Nitroglycerin-Induced Endothelial Dysfunction and Tolerance Involve Adverse Phosphorylation and S-Glutathionylation of Endothelial Nitric Oxide Synthase

Beneficial Effects of Therapy With the AT₁ Receptor Blocker Telmisartan

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Objective—Continuous administration of nitroglycerin (GTN) causes tolerance and endothelial dysfunction by inducing reactive oxygen species (ROS) production from various enzymatic sources, such as mitochondria, NADPH oxidase, and an uncoupled endothelial nitric oxide synthase (eNOS). In the present study, we tested the effects of type 1 angiotensin (AT₁)-receptor blockade with telmisartan on GTN-induced endothelial dysfunction in particular on eNOS phosphorylation and S-glutathionylation sites and the eNOS cofactor synthesizing enzyme GTP–cytochrome I.

Methods and Results—Wistar rats were treated with telmisartan (2.7 or 8 mg/kg per day PO for 10 days) and with GTN (50 mg/kg per day SC for 3 days). Aortic eNOS phosphorylation and S-glutathionylation were assessed using antibodies against phospho-Thr495 and Ser1177 or protein-bound glutathione, which regulate eNOS activity and eNOS-dependent superoxide production (uncoupling). Expression of mitochondrial aldehyde dehydrogenase was determined by Western blotting. Formation of aortic and cardiac ROS was assessed by fluorescence, chemiluminescence, and 3-nitrotyrosine/malondialdehyde-positive protein content. Telmisartan prevented endothelial dysfunction and partially improved nitrate tolerance. Vascular, cardiac, mitochondrial, and white blood cell ROS formation were significantly increased by GTN treatment and inhibited by telmisartan. GTN-induced decrease in Ser1177, increase in Thr495 phosphorylation or S-glutathionylation of eNOS, and decrease in mitochondrial aldehyde dehydrogenase expression were normalized by telmisartan.

Conclusion—These data identify modification of eNOS phosphorylation as an important component of GTN-induced endothelial dysfunction. Via its pleiotropic “antioxidant” properties, telmisartan prevents, at least in part, GTN-induced oxidative stress, nitrate tolerance, and endothelial dysfunction. (Arterioscler Thromb Vasc Biol. 2011;31:2223-2231.)

Key Words: endothelial function ■ nitric oxide synthase ■ nitroglycerin ■ superoxide

Organic nitrates are still widely used in the therapy of patients with coronary artery disease; however, these drugs are also known to have side effects, such as tolerance development and induction of endothelial dysfunction in coronary and peripheral conductance and resistance vessels (for review, see1).

An early adaptation to long-term nitrate therapy involves neurohormonal adjustments, including activation of the renin-angiotensin system, retention of sodium and water, and volume redistribution, which concur to impair the vasodilator and preload reducing effects of the organic nitrates. Several studies have now consistently demonstrated that treatment with organic nitrates, such as nitroglycerin (GTN)2 and isosorbide-5-mononitrate,3 leads to development of nitrate tolerance and endothelial dysfunction via mechanisms that involve an increased vascular production of reactive oxygen species (ROS).

Modifications of several enzymatic systems, including activation of the vascular NADPH oxidase,4 uncoupling of the mitochondrial respiratory chain,5 and uncoupling of the endothelial nitric oxide synthase (eNOS),6 have been identified as potential sources of this increased ROS bioavailability. Interestingly, an inappropriate activation of the renin-angiotensin axis might play a role in all these phenomena, as angiotensin II is a potent activator of the NADPH oxidase in smooth muscle and endothelial cells and it is able to stimulate superoxide production in mitochondria and to cause eNOS uncoupling. On the basis of these considerations, it has been...
previously hypothesized that GTN treatment with a type 1 angiotensin (AT₁) receptor blocker might prevent, or at least ameliorate, the development of endothelial dysfunction and tolerance.

In agreement with this hypothesis, several clinical and preclinical studies have demonstrated that chronic AT₁ receptor blockade⁷ or treatment with angiotensin converting enzyme inhibitors⁸ prevented tolerance development and concomitantly prevented the activation of the NADPH oxidase and reduced oxidative stress.⁹ Although the existence of negative studies also needs to be acknowledged,¹⁰,¹¹ these data suggest that an inappropriate activation of the renin-angiotensin axis might play a key role in determining the vascular abnormalities observed in response to nitrate therapy.

