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Oxidative stress and endothelial dysfunction

G. Muller, C. Goettsch, H. Morawietz
Department of Vascular Endothelium and Microcirculation (Head: Prof. Henning Morawietz),
University of Technology Dresden, Germany

Keywords
Endothelial dysfunction, lipoproteins, nitric oxide, oxidative stress

Summary
This review focuses on the role of vascular oxidative stress in the development and progression of endothelial dysfunction. We discuss different sources of oxidative stress in the vessel wall, oxidative stress and coagulation, the role of oxidative stress and vascular function in arteries and veins, the flow-dependent regulation of reactive oxygen species, the putative impact of oxidative stress on atherosclerosis, the interaction of angiotensin II, oxidative stress and endothelial dysfunction, and clinical implications.

Hämostaseologie 2007; 27: 5–12

Schlüsselwörter
Endotheliale Dysfunktion, Lipoproteine, oxidativer Stress, Stickstoffmonoxid

Zusammenfassung
Diese Übersichtsarbeit fokussiert auf die Rolle von vaskulärem oxidativen Stress in der Entwicklung und Progression von endothelialer Dysfunktion. Wir diskutieren unterschiedliche Quellen reaktiver Sauerstoffspezies in der Gefäßwand, oxidativen Stress und Koagulation, die Rolle von oxidativem Stress und Gefäßfunktion in Arterien und Venen, die flussabhängige Regulation von reaktiven Sauerstoffspezies, den möglichen Einfluss von oxidativem Stress auf die Atherosklerose, die Interaktion von Angiotensin II, oxidativem Stress und endothelialer Dysfunktion und klinische Implikationen.

Oxidativer Stress und endotheliale Dysfunktion

The endothelium as the largest organ in the body is located between the blood stream and the vessel wall. Because acetylcholine requires an intact endothelial cell layer for vasodilatation (26), the importance of endothelial function for vascular homeostasis has been increasingly recognized (21). Endothelial dysfunction was initially identified as an impaired vasodilatation in response to acetylcholine or bradykinin. The first clinical description of endothelial dysfunction was in the forearm vasculature of hypertensive patients (75). The initial state of endothelial dysfunction is also considered as an early stage of atherosclerosis, finally leading to clinical manifestations like coronary artery disease (61). Established risk factors of atherosclerosis are associated with oxidative stress and endothelial dysfunction (Fig. 1).

The concept of endothelial dysfunction now also includes a functional shift toward a proinflammatory and prothrombotic state of the endothelium. A rationale for this concept is the finding that endothelium-derived nitric oxide (NO) does not only mediate endothelium-dependent vasodilation, but also antiinflammatory and antithrombotic processes (54) like

- reducing leukocyte adhesion,
- platelet adhesion and aggregation, and
- expression of plasminogen activator inhibitor (PAI-1).

Therefore, endothelial formation of NO by the endothelial NO synthase (eNOS) is a critical determinant of endothelial function. The NO availability can be limited by enhanced formation of reactive oxygen species (ROS) like superoxide anions. NO and superoxide anions form in a very rapid reaction peroxynitrate thus reducing the amount of available NO (37). An increased formation of ROS has been termed oxidative stress and is considered as a major determinant of endothelial dysfunction (40).

Therefore, this review will focus on the impact of vascular oxidative stress in the development and progression of endothelial dysfunction. We will discuss different sources of oxidative stress in the vessel wall, oxidative stress and coagulation, the role of oxidative stress and vascular function in arteries and veins, the flow-dependent regulation of ROS, the putative impact of oxidative stress on atherosclerosis, the interaction of angiotensin II, oxidative stress and endothelial dysfunction, and clinical implication of augmented oxidative stress.

Oxidative stress

Sources in the vessel wall

Oxygen-derived radicals like superoxide anions (O_2^-) can be generated by a variety of enzymatic mechanisms in the vessel wall. Molecular sources of O_2^- include (79)

- enzymes of the respiratory chain,
- xanthine oxidase,
- uncoupled eNOS,
- cyclooxygenase,
- lipoxygenase,
- cytochrome P450 monooxygenase, and
- specific NADPH oxidase complexes.

In every cell type of the vessel wall, specific NAD(P)H oxidase complexes have been identified as major sources of O_2^- formation (36). In endothelial cells, a NADPH oxidase similar to the complex in granulocytes was initially shown to be a main source of O_2^- formation (34, 46, 81). This classical NAD(P)H oxidase complex in granulocytes and endothelial cells involves four essential subunits, membrane-bound subunits gp91phox and p22phox and initially cytosolic subunits p47phox and p67phox. After activation by phosphorylation of cytosolic subunits these subunits translocate from the cytosol to the membrane and form an active NADPH oxidase complex.

