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A nanomedical approach to understanding the mechanism of endothelial function and dysfunction- Clinical implications

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Abstract

A dysfunctional endothelium is the first step toward many diseases of modern civilization, including hypertension, coronary atherosclerosis, diabetes, obesity, heart failure, as well as aging. The development of new nanomedical devices and nanosensors allows in situ monitoring and measuring of the molecular processes in a single endothelial cell. It appears that the first step in triggering the dysfunction of endothelial cell is diminishing the release of cytoprotective molecule nitric oxide (NO). This process is coupled with the enhanced production of the cytotoxic molecules, superoxide (O_2^-) and peroxynitrite ($ONOO^-$). There are two major sources of the O_2^- in endothelial cells: NADPH oxidase and uncoupled endothelial nitric oxide synthase (eNOS). NO is an efficient scavenger of O_2^- which produces $ONOO^-$. Peroxynitrite is a powerful oxidant and is the main component of nitroxidative stress. It appears that the damaging effects to the biological milieu are not dependent on the absolute level of $ONOO^-$ by endothelium, but rather on the ratio of NO concentration, [NO], to the concentration of $ONOO^-$, [$ONOO^-$]. [NO]/ [$ONOO^-$] ratio can be a precise indicator of a level of endothelial dysfunction. Endothelial function and eNOS coupling can be partially restored by several currently available drugs like statins, ACE inhibitors, and β-blockers and also vitamin D_3. The restoration of functional endothelium can significantly improve a function of the cardiovascular system and inhibit progression of vascular damage due to diabetes, atherosclerosis, and aging.

Introduction

Functional endothelium plays a crucial role in maintaining an optimal performance of the cardiovascular system [1]. Nitric oxide (NO) is one of the most important messengers produced by functional endothelium [2,3]. NO is a regulatory and cytoprotective molecule and plays two crucial roles in the vasculature: it stimulates vascular smooth muscle relaxation and prevents the adhesion of blood components like platelets, leukocytes and also other biological molecules (like LDL) to the membrane of endothelial cells [4-10].

NO release in the vasculature is generated mainly by endothelial nitric oxide synthase (eNOS) and can be stimulated by shear stress induced calcium flux and by different agonists like acetylcholine, nor epinephrine and others. In blood, NO can be

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coordinated to hemoglobin, or it can be scavenged by superoxide (O_2^-) – a second messenger and powerful oxidant. Therefore, the half-life of NO can vary significantly from 2-4 s under laminar blood flow, to much less than 1 s under turbulent blood flow. As a result, the bioavailability of NO in the vasculature is limited not only by the expression and efficiency of eNOS but also by the level of reactive oxidative species (ROS), as well as laminar vs. turbulent flow. Superoxide anion is a major primary component of the ROS in addition to peroxynitrite ($ONOO^-$), hydrogen peroxide and possibly hydroxyl radical ($OH^•$) [11-14].

The peroxidation is the main product of rapid scavenging of NO by O_2^-, which is also the main component of nitroxidative stress. $ONOO^-$ is a cytotoxic vasoconstrictor that can impair several biological processes, cause the inhibition of several enzymes, and cause nitrosylation of proteins, as well as can trigger apoptosis, necrosis, and cell death [15-17]. In normal endothelium, the production of $ONOO^-$ is relatively low and this molecule can be isomerized to produce harmless nitrate (NO^{3-}). It has been suggested that at high levels of $ONOO^-$, the isomerization process can be homolytic and may lead to the production of highly aggressive, radicals like $OH^•$ and $NO_2^•$. Therefore, a proper balance between bioavailable NO and ROS (mainly O_2^- and $ONOO^-$) is required for the optimal function of endothelium. However, under pathological conditions of diabetes, atherosclerosis, hypertension, ischemia, aging, heart failure and others, a significant and unfavorable shift in the balance between NO, O_2^-, and $ONOO^-$ has been observed [10,18-23].

