Angiotensin II and its direct effects on rat neutrophils

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Angiotensin II and its direct effects on rat neutrophils

by

Eric Thomas Weatherford

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

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Major: Zoology

Program of Study Committee:
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Iowa State University

This is to certify that the master's thesis of

Eric Thomas Weatherford

has met the thesis requirements of Iowa State University

Signatures have been redacted for privacy
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Neutrophils are thought to cause tissue damage following hemorrhagic shock. The initial injury resulting from trauma can initiate a cascade of events leading to a non-specific inflammatory response. This inflammatory response results in the priming of neutrophils, which, upon activation by a secondary stimulus, release their armament of cytotoxic substances indiscriminately. This results in tissue damage away from the initial insult. The renin-angiotensin system and its major signaling molecule angiotensin II (angII) play an important role in stimulating mesenteric vasoconstriction and ischemia which is thought to be a key step in provoking the systemic inflammatory response. Evidence for a stimulatory effect of angII directly on neutrophils suggests that it might play an additional role in the inflammatory response following trauma.

In this study, I planned to confirm results obtained in other labs which have implicated angII as a stimulator of ROS production and CD11b up regulation. Rat neutrophils were isolated and incubated with angII, fMLP, PMA, or losartan followed by angII. Reactive oxygen species production was quantified using dihydrorhodamine 123 oxidation to fluorescent rhodamine 123, and the effects of angII on CD11b expression were detected using FITC conjugated anti-CD11b antibodies. The mean fluorescent intensity of rhodamine 123 and FITC was measured using flow cytometry. Angiotensin II at concentrations of 500 nM, 1 nM, and 0.5 nM stimulated increased expression of CD11b but had no effect on ROS production. This data confirms the effect of angII on CD11b expression, but contradicts previous findings by others in regards to the effect on ROS production. Despite the lack of ROS production, stimulation of increased CD11b expression still implicates angII as a molecule capable of modulating neutrophil
function (CD11b is a pivotal player in neutrophil mediated tissue damage). Combined with its established vascular effects and evidence that angII concentrations can remain elevated several days after traumatic insult, the observed CD11b elevation adds to the data suggesting that angII might be an important target for therapeutic intervention in the prevention of MODS.
Multiple Organ Dysfunction Syndrome (MODS)

Multiple organ dysfunction syndrome is a pattern of progressive altered organ function that may result in organ failure, and potentially, death. It is a major complication of hemorrhagic shock, trauma, sepsis, severe burn, and pancreatitis; and is the leading cause of death in the intensive care unit (Deitch, 1999; Murray, 1993; Brady, 2001). There is a considerable amount to learn about the pathogenesis of the syndrome, but many agree that MODS can be caused by tissue damage resulting from a dysregulated inflammatory response initiated by injury and/or infection. Systemic inflammatory response syndrome (SIRS) is a normal response to a variety of insults, including those already mentioned. It is a state of systemic hyperinflammation that involves numerous inflammatory pathways and effector cells. Over exuberance of that inflammatory response contributes to further tissue damage not only at the initial site of the injury or infection, but also at sites not originally affected.

Two-Hit Model

A two-hit model has been developed to explain the development of MODS post trauma. This model describes an initial priming event, such as hemorrhagic shock, that can prime the inflammatory response and leave a patient vulnerable to a secondary stimulus resulting in amplified SIRS (Partrick and Moore, 1996). If exposure to a secondary stimulus can be avoided, tissue damage resulting from the original trauma may resolve itself. As the inflammatory response proceeds, counter-regulatory pathways are activated to prevent tissue damage caused by inflammatory cells such as neutrophils in areas not originally injured. Depending on the intensity of these pathways, they may lead to severe immunosuppression leaving the patient susceptible to infection, or alternatively, may not be sufficient to prevent
tissue damage due to intensified SIRS. Thus, preventing amplified SIRS and a non-specific inflammatory response are important to averting the development or progression of MODS after initial injury.

