Preserved Endothelial Function After Long-Term Eccentric Isosorbide Mononitrate Despite Moderate Nitrate Tolerance

Senta Müller, DVM,* Ute Laber, MD,* Jost Müllenheim, MD,† Wilfried Meyer, PhD,‡ Georg Kojda, PHARMd, PhD*
Duesseldorf and Hannover, Germany

OBJECTIVES
We sought to investigate the effects of orally administered, long-term, eccentric isosorbide mononitrate (ISMN) on endothelial function.

BACKGROUND
Previous studies have shown that nitrate tolerance induced by continuous transdermal glyceryl trinitrate (GTN) is associated with increased vascular superoxide production and endothelial dysfunction. In contrast, it is unclear whether vascular superoxide increases during eccentric administration of oral nitrates, which is a widely used therapeutic dosing regimen.

METHODS
New Zealand White rabbits were randomly classified into three groups (n = 11005, each) that received either placebo, ISMN at 2 mg/kg body weight per day (ISMN-2), or ISMN at 200 mg/kg body weight per day (ISMN-200) in an eccentric, twice-daily scheme for four months. Animals were sacrificed 3 h after application of the last ISMN dose.

RESULTS
The continuously present, lowest ISMN plasma levels (ng/ml) were 4.8 ± 0.2 in ISMN-2 and 14.5 ± 4 in ISMN-200 (p < 0.026). Treatment with ISMN had no effect on aortic reactivity to phenylephrine, acetylcholine, or the nitric oxide (NO) donor S-nitroso-N-acetyl-D,L-penicillamine, while the half-maximal effective concentration of ISMN (EC50-value in logM) was shifted from 5.23 ± 0.03 (placebo) to 4.69 ± 0.04 (ISMN-200) (p < 0.0001 by analysis of variance). This moderate in vivo nitrate tolerance was not associated with increased aortic superoxide production (5 µmol/l lucigenin). The cumulative (20-min) lucigenin signals (cpm/mg) were 211 ± 34 (ISMN-200) and 230 ± 22 (placebo) (p < 0.415).

CONCLUSIONS
Long-term treatment with high-dose, eccentric ISMN does not increase vascular superoxide production and/or impair endothelium-dependent vasorelaxation, despite the development of moderate nitrate tolerance. Thus, it is unlikely that long-term anti-ischemic treatment with ISMN aggravates endothelial dysfunction in coronary artery disease. (J Am Coll Cardiol 2003;41:1994–2000) © 2003 by the American College of Cardiology Foundation

Nitrate tolerance is a multifactorial phenomenon that also includes a so-called pseudotolerance that involves increased circulating levels of vasoconstrictors (1). Mechanisms most likely involved in true nitrate tolerance include impaired vascular bioactivation of nitrates, increased plasma volume, and neurohormonal counter-regulation leading to vascular supersensitivity to endogenous vasoconstrictors and to increased vascular superoxide production (2–6). These mechanisms reduce the generation and bioavailability of nitric oxide (NO), which is the pharmacologically active metabolite of organic nitrates. The increase of vascular superoxide production in nitrate tolerance (7) holds the potential to facilitate the pathogenesis of atherosclerosis, which is the major cause of coronary artery disease (8). Subsequently, careful reconsideration of the safety of long-term nitrate therapy has been proposed (9).

Earlier studies have shown that an eccentric dosing regimen, including a nitrate-free interval of 10 to 12 h, is a useful therapeutic approach to prevent the development of nitrate tolerance. This has been proven for glyceryl trinitrate (GTN) patches (10), standard-formulation isosorbide mononitrate (ISMN) (11,12), and sustained-formulation ISMN (13). It is unclear whether long-term nitrate treatment with such a dosing regimen increases vascular oxidative stress. Furthermore, our current understanding of the mechanism of nitrate tolerance is largely based on investigations with GTN (5). However, a recent clinical study suggested that the development of nitrate tolerance and increased free radical production might differ among nitrates (14). Other clinical and experimental investigations failed to demonstrate increased vascular oxidative stress in the setting of nitrate tolerance induced by GTN (15,16). Finally, it seems possible that a lack of vascular oxidative stress during standard nitrate therapy might initiate vasoprotective actions of nitrate-derived NO against atherosclerosis (e.g., by improving endothelial function) (17,18).

