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Obesity as a Risk Factor for Preeclampsia: Role of Inflammation and the Innate Immune System

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OBESITY AS A RISK FACTOR FOR PREECLAMPSIA: ROLE OF INFLAMMATION AND THE INNATE IMMUNE SYSTEM

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Dedication

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List of Abbreviations

A	adipocytes
ACOG	American College of Obstetricians and Gynecologists
BMI	body mass index
CAM	cellular adhesion molecule
COX	cyclooxygenase
COX-1	cyclooxygenase-1
COX-2	cyclooxygenase-2
CRP	C-reactive protein
CVD	cardiovascular disease
DBP	diastolic blood pressure
DMEM	dulbecco's modified eagle medium
ddH_2O	double distilled water
EC	endothelial cells
$Fe_2(SO_4)_3$	Iron III sulfate hydrate
FFA	free fatty acid
HBSS	hank's balanced salt solution
Hg	mercury

HRP	horseradish peroxidase
HX	hypoxanthine
H_2O_2	hydrogen peroxide
ICAM-1	intercellular adhesion molecule-1
IL-1	interleukin-1
IL-6	interleukin-6
IL-8	interleukin-8
IL-10	interleukin-10
IκB	inhibitory-kappa B
KCL	potassium chloride
LA	linoleic acid
LPS	lipopolysaccharide
LDL	low-density lipoprotein
M199	Media 199
MPO	myeloperoxidase
NF-κB	nuclear factor-kappa B
NSAIDs	non-steroidal anti-inflammatory drugs
O_2	superoxide
PAF	platelet activating factor
PASM	placental arterial smooth muscle
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PECAM-1	platelet endothelial cell adhesion molecule-1

PG	prostaglandin
PGI_2	prostacyclin
PMA	phorbol-12-myristate acetate
pNPP	p-nitrophenyl phosphate substrate
ROS	reactive oxygen species
SBP	systolic blood pressure
SOD	superoxide dismutase
STBM	syncytiotrophoblast microfragments
TNFα	tumor necrosis factor α
TX	thromboxane
TXA_2	thromboxane A_2
TXB_2	stable metabolite of thromboxane A_2
VCAM-1	vascular cell adhesion molecule-1
VL	vessel lumen
VSM	vascular smooth muscle
vWF	von Willebrand factor
XO	xanthine oxidase

List of Units of Measurements

L	liter
ml	milliliter
µl	microliter
m	meter
mm	millimeter
µm	micrometer
g	gram
mg	milligram
µg	microgram
ng	nanogram
pg	picogram
M	molar (moles/ liter)
mM	millimolar (millimoles/ liter)
µM	micromolar (miromoles/ liter)
˚C	degrees Celsius
h	hour
rpm	revolutions per minute
IU	international unit

Abstract

OBESITY AS A RISK FACTOR FOR PREECLAMPSIA: ROLE OF INFLAMMATION AND THE INNATE IMMUNE SYSTEM

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

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Obesity is a known risk factor for preeclampsia, but the reason for this risk is unknown. We sought to demonstrate how obese individuals are predisposed to preeclampsia by mechanisms involving inflammation and the innate immune system. First, we used immunohistochemical studies to identify neutrophil infiltration, NF-κB activation and COX-2 expression in vascular tissue of obese women. We then demonstrated similar neutrophil infiltration and vascular inflammation in preeclamptic women.

We used in vitro experiments to test if neutrophils and their products, reactive oxygen species (ROS) and tumor necrosis factor-alpha (TNFα) can activate NF-κB and

cause expression of its inflammatory products, COX-2, thromboxane (TX) and IL-8. Co-culture of neutrophils or treatments of ROS or TNFα caused activation of NF-κB and expression of COX-2, TX and IL-8 in vascular smooth muscle cells.

This investigation is the first to demonstrate activation of NF-κB and expression of COX-2 coincident with neutrophil infiltration in systemic vascular tissue of obese and preeclamptic women. These data implicate neutrophils as a cause of vascular inflammation. They also suggest that if an obese woman's vasculature was in an inflamed state she is at increased risk for preeclampsia when exposed to the additional burden of oxidative stress and neutrophil activation imposed by the placenta, causing her to develop vascular dysfunction and clinical symptoms of PE (hypertension and edema).

CHAPTER 1

GENERAL INTRODUCTION

A. Obesity

Obesity is a major contributor to morbidity and mortality in Western societies through its contribution to the development of hypertension, heart disease, type II diabetes and preeclampsia. The World Health Organization and the National Institutes of Health define body type categories by body mass index (BMI) in kg/m^2. Underweight is defined as a BMI less than 18.5; normal weight as a BMI from 18.5 to 24.9; overweight as a BMI from 25.0 to 29.9 and obese as a BMI greater than or equal to 30. Many studies have shown that the rates of obesity in children and adults increased dramatically in the 1990s in the United States and other countries [1-5]. A recent study showed that in the United States about 27% of men and 34% of women are obese supporting the idea that obesity is a major public health problem that may be important in the increasing occurrence of diseases [2].

The major health consequences of obesity are predictable from an understanding of its pathophysiology. Obesity is a multisystem disorder with multiple causes. Body weight is determined by an interaction between genetic, environmental and psychosocial factors

acting through energy intake and expenditure [6]. Obesity is defined as a disease in which excess body fat has accumulated such that health may be adversely affected. These changes are to a certain extent dependent on the regional distribution of adipose tissue. For example, the intra-abdominal visceral deposition of adipose tissue is a major contributor to the development of hypertension, insulin resistance, and Type II diabetes [6].

Obesity is a known risk factor for preeclampsia, but preeclampsia is a complex disorder that has puzzled investigators for years. In order to study how obesity is a risk factor for preeclampsia, an understanding of the physiological consequences of obesity is essential. Leukocytes have been shown to be activated in obese individuals [7] and are known to infiltrate vascular tissue which could cause vascular dysfunction

i. Monocytes and Macrophages

Monocytes and macrophages play integral roles in the inflammatory response. The majority of studies that have been done on adipose tissue and inflammation point towards the involvement of monocytes and macrophages.

Monocytes constitute 3-8 percent of leukocytes in the circulating blood [8]. Monocytes spend only about a day and half in the blood, then migrate into the connective tissue, where they differentiate into tissue macrophages.

Macrophages are the principal phagocytes in normal connective tissue. They possess the capacity to destroy bacteria, and phagocytize dead cells and debris resulting from injury [8]. In addition, they serve important immune and scavenger functions. They are

mediators of the innate immune response, and are important participants in adaptive immunity serving as antigen presenting cells for lymphocytes [9].

Monocyte numbers have been shown to be elevated in patients with obesity [10]. Monocytes in obese individuals were shown to have elevated levels of oxidative burst and reduced ability for monocyte maturation [7]. Cottam et al. found that the monocyte CD14+/CD16+ subset was greatly expanded in chronic inflammatory states, similar to levels seen in morbid obesity [11, 12]. The CD14+/CD16+ cell population represents a proinflammatory subtype that exhibits features of tissue macrophages [12] and produces inflammatory cytokines such as tumor necrosis factor-α (TNFα) and interleukin-10 (IL-10).

The involvement of macrophages and monocytes in the process of inflammation is further confirmed by recent studies relating macrophages to atherosclerosis, an inflammatory disorder [13]. Both macrophages and monocytes are key to the initiation and progression of atherosclerosis [14]. Recruitment of monocytes into the artery wall is an early event of atherosclerosis and in the intima monocytes develop into macrophages. The uptake of modified lipoproteins by macrophages leads to the accumulation of cholesterol esters and formation of macrophage-derived foam cells, the hallmark of the fatty streak [14].

Several studies have shown that macrophage infiltration into adipose tissue in obesity could be integral to the process of inflammation [9, 15], and therefore cardiovascular disease. Weisberg et al. chose to profile gene expression in the white adipose tissue of mice of varying degrees of obesity in order to identify correlations between gene expression and the degree of obesity [9]. They examined macrophage involvement in obesity

by staining for the macrophage antigen, CD68. Immunohistochemical analysis of mouse subcutaneous adipose tissue showed that BMI and adipocyte size were strong predictors of the percentage of CD68-expressing macrophages. Transcriptional profiling indicated that macrophage genes were coordinately upregulated in direct proportion to body weight in several models of obesity. This study suggests that a large number of macrophage genes are upregulated in obesity and positively correlated with the amount of adiposity.

ii. Neutrophils

The involvement of neutrophils in obesity has not been extensively studied in comparison to macrophages and monocytes. Nieman et al. demonstrated that neutrophils had an increased ability for oxidative burst and phagocytosis, and both of these variables correlated with BMI [10]. However, the actual role of neutrophils in obese individuals has not been determined, which is a major focus of this thesis.

The primary role of neutrophils or polymorphonuclear leukocytes is the killing and phagocytosis of pathogens for host defense [16]. Neutrophils are part of the innate immune system responding to bacterial infection by releasing reactive oxygen species (ROS), myeloperoxidase (MPO) and proteolytic enzymes to fulfill this function [16]. Lipopolysaccharide (LPS) on the surface of bacteria activate neutrophils. Interleukin-1 (IL-1), interleukin–8 (IL-8) and oxidized lipids also activate neutrophils [17-19], whereas cytokines such as TNFα or interleukin-6 (IL-6) modulate neutrophils for enhanced activation [20].

Neutrophils have been implicated in many inflammatory diseases because unchecked neutrophil activity causes tissue damage leading to inflammatory conditions [21]. Many of the neutrophil mediators of inflammation exist presynthesized and stored in secretory granules, which are released only upon neutrophil activation at sites of inflammation [22]. Near sites of inflammation, neutrophils undergo a series of morphologic changes associated with adhesion to the endothelium. These include assuming a spherical shape and rolling along the blood vessel walls, cessation of movement and flattening, membrane ruffling, and modulation of membrane receptors [22, 23]. Many recent studies have concentrated on the molecules that mediate the selective adhesion of neutrophils at the sites of inflammation. For example, rolling of neutrophils along endothelium is supported by P-selectin and E-selectin glycoproteins [22, 24] and flattening and adherence of neutrophils to endothelium is supported by intercellular adhesion molecule-1 (ICAM-1) on endothelial cells [25] and L-selectin on neutrophils [26]. Neutrophil transendothelial migration across the vessel wall into the intimal space involves IL-8 [27] and platelet endothelial cell adhesion molecule-1 (PECAM-1) [28].

iii. Vascular Endothelial Cell Damage

The vascular endothelium, located at the interface of blood and tissue, is able to sense changes in hemodynamic forces and bloodborne signals and react by synthesizing and releasing vasoactive substances. A balance between endothelium-derived relaxing and

contracting factors [29] maintains vascular homeostasis. An inflammatory disease can disrupt this homeostasis and lead to endothelial cell dysfunction.

Endothelial cell dysfunction implies diminished production or availability of nitric oxide or prostacyclin or an imbalance in the relative contribution of these endothelium-derived relaxing factors and contracting factors, such as endothelin-1, angiotensin, and antioxidants [30]. When endothelial cells undergo inflammatory activation there is an increased expression of selectins, vascular cell adhesion molecule-1 (VCAM-1), and ICAM-1, which promotes the adherence of leukocytes [29]. Ferri et al. demonstrated that circulating levels of VCAM-1, ICAM-1 and E-selectin were all increased in obese men [31]. Another study found augmented levels of circulating von Willebrand factor (vWF), a marker of in vivo endothelial damage [32], in obese persons [33]. In other studies, circulating levels of endothelin-1 [34] and tissue plasminogen activator-1 [35] were increased while endothelium-dependent vasodilation [36] was reduced in normotensive obese individuals. To further confirm the essential role of obesity as a main promoter of endothelial activation, Ferri et al., demonstrated marked reductions of soluble adhesion molecules levels after weight loss due to caloric restriction [34]. These studies indicate that the endothelial vasodilatory substances are suppressed as well as reduced in number which may be why obese women are at risk for preeclampsia.

iv. Inflammation

Inflammatory responses are stimulated by tissue injury, as well as by immune activation. The inflammatory response involves the sequential release of mediators to cause recruitment of circulating leukocytes, which become activated at the inflammatory site and release further mediators [37]. The inflammatory response encompasses activation of monocytes and granulocytes, and activation of complement and clotting systems [38]. Central to the process of inflammation is a dramatic increase in endothelial cell surface expression of molecules that support the adhesion of blood leukocytes. Expression of endothelial cell adhesion molecules can be stimulated by endotoxin, interleukin-1 (IL-1), TNFα or oxidative stress [39, 40].

Obesity and obesity-related disorders including atherosclerosis and diabetes are associated with a low-level chronic inflammatory state [41]. Obese individuals at high risk of such inflammatory diseases are reported to have high levels of several inflammatory markers such as interleukin-6, TNFα and C-reactive protein (CRP) [42]. Interleukin-6 is a regulator of CRP production, the most widely examined circulating inflammatory marker. TNFα, an adipocytokine, may also play a role in the regulation of CRP synthesis and induction of IL-6. CRP, secreted by the liver in response to a variety of inflammatory cytokines, increases rapidly in response to inflammation and trauma [43]. CRP levels correlate with body weight and percentage body fat [44]. Several other studies have also shown that increased body mass index correlates with increases in systemic circulating levels of inflammatory proteins such as IL-6, P-selectin, VCAM-1, fibrinogen and

angiotensinogen [45]. Cook et al. observed that higher adiposity indicates higher CRP levels in children [46]. Visser et al. demonstrated that higher BMI is associated with higher CRP concentrations in adults between the ages of 17 to 39 years [47]. Many studies on BMI and CRP levels confirm a state of low-grade systemic inflammation in overweight and obese persons.

Both obesity and preeclampsia share common inflammatory features. Obesity is associated with circulating markers of inflammation, including C-reactive protein, IL-6, IL-8, ICAM-1 and TNFα [31, 42, 44, 45, 48]. Preeclampsia also shares similar elevations in these circulating inflammatory markers [49-53] which may help explain why obese women are at risk for preeclampsia (Table 1).

Table 1: Circulating Inflammatory Markers in Obesity and Preeclampsia.

Inflammatory Marker	Obesity	Preeclampsia
CRP	+	+
IL-6	+	+
IL-8	+	+
ICAM-1	+	+
TNFα	+	+
NF-κB	ND	ND
COX-2	ND	ND

(+), present; ND, not determined

v. Hypertension

Hypertension is a type of cardiovascular disease. Current guidelines for the diagnosis and management of hypertension have defined cardiovascular risk by the elevation of systolic blood pressure (SBP) and/or the elevation of diastolic blood pressure (DBP) [54]. Hypertension is defined as blood pressure $\geq$ 140/90 mm Hg.

Obesity and hypertension are frequently associated with one another, and changes in body weight are usually accompanied by consensual changes in blood pressure [55]. Obesity hypertension is considered a special form of hypertension [56]. The association between obesity and hypertension is supported by a wealth of epidemiological data [57, 58]. In some prospective studies, weight reduction is associated with a reduction in blood pressure [59, 60]. The Framingham Heart Study suggests that over 70% of the cases of hypertension can be directly attributed to obesity [61]. In Norway, two large health surveys were conducted and the association between change in BMI and its impact on blood pressure during an 11-year follow up study was determined. Their results indicate that people who increase their BMI are at risk for increased hypertension [62].

Other studies found that cardiac, systolic and diastolic function are impaired in obese individuals [63]. Masuo et al. studied the mechanism behind weight gain and elevated blood pressure. They discovered that weight gain-induced sympathetic overactivity is tightly linked to weight-gain induced blood pressure elevation [64].

Obesity places an extra burden on cardiovascular function. The increase in body weight is associated with an increase in both lean and fat mass. This, and the associated

increase in total blood volume, is in turn accompanied by an increase in stroke volume and cardiac output. An increase in circulatory preload and afterload can lead to left ventricular dilation and eccentric hypertrophy which can lead to heart attack. An increase in systemic vascular resistance in obese individuals leads to a rise in blood pressure which can result in hypertension [6].

Obesity and hypertension are strongly linked, so to investigate the effect of obesity on future risk of hypertension in pregnancy (preeclampsia) is an important public health concern.

B. Obesity as a Risk Factor for Preeclampsia

Obesity is a major risk factor for preeclampsia. Maternal body mass index has been suspected to be associated with preeclampsia. A number of studies have examined the relation between maternal prepregnancy BMI and preeclampsia, yet none of them has determined the mechanism of how BMI increases the risk of preeclampsia. Most of the current data suggest that obesity may act through its association with hypertension, insulin resistance, chronic inflammation and oxidative stress to increase the risk of preeclamspia [65-68]. These abnormalities may act independently or interact together to promote preeclampsia. Previous studies have shown that prepregnancy obesity is a risk factor for preeclamspia and that obese pregnant women have a 26% incidence of preeclampsia [69-72].

Several studies provide evidence to support the association between obesity and the systemic manifestations of preeclampsia [71, 73, 74]. First, women with preeclampsia have

higher levels of serum lipids compared to pregnant controls [75-80]. Second, byproducts of lipid peroxidation are elevated in women with preeclampsia [81, 82] and it is possible that these byproducts might be associated with endothelial cell damage and vasoconstriction. Third, placental vessels of women with preeclampsia had atherosclerotic like changes, including deposition of fibrinoid material and foam cells [83]. Finally, elevated lipid level is a component of insulin resistance syndrome [84] and insulin resistance is associated with preeclampsia [85, 86].

There is also an association between the degree of obesity and the intensity of other pregnancy complications including gestational diabetes, postpartum infection and preeclampsia. In one study overweight women experienced increased rates of gestational diabetes and cesarean delivery when compared with normal weight women [87]. As overweight women became obese, they had higher risks for preeclampsia [87]. Bodnar et al demonstrated that the risk of preeclampsia rises sharply from BMI values of 15 to 30 [88]. Women with a BMI of 24 were 70% more likely to develop preeclampsia than women with a BMI of 21, and obese women had a 2-fold higher risk of developing preeclampsia compared to normal weight or overweight women [88]. Bodnar also showed that the sharp rise in risk across most of the BMI distribution indicates that the risk of preeclampsia increases even within BMI categories.

Another possible mechanism to link obesity as a risk factor for preeclampsia is by increased diastolic and systolic blood pressures in obese individuals because hypertension is a risk factor for preeclampsia. Investigators have shown that risk of preeclampsia increases significantly with increased body mass index coincident with increased diastolic

and systolic blood pressure [89]. Women with gestational hypertension are often obese and are at significant risk for preeclampsia [90].

C. Pathophysiology and Proposed Causes of Preeclampsia

Preeclampsia is a pregnancy-specific disorder affecting 6-8% of all pregnancies. It is diagnosed as maternal hypertension ($\geq$ 140/90 mm Hg) with proteinuria (> 0.3 g/24 h)[91], but it is also characterized by pathological edema and coagulation abnormalities. Preeclampsia can affect virtually every organ in the body [91]. Preeclampsia remains the leading cause of intrauterine growth restriction (IUGR), death and prematurity for the fetus, and the leading cause of renal failure, pulmonary edema, stroke and death for the mother [92]. The pathophysiology of preeclampsia is complex and remains unclear. There are many risk factors for preeclampsia, but their common theme is oxidative stress (Figure 1).

There are various hypotheses proposed to explain the occurrence of preeclampsia. One focuses on abnormal placentation resulting in placental vascular insufficiency [92]. Placental insufficiency results in release of placental substances that circulate to cause endothelial cell dysfunction and other clinical symptoms of preeclampsia [93]. According to this 2-stage model, the first stage is due to decreased placental perfusion, which then results in the maternal syndrome of preeclampsia. However, the linkage between the maternal and fetal-placental compartments remains unclear [94]. Many researchers believe that oxidative stress could be the link between reduced placental perfusion and

preeclampsia because there is an abundant amount of evidence for oxidative stress in preeclampsia [93, 95-97].

The following literature review will briefly summarize the role of placental oxidative stress in the pathogenesis of preeclampsia with a focus on neutrophil activation, endothelial cell dysfunction, vascular smooth muscle (VSM) dysfunction and inflammation.

i. Placental Oxidative Stress in Preeclampsia

While multicellular organisms require oxygen for survival and production of energy, oxygen can also be toxic. Oxygen mediates its toxic effects through the production of free radical species. A free radical is any molecule capable of independent existence that contains one or more unpaired electrons [98]. Free radicals are highly reactive species because they will attack nearby atoms or molecules for electrons to complete their outer electron orbital. Reactive oxygen species are a byproduct of free radicals and oxidative stress results from excessive production of ROS and/or a deficiency in the protective antioxidant systems [99]. Oxidase enzymes also yield ROS byproducts. Two examples are xanthine oxidase which produces superoxide, ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) and cyclooxygenase (COX), which produces ROS during arachidonic acid metabolism [100]. Stimulated neutrophils are also a source of oxygen radicals through the activity of NADPH oxidase [101].

Oxidative metabolism and other physiological processes produce free radicals and it is necessary to inhibit free radical damage and maintain oxidant-antioxidant balance. Women with normal pregnancies have a certain amount of oxidative stress with increased lipid peroxidation, but it is offset by an increase in antioxidant protection [96, 97, 102]. In preeclampsia, however, there is a decrease in antioxidants with an increase in oxidative stress and lipid peroxidation [96, 97, 102]. Some important cellular antioxidant enzymes are superoxide dismutase (SOD) and catalase (Table 2). They function together to eliminate ROS generated during oxidative metabolism, which can damage cellular membranes, proteins and DNA. SOD dismutates $O_2^{\bullet-}$ to H_2O_2 and molecular oxygen and is thus significant because it quenches $O_2^{\bullet-}$ produced during oxidative metabolism. Catalase functions in coordination with SOD to catalyze conversion of H_2O_2 to water and molecular oxygen.

An important event of preeclampsia is thought to be abnormal placental implantation [103]. Failure of spiral artery remodeling in the placental bed of pregnancies affected by preeclampsia was first demonstrated by Brosens et al [104] and later associated with a partial failure of placental trophoblast invasion [105]. Preeclamptic placentas show an abnormal expression of integrin molecules that regulate cell-cell and cell-matrix interactions [106, 107]. As a result, trophoblastic invasion is inhibited, and spiral artery remodeling is limited creating a vasoconstrictive placental bed. Defective spiral artery remodeling in preeclampsia results in reduced uteroplacental perfusion [108], which could result in the production of ROS due to ischemia.

Lipid peroxidation occurs in the placenta during normal pregnancy [109], but is elevated in preeclampsia as compared to normal pregnancy [82, 110-112] Lipid peroxides (LOOH) are formed when polyunsaturated fatty acids interact with free radicals. Uncontrolled lipid peroxidation can result in cellular dysfunction and damage. There is significantly greater lipid peroxide, as well as thromboxane A_2 (TXA_2), production by trophoblast cells isolated from preeclamptic placentas as compared to normal placentas [113]. Lipid peroxides also influence enzyme activity to alter the balance between vasoconstrictors and vasodilators. Specifically, lipid peroxides stimulate cyclooxygenase (COX) to generate prostaglandins and thromboxanes, including the potent vasoconstrictor TXA_2 [96]. At the same time, lipid peroxides inhibit the PGI_2 synthase enzyme, so the end result is elevated levels of the vasoconstrictor TXA_2 and decreased levels of the vasorelaxant prostacyclin (PGI_2) (Figure 2). Other studies [111, 114-116] have confirmed the original report by Walsh [117] that more TXA_2 and less PGI_2 is produced by preeclamptic placentas as compared to normal placentas.

The generation of ROS from enzymes and other biochemical processes are sources of placental oxidative stress during preeclampsia. ROS perpetuate oxidative stress by stimulating lipid peroxidation and isoprostane formation. Walsh et al demonstrated both elevated lipid peroxidation and increased 8-isoprostane production and release from preeclamptic placentas [110, 118]. Lipid peroxides and isoprotanes are secreted by the placenta into the intervillous space, which is bathed by maternal blood, so placental secretion of lipid peroxides may be the link between placental oxidative stress and maternal vascular cell dysfunction in preeclampsia

ii. Neutrophils as the Link Between Placental Oxidative Stress and Maternal Vascular Cell Dysfunction in Preeclampsia

Placental oxidative stress may directly or indirectly lead to oxidative stress in the maternal circulation. Maternal leukocytes are activated in preeclampsia [119-121]. Oxidized lipids secreted by the placenta could activate maternal leukocytes during passage of maternal blood through the intervillous space of the placenta [96, 97](Figure 3). Release of ROS and cytokines from activated leukocytes could then contribute to maternal endothelial cell activation, and subsequent leukocyte adhesion [97]. Thus, oxidative stress of the preeclamptic placenta may be transferred to the maternal circulation by circulating leukocytes to induce cell dysfunction in many organ systems. Next, this review will focus on the effects of oxidative stress on neutrophils, endothelial cells, and vascular smooth muscle and, finally, their linked role in the pathogenesis of preeclampsia.

a. Neutrophils in Preeclampsia

A great deal of evidence suggests that activated neutrophils play a significant role in the pathogenesis of preeclampsia. Neutrophils are likely mediators in preeclampsia for several reasons, including: 1) they comprise 60% of the leukocytes, 2) their numbers increase in pregnancy [91], 3) their numbers further increase in preeclampsia [122], and 4) they produce toxic substances (ROS, myeloperoxidase (MPO), TXA_2, and TNFα) which could

cause endothelial and vascular smooth muscle cell dysfunction leading to clinical symptoms associated with preeclampsia.

There are several studies that support neutrophil activation in preeclampsia. Measurement of plasma neutrophil elastase was the first technique used to identify neutrophil activation in preeclampsia [119]. An increase in neutrophil elastase is seen in normal pregnancy [119], with a further increase in preeclampsia [119]. Elastase, a neutrophil protease, is a potent enzyme released as a host-defense mechanism upon neutrophil activation [123] and the presence of this protease in plasma indicates neutrophil activation. Likewise, neutrophil surface markers of activation, such as CD11b, [65, 121] and release of intracellular ROS [121] have also been used to document neutrophil activation in preeclampsia. Barden et al. [65] reported that preeclamptic patients have a higher basal expression of neutrophil glycoprotein, CD11b, in comparison with normal pregnant patients. Other studies showed that leukocytes of preeclamptic women have reduced expression of L-selectin, which is involved in the initial adhesion process and consistent with activation [121].

Adhesion of neutrophils to endothelium requires expression of adhesion molecules on endothelium, as well as neutrophils. There is evidence of up-regulation of endothelial membrane-bound cell adhesion molecule (CAM) expression as evidenced by shedding of soluble isoforms of these molecules into the circulation [124]. Increased soluble isoforms of CAMs have been reported in plasma of preeclamptic women [125, 126], and increased expression of ICAM-1 has been reported on the vasculature [127]. The cause of increased expression of CAMs on endothelial cells could be due to their up-regulation by cytokines,

including interleukins and TNFα [128]. In addition to the up-regulation of CAM expression on endothelial cells, TNFα has been shown, in vitro, to impair vascular endothelial function by causing neutrophils to directly adhere to endothelial cells [129].

There is evidence that oxidized lipids are the cause of neutrophil activation in preeclampsia. Hyperlipidemia is known to cause endothelial dysfunction [130] and preeclamptic women have hyperlipidemia [77, 131]. Polyunsaturated fatty acids in triglycerides and low-density lipoproteins (LDL) are susceptible to oxidation. Oxidized lipids and oxidized LDL have been identified as potent stimuli of leukocyte activation and adhesion to endothelium [17, 20]. The placenta is a rich source of linoleic acid [132] and oxidized linoleic acid stimulates neutrophil production of thromboxane, TNFα and superoxide [133]. One source of oxidized lipids in preeclampsia is the placenta. Placentas of preeclamptic women produce increased amounts of lipid peroxides [96]. As lipid peroxides are secreted into the intervillous space on the maternal side, leukocytes would be activated as they circulate through. The activated leukocytes then enter the maternal circulation where they generate oxidative stress and could be a cause of endothelial cell dysfunction [102]. In the next section, I will discuss endothelial cell dysfunction in preeclampsia and how neutrophils could be associated with it.

b. Endothelial Cell Dysfunction in Preeclampsia

The potential role for the endothelium in preeclampsia is evident when one considers the diverse pathophysiological changes of the disorder. Normal vascular

endothelial cells produce substances, such as nitric oxide, prostacyclin, and endothelial-derived hyperpolarizing factor that buffer responses to pressor agents [134]. In combination with other endothelial-derived proteins that activate circulating pro-anticoagulants, they serve to prevent intravascular coagulation. In addition, endothelial cells express molecules that recruit inflammatory cells to endothelium [134] and when appropriately activated, they serve as the signaling system to initiate response to local tissue insult.

In normal pregnancy, endothelial function is especially important to explain physiological changes, such as reduced blood pressure. Experiments suggest that the decrease in blood pressure in normal pregnancy may be explained by increased endothelial-derived vasodilators [135].

Endothelium can change its properties in response to several types of signals. For example, with inflammation, antigens are expressed on the endothelial surface that recruit, sequester, and transfer circulating cells to the appropriate tissue. This is an example of the process of endothelial activation. Another example occurs with changes associated with physical injury, including disruption of blood vessels. In this case, platelet activation, production of vasoconstrictors, and procoagulant compounds are expressed [136]. While both of these responses cause endothelial activation, circulating factors can also yield an inappropriate response. This type of response, in which normal responses are inappropriate to the stimuli, is termed, endothelial dysfunction [134].

Substantial evidence suggests that endothelial dysfunction is a central pathophysiological feature of preeclampsia. One hypothesis proposes that endothelial defect could be due to the reduced release of protective materials produced by the placenta,

coincident with increased release of cytokines, procoagulants, and products of oxidative stress [134]. The concentrations of TNFα and its soluble receptor are increased in the blood of women with preeclampsia [51, 137], and the placenta produces increased amounts of TNFα [138]. Other cytokines, such as interleukin-2, affect endothelial cell prostaglandin production, which may exacerbate the PGI_2 and TXA_2 imbalance, which is characteristic of preeclampsia [97, 117]. Decreased PGI_2 production in preeclampsia indicates that there is endothelial cell dysfunction because endothelial cells are the primary source of PGI_2.

Another explanation for endothelial cell dysfunction involves neutrophils [139]. There is evidence of activation of neutrophils in preeclampsia [139]. Secondary to the activation of neutrophils is their release of free radicals. Free radicals could generate endothelial oxidative stress when activated neutrophils come in contact with the maternal endothelium. This would cause dysfunction of the endothelium. Placental activation of neutrophils could provide a possible mechanism by which placental oxidative stress could alter systemic endothelial function in preeclampsia. Another possible placental mechanism that alters endothelial function is the increased release of small fragments of placenta into the circulation of preeclamptic women [139, 140].

The procoagulant state of preeclampsia also reflects endothelial dysfunction. Studies have shown that elevated plasma levels of tissue factor, von Willebrand factor, platelet activating factor (PAF), cellular fibronectin and thrombomodulin strongly suggest endothelial dysfunction in preeclampsia [141]. Endothelial cells release endothelin and von Willebrand factor following endothelial cell damage. Von Willebrand factor then initiates platelet activation, while endothelin causes vasoconstriction of vascular smooth muscle [141].

Investigators have also measured plasma levels of soluble adhesion molecules as an indication of determine endothelial cell dysfunction in preeclampsia [49, 65, 126]. Both ICAM-1 and VCAM-1 are expressed on endothelial cells. With endothelial dysfunction these proteins are shed into the circulation. Several studies have found elevated concentrations of ICAM-1 and VCAM-1 in preeclamptic plasma [125, 126, 142, 143].

There is good evidence to support a significant role for endothelial cell dysfunction in preeclampsia, but endothelial cells are not the only part of the vasculature that is dysfunctional. In the next section, I will review recent evidence of vascular smooth muscle dysfunction and show its association with neutrophils.

c. Neutrophils and Vascular Smooth Muscle Dysfunction

Vascular smooth muscle plays a critical role in both normal pregnancy and preeclampsia. Since the endothelium modulates vascular smooth muscle tension, most preeclampsia research has focused on endothelial cell physiology, despite the obvious role of vascular smooth muscle. Only recently have studies been done on vascular smooth muscle functioning during preeclampsia. One study demonstrated that placental extracts from preeclamptic women lead to increased oxygen consumption by porcine carotid arteries as compared to placental extracts from normal pregnant women [144]. Another study treated vascular smooth muscle with preeclamptic serum and measured changes in vascular smooth muscle oxygen consumption. They found an increase in oxygen consumption,

which is indicative of muscle contraction. When the preeclamptic serum was removed, the oxygen consumption returned to control levels [145].

Leik and Walsh found significant amounts of neutrophil infiltration into the maternal systemic vasculature of preeclamptic women [127]. They also found that this neutrophil infiltration was associated with inflammation of the vascular smooth muscle as indicated by increased expression of IL-8 and ICAM-1. IL-8 is a potent neutrophil chemokine for neutrophils. Increased expression of vascular smooth muscle IL-8, thus, establishes a concentration gradient for IL-8 from the circulation to the vascular smooth muscle to attract neutrophils to infiltrate the vasculature [127]. Increased expression of ICAM-1 not only provides the cell adhesion molecule for neutrophil infiltration, but also corroborates the IL-8 data that there is inflammation of the vascular smooth muscle because ICAM-1 is expressed by a variety of tissues under conditions of inflammation [146]. Normal pregnant women did not express ICAM-1 in vascular smooth muscle [127]. Neutrophil infiltration is a plausible reason for endothelial and vascular smooth muscle dysfunction in preeclampsia because neutrophils produce toxic substances, such as ROS, TNFα, TX and proteolytic enzymes, which may explain some of the clinical symptoms of preeclampsia [127].

D. Inflammation in Preeclampsia

Both normal pregnancy and preeclamptic pregnancy are associated with a systemic inflammatory response. Preeclampsia develops when the normal inflammatory response in

pregnancy becomes exaggerated in response to excessive stimuli and triggers endothelial dysfunction leading the maternal system to decompensate [147].

Endothelial activation is an intrinsic part of the inflammatory response and mediates its characteristic features of a locally increased blood supply to the inflamed area with increased capillary permeability, leukocyte adherence before extravasion, chemotaxis, and phagocytosis [38]. Thus, markers of inflammation that are changed in normal pregnancy are affected more severely in preeclampsia including increased leukocyte activation, increased complement activation, increased platelet activation, increased clotting activation, and increased systemic endothelial activation [148].

There is substantial evidence that there is systemic activation of maternal inflammatory cell responses in preeclampsia. Granulocytes, monocytes and lymphocytes are all activated [119, 121] and subsequently, activated leukocytes could activate endothelium [149], which as mentioned before is integral to the inflammatory response. There are also increased circulating levels of TNFα, TNFα soluble receptors [51, 137, 150], and IL-6 [53]. Other studies have looked at the extent and nature of the maternal inflammatory response in preeclampsia using flow cytometric techniques to analyze surface antigen expression and production of intracellular reactive oxygen in peripheral blood leukocyte subsets [147]. They have found that in preeclampsia there is an increase in leukocyte surface antigen expression. Likewise, intracellular ROS were significantly increased in granulocytes, monocytes, and lymphocytes of preeclamptic women as compared to normal pregnant women [147].

There is an abundant amount of evidence pointing towards the placenta as being the inflammatory stimulus in preeclampsia. According to Redman and Sargent [120], there are three possibilities that could account for the placenta being involved in the inflammatory response during preeclampsia: 1) dissemination of inflammatory cytokines released by syncytiotrophoblast, 2) placental oxidative stress, and 3) placental debris. The presence of syncytiotrophoblast microfragments (STBM) has been detected in higher amounts in the plasma of preeclamptic women as compared to normal pregnant women [151]. STBM have been shown to be directly damaging to the endothelium [140], which is preliminary evidence that they are pro-inflammatory.

The systemic inflammatory response is related to oxidative stress in preeclampsia. ROS are used as second messengers to propagate proinflammatory and growth stimulatory signals. As a consequence, oxidative stress and inflammation are interrelated [152]. An inflammatory response generates ROS, and conversely, ROS stimulate an inflammatory response. Since oxidative stress of preeclampsia is not localized to the placenta, but spread throughout the maternal circulation [95], the maternal compartment would also be expected to be in an inflammatory state.

i. NF-κB

Nuclear factor-κB (NF-κB) is considered the hallmark of the inflammatory response, suggesting a possible role in obesity and preeclampsia. NF-κB is a ubiquitous, dimeric transcription factor that when activated becomes a heterodimer, consisting of two

proteins, p65 subunit and a p50 subunit. NF-κB is found in the cytoplasm and is bound to IκBα and IκBβ, which prevent it from entering the nuclei [153]. When a cell is appropriately stimulated, specific kinases phosphorylate IκB, causing its degradation [154]. The release of NF-κB from IκB results in the translocation of the p65 and p50 subunits to the nucleus and their binding to specific sequences in the promoter regions of target genes (Figure 4).

Many stimuli activate NF-κB, including cytokines, oxidative stress [155], and circulating lipid peroxides [156-158]. Ironically, things that activate NF-κB are also regulated by NF-κB. Thus, NF-κB plays a pivotal role in inflammatory responses through the regulation of genes encoding proinflammatory cytokines, adhesion molecules, chemokines, growth factors and inducible enzymes [37]. Proinflammatory cytokines, such as interleukin-1β, TNF-α, IL-6, IL-8 and granulocyte-macrophage colony-stimulating factor [39] attract leukocytes to sites of inflammation. These cytokines play important roles in the inflammatory process. NF-κB activation triggers a cascade of events, such as expression of ICAM-1, which binds leukocytes to endothelial cells, and production of chemokines, such as IL-8, which attracts leukocytes to the inflammation site [159]. Adhesion molecules play a key part in the initial recruitment of leukocytes to sites of inflammation [127, 160].

The activation of NF-κB leads to a coordinated increase in the expression of many genes, such as cyclooxygenase-2 (COX-2), the inducible form of cyclooxygenase, whose products mediate inflammatory responses. NF-κB should be considered as an amplifying and perpetuating mechanism that can exacerbate the disease-specific inflammatory process through the coordinated activation of several inflammatory genes [39]. Next, I will consider one of these inflammatory gene products, COX-2.

ii. COX-2

Cyclooxygenase (COX) or prostaglandin (PG)H_2 synthase, catalyzes the first committed step in the biosynthesis of PGs by converting arachidonic acid to PGG_2 and PGH_2 [161]. Cyclooxygenase exists in two isoforms, COX-1 and COX-2, which are encoded by two separate genes. Both forms metabolize arachidonic acid to PGH_2, the common substrate for thromboxane A_2 (TXA_2), prostacyclin (PGI_2), PGD_2, $PGF_{2\alpha}$ and PGE_2 synthesis [162, 163]. PGs play a central role in inflammation, as well as in regulating other critical physiological responses. In humans, prostaglandins are involved in diverse functions, including blood clotting, ovulation, wound healing, blood vessel tone, and immune responses [162]. In pregnancy, prostaglandins are involved in the vasodilation of normal pregnancy, the most likely one being PGI_2 because of its potent relaxing effect on the smooth muscle of blood vessels and its ability to lower systemic arterial blood pressure [164-166].

Platelets, leukocytes and trophoblast cells of the placenta produce thromboxane (TX). Thromboxane is a potent stimulator of platelet aggregation, vasoconstriction, and uterine contractility [102, 161] and is associated with clinical symptoms of preeclampsia. In preeclampsia, there is an imbalance of increased thromboxane and decreased prostacyclin [102]. Prostacyclin opposes the actions of thromboxane, because it is a potent vasodilator, an inhibitor of platelet aggregation, and an inhibitor of uterine contractility [102].

The COX-1 enzyme is produced constitutively in cells, whereas COX-2 is induced at sites of inflammation. Early studies have revealed that while both enzymes carry out

essentially the same catalytic reaction and have similar primary protein structures [167], many of the inflammatory effects of COX appear to be mediated by COX-2, while many of the 'housekeeping' effects of COX appear to be mediated by COX-1. Thus, COX-1 appears to act in a variety of settings to produce homeostatic or maintenance levels of PGs, whereas induction of COX-2 expression is responsible for increased PG production seen in inflamed tissues [168, 169]. COX-2 is regulated by NF-κB, and it is expressed acutely at sites of inflammation by various stimuli, such as cytokines, mitogens and prostaglandins [170]. COX-2 leads to increased production of prostaglandins and thromboxane that mediate inflammation. COX-2 is induced during inflammatory responses [171] and plays a role in other inflammatory diseases. COX-2 expression in monocytes is associated with increased circulating oxidized lipids [172]. All of these data suggest a role for COX-2 in the pathogenesis of obesity. COX-2 expression in peripheral blood mononuclear cells (PBMCs) is positively correlated with plasma concentrations of oxidized LDL, suggesting that elevated COX-2 expression in monocytes is a response to systemic oxidative stress and inflammation [172]. Recently, Vaughan et al. showed that neutrophil release of TX and superoxide in response to oxidative stress is mediated by COX-2 and release of TNFα is mediated by TX, a COX-2 metabolite [133, 173].

Since COX-2 is associated with inflammation, it has become a major target for use of non-steroidal anti-inflammatory drugs (NSAIDs). Many studies have shown that the broad range of classical NSAIDs inhibit both COX-1 and COX-2 with a general tendency toward COX-1 selectivity [168]. Recently, more selective COX-2 inhibitors have been developed. Their advantage is that they are associated with fewer gastrointestinal side

effects in comparison to classical NSAIDs. This has provided the rationale for developing specific COX-2 inhibitors, including rofecoxib (Vioxx^{TM}), celecoxib (Celebrex^{TM}), and valdecoxib (Bextra^{TM}). COX-2 inhibitors have the potential to produce many beneficial effects, including reduced endothelial dysfunction [174], which is present in preeclampsia.

E. Purpose of Investigation

Obesity is a recognized risk factor for preeclampsia, but the mechanism by which obesity increases the risk is unclear. We propose that oxidative stress, neutrophil infiltration and vascular inflammation are common factors, and the presence of these factors in obese women puts them at risk of developing preeclampsia when they become pregnant because of the additional oxidative stress imposed by the placenta. Placental secretion of oxidized lipids results in further neutrophil activation and vascular inflammation sufficient to cause vascular cell dysfunction resulting in edema, proteinuria and hypertension (Figure 5).

F. Overall Hypothesis

Obesity is a risk factor for preeclampsia because it is associated with neutrophil infiltration into vascular tissue which causes inflammation and vasoconstriction by release of toxic substances, such as ROS, TNFα and TX. (Figures 5 and 6). When exposed to the

additional burden of oxidative stress and neutrophil activation imposed by the placenta, her risk of developing preeclampsia increases.

Specific Aims:

1) Determine the extent of neutrophil infiltration, activation of NF-κB and expression of COX-2 in vascular tissue of obese individuals and determine if these are correlated with BMI and blood pressure

2) Determine the extent of neutrophil infiltration, activation of NF-κB and expression of COX-2 in vascular tissue of women with preeclampsia and compare it to that of obese women

3) Determine if activated neutrophils or neutrophil products (ROS, TNFα) will activate NF-κB in vascular smooth muscle cells

4) Determine if neutrophil products (ROS, TNFα) will induce vascular smooth muscle expression of NF-κB regulated compounds, COX-2, IL-8 and TX, which would demonstrate inflammation

G. Significance of this Research

If we find that neutrophil infiltration, activation of NF-κB and expression of COX-2 correlate with BMI and BP and that preeclamptic women show similar infiltration and inflammatory markers to obese women, this would provide strong evidence that obese women are at increased risk of preeclampsia because their vasculature is already inflamed and susceptible to hypertension. Neutrophil infiltration in obese and preeclamptic women could explain vascular cell dysfunction. If our assumptions are correct in linking obesity and preeclampsia via an inflammatory mechanism then such findings would suggest novel treatments for obesity related hypertension and preeclampsia using antioxidants, NF-κB inhibitors, leukocyte cell adhesion molecule inhibitors or COX-2 inhibitors. For example the use of antioxidants in combination with COX-2 inhibitors to reduce oxidative stress and to reduce neutrophil activation and infiltration would consequently reduce the activation of NF-κB and induction of COX-2 expression. COX-2 may be a major player because it regulates neutrophil production of ROS, TNFα and TX under conditions of oxidative stress. COX-2 inhibition might stop the vicious cycle of oxidative stress, neutrophil infiltration and vascular inflammation.

