Adverse Effects of Nitroglycerin Treatment on Endothelial Function, Vascular Nitrotyrosine Levels and cGMP-Dependent Protein Kinase Activity in Hyperlipidemic Watanabe Rabbits

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OBJECTIVES
With the present studies we sought to determine how treatment with nitroglycerin (NTG) affects endothelial function, oxidative stress and nitric oxide (NO)-downstream signaling in Watanabe heritable hyperlipidemic rabbits (WHHL).

BACKGROUND
In vitro experiments have demonstrated potent antiatherosclerotic effects of NO suggesting that treatment with NO-donors such as NTG could compensate for the diminished availability of endothelial NO. Nitric oxide may, however, not only be scavenged by reaction with endothelium-derived superoxide but also form the potent oxidant and inhibitor of vascular function, peroxynitrite (ONOO⁻).

METHODS
Watanabe heritable hyperlipidemic rabbits were treated for three days with NTG patches. Normolipidemic New Zealand White rabbits (NZWR) served as controls. Endothelial function was assessed ex vivo with organ chamber experiments and vascular superoxide was quantified using lucigenin (5 and 250 μM) and CLA-enhanced chemiluminescence. Vascular ONOO⁻ formation was determined using nitrotyrosine antibodies. The activity of the cGMP-dependent kinase (cGK-I) was assessed by determining the phosphorylation of vasodilator-stimulated phosphoprotein VASP (P-VASP).

RESULTS
Nitroglycerin treatment caused endothelial dysfunction in NZWR and WHHL, associated with an increase in superoxide and ONOO⁻ production and a substantial drop in cGK-I activity. In vivo NTG-treatment decreased lipophilic antioxidants (α- and β-carotene) in NZWR and WHHL. Treatment of NZWR with NTG also decreased plasma extracellular superoxide dismutase (EC-SOD)-activity.

CONCLUSIONS
Nitroglycerin treatment of WHHL with exogenous NO worsens rather than improves endothelial dysfunction secondary to increased formation of superoxide and/or peroxynitrite leading to decreased cGK-I activity. The decrease in plasma levels of α- and β-carotene may be at least in part due to a decrease in EC-SOD activity.

Coronary artery disease is associated with endothelial dysfunction, a phenomenon, which has been attributed to a decreased availability of the endothelium-derived relaxing factor, nitric oxide (NO). In vitro, NO has been shown to possess potent antiatherosclerotic properties. Thus, one may conceptualize that treatment with an exogenous source of NO (e.g., nitroglycerin [NTG]) could compensate for the diminished endothelial availability of NO in atherosclerosis, thereby preventing the consequences of endothelial dysfunction such as increased propensity of vasoconstriction and platelet activation. Nitric oxide, however, rapidly reacts with vascular cell derived superoxide (O₂⁻) leading to the formation of peroxynitrite (ONOO⁻), a potent oxidant and potential mediator of vascular injury (1). Therefore, despite potent antiatherosclerotic properties demonstrated in vitro, it remains to be established whether in the setting of increased endothelial O₂⁻ production such as hypercholesterolemia treatment of hypercholesterolemic animals with NO in vivo may have beneficial or deleterious effects with respect to the atherosclerotic process.

When given acutely, NTG has potent vasodilatory and platelet-inhibitory effects and is successfully used for the treatment of angina pectoris and patients with post-myocardial infarction angina. Chronic NTG treatment, however, is limited due to the rapid development of nitrate tolerance. Recently, we have defined a new mechanism partially responsible for tolerance and cross-tolerance to other endothelium-dependent and -independent vasodilators (2). Chronic NTG treatment of rabbits and rats led to tolerance and cross-tolerance development associated with increased O₂⁻ production due to activation of an NADH-driven oxidase (3) and due to NOS III uncoupling (4). Early stages of atherosclerosis are also associated with increased endothelial O₂⁻ production in hypercholesterolemic animals.
and in patients, a phenomenon which seems to be related, at least in part, to the activation of NADH-dependent oxidases (3) and an uncoupled NOS III (4). Hyperlipidemia as well as nitrate tolerance have been recently shown to be associated with decreased cyclic guanosine monophosphate-dependent protein kinase activity as indicated by marked decreases in the phosphorylation of vasodilator-stimulated phosphoprotein (P-VASP) (5–7). Reduction of oxidative stress by concomitant treatment of hyperlipidemic animals with AT1 receptor blockers (5) or of NTG-treated animals with vitamin C (6) resulted in a marked improvement in endothelial function, a restoration of P-VASP.

