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The renin-angiotensin system and immune function

by

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Abstract

The renin-angiotensin system (RAS) has been implicated in vascular inflammation and atherosclerosis. Angiotensin II via the ATR1 can activate monocytes to produce inflammatory factors and increase adhesion. ATR1 expression is partly regulated by alternate splicing of the ATR1 gene. The RAS may also regulate immune function as part of the stress response: a model is proposed.

ATR1 expression in two monocyte cell lines (U937 and THP-1) compared to a human microvascular endothelial cell line (HMEC1) was investigated. Western blot showed ATR1 protein expression in all cell types. PCR protocols targeted to the terminal protein-coding exon common to all transcript variants confirmed mRNA expression of the ATR1 gene in EC and monocytes. The 5 known splice variants were not identified in monocytes. 5'-RLM RACE was used to identify the 5' untranslated ATR1 exons in monocytes. These data suggest a novel monocyte-specific splice variant, which may function in the cardiovascular disease process.

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List of Symbols, Nomenclature, and Abbreviations

µg: microgram

µL: microlitre

µM: micromoles per litre

5'RLM-RACE: 5-prime RNA ligase mediated-rapid amplification of cDNA ends

5'UTR: 5-prime untranslated region

ACE: angiotensin converting enzyme

ACEi: angiotensin converting enzyme inhibitor

ADH: antidiuretic hormone

Ang II: angiotensin II

ANOVA: analysis of variance

AP-1: activator protein-1

ARB: angiotensin receptor blocker

ATR1: angiotensin II receptor type 1

ATR2: angiotensin II receptor type 2

ATR3: angiotensin II receptor type 3

ATR4: angiotensin II receptor type 4

AUG: adenine uracil guanine (RNA translation start codon)

Ca^{2+}: calcium ions

cDNA: complimentary/cloned deoxyribonucleic acid

CIP: calf intestine alkaline phosphatase

CNS: central nervous system

DNase: deoxyribonuclease

EC: endothelial cell

ECF: extracellular fluid volume

ERK: ectracellular

ET-1: endothelin-1

EtBr: ethidium bromide

FACS: fluorescence activated cell sorting

FAK: focal adhesion kinase

FBS: fetal bovine serum

GAPDH: glyceraldenhyde phosphate dehydrogenase

GC-rich: guanine cytosine-rich

H_2O_2: hydrogen peroxide

HK-2: immortalized kidney proximal tubule epithelial cell line human kidney-2

HMEC1: human microvascular endothelial cell line-1

HPA: hypothalamic pituitary adrenal

ICAM-1: intracellular adhesion molecule-1

IFNγ: interferon gamma

IL-1: interleukin-1

IL-12: interleukin-12

IL-6: interleukin-6

IL-8: interleukin-8

IP-1: inducible protein-1

Iκβ: inhibitory protein kappa beta

JAK: Janus kinase

JNK: c-Jun NH_2-terminal kinase

KC: keratinocyte chemoattractant

MAPK: mitogen activated protein kinase

MCP-1: monocyte chemotactic protein-1

MCP-2: monocyte chemotactic protein-2

MIP-1α: macrophage inflammatory protein-1 alpha

ml: millilitre

mM: millimoles per litre

mRNA: messenger ribonucleic acid

N: sample number

NCBI: National Center for Biotechnology Information

NF-κB: nuclear factor-kappa-light-chain-enhancer of activated B cells

ng: nanogram

NK cells: natural killer cells

NO: nitric oxide

NOS-2: nitric oxide synthase-2

OPN: osteopontin

ORF: open reading frame

PCR: polymerase chain reaction

PKB: protein kinase B

PKC: protein kinase C

PLC: phospholipase C

pmol: picomoles

PNS: parasympathetic nervous system

PRA: plasma renin activity

PRR: proreninreceptor

PTK: protein tyrosine kinase

Pyk2: proline-rich tyrosine kinase 2

qPCR: real-time, quantitative polymerase chain reaction

RANTES: regulated upon activation, normal T-cell expressed and secreted (also known as chemokine (C-C motif) ligand 5 or CCL5)

RAS: renin angiotensin system

RNA: ribonucleic acid

RNase: ribonuclease

ROS: reactive oxygen species

RT: reverse transcription

RT-PCR: reverse transcripton-polymerase chain reaction

SE: standard error

SNS: sympathetic nervous system

STAT: signal transducers and activators of transcription

STAT1: signal transducers and activators of transcription 1

TAP: tobacco acid phosphatase

TE Buffer: Tris and ethylenediaminetetraacetic acid (EDTA) buffer

TGF-1β: transforming growth factor-1 beta

TGF-1β: transforming growth factor-1beta

THP-1: human acute monocytic leukemia cell line

TNFα: tumor necrosis factor alpha

U: unit

U937: human leukemic monocyte lymphoma cell line

UV: ultraviolet

VCAM-1: vascular cell adhesion molecule-1

VEGF: vascular endothelial cell growth factor

VSMC: vascular smooth muscle cell

ΔCt: corrected target gene expression that is the difference in cycle number at which the target gene and internal reference gene are detected

ΔΔCt: relative expression of corrected target gene expression compared to a control or other reference sample: difference between the corrected gene expressions of two samples

I. Introduction

1. Background

Conventionally, the renin-angiotensin system (RAS) acts in the preservation of extracellular fluid (ECF) volume and blood pressure through receptor-mediated activity of its major circulating effector, angiotensin II (Ang II). The classical pathway of Ang II generation is as follows. Renin catalyzes the breakdown of its major target angiotensinogen into angiotensin I (Ang I), which is the rate limiting step of Ang II synthesis. Ang I is further cleaved into circulating Ang II by tissue and plasma angiotensin converting enzyme (ACE), which is found ubiquitously throughout the body, or chymase (105). There are various other angiotensin breakdown products includin Ang III, Ang IV, Ang-(1-7) and Ang-(1-9) (105), with separate functions, though the precise physiological significance of these peptides has not been entirely defined.

Renin is derived from its precursor protein, prorenin, both of which are reported to have angiotensin-independent hormonal activity via the widely expressed prorenin receptor (140). Prorenin is secreted from kidney juxtaglomerular cells constitutively (47) while the secretion of mature renin from juxtglomerular cells can be regulated acutely. Decreased afferent arteriolar blood pressure, as recognized by intra-renal barroreceptors, causes mature renin secretion to increase (207). Sympathetic stimulation of the juxtaglomerular cells and reduced tubular sodium and chloride concentrations across the macula densa also cause an increase in the release of mature renin from the juxtaglomerular cells (207). A number of less influential factors also regulate renin release including levels of circulating catecholamines, porstaglandins, antidiuretic hormone (ADH), and angiotensin II (207).

Ang II increase blood pressure directly by stimulating vasoconstriction of arteriolar vascular smooth muscle cells in the periphery (71). Ang II increases blood volume by stimulating tubular sodium and chloride reabsorption, potassium

excretion and hence water retention directly and via the stimulation of aldosterone secretion from the adrenal cortex (71). Ang II also stimulates ADH secretion from the posterior pituitary which increases water in the renal collecting duct (71). These classical, hemodynamic functions of circulating Ang II and the RAS are well accepted. However, it is becoming increasingly apparent that the RAS has other regulatory roles that function independently or in conjunction with this regulation of hemodynamic homeostasis.

It has been discovered that local, tissue renin-angiotensin (RA) systems, which generate their own Ang II, exist independently of the circulating RAS in most tissues throughout the body. These local systems have all the necessary RAS components, including angiotensin receptors, to function autonomously (151). A local RAS has been described in the central nervous system (CNS) and peripheral nervous system, heart, vasculature, kidney, placenta, adrenal glands, pancreas, gonads, and various other endocrine glands, the spleen, adipose tissue, skin, and a variety of immune cells (151). It is suggested that local RAS function to amplify the actions of circulating Ang II (11). However, local RAS have also been shown to mediate functions independent of circulating Ang II. For example, locally generated Ang II in the myocardium is responsible for regulating cardiac myocyte growth and proliferation (151). In the CNS, local RAS is partly responsible for regulating drinking and food intake, maintaining the blood-brain barrier, induction of genes involved in neuroplasticity, as well as blood pressure control in the cerebral vasculature (151). Local RAS function in the male reproductive tract is thought to be involved in fertility (151). The local RAS have also been implicated in various pathological conditions including hypertension and congestive heart failure (11).

More recently, the RAS has been implicated in affecting the function of leukocytes, particularly in the context of atherosclerosis. By action on cells of the vascular wall as well as on leukocytes directly, Ang II appears to contribute to the processes of vascular inflammation and atherogenesis. Though some of the

mechanisms of the interaction between the RAS and inflammatory processes have been detailed, much is still unknown about the function of the RAS in leukocytes. Furthermore, the activity of the RAS with respect to immune function has largely been investigated only with respect to the pathophysiology of inflammatory processes. The physiological relevance of RAS activity in the immune system has not been investigated.

It is well recognized that there is an increase in renin-angiotensin system (RAS) activity during the stress response, which likely functions to maintain threatened fluid and sodium homeostasis, among mediating other stress-induced physiological adjustments. As such, the RAS should be considered a central mediator of the acute stress response.

There are marked changes in immune function during the stress response. Both glucocorticoids and catecholamines appear to play a role in controlling these immune changes. However, the entire mechanism behind stress-induced immune alterations is not yet fully understood. Given the evident increase in its activity during the stress response, the RAS may also play an important role in regulating stress-induced modulation of immune function that occurs normally as a non-pathological physiological process. However, prolonged or recurrent activation of the RAS by chronic or recurrent stress could lead to vascular inflammation and atherosclerosis.

2. Objectives of thesis

Outside of its apparent role in the pathophysiology of vascular inflammation and atherosclerosis, little is understood of the physiological relevance of the RAS in immune cells. As such, in a literature review, this thesis explores the potential physiological role for the expression of RAS components in immune cells in the stress response. Hypothesis: RAS mediates immune cell function as part of the stress response.

The activity of the RAS in immune cells is a new area of research and much attention has been paid to Ang II signaling in atherosclerosis. Monocytes are critically involved in the atherogenic process and much is unknown of the activity of Ang II on monocytes. This thesis explores one aspect of RAS function in monocytes: the expression and alternate splicing of the angiotensin II type 1 receptor gene. Hypothesis: monocytes express a novel ATR1 mRNA splice variant.

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II. Literature Review[1]

1. Ang II receptors

The actions of circulating Ang II are mediated by cell surface angiotensin receptors. Three Ang II receptor subtypes have been identified in humans: angiotensin II receptors type 1 (ATR1), type 2 (ATR2), and type 4 (ATR4) (48, 214). ATR1 and ATR2 are the predominant subtypes and ATR1 mediates most of the known functions of Ang II though ATR2 mediates some important functions as well (214). The exact role of ATR4 has not yet been defined though it has been implicated in memory consolidation and cellular proliferation (214). The ATR1 is a 41 kDa unglycosylated), 7 transmembrane spanning G-protein coupled receptor that is widely expressed among various tissues and cell types. The pro-inflammatory (and pathological) functions of Ang II are thought to be mediated mostly through the ATR1 (35, 190).

The expression of Ang II receptors has been identified in most human tissues (9) and can be stimulated by circulating and locally generated Ang II. The widespread expression of Ang II receptors alone suggests that the RAS functions outside of blood pressure and volume control. Specifically, the expression of angiotensin receptors in immune cells has garnered much attention in recent years because of the evident involvement of the RAS in vascular inflammation and the atherosclerotic process (20, 35, 190). The role of monocytes/macrophages has particular relevance in the atherosclerotic process and many studies have begun to elucidate the mechanisms behind RAS activity in these specific cell types.

2. ATR1 expression in immune competent cells

Several studies have demonstrated the expression of angiotensin receptors on various leukocyte subsets indicating that Ang II may have some effect on immune

[1] Part of this literature review has been submitted for publication as a scholarly review in the American Journal of Physiology: Renal Physiology under the title 'The renin-angiotensin system as a central mediator in the immune response to stress' (M. Groeschel and B. Braam)

cells directly. Shimada and Yazaki (183) were the first to identify binding sites for Ang II in unfractionated human mononuclear cells by autoradiography. Ang II binding to leukocytes was also later demonstrated in a variety of studies (117). Most of the pro-infammatory effects of Ang II are thought to be mediated through the ATR1 (ATR1a in rodents), though some have been attributed to the ATR2 (20). Activation of ATR2 is also thought to oppose many of the physiological effects of ATR1 activation(151, 190). The ATR1 has been identified in a wide range of normally functioning leukocyte subsets from healthy animals and subjects, from hypertensive patients and in cultured cell lines. Table 1 summarizes the findings of ATR1 expression in leukocyte subsets at various cellular levels. ATR1 mRNA expression has been identified in monocytes/macrophages (79, 138, 145, 160, 189, 196), granulocytes (118, 160), neutrophils (90), T lymphocytes (138, 160, 165), and B lymphocytes (138, 160, 165). Total cellular ATR1 protein (isolated from whole cell lysates) has been identified in monocytes/macrophages (1, 95, 179), T-lymphocytes (95), and natural killer (NK) cells (95). Cell surface expression of the ATR1 has been shown in monocytes/macrophages (90, 135, 160, 179), granulocytes (118, 160), neutrophils (90), T-lymphocytes (160), and B-lymphocytes (160). Rasini *et al.* (160) showed that ATR1 was expressed at the mRNA level and a the cell surface protein level to varying degrees in freshly isolated human polymorphonuclear leukocytes, monocytes, B-lymphocytes, and T-lymphocytes by RT-PCR and flow cytometric analysis.

3. The ATR1 gene and alternate splicing

The human ATR1 gene has been mapped to chromosome 3q (44), spans over 60 kb, and is composed of at least 4 exons(64). Exons 1, 2, and 3, make up the 5'untranslated region (5'UTR) and exon 4 harbours the entire open reading frame (ORF) and protein coding sequence of the ATR1 (64). There are at least 4 well described mRNA transcript splice variants, which differ only by way of inclusion or exclusion of exons 2 and 3 (63). ATR1 splice variant 1 contains exons 1 and 4, variant 2 contains exons 1, 2, and 4, variant 3 contains exons 1, 3, and 4, and variant 4 contains exons 1, 2, 3, and 4 (63) (Figure 1). Using a human liver cDNA

Table 1. Studies that have documented identification of ATR1 mRNA, total cellular ATR1 protein, and cell surface ATR1 protein in leukocyte subsets from various sources.

Cell Type	mRNA (RT-PCR or qPCR)	Total cellular or cytosolic protein (western blotting)	Surface protein (flow cytometry or immunohistochemistry)
Monocytes/ macrophages	Human primary (79, 160, 189) Mouse primary (138) Human cell line THP-1 (122, 196)	Human primary (1, 95) Human cell line THP-1 (179)	Human primary (135, 160) Human cell line THP-1 (179) Rat primary (90)
T-lymphocytes	Human primary (160, 165) Mouse primary (138)	Human primary (95)	Human primary (160)
B-lymphocytes	Human primary (160, 165) Mouse primary (138)		Human primary (160)
Granulocytes	Human primary (118, 160)		Human primary (118, 160)
Neutrophils	Rat primary (90)		Rat primary (90)
NK cells		Human primary (95)	

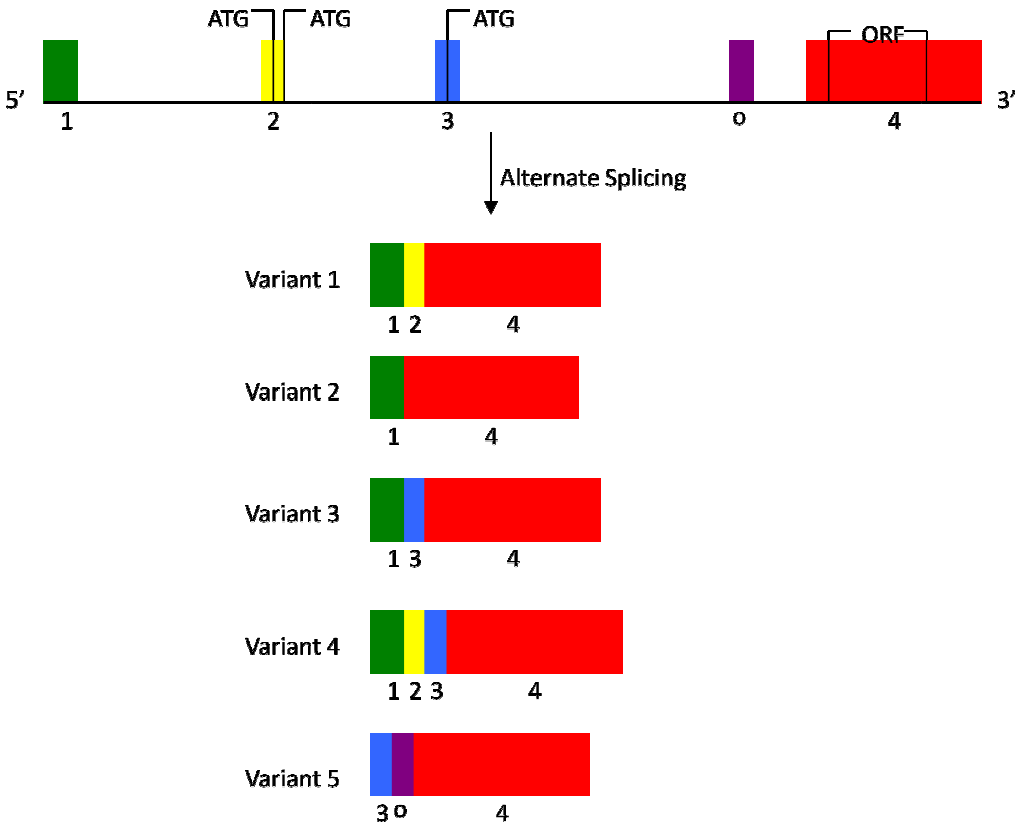

Figure 1. Schematic representation of the human ATR1 gene and the potential ATR1 mRNA transcript variants. Variants are generated from alternate splicing of the immature transcript. ATGs denote the beginnings open reading frames upstream of the open reading frame that translates to the functional ATR1 protein (ORF) contained entirely within the terminal exon (exon 4). Transcript variants 1 through 4 have been well characterized however the existence of other variants that contain exon o, such as variant 5, is still disputed. Translatability of the variants is impacted by presence/absence of exons 1 and 2. The start codon in exon 3 is in frame with the downstream ATR1 coding ORF and imparts an N-terminal extension of 32 amino acids to the ATR1 protein.

library, Guo *et al.* [32] demonstrated the existence of an additional ATR1 mRNA splice variant that harboured another exon (exon o) that encoded 155 base pairs of the 5'UTR sequence (Figure 1). However, mRNA transcripts harbouring this exon are often undetected and appear to be extremely rare (63). Additional variants have also been described including NCBI catalogued ATR1 transcript variant 5 (Figure 1), which contains exons 3, o, and 4, however their full nature has not yet been determined.

