Cardioprotective effects of glyceryl trinitrate: beyond vascular nitrate tolerance

Tamás Csont, Péter Ferdinandy*

Cardiovascular Research Group, Department of Biochemistry, University of Szeged, Dóm tér 9, H-6720 Szeged, Hungary

Abstract

Organic nitrates have been used for the treatment of ischemic heart diseases for more than 100 years and these drugs are still amongst the most frequently prescribed and applied drugs worldwide. Development of tolerance against the hemodynamic effects of nitrates during sustained therapy, however, limits their clinical application. Moreover, recent clinical studies have suggested that long-term nitrate treatment does not improve or may even worsen cardiovascular mortality, possibly due to the development of vascular nitrate tolerance. In agreement with these clinical findings, nitrate tolerance has been shown to increase superoxide and peroxynitrite production leading to vascular dysfunction. Nevertheless, nitrates exert a direct myocardial anti-ischemic effect that is independent from their vascular actions. The direct myocardial effect of glyceryl trinitrate (GTN) has been shown to be preserved even in the state of vascular nitrate tolerance. Moreover, no oxidative stress was observed in hearts isolated from rats with vascular nitrate tolerance, while increased systemic peroxynitrite formation was detected in the plasma in the same animals. The different effects of nitrates on the heart and vasculature are not well characterized; however, tissue specific differences in the metabolism and cellular signaling of nitrates might be a plausible explanation. These data suggest that sustained nitrate treatment increases oxidative stress in the extracardiac vasculature, thereby promoting the development of vascular nitrate tolerance. However, the direct myocardial anti-ischemic effect of nitrates seems to be preserved beyond the development of vascular nitrate tolerance. These new findings may open new perspectives in the clinical use of organic nitrates and suggest that the development of either cardioselective nitrates or nitrate-antioxidant hybrid drugs may replace classical nitrates in the therapy of ischemic heart disease.

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Abbreviations: cGMP, cyclic guanosine monophosphate; GTN, glyceryl trinitrate; K_ATP, ATP-sensitive potassium channel; NO, nitric oxide; PKG, cGMP-dependent protein kinase.

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1. History of the therapeutic use of glyceryl trinitrate

Glyceryl trinitrate (GTN), the “first” organic nitrate, was originally used in the explosive industry after Alfred Nobel invented a method of controlled detonation of GTN. More than 120 years ago, Murell (1879) reported that GTN administered orally relieved anginal pain and prevented subsequent attacks. GTN soon became generally accepted for the treatment and prevention of angina based on Murell’s empiric observation. However, development of tolerance towards GTN was soon recognized (Stewart, 1888). Later, new organic nitrate compounds were introduced and a variety of formulations were developed and used successfully for the treatment of ischemic heart diseases (Berlin, 1987). Detailed descriptions of current recommendations for nitrate use in stable and unstable angina, myocardial infarction, and heart failure are given in ACC/AHA Guidelines (Hunt et al., 2001; Braunwald et al., 2002; Gibbons et al., 2002; Antman et al., 2004). Nevertheless, nitrate tolerance still remains an unresolved problem, which limits the clinical application of organic nitrates.

2. Chemistry and formulation

The common characteristic of organic nitrates is their nitric acid ester of mono- or polyalcohol structures (Fig. 1). These compounds may contain only one or—in the majority of cases—more than one nitrate ester groups. The most well known representative of this class is GTN, often called nitroglycerin, although this is a chemically incorrect nomenclature as organic nitrates contain nitrate (-C-O-NO₂), but not ‘nitro’ groups (-C-NO₂).

Organic nitrate compounds are either oily liquids (GTN) or solids (higher molecular mass nitrate esters). Several organic nitrate esters are explosive in their pure form. Therefore, an inert carrier, such as lactose, is used to prevent spontaneous disintegration in medical formulations. The fully nitrated representatives are lipophilic, while the less completely nitrated compounds are more hydrophilic. Organic nitrates are generally stable in neutral or weakly acidic aqueous solutions. However, under strong alkaline conditions they undergo chemical modifications (Wang et al., 2002).

The chemical structures of the clinically most relevant organic nitrates are shown on Fig. 1. GTN is perhaps the most widely used and studied drug of the organic nitrate family. GTN is available in a large variety of formulations.
including tablets, i.v. injections, patches, sprays, and ointments. Other nitrates used in clinical practice include isosorbide-dinitrate, its active metabolite isosorbide-mononitrate, pentaerythrytol tetranitrate, erythrytyl-tetranitrate, and nicorandil, a nitrate with a well characterized ATP-sensitive potassium channel (K$_{ATP}$) opening effect. Besides these drugs, a number of nitrate esters are used only for research purposes. Moreover, recent efforts are being made for the development of bifunctional or hybrid drugs, for example, nitrate esterified non-steroid and steroid anti-inflammatory agents (Wang et al., 2002), or nitrate esters of antioxidant molecules (Wang et al., 2002). At present, to the best of our knowledge, no data in the literature are available on any attempt to develop organic nitrate molecules with tissue selectivity or organ specificity.

