Published in final edited form as:

Reprod Sci. 2010 February ; 17(2): 116–124. doi:10.1177/1933719109348252.

Neutrophil Infiltration and Systemic Vascular Inflammation in Obese Women

Tanvi J. Shah, Ph.D., Courtney E. Leik, Ph.D., and Scott W. Walsh, Ph.D.

Departments of Obstetrics and Gynecology, and Physiology and Biophysics Virginia Commonwealth University Richmond, VA

Abstract

Obesity has become epidemic worldwide and is especially pronounced in women of reproductive age which is important because obesity is a major risk factor for preeclampsia and chronic hypertension. We hypothesized that vascular inflammation is critical to the pathophysiology of hypertension in obese individuals because obesity and hypertensive disorders share common features related to inflammation. To study this, we collected subcutaneous fat biopsies from normal weight, overweight and obese women and stained the tissues for CD66b, a neutrophil marker, and for activated nuclear factor- κ B (NF- κ B) and cyclooxygenase-2 (COX-2) as markers of inflammation. We found that the number of neutrophils per vessel and the percentage and intensity of vessel staining for CD66b, NF- κ B and COX-2 were greatest in obese women and least in normal weight women, and that neutrophil infiltration and vascular inflammation significantly correlated with BMI and blood pressure. These data may help explain the relationship between obesity and hypertensive disorders.

Keywords

obesity; neutrophils; inflammation; hypertension; cyclooxygenase-2; nuclear factor-kappa B

INTRODUCTION

The U.S. Centers for Disease Control and Prevention ranks obesity as the number one health threat facing America. Obesity has become epidemic and is especially pronounced in women of reproductive age. In the USA, 28% of women aged 20–39 years old are obese and 54% are either obese or overweight¹. Obesity is a key risk factor for the development of hypertension^{2–4}. The obesity epidemic among women of reproductive age is especially important because maternal overweight is a major risk factor for preeclampsia^{5, 6}.

Although obesity is a known risk factor for hypertension, the reason is not known. We considered the idea that vascular inflammation is critical to the pathophysiology of hypertension in obese individuals because obesity and hypertensive disorders share common features related to inflammation. Obesity is associated with oxidative stress^{7, 8}, as well as with circulating markers of inflammation^{9–11}. Chronic hypertension and preeclampsia are also associated with oxidative stress^{12–15} and circulating markers of inflammation^{16–19}. Obese and hypertensive individuals also share common features of dyslipidemia, hyperinsulinemia, insulin resistance and impaired endothelial function^{3, 20–22}. All of these features are related

Correspondence: Scott W. Walsh, Ph.D. Virginia Commonwealth University Medical Center Department of Obstetrics and Gynecology P.O. Box 980034 Richmond, VA 23298-0034 (804) 828-8468 - Phone (804) 828-0573 - Fax swwalsh@vcu.edu.

Presented at the 53rd Annual Meeting of the Society for Gynecologic Investigation, March 22–25, 2006, Toronto, Canada

to inflammation and altered vascular function which is consistent with the premise that obesity is a risk factor for hypertension because of vascular inflammation.

To study the relationship between obesity and vascular inflammation, we collected subcutaneous fat biopsies from normal weight, overweight and obese women and used immunohistochemistry to evaluate the state of vascular inflammation. We examined neutrophil infiltration, and, because neutrophils release toxic compounds that can cause inflammation, we also determined vascular activation of NF- κ B and expression of COX-2 as markers of inflammation. Data were analyzed according to weight categories and correlated with body mass index (BMI) and blood pressure.