**Neutrophil Mediated Tissue Damage in Progression to MODS**

Neutrophil priming and activation is the cellular analogue to the two-hit model described previously. Priming is the enhancement of a cell response to a stimulus after previous exposure to another agonist. The first hit (trauma) in the two hit model is recognized by some as the priming event for neutrophils. Post-injury neutrophils are primed for increased superoxide production and cytokine and protease release and have increased CD11b expression as well as delayed apoptosis (Botha, 1995; Zallen, 1999; Quaid, 2001; Nolan, 2000). Together, these effects lead to an increased population of more adhesive, cytotoxic neutrophils that can sequester in tissues and damage them when exposed to a second hit or activating stimulus. The time window for neutrophil priming is debated but appears to occur three to six hours after injury (Botha, 1995). Nearly any molecule that can stimulate neutrophils can act as a priming agent depending on its concentration and the time of exposure.

In order for neutrophils to damage tissue, it is essential that they can adhere to their target. These interactions are mediated by adhesion molecules on the neutrophils and endothelial cells. It has been shown that delivery of monoclonal antibodies to CD11a and CD11b by intravenous or intratracheal injection can prevent lung injury (Mulligan, 1995). Also, CD18-ICAM-1 interactions were found to mediate neutrophil cytotoxicity (Carlton, 1998). Neutrophils following trauma are found to have increased expression of CD11b and extracellular $O_2^{-}$ production three, six, twelve, and twenty-four hours after injury. (Botha,
1995). In the same study, Botha et al. found a sharp increase in circulating neutrophils at three hours followed by a large decrease at six and twelve hours, which suggested neutrophil sequestration. Autopsy findings in patients dying from MODS have shown neutrophil infiltration in tissues, especially in the lung (Pape, 1994). Another study found neutrophils and tissue damage in organs not originally injured by trauma in a group of patients where one of the causes of death was MODS (Nuytinck, 1988).

Once neutrophils have reached their target, they can release ROS and proteases which together are primarily responsible for the killing of microorganisms. However, these products may be destructive to tissue at sites where the neutrophils release them. Approximately 50% to 70% of the ROS production by neutrophils is contained within intracellular compartments; the remaining 30% to 50% is released into the extracellular space (Brown, 2004). When priming has occurred, any given activating stimulus will invoke greater results including a shift to increased extracellular release. The released proteases and ROS of this intense degranulation can damage host tissues. Laboratory and clinical evidence for this exists. In animal models, neutrophil proteases have been shown to play an important role in tissue damage after hemorrhagic shock (Deitch, 2003). In trauma patients, observations of increased neutrophil myeloperoxidase and the presence of sequestered neutrophils in damaged tissue implicate neutrophils as harmful contributors following injury. Additionally, neutrophils taken from these patients release increased amounts of superoxide (Biffl, 1996).

**Linking the RAS and MODS**

Hemorrhagic shock results from blood volume loss exceeding that for which the body can compensate. The causes of the blood loss can be trauma, surgery, or other disease
processes. The body has compensatory mechanisms that are engaged by blood loss, many as a result of baroreceptor responses. One of those mechanisms involves the renin-angiotensin system (RAS). The juxtaglomerular apparatus of the kidney is the day to day regulator of this system and detects changes in blood pressure and filtrate sodium. The apparatus responds to low blood pressure or low filtrate sodium by secreting renin. Activation of the apparatus is also mediated by the sympathetic division of the autonomic nervous system (DiBona, 2001). Activation of the juxtaglomerular apparatus results in renin release. Renin cleaves circulating angiotensinogen peptide to angiotensin I. Angiotensin I is then cleaved by angiotensin converting enzyme (ACE) to the octapeptide angII. Angiotensin II has a number of effects that counteract the blood pressure lowering effects of hemorrhage: it directly stimulates vasoconstriction, it increases sympathetic activity, it causes aldosterone secretion, and it triggers the thirst response. In the case of vasoconstriction, angII has been found to be selective for mesenteric resistance arterioles and is responsible for disproportionate increases in mesenteric inflow resistance relative to that of the rest of the body in response to hemorrhage (Aneman, 1997 & 2000; Toung, 2000). The resulting redistribution of blood flow can readily result in gastrointestinal mucosal damage from inadequate blood flow while providing some protection for the brain and heart. The main mechanism that leads to tissue injury through ischemia is probably the depletion of cellular energy stores and the build up of toxic metabolites due to decreased flow. Redistribution of blood flow may well be beneficial for short-term survival; however, elevations in ICU patient angII levels have been shown to persist for up to five days following resuscitation from the initial insult (Boldt, 1998). Thus, selective vasoconstriction, hypoperfusion, ischemia, and resultant tissue injury might still occur days after the hemorrhagic insult. Although this
response is meant to preserve the organism as a whole, persistent hypoperfusion of the gut can cause negative consequences for the rest of the body.

