Vitamin C attenuates nitrate tolerance independently of its antioxidant effect

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Abstract In LLC-PK$_1$ kidney epithelial cells, a 5-h pretreatment with glyceryl trinitrate (GTN) resulted in substantial desensitization of the intracellular cyclic GMP response to a subsequent 10-min challenge with GTN (1 μM). GTN-tolerant cells were fully sensitive to the spontaneous nitric oxide (NO) donor spermine NONOate, which does not require enzymatic bioactivation. Cyclic GMP stimulation by GTN was up to 3.1-fold higher when vitamin C (1–10 mM) was present during the pretreatment period. In contrast, other oxygen radical scavengers such as tiron or dimethylsulfoxide and the NO scavenger PTIO left tolerance induction unaltered. Together, our results suggest that reactive oxygen species or NO do not contribute to the development of nitrate tolerance. Tolerance reduction by vitamin C may be due to a stabilizing effect on enzymes involved in the bioconversion of GTN to NO.

Key words: Nitrate tolerance; Nitric oxide; Cyclic guanosine monophosphate; Antioxidant; Vitamin C; Ascorbic acid

1. Introduction

Nitric acid esters such as glyceryl trinitrate (GTN) have been used for more than one hundred years in the therapy of myocardial ischemia and its principal symptom angina pectoris. The cellular mechanism underlying the antianginal effect of organic nitrates involves bioactivation of, and nitric oxide (NO) release from, these compounds which have therefore been coined NO prodrugs or NO donors. NO then activates soluble guanylyl cyclase which generates the vasodilatory second messenger cyclic GMP [1–3].

Sustained treatment of cardiovascular diseases with organic nitrates has long been known to induce tolerance to the hemodynamic and anti-ischemic effects of these drugs in humans and animals [4–6]. Despite its first description early this century, nitrate tolerance still poses an unsolved serious clinical problem with the basic mechanisms being only ill defined. Several lines of evidence point to down-regulation of enzymatic NO release from organic nitrates as being responsible for their diminished antianginal and vasodilatory action. [7–9]. Furthermore, irreversible blockade of the NO-sensitive soluble guanylyl cyclase has been demonstrated under conditions of nitrate tolerance [10,11]. Recently, it has been shown that prolonged exposure of rabbits to GTN is associated with enhanced superoxide production in the blood vessel wall [12] suggesting that oxidative stress may cause vascular desensitization to nitrates. In agreement with this hypothesis, co-administration of GTN and vitamin C in vivo has been found to counteract the development of tolerance [13,14]. However, conflicting results have been published as to whether antioxidants in general are capable of inhibiting vascular tolerance [15,16] leaving open the question of possible mechanisms underlying tolerance and tolerance reversal by vitamin C.

Using a cultured kidney epithelial cell line (LLC-PK$_1$), the present study investigates whether vitamin C as well as other antioxidants/radical scavengers are capable of reducing tolerance induction to GTN with respect to intracellular cyclic GMP accumulation. LLC-PK$_1$ cells have been established as a model for studying molecular mechanisms and pathways involved in organic nitrate-induced activation and desensitization of the guanylyl cyclase/cyclic GMP system [7,17].

2. Methods

2.1. Materials

LLC-PK$_1$ cells (ATCC CL 101) were obtained from the American Type Culture Collection (Rockville, MD, USA). Fetal calf serum, Ham's F-12 medium and penicillin-streptomycin were purchased from Gibco (Eggenstein, Germany). GTN was a gift from Schwarz Pharma AG (Monheim, Germany). 1,2-Dihydroxybenzene-3,5-disulfonate (tiron), vitamin C and all other reagents were obtained from Sigma (Deisenhofen, Germany). 2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) and spermine NONOate (Sper/NO) were obtained from Alexis Deutschland GmbH (Grünberg, Germany).

2.2. Cell culture

LLC-PK$_1$ cells were maintained and subcultured in Ham's F-12 medium, supplemented with 15% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were grown in a humidified incubator at 37°C and 5% CO_2.

2.3. Incubation procedure and cyclic GMP determination

Cells grown to confluence in 35-mm culture dishes were washed twice with phosphate-buffered saline. Cells were preincubated for 5 h with 1 ml of a basal salt solution containing (mM): NaCl, 130; KCl, 5.4; $CaCl_2$, 1.8; $MgCl_2$, 0.8; glucose, 5.5; and HEPES-NaOH, 20, buffered to pH 7.3) in the presence or absence of GTN (1 μM) and vitamin C, tiron, DMSO or PTIO. After the preincubation period, cells were washed twice with 2 ml phosphate buffered saline and incubated with the basal salt solution containing isobutylmethylxanthine (0.5 mM). After 10 min, GTN was added and the incubation was continued for another 10 min. The final assay volume was 1 ml. Supernatants were aspirated and cyclic GMP levels were determined by radioimmunoassay after addition of ethanol to the cells and subsequent evaporation as previously described [18].