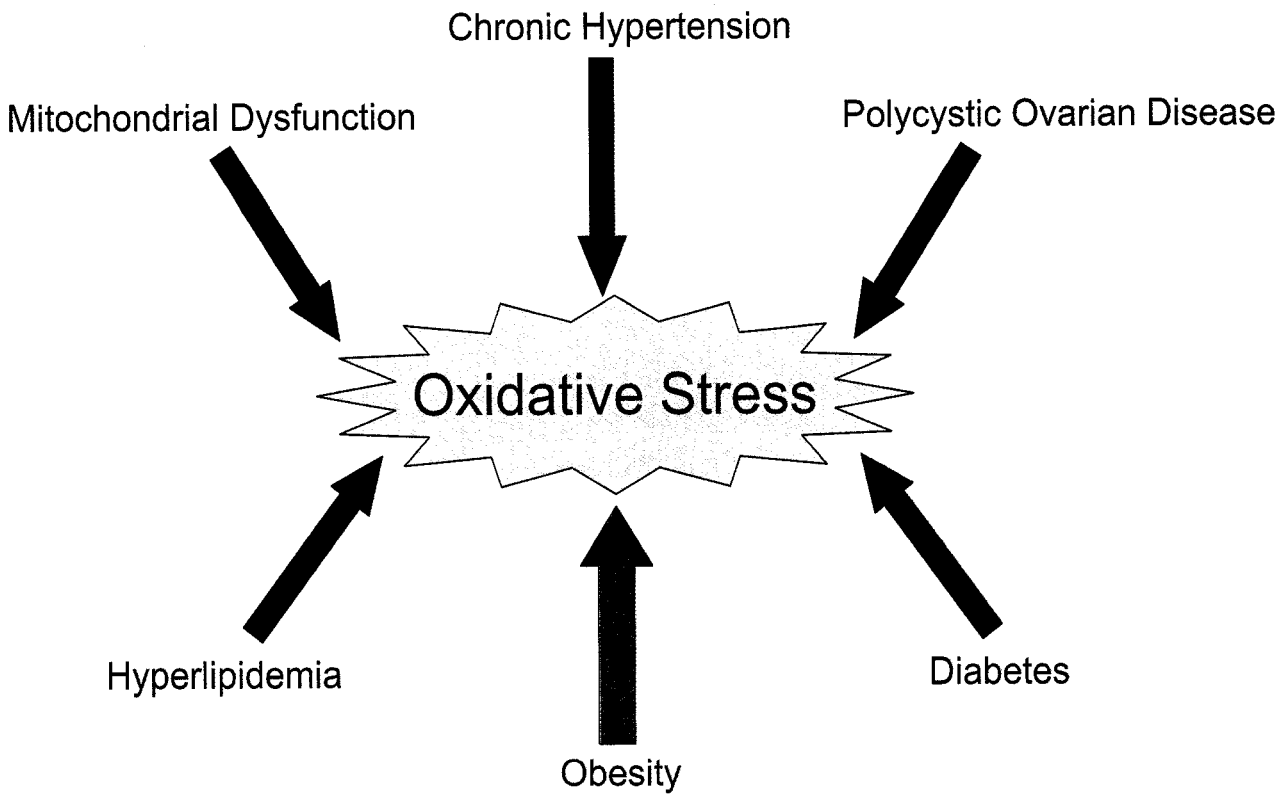


Figure 1. Risk factors for preeclampsia have a common theme of oxidative stress.

Preeclampsia is a complex disease associated with many risk factors, including mitochondrial dysfunction, chronic hypertension, polycystic ovarian disease, hyperlipidemia, diabetes and obesity. Oxidative stress is associated with each of these risk factors.

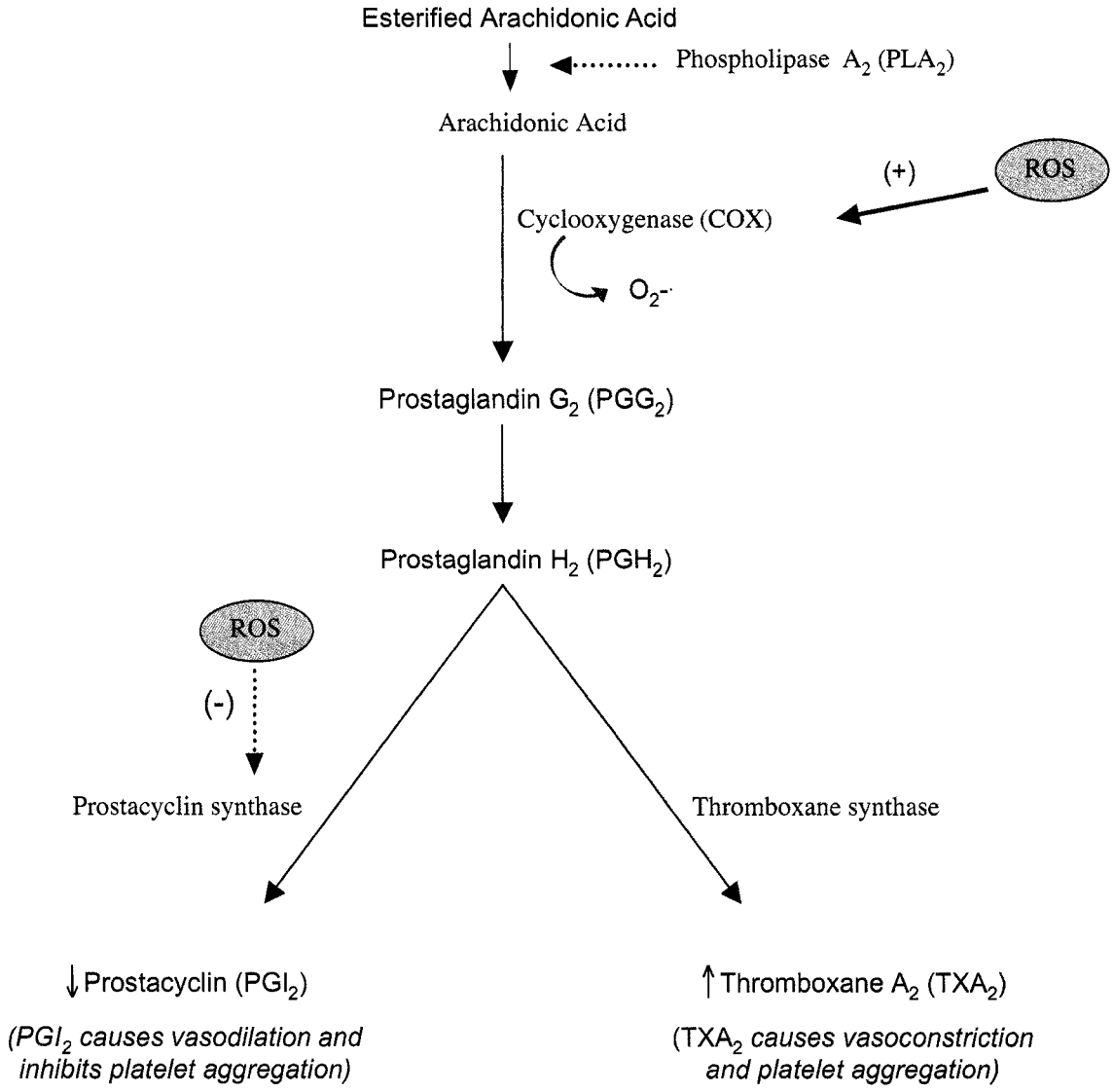


Figure 2. Mechanism whereby oxidative stress promotes a vasoconstrictive environment in vascular tissue by affecting AA metabolites.

ROS activate cyclooxygenase to increase thromboxane, a potent vasoconstrictor, but at the same time inhibit prostacyclin synthase to decrease prostacyclin, a potent vasodilator.

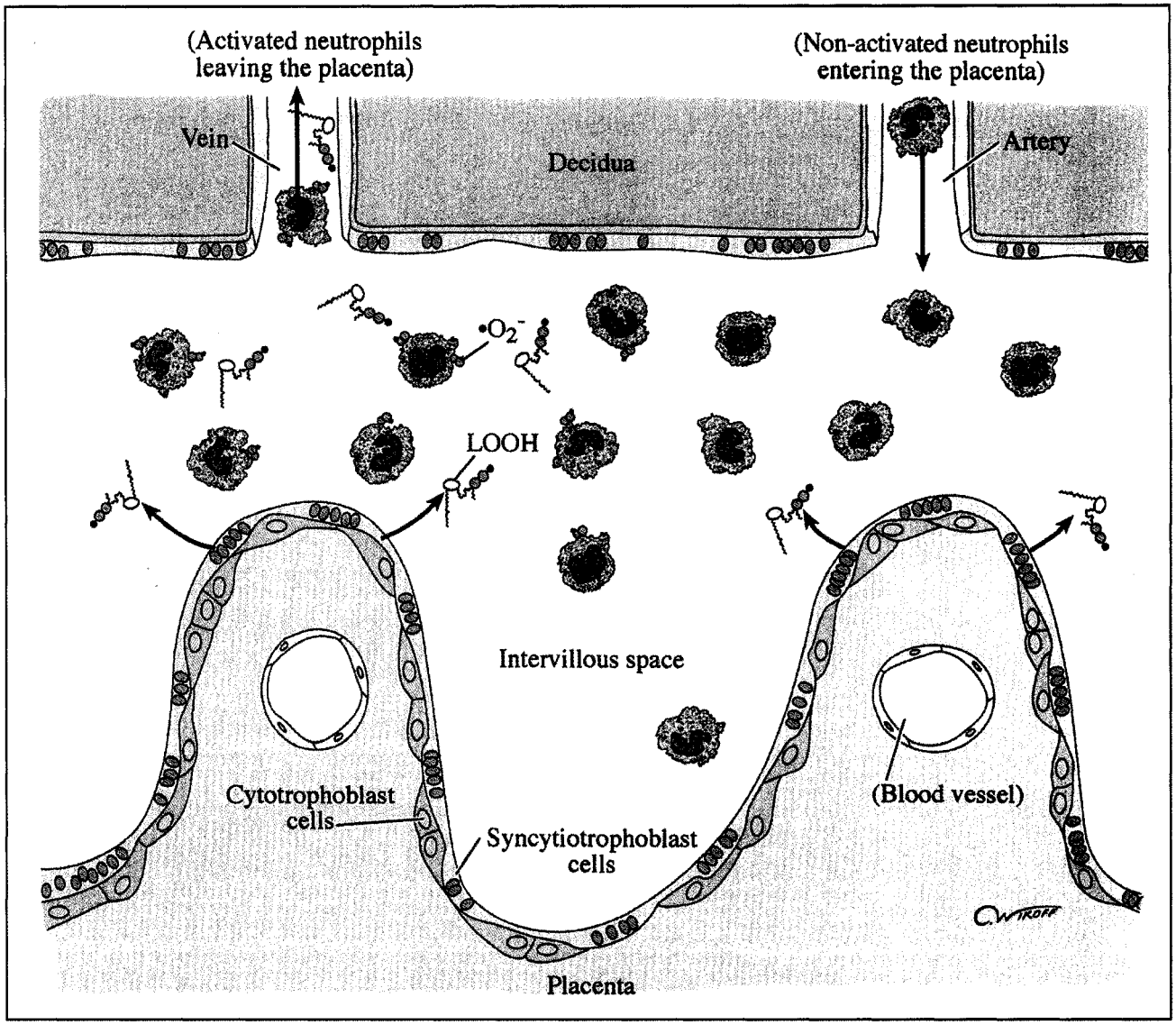


Figure 3. Mechanism for neutrophil activation in the intervillous space in preeclampsia.

Mechanism whereby placental secretion of lipid peroxides (LOOH) activates neutrophils as they circulate through the intervillous space. LOOH are potent activators of neutrophils, so as neutrophils return to the maternal circulation they could transmit the oxidative stress of the placenta to the maternal vasculature. If the maternal vasculature were already in an inflamed state, such as obesity, this additional neutrophil insult could be enough to cause preeclampsia. (Used with permission of artist. From Walsh, S.W. The role of oxidative stress and antioxidants in preeclampsia. Contemporary OB/GYN, 1997; 42:113-124.)

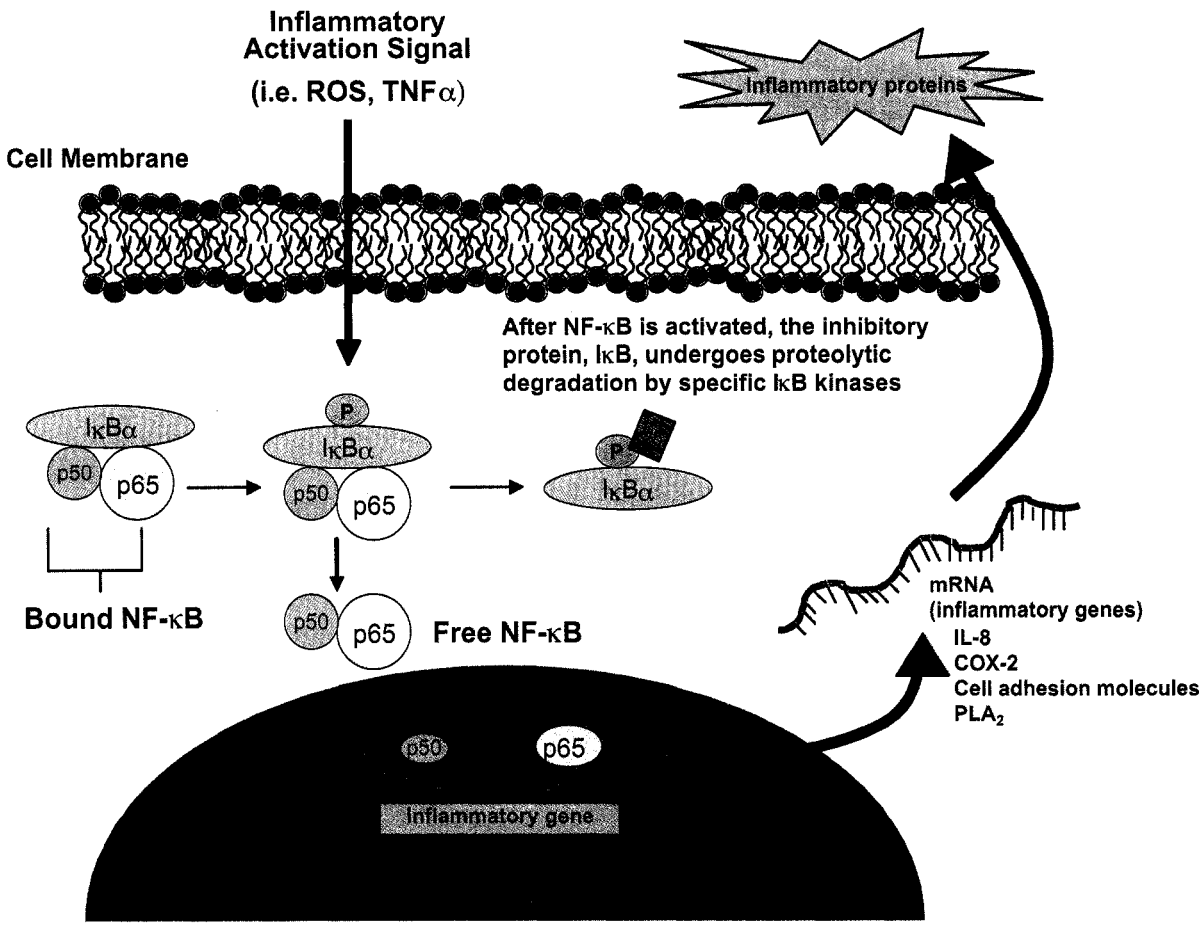


Figure 4. Activation of NF-κB during inflammation.

Activation of NF-κB (a heterodimer of p50 and p65 subunits) involves the phosphorylation and subsequent proteolytic degradation of the inhibitory protein IκB by specific IκB kinases. The free NF-κB subunits then pass into the nucleus, where they bind to sites in the promoter regions of genes for inflammatory proteins such as cytokines, enzymes and adhesion molecules.

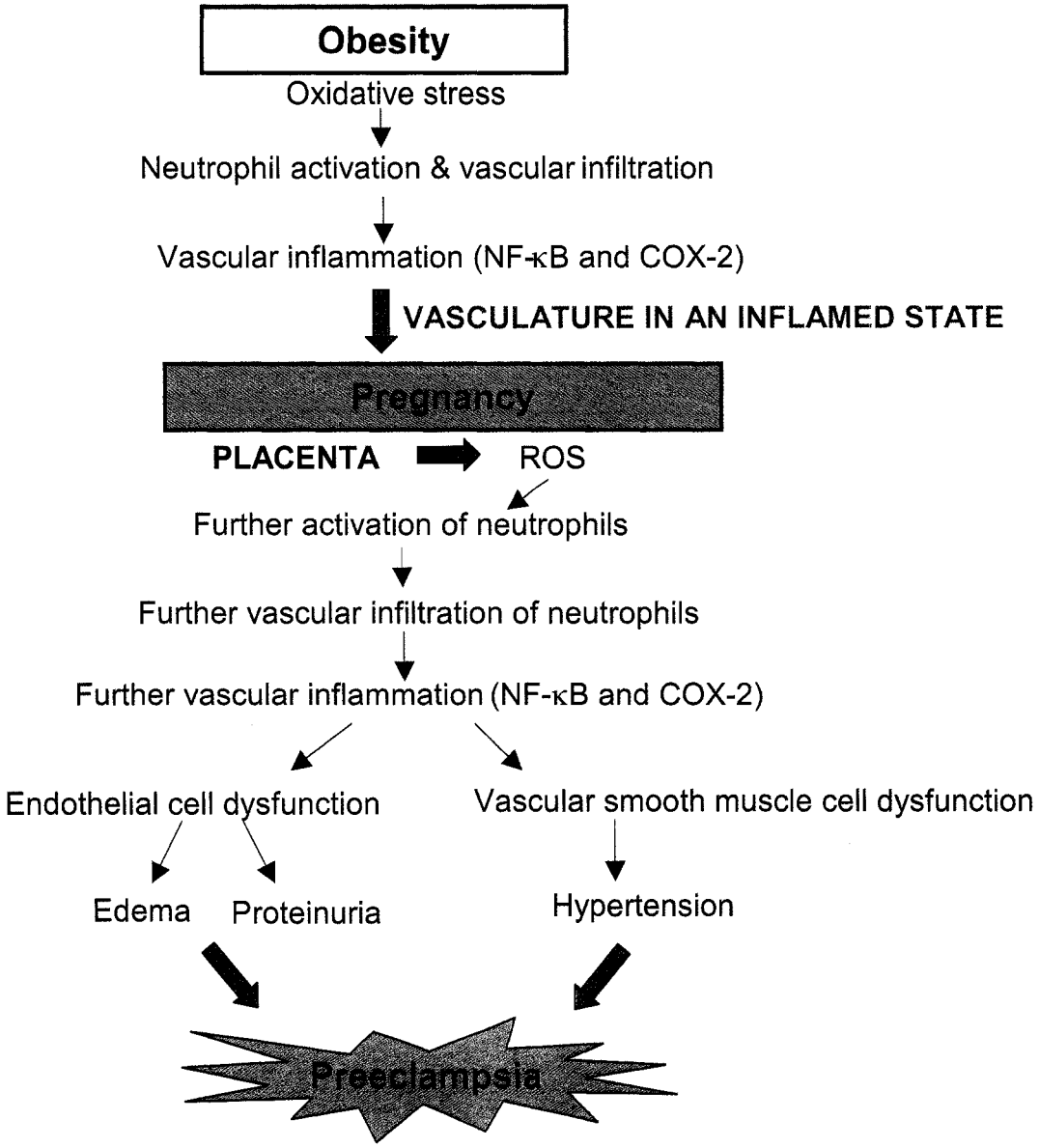


Figure 5. Proposed mechanism of why obese women are at increased risk for preeclampsia.

Obese women experience oxidative stress which activates neutrophils leading to vascular infiltration. This leads to vascular inflammation as indicated by NF-κB activation and COX-2 expression. When obese women become pregnant, the placenta secretes ROS. This increased oxidative stress causes further activation and infiltration of neutrophils which leads to further vascular inflammation and vascular cell dysfunction. Edema and proteinuria may result from endothelial cell dysfunction, whereas hypertension may result from vascular smooth muscle cell dysfunction. Consequently, all of these factors put obese women at increased risk for developing preeclampsia.

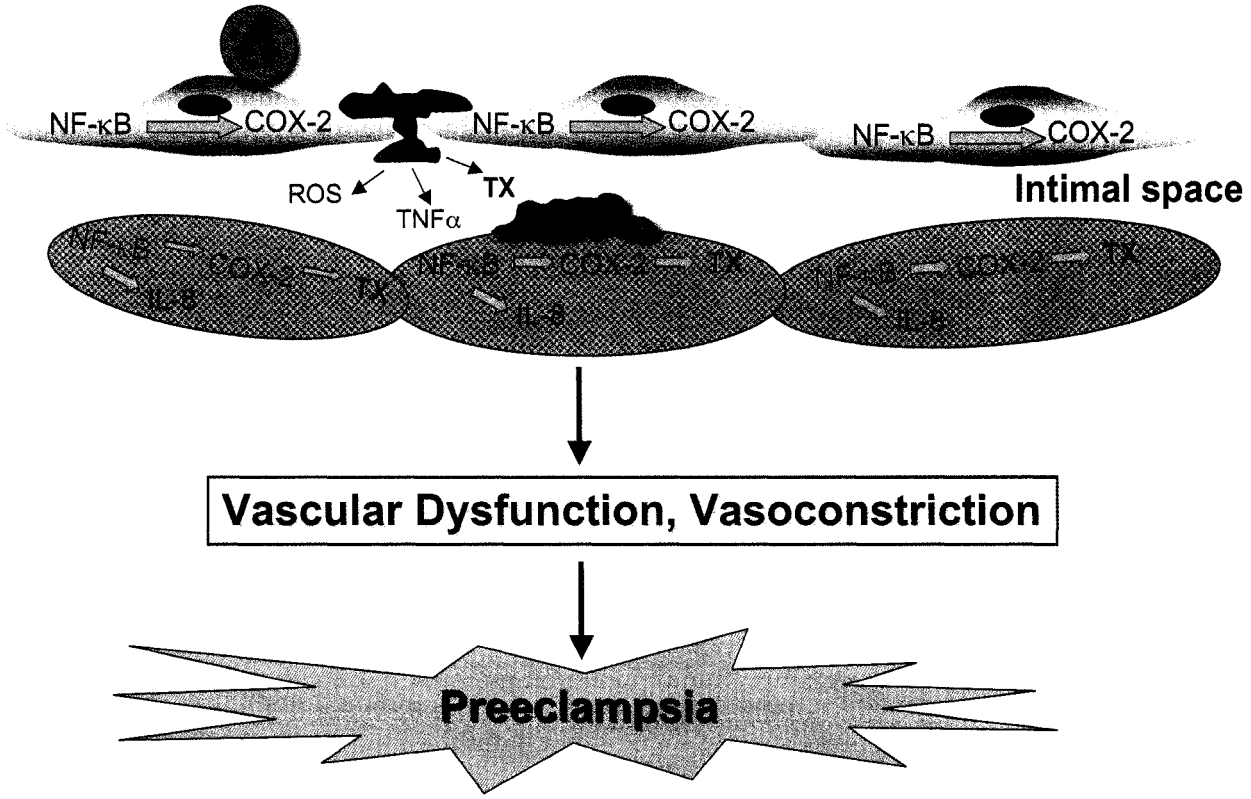
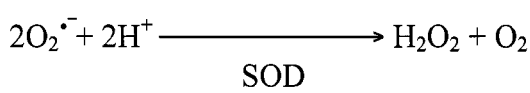
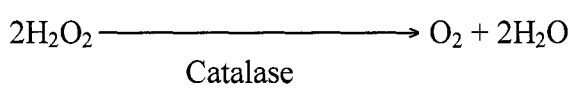
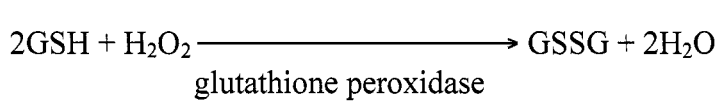
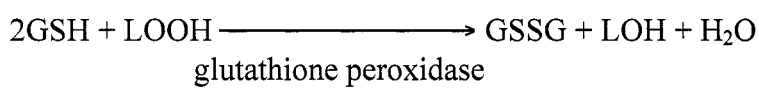


Figure 6. A visual representation of our hypothesis of activation of NF-κB and expression of COX-2 in maternal systemic vasculature coincident with infiltration of neutrophils in women with preeclampsia.

Activated neutrophils adhere and flatten onto endothelial cells, where we speculate that their release of ROS and TNFα cause activation of endothelial NF-κB and expression of COX-2. As neutrophils infiltrate into the intimal space and adhere to vascular smooth muscle cells, we speculate that they cause vasoconstriction by release of ROS and TX, and by activation of NF-κB in the vascular smooth muscle which leads to COX-2 expression and an increase in vascular smooth muscle TX. NF-κB activation also leads to an increase in IL-8 in the vascular smooth muscle which would further increase neutrophil infiltration. As a consequence of these events, these women develop preeclampsia.

Table 2. Cellular Antioxidant enzymes and their actions.

Antioxidant Enzyme	Action
Superoxide Dismutase (SOD)	Reduces superoxide radical to hydrogen peroxide and molecular oxygen 
Catalase	Converts hydrogen peroxide to molecular oxygen and water 
Glutathione peroxidase	Uses glutathione (GSH) as a hydrogen donor to convert glutathione into glutathione disulfide (GSSG) to convert hydrogen peroxide to water and lipid peroxide to water and a fatty acid alcohol  

CHAPTER 2

MATERIALS AND METHODS

A. Immunohistochemical Staining (Studies 1 and 2)

i. Collection of Fat

Subcutaneous fat biopsies (approximately 1 cm x 1 cm x 1 cm) were collected at cesarean section or abdominal surgery. Biopsies were placed in 10% neutral buffered formalin in the operating room. The tissue was further processed in the laboratory. Formalin-fixed samples were cut into smaller pieces. These pieces were then placed in Tissue Tek cassettes, labeled with patient's initials, patient type and date of collection. The biopsies were then returned to 10% neutral buffered formalin and placed on a rotating shaker for five days. On the fifth day, the biopsies were taken out of formalin and fixation was confirmed by observation and touch. Formalin-fixed samples were rinsed with ddH_2O and placed in 100 mM phosphate buffer, pH=7.5 until paraffin embedding.

ii. Protocol for Paraffin Embedding, Formalin Fixation and Immunostaining

Formalin-fixed tissue was used for NF-κB, COX-2, and CD66b staining. Tissue was dehydrated in a graded alcohol series (70%, 80%, 95%, 95%, 100%, 100%), cleared in CitriSolv (Fisher Scientific, Malvern, PA), and paraffin-embedded overnight using an automated tissue processor (Shandon Citadel 2000 Tissue Processor, Shandon Scientific Limited, Cheshire, England). The next morning, the paraffin embedded samples were placed on metal embedding trays and filled with warm paraffin. Plastic embedding rings which were labeled with patient information, patient group and date of collection were placed on top and filled with warm paraffin. The trays and embedding rings were then placed on ice until cool. Once cooled the tissue blocks were gently removed from the tray.

Paraffin embedded tissue was cut in 10 μm sections using a microtome (820 Spence Microtome, American Optical Company). Sections were floated on a preheated 42 °C ddH_2O bath (Flotation Bath Model 135, Fisher Tissue Prep, Fisher Scientific, Malvern, PA), separated into tissue sections of two, and placed on prelabeled (patient's information, patient's group, date of collection) Superfrost Plus glass slides (Fisher Scientific, Malvern, PA), that were pre-treated with Vectabond (Vector Laboratories, Burlingame, CA) to increase adhesion of the tissue to the slides. Slides were dried on a 37 °C slide warmer (C.S. & E Slide Warmer No. 26020, Clinical Scientific Equipment Co., Melrose Park, Illinois), and then placed in a 37 °C oven overnight.

On the day of staining, tissue sections were cleared three times with Histoclear (National Diagnostics, Atlanta, Georgia) to remove paraffin (five minutes each) and then

hydrated in a graded alcohol series (100%, 100%, 95%, 95%, 85%, 50%, 0%; two minutes each). Tissue sections were then placed in 100 mM phosphate buffer, pH=7.5 for ten minutes. The tissue sections were incubated in 3% H_2O_2 in methanol for thirty minutes to quench endogenous tissue peroxidase. Slides were then washed in 100 mM phosphate buffer, pH=7.5 for five minutes and Grade 1 ultrapure water for six minutes.

Antigen retrieval was performed using a beaker filled with 600 ml of 10mM citrate buffer and heated on a hot plate to a gentle boil. Slides were placed in the solution for 15 minutes. After heating, the beaker was removed from the hot plate and allowed to cool for 15-20 minutes. Slides were rinsed with phosphate buffer saline (PBS, 1X, pH=7.4, Invitrogen Corporation, Grand Island, NY) and incubated with primary antibodies for 30 minutes followed by three washes in PBS with Tween (0.05%) 2 minutes each.

The primary antibodies and titers were: 1) rabbit anti-human polyclonal antibody specific for the free p65 subunit of NF-κB (1:200, Zymed Laboratories, San Francisco, CA); 2) mouse IgG anti-human monoclonal antibody specific for COX-2 (1:400, Zymed Laboratories, San Francisco, CA); 3) mouse IgM anti-human monoclonal antibody specific for CD66b (1:50, BD BioSciences, San Diego, CA); 4) mouse IgG monoclonal isotype used as a negative control (pre-diluted, Zymed Laboratories, San Francisco, CA) and 5) a mouse IgM monoclonal isotype standard used as a negative control for CD66b (pre-diluted, BD BioSciences, San Diego, CA).

CD66b is a granulocyte-specific membrane antigen that is upregulated and released upon granulocyte activation. It is present in the membrane of specific and gelatinase-containing granules of neutrophils [175]. Granulocytes are comprised of neutrophils,

eosinophils, and basophils. Our data represent primarily neutrophils because neutrophils comprise 96% of the granulocyte population. In addition, neutrophils respond to inflammation [8], whereas eosinophils and basophils respond to infection caused by parasites in the body and various forms of allergy.

After incubation with primary antibodies, slides were incubated with horseradish peroxidase (HRP) polymer conjugate (ready-to-use) (Zymed SuperPicture Kit, Zymed Laboratories, San Francisco, CA) for 10 minutes followed by three washes in PBS with Tween, 2 minutes each. Finally, slides were stained with diamino benzamine (DAB) substrate for five minutes (Zymed Laboratories) followed by 3 rinses in PBS with Tween, 2 minutes each.

Counterstaining was performed on tissue sections once immunohistochemical staining was finished. Slides were placed in Alcian Blue (Recipe from Bancroft, John D., Theory and Practice of Histological Techniques, 1990) for five minutes and then rinsed in tap water. Tissue sections were then covered with methyl green and placed in a 60°C oven for three minutes. Slides were rinsed in ddH_2O for 1 minute and dipped 5 times in 0.05% acetic acid in acetone. Slides were then placed in 50% ethanol and tissue was dehydrated in alcohol series (50%, 85%, 95%, 95%, 100%, 100%; two minutes each).

Tissue sections were cleared two times with Histoclear (National Diagnostics, Atlanta, Georgia) for three minutes each. To preserve the tissue staining, 2 drops of Vectamount (Vector Laboratories, Burlingame, CA) were dropped on slides and a cover slip was gently placed on top of the slide.

iii. Data Analysis

To quantify staining, all vessels on each tissue section were evaluated by a visual score and optical density. Lumen width was measured for each vessel. Vessels between 10 μm and 200 μm, which represent resistance-sized vessels, were analyzed for staining. Vessels stained for NF-κB and COX-2 were graded using a visual score ranging from zero to three (absent to intense staining, Figure 20) and also were evaluated by density of staining using image analysis software (IP Lab, Scanalytics, Inc., Fairfax, VA). The visual score was correlated to density measurements to verify objectivity of the visual score. For NF-κB and COX-2, the percent of total vessels stained was evaluated, as well as the percent of vessels with neutrophils stained and the percent of vessels with vascular smooth muscle stained. Neutrophils stained for both NF-κB and COX-2. Likewise NF-κB and COX-2 staining was present in the vascular smooth muscle.

For CD66b staining each vessel was graded using a visual score ranging from zero to three based on overall staining intensity and neutrophil infiltration. Diffuse staining for CD66b was also evaluated using image analysis software, because the CD66b protein is secreted from activated neutrophils. In addition to visual scores and density measurements, CD66b staining of neutrophils was recorded as percent of vessels with staining and percent of vessels with staining present within the lumen, adhered to the endothelium, present within the intima and outside of the vessel. The number of stained neutrophils was counted in each of these locations.

B. Placental Arterial Smooth Muscle (PASM) Cell Isolation

Placentas were collected at the time of term delivery from normal pregnant women at MCV Hospitals, Virginia Commonwealth University Health System. Informed consent was obtained prior to delivery. This study was approved by the Virginia Commonwealth University Office of Research Subjects Protection.

PASM cells were isolated as previously described [176]. In a sterile environment, the chorionic plate arteries were identified and excised from the chorionic plate. The vessels were rinsed three times in a solution of Hank's Balanced Salt Solution (HBSS) (1X, with phenol red, Invitrogen Corporation, Grand Island, NY) containing 2x strength antibiotic/antimycotic (100 U/ml of penicillin, 100 μg/ml streptomycin, 25 μg/ml amphotericin B, Life Technologies, Long Island, NY). Next the vessels were placed in a sterile culture plate and cut longitudinally to expose the lumen. Vessels were dissected into small pieces of tissue (approximately 3-5 mm) and placed in a separate 100 mm culture plate with the lumen facing down. Approximately 20-25 explants were placed in each culture plate. Dulbecco's Modified Essential Media (DMEM, Life Technologies, Long Island, NY) supplemented with 10% fetal bovine serum (FBS, Life Technologies, Long Island, NY) (5 ml) was carefully added to the culture plate so as not to disturb adhered explants. Culture plates were placed in a 37°C incubator (5% CO_2).

Media were removed and replaced with fresh media twice a week. At this time, culture growth and cell morphology were examined using an inverted light microscope. Cell cultures became confluent in approximately 4 weeks. At confluence, cells were placed

in Dulbecco modified eagle medium (DMEM-0) without serum for 24 hours. This was performed to eliminate any contaminating cells, such as fibroblasts, endothelial cells or leukocytes which do not survive in medium without serum. The placental arterial smooth muscle (PASM) cells were then prepared for freezing. First, the PASM cells were rinsed with DMEM-0 to remove trypsin inhibitor proteins. Next, 2 ml of 0.25 % Trypsin-EDTA (1X) (Invitrogen Corporation, Grand Island, NY)) was added to the cells and plates were placed in an incubator for 3 minutes. Cells were observed under a microscope to determine if cells were lifted off the plate. Cells were collected into a 50 ml centrifuge tube by rinsing the culture dish with HBSS and then centrifuged at 1300 rpm for ten minutes. The supernatant was aspirated and the pellet was resuspended in 3-4 ml of M199. Then 1 ml of the cell suspension was added to a T-75 flask. Following seeding into the T-75 flask, 8 ml of M199 with 10% FBS were added to the T-75 flask and placed in the incubator. Cells were grown to confluence and then trypsinized. A cell count was performed by diluting 100 μL of cell suspension with 300 μL of trypan blue and then counting the cells with a hemocytometer. Cells were diluted to 1 million cells/ml and then frozen in 1 ml aliquots with 200 μL DMSO/vial (Sigma Chemical Company, St. Louis, Missouri). Vials were placed in a Nalgene Cryo Freezing Container and put in -20°C freezer for 1-2 hours. Once frozen vials were placed in a -80 °C and the following day placed in liquid nitrogen.

C. Transfection (Study 3)

PASM cells (passages 3-6) were used for the transfection experiments. Reporter vectors were obtained from Dr. A.A. Fowler in the Department of Internal Medicine, Virginia Commonwealth University. Transient transfection studies were done with a pGL3 luciferase reporter construct containing the NF-κB binding region of the human IL-8 promoter upstream from the firefly luciferase gene (Figure 7, top panel). Transfection of this reporter construct into the cells allowed determination as to whether a particular treatment activated NF-κB, because activation of the NF-κB binding site of the reporter would result in expression of firefly luciferase with an increase in luminescence. Development of this reporter construct, designated BF^2, was previously described [177]. The identity of the NF-κB binding site on BF^2 was confirmed using a BF^2 mutant, which was created by site-directed mutagenesis, to introduce a four-point transversion mutation into the native NF-κB site. For validation, negative controls received plasmid bearing the luciferase gene, but lacking an upstream promoter (pGL3-basic), whereas positive controls received a plasmid (pGL3-SV40) bearing the strong viral promoter SV40 linked to (*luc* +). pRL-TK, which contains a weak herpes simplex virus thymidine kinase promoter region upstream from Renilla luciferase gene, was cotransfected as a control for the transfection procedure (Figure 7, bottom panel). pRL-TK produced a low level of luminescence. Luminescence produced by BF^2 was indexed to luminescence produced by pRL-TK.

For the experiment, 24-well plates were seeded with 40,000 cells/well, supplemented with 10% FBS, and grown for 2 days so that on the day of the experiment

cells were 80% confluent. Reporter constructs were dissolved in Buffer EC (0.15 μg DNA/ 60 μl buffer) according to manufacturer's protocol for transfection (Qiagen, Valencia, CA). An enhancer (1.6 μl/0.15 μg DNA) (Qiagen, Valencia, CA) was added to the mixture and then vortexed for 1 second. The mixture was incubated at room temperature for 2-5 minutes and then spun down for a few seconds to remove drops from the top of the tube. The Effectene transfection reagent (1.5 μl/0.15 μg DNA) was added to the DNA-enhancer mixture and then vortexed for 10 seconds. The samples were incubated for 5-10 minutes at room temperature to allow complex formation (Figure 8). While the complex formation was taking place, growth medium was aspirated from the plates and wells were washed once with 1ml PBS. Then 350 μl M199 was added to the cells in each well. Following the incubation, M199 medium (350 μl /0.15 μg DNA) was added to the reaction tubes containing the transfection complexes. The tubes were mixed by pipetting up and down twice and immediately 415 μl of the transfection complexes were added to the appropriate wells in the 24-well plate. The plate was gently swirled to ensure uniform distribution of the complexes. Then cells were incubated for 6 hours at 37°C to allow the vector to be incorporated into the cells. Following the incubation, cells were washed with 1 ml PBS and then 1 ml of the treatment solution was added to the appropriate wells and incubated for 16 hours (Figure 8). The following day cells were washed with 1 ml PBS and then 20 μl Passive Lysate Buffer was added to each well to lyse the cells. Plates were then placed in the -80°C freezer until the dual luciferase assay.

To quantify luminescence from the transfected cells we used a Dual Luciferase Reporter (DRL) Assay system (Promega, Madison, WI) according to manufacturer's

instructions (Figure 9). A Fluostar Optima spectrophotometer (BMG LabTech, Durham, NC) was used for measurement. Before beginning, injectors 1 and 2 were set to dispense 100 µl Luciferase Assay Reagent II or Stop & Glo® Reagent, respectively. Plates were taken out of the -80°C freezer, thawed at room temperature and then luminescence was measured using the automated spectrophotometer.

D. Cell Culture (Study 4)

i. Cell Harvesting

PASM cells (passages 3-6) were used in experiments to determine if neutrophil products, ROS or TNFα, would induce COX-2 expression and increase production of TX and IL-8. The cells that were used in this experiment were taken out of vials that were stored in liquid nitrogen. The cells were seeded in a T-75 flask (1,000,000/flask) and grown to confluence. Cells from the T-75 flask were harvested and then passed to eight T-25 flasks, grown to confluence and given specific treatments. The day following the treatments, the cells were harvested in a sterile environment. The media was aspirated off after first collecting 1 ml samples and the T-25 flasks were rinsed with 2 ml of HBSS solution for approximately 30 seconds. Then 2.5 ml of 0.25% Trypsin-EDTA (1X) (Invitrogen Corporation, Grand Island, NY) was added to each plate and plates were placed in the incubator for 3-5 minutes. The trypsinized cells were transferred into 15 ml centrifuge tubes, the flasks were rinsed with 2 ml HBSS which was transferred to the

centrifuge tubes, and 1 ml FBS was added to neutralize the trypsin. Following centrifugation for 10 minutes, cells were resuspended in 2 ml of M199 and counted on a hemocytometer. Cell viability was confirmed by Trypan blue exclusion staining. The equation for determining the number of cells was:

{(Average # of cells/4) x 10,000}= # cells/ml

Following the cell count, cells were centrifuged for 10 minutes and the supernatant was vaccumed off. Each pellet was resuspended in 500 µl of TNE-buffer (10mM Tris, pH 8.0, 1% NP-40, 0.15M NaCl, 1mM EDTA, Zymed Laboratories, San Francisco, CA) and placed in a -20°C freezer until used.

ii. COX-2 ELISA

The detection of human COX-2 in PASM cells was performed using a COX-2 Enzyme Linked Immunosorbent Assay (ELISA) kit (COX-2 ELISA Kit, Zymed Laboratories, San Francisco, CA). This assay is an enzyme-linked immunoabsorbant sandwich assay used for quantitative detection of COX-2 in cell culture media. A standard curve was prepared from a COX-2 standard (550 ng/ml) by making eight serial dilutions resulting in standards ranging in concentrations between 275 ng/ml and 2.15 ng/ml. Tube 9 was a buffer blank with a concentration of 0 ng/ml. Once the standards were made, 100 µl each of the blank (tube-9), standards (tubes 1-8) and samples were put into appropriate wells of a 96-well plate. The plate was incubated for 1 hour at 37°C. Following the incubation each well was washed vigorously three times with the wash buffer (40X,

provided by the kit) for 15-30 seconds. Then 100 µl of labeled antibody was pipetted into each of the wells and test samples and the plate was incubated for 30 minutes at 4°C. The wells were then washed and 100 µl of TMB buffer was pipetted into each well and the plate was incubated for 30 minutes in the dark. The liquid turned blue after the addition of the TMB buffer. Following the incubation, 100 µl of Stop solution was added to each well and the liquid was mixed by tapping the side of the plate. The liquid turned yellow upon addition of the Stop solution. Within 30 minutes of the addition of the Stop solution, the absorbance was read at 450 nm using the Fluostar Optima spectrophotometer (BMG LabTech, Durham, NC).

iii. Thromboxane B_2 ELISA

A Thromboxane B_2 Immunoassay (R& D Systems, Minneapolis, MN) was used to measure the amount of TXB_2 present within cell culture media using a competitive binding technique in which TXB_2 present in a standard or in a sample competes with a fixed amount of alkaline phosphatase-labeled TXB_2 for sites on a rabbit polyclonal antibody specific for TXB_2. A standard dilution series was produced by first pipetting 900 µl of M199 into a tube containing 10,000 pg/ml TXB_2. Then serial dilutions were made to achieve concentrations ranging from 3,333 pg/ml to 13.7 pg/ml. M199 (150 µl) was added to the non-specific binding (NSB) well, 100 µl of M199 was added to the zero-standard (B_0) well and 100 µl of each standard was added to appropriate standard wells. For the sample wells, 50 µl of M199 and 50 µl of sample was added to each well. The addition of

50 µl of TXB_2 Conjugate (alkaline phosphatase-labeled TXB_2) was added to all wells and 50 µl of TXB_2 Antibody Solution (rabbit polyclonal antibody) was added to each well (excluding the NSB well). Following the application of all reagents, the plate was incubated for 2 hours at room temperature on a microplate shaker set at 500 ± 50 rpm. After the incubation, each well was aspirated and washed with a 1X wash buffer (provided by kit), repeating the process twice for a total of three washes. Following the washes, 200 µl of TXB_2 pNPP (p-nitrophenyl phosphate substrate) was added to all wells and the plate was incubated for 45 minutes at room temperature. After 45 minutes, 50 µl of Stop Solution (trisodium phosphate) was added to each well and the optical density was immediately determined using the Fluostar Optima spectrophotometer (BMG LabTech, Durham, NC) set at 405 nm.

iv. IL-8 ELISA

Cell culture media were analyzed for IL-8 by commercially available ELISA reagents (R & D Systems, Minneapolis, MN). Solutions were made according to manufacture's instructions. To begin the assay, the plate was prepared the evening before by adding 100 µl of capture antibody to each well of a high affinity EIA 96-well plate. The plate was sealed and incubated overnight to allow the antibody to bind to the plate. The following day the remaining solution was aspirated and the plate was washed with buffer three times. Plates were blocked for one hour with 300 µl blocking solution per well and then washed three times. Following the blocking step, 100 µl of the standard (range 5,000

pg/ml to 9.25 pg/ml) or sample was added to the appropriate wells and allowed to incubate at room temperature for two hours. The plate was washed three times and then 100 µl of biotinylated detection antibody was added to the wells and incubated for two hours. The plate was washed three times and then 100 µl of streptavidin horseradish peroxidase (HRP) was added to each well for 20 minutes, followed by three washes and then 100 µl of substrate solution (H_20_2 + tetramethylbenzidine, 1:1) was added to each well and allowed to incubate for 20-30 minutes at room temperature, but out of direct light. Then 50 µl stop solution (0.5 M H_2S0_4) was added to each well and measurements were made using the Fluostar Optima spectrophotometer (BMG LabTech, Durham, NC) set at 450 nm.

v. BCA Protein Assay

The BCA Protein Assay (Pierce, Rockford, IL) is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. A set of diluted albumin (BSA) standards from 2,000 µg/ml to 0 µg/ml was produced according to the protocol. Once the standards were made, 25 µl of each standard or unknown sample was pipetted into a microplate well. Then 200 µl of the working reagent (50 ml Reagent A: 1 ml Reagent B) was added to each well and mixed thoroughly on a plate shaker for 30 seconds. The plate was then covered and incubated at 37°C for 30 minutes. After the incubation the plate was cooled to room temperate and the absorbance was measured on the Fluostar Optima spectrophotometer (BMG LabTech, Durham, NC) set at 562 nm.