3. Is glyceryl trinitrate cardioprotective or an oxidative stressor?

Organic nitrates predominantly used for the treatment and prevention of cardiovascular diseases including coronary artery disease (stable, unstable, and vasospastic angina), acute myocardial infarction, and congestive heart failure (Abrams, 1996; Thadani, 1997b; Parker & Parker, 1998; Hunt et al., 2001; Braunwald et al., 2002; Gibbons et al., 2002; Antman et al., 2004). Nitrates are remarkably effective in restoring the balance of myocardial oxygen supply-demand in the ischemic heart. Their pronounced effect on vascular smooth muscle is believed to be a key mechanism in their anti-ischemic effects (for reviews, see Harrison & Bates, 1993; Abrams, 1996; Thadani, 1997b). The preferential effect of nitrates is the dilatation of the systemic veins, thus reducing venous refill of the heart. Nitrates, however, also cause arterial vasodilatation leading to decreased afterload. Large coronary arteries may also be dilated due to nitrates. These effects of nitrates result in an attenuated myocardial load, decreased oxygen demand, and increased oxygen supply, that is, an anti-ischemic effect. In addition, in recent studies, a direct cardioprotective effect of nitrates (independent of their vascular actions) has been implicated (Ferdinandy et al., 1995a; Szilvassy et al., 1997; Csont et al., 1999). Moreover, nitrates are relatively “safe” drugs (no severe side effects), thereby organic nitrates have become one of the most frequently prescribed and employed drugs world-wide (Abrams, 1996; Thadani, 1997b). However, development of tolerance to the vascular effects of nitrates due to sustained administration limits their application. Moreover, while meta-analysis studies initially suggested a reduction in mortality due to GTN treatment following acute myocardial infarction (Yusuf et al., 1988; Held, 1992), recent clinical studies (Devita et al., 1994; Ishikawa et al., 1996; Nakamura et al., 1999; Kanamasa et al., 2002) suggest that long-term nitrate treatment does not improve and may even worsen cardiovascular mortality.

What is behind the deleterious effects of organic nitrates? Although the background of nitrate-induced increase in cardiovascular mortality is still not known, the possible role of nitrate tolerance has been suspected (Devita et al., 1994; Nakamura et al., 1999). Experimental evidence on the cellular mechanism of some of the adverse effects of nitrates has been described only in the past few years. Development of experimental vascular nitrate tolerance due to GTN treatment has been shown to increase the formation of reactive oxygen species (Munzel et al., 1995; Dikalov et al., 1998b) including peroxynitrite in the vasculature, which leads to vascular dysfunction (Dikalov et al., 1998a; Mihm et al., 1999). In the light of these observations, treatment of patients with organic nitrate compounds may lead to side effects possibly due to generation of reactive oxygen species in the vasculature. Some authors have recently concluded that vascular oxidative stress could be the unifying mechanism for nitrate tolerance (Gori & Parker, 2002a).

Is it safe to use organic nitrates or their deleterious effects (i.e., oxidative stress) overcome the benefits they provide? To date there are not enough data to answer this question with certainty. Appropriately designed clinical trials examining the safety and efficacy of organic nitrates in the therapy of diseases for which nitrates are commonly used are needed. It has to be noted, however, that most of the studies investigating the mechanism of organic nitrate action and tolerance development have looked at their vascular effects. There is only limited information available so far to evaluate the effect of nitrates directly on the heart. However, differences in the tissue specific action of nitrates may be the key missing information relevant to the unwanted effects of nitrates.

Here we attempt to review the direct cardioprotective effect of GTN on the myocardium and provide a hypothesis to explain the contradictory findings regarding the therapeutic efficacy of GTN in the state of vascular nitrate tolerance from a cardiac and not vascular point of view.

4. Mechanism of action of nitrates in the vasculature

As we have already pointed out, organic nitrates are generally believed to exert their anti-ischemic effect by causing vasodilatation in the vasculature (Fig. 2). Therefore, in order to compare the vascular and myocardial effects of nitrates, here we give a brief overview of the most important knowledge regarding the cellular mechanism of action of organic nitrates in vascular tissues.

4.1. Biotransformation

It is generally believed that all organic nitrates exert their biological effects via the release of nitric oxide (NO). Unlike other NO releaser compounds, so called spontaneous NO releasing nitrovasodilators (such as sodium nitroprusside),
organic nitrates require enzymatic bioconversion to deliver NO, the biologically active principle. Some reports, however, implicate spontaneous NO release from GTN in aqueous solutions in the absence of any cellular components (Pataricza et al., 1998).

Although the enzymes responsible for the in vivo metabolism involving denitration and reduction of organic nitrates have not been clearly identified, a growing number of potential enzymes have been suggested including an uncharacterized vascular microsomal enzyme system (Chung & Fung, 1990), cytochrome P-450 like enzymes (McGuire et al., 1998), glutathione-S-transferase (Kurz et al., 1993), and recently mitochondrial aldehyde dehydrogenase (Chen et al., 2002). A very recent review proposes that instead of one enzyme, likely many -SH containing, non-specific and/or unrelated enzymes/proteins are involved in the metabolism of organic nitrates (Fung, 2004). Moreover, simple thiols can react with GTN to generate NO (Chong & Fung, 1991). Nevertheless, only little is known about the tissue specific expression, activity, coenzyme demand, and regulation of the nitrate metabolizing enzymes yielding NO release.