The aim of the present study therefore was to test whether in vivo treatment of hypercholesterolemic animals with NTG: 1) beneficially or detrimentally influence endothelial dysfunction, 2) beneficially or detrimentally may influence oxidative stress parameters in plasma such as the extracellular superoxide dismutase (EC-SOD) and antioxidant α- and β-carotene, and 3) how these changes may influence the activity of the cyclic guanosine monophosphate-dependent protein kinase-I (cGK-I).

### METHODS

#### Animal model and vessel preparation.
Forty male New Zealand White rabbits (NZWR) and 40 male Watanabe heritable hyperlipidemic (WHHL) rabbits (age three months) weighing 2 to 4 kg were studied. Four groups were formed: an untreated control group (no NTG, n = 20); control animals treated with transdermal NTG for three days (0.5 mg/h; for three days [2]; n = 20); a WHHL group (n = 20), and a WHHL group treated with transdermal NTG (n = 20). On the day of the study, blood samples were drawn for determination of plasma lipids and lipoprotein content. Aortic rings were suspended in individual organ chambers and relaxations to acetylcholine (ACh) and NTG were tested as described (2).

#### Oxidative fluorescent microtopography.
The oxidative fluorescent dye hydroethidine (2 × 10⁻⁶ M) was used to evaluate the in situ concentration of O₂⁻ as described recently (6).

#### Estimation of basal vascular superoxide production.
Relative rates of O₂⁻ production in intact vascular tissue were measured using lucigenin (5 µM [8] and 250 µM [2]) and CLA-derived chemiluminescence (9).

#### Determination of plasma EC-SOD activity.
The superoxide dismutase (SOD) enzymatic activity was determined with the direct spectrophotometric method employing KO₂ (10) the cyanide-sensitive isoenzymes CuZn-SOD and EC-SOD and the resistant Mn-SOD, 3 mM cyanide was used. To compensate for hemolysis during tapping and handling of the samples, the plasma hemoglobin contents were determined. This allowed calculation of CuZn-SOD released to plasma due to hemolysis using the CuZn-SOD content per mg hemoglobin of rabbit erythrocytes, and the results were subtracted from the measured plasma SOD activities. CuZn-SOD accounts for <4% of the plasma SOD activity in rabbits (11) and the cyanide-sensitive activities of the plasma samples were therefore assumed to derive from EC-SOD.

#### Immunohistochemistry.
Aortic sections (n = 4 from each group) were stained with a monoclonal antiserum to nitrotyrosine using a previously described method (12). The grades were assessed by two independent, blinded observers: Grade 0 = no staining, Grade 1 = focal staining (few scattered cells representing <25% of the total number of cells), Grade 2 = diffuse weak staining (most of the cells show weak staining), Grade 3 = diffuse moderate staining and Grade 4 = diffuse strong staining. In case of disagreement the average value of the grading was taken.

#### Detection of cGK-I expression, cGK-I activity and VASP serine239 phosphorylation (P-VASP).
Aortic segments from NZWR and WHHL with and without NTG treatment were frozen and homogenized in liquid nitrogen. SDS-PAGE electrophoresis and electroblotting was performed as described. Immunoblotting was performed with a polyclonal antibody against cGK-I and a mouse monoclonal antibody (16C2) specific for P-VASP, as described recently (5).

#### Analytical measurements in plasma.
Lipids were determined using enzymatic tests (Boehringer Mannheim, Mannheim, Germany). Lipophilic antioxidants such as α-carotene, β-carotene were measured as described (13).