The severity of ROS damages to cell function does not depend only on the absolute accumulation of $ONOO^-$ and/or O_2^-, but rather on the relative level of these two toxic molecules compared to cytoprotective NO [24,25]. Based on our nanomedical studies, which allows for the simultaneous measurements of NO, O_2^- and $ONOO^-$ concentrations produced by a single endothelial cell, we were able to conclude that the ratio of NO concentration, [NO], to the concentration of $ONOO^-$, [$ONOO^-$], or [NO] to the sum of [O_2^-] and [$ONOO^-$] accurately reflects and correlates with the function/dysfunction of endothelial cells in health and disease [26-31]. In review, presented here, we summarize our findings and findings of others concerning the role of NO and $ONOO^-$ imbalance in dysfunctional endothelium - a common denominator of many vascular diseases.

Nanomedical Approach to the Study of Endothelial Dysfunction

Nanomedical systems of analysis found a unique application for the in situ monitoring of signaling molecules in a single endothelial cell [3,29,32-37]. Nitric oxide, superoxide, and peroxynitrite are short-living, highly reactive species. The short half-life of NO (a few seconds) and the even shorter half-life of O_2^- and $ONOO^-$ (less than 1 second) makes the in situ monitoring and measurement of these molecules, in vivo or in vitro, very challenging. Additionally, a diffusion controlled propagation rapidly decreases NO concentration in the vicinity of endothelium and creates additional problems with its detection. The concentration of NO required to trigger smooth muscle relaxation is on the nanomolar level (8-15 nM). In order to deliver this concentration to the target (smooth muscle cells) in a relatively short time, the surface concentration of NO on endothelial cell membrane has to be much higher (about 100-500 nM). Our modeling experiments with NO concentration was around 300±50 nM on the cell membrane. The diffusion process decreased [NO] and reached the level of 5-15 nM at a distance of 100 μm [27].

Electrochemical nanosensors developed in our laboratories have a detection limit of 1-3 nM, and a linear concentration response from nanomolar to micromolar level. The most important features of these nanosensors are the small size and the capability to simultaneously measure the real concentration of bioavailable NO, O_2^-, and $ONOO^-$ in near real-time in a single cell (usually better than 10 μs) [3,14,16,26,36,38-43]. The precise location of sensors relative to the endothelial cell membrane is of great importance to achieve reproducible data. These minute sampling volumes, along with the sensitivity and the precision of the nanosensors (located in a very close proximity to the cell membrane) cannot currently be matched by any other bioanalytical techniques.

In order to maintain the reproducibility of the measurements, it is necessary to position the nanosensors in well-defined X,Y,Z coordinates, as close as possible to the membrane of a single endothelial cell, without touching the surface. Each sensor samples a volume of picoliter to femtoliters and can be positioned about 3-5 μm above the cell membrane with a precision better than 1 μm. Atypical response of sensors (current/concentration vs. time) to stimulated NO, O_2^- and $ONOO^-$ release by normal and dysfunctional endothelial cells are shown in **Figure 1** (these unpublished data were acquired in TM labs). The bioavailable maximal concentration of NO decreases while the maximal concentrations of $ONOO^-$ and O_2^- increase significantly in dysfunctional endothelium (**Figure 2**).

Mechanism of NO, O_2^- and $ONOO^-$ generated by normal and dysfunctional endothelium

NO stimulates soluble guanylate cyclase to form cGMP, which triggers smooth muscle relaxation and increases the diameter of the vascular lumen. NO can be generated not only by endothelium but also by platelets and leukocytes. Therefore, NO is the first line of protection against atherogenesis and the formation of atherosclerosis [9,13,44,45].

NO is produced from two substrates L-arginine and oxygen. In this process, L-arginine is oxidized in a 5-electron transfer reaction to form L-citrulline and NO (**Figure 3**). A dimeric form of eNOS is a catalyst for NO synthesis. An important cofactor of this process is tetrahydrobiopterin, BH_4 [46,47]. In order to produce NO, the dimeric form of eNOS is stabilized (coupled) by both substrates L-arginine and O_2, as well as the cofactors. With insufficient levels of any of the substrates or cofactors, the dimeric form of eNOS is destabilized (uncoupled) and starts to concomitantly produce O_2^- in a one-electron transfer to oxygen and NO in a five electron oxidation of L-arginine [11,171,41,48-50].