4.2. Smooth muscle relaxation by glyceryl trinitrate

Organic nitrates mimic the effects of endogenous NO. One of the most pronounced effects of NO is smooth muscle relaxation (Fig. 2; Moncada et al., 1991). NO diffuses through plasma membranes and directly activates soluble guanylate cyclase which in turn catalyses the formation of cyclic guanosine monophosphate (cGMP) from guanosine 5'-triphosphate (GTP; Murad, 1986). The second messenger cGMP activates a cGMP-dependent protein kinase, protein kinase G (PKG), with subsequent alterations in the phosphorylation state of various proteins (e.g., myosin light chain kinase, phospholamban, etc.; Karczewski et al., 1992; Carvajal et al., 2000). This finally leads to alterations in Ca\(^{2+}\) level of smooth muscle cells, dephosphorylation of myosin light chain (Waldman & Murad, 1987; Carvajal et al., 2000), activation of K\(_{ATP}\), and a resultant smooth muscle cell relaxation that leads to vasodilatation in the vasculature. Veins are more susceptible to the vasodilator effect of organic nitrates than arteries. Small arterioles (diameter <100 µm) in the heart, however, have been reported to be rather insensitive to nitrates (Harrison & Bates, 1993). Although organic nitrates are still considered to act predominantly via cGMP, it is now well recognized that NO, as well as nitrates, may exert a number of cGMP independent actions (for review, see Davis et al., 2001). These may include interaction with certain metal containing proteins, post-translational modification of proteins, or involvement in the formation of reactive oxygen species (Davis et al., 2001). To understand better how nitrates exert their effects more attention should be paid to these novel mechanisms.

Another important issue is whether tissue- or cell type-specific nitrate actions exist. It seems to be clear that nitrates exert biological effects on several cell types, and these actions are believed to be mediated by NO. However, comparative studies for the evaluation of the actions of nitrates (i.e., biotransformation and cellular mechanism of action) in different cell types and the characterization of these potential differences are lacking (Fig. 2). There is evidence showing, however, that the intensity of nitrate action varies among different types of vessels (Harrison & Bates, 1993).

4.3. Nitrates in non-cardiovascular tissues

Relaxation of smooth muscle cells in different organ systems, including the vascular system, respiratory tract, gastrointestinal tract, genitourinary tract, etc., results in a variety of functional responses due to nitrate treatment, which are all based on the same mechanism as seen in the vascular tissue. Moreover, exogenous NO may also affect the physiologic function of other cell types including platelets, immune system and nervous system (Moncada et al., 1991). Besides cardiovascular applications, organic nitrates are also implicated for cervico-uterine relaxation (Smith & Brien, 1998), the therapy of glaucoma (Wizemann & Wizemann, 1980), impotence (de Mey, 1998), anal fissure (Ehrenpreis et al., 2001), dyskinesia of the sphincter of Oddi (Luman et al., 1997), achalasia (Wong et al., 1987), osteoporosis (Jamal et al., 1998), and cancer pain management (Lauretti et al., 2002).

5. Vascular nitrate tolerance: limitations of nitrate treatment?

The term nitrate tolerance means that after prolonged, continuous, or high dose nitrate treatment, the clinical, or hemodynamic response to organic nitrates (i.e., vasodilatation with a subsequent decrease in blood pressure, or relief of anginal pain) is attenuated or abolished. Development of tolerance against the beneficial clinical effects of GTN was soon recognized (Stewart, 1888) after the drug was intro-
duced for the treatment of angina (Murell, 1879). Despite extensive research on this field, the mechanism of nitrate tolerance is still not clearly understood. Thus, development of tolerance to the hemodynamic effects of organic nitrates remains a major problem in their clinical application. Recent review articles provide a detailed overview of the present knowledge on the development of nitrate tolerance in the vasculature from diverse approaches (Thadani, 1997a; Parker & Parker, 1998; Gori & Parker, 2002a, 2002b; Fung, 2004).

5.1. Proposed cellular mechanisms of vascular nitrate tolerance

The mechanism of tolerance development has been studied extensively and a number of hypotheses suggested as possible mechanisms, although, a consensus has not been reached to date. Depletion of -SH moieties, volume expansion theory, counterregulatory neurohormonal activation, abnormalities in organic nitrate biotransformation or NO signal transduction, and the free radical hypothesis were all proposed as causative mechanisms of the development of nitrate tolerance (Abdollah et al., 1987; Parker & Parker, 1998; Gori & Parker, 2002a, 2002b; Fung, 2004). These theories are briefly discussed below.