A crucial role in the complex plays the subunit gp91phox mediating the electron transfer from NADH/NADPH to oxygen.

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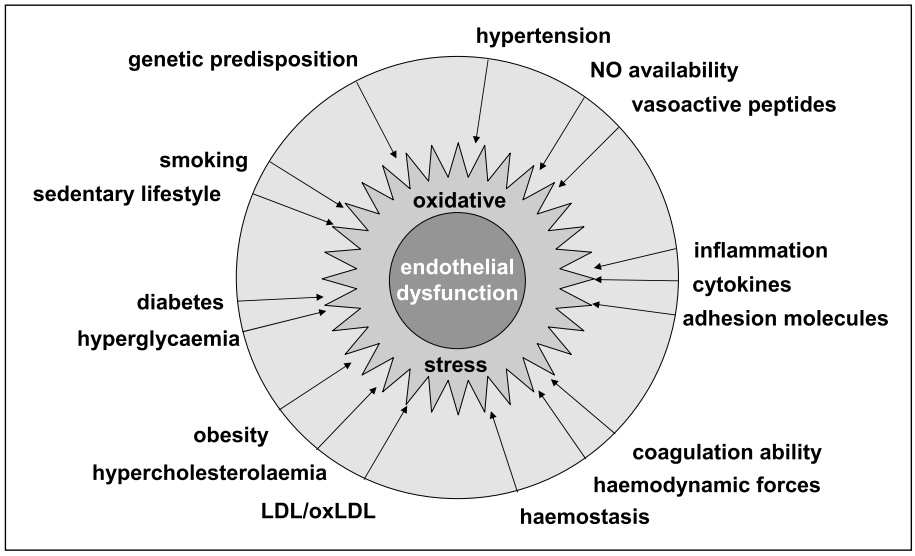

Fig. 1 Risk factors of oxidative stress and endothelial dysfunction.

Up to seven novel isoforms of gp91phox have been described in the preceeding five years and termed the Nox family of NADPH oxidase subunits (52, 53, 79). Recently, one of these novel NAD(P)H oxidase complexes containing Nox4 (29) and p22phox (5) have been described as a major source of O_2^- in endothelial cells (2, 86).

Superoxide anions can be converted into other ROS including hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^\bullet$). Reactive oxygen species play an important role as second messengers (18). In a healthy endothelium the cellular ROS formation can be balanced by antioxidative processes that include scavanging of ROS (i.e. by α-tocopherol, β-carotene, ascorbate, glutathione) or enzymatic degradation (i.e. by superoxide dismutases, catalase, and glutathione peroxidase). If this equilibrium is changed by increased ROS formation or by reduced antioxidative capacity augmented oxidative stress is formed. Oxidative stress has been described as risk factor in the pathogenesis of cardiovascular diseases and diabetes mellitus (9).

Influence on coagulation

There is a close relationship between ROS formation and the activation of the coagulation system during the pathogenesis of cardiovascular diseases. Due to injured endothelium or vascular disorders an activation of the intrinsic and/or extrinsic coagulation system or a decreased fibrinolytic activity can be found, resulting in a thrombotic phenotype in vascular pathology (6, 33). Quantification of coagulation factors and inhibitors can be useful tools in the determination of the individual risk (59). The involvement of intracellular NAD(P)H oxidase activity in platelet aggregation induced by collagen was shown by cell permeable superoxide dismutase (SOD) mimetics (11). Low flux extracellular superoxide can act as a procoagulatory stimulus by inducing endothelial NAD(P)H oxidase and tissue factor in human endothelial cells (44).

Tissue factor (TF) is thought to be the primary link between the coagulation and the vascular system because it is induced on the surface of vascular cells initiating the extrinsic clotting cascade leading to thrombin formation (41). The TF coagulant activity is suppressed by a nitric oxide-dependent pathway involving protein disulfide isomerase, linking the regulation of TF thrombogenicity to oxidative stress in the vasculature (3).

Initial reactions of blood coagulation can be blocked by the tissue factor pathway inhibitor (TFPI). The anticoagulant activity of TFPI is reduced by components of oxLDL. These inhibiting components were identified as oxidation products of δ-9 unsaturated phospholipids which impair the function of TFPI through specific association with its C-terminal basic region (73).