E. Statistical Analysis

i. Immunostaining

Visual score data for studies 1 and 2 were analyzed by Kruskal-Wallis test with Dunn's post hoc test to determine differences between patient groups. For density measurements, one-way analysis of variance (ANOVA) was used with Newman-Keuls post-hoc test. Regression analysis was performed to correlate visual scores with density measurements.

Statistical analysis was done for percent of vessels with staining for CD66b, NF-κB and COX-2. One-way ANOVA was used with Newman-Keuls post-hoc test to determine differences between patient groups. For CD66b staining data, statistical analysis was also performed for the percent of vessels with staining in the following vessel locations: 1) within the lumen, 2) adhered to the endothelium, and 3) within the intima.

ii. Transfection and Cell Culture

Data were analyzed for studies 3 and 4 by ANOVA and Student-Newman-Keuls post hoc test was used to determine differences between treatment groups. Data that had unequal variances were analyzed by Kruskal-Wallis test with Dunn's post hoc test.

Statistical analysis was performed using Prism GraphPad (San Diego, CA). Bar graph data are reported as mean ± SE. P less than 0.05 was considered statistically significant. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

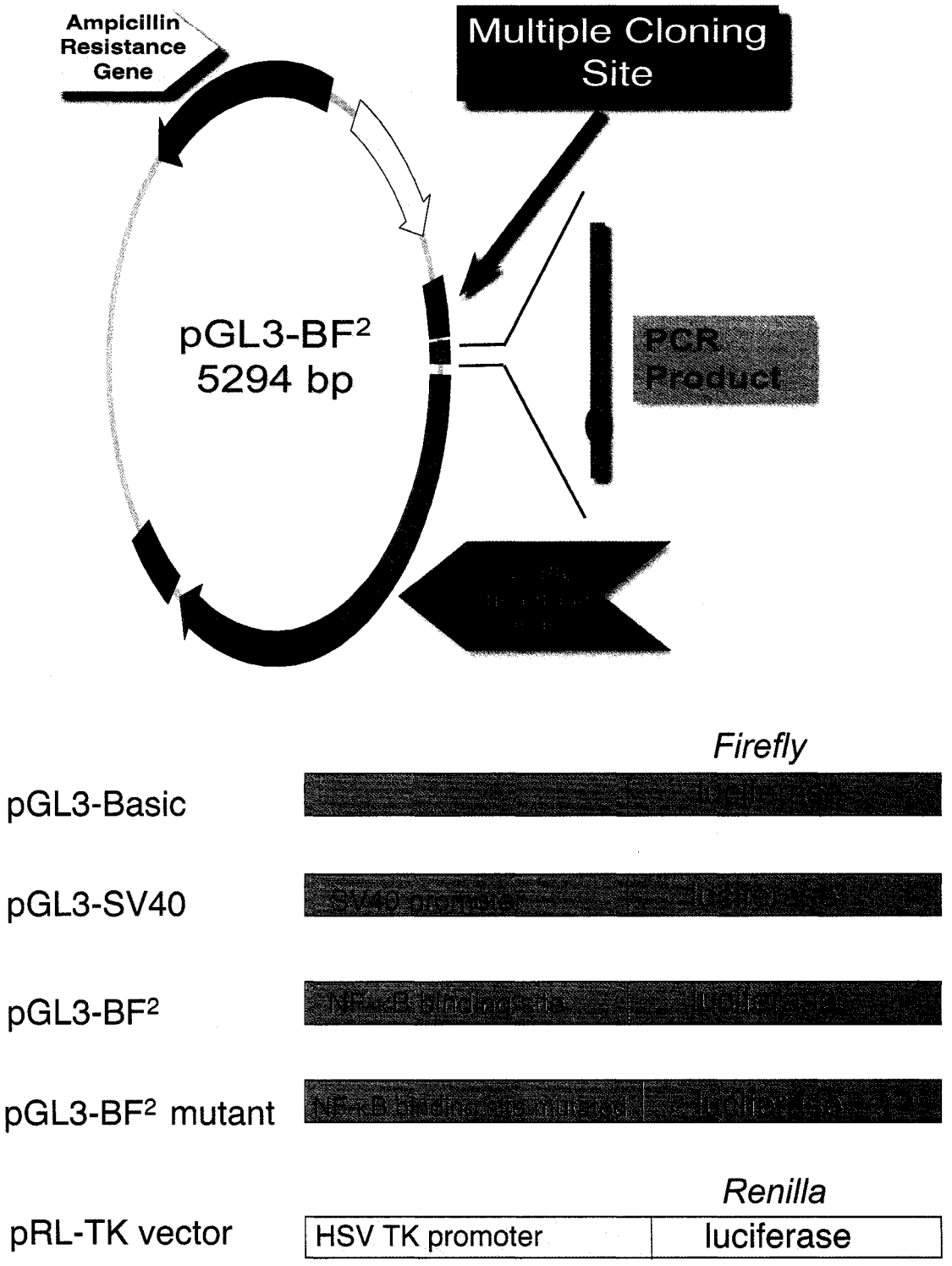


Figure 7. NF-κB reporter plasmid (top panel) and experimental plasmids (bottom panel).

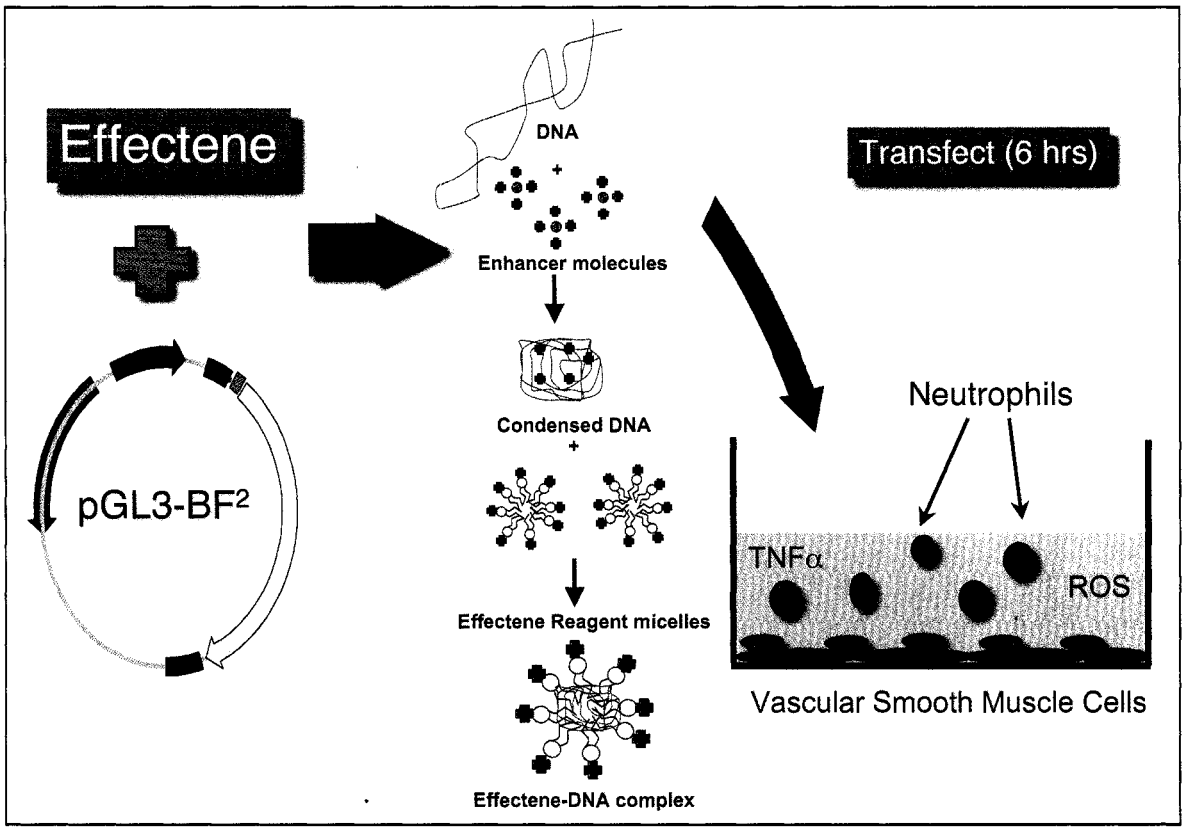


Figure 8. Transfection experiment.

The Effectene Transfection Reagent is an innovative non-liposomal lipid formulation that is used in conjunction with a special DNA-condensing enhancer and optimized buffer to achieve high transfection efficiencies. The enhancer first condenses the DNA molecules and Effectene Reagent subsequently coats them with cationic lipids to form micelles. Then the effectene-DNA complex is transfected into the cells for 6 hours. Following transfection, neutrophils, ROS or TNFα treatments are added to the vascular smooth muscle cells for 16 hours.

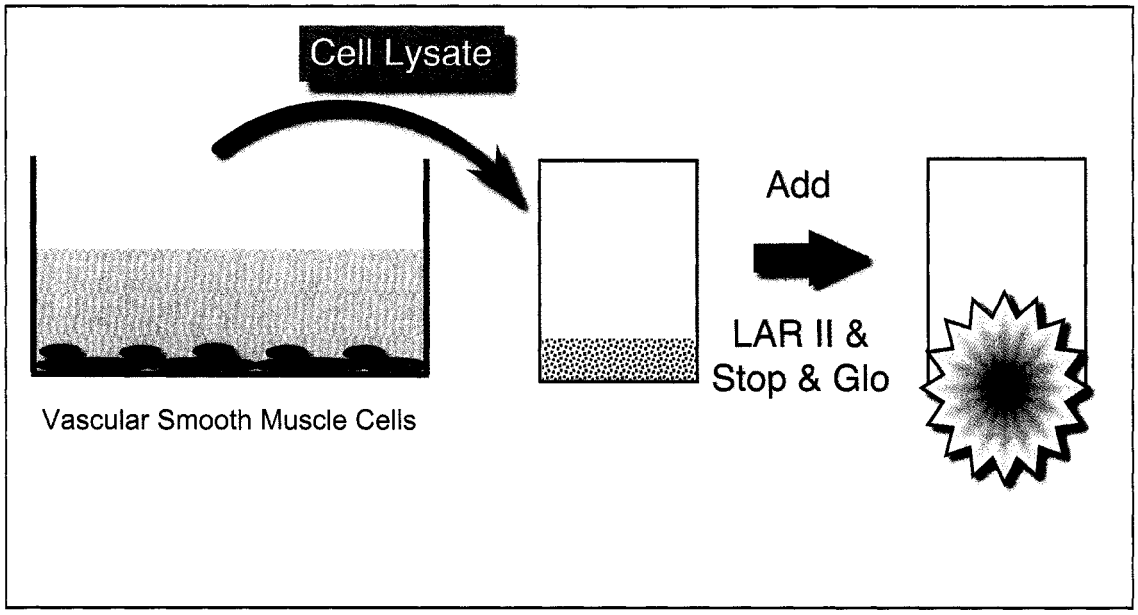


Figure 9. Assessing NF-κB activation by firefly luminescence.

The day following treatments, cells are lysed with passive lysate buffer, which ensures that the vector gets into the media. Cell lysates (20 μl) are placed into wells of a 96-well plate, and luminescence is measured with an automated luminometer. Luciferase Assay Reagent II is added to measure activation of the firefly luciferase stimulated by treatment. After quantifiying the firefly luminescence, the reaction is quenched by adding Stop & Glo reagent and the Renilla luciferase reaction is measured as a control for cell viability and transfection efficiency. Firefly luciferase is normalized to Renilla luciferase when calculating the results and the data are expressed as relative light units (RLU).

CHAPTER 3

NEUTROPHIL INFILTRATION AND SYSTEMIC VASCULAR INFLAMMATION IN OBESE WOMEN

A. Introduction

Obesity is a risk factor for many diseases including hypertension, type II diabetes and preeclampsia. A recent study showed that neutrophil infiltration and vascular inflammation occur in the systemic vasculature of women with preeclampsia [127]. We hypothesized that obese women might be at increased risk of developing preeclampsia because their vasculature is already in an inflamed state with neutrophil infiltration. To evaluate if this might put obese women at increased risk for developing a hypertensive disorder such as preeclampsia, we determined if neutrophil infiltration and vascular inflammation were correlated with BMI and if blood pressure correlated with neutrophil infiltration and vascular inflammation. To test this, we used immunohistochemical staining of vascular tissue in subcutaneous fat of obese women, as compared to overweight and normal weight women. We stained for neutrophils, and as markers of inflammation, we stained for NF-κB and COX-2.

B. Materials and Methods

i. Study Subjects

Subcutaneous fat biopsies were collected from patients at MCV Hospitals, Virginia Commonwealth University Medical Center. All patients were nonpregnant. Fat biopsies were collected at the time of abdominal surgery and each patient was categorized as normal weight, overweight or obese. The categories for each group were: normal weight, BMI 18.5 to 24.9 (n= 5); overweight, BMI 25 to 29.9 (n= 7) and obese, BMI greater than or equal to 30 (n= 10). The clinical data for patient groups are summarized in Table 3. The Office of Research Subjects Protection of Virginia Commonwealth University approved this study.

C. Results

CD66b (Neutrophils)

The visual score for CD66b represented intensity of staining as well as quantity of neutrophils. The visual score for CD66b was significantly correlated with BMI ($r = 0.62$, $P < 0.01$, Figure 10, top panel). When the data were organized into the weight classifications, the obese patients and overweight patients had significantly more neutrophil activation than normal weight patients (2.1 ± 0.2 vs. 1.4 ± 0.4 vs. 0.3 ± 0.1, $P < 0.01$, $P < 0.05$,

respectively, Figure 10, lower panel). Optical density (OD) was also significantly correlated with BMI ($r = 0.57$, $P < 0.01$, Figure 11, top panel). CD66b OD for obese patients and overweight patients was significantly higher than OD for normal patients (141 ± 6.6 vs. 128.6 ± 9.7 vs. 99 ± 10.3 OD, $P < 0.01$, $P < 0.05$, respectively, Figure 11, lower panel).

The percentage of vessels stained for CD66b was significantly correlated with BMI ($r = 0.53$, $P < 0.05$, Figure 12, top panel). Obese patients and overweight patients had significantly more vessels stained for CD66b than normal patients (73 ± 7 % vs. 57.3 ± 14. % vs. 18 ± 5 %, $P < 0.01$, $P < 0.05$, respectively, Figure 12, lower panel). The percent of neutrophils flattened and adhered to the endothelium was significantly correlated with BMI ($r = 0.53$, $P < 0.05$, Figure 13, top panel). In obese and overweight patients as compared with normal patients there was significantly greater adherence and flattening of neutrophils along the endothelium (52 ± 6 % vs. 40.3 ± 10.9 % vs. 8 ± 1 %, $P < 0.01$, $P < 0.05$, respectively, Figure 13), however infiltration into the intima was not significantly correlated with BMI nor was there a difference in intimal staining among the groups (48 ± 4 % vs. 37 ± 10 % vs. 21 ± 11 %, respectively, $P > 0.05$, Figure 14). The number of neutrophils/vessel was significantly correlated with BMI ($r = 0.48$, $P < 0.05$, Figure 15, top panel). Obese patients had significantly more neutrophils per stained vessel as compared with normal weight patients (6.5 ± 0.7 vs. 2.1± 0.65, respectively, $P < 0.05$, Figure 15, lower panel).

To assess the risk of cardiovascular disease, we determined whether diastolic blood pressure was correlated to CD66b staining. Diastolic blood pressure was

significantly correlated with CD66b visual score ($r = 0.56$, $P < 0.01$, Figure 16), optical density ($r = 0.49$, $P < 0.05$, Figure 17), % vessels stained ($r = 0.51$, $P < 0.05$, Figure 18) and % endothelial staining ($r = 0.56$, $P < 0.01$, Figure 19). Systolic blood pressure showed similar trends, but did not reach statistical significance for our sample size.

Figure 20 shows representative examples of assessment scoring ranging from 0-3. Figure 21 shows representative staining of vessels from normal, overweight and obese patients for CD66b. Normal patients had little or no staining, overweight patients had some brown staining, but obese patients had intense staining for neutrophils along the endothelium and vascular smooth muscle. Figure 22 shows additional examples of obese patients with intense staining of neutrophils: a) along the endothelium and within the vascular smooth muscle, b) along the endothelium and within the lumen, c) and d) throughout the vessel, e) along the endothelial cells and in the vascular smooth muscle, and f) within the lumen, along the endothelium and out into the vascular smooth muscle.

NF-κB

Visual score for NF-κB represented intensity of staining. The visual score for NF-κB was significantly correlated with BMI ($r = 0.71$, $P < 0.001$, Figure 23, top panel). When the data were organized into the weight classifications the obese patients had significantly more NF-κB activation than overweight or normal weight patients (2.7 ± 0.2 vs. 1.1 ± 0.3 vs. 0.4 ± 0.1, respectively, $P < 0.001$, Figure 23, lower panel). The visual score was verified by optical density measurements. Optical density was also significantly correlated

with BMI ($r = 0.68$, $P < 0.001$, Figure 24, top panel). NF-κB OD for obese patients was significantly higher than OD for overweight or normal patients (160 ± 4.9 vs. 127 ± 6.0 vs. 102 ± 8.0 OD, respectively, $P < 0.001$, Figure 24, lower panel).

The percentage of vessels stained for NF-κB was significantly correlated with BMI ($r = 0.62$, $P < 0.01$, Figure 25, top panel). Obese patients and overweight patients had significantly more vessels stained for NF-κB than normal patients (90.9 ± 2.4 % vs. 62.1 ± 11.1% vs. 27.1 ± 8.2, $P < 0.001$, $P < 0.01$, respectively, Figure 25, lower panel).

Leukocytes also stained for NF-κB. The number of leukocytes that stained for NF-κB/vessel was significantly correlated with BMI ($r = 0.70$, $P < 0.001$, Figure 26, top panel). Obese patients and overweight patients had significantly more vessels with leukocytes stained for NF-κB than normal weight patients (5.6 ± 0.4 % vs. 3.0 ± 0.5 % vs. 1.3 ± 0.4, $P < 0.001$, $P < 0.05$, respectively, Figure 26, lower panel).

To assess the risk of cardiovascular disease with respect to NF-κB, we determined whether blood pressure was correlated to NF-κB staining. Diastolic blood pressure was significantly correlated with NF-κB visual score ($r = 0.62$, $P < 0.01$, Figure 27), optical density ($r = 0.56$, $P < 0.01$, Figure 28), % vessels stained ($r = 0.68$, $P < 0.001$, Figure 29), and leukocytes/vessel ($r = 0.46$, $P < 0.05$, Figure 30). Systolic blood pressure showed similar trends, but did not reach statistical significance for our sample size.

Figure 31 shows representative staining for NF-κB in vessels of normal, overweight and obese patients. There was little or no staining in vessels of normal or overweight patients, but obese patients had intense staining for NF-κB in the endothelium

and vascular smooth muscle. NF-κB staining of neutrophils, and other leukocytes, is also evident along the endothelium of obese patients. Figure 32 shows representative staining for NF-κB in vessels of obese patients.

COX-2

Visual score for COX-2 represented intensity of staining. The visual score for COX-2 was significantly correlated with BMI ($r = 0.64$, $P < 0.01$, Figure 33, top panel). When the data were organized into the weight classifications, the obese patients had significantly more expression of COX-2 than overweight or normal weight patients (1.7 ± 0.3 vs. 0.84 ± 0.3 vs. 0.59 ± 0.2, respectively, $P < 0.05$, Figure 33, lower panel). Optical density was also significantly correlated with BMI ($r = 0.59$, $P < 0.01$, Figure 34, top panel). COX-2 OD for obese patients was significantly higher than for overweight patients or normal patients (132 ± 8.4 vs. 109 ± 8.5 vs. 110 ± 2.2 OD, respectively, $P < 0.05$, Figure 34, lower panel).

The percentage of vessels stained for COX-2 was significantly correlated with BMI ($r = 0.64$, $P < 0.01$, Figure 35, top panel). Obese patients had significantly more vessels stained for COX-2 than normal patients (68.7 ± 9.4 % vs. 33.9 ± 12.0 %, respectively, $P < 0.05$, Figure 35, lower panel).

Leukocytes in the vessels stained for COX-2. The number of leukocytes that stained for COX-2/vessel was significantly correlated with BMI ($r = 0.60$, $P < 0.01$, Figure 36, top panel). Obese patients had significantly more vessels with leukocytes stained for

COX-2 than normal weight patients (2.6 ± 0.4 % vs. 1.2 ± 0.2%, respectively, $P < 0.05$, Figure 36, lower panel).

As for CD66b and NF-κB, we determined whether blood pressure was correlated with COX-2 staining. Diastolic blood pressure was positively correlated with COX-2 visual score, although the "r" value did not reach statistical significance ($r = 0.40$, $P > 0.05$, Figure 37). Diastolic blood pressure was significantly correlated with optical density ($r = 0.47$, $P < 0.05$, Figure 38), % vessels stained ($r = 0.49$, $P < 0.05$, Figure 39), and leukocytes/vessel ($r = 0.49$, $P < 0.05$, Figure 40). Systolic blood pressure showed similar trends, but did not reach statistical significance for our sample size.

Figure 41 shows representative staining for COX-2 in vessels of normal, overweight and obese patients. There was little or no staining in vessels of normal or overweight patients, but obese patients had intense staining for COX-2 in the endothelium and vascular smooth muscle. COX-2 staining of neutrophils, and other leukocytes, is also evident along the endothelium of obese patients. Figure 42 shows representative staining for COX-2 in vessels of obese patients.

D. Discussion

The percentage of vessels stained for CD66b was significantly greater in obese women compared to normal weight women. There were more neutrophils adhered and flattened along the endothelium and within the intima in obese women compared to

normal weight women. The percentage of neutrophils present within the different portions of the vessel was higher for obese women, as were the number of neutrophils per vessel and total number of neutrophils. Diffuse vessel staining was also present in obese women and, most likely, reflected secretion of CD66b from activated neutrophils.

Staining for NF-κB, a transcription factor involved in inflammation, and staining for COX-2, an enzyme regulated by NF-κB, was significantly greater in obese women as compared to overweight or normal weight women. NF-κB and COX-2 staining were observed in the vascular smooth muscle, in addition to endothelium, in obese women. This finding is significant because it demonstrates inflammation of vascular smooth muscle in obesity. Neutrophils also stained for NF-κB and COX-2 demonstrating that inflammation is systemic, as well as localized to the vasculature. Previous studies have shown increased expression of inflammatory markers, such as CRP [42, 44, 47], but our data are new in that they demonstrate increased activation of a specific and major transcription factor and increased expression of an important enzyme involved in inflammation. Our data demonstrate that obesity is an inflammatory disease associated with neutrophil infiltration and immune dysfunction.

Our data may explain previous findings in the field of obesity. Keaney et al demonstrated that body mass index was highly associated with systemic oxidative stress[178]. Oxidative stress in obese individuals may result from release of ROS by activated neutrophils, which we demonstrated in this study to be so prevalent in the circulation and vasculature of obese women.

In summary, the present study is the first to provide in vivo evidence of vascular smooth muscle inflammation and neutrophil infiltration into systemic vascular tissue in obesity. These new data indicate that obesity is an inflammatory disease associated with immune dysfunction. We also found evidence that might explain why obesity is a risk factor for preeclampsia because the degree of neutrophil infiltration and the amount of vascular inflammation was positively correlated, not only with weight, but also with diastolic blood pressure. Therefore, obese women who become pregnant are already predisposed to hypertension and vascular dysfunction. In the next chapter, we will demonstrate that the vascular phenotype of obese women is characteristic of preeclamptic women, but not of normal pregnant or normal nonpregnant women matched for BMI.

Table 3. Clinical data for patient groups.

	Normal Weight (n = 5)	Overweight (n = 7)	Obese (n = 10)
BMI (kg/m^2)	22.9 ± 0.78	27.8 ± 0.72	39.8 ± 2.1***
Age	41.0 ± 4.6	38.6 ± 2.9	41.1 ± 2.0
Systolic Blood Pressure (mm Hg)	111.2 ± 3.9	120.6 ± 6.2	131.4 ± 5.2
Diastolic Blood Pressure (mm Hg)	60.4 ± 2.7	74.1 ± 4.8	83.3 ± 2.4**

Values are mean ± SE.
*** $P<0.001$ compared to overweight and normal weight
** $P<0.01$ compared to normal weight

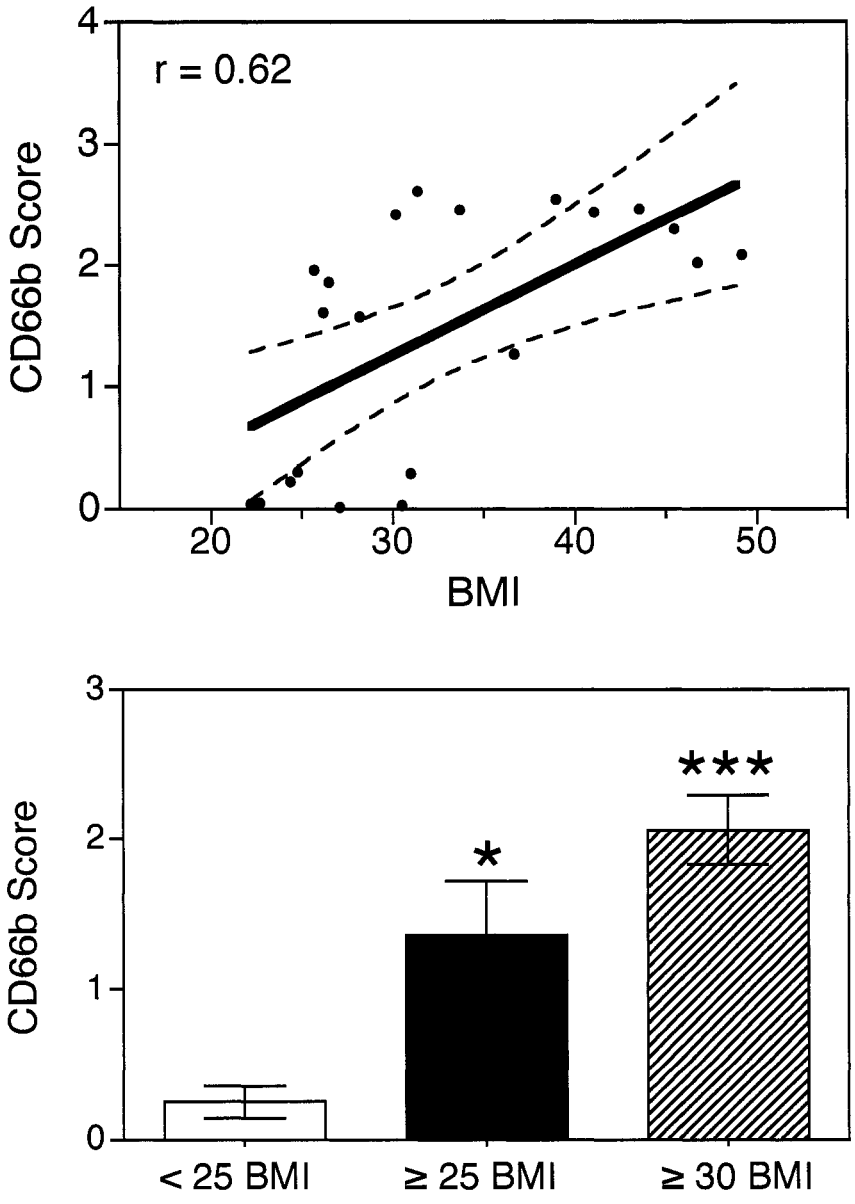


Figure 10. Summarized visual score results for CD66b staining.

Visual score was significantly correlated with BMI ($r = 0.62$, ** $P < 0.01$, Top panel). When evaluated according to weight categories, obese patients and overweight patients had significantly greater CD66b staining as compared to normal weight patients (Lower panel, *** $P < 0.001$, * $P < 0.05$). The visual scores for CD66b were based on both intensity and quantity of neutrophil infiltration.

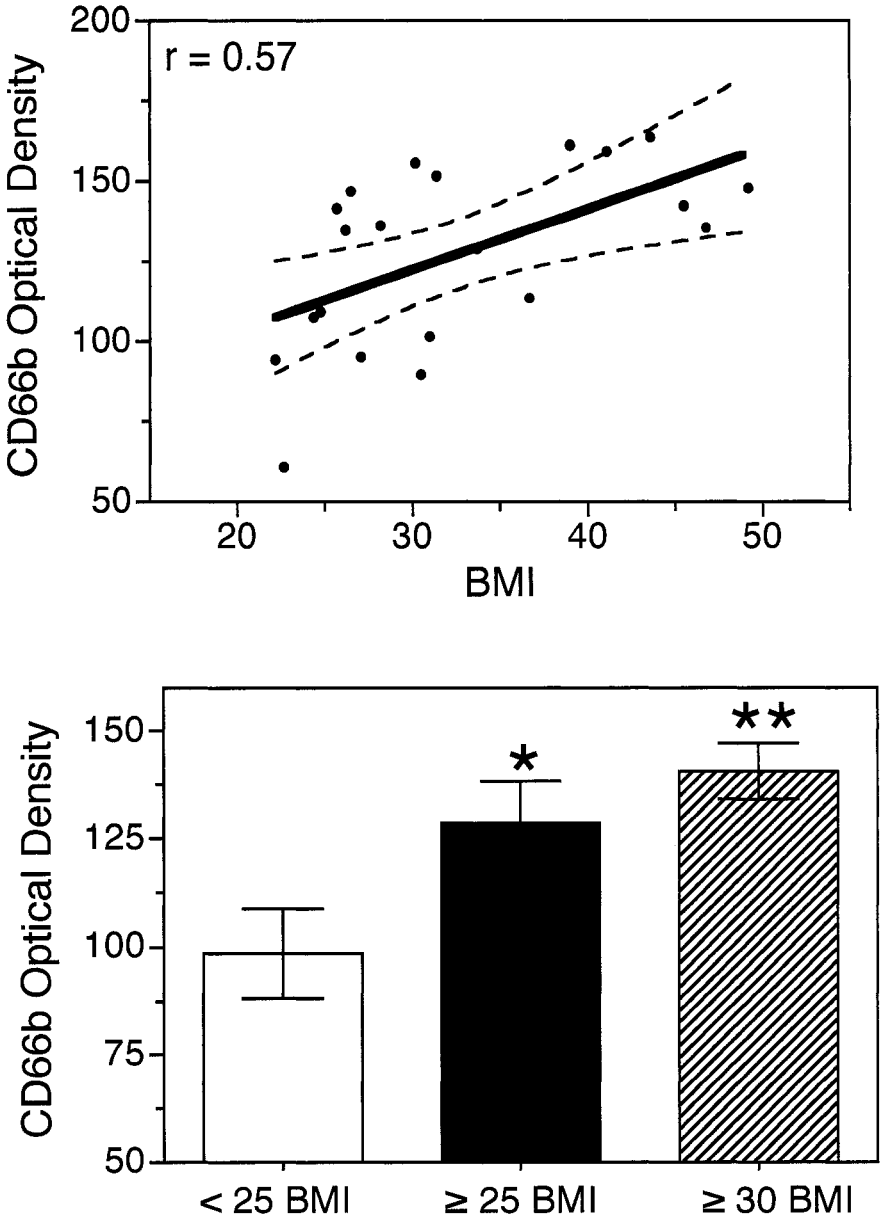


Figure 11. Summarized optical density measurements for CD66b staining.

Optical density measurements were significantly correlated with BMI ($r = 0.57$, ** $P < 0.01$, Top panel). Obese and overweight patients had significantly higher optical density measurements compared to normal weight patients (Lower panel, ** $P < 0.01$, * $P < 0.05$).

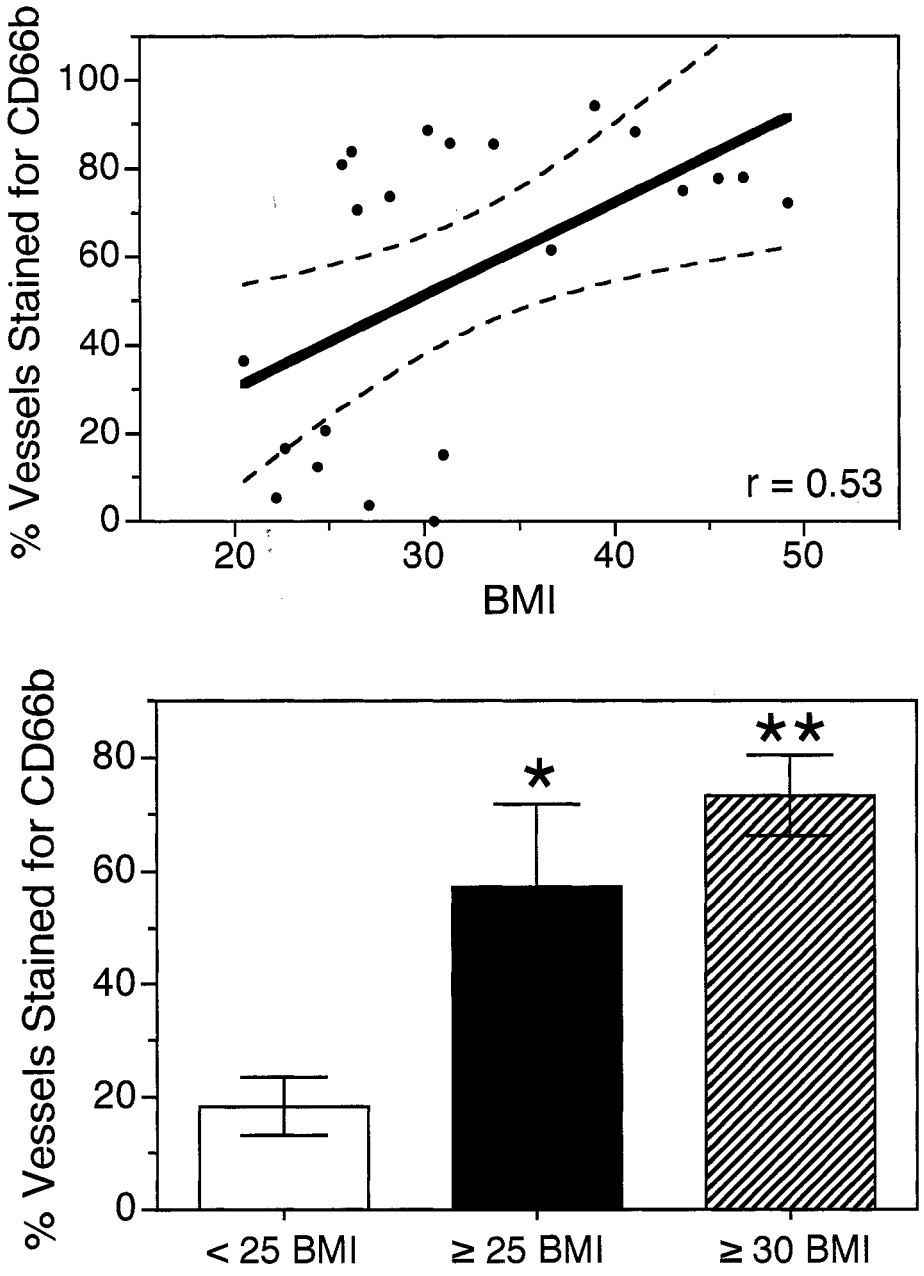


Figure 12. Percent of vessels stained for CD66b.

Percent of vessels stained for CD66b were significantly correlated with BMI ($r = 0.53$, * $P < 0.05$, Top panel). Obese and overweight patients had significantly more percent vessels stained compared to normal weight (Lower panel, ** $P < 0.01$, * $P < 0.05$).

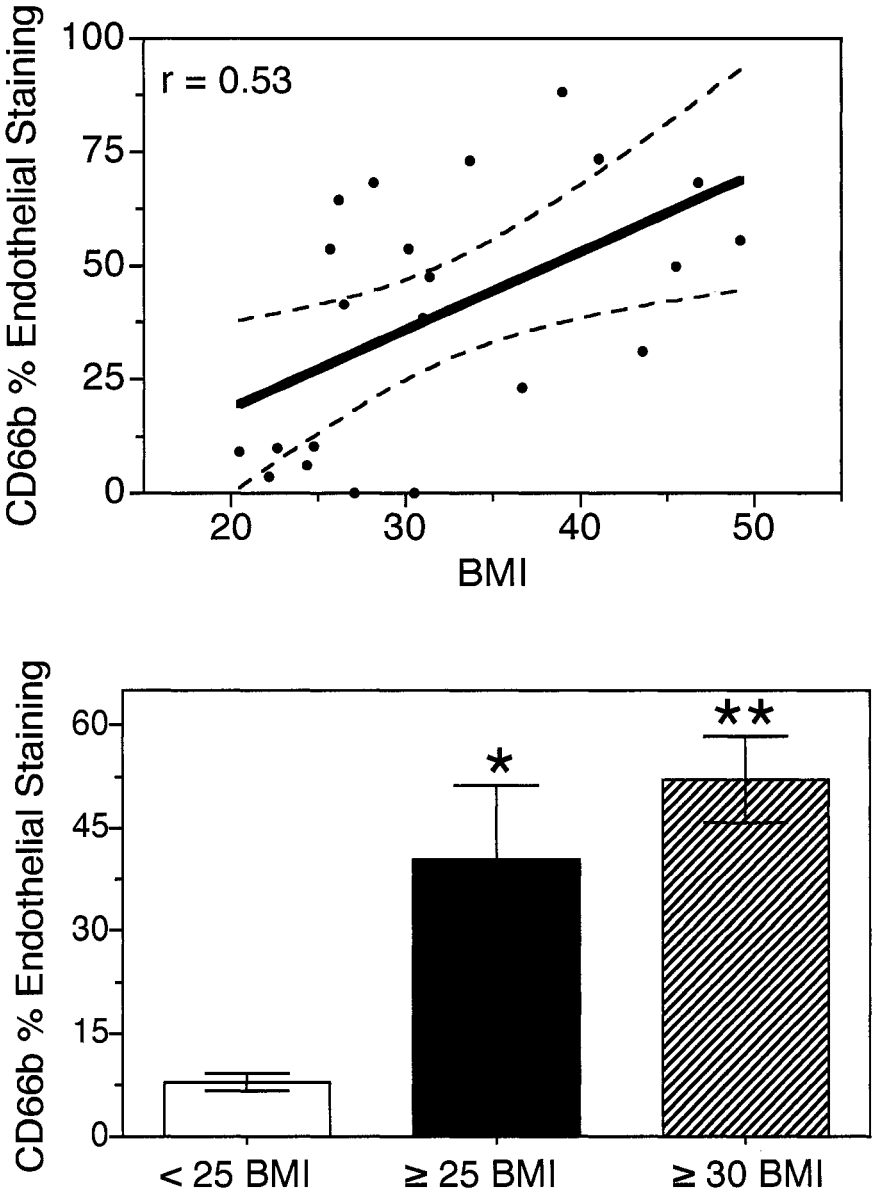
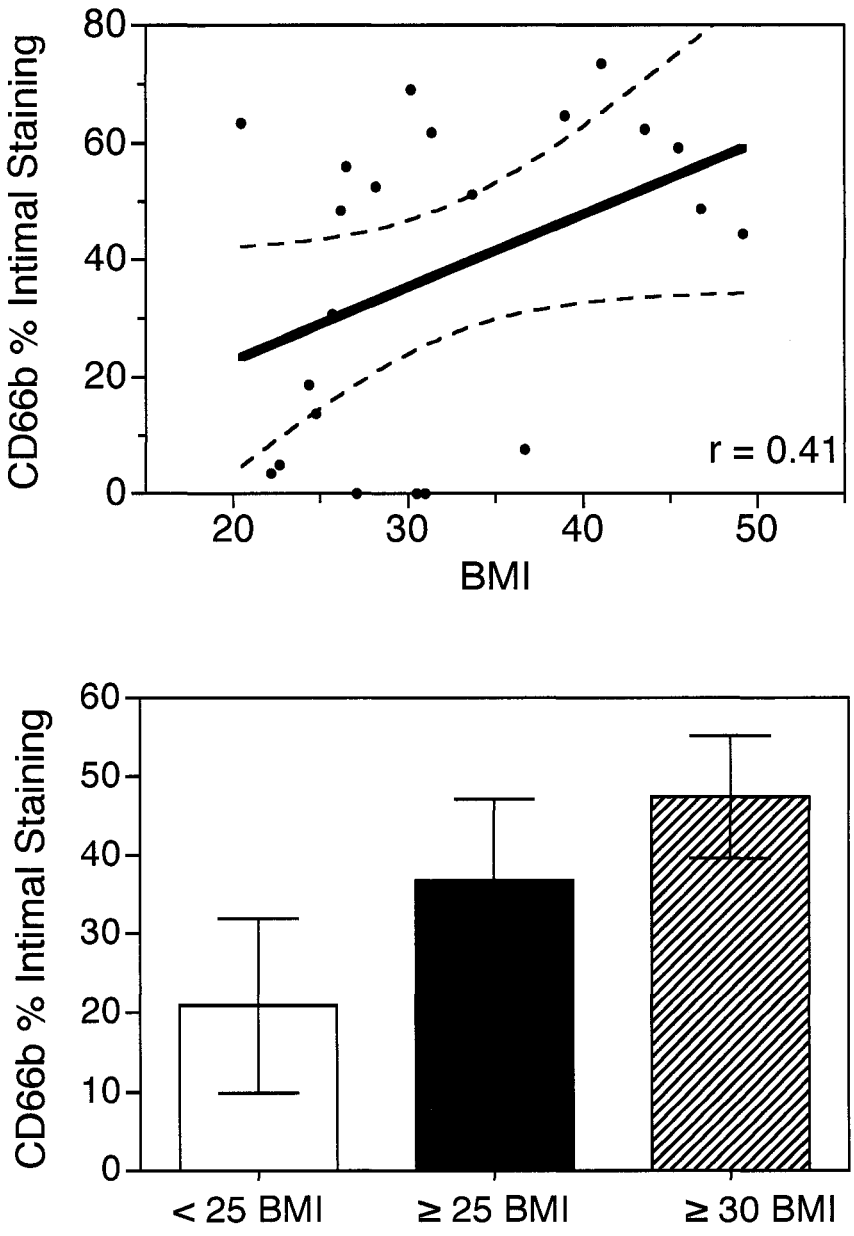


Figure 13. Percent of vessels with neutrophils adhered and flattened onto endothelial cells.