Hemorrhagic shock initiated gut hypoperfusion elicits SIRS with gut-origin inflammatory mediators entering the blood stream via the lymph (Deitch, 1992). Not only does ischemia from gut hypoperfusion increase intestinal mucosa barrier permeability, but it also results in gut ischemic injury, the majority of which occurs upon reperfusion of the ischemic gut (Shi, 2002; Parks, 1986). Coupled, these factors most likely amplify SIRS by allowing the translocation of bacterial by-products and release of other proinflammatory mediators into the lymph. Two such mediators released from the ischemic gut are IL-6 and TNF-α (Grotz, 1999). Others may be lipid mediators such as PAF that are derived from gut phospholipase A₂ activity (Gonzalez, 2001; Partrick, 1997). From the lymph, these mediators enter the bloodstream where they can exert their effects on cells not subjected to ischemic conditions. Evidence to support the role of gut derived lymph as a source of inflammatory mediators in SIRS and the development of MODS has been presented by groups who have shown that mesenteric lymph duct ligation or diversion reduces priming of neutrophils for enhanced cytotoxicity and reduces tissue damage (Adams, 2001; Dayal, 2002; Gonzalez 2002).

The RAS is thus linked to MODS following hemorrhagic shock by the mesenteric lymph and by events in the post-ischemic gut. Hemorrhagic shock mesenteric lymph contains mediators that can lead to a primed state within the organism. These mediators enter the lymph from ischemic gut tissue resulting from hypoperfusion created by selective mesenteric vasoconstriction stimulated by angII. These mediators lead to a primed state through the stimulation of increased adhesion molecule expression on both neutrophils and
endothelial cells, along with priming neutrophils for enhanced cytotoxicity (Adams, 2001; Dayal, 2002; Gonzalez, 2001 & 2002). In addition, entry of neutrophils into the post-ischemic gut also leads to priming of these cells. This is indicated by findings showing that neutrophils isolated from the aorta are not primed, whereas those isolated from the portal vein are primed (Biffl, 1996).

Increased adhesion molecule expression allows for firm adhesion of neutrophils to the endothelium. Firmly adhered neutrophils can then extravasate into the underlying tissues where they may come into contact with an activating stimulus. Some durations of gut ischemia and reperfusion alone result in the sequestration of neutrophils in the lung with a low incidence of injury and mortality. However, following the same duration of gut ischemia and reperfusion, a low dose of lipopolysaccharide results in an increase in neutrophil sequestration, injury, and mortality (Koike, 1992). Because the sequestered neutrophils are primed, the activating stimulus can cause the extracellular release of cytotoxic substances that are normally retained within the intracellular phagosomes. Extracellular release of these substances can cause tissue damage. There is also potential for release of these substances by adherent neutrophils at the endothelial surface resulting in vascular injury.

Much of the current research aimed at prevention of MODS is directed at modulating the inflammatory response following trauma in order to minimize neutrophil mediated tissue damage. Our lab is focusing on resuscitation methods following hemorrhagic shock using the ACE inhibitor enaliprilat. Through ACE inhibition we may interrupt vasoconstriction in mesenteric resistance arterioles and avoid resultant gut hypoperfusion that might persist days after the inciting event. By preventing persistent gut hypoperfusion, we may be able to minimize the inflammatory cascade initiated by gut ischemia and reperfusion. Although the
effects of angII stimulating an inflammatory response (through modulation of gut perfusion) have been well characterized, the role of angII in directly mediating the function of the neutrophils has not.
CHAPTER 2. INTRODUCTION