In an animal model of coronary artery occlusion and reperfusion ROS induced significant levels of TF-mRNA and procoagulant activity. These effects were abolished by NO and radical scavengers (30, 32). The enhanced thrombogenicity of the vasculature in pulmonary hypertension is partly induced by Rac-dependent binding of NFκB to a specific enhancer element in the TF promoter after thrombin induction (17).

Furthermore, the interaction between ROS and integrins during the process of blood coagulation represents a promising target for the therapeutic intervention in myocardial infarction or stroke-related thrombosis. This might involve $\alpha_{2b}\beta_3$ antagonists and HMG CoA reductase inhibitors (35).

In addition, acetylsalicylic acid directly affects neutrophils, erythrocytes, and platelets thus protecting the endothelium from oxidative stress and reducing endothelial dysfunction. In particular, it has antioxidant activity, enhances fibrinolysis, and suppresses plasma coagulation and platelet-dependent inhibition of thrombin formation (63). Acetylsalicylic acid reduced oxLDL-mediated lectin-like oxLDL receptor LOX-1 expression and superoxide anion generation in human coronary artery endothelial cells. It has also been shown to prevent hydrogen peroxide-induced caspase and NFκB activation in a dose-dependent manner through inhibition of phosphorylation and degradation of IκB (49).

These data support a link between increased formation of ROS and the coagulation cascade.

Effect on vascular function

It is known that raised levels of superoxide anions (O_2^-) or other biomarkers of oxidative stress in human vessels occurs in conjunction with endothelial dysfunction (12, 22). A comparative analysis of endothelial function and oxidative stress in patients with severe coronary artery disease (CAD) undergoing coronary artery bypass graft surgery and patients undergoing surgery for removal of varicose veins was recently per-

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formed (4). They showed a decreased relaxation and an increase superoxide production in saphenous veins of CAD patients compared to control patients. They described LDL cholesterol as a significant predictor of both endothelial dysfunction and oxidative stress. LDL cholesterol and oxidized LDL cholesterol can affect the trafficking of eNOS to the caveolae (83), the uncoupling of eNOS resulting in

- increased superoxide production (91) and
- the induction of NAD(P)H oxidase (81).

The loss of endothelium-derived nitric oxide (NO) is a hallmark of arterial dysfunction (47). The potent vasoconstrictor endothelin-1 (ET-1) has been shown to be more potent in veins than in arteries. The degree of desensitization of the contractile response is lower in veins than in arteries as well (23).

Rats treated with NO synthase inhibitor L-NNA showed increased oxidative stress but maintained the contractile function of ET-1. In this study, the vasoconstrictor efficiency was maintained in veins and reduced in arteries (89). Furthermore, Guzik et al. studied risk factors in arteries and the corresponding venous circulation (39). They identified a different superoxide production and expression of NAD(P)H oxidase subunits in veins and arteries.

Flow-dependent regulation of ROS

Endothelial cells in vivo are constantly exposed to shear stress by the flowing blood. Oscillatory shear stress induced the ROS generation in endothelial cells (15). The increased endothelial O_2^- formation in response to oscillatory shear stress involved a $p47^{phox}$-containing NAD(P)H oxidase complex (42, 43) and xanthine oxidase (62). Short-term application of pulsatile shear stress augmented O_2^- formation as well. An increased endothelial NO synthase (eNOS) expression has been shown by long-term shear stress in endothelial cells (72). Short-term and long-term endothelial NO formation by shear stress seems to involve different mechanisms. Shear stress-induced NO production of an endothelium-intact arterial segment, as assessed by changes in the tone of a preconstricted endothelium-denuded detector ring, was biphasic and consisted of an initial transient Ca^{2+}-dependent phase followed by a Ca^{2+}-independent plateau phase (7).

- The first phase represents a functional activation of eNOS,
- the second phase is accompanied by an upregulation of eNOS expression.

Shear stress-dependent upregulation of eNOS blocked activation of the caspase cascade in response to apoptosis-inducing exogenous oxygen radicals in endothelial cells (16). Therefore, a major vasoprotective mechanism of shear stress could be the formation of NO (24).

We recently showed a short-term induction, but a NO-dependent downregulation of superoxide anion formation during exposure to laminar shear stress in primary cultures of human endothelial cells (20). The downregulation of superoxide anion formation by long-term laminar shear stress support an increased flow-dependent NO availability in human endothelial cells as well.

