ET-1 SYSTEM ACTIVITY, CARDIOVASCULAR DISEASE RISK FACTORS AND PHARMACOLOGIC INTERVENTION

By

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ABSTRACT

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ET-1 System Activity, Cardiovascular Disease Risk Factors

and Pharmacologic Intervention

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

Endothelin (ET)-1 is a potent vasoconstrictor peptide produced and released by the vascular endothelium. In combination with the endothelial vasodilator nitric oxide, ET-1 plays a central role in the regulation of vascular tone. In addition to its vasoregulatory actions, there is considerable evidence supporting the involvement of ET-1 in the pathogenesis of atherosclerotic vascular disease and its associated risk factors, most notably elevated blood pressure. Nebivolol, a third generation beta(β)-blocker with high selectivity for β_1-adrenergic receptors, has proven to be highly effective in treating hypertension. A distinguishing feature of nebivolol from other beta-blockers is its positive effects on hemodynamic profile, particularly nitric oxide bioavailability. The effects of nebivolol, however, on ET-1-mediated vasoconstrictor tone are unclear. Accordingly, the primary hypotheses associated with this dissertation are: 1) chronic nebivolol treatment will reduce ET-1-mediated vasoconstrictor tone in adult humans with elevated blood pressure; and 2) reduced ET-1 vasoconstrictor activity contributes to the improvement in endothelial vasodilator function associated with nebivolol. To address these hypotheses, we employed a 3-month randomized, double-blind placebo controlled study to determine the effects of nebivolol, metoprolol and placebo on ET-1 vasoconstrictor tone in adults with suboptimal blood pressure. Venous occlusion plethysmography was used to measure forearm blood flow (FBF) responses to intra-arterial acetylcholine, sodium nitroprusside, and

selective and non-selective ET-1 receptor blockade. FBF responses to acetylcholine were also determined with the co-infusion of non-selective ET-1 receptor blockade. The results of this study indicate that although both nebivolol and metoprolol reduced blood pressure to a similar extent: 1) nebivolol, but not metoprolol, treatment reduced both ET_A and ET_B receptor mediated ET-1 vasoconstrictor tone in adult humans with elevated blood pressure; and 2) the reduction in ET-1-mediated vasoconstrictor tone contributes to the nebivolol-induced enhancement in endothelial vasodilator function. Collectively, these findings demonstrate direct effects of nebivolol, independent of reducing blood pressure, on ET-1 system activity. Diminished ET-1 system activity represents a favorable pleiotropic effect of chronic nebivolol treatment independent of blood pressure lowering.

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HYPERTENSION AND PREHYPERTENSION

INTRODUCTION

Hypertension, defined in humans as a systolic blood pressure (SBP) ≥ 140 mmHg and/or diastolic blood pressure (DBP) ≥ 90 mmHg (33), is an epidemic that globally affects ~40% of the adult population over the age of 25 (104) and is estimated to be the cause of more than 7.5 million or ~15% of total deaths per year (130). Accordingly, hypertension has been deemed the most important contributor to the global burden of morbidity and mortality (71). While awareness has grown and pharmacological interventions have continued to evolve to be safer and more effective, prevalence of the disease has risen from 600 million people in 1980 to over 1 billion in 2008 (104), a number expected to exceed 1.5 billion by the year 2025 (89). These statistics are not overly surprising as hypertension is known to be a primarily symptomless disease affecting both men and women regardless of age, race, ethnicity, or socioeconomic status (130). Indeed, the risk of becoming hypertensive in developed countries over the course of a lifetime currently exceeds 90% (131). Within the United States, hypertension is the most common primary diagnosis, estimated to affect roughly 65 million persons (48) or one third of the adult population (142). Of the 65 million, nearly one-third are not aware that they have the disease and of the two-thirds that know of their disease state, only half have their elevated blood pressure controlled (207). Collectively, the aforementioned epidemiological data has placed hypertension at the forefront of global health concerns by clinicians, scientists, and economists.

The impact of hypertension on public health was first realized as a result of large public health studies such as the Seven Countries Study (96) and the Framingham Heart Study (92). These seminal studies provided insight into associations and risk factors that exist between elevated blood pressure and cardiovascular disease (CVD) morbidity and mortality. The impact

of these studies within the medical community resulted in an effort to lower blood pressure in at-risk individuals through increased public awareness and the development of clinical standards for the detection and treatment of elevated blood pressure (134). Accordingly, advisory committees, including the National Heart, Lung, and Blood Institute (NHLBI) and International Hypertension Society (ISH), were established by the National Institutes of Health (NIH) and World Health Organization (WHO). These committees were primary contributors that worked cooperatively on the United States Joint National Committee (JNC), on the Prevention, Detection, Evaluation, and Treatment of High Blood Pressure, a publication that promotes clinical standards for the detection and treatment of elevated blood pressure (33, 134). Indeed, the JNC is continually updated based on current observational and clinical studies (134). For nearly forty years this report has acted as the gold standard reference aiding physicians in identification, cardiovascular risk assessment, and disease management of patients with elevated blood pressure (134).

In the original JNC report, published in 1976, definitive categories were assigned to blood pressure. The publication suggested that blood pressure between 140/90 mmHg and 160/95 mmHg was to be simply monitored and preliminary treatment was not suggested until an individual's blood pressure exceeded 160/95 mmHg (134). Between the original JNC 1 publication and the JNC 3 publication, a substantial amount of novel data was published regarding health risk, outcomes, and treatment associated with elevated blood pressure. In aggregate, results from such studies provided evidence that persistent blood pressure above 140/90 mmHg required treatment regardless of age (3, 134). Accordingly, the JNC 3 publication, published in 1984, established blood pressure of 140/90 mmHg as the threshold used to define hypertension and initiate pharmacologic intervention{Moser, 2006 #1924}. Of note, the definition of blood pressure ≥140/90 mmHg as defining hypertension is one of the only

consistent features of the JNC reports over the years. Further insight from studies such as the Systolic Hypertension in the Elderly Program (SHEP) study (3, 134) led to SBP being viewed as a more relevant marker of blood pressure associated morbidity and mortality than DBP (93, 134), a theme that could not be overlooked in the JNC 6 report, published in 1997. In addition to a greater focus given to SBP, the JNC 6 report redefined the classifications of blood pressure to include: optimal (SBP<120 and DBP<80); normal (SBP<130 and DBP<85); high-normal (SBP 130-139 or DBP 85-89); Stage I hypertension (SBP 140-159 or DBP 90-99); Stage II hypertension (SBP<160-179 or DBP 100-109); and Stage III hypertension (SBP>180 or DBP ≥110) (90, 93). With the seventh and newest publication of the JNC report, Stage II and III as well as normal and high-normal blood pressure classifications were consolidated with the normal and high-normal classifications being relabeled as "prehypertension" (SBP=120-139 or DBP=80-89)(90). This change resulted from peer-reviewed research demonstrating individuals with blood pressure in the prehypertensive range were at a greater risk of experiencing adverse cardiovascular consequences, such as myocardial infarction (70, 199). While prehypertension was not defined as a new disease category, the title change was made to bring attention to individuals that were in need of careful follow-up and changes in lifestyle, if appropriate (33, 104, 130, 134).

CARDIOVASCULAR DISEASE RISK AND ELEVATED BLOOD PRESSURE

Cardiovascular disease is an umbrella term which includes diseases of the heart and blood vessels (104, 130). CVD-associated death represents greater than half of the total deaths due to non-communicable diseases worldwide and has overtaken communicable diseases as the world's major disease burden (104, 173). As the leading cause of death globally, CVD accounts

for 17.3 million deaths per year, a number expected to grow to greater than 23.6 million by 2030 (104). In addition to mortality statistics, the economic burden CVD is estimated to have globally over the next 20 years is immense. In fact, if current preventative and treatment methods do not evolve, it is projected to cost as much as $47 trillion worldwide over the next 25 years (104). In the United States, it is estimated that from 2010 to 2030 the annual direct and indirect medical cost of CVD will increase from $275 billion to over $800 billion, and $200 billion to over $275 billion, respectively (104). The prevention of CVD through the identification and attenuation of significant risk factors is a major ongoing mission of both government and independent institutions such as the NIH, American Heart Association (AHA) and American College of Cardiology (ACC). Insight into risk factors of CVD have been gained through large comprehensive studies such as the Framingham Heart Study (92) and Seven Countries Study (96) of the 1960s and more recently the World Health Organization multinational monitoring of trend and determinants in cardiovascular disease (MONICA) (189) project and the International Heart (INTERHEART) study (130). Such studies have identified and confirmed amendable principal factors that are directly and independently associated with a persons' risk of developing CVD. These risk factors include hypertension, obesity, dyslipidemia, hyperglycemia, tobacco use and physical inactivity (90, 104, 130). Of these risk factors, hypertension is the leading attributable risk factor for CVD and mortality worldwide (104).

Indeed, there have been a multitude of studies to demonstrate that hypertension is associated with CVD (39, 69, 129, 130, 209). For example, in the third National Health and Nutrition Examination Survey (NHANES), an examination of nearly 17,000 subjects, hypertensive adults (age: 18-64 years) had greater than four times the risk of CVD mortality (95% confidence interval (CI): 1.83-9.80) compared to normotensive subjects. Of note, these

results were adjusted for traditional CVD risk factors including age, gender, race, physical activity, smoking status, obesity, and hypercholesterolemia (69). Furthermore, the Goettingen Risk, Incidence, and Prevalence Study (GRIPS) demonstrated that in a 10-year follow-up of 5,700 adult males, those with hypertension at baseline had twice the risk ($p<0.001$) of myocardial infarction (MI) compared to those with normal blood pressure (39). Moreover, an analysis by Sipahi et al. (172) demonstrated that two years after baseline measurements, adults with blood pressure in the hypertensive range had significantly larger atheroma volumes compared to adults with normal blood pressure ($p<0.01$), indicating an exacerbated progression of coronary atherosclerosis in hypertensive adults.

In addition to the relation demonstrated between CVD and hypertension, a number of large observational studies have shown that a significant continuous, consistent and independent relation exists between the risk of CVD morbidity and mortality and blood pressure levels below the classical definition of hypertension (68, 83, 112, 113, 123, 149, 198). Importantly, prehypertension is not only associated with an increased risk for the development of clinical hypertension, but is also an independent risk factor for the development of CVD (69, 106, 123). For example, in a meta-analysis of over one million adults, Lewington et al. (112) showed a doubling in CVD risk for every 20/10 mmHg increase in blood pressure starting at 115/75 mmHg. Furthermore, in a longitudinal analysis based on over 6,800 Framingham Heart Study participants with prehypertension (ranging in age from 35 to 64 years), Vasan et al. (198) showed a stepwise increase in cardiovascular event rates over a period of 10 years. Moreover, blood pressure in the prehypertensive range is also associated with a 76% increased risk of myocardial infarction and a 36% increased risk of heart failure in an eight-year follow up study of over 60,000 postmenopausal women (83).

SUMMARY

Unequivocal evidence indicates elevated blood pressure poses a significant and independent global healthcare burden (33, 78, 105, 119, 129). Additionally, through epidemiological and investigational research, it is generally understood that CVD, the leading cause of death globally (104), is a prominent sequelae of elevated blood pressure (69, 94, 113, 212). Thus, attenuating the increased risk of developing CVD caused by elevated blood pressure, as well as elevated blood pressure associated morbidity and mortality requires continued investigation into the etiologies of CVD and elevated blood pressure and what role blood pressure lowering interventions may play in preventing CVD pathogenesis.

THE VASCULAR ENDOTHELIUM

The vascular endothelium is a continuous monolayer of cells on the luminal surface of all blood vessels. Once thought to be a quiescent interface between blood and the vascular smooth muscle, the vascular endothelium is now recognized as an important physiologically active regulatory organ (26). Due to its location, the vascular endothelium continuously receives both chemical and physical stimuli. The ability of the vascular endothelium to respond to various stimuli quickly and acutely is imperative to the overall health and homeostasis of the vasculature (61, 200). The functions of the vascular endothelium are primarily mediated through autocrine and paracrine signaling with the release of a variety of vasoactive substances (139). Such vasoactive substances are tightly regulated by the vascular endothelium and play a major role in endothelium-dependent control of metabolic, structural and immunological functions within the vessel (61). Vascular endothelial functions include, but are not limited, to the regulation of vascular tone via nitric oxide (NO) and endothelin production, fibrinolysis, thrombosis,

angiogenesis, and the local suppression of inflammation (49). Certainly, the factors that contribute to the development and progression of CVD are largely mediated through damage to the vascular endothelium resulting in dysfunction (46, 121, 170, 193, 197). Thus, the maintenance and health of the vascular endothelium is paramount to maintaining vascular homeostasis and attenuating CVD risk (46, 114, 121, 141).

THE VASCULAR ENDOTHELIUM AND THE ENDOTHELIN SYSTEM

As one of the primary functions of a healthy vascular endothelium, the maintenance of vasomotor tone is accomplished by the tight regulation of both vasodilatory and vasoconstricting agents (49). The predominant vasoconstrictor agents produced by the vascular endothelium are a family of molecules known as endothelins (ETs). The endothelins are comprised of four peptides named according to the order in which their genes were sequenced: ET-1, ET-2, ET-3 and ET-4 (124). Of the four peptides, ET-1 is the major vascular isoform and the most extensively studied (162, 168). Initial animal (214, 215) and human studies (214) by Yanagisawa and colleagues demonstrated endothelin-1 to be the most potent vasoconstrictor peptide synthesized by the endothelium. Subsequent studies showed the effect of ET-1 on vascular tone was dependent on receptor type activation, ET_A or ET_B, and its location on either the endothelium or vascular smooth muscle (45). Clinically, elevations in ET-1 system activity have been associated with a myriad of cardiovascular pathologies that include hypertension (45, 85, 166), coronary artery disease (154), myocardial infarction and congestive heart failure (95). Relations that have promoted investigation into the role elevated ET system activity may play in the etiology of CVD and how attenuating elevated ET system activity, and therefore

reestablishing vascular tone homeostasis, may lessen the risk of CVD development and progression.

COMPONENTS OF THE ENDOTHELIN SYSTEM

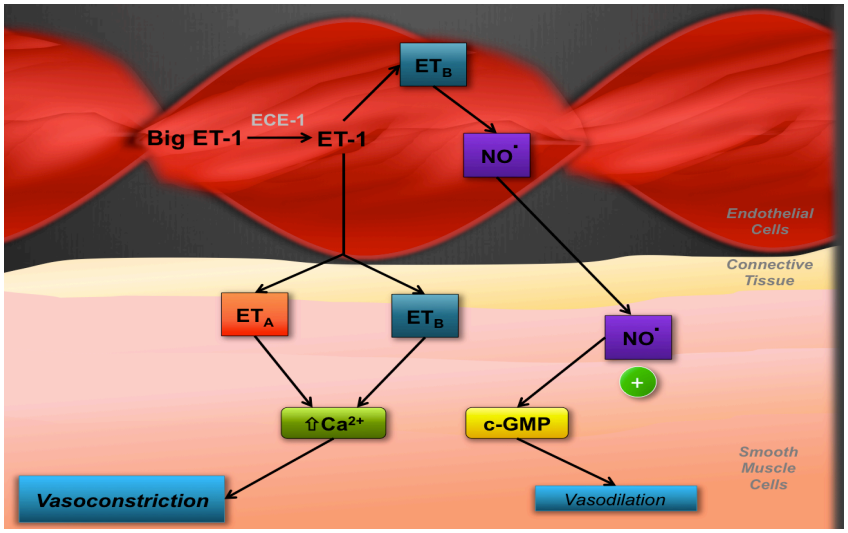
Endothelin-1

ET-1 is constitutively released by the vascular endothelium with the rate limiting step to its synthesis occurring at the level of transcription (133, 215). Downregulation of ET-1 gene transcription is known to be driven by shear stress, NO, prostacyclin, and atrial natiuritic peptide (174). Conversely, epinephrine, angiotensin II, thrombin, inflammatory cytokines, transforming growth factor-β, vasopressin and bradykinin are factors responsible for upregulation (133, 174). Transcription of the 5 exon ET-1 gene resulting in ET-1 mRNA is quickly translated to the 212-amino acid protein, prepro-ET-1 (133, 156). Prepro-ET-1 is translocated from the nucleus to the cytoplasm where proteolytic cleavage by endopeptidase yields the intermediate molecule big ET-1 (156, 174). Big ET-1 is then cleaved once more by endothelin converting enzyme to the 21-amino acid peptide ET-1 (174). Once in active form, the vast majority (>80%) of ET-1 is released from the cytoplasm abluminally towards the vascular smooth muscle where local levels of ET-1 within the vascular wall are greater than 100 fold that found in circulation (179).

Endothelin Receptor Subtype A and Subtype B

The effects of ET-1 on the human vasculature are constituted through its binding to two distinct receptor subtypes: ET_A receptors expressed exclusively on vascular smooth muscle cells and ET_B receptors expressed on both vascular smooth muscle and endothelial cells (164). While both ET_A and ET_B receptor subtypes are of the 7-transmembrane domain G-protein coupled

receptor family, the binding affinity of ET-1 is highest, ~100 fold greater, for the ET_A receptor (11). This is an important physiological phenomenon since ET_A receptors are primary recognized for vasoconstriction and vascular smooth muscle growth promotion and ET_B receptors for vasodilation/inhibition of vasoconstriction and clearance of the ET-1 peptide (11, 168). The primary vasomotor effects of both ET_A and ET_B receptors are coupled to the phospholipase-C cascade, which, when activated on the vascular smooth muscle, results in increased intracellular calcium causing phosphorylation of myosin kinase and subsequent smooth muscle cell contraction (11, 133). Within endothelial cells, phospholipase C-mediated increase in intracellular calcium levels results in the activation of calcium sensitive endothelial nitric oxide synthase, resulting in synthesis of the potent vasodilators NO and prostacyclin (45, 133). Thus, the ET-1 peptide has dueling effects on vascular tone and the dysfunction associated with ET-1 system activity and increased vasoconstrictor tone may, at least in part, be due to altered expression of the ET receptors on the cellular membranes of both the endothelium and smooth muscle.



Overview of the Endothelin-1 System. ET, endothelin receptor; ECE-1, endothelin converting enzyme; NO, nitric oxide; Ca2+, calcium; c-GMP, cyclic-guanine monophosphate.

Secondary responses of ET-1-mediated ET_A activation include: 1) phosphorylating the mitogen-activated protein kinase cascade and the subsequent transcription of vascular smooth muscle growth factors (11); 2) inducing inflammation in the vessel wall through the stimulation of cytokines, such as IL-6, and pro-inflammatory factors such as NF-kB (165, 180); 3) increasing cell adhesion molecule expression (120); and 4) stimulating platelet aggregation (98). Indeed, ET-1 expression has been shown to be upregulated in atherosclerotic lesions (50) and, in animal models, selective ET_A receptor blockade has been demonstrated to attenuate atherosclerotic progression (120). A study by Barton and colleagues (10) assessed atherosclerosis in apoE-knockout mice fed a western diet with and without ET_A receptor antagonism. Results of this study demonstrated that in the mice given chronic ET_A receptor antagonism, atherosclerosis in the aorta was inhibited by ~30% ($P<0.05$) without changing cardiometabolic or hemodynamic characteristics. Thus, in addition to its effects on vascular tone, ET-1 activation of the ET_A receptor is a mediator of atherosclerotic pathogenesis.

In contrast to the secondary actions of ET_A receptor activation, the primary secondary action of ET_B receptors has been demonstrated to be initiating the internalization and degradation of the ligand receptor complex, thereby removing active ET-1 from the system (45, 47). In an *in vivo* study by Dupuis et al. (47) the contribution of ET_A and ET_B receptors to ET-1 clearance was assessed in dogs. Interestingly, ET_A receptor blockade with BQ-123 resulted in no significant difference in ET-1 peptide clearance while ET_B receptor blockade with BQ-788 completely abolished ET-1 clearance (from 33.5 ± 6.6 to $-0.3 \pm 2.6\%$; $p<0.05$). Thus, establishing the clearance of ET-1 to be exclusively mediated by the ET_B receptor.

ENDOTHELIN-1 SYSTEM DYSFUNCTION AND ELEVATED BLOOD PRESSURE

Since the discovery of ET-1, a number of studies have demonstrated its effects on the cardiovascular system as well as its link to cardiovascular pathologies, such as hypertension (45, 180). Early animal (65) and human studies (202) demonstrated that the infusion of ET-1 significantly increased mean arterial blood pressure. For example, Vierhapper and colleagues (202) showed that the stepwise exogenous application of ET-1 increased mean arterial blood pressure in healthy men from 87.1±7.3 mmHg to 92.6±8.2 mmHg ($p<0.01$). While studies such as these provided a rational to link ET-1 to hypertension, confounding reports of ET-1 circulating plasma levels in hypertensive adults clouded the understanding of their relation (27, 40). However, as mentioned above, the development of pharmacologic agents selective for ET_A receptor blockade and nonselective for the blockade of both the ET_A and ET_B receptors, *in vivo*, provided the ability to assess local ET-1 system activity. In a seminal study by Cardillo and colleagues (27), the role of ET-1 system activity on the vasculature was assessed utilizing an isolated forearm blood flow (FBF) model to determine the responses to selective and non-selective ET-1 receptor blockade in both hypertensive and normotensive adults. The results of this study definitively demonstrated ET-1 system activity is elevated in persons with hypertension and that elevated ET-1 system activity may be an underlying factor in the increased vascular tone observed in this population (27). In a follow-up study, Weil et al. (208) assessed whether the relation between increased ET-1 system activity and hypertension extended to individuals with blood pressure in the prehypertensive range. Utilizing the same isolated FBF model, Weil and colleagues demonstrated that individuals with prehypertension had elevated ET-1 system activity which may contribute, at least in part, to the increased risk of CVD in the prehypertensive population (208).