Percent of neutrophils adhered and flattened onto the endothelium were significantly correlated with BMI ($r = 0.53$, $^*P < 0.05$, Top panel). Obese patients and overweight patients had significantly more neutrophils on the endothelium compared to normal weight patients (Lower panel, **, $P < 0.01$, * $P < 0.05$).



Percent 14. Percent of vessels with neutrophils infiltrated into the intimal space.

Percent of neutrophils infiltrated into the intimal space was not significantly correlated with BMI and there was no significant difference among weight groups.

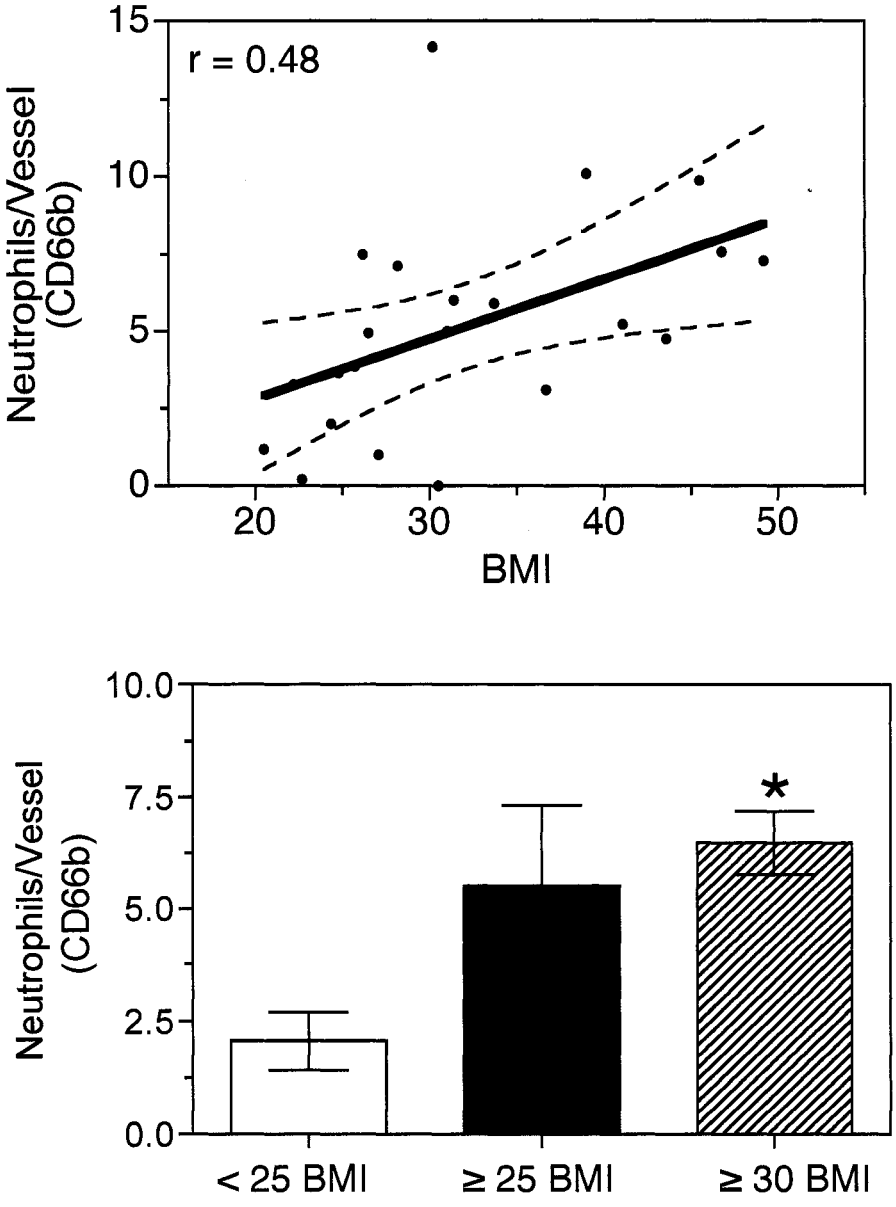


Figure 15. Total number of neutrophils per vessel stained for CD66b.

The number of neutrophils per stained vessel was significantly correlated with BMI ($r = 0.48$, * $P < 0.05$, Top panel). The number of neutrophils per vessel was significantly higher for the obese patients compared to the normal weight patients (Lower panel, * $P < 0.05$).

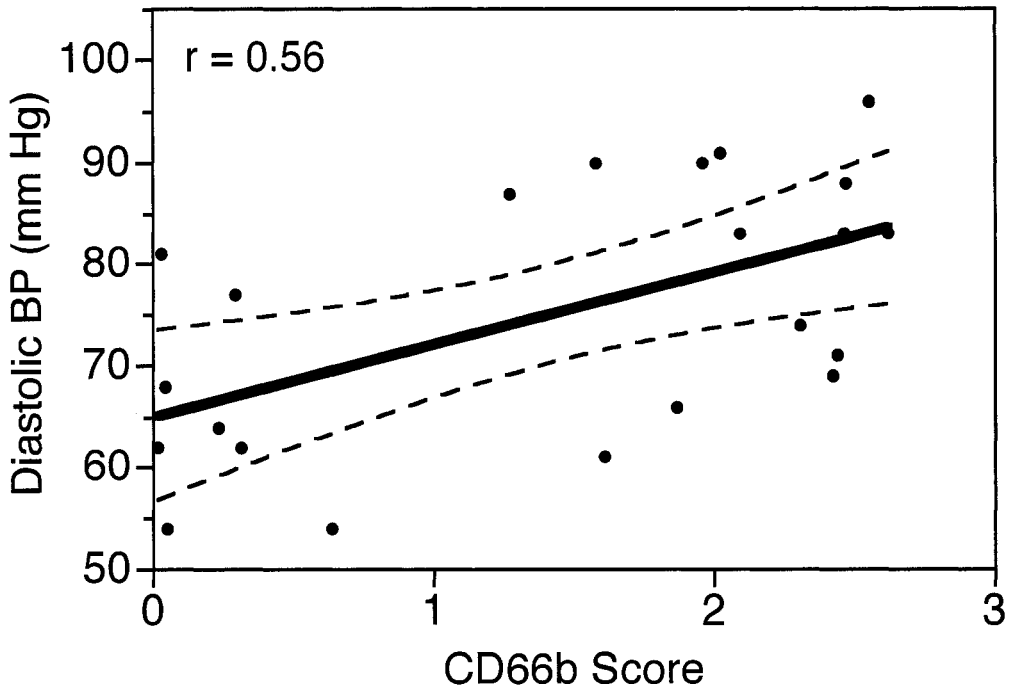


Figure 16. Diastolic blood pressure correlation with CD66b visual score.

Diastolic blood pressure was significantly correlated (r = 0.56) with CD66b visual score. ** $P < 0.01$

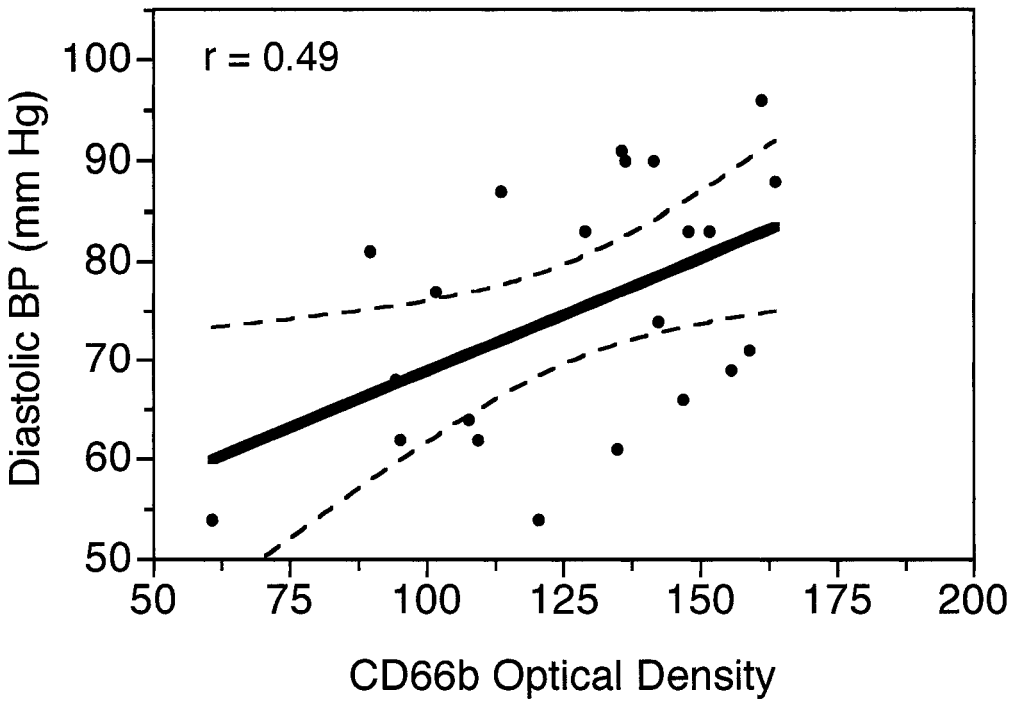


Figure 17. Diastolic blood pressure correlation with CD66b optical density.

Diastolic blood pressure was significantly correlated (r = 0.49) with CD66b optical density. * $P < 0.05$

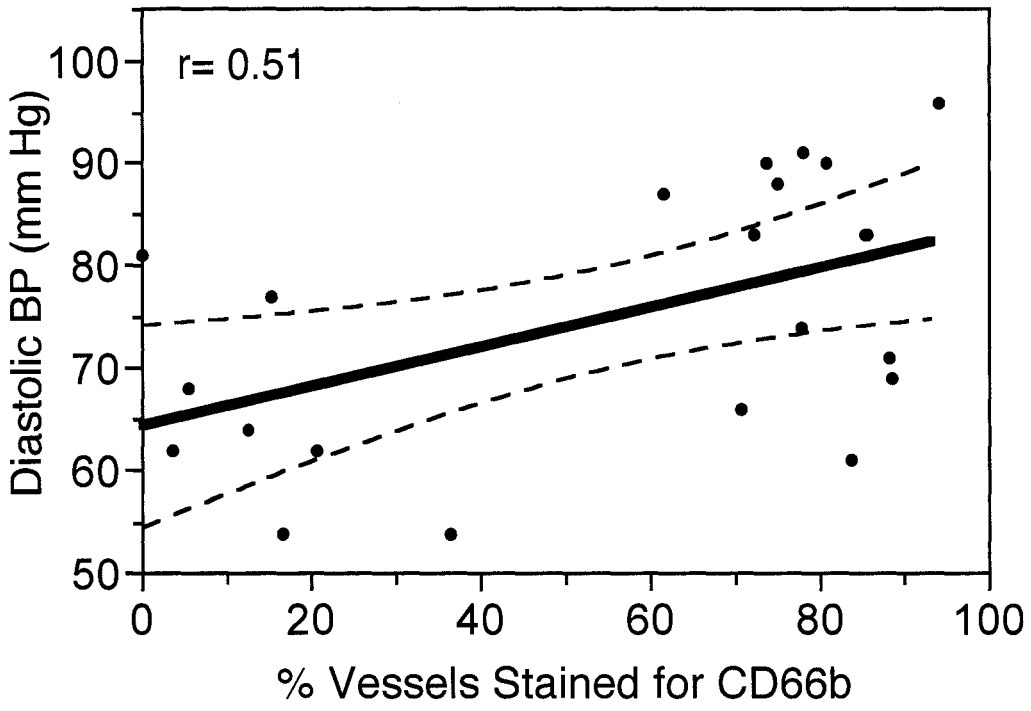


Figure 18. Diastolic blood pressure correlation with percent vessels stained for CD66b.

Diastolic blood pressure was significantly correlated ($r = 0.51$) with CD66b % vessels stained. * $P < 0.05$

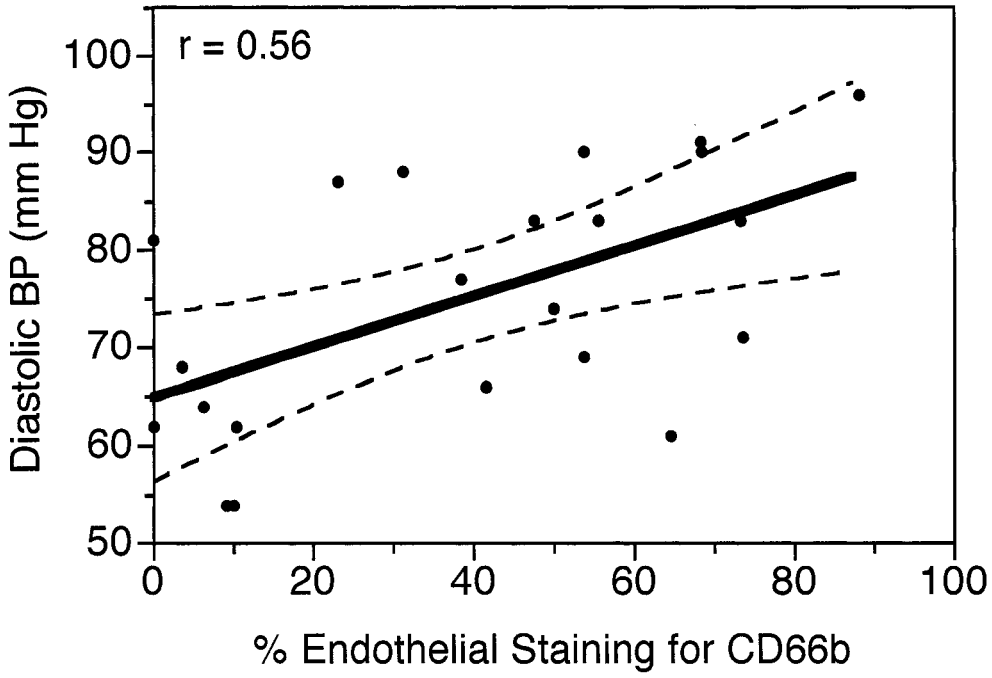


Figure 19. Diastolic blood pressure correlation with percent of vessels with neutrophils adhered and flattened onto endothelial cells.

Diastolic blood pressure was significantly correlated ($r = 0.56$) with the percent of vessels that had neutrophils flattened and adhered to the endothelium. ** $P < 0.01$

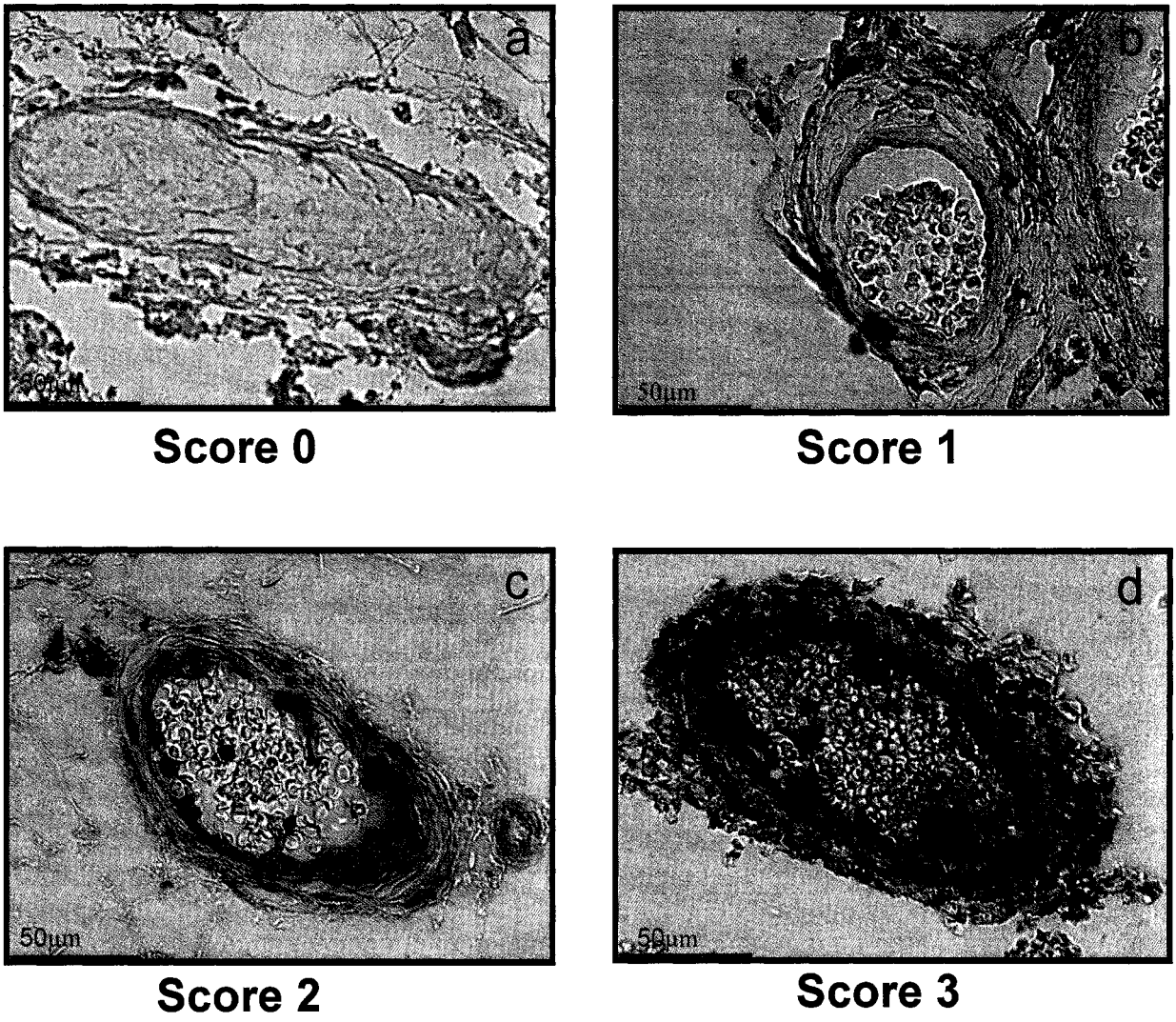


Figure 20. Examples of visual scoring.

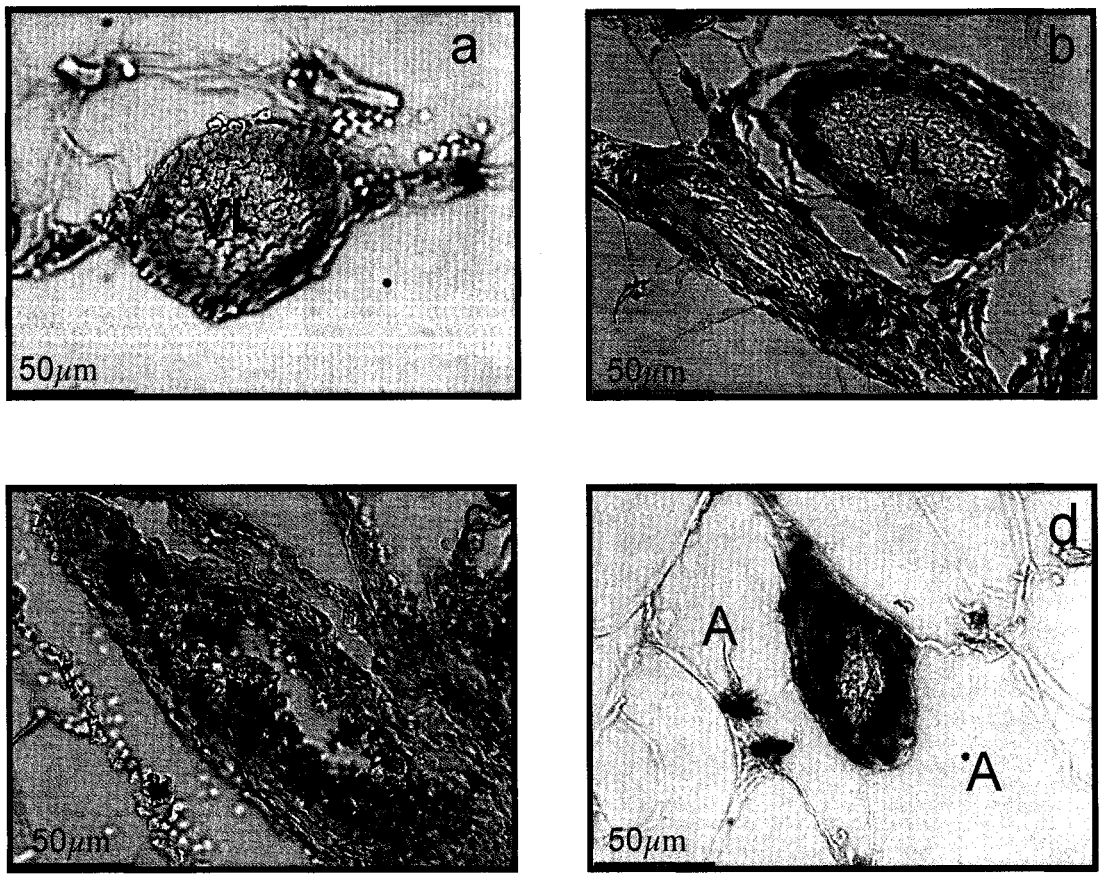


Figure 21. CD66b staining of neutrophils in representative sections from the patient groups.

a) IgM negative control, b) Normal weight patient showing no CD66b staining, c) Overweight patient showing some CD66b staining along the endothelium and vascular smooth muscle, d) Obese patient showing intense brown staining for CD66b along the endothelium and vascular smooth muscle. Magnification X400.

(A- adipocyte, VL- vessel lumen)

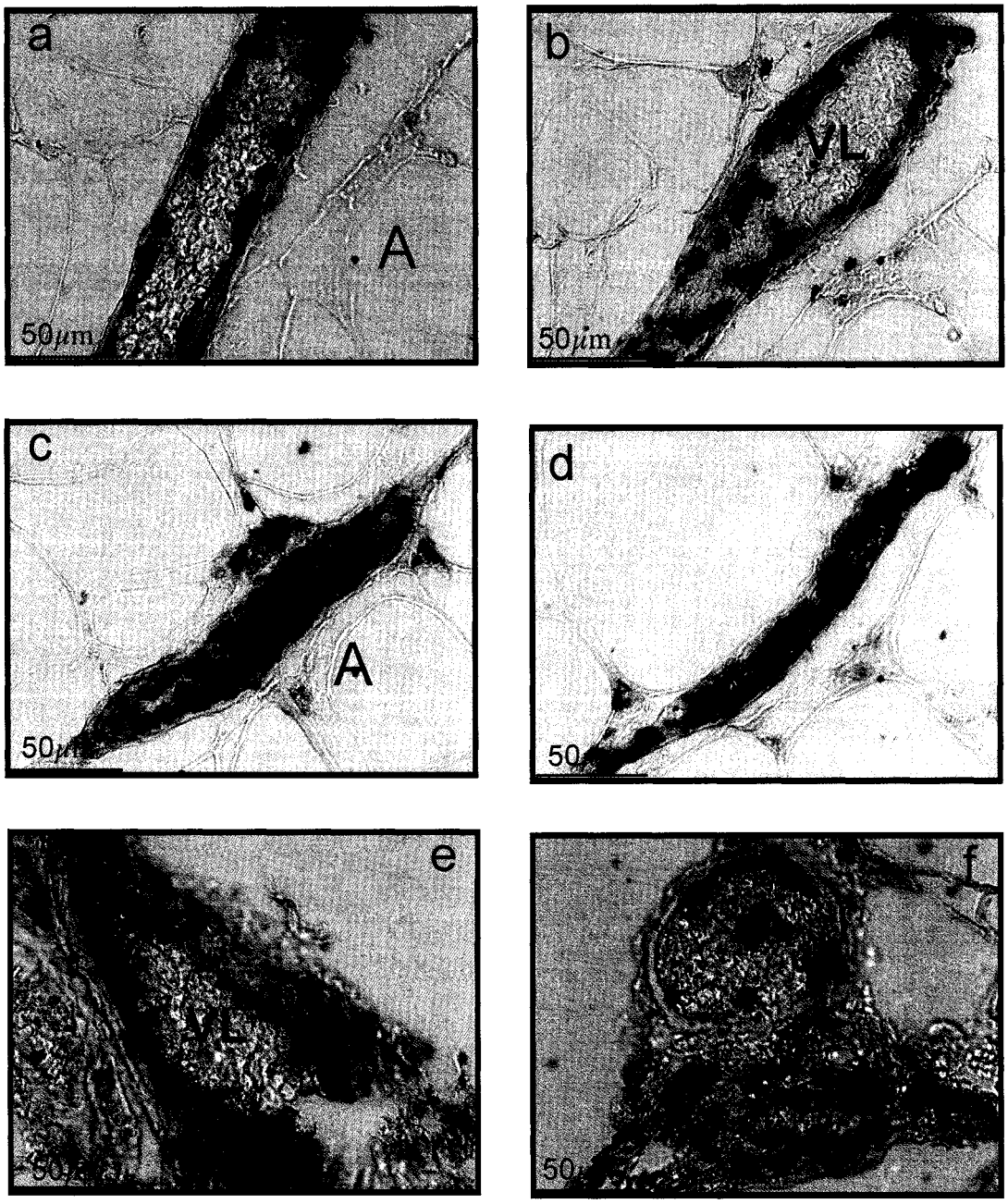


Figure 22. Representative sections of CD66b staining of neutrophils in various vessel locations in obese patients.

a) This section shows brown stained neutrophils along the endothelium and within the vascular smooth muscle, b) This longitudinal section of a vessel shows extensive neutrophil involvement along the endothelium and within the lumen, c) This vessel is an example of massive neutrophil staining throughout the vessel, d) This section also shows massive neutrophil staining throughout the vessel. e) This vessel section is an example of neutrophil staining along the endothelial cells and in the vascular smooth muscle. f) These vessels show extensive neutrophil infiltration within the lumen, along the endothelium and out into the vascular smooth muscle. Magnification X400.

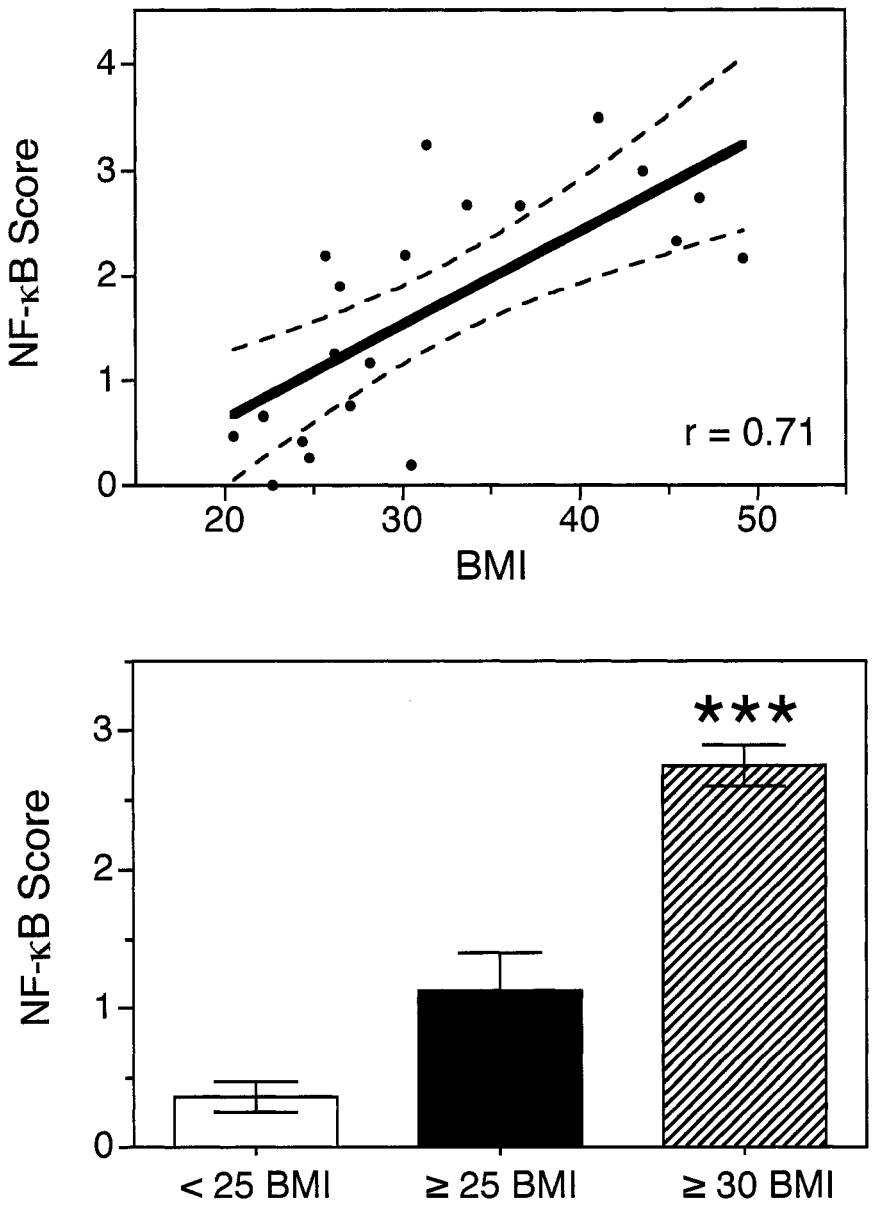


Figure 23. Summarized visual score results for NF-κB staining.

Visual score was significantly correlated with BMI (r = 0.71, *** P < 0.001, Top panel). Obese patients had significantly greater NF-κB staining as compared to normal weight patients and overweight patients (Lower panel, *** P < 0.001).

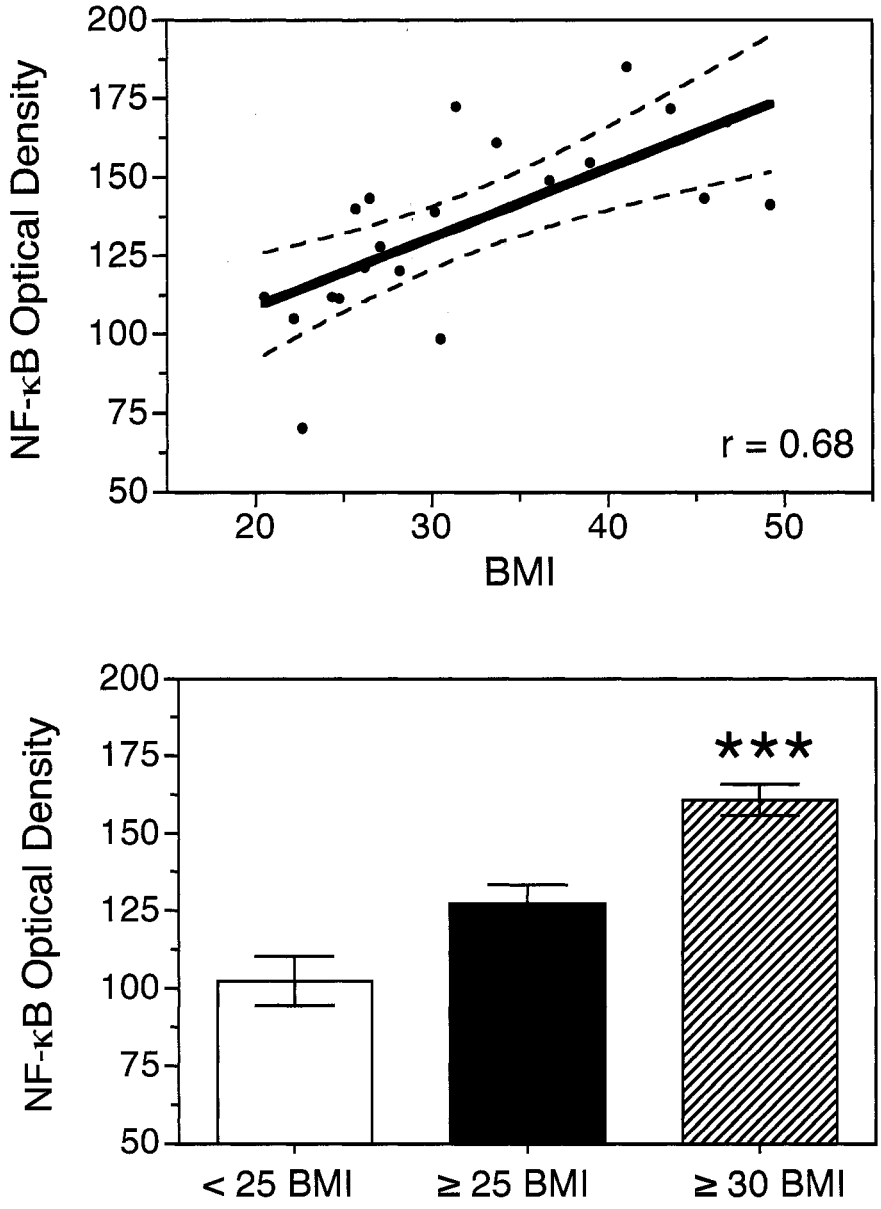


Figure 24. Summarized optical density measurements for NF-κB staining.

Optical density measurements were significantly correlated with BMI (r = 0.68, *** P < 0.001, Top panel). Obese patients had significantly higher optical density measurements compared to normal weight patients and overweight patients (Lower panel, *** P < 0.001).

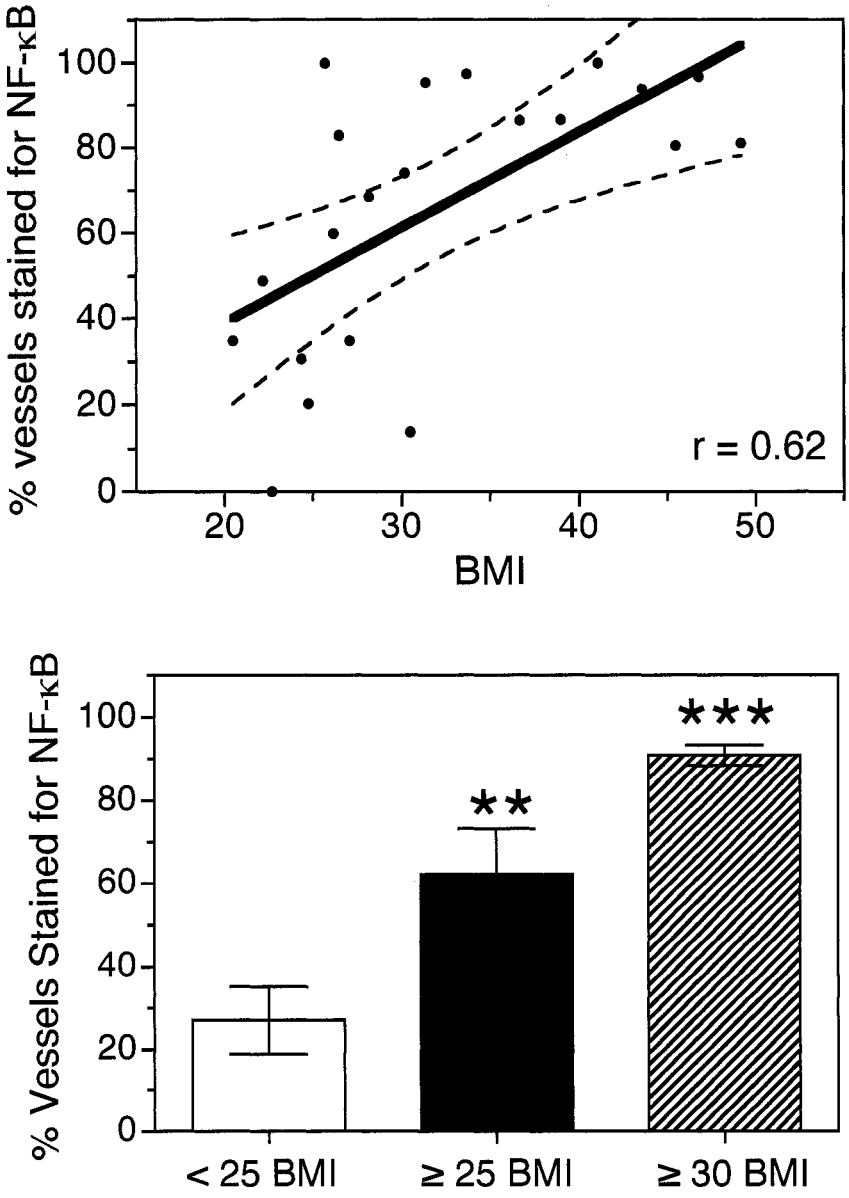


Figure 25. Percent of vessels stained for NF-κB staining.

Percent of vessels stained for NF-κB were significantly correlated with BMI ($r = 0.62$, ** $P < 0.01$, Top panel). Obese patients and overweight patients had significantly more percent vessels stained compared to normal weight patients (Lower panel, *** $P < 0.001$, ** $P < 0.01$).

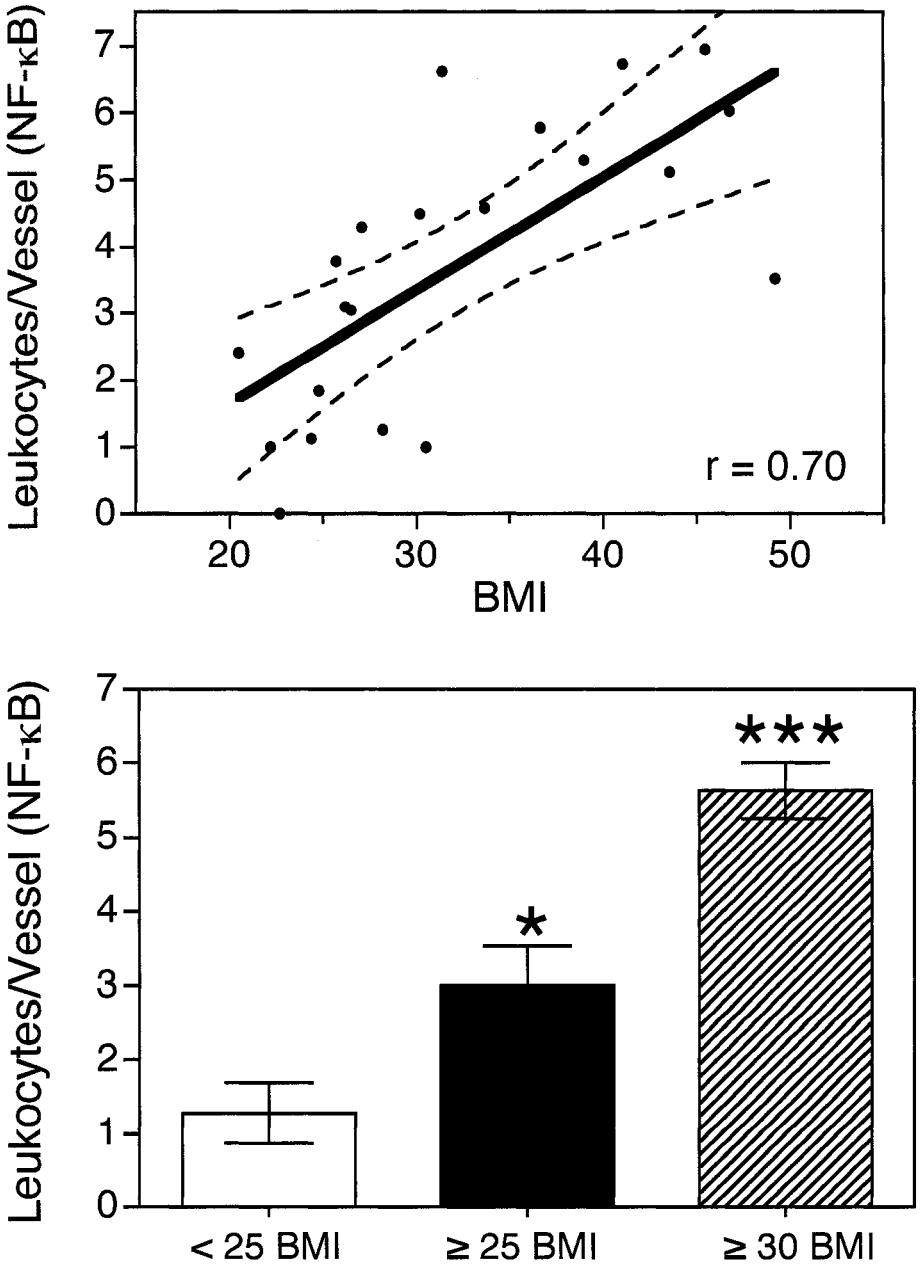


Figure 26. Total number of leukocytes per vessel that stained for NF-κB.

The number of leukocytes per vessel that stained for NF-κB was significantly correlated with BMI ($r = 0.70$, *** $P < 0.001$, Top panel). Obese and overweight patients had significantly more leukocytes that stained for NF-κB per vessel compared to normal weight patients (Lower panel, *** $P < 0.001$, * $P < 0.05$).

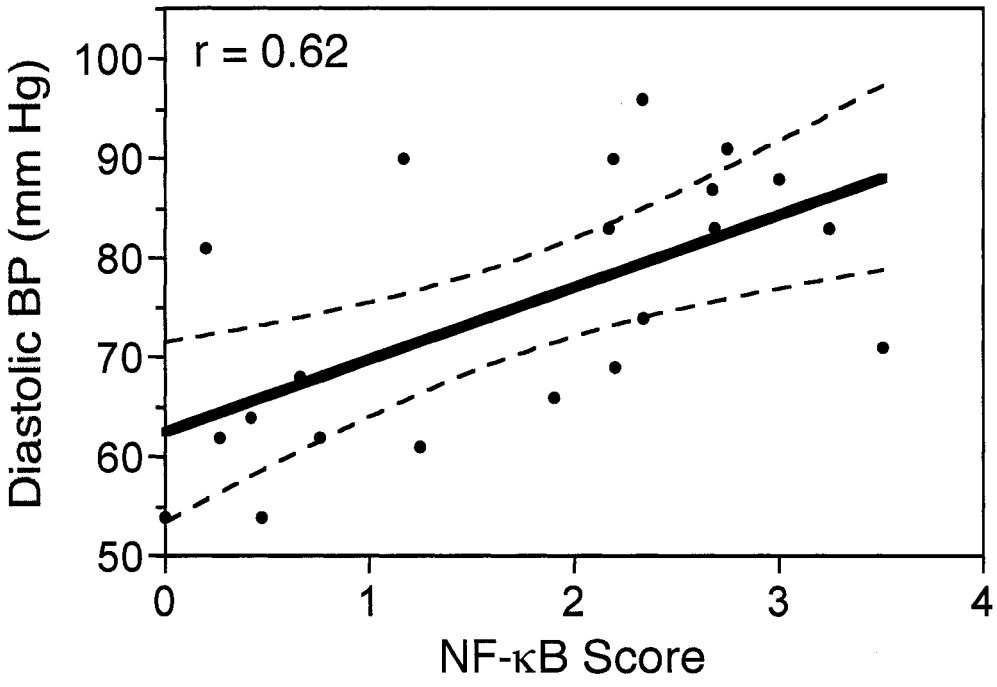


Figure 27. Diastolic blood pressure correlation with NF-κB visual score.