The increased O_2^- generation under these conditions is most probably mediated by an activation of NADPH oxidase complexes with preformed subunits. In contrast, long-term exposures to shear stress downregulates NADPH oxidase subunit gp91phox in the same order of magnitude like the shear stress-dependent downregulation of O_2^- formation. NO synthase inhibitor L-NAME was not capable to affect the shear stress-dependent induction of O_2^- generation after 2h, but prevented downregulation of gp91phox expression and superoxide anion formation in response to long-term shear stress. This mechanism seems to involve an NO-dependent regulation of expression of subunits of the NAD(P)H oxidase complex.

The in vivo relevance of the downregulation of endothelial superoxide anion formation by long-term laminar shear stress observed in this study is supported by studies in porcine coronary arterioles (85). Furthermore, cessation of flow in flow-adapted rat or mouse aorta increased generation of reactive oxygen species (60). Increased blood flow in mice subjected to voluntary training reduced vascular superoxide release, Nox1 and $p47^{phox}$ expression (56).

Chronic exercise training of patients with coronary artery disease before coronary artery bypass grafting surgery increased flow and decreased generation of reactive oxygen species and expression of $gp91^{phox}$ in internal mammary arteries (1). Therefore, the flow-dependent regulation of oxidative stress might contribute to the regulation of endothelial NO/O_2^- balance and the antiatherosclerotic and vasoprotective potential of laminar shear stress.

Atherosclerosis

Oxidative stress has been implicated in the initiation and progression of hypertension and atherosclerosis (36). Increased superoxide generation by NADPH oxidase has been associated with endothelial dysfunction and clinical risk factors of atherosclerosis (38). Expression of NADPH oxidase subunits has been associated with the severity of atherosclerosis (84). Superoxide anion rapidly reacts with nitric oxide (NO) forming peroxynitrite. Since NO is an important mediator of endothelium-derived relaxation, a reduced NO availability by peroxynitrite formation results in endothelial dysfunction and development of atherosclerosis (25).

The impact of peroxynitrite on endothelial function is further potentiated by inhibition of the vasodilator prostacyclin (96). NO can mediate antiatherosclerotic effects. NO has been shown to inhibit (28, 48, 77)

- thrombocyte aggregation,
- endothelial adhesion molecule expression, and
- smooth muscle cell proliferation.

Chronic treatment with nitric oxide-releasing acetylsalicylic acid has been shown to reduce in hypercholesterolaemic animals (68)

- low-density lipoprotein oxidation,
- oxidative stress, and
- atherosclerosis.

The antiatherosclerotic effects of NO are diminished by inactivation with superoxide anions, too.

Another proatherosclerotic potential of augmented vascular O_2^- formation is the in-

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creased oxidative modification of low-density lipoprotein (LDL) (14). Oxidized LDL (oxLDL) contributes to the pathogenesis of atherosclerosis. It interferes with the endothelium-dependent relaxation by reducing expression of endothelial nitric oxide synthase (55). OxLDL induces chemotactic factors and expression of adhesion molecules and the expression of scavenger receptors on macrophages (13, 51, 94). OxLDL promotes infiltration of macrophages into the intima and unlimited uptake of oxLDL by these macrophages via scavenger receptors. This process leads to foam cell formation and the development of atherosclerotic plaques (93). Furthermore, oxLDL stimulates vascular smooth muscle cell proliferation (10). This intimal thickening further reduces the lumen of blood vessels leading to further potentiation of endothelial dysfunction, hypertension, and atherosclerosis.

Cardiac risk factors lead to the induction of endothelial dysfunction which induces the pathology of atherosclerosis, a chronic inflammatory disease (27). One major cause is the formation of reactive oxygen species which leads to an imbalance of intracellular oxidative stress and anti-oxidative acting enzymes. Increased circulation levels of native low-density lipoprotein (nLDL) can be oxidized by an oxidative stress to oxLDL. OxLDL itself has been described as a potent inducer of superoxide anions and therefore as a cause of oxidative stress. We showed an increased ROS generation in response to oxLDL in human endothelial cells. This induction of endothelial radical formation could be blocked by the novel Nox inhibitor VAS2870 (87).

Another mechanism might involve the regulation of the vascular tone by affecting the synthesis of vasoactive substances like endothelin. We showed that oxLDL induces (71)

- endothelin-converting enzyme-1,
- prepro-endothelin-1, and
- the release of endothelin-1 peptide (ET-1).

