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NITRIC OXIDE INSUFFICIENCY AND ARTERIAL THROMBOSIS

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Nitric oxide (NO) is a simple heterodiatomic free radical found throughout nature that serves a variety of important biological functions (1). In the normal mammalian cardiovascular system, the endothelial cell is the principal source of NO (2). The endothelial isoform of NO synthase (eNOS) catalyzes the five-electron oxidation of L-arginine to L-citrulline, forming NO in the process. This oxidoreductase requires several important cofactors for activity, including flavin adenine mono- and dinucleotides (FMN and FAD, respectively), tetrahydrobiopterin, NADPH, calcium/calmodulin and a prosthetic heme moiety. The essential functions of endothelial NO include evoking vascular smooth muscle relaxation, maintaining endothelial impermeability, inhibiting leukocyte adhesion to the endothelium, preventing vascular smooth muscle cell proliferation, and, importantly, impairing platelet function.

Nitric Oxide and Platelet Function

Nitric oxide inhibits platelet activation, adhesion, and aggregation by influencing several signalling pathways, including activation of guanylyl cyclase and increasing intracellular cyclic GMP (3); inhibition of phosphatidylinositol-3 kinase (4); and inhibition of capacitative cation influx and agonist-dependent increases in intracellular calcium (5). These molecular events lead to an impairment of platelet activation, adhesion, secretion, fibrinogen-binding to glycoprotein lib/IIIa (6), and, ultimately, aggregation. In addition NO promotes platelet disaggregation. In conjunction with its vasorelaxing actions, these antiplatelet effects of NO maintain blood fluidity and tissue perfusion.

The platelet itself is also an important source of NO. We recently demonstrated that platelet-derived NO can be measured with a NO-

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selective electrode, and found that this source of NO limits platelet accrual to the growing platelet thrombus (7). To confirm the relevance of these in vitro observations to in vivo phenomena, we recently showed that endothelial NO synthase is expressed in normal megakaryocytes, and that endothelial NO synthase (−/−) mice have shortened bleeding times as a result of the complete absence of platelet-derived NO (8). Transfusion of platelets from endothelial NO synthase (+/+) mice into null mice rendered transiently thrombocytopenic by carbiplatin significantly prolonged the bleeding time toward normal.

Oxidative Inactivation of Nitric Oxide

Nitric oxide is a reactive free radical that can participate in a variety of redox reactions in the vasculature that mediate its biological actions, prolong its half-life, or inactivate it. Examples of the first two types of reactions are the reaction of NO with heme iron (i.e., nitrosylation), which is responsible for activation of guanylyl cyclase; and the reaction of NO with thiol groups in the presence of oxygen, which leads to the formation of the stable, naturally occurring NO-donors, S-nitrosothiols (2,9,10). Oxidative inactivation of NO can occur by its reaction with oxygen to form nitrite and nitrate; and the reaction of NO with superoxide or lipid peroxyl radicals to form peroxynitrite ($OONO^-$) and lipid peroxynitrites (LOONO), respectively. The formation of nitrite, nitrate, peroxynitrite, and lipid peroxynitrites are examples of common oxidative reactions that inactivate NO.

In order to minimize the oxidative stress imparted by reactive oxygen species (ROS) and to limit their ability to inactivate NO, several antioxidant defense mechanisms have evolved. In addition to naturally occurring antioxidants, such as vitamins C and E, antioxidant enzymes are critical for limiting oxidative stress: superoxide dismutases, catalase, and the glutathione peroxidases reduce superoxide anion, hydrogen peroxide, and lipid peroxides, respectively, and are found in essentially all cell types, including cells of the cardiovascular system. Deficiencies of these antioxidant enzymes increase oxidative stress and the flux of ROS, promoting free radical injury to cells and tissue. With regard to the biological actions of NO and its redox chemistry, we have recently demonstrated that the cellular isoform of glutathione peroxidase is essential for limiting cellular lipid peroxyl radical formation and NO inactivation through the formation of lipid peroxynitrite, and for catalyzing both the liberation of NO from S-nitrosothiols and transnitrosation reactions (11).