Angiotensin II has increasingly been found to initiate an inflammatory response. It can stimulate the expression of various molecules associated with inflammation. Thus far, evidence has shown that major targets of angII in mediating inflammation are endothelial cells and vascular smooth muscle cells. Vascular smooth muscle cells stimulated by angII express cytokines IL-6 and IL-8 along with the adhesion molecule VCAM-1 (Han, 1999; Ito, 2003; Brasier, 2000). Endothelial cells are activated by angII to express VCAM-1 and ICAM-1, release TNF-α, and produce superoxide (Arenas, 2003; Pueyo, 2000; Morrissey, 1998; Tsutamoto, 2000). Additionally, endothelial cell PLA2 is activated by angII through AT1 receptors (Pueyo, 1996). This activation can ultimately result in the formation of the inflammatory mediators LTB4 and PAF. Production of the latter by endothelial cells when stimulated with angII has been confirmed (Camussi, 1983). Both of these molecules perform significant roles in the inflammatory response that results after trauma.

Each of the products that results from angII stimulation of endothelial and vascular smooth muscle cells affects neutrophil function in various ways. Platelet-activating factor and LTB4 have been found to prime neutrophils for superoxide and elastase release after a secondary stimulus and increase neutrophil adhesiveness for endothelial cells through upregulation of the CD18/CD11b integrin complex, the ligand for ICAM-1 (Partrick, 1997). Additionally, angII has been found to mediate leukocyte adhesion in arterioles through stimulating increased endothelial cell expression of P-selectin, E-selectin, ICAM-1, and VCAM-1 (Alvarez, 2004). The inflammatory cytokine IL-6 can inhibit neutrophil apoptosis and enhance neutrophil priming, and IL-8 is a well known chemotactic factor for neutrophils (Biffl, 1996; Biffl, 1996). Tumor necrosis factor alpha delays apoptosis of adherent
neutrophils and has many other proinflammatory effects (Kilpatrick, 2002). Taken together, the production of these molecules can lead to a larger population of primed, activated, and more adhesive neutrophils that may contribute to the systemic inflammatory response after severe trauma.

Along with the indirect effects of angII exerted through endothelial cells and vascular smooth muscle cells, there is evidence that angII has direct effects on neutrophils as well. The AT1 receptor has been identified on neutrophils, and AT1 receptor antagonism in hypertensive rats led to a decrease in neutrophil CD18/CD11b expression (Ito H, 2001). Angiotensin II stimulates superoxide production and induces neutrophil migration in vitro in human neutrophils (Elferink, 1997; Paragh, 2002; El Bekay, 2003). It also stimulates activation of NFκB which can lead to the inhibition of apoptosis in neutrophils and to cytokine production (El Bekay, 2003). The combination of increased CD18/CD11b expression, NFκB activation, and superoxide production could lead to a larger, more adhesive, and cytotoxic population of neutrophils.

Primed, activated, adhesive, and long lived neutrophils involved in the systemic-inflammatory response following trauma are believed to be significant contributors to the tissue damage associated with MODS. The neutrophils damage tissue by releasing cytotoxic substances such as reactive oxygen species and proteolytic enzymes. These substances are part of a neutrophil’s normal armament for killing invaders, but are damaging to body tissues if excessively released into the neutrophil’s surroundings, as can happen when neutrophils become overly activated following trauma. The release of cytotoxic substances by overactive neutrophils adds to the tissue damage incurred by trauma and can create damage in organs not originally affected by the trauma. As the amount of damage in an organ system
increases, the function of that organ system decreases, and when more than one organ system fails to function properly, MODS results. Multiple organ dysfunction syndrome is often recognized as a complication of severe trauma, and angII levels are known to be elevated after severe trauma (Boldt, 1998). The timing of its elevation along with its known effects suggests that angII could promote or worsen MODS not only through blood flow effects but also through direct and indirect pro-inflammatory effects on neutrophils. It is my hypothesis that angII has a direct stimulatory effect on neutrophils.
CHAPTER 3. MATERIALS AND METHODS

This research was conducted in accordance with the National Institutes of Health guidelines for experiments involving animals, the provisions of the USDA Animal Welfare Act, the Animal and Plant Health Inspection Services Guide for the Care and Use of Laboratory Animals, and the U.S. Interagency Research Animal Committee Principles for the Utilization and Care of Research Animals. The protocols were approved by the Committee on Animal Care at Iowa State University.