The concomitant production of NO and O_2^-, by eNOS, results in the generation of $ONOO^-$. The reaction between NO and O_2^- is a highly efficient diffusion controlled process ($k=5x10^9 M^{-1}s^{-1}$). The $ONOO^-$ that is generated in this process is one of the most powerful oxidants in the biological milieu, much stronger than O_2^- or NO [14,19,24,25]. Therefore, uncoupled eNOS can generate cellular and intracellular oxidative/nitroxidative stress in the endothelium, which trigger a cascade of events leading to destruction of the cardiovascular system. In addition to uncoupled eNOS, NADPH oxidase is also a potent source of O_2^- in endothelium. In normal functional endothelium, NADPH oxidase produces relatively low levels of O_2^-, therefore its contribution to $ONOO^-$ production is minimal. However, in dysfunctional endothelium, eNOS is the dominant source of O_2^- (60-70%) while NADPH accounts for 30-40%, leading to the high production of

$ONOO^-$ [26,53,55].

$ONOO^-$ is a short-living ion that can be protonated (pKa of 6.8) to form a diffusible peroxynitrous acid (ONOOH). $ONOO^-$/ONOOH has an especially devastating cytotoxic effect causing: nitrosylation, nitration, apoptosis, necrosis, lipid peroxidation, enzyme inactivation, and DNA change. Therefore, high level of $ONOO^-$/ONOOH is a common denominator of several diseases including hypertension, diabetes, atherosclerosis, heart attack, stroke, Parkinson's, Alzheimer's, aging, hypovolemia, heart failure and others [11,12,16,17,18,20,45,56]. $ONOO^-$ is produced at the expense of NO. Therefore, high levels of $ONOO^-$ are always accompanied by low levels of bioavailable NO. As a net effect, there is a decrease in the efficiency of the cardiovascular system. This is not only due to high nitroxidative stress, but also the deficiency in NO signaling. Additionally, a decrease in NO may accelerate the adhesion and aggregation of the various biological components in the blood, such as platelets, leukocytes and LDL, among others.

We found that absolute values of NO and $ONOO^-$ concentrations do not necessarily reflect on cardiovascular function. Rather, it is the ratio of NO concentration, [NO], to $ONOO^-$ concentration, [$ONOO^-$] that provides the most accurate correlation between the function of endothelium and the cardiovasculature. This ratio, [NO]/[$ONOO^-$] in functional endothelium varies from 2-6. At [NO]/[$ONOO^-$] below 2.0, the endothelium can become partially dysfunctional, and at a level below 1.0, endothelium is significantly dysfunctional. We used [NO]/[$ONOO^-$] ratio to quantify endothelial function/dysfunction [10,25,30,57-59]. This was possible due to nanomedical systems which allows us the precise and simultaneous measurements of the real concentrations (expressed in the same units) of NO and $ONOO^-$ by nanosensors. Both ratio of [NO] to [$ONOO^-$] and a ratio of [NO]/[$ONOO^-$]+[O_2^-] can be used for characterization of endothelial function/dysfunctional and coupling/uncoupling of eNOS syntheses.