Due to the direct link between ET-1 and blood pressure, a number of clinical trials have sought to identify the effectiveness, safety, and tolerability of both selective and non-selective ET-1 receptor blockade in treating elevated blood pressure (101, 168). In patients with essential hypertension, the nonselective antagonist bosentan and the ET_A antagonist darusentan reduce blood pressure to a similar extent as the angiotensin converting enzyme inhibitor enalapril (100, 136). Despite these encouraging data, these drugs are not used for chronic control of elevated blood pressure due to the relatively high liver toxicity and greater incidence of other less severe side effects such as headache and peripheral edema (168). It is unknown, however, if there are current well tolerated US Food and Drug Administration (FDA) approved BP medications that may attribute their BP lowering effect, at least in part, through reducing ET-1 system activity. Accordingly, future studies into the mechanisms of well tolerated blood pressure medications, demonstrated to directly influence the vascular endothelium, are needed to assess whether there beneficial action is mediated, at least in part, by reducing ET-1 system activity.

THE VASCULAR ENDOTHELIUM AND FIBRINOLYSIS

Constant contact with circulating blood exposes the vascular endothelium to a variety of pathogenic events that disrupt endothelial cell function (203). One result of vascular endothelium disruption is the activation of an intricate physiological mechanism that results in the formation of fibrin-rich thrombi (61, 200). To maintain fluidity and prevent the progression of atherosclerotic vascular disease, the vascular endothelium is responsible for the lysis of potentially occlusive fibrin-rich thrombi by a process known as fibrinolysis (84, 204). Fibrinolysis is the enzymatic process by which fibrin within a thrombus is proteolytically broken down into soluble products (117, 118, 200, 204). Consistent with most enzymatic systems, fibrinolysis is dependent on the synthesis, availability, activation and clearance of various

substrate components including: plasminogen, tissue-type plasminogen activator (t-PA), and plasminogen activator inhibitors (PAI) (84).

COMPONENTS OF THE FIBRINOLYTIC SYSTEM

Plasminogen

Plasminogen is a single-chain glycoprotein that is the inactive precursor of the fibrinolytic enzyme plasmin (181). Primarily synthesized in the liver, plasma plasminogen concentrations in the healthy adult human are approximately 1.5 to 2.0 μmol/L (37, 153). The sequencing of plasminogen revealed the enzyme has a typical serine protease structure consisting of 791 amino acids and containing an N-terminal preactivation peptide domain, kringle domains, and catalytic protease domains (77, 147, 201). Each of these domains have been demonstrated to have important functional properties for the binding of plasminogen to fibrin and the activation of plasminogen to the active enzyme plasmin, a peptide that functions to enzymatically cleave fibrin (55, 147). Conversion of plasminogen to plasmin is accomplished by the cleavage of a single arginine-valine bond (80, 181, 200). The mediator of the arginyl-valine bond cleavage that converts plasminogen to plasmin is the serine protease t-PA (138, 200).

Tissue-Type Plasminogen Activator

t-PA is an active single-chain serine protease containing 562 amino acids that is synthesized and released luminally by the vascular endothelium (204). t-PA is functionally thought to be the most important endogenous protein that converts plasminogen to plasmin (138, 200). Through binding interactions in the circulation, t-PA can be cleaved from a single-chain to a double-chained molecule (116, 200). In the absence of fibrin, the double-chain form of t-PA

has demonstrated a greater catalytic efficiency compared to the single-chain form of the molecule (28, 116, 200). However, when bound to fibrin, both the single and double chain forms of t-PA have similar affinities and kinetic properties for plasminogen (116). Yet, the physiological significance of the double-chain t-PA molecule's activity in the absence of fibrin, the primary co-factor of t-PA, is currently unclear. Another fundamental component of t-PA efficacy is the location and concentration of t-PA binding proteins (72, 73, 75, 155). It has been demonstrated that the K^m of t-PA when bound to fibrin is decreased several hundred times, effectively maximizing the catalytic efficiency of the enzyme (80, 116). Through chemoilluminescence imaging, t-PA binding proteins have been determined to be most abundant on the surface of vascular endothelial cells with the greatest density being proximal to developing thrombi. This factor provides for optimal localization of fibrinolytic activity (74). Thus, the bond kinetics between t-PA, fibrin, and plasminogen play a critical role in the effectiveness and specificity of the fibrinolytic system to catabolize fibrinous thrombi (80, 200).

The t-PA enzyme has been demonstrated to have an *in vivo* half-life of four minutes (200) and is predominately cleared from the circulation through t-PA clearance receptors found on the surface of hepatocytes (29, 30). Additional t-PA clearance has been demonstrated by the vascular endothelium, but on a much lesser scale (29). Of note, hepatocyte t-PA clearance receptors are suggested to be moderately selective for different naturally occurring t-PA isoforms, but the mechanisms defining these phenomenons have not been elucidated (23, 29, 143). Along with hepatic and vascular endothelial clearance, facultative release from the vascular endothelium and t-PA inhibition are the primary determinates of active t-PA levels within the circulation (29, 30, 200).

The ability of the healthy vascular endothelium to release stored t-PA quickly and acutely is essential for endogenous fibrinolytic focality (86, 99, 137, 169). This is accomplished through intracellular storage vesicles called Weibel-Palade bodies, which release t-PA in significant concentration when the cell receives the proper stimuli (76, 135, 160). The release of t-PA from vascular endothelial Weibel-Palade bodies is accomplished through both pharmacological and mechanical mechanisms. For example, it has been demonstrated that the fibrinolytic cofactor thrombin, the serine protease responsible for the conversion of fibrinogen to fibrin (200), induces the vascular endothelium to release t-PA (111, 135, 163). Additionally, Tappy et al. (185) showed that hypoxia induced a 300% increase in the release of t-PA from the vascular endothelium in the isolated perfused pig ear. Moreover, shear stress has also been shown to be an activating factor of t-PA from the vascular endothelium (41). Such mechanisms significantly increase the availability of active t-PA at or near the thrombus stimulation site *in vivo* (41, 135, 200).

Plasminogen Activator Inhibitor-1

In the circulation, t-PA is primarily found in an inactive state irreversibly bound to PAI-1 (4). PAI-1 is a serine protease inhibitor consisting of 379 amino acids that is responsible for binding to and irreversibly inhibiting t-PA (28, 55, 167, 200). PAI-1 is generally accepted as being the primary endogenous inhibitor of fibrinolysis (4, 176), a finding based on studies such as that by Kluft and colleagues (97) who showed that PAI-1 is responsible for 60% of *in vivo* t-PA inhibition.

Although synthesized in a variety of cells such as hepatocytes, monocytes, and smooth muscle cells (28, 176, 200), PAI-1 is primarily synthesized and released, *in vivo,* from vascular

endothelial cells (31, 176, 182, 200). In healthy individuals, plasma levels of PAI-1 are ~20 ng/mL, but can fluctuate widely (normal range: 0.5-47ng/mL) due to strong diurnal variation in activity and a short half-life (~7 minutes) a factor of rapid clearance by the liver (191, 200). In the circulation, 93% of PAI-1 is stored by platelets, however, when released from platelets, PAI-1 is predominately in the inactive form. Notably, active PAI-1 release from platelets accounts for less than 5% of total PAI-1 activity in the blood (15). The upregulation and release of PAI-1 from endothelial cells is understood to be in response to signaling from endotoxin, lipoproteins, thrombin, hormones and growth factors (167). Therefore providing additional mechanisms for the regulation of fibrinolysis mediated through physiological stimuli on the endothelium (200).

ACTION AND ASSESSMENT OF FIBRINOLYSIS

The stimuli accounting for local fibrinolytic activation are complex. The initial aggregation of platelets to the site of endothelial damage causes the release of PAI-1 to allow for the development of a microthrombi (55, 200). As fibrinolysis is partially mediated by properties inherent to the thrombus, the growth of the thrombi is understood to be a stimulus for the initiation of endogenous fibrinolytic activity (7). Indeed, it has been shown that thrombin-dependent conversion of fibrinogen to fibrin is a stimulus that causes both the release of t-PA and inhibition of PAI-1 release from vascular endothelial cells (141). When bound to fibrin, t-PA undergoes a conformational change that prevents the binding and subsequent inhibition of t-PA activity by PAI-1 (36, 200). When not bound to fibrin, circulating t-PA is rapidly inhibited by PAI-1 (187). The acute release of t-PA along with plasminogen's high affinity for fibrin localizes fibrinolysis to thrombi (55, 200). Therefore, the effectiveness of endogenous

fibrinolysis is highly dependent on the ability of the vascular endothelium to rapidly and acutely release t-PA at or near the site of thrombosis (135, 169).

In the effort to measure endothelial t-PA release, Jern et al. (87) showed that the isolated forearm model can directly assess the release rates of t-PA from the endothelium without confounding factors, such as liver clearance or shifts between complexed t-PA/PAI-1 and free t-PA. In this model, the net release or uptake of t-PA and PAI-1 is determined measuring plasma t-PA concentrations, FBF, and arterial hematocrit values. This is expressed in the following formula:

$$\text{Net release} = (C_V - C_A) * (\text{FBF} * [101\text{-Hematocrit}/100])$$

Where C_V = venous plasma concentration, C_A = arterial plasma concentration, and $(C_V - C_A)$ = arteriovenous concentration gradient. This isolated forearm model is advantageous because, as an intact vascular bed, it has preserved innervation, circulation, blood flow pulsatility, and cell-to-cell interaction (87, 88). This is important because it is the local endothelial release rate of t-PA and not systemic plasma concentrations of t-PA and PAI-1 that determines endogenous thrombolytic capacity (24, 194, 195).

FIBRINOLYTIC DYSFUNCTION AND ELEVATED BLOOD PRESSURE

It is incontrovertible that the healthy vascular endothelium plays an important role in hemostasis. Collectively, prospective and cross-sectional scientific evaluations of elevated blood pressure and endothelial dysfunction provide substantial evidence correlating these two physiological variables (14, 52, 83, 103, 146, 150, 170, 183, 188). A study by Giannarelli et al. (62) demonstrated that, in hypertensive subjects, the endothelial dependent vasodilator

acetylcholine does not cause an increase in t-PA release, paradoxical to the response in subjects with normal blood pressure. The mechanism behind this finding was later clarified to be a reduction in the vascular endothelium's ability to release t-PA mediated by a lessened adrenergic-stimulated response (63). Furthermore, a study by Hrafnkelsdottir et al. (82) reported a significant impairment to desmopressin-induced endothelial t-PA release in adults with hypertension. Thus providing further evidence that endothelial fibrinolytic dysfunction is a consistent characteristic of elevated blood pressure (14, 52, 63, 103, 188). As elevated blood pressure is the primary modifiable risk factor for CVD, the aforementioned relation is consistent with studies demonstrating a significant association between impaired vascular endothelium-dependent fibrinolytic system activity and CVD risk (66, 81, 110, 128, 140, 151, 197, 210).

SUMMARY

The vascular endothelium is a primary component of the cardiovascular system and, when healthy, contributes to homeostasis through the regulation of vasomotor tone (49) and fibrinolysis (7, 46, 117, 200, 204). Due to its anatomical location, the vascular endothelium is commonly exposed to pathogenic agents and stressors, such as elevated blood pressure, that have the propensity to cause damage and dysfunction (46, 103, 175, 186, 188). Resultantly, endothelin-1 system and fibrinolytic system dysfunction has been demonstrated to be independently associated with elevated blood pressure, the progression of atherosclerotic vascular disease, and increased risk for CVD (64, 121, 159, 204).

β-*ADRENERGIC RECEPTOR ACTION AND BLOCKADE*

Blood pressure is a tightly regulated physiological variable that requires the cooperation of several systems including the autonomic nervous and endocrine systems (12). When deranged, these mechanisms and systems are thought to have a role in the development and progression of elevated blood pressure (12, 131). Over the last half century, a diligent effort to dissect the etiologies of elevated blood pressure has led scientists and physicians to develop and prescribe pharmaceuticals that attenuate and reverse the negative effects of elevated blood pressure (90, 134, 177). One such pharmaceutical family is the β-adrenergic receptor blockers also referred to as "β-blockers". This drug family works by blocking the stimulation of β-adrenergic receptors by the catecholamines epinephrine and norepinephrine (19, 20, 126). Though there is little evidence to suggest circulating catecholamines have a clear role in the etiology of hypertension, the effects of β-blockers on the central and peripheral cardiovascular system result in significant reductions in blood pressure and, therefore, are considered to provide an important therapeutic role (5, 9, 12, 213).

β-blockers elicit effects on β_1, β_2, and β_3-adrenergic receptors (6, 19). β_1, β_2, and β_3-adrenergic receptors are G protein-coupled receptors that, when stimulated, propagate intracellular cascades that result in numerous cellular responses dependent on the class of β-adrenergic receptor and tissue in which β-adrenergic receptor is located (178, 196). β_1-adrenergic receptors are predominant in cardiomyocytes, where they comprise 80% of adrenergic receptors. Activation of β_1-adrenergic receptors leads to an increase in intracellular calcium via the activation of the adenylyl cyclase-cyclic adenosine monophosphate (cAMP)-protein kinase A (PKA) pathway (19, 196). Increased intracellular calcium is known to induce the activation and upregulation of contractile proteins, such as troponin I, resulting in increased heart rate

(chronotropy) and force of contraction (inotropy) (19, 38, 196). Thus, the effects of β_1-adrenergic receptor blockade are primarily through decreasing heart rate and force of contraction (19, 126, 171, 196). Ultimately, blockade of β_1-adrenergic receptors has been shown to reduce cardiac output resulting in an overall reduction in blood pressure (91, 126).

In the cardiovascular system, β_2-adrenergic receptor activation results in vascular smooth muscle relaxation through the inhibition of calcium dependent pathways (19, 126, 178). In contrast to the effects of β-adrenergic receptor activation in the heart, activation of β-adrenergic receptors in the vascular smooth muscle, where β_2-adrenergic receptors predominate, leads to cAMP-dependent inhibition of myosin light chain kinase (MLCK) (19, 38, 117, 178). MLCK activation is necessary for vascular smooth muscle contraction, therefore its inhibition results in vascular smooth muscle relaxation (19, 38, 178). Thus, blockade of β_2-adrenergic receptors has been shown to increase total peripheral resistance (126).

Recent studies have demonstrated β_3-adrenergic receptors are located in both the coronary system and peripheral vasculature (43, 44, 132, 152). Investigation into the β_3-adrenergic receptors is still in its infancy and knowledge of mechanisms, location density, and kinetics are ongoing (122). However, recent studies have provided evidence that activation of β_3-adrenergic receptors in both the atria and ventricles are antipathetic to those of the β_1 and β_2-adrenergic receptor activation (59, 132). Furthermore, the effects of β_3-adrenergic receptor activation, ubiquitously found both on the endothelium and vascular smooth muscle, have been demonstrated to induce vasodilation (44, 51). The primary mechanism for the observed vasodilation has been demonstrated to be through increased synthesis and release of nitric oxide (NO) by the vascular endothelium resulting in activation of the cGMP pathway, thus reducing cytoplasmic calcium in the vascular smooth muscle (152, 196). From a medical intervention

standpoint, unlike the focus on receptor blockade associated with the β_1 and β_2-adrenergic receptors, the recent focus regarding β_3-adrenergic receptors has been on their activation, which has been shown to reduce peripheral resistance and blood pressure by increasing bioavailability of the potent vasodilatory molecule NO (32, 57, 126, 196).

β -ADRENERGIC RECEPTOR BLOCKER CLASSIFICATION

There are currently 17 β-blockers approved by the FDA (Table 1) (126). Although each of these 17 β-blockers has a specific and unique pharmacological mechanism, they have been broadly classified based on β_1 vs. β_2 receptor selectivity (cardioselectivity) and ancillary activity. This classification includes: first generation or nonselective β-adrenergic antagonists; second generation or selective β_1-adrenergic antagonists; and third generation or selective β_1-adrenergic antagonist with ancillary properties (115, 126, 127).

Table 1. Characteristics of FDA Approved β-Blockers

Drug	β_1 / β_2 Selectivity	Ancillary Properties
Nonselective β-adrenergic antagonists		
Carteolol	0	N/A
Nadolol	0	N/A
Penbutolol	0	N/A
Pindolol	0	N/A
Propranolol	0	N/A
Sotalol	0	Additional antiarrhythmic properties
Timolol	0	N/A
Selective β-adrenergic antagonists		
Acebutolol	+	N/A
Atenolol	+	N/A
Betaxolol	++	N/A
Bisoprolol	++	N/A
Esmolol (IV only)	++	N/A
Metoprolol	++	N/A
Selective β_1-adrenergic antagonists with ancillary properties		
Nebivolol	+++	Endothelium-dependent, nitric-oxide-mediated vasodilation
Labetalol	+	α_1-adrenergic blocking activity; direct β_2 vasodilatory activity
Carvedilol	0	α_1-adrenergic blocking activity; vasodilation

Basic characteristics of FDA approved β-adrenergic receptor blockers. 0 indicates equal selectivity; + indicates moderate β_1 over β_2 selectivity; ++ indicates high β_1 over β_2 selectivity; + indicates very high β_1 over β_2 selectivity. Adopted from Mason et al. *J Cardiovasc Pharmacol* 2009;54:123–128.

First generation β-blockers were shown to be a useful treatment for reducing CVD morbidity and mortality (5, 54). Propranolol, specifically, was accepted as a premier treatment for cardiac arrhythmia (54), angina pectoris (126), hypertension (56) and hypertrophic cardiomyopathy (56, 144). In a study published in 1969, Prichard et al. (8) detailed the benefits of propranolol as a potent blood pressure reducer. Furthermore, the results of the β-Blocker Heart Attack Trial (BHAT) demonstrated that a regimen of propranolol reduced sudden CVD associated death by 28% compared with a non-treatment group (25). Despite the beneficial evidence regarding the affect of first generation β-blocker treatment on reducing blood pressure, a number of studies showed adverse effects, such as increased triglycerides and bronchospasm, that potentially outweighed β-blocker benefit (107-109). Indeed, propranolol was demonstrated to induce a reduction in plasma high density lipoproteins and an increase in low density lipoproteins, thereby increasing a subject's overall risk for CVD (108). These deleterious effects observed with propranolol treatment lead to further pharmacological and biochemical advancements and the eventual development of the second generation of β-blockers.

Second generation β-blockers are known as being selective receptor blockers due to their β_1-adrenergic receptor antagonist properties, as well as having little or no effect on β_2-adrenergic receptors (19, 126). Metoprolol is an example of a second generation β-blocker that has been shown to: 1) significantly reduce the pathogenesis of common risk factors for CVD (13, 53); 2) be effective in treating congestive heart failure (2); and 3) reduce mortality in heart failure patients (79) without causing the most notable negative effects associated with non-selective β-blockers (211). While second generation β-blockers are still widely prescribed to attenuate the risk factors of CVD, such as hypertension, they still are associated with limitations that dampen

their therapeutic effectiveness. For example, at high doses second generation β-blockers demonstrate a decline in their selectivity towards β_1-adrenergic receptors (126, 127).

Nebivolol, labetalol and carvedilol are all considered third generation β-blockers and therefore have beneficial ancillary properties beyond selective β_1-adrenergic receptor blockade (126). Indeed, in comparison with the second generation β-blockers bisoprolol and metoprolol, nebivolol has three to four times the selectivity for β_1-adrenergic receptors (126, 192). While the ancillary properties of all three third generation drugs predominately involve peripheral vascular vasodilation, the specific mechanisms each drug utilizes to elicit its effect differs greatly. Notably, labetalol provokes a reduction in total peripheral resistance by eliciting vasoconstriction through α_1-adrenergic antagonist and direct β_2-adrenergic agonist properties, carvedilol's vasodilatory effects are through α_1-adrenergic antagonist activity, and nebivolol's are due, at least in part, to eliciting endothelium-dependent nitric-oxide-mediated vasodilation (115, 125, 126). In conjunction with their coronary cardioprotective properties, the aforementioned ancillary properties of third generation β-blockers has made them important and effective contributors to the treatment of hypertension and reduction of CVD risk (21, 54, 126).

NEBIVOLOL AND VASCULAR ENDOTHELIAL FUNCTION

In addition to the demonstrated effects nebivolol has on the coronary system through β_1-adrenergic receptor blockade, it has been shown to have hemodynamic effects that are unlike those of other β-blockers (22, 44, 59, 161). Specifically, nebivolol effects vasodilation through two primary mechanisms: 1) via the endothelium-dependent nitric oxide-mediated pathway (16, 18, 35, 102, 157) and 2) through the scavenging of oxygen free radicals (122, 206). A study by Cockcroft et al. (35) found that nebivolol elicited a ~90% increase in FBF from baseline values.