More recently, data from our laboratory emphasized the role of mitochondrial oxidative stress and mitochondrial aldehyde dehydrogenase (ALDH-2) in GTN-induced nitrate tolerance⁵,¹²,¹³ and described the existence of cross-talk between mitochondrial ROS and NADPH oxidases,¹⁴ whereby GTN-induced mitochondrial ROS release would trigger NADPH oxidase activation and further ROS production from extramitochondrial sources. Within these studies, we identified tolerance and endothelial dysfunction as distinct phenomena, the former depending on mitochondrial processes and the latter being determined by NADPH oxidase activation and uncoupling of eNOS. Furthermore, we reported an inhibitory effect of GTN on the activity of ALDH-2,⁵ an enzyme with potent cardioprotective and antischismic properties, in particular during development of myocardial infarction.¹⁵,¹⁶

With the present study, we sought to further investigate the mechanisms underlying eNOS dysfunction in nitrate tolerance by quantifying eNOS phosphorylation at Ser1177, as a calcium-independent mechanism of eNOS activation and by quantifying eNOS phosphorylation at Thr495, as well as the S-glutathionylation at cysteines of the reductase domain, all of which have been proposed to be involved in eNOS uncoupling at the enzymatic level.¹⁷,¹⁸ We also sought to determine the effects of chronic GTN treatment on the expression of the GTP-cyclohydrolase I (GCH-I) and dihydrofolate reductase, 2 enzymes critically involved in the regulation of the intracellular levels of the eNOS cofactor tetrahydrobiopterin (BH₄), which is also of critical importance in the eNOS uncoupling process.¹⁹,²⁰ eNOS uncoupling or the capacity of the enzyme to produce superoxide was addressed by stimulation of tolerant vascular tissue with calcium ionophore as described.⁶ It is clinically important that we studied the impact of concomitant treatment with the AT₁ receptor blocker telmisartan on vascular superoxide production by eNOS, and in particular on superoxide production within the vascular wall and mitochondria, where the GTN bioactivation process takes place.

Materials and Methods

Animals and In Vivo Treatment

All animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the US National Institutes of Health, and approval was granted by the ethics committee of the University Medical Center Mainz. For experimental details, see the supplemental material, available online at http://atvb.ahajournals.org.

Isometric Tension Studies

Vasodilator responses to acetylcholine and GTN were assessed with endothelium-intact isolated rat aortic rings mounted for isometric tension recordings in organ chambers, as described previously.¹² The rat aorta was preconstricted with phenylephrine. In some experiments, vasoconstriction was tested by high calcium ionophore (A23187, >1 μmol/L) concentrations, and the effect of sepiapterin (100 μmol/L) and polyethylene-glycolated superoxide dismutase (100U/mL) preincubation of the vessels for 1 hour was assessed.²¹

Detection of Oxidative Stress in Blood, Cardiac Tissue, and Aorta; Western Blot; and Dot Blot Analysis

ROS measurement was performed according to published protocols.²¹–²³ Isolated aortic tissue was frozen and homogenized in liquid nitrogen. Proteins were separated by SDS-PAGE and blotted onto nitrocellulose membranes.²⁴ Cardiac and aortic oxidative stress was also assessed by dot blot analysis of homogenized tissues, modified from a previous report.²⁵ For experimental details, see the supplemental material.

eNOS Immunoprecipitation and S-Glutathionylation

M-280 sheep anti-rabbit IgG-coated beads from Invitrogen (Darmstadt, Germany) were used, along with a monoclonal mouse eNOS (Biosciences) antibody. The beads were loaded with the eNOS antibody and cross-linked according to the manufacturer’s instructions. Next, aortic or EA.hy 926 homogenates were incubated with the eNOS antibody beads, precipitated with a magnet, washed, transferred to gel, and subjected to SDS-PAGE followed by a standard Western blot procedure using a monoclonal mouse antibody against S-glutathionylated proteins from Virogen (Watertown, MA) at a dilution of 1:1000 under nonreducing conditions. Disappearance of the signal on incubation with 2-mercaptoethanol served as a control. After the membrane was stripped, the bands were stained for eNOS to allow normalization of the signals.