Diastolic blood pressure was significantly correlated (r = 0.62) with NF-κB visual score. ** $P < 0.01$

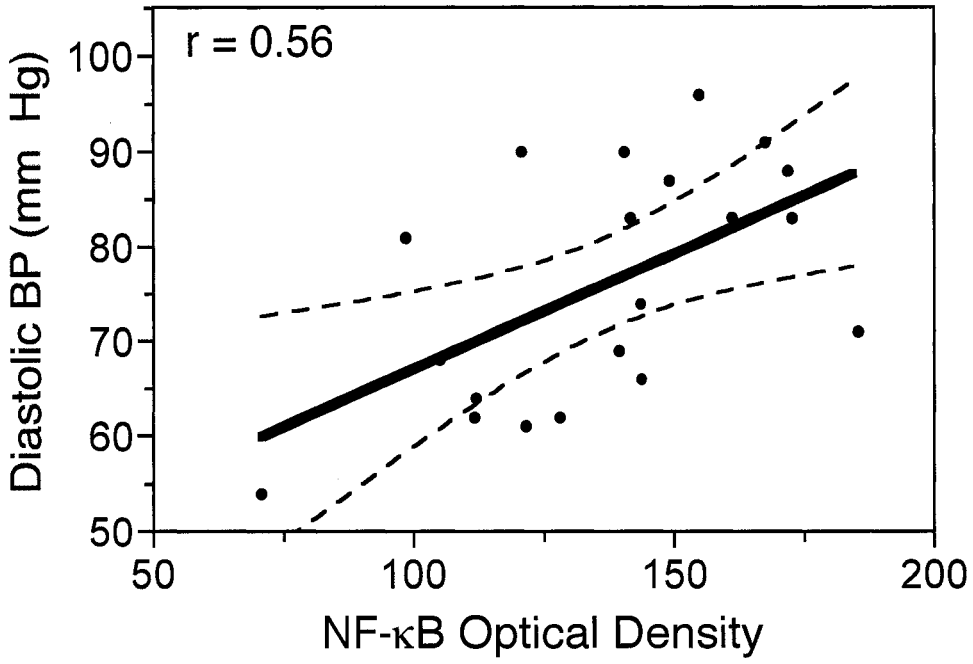


Figure 28. Diastolic blood pressure correlation with NF-κB optical density.

Diastolic blood pressure was significantly correlated (r = 0.56) with NF-κB optical density. ** $P < 0.01$

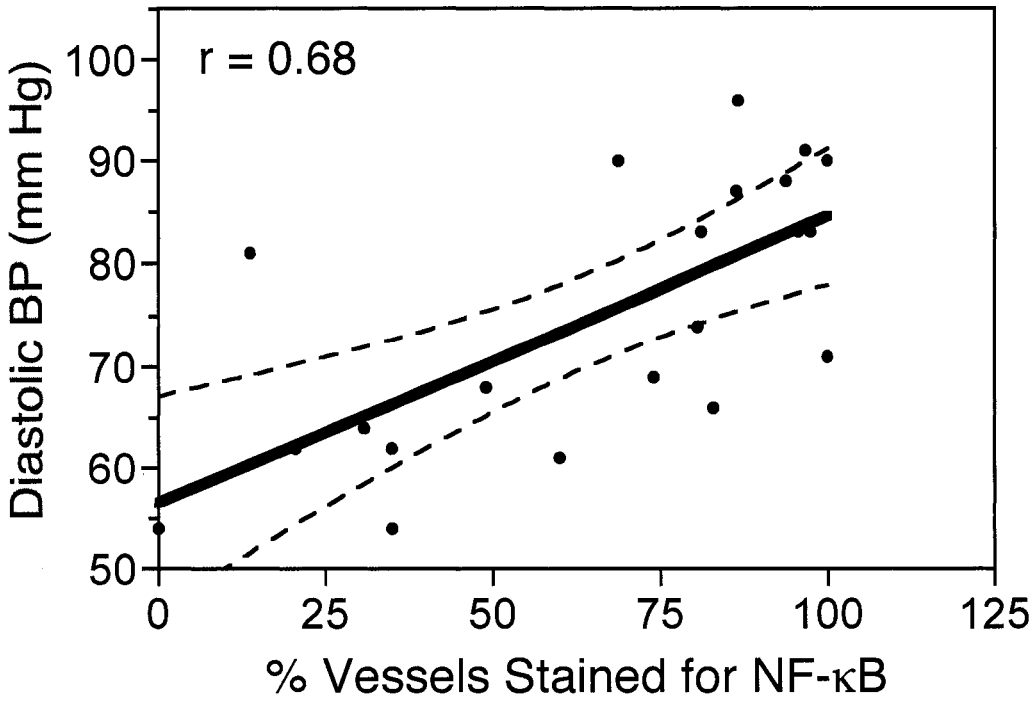


Figure 29. Diastolic blood pressure correlation with percent vessels stained for NF-κB.

Diastolic blood pressure was significantly correlated (r = 0.68) with NF-κB % vessels stained *** $P < 0.001$

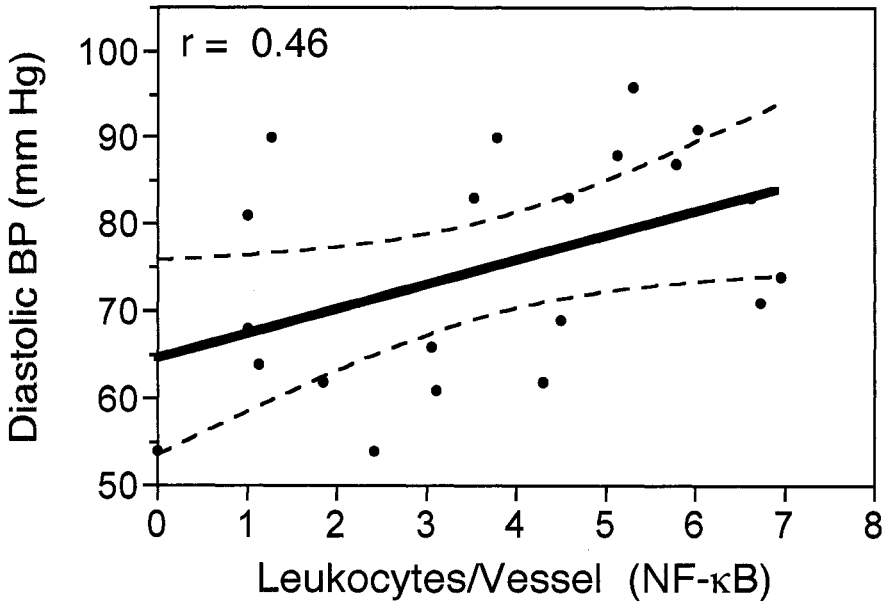


Figure 30. Diastolic blood pressure correlation with leukocytes stained for NF-κB per vessel.

Diastolic blood pressure was significantly correlated (r = 0.46) with the number of leukocytes that stained for NF-κB per vessel. * $P < 0.05$

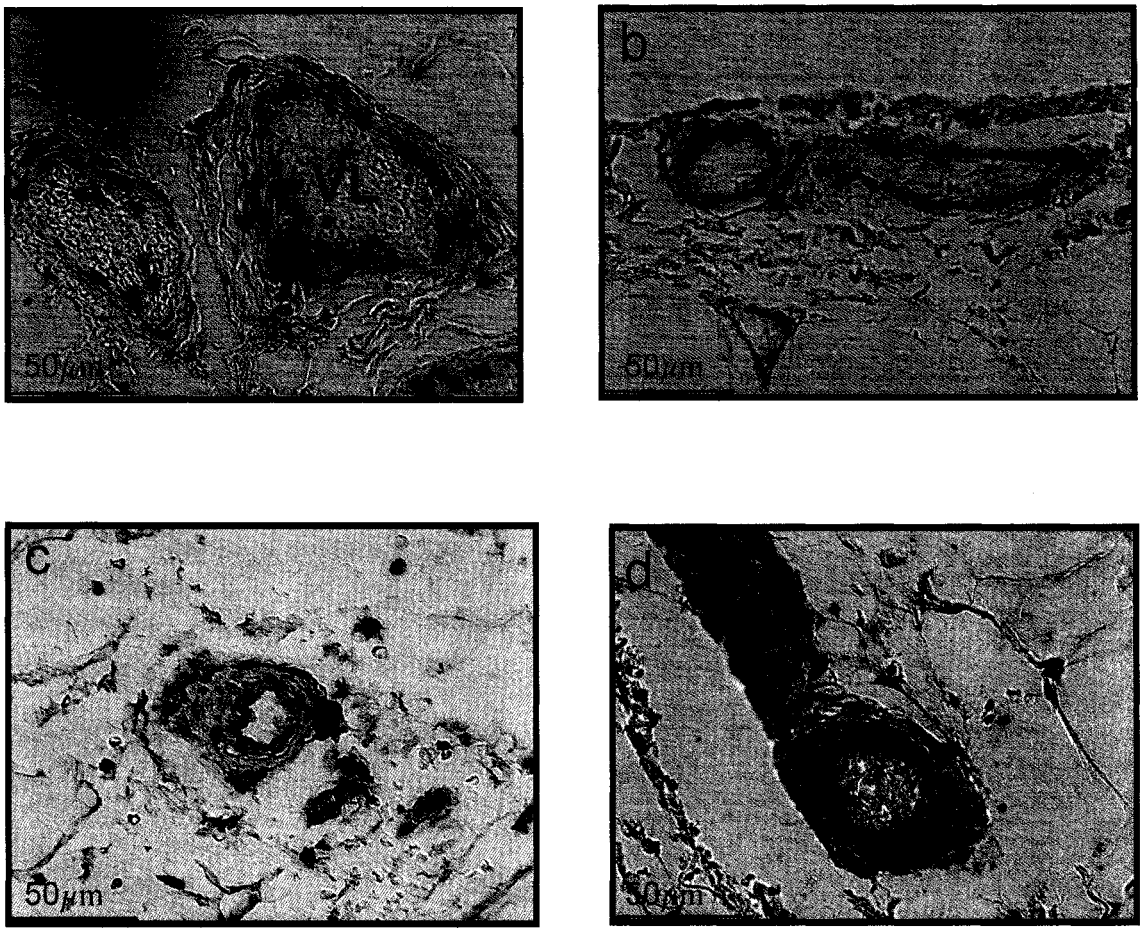


Figure 31. NF-κB staining in representative sections from the patient groups.

a) IgG negative control, b) Normal weight patient showing no NF-κB staining, c) Overweight patient showing some NF-κB staining along the endothelium and vascular smooth muscle, d) Obese patient showing intense brown staining for NF-κB along the endothelium and vascular smooth muscle. Magnification X400.

(VL- vessel lumen)

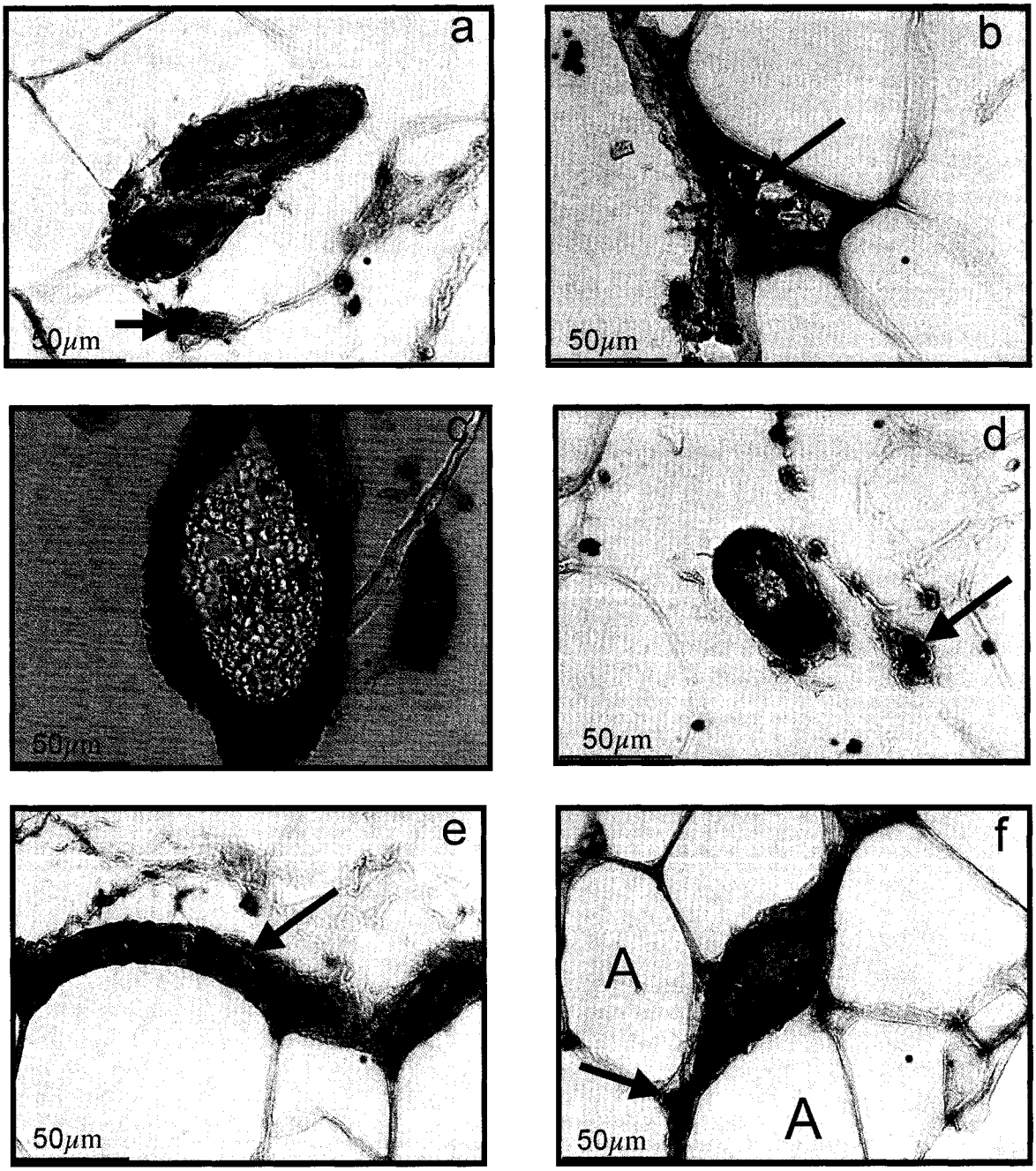


Figure 32. Representative sections of NF-κB staining in vessels of obese patients.

a) This section shows brown staining along the endothelium and within the vascular smooth muscle, b) This vessel shows brown staining within the lumen, along the endothelium and within the vascular smooth muscle, c) These vessels show brown staining along the endothelium and within the vascular smooth muscle, d) This section also shows brown staining along the endothelium and out into the vascular smooth muscle, e) This vessel shows massive brown staining throughout the vessel, f) This vessel shows brown staining along the endothelium and out into the vascular smooth muscle. Leukocytes also stained for NF-κB (arrows). Magnification X400.

(A- Adipocyte, VL-Vessel Lumen)

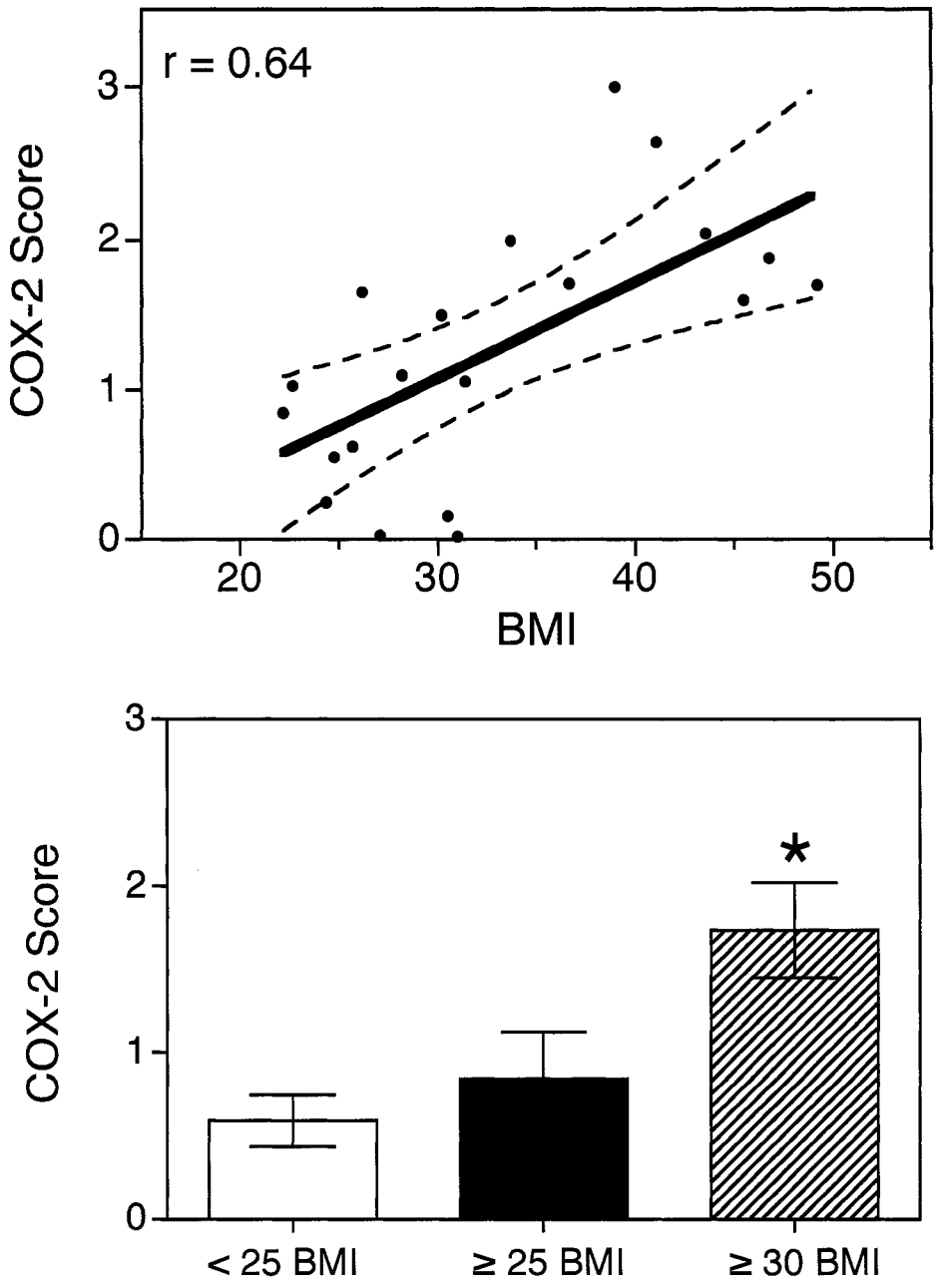


Figure 33. Summarized visual score results for COX-2 staining.

Visual score was significantly correlated with BMI (r = 0.64, ** $P < 0.01$, Top panel). Obese patients had significantly greater COX-2 staining as compared to normal weight or overweight patients (Lower panel, * $P < 0.05$).

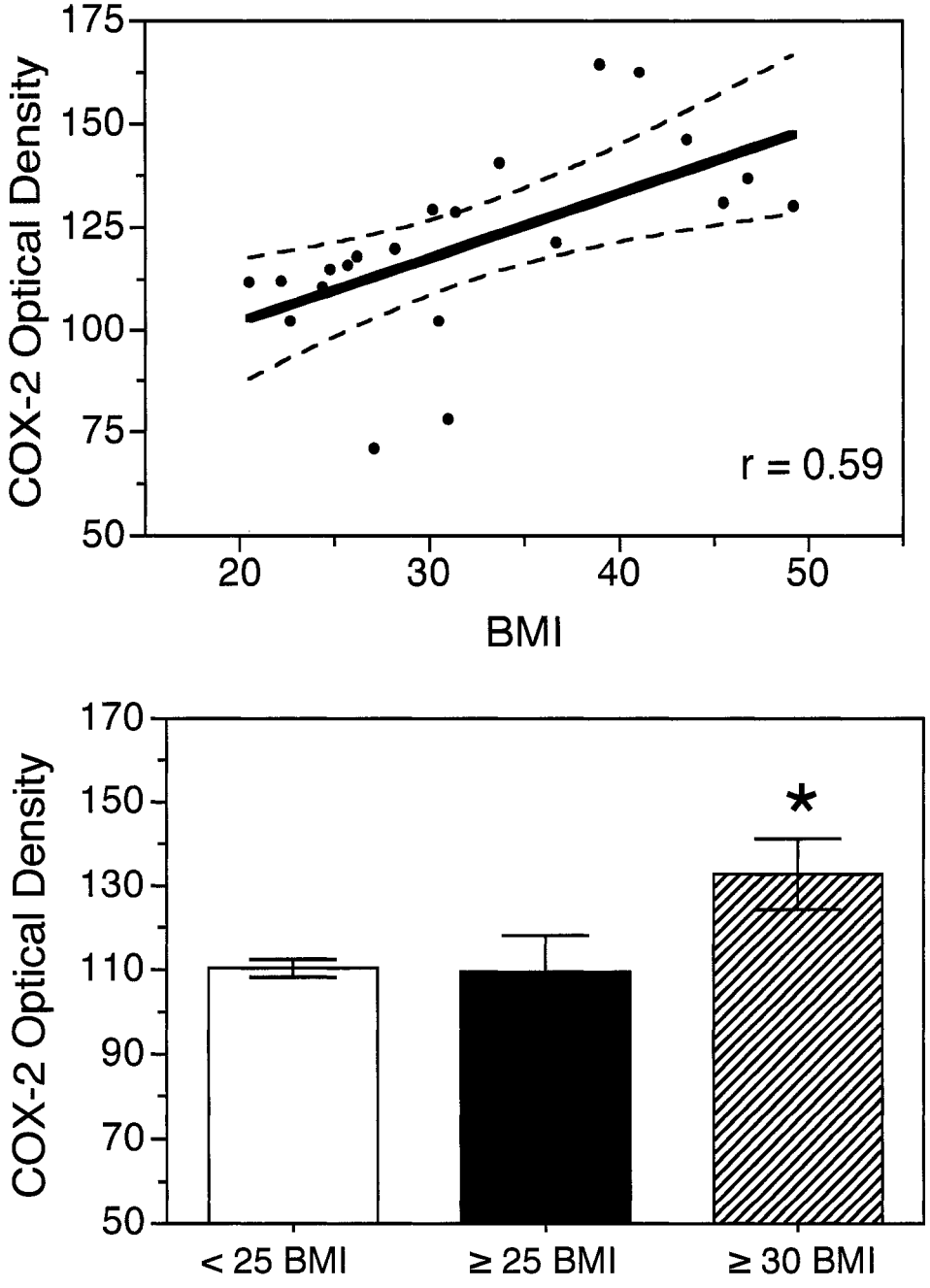


Figure 34. Summarized optical density measurements for COX-2 staining.

Optical density measurements were significantly correlated with BMI ($r = 0.59$,** $P < 0.01$, Top panel). Obese patients had significantly higher optical density measurements compared to normal weight patients and overweight patients (Lower panel, * $P < 0.05$).

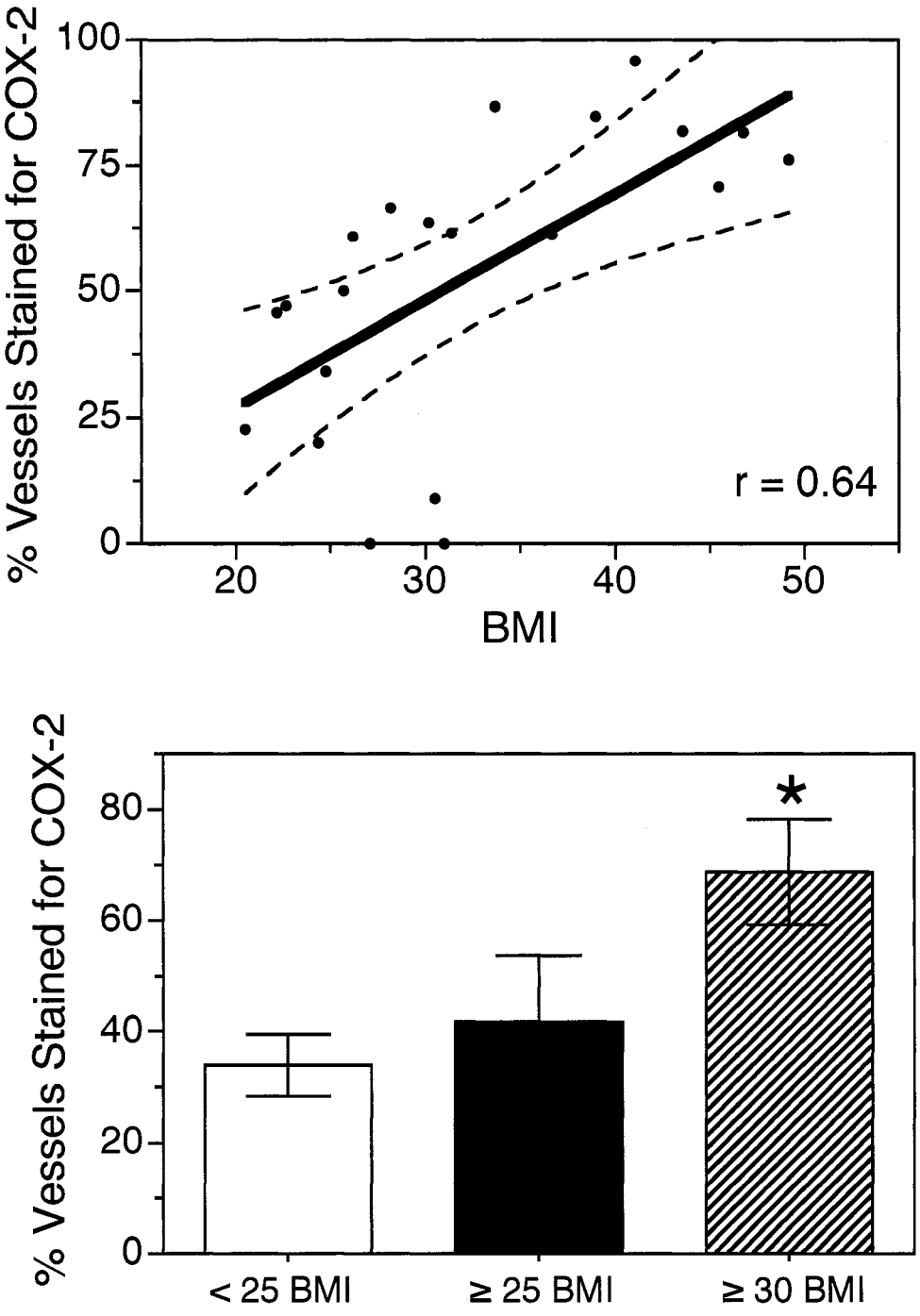


Figure 35. Percent of vessels stained for COX-2.

Percent of vessels stained for COX-2 were significantly correlated with BMI ($r = 0.64$, ** $P < 0.01$, Top panel). Obese patients had significantly more percent vessels stained compared to normal weight patients (Lower panel,* $P < 0.05$).

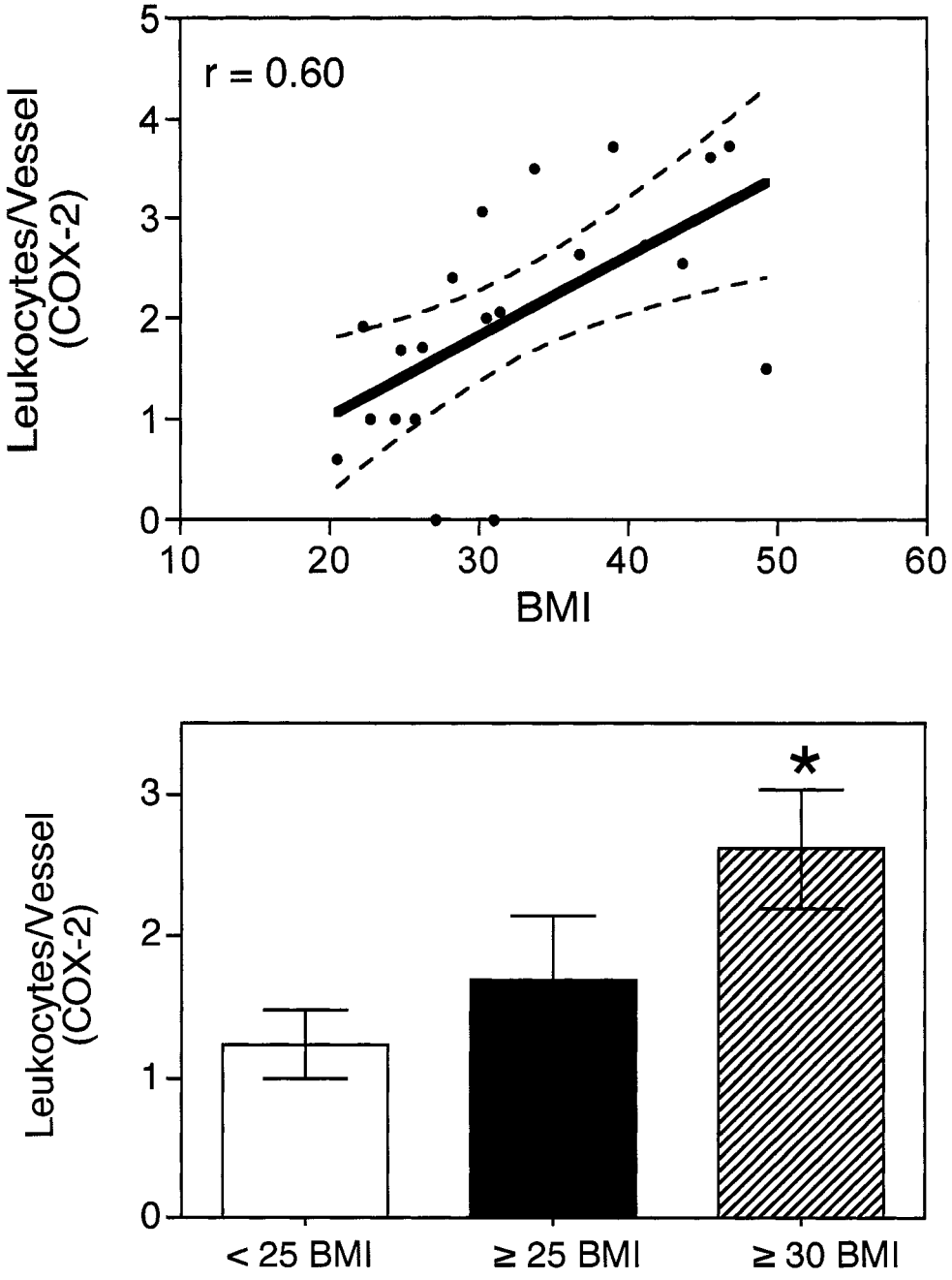


Figure 36. Total number of leukocytes that stained for COX-2 per vessel.

The number of leukocytes that stained for COX-2 per vessel was significantly correlated with BMI ($r = 0.60$, ** $P < 0.01$, Top panel). Obese patients had significantly more leukocytes stained for COX-2 per vessel compared to normal weight patients (Lower panel, * $P < 0.05$).

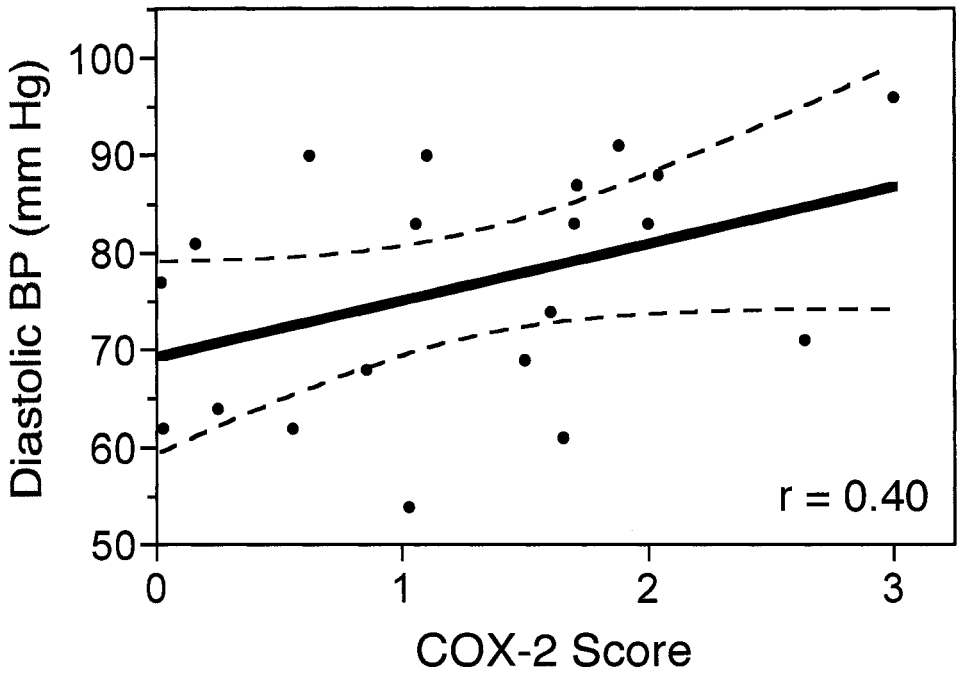


Figure 37. Diastolic blood pressure correlation with COX-2 visual score.

Diastolic blood pressure was positively correlated with COX-2 visual score ($r = 0.40$), but the correlation did not reach statistical significance. $P > 0.05$

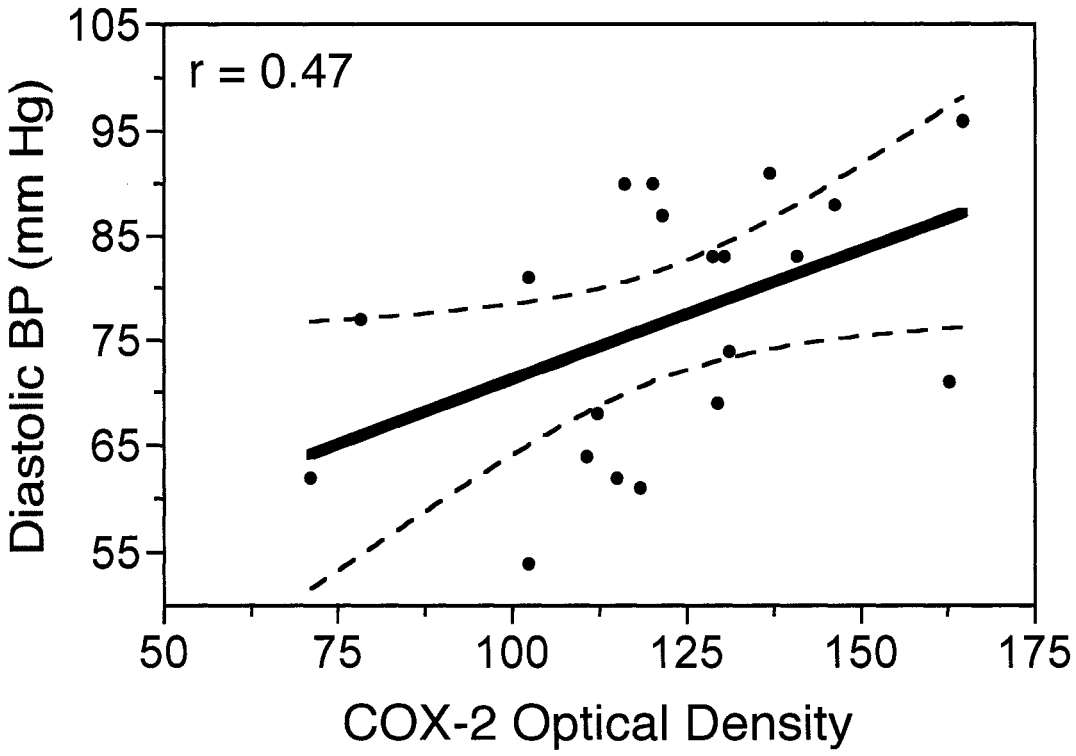


Figure 38. Diastolic blood pressure correlation with COX-2 optical density.

Diastolic blood pressure was significantly correlated ($r = 0.47$) with COX-2 optical density. * $P < 0.05$

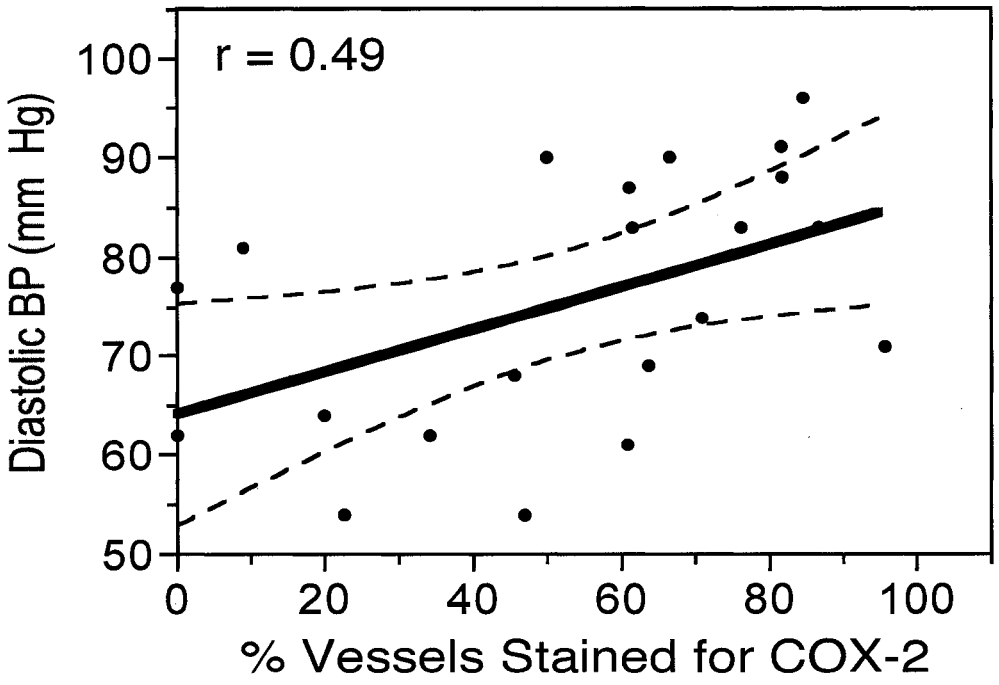


Figure 39. Diastolic blood pressure correlation with percent vessels stained for COX-2.

Diastolic blood pressure was significantly correlated ($r = 0.49$) with % vessels stained for COX-2. * $P < 0.05$

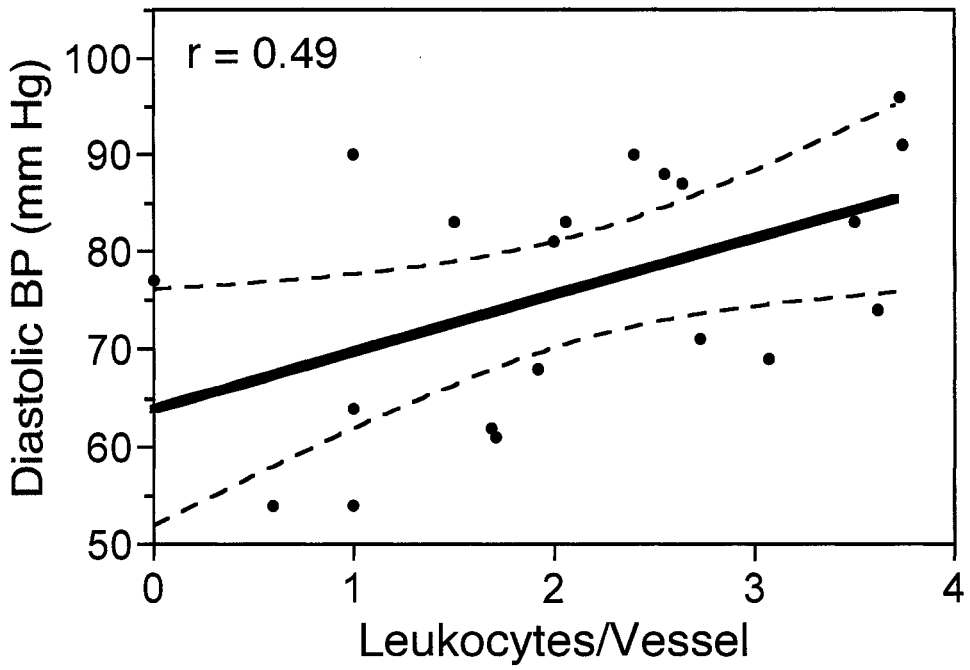


Figure 40. Diastolic blood pressure correlation with number of leukocytes stained for COX-2 per vessel.

Diastolic blood pressure was significantly correlated (r = 0.49) with the number of leukocytes stained for COX-2 per vessel.* $P < 0.05$

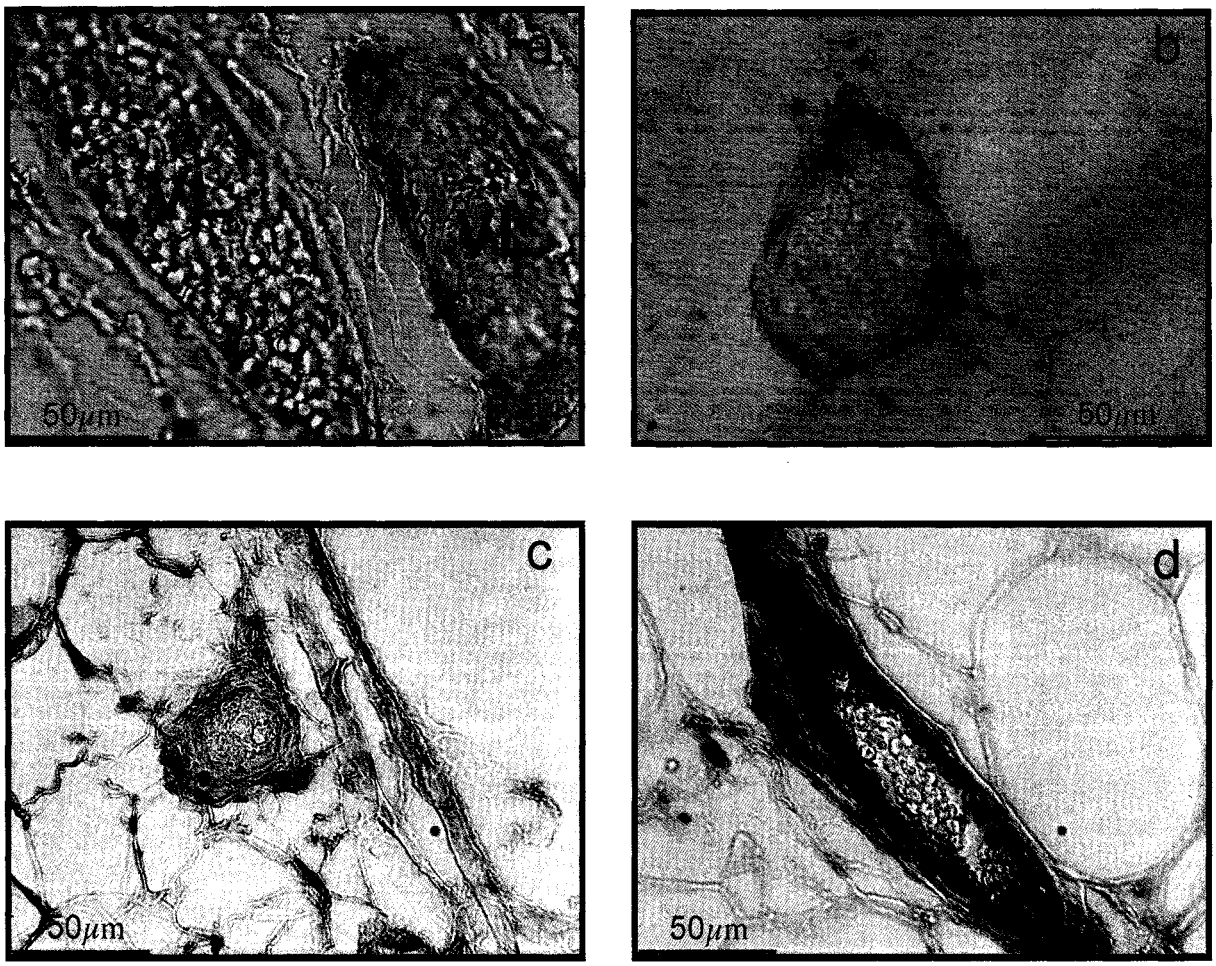


Figure 41. COX-2 staining in representative sections from the patient groups.

a) IgG negative control, b) Normal weight patient showing no COX-2 staining, c) Overweight patient showing some COX-2 staining along the endothelium and vascular smooth muscle, d) Obese patient showing intense brown staining for COX-2 along the endothelium and vascular smooth muscle. Magnification X400.