From the *Institut fuer Pharmakologie und Klinische Pharmakologie and †Institut fuer klinische Anaesthesiologie, Heinrich-Heine-Universitat, Duesseldorf; and ‡Institut fuer Anatomie, Tierarztliche Hochschule, Hannover, Germany.
Manuscript received April 15, 2002; revised manuscript received October 8, 2002, accepted November 11, 2002.
Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>ACh</td>
<td>acetylcholine</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>AoP</td>
<td>aortic pressure</td>
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<tr>
<td>C_{min}</td>
<td>minimal plasma concentration (17 h after dosing)</td>
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<tr>
<td>C_{max}</td>
<td>maximal plasma concentration (3 h after dosing)</td>
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<tr>
<td>EC_{50}</td>
<td>half-maximal effective concentration</td>
</tr>
<tr>
<td>GTN</td>
<td>glyceryl trinitrate</td>
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<tr>
<td>ISMN</td>
<td>isosorbide mononitrate</td>
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<tr>
<td>NO</td>
<td>nitric oxide</td>
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<tr>
<td>SNAP</td>
<td>S-nitroso-N-acetyl-D,L-penicillamine</td>
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According to these differential reports, we hypothesized that eccentric ISMN dosing that resembles the clinically used treatment might not be associated with unfavorable changes of vascular function, such as increased superoxide production. We sought to determine whether long-term treatment with eccentrically administered, high-dose ISMN induces nitrate tolerance, and whether this is associated with increased vascular superoxide production and endothelial dysfunction.

METHODS

Study animals. A total of 30 New Zealand White rabbits (10 to 12 weeks old; mean body weight 2,105 ± 47 g) were housed individually, as described previously (17). The rabbits were randomly classified into three groups of 10 animals and were fed a standard diet (control) and a diet supplemented with ISMN to achieve a daily dosage of ISMN at 2 mg/kg body weight per day (ISMN-2) or ISMN at 200 mg/kg body weight per day (ISMN-200) for 16 weeks. The dosage of ISMN was given in two identical portions in the morning (8:00 AM) and in the early afternoon (3:00 PM). Body weight was determined weekly, and the animals were supervised by a veterinarian. The animals were sacrificed 3 h after the last application of ISMN in the morning.

Permission for this study was provided by the regional government of Germany (AZ 23.05-230-3-77/99, AZ 23.05-230-3-52/99), and the experiments were performed according to the guidelines for the use of experimental animals, as given by “Deutsches Tierschutzgesetz,” and the “Guide for the Care and Use of Laboratory Animals” of the U.S. National Institutes of Health.

Vasorelaxation studies. Rabbits were anesthetized by injection of a mixture of xylazine (5 mg/kg) and ketamine (25 mg/kg) into the tibialis muscle. The animals were killed by exsanguination in deep anesthesia, and the entire thoracic and abdominal aorta was dissected free. Preparation of four thoracic ring segments and equilibration were performed in Krebs-Henseleit buffer, as described previously (17). The function of the endothelium was examined by cumulative addition of acetylcholine (ACh) (0.01 to 10 μmol/l) after submaximal pre-contraction with 0.2 μmol/l phenylephrine. This was followed by a cumulative application of phenylephrine (0.01 to 10 μmol/l). Thereafter, the aortic rings were divided into subgroups, and the vasorelaxations to different type of NO donors, such as S-nitroso-N-acetyl-D,L-penicillamine (SNAP) (1 nM to 10 μmol/l) and ISMN (10 nM to 1 mM), were studied by cumulative application after pre-contraction with phenylephrine (0.2 μmol/l). Potassium chloride, ACh, phenylephrine, and each NO donor was studied in the endothelium intact and in the endothelium-denuded thoracic rings from each animal.

Determination of aortic superoxide production. Aortic superoxide production was determined as described previously (19). Briefly, equilibrated segments of the thoracic aorta were incubated at 37°C in albumin buffer (pH 7.4) of the following composition (in mM): Na^+ 144.93, K^+ 7.23, Cl− 138.77, H2PO4− 4.55, HPO42− 8.03, glucose 5.55, and bovine serum albumin (0.1% weight/volume). This buffer was enriched with lucigenin (5 μmol/l), and superoxide production was calculated from chemiluminescence measurements (Packard Luminometer Analyzer, Picolite A6112, Packard, Downers Grove, Illinois).