Cell Culture

Human endothelial EA.hy 926 cells²⁶ were grown in Dulbecco’s modified Eagle’s medium (Sigma) with 10% fetal calf serum, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 1× HAT (hypoxanthine, amethopterin/methotrexate, thymine) as previously described.²⁷ Experiments were performed in 6-well plates by adding GTN (10 μmol/L) plus telmisartan (0, 5, or 50 μmol/L) to the cells once daily for 3 subsequent days.

Statistical Analysis

For experimental details, see the supplemental material.

Results

Plasma Levels of Telmisartan

The successful treatment regimen was documented by a dose-dependent increase in telmisartan plasma levels (Supplemental Figure I).
Vascular Reactivity

GTN therapy led to endothelial dysfunction and nitrate tolerance (blunted acetylcholine and GTN response, Figure 1A) and to an inhibition of the activity of the cGMP-dependent kinase I (P-VASP, Figure 1B). Therapy with telmisartan almost completely corrected endothelial dysfunction (Figure 1A) and partially improved tolerance (Figure 1C). Importantly, AT1-receptor blockade prevented the downregulation of the GTN reductase (ALDH-2, D). The data are mean±SEM of 24 to 38 (ACH) or 24 to 40 (GTN) aortic rings from 10 to 12 animals/group. *P<0.05 vs control, #P<0.05 vs GTN, $P<0.05 vs T2.7.

Vascular and Cardiac Sources of Oxidative Stress

Vascular ROS formation as assessed by dihydroethidine staining was markedly increased in the setting of nitrate tolerance and completely normalized by telmisartan treatment (Supplemental Figure II). This GTN-induced vascular ROS formation was largely suppressed by the protein kinase C inhibitor chelerythrine, pointing to a role for vascular NADPH oxidases (Figure 2A). GTN-induced increases in mitochondrial superoxide production were greatly inhibited by therapy with telmisartan (Figure 2B).

Cardiac oxidative stress, as assessed by 3-nitrotyrosine and malondialdehyde staining and dot blot analysis, was substantially increased by GTN treatment and stepwise normalized by T2.7 and T8 therapy (Supplemental Figure III). Evidence for increased vascular oxidative stress in response to GTN therapy was further supported by increased aortic levels in 3-nitrotyrosine- and malondialdehyde-positive proteins in aortic homogenates (Figure 2C and 2D). GTN-induced increases in aortic 3-nitrotyrosine and malondialdehyde staining were greatly inhibited by therapy with telmisartan (Figure 2C and 2D).
The expression of the NADPH oxidase isoforms NADPH oxidase (Nox)-1 and NADPH oxidase (type 2, phagocytic [gp91phox] isoform) (Nox2) was not significantly modified by GTN treatment (Figure 2E and 2F). In contrast, therapy with telmisartan suppressed the expression of Nox1 and Nox2 below control levels (Figure 2E and 2F). A phorbol ester–induced oxidative burst of white blood cells, reflecting NADPH oxidase activity of inflammatory cells, was increased in the setting of nitrate tolerance and normalized by telmisartan treatment (Supplemental Figure IV).

**Effects of Telmisartan Treatment on GTN-Induced Deterioration of eNOS Function**

GTN therapy for 3 days decreased eNOS phosphorylation at Ser1177 (Figure 3A and Supplemental Figure V) and increased phosphorylation of eNOS at Thr495 (Figure 3B and Supplemental Figure V). Inhibition of eNOS Ser1177 phosphorylation and upregulation of Thr495 phosphorylation was prevented by telmisartan therapy. GTN treatment markedly depressed the expression of GCH-1, the important BH₄-synthesizing enzyme, a phenomenon that was reversed by AT₁-receptor blockade (Figure 3C). Reproducing previous observations in the setting of diabetes, we observed a compensatory increase in the BH₂-recycling enzyme dihydrofolate reductase (Figure 3D). Treatment with telmisartan normalized the expression of the dihydrofolate reductase. S-Glutathionylation at cysteines of the reductase domain represents another and novel regulatory pathway for eNOS function. Treatment of cultured endothelial cells with GTN resulted in an increase in glutathione binding to eNOS, which was significantly improved by concomitant telmisartan treatment (Figure 4A). Similar results were obtained under in vivo conditions, where chronic GTN infusion increased the glutathione-positive eNOS staining and telmisartan therapy normalized S-glutathionylation of eNOS (Figure 4B). 2-Mercaptoethanol completely abolished the signal by reductive replacement of eNOS-bound glutathione.

