ISOLATING, CHARACTERIZING AND ENUMERATING CELL-DERIVED MICROPARTICLES: RELEVANCE IN HEALTH AND DISEASE

by

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Isolating, Characterizing and Enumerating Cell-Derived Microparticles: Relevance in Health and Disease

Dissertation directed by Professor Christopher A. DeSouza, Ph.D.

Microparticles are small (0.1-1.0 μm in diameter) extracellular vesicles derived from the plasma membrane of activated and/or apoptotic cells. Once thought to be inert cellular debris, microparticles are now recognized as important paracrine/endocrine vectors involved in the transcellular exchange of physiological and pathological information. Moreover, microparticles play key roles in the development of atherosclerotic cardiovascular disease and may serve as sensitive biomarkers of disease risk, progression, severity and outcome. Critical to the utility of microparticles as biomarkers of vascular health and disease is the isolation, characterization and enumeration of these submicron vesicles. A central feature of this dissertation was the development of flow cytometry protocols to accurately quantify microparticles, their cellular origin and, in some cases, their stimulus for release. These technically challenging approaches were conducted in both biologic samples (plasma) and culture media. Studies associated with this dissertation included: 1) determining whether circulating concentrations of endothelial cell-derived microparticles (EMPs) differ in middle-aged men compared with women; 2) determining, *in vitro*, whether high glucose (in the diabetic range) induces microparticle release from endothelial cells and, if so, whether high glucose-derived EMPs effect endothelial cell surface expression of key adhesion molecules involved in atherogenesis; and 3) determining whether circulating microparticles from various cell types are elevated in overweight and obese adults; if so, whether elevations in circulating microparticles are a potential biomarker of endothelial dysfunction; and if regular aerobic exercise affects circulating microparticles in overweight and obese adults. The results of these studies demonstrate, for the first time, that: 1) circulating concentrations of activation- and apoptosis-derived EMPs do not differ between middle-aged men and women; 2) high glucose stimulates marked increase in EMPs in addition

these EMPs significantly increase the expression of E-selectin, ICAM-1, VCAM-1 and PECAM-1 on the surface of endothelial cells; and 3) circulating levels of EMPs, platelet-, monocyte- and leukocyte-derived microparticles are significantly higher in overweight/obese compared with normal weight adults. In addition, circulating microparticles (EMPs, PMPs and MMPs) are inversely related to endothelial vasodilator function; and regular aerobic exercise training, independent of weight loss, reduces circulating levels of these microparticles in previously sedentary overweight/obese adults.

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CHAPTER I

LITERATURE REVIEW

ISOLATING, CHARACTERIZING AND ENUMERATING CELL-DERIVED MICROPARTICLES: RELEVANCE IN HEALTH AND DISEASE

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INTRODUCTION

Microparticles are a heterogeneous population of small (0.01-1.0 μm in diameter) vesicles formed from the outward budding of the cellular plasma membrane and are released into the extracellular space as a result of cellular activation and/or apoptosis.[1] It is now apparent that microparticles are present in various bodily fluids such as: blood, urine, saliva and cerebrospinal fluid.[2-3] Interestingly, they have also been observed incorporated into atherosclerotic plaque, suggesting potential pathological effects.[4-5]

Although nearly all eukaryotic cells have the capacity for vesicle shedding, endothelial-, platelet-, monocyte- and leukocyte-derived microparticles have garnered the most interest due to their physiological and pathophysiological role in various disease processes.[6-7] For example, numerous studies have demonstrated that high circulating numbers of microparticles are associated with an increased incidence and severity of cardiovascular and cerebral events.[8-9]

The synthesis and release of microparticles are tightly regulated and selective processes due, in part, to their integral role in intercellular signaling, inflammation, cell adhesion and coagulation.[10-11]After release from their parent cell, microparticles travel through biological fluids and become internalized by different cell types downstream, thereby influencing their phenotype.[12-13] The following review of literature is intended to describe: the process of microparticle formation; the isolation, characterization and detection of endothelial, platelet, monocyte and leukocyte derived microparticles; and the role of microparticles in cardiovascular disease (CVD) risk, development and progression and, in turn, their potential as novel biomarkers of CVD.

MICROPARTICLE FORMATION

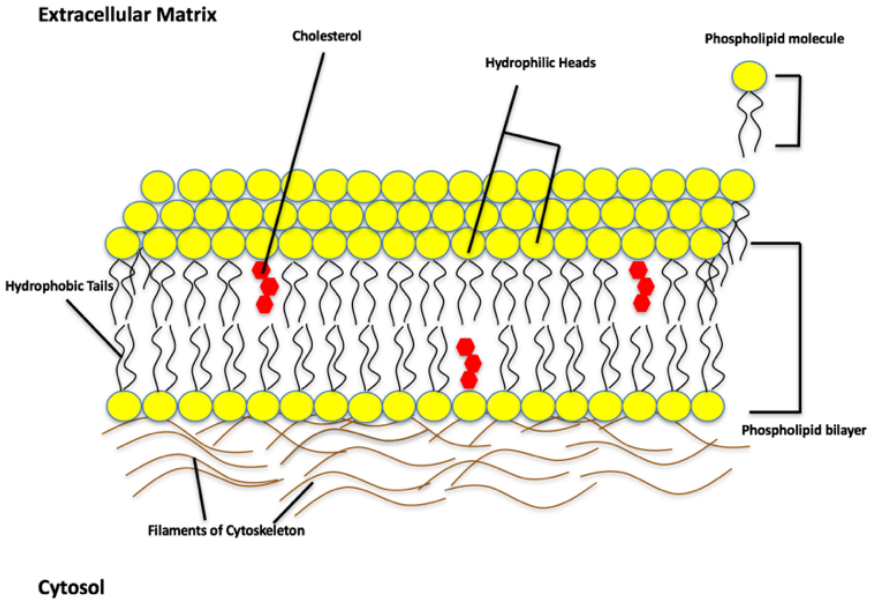
The stability of the cell membrane is dependent upon its structural and functional features, including the specific distribution of certain isoforms of lipids, as well as enzymes that maintain this lipid topology and the cytoskeleton scaffold. During different physiological /pathophysiological processes, an alteration in transmembrane ion flux disrupts the enzymatic activity of the lipid transporters aminophospholipid translocase (flippase), floppase and/or scramblase; along with activating cytoskeletal proteases, thereby destabilizing the cell membrane and releasing microparticles into the extracellular fluid. The following section will summarize key components of the cell membrane and critical features in microparticle formation.

Cell Membrane Components

In eukaryotes, the cell membrane is a 5-10 nm semi-permeable membrane composed of lipids, carbohydrates and proteins. It functions as a physical barrier, separating the intracellular and extracellular environment.[14,15] Cell membranes contain a wide diversity of lipids, most of which are amphipathic molecules; that is, they contain both hydrophilic and hydrophobic regions.[16] There are three main types of membrane lipids: Phosphoglycerides, sphingolipids and cholesterol.[16] Because most membrane phospholipids are built on a glycerol backbone, they are called phosphoglycerides.[17] Membrane phosphoglycerides have an additional group linked to the phosphate, most commonly choline, ethanolamine, or serine.[18] Each of these groups is small and hydrophilic and together with the negatively charged phosphate to which it is attached forms a highly water-soluble domain at one end, called the head group.[19] As represented in Figure 1, the polar phosphate heads of each molecular layer (or leaflet) are directed outward, either facing the cytoplasm or the extracellular environment.[20] As such, the hydrophobic fatty acyl chain tails are protected from contact with the aqueous environment.[21] Table 1 shows the relative lipid

composition of a typical nucleated mammalian cell. Under basal conditions, the negatively charged aminophospholipids, phosphatidylserine and phosphatidylethanolamine, are concentrated in the inner leaflet.

Figure 1: The plasma membrane is a lipid bilayer. The core of the membrane contains layer of phospholipids orientated with their water-soluble head groups facing the outer surfaces and their hydrophobic fatty acid tails facing the interior.



Whereas, the neutrally charged cholinephospholipids phosphatidylcholine and sphingomyelin are segregated on the outer leaflet.[7] This asymmetric distribution of phospholipids is ubiquitous in the cell membrane and is tightly regulated by the ATP-dependent enzymes flippase and floppase, and the ATP independent enzyme, scramblase. [7,22,23,24]

Table 1: Relative Mammalian Cell Lipid Composition

Lipid	Percentage of Total Lipids
Phosphatidylcholine	45-55%
Phosphatidylethanolamine	15-25%
Phosphatidylserine	5-10%
Sphingomyelin	5-10%
Cholesterol	10-20%

Adapted from Vance et al. 2014 [21]

Lipid Membrane Remodeling: Flippase

The role of flippase is to ensure that the majority of the phosphatidylserine and phosphatidylethanolamine lipids are on the inner leaflet of the membrane.[7,25,26] Flippases hydrolyze ATP to facilitate unidirectional transport of phosphatidylserine and phosphatidylethanolamine into the inner leaflet of the membrane (Figure 2). [27] Recently, the P4 ATPase was identified as the primary flippase in mammals.[28] The activity of P4 ATPase becomes blunted with increasing concentrations of calcium.[27] Therefore, physiological/pathophysiological processes such as cellular activation and apoptosis perturb membrane asymmetry by increasing concentrations of calcium and inhibiting P4 ATPase from translocating phosphatidylserine and phosphatidylethanolamine to the inner leaflet of the membrane.[29] Because phosphatidylserine is normally confined to the inner leaflet of the cell membrane, its appearance on the cell surface is considered the most prominent feature of the loss of trans-bilayer asymmetry. [30]

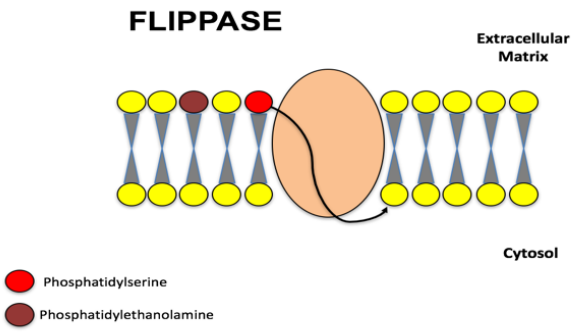


Figure 2: The ATP dependent transporter flippase, responsible for the transport of phosphatidylserine and phosphatidylethanolamine into the inner leaflet of the cell membrane.

Lipid Membrane Remodeling: Floppase

A second class of ATP-dependent transporter, floppases, mediate membrane asymmetry by transporting lipids in the opposite direction of flippases, thus outward.[31] Floppases are

members of the ABC transporter family, redistributing phosphatidylcholine and sphingomyelin ensuring that they are the dominant species on the external face of the membrane [7] (Figure 3). This outward lipid movement is much slower than the inward movement of phosphatidylserine and phosphatidylethanolamine by flippase.[22] Furthermore, it has been demonstrated that flippase and floppase operate independently, suggesting their concerted action is responsible for a dynamic asymmetric steady state distribution of the phospholipids phosphatidylserine, phosphatidylethanolamine, phosphatidylcholine and sphingomyelin.

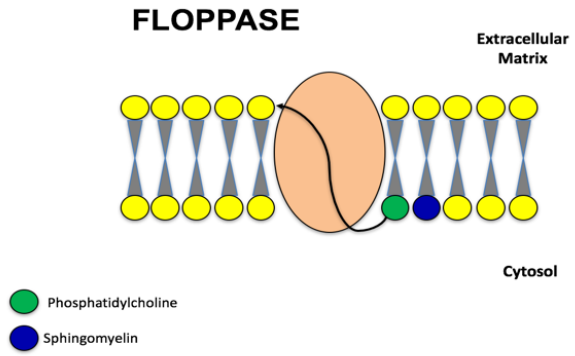


Figure 3: The ATP dependent transporter floppase, responsible for the transport of phosphatidylcholine and sphingomyelin to the outer leaflet of the cell membrane.

Lipid Membrane Remodeling: Scramblase

A third enzyme, scramblase, mediates trans bilayer phospholipid movement in a non-selective and energy independent manner. [32] Scramblase participates in the bidirectional distribution of all major phospholipids across the bilayer of the membrane (Figure 4). [23,24,33] The process of lipid scrambling is most clearly identified by the surface exposure of phosphatidylserine. [34] Importantly, an increase in the intracellular calcium concentration, as evoked by either cellular activation or apoptosis is a requisite for the scrambling process. [27,35–37]

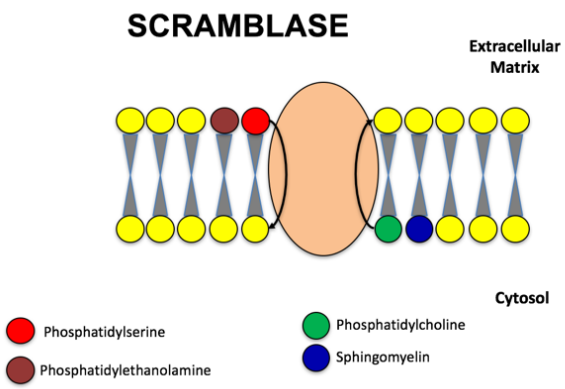


Figure 4: The ATP independent transporter scramblase, responsible for the unidirectional transport of all major phospholipids.