5.1.1. Sulphydryl-depletion hypothesis

Depletion of SH groups as a cause for the development of nitrate tolerance was suggested by Needleman and Johnson (1973). According to this theory, the loss of efficacy of nitrates during sustained treatment is due to the depletion of reduced -SH moieties necessary for the biotransformation of nitrates to NO. Although some subsequent studies have supported (at least partially) this hypothesis (May et al., 1987; Packer et al., 1987), others have failed to prevent nitrate tolerance with sulphydryl groups in vitro (Gruetter & Lemke, 1986; Abdollah et al., 1987), and also failed to show an association of nitrate tolerance with depletion of thiols in vascular tissues (Boesgaard et al., 1994).

5.1.2. Plasma volume expansion theory

A number of studies have reported that nitrate therapy leads to an expansion of plasma volume (Dupuis et al., 1990; Parker et al., 1991, 1992). According to the plasma volume expansion hypothesis, nitrate-induced increase in circulating plasma volume and filling pressure reverses the effect of nitrates on ventricular preload, thereby leading to the development of nitrate tolerance. The role of plasma volume expansion as a cause of nitrate tolerance is unclear. Attempts to prevent nitrate tolerance with diuretic therapy resulted in conflicting findings (Parker et al., 1992, 1996; Mohanty et al., 1995).

5.1.3. Counterregulatory neurohormonal activation

Nitrates are potent venous and arterial dilators, and exposure to nitrates may result in activation of compensatory mechanisms, that is, release of vasoconstrictor hormones, such as angiotensin, catecholamines, and endothelin (Dupuis et al., 1990; Parker et al., 1991). For instance, Packer et al. (1987) showed neurohormonal activation in patients with congestive heart failure treated with intravenous GTN. In healthy volunteers, an increase in plasma renin activity, norepinephrine, and arginine vasopressin levels occur within 24 hr of treatment with GTN patches (Parker et al., 1991). Still, there are no consistently convincing data showing that treatment with ACE inhibitors or other neurohormonal blockers prevents these clinical phenomena (Katz et al., 1991; Muiesan et al., 1993; Parker & Parker, 1993).

5.1.4. Decreased bioavailability of nitric oxide

Decreasing cellular levels of NO following sustained GTN administration has been proposed as a mechanism of the development of nitrate tolerance. Both reduced biotransformation of GTN and increased degradation of NO may result in decreased NO bioavailability, and subsequent impairment of vascular relaxation to GTN. Several studies demonstrated that tolerance is accompanied by reduced formation of glycyl 1,2-dinitrate, a metabolite of GTN biotransformation. However, an agreement has not been achieved, because of negative reports (Laursen et al., 1996; Csont et al., 1998). Increased degradation of NO by vascular superoxide has been proposed as a mechanism for the development of vascular nitrate tolerance (Munzel et al., 1995).

5.1.5. Abnormalities of cyclic guanosine monophosphate signalling

Nitrate tolerance may also be mediated by decreased activity of soluble guanylyl cyclase, increased activity of phosphodiesterases (the enzymes responsible for the catalysis of cGMP), or decreased activity of PKG. The former two mechanisms would reduce the level of cellular cGMP, while the latter would impair the effects of cGMP. Studies have presented both supportive and not supportive data for the involvement of these phenomena in the development of nitrate tolerance (Gori & Parker, 2002a, 2002b).

5.1.6. Free radical hypothesis

The free radical hypothesis states that the development of nitrate tolerance during the administration of GTN is due to an increase in vascular free radical production. This phenomenon was first observed by Munzel et al. (1995), demonstrating increased superoxide production in nitrate tolerant vessels. Recent publications consider this hypothesis as one of the most likely mechanism of the development of nitrate tolerance (Gori & Parker, 2002a, 2002b; Laight, 2003). Moreover, administration of antioxidant molecules has been shown to reverse vascular nitrate tolerance partially or completely both in animals and humans (Bassen et al., 1998; Dikalov et al., 1999).
5.2. Prevention or treatment of vascular nitrate tolerance

Organic nitrates are available in a wide range of formulations and delivery systems. Nitrate tolerance most often occurs with transdermal and intravenous routes of administration. The only widely accepted method to prevent tolerance development is appropriate dosage management to ensure a low nitrate, or nitrate-free interval during each day (Thadani, 1997a; Parker & Parker, 1998). Intermittent use of transdermal nitrate patches (7.5 mg GTN per 12 hr; 12 hr nitrate-free period during the night) is effective (Thadani, 1997a). However, concomitant use of an alternative anti-ischemic agent (β-blockers or Ca²⁺ antagonists) during the night and early morning is recommended to avoid nitrate rebound. Similarly, twice-a-day eccentric isosorbide-5-mononitrate (20 mg in the morning, and 7 hr later), or once-a-day slow-release isosorbide-5-mononitrate (120 or 240 mg) administered orally is also effective (Thadani, 1997a).