Functional consequences of these regulatory eNOS modifications were made obvious by an increased susceptibility of tolerant aorta to calcium ionophore-induced vasoconstriction...
Figure 5. Direct and indirect evidence for endothelial nitric oxide synthase (eNOS) uncoupling in tolerant rats and recoupling by telmisartan therapy. A and B, Vasoconstriction at high calcium ionophore (A23187) concentrations (A) and vasodilatory effects of sepiapterin plus superoxide dismutase under these conditions (B) were assessed by isometric tension recordings. C and D, Calcium ionophore-induced oxidative burst in whole blood (C) and effects of L-NAME on this reactive oxygen species (ROS) formation (D) were determined by 8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4-(2H,3H)dione sodium salt (L-012) enhanced chemiluminescence (ECL). E and F, Aortic ROS formation (E) and effects of L-NAME on this ROS formation (F) were measured by luminol/peroxidase ECL and dihydroethidine (DHE)-derived fluorescence microtopography, respectively. G, Three representative fluorescent stainings are shown for each condition. Ctr indicates control; GTN, nitroglycerin. The data are mean±SEM of aortic rings from 3 to 4 animals/group (A and B), blood samples from 2 to 3 animals/group (C and D), and aortic rings from 4 animals/group (E and F). *P<0.05 vs control, #P<0.05 vs GTN. EtOH indicates ethanol.
and more pronounced vasodilatory effects of sepiapterin pretreatment (probably by recoupling of the dysfunctional eNOS), effects that were normalized in the telmisartan group (Figure 5A and 5B). Calcium ionophore also increased the burden of oxidative stress in whole blood from tolerant rats to a higher extent as compared with the control and the telmisartan-cotreated groups, which, together with the distinct effects of the nitric oxide synthase inhibitor L-NAME, nitroarginine methyl ester points to uncoupled, superoxide-producing nitric oxide synthase in the setting of nitrate tolerance (Figure 5C and 5D). Similar observations were made for the effects of calcium ionophore on vascular ROS formation and effects of the nitric oxide synthase inhibitor L-NAME-nitroarginine methyl ester, supporting an uncoupling of eNOS in tolerant aortic tissue (Figure 5E and 5G).

**Discussion**

The present study provides substantial new insight to explain why the organic nitrate GTN causes endothelial dysfunction and nitrate tolerance. We demonstrate for the first time that GTN treatment downregulates the expression of the GCH-1, the most important enzyme in synthesizing BH4, the eNOS cofactor, which couples NO production to oxygen consumption. Likewise, increased eNOS S-glutathionylation, phosphorylation at Thr495, or decreased phosphorylation at Ser1177 may contribute to eNOS uncoupling and endothelial dysfunction. Nitrate tolerance was associated with a decreased expression of the ALDH-2, as well as increased ROS production within mitochondria, although a limitation of the present study was the use of photometric ROS detection methods (eg, chemiluminescence and fluorescence microscopy) instead of more reliable high-performance liquid chromatography–based or electron paramagnetic resonance-based techniques. Importantly, all of these GTN-induced negative side effects could be reversed by in vivo treatment with the AT1-receptor blocker telmisartan, suggesting an important role of the renin-angiotensin system in contributing to tolerance and endothelial dysfunction in response to chronic GTN treatment.

As a consequence of vasodilation, nitrate therapy has been shown to activate the circulating and local renin angiotensin system, which will compromise its vasodilator potency. A causal role of the renin-angiotensin system in nitrate tolerance has also been suggested from studies in patients and in experimental animals where concomitant treatment with an angiotensin converting enzyme inhibitors and AT1-receptor blockers were able to improve tolerance and to improve endothelial dysfunction, which was linked to a reduction of oxidative stress caused by the NADPH oxidase.

So far, we do not know to what extent other superoxide sources, such as an uncoupled eNOS or mitochondrial superoxide production induced by GTN treatment, are being modified by concomitant AT1-receptor blockade. In addition, because one of the major side effects of GTN therapy is endothelial dysfunction, we sought to investigate whether the eNOS phosphorylation and S-glutathionylation pattern, which largely determines eNOS function, is adversely influenced by GTN treatment and whether treatment with telmisartan is able to restore eNOS function by normalizing its phosphorylation/S-glutathionylation.

