Systemic Regulation of Vascular NAD(P)H Oxidase Activity and Nox Isoform Expression in Human Arteries and Veins

Tomasz J. Guzik, Jerzy Sadowski, Boguslaw Kapelak, Andrzei Jopek, Pawel Rudzinski, Ravi Pillai, Richard Korbut, Keith M. Channon

Objective—Impaired endothelial function, characterized by nitric oxide scavenging by increased superoxide production, is a hallmark of vascular disease states. However, molecular mechanisms regulating superoxide production in human blood vessels remain poorly defined.

Methods and Results—We compared endothelial function, vascular superoxide production, and the expression of NAD(P)H oxidase subunits in arteries and veins from patients undergoing coronary bypass surgery (n=86). Superoxide release was similar in arteries and veins. Inhibitor studies revealed that the NAD(P)H oxidase system was a quantitatively and proportionately greater source of superoxide in veins, whereas xanthine oxidase also contributed significantly to superoxide production in arteries. Moreover, NAD(P)H oxidase molecular composition differed in veins and arteries; veins expressed more nox2 and p22phox, whereas the relative expression of nox4 was greater in arteries. However, there were strong correlations between p22phox and nox4 expression and between superoxide production, NAD(P)H oxidase activity, and endothelial function in arteries and veins from the same patient.

Conclusions—In individuals with coronary artery disease, changes in vascular superoxide production, endothelial function, and NAD(P)H oxidase activity and expression are related in veins and arteries. These findings highlight the importance of systemic effects on the molecular regulation of the NAD(P)H oxidases in human vascular disease. (Arterioscler Thromb Vasc Biol. 2004;24:1-7.)

Key Words: endothelium ■ oxidant stress ■ reactive oxygen species ■ nitric oxide ■ NAD(P)H oxidase

Oxidative stress plays an important role in the pathogenesis of atherosclerosis, hypertension, and other vascular diseases. In particular, overproduction of superoxide anion may be detrimental because of its rapid interaction with nitric oxide (NO), which leads to the loss of NO bioavailability and reduces its anti-atherogenic effects. Superoxide also regulates redox-sensitive signaling pathways, acts as a direct vascular smooth muscle cell (VSMC) mitogen, and modulates vessel remodeling and plaque stability. Recent studies indicate that patients with endothelial dysfunction in whom arterial superoxide production is increased are at highest risk for vascular morbidity and mortality. The sources of vascular superoxide include NAD(P)H oxidases, xanthine oxidase, cyclooxygenases, nitric oxide synthases, or mitochondrial oxidases. In particular, NAD(P)H oxidases have been identified as a major enzyme system involved in the generation of vascular oxidative stress. Recent studies have revealed several molecular homologs of the NAD(P)H oxidase large subunit (termed nox-nonphagocytic oxidase). The molecular composition of vascular NAD(P)H oxidases appears to vary in different cell types and at different stages of atherosclerotic plaques. However, the molecular regulation of the NAD(P)H oxidases within atherosclerotic plaques may be more relevant to plaque events, such as rupture, rather than reflecting systemic changes related to global disease progression or pathogenesis. Further understanding of the relevance of the NAD(P)H oxidases to human vascular disease pathogenesis requires investigation of the relationships between the molecular regulation of the NAD(P)H oxidases in the vascular wall and established features of disease such as vascular superoxide production and endothelial dysfunction. Importantly, systemic factors, such as oxidized low-density lipoprotein, angiotensin II, pro-inflammatory cytokines, and diabetes, are major contributors to atherosclerotic risk and progression. These systemic factors likely underlie the observed correlations between endothelial function in coronary and peripheral arterial circulations and also suggest that functional and molecular markers of vascular disease in arteries may be accompanied by corresponding systemic changes in the venous circulation.

Accordingly, we aimed to characterize the contribution of candidate oxidase systems, in particular the NAD(P)H oxi-
to total superoxide production in paired human arteries and veins from patients with coronary artery disease. Furthermore, we sought to define the quantitative relationships between endothelial function, superoxide production, and the molecular composition of the vascular NAD(P)H oxidases in paired veins and arteries. We find that expression of individual NAD(P)H oxidase subunits are closely correlated in veins and arteries and are, in turn, related to vascular superoxide production, providing evidence for molecular regulation of vascular NAD(P)H oxidases at a systemic level in human atherosclerosis.