#### Materials.
All chemicals were purchased from Sigma (Deisenhofen, Germany) or Merck (Darmstadt, Germany).

#### Statistical analysis.
Results are expressed as mean ± SEM. The ED₅₀ value for each experiment was obtained by logit transformation. To compare O₂⁻ production, cGK-I expression and P-VASP in normal and hyperlipidemic vessels with and without NTG-treatment, two-way analysis of variance was employed. To compare the EC-SOD levels from NZWR before and after NTG treatment a paired Student t-test was used. Comparisons of vascular responses were performed using multivariate analysis of variance. Scheffe post-hoc test was used to examine differences
between groups when significance was indicated. With respect to plasma parameters, group differences in variables were analyzed by a two-way analysis of variance. Spearman’s rank correlation coefficients were calculated to evaluate relationships between variables. The p values < 0.05 were considered statistically significant.

RESULTS

Effects of NTG treatment on vasodilator responses to ACh and NTG. Treatment of NZWR with NTG for three days caused a significant degree of endothelial dysfunction and worsened endothelial dysfunction in WHHL (Fig. 1A, Table 1). The degree of tolerance achieved with NTG was comparable in both animal groups (Fig. 1B, Table 1).

Effects of NTG treatment on hydroethidine staining in endothelial and smooth muscle cells of NZWR and WHHL. Hydroethidine staining in endothelial and in smooth muscle cells was higher in WHHL compared to controls. NTG treatment increased hydroethidine staining in NZWR as well as in WHHL (Fig. 2).

Table 1. Effects of NTG Treatment on Potency (ED\textsubscript{50}) and Efficacy (Maximal Relaxations) to Endogenous and Exogenous Nitrovasodilators in Aortas from Untreated and NTG-Treated Animals

<table>
<thead>
<tr>
<th>Group</th>
<th>Potency ED\textsubscript{50} (-logM)</th>
<th>Efficacy Max. Relaxation (%)</th>
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<tr>
<td></td>
<td>ACh</td>
<td>NTG</td>
</tr>
<tr>
<td>Control</td>
<td>7.33 ± 0.07</td>
<td>7.65 ± 0.10</td>
</tr>
<tr>
<td>Control + NTG</td>
<td>6.98 ± 0.05*</td>
<td>7.14 ± 0.13*</td>
</tr>
<tr>
<td>WHHL</td>
<td>7.08 ± 0.09</td>
<td>7.56 ± 0.04</td>
</tr>
<tr>
<td>WHHL + NTG</td>
<td>6.91 ± 0.10*</td>
<td>6.98 ± 0.11*†</td>
</tr>
</tbody>
</table>

\*p < 0.05 vs. control, †p < 0.05 vs. WHHL. ED\textsubscript{50} are concentrations, which produced 50% of maximal relaxation to each drug. Each value is the mean ± SEM of 10 to 12 experiments.

ACh = acetylcholine; NTG = nitroglycerin; WHHL = Watanabe heritable hyperlipidemic rabbits.

Effects of NTG treatment on lucigenin and CLA chemiluminescence. The Lucigenin-derived chemiluminescence (LDCL) was more than two-fold higher in WHHL and NTG-treated NZWR compared to control. Treatment of hyperlipidemic WHHL with NTG reduced the vascular LDCL (250 μM) to control levels (Fig. 3). A similar pattern was observed using lucigenin concentrations of 5 μM. Using CLA, however, we failed to detect a significant decrease in the chemiluminescence signals of aortas from WHHL treated with NTG.

Effects of NTG treatment on EC-SOD activity. Three day NTG treatment reduced the plasma EC-SOD activity significantly from 1,279 ± 39 U/ml to 1,129 ± 36 U/ml (p < 0.05).

