Chronic therapy with isosorbide-5-mononitrate causes endothelial dysfunction, oxidative stress, and a marked increase in vascular endothelin-1 expression

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Aims

Isosorbide-5-mononitrate (ISMN) is one of the most frequently used compounds in the treatment of coronary artery disease predominantly in the USA. However, ISMN was reported to induce endothelial dysfunction, which was corrected by vitamin C pointing to a crucial role of reactive oxygen species (ROS) in causing this phenomenon. We sought to elucidate the mechanism how ISMN causes endothelial dysfunction and oxidative stress in vascular tissue.

Methods and results

Male Wistar rats (n = 69 in total) were treated with ISMN (75 mg/kg/day) or placebo for 7 days. Endothelin (ET) expression was determined by immunohistochemistry in aortic sections. Isosorbide-5-mononitrate infusion caused significant endothelial dysfunction but no tolerance to ISMN itself, whereas ROS formation and nicotinamide adenine dinucleotidephosphate (NADPH) oxidase activity in the aorta, heart, and whole blood were increased. Isosorbide-5-mononitrate up-regulated the expression of NADPH subunits and caused uncoupling of the endothelial nitric oxide synthase (eNOS) likely due to a down-regulation of the tetrahydrobiopterin-synthesizing enzyme GTP-cyclohydrolase-1 and to S-glutathionylation of eNOS. The adverse effects of ISMN were improved in gp91phox knockout mice and normalized by bosentan in vivo/ex vivo treatment and suppressed by apocynin. In addition, a strong increase in the expression of ET within the endothelial cell layer and the adventitia was observed.

Conclusion

Chronic treatment with ISMN causes endothelial dysfunction and oxidative stress, predominantly by an ET-dependent activation of the vascular and phagocytic NADPH oxidase activity and NOS uncoupling. These findings may explain at least in part results from a retrospective analysis indicating increased mortality in post-infarct patients in response to long-term treatment with mononitrates.

Keywords

Organic nitrate therapy • Nitrate tolerance • Endothelial dysfunction • Reactive oxygen and nitrogen species • NADPH oxidase • S-glutathionylation of endothelial nitric oxide synthase • Endothelin-1 • Bosentan therapy
Introduction

Despite their potent anti-ischaemic effects when given acutely, the efficacy of organic nitrates is rapidly lost upon chronic administration due to the development of tolerance, i.e. the loss of haemodynamic effects that invariably occurs when nitrate therapy is protracted for longer as 12–14 h (for review, see Munzel et al.1,2). While organic nitrates have long been considered to have neutral implications beyond their haemodynamic effects, recent research points out that these drugs induce oxidative stress and endothelial dysfunction,3 which has been described and well characterized in response to treatment with nitroglycerin (GTN).4,5 These phenomena have also been attributed an important role in the development of nitrate tolerance. Beyond the activation of counterregulatory mechanisms such as neurohormonal activation, an increase in vasopressin levels, and signs for intravascular volume expansion,1,2 a number of specific abnormalities have been shown to occur in the setting of prolonged nitrate therapy. These abnormalities include the desensitization of the GTN-bioactivating enzyme ALDH-2, the desensitization of the soluble guanylyl cyclase,4 an increase in phosphodiesterase 1A1 activity,5 an increase in endothelin (ET) expression,6 and a stimulation of vascular production of reactive oxygen species (ROS)7 along with an inhibition of the mitochondrial ALDH-2.8,9 These phenomena contribute substantially to tolerance but also to endothelial dysfunction as observed in response to chronic treatment with GTN in experimental animals,6,7 and in humans.10 In 2010, Zweier and colleagues11 have proposed a new mechanism of endothelial nitric oxide synthase (eNOS) regulation that is based on S-glutathionylation of a cysteine in the reductase domain. Recently, we were able to detect S-glutathionylated eNOS in vascular cells/tissue from GTN-treated rats and endothelial cells.12 Notably, while GTN and other nitrates such as isosorbide-5-mononitrate (ISMN) and pentaerythrityl tetrinitrate have traditionally been considered to compose a homogeneous class, several important differences have been demonstrated across these different compounds. For instance, pentaerythrityl tetrinitrate has not been associated with either tolerance or endothelial dysfunction in both animal and human studies.13 In the USA, the most popular nitrate in addition to GTN is the ISMN. Animal experiments revealed that eccentric treatment with ISMN does not cause endothelial dysfunction and does not stimulate vascular superoxide production.14 These animal data contrast however with a recent human report demonstrating that ISMN treatment for 5 days causes endothelial dysfunction,15 an effect that was likely due to increased bioavailability of ROS, as it was reversed by the intra-arterial application of vitamin C.16 Based on this background, we investigated in a well-characterized animal model of nitrate tolerance whether chronic ISMN therapy causes endothelial dysfunction, stimulates vascular ROS production, and uncouples eNOS by tetrahydrobiopterin (BH4)- and/or S-glutathionylation-dependent mechanisms. In this context, we used nicotine-induced adenine dinucleotide phosphate (NADPH) oxidase (Nox2)-deficient mice to define its role in ISMN-mediated endothelial dysfunction. In addition, since GTN therapy has been demonstrated to cause autocrine stimulation of vascular ET production,6 we studied whether changes in ET bioavailability also occur in response to chronic ISMN treatment.

