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The effect of peroxynitrite decomposition catalyst MnTBAP on aldehyde dehydrogenase-2 nitration by organic nitrates: Role in nitrate tolerance

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Isosorbide-5-mononitrate (IS-5-MN) (PubChem CID: 27661)

ABSTRACT

Bioconversion of glyceryl trinitrate (GTN) into nitric oxide (NO) by aldehyde dehydrogenase-2 (ALDH-2) is a crucial mechanism which drives vasodilatory and antiplatelet effect of organic nitrates *in vitro* and *in vivo*. Oxidative stress generated by overproduction of free radical species, mostly superoxide anions and NO-derived peroxynitrite, has been suggested to play a pivotal role in the development of nitrate tolerance, though the mechanism still remains unclear. Here we studied the free radical-dependent impairment of ALDH-2 in platelets as well as vascular tissues undergoing organic nitrate ester tolerance and potential benefit when using the selective peroxynitrite decomposition catalyst Mn(III) tetrakis (4-Benzoic acid) porphyrin (MnTBAP). Washed human platelets were made tolerant to nitrates *via* incubation with GTN for 4 h. This was expressed by attenuation of platelet aggregation induced by thrombin (40 U/mL), an effect accompanied by GTN-related induction of cGMP levels in platelets undergoing thrombin-induced aggregation. Both effects were associated to attenuated GTN-induced nitrite formation in platelets supernatants and to prominent nitration of ALDH-2, the GTN to NO metabolizing enzyme, suggesting that GTN tolerance was associated to reduced NO formation *via* impairment of ALDH-2. These effects were all antagonized by co-incubation of platelets with MnTBAP, which restored GTN-induced responses in tolerant platelets. Comparable effect was found under in *in vivo* settings. Indeed, MnTBAP (10 mg/kg, i.p.) significantly restored the hypotensive effect of bolus injection of GTN in rats made tolerants to organic nitrates *via* chronic administration of isosorbide-5-mononitrate (IS-5-MN), thus confirming the role of peroxynitrite overproduction in the development of tolerance to vascular responses induced by organic nitrates.

Abbreviations: GTN, glyceryl trinitrate; NO, nitric oxide; ALDH-2, aldehyde dehydrogenase-2; $O_2^{\bullet -}$, superoxide anions; PN, peroxynitrite; cGMP, cyclic guanosine monophosphate; PLTs, platelets; MnTBAP, Mn(III)tetrakis(4-Benzoic acid) porphyrin; IS-5-MN, isosorbide-5-mononitrate; ISDN, isosorbide dinitrate; SMC, smooth muscle cells; EC, endothelial cells; PTMs, post-translational modifications.

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In conclusion, oxidative stress subsequent to prolonged use of organic nitrates, which occurs *via* nitration of ALDH-2, represents a key event in GTN tolerance, an effect counteracted both *in vitro* and *in vivo* by novel peroxynitrite decomposition catalyst.

Introduction

Organic nitrates, such as glyceryl trinitrate (GTN), isosorbide dinitrate (ISDN) and mononitrate (IS-5-MN) are currently used in the treatment of coronary artery disease (*e.g.*, stable-effort angina, unstable angina, acute myocardial infarction) and in other cardiovascular disorders such as congestive heart failure, pulmonary edema, and severe arterial hypertension [1–4].

The beneficial clinical effect of GTN and other organic nitrates is due to preferential dilation of large conductance veins and large arteries while arterioles are dilated only by much higher concentrations of nitrates [5–7]. On the other hand, *in vitro* and *in vivo* antiplatelet properties of organic nitrate ester have been shown to contribute to their beneficial effect in cardiovascular disorders [8].

GTN and other nitrates are pro-drugs, which require previous bio-activation, to release nitric oxide (NO) or S-nitrosothiol, an effect accompanied by enhanced cGMP levels in target cells, including vascular smooth muscle cells (SMC) and platelets, thereby leading to vasodilatation and inhibition of platelet aggregation [9].

Despite organic nitrates still maintain their role in treating many acute cardiovascular diseases, the chronic use of such compounds has some clear limitations due to the development of tolerance, a complex phenomenon that involves neurohormonal counter-regulation and intrinsic vascular processes [10]. Although intermittent nitrate therapy which allows a daily nitrate washout interval, has been shown to be effective in the prevention of nitrate tolerance, this regimen is limited by its inability to provide a continuous and uninterrupted therapeutic effect [11–13]. Moreover, experimental and clinical investigations suggest that GTN-, ISDN-, and IS-5-MN-induced tolerance is associated with the expression of potentially deleterious modifications such as increased oxidative stress, endothelial dysfunction, and sympathetic activation [14–16]. Therefore, there is increasing awareness that nitrate tolerance cannot simply be considered as a loss of the beneficial effects of nitrate but also as a condition with potential extra harmful consequences [17].