Restoration of and protection against endothelial dysfunction

There are at least four different approaches for the protection/prevention and restoration of endothelial function. First, and the most important, is the prevention of eNOS uncoupling in functional endothelium. The prevention can be realized by increasing the level of eNOS substrate L-arginine and/or oxygen, and cofactors like tetrahydrobiopterin. Also, a decrease of expression of eNOS in endothelium, and the decrease of the expression NADPH oxidase is helpful in the prevention of eNOS uncoupling. Paradoxically, the decrease in eNOS expression improves eNOS coupling and efficiency due to relative increase in the ratio of substrates & cofactors in relation to eNOS [5,8,10,12,22,30,32,60,61]. At least partial restoration of endothelial function can be achieved by treatment with statins, β-blockers, ACE inhibitors or other drugs [12,20,26,28 ,29,35,41,50,54,56,58,62,71]. The pleiotropic effect of statins, β-blockers, and ACE inhibitors include a stimulated release of NO followed by a decrease of eNOS expression and increase in relative availability of substrates and cofactors and increase in eNOS coupling. Also, treatment with an elevated level of L-arginine and/or the precursor of BH_4 (sepiapterin) or vitamin D_3 can partially restore eNOS coupling and increase the NO bioavailability in dysfunctional endothelium [32,37,39,60]. The third, and oldest, treatment of dysfunctional endothelium would be supplementation of NO donors in the form of nitroglycerine, nitrates, nitroso albumine or in gaseous forms of NO or O_2 [42,72]. The last, and least effective manner of treatments appears to be supplementation of antioxidants for scavenging of O_2^- and $ONOO^-$ [19,40,53]. The antioxidants can be effective at a low level of eNOS uncoupling, however, with advanced uncoupling observed in a disease state, very large doses of antioxidants would be required to efficiently scavenge O_2^- and/or $ONOO^-$. This high doses (hundreds of grams, daily) of antioxidants are neither physiologically acceptable nor deliverable.

Therefore, the scavenging of the high levels of reactive oxygen species (ROS) in cardiovascular diseased state has minimal effect on the progression of the disease in advanced stages. Several of the existing drugs used for the treatment of cardiovascular diseases shown the pleiotropic effects on endothelial function. Nebivolol and carvedilol, third-generation of β-adrenoceptor improved the release of NO by the endothelium, decreased $ONOO^-$ production and improved microcirculation [62]. Nebivolol reduced nitroxidative stress and restored NO bioavailability in endothelium of African Americans [41]. Endothelial cell dysfunction contributes to insulin resistance in diabetes and is characterized by reduced NO and increased $ONOO^-$. Saxagliptin treatment restored NO and reduced $ONOO^-$ concentrations in obese rats [63]. Also, saxagliptin (dipeptidyl peptidase-4 inhibitor) enhances NO release and reduced blood pressure and sICAM-1 levels in hypertensive rats. Nanomedical studies show that adverse balance of NO/$ONOO^-$ in dysfunctional endothelium can be reversed by statins [50-52].

Amlodipine and atorvastatin showed synergistic effects in reversing LDL-induced endothelial dysfunction [65]. Aspirin decreased the activity of inducible nitric oxide synthase (iNOS) and increased NO production by eNOS [44]. S-nitro so albumin partially restored endothelial function and reduced ischemia/reperfusion injury in the pig heart after unprotected warm ischemia [42-45]. Also, S-nitroso albumin attenuated ischemia/reperfusion injury after cardioplegic arrest [21]. Nebivolol can favorably change the kinetics and balance of NO and $ONOO^-$ release in human endothelial cells [26]. Angiotensin II receptor blockers improved NO production in different eNOS variants [26]. Amlodipine increased endothelial NO and decreased nitroxidative stress disproportionately to blood pressure changes [54]. The chronic treatment with vasopeptidase inhibitor (AVE 7688) and ramipril improved endothelial function in diabetic rats [73]. A significant restoration of endothelial function was observed after long-term treatment of adult hypertensive rats with raloxifene [68]. Also, the combination of eicosapentaenoic acid and statins treatment restored the function of human umbilical vein endothelial cells (HUVECs) exposed to oxidized LDL [69,74,75]. Nebivolol improved endothelial function more significantly in Mexican Americans than in non-Hispanic white donors [15]. The synergistic effect of two or three different drugs in the restoration of endothelial function was also observed. Nebivolol and valsartan increased nitric oxide release from human endothelial cells in a synergistic fashion [76].