This increase was attenuated by the infusion of the NO synthase inhibitor N^G-monomethyl L-arginine (L-NMMA), indicating the observed increase in FBF was due to increased NO bioavailability. Moreover, an *in vitro* study by Wagenfeld et al. (206) provided data indicating the vasoconstricting effects of hydrogen peroxide on porcine ciliary arteries were dampened with the presence of nebivolol, demonstrating its anti-oxidative properties. While the exact mechanisms for the established increase in peripheral vascular vasodilation with nebivolol are still not fully understood, studies suggest it may be due to the agonistic properties of nebivolol on β_3-adrenergic receptors (51). The elucidation of this theory and investigation into additional putative benefits of nebivolol on endothelium-dependent vascular function are cause for future studies.

SUMMARY

For more than half a century, β-blockers have been successfully used to reduce blood pressure (126). However, first generation β-blockers have the propensity to exacerbate other metabolic risk factors of CVD (3, 127, 205). While second generation β-blockers yielded significant advancements in reducing negative metabolic effects, selectivity is generally inversely related with dose concentration (42, 184). Thus, more recent drug design has produced a third generation with: increased effectiveness and sensitivity for coronary specific β_1-adrenergic receptor blockade (42, 148); limited adverse side-effects commonly associated with non-specific β_2-adrenergic receptor blockade (60, 108); and ancillary benefits that further reduce the risk of CVD (44, 148, 157, 158).

FUTURE DIRECTIONS

While there are a large number of observational and clinical studies detailing the pathologies of hypertension, the current methods of treatment are often debated (17, 190, 196). It is important to note that only 27% of Americans with hypertension are currently taking a medication to lower their blood pressure to below 140/90 mmHg (1). This leaves more than 70% of the hypertensive population with uncontrolled or poorly controlled blood pressure (1). Enticing features in using third generation β-blockers, such as nebivolol, as an interventional therapy for hypertension are: overall safety (34, 67); effectiveness both as a monotherapy and combined with other antihypertensive drugs (190); and efficacy in the prevention and treatment of CVD (145, 158). However, the mechanisms behind these features have yet to be fully elucidated (58, 148).

Areas of particular interest for future study are the effects of nebivolol on both the endothelin and fibrinolytic systems. At present, it is unknown what effect, if any, nebivolol has on: 1) attenuating elevated ET-1 levels or; 2) the capacity of the vascular endothelium to release t-PA in adults with elevated blood pressure. Insight into the effects of nebivolol on these systems may further elucidate the beneficial vascular effects and observed reduction in risk for CVD morbidity and mortality associated with this third generation β-blocker.

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IMPAIRED ENDOGENOUS FIBRINOLYTIC CAPACITY IN PREHYPERTENSIVE MEN

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Running Head: Prehypertension and Endothelial Fibrinolysis

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What is known about topic

- Nearly 50% of blood pressure-related deaths occur in individuals with systolic blood pressure in the prehypertensive range (systolic BP 120-139 mmHg systolic and/or diastolic BP 80-89 mmHg).
- Mechanisms responsible for the increased cardiovascular risk in adults with blood pressure in the prehypertensive range are not fully understood.

What this study adds

- The capacity of the endothelium to release tissue-type plasminogen activator (t-PA), the primary activator of endogenous fibrinolysis, is markedly impaired in middle-aged men with blood pressure in the prehypertensive range.
- The level of impairment in endothelial t-PA release in prehypertensive men is similar to that observed in hypertensive men.
- Diminished endothelial fibrinolytic capacity may underlie the increased cardiovascular risk with prehypertension.

ABSTRACT

Prehypertension (BP 120-139/80-89 mmHg) is associated with an increased risk for future atherothrombotic events. Although the mechanisms underlying this elevated risk are not completely understood, one possibility is that prehypertension is associated with impaired endothelial fibrinolytic capacity. We tested the hypothesis that vascular endothelial release of t-PA is impaired in prehypertensive men. Net endothelial release of t-PA was determined, *in vivo*, in response to intrabrachial infusions of bradykinin (12.5, 25, 50 ng/100 mL tissue/min) and sodium nitroprusside at (1.0, 2.0, 4.0 μg/100 mL tissue/min) in 42 middle-age and older men: 16 normotensive (BP range: 100-119/57-79 mmHg); 16 prehypertensive (BP range: 120-139/76-89 mmHg); and 10 hypertensive (BP range: 140-150/74-100 mmHg). Net release of t-PA antigen was ~25% lower ($P < 0.05$) in the prehypertensive (-0.9 ± 0.8 to 42.4 ± 5.3 ng/100 mL tissue/min) compared with the normotensive (0.5 ± 1.0 to 53.9 ± 6.5 ng/100 mL tissue/min) men. There was no significant difference in t-PA release between the hypertensive (-1.8$\pm$1.6 to 40.8$\pm$6.6 ng/100 mL tissue/min) and prehypertensive groups. Sodium nitroprusside did not significantly alter t-PA release in any group. These data indicate that endothelial t-PA release is diminished in prehypertensive men. Further, the level of impairment in t-PA release seen with clinical hypertension is already apparent in the prehypertensive state. Impaired endothelial fibrinolytic function may underlie the increased atherothrombotic risk associated with blood pressure in the prehypertensive range.

Key Words: Endothelium, tissue-type plasminogen activator, blood pressure, hypertension

INTRODUCTION

Aside from age, elevated blood pressure (BP) is the most predominant cardiovascular disease (CVD) risk factor worldwide [1]. It is well-established that blood pressure-related CVD risk is not limited to values in the clinically hypertensive range (systolic BP > 140 mmHg and/or diastolic BP >90 mmHg), but also involves blood pressures in the prehypertensive range (systolic BP 120-139 mmHg systolic and/or diastolic BP 80-89 mmHg) [2]. Several epidemiological studies have demonstrated that prehypertension is linked not only with an increased risk for developing clinical hypertension but also increased prevalence of myocardial infarction, stroke and congestive heart failure [3]. Moreover, it is estimated that nearly 50% of blood pressure-related deaths occur in individuals with systolic blood pressure in the prehypertensive range [4]. Considering ~70 million adults in the United States are thought to have resting blood pressures in the prehypertensive range, prehypertension represents an important public health issue [5].

Endothelial dysfunction plays a vital role in the initiation, development and progression of atherosclerosis [6, 7]. In addition to vasomotor regulation, a prominent thromboresistant property of the vascular endothelium is its modulatory influence on fibrinolysis. Endothelial cells are the principal site of synthesis and release of tissue-type plasminogen activator (t-PA), the primary activator of the fibrinolytic system [8]. Experimental and clinical data indicate that it is the capacity of the endothelium to release t-PA rapidly and acutely from intracellular storage pools that determines the efficacy of endogenous fibrinolytic activity [9, 10]. Diminished capacity of the endothelium to release t-PA is associated with the pathogenesis of coronary artery disease and associated atherothrombotic events [11, 12] as well as a variety of CVD risk factors such as cigarette smoking[13] and hypertension [14]. For example, Hrafnkelsdottir et al. [14] reported that endothelial

t-PA release is ~40% lower in hypertensive compared with normotensive middle-aged adults. Currently, it is unknown whether blunted endothelial t-PA release observed with clinical hypertension is evident in the prehypertensive state. If so, impaired endothelial fibrinolytic capacity may contribute to the increased vascular risk with prehypertension.

Accordingly, we tested the hypothesis that the capacity of the endothelium to acutely release t-PA is impaired with prehypertension. To address this hypothesis, we employed an isolated forearm model to assess endothelial t-PA release *in vivo* in normotensive, prehypertensive and hypertensive middle-aged and older men.

METHODS

Subjects

Forty-two adult men with normal and elevated blood pressure were studied: 16 normotensive (BP range: 100-119/57-79 mmHg); 16 prehypertensive (BP range: 120-139/76-89 mmHg); and 10 hypertensive (BP range: 140-150/74-100 mmHg). Blood pressure classification was based on JNC-7 guidelines [15], determined by the average of two or more seated BP readings from two separate visits [15]. All subjects were free of overt cardiovascular and metabolic disease as assessed by medical history, physical examination, fasting blood chemistries, and electrocardiograms and blood pressure at rest and during incremental exercise performed to exhaustion. None of the subjects smoked, were taking medications (including vitamins), or performed regular physical exercise for at least 1 year before the start of the study. Prior to participation, all of the 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. All of the procedures were performed according to institutional guidelines.

Measurements

Body Composition

Body mass was measured to the nearest 0.1 kg using a medical beam balance (Detecto, Webb City, MO). Percent body fat was determined by dual energy x-ray absorptiometry (Lunar Radiation Corporation, Madison, WI). Body mass index (BMI) was calculated as weight (kilograms) divided by height (meters) squared. Minimal waist circumference was measured according to previously published guidelines [16].

Treadmill Exercise Test

To assess aerobic fitness, subjects performed incremental treadmill exercise using a modified Balke protocol. Maximal oxygen consumption ($\dot{V}O_2max$) was measured using on-line computer-assisted open circuit spirometry[36].

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 at Boulder. Insulin resistance was estimated using the homeostasis model assessment (HOMA-IR) derived from fasting glucose and insulin concentrations [17].

Intra-Arterial Fibrinolytic Protocol

All measurements were performed in a temperature-controlled room between 7 and 10 AM after an overnight fast as previously described by our laboratory [8]. Briefly, an intravenous catheter was placed in a deep antecubital vein of the non-dominant arm. Thereafter, a 5-cm, 20-

gauge catheter was introduced into the brachial artery of the same arm under local anesthesia (1% lidocaine). Forearm blood flow (FBF) was measured using strain-gauge venous occlusion plethysmography (D.E. Hokanson, Bellevue, WA) and presented as mL/100 mL forearm volume/min. Following the measurement of resting blood flow for 5 minutes, bradykinin was infused intra-arterially at rates of 12.5, 25, 50 ng·100 ml tissue^{-1}·min^{-1} and sodium nitroprusside at 1.0, 2.0, 4.0 μg·100 ml tissue^{-1}·min^{-1} for 5 min at each dose as previously described [8]. To avoid an order effect, the sequence of drug administration was randomized.

Net endothelial release of t-PA antigen and plasminogen activator inhibitor (PAI)-1 antigen in response to bradykinin and sodium nitroprusside was calculated according to Jern et al. [18] using the following equation:

$$\text{Net Release} = (C_V\text{-}C_A) \times (\text{FBF} \times [101\text{-hematocrit}/100])$$

where C_V and C_A represent the concentration in the vein and artery, respectively. For both t-PA and PAI-1, a positive difference indicated a net release and a negative difference, net uptake. Arterial and venous blood samples were collected simultaneously at baseline and the end of each drug dose. Enzyme immunoassay was used to determine t-PA and PAI-1 antigen concentrations. Hematocrit was measured in triplicate using the standard microhematocrit technique and corrected for trapped plasma volume within the erythrocytes [19]. The total amount of t-PA antigen released across the forearm in response to bradykinin was calculated as the incremental area under each curve using a trapezoidal model. In order to avoid confounding effects from potential infection or acute inflammation on fibrinolytic function, all subjects were free of recent infection/inflammation (> 2 weeks) as determined by questionnaire [20].

Statistical Analysis

Differences in subject baseline characteristics and area under the curve data were determined by between-groups analysis of variance (ANOVA). Group differences in FBF and endothelial t-PA and PAI-1 antigen release in response to bradykinin and sodium nitroprusside were determined by repeated-measures ANOVA. When indicated by a significant F value, a *post hoc* test using the Newman-Keuls method was performed to identify differences between the groups. Relations between variables of interest were assessed by linear regression analysis. All data are expressed as mean ± SEM. Statistical significance was set *a priori* at $P<0.05$.

RESULTS

Selected subject characteristics are presented in Table 1. There were no differences in age, anthropometric or metabolic variables between the normotensive and prehypertensive groups. By design, systolic and diastolic blood pressures were greater ($P<0.05$) in the prehypertensive compared with the normotensive group. Aside from blood pressure, BMI, and plasma glucose and insulin concentrations were highest in the hypertensive men,

The FBF responses to bradykinin and sodium nitroprusside were not significantly different between groups (Figure 1). FBF in the non-infused arm remained constant throughout the infusion protocols and did not differ significantly between groups.

Basal endothelial t-PA antigen release was not significantly different amongst the groups. However, compared with the normotensive men, the capacity of the endothelium to release t-PA in response to bradykinin was significantly blunted in the prehypertensive and hypertensive men (Figure 2). Net release of t-PA antigen was ~25% lower ($P < 0.05$) in the prehypertensive (from -0.9 ± 0.8 to 42.4 ± 5.3 ng/100 mL tissue/min) compared with the normotensive (from 0.5 ± 1.0

to 53.9 ± 6.5 ng/100 mL tissue/min) men. As a result, the total amount of t-PA antigen released (area under the curve to all doses of bradykinin) was markedly lower (~35%; $P < 0.05$) in the prehypertensive (220 ± 24 ng/100 mL tissue) than normotensive (341 ± 34 ng/100 ml tissue) group (Figure 2). Interestingly, net release of t-PA antigen (from -1.8±1.6 to 40.8±6.6 ng/100 mL tissue/min) and total amount of t-PA antigen released (213±47 ng/100 mL tissue) in response to bradykinin in the hypertensive group, although lower ($P<0.05$) than normotenisve controls, was not significantly different from the prehypertensive group. There was an inverse relation between systolic BP and total t-PA release to bradykinin ($r = -0.36$, $P<0.05$). Infusion of sodium nitroprusside did not stimulate significant changes in t-PA release in the normotensive (from 0.4 ± 0.8 to -0.6 ± 3.1 ng/100 mL tissue/min), prehypertensive (from -0.5 ± 0.9 to 3.4 ± 5.7 ng/100 mL tissue/min) and hypertensive (from 0.8±1.3 to 2.1±3.0 ng/100 mL tissue/min) adults. Neither bradykinin nor sodium nitroprusside elicited significant changes in PAI-1 antigen release in any group (data not shown).

DISCUSSION

The novel findings of the present study are as follows: 1) the capacity of the endothelium to release t-PA is diminished in middle-aged men with blood pressure in the prehypertensive range; and 2) the level of impairment in endothelial t-PA release in prehypertensive men is similar to that observed in hypertensive men. To our knowledge, this is the first study to examine the influence of blood pressure in the prehypertensive range on endothelial fibrinolytic function.

The vascular risks associated with prehypertension cannot be overlooked. Several epidemiological studies have demonstrated significantly increased risk of cardiovascular disease

and associated acute vascular events in prehypertensive compared with normotensive adults [21] [22] [23] [24] [25]. For example, multivariate-adjusted analyses of the original Framingham Heart Study cohort revealed that compared with normotension, prehypertension was associated with substantially greater risk of myocardial infarction (hazard ratio [HR]: 3.5)[21]. Moreover, a recently conducted meta analysis involving over one million individuals, spanning 20 prospective cohort studies, provided further confirmation of the elevated risk of CVD-related mortality with prehypertension [2]. The mechanisms for the increased vascular risk with blood pressure in the prehypertensive range are unclear. We [26, 27] and others [28] have reported that endothelial vasomotor function is markedly impaired in prehypertensive adults. Indeed, Weil et al. [27] showed, in a similar population to that of the present study, that prehypertension is associated with a ~30% reduction in nitric oxide-mediated endothelium-dependent vasodilation. In addition to reduced nitric oxide bioavailability, the same authors, in a separate study, also reported that endothelin-1 vasoconstrictor tone is elevated (~20%) in the prehypertensive state [26]. The results of the present study complement and significantly extend these previous findings by demonstrating that the capacity of the endothelium to release t-PA in prehypertensive men is severely blunted (~35%). Although the potential clinical consequence of reduced t-PA release with prehypertension is outside the scope of this study, both animal [29, 30] and clinical studies [31] have highlighted the deleterious impact of diminished endothelial release of t-PA on cardiovascular health. Studies involving t-PA deficient mice, for example, demonstrated accelerated rates of atherosclerotic fibrin deposition and extensive myocardial tissue necrosis in these animals [29, 30]. In humans, reduced capacity of the endothelium to release t-PA has been linked to atheromatous plaque development and higher rates of myocardial infarction[31]. Thus, endothelial fibrinolytic dysfunction may be a contributing mechanism underlying the increased

risk of thrombosis and acute cardiovascular events with prehypertension [3].

Interestingly, in the current study there was no difference between the prehypertensive and hypertensive groups in endothelial t-PA release. Both groups demonstrated significantly lower (~35%) capacity of the endothelium to release t-PA than their normotensive counterparts. Of note, the impairment in endothelial t-PA release observed in the hypertensive group is in line with that previously described [14]. Hrafnkelsdottir and colleagues [14] demonstrated that release of t-PA from the endothelium was significantly blunted in hypertensive compared with normotensive adults, independent of other risk factors. The results of the present study extend these findings by showing that the impairment in t-PA release with hypertension appears to be born in the prehypertensive state. This may account, in part, for the increase in vascular risk starting at blood pressure in the prehypertensive range [3]. Moreover, it provides further rationale for a more aggressive approach towards risk recognition and blood pressure control in the prehypertensive population.

The mechanisms underlying the impairment in endothelial t-PA release in adult males with blood pressure in the prehypertensive range are unclear. It has been demonstrated that elevations in blood pressure are associated with increased inflammation and oxidative stress [32-35]. Our group [36], as well as others [37], have shown that elevations in oxidative stress is associated with lower endothelial t-PA release. For example, Van Guilder et al. [36] demonstrated that endothelium t-PA release was potentiated after acute intra-arterial vitamin C administration and chronic oral vitamin C supplementation in overweight and obese adults. While not assessed in this study, it is possible that oxidative stress may contribute to impaired endothelial t-PA release with prehypertension, and is worthy of future study. It is important to emphasize that aside from elevated blood pressure, subjects in the present study were free of clinically evident coronary

artery disease and other cardiometabolic risk factors that have been shown to adversely influence endothelial t-PA release such as obesity and dyslipidemia. Thus, we believe that the observed impairment in t-PA is a primary consequence of chronic elevation in blood pressure.

There are a few experimental considerations regarding the present study that merit discussion. Firstly, the experimental design of our study was cross-sectional. Therefore, we cannot discount the possibility that variables such as genetics and/or lifestyle behaviors may have influenced our results. In the effort to minimize the influence of lifestyle behaviors, we studied sedentary adults who were non-smokers and not currently taking any medications or supplements that have been shown to influence endothelial fibrinolytic function. Secondly, it is important to emphasize that the present study involved men only. We have previously reported that postmenopausal women demonstrate markedly higher (~50%) endothelial t-PA release than middle-aged and older men [38]. It is possible that these gender differences may exist amongst prehypertensive adults. As such, studies are ongoing in our laboratory to examine the influence of prehypertension on endothelial t-PA release in middle-aged and older women. In addition, it is unknown whether prehypertension adversely affects endothelial fibrinolytic function in younger adults.

In conclusion, the results of this study indicate that the capacity of the endothelium to release t-PA is diminished in adult males with blood pressure in the prehypertensive range. Moreover, the level of impairment in t-PA release seen with clinical hypertension is already apparent in the prehypertensive state. Blunted endothelial fibrinolytic function may be an underlying factor contributing to the increased risk of cardiovascular disease and acute vascular events with prehypertension.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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FIGURE LEGEND

Figure 1. Forearm blood flow responses to bradykinin (A) and sodium nitroprusside (B) in normotensive, prehypertensive and hypertensive men. Values are mean ± SEM.

Figure 2. Net release rate (A) and total amount (B) of tissue-type plasminogen activator (t-PA) antigen released across the forearm in response to bradykinin and sodium nitroprusside (C) in normotensive, prehypertensive and hypertensive men. Values are mean ± SEM; *$P < 0.05$ vs. normotensive.

Table 1. Selected Subject Characteristics

Variable	Normotensive (n=16)	Prehypertensive (n=16)	Hypertensive (n=10)
Age (years)	52±3	52±2	57±2
Body mass (kg)	82.5±2.9	84.8±2.4	89.6±2.9
Body fat (%)	23.1±1.9	26.6±1.4	30.2±1.5*
Waist Circumference (cm)	91.7±2.3	95.4±2.1	100.6±2.0*
BMI (kg/m^2)	25.8±0.8	26.9±0.7	29.7±.0.9*†
Systolic BP (mmHg)	114±2	128±2*	145±2*†
Diastolic BP (mmHg)	73±2	83±2*	90±2*†
VO_2 max (mL/kg/min)	35.5±1.7	35.4±1.6	30.0±1.3*†
Total cholesterol (mmol/L)	4.6±0.1	4.9±0.2	4.6±0.2
HDL cholesterol (mmol/L)	1.1±0.1	1.1±0.1	1.3±0.1
LDL cholesterol (mmol/L)	3.0±0.1	3.1±0.2	2.7±0.2
Triglycerides (mmol/L)	1.1±0.1	1.5±0.2	1.5±0.3
Glucose (mmol/L)	4.9±0.1	5.1±0.1	5.2±0.1*
Insulin (pmol/L)	34.5±3.2	40.4±2.8	53.4±7.4*†
HOMA-IR	1.3±0.1	1.5±0.1	2.2±0.3*†

Values are mean ± SEM. BMI, body mass index; BP, blood pressure; VO2 max, maximal oxygen consumption; HDL, high-density lipoprotein; LDL, low-density lipoprotein; HOMA-IR, homeostasis model assessment.