The exact mechanisms of nitrate bioactivation and the development of nitrate tolerance still remain unclear. Many pathophysiological events for the development of nitrate tolerance have been proposed over the last few years including neurohumoral adjustments, such as increases in catecholamine levels [18], increases in plasma levels of vasopressin [19], increases in intravascular volume [20] and activation of the renin–angiotensin system [21]. On the other hand, nitrate-induced oxidative stress, and free-radical dependent impairment of GTN-bioconverting machinery has largely been studied, though the exact mechanism remains to be better clarified [10,22–24].

The mitochondrial isoform of aldehyde dehydrogenase 2 (ALDH-2) has been proposed to be the enzyme responsible for catalyzing the bioactivation of GTN [25,26] leading to the formation of NO. Thus, NO activates GC-cGMP-PKG signal transduction mechanism to induce vasodilatation and inhibition of platelet aggregation [27]. ALDH-2 has also been shown to be highly modulated by free radical species generated during the development of nitrate tolerance [28,29]; indeed, its activity was found more than 50% lower in vessels from rats treated with GTN under *in vivo* settings than in vessels from sham-treated controls [30]. Since GTN releases NO, the formation of peroxynitrite from NO and superoxide is expected as reported in studies showing increased levels of tyrosine-nitrated proteins in tolerant tissue resulting by an enhancement of peroxynitrite formation *in vivo* [31]. Thus, these data suggest that peroxynitrite may represent a key event in GTN tolerance [32,33]. We hypothesized that nitration of ALDH-2 by peroxynitrite is a putative key mechanism which drives nitrate tolerance and on the basis of the preliminary data, this study aims: (1) to demonstrate the ROS-dependent inactivation of ALDH-2 in GTN-tolerant platelets and to clarify the role of free radicals in rats undergoing chronic exposure of organic nitrate; (2) to study the effect of peroxynitrite decomposition catalyst (MnTBAP) on ALDH-2 nitration in GTN tolerant SMC and platelets and to evaluate the rats response to chronic nitrate treatment in the presence of free radicals scavenger.

Materials and methods

GTN-induced tolerance in human platelets

All the patients participating to the study signed an informed consensus according to the European Legislation and the protocol was previously submitted to the approval of the Regional Ethical Committee. In addition, the study protocol was performed according to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in *a priori* approval by the institution's human research committee.

Unless specified, all reagents were purchased from Sigma–Aldrich.

Washed platelets preparation procedures have been described in detail elsewhere [8]. Briefly, blood samples from healthy volunteers (who had not taken drugs for at least 15 days prior blood collection) were collected by venopuncture into a plastic flask containing 3.15% sodium citrate (1:9, v/v) and immediately centrifuged at $800 \times g$ for 10 min to 37 °C to obtain the platelet rich plasma (PRP). To prepare washed platelets, a cocktail of protease inhibitor (*Sigma–Aldrich*) and indomethacin (10 μM) was added to the PRP. The mixture was incubated for 15 min at 37 °C to prevent the formation of cyclooxygenase products and then was added to the tenth part of a solution of ACD (sodium citrate dibasic, citric acid and glucose in ratio 1.25–0.75–1) and centrifuged at $800 \times g$ for 10 min. The supernatant was removed and the platelet pellet resuspended in equivalent volume of calcium-free oxygenated (95% O_2/5% CO_2) modified Tyrod's buffer (NaCl 136 mM, KCl 2.7 mM, $NaH_2PO_4 \times H_2O$ $MgCl_2 \times 6\ H_2O$ 2 mM, Hepes 10 mM, Glucose 0,1% and Albumin 0,2%), adjusted to pH 7.35 and osmolality ~300; platelets were again centrifuged at $800 \times g$ for 10 min and washed as before. The platelet count was adjusted to approximately $1.5–2 \times 10^8$ platelets/mL [34].

Platelet aggregation

A suspension of washed platelets was incubated at 37 °C for 4 min in a Payton dual channel aggregometer [35,36] with continuous stirring at 1000 rpm and then stimulated with thrombin (40 mU/mL) to give a submaximal aggregation (80–90%). When required, glyceryl trinitrate (GTN; 40–80–200 μM) was added to platelet suspension 3 min before the thrombin administration to produce a dose-dependent inhibition of platelet aggregation.

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