**Blood Sample Collection**

Blood was obtained from male retired breeder Sprague-Dawley rats (retired breeder, RB) and a second group of male rats weighing 250-350 g (young rats, YR). Samples were collected using a modified version of the hetastarch exchange technique described previously (Williams, 1987). Rats were anesthetized with 25 mg/kg ketamine intramuscularly followed five minutes later by a subcutaneous injection of 50 mg/kg sodium pentobarbital. One percent lidocaine was used at all incision sites. A 1.5 cm midline incision beginning at the top of the sternum continuing towards the chin was made. The jugular vein and carotid artery on opposing sides were dissected out and cannulated.

Samples were obtained via a 50 ml syringe containing 2.5 ml of 2X acid citrate dextrose. A second 50 ml syringe containing 6% hetastarch in normal saline was connected to the jugular vein catheter. An initial 2.5 ml sample of blood was drawn from the carotid artery into the collection syringe followed by administration of a 2.5 ml bolus of hetastarch into the jugular vein. Blood continued to be collected in 2.5 ml increments, each one followed by 2.5 ml of hetastarch. The exchange was continued until the animal expired, or no additional blood/hetastarch mixture was obtainable.
Neutrophil Isolation

The isolation of neutrophils was carried out using a protocol described by Johnson et al. (1999). The red blood cells within the blood/hetastarch mixture were allowed to sediment for 35 minutes. The leukocyte rich upper layer was transferred to a 50 ml tube and centrifuged at 200g at 20°C for 12 minutes. The pellet was resuspended in 8 ml of Ca++ and Mg++ free phosphate buffered saline (PBS) and layered over 5 ml of NycoPrep™ 1.077A in a 15 ml tube. The gradient was centrifuged at 600g at 20°C for 20 minutes. The top layer of leukocytes at the interface between the PBS and Nycoprep, consisting of lymphocytes and mononuclear cells, was removed and the pellet washed twice by the addition of PBS and centrifugation at 350g at 4°C for 10 minutes. After the second wash, the pellet was subjected to hypotonic lysis for 35 seconds and then washed three times in PBS. After the final wash, cells were suspended in 2 ml HBSS with Ca++ and Mg++. Neutrophils were enumerated using a hemocytometer with >95% being viable as determined by trypan blue exclusion.

Following enumeration, the cell suspension was diluted with the appropriate volume of HBSS to achieve a concentration of 1x10^6 cells/ml. The final cell suspension was ≥90% neutrophils as determined by nuclei staining with 2 ng/ml Hoechst 33342 and observation through fluorescence microscopy.

Analysis of ROS Production

Isolated neutrophils were analyzed for ROS production using the non-fluorescent, cell permeable dye dihydrorhodamine 123 (DHR123). Upon oxidation of DHR123 by hydrogen peroxide, and to a lesser extent peroxynitrite, the fluorescent product rhodamine 123 (Rho123) localizes to mitochondria. Fluorescence of Rho123 can be excited using a wavelength of 488 nm and detected at 525 nm. The mean fluorescent intensity of 5,000
events was measured using the 525 nm band pass filter of a BD FACScan flow cytometer and analyzed using the BD LYSYS software. Only neutrophils were gated as determined by the forward-angle and right-angle light scatter signals. Background fluorescence was determined by analyzing samples loaded with DHR123 but not stimulated to produce ROS. Signals were adjusted so the peak fluorescence of the non-stimulated neutrophils was in the first decade of a logarithmic scale. Routine maintenance of the machine was done by employees of the flow cytometry facility at Iowa State University.

An aliquot of 720 µl of cell suspension was placed in a 37°C water bath for 10 minutes and then loaded with 50 µM DHR123 for 10 minutes. The DHR123 loaded cell suspension was then divided into seven 100 µl aliquots to which 400 µl of pre-warmed HBSS with Ca++ and Mg++ was added. The appropriate agent was then added to each cell suspension and incubated at 37°C for 30 minutes. Samples were placed on ice immediately after the final incubation and analyzed within 30 minutes. In experiments comparing RB and YR rats, the agent and final concentration of each were as follows: phorbol myristate acetate (PMA) 1000 nM, formyl-methionyl-leucyl-phenylalanine (fMLP) 1000 nM, angiotensin II (angII) 1000 nM, losartan 10 µg/ml. In experiments involving YR rats where the concentration of angII varied, the agent and final concentration of each were as follows: fMLP $1 \times 10^5 \mu$M; angII 1000 nM, 500 nM, 1 nM, 0.5 nM; losartan 10 µg/ml. Both groups also included one aliquot of cells that was pre-incubated for 5 minutes with losartan (angII receptor subtype 1 antagonist), followed by angII incubation.

