SUPEROXIDE DISMUTASE ACTIVITY AND EXPRESSION IN HUMAN VENOUS AND ARTERIAL BYPASS GRAFT VESSELS.

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Venous bypass grafts are more prone to accelerated atherosclerosis than arterial grafts, which is partly related to increased oxidative stress and diminished nitric oxide bioavailability. In veins superoxide production is dependent primarily on nox2 NAD(P)H oxidase expression, while in arteries nox4 appears to play an important role. This may in part explain differences in susceptibility to graft failure. Net levels of oxidative stress are however determined in parallel by the production as well as by degradation of free radicals (eg. by superoxide dimutases, catalases, thioredoxins etc). The differences in superoxide dismutase (SOD) expression and activity in human bypass conduit vessels remain unclear. Accordingly, we aimed to compare SOD activity and protein levels as well as its functional effects on superoxide production in segments of human internal mammary arteries (IMA) and saphenous veins (HSV) from patients undergoing bypass graft surgery (n=24). SOD activity was assessed by inhibition of pyrogallol autoxidation, Cu-Zn SOD and Mn SOD protein levels were studied by immunoblotting. Basal superoxide release was detected by lucigenin (5µM) enhanced chemiluminescence. Total SOD activity did not differ significantly between HSV and IMA. Similarly, no difference was observed in SOD activity in the presence of KCN (Mn-SOD). Human bypass conduit vessels show amounts of Cu-Zn SOD or Mn-SOD protein levels. In both HSV and IMA segments superoxide production was more than doubled in the presence of SOD inhibitor - DETC. Conclusions: These studies suggest that the differences in oxidative stress between human arteries and veins are unlikely to be caused by SOD activity. However SOD plays and important role in amelioration of oxidative stress in both types of vessels.

Key words: endothelium, oxidant stress, reactive oxygen species; superoxide dimutase; bypass grafts.
INTRODUCTION

Oxidative stress plays important roles in vascular physiology and pathology (1-3). Previous studies have shown that superoxide anion is produced by oxidases present in human vascular cells. These include NAD(P)H oxidases, xanthine oxidase and eNOS (4). However, net levels of oxidative stress are determined in parallel by production and biodegradation of free radicals (5) as well as by genetic susceptibility (6). The major anti-oxidant systems in the vasculature include superoxide dismutases, catalase, glutathione peroxidase and recently more and more appreciated thioredoxin (7). While all of the above enzymes play important roles in the modulation of oxidative stress, the primary role in the metabolism of superoxide anion radical is exerted by superoxide dismutases (5). There are 3 major forms of superoxide dismutases. MnSOD-present in mitochondria; CuZn SOD in cytoplasm, and extracellular SOD (EC SOD) secreted by vascular cells into the extracellular matrix (5). They catalyse the reaction of superoxide dismutation to hydrogen peroxide, which then undergoes further reactions and exerts numerous biological functions. This process leads not only to the removal of superoxide from the vascular wall, but also generates an important regulatory molecule - hydrogen peroxide (5). Therefore, the expression and activity of superoxide dimutases may be critical in the regulation of vascular function and may be critical for cellular defense from injury and ischemia reperfusion (8, 9). The reaction of superoxide (O$_2^-$) with NO is however approximately three times faster than the elimination of superoxide by superoxide dismutases, which could question the role of superoxide dimutases in protecting nitric oxide bioavailability (4). Animal models have however clearly indicated that the inhibition of superoxide dismutases by DETC leads to a very significant attenuation of endothelium dependent NO-mediated vasorelaxations (10). Similar findings have been reported in Cu-Zn SOD deficient mice (11). All these data show, that in spite of much slower rate of the reaction between SOD and superoxide, superoxide dismutases (including intracellular Cu-Zn SOD) are important in the regulation of NO bioavailability. Differences in SOD activity can also have impact on therapeutic approaches, as gene therapy using SOD as well as therapeutic use of SOD mimetics have been recently proposed to decrease levels of oxidative stress and improve endothelial dysfunction and diminish the development of atherosclerosis (12).

Our previous work has revealed several differences in the mechanisms of superoxide production between human arteries and veins (13, 14). These findings were described using bypass graft conduit vessels as model systems. The differences between studied vessels are important for understanding of vascular pathology, but also because these vessels are widely used for surgical treatment of coronary artery disease (15). Oxidative stress appears to be more pronounced in human saphenous veins than in mammary arteries, which could
partially explain better prognosis in arterial revascularisations (13, 14). However, the contribution of oxidative stress to this process is unknown. Limited number of studies have looked at the effects of superoxide dismutase inhibition on oxidative stress parameters in human blood vessels (16, 17). Those studies have found no significant differences in the increases of superoxide production following SOD inhibition in human arteries and veins. These studies were inconclusive as one found greater SOD activity in arteries while another reported lack of difference. However there was an important drawback in both studies, which used 250 µM-500 µM concentrations of lucigenin that can artificially generate superoxide by redox cycling in the presence of tissues and therefore change the potential differences in superoxide dismutase activity in studied vessels.

