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Review

Novel approaches to improving endothelium-dependent nitric oxide-mediated vasodilatation

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Abstract:
Endothelial dysfunction, which is defined by decreased endothelium-dependent vasodilatation, is associated with an increased number of cardiovascular events. Nitric oxide (NO) bioavailability is reduced by altered endothelial signal transduction or increased formation of radical oxygen species reacting with NO. Endothelial dysfunction is therapeutically reversible and physical exercise, calcium channel blockers, angiotensin converting enzyme inhibitors, and angiotensin receptor antagonists improve flow-evoked endothelium-dependent vasodilation in patients with hypertension and diabetes. We have investigated three different approaches, with the aim of correcting endothelial dysfunction in cardiovascular disease. Thus, (1) we evaluated the effect of a cell permeable superoxide dismutase mimetic, tempol, on endothelial dysfunction in small arteries exposed to high pressure, (2) investigated the endothelial signal transduction pathways involved in vasorelaxation and NO release induced by an olive oil component, oleanolic acid, and (3) investigated the role of calcium-activated K channels in the release of NO induced by receptor activation. Tempol increases endothelium-dependent vasodilatation in arteries from hypertensive animals most likely through the lowering of radical oxygen species, but other mechanisms also appear to contribute to the effect. While oleanolic acid leads to the release of NO by calcium-independent phosphorylation of endothelial NO synthase, endothelial calcium-activated K channels and an influx of calcium play an important role in G-protein coupled receptor-evoked release of NO. Thus, all three approaches increase bioavailability of NO in the vascular wall, but it remains to be addressed whether these actions have any direct benefit at a clinical level.

Key words: endothelium, nitric oxide, oleanolic acid, tempol, calcium-activated K channels

Abbreviations: BK_{Ca} – large-conductance calcium-activated K channels, EDHF – endothelium-derived hyperpolarizing factor, NO – nitric oxide, eNOS – endothelial NO synthase, O_2^- – superoxide anion, SOD – superoxide dismutase

Introduction

The endothelium regulates tone at rest and during exercise, the thrombotic and adhesive properties of the

vascular wall, the architecture of the vascular wall, and vascular permeability. Disturbances of these principal endothelial functions are termed endothelial dysfunction. Endothelium-dependent vasodilatation induced by increased blood flow and receptor-specific agonists, such as acetylcholine and bradykinin, are reduced in the presence of classical vascular risk factors including hypertension, hypercholesterolemia, diabetes, smoking, aging and by atherosclerotic disease and inflammation. Moreover, endothelial dysfunction in the coronary circulation [6, 63, 64, 80, 94] and the forearm [72] is associated with progression of ischemic heart disease, and endothelial dysfunction in the microvasculature is considered to be an independent risk factor for ischemic heart disease [43]. Therefore, disturbed endothelial function is of prognostic significance.

Early studies have suggested that endothelial dysfunction may be therapeutically reversible [17], and physical exercise, calcium channel blockers and angiotensin converting enzyme inhibitors and angiotensin receptor antagonists were found to improve flow-evoked endothelium-dependent vasodilation in patients with hypertension and diabetes [18, 40, 71, 81]. It is controversial whether lipid-lowering statins improve endothelium-dependent vasodilation in patients [18, 19, 96, 100] or have a minor or no effect on endothelium-dependent vasodilation [8, 30, 35, 103]. The latter can be ascribed to different clinical designs (see [44]), but also the potency, structure, and pleiotropic effects of each statin [67], which requires further investigation. However, correction of endothelial dysfunction in hypertension was found to have prognostic significance [58], hence supporting that there is an unmet need for drugs that specifically improve endothelial function.

A key mechanism underlying endothelial dysfunction and increased expression of adhesion molecules and chemoattractants is the loss of endothelial nitric oxide (NO) bioavailability. NO bioavailability is decreased either by decreased formation or by enhanced removal of NO. The presence of classical cardiovascular risk factors is associated with enhanced generation of radical oxygen species where superoxide anions (O_2^-) play a pivotal role by reacting with NO resulting in the formation of peroxynitrite ($ONOO^-$), and hence decreasing bioavailability of NO (see [15]). Moreover, we have found, by simultaneous measurements of relaxation and release of NO that endothelial cell signal transduction is altered in hypertension [92]. Some of the specific treatments of endothelial dysfunction have recently been reviewed, including endothelin receptor antagonists [48], L-arginine [11], tetrahydrobiopterin analogues [3], 1-methylnicotinamide [5] and phosphodiesterase type 5 inhibitors (e.g., sildenafil and tadalafil) [87] (Fig. 1). The following is a review of three novel approaches that we have investigated with the aim of correcting endothelial dysfunction in cardiovascular disease (Fig. 2). Thus, (1) we evaluated the effect of a cell permeable superoxide dismutase mimetic, tempol, on endothelial dysfunction in small arteries exposed to high pres-