Cytoskeleton Disruption and Release of Microparticles

In addition to flippase, floppase and scramblase, the integrity of the cytoskeleton scaffold is critical for membrane stability. During cellular activation, calpain, a calcium dependent cysteine protease plays an important role in the degradation of cytoskeletal filaments. [38] Normally found inactive in the cytosol, when calcium levels rise, calpain translocates to the inner leaflet of the membrane and becomes activated.[39] Once activated, calpain cleaves talin and actin filaments on the plasma membrane and promotes cytoskeletal rearrangement.[40] This cytoskeletal rearrangement destabilizes the membrane, facilitating the budding and release of microparticles.[41]

Compared to cellular activation, during apoptosis, caspases are the central proteases responsible for cytoskeletal degradation and microparticle release.[42] Caspase-3 cleaves rho associated kinase I, in turn converting the GDP-inactive form to the GTP-bound active form of rho associated kinase I. Once activated, rho associated kinases phosphorylate myosin light chains (MLC) which promote cellular contraction of cortical actin within the cell and the degradation of cytoskeletal filaments.[43–47] In addition to phosphorylation of MLC, rho associated kinase I also inactivates myosin light chain phosphatase, thereby sustaining myosin light chain

phosphorylation, upsetting the normal membrane architecture and triggering the formation of apoptotic microparticles. [48]

Summary of Components and Mechanisms of Microparticle Formation

The resting phospholipid asymmetry is maintained by an active transmembrane enzymatic balance involving flippase, floppase and scramblase equilibrium.[49] Cellular activation and apoptosis is associated with a release of intracellular calcium by the endoplasmic reticulum.[50] This sudden increase in cytosolic calcium disrupts membrane stability, characterized by phosphatidylserine externalization, stimulation of scramblase and floppase and the inhibition of flippase. [38,39] Depending on the stimulus, cytosolic enzymes calpain or caspase-3 become activated, thereby leading to the cleavage of cytoskeleton filaments. As a result, shedding and 'blebbing' of the plasma membrane occurs followed by the release of microparticles into the extracellular fluid (Figure 5). [51]

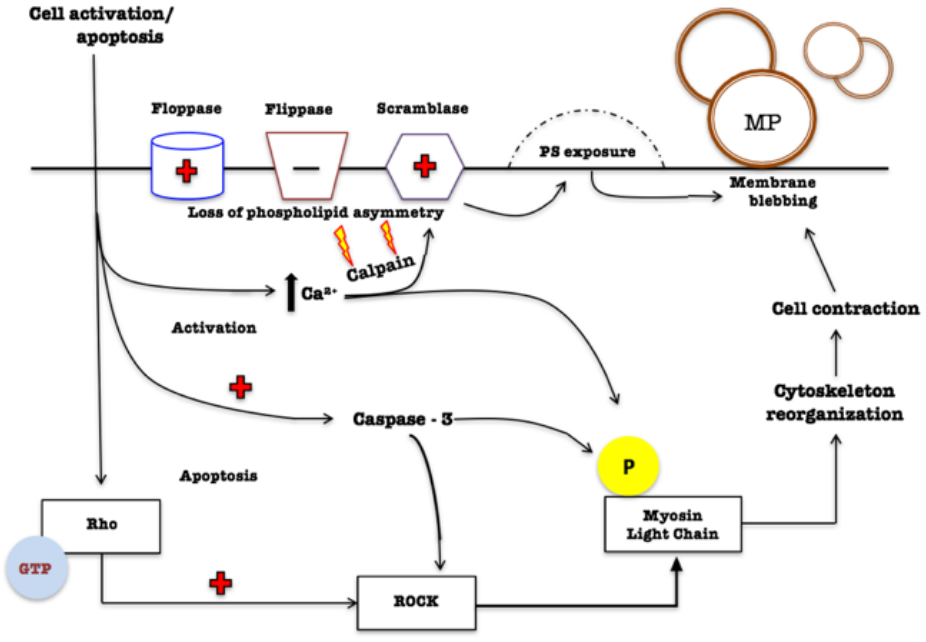


Figure 5: Distinct Pathways Leading to Microparticle Formation and Release. Cellular activation or apoptosis causes an increase in intracellular calcium. Phospholipid asymmetry is disrupted after flippase is inhibited and floppase and scramblase are maintained, causing the externalization of phosphatidylserine. Depending on the stimulus, either calpain or caspase will be activated, thereby phosphorylating myosin light chain and leading to the cleavage of cytoskeleton filaments, cell contraction and membrane blebbing

Adapted from Chironi et al. [49]

MICROPARTICLE ISOLATION AND CHARACTERIZATION

The presence of microparticles in the blood of healthy persons is well documented, and elevated numbers of specific subspecies are observed in numerous different cardiovascular and autoimmune diseases and cancer. However, different studies report a wide range of pre-analytical and analytical procedures, all of which can affect circulating microparticle number, and in turn, make it difficult to compare data across different groups/centers. Table 1 demonstrates important pre-analytical and analytical/technical parameters that should be considered when isolating and characterizing microparticles.

Table 2: Parameters in Microparticle Isolation and Characterization

Pre-Analytical Parameters	
Blood Sampling	• Needle 19-21 gauge • Tourniquet only to localize the vein • Smooth Draw • Discard the first mL • Collection at the same time period
Anticoagulant	• Sodium citrate (buffered or non-buffered)
Transportation	• Without any agitation or within special transportation boxes to avoid microparticle generation
Delay Before First Centrifugation/Analysis	• < 2 hours before first centrifugation and immediately after second
Centrifugation	• Double centrifugation • 1st spin to generate platelet poor plasma (1,500 x g for 10 min) • 2nd spin to generate platelet free plasma (13,000 x g for 2 min) • Light brake speed
Storage	• Store at -80 °C • Avoid repeated freeze thaws • All samples should be stored for similar length of time

	• Controls treated in same conditions as patients.
Analytical/Technical Parameters	
Flow Cytometer	• Intrinsic Resolution Capabilities
Size Gating	• Size Calibration Beads
Fluorescence Gating	• Proper use of isotype control • Multiple antibody overlap
Microparticle Enumeration	• Absolute counting beads

Adapted from Mullier et al.

MICROPARTICLE SUBSPECIES

Endothelial Derived Microparticles

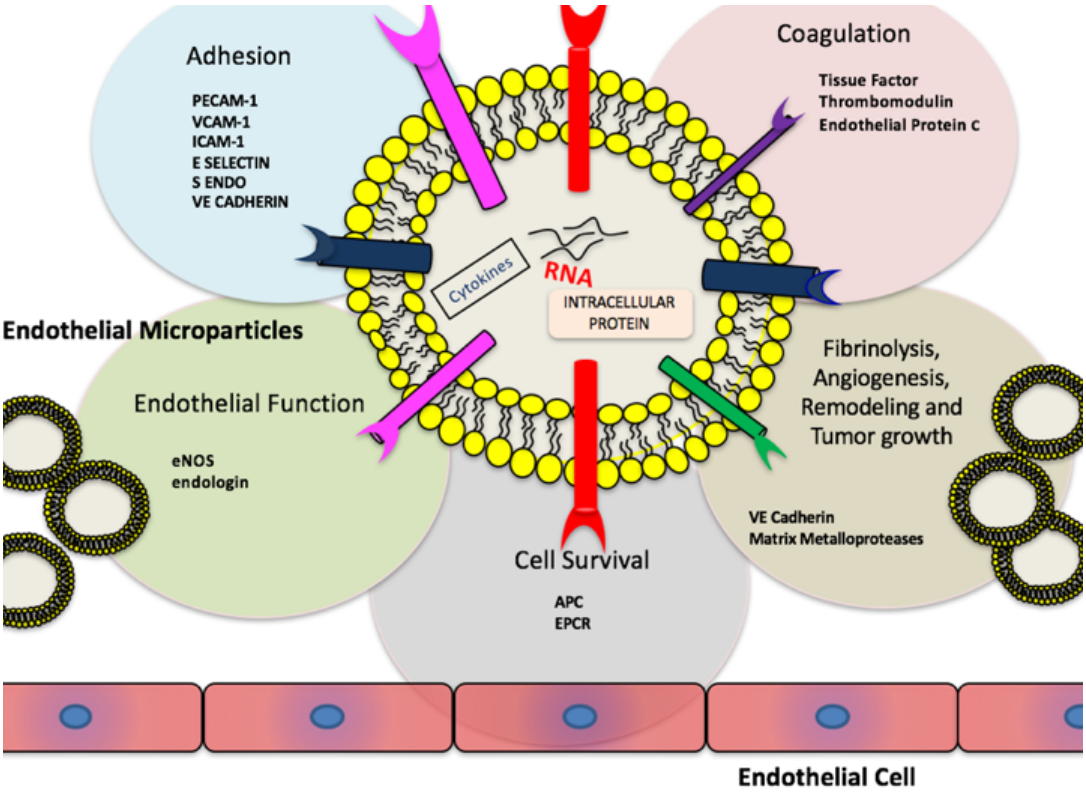
Endothelial derived microparticles are small membrane derived vesicles released constitutively or as a consequence of endothelial cell activation or apoptosis. [26] Proteins expressed on endothelial derived microparticles include: E-selectin, vascular endothelial cell adhesion molecule-1 (VCAM-1), endologin, intercellular cell adhesion molecule (ICAM-1), platelet endothelial cell adhesion molecule-1 (PECAM-1), S-endo or αv integrin. [52–56] Endothelial derived microparticles have been implicated in the etiology of a host of physiological and pathological processes including: coagulation, inflammation, oxidative stress**,** cell adhesion, proteolysis, angiogenesis and tumor growth [54,57–59] (Figure 6). Interestingly, Jimenez et al. reported that endothelial cells release quantitatively and phenotypically distinct microparticles depending on their stimulus of release.[60,61] Activated endothelial cells released microparticles are enriched in the expression of E-selectin, ICAM-1 and VCAM-1, whereas, low levels of these inducible markers are expressed on microparticles shed from apoptotic endothelial cells. [60] Moreover, apoptotic but not activated endothelial cells released microparticles that were enriched in the expression of PECAM-1, endologin and VE-cadherin. These results indicate that endothelial cells have the ability to alter endothelial derived microparticle phenotype in a stimulus dependent manner.[60,62] In

addition to the surface expression of endothelial proteins, endothelial derived microparticles have been reported to contain biologically active information including intracellular protein, microRNA and cytokines. Peterson and colleagues recently demonstrated the protein content of endothelial derived microparticles are distinct based on the stimulus of release. [63] For example, after incubating endothelial cells with plasminogen activator inhibitor-1 (PAI-1) or tumor necrosis factor α (TNF-α), the proteome of the endothelial derived microparticles showed that although some common overlap in protein content is evident, depending on the stimulus used (PAI-1 or TNF-α), proteins are selectively packaged inside microparticles. [63] Together, these data indicate all endothelial derived microparticles are not the same. Endothelial cells have the ability to release phenotypically distinct microparticles with a selectively packaged proteome that is representative of the health of their parent cell.

Endothelial derived microparticle formation have been reported both in vitro and in vivo in response to a number of pro-inflammatory, pro-oxidative, pro-apoptotic and pro-thrombotic stimuli. [49,64] For example, Sapet et al. showed that endothelial cells stimulated with thrombin induced a time and dose dependent release of microparticles contingent upon the activation of rho associated protein kinase II.[65] Pre-incubation of endothelial cells with either a selective rho associated protein kinase II or caspase-2 inhibitor, abrogated not only the mRNA expression of rho associated protein kinase II but also prevented its cleavage, thereby inhibiting microparticle formation. These findings suggest an important signaling pathway emphasizing the proteolytic activity of caspase-2 and the integral role of rho associated protein kinase II in thrombin induced microparticle formation. Szotowski and colleagues [66] demonstrated the causal role of reactive oxygen species (ROS) and apoptosis in endothelial derived microparticle generation with TNFα.

Interestingly, pre-treatment with the antioxidant pyrrolidine dithiocarbamate blunted the release of the microparticles, possibly through suppression of ROS formation and apoptosis. [66]

Endothelial derived microparticles role in cell-cell communication and intercellular exchange was recently elucidated by their ability to reduce ICAM-1 expression in a microRNA-222-dependent manner. These data suggest endothelial derived microparticles can protect endothelial cells from apoptosis and promote an anti-inflammatory effect through the transfer of microRNA-222, in turn, reducing ICAM-1 expression.[67] MicroRNAs are short single stranded noncoding RNAs that target gene expression post-transcriptionally by the degradation of messenger RNA and/or blocking translation. [68] Furthermore, ICAM-1 is a target of microRNA-222 implicating an important role for microparticle packaged microRNA in intercellular communication and vascular health.[67,69] In summary, endothelial derived microparticles reflect a subtle balance between cell stimulation, proliferation and death. [51] Their ability to encapsulate cargo along with transfer of physiologically important bioactive molecules to distal cells makes them promising prognostic and diagnostic targets for CVD treatment.



Adapted from Dignat George et al. [62]

Figure 6 Schematic Representation of Endothelial Microparticles. Summary of various molecules expressed by endothelial microparticles and their associated biological effects. Coagulation, fibrinolysis, angiogenesis, remodeling, tumor growth, cell survival, endothelial function and cell adhesion have all been linked to endothelial microparticles surface antigen expression

Platelet Derived Microparticles

Platelet derived microparticles are formed as a result of platelet activation and/or apoptosis.[70,71] In terms of protein composition, platelet microparticles shed from activated platelets predominately express α-granule derived factor Va and Xa. [72] A vast majority of platelet microparticles express the surface activation markers glycoprotein (GP)-Ibα (CD42b), P selectin (CD62P), β_3 integrin (GPIIIa, CD61), α_{IIb} integrin (GPIIb, CD41), lysosomal-associated membrane protein-3 (LAMP3, CD63), and thrombospondin-1 (TSP-1). Platelet microparticles

also carry platelet activating factor (PAF), β-amyloid precursor, anticoagulant protein C/S, CXCL7 and CCL5, all which may exert potent biological effects in recipient cells downstream.[73,74] In vitro, numerous physiological agonists including thrombin, collagen, and high shear stress have been shown to elicit microparticle formation and release from platelets.[75,76,77] Importantly, increased intracellular concentration of calcium is a prelude to platelet activation. This in turn activates calpain, the protease responsible for the cytoskeletal protein degradation of talin, actin-binding protein and myosin. As a result, cytoskeletal rearrangement occurs, the cell contracts and microparticle are released from the platelet.[78,79]

Because platelets lack a nucleus, they cannot undergo programmed cell death or DNA fragmentation.[80,81] Nevertheless, platelets contain many proteins required for apoptosis including caspase-9, caspase-3, and activators of caspase apoptotic protease activating factor-1 and cytochrome c.[82,83] . Wolf et al. showed treatment of platelets with the caspase activator cytochrome c not only recapitulated apoptotic events, but also included the sequential activation of procaspase-9 and procaspase-3 and the proteolysis of caspase substrates.[82] These data indicate that the intrinsic pathway of apoptosis is functional in platelets and may play an important role in platelet derived microparticle formation and release.[84]

The cellular dynamics underlying microparticle formation and release in platelets are similar to that in endothelial cells, monocytes and leukocytes. After an increase in intracellular calcium, caspase 3 cleaves rho associated kinase protein I, in turn phosphorylating MLC and inhibiting MLC phosphatase, thereby facilitating platelet microparticle release.[48] Numerous functions of platelet microparticles in the circulation have been elucidated. Mause et al. demonstrated a pro-inflammatory and pro-atherogenic potential of platelet microparticles after observing their role as a transcellular delivery system for chemokine ligand 5 (CCL5),

suggesting they play a causal role in enhancing monocyte recruitment in inflammation and atherosclerosis.[85] Additionally, because a requisite of microparticle formation is externalization of phosphatidylserine, platelet microparticles have been implicated in providing catalytic sites for prothrombinase complexes to generate thrombin needed for the conversion of fibrinogen to fibrin in formation of clots.[86] Moreover, microparticles from platelets are enriched in binding sites for activated factor V, VIIIa and IXa, and they provide the membrane surface for thrombin formation.[86–88] Therefore, the release of microparticles may have important consequences for the expression and dissemination of platelet derived procoagulant activity in vivo. [86,89]

Besides their role in coagulation, Merten and colleagues [90] demonstrated a role for platelet microparticles in cellular adhesion. In a rabbit model of arterial endothelial injury, infusion of platelet microparticles caused a 3- to 5- fold increase in microparticle adhesion to the injured site compared with the uninjured site.[90] This suggests platelet microparticles can act as a substrate for further platelet binding.[90] Platelet microparticles have also been implicated in the modulation of immune responses.[91] Recently, Dinkla et al.[92] demonstrated that platelet microparticles inhibit the differentiation of regulatory T cells (T-regs) into IL-17 and IFN-γ-producing cells. The binding of platelet microparticles to T-regs and the subsequent inhibition of T-reg differentiation, appeared to be P-selectin and CXCR3 dependent. [92] These findings suggest that platelet microparticles can actively regulate the immune response at sites of vascular injury.[92] In summary, platelet microparticles play an important role in coagulation, hemostasis, cellular adhesion and modulation of immune cells. Their ability to retain properties of their parent cell and their capacity to confer inflammatory signals to distal target cells makes potent mediators of cell communication and integral effectors of disease.[93]

Monocyte Derived Microparticles

Monocyte derived microparticles are shed as a result of monocyte activation or apoptosis. Expressing cell specific antigens lipopolysaccharide (LPS) receptor (CD14), tissue factor and P-selectin glycoprotein ligand-1(PSGL-1), numerous roles of monocyte derived microparticles have been elucidated in the circulation including atherogenesis, inflammation and cellular activation. [94–96]

The biological relevance of monocyte derived microparticles have been reported both in vivo and in vitro. In vivo, APOE$^{-/-}$ mice fed a high fat/cholesterol rich diet for eight weeks and simultaneously treated with intravenous monocyte derived microparticles reported significantly increased monocyte and T-cell infiltration into the vessel wall and enhanced plaque formation compared with mice that received vehicle.[94] In vitro, monocyte derived microparticle treatment provoked an increased endothelial cell and macrophage uptake of monocyte derived microparticles, thereby increasing the generation of intracellular ROS.[94] Additionally, macrophages pre-treated with monocyte derived microparticles demonstrated enhanced migration to MCP-1 (monocyte chemoattractant protein-1) and increased IL-6. [94] Moreover, co-incubation of monocyte derived microparticles with endothelial cells resulted in significantly higher expression of ICAM-1.[94] These results establish several pleiotropic pro-inflammatory effects of monocyte derived microparticles, providing evidence for monocyte derived microparticles role as paracrine messengers promoting vascular inflammation during atherogenesis.