Figure 1. Effects of three days nitroglycerin (NTG) treatment on acetylcholine (ACh) (A) and NTG concentration-response curve (B). Data are expressed as mean ± SEM of 8 to 10 experiments. *p < 0.05 versus control, †p < 0.05 versus WHHL. Black circles = control; black triangles = control + NTG; open circles = WHHL; open triangles = WHHL + NTG. WHHL = Watanabe heritable hyperlipidemic rabbits.

Figure 2. Effect of three days nitroglycerin (NTG) treatment on vascular superoxide in aortas from New Zealand white rabbits (NZWR) and Watanabe heritable hyperlipidemic rabbits (WHHL) as detected with hydroethidine. Vessels were labeled with the dye hydroethidine, which produces a red fluorescence when oxidized to ethidium bromide by superoxide. Data are representative for n = 4 experiments. A = adventitia; E = endothelium; M = media.
Effects of NTG treatment on vascular nitrotyrosine staining. Immunohistochemistry with a monoclonal antibody showed very low nitrotyrosine staining in vessels from NZWR, which was slightly but not significantly increased by in vivo treatment of NZWR with NTG. In vessels from WHHL we found a significant stronger nitrotyrosine staining, which was further increased by NTG treatment, especially in the endothelium and subendothelial space (Figs. 4 and 5).

Effects of NTG treatment on plasma lipid and lipophilic antioxidant levels. Plasma lipid levels were elevated in WHHL compared to control animals (Table 2). No difference in total lipids between WHHL rabbits and WHHL treated with NTG was found. Lipophilic antioxidants such as lipid-normalized α-carotene and β-carotene were significantly lower in WHHL compared to controls. NTG treatment caused a further decrease in α-carotene and β-carotene (Fig. 7).

DISCUSSION

The present study shows that early stages of atherosclerosis are characterized by endothelial dysfunction associated with increased vascular O$_2^-$ production. In vivo treatment of
WHHL with NTG increased rather than decreased oxidative stress and simultaneously worsened endothelial function. Accordingly we found that cGK-I activity as assessed with P-VASP was substantially reduced in aortas from WHHL treated with NTG. Treatment of WHHL with NTG also caused a marked increase in nitrotyrosine staining in the endothelium and the subendothelial space compatible with increased ONOO⁻ formation, a phenomenon, which was associated with a consumption of α- and β-carotene in rabbit plasma.

**Increased superoxide production in atherosclerosis.** Hypercholesterolemia has been shown to be associated with endothelial dysfunction in vivo. The mechanisms underlying this phenomenon are multifactorial but likely involve at least in part enhanced degradation of NO secondary to an activation of O₂⁻ producing enzymes such as the xanthine oxidase (14), the NAD(P)H-oxidase (15) and an uncoupled NOS III (5). Decreased vascular NO bioactivity secondary to increased endothelial production of reactive oxygen species may have important implications with respect to the progression of the atherosclerotic process. Nitric oxide has been shown to possess multifaceted antithrombotic properties. It inhibits platelet aggregation, prevents the expression of adhesion molecules by the endothelium, inhibits the adhesion of neutrophils to the endothelium via its ability to scavenge lipid peroxyl radicals and in turn inhibit NFkB-mediated gene expression of VCAM-1 and to a lesser extent ICAM. Further evidence for potential antithrombotic properties of NO was provided by experiments showing that inhibition of NOS III dramatically increases neointimal formation in cholesterol-fed rabbits (16). Thus, one may conceptualize that exogenous NO (e.g., via administration of organic nitrates) could compensate for the diminished endothelial net NO release in atherosclerosis.

**Figure 6.** Effects of nitroglycerin (NTG) treatment on the expression of the cGMP dependent protein kinase (cGK-I) and the vasodilatory stimulated phosphoprotein (P-VASP) in NZWR and WHHL. A, upper panel shows original blots of aortas from NZWR and WHHL with and without NTG treatment. B, the lower panel shows the densitometric quantification. Data are presented as mean ± SEM from 4 experiments *p < 0.05 versus control. Abbreviations as in Figure 2.