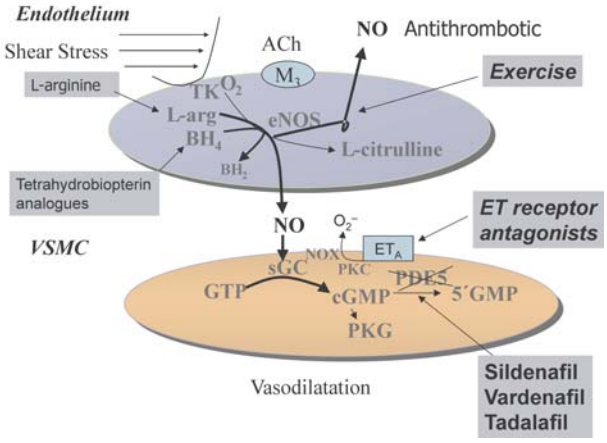

Fig. 1. Specific treatments to restore endothelium-dependent vasodilatation (grey boxes). sGC – soluble guanylyl cyclase; VSMC – vascular smooth muscle cell

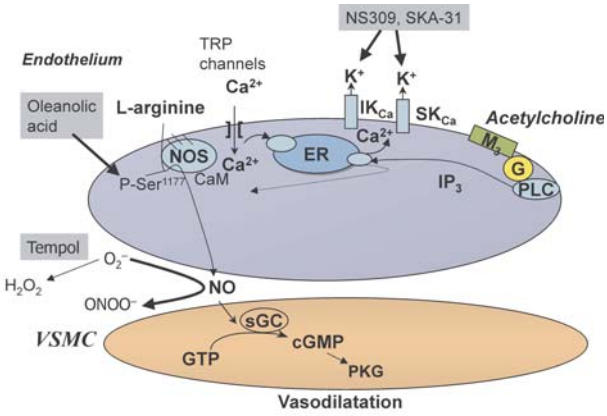

Fig. 2. Novel approaches to improve endothelium-dependent nitric oxide-mediated vasodilatation (grey boxes). SK_{Ca} – small conductance calcium-activated K channels; IK_{Ca} – intermediate calcium-activated K channels; sGC – soluble guanylyl cyclase; TRP – transient receptor potential channels; VSMC – vascular smooth muscle cell

sure, (2) investigated the endothelial signal transduction pathways involved in vasorelaxation and NO release induced by an olive oil component, oleanolic acid, and (3) investigated the role of calcium-activated K channels in the release of NO induced by receptor activation.

The superoxide dismutase mimetic tempol and endothelial dysfunction

The level of radical oxygen species is determined by the radical oxygen species generating enzymes in the extracellular and intracellular compartments depending on location, e.g., NAD(P)H/NADH oxidase in the cell membrane, xanthine oxidase, myeloperoxidase, cytochrome P450 in cytoplasm, and mitochondrial oxidation and antioxidant enzymes e.g., superoxide dismutases (SODs), glutathione peroxidase, heme oxygenase, thioredoxin peroxidase/peroxiredoxin, catalase, and paraoxonase [42]. The increased radical oxygen species levels are further enhanced by uncoupling of endothelial eNOS, which ceases to produce NO and instead switches to superoxide production [49, 104]. Thus, there are many sources in the vascular wall leading to radical oxygen species generation, making scavenging of radical oxygen species an attractive therapeutic approach for treating endothelial dysfunction associated with cardiovascular disease.