**Analysis of CD11b Expression**

Expression of CD11b was analyzed using anti-CD11b mouse IgA monoclonal antibody conjugated to fluorescein isothiocyanate (FITC). All samples were compared to
FITC conjugated IgA isotype controls to account for non-specific antibody binding. Five microliters of 0.5 mg/ml antibody was added to each sample tube. Samples were incubated on ice for 45 minutes and then mean fluorescent intensity was measured via flow cytometry using the 525 nm band pass filter. Sample treatments were identical to those in the ROS analysis except the antibody was added following the final incubation.

**AngII Solution Activity**

To determine that angII suspended in solution was not being degraded, rats were infused with 100 µl of 4 µM angII or vehicle 0.9% NaCl via the jugular vein catheter, and mean arterial pressure (MAP) was monitored. Another group was infused with 100 µl of losartan before infusion with angII. In addition to placement of carotid and jugular catheters, a catheter with a pressure transducer was placed in the right femoral artery. Mean arterial pressure was monitored using a Digimed Blood Pressure Analyzer. Readings were taken before infusion and recorded every ten seconds for two minutes after infusion.

**Chemicals**

Dihydrorhodamine 123 was purchased from Molecular Probes; NycoPrep™ 1.077A from Accurate Chemicals; anti-CD11b mouse IgA monoclonal antibody and FITC conjugated IgA isotype control from BD Biosciences Pharmingen. All other chemicals were from Sigma-Aldrich.

**Statistical Analysis**

Data was analyzed using Student’s t-test and the JMP statistical analysis software from the SAS Institute. Probabilities less than 0.05 were considered significant.
CHAPTER 4. RESULTS

ROS Production

The measurement of ROS production is a common method for measuring the ability of an agonist to activate neutrophils. I used DHR123 because it has been shown that a greater percentage of the superoxide produced by rat neutrophils is intracellular, and DHR123 is a common dye used for the detection of cell associated ROS (Johnson, 1999). I found that at a concentration of 1000 nM, angII had no effect on ROS production by rat neutrophils in suspension (Figure 1A). In addition, varying angII concentrations less than 1000 nM and varying angII incubation times did not have any effect on ROS production (Figure 1B). Both receptor dependent (fMLP) and independent (PMA) activation of ROS production were tested. The PMA response was significantly greater than ROS production by neutrophils when incubated with media. However, fMLP at a concentration of 1000 nM did not stimulate significant ROS production. On the other hand, greater concentrations of fMLP (1x10^5 nM) do stimulate ROS production that may be more equivalent to those induced by PMA.

Reactive oxygen species production due to fMLP activation was not significantly different from unstimulated neutrophils (Figure 1A). Neutrophils from retired breeder and young rats were compared to eliminate the possibility that the lack of angII effects was due to dampened neutrophil ROS production sometimes observed in elderly individuals (Alvarez, 2001). In my assays, neutrophil responses from retired breeder rats showed no significant differences from those of young rats in their responses to any of the agonists used.
Figure 1. Reactive oxygen species production in response to angII.
Neutrophils were loaded with DHR123, incubated with the agonist indicated, and mean fluorescent intensity of rhodamine123 was detected using flow cytometry to determine ROS production. A) 1000 nM angII had no effect on ROS production. PMA was the only agonist to incite significant neutrophil ROS production in comparison to unstimulated cells; n=4, **P<0.001 compared to media. B) Stimulation of ROS release from YR neutrophils was not dependent on the concentration of angII or the incubation time. fMLP at a concentration found to stimulate ROS production similar to PMA was used as a positive control. n=3; OMedia, ▲AngII 0.5 nM, ◆AngII 1nM, ■AngII 500 nM, ◇AngII 1,000 nM, ●fMLP 1x10^5 nM
**CD11b Expression**

Determining the level of expression of CD11b is another method used to test the effects of potential agonists on neutrophils. Because priming leads to increased expression of CD11b and by itself does not stimulate ROS production, an agonist at a specific concentration may still exert effects that are not detected by measuring ROS production. Therefore, I used anti-CD11b FITC conjugated antibodies and flow cytometry to assess whether angII at 1000 nM might stimulate expression of this adhesion molecule while having no effect on ROS production.