Determination of plasma concentrations of ISMN. Plasma concentrations of ISMN were determined with support of ACC Gmbh, Leidersbach, Germany. Briefly, blood samples were taken by puncturing the middle ear artery with syringes containing heparin (15 U/ml) at 8:00 AM and 3 h after application of ISMN at 11:00 AM. Blood samples were rapidly centrifuged at 4°C and 1,000 g for 10 min. The supernatant was frozen at −20°C and stored until use. The ISMN was determined by gas chromatography/mass spectrometry (HP6890, Hewlett-Packard, Waldbronn, Germany) after liquid-liquid extraction with ethyl acetate.

Hemodynamic studies. We investigated a total of eight New Zealand White rabbits. The mean body weight was 2,952 ± 42 g; the age was 20 to 24 weeks. The rabbits were anesthetized by intravenous propofol (10 mg/kg) and were orally intubated (internal diameter 3.0 mm). Anesthesia was maintained by continuous infusion of piritramide (8 mg/kg per h) and midazolam (5 mg/kg per h). A continuous infusion of NaCl (0.9%) was initiated (4 ml/kg per h), and a 20-gauge Teflon catheter was advanced from the right carotid artery into the aortic arch and connected to a Statham transducer (PD23, Gould, Cleveland, Ohio) for PC-supported measurement of aortic pressure (AoP). Another 20-gauge Teflon catheter was inserted into the right jugular vein and used to accomplish the infusions. The ISMN (n = 5) was dissolved in isotonic NaCl, which was used for control experiments (n = 3). Drug treatment was started after 20 min of equilibration to steady-state conditions. Both ISMN and GTN were cumulatively given as a bolus into the central vein. Hemodynamic measurements were obtained during 5 min after injection. Animals were sacrificed at the end of the experiments by injecting 100 mg/kg thiopental.
RESULTS

Plasma levels of ISMN. We measured plasma levels of ISMN in blood samples before (C_min) and 3 h after application (C_max) of ISMN (Fig. 1). In the ISMN-2 group, the mean C_max value was only slightly higher than the C_min value (p > 0.05), whereas in the ISMN-200 group, there was a more than 100-fold difference (p < 0.001). Thus, even a low dose of eccentric ISMN results in detectable plasma levels at 14 h after application. The initially much higher peak concentration of ISMN in plasma of the ISMN-200 group drastically declined to a C_min value that was only three-fold greater than the C_min value in the ISMN-2 group.

The C_max value measured after intravenous application of ISMN at 1 mg/kg body weight per day was 9,651 ± 3,457 ng/ml (n = 5) and that of ISMN at 2.3 and 10 mg/kg body weight per day resulted in peak plasma concentrations of 53,075 ± 15,081 ng/ml and 293,337 ± 81,467 ng/ml (n = 5 each), respectively. Regression analysis showed that the plasma concentration linearly correlates with the dose (r = 0.7933, p < 0.004).

Induction of nitrate tolerance in vivo. As shown in Figure 2, the concentration response curve for ISMN is shifted to the right in the ISMN-200 group (p < 0.0001 by ANOVA), whereas the maximal relaxant response is maintained. The pD2 values (half-maximal effective concentration [EC50] in −logM) were 5.23 ± 0.03 for the control group, 5.22 ± 0.04 for ISMN-2, and 4.69 ± 0.04 for ISMN-200 (p < 0.001 vs. control). Removal of the endothelium significantly improved the ISMN response in all groups (p < 0.001 vs. intact endothelium for all groups). The pD2 values (EC50 in −logM) were 5.52 ± 0.03 for the control group, 5.53 ± 0.02 for ISMN-2, and 5.03 ± 0.04 for ISMN-200. In striking contrast, removal of the endothelium did not improve nitrate tolerance. There was a similar rightward shift in the concentration response curve for ISMN from the control to ISMN-200 group (p < 0.0001) and a significantly lower pD2 value in the ISMN-200 compared with control group (p < 0.001). These data show that long-term oral treatment with high doses of eccentrically administered ISMN induces nitrate tolerance in vivo, which is independent of the vascular endothelium.