### Table 2. Lipid Profile of Controls and Hypercholesterolemic (WHHL) Rabbits With and Without NTG Treatment

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Control + NTG</th>
<th>WHHL</th>
<th>WHHL + NTG</th>
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<tbody>
<tr>
<td>Total cholesterol</td>
<td>91 ± 19</td>
<td>48 ± 8</td>
<td>577 ± 30*</td>
<td>574 ± 31*</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>122 ± 12</td>
<td>166 ± 24</td>
<td>333 ± 21*</td>
<td>291 ± 14*</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>23 ± 1</td>
<td>23 ± 6</td>
<td>13 ± 1*</td>
<td>13 ± 2*</td>
</tr>
</tbody>
</table>

Data are mean ± SEM of 8 to 12 samples. *p < 0.05 vs. controls. Abbreviations as in Table 1.
NO and O$_2^-$ ($\kappa = 6.7 \times 10^9$ M$^{-1}$s$^{-1}$) is about 3 times faster than the dismutation of O$_2^-$ to H$_2$O$_2$ by SOD. The reaction product between NO and O$_2^-$ not only lowers the steady state concentration of NO but at the same time yields ONOO$^-$, a product of potential pathophysiological significance. The ONOO$^-$ in high concentrations is cytotoxic and may cause oxidative damage to proteins, lipids and deoxyribonucleic acid. The ONOO$^-$ formation has been shown to cause endothelial dysfunction and nitration of tyrosine residues that may lead to a functional inhibition of the prostacyclin synthase (17). Indeed, with the present studies we provide evidence for increased vascular ONOO$^-$ formation by the demonstration of a broad nitrotyrosine band in vessels from WHHL treated with NTG. Interestingly nitrotyrosine formation was restricted to the endothelium and the subendothelial space.

There is a growing body of evidence showing that increased vascular ONOO$^-$ formation may also have deleterious consequences for the function of the NOS III by oxidizing the cofactor tetrahydrobiopterin (BH$_4$) to dihydrobiopterin (BH$_2$) (18). The resulting intracellular BH$_4$ deficiency may lead to an uncoupling of NOS III (18). Thus, NTG therapy may switch NOS III from a NO to a O$_2^-$ producing enzyme, which may further increase oxidative stress in vascular tissue in a positive feedback fashion (19). Indeed, an uncoupled NOS III has recently been demonstrated in an animal model of nitrate tolerance since an inhibitor of NOS III, NG-nitro-L-arginine was able to significantly reduce vascular O$_2^-$ in tolerant vessels (4). In addition, supplementation of NTG-treated rats with BH$_4$ was able to reverse NTG-induced endothelial dysfunction (20). Thus, the demonstration of endothelial dysfunction in our animal model goes parallel with data obtained from studies where NTG treatment of patients (21) and also healthy volunteers (22) was able to cause endothelial dysfunction. The concept of an uncoupled NOS in the setting of tolerance has been recently suggested by studies with chronically NTG-treated volunteers, where endothelial dysfunction but also nitrate tolerance was corrected by applying folic acid (22), a compound that restores NOS III function in vitro by increasing depleted intracellular BH$_4$ levels (23). Evidence for an uncoupled NOS III has been recently demonstrated also in two experimental models of atherosclerosis (5,18) as well as in hyperlipidemic patients (24). It is therefore tempting to speculate that a ONOO$^-$-induced endothelial dysfunction during NTG therapy is at least in part responsible for the worsening of endothelial function in both animal groups and that this is secondary to NOS III uncoupling.

In contrast to data obtained from the lucigenin assay, we found that the CLA-derived chemiluminescence signal from hyperlipidemic animals treated with NTG was not different from WHHL aortas without NTG treatment. This observation, however, is not surprising since CLA represents a chemiluminescent substance which may detect O$_2^-$ as well as ONOO$^-$ (25). Therefore, although the interaction between O$_2^-$ and NO decreases O$_2^-$ steady state levels, the simultaneously formed ONOO$^-$ may keep the CLA signal elevated.

