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Nitrate Tolerance Is Specific for Nitric Acid Esters and Its Recovery Requires an Intact Protein Synthesis

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Using cultured LLC-PK$_1$ cells the present study investigates mechanisms leading to nitrate tolerance and its reversal. A 5-h pretreatment with glyceryl trinitrate (GTN, 0.01-100 μM) resulted in desensitization of the intracellular cyclic GMP response to a subsequent 10-min challenge with GTN (1 μM). The spontaneous donor of nitric oxide (NO) spermine NONOate, which releases NO independently of enzymatic catalysis, did not induce tolerance to its own cyclic GMP stimulatory effect and remained fully effective in GTN-tolerant cells. Tolerant cells regained sensitivity to GTN after a 30-h incubation in media. Recovery of the cyclic GMP response was blocked in the presence of cycloheximide (10 μM) suggesting that de novo protein synthesis is necessary for tolerance reversal. Our results demonstrate that nitrate tolerance is specific for nitric acid esters and possibly due to down-regulation of enzymes involved in bioactivation of, and NO generation from, organic nitrates.

Organic nitrates such as glyceryl trinitrate (GTN) have been used for the therapy of myocardial ischemia and its principal symptom angina pectoris for more than one hundred years. The cellular mechanism underlying the antianginal effect of organic nitrates involves the conversion of these compounds into the nitric oxide (NO) free radical. NO then stimulates the soluble guanylyl cyclase, leading to increased intracellular levels of the vasodilatory second messenger cyclic GMP (1-3). Prolonged exposure to organic nitrates has long been known to induce tolerance to the cardiovascular effects of these drugs in humans and experimental animals (4-6). This desensitization phenomenon poses an as yet unsolved clinical problem with the underlying events being only poorly defined. Different mechanisms have been proposed to account for nitrate tolerance. At the cellular level, down-regulation of enzymes that participate in the bioactivation of nitric acid esters may entail diminished NO generation from these compounds (7-9). Sustained or irreversible inhibition of the NO-sensitive soluble guanylyl cyclase has also been demonstrated under conditions of nitrate tolerance (10, 11). Other authors have recently proposed that increased formation of oxygen radical formation during nitrate therapy causes a reduced response of vascular tissue to organic NO donors (12, 13).

The present study investigates mechanisms of tolerance, cross-tolerance and tolerance reversal at the cellular level using two different NO donors, GTN and spermine NONOate (Sper/NO). Whereas NO generation from GTN requires enzymatic catalysis presumably by cytochrome P450 (3, 8, 14, 15), Sper/NO releases NO spontaneously and independently of any co-factors or enzymes (16, 17). Most investigations published thus far have looked at mechanisms of tolerance induction rather than reversal, partly since commonly employed models such as isolated blood vessels have a limited life span of only several hours and thus do not survive long enough to study the day-long recovery period after tolerance induction (2, 6, 18). The cell line (LLC-PK$_1$) used in this investigation is an established model to study basic mechanisms involved in organic nitrate-induced desensitization of the guanylyl cyclase/cyclic GMP system (19, 20). The advantage of cultured cells lies in their infinite life allowing the exploration of long-term phenomena in general and in particular of the biological processes leading to recovery from tolerance.

MATERIALS AND METHODS

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, penicillin-streptomycin and L-glutamine were purchased from Gibco (Eggenstein, Germany). GTN was a gift from Schwarz Pharma AG (Monheim, Germany). Sper/NO was obtained from Alexis Deutschland GmbH (Grünberg, Germany). 3-Morpholinosydnonimine (linsidomine, SIN-1) was kindly provided by Hoechst AG (Frankfurt/Main). All other reagents were purchased from Sigma (Deisenhofen, Germany).

Cell culture. LLC-PK$_1$ cells were maintained and subcultured in Ham's F-12 medium, supplemented with 15% fetal calf serum, 100

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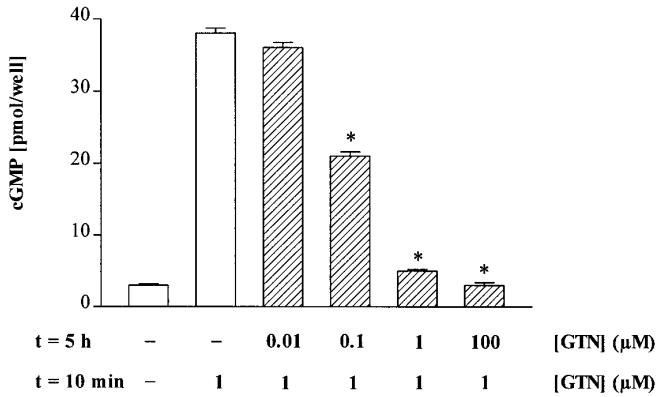