Methods

A more detailed description of the Methods section is provided in the Supplementary material online.

Materials

For isometric tension studies, GTN was used from a Nitrolingual infusion solution (1 mg/mL) from G.Pohl-Boskamp (Hohenlockstedt, Germany). All other chemical ingredients including 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH) and ISMN 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 US National Institutes of Health and approval was granted by the Ethics Committee of the University Hospital Mainz. Wistar rats or mice were anaesthetized by isoflurane, and a subcutaneous osmotic minipump (model 2001 for rat and model 1007D for mice, ALZET, Cupertino, USA) containing ISMN (75 mg/kg/day), bosentan (80 mg/kg/day), or the vehicle alone (dimethyl sulfoxide) was implanted as described recently.16 After 7 days of treatment, rats were killed by exsanguination in isoflurane anaesthesia, and the blood, aorta, and heart were collected.

Isoameric tension studies

Concentration–relaxation curves and concentration–constriction curves in response to increasing concentrations of ISMN, acetylcholine (ACh), and GTN or KCl, phenylephrine (Phe), and angiotensin-II (AT-II) were performed as described.6,17

Reactive oxygen species formation

Reactive oxygen species formation was measured by oxidative burst of leucocytes in whole blood or NADPH oxidase activity in the heart and aorta by ECL.18–21 For fluorescence (dihydroethidine (DHE), 1 μM) oxidative microtopography, isolated aortic rings were OCT-embedded (Tissue Tek, USA) and stained with DHE (1 μM) with or without L-NG-nitroarginine methyl ester (l-NAME) or apocynin as reported.16,19–22 Antioxidant capacity in serum was measured using a DPPH assay.21

Western blot analysis

Western blot analysis was performed as described previously.16,19–21

Endothelial nitric oxide synthase 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, USA) antibody as described.15 The beads were loaded with the eNOS antibody and cross-linked according to the manufacturer’s instructions. Next, the aortic homogenates were incubated with the eNOS antibody beads, precipitated with a magnet, washed and transferred to gel, and subjected to SDS–PAGE followed by a standard western blot procedure using a monoclonal mouse antibody against glutathionylated proteins from Virogen (Watertown, MA, USA) at a dilution of 1:1000 under non-reducing conditions.

Immunohistochemistry

The immunohistochemistry protocol was published previously.23 Aortic segments were OCT-embedded (Tissue Tek, USA) and frozen in liquid nitrogen [for anti-ET-1, 1:500, Meridian Life Science, USA] or fixed in paraformaldehyde (4%), paraffin-embedded [for
anti-big ET-1 (big ET-1), 1:10, IBL Co., Japan], and stained with primary antibodies; following the species of primary antibodies, appropriate biotinylated secondary antibodies were used after dilution following the manufacturer’s instructions. For immunochemical detection, ABC reagent (Vector) and then DAB (peroxidase substrate Kit, Vector) reagent as substrates were used.