**NTG therapy decreases the activity of the cGMP-dependent protein kinase in hyperlipidemic WHHL.** Studies with cGK-I deficient mice have recently been shown to cause a complete disruption of the NO/cGMP signaling pathway in the vascular smooth muscle (26) indicating that the activity and/or expression of cGK-I critically modulate NTG-induced vasorelaxation. To assess whether the expression of cGK-I was affected by hyperlipidemia and/or NTG treatment, we analyzed the expression of this protein by Western blotting. We could not detect any changes in cGK-I expression in aortas from NTG-treated NZWR or WHHL compared to the control group indicating that a down-regulation of cGK-I does not significantly contribute to tolerance and cross-tolerance in our model.
To assess the activity of cGK-I in intact aortic tissue from tolerant and non-tolerant animals, we studied the phosphorylation of the 46/50-kDa vasodilator-stimulated phosphoprotein (VASP) at serine239. The VASP is a well-characterized substrate for cGK-I and cAMP-dependent protein kinase (cAK) in platelets, endothelial and vascular smooth muscle cells. Activation of cGK-I in vascular tissue can be analyzed by specific monoclonal antibodies directed against the phospho-VASP form at serine239 (P-VASP) (5). In the present study, we found in vessels from WHHL a striking reduction in P-VASP as shown previously (5). Likewise, as shown before, treatment of animals and patients with NTG resulted in a marked decrease in vascular P-VASP (6,7) levels. Treatment of hypercholesterolemic animals with NTG almost completely abolished vascular P-VASP levels. The decrease in P-VASP induced by chronic NTG treatment was not due to decreased availability of this cGK-I substrate, since total VASP expression at the protein level was not different in tolerant and non-tolerant aorta. Recently we found that endothelial dysfunction in WHHL was associated with increased vascular O$_2^-$, as well as with decreased P-VASP. In this particular study, treatment of WHHL with the AT1 receptor blocker irbesartan improved endothelial dysfunction, reduced vascular O$_2^-$ and simultaneously increased vascular NO-bioavailability and increased P-VASP (5). These observations indicate again, that the level of vessel P-VASP closely follows changes in endothelial function and vascular oxidative stress. It is therefore reasonable to conclude that the decrease in P-VASP in control and WHHL treated with NTG is at least in part secondary to NTG-induced increases in reactive oxygen species within the vascular tissue.

**Effects of in vivo NTG treatment on plasma: α- and β-carotene.** In the present study we also found that plasma levels of two antioxidants, α- and β-carotene, were significantly decreased in WHHL after NTG-treatment. These data are in agreement with our recent finding that both α- and β-carotene are significantly lower in hyperlipidemic patients and patients with coronary artery disease than in healthy controls (27). Interestingly, Panasenko et al. (28) have shown that ONOO− can cause a marked loss of α- as well as β-carotene. These findings indicate that carotenoids can efficiently react with ONOO− and perform the role of scavengers of ONOO− in vivo. Therefore the consumption of α- as well as β-carotene in plasma may be a consequence of the observed increase in ONOO− formation within vascular tissue in response to chronic NTG treatment. In order to further differentiate between a direct effect of NTG on carotene levels and consumption of carotenes due to enhanced oxidative stress in plasma we examined the effect of in vivo NTG treatment on plasma EC-SOD activity. Interestingly, treatment of NZWR for three days resulted in a significant decrease in plasma EC-SOD. The reason for this finding is not clear, but may be related to NTG-induced formation of reactive oxygen species.

**Conclusions.** Taken together, the present studies demonstrate that in vivo treatment of hyperlipidemic animals with NTG increases rather than decreases reactive oxygen species in vessels and in plasma leading to nitrate tolerance, to a decrease cGK-I activity and to a strong increase in ONOO− formation within the endothelial cell layer. Thus, NTG-induced oxidative stress may explain, at least in part, why NTG-treatment worsens endothelial function as well as prognosis in patients with coronary artery disease.

**REFERENCES**