The results of the present study go along with our previous observation that GTN treatment causes tolerance and endothelial dysfunction, as evidenced by the significant shift to the right of the acetylcholine and GTN dose-response relationship in aortic rings (Figure 1). Tolerance and endothelial dysfunction was paralleled by a marked increase in vascular dihydroethidine staining and mitoSOX staining, compatible with increased ROS production within mitochondria, as shown before. In addition to increased production of ROS in mitochondria, we found that in vivo tolerance is associated with a significant downregulation of the expression of ALDH-2, the GTN bioactivating enzyme, which will contribute to tolerance to GTN as well.

Previously, by using ALDH-2 and NADPH oxidase knock-out animals, we could nicely dissect that tolerance is more a problem related to mitochondrial superoxide production, whereas GTN-induced endothelial dysfunction is more likely to involve the NADPH oxidase from vascular or inflammatory cells. With the present study, we can show that the expression of the NADPH oxidase Nox1 and Nox2 is not changed at all as described previously for the setting of nitrate tolerance, but that NADPH oxidase activity in inflammatory cells, as evidenced by the increased oxidative burst of whole blood in response to phorbol esters (direct activators of protein kinase C), was markedly stimulated by GTN treatment.

We can also provide substantially more mechanistic insight into the mechanism underlying endothelial dysfunction due to
changes of multisite phosphorylation/glutathionylation of eNOS (Figure 6).

1. Phosphorylation of the enzyme at Ser1177 was markedly inhibited by GTN treatment, indicating that Akt-dependent eNOS activation is greatly reduced and therefore the NO producing activity of the enzyme is reduced. Overall, Ser1177 appears to be the most important regulatory eNOS phosphorylation site, and prevention of Ser1177 phosphorylation reduces both basal and stimulated NO synthesis.17

2. We also observed an increase in eNOS phosphorylation at Thr495. The inhibitory effect of eNOS Thr495 phosphorylation on NO synthesis appears to be due to phosphorylation of this residue interfering with binding of Ca

\(^{2+}/\text{calmodulin}\) to eNOS. Interestingly, protein kinase C, which activates the NADPH oxidase, has also been shown to cause an increase in Thr495 phosphorylation and thereby impairs the coupling of L-arginine metabolism to efficient nitric oxide production.29 Thus, protein kinase C, which has been shown to be activated in endothelial cells in response to GTN treatment,20 can actually cause eNOS uncoupling via phosphorylation of eNOS at Thr495.

3. According to a recent report by Chen et al, eNOS is adversely regulated and uncoupled (leading to superoxide formation) by S-glutathionylation at 1 or more cysteine residues of the reductase domain.18 On the basis of our present observations, eNOS S-glutathionylation is largely increased in GTN-treated endothelial cells and aortic tissue from GTN-infused rats, probably contributing to eNOS uncoupling and endothelial dysfunction in the setting of nitrate tolerance.

4. Direct evidence for uncoupling of eNOS in response to GTN treatment is based on the combined effects of calcium ionophore-dependent vasoconstriction and relaxation by sepiapterin, a precursor of BH4, which has been reported to recouple a dysfunctional eNOS.21 Further support for this conclusion comes from the suppression of ROS formation by the nitric oxide synthase inhibitor L-N\(^{G}\)-nitroarginine methyl ester in whole blood and aorta from tolerant rats; differential effects of L-N\(^{G}\)-nitroarginine methyl ester (increase in ROS when eNOS is functional and decrease in ROS when eNOS is uncoupled) represent a direct read-out for the degree of eNOS uncoupling.21,23

In addition to the observed adverse changes in eNOS phosphorylation, the present study also demonstrated that chronic GTN treatment leads to a significant downregulation of the BH4-synthesizing enzyme GCH-1. This observation goes in parallel with previous findings where decreased expression of GCH-1 and eNOS uncoupling were observed in the setting of diabetes mellitus,23 with the possible explanation of proteosomal degradation of the GCH-1 on oxidative activation of the proteasome 26S.30,31 Likewise, we established an increase in the expression of dihydrofolate reductase, which may be interpreted as a compensatory but insufficient increase to restore intracellular BH4 levels and therefore to prevent eNOS uncoupling. Another mechanism of vascular dysfunction in tolerant tissue may be the desensitization of the soluble guanylyl cyclase by either oxidative inhibition or chronic "overload" with NO from nitrate therapy. However, a previous study by our group could not establish a diminished guanylyl cyclase expression or sodium nitroprusside-stimulated activity in response to GTN treatment.32 In the present study, the basal aortic levels of cGMP were somewhat decreased in the GTN treatment group and dose-dependently increased by telmisartan therapy (not shown).