FIG. 1. Effect of a 5-h pretreatment with glyceryl trinitrate (GTN, 0.01-100 μM) on subsequent cyclic GMP accumulation by GTN (1 μM, 10 min) in LLC-PK$_1$ cells. Values are means ± SEM of n = 6 observations. *$P < 0.05$ (Ordinary one-way ANOVA plus Bonferroni test) *vs.* non-tolerant control stimulation with 1 μM GTN.

U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine. The cells were grown in a humidified incubator at 37°C and 5% CO_2.

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 with 1 ml of a balanced 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 NO donors. After the preincubation period (tolerance induction), cells were washed twice with 2 ml phosphate buffered saline and incubated with the balanced salt solution containing isobutylmethylxanthine (0.5 mM). After 10 min, NO donors were 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 (8). In experiments assessing recovery from tolerance, cells were washed twice with 2 ml phosphate buffered saline after pretreatment with GTN (tolerance induction) and then incubated for 30 h with media in the presence or absence of cycloheximide (recovery period). After recovery, cells were washed and cyclic GMP stimulation by a 10-min challenge with GTN was determined.

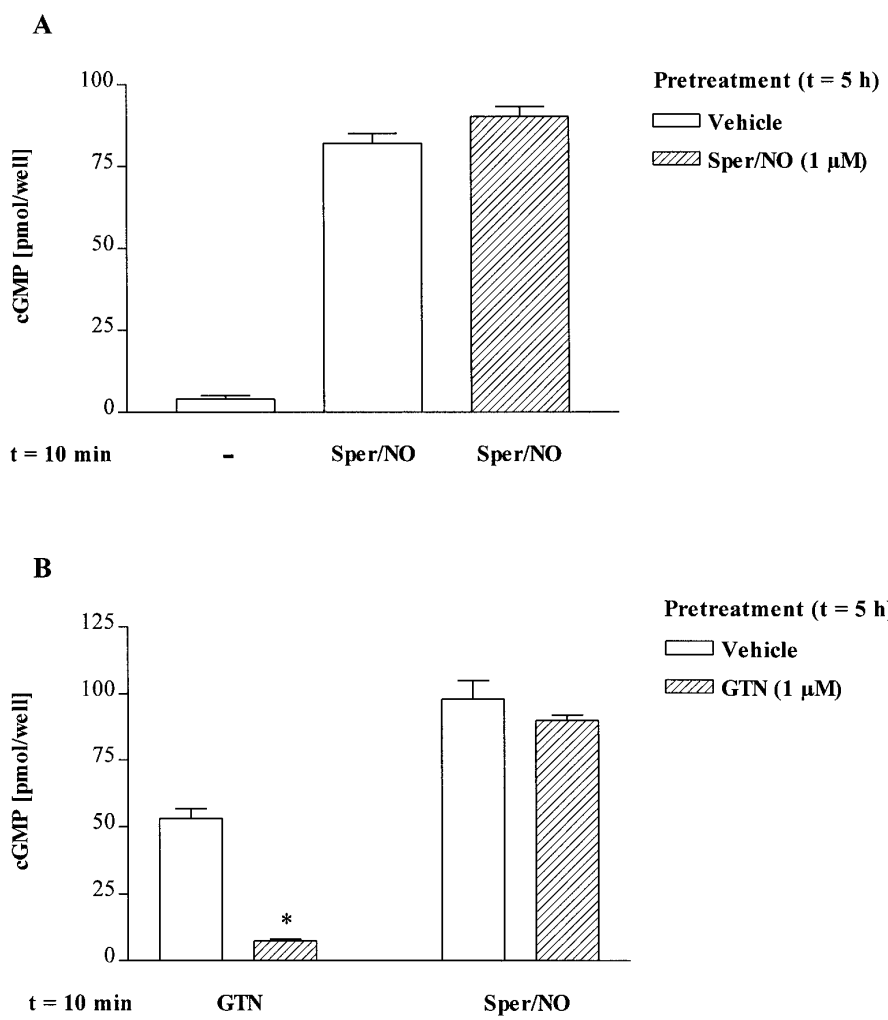

FIG. 2. Effect of a 5-h pretreatment with spermine NONOate (Sper/NO, panel A) or glyceryl trinitrate (GTN, panel B) on subsequent cyclic GMP accumulation by Sper/NO (1 μM, 10 min) in LLC-PK$_1$ cells. Values are means ± SEM of n = 6 observations. *$P < 0.05$ (Student's two-tailed *t* test) *vs.* corresponding non-tolerant control (vehicle).