A noninvasive, nanomedical methods of measurement of endothelial function has already helped to understand the fundamental mechanism which can lead to dysfunction of endothelium and dysfunction of the cardiovascular system. The elucidation of the mechanism of endothelial dysfunction with nanosensors has already accelerated and development of several treatments for the restoration of endothelial function in dysfunctional a cardiovascular system. These treatments can help to restore the function of endothelium damaged by hypertension, atherosclerosis, diabetes, and aging. However, we

recently found that the most efficient agent for the prevention/restoration of dysfunctional endothelium can be an active metabolite of vitamin D_3, 1, 25-dihydroxy vitamin D_3. This molecule efficiently stimulates the production of bioavailable NO and decreases the concentration of $ONOO^-$ in dysfunctional endothelium (cellular model of hypertension). Most importantly, vitamin D_3 effectively decreases the expression of eNOS and NADPH oxidase and effectively restores endothelial function [32].

Figures

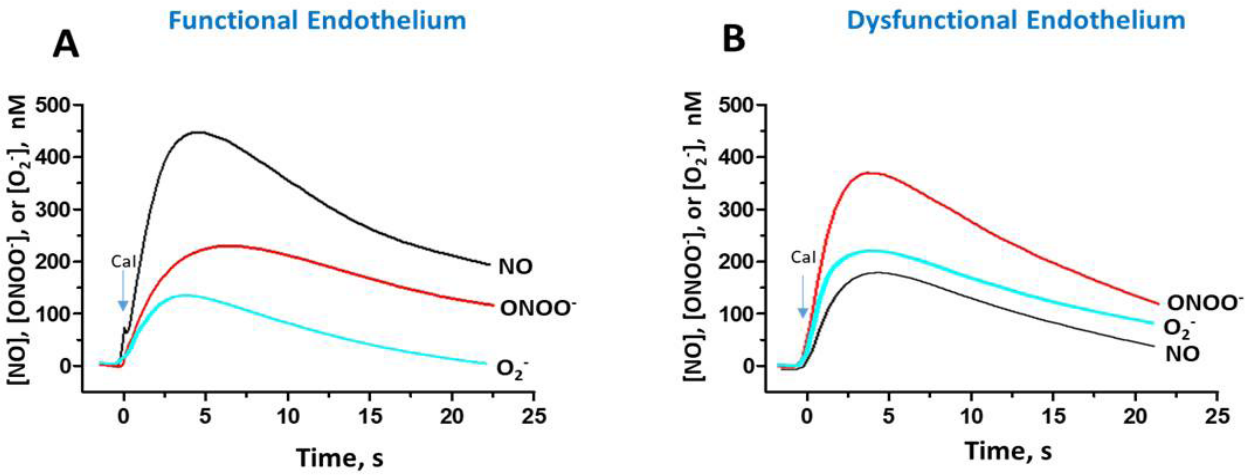

Figure 1: Typical amperograms showing a plot of concentrations (proportional to current) versus time, recorded with nanosensors. The release of NO, $ONOO^-$ and O_2^- was recorded from a single functional (A) and dysfunctional (B) HUVECs. The dysfunction of the HUVECs was triggered with 300 mg/dL D-glucose treatment for 2 hours. In dysfunctional endothelium, the [NO] level was about 60% lower, while [$ONOO^-$] was about 70% higher than in functional endothelium. The release of NO, $ONOO^-$ and O_2^- was stimulated with 1 µM of calcium ionophore A23187 (CaI). (Unpublished data collected in TM laboratory).