With the present study, we can demonstrate that chronic treatment with telmisartan was able to normalize endothelial dysfunction, to partially improve nitrate tolerance, and to normalize hypersensitivity to vasoconstrictors. The improvement of endothelial dysfunction is likely due to a normalization of eNOS phosphorylation at Ser1177 and Thr495, as well as to S-glutathionylation and an increase in the expression of the GCH-1. Correction of endothelial dysfunction was associated with a marked reduction of oxidative stress in vascular tissue, as evidenced by the dihydroethidine staining and by a substantial reduction of vascular and myocardial nitrotyrosine positive and malondialdehyde positive proteins. Increased vascular nitrotyrosine staining (reflecting increased production of the NO and O\(^{2-}\) reaction product peroxynitrite) occurs in response to GTN treatment and has been shown to cause tyrosine nitration of the prostacyclin synthase, which in turn shuts down the formation of the important vasodilator prostacyclin.33

Telmisartan is highly efficient in decreasing this peroxynitrite formation, probably by decreasing superoxide formation and accordingly the diffusion-controlled reaction of superoxide and nitric oxide. This observation is in good agreement with previous findings in diabetic rats, where AT1-receptor blocker therapy with telmisartan prevented superoxide formation nitrination.23

An interesting and important observation was that AT1-receptor blockade was able to prevent the downregulation of the GTN bioactivating enzyme ALDH-2,34 in response to chronic treatment with the organic nitrate. In addition, we found that telmisartan was able to substantially reduce superoxide production within mitochondria. Because GTN-induced ROS production represents an important mechanism for the inhibition of GTN bioactivation,2 it is conceivable that the pleiotropic "antioxidant" effects of telmisartan represent an important mechanism to restore GTN sensitivity in nitrate-tolerant vascular tissue.5 The possibility cannot be excluded, however, that other beneficial side effects of telmisartan, such as peroxisome proliferator–activated receptor-\(\gamma\) agonist properties, are responsible for the beneficial profile of this drug in the setting of nitrate tolerance. Peroxisome proliferator–activated receptor-\(\gamma\) agonists have been reported to be highly effective in ameliorating oxidative stress in various disease states,35 and therefore this pleiotropic action of telmisartan could represent an additional antioxidant effect.

**Conclusion**

With the present studies, we discovered several new mechanisms by which GTN treatment may cause endothelial dysfunction, such as the induction of an abnormal phosphorylation/S-glutathionylation pattern leading to decreased NO and increased superoxide production by the enzyme. We also
established an additional mechanism, such as decreased expression of the GCH-1, the key enzyme in the synthesis of the eNOS cofactor BH4. Tolerance was associated with decreased expression of the ALDH-2, along with a marked increase in ROS production within mitochondria and a hypersensitivity to vasoconstrictors. Importantly, almost all of these GTN-induced vascular side effects were corrected by in vivo treatment with telmisartan, suggesting that a combination therapy consisting of the AT1-receptor blocker and GTN may be suited for long-term therapy of patients with coronary artery disease. Finally, it might be a limitation of the present study that healthy animals were used because organic nitrates, at least in the clinical situation, are prescribed to patients with cardiovascular complications. Based on previous studies, one may assume that the adverse effects of chronic GTN treatment may be even more pronounced in diseased individuals and animals because GTN treatment had synergistic adverse effects in hyperlipidemic rabbits (additive increase in 3-nitrotyrosine levels in the endothelium and subendothelial space) and, in a clinical study, synergistic increase in 3-nitrotyrosine levels in the endothelium and subendothelial space.36 And, in a clinical study, synergistically increased the DNA damage in diabetic and dyslipidemic coronary artery disease patients.37

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Supplement Material

Nitroglycerin-induced endothelial dysfunction and tolerance involves adverse phosphorylation and S-glutathionylation of endothelial nitric oxide synthase – beneficial effects of therapy with the AT\textsubscript{1}-receptor blocker telmisartan