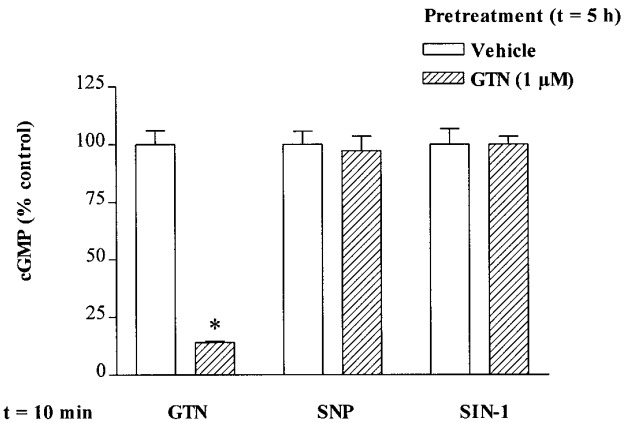

FIG. 3. Effect of a 5-h pretreatment with glyceryl trinitrate (GTN, 1 μM) on subsequent cyclic GMP accumulation by sodium nitroprusside (SNP, 1 μM, 10 min) and linsidomine (SIN-1, 10 μM, 10 min) in LLC-PK_1 cells. Values are means ± SEM of n = 6 observations. *$P < 0.05$ (Student's two-tailed t-test) *vs.* corresponding non-tolerant control (vehicle).

RESULTS

A 5-h pretreatment of cells with GTN (0.01-100 μM) reduced intracellular cyclic GMP stimulation by a subsequent 10-min challenge with GTN (1 μM) and led to complete desensitization in a concentration-dependent fashion (Fig. 1). Sper/NO (1 μM) did not induce tolerance to its own cyclic GMP stimulatory action (Fig. 2A) and remained fully active under conditions of nitrate tolerance (Fig. 2B). Similarly, the nitrovasodilators sodium nitroprusside (SNP) and SIN-1 were without cross-tolerance in GTN-tolerant cells (Fig. 3). GTN-tolerant cells regained sensitivity to GTN during a 30-h incubation in media after GTN washout (recovery period) (Fig. 4). Recovery of the cyclic GMP response to GTN was completely abolished when cycloheximide (10 μM) was present during the 30-h incubation (Fig. 4). In control experiments, a direct inhibitory effect of cycloheximide on basal or GTN-dependent cyclic GMP accumulation was excluded (not shown).

DISCUSSION

The basic mechanisms leading to diminished antianginal activity after prolonged or repeated administration of organic nitrates, a clinically relevant phenomenon known as nitrate tolerance, are still unknown. In the present study an established cell culture model of nitrate tolerance is used to explore GTN-induced desensitization of the cyclic GMP response which in vivo precedes and is responsible for the reduced vasodilatory action of organic nitrates under conditions of tolerance.

Desensitization of cyclic GMP stimulation occurred after pretreatment of cells with GTN at therapeutically relevant nano- and micromolar concentrations (21, 22). After a 5-h pretreatment with GTN, the capacity of LLC-PK_1 cells to respond with cyclic GMP increases to a subsequent challenge with the same compound was virtually abolished, i. e. the cells were completely tolerant. When trying to characterize the cellular events leading to tolerance, most investigations in the past have focussed on this time period of tolerance development using in vitro models such as isolated vascular tissues (2, 6, 18). However, in order to really understand what causes nitrate tolerance, it seems equally important to gain insight into pathways leading to restoration of the original sensitivity during the recovery period that corresponds with the nitrate-free intervall in therapy (18). Isolated organs have a limited survival time under experimental conditions and are therefore not a suitable model for studies lasting 24 h and more. In contrast, cultured cells offer the advantage of staying alive and showing unimpaired biochemical functions for days or weeks and thus represent an ideal system to study recovery from nitrate tolerance at the second messenger level.