Endothelium-dependent vasodilation. The maximal relaxant responses to ACh were 82.0 ± 3.1% in the control group, 85.7 ± 3.0% in ISMN-2, and 76.8 ± 3.8% in ISMN-200 (Fig. 3A). These responses to ACh are not significantly different (p > 0.05 by ANOVA). Likewise, the EC50 values for ACh were comparable among the different groups (data not shown). Thus, long-term treatment with
high doses of ISMN has no influence on endothelial function.

**NO-dependent vasodilation.** In all groups, a maximal vasodilation to the NO donor SNAP was observed (Fig. 3B). The pD₂ values (−logM) were 7.43 ± 0.03 (control group), 7.34 ± 0.04 (ISMN-2), and 7.24 ± 0.08 (ISMN-200) and were not significantly different (p > 0.05 by ANOVA). These data indicate that the aortic response to exogenous NO was not changed by ISMN treatment, not even in the setting of nitrate tolerance.

**Vasoconstriction to phenylephrine.** Maximal vasoconstrictions (Fig. 4) at 10 μmol/l phenylephrine were similar and amounted to 132.95 ± 5.36 mN (control group), 131.06 ± 5.21 mN (ISMN-2), and 144.25 ± 6.76 mN (ISMN-200) (p > 0.05 by ANOVA). A similar situation was found if the maximal vasoconstrictions to phenylephrine were related to the maximal vasoconstrictions to 80 mM KCl (data not shown). The EC₅₀ values (−logM) were also identical in the control group (6.29 ± 0.08), ISMN-2 group (6.45 ± 0.08), and ISMN-200 group (6.54 ± 0.08) (p > 0.05 by ANOVA). Thus, alpha-adrenergic vasoconstriction was not changed by long-term treatment with ISMN.

**Vascular superoxide production.** The maximal levels of the lucigenin signals shown in Figure 5 (in counts/mg aorta) were 229.72 ± 22.37 (control group), 168.44 ± 22.21 (ISMN-2), and 211.72 ± 34.34 (ISMN-200). There was no significant difference between the groups (p > 0.05 by ANOVA). These data show that long-term oral ISMN treatment does not increase vascular superoxide production, not even in the setting of moderate nitrate tolerance.

**Effect of increasing doses of ISMN on mean AoP.** In a separate set of experiments in rabbits, we found a significant reduction in mean AoP of 17.0 ± 3.9% (n = 5) at a dose of 1 mg/kg body weight per day of ISMN (Fig. 6). At this concentration, the heart rate was not changed. Increasing the dosage of ISMN resulted in a further decrease of mean AoP, which was associated with a significant increase in heart rate (p < 0.05 by ANOVA). As expected, GTN was much more potent than ISMN (approximately 1,000-fold), but produced a reduction of mean AoP to a similar extent.
Effect of ISMN on Endothelial Function

DISCUSSION

The new finding of this study is that long-term oral ISMN treatment given in an eccentric dosing regimen does not increase vascular superoxide production and has no effect on vascular responses to phenylephrine, ACh, and the NO donor SNAP. This holds true also in the setting of moderate nitrate tolerance, which was evident in the ISMN-200 group. These results suggest that long-term oral anti-ischemic pharmacotherapy of coronary artery disease with eccentrically administered ISMN is not associated with aggravation of endothelial dysfunction.

Recent investigations in rabbits treated with GTN patches continuously releasing the drug for three days showed a development of severe nitrate tolerance, where both endothelium-dependent and NO-induced vasorelaxations were markedly impaired. This was associated with other changes of vascular reactivity, including increased vascular superoxide production induced by angiotensin II-stimulated activation of reduced nicotinamide adenine dinucleotide/reduced nicotinamide adenine dinucleotide phosphate (21). Another source of vascular superoxide in nitrate tolerance might be endothelial NO synthase (22), although this has been questioned recently (23). Although the angiotensin receptor blocker losartan abolished vascular superoxide production and normalized endothelium-dependent vasodilation, it did not completely restore the vascular sensitivity to GTN (24). Our data indicate that oral, eccentric ISMN induces a more moderate form of nitrate tolerance than transdermal GTN, which is not associated with increased vascular superoxide production. This form of nitrate tolerance is characterized by a diminished vascular response to the organic nitrate ISMN, but not to endothelium-dependent or NO-induced vasodilation.