I found that angII at a concentration of 1000 nM did not have any effect on CD11b expression (Figure 2A & B). At lower concentrations (500-0.5 nM), angII appears to provoke increased CD11b up regulation similar to that of $1 \times 10^5$ nM fMLP (Figure 2B). As seen in Figure 2A, fMLP stimulated strong up-regulation of CD11b, but at the concentration used (1000 nM), was not sufficient to arouse ROS production. At a concentration of 1000 nM, fMLP induced a similar response to that of 1000 nM PMA which contrasts the results seen for ROS production. The response to angII has similar characteristics to that of a higher concentration of fMLP ($1 \times 10^5$ nM) that stimulates ROS production and CD11b up-regulation. But higher concentrations of angII results only in CD11b up-regulation and not in ROS production.
Figure 2. CD11b expression in response to angII.
Anti-CD11b FITC conjugated antibodies were used to detect expression using flow cytometry. A) fMLP and PMA provoked significant CD11b upregulation, whereas 1000 nM angII had no effect. n=4, **P<0.001 compared to media. B) AngII stimulates increased expression of CD11b on YR neutrophils at concentrations of 0.5 nM, 1 nM, and 500 nM when compared to CD11b expression on neutrophils incubated with angII concentration of 1 µM, or without angII. n=3; ○Media, ▲AngII 0.5 nM, ◆AngII 1 nM, ■AngII 500 nM, ◊AngII 1,000 nM, ●fMLP 1x10⁵ nM
**AngII Solution Activity**

Due to the short half-life of angII *in vivo*, I wanted to confirm the activity of my angII by infusing rats with the angII solution while monitoring changes in MAP. Angiotensin II infusion of 400 pmoles produced a rapid increase of the MAP in retired breeder and young rats (Figure 3). The intravascular volume of the rat in ml is approximately 5-8% V/W of body weight (Williams, 1987). Since the rats used were about 350 g, the total intravascular volume would be 17.5 – 28 ml. Assuming all of the angII solution enters the entire volume, and normal levels are approximately 100 pM, the concentration of angII in the rat after infusion would be 14.1 – 23.1 nM. Vehicle (saline) alone had no effect on the MAP and prior infusion of losartan abrogated the effect of angII. The MAP peaked at ten seconds and gradually declined until a return to baseline two minutes after infusion. The MAP changes in RB to YR followed similar trends following angII infusion and did not show any observable differences (Figure 3A & B). This confirms the biological activity of the angII solution that I used in these studies.
Figure 3. MAP in response to angII infusion.
A) & B) AngII infusion led to an increase of MAP within ten seconds in both groups of rats; n=2. The MAP peaked and gradually declined back to baseline two minutes after infusion with angII. In contrast, infusion of angII after pre-treatment with losartan had no effect. Losartan or saline had little or no effect on MAP.

AngII, □ Saline, ▲ Losartan, ○ Losartan + AngII
CHAPTER 5. DISCUSSION AND CONCLUSION

Angiotensin II is a molecule that can exert a number of effects, one of which is to mediate some inflammatory reactions. Thus far, the majority of studies on this effect have focused on endothelial and vascular smooth muscle responses. Recently, more groups have begun to investigate the direct effect of angII on the immune cells involved in inflammatory processes. Most of this research has been done with a focus on monocytes, but now, neutrophils and the role that angII has in regulating their function is being explored.