MATERIALS and METHODS

Study Subjects

Subcutaneous fat biopsies were collected from non-pregnant women at MCV Hospitals, Virginia Commonwealth University Medical Center. Subcutaneous fat biopsies were used because subcutaneous fat is a highly vascularized tissue representative of the systemic vasculature. Fat biopsies (approximately 2 cm $\times$ 2 cm $\times$ 2 cm) were collected at the time of abdominal surgery at the incision site from women undergoing removal of fibroids or tissue biopsies. Patients were categorized by weight into the following groups: normal weight (BMI 18.5 – 24.9, n = 5), overweight (BMI 25 – 29.9, n = 7) and obese (BMI $\geq$ 30, n = 10). Blood pressure was recorded for each subject at pre-operation admittance by surgery registered nurses using a Drager Medical Infinity Delta Sphygmomanometer placed on the left arm with the subject supine and the sphygmomanometer at heart level. Cuff size varied depending on the size of the patient's arm. The first phase was used for systolic blood pressure and the fifth phase for diastolic blood pressure. Exclusion criteria included infection, active STDs, diabetes and inflammatory disease because these could impact vascular inflammation. Fat biopsies were placed immediately in 10% neutral buffered formalin. Informed consent was obtained prior to surgery. The Office of Research Subjects Protection of Virginia Commonwealth University approved this study.

Immunohistochemistry

Tissue was formalin-fixed, paraffin embedded and cut into 10 μ m sections. Tissues were stained for CD66b, NF- κ B and COX-2. CD66b is a granulocyte-specific membrane antigen that is upregulated and secreted upon granulocyte activation. CD66b staining primarily represents neutrophils because they comprise 96% of the granulocyte population. The NF- κ B antibody was directed against epitopes on the free p65 subunit, so our staining results reflect activated NF- κ B.

On the day of staining, tissue sections were incubated in 3% H₂O₂ in methanol for thirty minutes to quench endogenous tissue peroxidase. Antigen retrieval was performed by placing the slides in 10 mM citrate buffer heated in a pressure cooker at low setting for 5 minutes. Tissues were stained with specific antibodies at room temperature using a Zymed SuperPicture Kit (Zymed Laboratories, San Francisco, CA) and DAB reagent which results in a brown stain. Slides were counterstained with alcian blue and methyl green. Tissues were stained with: 1) a mouse IgM anti-human monoclonal antibody specific for CD66b (1:50, BD BioSciences, San Diego, CA); 2) a rabbit anti-human polyclonal antibody specific for the p65 subunit of NF- κ B (1:200, Zymed Invitrogen, San Francisco, CA); and 3) a mouse IgG anti-human monoclonal antibody specific for COX-2 (1:400, Zymed Invitrogen, San Francisco, CA). Negative controls for CD66b were stained with a mouse IgM monoclonal isotype control (1:50, BD BioSciences, San Diego, CA) and negative controls for NF- κ B and COX-2 were stained with a mouse IgG monoclonal isotype control (pre-diluted, Zymed Invitrogen, San Francisco, CA). Neutrophils

isolated from whole blood by Histopaque density centrifugation served as a positive control for CD66b. Lymphoma tissue was used as the NF- κ B positive control and colon carcinoma tissue was used as the COX-2 positive control as recommended by Zymed Invitrogen.

Data Analysis

We analyzed vessels between 10 μ m and 200 μ m for % of vessels with staining, optical density of staining and number of neutrophils per vessel. All vessels in the tissue section were analyzed at 400X and the average values for each patient were used for statistical analysis. Lumen diameter and optical density of staining were measured for each vessel using image analysis software (IP Lab, Scanalytics, Inc., Fairfax, VA).

Statistical Analysis

Patient demographic data, density of staining, and % of vessels stained were analyzed for each antigen according to weight class by one-way ANOVA with Newman-Keuls post-hoc test or Kruskal-Wallis with Dunn's Multiple Comparison Test if data were not normally distributed or variances were not equal. Regression analysis was performed to correlate vessel staining with BMI or blood pressure, and to correlate NF- κ B and COX-2 staining with CD66b staining. Sample size to evaluate a significant difference between antigen staining of vessels in obese versus normal weight groups with an alpha error of 0.05 (two-tailed test) and a power of 0.8 was estimated to be between 5 – 8 based on the variation and difference in means for CD66b and NF- κ B in preliminary studies. Bar graph data are reported as mean $\pm$ SE. A statistical software application was used (Prism 4 for Macintosh, GraphPad Software, Inc., San Diego, CA).