It is known that nitrate tolerance develops when constant plasma concentrations are maintained for more than a day (6). Thus, an eccentric dosing regimen with a daily nitrate-free interval of 10 to 12 h is recommended. In our study, the rabbits received an ISMN-enriched chow twice daily so that the intervals between nitrate application were 7 to 17 h. Nevertheless, this dosing regimen was associated with the development of nitrate tolerance in the high-dose group of ISMN-200. The daily dose in the ISMN-200 group is considerably higher than that used to treat coronary artery disease (6,25). Measurements of ISMN plasma concentrations shortly before administration of the morning dose in the ISMN-200 group showed values that were more than twofold higher than the peak ISMN plasma concentration in ISMN-2 (Fig. 1) measured 3 h after ISMN administration. Thus, there is a threshold constant plasma concentration in eccentric dosing regimens above which ISMN induces nitrate tolerance in rabbits. Constant plasma concentrations of ISMN in eccentric dosing regimens below this threshold are not associated with the development of nitrate tolerance, as indicated by the results obtained in the ISMN-2 group.

The moderate nitrate tolerance found in our study was not mediated by increased vascular superoxide production or a reduced vascular response to NO and is independent of the presence of vascular endothelium. Furthermore, we found that it is most likely not due to a smaller hypotensive effect of ISMN as compared with GTN (see subsequent text). This implies that other mechanisms, such as a reduced metabolic conversion of nitrates to NO and the respective denitrated metabolites, seem to be involved (2,26). This well-known hypothesis has been recently reinforced by investigations in animals and humans. Rats treated with GTN patches develop a moderate form of nitrate tolerance that is independent of the presence of vascular endothelium—the predominant source of superoxide in nitrate tolerance (16). Our results closely resemble those of a recent clinical study that provided evidence that a reduced bioactivation of GTN occurs in nitrate tolerance induced by short-term intravenous GTN in humans (27). Comparable to our data, Sage et al. (27) found a rightward shift in the nitrate dose-response curve, but no apparent reduction in maximally induced vasodilation. In addition, there was also no cross-tolerance to endothelium-dependent and endothelium-independent NO-induced vasodilations. Unfortunately, we did not investigate cross-tolerance to GTN. Its occurrence would have extended our observation by providing some evidence for a moderate form of tolerance to GTN, which is not associated with increased vascular oxidative stress.

As shown by studies in isolated coronary arteries, the reduction of nitrate bioactivation might be mediated by NO.
Tolerance Severity

Severe Nitrate Tolerance
(e.g. by continuous transdermal GTN)
strong impairment of vasodilation by nitrates
impairment of vasodilation by NO
endothelial dysfunction
increased vascular oxidative stress

Moderate Nitrate Tolerance
(e.g. by eccentric high-dose ISMN)
selective impairment of vasodilation by nitrates
normal endothelial function
lack of vascular oxidative stress

No Nitrate Tolerance
(e.g. by eccentric normal-dose Nitrates)
maintained vasodilation by nitrates
normal endothelial function
lack of vascular oxidative stress

Vascular Impairment

- activation of the renin-angiotensin system
- activation of vascular protein kinase C
- activation of endothelial NADPH-oxidase
- modulation of the NO-signal transduction

selective inhibition of enzymatic nitrate bioactivation
normal-vascular function

Figure 7. Chart of the hypothesis that nitrate tolerance is a dynamic event where increasing severity is mediated by strikingly different molecular mechanisms. Severe nitrate tolerance increases vascular oxidative stress and endothelial dysfunction, whereas moderate forms of nitrate tolerance seem to be restricted to a specific impairment of bioactivation of nitrates to nitric oxide (NO). GTN = glyceryl trinitrate; ISMN = isosorbide mononitrate; NADPH = reduced nicotinamide adenine dinucleotide phosphate.