*P<0.05 vs normotensive
†P<0.05 vs prehypertensive

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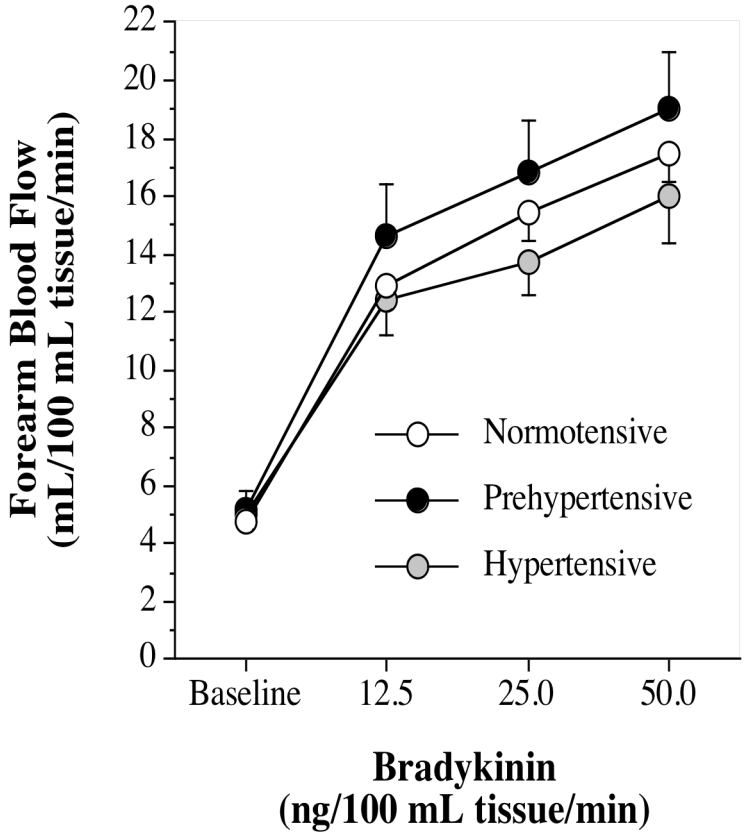
Forearm Blood Flow
(mL/100 mL tissue/min)
22
20
18
16
14
12
10
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2
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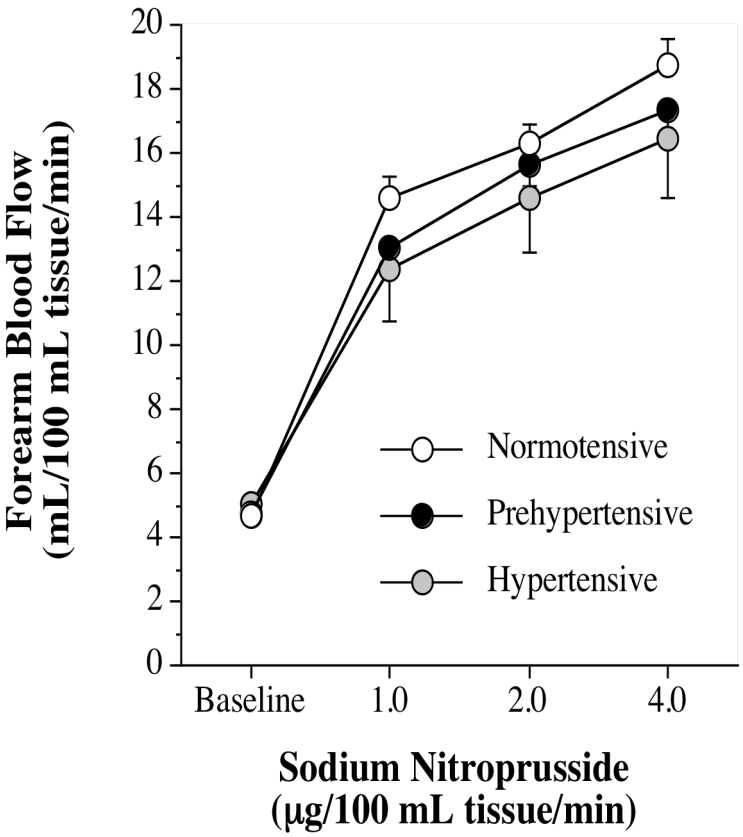
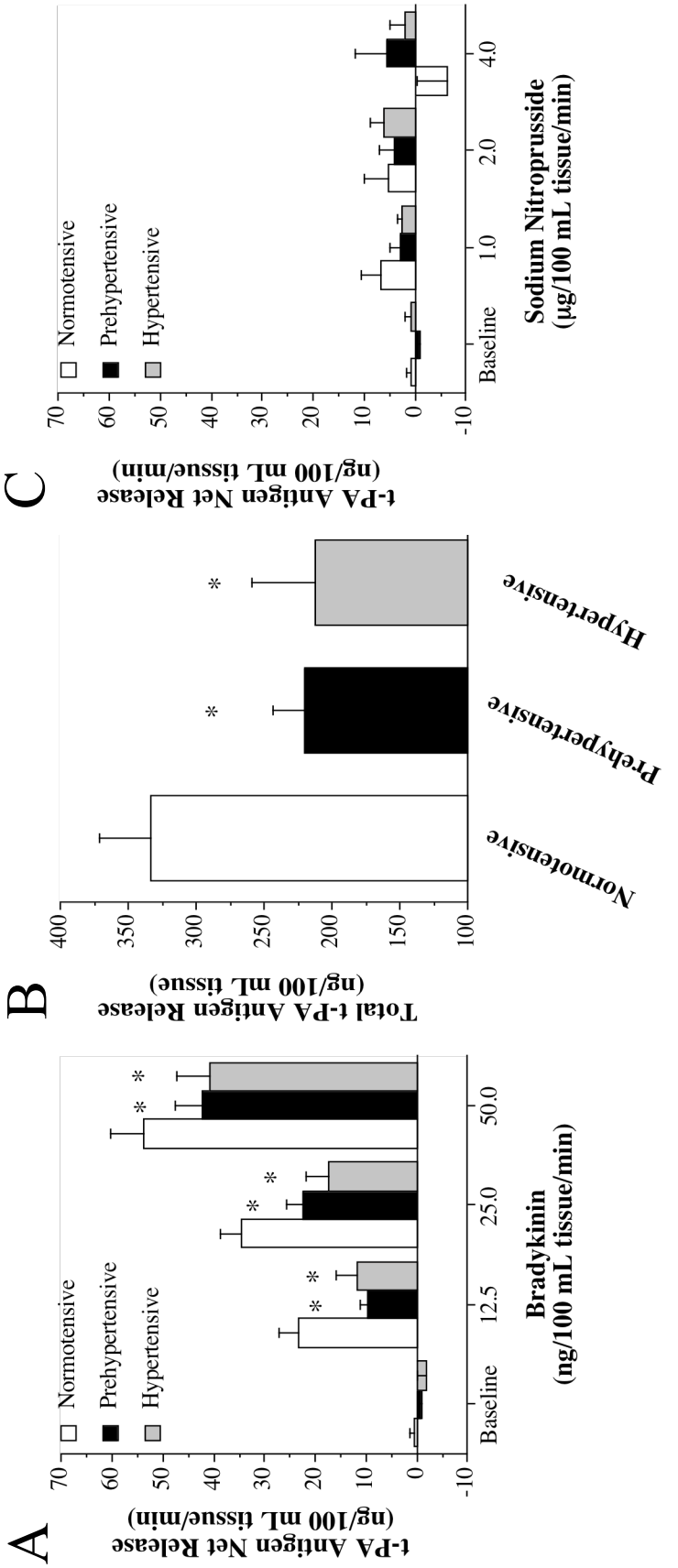
Normotensive
Prehypertensive
Hypertensive
Baseline
12.5
25.0
50.0
Bradykinin
(ng/100 mL tissue/min)

B


Forearm Blood Flow
(mL/100 mL tissue/min)
20
18
16
14
12
10
8
6
4
2
0
Normotensive
Prehypertensive
Hypertensive
Baseline
1.0
2.0
4.0
Sodium Nitroprusside
(μg/100 mL tissue/min)


A
t-PA Antigen Net Release
(ng/100 mL tissue/min)
Normotensive
Prehypertensive
Hypertensive
Baseline
12.5
25.0
50.0
Bradykinin
(ng/100 mL tissue/min)
B
Total t-PA Antigen Release
(ng/100 mL tissue)
Normotensive
Prehypertensive
Hypertensive
C
t-PA Antigen Net Release
(ng/100 mL tissue/min)
Normotensive
Prehypertensive
Hypertensive
Baseline
1.0
2.0
4.0
Sodium Nitroprusside
(µg/100 mL tissue/min)

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WHITE BLOOD CELL COUNT AND ENDOTHELIN-1 VASOCONSTRICTOR TONE IN MIDDLE-AGED AND OLDER ADULTS

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Running Title: WBC and ET-1 Vasoconstriction

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ABSTRACT

Background. Higher white blood cell (WBC) count is associated with impaired endothelium-dependent vasodilation. However, the influence of higher WBC count on endothelin (ET)-1 vasoconstrictor activity is currently unknown. We tested the hypothesis that adults with elevated WBC count demonstrate enhanced ET-1 system activity. **Methods.** Thirty-four healthy adults were studied: 17 with WBC count < $5.0x10^9$ cells/L (lower WBC; 9M/8F; age: 53±2 yr) and 17 with WBC count > $5.0x10^9$ cells/L (higher WBC; 10M/7F; 54±3 yr). Forearm blood flow (FBF) responses to intra-brachial infusion of ET-1 (5 pmol/min for 20 min) and selective ET_A receptor blockade (BQ-123; 100 nmol/min for 60 min) were measured by venous occlusion plethysmography. **Results.** The vasoconstrictor response to ET-1 was significantly blunted (~60%) in the higher WBC group versus the lower WBC group. The FBF responses to selective ET_A receptor blockade were also significantly different ($P<0.05$) between the groups. In the lower WBC group, resting FBF increased marginally (~5%) to BQ-123, whereas the increase in FBF to BQ-123 was significantly greater (~15%) in higher WBC group. Furthermore, there was a significant relation between WBC count and FBF response to ET-1 (r=-0.43) and BQ-123 (r=0.41). **Conclusions.** Relative elevations in WBC count in middle-aged and older adults, independent of adiposity and other cardiometabolic risk factors, are associated with enhanced ET-1-mediated vasoconstrictor tone. Elevated ET-1 system activity may be a mechanism linking higher WBC count with increased cardiovascular risk.

Key Words: endothelin, vasoconstriction, white blood cell

INTRODUCTION

White blood cells (WBC) play a critically important role in immunological responses to inflammatory and infectious processes [1]. The recognition that inflammation is a key component of atherosclerotic vascular disease and its clinical consequences sparked, and rekindled, interest in the potential link between WBC count and risk of cardiovascular disease [2, 3]. Indeed, several epidemiological and prospective studies have shown that elevations in WBC count, even within suggested normal ranges, are independently associated with coronary heart disease and stroke incidence and mortality [3-6]. A number of biochemical, biomechanical, electrical and rheological mechanisms have been proposed to underlie the pathologic link between WBCs and atherosclerotic vascular disease [3, 7]. Given the constant interaction between WBCs and the vascular endothelium, endothelial cell damage and dysfunction may be a primary causative factor.

Impaired endothelial cell function, particular vasomotor dysfunction, is an early component in the development of atherosclerosis. Diminished nitric oxide-mediated endothelium dependent vasodilation and enhanced endothelin (ET)-1 vasoconstrictor tone is recognized as an atherogenic endothelial phenotype associated with increased cardiovascular and cerebrovascular risk [8]. Elevations in WBC count have been shown to be inversely related to endothelial vasodilation in both healthy and diseased populations [9, 10]. It is currently unknown whether WBC count is also related to other endothelial vasomotor abnormalities. Accordingly, we tested the hypothesis that elevations in WBC count, within clinically normal range, are associated with increased ET-1 vasoconstrictor tone in middle-aged and older adults.

METHODS

Subjects

Thirty-four healthy sedentary adults (age range: 41-73 years) were studied: 17 (9 males/8 females) with WBC count < $5.0x10^9$ cells/L (lower WBC) and 17 (10 males/7 females) with WBC count > $5.0x10^9$ cells/L (higher WBC) [9]. The stratification used for WBC was based on a recent study demonstrating associated impairment in endothelial vasodilator function with WBC count > $5.0x10^9$ cells/L [9]. All subjects were normotensive (arterial blood pressure < 140/90 mmHg) and free of overt cardiovascular disease and metabolic abnormalities as assessed by medical history, physical examination, fasting blood chemistries and blood pressure at rest and during incremental exercise performed to exhaustion. None of the subjects smoked or were taking medications (including vitamins). No subjects had WBC count > $10.0x10^9$ cells/L, and all subjects were free of recent inflammation/infection (<2 weeks) as determined by questionnaire [11]. All of the women were at least 1 year postmenopausal and had never taken or had discontinued use of hormone replacement therapy at least 1 year before the start of the study. Prior to participation, all of the 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

Body mass was measured to the nearest 0.1 kg using a medical beam balance. Percent body fat was determined by dual energy X-ray absorptiometry (Lunar Corp., Madison, WI). Body mass index (BMI) was calculated as weight (kilograms) divided by height (meters) squared. Minimal waist circumference was measured according to published guidelines [12].

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 with on-line computer-assisted open circuit spirometry as described previously [13].

WBC Count and Metabolic Measurements

WBC count was determined by standard Coulter counter technique (Beckman Coulter Ac·T 5diff CP) by the Clinical and Translational Research Center (CTRC) core laboratory. Plasma lipid, lipoprotein, glucose and insulin concentrations were also determined by the CTRC core laboratory using standard methods. Plasma concentrations of C-reactive protein and oxidized LDL were determined by enzyme immuno-assay.

Intra-Arterial Infusion Protocol

All studies were performed between 7 and 10 AM after a 12-hour overnight fast in a temperature-controlled room. Under strict aseptic conditions a 5-cm, 20-gauge catheter was inserted into the brachial artery of the nondominant arm under local anesthesia (1% lidocaine). Heart rate and arterial blood pressure were continuously measured throughout the infusion protocol. Forearm blood flow (FBF) at rest and in response to each pharmacologic agent was measured using strain-gauge venous occlusion plethysmography (D. E. Hokanson, Bellevue, WA), as previously described by our laboratory [14]. Baseline FBF was measured for 5 minutes and for 5 minutes before each drug infusion thereafter. To rule out the possibility of nonspecific differences to vasoconstrictor agents between WBC count groups, vascular responses to norepinephrine were determined. Norepinephrine was infused at a rate of 260 pmol/min for 5

minutes, and FBF was measured during the last 3 minutes. After a 20-minute rest period to allow FBF to return to baseline levels, ET-1 (Clinalfa, AG) was infused at a rate of 5 pmol/min for 20 minutes, and FBF was measured during the last 3 minutes. After a 30-minute rest period to allow resting FBF to return to baseline, BQ-123 (Clinalfa, AG), a selective ET_A receptor antagonist, was infused at a rate of 100 nmol/min for 60 minutes, and FBF was measured every 10 minutes. The selected dose of BQ-123 has been shown to completely inhibit the vasoconstrictor effect of endogenous ET-1 in the human forearm of healthy adults [15].

Statistical Analysis

Differences in subject baseline characteristics, WBC count and the magnitude of change in FBF to norepinephrine and ET-1 were determined by between-groups analysis of variance (ANOVA). Group differences in FBF responses to BQ-123 were determined by repeated-measures ANOVA. Relations between variables of interest were assessed by linear regression analysis. There were no significant gender interactions in any of the primary 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

Selected subject characteristics are presented in the table. By design, WBC count was significantly different (~30%) between the lower and higher WBC count groups. Aside from WBC count, there were no significant differences between the groups in any anthropometric, hemodynamic or metabolic variables.

The vasoconstrictor response to norepinephrine was not significantly different between

groups, as FBF was reduced by 24% in both groups (data not shown). In contrast, the vasoconstrictor response to ET-1 was markedly different (~60%; P=0.01) between the WBC groups. In the higher WBC group, there was a 5.1±2.5% reduction in resting FBF to ET-1 compared with a 12.2±1.2% reduction in the lower WBC group (Figure 1). The FBF responses to selective ET_A receptor blockade were also significantly different between the groups. In the lower WBC group, resting FBF increased marginally (~5%) to BQ-123, whereas the increase in FBF to BQ-123 was approximately three-fold greater (~15%) in higher WBC group (Figure 1). In the overall study population, WBC count was inversely related to the FBF response to ET-1 (r=-0.43, P=0.01) and positively correlated with the peak FBF response to BQ-123 (r=0.41, P=0.02) (Figure 2).

DISCUSSION

The primary new finding of the present study is that elevations in WBC count, within clinically normal range and independent of other cardiovascular risk factors, is associated with increased ET-1-mediated vasoconstrictor tone. To the best of our knowledge this is the first study to determine a potential link between WBC count and ET-1 system activity. Increased ET-1 vasoconstrictor tone may contribute to the heightened risk of cardiovascular disease and stroke reported with elevations in WBC count.

WBC count in both healthy and diseased adults has been shown to be associated with, and predictive of, cardiovascular and cerebrovascular events [4-6, 16-19]. For example, in the Atherosclerosis Risk in Communities Study [4] the incidence of coronary artery disease was almost two-fold higher in Caucasian and African American adults with WBC counts $>7.0x10^9$ cells/L compared with adults of similar age with WBC counts $< 4.8x10^9$ cells/L. Elkind and

colleagues [5] reported that elevations in WBC count were associated with an increased risk of ischemic stroke, independent of other risk factors, in a population cohort of the Northern Manhattan Study. Moreover, the risk of future stroke was estimated to increase by ~20% per $1.8x10^9$ cells/L increment in WBC count. Several studies have also noted the prognostic value of WBC count for morbidity and mortality following percutaneous coronary intervention [16-18]. The mechanisms underlying the vascular risk with elevated WBCs are not completely understood.

Increased ET-1 system activity has been linked with a number of cardiovascular risk factors associated with elevated WBC count, such as obesity [20] and hypertension [21], and is considered to be an important contributor to the etiology of atherosclerotic vascular disease [22]. In the present study, WBC count was associated with increase ET-1 system activity. Indeed, there were marked differences in the vascular responses to ET-1 and selective ET_A receptor blockade between the higher and lower WBC groups. The forearm vasoconstrictor response to exogenous ET-1 was significantly blunted in the adults with higher WBC count compared with lower WBC count. The reduced vasoconstrictor effect of the peptide in the higher WBC count group suggests greater endogenous ET-1 bioavailability and continuous receptor activation [15]. It has been shown that chronic exposure to ET-1 does result in a reduction in ET-1 receptors on vascular smooth cells [23]. Of note, it is unlikely that the observed WBC count-related differences in the ET-1 response were due to a nonspecific decline in contractile function of the vascular smooth muscle, because the vasoconstrictor response to norepinephrine was similar between the groups. The vascular response to exogenous ET-1 is often used as a bioassay of endogenous ET-1 production given the inaccuracy and unreliability of circulating ET-1 concentrations as an indicator of vascular ET-1 production [15]. Coupled with the blunted

response to ET-1, the higher WBC count group demonstrated a significantly greater vasodilator response to the selective ET_A receptor antagonist BQ-123 than the lower WBC count group suggesting enhanced ET_A receptor-mediated ET-1 vasoconstrictor tone in the higher WBC count group. Additionally, we observed significant correlations between WBC count and the vascular responses to both ET-1 and BQ-123 reflecting an association between WBC count and the ET-1 system. No other factors were associated with these blood flow responses in the present study.

The mechanisms responsible for the apparent WBC-related increase in ET-1 system activity are unclear and outside the scope of this cross-sectional study. However, it is important to note that aside from WBC count, there were no other group differences in anthropometric, cardiometabolic, inflammatory or oxidative factors that have been shown to influence ET-1 system activity between the groups [15, 24, 25]. As a result our findings suggests a primary link between WBCs and vascular function. Indeed, the results presented herein complement and extend the results of previous studies demonstrating that relative elevations in WBC count are associated with a reduction in endothelial vasodilator function [9, 10]. For example, a recent study by Walker et al. [9], involving a similar population of healthy, sedentary middle-aged and older adults stratified by the same WBC count criteria as the present study, demonstrated impaired nitric oxide-mediated endothelium-dependent vasodilation in adults with higher ($6.0\pm0.2x10^9$ cells/L) compared with lower ($4.1\pm0.1x10^9$ cells/L) WBC count. These findings and those presented herein indicate that relative elevations in WBC count, independent of other risk factors, are associated with a proatherogenic endothelial phenotype characterized by enhanced ET-1-mediated vasoconstriction and reduced nitric oxide-mediated vasodilation. Future studies are needed determine the underlying causes for this apparent deleterious interaction between WBCs and vascular endothelial function.

Given the inherent limitations of the cross-sectional design employed in the present study we are unable to dismiss that genetic and/or lifestyle factors may have influenced our results. To minimize the influence of lifestyle behaviors, we studied subjects who were non-smokers, not currently taking any medication that could influence WBC or endothelial vasomotor function, and did not differ in body composition or habitual physical activity. In addition, in an effort to isolate the primary influence of WBC, we studied adults free of other cardiometabolic risk factors that have been shown to influence WBC and endothelin-1 system activity such as hypertension [21] and type 2 diabetes [26, 27]. Although the plasma concentrations of CRP were slightly, but not significantly, higher in the WBC group, we do not believe that these higher CRP concentrations influenced our findings, and in turn, the interpretation the data. Indeed, in the present study we observed no relation between CRP and either WBC or the FBF responses to ET-1 or BQ-123. This finding is consistent with previous studies from our laboratory involving CRP and ET-1 system activity [28]. Unfortunately, due to a reduction in drug availability during the course of this study, we were unable to administer the selective ET_B receptor antagonist BQ-788 and therefore cannot comment on the influence of WBC on the vascular actions of the ET_B receptor and non-selective ET-1-receptor blockade.