RESULTS

Patient demographic data are given in Table 1. There was no significant difference for patient age. Obese and overweight patients had significantly higher diastolic blood pressures than normal weight patients. Differences in systolic blood pressures did not reach statistical significance, perhaps due to use of hypertensive medications by some of the overweight and obese subjects. Sixteen patients had normal diastolic pressures that ranged from 54 to less than 85 mmHg, 2 patients had pre-hypertensive pressures of 85 – 89 mmHg and four had hypertensive diastolic pressures of 90 mmHg or greater. One overweight and four obese patients had chronic hypertension, three of which were taking hypertensive medications. Four of the hypertensive patients were black and one was white.

An average of 36 vessels were evaluated for each patient. The average result for each patient was used for statistical analysis. Figure 1 shows the data for CD66b, NF- κ B and COX-2 for % vessels stained and optical density according to weight categories. Obese patients had significantly more staining for all three antigens than normal weight patients. Overweight patients had significantly more staining for CD66b and NF- κ B than normal weight patients. Percent vessel staining was significantly correlated with BMI for all three antigens (Fig. 2), as was optical density (CD66b, $r = 0.53$, $P < 0.05$; NF- κ B, $r = 0.68$, $P < 0.001$; COX-2, $r = 0.59$, $P < 0.01$). The % vessels stained for NF- κ B and COX-2 were significantly correlated with the % vessels stained for CD66b (Fig. 3). Vessel staining was not correlated with the age of the patient.

The number of neutrophils per vessel was associated with the weight of the patient (Fig. 4). Obese patients had significantly more neutrophils per vessel stained for CD66b than normal weight patients (6.5 ± 0.7 vs. 2.1 ± 0.7 , respectively, $P < 0.05$). The number of neutrophils/vessel was significantly correlated with BMI ($r = 0.48$, $P < 0.05$) and with diastolic blood pressure ($r = 0.53$, $P < 0.05$). NF- κ B and COX-2 staining also identified leukocytes, including neutrophils,

within the vessels. Most of the stained leukocytes were neutrophils, based on CD66b staining, but some could also be lymphocytes or monocytes. The number of NF- κ B stained leukocytes per vessel was significantly greater for obese and overweight patients than for normal weight patients. The number of leukocytes per vessel identified by NF- κ B and COX-2 were significantly correlated with BMI ($r = 0.70$ and $r = 0.60$, $P < 0.001$ and $P < 0.01$, respectively), as well as diastolic blood pressure ($r = 0.46$ and $r = 0.49$, $P < 0.05$, respectively).

Figure 5 shows the correlation of diastolic blood pressure with BMI and vessel staining for CD66b, NF- κ B and COX-2. Diastolic blood pressure was significantly correlated with BMI and vessel staining for all three antigens. As diastolic blood pressure increased, the percentage of vessels stained also increased. Systolic blood pressure was significantly correlated with vessel staining for CD66b ($r = 0.58$, $P < 0.01$) and activation of NF- κ B ($r = 0.54$, $P < 0.01$).

Figure 6 shows representative examples of vessel staining for CD66b (Row 1), NF- κ B (Row 2) and COX-2 (Row 3) for normal weight (Column 1), overweight (Column 2) and obese (Column 3) women. IgG negative controls for each antigen showed no brown staining (data not shown). Normal weight women had few vessels that stained for CD66b, NF- κ B or COX-2. Obese women had the most vessel staining with overweight women intermediate. Neutrophils as marked by CD66b were extensively present in the lumen, flattened and adhered along the endothelium and infiltrated into the intimal space in obese women. Staining for NF- κ B and for COX-2 was present in the endothelium and vascular smooth muscle and was the most intense in the obese patients. Leukocytes present in the vessels also stained for NF- κ B and, to a lesser extent, for COX-2.

DISCUSSION

In this study we evaluated systemic blood vessels in subcutaneous fat of normal weight, overweight and obese women for neutrophil infiltration and vascular inflammation. We found that neutrophil infiltration and vascular inflammation progressively increased with the weight group of the patients. Obese women had more staining than overweight women and overweight women more staining than normal weight women. When stratified according to BMI, the percentage of vessels with neutrophil infiltration and vascular inflammation was significantly correlated with the patient's BMI.