It has been demonstrated that ATR1 mRNA splice variants 1 through 4 are functionally distinct. All 4 of the well described ATR1 transcript variants contain exon 1, which was demonstrated to have both inhibitory and enhancing effects on ATR1 expression (43, 63). The sequence of exon 1 is GC-rich and may form stable stem-loop structures that have the potential to inhibit translation of the ATR1 transcripts. It has also been postulated that exon 1, acting at a transcriptional level, enhances promoter function of the ATR1 gene. At a translational level, it is thought that exon 1 contains a site for internal ribosome entry thereby allowing translation to initiate via a cap-independent mechanism (63). Splice variants containing exon 2 are translated at a reduced rate (43, 119, 209). Exon 2 contains 2 upstream AUG start codons the first of which is a consensus Kozak start site, and both of which are out of frame with the downstream protein coding ORF in exon 4 (63). It is thought that these upstream start codons could initiate a futile translation that prevents the ribosome from reaching the ATR1 translational start site in exon 4 (64). Splice variants containing exon 3 are rare and have the capacity to translate an additional 32 N-terminal amino acids onto the translational start site in exon 4 (64) – exon 3 contains an AUG start codon that is in frame with the downstream ORF in exon 4 (43, 63, 209). This long ATR1 isoform has a reduced affinity for Ang II (119).

A cell's responsiveness to Ang II can be controlled at a number of levels. The level of protein expression can be controlled by altering rates of ATR1 gene transcription. Surface expression of the ATR1 protein can be modulated by

controlling protein trafficking, reuptake, and degradation. Rates of ATR1 translation and protein synthesis can be controlled by modulating mRNA degradation. In addition, alternate splicing of the ATR1 transcripts may allow for a fine-tuning of Ang II responsiveness depending on which ATR1 splice variants are predominantly expressed in a given cell (63). That is, Ang II responsiveness can be fine tuned by regulating the relative levels of expression of the different ATR1 transcript variants given the differences in rates of translation of ATR1 between the transcript variants and the translation of the long ATR1 isoform (with reduced ATR1 affinity and hence reduced Ang II signaling) versus the short ATR1 isoform.

It has been shown by various PCR studies that the relative expression of the ATR1 splice variants is regulated in a tissue specific manner (43, 63, 119, 120). Each splice variant was found to be present in different amounts relative to the others within a given tissue. As such, each tissue may modulate alternate splicing of the ATR1 gene differently allowing the development subtle differences in Ang II responsiveness between tissues and cell types. To date, relative tissue specific expression of the ATR1 transcript variants has only been examined in the adrenal gland, aorta, heart, kidney, liver, and placenta(63). Ang II signalling is involved in many physiological and pathophysiological processes and virtually all tissues and cell types express the ATR1. As such, alternate splicing of the ATR1 mRNA may have some role to play in tissue and cell type specific responsiveness to Ang II.

Alternative splicing of the ATR1 gene in leukocytes, and specifically in monocytes, has not been examined to date. The studies that have confirmed ATR1 mRNA expression in monocytes by PCR have used primers targeted only to the terminal protein-coding exon (exon 4) common to all transcript variants (Table 2). The responsiveness of monocytes to Ang II signalling may be dependent upon cell-specific alternative splicing of the ATR1 gene, which may have a role in the pathogenesis of atherosclerosis under certain conditions and in susceptible individuals. Additionally, pro-inflammatory conditions, including hypertension

Table 2. Primer sequences and amplicon locations from studies that have identified expression of ATR1 mRNA in monocytes/macrophages by conventional RT-PCR.

Reference	Monocyte/ macrophage Source	Primer Sequence (5'-3')	Sense Primer Position	NCBI Reference Sequence	Exon #
(138)	Mouse primary	sGCATCATCTTTGTGGTGGG aATCAGCACATCCAGGAATG	376	rAGTR1a NM_177322.2	3 (terminal)
(145)	Human cell line THP-1	sGGCCAGTGTTTTTCTTTTGAATTTAGCAC aTGAACAATAGCCAGGTATCGATCAATGC	632	hAGTR1 v4 NM_031850.2	4 (terminal)
(196)	Human cell line THP-1	sGATGATTGTCCCAAAGCTGG aTAGGTAATTGCCAAAGGGCC	492	hAGTR1 v4 NM_031850.2	4 (terminal)
(79)	Human primary	sCGAACATGTCACTCAACCTC aTTATTGATTCACTCTTCTAC	1534	hAGTR1 v4 NM_031850.2	4 (terminal)
(189)	Human primary	sGCCAGTGTTTTTCTTTTGAATTTAGCAC aTGAACAATAGCCAGGTATCGAATCA	633	hAGTR1 v4 NM_031850.2	4 (terminal)
(160)	Human primary	sGATGATTGTCCCAAAGCTGG aTAGGTAATTGCCAAAGGGCC	492	hAGTR1 v4 NM_031850.2	4 (terminal)

Sense (forward) primer sequences are presented above the antisense (reverse) primer sequences; s = sense; a = antisense. All studies used primers targeted to the terminal protein-coding exon common to all ATR1 mRNA splice variants. In the human ATR1 transcripts, exon '4' is the terminal exon. The murine ATR1 gene only has 3 exons, hence exon '3' is the terminal exon.

and chronic kidney disease, may pathologically alter the pattern of alternate splicing in monocytes such that Ang II signaling is enhanced.

4. Ang II is a pro-inflammatory and pro-atherosclerotic molecule

As has already been mentioned, aside from its hemodynamic effects, Ang II has recently been implicated as an inflammatory mediator and much research has focused on its involvement in the pathophysiologies of vascular inflammation and atherosclerosis (reviews (20, 35, 190)). The pro-inflammatory and pro-atherosclerotic functions of Ang II are mostly mediated through the ATR1 (35, 190). It has been shown that knock-out of the AT1Ra gene in apolipoprotein E (apo E) deficient mice on a high cholesterol diet inhibited the formation (210) and progression (65) of atherosclerotic plaques perhaps by reducing vascular oxidative stress (210), endothelial cell dysfunction (210), and matrix metalloproteinase expression and apoptosis within atherosclerotic plaques (65). Interestingly, disruption of the AT2R gene in apo E deficient mice resulted in an exaggeration of atherosclerosis (91). These studies strongly support a role for the ATR1 in the pathogenesis of atherosclerosis. One study generated chimeric apo E-deficient mice lacking ATR1a in cells of the bone marrow (70). It was demonstrated that in mice lacking bone marrow ATR1a, aortic atherosclerotic lesion numbers were significantly decreased after infusion with Ang II compared to chimeric mice with ATR1a in the bone marrow but with or without ATR1a in the vasculature (70). The study also reported more stable plaques in bone marrow ATR1a-deficient mice with fewer bone marrow cells, that maintained characteristics of macrophages, within the plaques (70) indicating that, specifically, bone marrow cells containing the ATR1a are critically involved in the pathogenesis of atherosclerosis.

Atherogensis requires that leukocytes traffic to and infiltrate the vascular wall at specific sites. This process involves leukocyte margination (an attraction to and rolling along the vessel wall and adhesion to the endothelium through leukocyte-endothelial cell interactions) as well as an eventual transmigration (or diapedesis).

A vast number of *in vivo* studies have examined the effects of Ang II on each step of leukocyte margination, largely as means of supporting the hypothesis that Ang II is critically involved in vascular inflammation and atherogenesis. Studies using rat models have shown that increased levels of circulating Ang II via ATR1 activation increase leukocyte rolling (8, 154), adhesion (8, 154), and transmigration across vessel walls and infiltration of tissues (8, 121, 122, 127, 154, 219). Studies have also shown that treatment of rats with ATR1 antagonists (32, 45, 132, 163, 218, 220) and ATR1 knockout (153, 195) decreases leukocyte infiltration. Specifically, Ang II has been shown to enhance the margination of monocytes/macrophages (45, 121, 122, 127, 132, 195, 219, 220), neutrophils (80, 122, 133, 195), and T-cells (42, 78), though other leukocytes may be similarly affected.

5. Ang II induces leukocyte margination and transmigration by action on ECs and VSMCs

The action of Ang II on cells of the vascular wall, including ECs and VSMCs, may play a large part in bringing about Ang II-stimulated leukocyte margination and diapedesis and there appear to be three mechanisms at work. In general, Ang II acts on ECs and VSMCs to enhance the expression of chemokines, adhesion molecules, and cytokines (mediated by activation of the transcription factors NF-κB and AP-1 (32, 85, 112, 156, 168, 169, 190)), and to increase vascular permeability (Figure 2).

It has been shown that Ang II via ATR1 directly stimulates ECs to increase expression and release of various chemokines including MCP-1, MIP-1α, IL-8, IP-1, KC, RANTES (190), which serve as chemoattractant agents that bring circulating leukocytes close to the vessel wall being stimulated by Ang II. Increased chemotaxis to the vascular wall would allow more chances for contact between leukocytes and ECs, and hence increase the probability of transmigration.

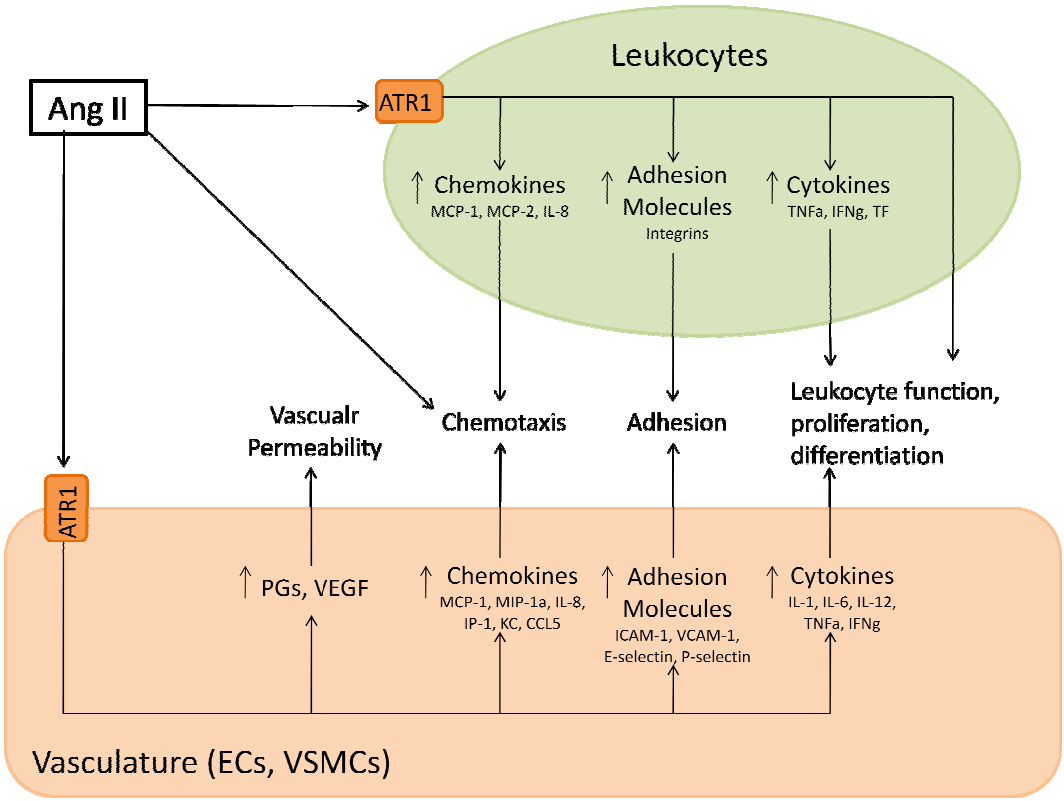

Figure 2. Increased circulating Ang II during the stress response induces leukocyte redistribution and enhances leukocyte function. Ang II induces leukocyte infiltration and redistribution by increasing vascular permeability, leukocyte adhesion to the vessel wall, and leukocyte chemotaxis. Vascular permeability increases in response to increased vascular production of prostaglandins (PGs) and vascular endothelial cell growth factor (VEGF). Ang II induced leukocyte adhesion results from increased expression of adhesion molecules and their ligands on both endothelial cells and leukocytes. Chemokines released from leukocytes and the vasculature serve as chemotactic signals for leukocytes. Also, Ang II is itself a chemotactic signal for leukocytes. In addition to stimulating leukocyte redistribution, Ang II directly enhances leukocyte function (phagocytosis, cytokine production), proliferation, and differentiation. In addition, Ang II stimulates leukocytes and endothelial cells and vascular smooth muscle cells to produce cytokines that also activate leukocytes.

It has also been shown that Ang II increases the expression of adhesion molecules including vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), and various selectins in the general vasculature (127, 190, 198, 219). Though changes in expression of adhesion molecules does not acutely regulate leukocyte adhesiveness, as does changes in conformation of the adhesion molecules, the action of Ang II may represent a more chronic form of control over leukocyte adhesion. I*n vitro*, Ang II via ATR1 directly enhances the expression of specific adhesion molecules in ECs including E-selectin (73), P-selectin (186), ICAM-1 (186), and VCAM-1 (156, 186). By increasing the expression of these adhesion molecules, Ang II effectively increases the number and duration of adhesive interactions between leukocytes and the vascular wall, again, giving more chances for leukocyte transmigration.

In addition to enhancing leukocyte adhesion and chemotaxis by action on ECs and VSMCs, Ang II has been shown to increase vascular permeability by increasing the expression and release of prostaglandins and vascular endothelial cell growth factor (VEGF) in the vascular endothelium (190). By increasing vascular permeability, Ang II allows for greater ease of passage of leukocytes across the vascular wall.

In summary, Ang II stimulates cells of the vascular wall to release chemokines that attract leukocytes to the vessel wall, adhesion molecules that allow leukocytes to adhere to the vessel wall, and to increase vascular permeability allowing leukocytes to pass across the vessel wall more easily. All three mechanisms likely contribute to increased margination and diapedesis seen with Ang II infusion *in vivo.* Importantly, the action of Ang II on ECs also stimulates the release of various cytokines including IL-1, IL-6, IL-12, TNFα, IFNγ along with other factors such as ET-1, OPN, and TGF-1 (190). These factors serve to activate and enhance the function of circulating and tissue-resident leukocytes, which may also contribute to the increase in margination and diapedesis seen with Ang II infusion *in vivo*. Cytokine-stimulated activation likely increases the

expression of adhesion molecule ligands on the leukocyte surface allowing for increased adhesion to the vascular wall.