In conclusion, the results of this study demonstrate that WBC count is associated with significant differences in FBF responses to exogenous ET-1 administration and ET-1 receptor antagonism in healthy, sedentary, non-smoking middle-aged and older adults. Increased ET-1-mediated vasoconstrictor tone may contribute to the increased risk of cardiovascular and cerebrovascular disease and events associated with relative elevations in WBC count. In addition, the apparent link between WBC count and vascular endothelial function may also underlie the reported prognostic value of WBC count to cardiovascular outcomes.

ACKNOWLEDGEMENTS

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FIGURE LEGEND

Figure 1. FBF responses to ET-1 (5 pmol/min for 20 minutes) (panel A) and BQ-123 (panel B) in the lower and higher WBC count groups. Values are mean ± SEM. *P<0.05 vs lower WBC count.

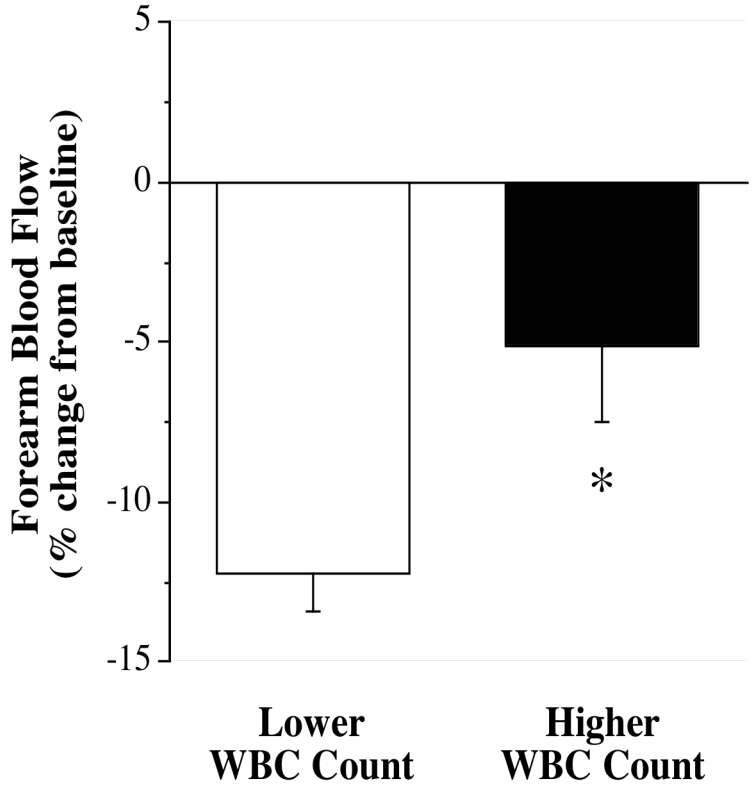
Figure 2. Relation between white blood cell count and peak FBF response to ET-1 (panel A) and BQ-123 (panel B) in the study population.

Table. Selected subject characteristics.

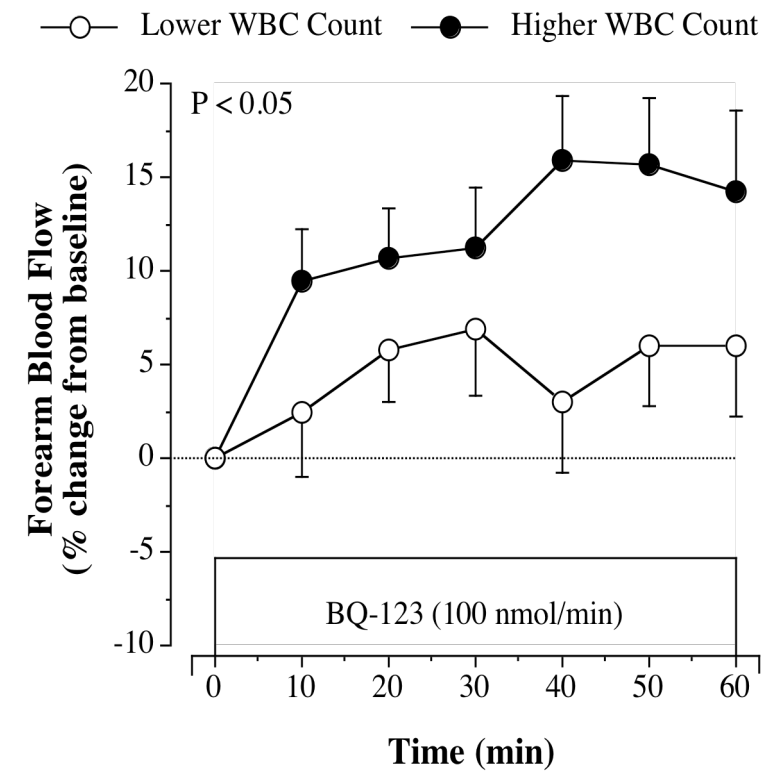
Variable	Lower WBC Count (n=17)	Higher WBC Count (n=17)
Age, yr	53±2	55±2
Gender, M/F	9/8	10/7
WBC count, 10^9 cells/L	4.4±0.2	6.3±0.2*
Body mass, kg	78.4±4.2	82.4±3.6
BMI, kg/m^2	26.7±0.9	27.9±1.0
Body fat, %	35.2±2.1	33.9±2.2
Waist circumference, cm	91.4±3.6	91.0±3.0
Systolic BP, mmHg	122±2	126±2
Diastolic BP, mmHg	76±2	79±1
$\dot{V}O_2$max, mL/kg/min	28.7±1.9	29.0±1.9
Total cholesterol, mmol/L	5.3±0.2	5.1±0.2
LDL-cholesterol, mmol/L	3.3±0.2	3.0±0.2
HDL-cholesterol, mmol/L	1.3±0.1	1.3±0.1
Triglycerides, mmol/L	1.5±0.1	1.6±0.2
Glucose, mmol/L	5.1±0.1	5.1±0.1
Insulin, pmol/L	47.3±5.4	55.7±8.9
C-Reactive Protein, mg/L	2.6±0.7	3.7±0.7
Oxidized LDL, U/L	64.1±3.7	60.2±4.4

Values are mean±SEM. WBC: white blood cell count; BMI: body mass index; BP: blood pressure; VO_2max: maximal oxygen consumption; LDL: low-density lipoprotein; HDL: high-density lipoprotein; *$P < 0.05$ vs. Lower WBC count

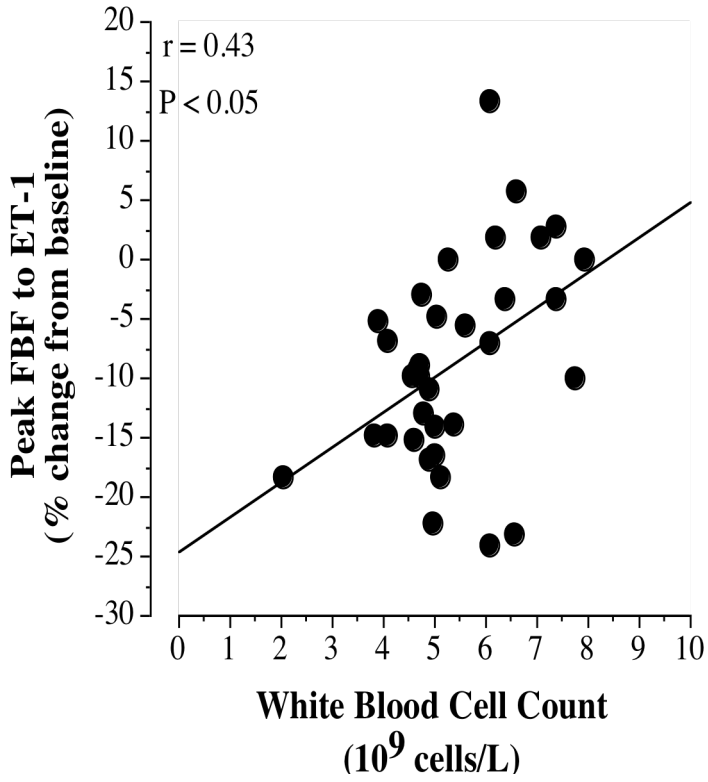
A


5
0
-5
-10
-15
Forearm Blood Flow
(% change from baseline)
*
Lower
WBC Count
Higher
WBC Count

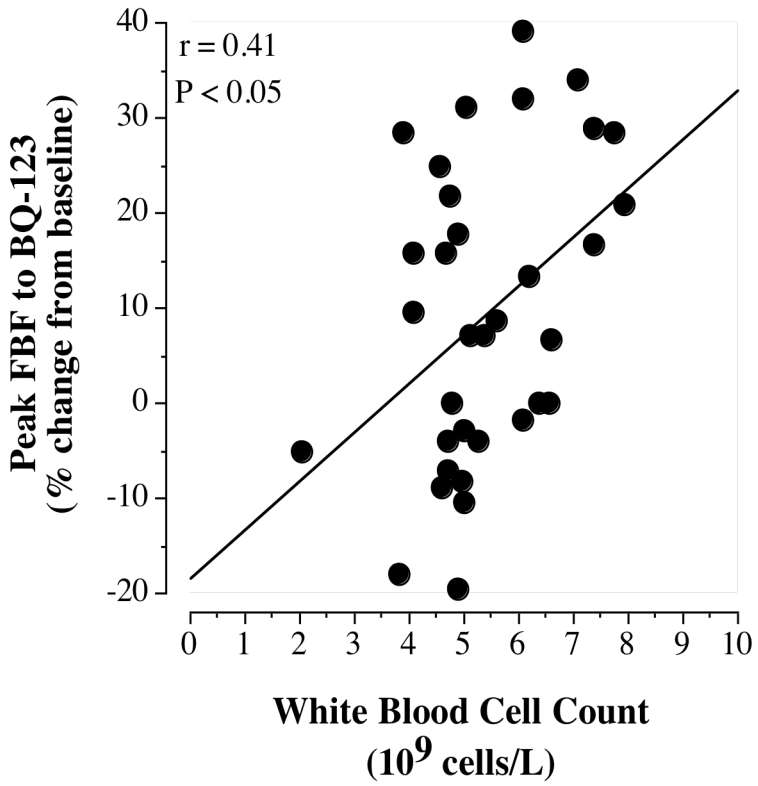
B


Lower WBC Count
Higher WBC Count
P < 0.05
20
15
10
5
0
-5
-10
Forearm Blood Flow
(% change from baseline)
BQ-123 (100 nmol/min)
0
10
20
30
40
50
60
Time (min)

A


r = 0.43
P < 0.05
Peak FBF to ET-1
(% change from baseline)
White Blood Cell Count
(10^9 cells/L)

B


r = 0.41
P < 0.05
Peak FBF to BQ-123
(% change from baseline)
White Blood Cell Count
(10^9 cells/L)

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IMPAIRED FASTING BLOOD GLUCOSE IS ASSOCIATED WITH INCREASED ENDOTHELIN-1 VASOCONSTRICTOR TONE

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Short Title: Fasting Glucose and Increased ET-1
Key words: endothelin-1, glucose, vasoconstriction

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ABSTRACT

Aim/Hypothesis: The experimental aim of this study was to determine whether ET-1-mediated vasoconstrictor tone is elevated in adult humans with impaired fasting blood glucose concentrations, independent of other cardiovascular risk factors. *Methods:* Forearm blood flow (FBF: plethysmography) responses to intra-arterial infusion of selective ET_A receptor blockade (BQ-123: 100 nmol/min for 60 min) and non-selective $ET_{A/B}$ blockade (BQ-123 + BQ-788: 50 nmol/min for 60 min) were determined in 28 middle-aged, sedentary adults (17 M/11 F): 14 with normal fasting blood glucose (age: 57$\pm$2 yr; 6F/8M; BMI: 29.2$\pm$0.9 kg/m2; glucose: 4.9$\pm$0.1 mmol/L) and 14 impaired fasting blood glucose (58$\pm$1 yr; 5F/9M; 29.6$\pm$1.1 kg/m2; 5.8$\pm$0.1 mmol/L) concentrations. *Results:* Selective ET_A receptor blockade elicited a significantly greater (~20%) increase in FBF in the impaired fasting glucose adults compared with the normoglycemia controls. $ET_{A/B}$ blockade resulted in a further 2-fold increase ($P<0.05$) in FBF above that elicited by ET_A receptor antagonism in the impaired fasting glucose but not normal fasting glucose adults. There was a positive correlation between fasting blood glucose levels and the peak vascular responses to ET_A ($r=0.44$; $P<0.05$) and $ET_{A/B}$ ($r=0.62$; $P<0.05$) blockade. No other anthropometric, hemodynamic or metabolic variable was correlated with the blood flow responses to ET-1 receptor blockade. *Conclusions/Interpretation:* ET-1-mediated vasoconstrictor tone is elevated in adults with impaired fasting blood glucose concentrations, independent of other cardiometabolic risk factors. Enhanced ET-1 system activity may underlie endothelial vasomotor dysfunction and increased cardiovascular risk in adults with impaired fasting blood glucose concentrations.

INTRODUCTION

Approximately 80 million adults in the United States have impaired fasting blood glucose concentrations [1], defined as fasting plasma glucose between 5.6-6.9 mmol/L [2]. It has recently been reported that middle-aged adults without diabetes, but with elevated fasting plasma glucose, are at an increased risk for coronary heart disease (4). For example, Alexander et al [3] demonstrated that adults with impaired fasting glucose are at a 50% higher risk of developing cardiovascular disease compared with adults with normal fasting glucose. The mechanisms responsible for this apparent increase in vascular risk are not fully understood. Glucose has been shown to adversely affect endothelial cell function, which may propagate the atherosclerotic process [4]. Several clinical studies have shown that impaired fasting glucose is associated with endothelial dependent vasodilator dysfunction [5, 6], a central feature of atherogenesis [7].

Endothelin-1 (ET-1) is a potent vasoconstrictor peptide released by the endothelium that contributes to the regulation of vascular tone and has been implicated in the etiology of atherosclerotic vascular disease [8]. Interestingly, *in vitro*, a high glucose environment results in an elevation in endothelin-1 converting enzyme, suggesting a link between glucose and the ET-1 system [9]. Currently, it is unknown whether ET-1 system activity is altered in adult humans with impaired fasting plasma glucose. If so, this may contribute mechanistically to impaired endothelial vasomotor function and increased cardiovascular risk in this population. Thus, the aim of this study was to determine whether ET-1-mediated vasoconstrictor tone is elevated in adult humans with impaired fasting blood glucose concentrations, independent of other cardiovascular risk factors.

METHODS

Subjects

Twenty-eight sedentary adults participated in this study: 14 (6F/8M) with normal plasma glucose (<5.6 mmol/L); and 14 (5F/9M) with impaired fasting plasma glucose (5.6-6.9 mmol/L) concentrations. Groups were stratified according to American Diabetes Association criteria [2]. Subjects were non-smokers and free of overt cardiovascular disease. Fasting plasma lipid, lipoprotein, glucose and insulin concentrations were determined using standard techniques. HOMA-IR was calculated as previously described [10]. All women were at least 1 year postmenopausal and not taking hormone replacement therapy. Written informed consent was obtained according to the guidelines of the University of Colorado at Boulder.

Intra-arterial Infusion Protocol

All studies were performed between 7 AM and 10 AM after a 12-hour overnight fast as previously described by our laboratory [11]. Briefly, following arterial catheterization, forearm blood flow (FBF: venous occlusion plethysmography) responses to BQ-123 (Clinalfa, AG), a selective ET_A receptor antagonist, infused for 60 minutes with FBF measured every 10 minutes. Thereafter, FBF was assessed every 10 minutes for an additional 60 minutes with the co-administration of BQ-123 and BQ-788 (Clinalfa, AG), a specific antagonist of ET_B receptors. Due to product availability, BQ-788 was infused in 7 of the 14 subjects in each group.

Statistical Analysis

Differences in subject characteristics were determined by between-group analysis of variance (ANOVA). Group differences in FBF responses to BQ-123 and BQ-123 + BQ-788

were determined by repeated-measures ANOVA. Relation between variables of interest was assessed by linear regression analysis. There were no significant main effects of gender on FBF responses to endothelin blockade of FBF x gender interactions, therefore the data were pooled and presented together. Data are expressed as means ± SEM. Statistical significance was set at $P<0.05$.

RESULTS

Subject characteristics are presented in the Table. There were no significant differences in baseline FBF between the normal (4.4±0.3 mL/100 mL of tissue/min) and impaired (4.0±0.3 mL/100 mL of tissue/min) fasting blood glucose groups. FBF responses to ET-receptor blockade are shown in the Figure. BQ-123 elicited a significantly greater (~20%) increase in FBF in the impaired fasting glucose than normal fasting glucose groups. Moreover, the addition of BQ-788 to BQ-123 resulted in a further 2-fold increase ($P<0.05$) in FBF in the impaired fasting glucose but not normal fasting glucose adults. In the overall study population, there was a strong and positive correlation between fasting blood glucose levels and the peak vascular responses to BQ-123 ($r=0.44$; $P<0.05$) and BQ-123 + BQ-788 ($r=0.62$; $P<0.05$). No other anthropometric, hemodynamic or metabolic variable was significantly correlated with the vascular responses to ET-1 receptor blockade.

DISCUSSION

The key finding of the present study is that ET-1 mediated vasoconstrictor tone is elevated in adults with impaired fasting blood glucose concentrations independent of other cardiometabolic risk factors. Moreover, the enhancement in ET-1 vasoconstriction is facilitated

by both the ET_A and ET_B receptors. To our knowledge, this is the first study to assess the influence of impaired fasting blood glucose concentrations on ET-1 system activity.

In a recent study, DeVan and colleagues [6] reported that endothelial vasodilator function is impaired in middle-aged adults with impaired fasting blood glucose. Indeed, brachial artery flow-mediated dilation was ~30% lower in the adults with impaired fasting glucose compared with healthy controls of similar age. The results of the present study compliment and extend these findings by demonstrating that endothelial vasomotor dysfunction with impaired fasting glucose is not limited to vasodilation. Indeed, the seminal findings presented herein demonstrate that ET-1-mediated vasoconstrictor tone is markedly higher in middle-aged adults with impaired fasting glucose. FBF responses to both selective and non-selective ET-1 receptor blockade were markedly higher (20% and 40%, respectively) in the adults with impaired fasting glucose compared with their normal fasting plasma glucose counterparts. Of note, $ET_{A/B}$ receptor blockade resulted in a further increase in FBF above that observed with ET_A blockade alone in the impaired fasting glucose adults only, demonstrating that the ET_B receptor also contributes to the elevation in ET-1 vasoconstrictor tone with impaired fasting blood glucose. It should be noted that we assessed ET-1 system activity by pharmacologically blocking both ET-1 receptors (located on vascular smooth muscle cells and endothelium) instead of relying on circulating plasma concentrations of ET-1. The physiological relevance of plasma ET-1 levels is questionable as ET-1 is predominantly (>80%) released abluminally toward the vascular smooth muscle [12]. Thus, circulating levels provide little information on the vascular effects of the peptide at the level of the vessel wall.

The mechanisms underlying greater ET-1 vasoconstrictor tone with impaired fasting glucose are not well understood. It is important to emphasize that there were no differences

between our groups with respect to body composition, blood pressure or plasma lipid and lipoproteins, all factors that are independently associated with increased ET-1 system activity and often coexist with the impaired fasting glucose condition. For example, overweight and obesity, a common co-morbidity with impaired fasting glucose, has been shown to adversely influence endothelial vasomotor regulation via increased ET-1 system activity [11]. The subjects in the present study, however, were remarkably similar anthropometrically; discounting the influence of body composition on our findings. Notably, the only variable in the present study that correlated with the vascular responses to ET-1 receptor blockade was fasting plasma glucose. Peak blood flow responses to both selective ET_A receptor (r=0.44) and non-selective $ET_{A/B}$ receptor (r=0.62) blockade were significantly and positively associated with plasma glucose concentrations. At the very least, these correlative data provide directional support that the observed group differences were indeed glucose-related. While not measured in the present study, it is possible that oxidative stress and inflammatory burden may underlie the impaired fasting glucose-related increase in ET-1-mediated vasoconstrictor tone. Both oxidative stress and inflammation have been shown to exacerbate ET-1 system activity [13] and are prevalent with the impaired fasting glucose condition [14]. Future studies are needed to determine whether the increase in ET-1 vasoconstriction with impaired fasting glucose is due, at least in part, to oxidative and inflammatory processes.

There are two experimental considerations regarding the present study that deserve mention. Firstly, given our cross-sectional study design, we cannot discount the possibility that genetic and/or lifestyle behaviors may have influenced our results. To minimize the influence of lifestyle behaviors, we studied sedentary adults who were non-smokers and not currently taking any medication that could influence endothelial vasomotor function. Moreover, to isolate the

primary influence of impaired fasting blood glucose concentrations, we studied adults of similar age who were free of other cardiometabolic abnormalities that are known to influence endothelial function, such as hypertension [15] and dyslipidemia [16]. Secondly, the present study has a modest sample size and all of the subjects were Caucasian. Thus, any generalizations to larger, more diverse populations require further study.