(VL- vessel lumen)

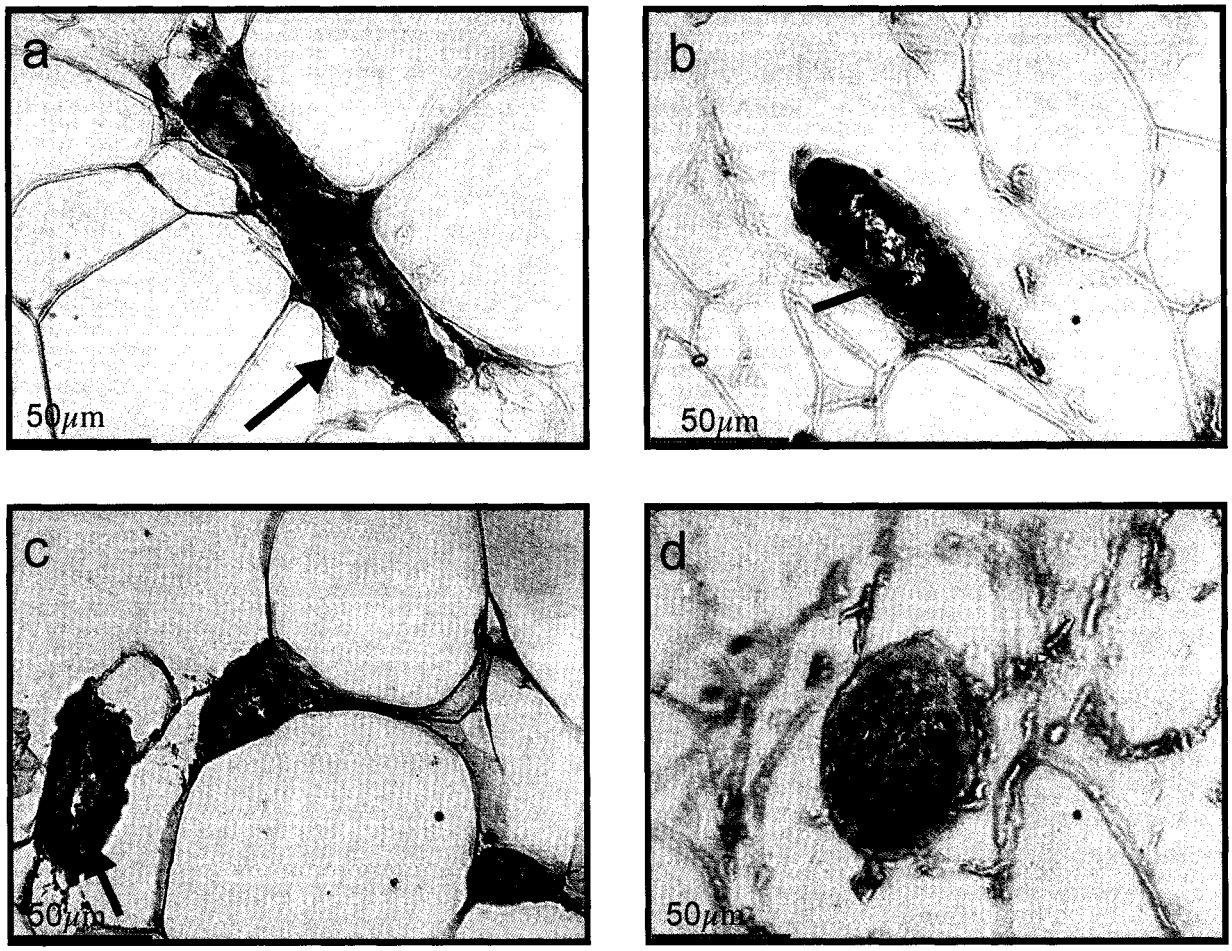


Figure 42. Representative sections of COX-2 staining in vessels of obese patients.

a) This section shows brown staining along the endothelium and within the vascular smooth muscle, b) This vessel shows brown staining within the lumen, along the endothelium and within the vascular smooth muscle, c) These vessels show brown staining along the endothelium and within the vascular smooth muscle, d) This section also shows brown staining along the endothelium. Leukocytes also stained for COX-2 (arrows). Magnification X400.

CHAPTER 4

ACTIVATION OF NF-κB AND EXPRESSION OF COX-2 IN ASSOCIATION WITH NEUTROPHIL INFILTRATION IN SYSTEMIC VASCULAR TISSUE OF WOMEN WITH PREECLAMPSIA [179]

Modified from Shah, TJ, "Activation of NF-κB and Expression of COX-2 in Association with Neutrophil Infiltration in Systemic Vascular Tissue of Women with Preeclampsia", Master's Thesis, May 2005, Virginia Commonwealth University

A. Introduction

In the previous chapter we demonstrated that the vasculature of obese women is in an inflamed state and predisposed to developing hypertension and vascular dysfunction. This could put them at risk of developing preeclampsia during pregnancy because of the additional inflammatory burden imposed by the placenta with its secretion of oxidized lipids into the intervillous space. The number of neutrophils increases during pregnancy [91], so more neutrophils would be present and susceptible to activation as they circulated through the intervillous space. This could lead to further inflammation as the activated neutrophils infiltrated the maternal vasculature. This process would worsen with growth of the placenta in the second and third trimesters of pregnancy when clinical symptoms of preeclampsia are manifest. If this scenario is true, then the vascular phenotype of obese women should be characteristic of preeclamptic women.

In this chapter we will demonstrate that neutrophil infiltration, activation of NF-κB and expression of COX-2 observed in systemic vascular tissue of obese women is characteristic of women with preeclampsia, but not of women with normal pregnancy or normal nonpregnant women.

B. Materials and Methods

The clinical data for normal non-pregnant, normal pregnant, and preeclamptic groups are summarized in Table 4. Systolic and diastolic blood pressures and proteinuria were significantly greater for preeclamptic patients than for other groups. The preeclamptic group also was characterized by significantly lower gestational age and infant birth weight as compared to normal pregnant patients. Normal non-pregnant patients were older than pregnant patients. There was no difference in BMI among groups.

C. Results

The visual score for CD66b represented intensity of staining, as well as quantity of neutrophils. The visual score for CD66b was significantly greater for preeclamptic patients than normal pregnant patients or normal non-pregnant patients (1.5 ± 0.3 vs. 0.3 ± 0.1 vs. 0.1 ± 0.05, respectively, $P < 0.01$, Figure 43). The visual score was verified by density measurements, which also indicated more intense CD66b staining for preeclamptic patients as compared with normal pregnant patients or normal non-pregnant patients (141.4 ± 18.0

vs. 98.9 ± 13.7 vs. 84.4 ± 7.4 OD, respectively, $P < 0.05$, Figure 44). There was a strong correlation between visual scores and density measurements, $r = 0.85$ (Figure 45).

The percentage of vessels stained for CD66b was significantly greater for preeclamptic patients than normal pregnant patients or normal non-pregnant patients (72.5 ± 10.8% vs. 33.8 ± 10.3% vs. 17.1 ± 8.8%, respectively, $P < 0.01$, Figure 46). In preeclamptic patients, as compared with normal pregnant patients and normal non-pregnant patients, there were more neutrophils present within the lumen (38 ± 12% vs. 25 ± 8% vs. 16 ± 8%, respectively, $P > 0.05$, Figure 47), there was significantly greater adherence and flattening of neutrophils along the endothelium (51 ± 9% vs. 25 ± 7% vs. 16 ± 8%, respectively, $P < 0.05$, Figure 48), there was significantly more infiltration into the intima (42 ± 12% vs. 11 ± 4% vs. 4 ± 3%, respectively, $P < 0.05$, Figure 49) and there were more neutrophils on the outside of the vessel (32 ± 15% vs. 6 ± 3% vs. 2 ± 1%, respectively, $P > 0.05$, Figure 50). Vessel staining for CD66b is summarized in Table 5.

Figure 51 shows representative staining of vessels from NNP, NP and PE patients. NNP and NP patients had little or no staining, but PE patients had intense staining for neutrophils along the endothelium and vascular smooth muscle. Figure 52 shows additional examples of PE patients with intense staining of neutrophils along the endothelium (a), within the intima (b), along the endothelium and vascular smooth muscle (c), and within the lumen and intima.

Vessels of preeclamptic patients had intense NF-κB staining whereas normal pregnant patients and normal non-pregnant patients had very light or no NF-κB staining (Visual score: 1.9 ± 0.2 vs. 0.6 ± 0.1 vs. 0.5 ± 0.3, respectively, $P < 0.001$, Figure 53).

Optical density (OD) measurements confirmed that preeclamptic patients had significantly greater NF-κB staining as compared to normal or normal non-pregnant patients (151.0 ± 5.9 vs. 107.5 ± 5.4 vs. 104.7 ± 13.2 OD, respectively, $P < 0.01$, Figure 54). The visual scores were highly correlated with the density measurements, $r = 0.89$ (Figure 55).

The percentage of vessels stained for NF-κB was significantly greater for preeclamptic patients than normal pregnant patients or normal non-pregnant patients (87.1 ± 4.9% vs. 39.2 ± 10.7% vs. 26.7 ± 12.7%, respectively, $P < 0.01$, Figure 56).

Neutrophils, as well as other leukocytes, stained for NF-κB. When there was vessel staining for NF-κB, neutrophils were present 76%-78% of the time. PE patients had significantly more vessels with neutrophils stained for NF-κB than NNP or NP patients (66.4 ± 4.6% vs. 30.5 ± 4.0% vs. 20.1 ± 13.1%, $P < 0.01$, Figure 57).

Vessels stained for NF-κB had staining in vascular smooth muscle cells, as well as in endothelial cells. When there was staining for NF-κB in endothelial cells, 85% of the time there was also staining in the vascular smooth muscle. PE patients had significantly more vessels with NF-κB staining in vascular smooth muscle than NNP or NP patients (72.2 ± 4.5% vs. 33.3 ± 5.3% vs. 22.6 ± 4.8%, $P < 0.001$, Figure 58). Although normal pregnant and normal non-pregnant patients had little or no NF-κB staining, when there was staining in endothelial cells there was usually staining in vascular smooth muscle cells.

Figure 59 shows representative staining for NF-κB in vessels of NNP, NP and PE patients. There was little or no staining in vessels of NNP or NP, but PE patients had intense staining for NF-κB in the endothelium and vascular smooth muscle. NF-κB

staining of neutrophils, and other leukocytes, is also evident along the endothelium of PE patients.

Vessels of preeclamptic patients had intense COX-2 staining whereas normal pregnant patients and normal non-pregnant patients had little or no COX-2 staining (Visual score: 1.6 ± 0.2 vs. 0.4 ± 0.1 vs. 0.3 ± 0.2, respectively, $P < 0.001$, Figure 60). Optical density (OD) measurements confirmed that preeclamptic patients had significantly greater COX-2 staining as compared to normal or normal non-pregnant patients (151.0 ± 6.1 vs. 90.2 ± 7.7 vs. 88.5 ± 6.3 OD, respectively, $P < 0.001$, Figure 61). The visual scores were highly correlated with the density measurements, $r = 0.92$ (Figure 62).

The percentage of vessels stained for COX-2 was significantly greater for preeclamptic patients than normal pregnant patients or normal non-pregnant patients (92.9 ± 2.9% vs. 21.1 ± 8.2% vs. 11.2 ± 9.1%, respectively, $P < 0.001$, Figure 63).

Neutrophils and other leukocytes stained for COX-2. When there was vessel staining for COX-2, neutrophils were present 75%-81% of the time. PE patients had significantly more vessels with neutrophils stained for COX-2 than NNP or NP patients (69.4 ± 5.4% vs. 16.6 ± 3.2% vs. 9.1 ± 1.0%, $P < 0.001$, Figure 64).

Similar to NF-κB, vessels stained for COX-2 had staining in vascular smooth muscle cells, as well as endothelial cells. When there was staining for COX-2 in endothelial cells, 84%-100% of the time there was also staining in the vascular smooth muscle. PE patients had significantly more staining for COX-2 in vascular smooth muscle than NNP or NP patients (77.7 ± 4.3 vs. 18.1 ± 10.3 vs. 11.2 ± 1.0, $P < 0.001$, Figure 65.

Figure 66 shows representative vessels stained for COX-2. Normal pregnant and normal non-pregnant patients had little or no COX-2 staining in either endothelial cells or vascular smooth muscle cells (Figure 66 b-c). In contrast, Figure 66 d-f shows intense staining for COX-2 along the endothelium and vascular smooth muscle of preeclamptic patients. Neutrophils stained for COX-2 can also be seen flattened and adhered along the endothelium.

D. Discussion

This investigation is the first to demonstrate NF-κB activation and induction of COX-2 expression in systemic vasculature of women with preeclampsia. Activation of NF-κB and expression of COX-2 were associated with neutrophil infiltration. Neutrophils also demonstrated activation of NF-κB and expression of COX-2. These data clearly place preeclampsia in the category of an inflammatory disease because NF-κB and COX-2 are hallmarks of inflammation. These data link neutrophil infiltration, NF-κB activation and COX-2 expression as a possible mechanism for explaining clinical symptoms of preeclampsia.

Eighty-seven to 92% of vessels in preeclamptic women stained for NF-κB and COX-2, which was significantly higher than the percent vessels stained in normal pregnant women or normal non-pregnant women. In almost every case there was staining in the vascular smooth muscle, as well as in endothelium. Since neutrophils also stained for NF-κB and COX-2, it was possible to determine that whenever there was vascular staining for

NF-κB or COX-2, there were also neutrophils present. Although these data do not exclude other mechanisms, they strongly implicate neutrophils as causative agents of vascular inflammation and dysfunction due to their release of inflammatory substances such as ROS, myeloperoxidase and TNF-α. Neutrophils also produce thromboxane, so neutrophils that have infiltrated the vasculature could be responsible for increasing vascular tone leading to hypertension.

A previous study demonstrated increased vascular expression of ICAM-1 and increased vascular smooth muscle expression of IL-8 [127]. NF-κB activation plays an important role in the up-regulation of ICAM-1 and IL-8, as well as COX-2, by binding to the promoter regions of their respective genes [39, 156]. NF-κB activation could, therefore, be a key factor in preeclampsia.

Initial activation of NF-κB in the maternal vasculature could be caused by circulating substances. Lipid peroxides, ROS and TNFα are elevated in the maternal circulation of preeclamptic women, and all are known to activate NF-κB in a variety of cell types [155, 156] including in endothelial cells [158].

Preeclamptic patients had more staining for COX-2 than normal pregnant patients and normal non-pregnant patients. These data strongly indicate inflammation in preeclampsia because COX-2 plays an important role in inflammatory diseases, including rheumatid arthritis and osteoarthritis [180]. COX-2 is induced during an inflammatory response [171], and its expression in monocytes is associated with increased circulatory levels of oxidized lipids [172].

In this chapter we demonstrated that the vascular phenotype of obese women which is characterized by neutrophil infiltration and vascular inflammation, as indicated by NF-κB activation and COX-2 expression, is also present in women with preeclampsia, but not in women with normal pregnancy or normal women who are not pregnant. Therefore, neutrophil infiltration and vascular inflammation may predispose obese women to develop preeclampsia when they become pregnant. In the next two chapters, we will determine if activated neutrophils or neutrophil products can cause activation of NF-κB and expression of COX-2 in vascular smooth muscle cells.

Table 4. Clinical data for patient groups.

	Normal Non-pregnant (n = 5)	Normal Pregnant (n = 6)	Preeclamptic (n = 7)
Maternal Age	42.4 ± 6.0***	22.3 ± 2.7	25.4 ± 4.7
Pre-pregnancy BMI	28.9 ± 4.0	30.8 ± 5.5	31.2 ± 9.0
Systolic Blood Pressure (mm Hg)	126.8 ± 10.4	126.3 ± 19.9	176.9 ± 11.0+
Diastolic Blood Pressure (mm Hg)	73.6 ± 11.2	75.2 ± 14.1	111.0 ± 9.6+
Proteinuria (mg / 24 h)	ND	ND	670 ± 352.8 (n = 3)
Dipstick	ND	ND	3.0 ±1.0 (n = 3)
Parity	NA	1.0 ± 0.6	1.0 ± 1.3
Gestational Age (wk)	NA	38.8 ± 1.5	33.3 ± 3.8‡
Infant Birth Weight (g)	NA	3187 ± 631.2	1864 ± 901.5‡

Values are mean ± SD. ND indicates not determined. NA indicates not applicable.
*** P<0.001 compared to normal pregnant or preeclamptic.
+ P<0.001 compared to normal pregnant or normal non-pregnant.
‡ P<0. 01 compared to normal pregnant.

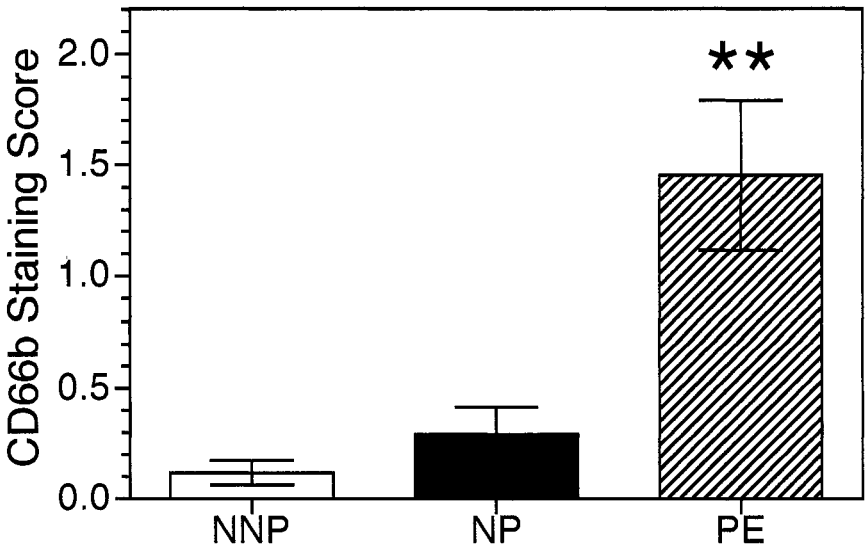


Figure 43. Summarized visual score results for CD66b staining.

Preeclamptic patients had significantly greater CD66b staining as compared to normal non-pregnant or normal pregnant patients. The visual score for CD66b was based on both staining intensity and quantity of neutrophil infiltration. Background had a density of approximately 60. **P < 0.01

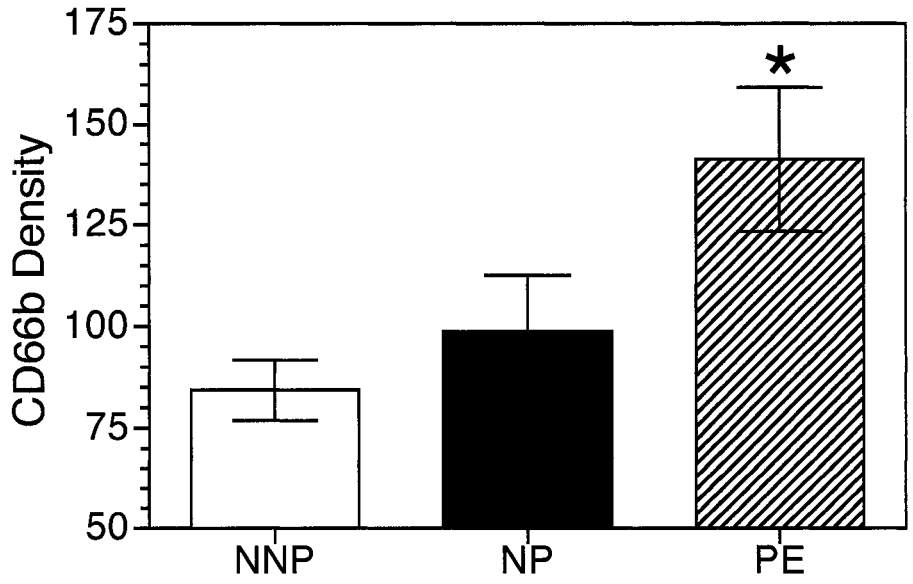


Figure 44. Summarized optical density measurements for CD66b staining.

Optical density of CD66b staining was significantly greater for preeclamptic patients as compared to normal non-pregnant or normal pregnant patients. Background had a density value of approximately 60. *P < 0.05

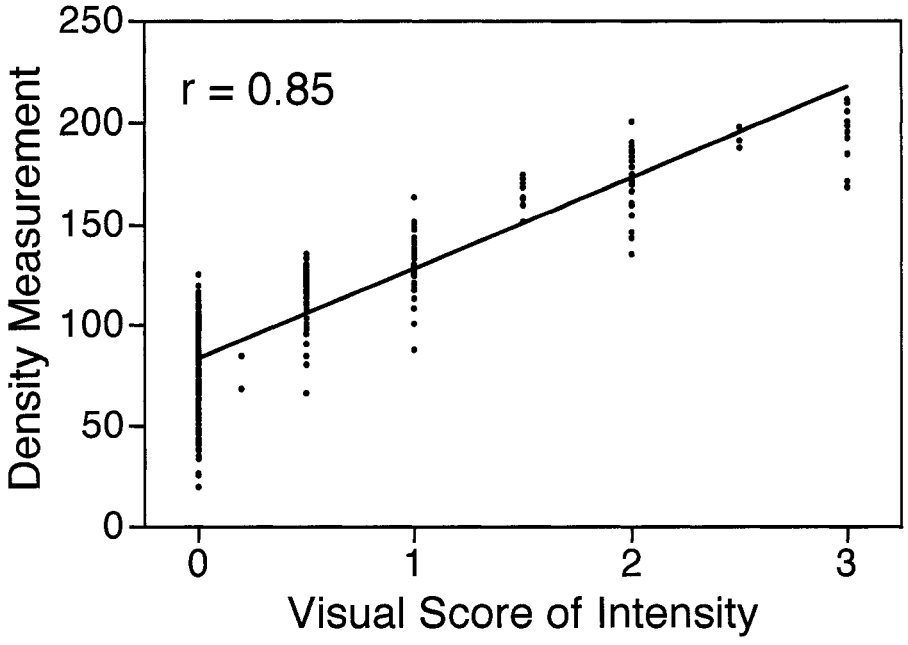


Figure 45. Correlation between visual scores and density measurements for CD66b staining.

There was a positive correlation (r = 0.85) between visual scores and density measurements. These data show that visual scoring was objective and precise.

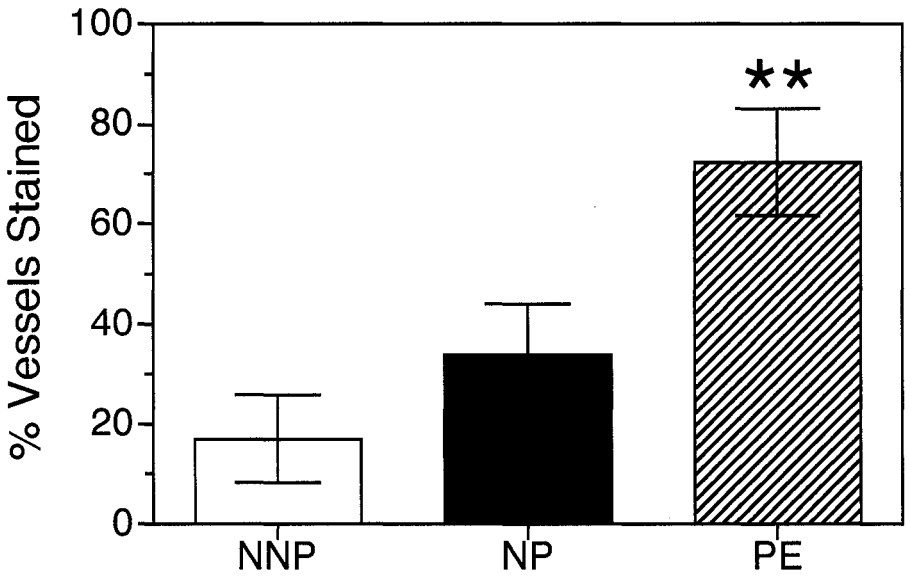


Figure 46. Percent of vessels stained for CD66b.

A mean of 72% of the vessels in preeclamptic patients stained for CD66b as compared to 34% for normal pregnant patients and 17% for normal non-pregnant patients. **P < 0.01

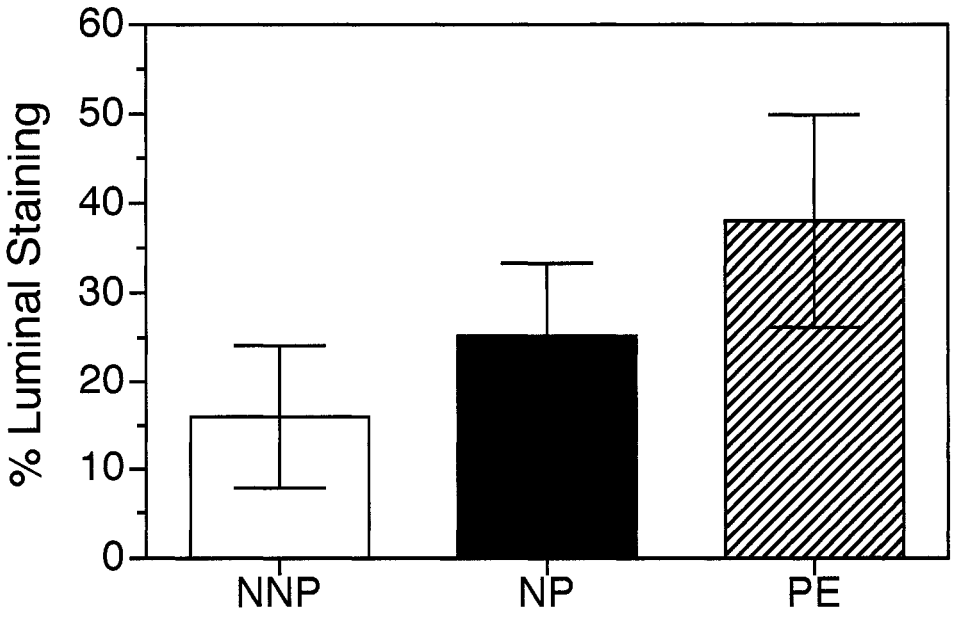


Figure 47. Percent of vessels with neutrophils within the lumen.

Thirty-eight percent of the vessels of preeclamptic patients had neutrophils present within the lumen as compared to 25% for normal pregnant patients and 16% for normal non-pregnant patients. Differences were not statistically significant. $P > 0.05$

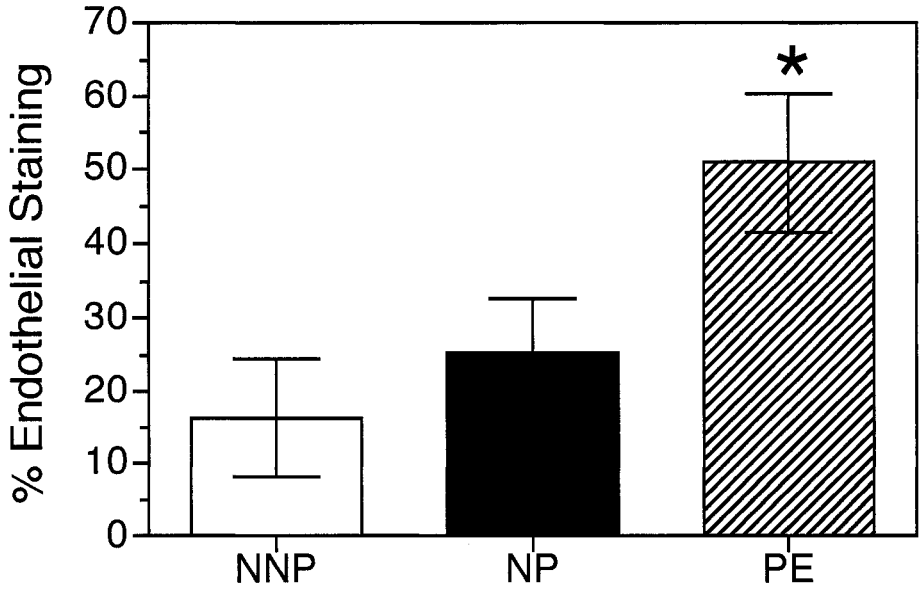


Figure 48. Percent of vessels with neutrophils adhered and flattened onto endothelial cells.

Over half of the vessels of preeclamptic patients had neutrophils adhered and flattened to the endothelium as compared to 25% for normal pregnant patients and 16% for normal non-pregnant patients. *$P < 0.05$

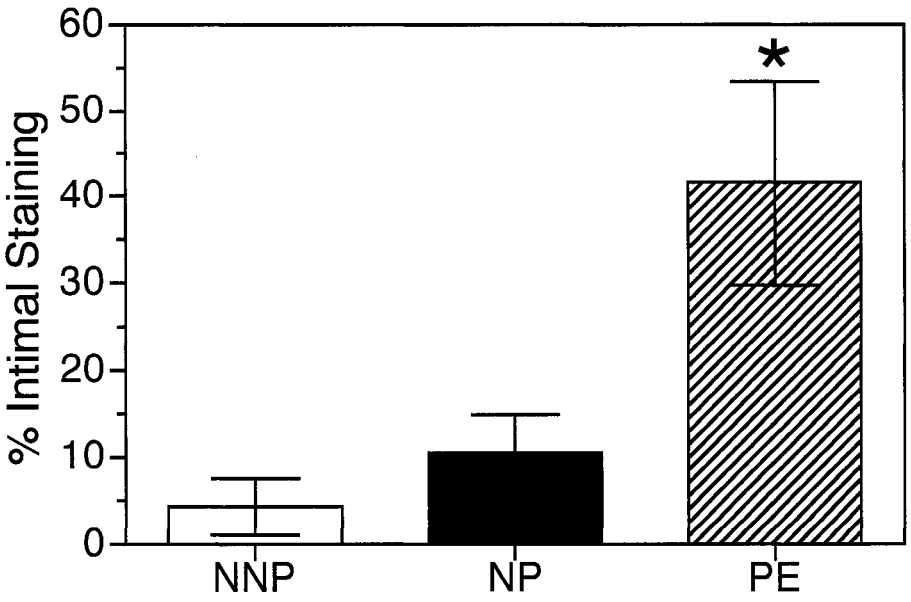


Figure 49. Percent of vessels with neutrophils infiltrated into the intimal space.

Over 40% of the vessels of preeclamptic patients showed neutrophil infiltration to the intima as compared to 11% for normal pregnant patients and 4% for normal non-pregnant patients. * $P < 0.05$

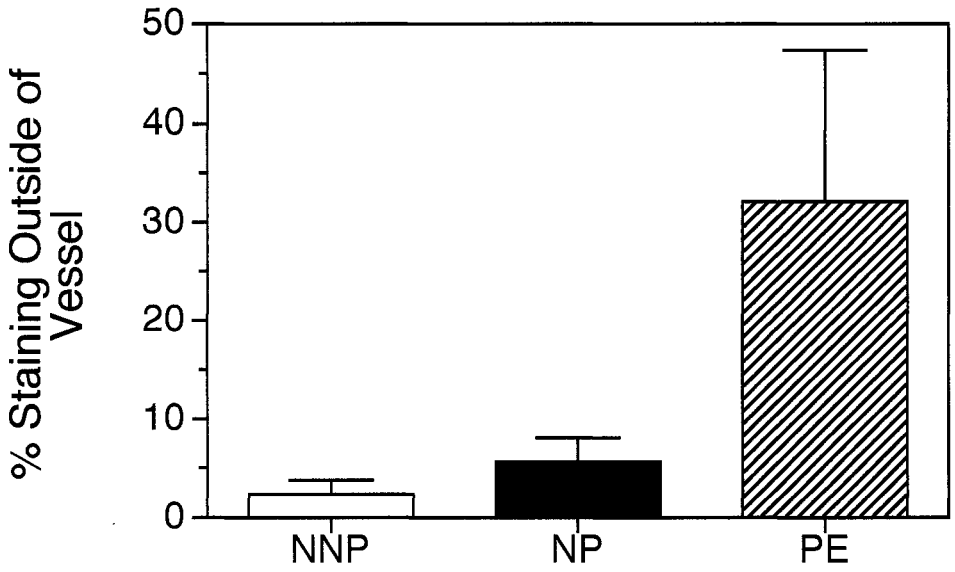


Figure 50. Percent of vessels with neutrophils present on the outside of the vessel.

Thirty-two percent of the vessels of preeclamptic patients had neutrophils present on the outside of the vessel as compared to 6% for normal pregnant patients and 2% for normal non-pregnant patients. Differences were not statistically significant. $P > 0.05$

Table 5. Summary of CD66b immunohistochemical staining for resistance sized vessels (10 μm - 200 μm)

	Normal Non-Pregnant (n = 5)	Normal Pregnant (n = 6)	Preeclamptic (n = 6)
Total Vessels with Stained Cells	17.1 ± 8.8%	33.8 ± 10.3%	72.5 ± 10.8%**
Vessels with Stained Cells in Lumen	16.0 ± 8.1%	25.2 ± 8.1%	38.1 ± 11.8%
Vessels with Stained Cells Adhered & Flattened on Endothelium	16.3 ± 8.2%	25.3 ± 7.3%	50.1 ± 9.5%*
Vessels with Stained Cells in Intima	4.3 ± 3.2%	10.6 ± 4.3%	41.6 ± 11.9 %*
Vessels with Stained Cells on Outside of Vessel	2.3 ± 1.5%	5.6 ± 2.5%	32.1 ± 15.3%

Values represent mean ± SEM. *P < 0.05, **P < 0.01 compared with normal pregnant and normal non-pregnant.

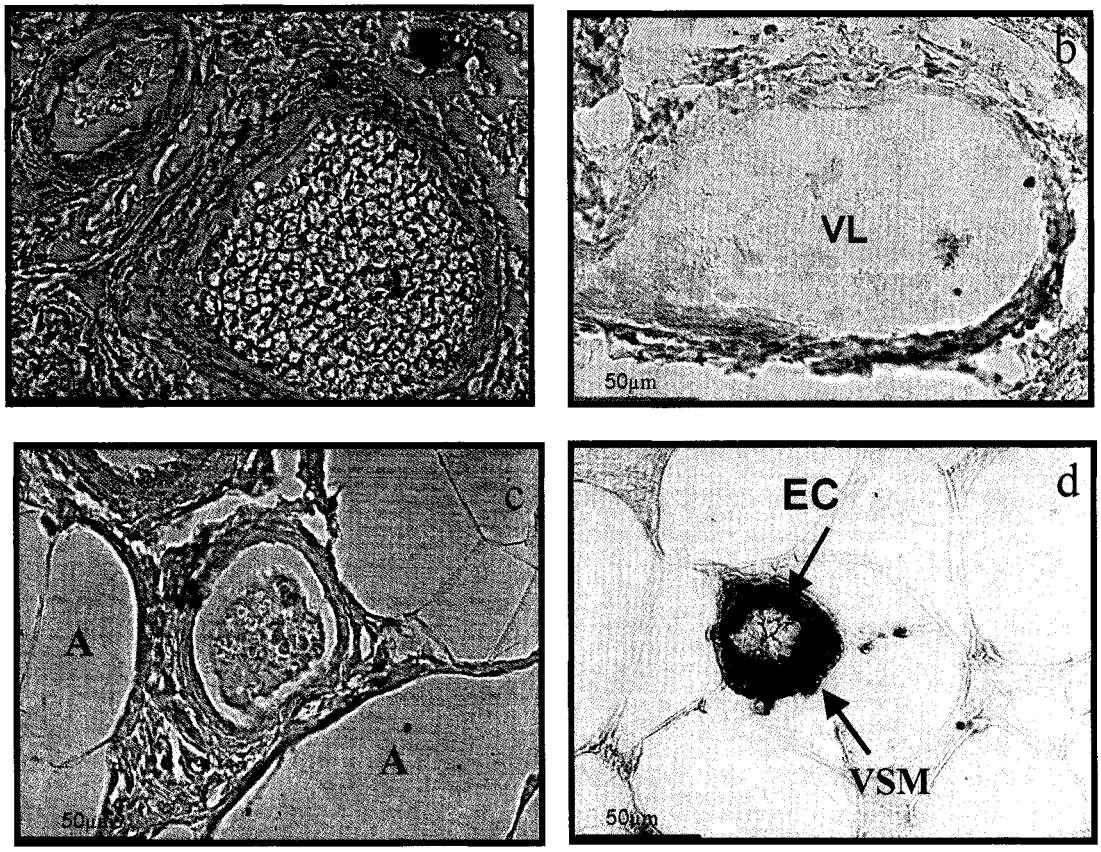


Figure 51. CD66b staining of neutrophils in representative sections from the patient groups.

a)) IgM negative control, b) Normal non-pregnant patient showing no CD66b staining, c) Normal pregnant patient showing no CD66b staining, d) Preeclamptic patient showing massive brown staining for CD66b along the endothelium and vascular smooth muscle. Magnification X400.

(A- adipocyte, VL- vessel lumen, VSM- vascular smooth muscle, EC- endothelial cells)

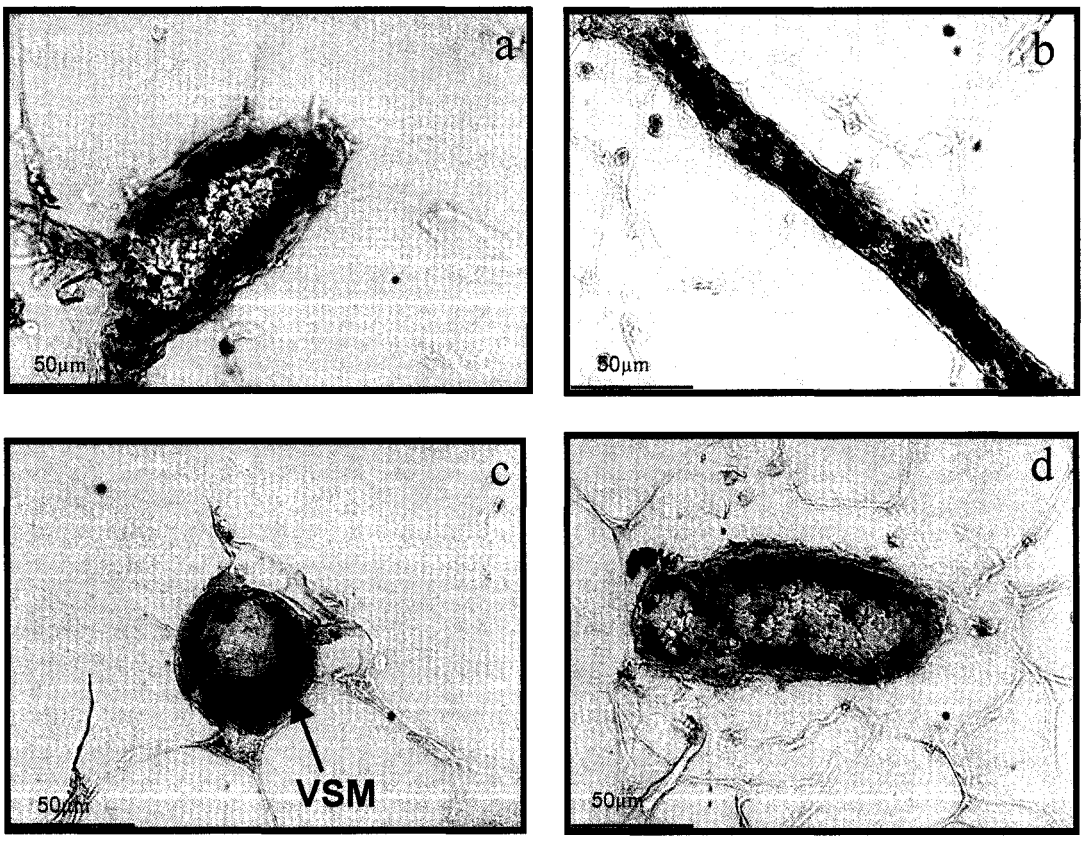


Figure 52. Representative sections of CD66b staining of neutrophils in various vessel locations in preeclamptic patients.

a) This section shows brown stained neutrophils along the endothelium and within the vascular smooth muscle, b) This longitudinal section of a vessel shows massive neutrophil involvement along the entire vessel, c) This vessel is an example of neutrophil staining in the intima, d) This section shows neutrophils stained in the lumen, along the endothelium and within the intima. Magnification X400.

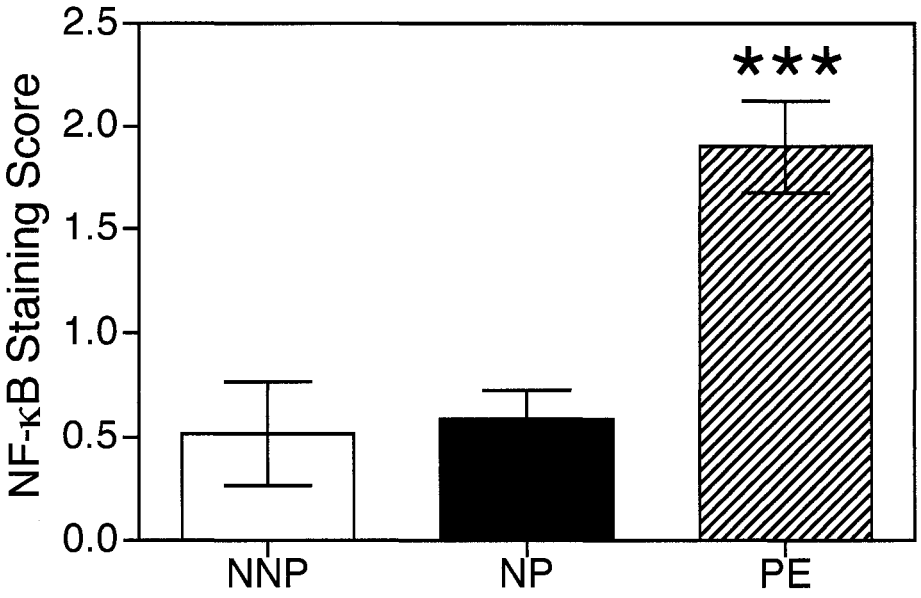


Figure 53. Summarized visual score results for NF-κB staining.

Preeclamptic patients had significantly greater NF-κB staining as compared to normal non-pregnant or normal pregnant patients. Staining was present in both vascular smooth muscle cells and endothelial cells. ***P < 0.001

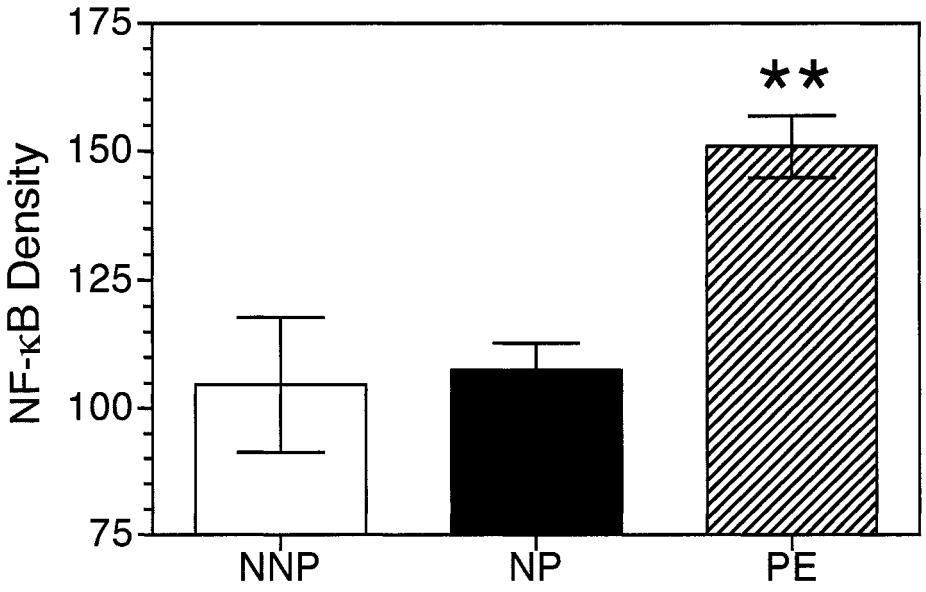


Figure 54. Summarized optical density measurements for NF-κB staining.