Monocyte derived microparticles role in inflammation and cellular activation were further elucidated after internalization by endothelial cells and the subsequent activation of phosphor-extracellular signal regulated kinase (ERK1/2) and nuclear factor (NF-κB).[95] This in

turn induced the expression of the cell adhesion molecules ICAM-1, VCAM-1 and E selectin.[95] Also, western blot analysis of monocyte derived microparticles showed high expression of IL-1β, and when internalized by endothelial cells, mediated endothelial cell activation through IL-1R.[95]

The role of monocyte derived microparticles in oxidative stress and ROS production has also been implicated after incubation with endothelial cells resulted in enhanced nitric oxide (NO) release without affecting superoxide anion (O_2^-) generation.[97] Mechanistically, this was linked to the activation of both phosphoinositide 2-kinase (PI3-kinase) and ERK1/2 through the regulation of caveolin-1 expression, independent of its phosphorylation. [97] Furthermore, monocyte derived microparticle treatment was able to activate protein kinase B (AKT) and P38 mitogen activated protein kinase (P38 MAPK) pathways along with increasing the nitration of several proteins, reflecting peroxynitrite production.[97]

Paradoxically, Bardelli et al.[98] reported both pro-inflammatory and anti-inflammatory effects of monocyte derived microparticles. After incubation with monocytes and macrophages, there was a significant increase in the release of TNFα and IL-6 , superoxide (O_2^-) anion production and NF-κB activation.[98] However, there was also a 2-fold increase in PPARγ (peroxisome proliferator-activated receptor gamma) protein expression after incubation, suggesting an anti-inflammatory role for monocyte derived microparticles too. [98] Some of the anti-inflammatory properties of PPARγ are attributed, at least partially, to its trans-repression ability. [98] PPARγ can physically interact with the p65 subunit of NF-κB, so preventing its nuclear translocation, or it can be sumoylated and indirectly inhibit NF-κB binding.

Monocyte derived microparticles role in cell-cell communication and intercellular exchange was elucidated by their ability to selectively package microRNA. Zhang et al.[99] showed

microRNA-150 is selectively packaged into monocyte derived microparticles and can be transferred to endothelial cells enhancing endothelial cell migration and vascular repair. One target of microRNA-150, is c-Myp, a transcription factor implicated in cell proliferation, apoptosis and tumorigenesis. [100] These results suggest potential mechanisms that may underlie vascular injury under various pathophysiological conditions, including obesity, hyperglycemia and chronic inflammation. [99]

To summarize, monocyte derived microparticles may have many regulatory effects, suggesting a dual role in the circulation with the capacity to directly increase monocyte cell activation, inflammation and proliferation but also serving an anti-inflammatory mechanism with the induction of PPARγ. Their ability to encapsulate cargo along with transfer of physiologically important bioactive molecules to distal cells makes them promising prognostic and diagnostic targets for CVD treatment.

Leukocyte Derived Microparticles

Leukocyte derived microparticles are released after leukocyte activation and/or apoptosis.[101] They express cell-specific antigens including Mac-1 (CD11b), CD66b, CD15 and leukocyte common antigen (CD45). These surface antigens have been implicated in various adhesive interactions of monocytes, macrophages and granulocytes; cellular migration, pathogen binding; and the regulation of cell growth and differentiation. [102–104] Leukocyte derived microparticles contain biologically active proteins such as IL-1β, CD40 ligand, major histocompatibility complex class I and II (MHC I & MHC II) and ICAM-1. [44,64,105] These encapsulated proteins are critical modulators of leukocyte activation, endothelial cell proliferation, intraplaque neovascularization and monocyte adhesion and migration.[4,64,105,106]

Interestingly, Leroyer and colleagues demonstrated leukocyte derived microparticles are highly abundant (>50%) and thrombogenic in atherosclerotic lesions.[64] These results were confirmed by Mayr et al.[105] demonstrating atherosclerotic plaques are primarily packed with leukocyte derived microparticles whose primary metabolite is taurine and lactate. These findings suggest an oxidative microenvironment created by monocytes and leukocytes in atherosclerotic plaques, and, indicate that leukocyte derived microparticles may still actively metabolize subcellular entities, even after being incorporated into plaques. [105]

In summary, leukocyte derived microparticles have been implicated in the increased expression of various adhesion molecules on endothelial cells, up regulation of inflammatory and chemotactic cytokines, and increased monocyte adhesiveness.[106–108] Moreover, their pro-thrombogenic potential along with incorporation into atherosclerotic plaques makes them targets for the manifestation of vascular disease.

CARDIOVASCULAR DISEASE

Cardiovascular disease (CVD) is an umbrella term that encompasses diseases affecting the heart and blood vessels. Globally, CVD is the leading cause of death, accounting for nearly 17.3 million deaths per year and is expected to grow to more than 23.6 million by 2030. [109] The vascular endothelium is a layer of cells lining the luminal surface of all blood vessels. [110] Originally considered a quiescent interface between blood and the vascular smooth muscle, it is now recognized as an important physiologically active regulatory organ.[111] Its location makes the vascular endothelium subject to deleterious processes and mechanical forces related to a host of cardiovascular disease risk factors which, in-turn, impairs cell function.[112] Endothelial cell dysfunction promotes a proatherogenic endothelial phenotype that is fundamental to the

initiation and progression of vascular disease.[113–117] The vascular endothelium is a central target of circulating microparticles. Microparticles are now recognized as potent regulators of endothelial function, and as long range vectors of biological information with the capacity to alter endothelial function.[51] Numerous cardiovascular and cerebrovascular pathologies have been associated with an increased number of circulating microparticles.[106,118] Therefore, increased number of circulating microparticles may provide insight and viable prognostic and diagnostic information of cardiovascular disease pathogenesis in several disease pathologies.[119]

MICROPARTICLES AND CARDIOVASCULAR DISEASE

Endothelial Derived Microparticles and Cardiovascular Disease

High numbers of circulating endothelial microparticles have been linked with a host of cardiovascular/cerebrovascular diseases such as: heart failure,[119–121] coronary artery disease (CAD),[9,122–124] ischemic stroke,[125,126] metabolic syndrome,[127] diabetes mellitus[47,128] and hypertension.[118] The utility of endothelial derived microparticles as predictive biomarkers for future events may represent viable information in CVD pathogenesis. [9,121,129] Moreover, the distinct surface expression and encapsulated biologically active cargo may elucidate information about the global health of the vascular endothelium.

Platelet Derived Microparticles and Cardiovascular Disease

Elevated levels of circulating platelet microparticles have been demonstrated in patients with acute coronary syndrome, diabetes mellitus, arterial thrombosis, hypertension, metabolic syndrome and hypercholesterolaemia.[118,130–134] Interestingly, platelet derived microparticles reflect the size of myocardium at risk in patients with ST-elevation myocardial infarction.[135] This

supports the view that platelet microparticles can serve as a marker of ongoing thrombus, with circulating levels correlating to the extent of ischemic and thrombotic burden. [135]

In patients with familial hypercholesterolemia and high cardiovascular disease risk, circulating platelet microparticles directly correlated with MRI detected lipid rich atherosclerotic plaques, while inversly correlating with MRI detected calcified plaques.[136] Mechanistically, platelet microparticles have been linked to the progression of atherosclerosis by facilitating the adherence of platelets to endothelial lesion sites, stimulating proliferation, transmigration and apoptosis of endothelial cells.[137] Studies *in vitro* show platelet microparticles possess the ability to stimulate growth of endothelial progenitor cells, thereby helping to facilitate the repair of damaged endothelial cells.[138,139] Therefore, platelet derived microparticles may represent valuable prognostic and diagnostic information about CVD pathogenesis.

Monocyte Derived Microparticles and Cardiovascular Disease

Numerous lines of evidence support the role of monocyte derived microparticles in cardiovascular disease pathologies. Hypertension, diabetes mellitus, and familial hypercholesteremia have all been linked to an increased number of circulating monocyte derived microparticles.[140,141] For example, Omoto et al.[140] reported the utility of monocyte derived microparticles as indicators of diabetic disease progression. In patients with diabetes, those whose condition was complicated with nephropathy, retinopathy or neuropathy, had higher circulating levels of monocyte derived microparticles compared with those without diabetic related complications.[140] In summary, circulating monocyte derived microparticles may represent a viable biomarker in the progression of cardiovascular pathogenesis.

Leukocyte Derived Microparticles and Cardiovascular Disease

Although they circulate in the plasma of healthy individuals, leukocyte derived microparticles only represent a minor portion (<10%) of the total microparticles in the blood. In patients with cardiovascular and metabolic disorders including atherosclerosis, venous thromboembolism, hypertension and diabetes, plasma levels of circulating leukocyte derived microparticles are significantly increased.[106] Numerous studies have reported both *in vitro* and *in vivo* evidence for the role of leukocyte derived microparticles in endothelial cell activation and inflammation. *In vitro* data from Mesri and collegues[108] demonstrate leukocyte derived microparticles can stimulate increased expression of the adhesion molecules ICAM-1, E-selectin and VCAM-1 on endothelial cells; up-regulate inflammatory cytokines IL-6 and IL-8; and increase monocyte adhesion to endothelial cells. Additionally, *in vivo* data suggests leukocyte derived microparticles ability to cooperate with locally released cytokines and leukocyte-endothelial cell intercellular signaling thereby stimulating a proadhesive, procoagulant and proinflammatory phenotype.[108,142] These findings suggest the integral role leukocyte derived microparticles have in endothelial dysfunction and their potential relevelence with increased leukocyte recruitement, platelet activation and fibrin deposition seen at the site of vascular injury.[108] Moreover, their presence in atherosclerotic lesions along with their increased prothrombotic potential suggest leukocyte derived microparticles relevance during the various stages of plaque progression.[106,143] In 2013, Sarlon-Bartoli and colleagues demonstrated plasma levels of leukocyte derived microparticles are associated with unstable plaque in asymptomatic patients with high-grade carotid stenosis.[5] After logistic regressions, leukocyte derived microparticles were identified as independent predictors of unstable plaques in asymptomatic patients. These data suggest the prognostic ability for leukocyte derived microparticles in patients with plaque vulnerability.[5]

SUMMARY AND FUTURE DIRECTIONS

It is clear that microparticles play an important role in a number of physiologic and pathologic processes. Elevations in the circulating number microparticles derived from endothelial, platelet, monocyte and leukocytes are of most clinical relevence due to their implications in cardiovascular disease development and progression. As a response to either cellular activation and/or apoptosis microparticles are blebbed from the plasma membrane, after selectively packaging biological information from their parent cell. Their capacity to serve as long range vectors of communication and their role in cell-cell communication with proximal or distal cells warrents further research. Many studies examining circulating levels of microparticles have significant design limitations and few studies have examined the influence of risk factors of CVD on circulating microparticles. Currently, there are few studies demonstrating the influence of traditional risk factors of CVD on circulating endothelial, platelet, monocyte and leukocyte microparticle number. This represents a critical lack of knowledge as a greater understanding of the factors and mechanisms contributing to the increased vascular risk associated with CVD risk factors may improve the identification, clinical management and treatment of CVD. Examining the influence of risk factors of CVD on the circulating levels of endothelial, platelet, monocyte and leukocyte derived microparticles may provide further insight into the link between risk factors of CVD and vascular endothelial cell dysfunction.

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CHAPTER II

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INFLUENCE OF SEX ON CIRCULATING ENDOTHELIAL MICROPARTICLE NUMBER AND microRNA EXPRESSION IN MIDDLE-AGED ADULTS

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ABSTRACT

The aims of this study were to determine: 1) if circulating concentrations of endothelial microparticles (EMPs) differ in middle-aged men compared with women; and 2) whether there are sex-related differences in microRNA expression in EMPs. Peripheral blood was collected from 30 sedentary adults: 15 men (52±2 yr) and 15 women (52±2 yr). EMPs were defined by markers of activation ($CD62e^{+}$) or apoptosis ($CD31^{-}$/CD42b^{-}) by flow cytometry. MicroRNA (miR-34a, 92a, 125a, and 126) expression in activation- and apoptosis-derived EMPs was measured by RT-PCR. Circulating activation- (33±8 vs. 39±9 MP/μL) and apoptosis- (49±14 vs. 42±11 MP/μL) derived EMPs were not significantly different between men and women. miR-125a expression (2.23±0.52 vs. 6.95±1.03 AU) was lower (~215%; $p<0.05$) in activation-derived EMPs; whereas, expression of miR-34a (1.17±0.37 vs. 0.38±0.09 AU) was higher (~210%; $p<0.05$) in apoptosis-derived EMPs from men than women. microRNA expression in circulating EMPs may provide novel insight into sex-related differences in cardiovascular disease.

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death in the United States. Between the ages of 45 and 65 years men have an ~50% higher incidence and prevalence of CVD and CVD-related events compared with women, despite marginal sex-associated differences in traditional cardiovascular risk factors [1]. To date, the mechanisms responsible for this sex-related difference in CVD risk and events are not well understood, resulting in substantial clinical and public health challenges.

Endothelial microparticles (EMPs) (0.1μm to 1.0 μm in size) are shed from endothelial cells as a result of cellular activation or apoptosis [2]. Recently, EMPs have emerged as prognostic and diagnostic markers of CVD due to their role in the initiation, development and progression of atherosclerosis [3]. Elevations in the circulating number of EMPs have been observed in a number of vascular pathologies, such as coronary artery disease, ischemic stroke and diabetes [4]. Moreover, EMPs have been shown to directly contribute to atherogenesis by inducing endothelial activation, inflammation and dysfunction [5]. EMPs are also known to carry and transfer microRNAs (miRNA or miR) to target tissues including endothelial cells [6]. miRNAs are ~22 nt non-coding RNAs involved in the regulation of a number of physiological and pathophysiological processes. Altered expression of vascular-related miRNAs, specifically miR-34a, miR-92a, miR-125a and miR-126, within EMPs has been linked with adverse vascular outcomes [6,7]. For example, *in vitro* and *in vivo* studies have demonstrated that lower EMP expression of miR-126 is associated with greater atherosclerotic plaque burden in mice and a higher incidence of atherosclerotic vascular disease in middle-aged adults [7,8]. Currently, it is unknown whether there are sex-related differences in circulating EMPs and their miRNA cargo in healthy, middle-aged adults.