Furthermore, a transient induction of the endothelin receptor type B (ET_B) in response to nLDL and oxLDL was found in human endothelial cells (67). These data support interactions of the LDL-cholesterol and the endothelin system.

Angiotensin II, oxidative stress and endothelial dysfunction

Angiotensin II (Ang II) has been suggested to be involved in the development and progression of endothelial dysfunction and atherosclerosis (50). Ang II receptor type 1 (AT_1) inhibitors show antiatherosclerotic effects in primates (88). Clinical studies support a reversal of endothelial dysfunction in patients with coronary artery disease by AT_1 inhibitors (76). Furthermore, ACE inhibitor therapy improves the prognosis of patients with coronary artery disease (95).

Growing evidence support a link between Ang II and oxidative stress. Chronic infusion of Ang II results in hypertension, augmented O_2^- formation and endothelial dysfunction in experimental studies (78). Therefore, Ang II-stimulated increase in vascular O_2^- formation might contribute to the development of endothelial dysfunction and atherosclerosis (8).

We found a dose-dependent bimodal regulation of expression of the limiting NAD(P)H oxidase subunit $gp91^{phox}$ and of corresponding O_2^- formation by Ang II in human endothelial cells (80). Angiotensin II induces superoxide anion formation and $gp91^{phox}$ in a dose-dependent manner via AT_1. At higher Ang II concentrations, superoxide anion formation and $gp91^{phox}$ expression is partially inhibited by an AT_2 receptor-mediated mechanism. The finding that Ang II-infusion does not induce vascular NAD(P)H oxidase activity in $gp91^{phox}$ knockout mice (92) further support an essential role of $gp91^{phox}$. Thus, differential stimulation of Ang II receptor subtypes results in contrary effects on endothelial gp91phox expression and NAD(P)H oxidase activity, respectively. Since both receptor subtypes have been reported to have a similar affinity to Ang II (92), higher threshold of AT_2-mediated repression might result from a lower expression of AT_2 receptors compared to AT_1 receptors (57). Therefore, vessel-specific ratio of endothelial AT_1 and AT_2 receptors could determine gp91phox expression and NAD(P)H oxidase activity at a certain Ang II concentration.

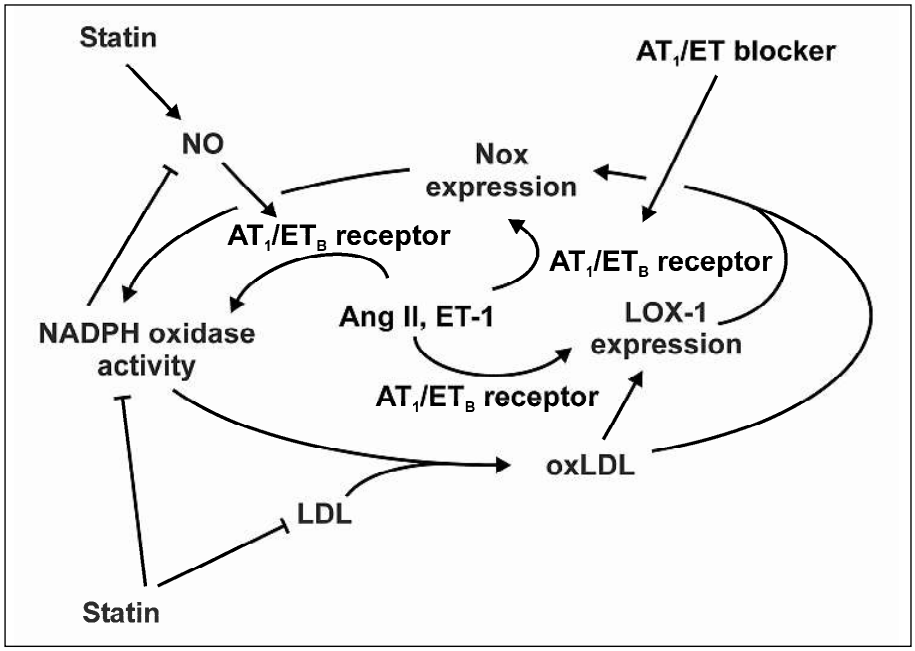

Fig. 2 Proatherosclerotic vicious cycle of locally increased angiotensin II (Ang II) and endothelin-1 (ET-1) levels, augmented oxidative stress, increasing oxidation of low-density lipoprotein (LDL) to oxidized LDL (oxLDL), augmented uptake of oxLDL by endothelial oxLDL receptor LOX-1 in response to Ang II and ET-1, and further potentiation of oxidative stress in response to oxLDL in the vessel wall.