Optical density of NF-κB staining in preeclamptic patients was significantly greater than in normal non-pregnant or normal pregnant patients. ** $P < 0.01$

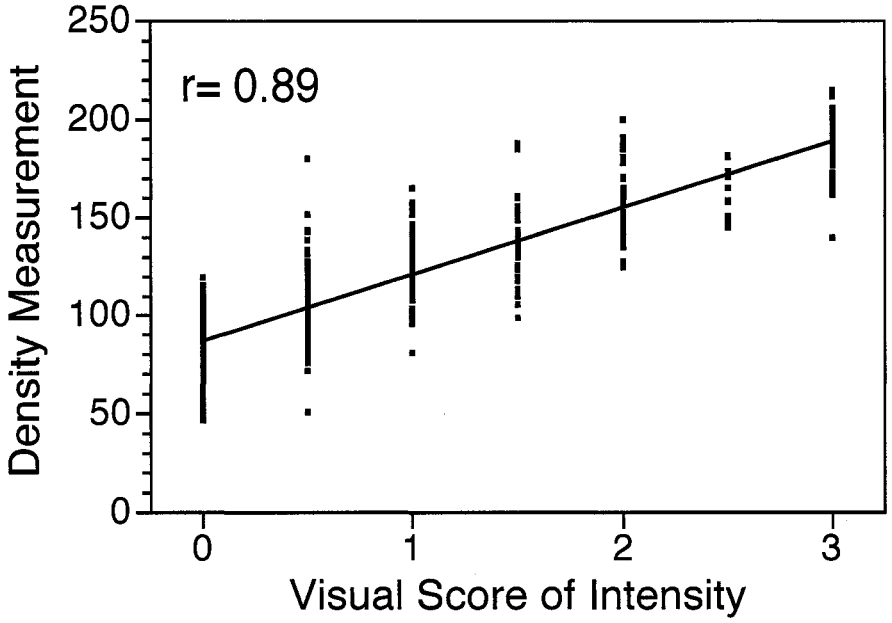


Figure 55. Correlation between visual scores and density measurements for NF-κB staining.

There was a positive correlation (r = 0.89) between visual scores and density measurements.

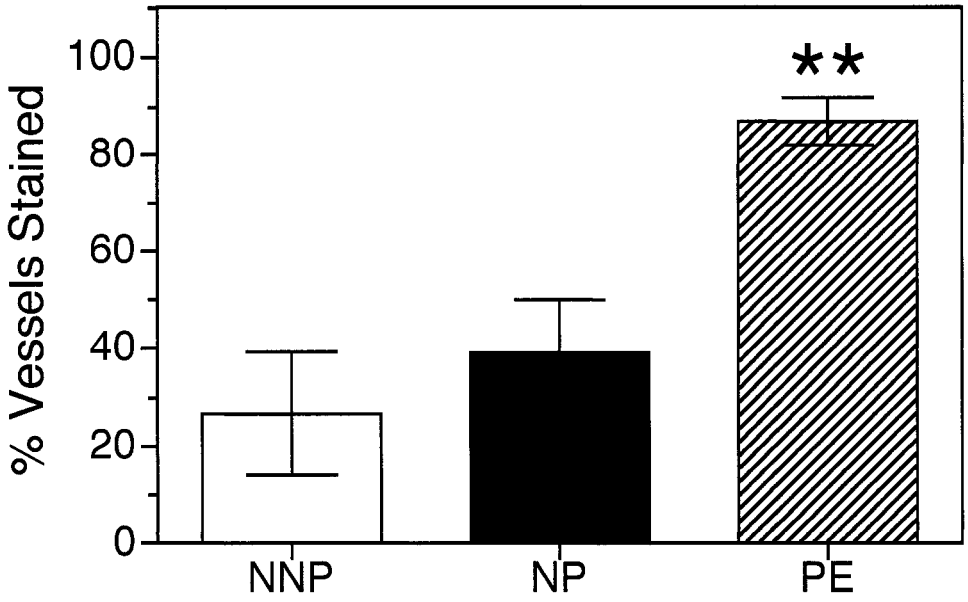


Figure 56. Percent of vessels stained for NF-κB.

A mean of 87% of the vessels in preeclamptic patients stained for NF-κB as compared to 40% for normal pregnant patients and 27% for normal non-pregnant patients. **P < 0.01

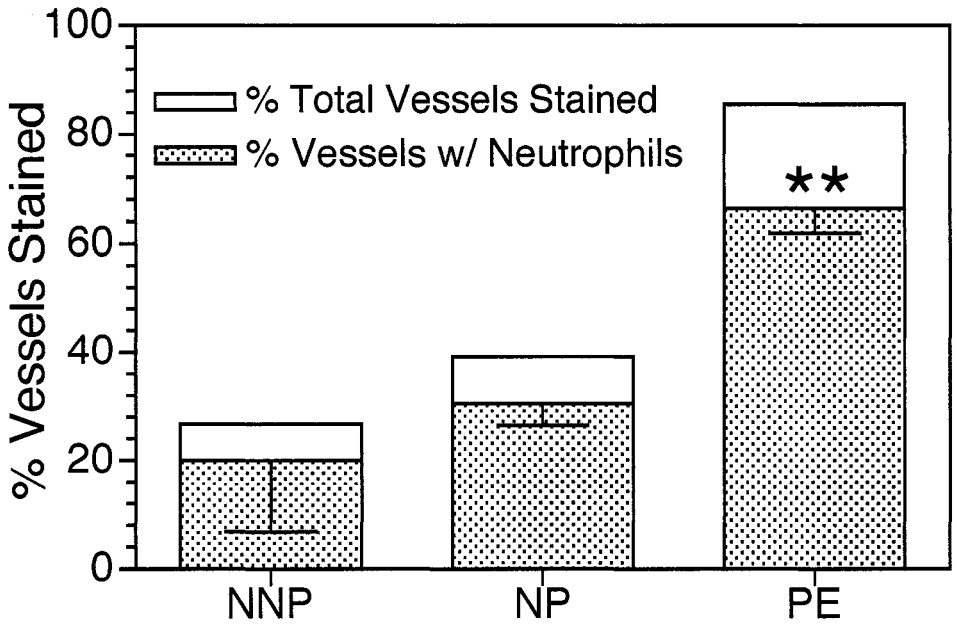


Figure 57. Percent of vessels stained for NF-κB that also had neutrophils stained for NF-κB.

When there was vessel staining for NF-κB, 76%-78% of the time neutrophils (and other leukocytes) were present as evidenced by their staining for NF-κB. Preeclamptic patients had significantly more vessels stained with neutrophils than normal pregnant or normal non-pregnant patients. $P < 0.01$

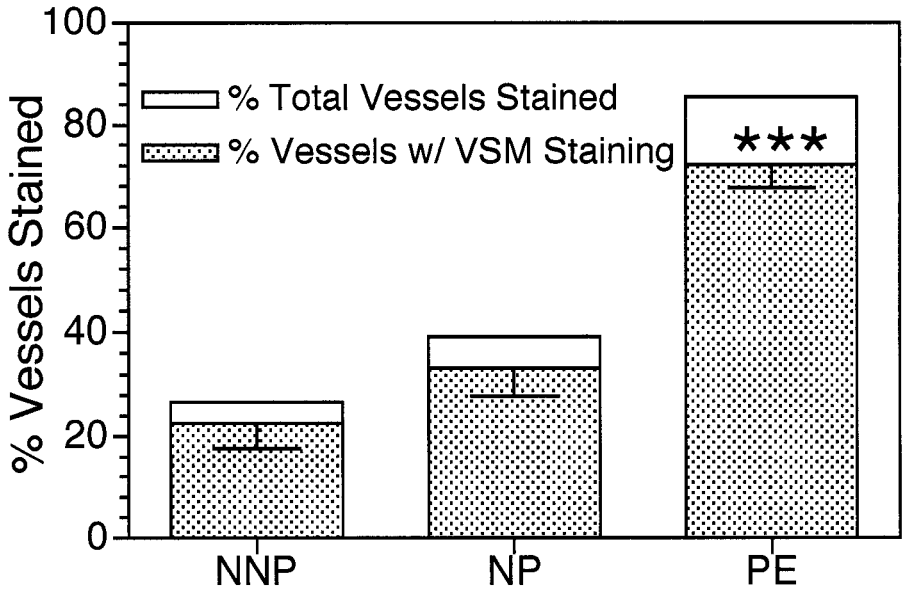


Figure 58. Percent of vessels with NF-κB staining that also had staining in vascular smooth muscle.

When there was vessel staining for NF-κB, 85% of the time there was staining in the vascular smooth muscle. Preeclamptic patients had significantly more vessels with vascular smooth muscle staining than normal pregnant or normal non-pregnant patients. $P < 0.001$

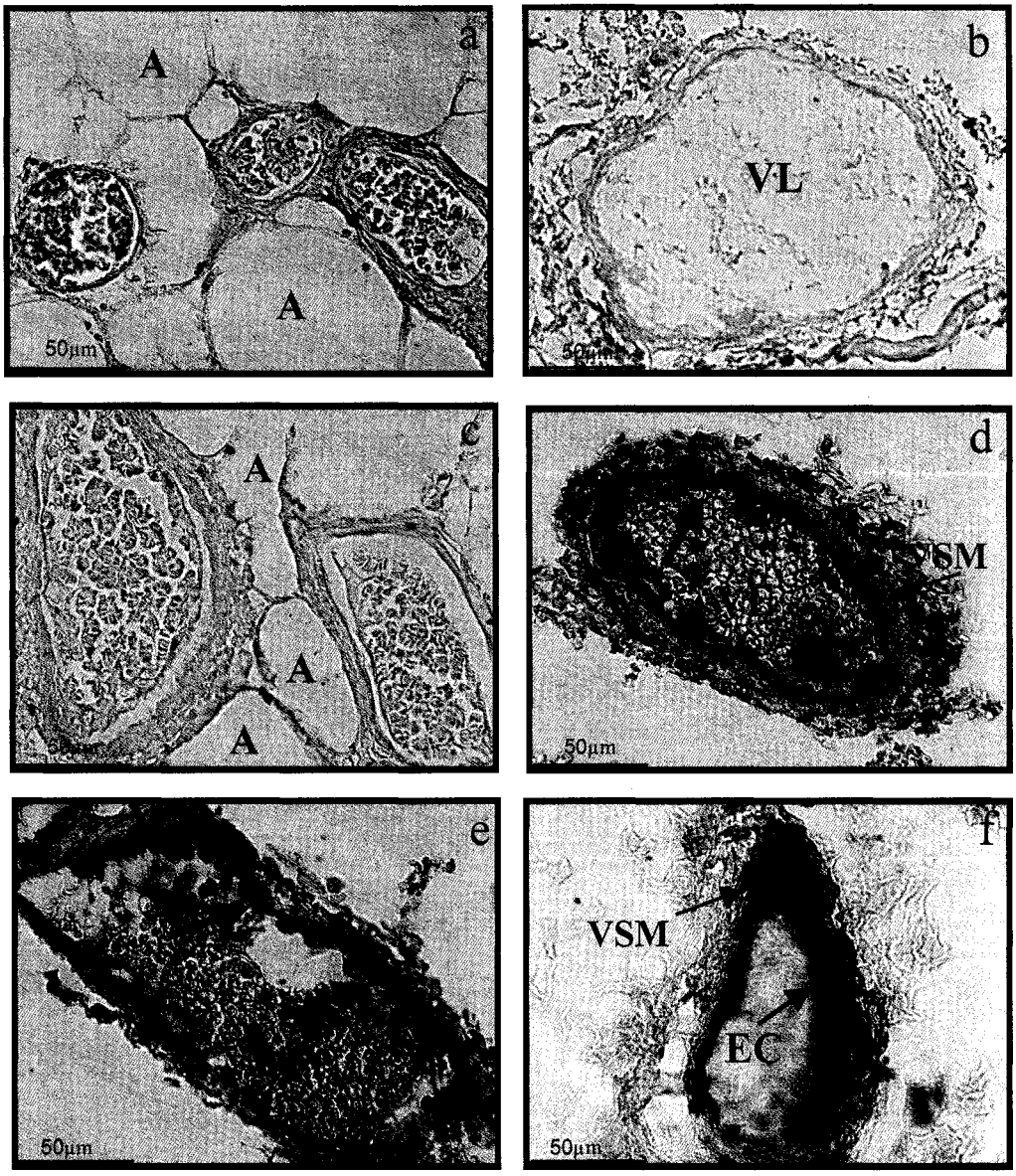


Figure 59. NF-κB immunohistochemical staining of vessels in subcutaneous fat of the patient groups.

a) IgG negative control, b) Normal non-pregnant patient showing no vessel staining, c) Normal pregnant patient showing some light brown vessel staining, d) Preeclamptic patient showing intense brown vessel staining of endothelial cells and brown staining in vascular smooth muscle. Neutrophil (and other leukocyte) staining for NF-κB is also apparent (bold arrow), e) Dark brown staining is present on endothelial cells and vascular smooth muscle in this preeclamptic patient. Neutrophil (and other leukocyte) staining for NF-κB is also present (bold arrows), f) Preeclamptic patient showing intense brown staining of endothelial cells which extends into vascular smooth muscle. X400

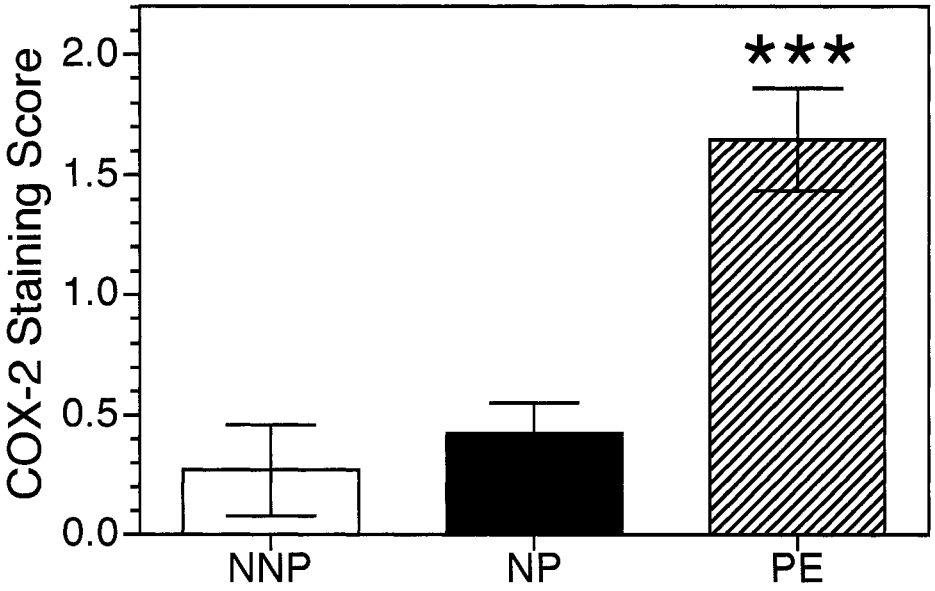


Figure 60. Summarized visual score results for COX-2.

Preeclamptic patients had significantly greater COX-2 staining as compared to normal non-pregnant or normal pregnant patients, primarily due to staining in vascular smooth muscle cells, as well as endothelial cells. There was no difference for COX-2 staining between normal non-pregnant and normal pregnant patients. ***$P < 0.001$

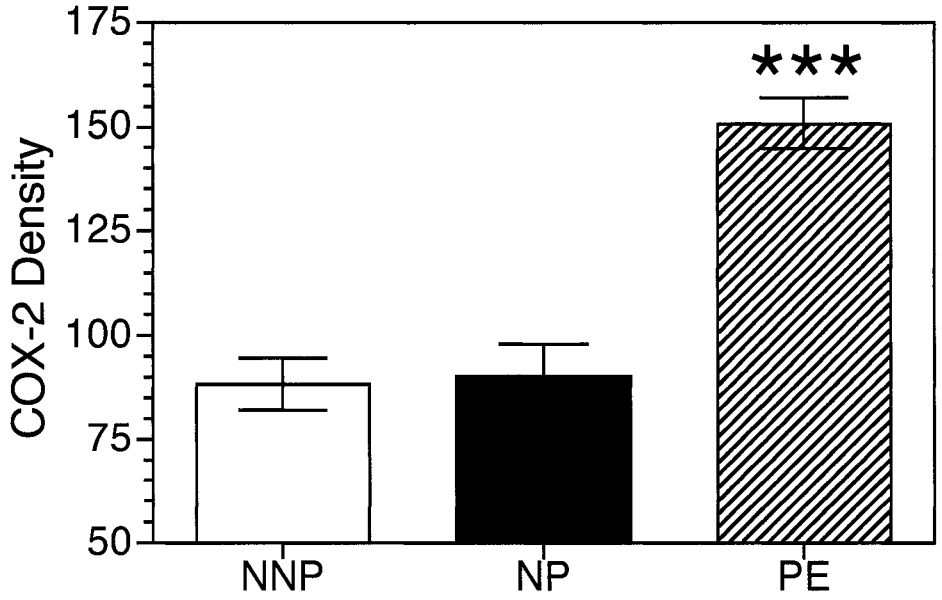


Figure 61. Summarized optical density measurements for COX-2.

Optical density of COX-2 staining in preeclamptic patients was significantly greater than in normal pregnant patients or normal non-pregnant patients. Staining in normal non-pregnant patients was limited to the endothelium, whereas in the normal pregnant patients and especially in the preeclamptic patients, staining was present in vascular smooth muscle, as well as endothelium. Density measurement, as opposed to visual score, did not reflect the spread of staining to vascular smooth muscle cells.
***$P < 0.001$

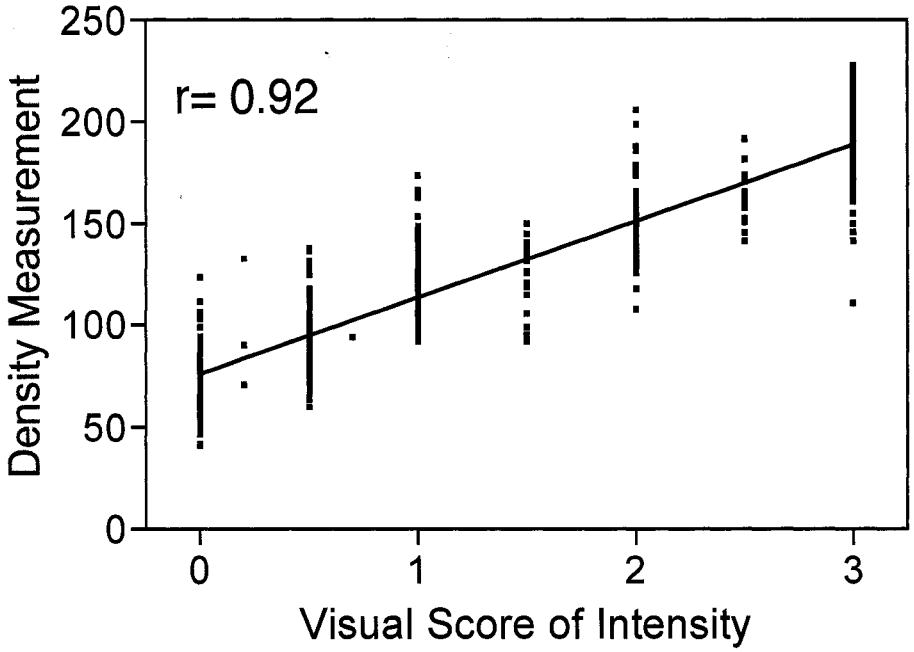


Figure 62. Correlation between visual scores and density measurements for COX-2 staining.

There was a positive correlation (r = 0.92) between visual scores and density measurements.

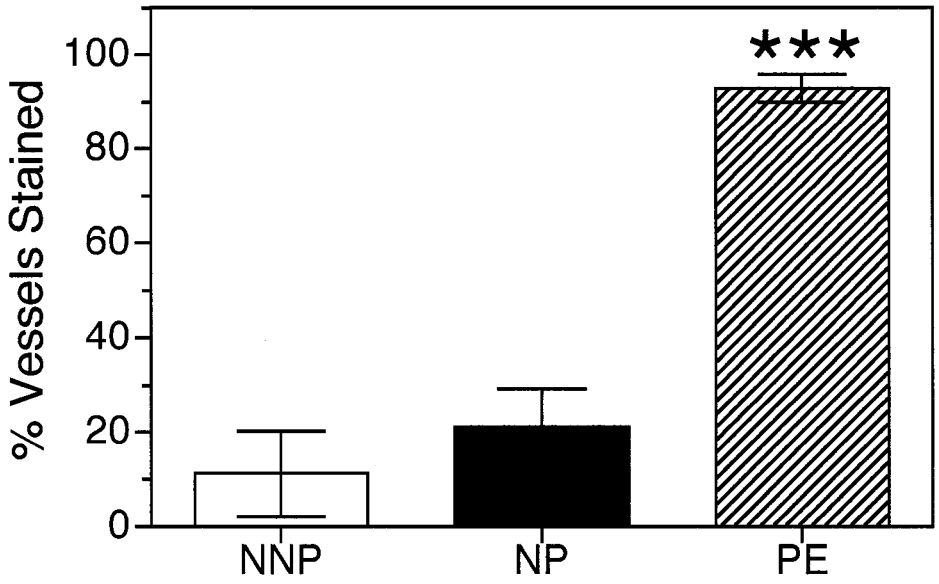


Figure 63. Percent of vessels stained for COX-2.

A mean of 92% of the vessels in preeclamptic patients stained for COX-2 as compared to 21% for normal pregnant patients and 11% for normal non-pregnant patients. **P < 0.01

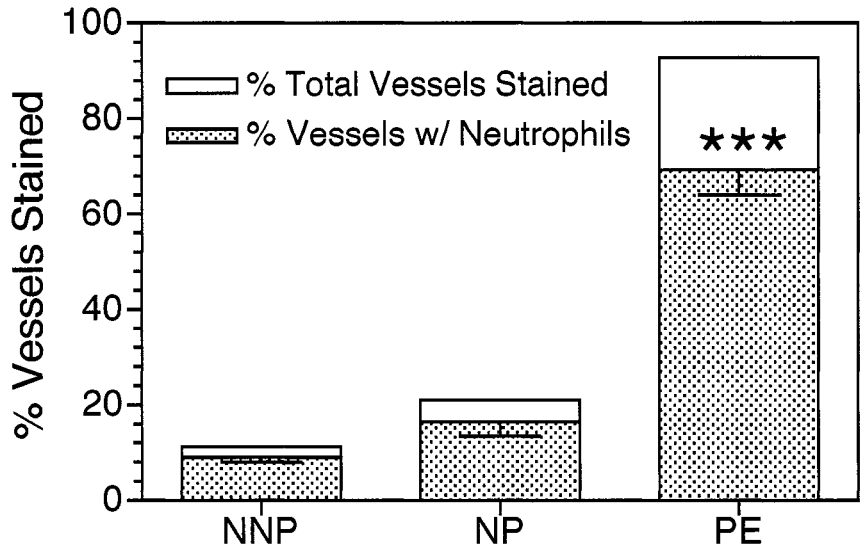


Figure 64. Percent of vessels stained for COX-2 that also had neutrophils stained for COX-2.

Seventy-five percent to 81% of the time when vessels stained for COX-2, there were also neutrophils (and other leukocytes) stained for COX-2. Preeclamptic vessels had more neutrophils stained for COX-2 than normal pregnant or normal non-pregnant patients. $P < 0.001$

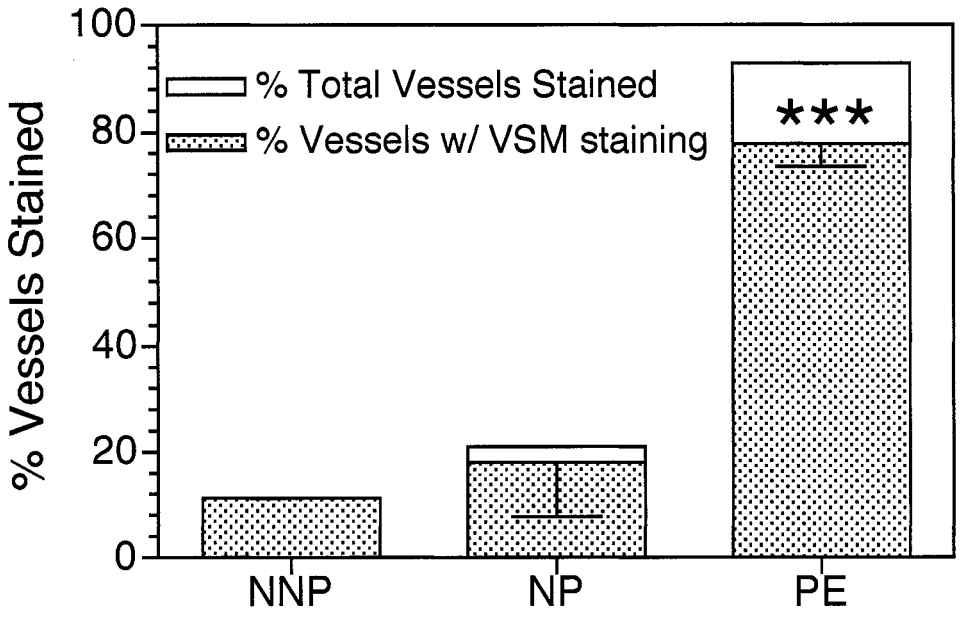


Figure 65. Percent of vessels with COX-2 staining that also had COX-2 staining in vascular smooth muscle.

When there was vessel staining for COX-2, 84%-100% of the time there was staining in the vascular smooth muscle. Preeclamptic patients had significantly more vessels with vascular smooth muscle staining than normal pregnant or normal non-pregnant patients. $P < 0.0001$

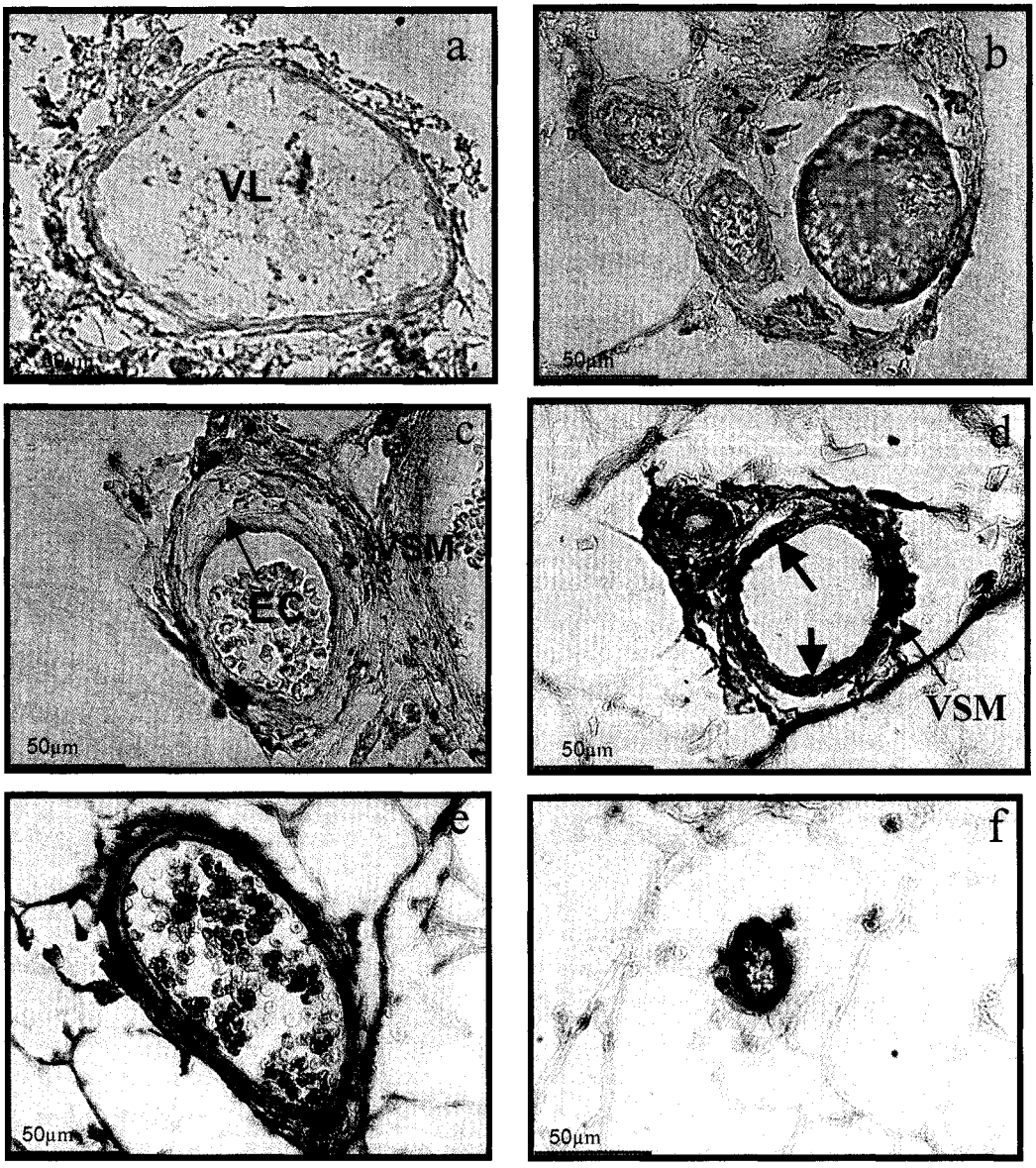


Figure 66. COX-2 immunohistochemical staining of representative vessels in subcutaneous fat of the patient groups.

a) IgG negative control, b) Normal non-pregnant patient showing no vessel staining, c) Normal pregnant patient showing light brown staining along the endothelium and in VSM d) Preeclamptic patient showing intense vessel staining on endothelium and VSM. Neutrophils adhered and flattened along endothelium are also stained for COX-2 (bold arrows), e) This preeclamptic section shows intense brown staining along endothelium and VSM, f) Preeclamptic patient showing intense VSM staining. X400

CHAPTER 5

ACTIVATION OF NF-κB IN VASCULAR SMOOTH MUSCLE CELLS BY NEUTROPHILS OR NEUTROPHIL PRODUCTS

C. Introduction

In the previous two chapters, we determined that obese women and preeclamptic women both have significant vascular infiltration of neutrophils in association with activation of NF-κB and expression of COX-2. This raised the question of whether neutrophils could be the cause of the activation of NF-κB. In this chapter we sought to determine if activated neutrophils or neutrophil products (ROS, TNFα) would activate NF-κB. For this experiment we co-cultured activated neutrophils with vascular smooth muscle cells transfected with an NF-κB reporter. We also exposed vascular smooth cells to ROS and TNFα.

B. Materials and Methods

i. Cell Culture

PASM cells were isolated and cultured as previously described (Chapter 2). Briefly, PASM cells were seeded into 24-well plates (40,000 cells/well) and grown for 2 days to 80% confluence. Cells were then transfected with the reporter constructs for 6 hours, after which treatments were applied for overnight. Cell viability was assessed by Trypan blue exclusion staining and MTT viability assay.

ii. Neutrophil Isolation

While PASM cells were being transfected, neutrophils were isolated. Healthy volunteers (n = 10) agreed to donate blood for the isolation of neutrophils. This protocol was approved by the Office of Research Subjects Protection, Virginia Commonwealth University. Whole blood (20 ml) was collected by vein puncture into two 16 x 100 mm sodium heparin vacutainer tubes (VWR Scientific Products, Pittsburgh, PA). The neutrophil isolation procedure was performed within one hour of blood collection.

Human peripheral blood neutrophils were isolated from whole blood as described by Boyum [181]. Histopaque 1119 (Sigma Chemical Company, St. Louis, Missouri) was layered below Histopaque 1077 (Sigma Chemical Company, St. Louis, Missouri) in a 50 ml conical tube (Corning Incorporated, Corning, New York). Whole blood was slowly

layered onto the upper gradient of Histopaque 1077 (Figure 67). The layered blood and Histopaque were centrifuged at 700 x g (Sorvall ® RC5 PLUS, Thermo Electron Corporation, Waltham, Ma) for 30 minutes at room temperature (25°C). Two distinct layers of leukocytes were obtained; the upper layer contained monocytes, lymphocytes and platelets, and the lower layer contained granulocytes.

The upper layers of plasma, monocytes, lymphocytes and Histopaque 1077 were aspirated to within 0.5 cm of the granulocyte layer and discarded. Granulocytes were aspirated and transferred to a new 50 ml conical vial. The granulocytes were washed to remove any remaining Histopaque by adding sterile PBS, pH 7.4 (Life Technologies, Grand Island, New York) and centrifuged for 10 minutes at 200 x g. PBS was aspirated and the washing procedure was repeated.

A pellet remained which contained neutrophils and residual erythrocytes. To lyse contaminating erythrocytes, 3 ml of ice-cold ddH_2O was added and the tube gently agitated. After exactly 30 seconds, 1 ml of ice-cold 0.6 M KCl was added to restore tonicity. The cell suspension was centrifuged at 200 x g for 4 minutes at 4°C to form a white pellet containing primarily neutrophils. Approximately 96% of the cells were neutrophils and only 4% were eosinophils and basophils.

Neutrophils were resuspended with 4 ml of Hanks' Balanced Salt Solution (HBSS) containing no Mg^{++} or Ca^{++} (Life Technologies, Grand Island, New York) and placed in a 15 x 100 mm non-adherent Teflon tube (Minisorp, Nunc, Rochester, NY). The Teflon tube was placed on a slant rack attached to an orbital rotator (50800 Rotomix Rotator, Thermolyne, Dubuque, IA) in an incubator gassed with 5% CO_2 while a cell count was

performed (Forma Scientific, Inc., Marietta, Ohio). Neutrophils were resuspended with DMEM/F12, 10% FBS and returned to the incubator until the experiment, approximately 4 hours later. At the time of the experiment, cells were resuspended to a concentration of 1 x 10^6 cells/ml of PBS.

iii. Protocol for Reactive Oxygen Species (ROS)

Hypoxanthine (HX, 0.0003 g, Sigma Chemical Company, St. Louis, Missouri) was added to a 50 ml conical vial containing 36 ml of M199 media (Life Technologies, Grand Island, New York) and sonicated for thirty minutes, followed by the addition of 4 ml FBS (final concentration 0.05 mM). Then 8 μl of xanthine oxidase (XO, activity of 10 units / 0.5 ml, Sigma Chemical Company, St. Louis, Missouri) was added for a final concentration of 0.004 units / ml. A 0.02 M ferric sulfate ($Fe_2(SO_4)_3$, Sigma Chemical Company, St. Louis, Missouri) solution was made by sterile filtering 25 ml of ddH_2O and then adding 0.200 g of $Fe_2(SO_4)_3$. A volume of 5 μl of this stock was added to the HX + XO solution for a final concentration of 2.5 μM. This was our ROS solution.

iv. Experimental Treatments and Reporter Vectors

The following firefly luciferase reporter vectors were used: 1) pGL3-Basic (no promoter – negative control); 2) pGL3-BF^2 (reporter plasmid bearing the NF-κB binding site; 3) pGL3-SV40 (positive control); 4) pGL3-BF^2 mutant. All cells were cotransfected

with Renilla luciferase reporter vector (pRL-TK) as a control for the transfection procedure. The transfected cells were incubated for 16 hours with the following treatments:

1. M199
2. Arachadonic Acid (AA, 50 μM) - a neutrophil activator
3. Neutrophils (5,000)
4. Neutrophils (1,250; 2,500; 5,000; 10,000) + AA
5. Neutrophils (5,000) + AA + SOD (3,000 units/mg) /catalase (5,000 units/ml)
6. Neutrophils (5,000) + AA + TNFα neutralizing antibody (500 μg/ml)
7. ROS (HX, 0.05 mM + XO, 0.002 units/mg + $(Fe_2(SO_4)_3$, 2.5 μM)
8. ROS + SOD/catalase
9. TNFα (1ng/1ml)
10. TNFα + TNFα neutralizing antibody
11. TNFα + SOD/catalase

SOD and catalase were used to quench ROS and TNFα antibody was used to neutralize the effects of TNFα.

C. Results

Neutrophils activated with arachidonic acid increased NF-κB activation in PASM cells in a dose-dependent manner (AA: 130.9 ± 53.5 RLU; 5,000 neutrophils: 43.9 ± 10.8 RLU; AA + 1,250 neutrophils: 72.4 ± 30.8 RLU; AA + 2,500 neutrophils: 115.5 ± 39.0 RLU; AA + 5,000 neutrophils: 238.4 ± 94.5 RLU; AA + 10,000 neutrophils: 610.6 ± 329.1 RLU, $P < 0.05$, Figure 68). The highest doses of neutrophils (10,000 and 5,000) significantly activated more NF-κB as compared to arachidonic acid alone, unactivated neutrophils or the lower doses of activated neutrophils (1,250 and 2,500) ($P < 0.05$, Figure 68). Co-treatment of activated neutrophils (5,000) with SOD/catalase or TNFα neutralizing antibody significantly decreased the ability of neutrophils to activate NF-κB (5,000 neutrophils: 43.9 ± 10.8 RLU; AA + 5,000 neutrophils: 256.4 ± 98.6 RLU; AA + 5,000 neutrophils + SOD/catalase: 41.6 ± 12.3 RLU; AA + 5,000 neutrophils + TNFα neutralizing antibody: 51.7 ± 21.3 RLU; $P < 0.05$, Figure 69). Activated neutrophils did not activate NF-κB when cells were transfected with the BF^2 mutant.

ROS significantly activated NF-κB as compared to control and this was significantly inhibited by co-treatment with SOD/catalase (Control: 161.3 ± 42.9 RLU; ROS: 710.6 ± 265.4 RLU; ROS/SOD/catalase: 177.2 ± 85.21 RLU, $P < 0.05$, Figure 70). TNFα also significantly activated NF-κB as compared to control and this was significantly inhibited by TNFα neutralizing antibody. Cells transfected with the BF^2 mutant did not respond to TNFα (Control: 454.6 ± 146.9 RLU; TNFα: 942.7 ± 200.7 RLU; TNFα +

TNFα neutralizing antibody: 368.4 ± 110.6 RLU; TNFα + BF^2 mutant: 2.7 ± 0.9 RLU, $P < 0.05$, Figure 71).

D. Discussion

This study showed that NF-κB is activated in vascular smooth muscle cells treated with neutrophils activated with arachidonic acid. Activated neutrophils caused activation of NF-κB in a dose-dependent manner from 1,250 to 10,000 neutrophils per well. Each well contained 70,000 to 80,000 PASM cells, so the ratio of neutrophils to PASM cells ranged from approximately 1:60 to 1:8. Given the extensive vascular infiltration of neutrophils we observed in obese and preeclamptic women (Chapters 3 and 4), these ratios appear reasonable for studying the effect of neutrophils on vascular smooth muscle.

To determine which neutrophil products were involved in NF-κB activation, we used SOD/catalase to inhibit ROS action and TNFα neutralizing antibody to inhibit TNFα action. Both SOD/catalase and TNFα neutralizing antibody inhibited NF-κB activation by activated neutrophils, indicating that both ROS and TNFα are mediators for the activation of NF-κB in vascular smooth muscle cells. To specifically determine if ROS or TNFα can activate NF-κB, we treated cells with ROS or TNFα. We found that they both caused significant activation of NF-κB as compared to control. ROS activation was significantly inhibited by SOD/catalase and TNFα activation was significantly inhibited by TNFα neutralizing antibody. The mechanism whereby TNFα activated NF-κB may have been by

increasing intracellular ROS because TNFα stimulates mitochondrial production of ROS [182]. The responses we observed were specific for NF-κB because cells that were transfected with a vector containing site direct mutation of the NF-κB binding site did not respond to treatment.

NF-κB is sensitive to cellular oxidative stress [157, 183] which is present in obese and preeclamptic women [102, 178]. TNFα is also elevated in obese and preeclamptic women [42, 51]. The present study demonstrated that both ROS and TNFα strongly activated NF-κB in vascular smooth muscle cells. A source of ROS and TNFα is activated neutrophils. In view of the fact that our previous studies showed extensive neutrophil infiltration into vascular tissue in obese and preeclamptic women (Chapters 3 and 4), neutrophil release of ROS and TNFα are reasonable causes of the NF-κB activation in vascular tissue that was observed in these women.

Since NF-κB is a transcription factor that regulates many inflammatory genes, in the next chapter we will determine if the neutrophil products that activated NF-κB, ROS and TNFα, will induce expression of inflammation gene products.

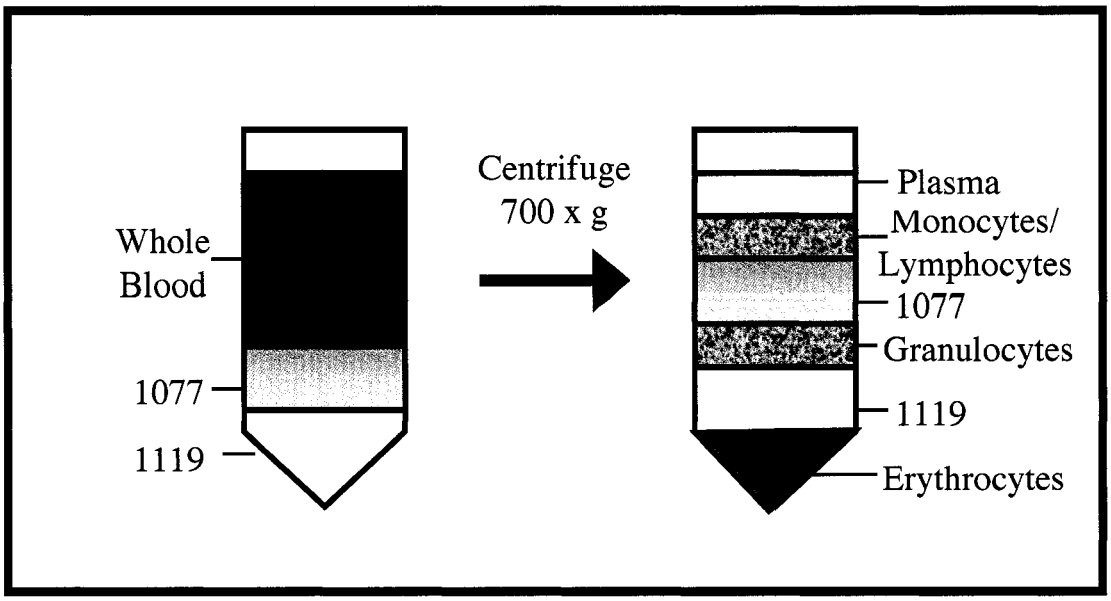


Figure 67. Neutrophil isolation from whole blood by histopaque density gradient separation.

Whole blood was layered upon two densities of histopaque. Centrifugation caused leukocytes to separate into layers by density, so that granulocytes (neutrophils) could be collected for experiments.

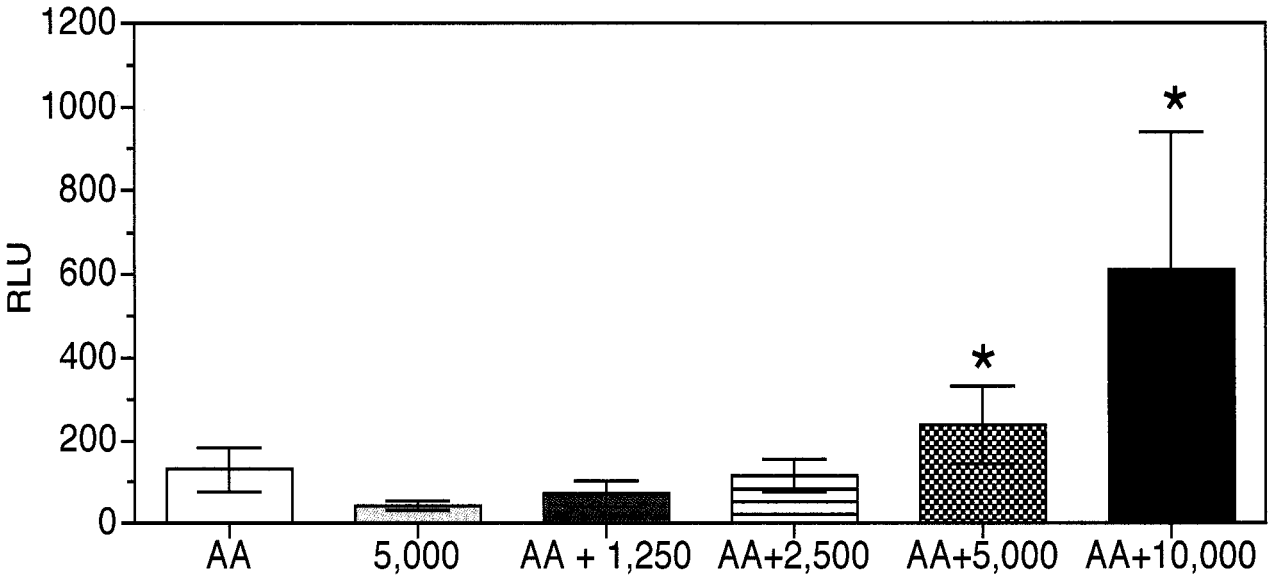


Figure 68. Activation of NF-κB in vascular smooth muscle cells by neutrophils.