In summary, the results of this study demonstrate that ET-1-mediated vasoconstrictor tone is elevated in adults with impaired fasting blood glucose concentrations, independent of other cardiometabolic risk factors. From a public health perspective, endothelial vasomotor dysfunction, characterized by ET-1 system hyperactivity, is a well-established vascular abnormality with type II diabetes that contributes to cardiovascular risk [17]. Our findings indicate that augmented ET-1 vasoconstrictor tone is already apparent in the impaired fasting glucose prediabetic state. This provides further support for early intervention in adults with impaired fasting blood glucose concentrations not only to reduce their risk of developing diabetes but cardiovascular abnormalities as well.

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FIGURE LEGEND

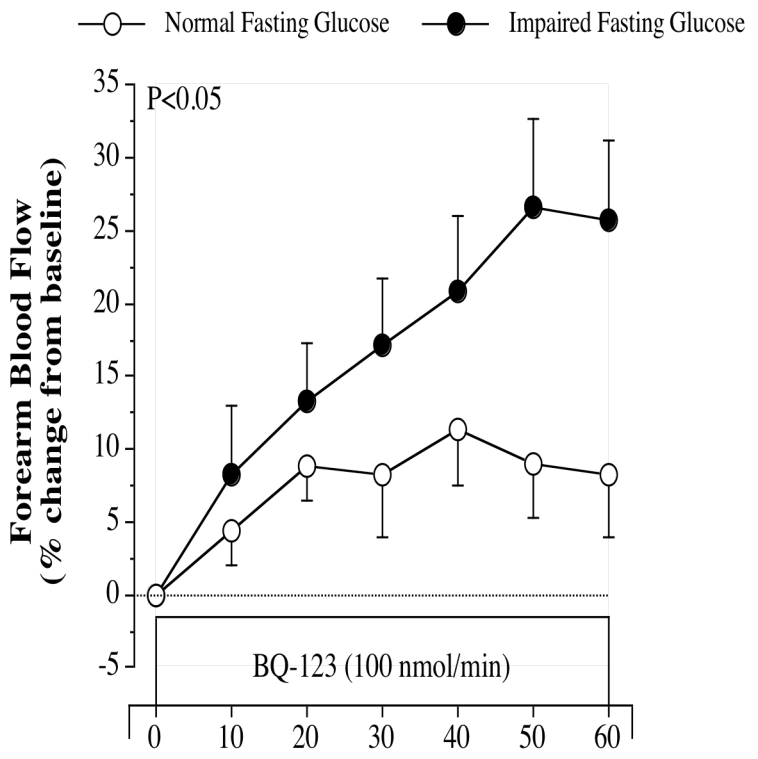
Figure. Forearm blood flow responses to BQ-123 (100 nmol/min), a selective ET_A receptor antagonist (panel A) and BQ-788 (50 nmol/min), a selective ET_B receptor antagonist (panel B), in normal fasting glucose and impaired fasting glucose adults. Values are mean ± SEM. The P value refers to the difference in the FBF response to ET_A and $ET_{A/B}$ blockade in the normal vs. impaired fasting glucose groups.

Table. Selected subject characteristics.

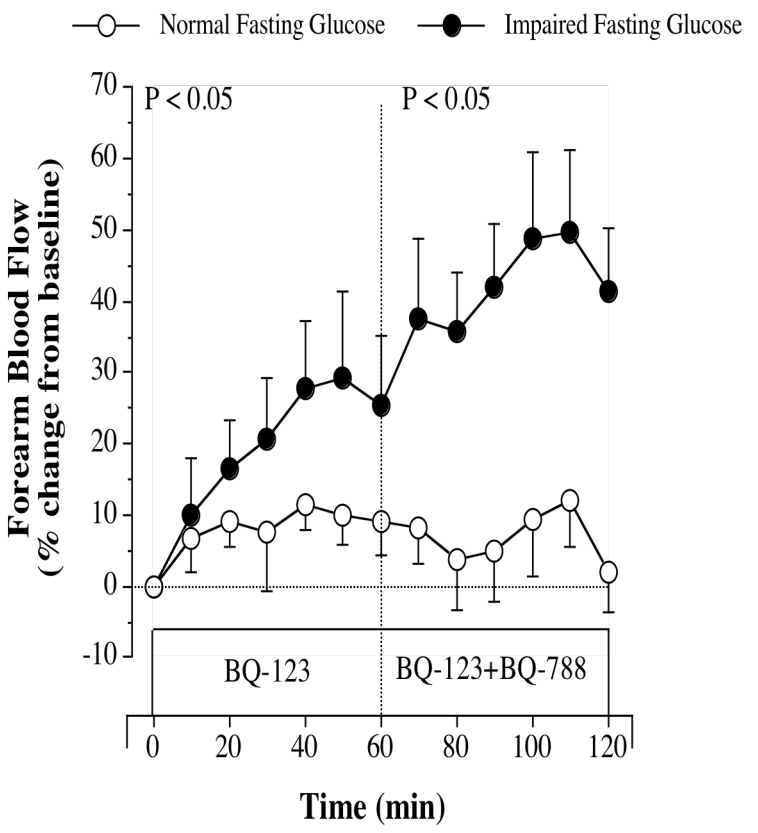
Variable	Normal Fasting Glucose (n=14)	Impaired Fasting Glucose (n=14)
Age (yrs)	57 ± 2	58 ± 1
Gender, M/F	8/6	9/5
Body Mass (kg)	84.9 ± 3.9	87.5 ± 3.9
Body Mass Index (kg/m^2)	29.1 ± 0.9	29.7 ± 1.0
Body Fat (%)	36.1 ± 1.5	35.4 ± 1.6
Waist Circumference (cm)	94.3 ± 3.4	99.1 ± 2.8
Systolic BP (mmHg)	124 ± 2	127 ± 3
Diastolic BP (mmHg)	79 ± 2	79 ± 2
Total Cholesterol (mmol/L)	5.0 ± 0.2	5.3 ± 0.3
LDL-Cholesterol (mmol/L)	3.0 ± 0.2	3.4 ± 0.2
HDL-Cholesterol (mmol/L)	1.3 ± 0.1	1.2 ± 0.1
Triglycerides (mmol/L)	1.5 ± 0.2	1.5 ± 0.2
Glucose (mmol/L)	4.9 ± 0.1	5.8 ± 0.1*
Insulin (pm/L)	49.8 ± 6.6	51.0 ± 6.6
HOMA-IR	1.9 ± 0.2	2.4 ± 0.3

BP = blood pressure; HDL=high-density lipoprotein; LDL=low-density lipoprotein; HOMA-IR= homeostasis model assessment of insulin resistance; values are mean ± SEM
*$P < 0.05$ vs. normal fasting glucose

A


Normal Fasting Glucose
Impaired Fasting Glucose
P<0.05
Forearm Blood Flow
(% change from baseline)
BQ-123 (100 nmol/min)
35
30
25
20
15
10
5
0
-5
0
10
20
30
40
50
60

B


Normal Fasting Glucose
Impaired Fasting Glucose
P < 0.05
P < 0.05
Forearm Blood Flow
(% change from baseline)
BQ-123
BQ-123+BQ-788
70
60
50
40
30
20
10
0
-10
0
20
40
60
80
100
120
Time (min)

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ENDOTHELIN-1 SYTEM ACTIVITY IN ADULTS WITH BORDERLINE HIGH LDL-CHOLESTEROL

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ABSTRACT

Background: Modest elevations in plasma low-density lipoprotein (LDL)-cholesterol have been shown to confer a significant increase in cardiovascular risk. Endothelin (ET)-1 is a vasoconstrictor peptide with proatherogenic properties. The experimental aim of this study was to determine whether ET-1 system activity is elevated in adults with borderline high LDL-cholesterol, independent of other cardiometabolic abnormalities.

Methods: Forearm blood flow (FBF; plethysmography) responses to intra-arterial infusion of ET-1, selective ET_A receptor blockade (BQ-123), and non-selective $ET_{A/B}$ blockade (BQ-123 + BQ-788) were determined in 40 middle-aged and older adults (45-70 years): 20 with optimal/near optimal LDL-cholesterol (<3.4 mmol/L) and 20 with borderline-high LDL-cholesterol (3.4-4.1 mmol/L).

Results: Both groups demonstrated a similar, non-significant (~10%) reduction in FBF to ET-1. BQ-123 and BQ-123+788 elicited a modest, but significant, increase in FBF (~15-20%) in each group. However, there were no group differences in the FBF responses to either selective ET_A or non-selective $ET_{A/B}$ receptor antagonism.

Conclusion: Borderline-high LDL-C is not associated with increased ET-1 mediated vasoconstrictor tone. Disrupted ET-1 system activity may not contribute to the increased cardiovascular risk burden with borderline-high LDL-cholesterol.

INTRODUCTION

Low-density lipoprotein (LDL) cholesterol is a major atherogenic fraction of total cholesterol [1, 2] and an important predictor of myocardial infarction in middle-aged adults [3]. In fact, the risk of cardiovascular disease is estimated to increase by ~30% for every 30 mg/dL rise in plasma LDL-cholesterol concentration, suggesting increased cardiovascular risk burden at levels below the clinically recognized elevated range of >130 mg/dL [1]. Indeed, the relative risk of heart disease is nearly two-fold higher in adults with borderline high LDL-cholesterol (3.4-4.1 mmol/L [30-159 mg/mL]) compared with adults with optimal/near optimal LDL-cholesterol levels (<3.4 mmol/L [130 mg/dL]) [1]. Moreover, nearly 40% of the adults in the United States have LDL-cholesterol levels in the borderline high range. Thus, understanding the mechanisms involved with the increased vascular risk with borderline high LDL-cholesterol has great public health importance.

Endothelin-1 is a powerful vasoconstrictor peptide that in addition to contributing to the regulation of vascular tone is also involved in the development and progression of atherosclerotic vascular disease [4, 5]. Indeed, enhanced ET-1 system activity elicits a proinflammatory vascular phenotype and ET-1 immunoreactivity is prominent in the walls of atherosclerotic human vessels [6, 7]. Moreover, ET-1 promotes fibrous tissue formation and inhibits endothelial nitric oxide synthesis [8, 9]. Increased ET-1 vasoconstrictor tone is common characteristic of numerous cardiovascular conditions and risk factors [10-12], including clinical hypercholesterolemia [13]. Whether this vascular impairment is already apparent in the borderline-high cholesterolemic state with its corresponding increase in vascular risk is unknown.

Accordingly, the experimental aim of this study was to determine whether ET-1 system activity is elevated in adults with borderline high LDL-cholesterol, independent of other cardiometabolic abnormalities. If so, this may contribute mechanistically to endovascular dysfunction and increased cardiovascular risk in this population.

METHODS

Subjects

Forty, sedentary middle-aged adults were studied: 20 with optimal/near optimal LDL-C (<130 mg/dL) and 20 with borderline-high LDL-C (130-159 mg/dL) [14]. All subjects were sedentary, non-obese, non-smokers, normotensive, non-medicated (including vitamins) and free of overt cardiovascular disease as assessed by physical examination, fasting blood chemistries and electrocardiogram and blood pressure at rest and during incremental exercise performed to volitional exhaustion. All women were at least 1 year postmenopausal and had never taken or had discontinued use of hormone replacement therapy at least 1 year before the start of the study. Written informed consent was obtained according to the guidelines of the University of Colorado at Boulder.

Intra-Arterial Infusion Protocol

All of the studies were performed between 7 AM and 10 AM after a 12-hour overnight fast in a temperature-controlled room as previously described [15]. Briefly, a 5-cm, 20-gauge catheter was inserted into the brachial artery of the nondominant arm under local anesthesia (1% lidocaine). Forearm blood flow (FBF) at rest and in response to each pharmacological agent was measured by strain-gauge venous occlusion plethysmography (D.E. Hokanson, Bellevue,

Washington). To rule out the possibility of nonspecific group differences to vasoconstrictor agents, vascular responses to norepinephrine were determined. Norepinephrine was infused at a rate of 260 pmol/min for 5 minutes, and FBF was measured during the last 3 minutes. After a 20-minute rest period to allow FBF to return to baseline levels, ET-1 (Clinalfa, AG) was infused at a rate of 5 pmol/min for 20 minutes, and FBF was measured during the last 3 minutes. After a 30-minute rest period to allow resting FBF to return to baseline, BQ-123 (Clinalfa, AG), a selective ET_A receptor antagonist, was infused at a rate of 100 nmol/min for 60 minutes. FBF was measured every 10 minutes throughout the infusion period. After 60 minutes of BQ-123 infusion, FBF was measured every 10 minutes during the combined infusion of BQ-123 and BQ-788. BQ-788, a selective ET_B receptor antagonist, was infused at a rate 50 nmol/min for 60 mins.

Statistical Analysis

Differences in subject baseline characteristics and the magnitude of change in FBF to norepinephrine and ET-1 were determined by between-groups analysis of variance (ANOVA). Group differences in FBF responses to BQ-123 and BQ-123+BQ-788 were determined by repeated-measures ANOVA. There were no significant gender interactions, therefore the data were pooled and presented together. Data expressed as means ± SEM. Statistical significance was set at $P < 0.05$.

RESULTS

Selected subject characteristics are presented in the Table. By design, total cholesterol and LDL-C levels were the only variables significantly different ($p<0.05$) between the groups. The vasoconstrictor response to norepinephrine was not significantly different between the groups optimal/near optimal (from 4.0 ± 0.3 to 3.0 ± 0.3 mL/100 mL tissue/min) and borderline

high (from 4.1 ± 0.3 to 3.0 ± 0.2 mL/100 mL tissue/min) LDL-C groups. Resting FBF was reduced by ~10% in response to ET-1 in both the optimal/near optimal (from 4.0 ± 0.2 to 3.6 ± 0.2 mL/100 mL tissue/min) and borderline high (from 3.9 ± 0.2 to 3.6 ± 0.3 mL/100 mL tissue/min) LDL-C groups (Figure). In response to BQ-123, there was a significant increase in FBF (~15%) from baseline in both groups. The addition of BQ-788 did not significantly alter FBF in either group (Figure). Importantly, there were no group differences in FBF responses to either BQ-123 (P=0.77) or BQ-123 + BQ-788 (P=0.51).

DISCUSSION

The ET-1 system not only plays a central role in the regulation of vascular tone, but is also involved in the etiology of atherosclerotic vascular disease [4, 15]. The primary finding of the present study is that borderline-high LDL-cholesterol is not associated with enhanced ET-1 system activity. Indeed, forearm vascular responses to exogenous ET-1, selective ET_A receptor antagonism and non-selective $ET_{A/B}$ receptor blockade were almost identical between adults with LDL-cholesterol in the optimal/near optimal and borderline-high levels.

Cardillo and colleagues [13] were the first to demonstrate a relation between hypercholesterolemia and ET-1 system activity. In a seminal study, employing the same isolated forearm model as used herein, Cardillo et al. [13] reported that ET_A receptor-mediated ET-1 vasoconstrictor tone was significantly elevated in middle-aged adults with high LDL-cholesterol concentrations (5.8±0.2 mmol/L [223±9 mg/dL]). The results of the present study compliment and extend these findings by demonstrating that ET-1 vasoconstrictor is not elevated at subclinical levels of LDL-cholesterol. This finding is in contrast to conditions such prehypertension [12] and impaired fasting glucose [16] where ET-1-mediated vasoconstrictor

tone is already elevated to comparable levels seen with clinical hypertension [17] and overt diabetes [18]. Interestingly, borderline high LDL-cholesterol is associated with diminished nitric oxide-mediated endothelial vasodilation [19], thus modest elevations in LDL-cholesterol appear to have differential effects on two key elements of endothelial vasomotor regulation. Moreover, given the inhibitory interaction between ET-1 and nitric oxide [20], lack of enhanced ET-1 system activity argues against an ET-1-related mechanism underlying endothelial vasodilator dysfunction with borderline high LDL-cholesterol.

It should be noted that we did not measure circulating plasma concentrations of ET-1. Since the vast majority of ET-1 produced by the endothelium (>80%) is released towards the vascular smooth [21], circulating levels of ET-1 do not appropriately reflect the vascular effects of the peptide [22]. Rather, we employed a pharmacological approach involving the intra-arterial infusion of exogenous ET-1 and ET receptor antagonists providing a more direct and specific biological assessment of ET-1 system activity *in vivo* [13, 17].

In conclusion, the results of the present study demonstrate that borderline high LDL-cholesterol is not associated with increased ET-1 vasoconstrictor activity. Although elevations in LDL-cholesterol in the borderline high range is linked with both diminished endothelial vasodilator function [19, 23] and increased cardiovascular risk burden [1], disrupted ET-1 system activity does not appear to be an contributing factor.

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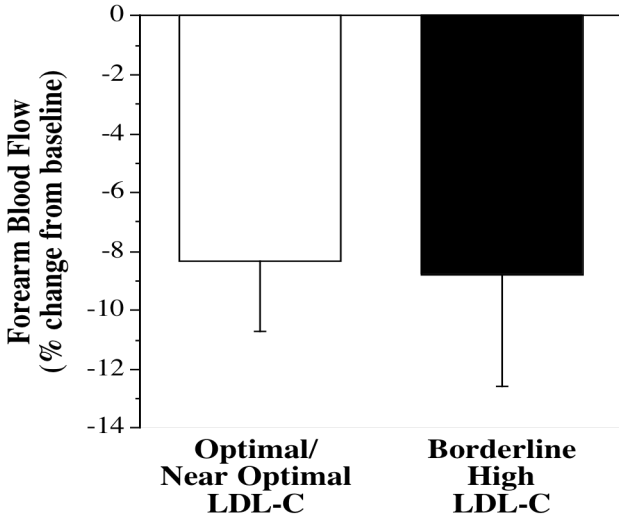
FIGURE LEGEND

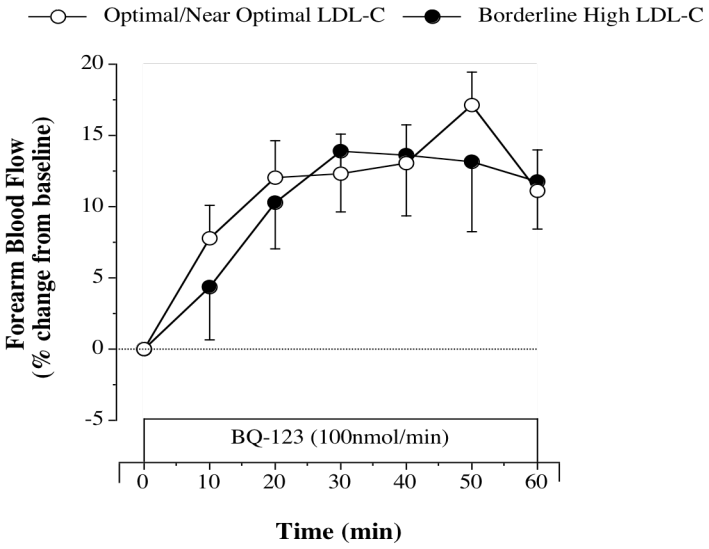
Figure. Forearm blood flow response to ET-1 (top panel), BQ-123, selective ETA receptor antagonism (middle panel) and BQ-123 + BQ-788, ETA and ETB receptor blockade (bottom panel) in the optimal/near optimal LDL-C and borderline high LDL-C groups.

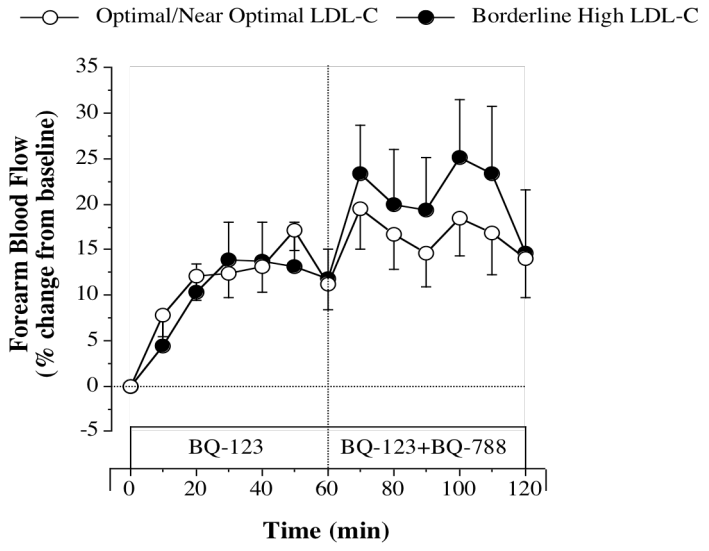
Table. Selected Subject Characteristics.