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Vicious cycle

Our data suggest a vicious cycle of vascular O_2^- formation, oxidative modification of LDL, endothelial oxLDL uptake by LOX-1 and subsequent oxLDL-mediated induction of gp91phox expression (79). This vicious cycle can be potentiated by Ang II. Because the proatherosclerotic vasoconstrictor endothelin-1 (ET-1) induces NAD(P)H oxidase and oxLDL uptake in human endothelial cells as well (19, 64), ET-1 could further promote the proposed vicious cycle (Fig. 2).

Ang II might activate the NAD(P)H oxidase complex by PKC-dependent phosphorylation of subunit p47phox, thus increasing directly NAD(P)H oxidase activity. Since proatherosclerotic effects of Ang II are mediated by AT_1 receptors, additional mechanisms might be involved. AT_1 receptor expression has been induced by high levels of LDL in vitro and reduced by HMG CoA reductase inhibitor therapy in vivo (69, 70).

Therefore, LDL not only serves as a substrate for oxidative modification, but also potentiates Ang II-mediated effects by induction of AT_1 receptor expression in the proposed vicious cycle. In addition, NO was shown to repress AT_1 receptor expression (90). Since increased NAD(P)H oxidase-dependent O_2^- formation could additionally reduce NO availability, this mechanism could further promote proatherosclerotic effects of Ang II mediated by the AT_1 receptor. AT_1 and ET receptor blockers have the potential to interfere with this vicious cycle and reduce the risk of endothelial dysfunction and atherosclerosis.

Clinical implications

The NAD(P)H oxidase expression has been studied in internal mammary arteries of patients undergoing elective coronary artery bypass grafting (80). Preoperative treatment with low-dose ACE inhibitors had no effect on vascular gp91phox expression. In contrast, therapy with AT_1 receptor antagonists reduced expression of gp91phox. This blood pressure-independent effect could be due to the retrospective determined rather low doses of ACE inhibitors prescribed by the referring physicians. These data could be the consequence of the bimodal dose-dependent regulation of gp91phox by Ang II we described in vitro.

As a consequence, local Ang II concentration might be decreased below the threshold of AT_2 receptor-mediated repression but remains above the threshold level of AT_1 receptor-mediated induction of gp91phox expression in some patients. In patients receiving similar ACE inhibitor dosages, Ang II-induced expression of endothelial oxidized low-density lipoprotein (oxLDL) receptor LOX-1 was reduced in internal mammary arteries (65).

Therefore, prescribed ACE inhibitor dosage seems to be crucial in reducing proatherosclerotic oxidative stress and uptake of oxLDL. Higher doses of ACE inhibitors show beneficial effects in patients with heart failure (74). In a HOPE sub study (SECURE), ACE inhibitors dose-dependently reduced the progression of atherosclerosis (58). Treatment of patients with AT_1 receptor blockers improved endothelium-dependent relaxation (31, 82). AT_1 receptor blockade has an antiatherosclerotic and antioxidative potential by reduction of oxidative stress in the vessel wall.

In our recent EPAS (Endothelial Protection, AT_1 blockade and Cholesterol-Dependent Oxidative Stress) trial, we tested in a clinical trial in PROBE (Prospective Randomized Open Label and Blinded Evaluation) design whether statin and angiotensin type 1 (AT_1) receptor blocker therapies independently or in combination influence endothelial expression of anti- and proatherosclerotic genes and endothelial function in arteries of patients with coronary artery disease (66). Statin and AT_1 blocker therapy independently and in combination improved endothelial expression quotient of anti- and pro-atherosclerotic genes (including NADPH oxidase subunit and eNOS expression) and endothelial function. A potentiation by interaction of both therapies was not observed. These data support beneficial effects of both therapies in the treatment of coronary artery disease.

The use of dietary antioxidants in randomized clinical trials for the prevention of cardiovascular diseases is still contradictory (45). Therefore, further clinical studies are needed to substantiate the so-called oxidative hypothesis of endothelial dysfunction and atherosclerosis.

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Correspondence to:
Prof. Dr. Henning Morawietz
University of Technology, Medical Faculty Carl Gustav Carus
Department of Vascular Endothelium and Microcirculation,
Fetscherstr. 74, 01307 Dresden, Germany
Tel. +49/(0)3 51/4 58-66 25, Fax -63 54,
E-mail: Henning.Morawietz@tu-dresden.de

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