Neutrophils activated by arachidonic acid (AA, 50 μM) activated NF-κB in a dose response manner. PASM cells treated with 10,000 and 5,000 activated neutrophils had significantly more NF-κB activation than the control (AA), 5000 neutrophils, AA + 1,250 neutrophils and AA + 2,500 neutrophils. * $P < 0.05$

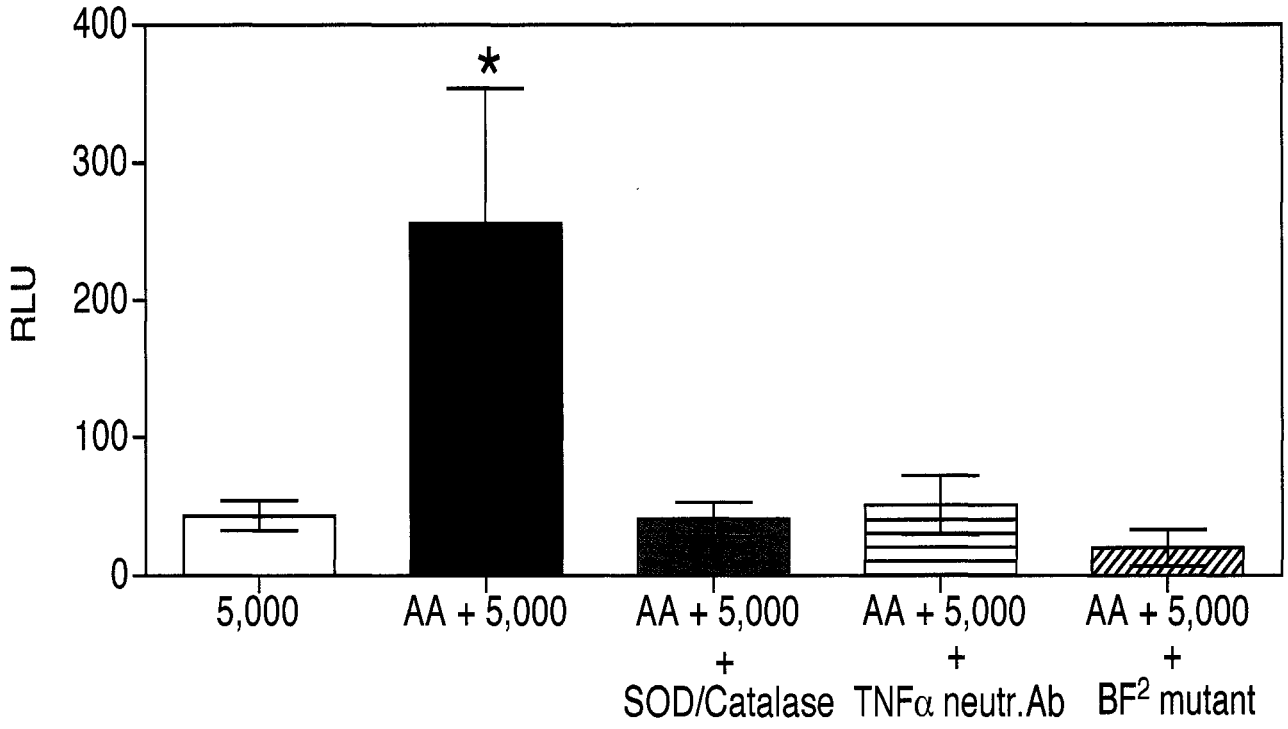


Figure 69. Activation of NF-κB in vascular smooth muscle cells in the presence of 5,000 neutrophils with or without inhibitors (SOD/catalase, TNFα neutralizing antibody) or in cells transfected with a mutant vector.

Activated neutrophils significantly activated NF-κB as compared to unactivated neutrophils (5,000 neutrophils). NF-κB activation was significantly inhibited by co-treatment with SOD/catalase or TNFα neutralizing antibody. Cells transfected with the mutant did not respond to activated neutrophils. * $P < 0.05$

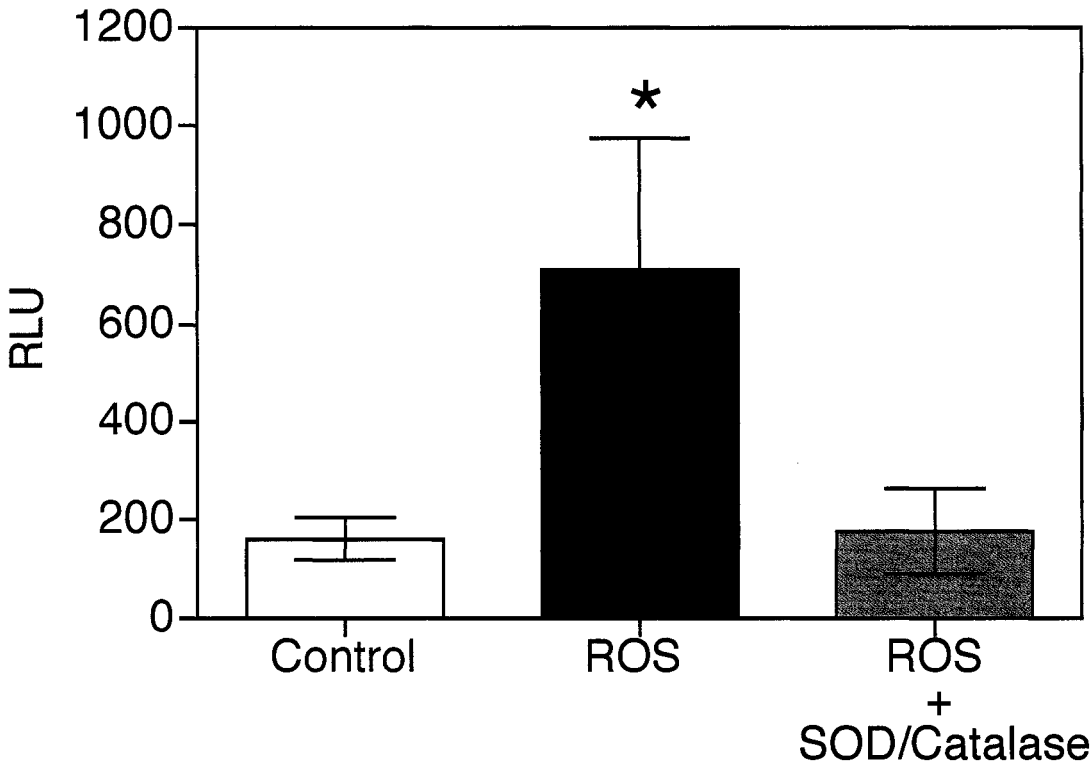


Figure 70. NF-κB activation in vascular smooth muscle cells in the presence of ROS or ROS + SOD/catalase.

ROS significantly activated NF-κB as compared to the BF^2 control and this was significantly inhibited by SOD/catalase. *$P < 0.05$

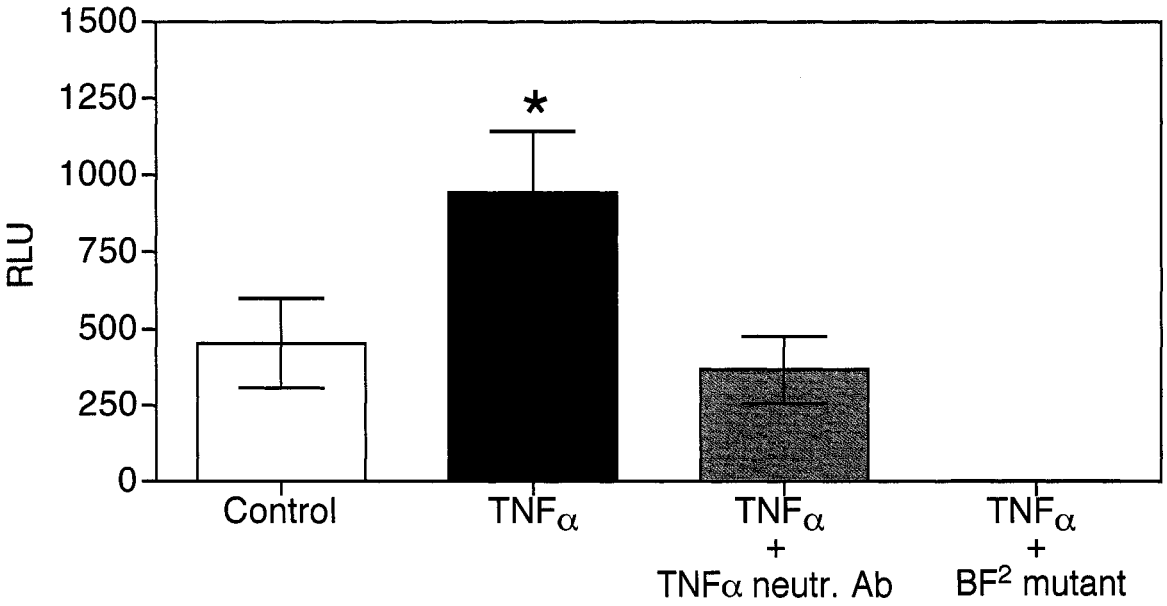


Figure 71. NF-κB activation in vascular smooth muscle cells in the presence of TNFα with or without TNFα neutralizing antibody or in cells transfected with the BF^2 mutant.

TNFα significantly activated NF-κB as compared to the BF^2 control and this was significantly inhibited by TNFα neutralizing antibody. Cells transfected with the BF^2 mutant did not respond to TNFα. * $P < 0.05$

CHAPTER 6

VASCULAR SMOOTH MUSCLE CELL EXPRESSION OF COX-2 AND PRODUCTION OF THROMBOXANE AND IL-8 IN RESPONSE NEUTROPHIL PRODUCTS

D. Introduction

In Chapters 3 and 4 we observed in obese and preeclamptic women significant vascular infiltration of neutrophils in association with vascular inflammation as indicated by activation of NF-κB and expression of COX-2. In Chapter 5, we demonstrated that neutrophils and neutrophil products activated NF-κB in cultured vascular smooth muscle cells, which provided support for the idea that neutrophils were a cause of vascular inflammation in obese and preeclamptic women. In this chapter, we performed a second set of in vitro experiments where we treated vascular smooth muscle cells with neutrophil products, ROS and TNFα, and then measured expression or production of inflammatory gene products regulated by NF-κB. We chose to examine COX-2, because it was expressed in the vasculature of obese and preeclamptic women, it is an enzyme that is regulated by NF-κB, it is induced during inflammation [171], and its products modulate inflammation. We chose to examine thromboxane (TX) because it is a product of COX-2 and it is a potent vasoconstrictor [102, 161]. An increase in TX production could explain

vasoconstriction and hypertension in obese and preeclamptic women. Finally, we chose to examine IL-8 because it is also regulated by NF-κB, it is a potent neutrophil chemokine that activates and attracts neutrophils, and it is expressed in vascular smooth muscle of preeclamptic women [127]. Its expression in vascular smooth muscle provides a chemotactic gradient from the circulation to the vascular smooth muscle to cause neutrophil infiltration. An increase in vascular smooth muscle IL-8 could explain why neutrophils infiltrate the vasculature of obese and preeclamptic women.

B. Materials and Methods

i. Cell Culture

PASM cells were isolated and cultured as previously described (Chapter 2). Briefly, PASM cells were seeded into a T-75 flask and grown to confluence. Cells were then passed into eight T-25 flasks for experimental treatments. It was necessary to use T-25 flasks in order to get enough cells to measure COX-2. Cell viability was confirmed by Trypan blue exclusion staining.

ii. Protocol to generate (ROS)

Hypoxanthine (HX, 0.0005 g, Sigma Chemical Company, St. Louis, Missouri) was added to a 50 ml conical vial containing 36 ml of M199 media (Life Technologies, Grand

Island, New York) and sonicated for thirty minutes, followed by the addition of 4 ml FBS (for a final concentration of 0.10 mM). Then 12 μl of xanthine oxidase (XO, activity of 10 units/0.5 ml) (Sigma Chemical Company, St. Louis, Missouri) was added for a final concentration of 0.006 units/ml. A 0.02 M ferric sulfate ($Fe_2(SO_4)_3$, Sigma Chemical Company, St. Louis, Missouri) solution was made by sterile filtering 25 ml of ddH_2O and then adding 0.200 g of $Fe_2(SO_4)_3$. A volume of 20 μl of this stock was added to the HX + XO solution for a final concentration of 10 μM.

iii. Experimental Design

To test the hypothesis that neutrophil products would cause expression of COX-2 and production of TX and IL-8 in vascular smooth muscle cells, PASM cells were incubated for 18 hours with the following treatments:

1. M199/10% FBS (Control)
2. ROS (HX, 0.10 mM, + XO, 0.006 units/ml,+ ($Fe_2(SO_4)_3$, 10 μM)
3. ROS + SOD (1mg/ml) and catalase (5,000 U/ml)
4. ROS + NS398 (20 μM) – COX-2 inhibitor
5. TNFα (2 ng/ml)
6. TNFα + NS398

C. Results

ROS significantly increased COX-2 expression as compared to control (228.2 ± 19.5 vs. 100 ± 0.0, respectively, $P < 0.01$, Figure 72). Both SOD/catalase and NS398 significantly inhibited COX-2 expression induced by ROS (88.2 ± 26.0 vs. 131.0 ± 24.9, respectively, $P < 0.01$, Figure 72).

ROS significantly increased production of TX as compared to control (1.9 ± 0.1 vs. 0.8 ± 0.1, respectively, $P < 0.05$, Figure 73). Both SOD/catalase and NS398 significantly inhibited production of TX induced by ROS (0.9 ± 0.2 vs. 1.2 ± 0.3, respectively, $P < 0.05$, Figure 73).

PASM cells treated with ROS produced significantly more IL-8 than control (7.4 ± 1.8 vs. 2.5 ± 0.6, respectively, $P < 0.05$, Figure 74). SOD/catalase and NS398 significantly inhibited production of IL-8 induced by ROS (3.6 ± 0.7 vs. 3.3 ± 0.6, respectively, $P < 0.05$, Figure 74).

Similar to ROS, TNFα significantly increased COX-2 expression as compared to control (320.8 ± 33.0 vs. 100 ± 0.0, respectively, $P < 0.001$, Figure 75). NS398 significantly inhibited COX-2 expression induced by TNFα (190.2 ± 59.2, $P < 0.001$, Figure 75).

TNFα did not significantly increase production of TX as compared to control (2.8 ± 0.7 vs. 1.7 ± 0.3, respectively, $P > 0.05$, Figure 76). NS398 prevented any increase in TX induced by TNFα (1.5 ± 0.2, $P > 0.05$, Figure 76).

PASM cells treated with TNFα produced significantly more IL-8 than control (6.6 ± 2.7 vs. 0.3 ± 0.1, respectively, $P < 0.05$, Figure 77). NS398 significantly inhibited production of IL-8 induced by TNFα (0.6 ± 0.1, $P < 0.05$, Figure 77).

D. Discussion

In the previous chapter, we demonstrated that activated neutrophils or products of activated neutrophils, ROS or TNFα, caused activation of NF-κB in vascular smooth muscle cells. Since NF-κB is a transcription factor that regulates many inflammatory genes, in this chapter we determined whether treatment of vascular smooth muscle cells with ROS or TNFα caused increases in inflammatory gene products. We showed that COX-2 expression was induced and TX and IL-8 were produced when vascular smooth muscle cells were treated with ROS or TNFα. Treatment with either SOD/catalase or NS398 inhibited COX-2 expression and production of TX and IL-8 induced by ROS. NS398 also inhibited expression of COX-2 and production of IL-8 induced by TNFα. Previous studies have suggested that IL-8 is regulated by AA metabolites, possibly by thromboxane, under conditions of oxidative stress [184, 185]. Inhibition of thromboxane and IL-8 in our study using a COX-2 inhibitor support these previous studies.

In this study, we showed that products of activated neutrophils significantly increased the expression of COX-2 and production of TX and IL-8 in vascular smooth muscle cells. Increased vascular smooth muscle expression of IL-8 would provide a chemotactic gradient from the circulation to the vascular smooth muscle for neutrophil

infiltration. These data support the idea that the increased expression of COX-2 in systemic vasculature that we observed in obese and preeclamptic women may be due to release of ROS and TNFα by activated neutrophils that have infiltrated the vasculature. They also suggest that increased vasoconstriction leading to hypertension may result as a consequence of COX-2 expression leading to an increase in thromboxane production by the vascular smooth muscle. These results further imply that neutrophils could be a cause of vascular inflammation, and thus, a primary link between obesity and preeclampsia. The vasculature of obese women is already in an inflamed state and susceptible to vasoconstriction, so the additional oxidative stress imposed by the placenta with its ability to further activate neutrophils could worsen the inflamed and vasoconstrictive state of the vasculature resulting in preeclampsia.

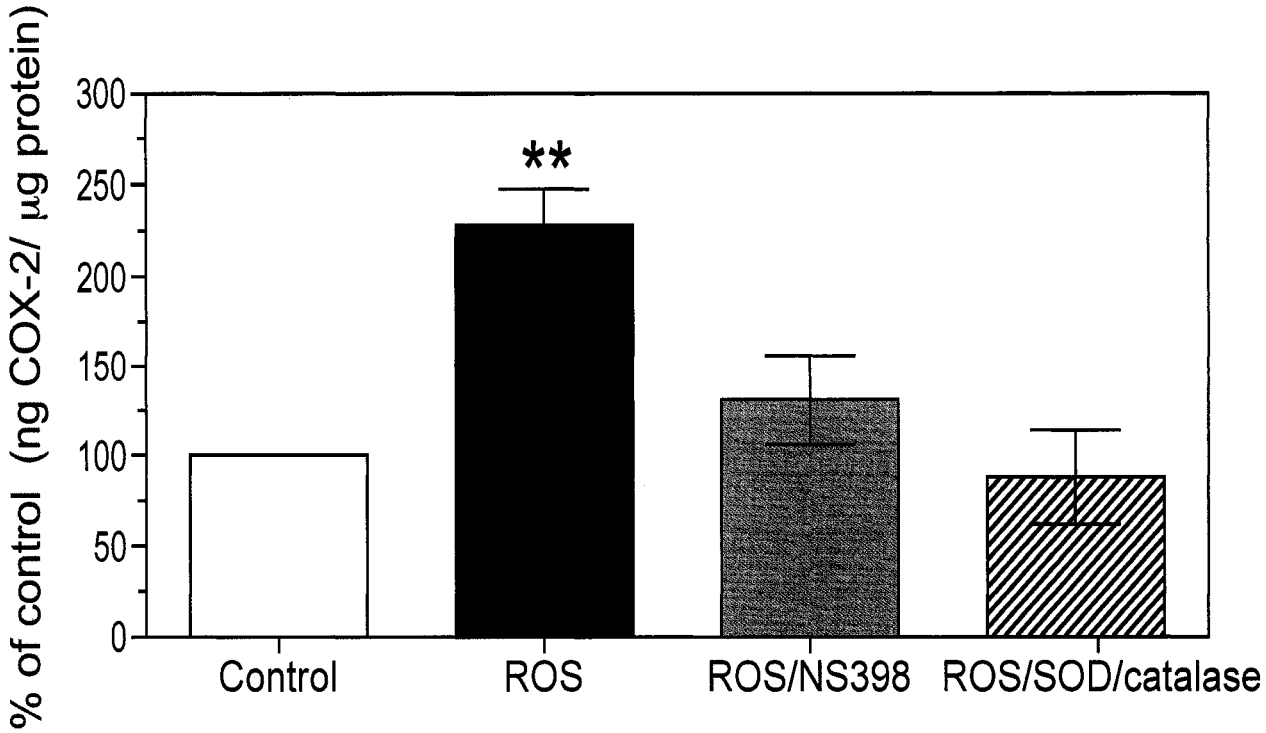


Figure 72. Induction of COX-2 expression in PASM cells exposed to ROS, ROS + NS398 or ROS + SOD/catalase.

ROS significantly stimulated expression of COX-2 as compared to control. NS398 or SOD/catalase significantly inhibited ROS stimulation of COX-2. Data are plotted as % of control because of variability between cell lines for COX-2 expression.
**$P < 0.01$ ($n = 5$)

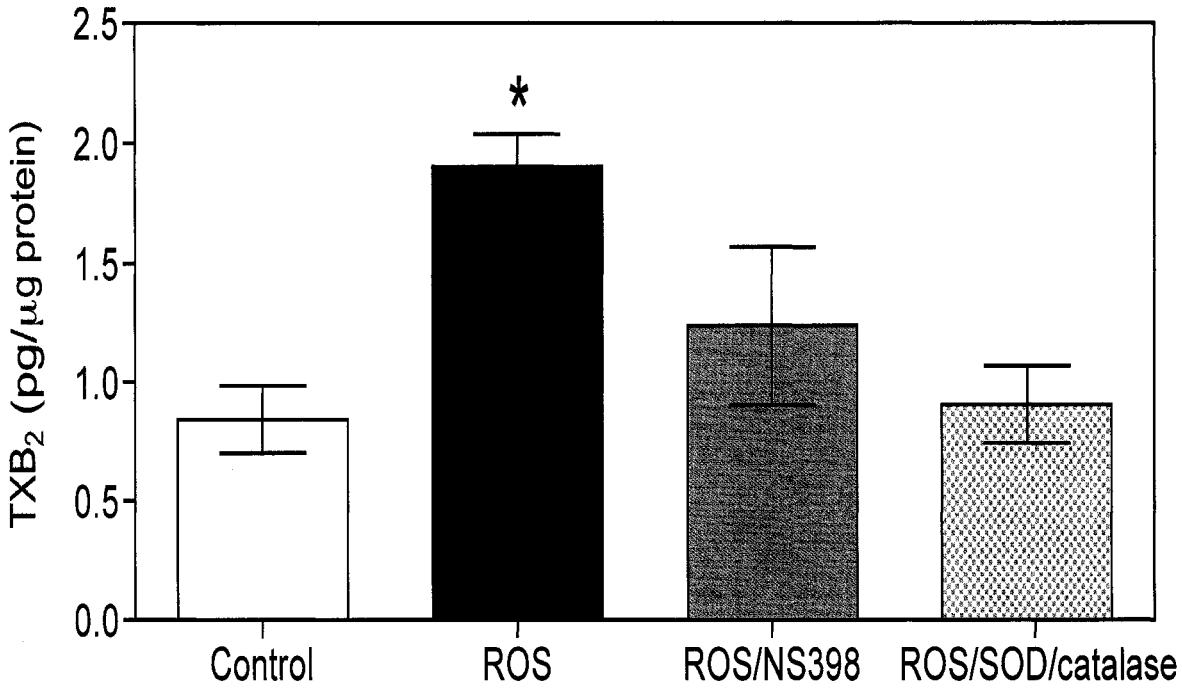


Figure 73. Production of thromboxane by PASM cells exposed to ROS, ROS + NS398, or ROS + SOD/catalase.

ROS significantly stimulated production of TX. NS398 or SOD/catalase significantly inhibited ROS stimulation of TX. * $P < 0.05$ ($n = 6$).

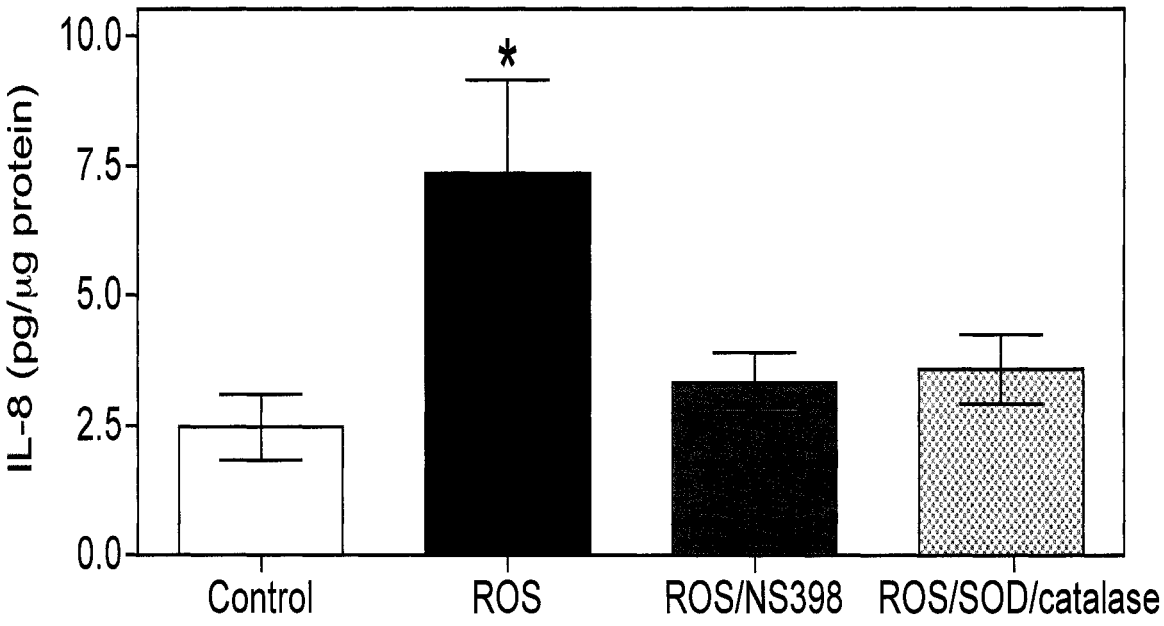


Figure 74. Production of IL-8 by PASM cells exposed to ROS, ROS + NS398 or ROS + SOD/catalase.

ROS stimulated production of IL-8. NS398 or SOD/catalase significantly inhibited ROS stimulation of IL-8. * $P < 0.05$ ($n = 7$).

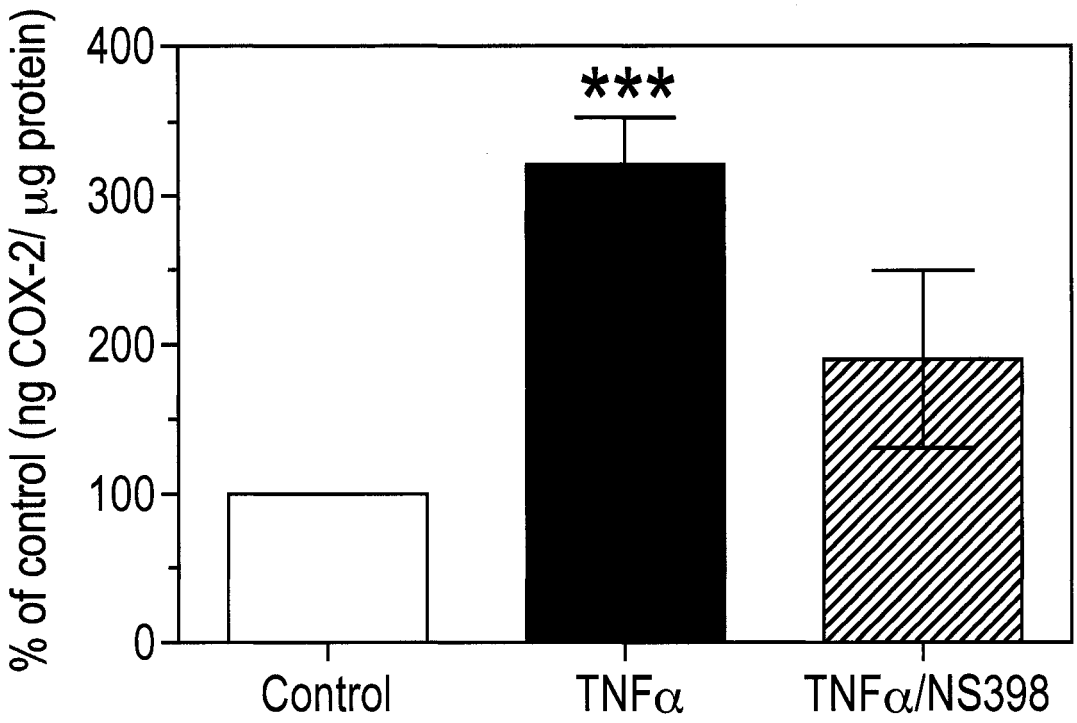


Figure 75. Induction of COX-2 expression in PASM cells exposed to TNFα or TNFα + NS398.

TNFα significantly stimulated expression of COX-2 as compared to control. NS398 significantly inhibited TNFα stimulation of COX-2. Data are plotted as % of control because of variability between cell lines for COX-2 expression. ***P < 0.001 (n=6)

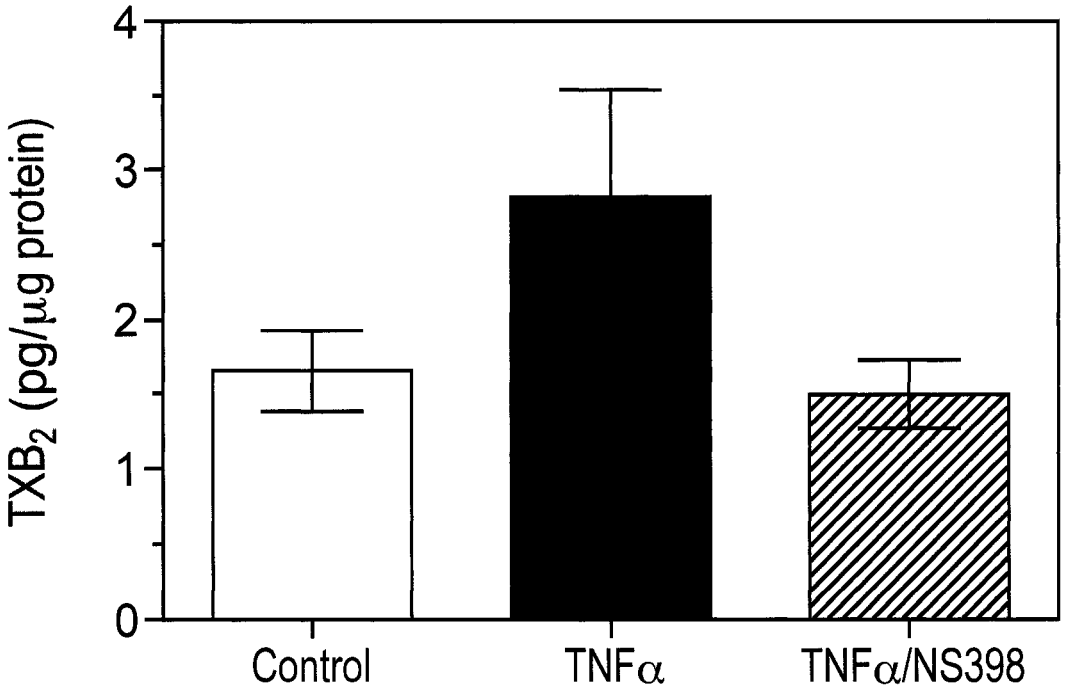


Figure 76. Production of thromboxane by PASM cells exposed to TNFα or TNFα + NS398.

TNFα did not significantly stimulate production of thromboxane. $P > 0.05$ ($n = 6$).

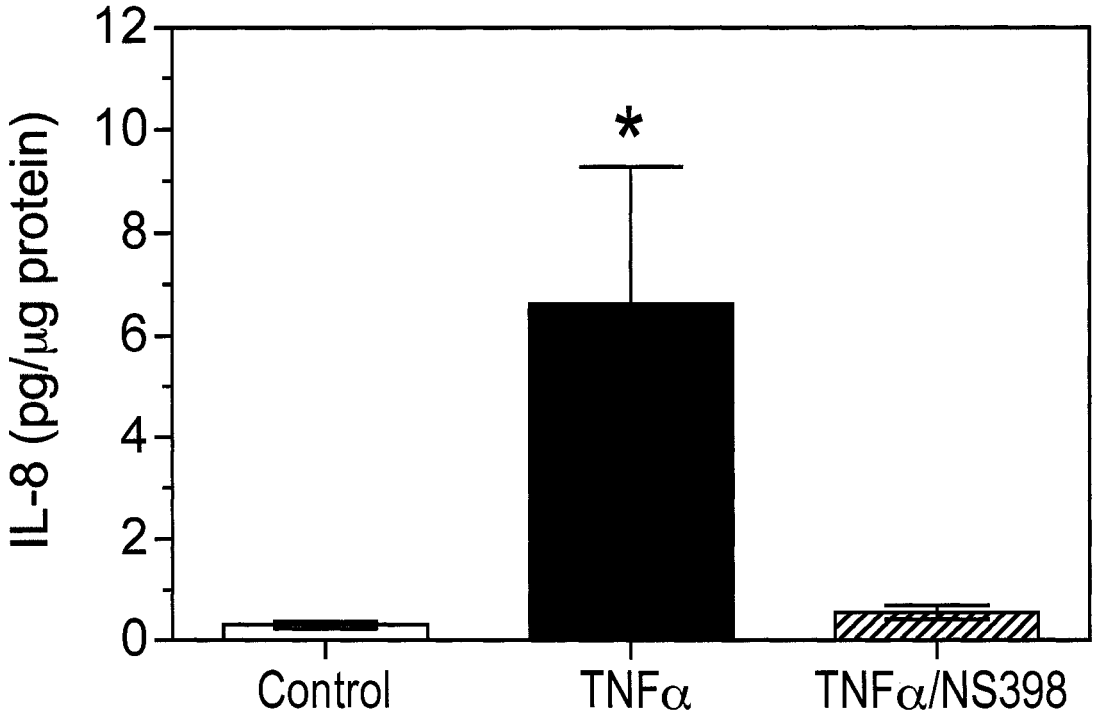


Figure 77. Production of IL-8 by PASM cells exposed to TNFα or TNFα + NS398.

TNFα stimulated production of IL-8. NS398 significantly inhibited TNFα stimulation of IL-8. *$P < 0.05$ ($n = 7$)

CHAPTER 7

GENERAL DISCUSSION

This investigation is the first to demonstrate a mechanism for how obesity is a risk factor for preeclampsia by implicating a role for inflammation and immune dysfunction. We began with the concept that obesity is already associated with a certain amount of oxidative stress, which causes neutrophil activation. We proposed that when obese women become pregnant the additional oxidative stress imposed by the placenta would further activate neutrophils resulting in widespread endothelial and vascular smooth muscle cell dysfunction as activated neutrophils infiltrated the vasculature.

We began our investigation by immunohistochemical staining of vessels from the systemic vasculature of obese and preeclamptic women for CD66b, a neutrophil marker, and NF-κB and COX-2, markers of inflammation. No previous studies had demonstrated a link between neutrophil infiltration and vascular inflammation in obesity or preeclampsia. We found that the vascular phenotype of obese and preeclamptic women was similar in that they both showed activation of NF-κB and expression of COX-2 in association with neutrophil infiltration. Inflammation was evident in the vascular smooth muscle, as well as the endothelium, and neutrophils were not only flattened and adhered on the endothelium, but also infiltrated into the intimal space.

To evaluate if obesity was a risk factor for a hypertensive disorder, such as preeclampsia, we determined if neutrophil infiltration and vascular inflammation were correlated with BMI and if blood pressure correlated with neutrophil infiltration and vascular inflammation. We found that neutrophil infiltration and vascular inflammation were significantly correlated with BMI, and that diastolic blood pressure was significantly correlated with neutrophil infiltration and vascular inflammation. This suggests that neutrophil infiltration could cause hypertension and be a link as to why obesity is a risk factor for preeclampsia.

We then asked the question if neutrophils or neutrophil products, such as ROS and TNFα, could induce vascular smooth muscle cell activation of NF-κB and cause vascular inflammation. To evaluate this, we co-cultured neutrophils with vascular smooth muscle cells that were transfected with a reporter vector for NF-κB, and then treated them with neutrophils or neutrophil products. We found that activated neutrophils and neutrophil products, ROS and TNFα, caused activation of NF-κB in vascular smooth muscle cells. Neutrophil activation of NF-κB was inhibited by SOD/catalase or TNFα neutralizing antibody, indicating that ROS or TNFα mediated the actions of the neutrophils. This indicated that infiltration of neutrophils with release of ROS and TNFα could be responsible for the vascular activation of NF-κB in obese and preeclamptic women.

In a second set of in vitro experiments, we treated vascular smooth muscle cells with neutrophil products, ROS or TNFα, and measured expression of COX-2 and production of TX and IL-8. TX is a product of COX-2 and a potent vasoconstrictor, whereas IL-8 is a potent chemotactic agent for neutrophils. Therefore, TX production by

vascular smooth muscle cells could result in vasoconstriction leading to hypertension and IL-8 production would exacerbate the infiltration of neutrophils. We found that ROS significantly stimulated vascular smooth muscle cell expression of COX-2 and production of TX and IL-8. NS398, a COX-2 inhibitor and SOD/catalase significantly inhibited ROS stimulation of COX-2, TX and IL-8. TNFα also significantly stimulated vascular smooth muscle cell expression of COX-2 and production of IL-8. NS398 significantly inhibited TNFα stimulation of COX-2 and IL-8. These data suggested that neutrophil products could be responsible for vasoconstriction and vascular smooth muscle cell inflammation of obese and preeclamptic women.

There were some limitations in our studies. One was that collection of fat biopsy samples from nonpregnant women was most often from women undergoing hysterectomy for uterine fibroids. Therfore, the women in the obesity study were older than the pregnant women of the preeclampsia study. Older, obese women may have more neutrophil infiltration and vascular inflammation than younger, obese women. Future studies will collect fat biopsy samples from younger, obese women to eliminate any age-related problems. Another limitation is that only one of our obese patients had hypertension. Although we observed significant correlations for neutrophil infiltration and vascular inflammation with diastolic blood pressure, the correlations did not reach statistical significance for systolic blood pressure. Future studies will increase our sample size for obese, hypertensive women which should increase our chance to see a statistically significant correlation for systolic blood pressure.

Our findings for obese women may explain why obesity predisposes to cardiovascular disease later in life. Compliant vasculature of a young, obese woman may protect her from hypertension, but as she ages and her vasculature becomes less compliant, the physical presence of increased numbers of neutrophils adhered and flattened onto endothelium could increase her total peripheral resistance by production of TX and ROS to cause hypertension. Development of preeclampsia in obese, younger women may be unmasking a risk for hypertension later in life since obese women show early signs of a pathological process that will likely exacerbate over time.

Investigators have found circulating levels of IL-8 to be increased in obese women [186] and previous work by our laboratory demonstrated increased vascular expression of ICAM-1 and increased vascular smooth muscle expression of IL-8 in women with preeclampsia [127]. NF-κB activation plays an important role in the upregulation of ICAM-1 and IL-8, as well as COX-2, by binding to the promoter regions of their respective genes [39, 156]. Lipid peroxides, ROS and TNFα are all elevated in the maternal circulation of preeclamptic women, as well as obese women [178, 187], and all are known to activate NF-κB in a variety of cell types [155, 156] including in endothelial cells [158]. In the present study, we showed that ROS and TNFα also activate NF-κB in vascular smooth muscle cells. NF-κB activation could, therefore, be a key factor in both obesity and preeclampsia.

Activation of NF-κB and expression of COX-2 in the vascular smooth muscle of obese women may result from ROS generated by neutrophils as they infiltrate into the intimal space. Oxidative stress as a result of hyperlipidemia may be responsible for initial activation of neutrophils and endothelium, but as the neutrophils flatten and adhere onto

endothelium they would cause endothelial dysfunction, and as they infiltrate, the oxidative stress would be transmitted to the vascular smooth muscle causing vascular smooth muscle dysfunction. This may explain why women who have conditions associated with hyperlipidemia, such as obesity, diabetes, insulin insensitivity, cardiovascular disease and polycystic ovarian disease have a higher risk for developing preeclampsia [69, 70, 188, 189]. Women who develop preeclampsia also have a higher fatty acid consumption (primarily palmitic, stearic and linoleic acids) compared to women with uncomplicated pregnancies [190].

The work presented in this thesis offers several options for potential preventative and therapeutic treatment of preeclampsia. One therapeutic target is inhibition of COX with traditional NSAIDs, such as aspirin, which would reduce vascular inflammation and possibly inhibit neutrophil transendothelial migration. Many clinical studies have shown that treatment with low-dose aspirin in women at high risk for preeclampsia reduced the incidence of preeclampsia [191, 192]. However, traditional NSAIDs are associated with gastrointestinal side effects and specific COX-2 inhibitors could provide a resolution to inflammation without causing these adverse gastrointestinal problems.

Recently concern has been raised that selective COX-2 inhibitors may increase cardiovascular events largely because the Vioxx Gastrointestinal Outcomes Research (VIGOR) trial showed an increase in myocardial infarction rate in rheumatoid arthritis patients treated long term with rofecoxib versus naproxen [193]. Neither aspirin nor placebo control were included in this study. Another study reported an increase risk of cardiovascular events with rofecoxib after 18 months of treatment of patients with

colorectal adenoma, however the increased risk was primarily due to a decrease in events in the placebo group rather than an increase in the rofecoxib group [194]. Not all trials show an increase in cardiovascular events with COX-2 inhibitors. Meta-analysis of 23 trials in which more than 28,000 patients were treated with rofecoxib showed no evidence for an increase in cardiovascular events [195]. Several other recent trials with selective COX-2 inhibitors also found no increase in cardiovascular events [174, 196-200].

Potential treatment of preeclamptic women with COX-2 inhibitors would be considerably different than current trials which have been long-term treatment in older subjects with chronic inflammatory conditions. Potential treatment of preeclamptic subjects would be in young, otherwise healthy women, for only 1-3 months. Potential benefit of COX-2 treatment for preeclampsia is suggested by studies showing that selective COX-2 inhibition improves endothelium-dependent vasodilation and reduces low-grade chronic inflammation and oxidative stress [201, 202]

Another therapeutic target is inhibition of NF-κB. NF-κB inhibitors are starting to become available. Multiple steps in the NF-κB activation cascade have been marked as targets for specific inhibition [203]. One other form of NF-κB inhibition is using aspirin and sodium salicylate [39].

The findings can also serve as a rationale for dietary modifications or therapeutic target. Dietary modifications include increasing the amount of antioxidants prior to and throughout pregnancy, which would offset or decrease oxidative stress and inflammation. A combination of antioxidant therapy and aspirin may be beneficial in treating preeclampsia. Both aspirin[204] and antioxidants [157, 183, 205] inhibit NF-κB activation. Aspirin

appears to inhibit NF-κB activation through a mechanism independent of its inhibition of COX [206]. Antioxidants are thought to block NF-κB by eliminating ROS that serve as intracellular activation signals [153]. Chappell et al found in a small study that antioxidant supplementation in women who were at risk of preeclampsia significantly decreased the occurrence of preeclampsia[207]. However, a recent much larger study found that supplementation with vitamin C and vitamin E did not prevent preeclampsia [208]. The doses of the antioxidants might have played a role in the outcome. Very high doses of vitamins C and E were used which may have increased, rather than decreased oxidative stress. The reason for this is women with preeclampsia have increased levels of free iron in their serum [209, 210] and vitamin C is a strong prooxidant in the presence of transitional metals [98].

In conclusion, this investigation demonstrated that infiltration of neutrophils into systemic vasculature is associated with NF-κB activation and COX-2 expression in obese women and women with preeclampsia. These data suggest that endothelial cells and vascular smooth muscle cells may be modified by neutrophil infiltration to favor activation of NF-κB and induction of COX-2 expression. These new data implicate both obesity and preeclampsia as inflammatory conditions associated with immune dysfunction. These findings could explain clinical symptoms of preeclampsia and explain why obese women are at increased risk of developing preeclampsia.

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