When intravenous nitrates are administered, an effort must be made to use non-tolerance-producing regimens (lower dose and/or intermittent dosing). GTN infusion may be initiated at a low rate (5–10 mcg/min) and increased by 5–20 mcg/min every 3–5 min until some symptom or blood pressure response noted. When patients have been free of manifestations of ischemia for 12–24 hr, an attempt should be made to reduce the dose of intravenous GTN and to switch to oral or topical nitrates. If ischemia recurs during continuous intravenous GTN therapy, responsiveness to nitrates can often be restored by increasing the dose and then, after symptoms have been controlled for several hours, attempting to add a nitrate-free interval. This strategy should be pursued as long as symptoms are not adequately controlled. In stabilized patients, intravenous GTN should generally be converted within 24 hr to a non-parenteral alternative administered in a non-tolerance-producing regimen to avoid the potential reactivation of symptoms (Braunwald et al., 2002).

Besides manipulations of the dosage regimen of GTN, several pharmacological interventions, such as angiotensin converting enzyme inhibitors, diuretics, sulphydryl-containing agents, antioxidants, etc., were studied to prevent the development of nitrate tolerance; however, sufficient evidence to prove the efficacy of these drugs is still missing (Parker & Parker, 1998).

5.3. Rebound, dependence

A rebound effect can be seen when nitrate exposure is discontinued, and consequently the incidence of ischemic episodes is increased. This phenomenon was first observed in munitions workers when they were removed from exposure to high doses of GTN. Unfortunately, this situation may occur during the nitrate-free period of intermittent nitrate therapy used to prevent the development of nitrate tolerance (Thadani, 1997a).

5.4. Adverse effects of glyceryl trinitrate treatment

Most often, side effects of nitrate treatment are related to vascular smooth muscle relaxation and include headache, dizziness, weakness, hypotension, and reflex tachycardia. These symptoms usually are not too severe and can be controlled by decreasing the therapeutic dose. High doses of organic nitrates may lead to a reduction in blood pressure to such an extent that coronary flow becomes insufficient to meet myocardial demands. In these cases nitrates provoke anginal attacks rather then prevent them (Thadani, 1997a; Parker & Parker, 1998).

6. Effect of glyceryl trinitrate on the heart: a direct myocardial protective action?

6.1. Glyceryl trinitrate in myocardial ischemia/reperfusion

Although the anti-ischemic effect of nitrates is traditionally believed to be based on the drug-induced decrease in preload and afterload, dilatation of stenotic coronary arteries, improvement of coronary collateral flow, and inhibition of platelet aggregation (for reviews, see Harrison & Bates, 1993), recent investigations have shown that organic nitrates also exert direct myocardial effects (Ferdinandy et al., 1995a; Szilvassy et al., 1997; Csont et al., 1999; Bolli, 2001).

Our group has previously demonstrated that a nonvasodilator concentration of GTN exerts a direct myocardial anti-ischemic effect independent of its vascular actions in isolated rat hearts (Fig. 3; Ferdinandy et al., 1995a) and in conscious rabbits (Szilvassy et al., 1997). In addition, this effect of GTN is not diminished by the development of

![Fig. 3. The “direct” cardioprotection of GTN is mediated by KATP channels and nitrate tolerance does not interfere with this effect of GTN. The graph shows the protection of the ischemic myocardium by glyceryl trinitrate (GTN) as measured by lactate dehydrogenase (LDH) release at the end of coronary occlusion in working hearts isolated from rats with or without vascular tolerance to GTN. Hearts were perfused in the presence of nonvasodilatory doses of GTN (10⁻⁷ M) and the nonselective KATP inhibitor glibenclamide (GL, 10⁻⁷ M), as well as the combination of GL and GTN, and their solvent (C), respectively. Data are mean ± SEM. *P<0.05 versus solvent-treated control group (n = 7 in each group). Modified from Csont et al. (1999).](image-url)
vascular tolerance to GTN (Ferdinandy et al., 1995a; Szilvassy et al., 1997). GTN selectively dilates coronary vessels >100 μm, therefore it has minimal effect on coronary resistance (Harrison & Bates, 1993). Accordingly, 10⁻⁷ M GTN did not change area of ischemic zone and failed to affect coronary flow, thus showing that GTN did not affect coronary circulation in isolated rat hearts (Ferdinandy et al., 1995a). GTN was also found to be cardioprotective in hearts isolated from GTN-tolerant rats (Ferdinandy et al., 1995a; Csont et al., 1999). Consequently, the anti-ischemic effect of GTN involves a direct action on the myocardium, and this effect of GTN is not diminished in the state of vascular nitrate tolerance.

6.2. Are the mechanisms of nitrate action the same in the heart as in smooth muscle cells?

The vascular effects of organic nitrates have been studied most extensively, and relatively little is known about the cardiac effects of nitrates. However, it has been shown that GTN is not metabolized in coronary arteries smaller in diameter than 100 μm (Harrison & Bates, 1993). Furthermore, recent findings suggest that organic nitrates, besides their vascular effects, exert direct effects on cardiac muscle cells (Mehta et al., 2002) and isolated hearts (Ferdinandy et al., 1995a; Csont et al., 1999). However, the involvement of cGMP in these cardiac effects of nitrates has been questioned (Csont et al., 1998). These studies suggest that the in vivo cellular mechanisms of action of organic nitrates are probably more complex than it was previously believed, and it is possible that nitrates trigger different biochemical mechanisms in the heart than in the systemic vascular elements. As an alternative, the mechanism triggered by nitrate treatment may be the same in the different tissues, however, these pathways are regulated in a distinct way.