Methods

Patients and Blood Vessels

Paired segments of human saphenous vein (HSV) and internal mammary artery (IMA) were obtained from 86 patients undergoing coronary artery bypass graft surgery. Vessels were harvested using a no-touch technique, before surgical distension, and before topical coronary artery bypass graft surgery. Vessels were harvested using a no-touch technique, before surgical distension, and before topical

Vascular Superoxide Production

Superoxide production was measured in intact vessel rings and from vascular homogenates using 2 independent assays: by lucigenin-enhanced chemiluminescence (5 μmol/L) and by ferricytochrome c reduction, using previously described and validated methods.4,7 Additionally, superoxide generation was measured in the presence of various oxidase inhibitors using lucigenin at 20 μmol/L. Superoxide production was expressed as relative light units (RLU) per second per mg vessel dry weight.

Isometric Tension Studies

NO-mediated endothelial function was assessed using isometric tension studies in response to acetylcholine, as described previously and expressed as a percentage of the precontracted tension.4 Saphenous vein and internal mammary artery segments were immediately washed and transported to the laboratory in ice-cold Krebs–Henseleit buffer. In vessels that showed endothelial dysfunction (defined as vasorelaxations), acetylcholine-mediated vasorelaxations were also measured after pre-incubation with polyethylene glycol (PEG)-SOD (500 IU/mL).

Western Immunoblotting

Portions of vascular homogenate (20 μg protein) were separated by 12% SDS-PAGE and transferred to nitrocellulose membranes. NAD(P)H oxidase subunits were detected using mouse monoclonal antibodies against p67phox or p47phox, (Transduction Laboratories) or by rabbit polyclonal antibodies against p22phox (generously provided by Dr Imajoh-Ohmi, Tokyo, Japan). Bands were visualized by chemiluminescence (Supersignal; Pierce) and quantified using National Institutes of Health Image software.

Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction

RNA was isolated from snap-frozen segments of saphenous vein and mammary artery using Tri-reagent, repurified using RNA easy kit (Qiagen) with DNAse digestion,3 and quantified using the Ribogreen fluorometric assay (Molecular Probes). cDNA was synthesized using ImProm Reverse Transcription System (Promega) using random primers. The cDNA synthesized from 20 ng total RNA was used in subsequent quantitative polymerase chain reaction (PCR) using the SYBR Green system (Quiagen) and Rotorgene 3000 fluorescent real-time PCR machine (Corbett Research). Mg++ concentrations were 1.5 mmol/L for all primers, except nox4 (4 mmol/L). Annealing temperatures were 58°C for all primers except nox4 (68°C). All primers were used as described by Sorescu et al,3 apart from primers for p22phox (forward: CCGCGGCGTCCGGCCTGATCCTCA; reverse: AGCGCACAGCCGCAAGTGAGTAGAT). For quantitative reverse-transcription (RT)-PCR, the MLN-51 gene transcript was used to normalize for RT and PCR efficiencies (forward: CAAGAGTGCTGAGGAGTCGG; reverse: TCATTAGCTTCGATTTCAG), although variability between samples was minimal (not shown). Quantification of specific mRNAs was determined relative to standard curves of total RNA isolated from THP1 cells as standards for nox2 and p22phox, or from human microvascular endothelial cells for nox1 or nox4.

Statistical Analysis

Results are expressed as means±SEM with n indicating number of patients. Statistical comparisons between the 2 groups were made using Student t test for independent or dependent samples, Wilcoxon and Mann–Whitney U test or using ANOVA followed by post hoc tests depending on distribution and variance analysis. P<0.05 was considered significant.