There are natural extra- and intracellular antioxidant defense mechanisms that scavenge radical oxygen species. For example, SOD catalyzes the conversion of O_2^- to H_2O_2, which can be turned into water by catalase or the glutathione peroxidase system. Although SOD is the first line of physiological defense against oxidative stress, the reaction of O_2^- with NO is about three times faster than its reaction with SOD [7]. However, the addition of an exogenous SOD mimetic, tempol, protects animals and mammalian cells from cytotoxicity induced by oxygen-free radicals like hydroxyl radicals, H_2O_2, and O_2^- [57, 78]. An additional property of tempol as antioxidant is that it can penetrate cell membranes, and hence react with both intracellular and extracellular oxygen-free radicals. These properties make tempol attractive for treatment of cardiovascular disease associated with oxidative stress (e.g., hypertension).

In rat small mesenteric arteries activated with a thromboxane analogue, increases in flow and acetylcholine induce endothelium-dependent vasodilatation, which, in the case of flow, appears to be mediated by NO, while acetylcholine evokes mainly endothelium-derived hyperpolarizing factor (EDHF) type relaxations [98]. Exposing isolated pressurized arteries to elevated pressure for one hour and then returning intraluminal pressure to normotensive levels results in impaired vasodilation in response to flow, while acetylcholine-evoked vasodilatation is conserved [24]. These findings suggest that bioavailability of NO is selectively decreased by high pressure and underlies impaired flow-mediated vasodilatation. Our findings are in line with those from previous studies in models of hypertension *in vivo* [36, 110] and in rat isolated skeletal arterioles [101], where elevated pressure was also shown to elicit arterial O_2^- production by activation of NADPH oxidase resulting in impaired endothelial function. Moreover, these results were further supported by the observations that vasodilatation to an NO donor, S-nitroso-N-acetylpenicillamine was also reduced, while vasodilation in response to a direct guanylyl cyclase activator, BAY 412272, were conserved, and O_2^- formation was increased in arterial segments exposed to high pressure in small mesenteric arteries [24]. These results suggest that O_2^-, by reaction with NO, reduces NO bioavailability and leads to impaired flow-induced NO-mediated dilatation in arteries exposed to high pressure; indeed, in the *in vitro* model of endothelial dysfunction in small arteries induced by elevated pressure, incubation of the SOD mimetic tempol conserved the vasodilatation most likely by lowering O_2^- levels in the arterial wall [24].

There have been numerous studies performed on arteries from different animal models showing that treatment or incubation with tempol restores agonist-induced endothelium-dependent vasodilatation. Thus, tempol restored acetylcholine and arachidonic acid relaxation in skeletal muscle arteries and coronary arteries from diabetic animals [38, 106], and acetylcholine relaxation in afferent arterioles from Dahl salt sensitive rats [65] and in carotid arteries from deoxycorticosterone acetate-salt hypertensive rats [65, 111]. That tempol can substitute for endogenous SOD is also supported by the observations that tempol treatment restores acetylcholine relaxation in carotid arteries from mice heterozygous for CuZn SOD [27, 108]. Therefore, to investigate whether tempol is also able

to restore endothelial function in small arteries exposed to high blood pressure *in vivo*, one-kidney one-clip hypertensive rats were treated with tempol [25]. In arteries from the renal hypertensive rats, flow-induced dilatation was blunted compared to normotensive and tempol-treated rats, while acetylcholine-induced dilatation remained normal [25]. As measured by dihydroethidium staining, there was an increased level of O_2^- in arteries from vehicle-treated rats, but not from tempol-treated rats [25]. These studies suggest that renal hypertension, similar to elevated pressure *in vitro*, selectively inhibits flow-induced NO-mediated vasodilatation by increased generation of O_2^- contributing to decreased NO bioavailability. Furthermore, the endothelial dysfunction observed can be corrected by tempol treatment.

The findings of less O_2^- in the arterial wall of arteries from hypertensive rats treated with tempol suggest that tempol is a SOD mimetic, but do not exclude other mechanisms also contributing to the vasodilator effect. Thus, similar to endogenous SOD, tempol not only reduces O_2^-, but may also increase H_2O_2 in blood vessels and the kidney medulla [21, 22] . In mouse cremaster arterioles, tempol (1 mM) induced a transient and catalase-sensitive vasodilatation, suggesting it was mediated by H_2O_2 [21]. Moreover, in mesenteric arteries from Cu, Zn-SOD$^{-/-}$ mice, tempol increased acetylcholine-induced catalase-sensitive vasodilatation and increased vascular H_2O_2 [108]. Although H_2O_2 can also induce vascular contraction [4, 39], these studies suggest H_2O_2 can contribute to the effects of tempol and that an enhanced vasodilatory effect by adding tempol should not only be attributed to dismutation of O_2^- anion and increased bioavailability of NO. Moreover, isolated patch clamp studies on vascular smooth muscle cells from normotensive and deoxycorticosterone acetate-salt hypertensive rats suggest that high tempol concentrations (~1 mM) increase a large-conductance calcium-activated potassium (BK_{Ca}) current [107]. Therefore, high concentrations of tempol appear to activate BK_{Ca} channels directly, whereas it remains to be addressed whether lower concentrations of NO, indirectly through formation of H_2O_2 or increased bioavailability, can lead to vasodilatation through activation of BK_{Ca} channels.