Accordingly, we aimed to directly measure superoxide dismutase activity in human arterial and venous bypass conduit vessels; compare their protein expression and functional effects of SOD inhibition on superoxide production.

MATERIALS AND METHODS

Patients and Blood Vessels

Excess segments of human saphenous vein (HSV) and internal mammary artery (IMA) were obtained from a total of 27 patients undergoing coronary artery bypass graft surgery. We and others have used this approach to study ‘paired’ artery and vein specimens, from the same patient, in several previous studies (16-18). Vessels were harvested using a no-touch technique, before surgical distension. The segment was immediately transferred to ice cold Krebs-Hensleit buffer, delicately flushed and carefully dissected to remove excess adventitial tissue, using microsurgical instruments. All vessels were collected before topical administration of drugs such as papaverine. The integrity of the endothelium after harvesting was checked by immunohistochemistry for the endothelial cell marker CD31, in preliminary studies. Patient characteristics and risk factor profile was typical for patients undergoing bypass graft surgery. All patients were treated with HMG-CoA reductase inhibitor.

Collection of tissue specimens was approved by the Local Research Ethics Committees and informed consent was obtained.

Superoxide Dismutase activity

Superoxide dismutase activity was measured by inhibition of pyrogallol autooxidation (19). Frozen vessel segments (-70°C) were ground in liquid nitrogen and suspended in 50 mM Tris buffer, pH 8.2. After centrifugation at 10000 g for 5 minutes, equal volumes of supernatants were mixed with 0.9 ml 50 mM Tris buffer pH 8.2 containing 1 mM DTPA, 10 µM catalase and 200 µM pyrogallol. The change of absorbance at 420 nm was measured every 5 sec for 5 minutes in 23°C using Beckman DU 640 BV spectrophotometer equipped with kinetic software package. The SOD activity was expressed as units/ mg protein, one unit determined as the amount of enzyme that inhibited the oxidation of pyrogallol by 50%. In parallel experiments, the activity of MnSOD was estimated after inhibition of CuZnSOD in samples by the addition of 5 mM KCN.
Western blotting

Portions of vascular homogenate were prepared in a homogenisation buffer (HEPES; 20mM; EDTA 0.01mM; protease inhibitors (Roche); PMSF 1mM; Triton X100 - 2% and SDS 0.5%). Protein content was measured using Bio-Rad system and samples were equalized for protein content. 20 µg protein portions of vascular homogenate were separated by SDS-PAGE (120mV) and transferred to nitrocellulose membranes for 4 hours at 4°C using 200 mAmp in a transfer buffer (25 mMTris; 190 mM Glycine; 20% methanol). MnSOD and Cu/Zn SOD were detected using anti-human SOD sheep polyclonal antibodies (Calbiochem, San Diego, CA). Membranes were incubated for 1 hour at room temperature in 1:500 final dilution of primary antibodies. Anti alpha actin antibody (mAb; Sigma, Germany) (1:2500) was used to check for equal protein loading and data were expressed per α-actin band. Goat horseradish peroxidase (HRP) conjugated anti-sheep IgG was used as secondary antibody to detect SODs (Calbiochem; San Diego, CA). Bands were detected using chemiluminescence substrate and developed on photographic membrane.

Vascular superoxide production

Superoxide production was measured by lucigenin-enhanced chemiluminescence (LGCL), using previously described and validated methods (6, 20). Briefly, intact vessel segments were equilibrated in Krebs-HEPES gassed with 95%O₂/5%CO₂ for 30 minutes at 37°C. Lucigenin-enhanced chemiluminescence from intact vessels was measured in buffer (2 ml) containing low concentration lucigenin (5 µM). In order to address the role of SOD in modulating superoxide release in human saphenous veins and internal mammary arteries, superoxide release was measured in the presence of SOD inhibitor - diethyldithiocarbamate - DETC (100 µM). Superoxide production was expressed as relative light units (RLU)/second/mg vessel dry weight.

Statistical analysis

Results are expressed as means ± SEM with n equal to the number of patients. Statistical comparisons between the two groups were made using Students t-test for independent or dependent samples. p values <0.05 were considered statistically significant.