It is well accepted that the enzymatic bioconversion of GTN to NO and the consequent increase in cGMP concentration is responsible for the vascular effects of GTN (Moncada et al., 1991). However, in the state of nitrate tolerance impaired conversion of GTN to NO has been reported in vascular tissue (Sage et al., 2000). In contrast, by a direct detection of NO with electron spin resonance spectroscopy, we have demonstrated that GTN is converted to NO in hearts isolated from either GTN-tolerant or non-tolerant rats (Csont et al., 1998, 1999).

Recent studies suggest that NO has cGMP-independent actions (Balligand & Cannon, 1997; Davis et al., 2001). We have previously shown that changes in cardiac NO content were not reflected by changes in cGMP level in the rat heart in vivo (Csont et al., 1998). Accordingly, we have found that while cardiac NO content significantly increased after GTN administration, myocardial cGMP content was not changed significantly in hearts isolated from either vascular GTN tolerant or non-tolerant rats (Csont et al., 1999). Similarly, Torfgard et al. (1991) have shown that administration of GTN does not affect cardiac cGMP content in the rat heart in vivo. This finding shows that the cardioprotective effect of GTN cannot be mediated by cGMP in the heart and confirms that the conversion of GTN to NO is not diminished in GTN-tolerance (Laursen et al., 1996; Csont et al., 1998).

A specific blocker of K<sub>ATP</sub> glibenclamide, was shown to abolish the direct anti-ischemic effect of GTN (Csont et al., 1999). Consequently, this effect of GTN may involve activation of K<sub>ATP</sub>. This is a plausible mechanism for the direct myocardial anti-ischemic effect of GTN (Fig. 4), since pharmacological activation of K<sub>ATP</sub> has been shown by several laboratories to mediate cardioprotection in the rat (Grover et al., 1989; Gross & Auchampach, 1992; Ferdinandy et al., 1995b). In agreement with these findings, NO has been shown to activate K<sub>ATP</sub> in rabbit ventricular myocytes (Han et al., 2002). Further studies indicated that mitochondrial rather than sarcolemmal K<sub>ATP</sub> are activated by NO donors (Sasaki et al., 2000; Xu et al., 2004). However, the exact mechanism by which NO may open K<sub>ATP</sub> in the myocardium is not clear (Csont et al., 1999; Xu et al., 2004). Interestingly, we have shown that the basal level of NO in the myocardium was significantly increased in the GTN-tolerant hearts as compared to the non-tolerant ones (Csont et al., 1999). These hearts, however, were not protected against ischemia. Nevertheless, the severity of ischemic damage diminished when NO level was increased by acute GTN-treatment in the hearts isolated from either GTN-tolerant or non-tolerant rats. This shows that the rapid increase in cardiac NO due to acute GTN administration may be responsible for activation of K<sub>ATP</sub> rather than the increased basal level of cardiac NO.

Despite these findings, involvement of K<sub>ATP</sub>-independent mechanisms in the direct myocardial cardioprotective effect of GTN cannot be excluded. The NO donor S-nitroso-N-acetyl-DL-penicillamine has been shown to reduce ischemia/reperfusion injury via decreasing the endogenous formation of peroxynitrite in isolated rat hearts (Yasmin et al., 1997). The direct antioxidant properties of NO or NO-generating drugs and their ability to reduce the detrimental actions of authentic peroxynitrite have also been observed by others in a variety of biological systems (Villa et al., 1994; Rubbo et al., 1996). Calcitonin gene-related peptide (CGRP) has also been reported to be involved in the cardioprotective action of GTN in Langendorff perfused rat hearts (Hu et al., 1999).

Although further investigations are needed to understand the mechanisms of action of the direct myocardial anti-ischemic effect of GTN, the fact that this effect of GTN is preserved even in the presence of vascular nitrate tolerance may open new perspectives in the therapeutic use and the development of new organic nitrate compounds.

6.3. Glyceryl trinitrate does not induce oxidative stress in the heart

Many previous studies (Munzel et al., 1995; Dikalov et al., 1998a, 1998b; Mihm et al., 1999) have suggested that...
nitrates and nitrate tolerance, our group has shown that the generation of cardiac superoxide, hydroxyl radical, and peroxynitrite, enzymatic superoxide synthesis by xanthine oxidoreductase and its detoxification by superoxide dismutase (SOD), as well as the non-enzymatic breakdown of superoxide and peroxynitrite, were not changed in the heart after in vivo GTN treatment resulting in the development of vascular nitrate tolerance (Csont et al., 2002). Furthermore, myocardial mechanical function was not altered either as a result of vascular nitrate tolerance (Csont et al., 2002). These findings clearly show that superoxide and peroxynitrite generation in the myocardium and in the coronary vasculature were unaffected by vascular GTN tolerance. However, in the same study, a significant increase in serum-free nitrotyrosine concentration was observed in nitrate-tolerant rats 12 hr after the last GTN injection (Csont et al., 2002). This shows a sustained, increased production of peroxynitrite in extracardiac tissues, possibly in the extracardiac vasculature. This is in accordance with previous findings (Munzel et al., 1995; Dikalov et al., 1998a, 1998b; Mihm et al., 1999) showing increased generation of reactive oxygen species in vascular tissue due to nitrate tolerance.