In humans, endothelial dysfunction is a marker of cardiovascular disease, and compromised flow-mediated dilatation is associated with a worse prognosis. In cross sectional studies, flow-mediated dilatation is linked with increased oxidative stress markers [95], and a main contributor to a reduced flow-mediated dilatation is increased O_2^- production and reduced bioavailability of NO [61]. Thus, a reduction in O_2^- generation would be a logical therapeutic approach. However, it currently remains unproven that a reduction in the level of oxidative stress *per se* improves outcome of cardiovascular diseases in humans. Potential therapies for lowering of radical oxygen species include existing drug treatment with angiotensin-converting enzyme inhibitors [53], statins [23], and the β-adrenoceptor antagonists, carvedilol and nebivolol [2]. Dietary polyphenols present in wine, green tea, and fruits are mild antioxidants and may also offer long-term benefits by lowering radical oxygen species [68]. Moreover, vitamin C and vitamin E were found to lower oxidative stress, but large-scale clinical studies have not supported an effect of vitamin C or vitamin E supplementation on cardiovascular disease or mortality [10, 51]. However, the drugs that affect O_2^- levels in humans, such as inhibitors of the renin angiotensin aldosterone system, some beta-adrenoceptor antagonists and statins, like tempol, have additional effects including lowering of blood pressure or the cholesterol concentration, which by themselves lower O_2^- production. It is therefore important that tempol is compared to existing cardiovascular drugs. However, as tempol clearly has a different mechanism of action from these compounds, it is possible that a combination of tempol and conventional therapy could result in improved endothelial function and patient outcome.

The olive oil component, oleanolic acid, and endothelial dysfunction

Olive oil is an integral ingredient of the Mediterranean diet, and accumulating evidence suggests that it may have healthy benefits in terms of reduction of cardiovascular risk factors [32, 99]. The cardioprotective effects of olive oil have been ascribed to its content of monounsaturated fatty acids such as oleic acid and the presence of other biologically minor constituents such as polyphenols, tocopherols and triterpenoids [32, 54]. Olive pomace oil, so-called *orujo* olive oil in Spain, is obtained from the residue that remains after virgin olive oil has been mechanically extracted. As a consequence of these extraction processes, po-

mace olive oil differs from virgin olive oil in the content of minor components. In this regard, despite the lack of polyphenols, pomace olive oil contains higher concentrations of triterpenic compounds, such as oleanolic acid, than virgin olive oil [70]. Recent research has demonstrated that chronic parenteral administration of oleanolic acid prevents hypertension in salt-sensitive rats [88]. Furthermore, ingestion of pomace olive oil with a high proportion of oleanolic acid delays progression of lipid peroxidation in rat liver microsomes [69]. These studies suggest that oleanolic acid may contribute to the cardioprotective effect of ingestion of a diet rich in olive oil.

In aortic segments from normotensive and hypertensive rats, oleanolic acid evokes endothelium-dependent relaxations sensitive to inhibition of eNOS [74]. Investigation of large and small mesenteric arteries from rats also revealed that oleanolic acid induces endothelium-dependent relaxations sensitive to inhibition of NO synthase [76]. Moreover, inhibition of cyclooxygenase with indomethacin and of EDHF-type relaxation with a combination of calcium-activated K channel blockers, apamin and charybdotoxin, failed to alter oleanolic acid relaxation. These findings suggest that oleanolic acid evokes endothelium-dependent vasodilatation mediated by NO, and simultaneous measurements of NO and relaxation revealed that oleanolic acid evokes relaxation by release of NO [76].