Obese women had the greatest amount of neutrophil involvement with the vessels. Neutrophils were observed to be flattened and adhered to the endothelium and infiltrated to the vascular smooth muscle. Overweight women showed similar types of neutrophil involvement, but not to the same degree. Obese women also showed the greatest amount of vascular inflammation as indicated by activation of NF- κ B and increased expression of COX-2. Activated NF- κ B and increased expression of COX-2 were present in the endothelium, as well as in the vascular smooth muscle, of obese women. Staining for NF- κ B and COX-2 were highly correlated with staining for CD66b suggesting that vascular inflammation was due to the infiltration of neutrophils because neutrophils release inflammatory substances. This is further supported in that vascular inflammation was positively correlated with the number of neutrophils per vessel. Number of neutrophils per vessel was also significantly related to weight category and correlated with BMI.

To assess the risk for hypertension, we determined whether blood pressure was correlated with vessel staining. We found that diastolic and systolic blood pressures were significantly correlated with neutrophil infiltration and vascular inflammation. Neutrophils produce several inflammatory mediators, such as TNF α , as well as substances that could cause vasoconstriction or enhance vessel reactivity to vasoconstrictors. One of these substances is reactive oxygen species (ROS). We recently reported that ROS markedly enhanced vessel reactivity to

angiotensin II via activation of the rhoA kinase (ROK) pathway²³. ROK has been shown to play a critical role in hypertension^{24, 25}, so neutrophil infiltration into the vasculature and release of ROS could result in increased blood pressure. Neutrophils might also cause vasoconstriction or enhance vessel reactivity by release of thromboxane or by inactivating nitric oxide, either by release of myeloperoxidase which consumes nitric oxide²⁶ or release of superoxide which combines with nitric oxide to form peroxynitrite.

The neutrophil infiltration and vascular inflammation observed in obese women in this study was very similar to that previously observed in women with preeclampsia^{27, 28}. The similar vascular phenotypes may explain why obesity is a risk factor for preeclampsia.

The present study is the first to report direct vascular inflammation and neutrophil infiltration in obese women, and to show that this correlates with increased weight and increased blood pressure. These findings may help explain the relationship between obesity and hypertensive disorders, such as chronic hypertension and preeclampsia.

Acknowledgments

Supported by a grant to SWW from the National Institutes of Health (HL069851)

REFERENCES

1. Flegal KM, Carroll MD, Ogden CL, Johnson CL. Prevalence and trends in obesity among US adults, 1999–2000. *JAMA* 2002;288:1723–1727. [PubMed: 12365955]
2. Mokdad AH, Ford ES, Bowman BA, et al. Prevalence of obesity, diabetes, and obesity-related health risk factors, 2001. *JAMA* 2003;289:76–79. [PubMed: 12503980]
3. Srinivasan SR, Myers L, Berenson GS. Changes in metabolic syndrome variables since childhood in prehypertensive and hypertensive subjects: the Bogalusa Heart Study. *Hypertension* 2006;48:33–39. [PubMed: 16769996]
4. Thomas F, Bean K, Pannier B, Oppert JM, Guize L, Benetos A. Cardiovascular mortality in overweight subjects: the key role of associated risk factors. *Hypertension* 2005;46:654–659. [PubMed: 16157786]
5. Bodnar LM, Ness RB, Markovic N, Roberts JM. The risk of preeclampsia rises with increasing prepregnancy body mass index. *Ann Epidemiol* 2005;15:475–482. [PubMed: 16029839]
6. O'Brien TE, Ray JG, Chan WS. Maternal body mass index and the risk of preeclampsia: a systematic overview. *Epidemiology* 2003;14:368–374. [PubMed: 12859040]
7. Hansel B, Giral P, Nobecourt E, et al. Metabolic syndrome is associated with elevated oxidative stress and dysfunctional dense high-density lipoprotein particles displaying impaired antioxidative activity. *J Clin Endocrinol Metab* 2004;89:4963–4971. [PubMed: 15472192]
8. Keaney JF Jr, Larson MG, Vasan RS, et al. Obesity and systemic oxidative stress: clinical correlates of oxidative stress in the Framingham Study. *Arterioscler Thromb Vasc Biol* 2003;23:434–439. [PubMed: 12615693]
9. Bray GA. Medical consequences of obesity. *J Clin Endocrinol Metab* 2004;89:2583–2589. [PubMed: 15181027]
10. Esposito K, Nicoletti G, Giugliano D. Obesity, cytokines and endothelial dysfunction: a link for the raised cardiovascular risk associated with visceral obesity. *J Endocrinol Invest* 2002;25:646–649. [PubMed: 12150343]
11. Straczkowski M, Dzienis-Straczowska S, Stepień A, Kowalska I, Szelachowska M, Kinalska I. Plasma Interleukin-8 Concentrations Are Increased in Obese Subjects and Related to Fat Mass and Tumor Necrosis Factor- α System. *J Clin Endocrinol Metab* 2002;87:4602–4606. [PubMed: 12364441]
12. Zhou L, Xiang W, Potts J, et al. Reduction in extracellular superoxide dismutase activity in African-American patients with hypertension. *Free Radic Biol Med* 2006;41:1384–1391. [PubMed: 17023265]