6. Specific actions of Ang II on different immune cell types

It is also becoming clear that Ang II acts on leukocytes directly to stimulate enhanced leukocyte activity and trafficking to and infiltration of the vascular wall, which may fit into a model for RAS-driven atherogenesis. Angiotensin receptors have been identified on many of the leukocyte subsets (Table 1). It follows that Ang II appears to affect the activity these same leukocyte subsets, including lymphocytes, granulocytes and monocytes/macrophages. Of all the leukocytes subsets, monocytes/macrophages appear to be the major leukocyte players in atherogenesis, however other leukocytes types, including lymphocytes and granulocytes, appear to have roles, though less important, in atherogenesis as well (182, 203).

a. Lymphocytes

Lymphocytes are categorized into three subsets: NK cells, T-cells, and B-cells. NK cells function in the defense against tumor and virally infected cells as part of the innate immune response. There are a variety of T-cell types that all function in cell-mediated immunity as part of the adaptive immune response and B-cells function namely in the production of immunoglobulins involved in humoral immune defense of the adaptive immune response. Evidence continues to accumulate that indicates that the activity of lymphocytes is also altered by Ang II directly. Crowley *et al.* (42) demonstrated that *in vitro* exposure of freshly isolated mouse splenic T cells to Ang II resulted in cytoskeletal actin rearrangements (specifically increased formation of F actin), an effect that was dependent on Rho kinase and was inhibited with ATR1 antagonism. Such cytoskeletal rearrangements are thought to be one of the hallmarks of T-cell activation, specifically important to the formation of synapses between T cells and antigen presenting cells (42). In addition, Sagawa *et al.* (171) showed that *in vivo* treatment of mice with an ATR1 antagonist suppressed T cell production of IFNγ

and Cheng *et al.* (34) demonstrated that ATR1 antagonism inhibits the production of TNFα and IFNγ in activated T cells.

Ang II has also been shown to augment lymphocyte chemotaxis. Guzik *et al.* (78) showed that T cell expression of CC chemokine receptor 5, which activates upon binding of certain cytokines such as RANTES and MIP-1α/β, was increased after mice were injected with Ang II. Increased expression of CC chemokine receptor 5 would sensitize lymphocytes to chemotactic signals thereby enhancing the speed with which lymphocytes traffic to sites of injury.

Lymphocyte proliferation is an important part of the adaptive immune response, particularly where T and B memory cells come across previously encountered antigens that trigger rapid memory cell expansion. Nataraj *et al.* (138) demonstrated that Ang II stimulates the proliferation of cell suspensions of splenic lymphocytes isolated from ATR1 wild type mice in a dose-dependent manner, an effect not seen in lymphocytes from ATR1 knockout mice and reduced by treatments with losartan and the ACE inhibitor, enalapril. The effects of Ang II on splenocyte proliferation via ATR1 were thought to be mediated by a calcineurin-dependent pathway, as Ang II sitmulated lymphocyte proliferation was inhibited by cyclosporine, a calcineurin inhibitor (138). However, cyclosporine also inhibits lymphokine production and interleukin release, which may have had an effect on Ang II stimulated proliferation.

While it is suggested in the study by Nataraj *et al.* (138) that Ang II stimulates lymphocyte proliferation directly, more studies suggest that Ang II augments the proliferation of lymphocytes that is stimulated by other signals. It has been shown that Ang II augments the proliferation of a nephritogenic T-cell clone, an effect mediated by ATR1 (202), and the mitogen and anti-CD3 stimulated proliferation of mixed splenic lymphocytes (138) and T and NK cells specifically (95). *In vivo* treatment of mice with olmesartan, an ATR1 specific antagonist, has also been

shown to suppress ovalbumin-stimulated T-lymphocyte proliferation in mice (171).

While Ang II appears to have a positive effect on lymphocyte proliferation on its own, Ang II may function to enhance the sensitivity of lymphocytes to infectious antigens and/or certain cytokines such that lymphocyte activation and memory cell expansion occurs more rapidly during the stress response. No studies have examined the effects of Ang II on B cell function and immunoglobulin production, however the presence of angiotensin receptors, ATR1 specifically, does point towards some role for Ang II in modulating B cell function. It is likely that Ang II activates B cells in a way that is similar to its activating effects on T-lymphocytes and it has been shown that stress enhances both the cellular and hummoral immune response to various immunological challenges. Increases in circulating Ang II during the stress response may well directly activate NK cells and B and T lymphocytes and/or augment the activation of T and B lymphocytes in response to other immune enhancing signals, in effect increasing the sensitivity and preparedness of the adaptive immune response to imminent immune challenges.

b. Granulocytes

There are three types of granulocytes or polymorphonuclear leukocytes: neutrophils, basophils, and eosinophils. Neutrophils make up the greatest proportion of the granulocytes and are also the most abundant type of all of the circulating leukocytes. Neutrophils are phagocytes that also release various proteins (peroxidases, proteases, etc) by degranulation that act on both host and foreign cells. In response to acute inflammation and the associated chemotactic signals, neutrophils are known to acutely traffic to affected tissue sites. Eosinophils and basophils are far less common and function namely to secrete similar proteins by degranulation. Like the other immune cells, granulocytes, neutrophils in particular, have been shown to respond to Ang II stimulation.

The generation of reactive oxygen species (ROS) is strongly associated with immune cell activation. It has been demonstrated that Ang II stimulates NADPH oxidase dependent (81) ROS production in neutrophils (149, 157), which is mediated by p38 MAP kinase, ERK (81, 157), and cytosolic phospholipase A2 (81). Ang II has also been shown to prevent the synthesis of heme-oxygenase-1, an antioxidant enzyme, in human neutrophils (6). Additionally, Dandona *et al.* (46) showed that ROS generation by neutrophils isolated from human subjects decreased significantly after subjects were treated with the ATR1 antagonist, valsartan.

ROS are thought to function as part of certain intracellular pro-inflammatory signaling pathways and have been linked to activation of NF-kβ. Ang II has been shown to increase cytosolic calcium ion levels, synthesis and activity of calcineurin, and NF-kβ binding activity in cultured human neutrophils (157). Dandona *et al.* (46) found that NF-kβ DNA binding activity in the neutrophils from ATR1 antagonist-treated subjects decreased and that expression of the protein IkB, which binds and inhibits nuclear translocation of NF-kβ, increased. Through binding of ATR1 on neutrophils, Ang II stimulates the production of ROS that may function to activate NF-kβ, though NF-kβ may activate through other Ang II-stimulated mechanisms, and enhance the expression of pro-inflammatory factors.

One of the functional consequences of Ang II stimulation of granulocytes appears to be chemotactic and/or chemokinetic in nature. Eferink and de Koster (62) characterized the chemotactic response of neutrophils to various concentrations of Ang II *in vitro.* It was found that a concentration of 10^{-10} M stimulated migration of neutrophils in a calcium-dependant way, while migration fell sharply with higher concentrations. As such, Ang II may enhance or inhibit the migration of neutrophils directly depending on the concentration of Ang II achieved in circulation. Eferink and de Koster (62) also found that Ang II concentrations above 10^{-10} M inhibited migration of neutrophils in response to IL-8, suggesting

that Ang II has a mediating role in the control of cytokine stimulated neutrophil chemotaxis. Ang II may also stimulate granulocyte adhesion to the vessel wall as candesartan, an ATR1 specific antagonist, treatment of neutrophils isolated from the blood of patients after an ischemic stroke has been shown to inhibit their adhesion to cultured vascular endothelial cells (80). As such, increased Ang II signaling in neutrophils may function as a chemotactic signal and to enhance the adhesion of neutrophils to the vessel wall thereby increasing the efficiency with which neutrophils traffic to sites of tissue damage or infection during the stress response.

The effects of Ang II on granulocyte phagocytosis has not been examined to date, though if Ang II signaling mechanisms operate in the same manner as they do with monocytes and lymphocytes it is feasible to postulate that Ang II may enhance the phagocytic activity of these cells. The effects of Ang II stimulation on granulocyte degranulation have also not been examined to date though Miselis *et al.* (129) have demonstrated that inhibition of ACE in freshly isolated unstimulated and opsonised zymosan stimulated neutrophils increased myeloperoxidase and lysozyme secretion by degranulation, suggesting that Ang II may inhibit granulocyte degranulation. Nonetheless, Ang II appears to have an overall stimulatory effect on the function and trafficking of granulocytes. Increased circulating Ang II during the stress response may enhance the activity of granulocytes in such a way that responsiveness to chemotactic signals is increased and trafficking to sites of injury or infection is more rapid if injury or infection were to occur at the time of the stress.

c. Monocytes

Monocytes function in the blood stream to phagocytose mobile pathogens and to replenish tissue resident macrophages, which function mainly as phagocytes, and dendritic cells, which function in antigen presentation. Monocytes can also move quickly to sites of infection in response to inflammatory signals. *In vitro* studies have shown that Ang II stimulates monocytes to increase the expression of

various cytokines and inflammatory mediators including of TNFα (68, 79, 122), IL-8 (179), MCP-1 (179), MCP-2 (196), and tissue factor (82, 137). These inflammatory mediators function to further stimulate monocytes and other immune cells. Ang II also stimulates monocytes to produce nitric oxide (178) and reactive oxygen species (215), which can activate pertinent transcription factors, such as NF-κB (157) and thereby perpetuate the inflammatory cascade.

Importantly, direct functional consequences of monocyte and macrophage stimulation with Ang II have also been demonstrated. Ang II has been shown to directly enhance the phagocytic activity of macrophages (123, 124, 165). It is also known that activation of monocytes leads to increased adhesion to vascular wall endothelium and eventual transmigration across the vessel wall and tissue infiltration. *In vitro* studies have shown that direct stimulation of monocytes with Ang II increases their adhesion to endothelial cell monolayers (1, 60, 79, 99, 191). ATR1 auto-antibodies, which bind ATR1 with greater affinity and specificity than Ang II, have been shown to enhance monocyte adhesion beyond that which is achieved with Ang II stimulation alone (60). AbdAlla *et al.* (1) showed that monocytes with increased levels of ATR1 homodimers (crosslinked by the action of factor XIIIA) showed elevated levels of ang II-stimulated adhesion, above that seen in monocytes with fewer or no ATR1 homodimers. Ang II stimulation of monocytes increases monocyte phagocytosis and adhesion to endothelial cells.

Few studies have examined the mechanisms by which Ang II induces monocyte adhesion. Mateo *et al.* (121) demonstrated that by incubating rat whole blood with Ang II for 4 hours, expression of the CD11b protein, one subunit of the amB2 integrin responsible for mediating monocyte adhesion, increased in monocytes. Piqueras *et al.* (154) reported that Ang II stimulation of whole blood did not result in any changes in the surface expression of CD11b/CD18 integrins or L-selectin, however the stimulation was performed at 25°C and for only 15 minutes. Increased expression of adhesion molecules such as integrins on monocytes may be partly responsible for increased the increased adhesion to endothelial cells

upon stimulation with Ang II. It has been shown that Ang II-induced monocyte adhesion to endothelial cells is mediated via the geranylgeranyl isoprenoid-dependent generation of ROS (150).

The effect of Ang II activation of ATR1 on monocytes appears to be two-fold. Ang II increases the function of monocytes in terms of cytokine production and phagocytosis. Additionally, Ang II induces monocyte adhesion to vessel wall endothelium by enhancing expression of integrins, subsequently enhancing the transmigration and redistribution of monocytes to various tissues. Increased circulating Ang II during the stress response may therefore be at least partly responsible for mediating the stress-induced activation and redistribution of monocytes, thereby increasing immune surveillance of certain tissues in preparation for immune challenges.

i. Angiotensin II signalling transduction in monocytes

There are a wide number of ATR1 intracellular signal transduction cascades, which mediate the numerous cell type-specific effects of Ang II. In general the ATR1 interacts with various G-proteins including Gq/11, Gi, G12 and G13(83) that stimulate various cascades including phospholipases C (PLC), A2 (PLA2), and D (PLD) upon activation(86). Various second messengers are produced by these interactions. For example, PLC cleaves phosphatydilinositol 4,5-bisphosphate into inositol 1,4,5-triphosphate, which stimulates calcium release, and diacylglyceral, which stimulated protein kinase C(83). Arachadonic acid, lysophosotide, phosphatidic acid, ROS and NO are other important second messengers. ATR1 has also been shown to activate various non-G-protein intracellular signalling pathways(86). ATR1 activates small GTP-binding proteins such as Ras, Rho, and Rac (83, 144). ATR1 also activates various protein kinases including receptor tyrosine kinases, non-receptor tyrosyine kinases such as janus kinase (JAK), serine/threionine kinases, the mitogen-activated protein kinase (MAPK) family including extracellular signal-related kinase (ERK), c-Jun N

terminal kinase (JNK), and p38MAPK, Akt/protein kinase B(PKB) and various protein kinase C isoforms (61, 75, 83, 188, 217).

The pro-inflammatory effects of Ang II in monocytes are primarily mediated by increased activity of the central inflammatory transcription factor, nuclear factor-kappa-light-chain-enhancer of activated B cells (NF-κB). Activation of ATR1 results in phosphorylation of inhibitor of κβ (Iκβ) proteins, which binds to and prevents nuclear translocation of NF-κB. Upon phosphorylation Iκβ dissociates from NF-κB, allowing NF-κB to translocate to the nucleus where it binds to specific response elements thereby mediating expression of certain genes. NF-κB controls the expression of many pro-inflammatory genes including cytokines, chemokines, and adhesion molecules, and its activation in response to Ang II-ATR1 interactions leads to increased expression of these inflammatory mediators. Numerous studies have shown that activation of monocyte/macrophage ATR1 by Ang II binding results in an activation of NF-κB (46, 82, 106, 179) and a decrease in the levels of the inhibitory protein Iκβ (46, 82).

Protein kinase C (PKC), protein tyrosine kinase (PTK), and mitogen-activated protein kinase (MAPK) pathways are thought to be the predominant intracellular signalling cascades involved in the regulation of transcription (24, 82, 130, 193). Activation of one or more of these pathways may be responsible for activation of NF-κB in monocytes (Figure 3). Specifically, Src kinase (a PTK), ERK 1/2 and p38 MAPK pathways are involved in Ang II/ATR1 stimulate monocyte migration (100), which may be the result of increased NF-κB activity. Additionally, the effects of cytokines on gene expression and cellular activation, proliferation, and differentiation in monocytes are mediated by the Janus Kinase/signal transducers and activators of transcription (JAK/STAT) family pathway following activation of cytokine receptors (146). STAT1 may be particularly important in mediating the pro-inflammatory effects of cytokines, and potentially Ang II, in monocytes (96). Upon activation, the receptor cytoplasmic domain of JAK is phospphorylated which recruits and tyrosine phosphorylates STAT.

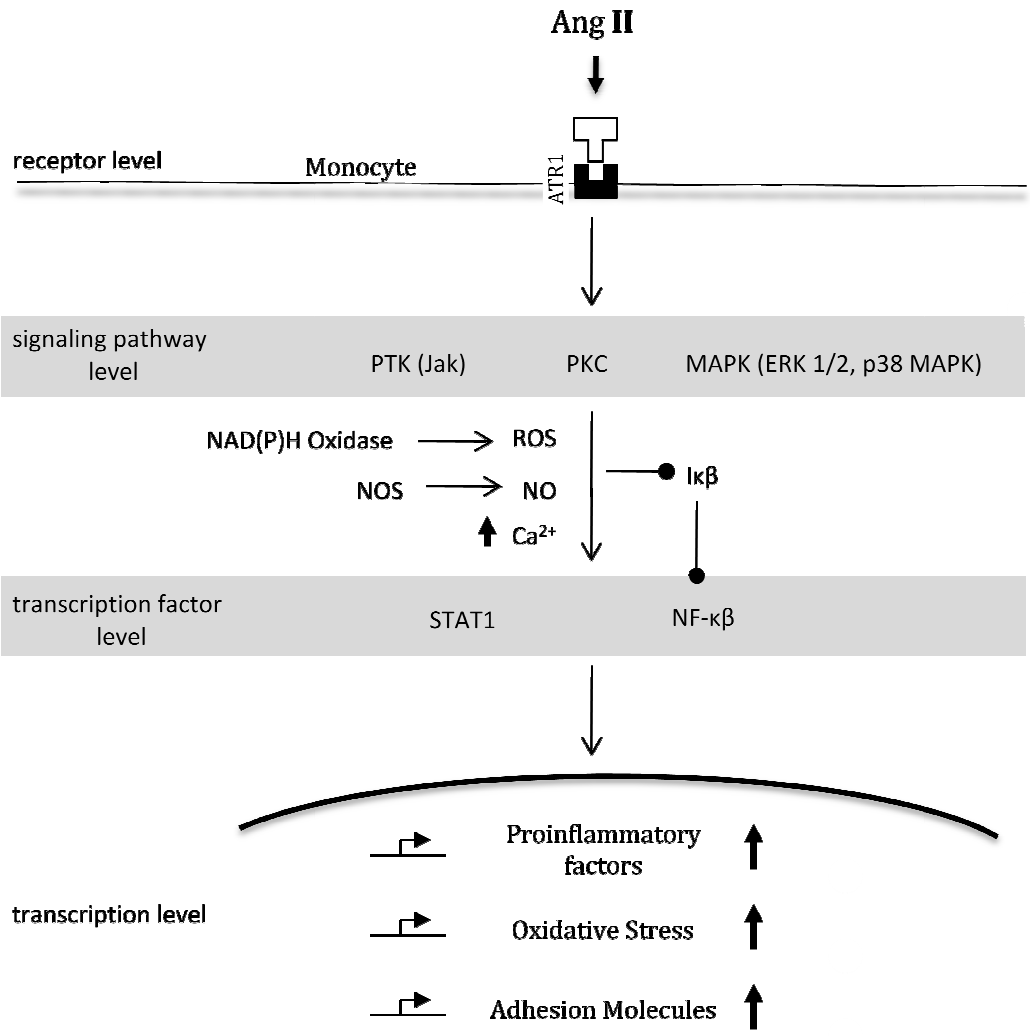

Figure 3. Potential pro-inflammatory ATR1 signalling transduction pathways in monocytes. Binding of Ang II to ATR1 on the monocyte cell surface activates various signalling pathways, including PTKs such as Jak, PKC, and MAPKs such as ERK1/2 and p38 MAPK. Following activation of these pathways, various second messengers are generated including ROS, NO, and increased intracellular Ca^{2+} , which may act to directly activate NF-κβ or may deactivate Iκβ thereby releasing NF-κβ. Jak specifically activates STAT1 via phosphorylation. Both activated NF-κβ and STAT1 translocate to the monocyte nucleus where they increase the transcription of proinflammatory genes, including cytokines and chemokines, as well as adhesion molecules and genes involved in oxidative stress.