Evidence gathered to date suggests that angII does in fact have a stimulatory effect on neutrophils. Elferink et al. (1997) demonstrated that human neutrophil migration in vitro is stimulated by angII. Others have shown that superoxide production from human neutrophils in vitro is stimulated by angII (El Bekay, 2003; Pargh 2002). El Bekay et al. (2003) carried out a series of experiments that identified potential signaling molecules involved in neutrophil responses to angII and confirmed the stimulation of ROS production by angII. However, they did not analyze CD11b or other adhesion molecule expression that may be induced. Ito et al. (2001) observed increased expression of the CD18/CD11b adhesion molecule on neutrophils from hypertensive rats and that treatment with AT1 antagonist reduced that expression to near normal levels. They also found that the AT1 receptor is expressed on the surface of rat neutrophils. Their study differed from the others mentioned because they utilized in vivo methods and did not assess differences in ROS production. It failed to eliminate the effects of angII on endothelial and vascular smooth muscle cells that have an indirect effect on neutrophils. The inflammatory molecules released by these cells in response to angII stimulation may account for the effects on CD11b upregulation, rather than a direct effect of angII. The evidence presented by these groups suggests a significant role
for angII in activating the cytotoxic potential of neutrophils. My goal was to confirm the results observed by these groups, but combine in vitro analysis of both CD11b expression and ROS production. If their results can be confirmed, it would point to another role of the RAS in the regulation of inflammatory responses involved in the development of MODS.

It is important to differentiate between studies of neutrophil responses in rats and humans. Neutrophils account for 20% of white blood cells in rats, whereas they make up 70% of the population in humans. Another difference, and perhaps of greater importance, is the disparity in the responses to different agonists. In this study, I noted that fMLP at a concentration of 1000 nM stimulated very weak ROS production. Human neutrophils, however, have a strong ROS production response to fMLP at the same concentration. Dayal et al. (2002) found aqueous extracts of mesenteric lymph from hemorrhaged rats primed rat neutrophils but the lipid extracts did not. Contrastingly, human neutrophils were primed by the lipid and not the aqueous extracts. This suggests that the same type of agonist does not necessarily have the same effect in different species. In addition, rat neutrophils produce much less extracellular superoxide compared to human neutrophils stimulated with fMLP, whereas the amount of intracellular production is not significantly different (Johnson, 1999). Therefore, it is not unreasonable to believe that angII stimulates different responses in rat neutrophils compared to human neutrophils. These effects may be simply due to differences in agonist receptor expression, but could potentially involve different signal transduction molecules.

I have shown in this study that angII does not stimulate ROS production, but does have an effect on CD11b expression in rat neutrophils. The CD11b expression response to angII is concentration and time dependent. The data presented here suggests that angII may
only stimulate increased adhesion molecule expression, but does not eliminate the possibility that it may be a priming agent at the concentrations tested. Here, angII had no effect on ROS production at different concentrations and various incubation times up to 30 minutes. It is possible that longer incubation times may be required for angII to stimulate or prime neutrophil ROS production. However, the onset of the respiratory burst is a highly kinetic event that usually occurs around 2.4 seconds following activation with common agonists (Wymann, 1987). Therefore, it is unlikely that longer incubation times would result in ROS production. Again, angII might still be able to potentiate the ROS response of neutrophils to other agonists, but most agonists that prime neutrophils can stimulate ROS production alone at higher concentrations.

Although I did not see activation of the respiratory burst measured by ROS production, the fact that CD11b up-regulation was elicited indicates that angII may still be able to mediate neutrophil responses and their contribution to tissue damage. Angiotensin II has been shown to regulate the expression of several different adhesion molecules and effect leukocyte endothelial cell interactions (Alvarez, 2001 & 2004). Because firm adhesion is important for neutrophils to extravasate into tissue and carry out cytotoxic functions, and CD18/CD11b-ICAM interactions play a major role in the process, elevated angII after trauma may potentiate tissue damage mediated by neutrophils by promoting CD11b upregulation.

Overall, angII may contribute to neutrophil mediated tissue damage following trauma and the progression of MODS in two ways. The first involves stimulation of selective resistance arteriole constriction and the resultant hypoperfusion and ischemia in the gut that creates an inflammatory mediator secreting organ. Those mediators can then enhance
neutrophil cytotoxic potential, causing tissue damage upon activation by a secondary stimulus. Second, by acting directly on neutrophils leading to up-regulation of CD11b and adhesion molecules on endothelial cells, angII can promote adherence of neutrophils in tissues allowing them to sequester and release their cytotoxic armament into the surrounding tissue. If this holds true, the interruption of angII effects through the use of ACE inhibitors or angII receptor antagonists in combination with other resuscitative strategies after trauma might provide a beneficial therapeutic intervention.
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