Results

Sources of Superoxide Production in Human Veins and Arteries

Quantification of basal superoxide release from intact vessel rings measured ex vivo using low-concentration lucigenin chemiluminescence (5 μmol/L) revealed no statistically significant differences between saphenous veins and mammary artery segments (16.8±1.7 versus 14.3±1.9 RLU/second per mg; n=41). Pre-incubation with SOD reduced chemiluminescence by almost 70% and addition of tiron by 80% in both arteries and veins, confirming specificity for superoxide. Superoxide production was also demonstrated by SOD-inhibitable dihydroethidium fluorescence in vessel sections, revealing superoxide production from endothelium, media, and adventitia (Figure I, available online at http://atvb.ahajournals.org). Systematic investigation of potential sources of superoxide revealed that in HSV and IMA superoxide production was greatly inhibited by flavin oxidase inhibitor diphenyliodonium (50 μmol/L) and by apocyinin
To address the importance of superoxide production for endothelial dysfunction in human vessels, we measured effects of superoxide on nitric oxide production on superoxide release, indicating superoxide NO scavenging. This effect was significant in arteries but not in veins (Figure 1). To address the importance of superoxide production for endothelial dysfunction in human vessels, we measured changes in acetylcholine-dependent vasorelaxations in response to pre-incubation with PEG-SOD. We found that PEG-SOD (500 U/mL) improved vasorelaxations, more markedly in IMA than in HSV (Figure II, available online at http://atvb.ahajournals.org).

NAD(P)H Oxidase Protein and Activity in Human Veins and Arteries

Because NAD(P)H oxidases appear to play a principal role in superoxide production in human arteries and veins, we next sought to investigate the presence of the NAD(P)H oxidase protein subunits and enzyme activity in vessel protein extracts. Western blotting revealed that both membrane-associated (p22phox) and cytoplasmic subunits (p67phox and p47phox) were more abundant in saphenous veins than in mammary arteries (Figure 2). In line with these findings, measurements of NAD(P)H oxidase activity in vascular homogenates using SOD-inhibitable ferricytochrome c reduction assay showed greater activity in HSV (Figure 2). To investigate the contribution of PKC signaling, known to be important in NAD(P)H oxidase activation, we used the PKC inhibitor chelerythrine. PKC inhibition significantly decreased basal and NADPH-stimulated superoxide production (Table II, available online at http://atvb.ahajournals.org) and abolished the difference in NADPH-stimulated oxidase activity between veins and arteries.

Systemic Relationships Between NAD(P)H Oxidase Activities and Endothelial Function in Human Arteries and Veins

There was marked variability in basal vascular superoxide production and in maximally stimulated NAD(P)H oxidase activity in saphenous veins and in mammary arteries from patients with coronary artery disease. However, in paired arteries and veins from individual subjects basal and stimulated superoxide production were significantly correlated...
Similarly, NO-mediated endothelial function, measured by vasorelaxations to ACh, revealed a strong correlation between arteries and veins (Figure 3C). These related findings in veins and arteries from individual patients suggest that vascular superoxide production and nitric oxide-mediated endothelial function are regulated by systemic factors in patients with coronary artery disease.

**Molecular Composition of Vascular NAD(P)H Oxidase in Human Arteries and Veins**

To investigate potential differences in the molecular composition of NAD(P)H oxidases between veins and arteries, expression of NAD(P)H oxidase subunit mRNA was analyzed using quantitative fluorescent RT-PCR (Figure 4). The expression of p22phox and nox2 (gp91phox) mRNA was greater in saphenous veins than in mammary arteries, in keeping with our earlier observations of increased p22phox protein levels in veins. Nox4 mRNA was readily detected in both veins and arteries, with a trend toward higher levels in arteries. Nox1 mRNA was not detected in veins or arteries except at very low levels in vessels from 2 patients. Finally, no expression of macrophage colony-stimulating factor (CSF) receptor (MCSFR) mRNA was detected in either saphenous veins or mammary arteries, suggesting that the presence of NAD(P)H oxidase subunit mRNA in the vascular wall was not significantly influenced by infiltrating leukocytes. These findings suggest that human blood vessels express NAD(P)H oxidases that are based principally on nox2 and nox4. In saphenous veins, a nox2 oxidase appears to predominate, and levels of p22phox expression are also higher, whereas a nox4 NAD(P)H oxidase is relatively more abundant in mammary arteries.
In view of our previous observation of strong relationships between superoxide production and endothelial function in veins and arteries, we next investigated relationships between the levels of NAD(P)H oxidase subunit mRNA expression in veins and arteries from individual patients. We observed a strong correlation between p22phox mRNA levels and nox4 levels in veins and arteries (Figure 5). In contrast, nox2 (gp91phox) mRNA levels showed no correlation between veins and arteries from the same patient.