Phosphorylated STAT dimereizes and translocates to the nucleus to activate specific gene expression. It has been shown that Ang II via ATR1 also activates the JAK/STAT pathway, which is also involved in many of the pro-inflammatory and proliferative effects of Ang II (86). Though not yet clearly defined, activation of ATR1 in monocytes may induce JAK/STAT signalling in addition to the induction of NF-κB.

A number of second messengers are activated when monocytes are stimulated with Ang II. Nitric oxide (NO) is produced via induction of nitric oxide synthase-2 (NOS-2) (178), reactive oxygen species are produced by induction of nicotaniamide adenine dinucleotide and/or reduced nicotinamide adenine dinucleotide phosphate oxidase-dependent mechanisms (64, 215), and changes in cytosolic calcium occur as well (110). ROS modulate the activity of other intracellular signalling molecules including tyrosine kinases and phosphatases, Ca^{2+} channels, MAPKs, and transcription factors such as the proinflammatory nuclear factor-kB (NF-kB) (39, 64, 197). NO and intracellular calcium may also modulate NF-kB and/or other signalling molecules involved in the inflammatory response in monocytes. Figure 1 illustrates potential signalling pathways that enhance the expression of pro-inflammatory genes following ATR1 activation of monocytes.

ATR1 activiation in monocytes may also mediate some important, non-transcriptional changes in cell function, particularly where cell-movement and migration are concerned (Figure 4). Proline-rich tyrosine kinase II (Pyk2), a member of the p125 focal adhesion kinase (FAK) family, and paxillin, a focal adhesion protein, are proteins that are directly associated with the cytoskeleton and are involved in cell movement and attachement (199, 221). Both Pyk2 and paxillin are highly expressed in monocytes and are phosphorylated by c-Src following stimulation of monocytes with Ang II (100, 109, 211). Upon phosphorylation, it is thought that Pyk2 translocates to focal adhesions, where it

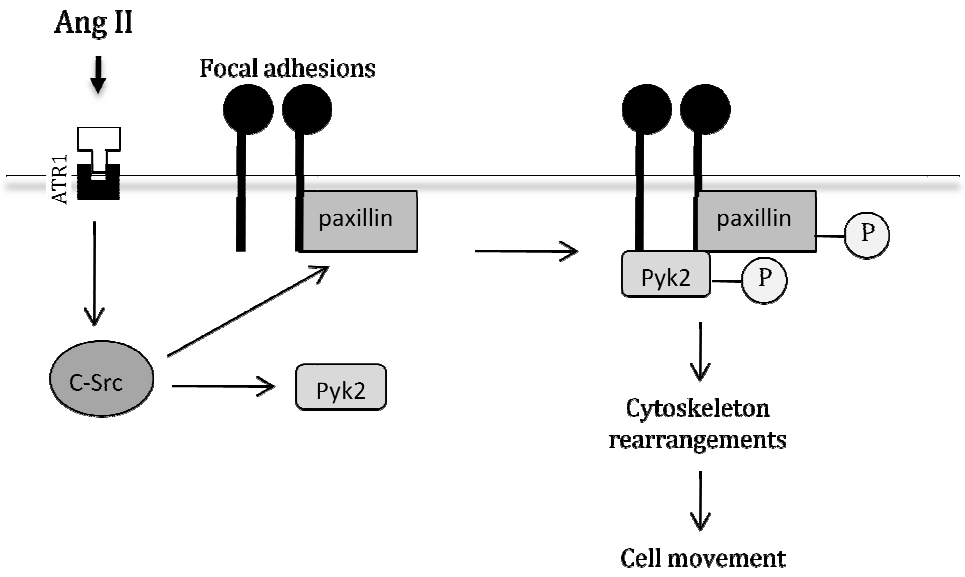

Figure 4. Non-transcriptional ATR1 signaling pathway that leading to cell movement in monocytes. Binding of Ang II to ATR1 activates the protein tyrosine kinase c-Src which phosphorylates both Pyk2, a cytoskeleton associated protein, and paxillin a focal adhesion associated protein. Upon phosphorylation, Pyk2 targets to focal adhesions where it is stabilized by phosphorylated paxillin. The movement of Pyk2 causes cytoskeleton rearrangements that culminates in cell movement.

colocalizes with paxillin resulting in cytoskeletal rearrangements and cell movement (100, 111).

7. Physiological relevance?

There is a large body of data that indicates that the RAS has function in inflammatory processes, atherosclerosis in particular. As illustrated, Ang II acts to enhance immune cell activity and migration by action on leukocytes directly as well as by action on cells of the vascular wall, which fits into a model for RAS-driven atherosclerosis. However, the expression of ATR1 throughout the spectrum of leukocyte subsets alone suggests that Ang II has some significant role in mediating the activity of these immune cells as part of a normal physiological process, outside of atherosclerosis. Indeed, Ang II affects the activity of all of these leukocyte types and it seems improbable that such mechanisms exist only to result in atherosclerosis. It is also known that Ang II can induce immune cell infiltration of a variety of tissues (review (190)), beyond the vascular wall where atherosclerosis takes place. We have hypothesized that the changes in leukocyte activity and trafficking that Ang II causes may have physiological relevance in the scheme of stress-induced immune enhancement.

8. The stress response

In general, stress describes a physiological and/or psychological response to a potentially harmful stimulus. There are multiple key events that are essential to the human stress response. First, there is the stressor event itself (real or imagined), a cognitive integration of the stressor and a neurological triggering mechanism that involves certain central nervous system (CNS) loci (locus ceruleus, limbic nuclei, hypothalamic nuclei). Second, there is an efferent physiological response leading to target organ activation. Finally coping mechanisms are activated (physiological and psychological) to deal with the stressor event (66). Biogenic (or sympathomimetic) stressors, including extremes of temperature, injury (hemorrhage), and physical exercise, bypass the cognitive appraisal mechanism and directly elicit a physiological stress response by action

on the affective and neurological triggering nuclei in the CNS (66). The physiological stress response mechanism that leads to target organ activation can itself be divided into three pathways: autonomic neural (initial and fast activation of the sympathetic nervous system (SNS), parasympathetic nervous system (PNS), and neuromuscular nervous systems), neuroendocrine (SNS activation of the adrenal medulla), and endocrine (activation of the hypothalamic-pituitary-adrenal (HPA), somatotropic, thyroid, and posterior pituitary axes requiring the greatest intensity of stimulation) (66).

The response to stress is dependent upon a large number of factors including the nature, duration, intensity, and number of occurrences of the stimulus, the time between subsequent stimuli, and the psychological state of the individual. The degree to which the stress axes are activated depends not only on the characteristics of the stressor event, but also on how the stressor is perceived. If coping mechanisms immediately available or there is preconceived knowledge of the stressor event, the stress response will be less or even absent (66).
Regarding their duration, stressful stimuli can be divided into four major categories(126): single, time-limited (acute) stressors; stressor sequences whereby one initiating event produces a cascade of physiological and behavioral changes over time; chronic intermittent stressors; and chronic persistent stressors. In general, in the absence of a habituation to the stressor, the more chronic the stressor, the more prolonged the activation of the three physiological stress response pathways will be. If coping mechanisms fail, prolonged activation of the stress pathways due to chronic stress results in dysregulation of the integrated stress response systems. As a consequence renal, cardiovascular, gastrointestinal and immune disturbances may result (181). Ultimately, activation of the physiological stress response pathways results in activation of many target organ systems that prepare the body to maintain homeostasis in the presence of a perceived or biogenic threat. Stress causes changes in renal, cardiovascular, metabolic, neural, and immune function that can become dysfunctional in chronic stress situations

9. Increased systemic RAS activity during the stress response

The idea that systemic RAS was involved in the stress response was founded on studies published during the 1970's that showed increased plasma renin activity (PRA) and increased levels of circulating Ang II in animals and humans exposed to various stressors. It was shown that PRA increased significantly in rats exposed to a novel environment as well as in caged rats exposed to the presence of a hungry cat (37). A study using baboons reported that PRA increased significantly on the day that Sidman avoidance tests were administered (baboons were trained to press a lever in response to a light signal to avoid an electric shock; if the lever was not pressed, a shock was delivered) at 1, 2, and 3 hours after the onset of the avoidance test (even before any shocks were delivered) and 30 minutes after it's termination (18). A series of small studies using healthy human male subjects demonstrated that PRA and plasma Ang II increased 30 minutes after an intense running exercise (103), immediately after subjects endured 20 minutes in an 85-90°C sauna (104), and 15 minutes after subjects performed mental arithmetic exercises for 20 minutes (102).

10. RAS activity is increased by the SNS response to stress

The increased renal renin levels, PRA, and plasma Ang II concentration that occur during the stress response are attenuated with prior β-adrenergic blockade (3, 72). Sympathetic activation during the stress response is also responsible for increased release of catecholamines from the adrenal medulla. Likewise, it appears that activation of the efferent sympathetic renal nerves that innervate the renin-secreting granular juxtaglomerular cells is largely responsible for increasing plasma renin during the stress response.

11. Different stress sensors can activate the RAS

Both psychosocial/psychological stressors (mental arithmetic anxiety, threatening presence, novel environments, etc.) and physical/biogenic stressors (extremes of temperature, pain, physical exertion, etc.) increase circulating levels of Ang II. Studies in rats showed increases in PRA and/or plasma Ang II after exposure to

psychosocial stressors such a novel environment or a hungry cat (37). Similary, studies in humans showed increased PRA, plasma renin concentrations, and/or plasma Ang II concentrations in response to various mentally stressful activites such as puzzle solving in naïve subjects (38) and mathematics tasks (59, 102). While it is easy to distinguish a strictly psychological stressor, it is often difficult to exclude a psychological component from apparently physical stressors, especially in humans. Nonetheless, studies in rats showed increases in PRA and/or circulating levels of Ang II after exposure to cold temperatures (4-8°C) (29, 216) and various time-courses of immobilization, which likely resulted in some amount of psychological stress (3, 72, 94). Similarly, studies using human subjects showed increased PRA, plasma renin concentrations, and/or plasma Ang II concentrations in response to exercise (76, 103) and hot temperatures (85-90°C) (104). Importantly, these changes in PRA and plasma Ang II concentrations are often reported to occur despite sodium loading (3, 37, 59) indicating that increases in PRA are not as a result of renal salt or water loss. On the basis of these studies it can be said that the stress response pathways that bypass cognitive appraisal and pathways that begin with cognitive appraisal, that is, stressful stimuli that have no physical component, both have the capacity to increase circulating RAS activity.

12. Systemic RAS activation in acute versus chronic stress

Most of the studies examining changes in circulating RAS activity during the stress response reviewed above have used single, time-limited acute stress protocols. These studies show that, *in* vivo, acute stress results in elevated circulating Ang II. Few studies have looked at the effects of persistent or intermittent chronic stressors on circulating RAS activity, perhaps because defining a model of chronic stress is difficult to achieve in experimental settings.

Aguilera *et al.* (4) demonstrated that chronic intermittent stress in the form of 2 hours of immobilization per day for 14 days significantly increases PRA and plasma aldosterone concentration as well as renin mRNA content in the kidneys 24 hours after the last exposure to stress. In this same study, it was shown that

acute stress in the form of 30 minutes of immobilization also resulted in significantly increased PRA and plasma aldosterone. Though the results of these experiments were not compared and statistically analyzed, the PRA 24 hours after chronic intermittent stress was measured at 2.7 ng/ml/hr, whereas PRA immediately after 30 minutes of immobilization (acute stress) was measured at 14 ng/ml/hr. Although PRA after both chronic and acute stress protocols were significantly higher compared to unstressed rats, the level of PRA after acute stress appears to be significantly higher than after chronic stress. However, given that the half-life of plasma renin in rats is on the order of minutes (measured at 3.0±0.4 minutes by Al-Merani *et al.* (5)), the increase in PRA seen with chronic intermittent stress suggests that some level of RAS activation above baseline persists even 24 hours after the administration of the last stressor event.

Two *in vivo* studies have tried to directly compare the effects of acute stressors to chronic stressors on circulating RAS activity. Yang *et al.* (216) exposed rats to compulsive cold water swimming for 20 minutes (acute stress) or to an ambient temperature of 4-8°C for 5 days (chronic stress) and showed that plasma Ang II levels were significantly higher in both stress groups compared to control rats. However, Ang II levels were significantly lower in the chronically cold stressed rats compared to the acutely cold stress rats: plasma Ang II reached only up to 134% of controls in the chronic stress group whereas plasma Ang II reached up to 900% of controls in the acute stress group. The differences in the stress protocols (forced swimming in the acute stress protocol versus no forced activity in the chronic stress protocol) may have impacted these results.

Cassis *et al.* (29) exposed rats to cold temperatures (4°C) for 7 days and took measurements of plasma Ang II concentrations after 4 hours (acute stress), 1, 3, and 7 days. It was found that plasma Ang II concentrations increased 10-fold immediately after 4 hours of cold exposure, returned to control levels at 1 and 3 days of cold exposure, and increased 2-fold at 7 days of cold exposure. However, the increase of plasma Ang II in the rats after 7 days of cold exposure was found

to be related to increased food intake, which increased in mice at 5 days of cold exposure and beyond. Pair fed mice showed no changes in plasma Ang II after 7 days cold exposure. Increased feeding may itself be directly responsible for increased RAS activation in sustained chronic stress situations. However, increased feeding may simply allow the animal to meet the increased energy demands of chronic stress, allowing SNS activation of the RAS to be sustained. If energy demands are not met, SNS activation of the RAS may cease.

It is known that animals exposed to the same stressful stimulus each day for several weeks show increased synthesis and storage of catecholamines in the adrenal medulla, increased basal levels of circulating catecholamines, and decreased release of catecholamines into the circulation following exposure of an identical stressor (reviewed by McCarty *et al.* 1988). However, these same chronically stressed animals show an exaggerated sympathetic-adrenal medullary response when exposed to a novel stressor, suggesting that a habituation to the chronic stressor and a sensitization of the sympathetic-adrenal medullary stress response system has taken place. A similar pattern of neuroendocrine adaptation may take place with the RAS in the rats exposed to the aforementioned chronic stress protocols.

It is clear that acute stress activates the RAS. The relationship between persistent and intermittent chronic stress and RAS activity is less obvious. Given that the physiological response to the stressor is sustained to some degree, chronic stress may be associated with sustained elevated levels of PRA and circulating Ang II that are below levels achieved during acute stress but are above unstressed, baseline levels.

13. The immune response to stress

Changes in immune function are an important part of the stress response. Acutely, stress enhances immune function (51), which is thought to be an adaptive response to stressful stimuli by preparing the immune system for imminent

immune challenges that may result from wounding (53-56). Chronic stress on the other hand is known to suppress immune function and it is well accepted that chronically stressed individuals are more susceptible to infection. Alterations in immune function during the acute and chronic stress responses is brought about as a result of a complex set of changes in the trafficking, distribution, maturation, and function of leukocytes. The mechanisms by which stress-induced immune alterations occur is not yet fully understood. However, stress-induced immune alterations are thought to be mediated largely by the major stress hormones of the HPA axis, glucocorticoids, and catecholamines, as well as by sympathetic nervous system neurotransmitters (16, 50, 147).

a. Immune cell alterations during the stress response

There are a number of time-dependent changes in the trafficking and distribution of leukocytes during the stress response. Overall circulating leukocyte numbers appear to rise initially during acute stress (22, 84, 128, 136, 176), with granulocytes and natural killer cells making up for most of this increase (13, 22, 128, 136, 176) while lymphocytes and monocyte numbers in the circulation decrease (54, 55, 176). As the stress response continues, circulating neutrophil numbers continue to rise (54), while there is a disproportionate decrease in the absolute number of helper T-cells, cytolytic T-cells, B-cells, natural killer (NK) cells and monocytes causing the total number of circulating leukocytes to fall below baseline (7, 176). Dhabhar and McEwan (50) have suggested that the initial increase in total circulating leukocyte numbers represents a mobilization of leukocytes from sites of storage, while the subsequent decrease in total circulating leukocytes represents a redistribution of lymphocytes, NK cells, and monocytes to sites important for first line defence against immune challenges, particularly the skin (53, 206) and lymph tissues (194).