Variable	Optimal/Near Optimal LDL-C (n=20)	Borderline-High LDL-C (n=20)
Age, yr	59±2	59±2
Sex, M/F	11/9	11/9
Body mass, kg	80.5±3.5	76.9±3.6
BMI, kg/m^2	27.2±0.8	26.3±0.9
Body fat, %	34.1±2.3	34.1±1.8
Waist circumference, cm	91.8±2.7	88.0±3.0
Systolic BP, mmHg	126±2	122±2
Diastolic BP, mmHg	78±1	78±2
Total cholesterol, mmol/L	4.7±0.1	5.6±0.1*
LDL-cholesterol, mmol/L	2.7±0.1	3.7±0.1*
HDL-cholesterol, mmol/L	1.4±0.1	1.3±0.1
Triglycerides, mmol/L	1.2±0.1	1.4±0.1
Glucose, mmol/L	5.2±0.1	5.1±0.1
Insulin, pmol/L	38.1±4.9	40.2±4.8

BMI indicates body mass index; BP, blood pressure; LDL, low-density lipoprotein; HDL, high-density lipoprotein; Values are mean±SEM. *P < 0.05 vs. optimal/near optimal LDL-C.


Forearm Blood Flow
(% change from baseline)
0
-2
-4
-6
-8
-10
-12
-14
Optimal/
Near Optimal
LDL-C
Borderline
High
LDL-C


Optimal/Near Optimal LDL-C
Borderline High LDL-C
Forearm Blood Flow
(% change from baseline)
20
15
10
5
0
-5
BQ-123 (100nmol/min)
0
10
20
30
40
50
60
Time (min)


Optimal/Near Optimal LDL-C
Borderline High LDL-C
Forearm Blood Flow
(% change from baseline)
35
30
25
20
15
10
5
0
-5
BQ-123
BQ-123+BQ-788
0
20
40
60
80
100
120
Time (min)

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CHRONIC NEBIVOLOL TREATMENT SUPRESSES ENDOTHELIN-1-MEDIATED VASCOCONSTRICTOR TONE IN ADULTS WITH ELEVATED BLOOD PRESSURE

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INTRODUCTION

Endothelin (ET)-1 is a potent vasoconstrictor peptide produced and released by the vascular endothelium (41, 47). In humans, the vascular actions of ET-1 are mediated by two distinct ET receptor subtypes: ET_A receptors located exclusively on vascular smooth muscle and ET_B receptors located on both the vascular smooth muscle and endothelial surfaces (20, 25, 34). In combination with the endothelial vasodilator nitric oxide, ET-1 plays a central role in the regulation of vascular tone (11, 42). In addition to its vasoregulatory actions, there is considerable evidence supporting the involvement of ET-1 in the pathogenesis of atherosclerotic vascular disease (20, 26, 36) and its associated risk factors, most notably elevated blood pressure (BP) (10, 23, 29, 31, 46). In a seminal series of studies, Cardillo et al. (9, 13) reported that adults with essential hypertension demonstrate higher forearm vasodilator responses to selective ET_A receptor blockade compared with normotensive controls. Moreover, when ET_A receptor blockade was combined with ET_B receptor blockade there was a further increase in the vasodilator response in the hypertensive adults whereas forearm blood flow remained unchanged in the normotensive controls. Collectively, these results indicated that vasoconstrictor tone to ET-1 is markedly elevated with hypertension and is mediated by both the ET_A and ET_B receptors. Further, in a follow-up study, blockade of ET-1 receptors improved acetylcholine-induced endothelium-dependent vasodilation in hypertensive patients, indicating that increased ET-1 vasoconstriction contributes to the vasodilator dysfunction associated with hypertension (9). Weil et al. (46) recently reported identical findings in adults with blood pressure in the prehypertensive range (systolic blood pressure: 120-139 mmHg and/or diastolic blood pressure: 80-89 mmHg). Thus, clear links have been established between ET-1 system activity and elevations in blood pressure.

Nebivolol, a third generation beta-blocker with high selectivity for β_1-adrenergic receptors, has proven to be highly effective in treating elevated blood pressure (3, 15, 17, 30). A distinguishing feature of nebivolol from other beta-blockers is its hemodynamic profile, specifically the unique ability to enhance both basal and stimulated nitric oxide release resulting in peripheral vasodilation, improved endothelial function and increased myocardial compliance (19, 28, 39, 45). Cockcroft et al. (18) demonstrated that the vasodilatory effects of nebivolol were attenuated by the infusion of the nitric oxide synthase inhibitor N^G-monomethyl L-arginine; indicating that nebivolol induced improvement in vasodilator function is mediated, in part, by increased nitric oxide bioavailability. However, the favorable vascular effects of nebivolol that contribute to its blood pressure lowering action may not be limited to nitric oxide. Indeed, there are *in vitro* data to suggest that nebivolol suppresses endothelial ET-1 production (6), but there is currently no *in vivo* clinical evidence that treatment with nebivolol reduces ET-1-mediated vasoconstrictor tone.

Accordingly, we tested the hypothesis that chronic nebivolol treatment will reduce ET-1-mediated vasoconstrictor tone in adult humans with elevated blood pressure. Moreover, that reducing ET-1 vasoconstrictor activity contributes to the improvement in endothelial vasodilator function associated with nebivolol. To address this hypothesis, we employed a 3-month randomized, double-blind placebo controlled study to determine the effects of nebivolol, compared with metoprolol and placebo, on ET-1 vasoconstrictor tone in adults with suboptimal blood pressure.

METHODS

Subjects

Thirty-nine middle-aged adults with elevated blood pressure (BP) (systolic BP $\geq$ 130 and/or diastolic BP $\geq$ 85 mmHg) participated in a double-blind, randomized, placebo controlled trial: 14 received nebivolol (8M/6F; 5 mg/day: Forest Laboratories, Inc.); 11 received metoprolol succinate (8M/3F; 100 mg/day; AstraZeneca LP); and 14 received placebo (9M/5F; 1 gelatin capsule/day; Forest Laboratories, Inc.). Resting blood pressure was determined by the average of two or more seated BP readings from two separate visits per American Heart Association guidelines (16). All subjects were free of overt coronary and metabolic disease as assessed by medical history, physical examination, fasting blood chemistries, and electrocardiograms and blood pressure at rest and during incremental exercise performed to exhaustion. In addition, all subjects presented with a resting HR > 50 beats per minute. None of the subjects smoked, were taking medications (including vitamins), or performed regular physical exercise for at least 1 year before the start of the study. All of the women were at least 1 year postmenopausal and had never taken or had discontinued use of hormone replacement therapy at least 1 year before the start of the study. After baseline testing, subjects were randomly assigned to 1 of the 3 experimental groups. Prior to participation, all of the 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. All of the procedures were performed according to institutional guidelines.

Measurements

Blood Pressure. Resting blood pressure measurements were performed in the sitting position on at least two separate days at least one week apart. Subjects were instructed not to ingest caffeine-containing beverages prior to all blood pressure measurements. The recordings were made under quiet, comfortable ambient (~24°C) laboratory conditions. To avoid the possibility of investigator bias, measurements were made with a semi-automated device (Dinamap, Crtikon, FL) that uses an oscillometric technique over the brachial artery. Recordings were made in triplicate in the upright sitting position. All measurements conformed to American Heart Association guidelines as established by the Council for High Blood Pressure Research (38).

*Body Composition***.** Body mass was measured to the nearest 0.1 kg using a medical beam balance (Detecto, Webb City, MO). Percent body fat was determined by dual energy x-ray absorptiometry (Lunar Radiation Corporation, Madison, WI). Body mass index (BMI) was calculated as weight (kilograms) divided by height (meters) squared. Minimal waist circumference was measured according to previously published guidelines (33).

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 at Boulder.

Intra-arterial Infusion Studies. All studies were performed between 7:00 am and 10:00 am after a 10-hour overnight fast in a temperature-controlled room. Under strict aseptic conditions a 5-cm, 20-gauge catheter was inserted into the brachial artery of the nondominant arm under local anesthesia (1% lidocaine). Heart rate and arterial blood pressure were continuously measured throughout the infusion protocol. Forearm blood flow (FBF) at rest and

in response to each pharmacological agent was measured in both the experimental (nondominant) and contralateral (dominant) forearm using strain-gauge venous occlusion plethysmography (D. E. Hokanson, Bellevue, WA), as previously described by our laboratory (44). Baseline FBF was measured for 5 minutes and for 5 minutes before each drug infusion thereafter. Following the measurement of resting blood flow, FBF was assessed in response to infusions of acetylcholine (ACh; IOLAB pharmaceuticals, Duluth, GA) at 4.0, 8.0, and 16.0 μg/100 mL tissue/min and sodium nitroprusside (SNP; Nitroprusside, Abbott Laboratories) at 1.0, 2.0, and 4.0 μg/100 mL tissue/min. Each dose of ACh and SNP was infused for ~5 minutes and sufficient time (~20 minutes) was allowed for FBF to return to resting levels between each vasoactive agent. To avoid an order effect, the sequence of drug administration was randomized. After the initial infusion of ACh and SNP and allowing FBF to return to baseline (~20 min), BQ-123 (Clinalfa, AG), a selective ET_A receptor antagonist, was infused at a rate of 100 nmol/min for 60 minutes. FBF was measured every 10 minutes throughout the infusion period. The selected dose of BQ-123 has been shown to completely inhibit the vasoconstrictor effect of ET-1 in the human forearm of healthy adults (12, 44). After 60 minutes of BQ-123 infusion, the FBF response to nonselective ET-1 receptor blockade was assessed by the coadministration of BQ-123 and BQ-788 (Clinalfa, AG) for an additional 60 minutes. BQ-788, a specific antagonist of ET_B receptors, was infused at a rate 50 nmol/min, a dose demonstrated to effectively inhibit ET_B receptors (10). Thereafter, the infusion of BQ-123 and BQ-788 was continued at the same dose and FBF was reassessed during co-administration of ACh as performed earlier.

Statistical Analysis

Differences in subject baseline characteristics were determined by between-groups analysis of variance (ANOVA). Group differences in FBF responses to acetylcholine, sodium nitroprusside, BQ-123, BQ-788 and BQ-123/BQ-788+Ach were determined by repeated-measures ANOVA. There were no significant gender interactions, therefore the data were pooled and presented together. All data are expressed as mean ± SEM. Statistical significance was set *a priori* at $P<0.05$.

RESULTS

Selected subject characteristics are presented in Table 1. There were no differences in age, anthropometric, metabolic, or hemodynamic variables between the groups. Table 2 shows the blood pressure responses amongst the groups. There were no differences in resting blood pressure between the nebivolol, metoprolol, and placebo groups. Both nebivolol and metoprolol treatment resulted in similar and significant reductions in systolic (~10%), diastolic (~15%), and mean arterial (~15%) blood pressure. There were no significant changes in blood pressure in placebo group.

Before beginning the respective interventions, FBF responses to selective ET_A receptor blockade with BQ-123 were similarly and significantly elevated (~30%) from baseline in all three groups. However, nebivolol, but not metoprolol or placebo, therapy resulted in a marked (~25%; $P<0.05$) reduction in FBF response to BQ-123 (Figure 1). The vasodilator response to BQ-123 were almost identical before and after either metoprolol or placebo treatment. The FBF responses to nonselective $ET_{A/B}$ receptor blockade with BQ-123 and BQ-788 were similar amongst the groups prior to treatment. There was a significant increase (~35%) in FBF beyond

that of ET_A receptor blockade (Figure 2). However, after 3-month of treatment, only nebivolol therapy significantly reduced (~40%) the FBF response to non-selective $ET_{A/B}$ receptor blockade (Figure 2). Neither metoprolol therapy nor placebo significantly altered the FBF responses to BQ-123/BQ-788 infusion.

FBF responses to the endothelium-dependent vasodilator acetylcholine were not significantly different (nebivolol: from 5.1±0.3 to 13.3±0.8 mL/100 mL tissue; metoprolol: from 5.5±0.4 to 14.9±1.3 mL/100 mL tissue; and placebo: from 4.7±0.2 to 12.9±0.9 mL/100 mL tissue) between the three groups before intervention. Nebivolol treatment resulted in a significant increase (~20%, $P \leq 0.05$) in the vasodilator response to acetylcholine (from 13.3±0.8 to 15.8±0.6 mL/100 mL tissue) (Figure 3). In stark contrast, there was no change in the FBF responses to acetylcholine in either the metoprolol (from 14.9±1.3 to 14.9±1.3 mL/100 mL tissue) or placebo (from 12.9±0.9 to 13.6±0.8 mL/100 mL tissue) groups (Figure 3). The co-infusion of acetylcholine with non-selective ET blockade (BQ-123 + BQ-788) resulted in significantly greater (~30%) vasodilator response in all three groups prior to intervention (Figure 4). FBF responses to acetylcholine in the presence and absence of BQ-123 + BQ-788 were no longer different following nebivolol but not metoprolol or placebo treatment (Figure 4).

DISCUSSION

The blood pressure lowering effects of nebivolol are well established (14, 22, 35). The seminal finding of the present study, however, is that in addition to, and independent of, lowering blood pressure, nebivolol markedly and favorably affects ET-1 system activity. Indeed, the results reported herein demonstrate that chronic nebivolol, but not metoprolol, therapy: 1) reduces ET-1-mediated vasoconstrictor tone in adults with elevated blood pressure; and 2)

reductions in ET-1 vasoconstriction underlie nebivolol-induced improvements in endothelium-dependent vasodilation. Diminished ET-1-mediated vasoconstrictor tone may represent an important endovascular pleiotropic effect of nebivolol, contributing to its favorable effect on overall cardiovascular risk (4). To the best of our knowledge, this is the first study using established pharmacological approaches to demonstrate nebivolol-specific effects on ET-1 system activity in adult humans.

In the present study, there was a similar and significant (~30%) increase in FBF responses to selective ET_A receptor blockade in all three groups prior to treatment. In addition, non-selective $ET_{A/B}$ receptor blockade resulted in a further increase (~35%) in FBF in all the groups. These findings are fully consistent with previous studies establishing enhanced ET-1 system activity in adults with blood pressure in both the hypertensive (13) and prehypertensive (46) range. For example, Cardillo et al. (13) demonstrated almost identical increases (35-55%) in FBF to selective and non-selective ET receptor blockade in adults with essential hypertension compared with marginal, non-significant changes in FBF in normotensive adults. Thus, we are confident that ET-1-mediated vasoconstrictor tone was abnormally high in our subjects with elevated blood pressure without a direct comparison to a normotensive control group.

In vitro, nebivolol has been shown to blunt endothelial production, and it turn release, of ET-1 (6). The results of the present study compliment and significant extend these findings by demonstrating that nebivolol reduces ET-1 mediated vasconstrictor tone in adults with elevated blood pressure. Indeed, after three months of nebivolol therapy, there was a marked reduction (~25%) in the vasodilator response to both ET_A and $ET_{A/B}$ receptor blockade. Of note, the nebivolol-induced reduction in ET-1 vasoconstrictor tone was independent of concomitant reductions in blood pressure. Blood pressure was equally and significantly reduced in adults

randomized to either the nebivolol or metoprolol treatment groups. However, metoprolol therapy had no effect on the vascular responses to either selective or non-selective ET-1 receptor blockade despite its blood pressure lowering effect. Moreover, there were no significant changes in body composition or cardiometabolic risk factors in response to nebivolol (or metoprolol) treatment. Collectively, this provides further evidence for a direct clinical effect of nebivolol on the ET-1 system. Several mechanisms may underlie this unique feature of nebivolol. Most notably, nebivolol has been shown to ameliorate prepro-ET-1 mRNA production in human coronary endothelial cells (6, 7). Prepro-ET-1 is the peptide transcribed from prepro-ET-1 mRNA that is posttranslationally modified to ET-1 (47). Diminishment of prepro-ET-1 would ultimately lead to less ET-1 formation. Other contributing factors may include greater nitric oxide bioavailability (2) and reduced oxidative mediators (24, 40). With respect to nitric oxide, nebivolol increases nitric oxide bioavailability by enhancing endothelial nitric oxide synthase activity through calcium (8, 27) and non-calcium dependent pathways (37). Nitric oxide, in turn, has a potent inhibitory influence on ET-1 at the level of transcription as well as endothelin converting enzyme activity (1, 5). Regarding oxidative stress, nebivolol has been shown to block NADPH oxidase, a known activator of the ET-1 system (32).

Concurrent with the nebivolol-induced reduction in ET-1-mediated vasoconstrictor tone, we also noted that the nebivolol-induced improvement in endothelium-dependent vasodilation is due, at least in part, to the reduction in ET-1 vasoconstriction. It is important to note that prior to intervention, the FBF responses to acetylcholine in all three groups were similar to that previously reported in prehypertensive (46) and hypertensive (13) adults, indicating that the ability to vasodilate was impaired in our study population. Consistent with previous studies (43), chronic nebivolol therapy significantly improved (~30%) Ach-mediated endothelium dependent

vasodilation. In stark contrast, there was no effect of metoprolol therapy (or placebo), on endothelial vasodilator function. It has been suggested that the nebivolol-induced improvement in endothelium-dependent vasodilation is largely due to an increase in nitric oxide bioavailability (2). A seminal finding of the present study is that reduced ET-1 vasoconstrictor tone appears to be a primary contributor to improved endothelial vasodilator function. Indeed, prior to intervention, the co-infusion of non-selective ET receptor blockade resulted in a significant increase (~35%) in Ach stimulated endothelial vasodilation in all 3 treatment groups. After the 3-month intervention period this effect was unchanged in the metoprolol and placebo groups; however, in the nebivolol group non-selective ET receptor antagonism no longer enhanced the FBF responses to Ach. Although we did not assess whether the nebivolol-induced improvement in Ach-mediated vasodilation was nitric oxide dependent, it is plausible that the reported increase in nitric oxide bioavailability with nebivolol is due, in part, to an uncoupling of ET-1-nitric oxide inhibition. Moreover, relieving ET-1-mediated vasoconstriction would allow nitric oxide, and other endothelium-derived relaxing factors, to act without opposition and dilate the vessel appropriately in response to stimulation. Thus, the unique vasomotor properties of nebivolol appears to involve both vasodilator and vascoconstrictor factors. To the best of our knowledge, this is the first study to assess the involvement of the ET-1 system in nebivolol-induced improvements in endothelial vasodilator function.

There are two experimental considerations regarding the present study that deserve mention. First, given the extended half-life of ET receptor agonists, our study design did not involve the singular administration of the selective ET_B receptor antagonist BQ-788 and therefore we cannot comment on the effects of nebivolol (or metoprolol) on the vascular actions of the ET_B receptor. Secondly, we did not measure circulating plasma levels of ET-1 in the

present study. ET-1 produced by the endothelium is predominantly (>80%) released abluminally toward the vascular smooth muscle (41); thus, the pathophysiological significance of circulating ET-1 levels is questionable as values reported are highly variable and misleading (13). Moreover, circulating plasma concentrations of the peptide do not necessarily reflect local vascular production but rather spillover into, and clearance from, the bloodstream (13). Intra-arterial infusion of ET-1 receptor antagonists offers a more direct, reproducible biological assessment of ET-1 system activity *in vivo* (13).

In conclusion, the results of this study indicate that nebivolol treatment reduces ET-1-mediated vasoconstrictor tone in adult humans with elevated blood pressure. Moreover, nebivolol-induced reduction in ET-1-mediated vasoconstrictor tone contributes to the favorable effects of nebivolol on endothelial vasodilation. Importantly, the direct effect of nebivolol on ET-1 system activity is independent of reductions in blood pressure and may be an important underlying factor contributing to the improvement in endovascular function and reduction in cardiovascular morbidity and mortality (21) associated with chronic nebivolol treatment.

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FIGURE LEGEND

Figure 1. FBF responses to acetylcholine in the nebivolol (panel A), metoprolol (panel B) and placebo (panel C) groups before and after 3 month intervention. Values are mean ± SEM; *P < 0.05 vs. before intervention.

Figure 2. FBF responses to selective ET_A receptor blockade with BQ-123 (100nmol/min) in the nebivolol (panel A), metoprolol (panel B) and placebo (panel C) groups before and after 3 month intervention. Values are mean ± SEM. The *P* value refers to the difference in the FBF responses to ET_A receptor blockade before vs. after intervention.

Figure 3. FBF responses to non-selective $ET_{A/B}$ receptor blockade with BQ-123+BQ-788 (100nmol/min and 50nmol/min) in the nebivolol (panel A), metoprolol (panel B) and placebo (panel C) groups before and after 3 month intervention. Values are mean ± SEM. The *P* value refers to the difference in the FBF responses to $ET_{A/B}$ receptor blockade before vs. after intervention.

Figure 4. FBF responses to acetylcholine in the presence and absence of non- selective $ET_{A/B}$ receptor blockade with BQ-123+BQ-788 (100nmol/min and 50nmol/min) in the nebivolol (5mg tablet taken orally once per day) group before (panel A) and after (panel B) 3 month intervention. Values are mean ± SEM; *P < 0.05 vs. saline.

Table 1. Selected Subject Characteristics.