Accordingly, the experimental aims of this study were to determine: 1) if circulating concentrations of EMPs differ in middle-aged women compared with men; and 2) whether there are sex-related differences in miRNA expression in circulating EMPs. EMPs and their miRNA content may provide novel insight into sex-related discrepancy in CVD risk in middle-aged adults.

METHODS

Subjects. A total of 30 healthy, sedentary middle-aged adults aged 45-65 years participated in the study: 15 men and 15 women. All subjects were non-obese (body mass index: $\leq$ 30 kg/m^2), normotensive, non-smokers, normolipidemic, non-medicated and free of overt cardiovascular, renal, metabolic and hematologic diseases as assessed by medical history, resting and exercise electrocardiograms, and fasting blood chemistries. All women were at least 1 year postmenopausal (mean: 5.3±1.4 yr) and not taking hormone replacement therapy. Prior to participation, all subjects had the research study and its potential risks and benefits explained fully before providing written informed consent according to the guidelines of the University of Colorado at Boulder.

Body Composition and Metabolic Measures. Body mass was measured to the nearest 0.1 kg using a medical beam balance. Body mass index (BMI) and minimal waist circumference was measured according to published guidelines [9]. Fasting plasma lipid, lipoprotein, glucose, and insulin concentrations were determined using standard techniques.

Circulating Microparticles. Peripheral blood from an antecubital vein was collected after an overnight fast into sodium citrate containing tubes and centrifuged at 1,500 x g for 10 minutes at

room temperature, plasma was collected and stored at -80°C for batch analysis. For the characterization and quantification of circulating EMP subspecies, all plasma samples were centrifuged at 13,000 x g for 2 minutes and 200μL was transferred to a TruCount tube (BD Biosciences, New Jersey, USA). EMP phenotype was determined using markers indicative of activation (CD62e) and apoptosis (CD31/CD42b) (BioLegend, San Diego, California). Samples were incubated with the flourochrome labeled antibodies for 20 minutes in the dark at room temperature. Following incubation, samples were fixed with 2% paraformaldehyde (ChemCruz Biochemicals, Santa Cruz, California) and diluted with RNAse free PBS. Microparticle size threshold was established using Megamix-Plus SSC calibrator beads (Megamix-Plus SSC beads, Biocytex, Marseille, France), and only events <1 μm in size and positively expressing markers of activation ($CD62E^+$) and apoptosis ($CD31^+/CD42b^-$) were counted and sorted by flow cytometry (BD Biosciences FACSAria I) [10]. A total of 25,000 activation- and apoptosis-derived EMPs were sorted directly into RNase free eppendorf microtubes containing 250μL of RNase free PBS. The concentration of activation- and apoptosis-derived EMPs were determined using the formula: ([number of events in region containing MPs /number of events in absolute count bead region] x [total number of beads per test / total volume of sample]) [11].

Microparticle miRNA. Total RNA was isolated from sorted activation- and apoptosis-derived EMPs using the miRNeasy FFPE Kit (Qiagen, Hilden, Germany) [12]. Samples were centrifuged at 21,000 x g for 30 min at 4°C to pellet EMPs. Supernatant was removed and RNA was extracted from pelleted EMPs, treated with 1/10 v/v DNase 1 for 15 minutes to remove genomic or cell free DNA, immobilized, washed and eluted in 18.5μL of RNAse free water [12]. To normalize for RNA content and extraction efficiency 3.5μL ($1.6x10^8$ copies/μL) of *Canorhabditis elegans* miR-39

(cel-miR-39) was added during the RNA isolation. Immediately after isolation 12μL of total RNA was reverse transcribed using the miScript Reverse Transcription Kit (Qiagen, Hilden, Germany) [13]. cDNA was PCR-amplified on the CFX96 (BioRad) RT-PCR platform using the miScript SYBR green PCR kit and miRNA specific primers (Qiagen, Hilden, Germany) [13]. All samples were assayed in duplicate. Relative expression per EMP for a given miRNA was normalized to cel-miR-39, calculated as $RE = 2^{-\Delta Ct} /MP=2^{-(Ct[miRNA]-Ct[cel\text{-}miR\text{-}39])} /MP$ and expressed as arbitrary units (AU) [13].

Statistical Analysis. Group differences in subject characteristics, circulating activation- and apoptosis-derived EMP number and EMP miRNA expression were determined by analysis of variance. Data are reported as mean±SEM. Statistical significance was set a priori at $P<0.05$.

RESULTS

Selected subject characteristics are presented in Table 1. Although none of the subjects were obese, the men demonstrated significantly higher body mass and waist circumference and lower percent body fat compared with the women. Other than high-density lipoprotein cholesterol, there were no significant differences in metabolic or hematologic variables between the groups.

There were no sex-related differences in circulating concentrations of activation- and apoptosis-derived EMPs. Concentrations of circulating activation- (33±8 vs. 39±9 MP/μL) and apoptosis- (49±14 vs. 42±11 MP/μL) derived EMPs were similar between the men and women (Figure 1). The EMP miRNA expression signature between the activated- and apoptosis-derived EMPs was not significantly different within each group, however, differential expression of

specific miRNAs in each subspecies was observed between the groups. In activation-derived EMPs, there were no significant differences in the expression of miR-34a (3.66±0.92 vs. 4.11±0.87 AU), miR-92a (2.63±0.86 vs. 3.49±0.96 AU) and miR-126 (1.32±0.29 vs. 1.55±0.35 AU) in men compared with women; however, miR-125a expression was markedly lower (~215%; $p<0.05$) in activation-derived EMPs from men (2.23±0.52 AU) compared with women (6.95±1.03 AU) (Figure 2).

With respect to apoptosis-derived EMPs, there were no significant sex-related differences in the expression of miR-92a (4.31±1.05 vs. 3.66±0.69 AU), miR-125a (5.36±1.79 vs. 6.94±2.36 AU) or miR-126 (1.32±0.36 vs. 1.23±0.25 AU). Expression of miR-34a, however, was significantly higher (~210%) in circulating apoptosis-derived EMPs from men (1.17±0.37 AU) compared with women (0.38±0.09 AU) (Figure 2).

Table 1: Selected subject characteristics

Variable	Women (n=15)	Men (n=15)
Age (years)	56±8	56±8
Body mass (kg)	69.5±12.8	80.8±9.3*
BMI (kg/m^2)	25.3±4.3	25.8±2.3
Body fat (%)	39.0±6.6	23.6±5.0*
Waist circumference (cm)	80.1±12.4	88.8±7.7*
Systolic BP (mmHg)	116±15	117±8
Diastolic BP (mmHg)	73±8	75±8
Total Cholesterol (mg/dL)	205±27	195±31
HDL-Cholesterol (mg/dL)	64±12	51±12*
LDL-Cholesterol (mg/dL)	123±27	128±27
Triglycerides (mg/dL)	91±35	86±27
Glucose (mg/dL)	85±8	89±8
Insulin (μU/mL)	8±4	8±4

BMI= body mass index; BP= blood pressure; HDL= high-density lipoprotein; LDL= low-density lipoprotein
Values are mean ± SD. *P<0.05 vs. women

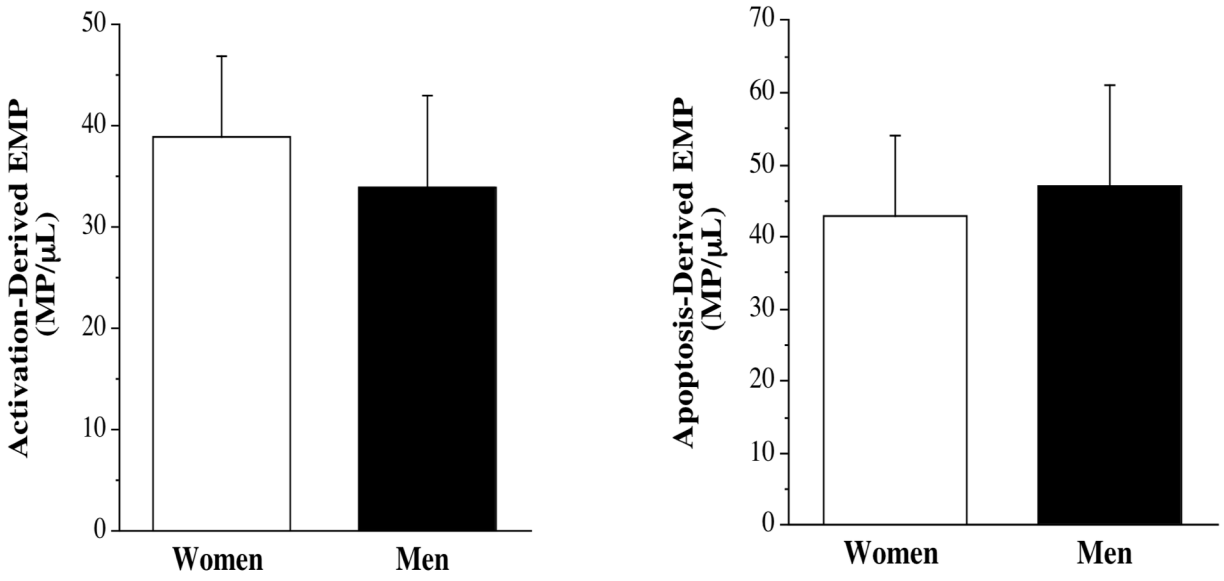

Figure 1. Circulating activation-derived (panel A) and apoptosis-derived (panel B) EMPs in middle-aged women and men. Values are mean ± SEM.

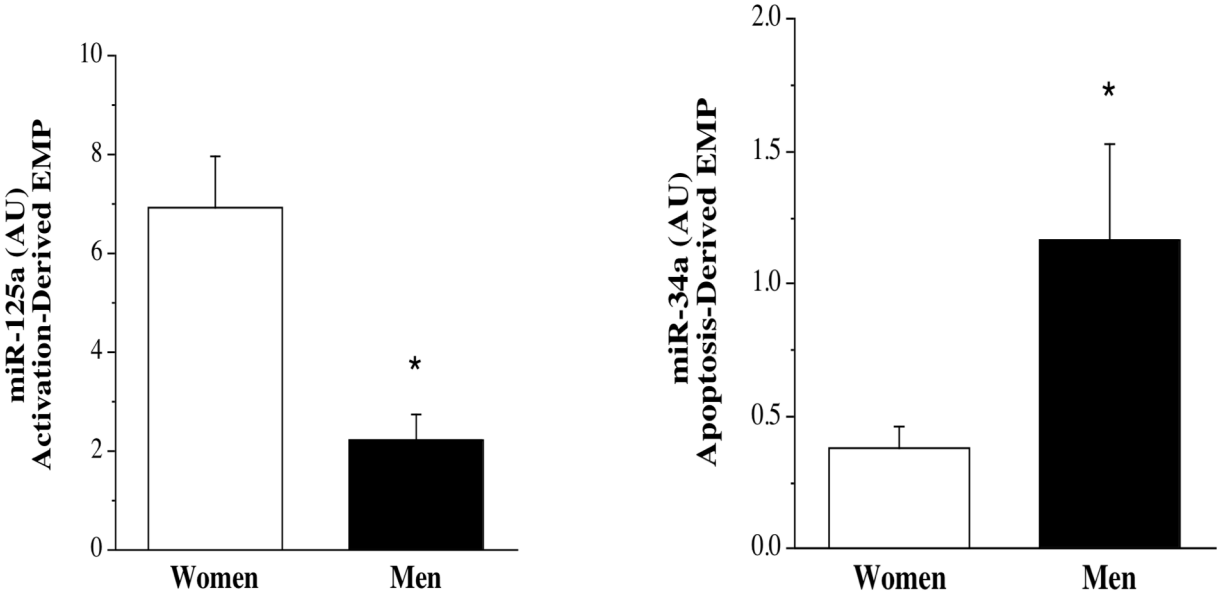

Figure 2: miR-125a expression in activation-derived EMPs (panel A) and miR-34a expression in apoptosis-derived (panel B) EMPs isolated from middle-aged women and men. Values are mean± SEM. P*<0.05.

DISCUSSION

Clinical interest in circulating EMPs has intensified due to their pathologic role in atherosclerotic vascular disease [14] and their value as a vascular biomarker of disease risk and severity [4]. Indeed, higher circulating EMP concentrations are significantly associated with increased CVD risk and events [15]. The novel findings of the present study are that: 1) circulating concentrations of EMPs are not significantly different in healthy, middle-aged women and men; however, 2) there are sex-related differences in the expression of miR-125 and miR-34a contained in activation- and apoptosis-derived EMPs, respectively. Differential expression of these vascular-related miRNAs have been linked to endothelial dysfunction and CVD risk [6,13]. Given their potential role in regulating target cell function and their link to vascular disease [16], circulating EMP miRNA content may contribute to the disparate CVD rates between middle-aged women and men.

Elevations in circulating EMPs are not only considered to be a consequence and biomarker of endothelial activation, injury and apoptosis but also causative agents in vascular damage, dysfunction and disease [17]. In addition, there is increasing evidence supporting their strong predictive and prognostic value with CVD risk, events and outcome [15]. It is now recognized that endothelial cells release phenotypically and quantitatively distinct EMPs in response to cell activation or apoptosis [18]. Thus, circulating concentrations of activation-derived ($CD62e^+$ microparticles) and apoptosis-derived ($CD31^+/CD42b^-$ microparticles) EMPs are considered to provide a sensitive index of endothelial health, and in turn, cardiovascular risk [19]. Elevations in both subspecies of EMPs have been linked to greater vascular risk and disease burden by promoting and generating a proinflammatory environment as well as supporting coagulation and thrombosis [20]. Given the potential role of EMPs in disease pathology, it was somewhat surprising that we observed no sex-related difference in circulating concentrations of

either activation- or apoptosis-derived EMPs in the present study. Levels of both species of EMPs were almost identical between the groups of middle-aged women and men. Interestingly, Toth et al. [21] reported significantly higher circulating concentrations of activation-derived EMPs in premenopausal women compared with men of similar age. Moreover, the sex-related difference in activated EMPs was most apparent during the luteal phase of the menstrual cycle, suggesting menstrual cycle-related influences on EMP release. All of the women in the present study were postmenopausal. The absence of menstrual cycle-induced disturbances of the endothelium [22] may account for the lack of higher circulating EMPs in our middle-aged women.