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Figure I. Telmisartan plasma levels. Telmisartan content in plasma was determined by LC-MS analysis. BLQ means below limit of quantification. Data are mean±SEM from at least 5 samples.
Figure II. Effects of telmisartan treatment on vascular oxidative stress in tolerant rats. Quantification of vascular ROS formation in aortic cryo-sections by fluorescent microtopography using the superoxide-sensitive dye DHE (1µM). Below each densitometric quantification, 3 representative fluorescent stainings are shown. The data are mean ± SEM of 17-22 aortic rings from 10-12 animals/group. *, p<0.05 vs. control; #, p<0.05 vs. GTN.
Figure III. Effects of telmisartan treatment on cardiac and blood oxidative stress in tolerant rats. Dot blot analysis with a 3NT- (A) or MDA-specific (B) antibodies was used to assess 3-nitrotyrosine or malondialdehyde content in cardiac proteins. Representative dot blot results are shown below the densitometric quantification. The data are mean ± SEM of heart pieces from 8-12 animals/group. *, p<0.05 vs. control; †, p<0.05 vs. GTN; ‡, p<0.05 vs. T2.7.
Figure IV. Effects of telmisartan treatment on blood oxidative stress in tolerant rats.

Oxidative burst in phorbol ester (PDBu, 10µM)-stimulated whole blood was measured by L-012 ECL. The data are mean ± SEM of aortic rings or blood samples from 8-12 (L-012) animals/group. *, p<0.05 vs. control; #, p<0.05 vs. GTN.
Figure V. Effects of telmisartan treatment on serine1177 and threonine495 phosphorylation of eNOS. The content of Ser1177-phospho-eNOS (A) and Thr495-phospho-eNOS (B) were assessed by Western blotting and specific antibodies and normalized on the β-actin levels. Representative blots are shown at the bottom of each densitometric quantification along with the respective loading control. The data are mean ± SEM of aortic rings from 6-8 animals/group. *, p<0.05 vs. control; #, p<0.05 vs. GTN; $, p<0.05 vs. T2.7.
Extended Material and Methods

Materials

For isometric tension studies, GTN was used from a Nitrolingual infusion solution (1mg/ml) from G.Pohl-Boskamp (Hohenlockstedt, Germany). For induction of nitrate tolerance, GTN was used from an ethanol solution (100g/l) from Unikem (Copenhagen, Denmark). L-012 (8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4-(2H,3H)dione sodium salt) was purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals including DPPH• and dihydroethidium (DHE) were of analytical grade and were obtained from Sigma-Aldrich, Fluka or Merck.

Animals and in vivo treatment

All animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the U.S. National Institutes of Health and approval was granted by the Ethics Committee of the University Medical Center Mainz. A total number of 60 male Wistar Rats (6 weeks old, 250g, Charles River Laboratories, Sulzfeld, Germany) were divided into 4 treatment groups: solvent (ethanol) controls (Ctr), GTN (in tolerance-inducing doses) plus placebo (GTN), GTN plus telmisartan 2.7mg/kg/d p.o. (+T2.7) or GTN plus 8mg/kg/d p.o. (+T8) therapy. In some experiments we also tested telmisartan 0.9mg/kg/d p.o. (+T0.9) therapy. For induction of nitrate tolerance and endothelial dysfunction, rats were injected for 3 subsequent days with a daily dose of GTN (50mg/kg s.c., in ethanol). Control animals were injected with the solvent. Telmisartan treatment by special chow (ssniff Spezialdiäten GmbH, Soest, Germany) was started 7 days before GTN-injection and continued for 3 days. After 10 days of total treatment duration, animals were sacrificed under isoflurane anesthesia. The successful administration of telmisartan was verified by liquid chromatography and mass spectrometry (courtesy of Boehringer Ingelheim, Ridgefield, USA).
Detection of oxidative stress in serum, cardiac mitochondria, membrane fractions and aorta

The serum antioxidant capacity was measured according to a previously published protocol \(^1\). Formation of vascular reactive oxygen species was determined using dihydroethidine (DHE, 1µM)-dependent fluorescent microtopography in aortic cryo-sections as reported elsewhere \(^2\). In some experiments the vessels were preincubated with the protein kinase C (PKC) inhibitor chelerythrine (1µM) to assess the role of activation of PKC for vascular ROS formation. GTN-dependent induction of mitochondrial oxidative stress in isolated aorta was assessed by mitochondria-targeted dihydroethidine (mitoSOX, 1µM)-dependent fluorescent microtopography in aortic cryo-sections. The specificity of mitoSOX for mitochondrial ROS was previously demonstrated \(^3\).