Aside from altering the distribution of leukocyte populations, stress is also know to affect the function of immune cells. Acute stress has been shown to enhance the acquired immune response by increasing both cellular immunity (15, 57, 172,

205), and humoral immunity, particularly antibody production (40, 152, 213). Acute stress has also been shown to enhance the innate immune response by increasing spleen macrophage phagocytic function (114), blood polymorphonuclear leukocyte phagocytosis (184), NK cell activity (141) as well as by increasing the proliferation of blood and spleen leukocytes (93, 113, 164, 184). Chronic stress, on the other hand, has well described immune suppressing effects and a variety of chronic stressors have been shown to suppress the skin cell-mediated immune response (12, 51), antibody production (69), NK cell activity (33, 89), leukocyte proliferation (33, 161), virus-specific T-cell and NK cell activity (19), and macrophage activity (23).

b. The role of endocrine factors in stress-induced immune alterations

The precise role of stress hormones in the control of stress-induced immune alterations is an active area of research that has largely focused on glucocorticoids and catecholamines. The role of glucocorticoids, in particular, remains controversial. Endogenous glucocorticoids at pharmacological concentrations and synthetic glucocorticoids have well described immune-suppressing effects (25). In addition, glucocorticoids are used clinically for their immune-suppressing and anti-inflammatory properties(25). However, it has been described that physiological concentrations of endogenous glucocorticoids can exert immunomodulatory, immune-enhancing, or immunosuppressive effects.

Most *in vitro* studies indicate that glucocorticoids inhibit the function of immune cells and blunt the inflammatory response by inhibiting the action of inflammatory transcription factor NF-κB (125) via induction of I κβ, which binds and sequesters NF-κB to the cytoplasm (10, 177). It has also been shown that activated glucocorticoid receptors directly bind and interfere with the function of both activator protein-1 (AP-1; another transcription factor that upregulates the transcription of various inflammatory genes including several of the interleukins) (180) and NF-κB (2, 27, 131, 162, 175, 177). However, studies have also shown that low levels of glucocorticoids administered *in vivo* enhance humoral immunity

and T-cell activation (50). These data suggest that low doses of endogenous glucocorticoids are permissive of immune enhancement.

Evidence also exists to suggest that catecholamines affect immune cell activity. Adrenergic receptors are found on lymphocytes and macrophages (115) and noradrenergic sympathetic nerve fibres run from the CNS to both primary and secondary lymph organs (67) where noradrenalin-releasing sympathetic nerve terminals synapse with neighbouring immune cells (173). Moreover, it has been shown that noradrenaline and small doses of adrenaline activate cellular NF-κB in monocytes in a time and dose dependent manner (14).

It is apparent that both glucocorticoids and catecholamines are important to the immune response to stress. Adrenalectomy in rats, which eliminates glucocorticoid and adrenaline stress responses, eliminates stress induced enhancement of skin cell-mediated immune responses (52). Enhancement of skin cell-mediated immunity is reduced by administration of low dose corticosterone or adrenaline however simultaneous administration of both hormones produces an additive increase in the cell-mediated immune response. As such, catecholamines may be partly responsible for the immune enhancement that occurs during acute stress, while basal glucocorticoids permit immune enhancement to take place and stimulated levels of glucocorticoids prevent immune overactivity and adverse outcomes such as autoimmune targeted tissue damage.

While it is clear that both glucocorticoids and catecholamines have critical roles to play in mediating stress-induced immune alterations, the precise endocrine and other mechanisms by which these alterations occur is not yet fully understood. Black (16) has hypothesized that psychologic stress produces an inflammatory response through the combined action of the major stress hormones noradrenaline and cortisol, together with the RAS, proinflammatory cytokines, and free fatty acids. Black also suggests that the entire stress-induced inflammatory process (and the engagement of the acute phase response of the innate immune system) is

initiated by activation of NF-κB in macrophages, visceral fat and endothelial cells. The control of the immune response to stress likely involves factors other than the glucocorticoids and catecholamines, and the activity of the RAS, which increases as part of the stress response, may be an important one of these factors.

14. Ang II is partly responsible for mediating acute enhancement of immune function during the stress response

It is becoming increasingly apparent that Ang II is an endocrine effector of the stress response, like the glucocorticoids and catecholamines. To support a role for Ang II in modulating the immune response to stress, evidence must show that 1) stress causes enhanced leukocyte trafficking and function, 2) Ang II causes enhanced leukocyte trafficking and function, and 3) blockade of Ang II activity prevents enhanced leukocyte trafficking and function caused by stress.

As previously described, much research shows that immune function is enhanced by stress acutely. During the stress response total circulating leukocyte numbers, particularly neutrophils, are initially increased as they are mobilized from storage sites. Monocytes and lymphocytes begin exiting the blood and are redistributed throughout the body (to the skin and lymph tissues) while circulating neutrophil numbers continue to rise. The activity of circulating and tissue leukocytes – phagocytic activity, cytokine production, antibody production, etc. – also increases to some extent and both innate and adaptive (cell-mediated and humoral) immune responses are enhanced during the stress response.

As reviewed in previous sections, there is much evidence to suggest that Ang II has the capacity to enhance immune function. Ang II, by acting on ATR1, induces increased expression of adhesion molecules on vascular endothelium and on circulating leukocytes, which increases the incidence of leukocyte adhesion, transmigration and infiltration of various tissue sites. Ang II also induces the production and release of chemokines and cytokines from ECs and VSMCs among other cell types that serve to attract leukocytes to the vascular wall at

relevant tissue sites through chemotaxis and enhance the function (phagocytosis, cytokine, proliferation, antibody production, etc.) of target leukocytes. Ang II also directly stimulates monocytes/macrophages, lymphocytes, and granulocytes to enhance their function through various mechanisms.

The studies providing this evidence have examined the effects of Ang II on leukocyte trafficking and function largely in the context of atherosclerosis. The relationship between increased circulating Ang II during the stress response and stress-induced alterations in immune cell trafficking and function has not been thoroughly examined. However, it may be reasonable to assume that increased circulating levels of Ang II during the stress response results in these same immune enhancing effects. One study has provided a clue that activation of ATR1 may be necessary for immune alterations during stress.

In 2003, Bergonzio *et al.* (21) showed that administration of cold-restraint stress (restraint in a 4°C environment for 2 hours) to spontaneously hypertensive rats (SHR) reduced gastric blood flow and produced acute gastric mucosal lesions, which was associated with increased expression of ICAM-1 in gastric arterial endothelium, neutrophil production of TNFα and neutrophil infiltration of the gastric mucosa. These effects were diminished when SHR were pretreated with candesartan, an ATR1 antagonist, for 14 days prior to the administration of the stress protocol. Candesartan treatment did increase gastric blood flow in unstressed animals however measurements of blood flow were not taken during cold-restraint stress. Indeed, changes in gastric and systemic hemodynamic parameters may well have affected neutrophil infiltration and the formation of gastric ulcers. However, the evidence indicating that Ang II directly alters the trafficking and function of immune cells in the *in vitro* studies described in the previous sections lends support to the hypothesis that activation of ATR1 (by increased circulating Ang II) during cold-restraint stress, is at least partly responsible for enhancing the observed gastric neutrophil infiltration by direct action on neutrophil and EC ATR1.

Though only one study has examined the relationship between stress, RAS activity, and immune cell function, we hypothesize that increased circulating Ang II and ATR1 activation on leukocytes, ECs, VSMCs, among other cell types during the stress response is critically involved in the immune-alterations that are observed during the acute stress response. Figure 5 provides an illustration of the potential pathways that mediate stress-induced immune alterations. Ang II, functioning as a major stress hormone, serves to enhance both the innate and adaptive immune responses in preparation for imminent immune challenges that may occur upon experience of the stressor.

15. Ang II: one of a group of stress hormones that modulate immune function

It has been shown that glucocorticoids, one of the major stress hormones generally understood to suppress immune cell function, are required for immune alterations during the stress response, particularly where leukocyte redistribution is concerned (50). Controversy still surrounds the action of glucocorticoids on immune cell function and trafficking: high doses and synthetic glucocorticoids always suppress immune function while low doses of endogenous glucocorticoids sometimes enhance immune cell function (50). This may be explained by the fact that some level of glucocorticoids is necessary to maintain basal ATR1 expression (3, 30). It has been shown in rat VSMCs that dexamethosone, upon binding the glucocorticoid receptor, translocates to the nucleus where the steroid-receptor complex binds to and activates a glucocorticoid response element in the upstream promoter region of the ATR1 gene thereby enhancing its expression (77). Without glucocorticoids then, ATR1 expression may be driven down to such a level that Ang II signaling is incapable of producing functional outcomes. Otherwise, glucocorticoids inhibit activation of NF-κB and therefore the expression of adhesion molecules and pro-inflammatory factors that are critically involved in the activation and redistribution of immune cells.

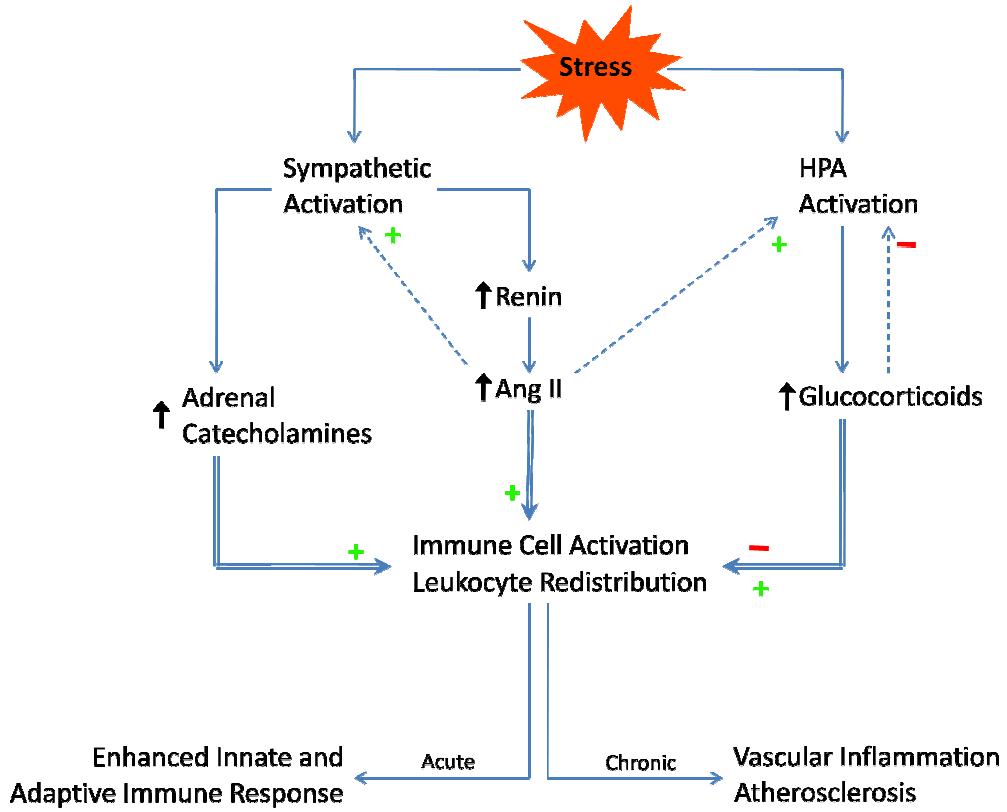

Figure 5. Endocrine pathways that mediate stress-induced immune alterations. Sympathetic activation in response to stressful stimuli increases the release of adrenal catecholamines into circulation and also stimulates the juxtaglomerular granular cells to release renin. Increased plasma renin activity results in increased circulating Ang II. Both catecholamines and Ang II act to activate immune cells and stimulate a redistribution of immune cells to sites (skin, lymph tissues) important in primary immune defence. Stressful stimuli also activate the HPA axis that results in increased circulating glucocorticoids, which have both stimulatory and inhibitory effects on immune cell activation and redistribution. Ang II also enhances sympathetic activity and activation of the hypothalamic pituitary adrenal (HPA) axis – Ang II is a major mediator of the stress response and should be considered a major stress hormone, like the glucocorticoids and catecholamines. Overall, acute stress stimulates enhanced innate and adaptive immune responses in preparation for imminent immune challenges that may be encountered during the stressful event. Chronic stress and chronic activation of these endocrine pathways may culminate in vascular inflammation and increased incidence of atherosclerosis.

Through these integrated signaling mechanisms, immune alterations during the stress response are tightly controlled. Increased circulating Ang II during the stress response enhances activation and redistribution of immune cells, effects that are dependent upon the expression of ATR1 which is under the control of glucocorticoids, and glucocorticoids, which are also increased during the stress response, may serve to buffer these immune-enhancing effects such that deleterious autoimmune outcomes do not result. There is undoubtedly interplay between the RAS, glucocorticoids, catecholamines, among other non-endocrine factors such as sympathetic activity in the control of immune function during the stress response, one that has not yet been entirely defined. Nonetheless, it is apparent that Ang II has an important and prominent role to play in the control of stress-induced immune alterations.

16. Could intermittent acute or chronic stress lead to Ang II mediated inflammation and atherosclerosis?

The hypothesis that repeated episodes of acute stress or chronic stress can promote atherogenesishas been around for some time (17). Various psychosocial stressors, including personality, job stress, social isolation, etc., have been identified as risk factors for the development of atherosclerosis (17, 107, 167, 212). There is also much evidence to suggest that chronic psychosocial stress is a risk factor specifically for the acceleration of coronary artery disease (58, 101, 108, 159, 187, 208). Given the pro-inflammatory activity of the RAS and the evident increase in RAS activity during the stress response, there may be a link between recurrent or chronic stress, recurrent or chronic activation of the RAS, and the development of atherosclerosis. Research has yet to directly examine the links between stress, RAS activity, and atherosclerosis.

Black and Garbutt (17) suggest that elevations in blood pressure via SNS activation, increases in the levels of corticosteroids and catecholamines, as well as a subsequent increase in the levels of proinflammatory cytokines during the stress response are responsible for mediating induction of adhesion molecules,

recruitment of leukocytes to the vessel wall, and endothelial damage. When unattenuated or persistently repeated, as in chronic and recurrent stress, this cascade of events leads to vascular inflammation and subsequent development of atherosclerotic plaques. Black and Garbutt also mention involvement of the circulating RAS in stress-promoted atherogenesis given that Ang II can accelerate atherosclerosis outside of its effects on vascular tone and blood pressure.

Though evidence is minimal, RAS activity appears to remain elevated in recurrent stress models (4) and may also remain elevated with chronic stress (29, 216). Recurrent and chronic stress may also increase sensitivity of the RAS (among other physiological response pathways) to other novel stressors, resulting in increases in a hyperactive RAS (126). Acute activation of the RAS during stress likely serves to prepare the immune system for imminent immune challenges and may be involved in the physiological processes of wound or vascular injury repair. However, recurrent activation, chronic activation, or sensitized over-activation of the RAS in recurrent or chronic stress situations may exacerbate typical vascular injury, leading to atherogenesis and adverse cardiovascular sequelae.

We propose at least four mechanisms that likely underlie RAS-mediated atherogenesis in recurrent/chronic stress situations. Recurrently or chronically active RAS results in recurrent or chronic activation of immune, endothelial, and vascular smooth muscle cells directly (36), which would predispose the vasculature to inappropriate inflammation. Recurrent or chronic activation of RAS may also positively feedback to other components of the stress response that affect cardiovascular function, including the HPA axis and glucocorticoid release (170, 200, 201), sympathetically stimulated release of catecholamines from the adrenal medulla (201), and sympathetic activity by facilitating norepinephirine release from sympathetic nerve endings and inhibiting re-uptake(74). Recurrent or chronic increases in blood pressure or blood pressure lability may also result from recurrent/chronic activation of RAS (92, 166) that may additionally contribute to

the development of atherosclerosis (hypertension is a known risk factor for atherosclerosis) (158). Finally, recurrent/chronic activation of RAS may increase the sensitivity of RAS and other physiological stress response systems to other, novel stressors as occurs with the adrenal medullary response (catecholamine release) (126), which would perpetuate the proinflammatory and atherogenic actions of the RAS response. Acting together, these four RAS-mediated effects likely contribute to the development of atherosclerosis in individuals experiencing recurrent or sustained chronic stress.

Given that maladaptive RAS activity during recurrent or sustained chronic stress may be contributing to acceleration of atherosclerosis and adverse cardiovascular outcomes, individuals with unresolving psychosocial stress may benefit from treatment with angiotensin converting enzyme inhibitors or angiotensin receptor blockers (ARBs). Administration of angiotensin receptor blockers or angiotensin converting enzyme inhibitors (ACEi) is well known to prevent acceleration of atherosclerosis and improve outcomes of patients with CAD (116, 143, 185). It has been shown that administration of ARBs prevents the development of gastric inflammation and ulceration in chronically cold stressed rats [195, 219] and a similar benefit may exist where stress-induced atherosclerosis is concerned.