Increase in serum nitrotyrosine was found 1 hr after administration of a single dose of GTN (Csont et al., 2002). However, this increase was not observed 5 and 12 hr after treatment in non-tolerant rats. This finding suggests that GTN treatment in the absence of nitrate tolerance leads to a transient increase in extracardiac peroxynitrite formation. This is in accordance with findings of Dikalov et al. (1998a), showing that the biotransformation of GTN is accompanied by superoxide formation in endothelial and smooth muscle cells in the absence of nitrate tolerance. The reason why nitrate tolerance extends the time frame of systemic peroxynitrite formation is not clear; however, increased accumulation of tissue GTN is a plausible explanation (Torfgard et al., 1991).

Contrary to our findings, Sydow et al. (2004) has recently shown an increased formation of reactive oxygen species in cardiac mitochondria isolated from rats subjected to a 3-day treatment with a high dose of GTN that accompanied by the development of nitrate tolerance in isolated aortic rings. However, in this study isolated mitochondria were investigated without measuring reactive oxygen species in whole heart tissue, which would reflect in vivo conditions. Unfortunately, mitochondrial antioxidant mechanisms have not been studied either. Moreover, in vivo or ex vivo assessment of cardiac performance parallel to increased oxidative stress observed in cardiac mitochondria is missing in this study (Sydow et al., 2004).

Based on these observations, we hypothesized that short-term administration of GTN results in beneficial vascular effects (systemic vasodilatation) against cardiac ischemia, while sustained application is associated with increased superoxide production in the vasculature that contributes to the development of vascular nitrate tolerance. However, in the heart, GTN exerts a cardioprotective effect during both acute treatment and sustained treatment that leads to the development of vascular nitrate tolerance. Therefore, we strongly believe that tissue-specific differences in either the bioactivation or the cellular signaling of GTN exist, and these mechanisms in the cardiac tissue are distinct from that seen in the vasculature.

6.4. The phenomenon “cardiac nitrate tolerance” does not exist

Similarly to the development of vascular nitrate tolerance, that is, diminished vascular effects of GTN due to long-term treatment, an obvious question arises: is there cardiac nitrate tolerance, that is, diminished direct cardioprotective effect of GTN due to long-term use? The facts that the direct cardioprotective effect of GTN is not diminished in vascular nitrate tolerance and that GTN does not induce oxidative stress in the heart show that the phenomenon “cardiac nitrate tolerance” does not exist (Fig. 4).

6.5. Glyceryl trinitrate tolerance and cardiac adaptation to ischemic stress

Brief episodes of ischemia and reperfusion protect the myocardium from the injury induced by subsequent episodes of sustained ischemia. This adaptive response of the heart to ischemic stress is termed ischemic preconditioning (Yellow & Downey, 2003). The initial phase of cardioprotection, termed “early” or “classical” preconditioning, appears within minutes following the preconditioning stimuli and lasts for 2–3 hr. On the other hand, a “second window” of protection (also known as delayed or late preconditioning) reappears 12–24 hr later and lasts for 3–4 hr.

Fig. 4. Proposed cellular mechanism for the effect of GTN in the heart. The cardioprotective effect of GTN is mediated by $K_{ATP}$. This effect of GTN is not diminished in vascular nitrate tolerance. GTN does not lead to increased oxidative stress (peroxynitrite, ONOO$^-$) formation in the heart. Therefore, there is no “cardiac nitrate tolerance”. Thick arrows: dominant signaling pathways. Thin arrows: negligible pathways in the heart.
days. Although preconditioning provides a remarkable cardioprotection, its effectiveness is attenuated in some pathological conditions, such as hyperlipidemia, diabetes, heart failure, and aging (for reviews, see Ferdinandy, 2003; Ferdinandy et al., 1998). Several recent studies have demonstrated that ischemic preconditioning may be triggered by a diversity of tools including the clinically more relevant pharmacological induction of preconditioning.

NO derived from either endogenous or exogenous sources has been suggested to play an important role in cardiac adaptation to ischemic stress both as a trigger or a mediator of protection (for reviews, see Bolli, 2001; Ferdinandy & Schulz, 2003). Although the mechanism is not yet clear, GTN has been shown to trigger both early and delayed cardioprotection in animal models (Bilinska et al., 1996; Banerjee et al., 1999; Hu et al., 1999; Hill et al., 2001; Du et al., 2004), as well as in humans (Leesar et al., 2001). The latter observation may have a great clinical relevance.