In the present study, we focused on a specific subset of vascular-related miRNAs: miR-34a, miR-92a; miR-125a; and miR-126. Altered expression of these miRNAs have been implicated in key features of the atherosclerotic disease process such as: endothelial cell senescence and apoptosis (miR-34a); inflammation (miR-92a, miR-126); and vascular vasomotor regulation (miR-125a). Moreover, recent studies indicate that miRNAs carried by EMPs are more biologically active and predictive of atherosclerotic disease compared with freely circulating miRNAs in plasma [8]. For example, Jansen et al., [7] reported that the miRNA signature in circulating EMPs could directly affect the vascular endothelium, promoting a pro-atherogenic endothelial phenotype. In addition, miRNA expression in EMPs has been shown to be independently predictive of adverse cardiac events in patients with coronary artery disease [8]. In the present study, we observed sex-related differential expression of miRNAs in activation- and apoptosis-derived EMPs. To our knowledge this is the first study to observe sex differences in the expression of key vascular related miRNAs contained in circulating EMPs. In circulating activation-derived EMPs, expression of miR-125a was markedly lower (~215%) in microparticles from men than women. Lower expression of miR-125a has been linked to

endothelial dysfunction [16] and atherosclerosis [13]. Interestingly, miR-125a is an important negative regulator of the vasoconstrictor peptide endothelin-1 (ET-1) [16]. It is plausible that lower EMP miR-125a expression may contribute to greater endothelium-mediated ET-1 vasoconstrictor tone reported in middle-aged men compared with women [23]. In circulating apoptosis-derived EMPs, expression of miR-34a was substantially higher (~210%) in the microparticles from the men than women. miR-34a has been shown to influence endothelial cell senescence and apoptosis [24]. Zhou and colleagues [25] eloquently demonstrated *in vivo* that microparticle transfer of miR-34a enhances cellular apoptosis by altering the expression of the anti-apoptotic molecule Bcl-2. Endothelial cell senescence and apoptosis are fundamental features in the atherosclerotic process [24]; it is plausible that sex-related differences in EMP miR-34a content may influence the senescent and apoptotic propensity of the endothelium and, in turn, atherogenic consequences. To address this intriguing postulate, we are currently determining whether EMPs from middle-aged men induce a more proatherogenic endothelial phenotype, specifically a phenotype more susceptible to senescence and apoptosis. Future studies are also needed to expand the microRNA expression profile in circulating EMPs in middle-aged women and men to identify other microRNAs that may be differentially expressed with potential links to CVD risk.

In conclusion, the results of the present study demonstrate that although circulating EMP number (both activation- and apoptosis-derived EMPs) do not differ between middle-aged men and women, microRNA expression in circulating EMPs appears to be differentially expressed in men and women. Indeed, it is important to emphasize that, although a number of pathologic, pharmacologic, and physiologic factors can influence EMP number as well as miRNA content [3,4,15], all of the adults in the present study were free of overt disease, non-medicated, nonsmokers, and sedentary. In addition, all women were postmenopausal and not taking estrogen replacement

therapy, thus decreasing the potential confounding effect of estrogen-mediated effects on EMP number and miRNA expression. Sex-related differences in EMP microRNA cargo may significantly influence how EMPs affect various target tissue function, especially endothelial cells and, in turn, influence vascular risk.

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CHAPTER III

In Review: *Diabetes and Vascular Disease Research*

HIGH GLUCOSE-INDUCED ENDOTHELIAL MICROPARTICLES INCREASE ADHESION MOLECULE EXPRESSION ON ENDOTHELIAL CELLS

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ABSTRACT

The experimental aim of this study was to determine, in vitro, the effects of glucose-induced endothelial-derived microparticles (EMPs) on endothelial cell expression of E selectin, intercellular cell adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 and platelet cell adhesion molecule-1 (PECAM-1). Human umbilical vein endothelial cells (HUVECs) were cultured and incubated with media containing either 5 mM D-glucose (normoglycemic condition) or 25 mM D-glucose (concentration representing a hyperglycemic state) for 48 hr to generate EMPs. EMP identification ($CD144^+$) and concentration was determined by flow cytometry. HUVECs (3×10^6 cells/condition) were treated with either normal glucose-derived (ngEMPs) or high glucose-derived EMPs (hgEMPs) for 24 hr and surface expression of E-selectin (CD62E-PE), ICAM-1 (CD54-FITC), VCAM-1 (CD106-APC) and PECAM-1 (CD31-BV) was assessed by flow cytometry and reported as mean fluorescent intensity (MFI). hgEMPs induced significantly higher surface expression of E-selectin (2614±132 vs. 2010±204 MFI), ICAM-1 (2110±81 vs. 1688±152 MFI), VCAM-1 (3589±431 vs. 2134±386) and PECAM-1 (4237±395 vs. 2525±269 MFI) on endothelial cells than ngEMPS. Microparticle-induced cell adhesion molecule expression provides potential novel mechanistic insight regarding the increased risk of atherosclerotic vascular disease associated with high glucose conditions.

INTRODUCTION

Hyperglycemia is associated with an increased risk and prevalence of atherosclerotic cardiovascular disease (CVD) [26]. A major factor underlying the increased CVD burden associated with hyperglycemia is glucose-induced endothelial cell activation and dysfunction [27,28]. A hallmark characteristic of endothelial cell activation, and in turn atherogenesis, is increased surface expression of cell adhesion molecules [29,30]. Indeed, the adherence and subsequent transendothelial migration of circulating leukocytes and other inflammatory cells into the intima is predominantly mediated by the surface expression of adhesion molecules such as E-selectin, intracellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1 and platelet endothelial cell adhesion molecule (PECAM)-1 on the vascular endothelium. Morigi et al. [31] and others [32,33] have demonstrated, in vitro, that high glucose concentrations upregulate the surface expression of these adhesion molecules, establishing glucose as a potent promoter of leukocyte adhesion and intimal infiltration [32]. High glucose also stimulates microparticle release from endothelial cells [34]. These endothelial-derived microparticles (EMPs) can confer deleterious autocrine effects on the endothelium [35-37]. For example, hyperglycemic-related EMPs have been shown to promote endothelial cell inflammation and apoptosis [36,38,39]. Moreover, EMPs have emerged as mediators and biomarkers of diabetes-related CVD risk and development [40,41]. The effects of glucose-derived EMPs on endothelial adhesion molecule expression are not well defined. Accordingly, the aim of this study was to determine the effects of glucose-stimulated EMPs on endothelial cell surface expression of adhesion molecules.

METHODS

Cell Culture, EMP Generation and Enumeration. Human umbilical vein endothelial cells (HUVECs) were purchased from Life Technologies (ThermoFisher, Waltham, MA), and cultured in endothelial growth media (EBM-2 Bulletkit, Clonetics Lonza, Walkersville, MD), supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin (Life Technologies, Carlsbad, CA) under standard cell culture conditions (37 °C and 5% CO_2). HUVECs were cultured to the 3rd passage after reaching ~90% confluence and treated with media (RPMI 1640) containing 25mM mannitol (to control for osmolality) and either 5mM D-glucose (normal glucose) or 25mM D-glucose (high glucose; representative of a diabetic glycemic state) for 48 hr as previously described [39]. Thereafter, the supernatant from each condition was collected, centrifuged at 13,000 x g at room temperature for 2 minutes to pellet and discard cellular debris, transferred to TruCount™ tubes (BD Biosciences, San Jose, CA) and incubated with CD144-PE (VE-Cadherin) for EMP determination. EMP size threshold was established using Megamix-Plus SSC calibrator beads (Biocytex, Marseille, France)[42] EMP concentration (MP/μL) in the media from both conditions was determined by flow cytometry using the formula: ([number of events in region containing EMPs/number of events in absolute count bead region] x [total number of beads per test/ total volume of sample]).

EMP Treated HUVECS: HUVECs were cultured as described above. Media containing EMPs harvested from either the normal glucose or high glucose conditions were centrifuged at 20,500 x g for 30 minutes at 4°C to pellet EMPs [36,39,43]. The pelleted EMPs were then re-suspended in EBM-2 at a concentration of $1.0x10^7$ MPs/mL. HUVECs were then treated with an equal number of

EMPs derived from either the normal glucose (ngEMPs) or high glucose (hgEMPs) conditions for 24 hr.

Endothelial Cell Adhesion Molecule Surface Expression: After EMP treatment, harvested HUVECs were incubated with CD62E-PE (E-selectin); CD54-FITC (ICAM-1); CD106-APC (VCAM-1); and CD31-BV (PECAM-1). The mean fluorescent intensity (MFI) for each antibody was determined by flow cytometry.

Statistical Analysis. Differences in glucose-treatment derived EMP number and adhesion molecule expression were determined by two-tailed, unpaired Student's t-test. Data are reported as mean±SEM for 4 independent HUVEC experiments. Statistical significance was set a priori at $P<0.05$.

RESULTS

The hyperglycemic condition elicited markedly higher (~260%; $P<0.05$) EMP release compared with the normoglycemic condition (136±38 vs. 37±13 MP/μL). hgEMP exposure induced greater endothelial cell expression of each adhesion molecule. Surface expression of E-selectin (2614±132 vs 2010±204 MFI), ICAM-1 (2110±81 vs 1688±152 MFI), VCAM-1 (3590$\pm$431 vs 2134$\pm$386 MFI) and PECAM-1 (4237±395 vs. 2525±269 MFI) were significantly higher in the hgEMP compared with ngEMP treated cells (Figure).

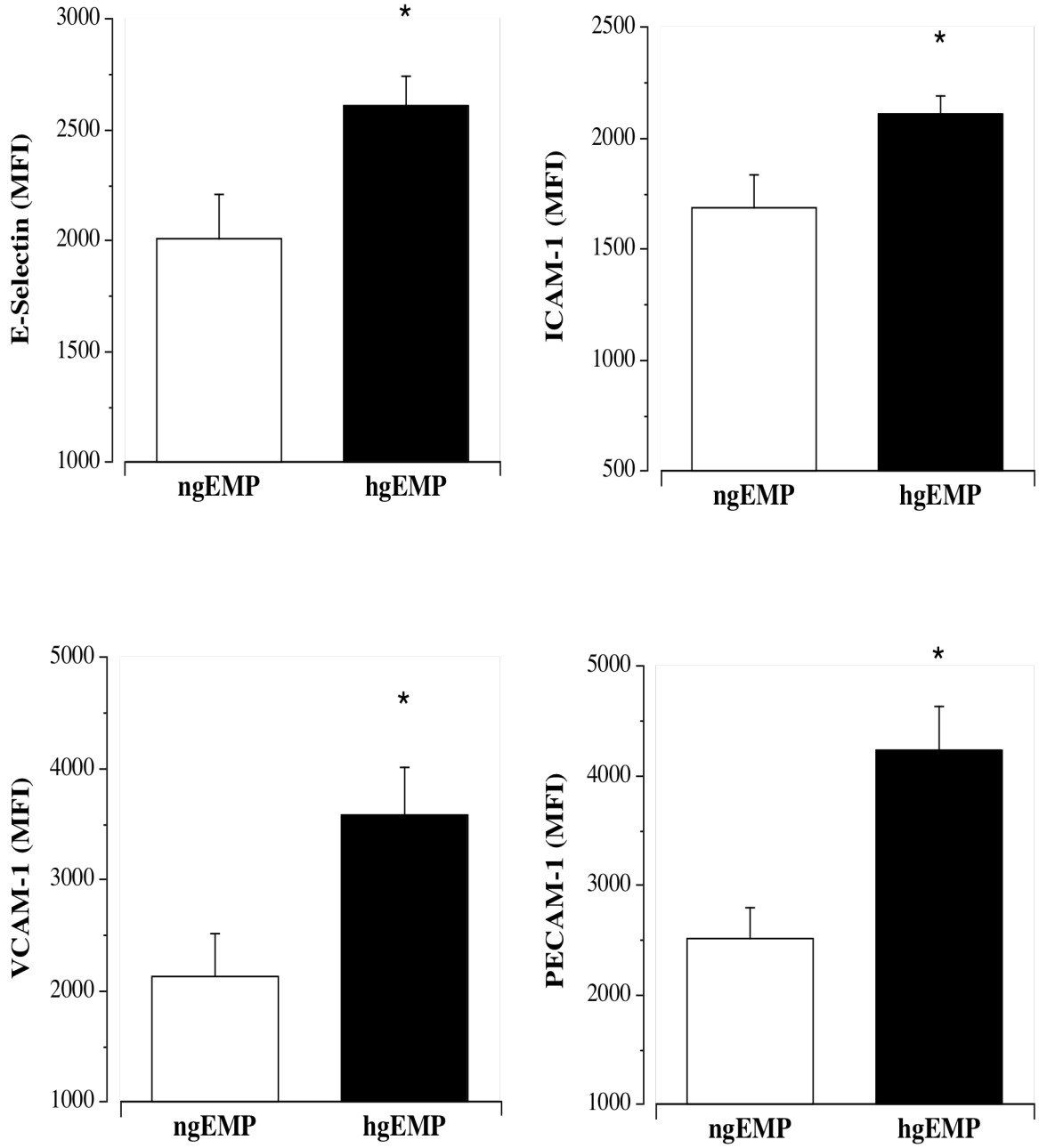

Figure: Effect of EMPs generated under normal (ngEMPs) and high glucose (hgEMPs) concentrations on endothelial cell surface expression of E-selectin, ICAM-1, VCAM-1 and PECAM-1. Values are mean ± SEM (n=4). *P<0.05.

DISCUSSION

The novel finding of the present study is that high glucose-derived EMPs markedly increase endothelial cell surface expression of E-selectin, ICAM-1, VCAM-1 and PECAM-1. Elevated endothelial surface expression of these adhesion molecules is known to be an integral, precipitating event in the pathogenesis of atherosclerosis as well as an important contributor to plaque development and rupture [44,45]. Several experimental [33,46,47] and clinical [48,49] studies have demonstrated that hyperglycemia is associated with enhanced expression of cell adhesion molecules. While the direct effects of glucose on cell adhesion molecules is clear; to our knowledge, this is the first study to demonstrate that EMPs produced in response to high glucose stimulation also promote overexpression of adhesion molecules on the surface of endothelial cells.

Increased expression of cell adhesion molecules on the surface of endothelial cells has been shown to facilitate greater luminal leukocyte interaction, adhesion and migration into the subendothelial space, in turn, increasing the propensity for atherosclerosis [30]. Indeed, the tethering and rolling of leukocytes on the endothelial surface is controlled by selectins such as E-selectin, whereas leukocyte adhesion is mediated largely by immunoglobulins (e.g. ICAM-1 and VCAM-1) and transmigration through gap junctions is facilitated by PECAM-1 [50]. The pathologic consequence of this coordinated, complex interaction on the endothelial cell surface depends on the extent of adhesion molecule availability [48,49]. In the present study, hgEMPs significantly increased the expression of E-selectin (~30%), ICAM-1 (~25%), VCAM-1 (~70%) and PECAM-1 (~70%) on the surface of endothelial cells. This finding is consistent with the notion that glucose-stimulated EMPs likely contribute to the increased risk of atherosclerotic vascular disease associated with hyperglycemic conditions [37]. The mechanisms underlying the

hgEMP-induced increase in the surface expression of adhesion molecules are not clear. However, EMPs are important transport vectors for a myriad of proteins, cytokines and miRNAs that can disrupt target cell homeostasis. For example, Jansen et al.[36] demonstrated that EMPs derived under high glucose conditions (similar to the present study) are pro-oxidative, with a cargo rich in NADPH oxidase and reactive oxygen species. This atherogenic microparticle phenotype was suggested to promote endothelial activation and adhesion molecule expression. Indeed, in their study, Jansen and colleagues [36] reported an increase in endothelial cell ICAM-1 and VCAM-1 protein levels in response to high glucose-derived EMPs. Our findings confirm increased expression of these adhesion molecules and significantly extend these findings demonstrating, for the first time, that the increased expression involves greater surface density of not only ICAM-1 and VCAM-1 but also E-selectin and PECAM-1 [51]. Collectively, these findings provide further insight regarding the proatherogenic effects of EMPs that are produced under hyperglycemic conditions.