III. Materials and Methods

1. Cell culture

An imortalized human microvascular endothelial cell line (HMEC1), and two immortalized human monocyte cell lines (U937 and THP-1) derived from leukemia patients were purchased from ATCC (Manassa, VA, USA). HMECs were cultured in MCDB 131 medium (Gibco, Invitrogen, Grand Island, New York, USA) supplemented with 10% fetal bovine serum (FBS) (VWR), 10ng/mL epidermal growth factor (VWR), 10mM hydrocortisone (Sigma-Aldrich, Oaksville, ON), 10mM L-glutamine (Gibco) and 100IU/ml penicillin and 100ug/ml streptomycin (Gibco) on conventional fibronectin-coated culture plates. U937 cells were cultured in ATCC RPMI 1640 medium supplemented with 10% FBS, 100IU/ml penicillin and 100ug/ml streptomycin in conventional T-flasks. THP-1 cells were cultured in ATCC RPMI 1640 medium supplemented with 10% FBS, 100IU/ml penicillin and 100ug/ml streptomycin, and 50uM mercaptoethanol (Sigma-Aldrich) in conventional T-flasks. All cells were cultured at 37°C in 95% humidified air with 5% CO_2. Primary human monocytes were also isolated directly from the blood of healthy subjects using immune-magnetic bead separation with Dynabeads (Dynal, Invitrogen) and a MPC15 magnet (Dynal) following the manufacturer's instructions. Fluorescence-activated cell sorting (FACS) was used to assess the purity of freshly isolated monocyte populations.

2. RNA isolation

Total RNA isolation was performed using either TRIzol reagent (Invitrogen) or silica gel column RNA isolation kit (RNEasy mini-prep, Qiagen). Total RNA extraction from TRIzol solution was performed using the procedure recommended by the manufacturer. Total RNA extraction on silica-gel separation columns from 2.Mississauga, ON) following the procedure recommended by the manufacturer. Quality and quantity of the RNA was assessed using a spectrophotometer, measuring absorbance values of 1:200 RNA dilutions in water (to determine quantity) and TE buffer (to determine quality) at wavelengths of 260 and 280 nm.

RNA concentration was determined by dividing the A260 value measured in samples of RNA diluted in water by 25 and multiplying by the dilution factor (200). RNA quality was determined by the ratio of A260/A280 measured in RNA diluted in TE buffer: a ratio between 1.90 and 2.10 was considered acceptable. RNA was stored at -80^{o}C until further processing.

3. DNAse digestion

For use in PCR protocols utilizing the primer sets targeted to the terminal exon of the ATR1 transcripts that we designed total RNA was treated with DNAse by combining the desired mass of RNA (in an 8 ul volume) to 1 uL of 1U/uL DNAse enzyme (Fermentas, Burlington, ON) and 1 uL of 10x DNAse buffer (Fermentas) and incubating at 37^{o}C for 60 minutes. DNAse digestion was terminated by adding 1 uL of 25uM EDTA to the reaction and incubating at 70^{o}C for 10 minutes. DNAse digested and undigested total RNA was stored at -80^{o}C until further processing.

4. Reverse transcription

Reverse transcription (RT) was carried out using a cDNA synthesis kit (iScript, BioRad, Mississauga, ON) following the manufacturer's instructions. Reactions were prepared on ice using total RNA (5ug total HMEC1 RNA, and 10ug total U937, THP-1, and fresh monocyte RNA were used for each RT reaction). 4 µL of 5x iScript reaction mix (containing a blend of oligo(dT) and random primers along with the necessary buffers) and 1 µL of iScript (RNase H^{+} MMLV) reverse transcriptase were added to the total RNA and brought up to a final volume of 20 µL with nuclease-free water. Reactions were incubated for 5 minutes at 25°C followed by 30 minutes at 42°C. Reactions were terminated by incubating at 85°C for 5 minutes and stored at -20°C until further processing. Reactions without reverse transcriptase enzyme, substituted with 1uL nuclease-free water, were prepared to check for DNA contamination of the RT products during PCR.

5. Quantitative PCR

Quantitative polymerase chain reaction (qPCR) was carried out using pre-designed probe and primer sets (Applied Biosystems TaqMan® Assays-on-Demand™, Foster City, CA). Primer/probe sets were targeted to the known mRNA transcript splice variants. Assay Hs99999095_m1 was targeted to variant 1, Hs01096942_m1 was targeted to variant 2, Hs00258938_m1 was targeted to variants 3 and 4, Hs00241341_m1 was targeted to variants 1 and 4, and Hs00259315_m1 was targeted to variant 5 (Figure 6). A pre-designed probe and primers (Hs99999095) targeted to the glyceraldehydes phosphate dehydrogenase (GAPDH) gene was used as an internal reference. Primers and a probe targeted to the terminal, protein coding exon found in all ATR1 transcript variants (Figure 6) were designed as follows: ATR1 inner forward 5'- CCAA AGGGCAGTAAAGTTTTCG-3' and reverse 5'-GCAACTTGACGACTACTGCTTAGC-3', ATR1 probe 5'-CAACTGTGCTACACTTGCACCTGGTACTGC-3'.

qPCRs using the predisigned primer/probe sets were performed in a 25µL reaction volume by combining 7.5µL RT product (undiluted for ATR1 reactions; diluted 1:625 for GAPDH reactions), 12.5µL 2x TaqMan Universal Master Mix with AmpErase UNG (Applied Biosystems), 1.25µL of the appropriate target assay primer-probe mix, and 3.75µL of nuclease-free water. qPCRs using the primers and probe designed to target the terminal exon of all ATR1 transcripts were performed in a 25uL reaction volume by combining 7.5µL RT product (undiluted for ATR1 reactions; diluted 1:625 for GAPDH reactions), 12.5µL 2x TaqMan Universal Master Mix with AmpErase UNG (Applied Biosystems), 2.25µL each of the 10pmol/ul forward and reverse primers, 0.0625ul of the 100uM probe and brought up to final volume with nuclease free water. qPCR was carried out in an Applied Biosystems PRISM 7500 Sequence Detection System (Applied Biosystems) with an initial 10 minute denaturation step at 95°C, followed by 50 cycles of 15 seconds at 95°C and 1 minute at 60°C. Fluorescence was measured

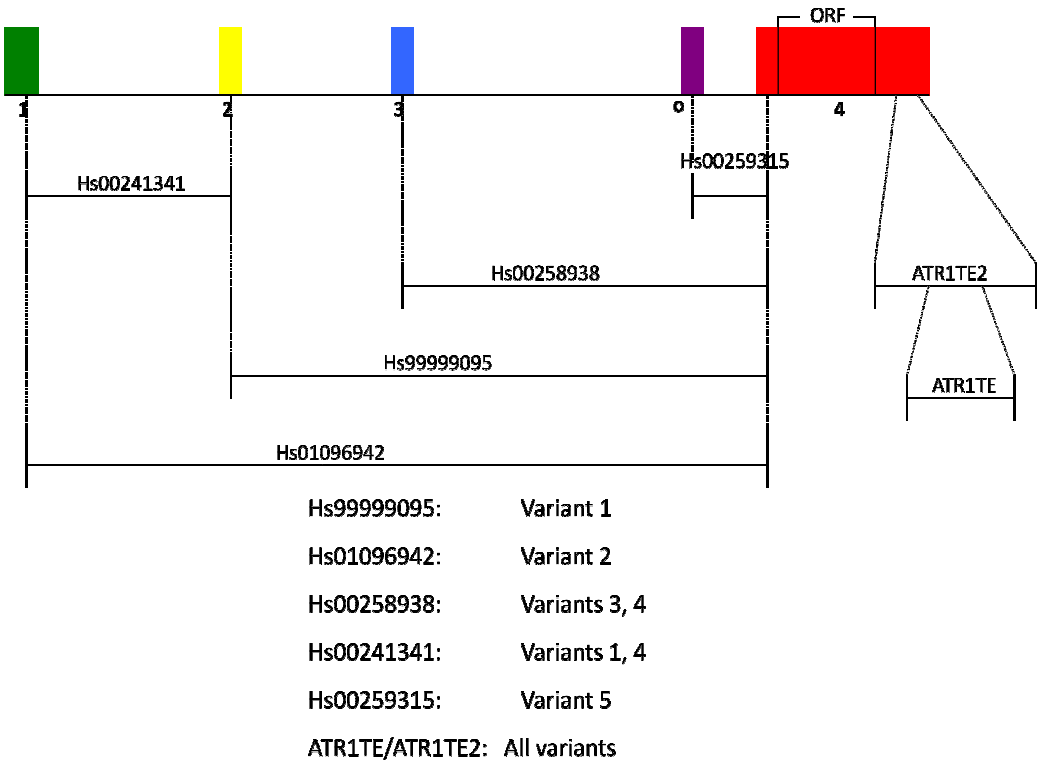

Figure 6. Schematic representation of primer/probe locations on the ATR1 coding sequence. The primer/probe sets developed by ABI (catalogue numbers Hs99999095, Hs01096942, Hs00258938, Hs00241341, and Hs00259315) were designed to cross exon boundaries where the forward primer localizes to the 5' exon and the reverse primer localizes to the 3' exon and the probe directly overlaps the corresponding exon boundary. The primer sets we have designed (ATR1TE2 and ATR1TE) localize to the terminal protein-coding exon (exon 4) common to all ATR1 transcript variants and do not cross any exon boundaries. The transcript variants targeted by each primer set is shown.

during the last 30 seconds of the 60°C step. Expression of each target gene in each sample was normalized to the endogenous control GAPDH (ΔCT).

6. Conventional single round and nested PCR

Single round RT-PCR was performed using a PCR kit (BioRad) in a 50uL reaction volume with 5uL 10x PCR buffer, 5 uL 2mM dNTP, 1.25 uL of 10pmol/ul forward primer, 1.25 ul of 10pmol/ul reverse primer, 3ul of 25mM MgCl, 0.2 uL 5U/ul TAQ polymerase, 7.5 ul RT product (undiluted for ATR1 reactions; 1:50 dilutions for GAPDH reaction), and brought up to 50 uL with nuclease-free water. Nested PCR targeted to the terminal exon of the ATR1 gene (Figure 2A) was performed using 1:100 dilutions of the outer (first round) PCR products to seed the inner (second round) PCRs, which otherwise followed the same reaction recipe as the inner RT-PCRs. Outer (first round) PCR products were purified on silica gel columns (PCR purification kit, Qiagen) prior to use in nested PCR. Primer sequences for target genes were as follows: ATR1 outer forward 5'- GACAGATGACGGCTGCTCG -3' and reverse 5'- AGACACTACTACT TGGGACCAGTGC -3', ATR1 inner forward 5'- CCAAAGGGCAGTAAAGTTTTCG-3' and reverse 5'- GCAACTTGACGACTACTGCTTAGC-3'. Only single round GAPDH RT-PCRs using GAPDH primers designed with the following sequences: forward 5'- GTGAAGGTCGGA GTCAACGG-3' and reverse 5'- GTGGTGAAGACGCCAGTGG-3'. Both single round and nested PCRs were run with an initial 10 minute denaturation step at 95°C followed by 40 cycles of 20 seconds of denaturation at 95°C, 30 seconds of annealing at 56°C, and 1 minute of extension at 72°C and a final 7 minute period of extension at 72°C. PCR products were run on 2% agarose gel with 1 µg/ml ethidium bromide and visualized under ultraviolet light.

7. Nested qPCR

Outer first round RT-PCRs for both ATR1 terminal exon and GAPDH were performed as described. Outer PCR products were purified on silica gel columns

(PCR purification kit, Qiagen) following the manufactures instructions. Inner second round qPCRs were performed using 1:100 dilutions of outer ATR1 terminal exon PCR products and 1:625 dilutions of outer GAPDH PCR products. Nested ATR1 terminal exon qPCRs were prepared by adding 7.5uL of the purified and diluted outer PCR products to 12.5µL 2x TaqMan Universal Master Mix with AmpErase UNG (Applied Biosystems), 2.25ul each of 10pmol/ul forward and reverse primers, 0.0625ul of 100uM probe. Reactions were brought up to a final volume of 25ul with nuclease free water. GAPDH qPCRs were prepared as described for single round (non-nested) qPCR. Thermal cycling and fluorescence measurements were performed as described for single round (non-nested) qPCR.

8. 5' RLM-RACE

5' RNA ligase mediated rapid amplification of cDNA ends (5' RLM-RACE) was performed using a commericial kit (FirstChoice® RLM-RACE Kit, Ambion, Applied Biosystems) following the manufacturer's instructions. In brief, 10ug total RNA isolated from cells with RNEasy, was first treated with calf intestine alkaline phosphatase (CIP) in a reaction volume of 20uL incubated at 37°C for 1 hour to cleave open ended 5' phosphate groups from degraded mRNA, rRNA, tRNA, and any DNA present. The CIP reaction was terminated and the CIP-treated RNA recovered with ammonium acetate and acid phenol:chloroform and isopropanol precipitation. The RNA pellet was washed once with 70% ethanol and resuspended in 11uL nuclease free water. 4uL of the CIP-treated RNA was then treated with tobacco acid pyrophosphatase (TAP) in a 10uL reaction volume incubated at 37°C for 1 hour to cleave the 5' 7-methylguanosine cap + 2 phosphate groups from intact mRNA leaving one 5' phosphate group still attached. 2uL of the CIP/TAP-treated RNA (or RNA treated with CIP only (TAP minus control)) was ligated to the given 5'RACE RNA adapter (5'-GCUGAUGGCGAUGAAUGAA CACUGCGUUUGCUGGUUUGAUGAA-3') at its 5'end with T4 RNA Ligase in a 10 uL reaction volume incubated at 37°C for 1 hour. 2uL of the ligated RNA was then used for reverse transcription with

random decamers and M-MLV reverse transcriptase in a reaction volume of 20uL incubated at 42°C for 1 hour.

RT products were put through a nested PCR protocol using two sets of primers. The outer 5'RLM-RACE PCR was carried out with the provided forward 5' RACE outer primer (5'-GCTGATGGCGATGAATGAACACTG-3') and a reverse outer primer targeted to the 5' region of the terminal exon of the ATR1 mRNA transcripts (5'-ACTATCACCACCAAGCTGTTTC-3'). The inner (nested) 5'RLM-RACE PCR was carried out with the provided forward 5'RACE inner primer (5'-CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG-3') and a reverse inner primer targeted to the 3' region of the terminal exon of the ATR1 mRNA transcripts (5'-AGCTTTGGGACAATCATCTTGG-3'). 1uL of the RT product was used to seed the outer PCR and 2uL of the outer PCR product was used to seed the inner (nested) PCR. Both outer and inner 5'RLM-RACE PCRs were run in a 50uL reaction volume using 1.25 U of Super Taq-Plus thermostable DNA polymerase (Ambion, Applied Biosystems) with an initial 3 minute 94°C denaturation step followed by 40 cycles of 30 seconds denaturation at 94°C, 30 seconds of annealing at 60°C, and 30 seconds of extension at 72°C, with a final extension period of 7 minutes at 72°C. 10uL of the inner PCR product was run on a 3% agarose gel containing 1ug/ml ethidium bromide and visualized under ultraviolet light.

IV. Results

1. ATR1 expression in monocytes

The ATR1 protein was readily detected in all cell types (data not shown). Two expected bands were seen with western blots for all cell types: one at 41kD and one at 55kD. Semi-quantification of the western blot bands showed that expression levels were HMEC1>HK-2>U937=THP-1>fresh monocytes (data not shown).