However, it needs to be clarified if the development of nitrate tolerance influences the cardiac adaptation induced by GTN. Our group has previously demonstrated that the protective effect of acute preconditioning induced by rapid ventricular pacing in conscious rabbits was abolished when the animals had been made tolerant to the vasodilator effect of GTN (Szilvassy et al., 1994). This finding was further confirmed and supplemented with a further observation, that despite the loss of preconditioning induced by rapid ventricular pacing in rabbits made tolerant to the vasodilator effect of GTN, a direct anti-ischemic effect of GTN was preserved (Szilvassy et al., 1997). Moreover, neither GTN nor application of preconditioning stimuli was effective in increasing cardiac cGMP level in the tolerant state (Szilvassy et al., 1997). Thus, it was concluded that GTN might elicit cardioprotection without involvement of cGMP, whereas maneuvers that make the heart unable to produce increased amounts of cGMP in response to an ischemic challenge render the heart incapable of adapting to repetitive ischemic insults (Szilvassy et al., 1994, 1997). Intermittent nitrate therapy with nitrate patches effectively prevented nitrate tolerance and the loss of the preconditioning effect on hemodynamic and electrophysiological changes due to rapid pacing-induced ischemia in conscious rabbits (Szilvassy et al., 1997). However, during nitrate-free periods, the heart was more vulnerable to an ischemic episode (Szilvassy et al., 1997).

In contrast to our findings, Hill et al. (2001) have reported that GTN induces delayed preconditioning against myocardial infarction in conscious rabbits despite the development of nitrate tolerance. In this study, the index ischemia (30 min coronary occlusion) was carried out 72 hr following the removal of the last GTN patch used for 28 days to induce nitrate tolerance, and they could still observe a reduction of infarct size (Hill et al., 2001). This long GTN-free interval, however, could have been sufficient for nitrate tolerance to cease. Unfortunately, the authors have not verified the presence of vascular nitrate tolerance at the time of coronary occlusion.

These results suggest, that GTN triggers both early and late preconditioning. However, the development of vascular tolerance to GTN, attenuates the protective effect of preconditioning.

The mechanism by which GTN tolerance leads to deterioration of ischemic preconditioning of the heart has not been extensively studied yet. The involvement of cardiac capsaicin-sensitive sensory neurons can be suspected, as the deterioration of the function of capsaicin-sensitive sensory neurons have been shown in hearts of guinea pigs with vascular nitrate tolerance (Oroszi et al., 1999). We have previously shown that depletion of neurotransmitters (NO and CGRP) from these sensory neurons by high dose capsaicin treatment leads to a loss of preconditioning in isolated rat hearts (Ferdinandy et al., 1997). Others have also shown that capsaicin-sensitive sensory neurons have been shown to play an important role in acute preconditioning (Li et al., 1996; Hu et al., 1999). Therefore, GTN-tolerance-induced deterioration of cardiac capsaicin-sensitive sensory neurons may lead to the loss of preconditioning. Another potential explanation for the loss of preconditioning in nitrate tolerance could be the development of adaptive responses that result in an impairment of the alterations of cardiac cGMP content. Hypoxia induced by rapid ventricular pacing increased cGMP in rabbit hearts. However, this elevation of cardiac cGMP was abolished in the hearts of rabbits subjected to continuous treatment with transdermal GTN for 7 days (Szilvassy et al., 1997). This is in agreement with findings indicating that cGMP elicits cardioprotection either as a trigger or a mediator of preconditioning (Horimoto et al., 2000; Kodani et al., 2002; Du et al., 2004; Qin et al., 2004). Activation of vasoconstrictor hormones (such as angiotensin and endothelin) in response to sustained nitrate treatment leading to the development of vascular nitrate tolerance may also participate in the mechanism of attenuated protection conferred by preconditioning in nitrate tolerance, since angiotensin is known to exert detrimental effects directly on cardiac muscle (Kim & Iwao, 2000), thereby counteracting the protective effect of preconditioning.

7. Concluding remarks

Recent results showing the pro-oxidant activities of GTN and the clinical results reporting negative data on cardiovascular mortality with GTN treatment made the medical community more cautious with the use of nitrates. However, experimental data of the last decade indicate that GTN exerts a “direct” cardioprotective effect irrespective the development of vascular nitrate tolerance and that GTN induces oxidative stress in the extracardiac vasculature but
not in the heart tissue. These results suggest that either cardioselective nitrates, nitrate compounds with additional antioxidant activity, or a combination therapy with nitrates and antioxidants may increase the safety of organic nitrates in the chronic treatment of ischemic heart disease. Unfortunately, no pharmaceutical advance has been made so far in the field of developing tissue specific organic nitrates. The development of nitrates with antioxidant properties, such as TEMPO-4-mononitrate (Wang et al., 2002; Laight, 2003) is in a rather premature phase, but it can be a promising approach. Combined treatment with GTN and the antioxidant vitamin C has been used successfully to attenuate the development of nitrate tolerance in animal studies and humans (Bassenge et al., 1998; Dikalov et al., 1999).

The new findings summarized in this review may open new perspectives in the clinical use of organic nitrates and suggest that the development of either cardioselective nitrates or nitrate-antioxidant hybrid drugs may replace classical nitrates in the therapy of ischemic heart disease.

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