NO Insufficiency and Childhood Stroke

Over the past 15 years, our research group has amassed abundant evidence for the antiplatelet actions of NO. Yet, until recently, we had no compelling evidence for the biological relevance of these in vitro phenomena. An experiment of nature first provided us with evidence for oxidative inactivation of NO leading to platelet-mediated arterial thrombosis (12). Our colleague, Alan Michelson, cared for two brothers who sustained childhood stroke and transient ischemic attacks. Routine evaluation for known causes of a prothrombotic state likely to be associated with this disorder was unrevealing. We studied the platelets of these brothers, as well as those of an unaffected sister, mother, and father, and found that their platelets were resistant to inhibition by NO. Mixing experiments in which we suspended the affected children's platelets in normal plasma and vice versa showed that the defect resided in their plasmas, which we found to contain approximately one-half of the normal activity of the plasma isoform of glutathione peroxidase (GPx). Adding back authentic GPx to their plasma restored sensitivity to platelet inhibition by NO. We concluded that plasma GPx deficiency led to increased lipid peroxide and derivative lipid peroxyl radical formation, which, in turn, inactivated NO by forming the lipid peroxynitrite derivatives (Figure 1).

More recently, in collaboration with Aida Inbal, we have identified seven families with childhood stroke in which there is a deficiency of plasma GPx activity. Pedigree analysis suggests that the defect is inherited as an autosomal dominant trait (13). We are currently sequencing the plasma glutathione peroxidase gene in order to identify the muta-

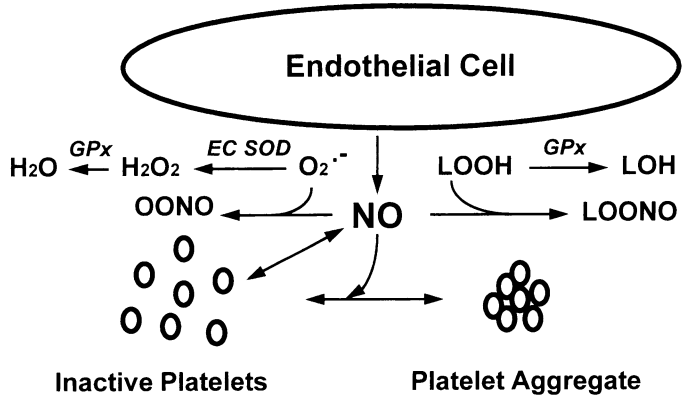

FIG. 1. Fates of vascular nitric oxide and effects on platelet function. Abbreviations used: Gpx, plasma glutathione peroxidase; EC SOD, extracellular superoxide dismutase; LOOH, lipid peroxides; LOH, lipid alcohols.

tion(s) that may be responsible for this reduced activity. From these data, we conclude that 1) NO bioactivity is dependent on the availability of adequate antioxidant defenses to minimize oxidative inactivation of the molecule, and 2) NO insufficiency states are accompanied by enhanced platelet activation and platelet-mediated arterial thrombosis.

NO Insufficiency and Coronary Atherothrombotic Disease

The families we have identified thus far with plasma GPx deficiency and arterial thrombosis in childhood are uncommon. Yet, we know that oxidative stress accompanies many common cardiovascular diseases, and that several of these have been associated with reduced NO activity (14). To address a more typical population of patients with arterial thrombotic risk, we studied a series of 37 and 50 consecutive patients undergoing cardiac catheterization for stable angina pectoris and acute coronary syndromes, respectively, and measured platelet-derived NO production (15) and plasma GPx activity in each individual. We found that patients with acute coronary syndromes had significantly less platelet-derived NO than did patients with stable angina pectoris (0.26 ± 0.05 pmol/10^8 platelets vs. 1.78 ± 0.36 pmol/10^8 platelets, $p = 0.0001$). Patients with acute coronary syndromes also had significantly less plasma glutathione peroxidase activity than did patients with stable angina (0.36 ± 0.04 U/ml vs. 0.94 ± 0.12 U/ml, $p = 0.01$). Furthermore, logistic regression analysis demonstrated that platelet NO production (odds ratio = 3.9, 1.3–11.1, $p = 0.01$) and heparin treatment (odds ratio = 6.4, 1.9–22.0, $p = 0.004$) were independent predictors of an acute coronary syndrome.