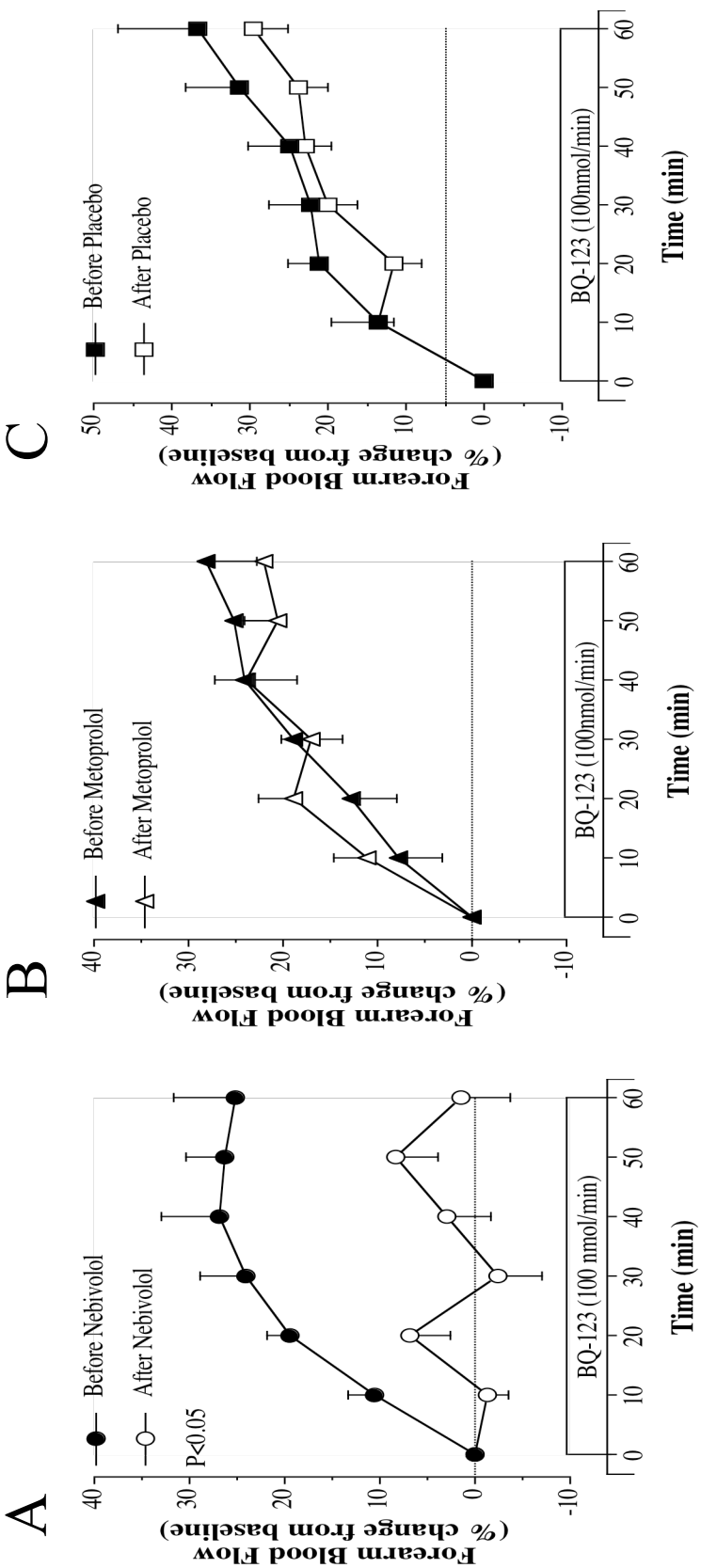
Variable	Nebivolol		Metoprolol		Placebo	
	Before	After	Before	After	Before	After
Sex, M/F	8 / 6	8 / 6	8 / 3	8 / 3	9 / 5	9 / 5
Age, yr	57 ± 1	57 ± 1	55 ± 1	55 ± 1	56 ± 1	56 ± 1
Body mass, kg	77.7 ± 2.7	78.4 ± 2.5	87.9 ± 4.9	89.9 ± 4.9	84.2 ± 4.8	84.6 ± 4.9
BMI, kg/m²	26.6 ± 0.7	26.9 ± 0.7	28.5 ± 1.4	29.2 ± 1.5	27.9 ± 1.3	28.0 ± 1.9
Body fat, %	33.6 ± 1.9	34.6 ± 1.9	33.2 ± 1.9	34.5 ± 1.7	35.2 ± 3.0	35.5 ± 3.0
Waist circumference, cm	88.3 ± 2.4	89.0 ± 2.4	95.5 ± 4.5	96.9 ± 4.4	91.6 ± 2.6	92.3 ± 2.7
Total cholesterol, mmol/L	5.2 ± 0.2	4.7 ± 0.2	4.9 ± 0.3	4.3 ± 0.3	5.4 ± 0.1	5.0 ± 0.3
LDL-cholesterol, mmol/L	3.1 ± 0.1	2.6 ± 0.1*	2.9 ± 0.3	2.7 ± 0.3	3.7 ± 0.2	3.2 ± 0.3
HDL-cholesterol, mmol/L	1.4 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	1.0 ± 0.1	1.3 ± 0.1	1.1 ± 0.1
Triglycerides, mmol/L	1.5 ± 0.3	2.2 ± 0.4	1.7 ± 0.3	1.3 ± 0.2	1.4 ± 0.1	1.2 ± 0.1
Glucose, mmol/L	4.9 ± 0.2	4.9 ± 0.2	5.0 ± 0.1	5.0 ± 0.2	5.0 ± 0.1	4.9 ± 0.1
Insulin, pmol/L	62.3 ± 5.6	57.2 ± 6.6	58.5 ± 7.1	71.1 ± 13.8	80.2 ± 9.3	65.3 ± 11.0
HOMA-IR	2.3 ± 0.2	2.1 ± 0.3	2.2 ± 0.3	2.4 ± 0.5	3.1 ± 0.4	2.5 ± 0.4

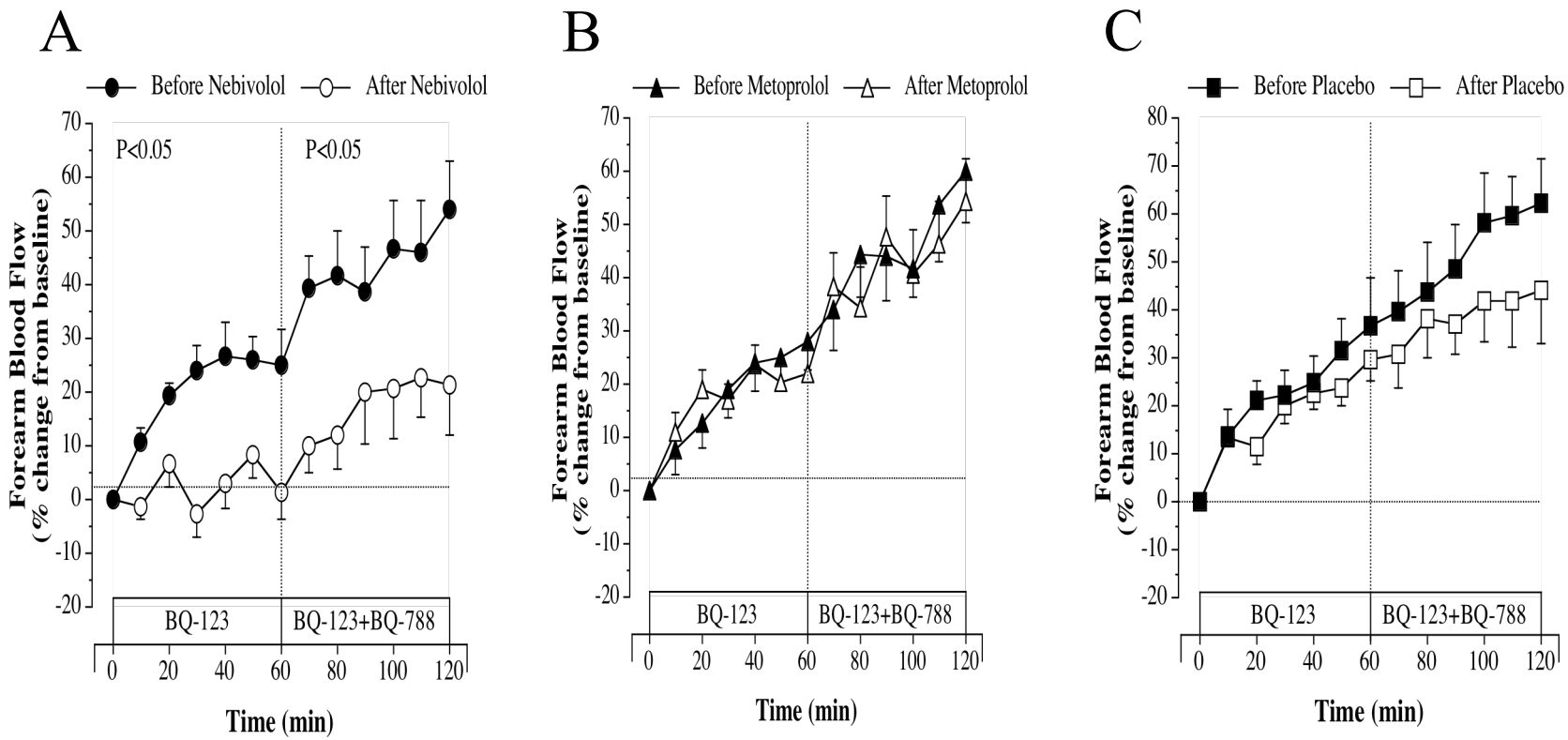
BMI indicates body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment-insulin resistance. Values are mean±SEM. *P < 0.05 vs. before intervention. †P < 0.05 vs.

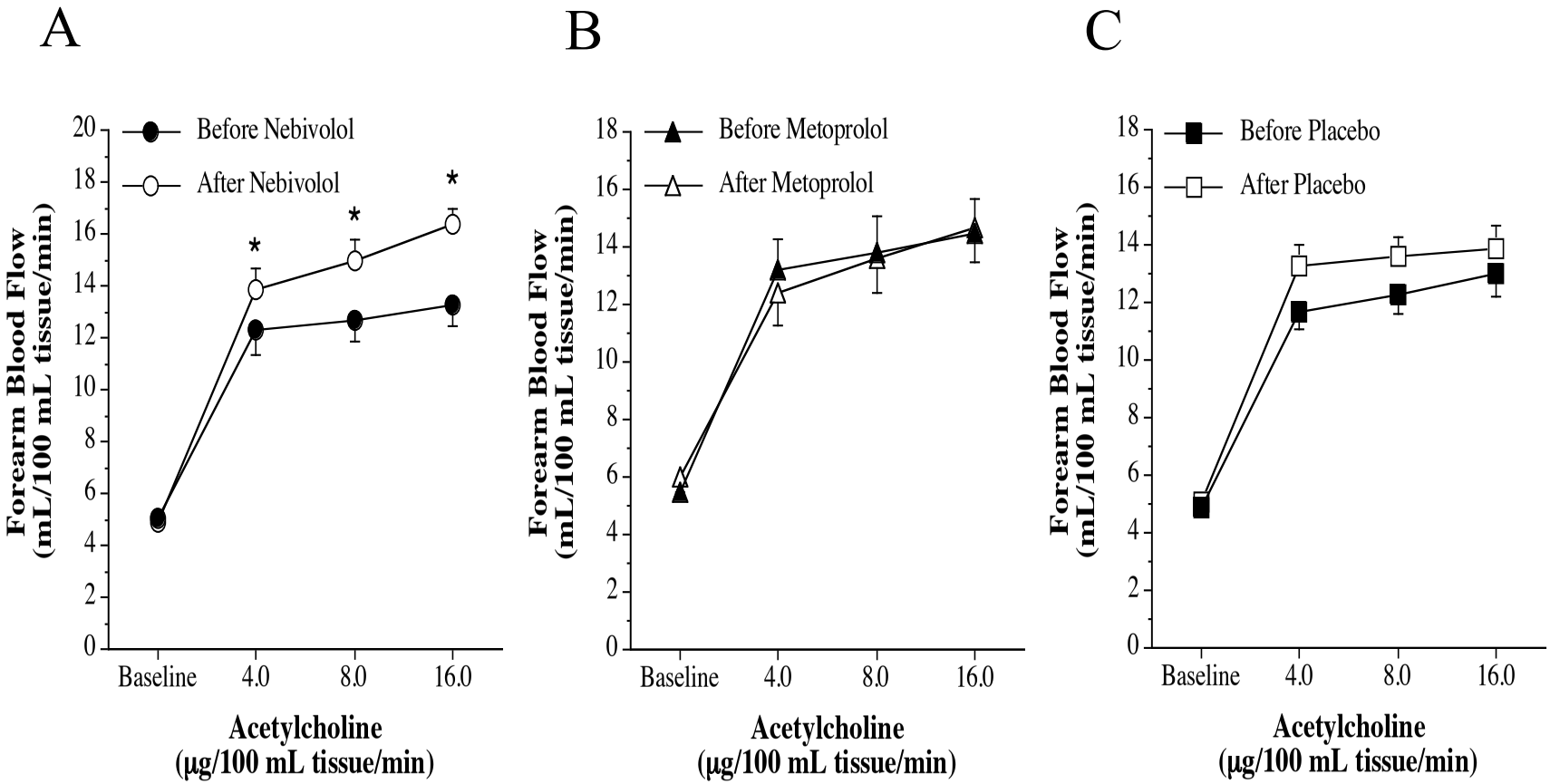
Table 2. Subject Blood Pressure.

Variable	Nebivolol		Metoprolol		Placebo	
	Before	After	Before	After	Before	After
Systolic BP, mmHg	144 ± 2	126 ± 2*	140 ± 2	125 ± 3*	139 ± 1	135 ± 2
Diastolic BP, mmHg	89 ± 1	77 ± 1*	90 ± 2	77 ± 1*	86 ± 2	83 ± 2
MAP, mmHg	108 ± 1	94 ± 2*	107 ± 2	93 ± 2*	104 ± 2	100 ± 2

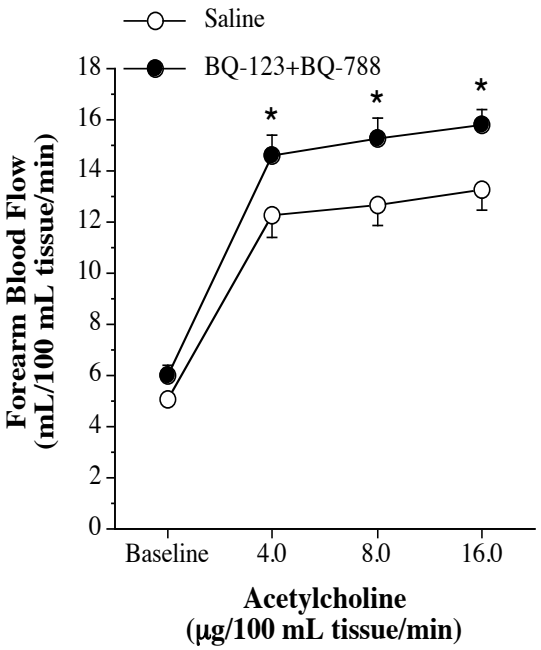
BP, blood pressure; MAP, mean arterial pressure. Values are mean±SEM. *P < 0.05 vs. before intervention. †P < 0.05 vs.


A
Before Nebivolol
After Nebivolol
P<0.05
Forearm Blood Flow
(% change from baseline)
40
30
20
10
0
-10
BQ-123 (100 nmol/min)
0
10
20
30
40
50
60
Time (min)
B
Before Metoprolol
After Metoprolol
Forearm Blood Flow
(% change from baseline)
40
30
20
10
0
-10
BQ-123 (100nmol/min)
0
10
20
30
40
50
60
Time (min)
C
Before Placebo
After Placebo
Forearm Blood Flow
(% change from baseline)
50
40
30
20
10
0
-10
BQ-123 (100nmol/min)
0
10
20
30
40
50
60
Time (min)

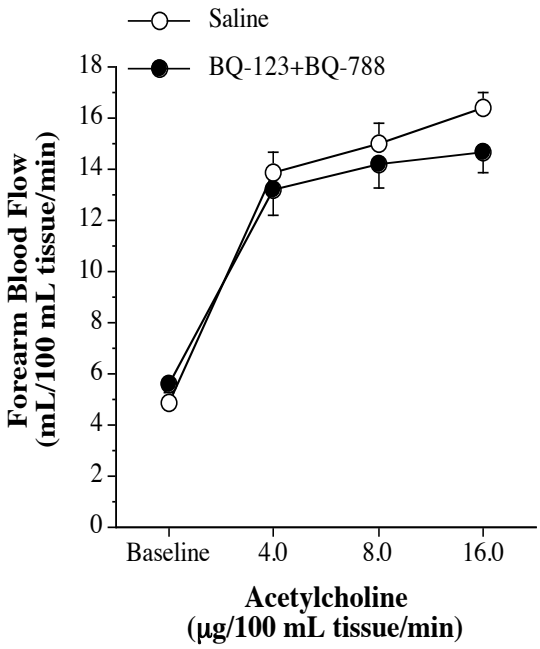

A
Before Nebivolol
After Nebivolol
P<0.05
P<0.05
Forearm Blood Flow
(% change from baseline)
BQ-123
BQ-123+BQ-788
Time (min)
B
Before Metoprolol
After Metoprolol
Forearm Blood Flow
(% change from baseline)
BQ-123
BQ-123+BQ-788
Time (min)
C
Before Placebo
After Placebo
Forearm Blood Flow
(% change from baseline)
BQ-123
BQ-123+BQ-788
Time (min)


A
B
C
Before Nebivolol
After Nebivolol
Before Metoprolol
After Metoprolol
Before Placebo
After Placebo
Forearm Blood Flow
(mL/100 mL tissue/min)
Baseline
4.0
8.0
16.0
Acetylcholine
(µg/100 mL tissue/min)
*

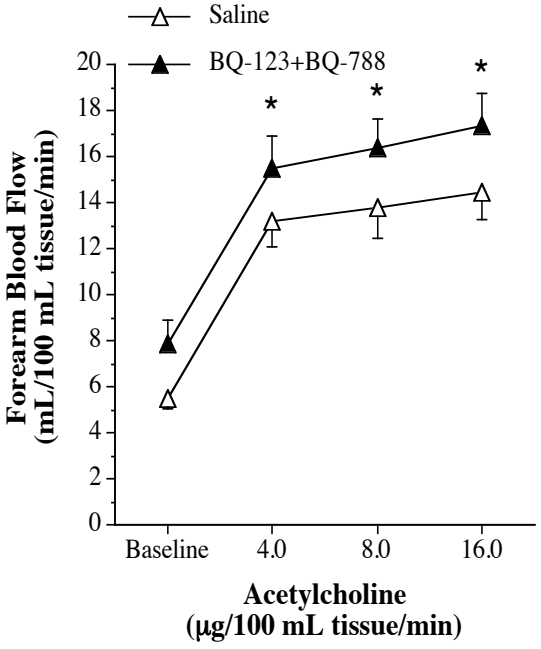
Before Nebivolol



After Nebivolol



Before Metoprolol



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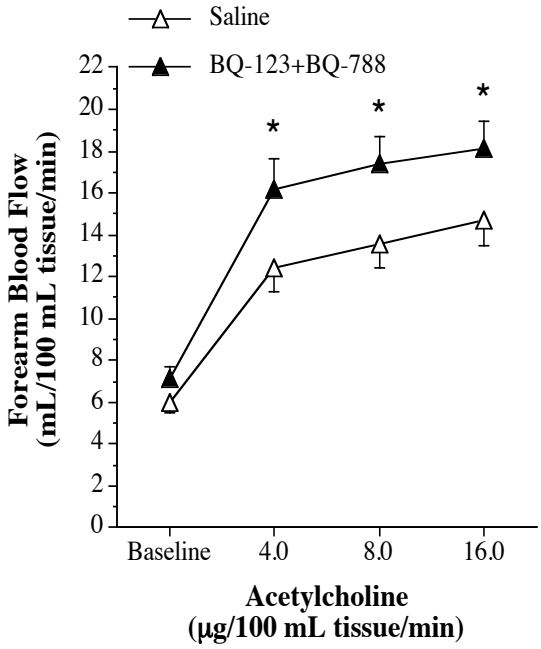

CONCLUSION

The primary new findings of the studies presented herein are as follows:

1. The capacity of the endothelium to release t-PA is diminished in adult males with blood pressure in the prehypertensive range. Additionally, the level of impairment in t-PA release seen with clinical hypertension is already apparent in the prehypertensive state.

2. Elevations in WBC count, within clinically normal range and independent of other cardiovascular risk factors, is associated with increased ET-1-mediated vasoconstrictor tone. Therefore, increased ET-1 vasoconstrictor tone may contribute to the heightened risk of cardiovascular disease and stroke reported with elevations in WBC count.

3. ET-1-mediated vasoconstrictor tone is elevated in adults with impaired fasting blood glucose concentrations, independent of other cardiometabolic risk factors. Thus, indicating that augmented ET-1 vasoconstrictor tone is apparent in the impaired fasting glucose prediabetic state.

4. Forearm vascular responses to exogenous ET-1, selective ET_A receptor antagonism and non-selective $ET_{A/B}$ receptor blockade were almost identical between adults with LDL-cholesterol in the optimal/near optimal and borderline-high levels. Consequently, borderline-high LDL-cholesterol is not associated with enhanced ET-1 system activity.

5. Independent of reducing blood pressure, chronic nebivolol, but not metoprolol, therapy reduces ET-1-mediated vasoconstrictor tone in adults with elevated blood pressure. Moreover, these reductions may, at least in part, underlie the known nebivolol-induced improvements in endothelium-dependent vasodilation.

In aggregate, these studies indicate that endothelial dysfunction, specifically elevations in ET-1 system activity and impaired fibrinolytic function, is omnipresent with many cardiovascular disease risk factors and is an appropriate target for both lifestyle and pharmacologic intervention aimed at reducing cardiovascular risk.

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