Using single round qPCR with predesigned primer/probe sets, expression of ATR1 mRNA transcript splice variants 1, 2, 3, 4, and 5 was readily detected in HMEC1 (Table 3). The levels of expression differed somewhat between the different splice variants though statistical comparison between levels of expression for each variant in qPCR has not been done. qPCR did not show expression of any of the ATR1 splice variants in U937 or THP-1 (Table 3). Single round conventional RT-PCR with the ATR1TE2 outer primers targeted to the terminal protein-coding exon common to all transcript variants showed a distinguishable PCR product at the expected 469bp size in HMEC1 (Figure 7). However, the same protocol revealed a distinguishable PCR product of only 300bp in size in U937 (Figure 7). Nested RT-PCR using the first round outer PCR products revealed a nested PCR product of the expected 115bp in U937 (Figure 7) as well as in freshly isolated monocytes and HMEC1 (not shown). Single round RT-PCR with the ATR1TE inner primers revealed a PCR product of the expected 115bp in HMEC1, U937, and THP-1 (Figure 7). The PCR bands have not been quantified but it appears that the levels of expression of the ATR1 terminal exon are HMEC1>THP-1>U937 (Figure 7). Only single round RT-PCR using the designed GAPDH primers was performed in all experiments: a single PCR product of the expected 300bp size was consistently observed. qPCR using the ATR1TE inner primers and probe confirmed this pattern of expression (Table 3). Nested qPCR with the ATR1 primers targeted to the terminal protein-coding exon

Table 3. Expression of ATR1 mRNA in HMEC1, U937, and THP-1

ATR1 mRNA qPCR target transcripts	**HMEC1**	**U937**	**THP-1**	**Hk-2**	**HUVEC**
Variant 1 (Hs99999095)	CT:30.36-30.48 dCT: 1.40	Undetected	Undetected	Undetected	CT: 36.01-36.84 dCT: 1.46
Variant 2 (Hs01096942)	CT :34.40-34.77 dCT: 1.57	Undetected	Undetected		
Variants 3 and 4 (Hs00258938)	CT 29.81-30.05 dCT: 1.47	Undetected	Undetected	CT: 37.05-38.11 dCT: 1.84	CT: 36.85-39.54 dCT: 1.54
Variant 1 and 4 (Hs00241341)	CT: 38.07-38.56 dCT: 1.78	Undetected	Undetected	Undetected	CT: 46.03-47.47 dCT: 1.88
Variant 5 (Hs00259315)	CT: 34.22-34.29 dCT: 1.46	Undetected	Undetected		
Terminal exon - all variants (ATR1TE)	CT: 35.12-35.97 dCT: 1.45	CT: 41.79-43.22 dCT: 1.71	CT: 41.35-42.00 dCT: 1.72		
Terminal exon - all variants (nested qPCR)	CT: 16.79-16.82 dCT: 0.50	CT: 13.62-13.67 dCT: 0.41	CT: 13.12-13.13 dCT: 0.40		

mRNA levels were measured by qPCR using primers and probes targeted to particular mRNA transcript variants and to the terminal exon common to all variants. The range of values for the detected cycle threshold (Ct) in ATR1 qPCRs are reported above averaged and GAPDH corrected cycle threshold (dCt) values.(dCT = ave ATR1 Ct/ave GAPDH Ct). Primer-probe catalogue numbers/names are given in brackets.

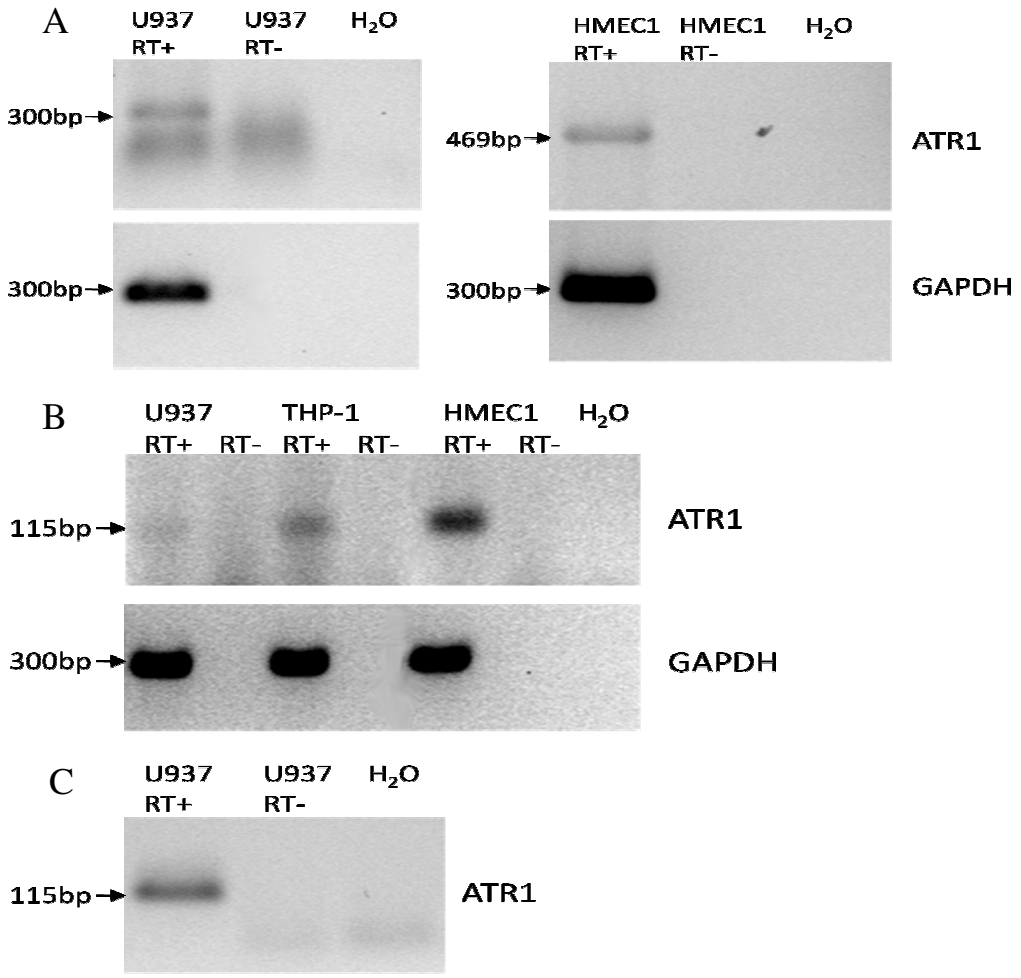

Figure 7. RT-PCR analysis of the expression of the ATR1 mRNA terminal protein-coding exon in monocytes and endothelial cells. (A) Single round RT-PCR using outer ATR1 terminal exon primers showing a 300bp product in U937 and a 499bp product HMEC1. For internal reference, single round RT-PCR targeted to the GAPDH gene is shown in the lower panel. (B) Single round RT-PCR using inner ATR1 terminal exon primers showing a 115bp product in U937, THP-1, and HMEC1. For internal reference, single round RT-PCR targeted to the GAPDH gene is shown in the lower panel. (C) Nested RT-PCR using the outer ATR1 terminal exon primers to amplify U937 cDNA and the inner ATR1 terminal exon primers to amplify PCR products generated in the outer PCR reaction. Nested RT-PCR yielded a product of the expected 115bp. Nested RT-PCR targeted to the GAPDH gene was not performed. Lanes marked with 'RT+' indicate that PCR reactions were performed on products (cDNA) from reverse transcritption (RT) reactions performed in the presence of reverse transcriptase enzyme. Lanes marked with 'RT-' indicate that PCR reactions were performed on products from RT reactions performed without reverse transcriptase enzyme (no cDNA generated) to eliminate the possibility that PCR products observed were the result of amplification of DNA contaminants. Lanes marked 'H2O' indicate that PCRs were performed without any template to ensure that PCR components were not contaminated with DNA

(used in the RT-PCR) and the relevant probe confirmed expression of the ATR1 terminal exon in HMEC1, U937, and THP-1 though the quantification of these results is not reliable due to issues with contamination or nonspecific primer/probe binding and amplification.

2. 5' RLM-RACE in monocytes

5' RLM-RACE revealed two bands in HMEC1 one at around 220bp and a fainter band around 300bp (Figure 8). No bands were visible in THP-1 samples (Figure 8). TAP minus controls for both HMEC1 and THP-1 were clean indicating that amplification occurred only on intact mRNA with ligated 5' adapters. Conventional RT-PCR using the 5'RACE adapter ligated and unligated mRNA showed expression of GAPDH in all samples (Figure 8) confirming the presence of RNA.

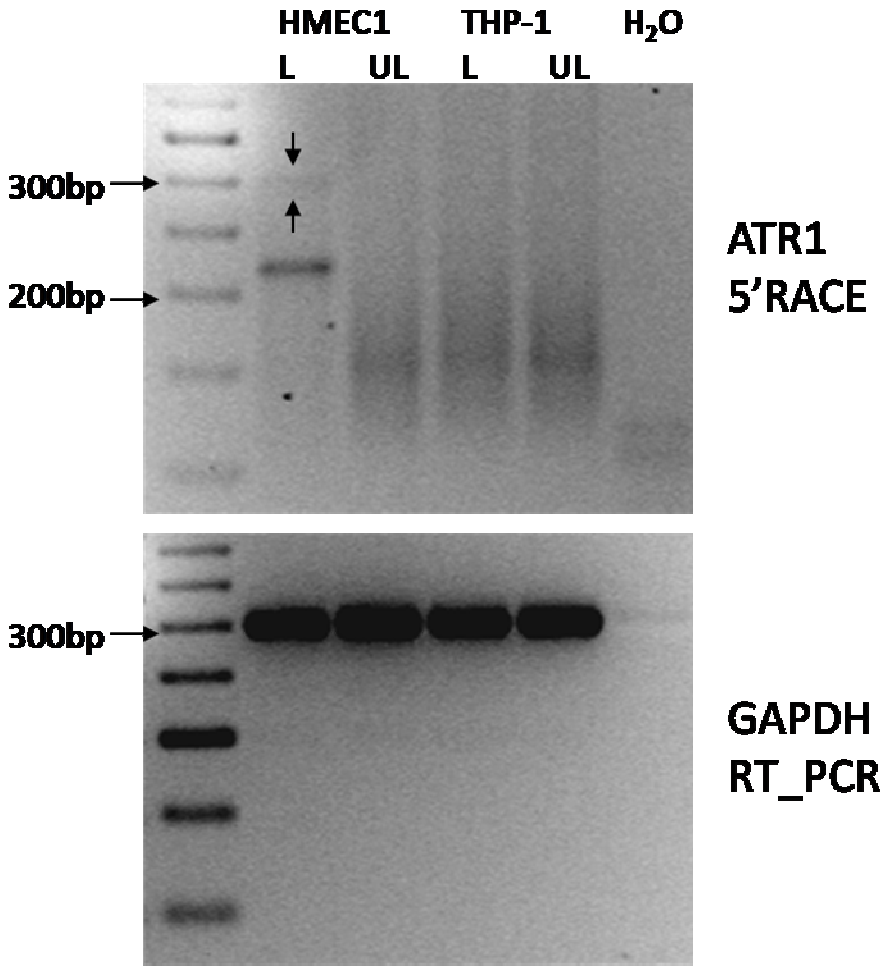

Figure 8. 5'RLM-RACE of the ATR1 (reverse primer targeted to the 5' end of the terminal protein-coding exon) in HMEC1 and THP-1. A ~220bp product and a faint ~300bp product (between vertical arrows) was observed following 5'RLM-RACE using total HMEC1 RNA. No products were observed in 5'RLM-RACE reactions using total RNA from THP-1. Lanes marked with 'L' indicates that total RNA was treated with tobacco acid pyrophosphatase (TAP) to cleave the 5'methyl guanosine cap from intact mRNA to allow ligation of the 5'RACE adapter to the 5' end of intact mRNA. Lanes marked with 'UL' indicates that total RNA was not treated with TAP such that ligation of the 5'RACE adapter should not have occurred. The absence of any bands in the UL lanes indicates that adapter ligation occurred only on intact mRNA and that the appropriate ATR1 sequences were amplified. The lane marked with 'H2O' indicates that the nested PCR protion of the 5'RLM-RACE was performed without addition of template to ensure that PCR components were free of contamination. Conventional single round RT-PCR using primers targeted to the GAPDH gene was also performed on the L and UL reverse transcription products (lower panel). Products of the expected 300bp were observed in all PCRs except those run without template ('H2O' lane).

V. Discussion

In the present study, we have investigated the expression of the human ATR1 gene in monocytes relative to other cell types. The ATR1 protein has been previously identified in monocytes/macrophages from whole cell lysates by western blotting and on the cell surface by immunohistochemistry and FACS (see Table 1 for references). In line with these reports, the ATR1 protein has been identified here in whole cell lysates from U937, THP-1, and freshly isolated monocytes by western blotting. Similarly, ATR1 mRNA has been previously identified in monocytes, namely by conventional RT-PCR (Table 2).

ATR1 mRNA was identified in HMEC1, U937, and THP-1 using conventional single round and nested RT-PCR targeted to the terminal protein-coding exon of the ATR1 gene. Interestingly, a PCR product of only around 300bp was seen in single round RT-PCR using the outer primers where a 469bp product was expected. The primers used were designed to target a sequence within the 3'UTR. This 300bp fragment may be the result of an amplification of an undesired target, while amplification of the correct ATR1 sequence was not sufficient for detection by EtBr electrophoresis and visualization under UV illumination. It is also possible that the ATR1 transcript in monocytes is lacking a 169bp sequence at the 3'UTR in between the forward and reverse primers used for the outer single round/nested RT-PCR. Using the inner ATR1 primers, single round RT-PCR in U937, THP-1, and HMEC1 and nested RT-PCR in U937 and THP-1 yielded PCR products of the expected 115bp size. The inner forward primer localized to a sequence that is 199bp away from the 5' edge of the outer 5' priming site. As such, a deletion of 169bp may have occurred in the monocyte ATR1 transcripts to yield an outer RT-PCR product of only 300bp instead of the expected 469bp. Quantitative PCR using the same primers as were used for conventional RT-PCR, confirmed ATR1 expression in U937, THP-1, and HMEC1. The results of the western blot, RT-PCR and qPCR analyses reported confirm the expression of ATR1 protein and mRNA in monocytes.

However, qPCR targeted to the 5'UTRs of the 5 NCBI-catalogued ATR1 mRNA splice variants yielded signal only in HMEC1. No qPCR signal was obtained for any of the 5 ATR1 variants in U937 or THP-1. These results suggest that both monocytic cell lines, U937 and THP-1, do not express any of these variants while HMEC1 express all 5 variants to varying degrees. It is also possible that expression of the known variants in monocytes is too low for single round qPCR to achieve a reliable amplification and detection. The expression profile of the known ATR1 transcript variants by qPCR or other techniques has not been examined in monocytes or other leukocytes to date. However, given that we and others have confirmed expression of the ATR1 terminal protein coding exon and the ATR1 protein itself, it is possible that monocytes predominantly express an ATR1 mRNA splice variant with a novel combination of 5' untranslated exons.

5'RLM-RACE using a reverse ATR1 gene specific primer targeted to the 5' end of the terminal protein-coding exon found in all known ATR1 mRNA transcript variants, was performed to determine the expression profile of ATR1 splice variants in HMEC1 and THP-1. Experiments using 5'RLM-RACE are quite preliminary. However, 5'RLM-RACE yielded a 220bp and a 300bp product in HMEC1. Using the 5'RLM RACE target size chosen here, the expected sizes of amplification products from NCBI catalogued ATR1 splice variants 1, 2, 3, 4, and 5 are 486, 402, 460, 544, and 360 base pairs respectively. Given that only the terminal protein coding exon was expressed, an amplification product of 144 base pairs is expected. Neither the 220bp or 300bp fragments generated here appear to correspond to any of the previously identified, NCBI catalogued splice variants based strictly on size. These fragments may represent the 5' end of a yet unidentified variant or amplification of a non-ATR1 sequence. DNA sequencing of these fragments will give further insight into their origin and the possibility that they represent 5' sequences from two distinct ATR1 transcript variants. 5'RLM-RACE yielded no products in THP-1 perhaps indicating that the 5' end of the terminal exon found in monocytes differs from that of the known ATR1

variants. It is also possible that expression of the ATR1 gene in monocytes is insufficient for detection with 5'RACE. It has been reported that ATR1 mRNA expression in monocytes is significantly lower compared to monocyte-derived macrophages (145, 196). Though statistical analysis has not been performed, single round qPCR targeted to the terminal protein coding exon performed in this study suggests that monocyte expression of ATR1 mRNA is considerably lower compared with endothelial cells (Figure 7). In any case, 5'RLM-RACE reaction conditions need to be optimized further and more trials run before any conclusions regarding ATR1 transcript variant expression in monocytes can be made.

The ATR1 is undoubtedly expressed in monocytes given the positive results of western blotting and RT-PCR and qPCR targeted to the terminal protein coding exon common to all ATR1 mRNA transcript variants. However, qPCR targeted to the 5'UTR of the known ATR1 transcript variants indicates that these variants are not expressed in monocytes, while they are expressed in other cell types such as endothelial cells. Interestingly, all of the studies that have identified ATR1 mRNA in monocytes by RT-PCR have used primers that were targeted to the terminal protein-coding exon (Table 2); no studies have identified ATR1 mRNA in monocytes with qPCR, even where this method is used to identify other gene transcripts in the same study. The results presented here indicate that a novel monocyte-specific ATR1 mRNA transcript variant may exist. Given the relation between hypertension, inflammation and vascular damage, such a splice variant may have a separate role in the cardiovascular disease process. Further trials with 5'RLM-RACE and DNA sequencing of the 5'RLM-RACE products is necessary to substantiate this hypothesis.

It has been suggested that certain physiological or pathophysiological stimuli modulate alternative splicing of the ATR1 gene, which may function to alter cell responsiveness to Ang II under certain conditions (64). Warnecke *et al.* (209) demonstrated that the ratio of transcripts containing exon 2 to those lacking exon

2 decreased in atria from failing hearts when compared to the atria from healthy hearts. TGFβ1 treatment of human lung fibroblasts was shown to upregulate transcript variants containing exons 1 and 4 and variants containing exons 1, 2, and 4, while variants containing exons 1, 3, and 4 were downregulated (119). Cytokine treatment of rat cardiac fibroblasts was shown to upregulate all of the known rat ATR1a splice variants (41). Splicing of the ATR1 transcripts and hence the expression of ATR1 protein and the responsiveness of monocytes to Ang II may be affected by hypertension, a known risk factor for the development of atherosclerosis and cardiovascular disease, chronic stress, and other chronic inflammatory conditions such as chronic kidney disease.