3. Results

A 5-h preincubation of cells with GTN (1 μM) substantially reduced cyclic GMP elevation upon a subsequent 10-min exposure to 1 μM GTN (Fig. 1). However, GTN-tolerant cells

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Abbreviations: DMSO, dimethylsulfoxide; GTN, glyceryl trinitrate; NO, nitric oxide; PTIO, 2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide; Sper/NO, spermine NONOate

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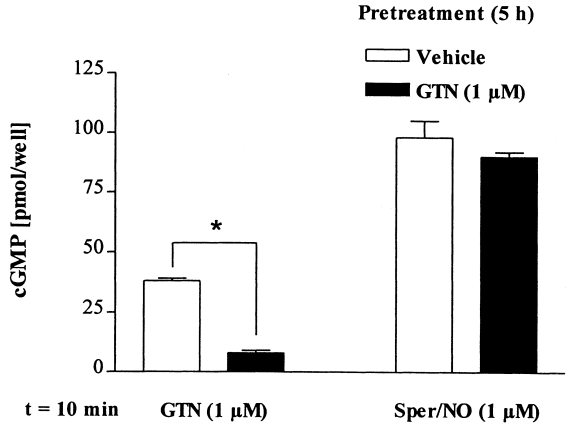

Fig. 1. Effect of a 5-h pretreatment with GTN on subsequent cyclic GMP accumulation by GTN and Sper/NO in LLC-PK_1 cells. Values are means ± S.E.M. of $n = 6$ observations. $^*P < 0.05$, vitamin C vs. vehicle (Student's two-tailed t-test).

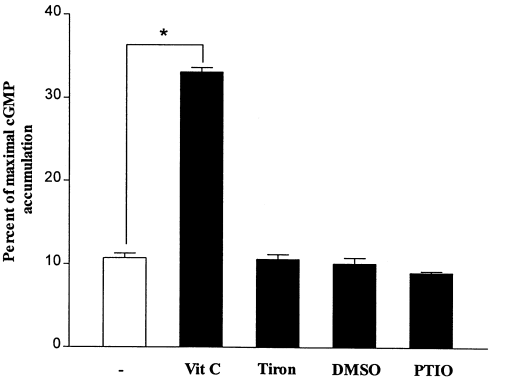

Fig. 3. Effect of vitamin C (Vit C, 10 mM), tiron (10 mM), DMSO (0.2% v/v), and PTIO (30 μM) on tolerance development to GTN in LLC-PK_1 cells. All cells were pretreated for 5 h with GTN (1 μM) in the absence (open column) or presence (solid columns) of the above mentioned compounds and subsequently challenged with 1 μM GTN. Values are means ± S.E.M. of $n = 6$ observations. $^*P < 0.05$ (ordinary one-way ANOVA plus Bonferroni test).

were fully responsive to Sper/NO (Fig. 1). Simultaneous incubation with vitamin C (1 and 10 mM) attenuated tolerance induction by GTN (Fig. 2). This effect was concentration-dependent in that 10 mM vitamin C allowed a significantly higher cyclic GMP stimulation by GTN in tolerant cells than 1 mM vitamin C ($P < 0.05$; ordinary one-way ANOVA plus Bonferroni test). Moreover, and as an additional control experiment, a 5-h incubation with vitamin C (1 and 10 mM) did not alter basal or GTN-induced cyclic GMP levels in non-tolerant cells (Fig. 2). Intracellular cyclic GMP concentrations in tolerant, vitamin C-treated cells did not differ from the respective vehicle-treated tolerant control at the end of the GTN-pretreatment period (data not shown). In cells pretreated with a combination of vitamin C and GTN, subsequent cyclic GMP-stimulation by GTN was 3.1-fold higher than in cells pretreated with GTN alone (Fig. 3). Under the same conditions, the intracellular superoxide anion scavenger tiron (10 mM) failed to attenuate nitrate tolerance (Fig. 3). Likewise, the intracellular scavenger of hydroxyl radicals and peroxynitrite DMSO (0.2% v/v) and the specific NO scavenger PTIO (30 μM) were without effect on the development of nitrate tolerance (Fig. 3).