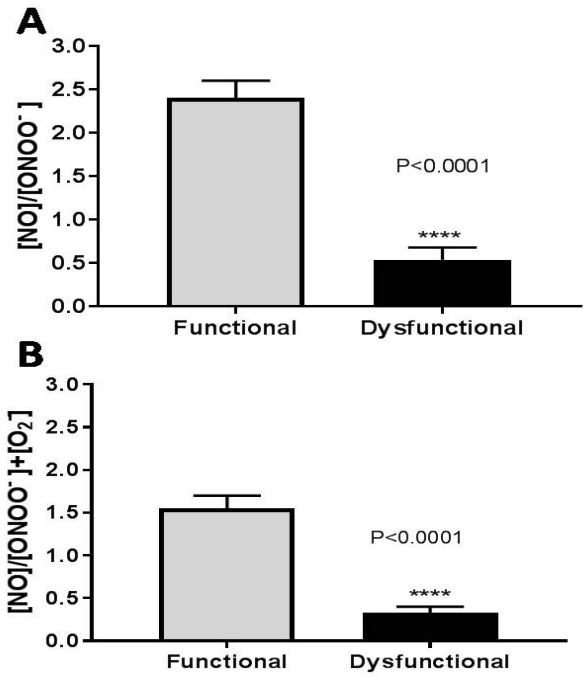

Figure 2: The ratio of maximal [NO]/[$ONOO^-$] concentrations in functional and dysfunctional HUVECs (A) and the ratio of maximal [NO]/[$ONOO^-$]+[O_2^-] in functional and dysfunctional HUVECs (B). The dysfunctional endothelium was obtained after treatment of functional endothelium with 300 mg/dL D-glucose for 2 hours. (Unpublished data collected in TM laboratory). Maximal concentrations were calculated from amperograms presented in Figure 1.

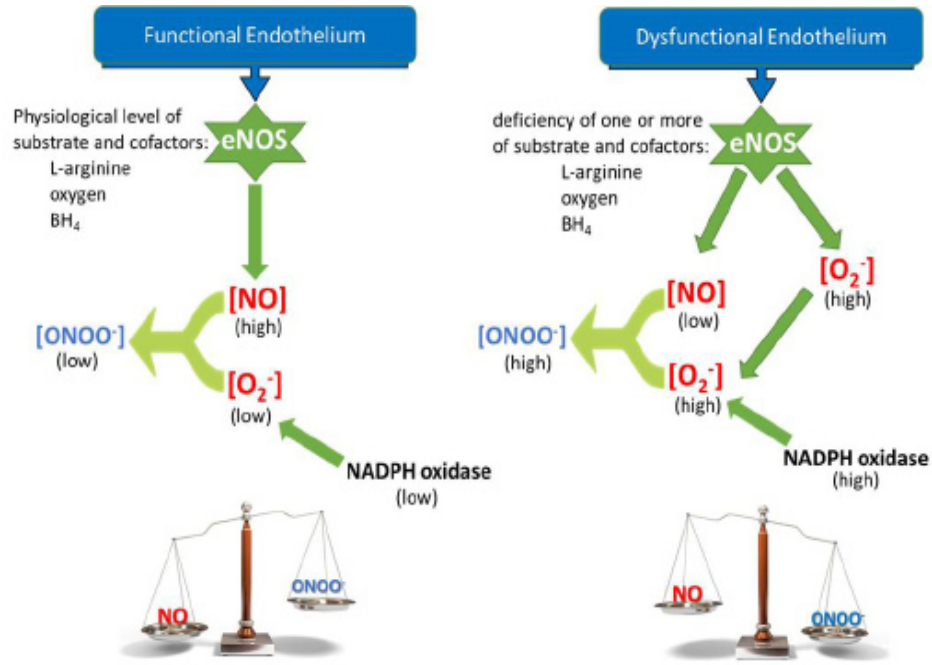

Figure 3: Schematic diagram showing the trigger mechanism which may lead to the balance/imbalance between nitric oxide concentration [NO] and peroxynitrite concentration [$ONOO^-$] in endothelial cells. Cells were stimulated with 1 µM of calcium Ionophore (CaI).****P<0.0001 vs. control group (unpaired, two-tailed Student's t-test). Data shown are the means ± S.D, n=5. eNOS (endothelial nitric oxide synthase), NADPH oxidase (nicotinamide adenine dinucleotide phosphate oxidase), BH_4 (tetrahydrobiopterin).

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