**Statistical analysis**

Results are expressed as mean ± SEM. One-way ANOVA (with Bonferroni’s or Dunn’s correction for comparison of multiple means) was used for comparisons of vasodilator potency and efficacy (see Supplementary material online, Tables S1 and S2), ROS detection by chemiluminescence or fluorescence as well as protein expression and antioxidant capacity based on the SigmaStat 3.5 Software. The EC50 value for each experiment was obtained by log-transformation. The isometric tension data were also analysed using two-way ANOVA with Bonferroni’s post hoc test using GraphPad Prism 5 Software. Haem oxygenase-1 promoter activity data were analysed using GraphPad Prism 5 Software and represent means ± SEM. Statistical differences were determined either by t-tests or by factorial analysis of variance (ANOVA) followed by Tukey’s multiple comparison test for comparison of multiple means. P-values <0.05 were considered significant. All used tests were two-sided.

**Results**

**Effects of isosorbide-5-mononitrate on endothelial function**

Treatment with ISMN for 7 days did not modify the responsiveness to ISMN, demonstrating an absence of tolerance to this organic nitrate (Figure 1A for rat; see Supplementary material online, Figure S1, for mouse). In contrast, a marked shift of the dose–response relationship to the endothelium-dependent vasodilator ACh was observed, compatible with endothelial dysfunction (Figure 1B). Likewise, a small but significant degree of GTN tolerance was established, indicated by the significant decrease in ED50 and maximal relaxation to GTN (Figure 1C; see Supplementary material online, Table S1). Constriction in response to KCl, Phe, and AT-II was significantly increased in the ISMN group (Figure 1D–F; see Supplementary material online, Table S2).

**Effects of isosorbide-5-mononitrate treatment on total serum antioxidant capacity and superoxide production in inflammatory cells**

Isosorbide-5-mononitrate treatment significantly decreased serum antioxidant capacity by ~50% (P < 0.05) and markedly stimulated the superoxide signal of whole blood in the presence of NADPH (200 μM). This assay mainly reflects superoxide production in inflammatory cells such as neutrophils and monocytes and involves the enzymatic source phagocytic NADPH oxidase (Figure 2A and B) since the signal is almost absent in whole blood from gp91phox knockout mice. Isosorbide-5-mononitrate and ET-1-stimulated ROS formation in whole blood and isolated neutrophils, which was suppressed by co-incubation with the ET receptor antagonist bosentan (Figure 2C and D).

**Effects of isosorbide-5-mononitrate treatment on myocardial and vascular nicotinamide adenine dinucleotidetriphosphate oxidase activity and expression**

Isosorbide-5-mononitrate treatment substantially increased NADPH oxidase-driven superoxide production in membrane preparations from the heart (Figure 3A) and the aorta (Figure 3B). Likewise, the increased DHE staining throughout the vasculature was substantially reduced by the inhibitor of the NADPH oxidase apocy- nin (Figure 3C and D), compatible with an activation of the NADPH oxidase in response to ISMN treatment. Accordingly, we found an increase in the expression of Nox1, Nox2, and Nox4 (see Supplementary material online, Figure S2A–C). The observed increase, however, was rather moderate. The increase in Nox4 expression was also observed in cultured endothelial cells [human umbilical vein endothelial cells (HUVECs)] in response to ISMN (see Supplementary material online, Figure S3). As a proof of concept for the role of NADPH oxidase in causing endothelial dysfunction, we treated gp91phox (the phago- cytic isoform of NADPH oxidase, also termed Nox2)-deficient mice with ISMN, observed accordingly a normalization of the activa- tion of leucocytes (oxidative burst in whole blood), and decreased serum antioxidant capacity, endothelial dysfunction (ACh-elicited relaxation), and membranous NADPH oxidase activity (Figure 4A–E).

**Effects of isosorbide-5-mononitrate treatment on the nitric oxide/cyclic guanosine monophosphate signalling pathway**

Isosorbide-5-mononitrate treatment significantly increased superoxide production by the endothelium. This increase was substan- tially decreased by using the eNOS inhibitor L-NAME compatible with eNOS uncoupling in vessels from ISMN-treated animals (Figure 5A and B). Isosorbide-5-mononitrate treatment significantly reduced the expression of the BH4-synthesizing enzyme GTP-cyclohydrolase-1 (GCH-1; Figure 5C). Endothelial nitric oxide synthesis 5-glutathionylation, which contributes to eNOS dysfunction/ uncoupling, was significantly increased by 43% in ISMN-treated tissue (Figure 5D). Isosorbide-5-mononitrate treatment decreased the activity of the cGK-I [assessed as phosphorylation of the vasodilator-stimulated phosphoprotein (VASP) at serine 239] (Figure 5E). The incubation of vessels from ISMN-treated animals with a polyethylene glycolated Cu,Zn superoxide dismutase (PEG-SOD) normalized the ACh–dose relationship (Figure 5F).