Endothelial NO is produced by eNOS, which is classically activated by agonists such as acetylcholine, histamine and bradykinin inducing an increase in intracellular calcium levels followed by calcium/calmodulin-dependent modulation [59]. In contrast to acetylcholine, oleanolic acid failed to increase endothelial cell calcium, and hence evoked relaxation and release of NO independent of endothelial cell calcium in rat superior mesenteric arteries [76]. However, over the past five to 10 years, a far more complex model of eNOS regulation has emerged involving post-translational mechanisms such as protein-protein interactions and tightly regulated multisite phosphorylation. Overall, Ser^{1177} is the most extensively studied and appears to be the most important of the regulatory eNOS phosphorylation sites [34]. Phosphorylation of eNOS-Ser^{1177} is associated with an increase in activation and NO production in response to a growing list of stimuli, including mechanical factors (e.g., shear stress) [12] and humoral factors (e.g., insulin) [60] even at resting levels of calcium [55, 60]. Estrogen, mechanical shear stress, and inhibitors of tyrosine phosphatase such as vanadate have been demonstrated to cause eNOS-Ser^{1177} activation and NO release by calcium-independent mechanisms [26, 66, 77]. eNOS-Ser^{1177} can be phosphorylated by several protein kinases, including Akt/protein kinase B, protein kinase A and AMP-activated protein kinase [12, 28, 37]. Therefore, in human umbilical cord endothelial cells, we investigated these pathways and found that oleanolic acid time-dependently increased phosphorylation of Akt kinase at $Serine^{473}$ and eNOS at $Serine^{1177}$, where this effect was blunted by inhibition of the upstream enzyme, phosphoinositide-3-kinase [76]. These findings suggest that oleanolic acid and acetylcholine increase vascular NO concentration by different signal transduction pathways, and that oleanolic acid by activation of phosphoinositide-3-kinase and phosphorylation of Akt can increase eNOS activity and NO production.

Several observations suggest that the effect of oleanolic acid on cell function is specific. Thus, structurally modified analogues of oleanolic acid bind with different potency to Kelch-like-ECH-associated protein 1, a cytoplasmic repressor of the transcription factor, NRF2 [29]. The structurally-related compounds, oleanolic acid, erythrodiol, maslinic acid, and uvaol, which only differ in substitution of one chemical group, cause relaxations with different potency and magnitude in rat aorta [29, 75], and caulophyllogenin, which is structurally related to oleanolic acid and only differs by a couple of hydroxyl substitutions, failed to induce relaxation in rat mesenteric arteries. Although these findings suggest the effects of oleanolic acid on endothelial cell function are specific, further studies are required to clarify the role of structural modifications of oleanolic acid for release of NO and endothelium-dependent relaxation as well as for the anti-inflammatory effects of the compound.

The clinical relevance of the NO releasing and relaxing activity of oleanolic acid depends on the systemic availability. In olive oil, the content of oleanolic acid is approximately 56 mg kg^{-1} and in orujo olive oil it is 416 mg kg^{-1} [73]. Recent studies reveal that, after ingestion of 40 mg oleanolic acid, plasma concentrations reached 12 ng ml^{-1} [89], which is below the oleanolic acid concentrations causing release of NO and relaxation. That systemic availability of oleanolic acid is important is also supported by the observation that intraperitoneal administration of oleanolic acid lowers blood pressure in hypertensive

rats [88], but there is no effect on blood pressure of oleanolic acid administered in the diet [73]. However, in the same study, pomace olive oil supplemented in oleanolic acid increased eNOS expression and improved vasorelaxation in aortic segments from spontaneous hypertensive rats [73]. The latter findings support that systemic availability of oleanolic acid by administration in the diet is sufficient to improve endothelial function.

Oleanolic acid is a novel approach to improving endothelial function and may explain some of the beneficial effects on cardiovascular disease of a Mediterranean diet. However, oleanolic acid and structurally similar compounds should be tested in other animal models to determine whether they improve endothelial dysfunction induced by other cardiovascular risk factors, and it remains to be addressed whether the *in vivo* effects of oleanolic acid are due to an effect on eNOS activity and/or anti-inflammatory effects of the compound.

Therapeutic potential of targeting calcium-activated K channels in the endothelium

By simultaneous measurements of relaxation and release of NO in arteries from renal hypertensive rats, we have found that treatment with superoxide dismutase or L-arginine *per se* does not restore NO bioavailability [92]. Moreover, treatment of renal hypertensive animals with tempol restores vasorelaxation, but fails to restore NO release induced by acetylcholine in rat coronary arteries [25]. These findings suggest that endothelial cell signal transduction is also altered in hypertension, and that a therapeutic potential to treat endothelial dysfunction would be modulation of the endothelial signal transduction pathways.