RESULTS

Superoxide dismutase activity in human arteries and veins

Since the superoxide dismutase (SOD) enzymes regulate superoxide levels in the vascular wall, we next aimed to investigate whether differences in SOD protein levels and activities could account for differences in the superoxide-mediated effects observed in venous and arterial conduit vessels (Fig.1). Experiments comparing Cu/ZnSOD and MnSOD protein levels using Western Blotting in HSV and IMA revealed no differences between these two conduit vessels (Fig.5 A and B). Similarly, both total SOD activity and MnSOD activity, measured in vascular homogenates using inhibition of pyrogallol auto-oxidation, was similar in both saphenous veins and mammary arteries (Fig.5 C).
Fig. 1. Superoxide dismutase (SOD) activity in human veins and arteries. Total SOD activity was assessed in vascular homogenates in human saphenous vein (HSV) and internal mammary artery (IMA) conduit vessels. Spectrophotometric determination of the inhibition of pyrogallol autooxidation (200 µM) was used. Parallel experiments were conducted in the presence of KCN (5 mM) to inhibit CuZn SOD. Results were calculated in units of activity per mg of total protein. Data are expressed as mean±SEM. NS - lack of significant difference between HSV and IMA.

Fig. 2. Superoxide dismutase protein levels and activity in venous and arterial bypass conduits Panel A. Western blots showing MnSOD and CuZnSOD proteins in human saphenous vein (HSV) and internal mammary artery (IMA) conduit vessels. α-actin band shows equal loading of protein in each sample. Panel B. Densitometric analysis of SOD bands in HSV, IMA (mean±SEM; paired n=6), expressed in relation to density of actin bands.
SOD protein levels in human venous and arterial conduit vessels

Since the superoxide dismutase (SOD) protein levels may have important effects on SOD activity in the vascular wall, we next aimed to investigate the differences in SOD protein levels between venous and arterial conduit vessels (Fig 2). Experiments comparing Cu/ZnSOD and MnSOD protein levels using Western Blotting in HSV and IMA revealed no differences between these two conduit vessels (Fig 5 A and B).

Effects of SOD inhibition on vascular superoxide release.

In order to study the functional effect of superoxide dismutase activity in human vessels we measured vascular superoxide production by LGCL in the presence and absence of SOD inhibitor DETC (100 µmol/L). This approach allowed not only to compare the functional effects of vascular SOD in human bypass conduits, but was also an indirect indice of SOD activity measured in intact vessels as opposed to vascular homogenates used by majority of available methods of SOD activity determination.

Vessels were preincubated with DETC for 30 minutes prior to assay. DETC increased ·O₂⁻ release in both HSV and in IMA (Fig 3). The degree of increase of superoxide release in response to DETC was similar in HSV and IMA, which was in line with SOD activity measurements.

DISCUSSION

Vascular oxidative stress is determined by both production and degradation of free radicals in the vascular wall. While significant amount of data is available...
about the activity and expression of oxidases in the vascular wall of human bypass conduit vessels, relatively little is known about the anti-oxidant enzyme systems and their role in the regulation of oxidative stress in human veins and arteries.

In the present study, we did not find any significant differences in the activity or expression of superoxide dismutases in the vascular wall of human saphenous veins and internal mammary arteries. This suggests, that the differences in SOD activity are unlikely to be a primary cause for the variable characteristics of oxidative stress in HSV and IMA. Our studies, however, show that in both human saphenous veins and internal mammary arteries, in patients with generalized atherosclerosis, SOD is important in limiting oxidative stress, as its inhibition by DETC caused significant increases in superoxide release from vascular rings. This is in line with studies showing the importance of superoxide dismutase activity in the regulation of NO-dependent endothelial function in both animal models and in humans. In animal models inhibition of SOD by DETC leads to a significant impairment of NO bioavailability (10), and transgenic overexpression of Cu-Zn SOD prevents the development of endothelial dysfunction caused by lipopolysaccharide (21). Landmesser et al. have nicely shown that in patients with coronary artery disease (CAD), vascular EC-SOD activity is substantially reduced. The close relation between endothelium-bound EC-SOD activity and endothelial function suggests that reduced SOD activity contributes to endothelial dysfunction in patients with CAD (22). In our study all vessels were obtained from patients with advanced atherosclerosis undergoing bypass graft surgery. SOD activity could be diminished in this group of patients and the differences in SOD activity could be observed in earlier stages of atherosclerosis or in physiologic conditions. Similarly, activity and expression of superoxide dismutases may differ in younger and elderly individuals (23). Indeed, in young hypercholesterolemic subjects, SOD activity is increased as a defense mechanism to counteract impairment of endothelial function, as the result of increased formation of oxygen free radicals (22). Moreover, although no significant differences in the expression of Cu-Zn SOD or Mn SOD between human conduit artery and vein was found, we cannot exclude a difference in extracellular superoxide dimutase (EC-SOD) protein levels (24), which we were unable to study. Indeed, Landmesser et al. have found that in human coronary arteries from patients with coronary artery disease EC-SOD was increased along with total SOD activity, while Cu-Zn SOD or Mn SOD was not different (22). It is, however, important to note, that both inhibition of pyrogallol autooxidation by vascular homogenates and effects of DETC on superoxide release were not different, indicating that total activity of SOD (which includes EC-SOD) is not different in both types of human vessels.