In summary, greater cell adhesion molecule expression on the surface of the endothelium heightens atherogenic potential and thrombotic risk [30]. In the present study we demonstrate that EMPs generated from high glucose exposure induce an increase in cell adhesion molecules on the surface of endothelial cells. Our results support the notion that hyperglycemic-related EMPs contribute to the pathogenesis of vascular disease associated with diabetes [36,37].

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CHAPTER IV

CIRCULATING MICROPARTICLES ARE ELEVATED IN OVERWEIGHT AND OBESE ADULTS AND ARE REDUCED WITH REGULAR AEROBIC EXERCISE

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ABSTRACT

Clinical interest in circulating microparticles have focused primarily on those derived from endothelial cells (EMPs), platelets (PMPs), monocytes (MMPs) and leukocytes (LMPs) because elevations in each of these microparticles have been linked with endothelial dysfunction, atherosclerosis and thrombosis [52,53]. We tested the hypothesis that: 1) circulating levels of EMPs, PMPs, MMPs and LMPs are elevated in overweight and obese adults, independent of other cardiovascular risk factors, compared with normal weight adults; 2) circulating microparticle concentrations are related to endothelial function; and 3) regular aerobic exercise reduces circulating levels of EMPs, PMPs, MMPs and LMPs in overweight and obese adults. Forty-six middle-aged (44-64 years) adults were studied: 16 normal weight (body mass index (BMI) >18.5 and < 25 kg/m^2; 10 males/ 6 females); 12 overweight (BMI $\geq$25 and <30 kg/m^2; 7 males/ 5 females) and 18 obese (BMI $\geq$30 kg/m^2; 12 males/6 females). Circulating EMPs (CD62E$^+$); PMPs (CD62P$^+$); MMPs (CD14$^+$) and LMPs (CD45$^+$) were determined by flow cytometery. Endothelium-dependent vasodilation was determined by forearm blood flow responses (via plethysmography) to intra-arterial infusion of acetylcholine and sodium nitroprusside. Twenty of the 30 overweight and obese adults completed a 3-month aerobic exercise training intervention. Circulating concentrations of EMPs, PMPs, MMPs and LMPs were significantly higher (~50-85%) in the overweight and obese compared with normal weight adults. FBF responses to acetylcholine were lower (~20%; P<0.05) in the overweight (from 4.4± 0.3 to 12.2 ±0.9 mL/100 mL tissue/min) and obese (from 4.3$\pm$0.2 to 10.3$\pm$0.6 mL/100 mL tissue/min) versus normal weight (from 4.0±0.3 to 15.2±0.8 mL/100 mL tissue/min) adults. EMPs (r=-0.46), PMPs (r=-0.53) and MMPs (r=-040), but not LMPs (r=0.12; P=0.45), were significantly inversely related with total blood flow response to acetylcholine. Aerobic exercise training reduced (~30-60%;

P<0.05) circulating EMPs, PMPs, MMPs and LMPs in the overweight and obese adults. These results indicate that circulating EMPs, PMPs, MMPs and LMPs are elevated in both overweight and obese adults. However, regular aerobic exercise can reduce circulating concentration of these microparticles in this at-risk population.

INTRODUCTION

It is estimated that ~70% of adults in the United States are either overweight or obese. Regardless of age, the public health ramifications and financial burden associated with overweight and obesity are enormous, representing a major health burden [54]. For example, >300,000 deaths annually are consequences of obesity related morbidity [55]. Indeed, obesity reduces the number of years lived free of cardiovascular disease (CVD) and increases the number of years lived with CVD [56], contributing to increased morbidity and mortality risk across all ages [55,57-60]. Endothelial cell activation, inflammation, dysfunction and death are considered to be major mechanistic factors contributing to greater CVD risk and vascular events with increased adiposity [61-64].

Circulating cell-derived microparticles are now recognized to be key mediators and indicators of vascular endothelial health and dysfunction [52,53]. Microparticles are small (100 to 1000 nm diameter) phospholipid vesicles released into the circulation by various cell types in response to a myriad physiologic and pathologic processes such as cellular activation, damage and apoptosis [65-67]. The notion that microparticles in the circulation were merely "inert cellular debris" changed when it became apparent that microparticles can both trigger a proatherogenic endothelial cell phenotype and serve as a prognostic and diagnostic biomarker of vascular risk [53,68-72]. Although most eukaryotic cells possess the capacity to release microparticles, clinical interest in circulating microparticles have focused primarily on those derived from endothelial cells (EMPs), platelets (PMPs), monocytes (MMPs) and leukocytes (LMPs) and because of their principal involvement in the initiation, development and progression of atherosclerotic vascular disease and its clinical consequences [73-76]. Elevations in each of these microparticles have been linked with atherosclerosis and thrombosis [77,78]. It has been suggested that EMPs and PMPs are

elevated with obesity. Esposito et al. [79] reported higher circulating levels of EMPs and PMPs in morbidly obese (BMI>40 kg/m^2) postmenopausal women. However, whether the higher levels of microparticles were due to increased adiposity per se is not clear as the subjects were profoundly hyperinsulinemic and insulin resistant. Moreover, circulating levels of C-reactive protein (CRP) were higher in the obese women and CRP has been shown to directly affect microparticle release [80]. Currently, there are no data regarding the primary influence of obesity on circulating microparticles.

Regular aerobic exercise reduces the risk of atherosclerotic vascular disease and acute cardiovascular events [81]. In overweight/obese adults, the prevalence of cardiovascular disease-related morbidity and mortality is substantially lower in those who engage in habitual physical activity [82-84], due, in part, to enhanced endothelial function [85]. The effect of habitual aerobic exercise on circulating microparticles, however, is not clear. Considering lowering circulating microparticle concentrations has been shown associated with a reduction in cardiovascular events [66,79,86], changes in microparticle number may contribute to the vasculoprotection conferred by regular aerobic exercise [87].

Accordingly, we tested the hypotheses that: 1) circulating levels of EMPs, PMPs, MMPs and LMPs are elevated in overweight and obese adults, independent of other CVD risk factors, compared with normal weight adults; 2) circulating microparticle concentrations are related to endothelial function; and 3) regular aerobic exercise reduces circulating levels of EMPs, PMPs, MMPs and LMPs in overweight and obese adults. To systematically test these hypotheses, we employed two experimental approaches. First, we utilized a cross-sectional model to examine the influence of overweight/obesity on the circulating levels of EMPs, PMPs, MMPs, and LMPs. We

then performed an intervention study to determine the effects of regular aerobic exercise on circulating levels of these microparticles in overweight/obese adults.

METHODS

Subjects: Forty six sedentary, middle-aged (44-64 years) adults participated in the study: 16 normal weight (body mass index (BMI) >18.5 and < 25 kg/m^2; 10 males/ 6 females); 12 overweight (BMI ≥25 and <30 kg/m^2; 7 males/ 5 females) and 18 obese (BMI ≥30 kg/m^2; 12 males/6 females). All subjects were free of overt cardiovascular disease as assessed by medical history, physical examination, fasting blood chemistry and ECG measured at rest and during incremental exercise performed to exhaustion. None of the subjects smoked, were taking medications (including vitamins or other dietary supplements) or performed regular aerobic physical activity for ≥ 1 year before the beginning of the study. All of the women were at least 1 year postmenopausal and had never taken or had discontinued the use of hormone replacement therapy before the start of the study.

Body Composition: Body mass was measured to the nearest 0.1 kg using a medical beam balance (Detecto, Webb City, MP, USA). Body mass index was calculated as the weight in kilograms divided by the height in meters squared. The percentage of body fat was determined by dual energy X-ray absorptiometry (Lunar Corp., Madison, WI, USA). Minimal waist circumference was measured according to published guidelines (Lohman et al. 1988)

Maximal Oxygen Consumption: To assess aerobic fitness, subjects performed incremental treadmill exercise with a modified Balke protocol. Maximal oxygen consumption ($VO_{2\,max}$) was

measured using online computer assisted open circuit spirometry, as previously described [88]. In addition, heart rate, rate of perceived exertion and total exercise time to exhaustion were recorded throughout the protocol.

Metabolic Measurements: Fasting plasma lipid, lipoprotein, glucose and insulin concentrations were determined using standard techniques by the clinical laboratory affiliated with the Clinical Translational Research Center at the University of Colorado Boulder.

Intra-arterial Infusion Protocol: All measurements were performed in a temperature controlled room between 7:00-8:30 am after an overnight fast, as previously described [89]. Briefly, a 5-cm gauge catheter was inserted into the brachial artery of the non-dominant arm under local anesthesia (1% lidocaine). FBF was measured via strain gauge venous occlusion plethysmography at rest and in response to each pharmacological agent. After the measurement of resting blood flow for 5 min, acetylcholine (IOLAB Pharmaceuticals, Duluth, GA, USA) was infused intra-arterially at rates of 4.0, 8.0 and 16 μg/100 mL tissue/min and sodium nitroprusside (Nitropress; Abbott Laboratories, Abbott Park, IL, USA) at 1.0, 2.0 and 4.0 μg/100 mL tissue/min. Flow was recorded four times each minute at rest and throughout each drug infusion protocol. Flows during the last minute of rest and each drug dose were measured and the mean value reported.

Circulating Microparticle Isolation and Characterization: Venous blood from an antecubital vein was collected into sodium citrate containing tubes and centrifuged at 1500 x g for 10 min at room temperature. Plasma was collected and stored at -80°C for batch analysis. For the

characterization and quantification of circulating microparticle subspecies, plasma samples were thawed, centrifuged at 13,000 x g for 2 min, and then transferred to a TruCount tube (BD Biosciences, New Jersey, USA) [90]. Thereafter, samples were incubated with flourochrome labeled antibodies CD62E-PE and CD62P-BV or CD14-APC and CD45-FITC (Biolegend, San Diego, California) for 20 min. Microparticle cell lineage was defined as: EMPs ($CD62E^+$); PMPs ($CD62P^+$); MMPs ($CD14^+$) and LMPs ($CD45^+$) [91]. Microparticle size threshold was established using Megamix-Plus SSC calibrator beads, and counts per μL of plasma was determined as follows: ([number of events in region containing MPs/number of events in absolute count bead region] x [total number of beads per test/total volume of sample]) [92].

Exercise Intervention: Twenty overweight (n=9) and obese (n=11) subjects participated in a 3-month home-based aerobic exercise training program that has been previously described by our laboratory [93]. Briefly, subjects were instructed to exercise 5-7 days per week for 45-60 min/day at 65-75% of their individual maximal heart rate, as determined from maximal aerobic testing. Most subjects walked, but as their fitness improved, some incorporated jogging in order to maintain their heart rate within the prescribed range. Exercise compliance was documented every two weeks with data downloaded from heart rate monitors (Polar Electro, Kempele, Finland) and a review of exercise logs. All twenty subjects completed the 3-month exercise intervention and were studied at least 24 hours after their last exercise training session to avoid any acute effects of exercise, while still representing their normal physiological state.

Statistical Analysis: Differences in subject baseline characteristics, circulating microparticles and area under the curve data were determined by between-group analysis of variance (ANOVA). When indicated by a significant F value, a post hoc test using the Newman-Keuls method was

performed to identify differences among the groups. Group differences in FBF responses to each vasoactive drug were determined by repeated measures ANOVA. Correlations between variables of interest were assessed by means of Pearson's correlation coefficient and linear regression analysis. Changes in the dependent variables resulting from the exercise intervention were assessed by repeated measures ANOVA. Importantly, there were no significant gender interactions in any of the key outcome variables; therefore, the data were pooled and presented together. All data are expressed as means ± SEM. Statistical significance was set a priori at $P < 0.05$.

RESULTS

Cross Sectional Study:

Subject characteristics are presented in Table 1. Body mass, body mass index, percentage body fat and waist circumference was significantly higher in the overweight and obese groups compared with the normal weight group. Although within clinically normal ranges, overweight/obese subjects demonstrated higher ($P<0.05$) resting systolic and diastolic blood pressure, triglycerides, plasma glucose, insulin concentrations, as well as HOMA-IR levels compared with the normal weight group. There were no differences amongst the groups in the plasma concentrations of total cholesterol, HDL-cholesterol, LDL-cholesterol or VO_2 max.

Circulating levels of EMPs, PMPs, MMPs and LMPs are presented in Figure 1. Circulating concentrations of EMPs were significantly higher (~50%) in the overweight (270 ± 28 EMP/ μL) and obese (285 ± 27 EMP/μL) compared with normal weight (187 ± 22 EMP/μL) adults. There was no significant difference in circulating EMPs between the overweight and obese groups. Circulating PMPs were highest ($P<0.05$) in the obese (173 ± 20 PMP/μL) compared with

the overweight (118±15 PMP/μL) and normal weight (53±4 PMP/μL) groups. In addition, PMP concentrations were significantly higher in the overweight than normal weight adults. Similar to EMPs, circulating concentrations MMPs and LMPs were significantly higher in the overweight (222±42 MP/μL and 166±16 MP/μL, respectively) and obese (219± 30 MMP/μL and 199±25 LMP/μL respectively) compared with the normal weight (121±21 MP/μL and 132±27 MP/μL; respectively) group.

FBF responses to acetylcholine were significantly lower (~20%) in the overweight (from 4.4± 0.3 to 12.2 ±0.9 mL/100 mL tissue/min) and obese (from 4.3±0.2 to 10.3±0.6 mL/100 mL tissue/min) groups compared with normal weight (from 4.0±0.3 to 15.2±0.8 mL/100 mL tissue/min) group (Figure 2). There were significant differences in the FBF responses to acetylcholine between the overweight and obese groups. Further, total FBF to acetylcholine (area under the curve) was ~40% lower in the overweight (59±7.0 mL/100 mL tissue) and obese (45± 4 ml/100 mL tissue) compared with normal weight (85±6 mL/100 mL tissue) (Figure 2). There were no significant differences between the groups in FBF responses to sodium nitroprusside (Figure 2). In the overall study population, EMPs (r=-0.46), PMPs (r=-0.53) and MMPs (r=-040) were significantly inversely related with total blood flow response to acetylcholine. There was no significant correlation between LMPs (r=0.12; P=0.45) and endothelial vasodilator function.