**Role for endothelin in isosorbide-5-mononitrate-induced endothelial dysfunction**

Isosorbide-5-mononitrate treatment substantially increased the expression of big ET-1 (Figure 6A) and ET-1 (Figure 6B) in the...
endothelial and adventitial cell layer. The increase in prepro-ET expression was also observed in cultured endothelial cells (HUVECs) in response to ISMN (see Supplementary material online, Figure S3).

Bosentan, the non-selective ET receptor blocker treatment normalized endothelial dysfunction in isolated aortic ring segments from ISMN in vivo treated rats ex vivo (Figure 7A), which went along with the suppression of ET-1-triggered ROS formation by bosentan in whole blood (Figure 2C). ET-1 and ISMN in vitro incubations also activated ROS formation in isolated neutrophils (Figure 2D). Bosentan in vivo therapy completely abolished endothelial dysfunction in the ISMN treatment group and improved ACh-dependent vasodilation in the control group (Figure 7B). As expected, ET-1-dependent

Figure 1 Effects of in vivo isosorbide-5-mononitrate (ISMN, 75 mg/kg/day for 7 days) therapy on vascular reactivity. Vascular sensitivity to isosorbide-5-mononitrate (A), the endothelium-dependent vasodilator acetylcholine (ACh, B), and nitroglycerin (GTN, C) as well as to the constrictors KCl (D), phenylephrine (Phe, E), and AT-II (F) were tested. Isosorbide-5-mononitrate treatment caused a substantial shift of the dose–response curve to acetylcholine to the right compatible with endothelial dysfunction. The sensitivity to vasoconstrictors was increased in the isosorbide-5-mononitrate group. Data are mean ± SEM from at least 6–12 independent experiments. The statistics were on the basis of one-way ANOVA comparison of EC50 values (half maximal relaxation/constriction) and efficacies (maximal relaxation/constriction) (see also Supplementary material online, Tables S1 and S2); *EC50: P < 0.05 vs. Ctr; †% Max.Rel. or Max.Con.: P < 0.05 vs. Ctr.
vasoconstriction was significantly shifted at least to three-fold higher concentrations by bosentan \textit{in vivo} therapy (Figure 7C). Isosorbide-5-mononitrate treatment increased leucocyte-dependent oxidative burst in whole blood compatible with an activation of superoxide-producing enzymes such as the nicotinamide adenine dinucleotidephosphate oxidase in neutrophils and monocytes. Effects of endothelin-1 and isosorbide-5-mononitrate in \textit{vitro} challenges on leucocyte-derived reactive oxygen species in whole blood (C) and isolated neutrophils (D). Data are mean ± SEM from six (A and B) and eight (C) independent experiments. *P < 0.05 vs. Ctr; °P < 0.05 vs. endothelin-1 or isosorbide-5-mononitrate treatment groups.

\section*{Discussion}

\subsection*{Differences across different organic nitrates}

The finding of the importance of NO as a mediator of endothelial function leads to the speculation that by providing an exogenous source of this free radical, organic nitrates might represent a therapy for endothelial dysfunction. Several flaws have been identified in this hypothesis. First of all, the discovery that endothelial dysfunction is caused by increased production of ROS such as superoxide$^{25}$ implies that by adding NO, the formation of the NO/superoxide reaction product peroxynitrite is favoured,$^{26}$ resulting in the increased bioavailability of a highly reactive intermediate with negative effects on eNOS,$^{27}$ prostacyclin synthase function$^{28}$ and NO signalling.$^{29}$ It is also likely that the induction of ROS production within tolerant tissue stimulates the expression of one of the most potent vasoconstrictors known, i.e. ET-1,$^{30,31}$ a phenomenon which has been observed in response to chronic GTN treatment.$^6$