**Discussion**

In this study, we find that NAD(P)H oxidases are important sources of superoxide production in human veins and arteries. The NAD(P)H oxidase system is quantitatively and proportionally a greater source of superoxide in veins, whereas xanthine oxidase appears to additionally contribute substantially to superoxide production in arteries. We find that increased vascular NAD(P)H oxidase activity is associated with increased protein levels of p22phox, p47phox, and p67phox, and increased p22phox and nox2 (gp91phox) mRNA expression. The NAD(P)H oxidase is predominantly nox2-based in saphenous veins, whereas a nox4-based oxidase appears proportionately more important in mammary arteries. Despite these differences in the functional and molecular characteristics of superoxide production between veins and arteries, several key findings strongly suggest that oxidative stress and endothelial dysfunction are regulated by similar systemic factors in human atherosclerosis. First, both superoxide production and endothelial dysfunction are significantly correlated in arteries and veins from individual patients. Second, the expression of both p22phox and nox4 mRNA are strikingly correlated in arteries and veins. Finally, protein kinase C signaling has a key role in regulating superoxide production in veins and arteries.

The NAD(P)H oxidases are multicomponent enzymes composed of membrane-associated proteins and cytosolic subunits and expressed in endothelial smooth muscle cells (SMC), and adventitial cells. In the phagocytic-type NAD(P)H oxidase, the membrane-associated proteins gp91phox and p22phox compose the flavocytochrome b558 complex, which forms the catalytic subunit of the oxidase. The cytosolic subunits, including p47phox, p67phox, and the G-protein Rac, provide regulatory function. Recently, homologues of the NAD(P)H oxidase gp91phox (nox2), termed nox1 and nox4, have been identified in VSMC and may underlie biologically important differences in NAD(P)H oxidase regulation and activity in the vascular wall in the development of atherosclerosis. Nox homologs may be differentially associated with various vascular disease phenotypes. Changes in nox1 expression directly alter cell proliferation in culture, and the treatment of SMC with angiotensin II or PDGF upregulates nox1 while downregulating nox4. In the rat carotid model of vascular injury, the expression of nox1, nox2, and p22phox is elevated early after injury, whereas nox4 increases later, coinciding with a reduction in the rate of SMC proliferation. Indeed, nox2 appears to play a particularly important role in the proliferative response, as demonstrated by inhibition of neointimal hyperplasia by specific peptide inhibition of nox2-containing NAD(P)H oxidases. Similarly, a nox2-containing NAD(P)H oxidase mediates the hypertrophic phenotype of VSMC in response to angiotensin II. These findings suggest that although nox1 and nox2 are involved in acute response to injury or to angiotensin II stimulation, nox4 is involved in maintaining the quiescent phenotype. The importance of nox1 in human vessels is less clear, because both our study...
and previous studies in human coronary arteries show only very low levels of expression. The previous observation of increased nox2 expression after vascular injury are interesting in the light of our observations that both p22phox and nox2 are more abundant in saphenous veins than in internal mammary arteries. The marked difference in the nox2/nox4 ratio could at least in part account for the difference in susceptibility to smooth muscle intimal hyperplasia leading to adverse vein graft remodeling and accelerated atherosclerosis in vein grafts, whereas mammary artery grafts are not susceptible to atherosclerosis.

We estimated the relative contribution of the individual vascular wall segments (endothelium-media-adventitia) to total superoxide production in human vessels. Semi-quantitative analysis of dihydroethidium fluorescence showed that endothelium accounts for approximately one quarter of total superoxide in the vessel wall in human veins and arteries, which is in agreement with our previous study in vascular homogenates. The media appeared to be a more important relative contributor in IMA, whereas the adventitia was the dominant source in veins.