I. Selected Subject Characteristics

Variable	Normal Weight (n=16)	Overweight (n=12)	Obese (n=18)
Age, yrs	57±1	57±1	55±1
Body mass, kg	69.6±2.5	83.5±2.8*	98.1±2.7*†
BMI, kg/m^2	23.2±0.4	28.2±0.2*	32.0±1.1*†
Body fat, %	26.2±2.4	34.2±2.2*	39.1±1.7*
Waist circumference, cm	79.1±2.3	94.6±2.1*	100.5±1.7*
VO_2 max, L/min	2.4±0.1	2.6±0.2	2.6±0.2
Systolic BP, mmHg	114±2	116±3	121±2*
Diastolic BP, mmHg	71±2	73±3	78±2*
Total cholesterol, mg/dL	194.4±6.8	200.8±9.7	205.6±8.7
LDL-cholesterol, mg/dL	116.7±7.1	128.9±8.4	121.8±8.0
HDL-cholesterol, mg/dL	60.6±3.2	53.7±3.0	57.1±4.4
Trigylcerides, mg/dL	87.3±9.1	95.3±9.8	130.7±13.3*†
Glucose, mg/dL	84.7±1.9	92.4±2.1*	92.6±2.2*
Insulin, μU/mL	7.0±0.6	9.0±0.7	8.7±1.0

Values are mean±SEM. BMI, body mass index; BP, blood pressure; LDL, low-density lipoprotein; HDL, high-density lipoprotein. *P<0.05 vs. normal weight; †P<0.05 vs. overweight.

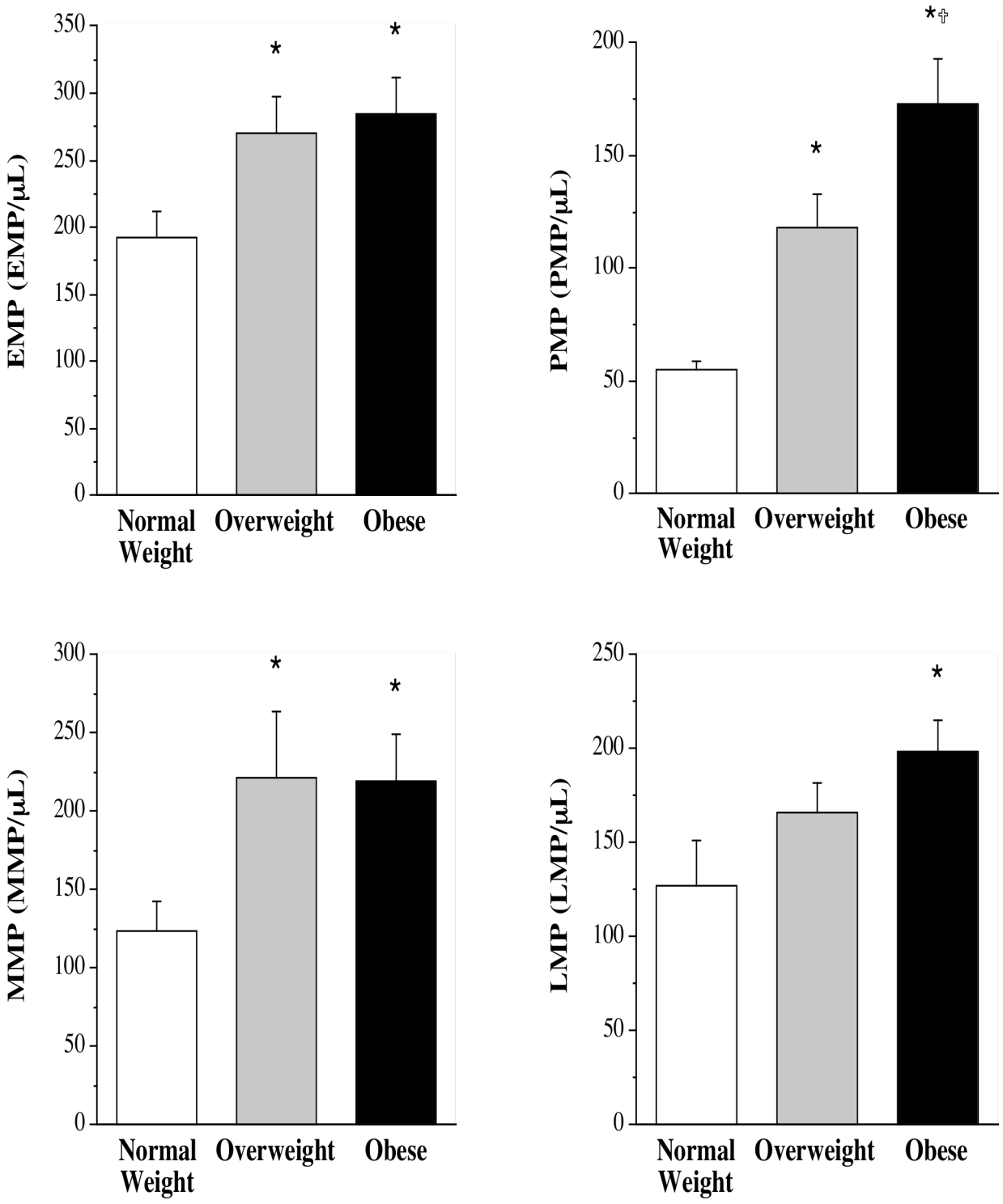

Figure 1. Circulating levels of EMPs (A), PMPs (B), MMPs (C) and LMPs (D) in normal weight, overweight and obese adults. Values are mean±SEM. *P<0.05.

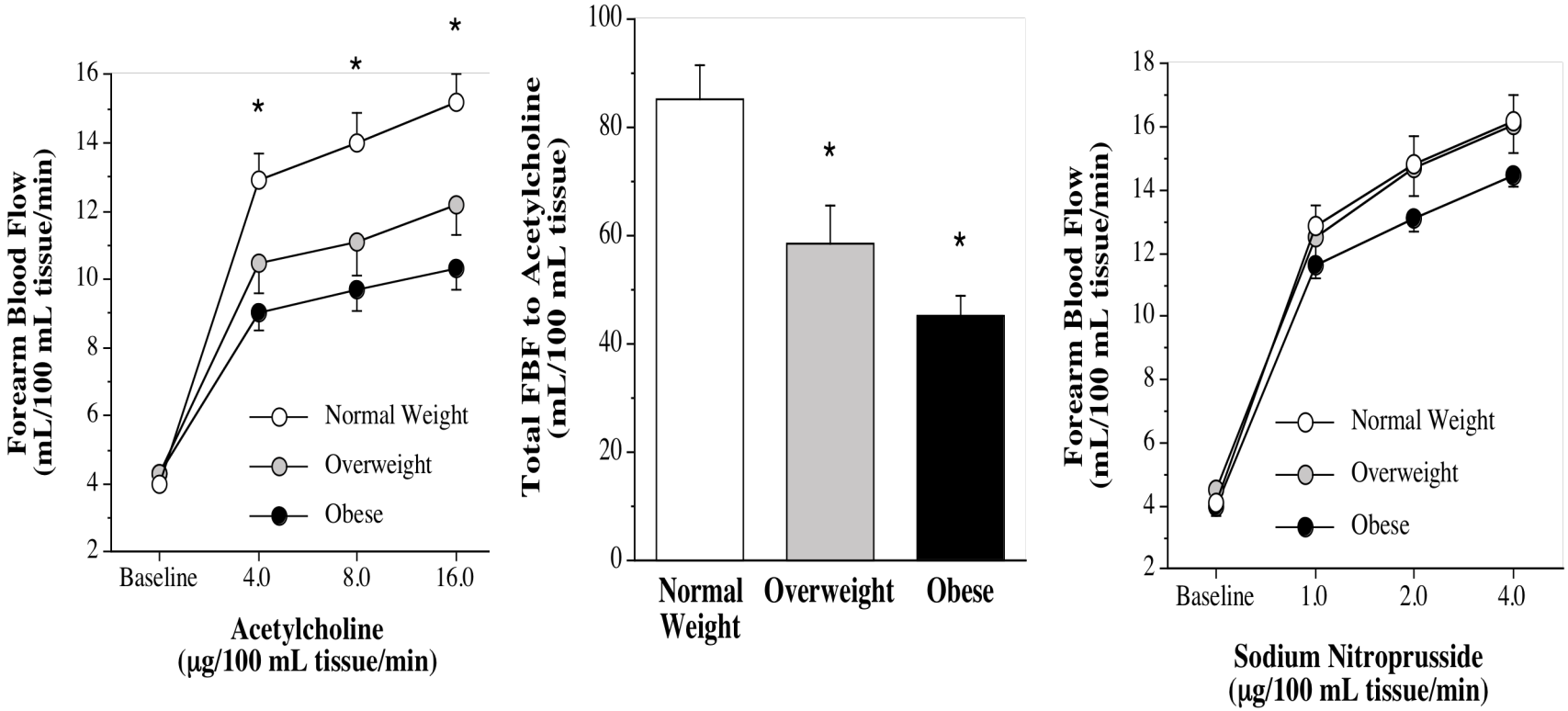

Figure 2: FBF response (A0 and total FBF (area under the curve; B) to acetylcholine and RBR response to sodium nitroprusside in normal weight, overweight and obese adults. Values are mean±SEM. *P<0.05.

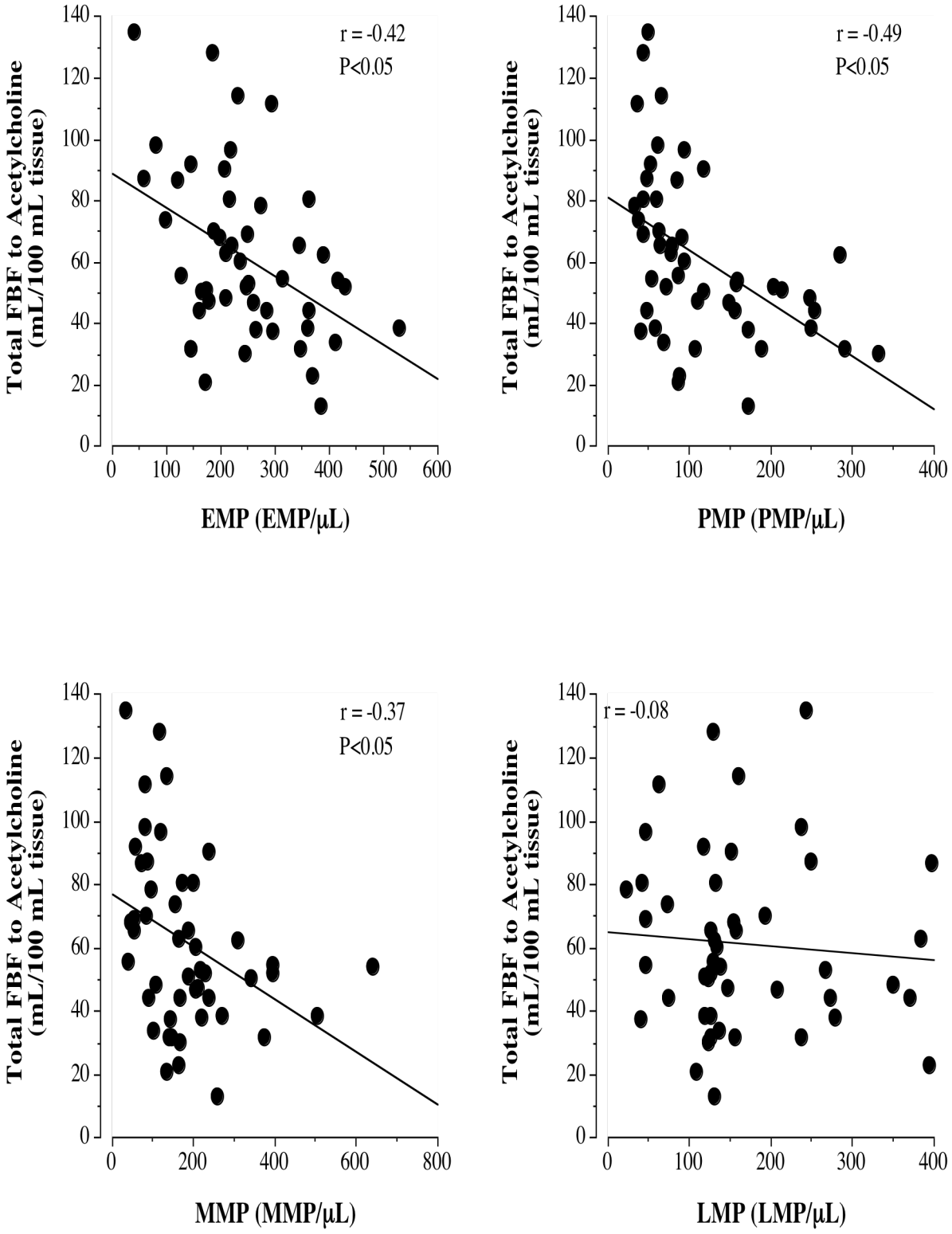

Figure 3. Relation between total FBF to acetycholine and circulating levels of EMPs (A), PMPs (B), MMPs (C) and LMPs (D) in overweight and obese adults.

Exercise Intervention Study:

All 20 overweight/obese adults completed the three-month exercise intervention study (Table 2). There were no significant changes in anthropometric or metabolic characteristics following the exercise intervention. However, aerobic exercise training significantly increased exercise time to exhaustion and significantly decreased heart rate at the same absolute submaximal workload (70% of baseline VO_2 max).

Regular aerobic exercise training significantly lowered circulating microparticle concentrations in both the overweight and obese adults (Figure 4). In the overweight adults, circulating concentrations of EMPs (from 292$\pm$34 to 169$\pm$24 EMP/μL), PMPs (from 125$\pm$19 to 57$\pm$8 PMP/μL), MMPs (from 232$\pm$56 to 151$\pm$32 MMP/μL) and LMPs (from 171$\pm$21 to 124$\pm$ 13 LMP/μL) were ~30-55% lower after the exercise intervention. Similarly, in the obese adults the exercise training intervention lowered (~40-60%; $P<0.05$) circulating concentrations of EMPs (from 311$\pm$36 to 188$\pm$28 EMP/μL), PMPs (from 168$\pm$26 to 72$\pm$13 PMP/μL), MMPs (from 252$\pm$43 to 148$\pm$28 MMP/μL) and LMPs (from 166$\pm$26 to 93$\pm$14 LMP/μL). Of note, in both the overweight and obese adults regular aerobic exercise lowered circulating concentrations of EMPs, PMPs, MMPs and LMPs to levels comparable to that observed in the normal weight adults.

Table 2. Selected Subject Characteristics of the Exercise Intervention Study

	Overweight (n=9)		Obese (n=11)	
Variable	Before Training	After Training	Befor Training	After Training
Body Mass, kg	86.1±3.3	85.7±3.2	95.3±3.5	95.3±3.7
BMI, kg/m^2	28.2±0.2	28.1±0.3	31.3±0.4	31.4±1.6
Body fat, %	33.2±2.4	33.2±2.3	39.3±2.3	38.9±2.6
Waist Circumference, cm	97.7±1.3	96.7±1.4	97.7±2.2	96.8±2.5
$VO_{2\,max}$, L/min	2.8±0.2	2.8±0.2	2.6±0.2	2.8±0.2
Systolic BP, mmHg	113±3	116±3	123±2	124±1
Diastolic BP, mmHg	71±3	73±3	79±2	77±2
Total cholesterol, mg/dL	193.9±12.1	174.8±13.1	200.2±11.9	187.9±11.1
LDL-cholesterol, mg/dL	124.1±10.0	114.4±10.8	115.4±10.8	112.1±9.2
HDL-cholesterol, mg/dL	51.2±2.9	45.0±3.3	55.4±6.4	53.7±6.2
Triglycerides, mg/dL	98.1±12.6	79.0±11.5	139.4±18.2	111.9±20.5
Glucose, mg/dL	91.9±2.7	90.8±2.4	91.4±2.7	92.2±2.4
Insulin, μU/mL	9.4±0.6	8.5±0.9	9.1±0.8	7.9±0.7
Treadmill Exercise Time, min	9.9±0.5	10.8±0.4*	9.8±0.2	10.9±0.4*
Submaximal heart rate, beats/min	152±3	144±3*	153±3	145±4*
Submaximal RPE	16±1	15±1	14±1	14±1

BMI, body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein; RPE, rating of perceived exertion. Values are mean±SEM. *P<0.05 vs. before training.