Other more recent findings by our group and others revealed that rather than being ‘simple’ NO donors, organic nitrates are a
quite heterogeneous group of compounds. For instance, GTN as well as pentaerythrithyl tetranitrate (PETN) undergo mitochondrial bioactivation by the ALDH-2, a redox-sensitive enzyme. Chronic treatment with GTN has been shown to stimulate superoxide production within mitochondria and therefore causes an oxidative stress-mediated inhibition and down-regulation of the enzyme, thereby concurring to tolerance. A cross-talk mechanism between mitochondria and the vascular NADPH oxidase has been shown to be responsible for the development of endothelial dysfunction in response to GTN treatment.

Although less is known regarding the bioactivation of other organic nitrates such as ISMN and its dinitrate ISDN, the cytochrome P450-dependent mechanisms within the endoplasmatic reticulum has been proposed as a possible enzyme. It also seems conceivable that vascular relaxations in response to ISDN and ISMN are mediated by the radical NO, while this mediator does not appear to be involved in the relaxation in response to GTN.

While there are numerous manuscripts addressing the effects of chronic GTN therapy on vascular function in preclinical and clinical studies, information on the effects of ISMN on vascular function is rather scarce. Muller et al. described that eccentric dosing of high concentrations of ISMN prevent the development of endothelial dysfunction and the stimulation of ROS production in the rabbit aorta. In contrast, as reported above, human data clearly showed that ISMN treatment for 7 days caused oxidative stress-induced endothelial dysfunction. Finally, while GTN has been consistently associated with endothelial dysfunction, superoxide bioavailability, ET-1 expression, and NADPH oxidase activation, PETN does not appear to have these deleterious effects.

**Summary of the present findings**

Isosorbide-5-mononitrate treatment for 7 days in rats caused, as observed in humans, a substantial degree of endothelial dysfunction as evidenced by the strong shift to the right of the dose–response relationship of the endothelium-dependent vasodilator acetylcholine. Interestingly, the ISMN sensitivity of the vasculature was not altered by the ISMN pre-treatment, while a small degree of cross-tolerance to GTN was observed. This clearly emphasizes that endothelial dysfunction, rather than tolerance development, represents the major side effect of chronic therapy with the mononitrate.

Previously, we have demonstrated that endothelial dysfunction to GTN is likely due to a cross-talk between mitochondria and the vascular NADPH oxidase since no endothelial dysfunction developed in response to treatment with this organic nitrate in an NADPH oxidase knockout animal. The present studies demonstrate that this superoxide source is activated also during ISMN treatment and that increased ROS production in mitochondria is not a prerequisite. Increased NADPH oxidase activity was

**Figure 3** Effects of in vivo treatment with isosorbide-5-mononitrate on nicotinamide adenine dinucleotidephosphate oxidase activity. Isosorbide-5-mononitrate increased the activity in the heart (A) and the aorta (B). Dihydroethidine staining revealed that the increases in superoxide were largely blocked by the nicotinamide adenine dinucleotidephosphate oxidase inhibitor apocynin (Apo) (C and D). Data are mean from four to eight independent experiments; *P < 0.05 vs. Ctr; **P < 0.05 vs. w/o apocynin.
observed in membrane preparations from the heart and from the aorta along with an increase in the expression of the NADPH oxidase subunits Nox1, Nox2, and Nox4. The increase in superoxide was observed throughout the vessel wall, also including the media, all of which was largely blocked by the NADPH oxidase inhibitor apocynin, an observation which points to the vascular NADPH oxidase as the leading superoxide source. Additional support comes from key experiments in gp91phox (Nox2) knockout mice clearly indicating that the phagocytic (but also vascular) Nox2 is a major source of ROS and its deletion prevents ISMN-dependent adverse effects and significantly improves ISMN-induced endothelial dysfunction. Interestingly, superoxide production of whole blood (reflecting NADPH oxidase activity of inflammatory cells such as neutrophils and monocytes) was clearly enhanced in response to ISMN treatment, indicating that ISMN is able to activate the enzyme in inflammatory cells and this effect was absent when gp91phox knockout mice were treated with ISMN. Moreover, ET-1 and ISMN in vitro challenges stimulated the oxidative burst in whole blood leucocytes, which was suppressed by bosentan. Finally, in contrast to the tетranitrate PETN, ISMN does not confer the induction of the antioxidant enzyme system haem oxygenase-1 as shown by the lack of ISMN-dependent increase in haem oxygenase-1 promoter activity (see Supplementary material online, Figure S5).