Our study adds further insights into the relationships between nox isoform expression and increased oxidative stress in human atherosclerosis. Azumi et al were the first to show the presence of a p22phox-based NAD(P)H oxidase in human coronary artery atherosclerotic plaque. Sorescu et al very elegantly demonstrated that nox2 and p22phox are greatly increased with the progression of human atherosclerotic plaques in coronary arteries, in part related to inflammatory cell infiltration, whereas nox4 was increased in early lesions and decreased in very severe lesions. A recent study has additionally shown that nox2 expression is increased by oscillatory versus pulsatile flow, which may be particularly relevant to the development of atherosclerotic plaques in regions of turbulent flow. Importantly, nox4 expression in this model coincided with increased oxidative stress and low-density lipoprotein oxidation. Azumi et al showed a direct spatial relationship between NAD(P)H oxidase-generated oxidative stress and oxidized low-density lipoprotein in atherectomy specimens of human atherosclerotic plaque that were increased in samples from patients with unstable angina. The importance of NAD(P)H oxidase for plaque stability is further emphasized by the finding that the shoulder region of plaques is a particularly intense area of reactive oxygen species production, in association with p22phox and Nox2 expression.

The differences in NAD(P)H subunit molecular composition, between arteries and veins, could also reflect the differences in the vascular cells that contribute to superoxide production. Although all layers of human artery and vein produce superoxide, the relative importance of endothelium, SMC, and adventitial cells may be subject to differential regulation. In veins, the predominance of nox2 expression suggests major contributions from the endothelium and adventitia, because these contain nox2-based oxidases. Adventitial superoxide production from a nox2-containing NAD(P)H oxidase directly contributes to endothelial dysfunction by NO scavenging. In human arteries, our observation of increased nox4 expression suggests that SMC could play a critical role. However, recent data show that human microvascular SMC express nox2 in response to angiotensin II stimulation, mediated by a c-Src pathway. This clearly illustrates that nox isoform expression in human vascular cells is regulated in a complex manner that can vary with cell type in different vessels and in response to different physiologic stimuli.

Apart from the NAD(P)H oxidases, xanthine oxidoreductase may be an additional source of vascular superoxide. Our study confirms the earlier findings of Spiekermann et al by showing that xanthine oxidase contributes to superoxide production, but principally in human arteries rather than in veins. Arachidonic acid metabolism may also contribute to vascular superoxide, as indicated by inhibition by indomethacin, although this effect may reflect the importance of arachidonic acid in NAD(P)H oxidase activation.

Although previous studies have emphasized the differences between endothelial function and oxidative stress in human veins and arteries, we have focused on potential relationships that suggest systemic regulatory factors. We find that NO bioactivity and oxidative stress, despite wide variations between individual patients, are closely correlated between veins and arteries. Whereas sources of superoxide anion in arteries and veins are not identical, they are clearly subject to systemic factors, such as diabetes, hypercholesterolemia, or angiotensin II. We have observed that nox4 expression was most closely correlated between arteries and veins. Although nox2 was expressed in veins and arteries, there was no correlation between the two. Nox1, in turn, was expressed at only low levels in a small number of patients, but when detected it was present in arteries and veins. It is important to point out that mRNA levels do not necessarily correspond directly to NAD(P)H oxidase activity, because of possible differences between mRNA levels and protein levels, and because of complex regulatory interactions between individual oxidase subunits. Nevertheless, the strong correlation of mRNA levels of p22phox and nox4 between human arteries and veins shows that systemic regulation of the NAD(P)H oxidase system in humans is evident at the molecular level.

There are multiple factors that could affect endothelial function and oxidative stress parameters in a systemic fashion. In a previous study, we found that clinical risk factors such hypercholesterolemia and diabetes are most strongly associated with NAD(P)H oxidase activity and reduced NO bioavailability (please see http://atvb.ahajournals.org). Genetic factors could also affect vascular NAD(P)H oxidase activity at a systemic level.

Our findings add further weight to recent evidence suggesting that the PKC pathway is an important regulator of NADPH oxidase activity in the vascular wall. Recently described, orally active, specific PKC-β inhibitors reverse the acute deterioration in endothelial function observed after a hyperglycemic challenge in humans. Our present data add further weight to suggest that the beneficial effects of PKC inhibition in human vascular disease may not be restricted to patients with diabetes mellitus.