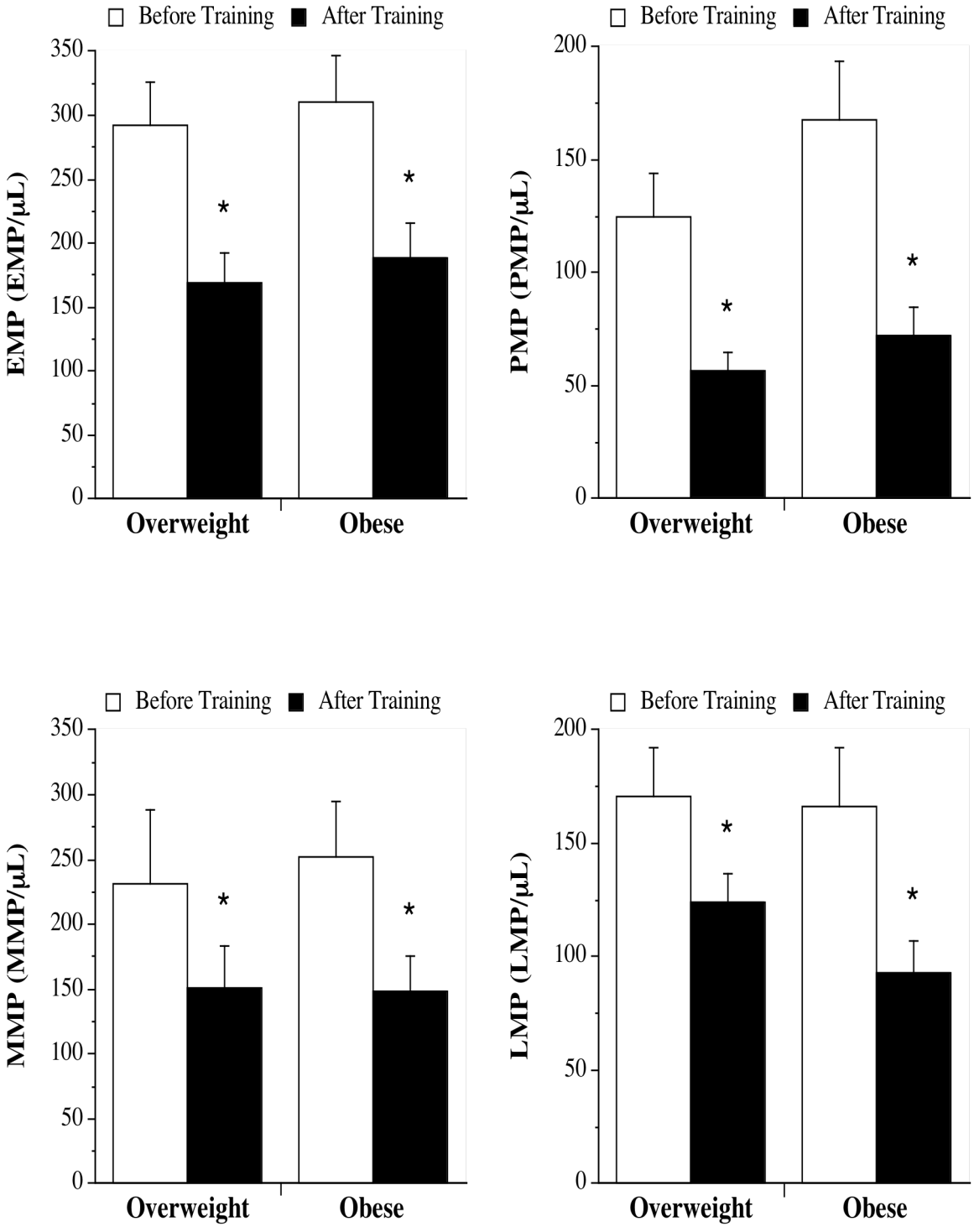

Figure 4. Circulating levels of EMPs (A), PMPs (B), MMPs (C) and LMPs (D) in overweight and obese adults before and after 3 months of aerobic exercise training. Values are mean±SEM. *P<0.05.

DISCUSSION

The primary new findings of the present study are that: 1) circulating concentrations of EMPs, PMPs, MMPs and LMPs are elevated in overweight and obese adults compared with their normal weight peers; 2) circulating levels of EMPs, PMPs and MMPs are inversely related with endothelial vasodilator function; and 3) regular aerobic exercise training, independent of weight loss, reduces circulating microparticle concentrations in previously sedentary overweight and obese adults. Increased circulating concentrations of microparticles may contribute to the increased risk of atherosclerosis and thrombosis with overweight and obesity. In addition, the association between elevations in EMPs, PMPs and MMPs and endothelial dysfunction suggest that these microparticles are potential circulatory biological marker of endovascular health. Given the known links between elevated circulating microparticles and increased vascular risk and events, it is clinically relevant that regular aerobic exercise can lower microparticle concentrations in overweight and obese adults.

Cell-derived microparticles represent a heterogeneous population of anucleod, submicron, plasma membrane vesicles [94]. Although released constitutively by most cells, the influence of microparticle bioavailability, and their source of origin, on vascular risk and disease has increased in clinical importance in the past decade. Elevations in circulating microparticles have been linked with a host of cardiovascular/cerebrovascular diseases such as: heart failure, [95,96] coronary artery disease (CAD), [97-99] ischemic stroke, [100,101] metabolic syndrome, [102] diabetes mellitus [103,104] and hypertension [105]. Moreover, microparticles have been shown to be etiologically involved in thrombosis formation [106], plaque instability [107-109] and plaque rupture [110]. Indeed, circulating microparticles have been shown to contribute etiologically to the progression of atherosclerosis through a variety mechanisms such as promoting inflammation and oxidative stress [53], optimizing

coagulation [111] and altering vascular endothelial cell function [112]. In the present study, we demonstrate for the first time that circulating microparticles are elevated in metabolically healthy overweight and obese adults. Indeed, concentrations of EMPs, PMPs, MMPs, and LMPs were higher in the overweight and obese compared with normal weight adults. Moreover, aside from PMP concentrations that were highest in the obese adults, there were no significant differences in circulating EMPs, MMPs and LMPs between the overweight and obese adults. This finding complements previous studies demonstrating similar levels of endothelial dysfunction in overweight and obese adults compared with normal weight adults [113]. In fact, in the present study the impaired vasodilator response to acetylcholine was not significantly different between the obese and overweight groups. Thus, it appears that adiposity-related elevations in circulating microparticles occur before the onset of clinical obesity and, as such, may contribute to the increased risk and prevalence of atherosclerosis in overweight adults.

The mechanisms responsible for elevated circulating concentrations of EMPS, PMPs, MMPs and LMPs in overweight and obese adults are not clear. A major, and predominant, factor underlying microparticle release from these various sources is parent cell activation [114]. Cellular activation triggers a host of upstream intracellular signaling cascades which results in upregulation p38 mitogen-activated protein kinase (MAPK) and/or caspase-2 activity [115]. Once activated, p38 MAPK and caspase-2 cleave Rho Kinase II inducing cytoskeletal and plasma membrane rearrangement resulting in microparticle vesiculation and release [115]. It is well established that increased adiposity is associated with endothelial cell, platelet, monocyte and leukocyte activation [116-118]. Indeed, there is strong evidence for adiposity-related endothelial cell activation in both animal and human studies [119-121]. In humans, obesity-related endothelial cell activation has been reported in both children and adults [122,123]. Moreover, the degree of platelet

activation has been reported to be higher in obese compared with overweight adults [117] and Coban et al. [124] demonstrated a robust positive correlation between BMI and platelet activation. Thus, our finding of higher PMPs in obese compared with overweight (and normal weight) adults is consistent with, and likely indicative of, greater platelet activation with increased levels of adiposity.

It has been suggested that circulating microparticles can serve as biomarkers of vascular health, particularly endothelial dysfunction. Werner and colleagues [97] demonstrated that circulating EMPs were associated with coronary endothelial function in patients with coronary artery disease. Moreover, the microparticles predicted the severity of endothelial dysfunction independent of traditional risk factors. In the present study, EMPs, PMPs and MMPs were inversely associated with the FBF responses to acetylcholine. Secondary analysis within the overweight and obese groups only also demonstrated an inverse relation between these microparticle subspecies and endothelial vasodilator function. Although we cannot be definitive given the relative small sample size, our findings suggest that the circulating levels of EMPs, PMPs and MMPs may serve as biomarkers of endothelial function in overweight and obese adults. Interestingly, there was no relation between LMPs and the FBF responses to acetylcholine. LMPs have been reported to stimulate the expression of proinflammatory genes in endothelial cells [112] and are densely packed in atherosclerotic lesions [110]. Endothelial dysfunction is not limited to vasodilation [125], it is possible that correlations between LMP and other measures of endothelial function may exist. For example, given the links between circulating LMPs and thrombosis; it is possible that LMPs may emerge as a biomarker of thrombotic risk and be associated with endothelial fibrinolytic rather than vasodilator dysfunction in overweight and obese adults.

Because circulating concentrations of microparticles have been shown to be higher in multiple cardiovascular diseases and are associated with disease severity and outcome [97,98,105,126] there is growing interest in determining the effects of lifestyle and pharmacological interventions on circulating levels. Indeed, it has been suggested that reductions in circulating microparticles in response to a treatment intervention could serve as a biomarker of risk/disease regression and treatment efficacy [127]. For example, pioglitazone, a PPAR-[redacted] ligand, has been shown to reduce circulating EMPs in adults with metabolic syndrome potentially contributing to their favorable vascular effects [128]. To date, most studies involving exercise and circulating microparticles have focused on the acute effects [129-131], with varied results. For example, acute aerobic exercise has been shown to cause a transient increase in microparticle counts in healthy adults but not individuals with cardiovascular disease [132]. In the present study, regular aerobic exercise (daily walking) markedly reduced circulating EMPs, PMPs, MMPs and LMPs in both overweight and obese adults, without a change in adiposity. Importantly, the observed changes in microparticle count occurred in response to a home-based, moderate-intensity aerobic exercise program that can be performed by most, if not all, middle-aged and older overweight and obese adults. The likely mechanisms responsible the reduction in circulating microparticles in response habitual aerobic exercise is a reduction in cell activation. Indeed, Byrkjeland et al. [133] demonstrated that chronic aerobic exercise reduces endothelial cell activation. Similar findings have been reported regarding aerobic exercise training and platelets [134]. Future studies are needed to determine the clinical consequences of exercise-induced reductions in circulating microparticles and their functional phenotype. It is plausible that numerical and functional changes in circulating microparticles may contribute to the cardioprotection conferred by regular aerobic exercise.

There are a few experimental limitations regarding this study that deserve mention. Firstly, considering our cross-sectional study design to address the influence of overweight and obesity on circulating microparticles we cannot dismiss the possibility that genetic and/or lifestyle factors might have affected the results of our group comparisons. However, to minimize the influence of lifestyle behaviors, all subjects were nonsmokers, were not taking medication, dietary supplements or vitamins that could influence circulating microparticles and did not differ in habitual physical activity. In addition, we studied carefully screened normal weight, overweight and obese adults to eliminate the confounding effects of clinically overt cardiovascular and metabolic disease and risk factors. Indeed, a variety of comorbidities that often accompany increased adiposity such as dyslipidemia, hyperglycemia and hypertension have been shown to be associated with elevations in circulating microparticles [105,126]. While other studies have suggested that circulating microparticles are increased with obesity [79,135-137], the presence of these risk factors or disease severely compromise their interpretation. This is the first study to determine the influence of increased adiposity per se on circulating microparticles. Secondly, with regard to the exercise intervention, the absence of a non-exercise control group does not allow us to completely rule out the possibility that our results are due to chance, experimental bias or both. However, the 3-month home-based aerobic exercise training program used in the present study has been repeatedly shown to confer important vascular benefits, independent of changes in body mass and/or cardiometabolic risk factors, in overweight and obese adults similar to those study herein [93,138,139]. Thus, we are confident that the exercise-induced changes in EMPs, PMPs, MMPs and LMPs were indeed a primary effect of the intervention.

In conclusion, the results of the present study demonstrate that overweight and obesity is associated with elevations in circulating EMPs, PMPs, MMPs and LMPs, independent of other

cardiovascular risk factors. Circulating levels of EMPs, PMPs, and MMPs are inversely related to endothelial function, suggesting that they may be a novel biomarker of vascular health. Elevations in circulating microparticles may contribute to the increased risk of atherosclerosis in both overweight and obese adults. Importantly, elevations in EMPs, PMPs, MMPs and LMPs with overweight and obesity are not irreversible. Regular aerobic exercise, without weight loss, significantly lowers the concentration of these microparticles in overweight and obese adults. Due to the potential mechanistic links between circulating microparticles and vascular disease, future studies are needed to characterize the functional phenotype associated with overweight and obesity in order to address their pathogenicity.

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CHAPTER V

CONCLUSION

Microparticles have attracted increasing attention from various fields in medicine and biology due to their role as active mediators of physiological and pathological processes. Now recognized as sensitive biomarkers of disease risk, progression, severity and outcome, it is of clinical importance to develop protocols aimed at accurately isolating, characterizing and quantifying these submicron vesicles. An essential feature of my dissertation was the development of technologically challenging protocols aimed at accurately identifying cellular origin, and in some cases, stimulus of release of microparticles from both biologic samples (human plasma) and culture media. The primary new findings of the studies presented herein are as follows:

1. Circulating concentrations of activation-derived and apoptosis-derived endothelial microparticles do not differ between middle-aged men and women. Microparticle-encapsulated miRNA, but not circulating levels of EMPs may provide novel insight into sex-related differences in cardiovascular disase.
2. High glucose stimulates marked increase in endothelial-derived microparticles in addition, these EMPs significantly increase the expression of E-selectin, ICAM-1, VCAM-1 and PECAM-1 on the surface of endothelial cells. These hyperglycemic-related EMPs may contribute to the pathogenesis of vascular disease associated with diabetes.
3. Overweight and obesity is associated with elevations in circulating EMPs, PMPs, MMPs and LMPs, independent of other cardiovascular risk factors. Circulating levels of EMPs, PMPs and MMPs are inversely related to endothelial function, suggesting that they may

be a novel biomarker of vascular health. Elevations in circulating microparticles may contribute to the increased risk of atherosclerosis in both overweight and obese adults. However, elevations in these microparticles are not irreversible as regular aerobic exercise, without weight loss, significantly lowers the concentration of EMPs, PMPs, MMPs and LMPs in previously sedentary, overweight and obese adults.

Collectively, the results of these studies demonstrate the utility of microparticles as reliable biomarkers of vascular health and disease, and as such, important vectors and targets for novel therapeutic approaches.

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