13. Touyz RM. Reactive oxygen species, vascular oxidative stress, and redox signaling in hypertension: what is the clinical significance? *Hypertension* 2004;44:248–252. [PubMed: 15262903]
14. Walsh SW. Maternal-placental interactions of oxidative stress and antioxidants in preeclampsia. *Semin Reprod Endocrinol* 1998;16:93–104. [PubMed: 9654611]
15. Walsh SW, Vaughan JE, Wang Y, Roberts LJ II. Placental isoprostane is significantly increased in preeclampsia. *FASEB J* 2000;14:1289–1296. [PubMed: 10877821]
16. Pitsavos C, Chrysoshoou C, Panagiotakos DB, Lentzas Y, Stefanadis C. Abdominal obesity and inflammation predicts hypertension among prehypertensive men and women: the ATTICA Study. *Heart Vessels* 2008;23:96–103. [PubMed: 18389333]
17. Bautista LE, Vera LM, Arenas IA, Gamarra G. Independent association between inflammatory markers (C-reactive protein, interleukin-6, and TNF-alpha) and essential hypertension. *J Hum Hypertens* 2005;19:149–154. [PubMed: 15361891]
18. Kupferminc MJ, Peaceman AM, Aderka D, Wallach D, Socol ML. Soluble tumor necrosis factor receptors and interleukin-6 levels in patients with severe preeclampsia. *Obstet Gynecol* 1996;88:420–427. [PubMed: 8752252]
19. Qiu C, Luthy DA, Zhang C, Walsh SW, Leisenring WM, Williams MA. A prospective study of maternal serum C-reactive protein concentrations and risk of preeclampsia. *Am J Hypertens* 2004;17:154–160. [PubMed: 14751658]
20. Ferrannini E, Balkau B, Coppock SW, et al. Insulin Resistance, Insulin Response, and Obesity as Indicators of Metabolic Risk. *J Clin Endocrinol Metab* 2007;92:2885–2892. [PubMed: 17504904]
21. Freeman DJ, McManus F, Brown EA, et al. Short- and long-term changes in plasma inflammatory markers associated with preeclampsia. *Hypertension* 2004;44:708–714. [PubMed: 15452036]
22. Kaaja R. Insulin resistance syndrome in preeclampsia. *Semin Reprod Endocrinol* 1998;16:41–46. [PubMed: 9654606]
23. Mishra NV, Walsh SW. Reactive oxygen species enhance vascular reactivity to angiotensin II via the rho A kinase pathway. *Reprod Sci* 2009;16(Supplement):71A. [PubMed: 19087983]
24. Uehata M, Ishizaki T, Satoh H, et al. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature* 1997;389:990–994. [PubMed: 9353125]
25. Asano M, Nomura Y. Comparison of inhibitory effects of Y-27632, a Rho kinase inhibitor, in strips of small and large mesenteric arteries from spontaneously hypertensive and normotensive Wistar-Kyoto rats. *Hypertens Res* 2003;26:97–106. [PubMed: 12661918]
26. Eiserich JP, Baldus S, Brennan ML, et al. Myeloperoxidase, a leukocyte-derived vascular NO oxidase. *Science* 2002;296:2391–2394. [PubMed: 12089442]
27. Leik CE, Walsh SW. Neutrophils infiltrate resistance-sized vessels of subcutaneous fat in women with preeclampsia. *Hypertension* 2004;44:72–77. [PubMed: 15148293]
28. Shah TJ, Walsh SW. Activation of NF-kappaB and expression of COX-2 in association with neutrophil infiltration in systemic vascular tissue of women with preeclampsia. *Am J Obstet Gynecol* 2007;196:48.e41–48.e48. [PubMed: 17240230]