These data suggest that oxidative inactivation of NO is found much more commonly in the general population, especially in the setting of enhanced oxidative stress. As yet, we have no reason to believe that plasma GPx deficiency is a common genetic abnormality that accounts for the observations in these patients with atherothrombotic disease. Rather, we believe that there are limits to which native antioxidant defense mechanisms can provide protection against ROS. Reactive oxygen species flux in excess of these limits will lead to oxidative stress that causes cell and tissue injury, one of the consequences of which may be inactivation of active-site selenocysteine or thiol groups in GPx that reduces enzyme activity. An alternative explanation is that excess oxidative stress can modulate antioxidant gene expression adversely. For example, we have recently shown that the oxidative stress accompanying hyperhomocysteinemia suppresses expression of the cellular isoform of GPx, potentiating the oxidative stress of this metabolic disorder and the resulting vascular injury (16).

CONCLUSIONS

Endothelial and platelet NO impair platelet-mediated thrombosis. We have shown that oxidative inactivation of NO promotes platelet activation and can lead to arterial thrombotic disorders. Plasma GPx appears to be an important extracellular antioxidant enzyme. Plasma Gpx deficiency promotes oxidative stress and NO inactivation, and leads to an NO insufficiency state that underlies both rare and common arterial thrombotic disorders.

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DISCUSSION

WOLF, Boston: Joe, what happens to the people with acute coronary syndrome when they have recovered? In other words, what happens to their NO production? Assuming that it returns toward normal, what is the mechanism of its occurring in the acute syndrome?

LOSCALZO: We haven't studied all the patients we showed here, Marshall, late after their infarction. We have studied about 30 of them and their glutathione peroxidase activity returns toward normal, but not completely. Our current hypothesis is that part of the cause for the reduction in activity is oxidation of the active site, selinocysteine, by reactive oxygen species in this highly inflammatory and oxidizing environment of an acute atherothrombosis. There were some patients who had persistent reduction and we are pursuing them for genetic causes.

TANNEN, Philadelphia: You indicated that glutathione peroxidase is made by the kidney and you showed in anephric patients that the levels were low. As you know, one of the major causes of death in people with chronic renal disease on dialysis is cardiovascular events. Do you think that there might be any association?

LOSCALZO: Yes, there very well may be. We have not looked at that yet. Clearly, there are other complications of uremia that modulate thrombotic risk and may offset that prothrombotic risk, such as the prolonged bleeding time and impaired platelet function. We have yet to study a large end-stage renal disease population in an effort to correlate changes in GPX activity with coronary or cerebrovascular events, but it is a wonderful question to ask.

STEVENSON, Stanford: As you know, "nitric oxide insufficiencies" can be considered as a contributing cause of pulmonary hypertension, which is potentially lethal. I wonder whether in these families there has been anything noted with respect to their birth histories. Are there fetal deaths or newborn deaths?

LOSCALZO: I don't have that information. The pedigrees I have represent the complete families, I think, but I have not asked that specific question and I should do that.

STEVENSON: That would be very interesting. The other thing I would like to speak with you afterward about is that since heme oxygenase is also an antioxidant enzyme, it would be very interesting to look at the heme oxygenase system in these particular patients because it is obviously perturbed and there might be some very interesting interactions between the nitric oxide synthase and heme oxygenase systems.

CAREY, Charlottesville: Could you tell us about the primacy of the peroxidase changes and reduction in nitric oxide release that you observed in the patients? Are these changes a secondary phenomenon or are they really primary?

LOSCALZO: My guess is, based on the few patients that we have studied late after their infarction in followup, that it is really a secondary phenomenon in most patients, but not all, and the oxidative stress that is manifest in the setting of the acute inflammatory response to an acute infarction may contribute, in part, to impairing enzyme activity. That is our current hypothesis for that pool of patients.