways leading to various biological responses. Obviously research from this thesis cannot be applied directly to a clinical trial. But the results definitely warrant a further

examination of the usefulness of adrenomedullin and PKA pathways in proliferative glomerulonephritis involving mesangial cells. Our results have also opened a number of new avenues for further research both in animal and cell culture models to identify the various mechanisms of proliferation, apoptosis and matrix production. Future research should also focus on the interaction of different cytokines and growth factors in relation to the signaling molecules.

In conclusion, this thesis work suggests an important role for adrenomedullin in mesangial cell turnover and matrix production. This work also demonstrates for the first time the involvement of some crucial components in the signaling system in the regulation of mesangial cell proliferation, apoptosis and hyaluronic acid production.

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