In our study, LLC-PK_1 cells regained sensitivity to cyclic GMP stimulation by GTN after a 30-h incubation in media following nitrate washout. Recovery of the cyclic GMP response was completely blocked when the translation inhibitor cycloheximide was present during the incubation. This finding suggests that de novo synthesis or up-regulation of proteins is required for the recovery from nitrate tolerance. Down-regulation of proteins as a possible cause of tolerance might explain all three currently discussed mechanisms of tolerance induction. Thus, irreversible inactivation of the soluble guanylyl cyclase (10, 11) could be overcome by de novo synthesis of this enzyme during the recovery period. Similarly, enzymes catalysing bioactivation of, and NO release from, nitric acid esters (8, 14, 15) may be down-regulated through sustained exposure to GTN and resynthesized during therapy intervals. Since the expression of antioxidant defense proteins such as ferritin or superoxide dismutase is modulated by NO donors (23-26) it is conceivable that repression of these proteins contributes to the

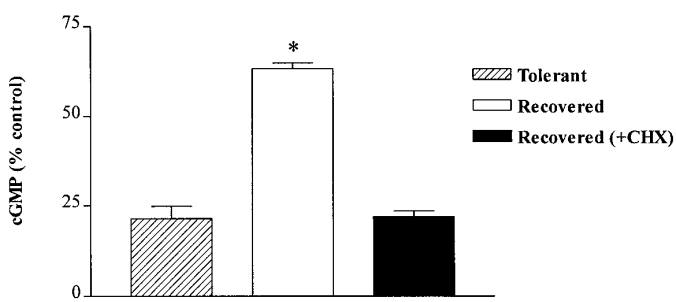

FIG. 4. Effect of cycloheximide (CHX, 10 μM) on the recovery of the cyclic GMP response to glyceryl trinitrate (GTN, 1 μM, 10 min). Cells were made tolerant by a 5-h pretreatment with 1 μM GTN and after nitrate washout allowed to recover during a 30-h incubation in media (in the presence or absence of cycloheximide as described under Materials and Methods). Values are means ± SEM of n = 6 observations. *$P < 0.05$ (Ordinary one-way ANOVA plus Bonferroni test) *vs.* tolerant cells.

increased superoxide formation observed under conditions of tolerance by some authors (12, 13). According to this hypothesis, renewed synthesis of antioxidant proteins during the recovery period would bring oxygen radical formation back to baseline values and thus restore sensitivity to nitrates.

In order to discriminate between the different potential sites of tolerance induction we have performed additional experiments using Sper/NO. Sper/NO belongs to a novel group of pure NO donors that have many advantages as research tools in cardiovascular pharmacology. NO release from Sper/NO is not catalyzed by exogenous thiols or enzymes and occurs spontaneously in aqueous solutions without giving rise to other radical species or by-products (3, 16, 17). In our study, Sper/NO did not cause desensitization to its own cyclic GMP stimulatory effect, and cells tolerant to GTN remained fully responsive to Sper/NO. This finding argues against down-regulation of soluble guanylyl cyclase in nitrate tolerant cells (10, 11) since under these circumstances cross-tolerance to other donors of NO or stimuli of cyclic GMP would occur. Likewise, increased formation of oxygen radicals by GTN (12, 13) should be expected to reduce cyclic GMP stimulation by Sper/NO, since superoxide reacts with NO to form peroxynitrite and thus blocks NO-dependent biological actions (27). The unaltered cyclic GMP response to Sper/NO in GTN-tolerant cells rather indicates that tolerance induction occurs at a site upstream of NO-dependent guanylyl cyclase activation, which might be the enzymatic bioconversion of GTN to NO. According to previous studies, enzymes such as cytochrome P-450 play a crucial role in mediating NO release from GTN and their down-regulation would specifically affect the biological activity of nitric acid esters (8, 14, 15). A spontaneous NO donor such as Sper/NO by-passes this bioactivation step and can therefore trigger cyclic GMP accumulation despite blockade of GTN metabolizing enzymes. In agreement with this, two other therapeutically used donors of NO that do not require enzymatic bioconversion, SNP and the active metabolite of molsidomine SIN-1, were found to be free of cross-tolerance in cells pretreated with, and tolerant to, GTN. This finding correlates with clinical observations documenting unaltered vasodilatory activity of SIN-1 and SNP in patients being tolerant to GTN (28-30).

Together, our results show that nitrate tolerance is a substance-specific phenomenon associated with nitric acid esters such as GTN but not seen with NO donors in general. The recovery from nitrate tolerance is dependent on the de novo synthesis or up-regulation of proteins. It is concluded that tolerance induction occurs at a site proximal to guanylyl cyclase activation and may involve down-regulation of enzymes catalyzing NO release from organic nitrates.

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