The inhibition by apamin and charybdotoxin, blockers of small- and intermediate-conductance Ca^{2+}-activated K channels, has been considered a unique characteristic of EDHF type relaxation [14, 31, 109, 112]. Acetylcholine-evoked hyperpolarization of the endothelial cell coincides with rises in intracellular calcium in endothelial cells in intact rat aorta [16, 102], and patch clamp experiments and RT-PCR have provided evidence for the presence of small, intermediate and large-conductance Ca^{2+}-activated K channels in endothelial cells of intact arteries [50, 52, 62]. Therefore, it was suggested that apamin and charybdotoxin may exert their effects mainly *via* Ca^{2+}-activated K channels located on the endothelial cells rather than on smooth muscle cells [9, 13, 31]. In the superior mesenteric artery, the combination of apamin and charybdotoxin also inhibits smooth muscle hyperpolarization and acetylcholine relaxation [20]. However, in contrast to small mesenteric arteries where acetylcholine mainly evokes EDHF type relaxations [47, 84, 98], acetylcholine evokes endothelium-dependent NO release and relaxation, which are blunted in the presence of a NOS inhibitor and abolished by combination with a NO scavenger, oxyhemoglobin, in rat superior mesenteric artery [86, 91, 92]. Therefore, we addressed whether agonist-evoked increases in NO concentration in intact segments of rat superior mesenteric arteries also involved activation of calcium-activated K channels of small and intermediate conductance. A combination of blockers of these channels, apamin and charybdotoxin markedly inhibited an acetylcholine-evoked increase in NO concentration and relaxation [91]. Despite causing contraction, noradrenaline also increases endothelial cell calcium and NO concentration [45], and a noradrenaline-evoked increase in NO concentration was also blunted in the presence of apamin and charybdotoxin [91]. These findings suggest that receptor-activated endothelial signal transduction leading to an increase in endothelial cell calcium is followed by activation of calcium-activated K channels, hyperpolarization and most likely amplified NO release.

Based on patch clamp studies and measurements of intracellular calcium in cultured endothelial cells, it has been suggested that the opening of calcium-activated K channels of small and intermediate conductance is coupled to increased calcium influx [82]. However, in intact arteries, acetylcholine and noradrenaline-evoked endothelial cell calcium levels remain unaltered in the presence of a combination apamin and charybdotoxin, which blocks these channels [41, 56, 76, 91]. Therefore, it remains to be addressed how opening of calcium-activated K channels of small and intermediate conductance are coupled to release of NO.

Knockout of endothelial calcium-activated K channels of small [97] and intermediate conductance [85] are associated with impaired endothelium-dependent vasodilatation and hypertension. The expression of small and intermediate conductance calcium-activated

K channels is reduced in angiotensin II-induced hypertension [46], while expression is unaltered in mesenteric arteries from diabetic rats, but the current is reduced [1]. Small molecules (NS309 and SKA31) modulating these channels are available [93, 105], and have recently been shown to induce hyperpolarization [1], release of NO in segments of rat mesenteric [90] and skeletal arteries [83], enhancement of receptor-activated EDHF-type relaxation in small arteries from mice [79], and induction of relaxation in human arteries [33] (Fig. 2). Therefore, given that opening of calcium-activated K channels of small and intermediate conductance are involved in both release of NO and EDHF-type relaxation, and that small molecules are available, provides these channels as attractive targets for exploring improvements in endothelium-dependent vasodilatation in cardiovascular disease.

Conclusion and perspectives

In summary, the three approaches that we have applied here show that: (1) tempol increases endothelium-dependent vasodilatation in arteries from hypertensive animals, most likely through a lowering of radical oxygen species, but that other mechanisms also appear to contribute to the effect, (2) oleanolic acid leads to the release of NO by calcium-independent phosphorylation of endothelial NO synthase, (3) endothelial calcium-activated K channels and influx of calcium play an important role in G-protein coupled receptor-evoked release of NO. Thus, all three approaches increase bioavailability of NO in the vascular wall, but it remains to be addressed whether they have any direct benefit at the clinical level.

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