FIGURE 1.

Percentage of vessels stained and optical densities of staining of resistance sized vessels in subcutaneous fat for CD66b, NF-κB and COX-2 according to weight categories. Vessel staining for all three antigens increased with increasing weight of the patients. Vascular infiltration of neutrophils indicated by CD66b and vascular inflammation indicated by NF-κB and COX-2 were highest in obese patients, next highest in overweight patients and lowest in normal weight patients. * P < 0.05, ** P < 0.01, *** P < 0.001 as compared to normal weight.

FIGURE 2.

Correlation of vessel staining with BMI. Vessel staining was significantly correlated with BMI for all three antigens. Dashed lines are the 95% Confidence Intervals for the regression lines.

FIGURE 3.

Correlation of vessel staining for NF-κB and COX-2 with staining for CD66b. The % vessels stained for NF-κB and COX-2 were highly correlated with CD66b staining.

FIGURE 4.

Number of leukocytes per vessel according to weight categories. Obese patients had significantly more leukocytes per vessel than normal weight patients as indicated by staining for CD66b and NF-κB. The number of leukocytes/vessel was significantly correlated with BMI for all three antigens ($r = 0.48$, $P < 0.05$; $r = 0.70$, $P < 0.001$ and $r = 0.60$, $P < 0.01$, respectively). * $P < 0.05$ as compared to normal weight, *** $P < 0.001$ as compared to overweight and normal weight.

FIGURE 5. Correlation of diastolic blood pressure with % vessel staining for CD66b, NF-κB and COX-2. Blood pressure was significantly correlated with neutrophil infiltration and vascular inflammation.

FIGURE 6.

Representative examples of antigen staining in resistance sized vessels of subcutaneous fat. Column 1, Normal Weight; Column 2, Overweight; Column 3, Obese. Row 1, CD66b: Panel a shows two vessels from a normal weight women with no neutrophil staining for CD66b. Panel b shows moderate staining of neutrophils with CD66b in the lumen, adhered on endothelium and infiltrated into a vessel of an overweight women. Obese women had the most staining for CD66b. Panel c shows extensive staining of neutrophils in the lumen, adhered on endothelium and infiltrated into two vessels of an obese women. Row 2, NF- κ B: Normal weight women had little staining for NF- κ B (Panel d), overweight women had staining in the endothelium and in the vascular smooth muscle of the majority of their vessels (Panel e), obese women had extensive and intense staining for NF- κ B in the endothelium and vascular smooth muscle of almost all of their vessels (Panel f). Row 3, COX-2: Both normal weight and overweight women showed moderate staining for COX-2 (Panels g and h). Obese women had the most intense staining for COX-2 and the staining was present in most vessels (Panel i). Leukocytes within the vessels also stained for NF- κ B and COX-2. A, adipocytes, VL, vessel lumen. All images are 400X. Red scale bar is 50 μ m.

Table 1

Patient Demographic Data

	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 (years)	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 ***
Race			
White	2	3	3
Black	2	4	7
Other	1		

Values are mean ± SE

* P < 0.05,

*** P < 0.001 compared to normal weight