Increased superoxide production, in particular in the endothelial cell layer, leads to increased formation of peroxynitrite, which in turn may cause eNOS uncoupling via oxidation of the important eNOS cofactor BH4 to the so-called BH3 radical. Intracellular BH4 depletion may be also a consequence of a decreased expression of the BH4-synthesizing enzyme, the GCH-1. Recently, we were able to demonstrate a down-regulation and an uncoupling of the eNOS in the setting of diabetes mellitus, in AT-II hypertension as well as in GTN-induced nitrate tolerance. Accordingly, we observed an uncoupling of eNOS as demonstrated by the reduction in the activity of the cGK-1 as indicated by the decreased phosphorylation of eNOS.
the VASP at serine 239. The importance of the oxidative stress concept was further demonstrated by the improvement of ISMN-induced endothelial dysfunction by PEG-SOD. The present data fit well with recent studies where Sekiya et al. demonstrated that ISDN, of which ISMN is a metabolite, causes endothelial dysfunction as demonstrated by a decrease in flow-mediated dilation of the brachial artery and also a worsening of the intima–media thickness, an observation suggestive of a proatherogenic action of ISDN.

Finally, oxidative stress in endothelial cells has been demonstrated to cause a marked stimulation of the expression of ET-1 in cultured endothelial and smooth muscle cells. Since ISMN treatment increased ROS production in the present animal model throughout the vessel wall, we tested whether we can observe an increase in the expression of ET-1 and big ET-1 in ISMN-treated animals as described previously by in vitro experiments with cultured endothelial and smooth muscle cells. Indeed, using immunohistochemistry, we clearly found an increase in the expression of ET-1 mainly in the endothelial cell layer and also in the adventitia. The important role of ET-1 in causing vascular dysfunction in response to ISMN was further substantiated on a more functional basis by a complete normalization of endothelial dysfunction and whole blood oxidative burst induced by bosentan in vivo therapy.

Conclusions and clinical implications

Taken together, the present studies indicate that the organic nitrate ISMN causes endothelial dysfunction, activates the vascular and phagocytic NADPH oxidase, and stimulates vascular ET-1 production. Although these findings look strikingly similar to previous observations in response to GTN, there are several fundamental differences:

(i) In contrast to GTN, ISMN is not bioactivated by mitochondrial ALDH-2, a process, which leads to a marked increase in mitochondrial ROS production.

(ii) Thus, the NADPH oxidase activation in response to ISMN is not dependent at all on the cross-talk between ROS-producing mitochondria and the enzyme.
**Figure 6** Effects of in vivo isosorbide-5-mononitrate treatment of vascular endothelin-1 and big-endothelin-1 expression. Immunohistochemistry revealed that big endothelin-1 (A) and endothelin-1 (B) staining substantially increased in the endothelial cell layer and to a lesser extent in the adventitia. Images are representative for $n = 4$ independent experiments.

**Figure 7** Effects of ex vivo and in vivo bosentan treatment on isosorbide-5-mononitrate-induced vascular dysfunction and whole blood oxidative stress. Bosentan (10 μM for 1 h) ex vivo incubation improved isosorbide-5-mononitrate-induced endothelial dysfunction (ACh, A). Bosentan (80 mg/kg/day for 7 days) in vivo therapy improved isosorbide-5-mononitrate-induced endothelial dysfunction (ACh, B) and vasoconstrictor sensitivity to endothelin-1 (ET-1, C) as well as the leucocyte-dependent oxidative burst in whole blood in response to a phorbol ester (PDBu, D) or the endotoxin zymosan A (E). Data are mean ± SEM from at least 8 to 12 (A), 4 to 14 (B–C) or 6 to 24 (D–E) independent experiments. *P < 0.05 vs. Ctr; *P < 0.05 vs. isosorbide-5-mononitrate.
Supplementary material

Supplementary material is available at European Heart Journal online.

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References


Mononitrate and endothelial function