(28) and thus represent a form of feedback inhibition. Interestingly, a very recent study identified the mitochondrial aldehyde dehydrogenase as an important enzyme for nitrate bioactivation and demonstrated its inhibition in the setting of in vitro nitrate tolerance (29). These findings further reinforce the view that nitrate tolerance is indeed at least partially mediated by impairment of nitrate bioactivation. Another mechanism of nitrate tolerance that does not involve increased vascular superoxide was suggested recently (30). This study provided evidence for an increased expression of the cyclic guanosine monophosphate-hydrolizing phosphodiesterase 1A1 in nitrate tolerance, whereas the cyclic guanosine monophosphate-hydrolizing phosphodiesterase V was not changed. As expected, tolerant rings also showed a reduced response to endogenous and exogenous NO. It is unlikely that this mechanism applies to our results, because both endothelium-dependent and NO-induced vasorelaxation was unchanged.

The rabbit model has been frequently used to investigate in vivo nitrate tolerance, and the results obtained in this model, in particular, the oxidant stress hypothesis of nitrate tolerance, has been confirmed in other experimental and clinical investigations where both increased superoxide production and effective prevention with vitamin C, tetrahydrobiopterin, and folic acid were demonstrated (27,31–34). Our investigation is the first to demonstrate that nitrate tolerance to high-dose, eccentric ISMN differs in some aspects from the severe form of nitrate tolerance that has been described for continuous, high-dose GTN (e.g., lack of increased vascular superoxide production and endothelial dysfunction). However, we cannot exclude the possibility that these vascular changes may occur in nitrate tolerance induced by a continuous, high-dose treatment regimen with ISMN.

It is well accepted that nitrate tolerance is a multifactorial phenomenon to which several different mechanisms contribute. In view of our results and those previously published, we suggest that nitrate tolerance is a dynamic event where increasing severity is mediated by strikingly different molecular mechanisms (Fig. 7). These differences are presumably important for patients receiving treatment with organic nitrates to prevent ischemic episodes, for their coronary endothelial function is already impaired. Recently reported data suggest that the risk of cardiovascular events in patients with coronary artery disease is dependent on the degree of endothelial dysfunction in these patients (35).

To estimate the hemodynamic efficacy of ISMN in our study, we measured ISMN plasma concentrations 3 min after intravenous ISMN injection in a group of instrumented rabbits whose AoP and heart rate were measured in parallel. There was a significant correlation between vasodilation and ISMN plasma concentration, suggesting that the plasma concentration of ISMN can predict the hemodynamic effects of ISMN in rabbits. Thus, we compared plasma concentrations after intravenous application and after oral application in the ISMN-2 and ISMN-200 groups. Three hours after oral administration, we found plasma ISMN concentrations in the ISMN-200 group that were approximately four-fold lower than those detected in rabbit plasma after injection of 1 mg/kg body weight ISMN. Thus, the effect of oral ISMN on blood pressure was presumably lower than the reduction in blood pressure after intravenous application of 1 mg/kg body weight ISMN (17 ± 3.9%) (Fig. 6). Nevertheless, our data suggest a blood
pressure-lowering effect of approximately 5% to 10% in the high-dose group of ISMN-200, a reduction that also occurs following therapeutic doses of transdermal GTN (6,14).

Taken together, we suggest that long-term anti-ischemic treatment with ISMN, even in doses inducing moderate nitrate tolerance, is unlikely to aggravate endothelial dysfunction in coronary artery disease. Our results also support the hypothesis that nitrate tolerance is a dynamic event where increasing severity is mediated by strikingly different molecular mechanisms. Severe nitrate tolerance, as caused by short-term, continuous, transdermal GTN, increases vascular oxidative stress and endothelial dysfunction, whereas moderate forms of nitrate tolerance, as initiated by long-term, eccentric, oral ISMN, seem to be restricted to a specific impairment of bioactivation of nitrates to NO.

Reprint requests and correspondence: Dr. Georg Kojda, Institut fuer Pharmakologie und Klinische Pharmakologie, Heinrich-Heine-Universitaet, Moorenstrasse 5, 40225 Duesseldorf, Germany. E-mail: kojda@uni-duesseldorf.de.

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