Acknowledgments

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References

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*Online supplement: www.ahajournals.org*

**Figure I. Analysis of the contribution of individual layers of the vascular wall to total superoxide production**

Superoxide production in endothelium/intima, media and adventitia was visualized by SOD inhibitable - dihydroethidium (2 μM) staining and subsequent quantification using Image Pro Plus was used as described before (Alp et al, 2003, J Clin Invest). Measurements were performed in paired vessel sections incubated without and with SOD. Values obtained from sections with SOD were considered as background and were subtracted from the measurements. For each vessel studied individual values were calculated as a % of total DHE in the section. *p<0.05 vs media; ¶ p<0.05 vs HSV.
Figure II. Effects of superoxide scavenging by SOD on vascular relaxations to acetylcholine in segments of HSV and IMA with endothelial dysfunction (maximal vasorelaxations <median) (n=8). Vessels were pre-incubated with PEG-SOD (500 IU/ml) for 30 minutes prior to the assessment of vasorelaxations to increasing doses of acetylcholine.
Table I. Dose-Responses of Oxidase Inhibitors in Human Blood Vessels
The effects of increasing concentrations of potential vascular oxidase inhibitors on basal vascular superoxide production was determined in segments of human saphenous vein (HSV) and internal mammary artery (IMA) (n=4-6 patients for each inhibitor). Superoxide production was measured under basal conditions and in consecutive vascular segments pre-incubated with increasing concentrations of diphenyliodonium (DPI), apocynin, oxypurinol or indomethacin. Superoxidce production was measured using lucigenin enhanced chemiluminescence. Values are given in RLU/sec/mg dry weight. *p<0.05 vs basal.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>HSV (mean ± SEM)</th>
<th>IMA (mean ± SEM)</th>
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<td></td>
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<td>basal inhibitor</td>
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<tr>
<td>DPI</td>
<td></td>
<td></td>
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<tr>
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<td>5.3±0.3*</td>
<td>8.9±2.1*</td>
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<tr>
<td>100μM</td>
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<td>5.2±0.7*</td>
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<td>Apocynin</td>
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<td>250μM</td>
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<tr>
<td>100μM</td>
<td>10.1±3.4*</td>
<td>9.7±0.3*</td>
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Table II. Effects of protein kinase C inhibition on superoxide production in saphenous veins and mammary arteries
Basal and NADPH-stimulated superoxide production was measured in intact segments of human saphenous vein (HSV) and internal mammary artery (IMA) (n=6-10 each) using lucigenin enhanced chemiluminescence (5 µM). Adjacent segments were preincubated with and luminescence was measured in the presence of increasing concentrations of the protein kinase C inhibitor chelerythrine (Chel) at 1 or 3 µM. * p<0.05 vs. control; ** p<0.01 vs. control; ¶ - <0.05 vs. HSV.

<table>
<thead>
<tr>
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<th>Saphenous Vein</th>
<th>Internal Mammary Artery</th>
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<tr>
<td></td>
<td>Basal</td>
<td>+ NADPH</td>
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<tr>
<td>Control</td>
<td>26.6±4.5</td>
<td>2766±364</td>
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<tr>
<td>+ Chel 1µM</td>
<td>14.0±2.5 **</td>
<td>1526±414 *</td>
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<tr>
<td>+ Chel 3µM</td>
<td>9.8±3.1 **</td>
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Table III. Major Clinical Risk Factors Associated with Endothelial Function
Type III Sums of Squares ANOVA was performed using clinical risk factors as categorical covariates, and maximal relaxation to increasing doses of ACh, expressed in % of precontracted tension, as the dependent variable.

<table>
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<th>Risk Factor (RF)</th>
<th>n with RF</th>
<th>n without RF</th>
<th>With RF</th>
<th>without RF</th>
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<td>28.7±2.7</td>
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<td>40</td>
<td>21.1±1.4</td>
<td>30.8±2.8</td>
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<td>Smoking</td>
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<td>71</td>
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<td>Diabetes Mellitus</td>
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