VI. Conclusions and Perspectives

The results presented here show that monocytes express the ATR1 at the mRNA and protein level. A novel, monocyte-specific mRNA splice variant may be responsible for this expression. Monocytes and macrophages are intimately involved in the atherogenic process. Changes in the expression profile of the ATR1 transcript variants within monocytes as brought about by chronic stress, hypertension, chronic kidney disease, or other chronic inflammatory conditions may contribute to increased Ang II signaling in these cell types and hence enhanced monocyte/macrophage activity in the context of atherogenesis. Further studies should aim to elucidate the expression profile of ATR1 transcript variants in monocytes and changes in this expression profile in response to conditions of chronic inflammation.

The RAS is intimately involved with the stress response and Ang II is a major stress hormone, like the glucocorticoids and catecholamines. RAS activity and circulating levels of Ang II increase in response to acute stressors as a result of sympathetic stimulation of juxtaglomerular cells which subsequently release renin thereby accelerating the proteolytic generation of Ang II. Recurrent stressors or chronic sustained stressors may also result in elevated levels of RAS activity and Ang II levels, but may also increase the responsiveness of the RAS to novel stressors. Ang II likely has a physiological purpose in the stress response by acutely maintaining extracellular fluid volume and blood pressure and by enhancing immune function.

The immune response to stress is complex. Acute stress results in a general enhancement of immune function: leukocytes are redistributed to sites important for fist line defence against infections and are stimulated to increase their activity. Ang II is now recognized to have potent pro-inflammatory effects and cells of the vascular wall, including endothelial cells and vascular smooth muscle cells, as

well as the spectrum of leukocytes express angiotensin II receptors. The actions of Ang II on immune and vascular cells stimulates chemotaxis, margination, and diapedesis and hence redistribution as well as direct enhancement of immune cell function, including phagocytosis and cytokine production. It is the view of the authors that increased RAS activity and increased circulating levels of Ang II, among other factors, are directly involved in mediating acute stress-induced immune enhancement. Such an enhancement primes the immune system to respond to immune challenges that may result from an encounter with a potentially harmful stimulus.

There appears to be a link between chronic stress and the development of atherosclerosis, however the precise mechanisms behind this link is unknown. While the role of the RAS during recurrent or chronic sustained stress is not fully understood, available evidence suggests that RAS activity may be sustained or sensitized to novel stressors in response to recurrent or chronic stressors. Inappropriately sustained or hyper-reactive RAS activity in recurrent or chronic stress may be involved in the development and acceleration of atherosclerosis through hemodynamic and pro-inflammatory effects and through effects on other pathways of the stress response. RAS inhibitory treatment may prove beneficial to individuals in situations of unresolving chronic stress.

The role of the RAS in the immune response to stress and in the development of chronic stress-associated atherosclerosis and cardiovascular disease is still unclear. However, available evidence suggests that chronic or repeated exposure to stress results in elevated levels of circulating Ang II. Along with other factors, including circulating lipid abnormalities and the effects of glucocorticoids, increased Ang II brought about by chronic stress may result in a state of chronic inflammation thereby contributing to atherogenesis. Further research exploring these links may provide valuable insight into the physiological and pathophysiological functions of the RAS.

VII. References

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Appendix A: The prorenin receptor is regulated by oxidative stress in kidney proximal tubule cells [2]

[2] This data is included in a manuscript entitled 'Reactive Oxygen Species (ROS) Maintain Renal Expression of ProRenin Receptor (PRR)' (S. Wesseling, M. Groeschel, B. Braam, and J.A. Joles) to be submitted for publication.

1. Introduction

The activity of renin is the rate limiting step of angiotensin II (Ang II) generation. Renin is derived through the cleavage of a 43-amino acid N-terminal propeptide from its precursor, prorenin both within kidney juxtaglomerular cells and in the circulation. However, prorenin is secreted constitutively from the kidney (47) and prorenin blood plasma concentration is approximately 10-fold higher than that of renin (47, 140). Prorenin has the ability to convert angiotensinogen into angiotensin I given that conformational change moves the prosegment out of and exposes the enzymatic site.

The prorenin receptor (PRR) is a 350-amino acid protein with a single transmembrane domain (140). The PRR colocalizes at the cell membrane with a vacuolar H+-ATPase (Ludwig 1998) that functions to maintain an acidic pH in intracellular vesicles and regulates cellular pH homeostasis(142).It is thought that the PRR is ubiquitously expressed during development and mediates essential non-renin-angiotensin system effects on cell survival (139). However, the PRR gene is expressed in specific tissues in developed humans with high levels of expression in the human brain, heart, placenta, vasculature, and retina as well as reduced expression in the liver, pancreas, and kidney as well as weak expression in the lung and skeletal muscle (26, 28, 49, 140).

The PRR binds prorenin with higher affinity than renin (134). Binding of prorenin to the PRR causes prorenin to become catalytically active without proteolytic cleavage of the pro-segment. Prorenin undergoes a reversible conformational change upon binding to PRR that exposes its active site (139), which converts angiotensinogen into angiotensin I at the cell surface. As such, the PRR, through activation of prorenin, can increase the generation of angiotensin II which may contribute to regulation of blood pressure and volume and hence the development of hypertension and cardiovascular disease. Moreover, renin or prorenin binding to the PRR activates intracellular signaling pathways, independent of angiotensin and blood pressure, that upregulate the expression of profibrotic genes, which

may contribute to cardiac and renal fibrosis, growth, and remodeling as well as vascular damage (87, 88, 97, 98, 139, 155, 174, 192, 204). Available evidence suggests that PRR plays an important role in endorgan damage in diabetic and inflammatory pathologies(139). Because of its involvement in cardiovascular and renal disease, the PRR may be an important target for pharmacologic therapy.

Reactive oxygen species (ROS) are known to contribute to the development of hypertension, vascular inflammation, and cardiovascular disease (148). Angiotensin signaling, via the angiotensin II receptor type 1, is involved in the generation of ROS. There are a number of mechanisms by which ROS lead to the development of these conditions including activation of the inflammatory nuclear transcription factor, NF-κB, in cells of the vascular wall and leukocytes (197). Given its apparent involvement in cardiovascular disease, the PRR may also be regulated by ROS. However, whether ROS regulates the expression of PRR is not known. We hypothesized that ROS upregulate the expression of the PRR in kidney proximal tubule cells. We examined changes in the mRNA expression of the PRR after stimulation of a kidney proximal tubule cell line with hydrogen peroxide (H_2O_2).

The PRR has pro-inflammatory and pro-fibrotic activity when activated by either renin or prorenin, which is likely involved in the development of kidney disease but may also play an important role in RAS-driven atherogenesis. This thesis also explores the regulation of kidney PRR by oxidative stress, which is known to contribute to vascular inflammation and atherosclerosis. Changes in the mRNA expression of the PRR after stimulation of a kidney proximal tubule cell line with hydrogen peroxide (H_2O_2) were examined. Hypothesis: ROS upregulates the expression of the PRR in kidney proximal tubule cells.

2. **Methods and Materials**

An immortalized epithelial cell line derived from human kidney proximal tubules (Ryan ea KI 1994), human kidney-2 (HK-2), was obtained from ATCC (Manassas, VA, USA). HK-2 cells were cultured at 37oC and 5% CO2, using Gibco RPMI 1640 medium (with 25mM HEPES buffer and L-glutamine) (Invitrogen, Grand Island, New York, USA) supplemented with 10% fetal bovine serum (FBS) (VWR), 5ng/mL epidermal growth factor(VWR 324831), 100 U/ml penicillin, and 100 µg/ml streptomycin.

Cells were grown to confluence in six-well plates and rested in Gibco RPMI 1640 medium supplemented only with 1% fetal bovine serum (FBS) overnight. Following starvation cells in each well were exposed to 0 (untreated), 1 or 10 µM $H_2O_2$for 4 or 24h. Each well = 1 N. For the 4hr stimulations, N = 19 for 0 µM H_2O_2, N = 13 for both 1 and 10 µM H_2O_2. For the 24 hr stimulations, N = 8 for 1 µM H_2O_2, and N = 10 for both 0 and 10 µM H_2O_2. After exposures cells were harvested in TRIzol reagent (Invitrogen). Total RNA extraction from TRIzol solution was performed using the procedure recommended by the manufacturer, quality and quantity assessed using a spectrometer and stored at -80^{o}C. Reverse transcription (RT) was carried out using the iScript cDNA synthesis kit (BioRad 170-8891) following the manufacturer's instructions. Reactions were prepared on ice using 5µg total RNA; 4 µL of 5x iScript reaction mix (containing a blend of oligo(dT) and random primers along with the necessary buffers) and 1 µL of iScript (RNase H^+ MMLV) reverse transcriptase were added and brought up to a final volume of 20 µL with nuclease-free water. Reactions were incubated for 5 minutes at 25°C followed by 30 minutes at 42°C. Reactions were terminated by incubating at 85°C for 5 minutes and stored at -20°C until further processing.

Gene expression was assessed by real-time, quantitative PCR (qPCR) using pre-designed probe and primer sets for the prorenin receptor (Hs00366626_m1) and GAPDH (Hs9999905_m1) as an endogenous control (Applied Biosystems TaqMan® Assays-on-Demand™, Foster City, CA). PCRs were performed in a

25µL reaction volume by combining 7.5µL RT product (undiluted for prorenin reactions; diluted 1:625 for GAPDH reactions), 12.5µL 2x TaqMan Universal Master Mix with AmpErase UNG (Applied Biosystems), 1.25µL of the appropriate target assay primer-probe mix, and 3.75µL of nuclease-free water. PCR was carried out in an Applied Biosystems PRISM 7500 Sequence Detection System (Applied Biosystems) with an initial 10 minute step at 95°C, followed by 45 cycles of 15 seconds at 95°C and 1 minute at 60°C. Fluorescence was measured during the last 30 seconds of the 60°C step. Expression of each target gene in each sample was normalized to the endogenous control GAPDH (ΔCt) and related to a reference (ΔΔCt), being RNA from untreated HK-2 cells. Gene expression is presented as ΔΔCt ± standard error (SE). Data was analyzed for statistical significance using single-factor ANOVA and the Neuman-Keuls post-hoc test.

3. Results

After 4hrs of stimulation with 1µM H_2O_2, PRR expression was 0.192 ± 0.4 ΔCt higher, though not significantly, than unstimulated cells. After 4hrs of stimulation with 10µM H_2O_2, PRR expression was significantly higher (1.603 ± 0.3 ΔCt, $p < 0.05$) higher than unstimulated cells and was also significantly higher ($p < 0.05$) than in cells stimulated with 1µM H_2O_2. After 24hrs of stimulation with 1µM and 10µM H_2O_2, PRR expression was 0.722 ± 0.1 and 0.280 ± 0.3 ΔCt lower than unstimulated cells respectively. However this drop n PRR expression was not statistically significant. * $p < 0.05$ versus untreated (0µM H_2O_2) and 1µM H_2O_2stimulated cells. See Figure A-1.

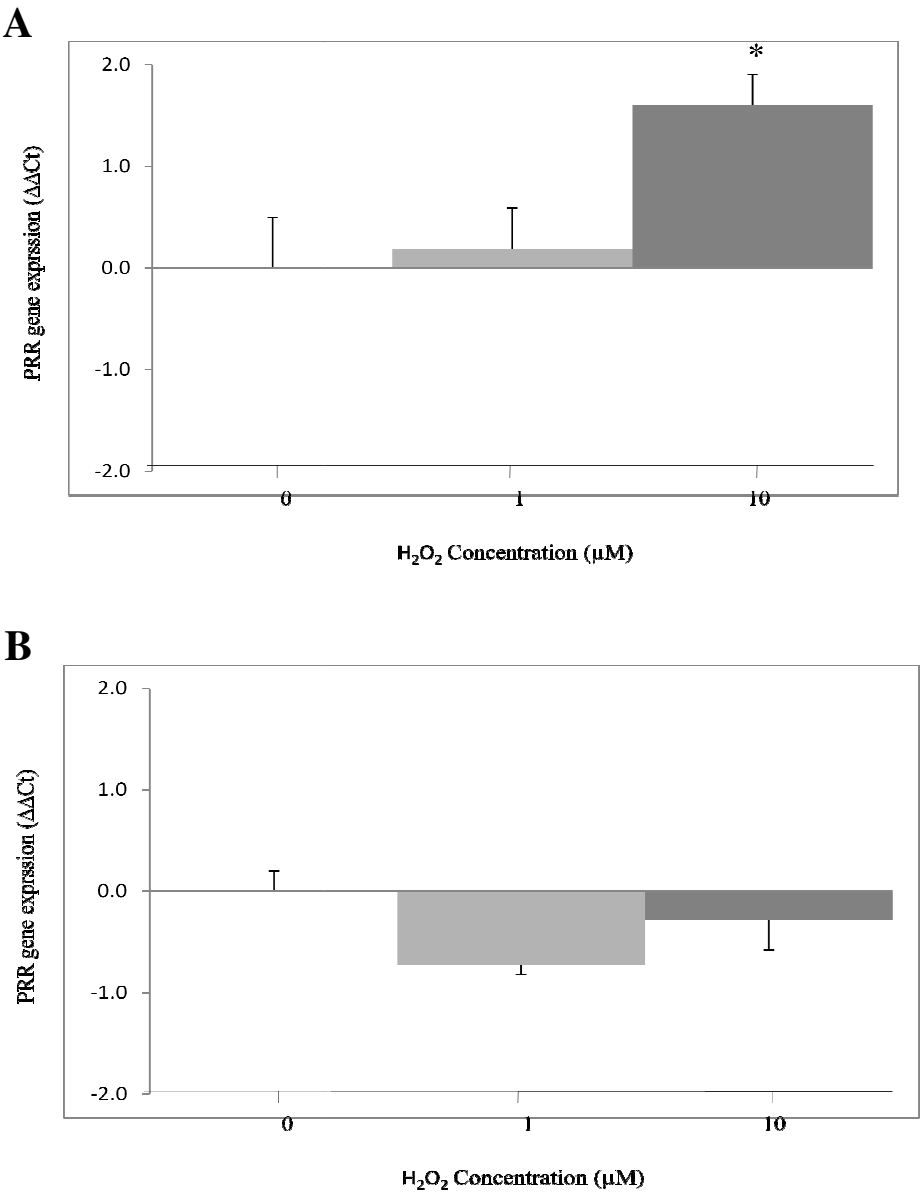

Figure A-1. Relative expression of prorenin receptor (PRR) mRNA in an immortalized human kidney proximal tubule cell line (HK-2) as measured by qPCR following (**A**) 4hrs and (**B**) 24hrs of stimulation with 0, 1, and 10 μM of H_2O_2. Gene expression for each concentration is presented as a difference from expression measured following stimulation with 0 μM of H_2O_2. For the 4hr stimulations, N = 19 for 0 μM H_2O_2, N = 13 for both 1 and 10 μM H_2O_2. For the 24 hr stimulations, N = 8 for 1 μM H_2O_2, and N = 10 for both 0 and 10 μM H_2O_2. * $p < 0.05$ versus untreated (0μM H_2O_2) and 1μM H_2O_2stimulated cells.

4. **Discussion**

In the short term (4 hours), stimulation with H_2O_2 appears to increase the expression of the PRR in kidney proximal tubule cells in a dose-dependent manner (Figure A-1). However, prolonged stimulation (24 hours) with H_2O_2 did not appear to increase or decrease PRR gene expression significantly (Figure A-1). The viability of HK-2 cells, which was not tested, may have been compromised by the prolonged length (24 hours) of direct stimulation with H_2O_2. Otherwise, ROS may in fact stimulate upregulation of the PRR gene in HK-2 cells over the short term, and may return to baseline if cells are continually stimulated.

Diabeteic and inflammatory pathologies that lead to cardiovascular and renal disease are associated with increased oxidative stress (31). Given that ROS appear to upregulate the PRR, at least in the short term, increased signalling via the PRR may contribute to the pro-fibrotic changes that are seen with renal and cardiovascular diseases. Though we have examined regulation of the PRR in kidney proximal tubule cells here, similar mechanisms of regulation by oxidative stress may be at work in cells of the vasculature where the PRR has a high level of expression in adults. Given that oxidative stress upregulates the PRR in the vasculature, enhanced generation of Ang I and hence Ang II, and through the pro-inflammatory and pro-fibrotic signalling pathways that follow activation of PRR may contribute to the development of atherosclerosis under circumstances of hypertension and other chronic inflammatory conditions. Future studies should examine the regulation of the PRR by oxidative stress in other cell types, including vascular endothelial and smooth muscle cells.

5. References

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