Some previous studies compared the activity of SOD in human saphenous veins and internal mammary arteries. They have, however, obtained contrasting results. Schmalfuss et al. has found that SOD activity along with the generation of superoxide anion (measured by very high lucigenin concentrations) were all
greater in the IMA than in the HSV. Interestingly, Western blot analysis showed no differences in SOD protein expression in IMA and HSV (16). Berry et al., in turn, have found no differences in either activity or protein for SOD (16). Our study brings some additional important insights. Firstly, we used low concentrations of lucigenin in the measurements of superoxide production. Secondly, we paid particular attention to using the same size (weight) vascular rings for both HSV or IMA, which was not discussed by previous studies. Our results of functional effect of SOD inhibition on superoxide release are in agreement with previous studies conducted using high concentrations of lucigenin, thus confirming the validity of the approach taken by Schmalfuss et al. (16) or Berry et al. (17). This may suggest that therapeutic approaches aimed on increasing endogenous antioxidant systems may be very useful in limiting vascular oxidative stress in humans. This approach may be even more important in the light of findings that exogenous antioxidant vitamins do not seem to fulfill this aim. Some novel therapeutic approaches have been proposed recently and could include L-tryptophan (melatonin precursor) which can be useful in re-establishing SOD activity in the model of inflammatory diseases (25). Other potential approaches could include pentoxifylline in inflammation (26), L-carnitine (27), ferulic acid (28) in alcoholic abusive disease, all of which have been shown to induce intrinsic anti-oxidant mechanisms. The mechanisms of SOD activation and regulation of expression are however not fully understood and require further investigation, as novel studies suggest neural component in this process (29). The determination of mechanisms of oxidative stress in human bypass conduit vessels have important practical implications. Indeed, it has been found that oxidative stress plays an important role in the pathogenesis of intimal hyperplasia in experimental venous bypass conduits (30). Oxidative stress is critical in endothelial dysfunction (2, 3) as well as in the regulation of other factors involved in atherogenesis - including platelet and coagulation regulation (31). The role of superoxide dismutases in this process is not clear. In the present study we show that although the differences in SOD activity can rather not account for differences in the susceptibility to develop graft disease between HSV and IMA, SODs play important limiting role in the oxidative stress pathology in both types of vessels. In previous studies we found that vascular superoxide production has important functional differences between venous and arterial conduits used in patients undergoing coronary artery bypass grafting (4, 14, 18, 20). Specifically, we found that the NAD(P)H oxidase system is quantitatively and proportionately a greater source of superoxide in veins, whereas xanthine oxidase appears to additionally contribute to superoxide production in arteries. We found 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 (14). The NAD(P)H oxidase is predominantly nox2-based in saphenous veins, whereas a nox4-based oxidase appears
portionately more important in mammary arteries. The importance of differential expression of NAD(P)H oxidase homologues has been shown recently by several investigators (32, 33). Another important difference includes differential response to angiotensin II, which is not observed in veins (17). Despite these differences in the functional and molecular characteristics of superoxide production between veins and arteries, several findings 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 (14). Second, the expression of both p22phox and nox4 mRNA are strikingly correlated in arteries and veins (14). Finally, protein kinase C signaling has a key role in regulating superoxide production in both veins and arteries (14). The present study adds to that, the superoxide dismutase activity is also a common feature of oxidative stress regulation in veins and arteries. It is also important to remember that systemic nature of oxidative stress may be greatly affected by both clinical risk factors for atherosclerosis including hypertension, hypecholesterolemia or diabetes (18, 20) as well as by genetic factors. The latter may include NAD(P)H oxidase subunit genetic polymorphisms (6), as well as polymorphisms within genes encoding SOD (34).

In conclusion, our study shows that although superoxide dismutase activity and expression do not seem to differ significantly between human conduit arteries and veins, SOD does play a regulatory role in oxidative stress, therefore novel antioxidant approaches are necessary to enhance SOD activity, and improve endothelial function. Some natural products which may be useful in limiting oxidative stress include flavonoids (35) or grapefruit seeds extract (36), as these have been suggested to increase SOD activity in various tissues. However, better understanding of the regulation of vascular antioxidants is necessary to introduce better antioxidant therapeutic strategies for atherosclerosis and graft failure.

REFERENCES


Received: May 3, 2005
Accepted: May 16, 2005

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