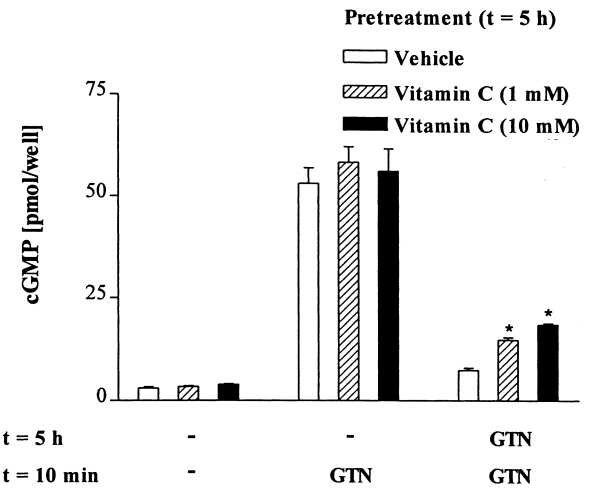

Fig. 2. Effect of vitamin C on GTN-induced tolerance in LLC-PK_1 cells. Cells were pretreated for 5 h, as indicated, in the absence (open columns) or presence (hatched and solid columns) of vitamin C. Values are means ± S.E.M. of $n = 6$ observations. $^*P < 0.05$ (ordinary one-way ANOVA plus Bonferroni test).

4. Discussion

The present study demonstrates attenuation of tolerance development to GTN by concurrent administration of vitamin C, whereas different oxygen and nitrogen radical scavengers failed to prevent GTN-induced desensitization of the cyclic GMP response. In control experiments, vitamin C did not change basal or GTN-induced cyclic GMP accumulation in non-tolerant cells. Moreover, vitamin C had no influence on cyclic GMP levels in tolerant cells when measured immediately after tolerance induction, i.e. prior to the rechallenge with GTN. Since vitamin C was washed out after the 5-h pretreatment period, preservation of the cyclic GMP response in tolerant cells by vitamin C cannot be due to increased extracellular metabolism of GTN during the 10-min stimulation period. Together, these data preclude a direct stimulatory effect of vitamin C on soluble guanylyl cyclase and argue against facilitation of non-enzymatic NO release from GTN via a chemical reaction with vitamin C. Our findings rather suggest that vitamin C reduces tolerance by interacting at a site upstream of NO-dependent guanylyl cyclase activation, which might be the enzymatic bioconversion of GTN to NO. That tolerance specifically affects NO release from nitric acid esters and leaves NO sensitivity of guanylyl cyclase unimpaired is further supported by our observation demonstrating an unaltered cyclic GMP response to spermine NONOate in GTN-tolerant cells. NO release from spermine NONOate occurs spontaneously in aqueous solution and does not require enzymatic catalysis [3].

According to previous studies, redox-active enzymes such as cytochrome P-450 play a crucial role in mediating NO release from GTN and appear to be down-regulated in tolerant vascular tissue [18–21]. Interestingly, NO at high concentrations has been shown to directly block cytochrome P-450 activity in cell-free systems [22]. In our study the specific NO scavenger PTIO [23,24] was unable to prevent desensitization of the cyclic GMP response indicating that in intact cells, tolerance develops independently of NO and that NO per se is not a relevant mediator of nitrate tolerance. This finding correlates

with clinical observations documenting lack of tolerance induction by spontaneous NO donors such as sodium nitroprusside or molsidomine/SIN-1 [25–27].

Superoxide anion radicals have recently been suggested to cause nitrate tolerance, possibly via reacting with NO to form peroxynitrite [12]. Peroxynitrite has a shorter half-life than NO and is a less potent activator of the soluble guanylyl cyclase/cyclic GMP system [12]. Superoxide originates during the redox process of nitrate bioactivation [28], and in addition to forming peroxynitrite is capable of inactivating organic nitrate converting enzymes such as cytochrome P-450 [13,29]. In the present investigation, however, the tolerance sparing effect of vitamin C was not reproduced by tiron, which is widely used as an intracellular superoxide anion scavenger [12,16,30]. Similarly, the scavenger of hydroxyl radicals and peroxynitrite anions DMSO [16,31,32] did not influence desensitization of the cyclic GMP response. These findings suggest that reactive oxygen species play no major role in the development of tolerance and that the preventive potential of vitamin C comprises mechanisms other than pure radical scavenging.

In addition to acting as an antioxidant, vitamin C is known to function as a co-factor in numerous enzymatic reactions and during processes leading to altered gene expression. Notably, vitamin C has been shown to maintain mRNA levels and to prevent down-regulation of cytochrome P-450-dependent enzymes including those isozymes which are able to catalyze NO formation from organic nitrates [33–35]. In those studies, preservation of cytochrome P-450 expression by vitamin C occurred independently of its antioxidant effect [36] which corresponds with our findings on tolerance prevention. That tolerance development may be successfully avoided by protecting cellular proteins from being down-regulated can also be concluded from a previous study showing the necessity of de novo protein synthesis for restoring cellular sensitivity to organic nitrates in tolerant cells [19].

In summary, our results show that vitamin C has a preventive potential on the development of nitrate tolerance. Its inhibitory action on the desensitization of the cyclic GMP response to organic nitrates appears not to be related to antioxidant effects and may result from preserving, at least in part, the enzymatic bioactivation of organic nitrates.

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