Effects of GTN and telmisartan treatment on activation of white blood cells reflecting activity of the NADPH oxidase in inflammatory cells was assessed by measuring the oxidative burst triggered by phorbol ester (PDBu, 10µM) in whole blood (1:50 in PBS) by L-012-enhanced chemiluminescence \(^4\).

ROS formation was also tested in calcium ionophore (A23187, 10µM)-stimulated whole blood (L-012, 100µM), particulate fractions (amplex red (50µM)/peroxidase (0.1µM) plus 100µM NADPH) and aorta (luminol (200µM)/peroxidase (0.1µM)) in phosphate buffered saline containing 1mM Ca\(^{2+}\)/Mg\(^{2+}\). The effect of the NOS inhibitor L-NAME (200µM) was tested on the ROS formation in all of these samples as well as DHE-derived fluorescence microtopography in order to assess the eNOS coupling state \(^5\).

Western Blot analysis

Isolated aortic tissue was frozen and homogenized in liquid nitrogen. Proteins were separated by SDS-Page and blotted onto nitrocellulose membranes \(^6\). After blocking, immunoblotting was performed with the following antibodies: monoclonal mouse α-actinin (100 kDa) or polyclonal rabbit β-actin (42 kDa) (1:2500, Sigma-Aldrich) as controls for
loading and transfer, polyclonal goat Nox1 (1:100, Santa Cruz Biotechnologies, USA) and monoclonal mouse Nox2 (gp91phox, 1:1000, BD Transduction Lab., San Jose, CA, USA), monoclonal mouse eNOS (1:1000, BD Biosciences), polyclonal rabbit phospho-Ser1177-eNOS (1:1000, Upstate Biotechnology, Billerica, MA, USA), polyclonal rabbit phospho-Thr495-eNOS (1:2000, Upstate Biotechnology), mouse monoclonal phospho-Ser239-VASP (1.5µg/ml, Millipore, Billerica, MA, USA), polyclonal rabbit ALDH-2 (1:2000, kindly provided by K.K. Ho and H. Weiner, Purdue University, West Lafayette, USA), monoclonal mouse GCH-1 (1 µg/ml, Abnova Corp., Germany), and monoclonal mouse DHFR (1 µg/ml, RDI Div. of Fitzgerald Ind., USA). Detection and quantification were performed by ECL with peroxidase conjugated anti–rabbit/mouse (1:10000, Vector Lab., Burlingame, CA) and anti-goat (1:5000, Santa Cruz Biotechnologies, USA) secondary antibodies. Densitometric quantification of antibody-specific bands was performed with a ChemiLux Imager (CsX-1400M, Intas, Göttingen, Germany) and Gel-Pro Analyzer software (Media Cybernetics, Bethesda, MD).

**Dot blot analysis**

Cardiac and aortic oxidative stress was also assessed by dot blot analysis of homogenized tissues, modified from a previous report 7. Briefly, 100 µl (0.2 µg/µl protein based on Bradford analysis) of the homogenized tissue sample was transferred to a Protran BA85 (0.45µm) nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) by a Minifold I vacuum Dot-Blot system (Schleicher&Schuell, Dassel, Germany). Each slot was washed with 250µl PBS and the membrane was dried for 15min at 60°C. For detection of nitrated protein, a mouse monoclonal 3-nitrotyrosine (3NT) antibody (Upstate Biotechnology, MA, USA) was used at a dilution of 1:1000. For detection of malondialdehyde (MDA)-positive proteins, a polyclonal rabbit antibody against MDA-bound proteins (Calbiochem, Darmstadt, Germany) was used at a dilution of 1:2000. Positive bands were detected by
enhanced chemiluminescence after incubation with a peroxidase-coupled secondary antibody (GAM-POX, 1:5000) (Vector Laboratories, CA, USA). All incubation and washing steps were performed according to the manufacturer’s instructions. Densitometric quantification of the dots was performed as described in the Western blot section.

Statistical Analysis

Results are expressed as mean±SEM. Two-way ANOVA (with Bonferroni’s or Dunn’s correction for comparison of multiple means) was used for comparisons of vasodilator potency and efficacy. One-way ANOVA (with the same post-hoc tests) was used for comparisons of ROS detection by chemiluminescence or fluorescence as well as protein expression and antioxidant capacity. P values < 0.05 were considered significant.
REFERENCES


