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A Common Pathway for Nitric Oxide Release from NO–Aspirin and Glyceryl Trinitrate

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NO-Aspirin (NCX-4016) releases nitric oxide (NO) in biological systems through as yet unidentified mechanisms. In LLC-PK1 kidney epithelial cells, a 5-h pretreatment with glyceryl tinitrate (GTN, 0.1–1 μM) significantly attenuated the cyclic GMP response to a subsequent challenge with both NO-aspirin or GTN. Similarly, NO-aspirin (10–100 μM) was found to induce tolerance to its own cyclic GMP stimulatory action and to that of GTN. In contrast, cyclic GMP stimulation by the spontaneous NO donor SIN-1, which releases NO independently of enzymatic catalysis, remained unimpaired in cells pretreated with GTN or NO-aspirin. The observed cross-tolerance between NO-aspirin and GTN cells indicates that bioactivation pathways of organic nitrates, which have been shown to involve cytochrome P450, may also be responsible for NO release from NO-aspirin. Prolonged treatment with NO-aspirin causes down-regulation of the cellular cyclic GMP response, suggesting that tolerance may occur during therapy with NO-aspirin. © 2000 Academic Press

Key Words: **nitric oxide; nitrate tolerance; cGMP; aspirin; glyceryl trinitrate; cultured cells; nonsteroidal anti-inflammatory drugs; linsidomine; SIN-1; nitric oxide donor.**

The major limitation to the long-term use of nonsteroidal anti-inflammatory drugs (NSAIDs) is the increased risk of gastrointestinal bleeding and development or exacerbation of gastric ulcers (1, 2). The mechanism by which NSAIDs cause intestinal damage is thought to be due to topical irritation of the epithelium because of their acidic nature as well as inhibitory action on the synthesis of gastroprotective prostaglandins (1, 3).

Abbreviations used: GTN, glyceryl trinitrate; NO, nitric oxide; NO-ASA, NO-aspirin, 2-acetoxybenzoate-2-[1-nitroxy-methyl]-phenyl-ester; NSAID, non-steroidal anti-inflammatory drug; PTIO, 2-Phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide; SIN-1, linsidomine, 3-morpholinosydnonimine.

Nitric oxide (NO) is recognized as a critical mediator of the gastrointestinal mucosal defense, exerting many of the same actions in the stomach as prostaglandins. In particular, NO functions as a smooth muscle relaxing agent and is thus thought to counteract the reduction in gastric blood flow caused by inhibitors of prostaglandin synthesis such as NSAIDs (4–6). Cytoprotective effects by NO may also occur independently of vasodilation through increased expression of stress proteins such as ferritin or heme oxygenase-1 (7, 8).

Based on these assumptions and with the intention to reduce side effects under NSAID therapy, the new class of NO releasing NSAIDs was developed (9). In various models of pain, inflammation and thrombosis, NO-NSAIDs produced effects similar, sometimes even superior to NO-free control compounds (9, 10). At the same time, NO-NSAIDs exhibited greatly reduced gastric or renal toxicity (9, 11). Clinical trials are currently under way to examine the safety and efficacy of several NO-NSAIDs in humans.

Despite a significant number of studies investigating the pharmacological profile of NO-NSAIDs there is little information available on pathways of bioactivation and NO release mechanisms. Since all NO-NSAIDs are nitric acid esters it may be assumed that NO formation from these compounds is mediated through processes similar to those responsible for NO generation from glyceryl trinitrate (GTN) and other organic nitrates. Previous studies have shown that NO release from organic nitrates is dependent on enzymatic catalysis involving cytochrome P450 (12–17). Prolonged treatment with nitric acid esters leads to specific down-regulation of these NO releasing pathways, a phenomenon that may contribute to nitrate tolerance and reduced antianginal activity of these drugs under clinical conditions (12, 16, 18). Using a cultured kidney epithelial cell line (LLC-PK_1), the present study investigates whether NO release from the aspirin derivative NCX-4016 (NO-aspirin) requires similar enzymatic catalysis, i.e., is likewise susceptible to down-regulation

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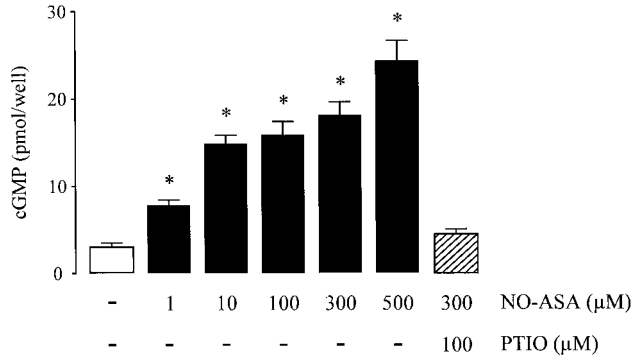

FIG. 1. Effect of NO-aspirin (NO-ASA) on cGMP accumulation (10 min) in LLC-PK1 cells. Values are means ± S.E.M. of $n = 6$ observations. *$P < 0.05$, NO-aspirin vs vehicle (one-way ANOVA plus Bonferroni test).

and may thus be affected by nitrate tolerance or cross-tolerance. Stimulation of the second messenger cGMP as a measure of NO release from NO-aspirin is assessed after various tolerance inducing treatments and compared to two other clinically employed NO donors, GTN and linsidomine (SIN-1) under the same conditions. LLC-PK_1 cells have been established as a model for studying molecular mechanisms and pathways involved in organic nitrate-induced activation of the guanylyl cyclase/cGMP system (19, 20).

MATERIALS AND METHODS

Materials. LLC-PK1 cells (ATCC CL 101) were obtained from the American Type Culture Collection, Rockville, MD, USA. Fetale bovine serum, Ham's F-12 medium, Dulbecco's modified Eagle medium and penicillin-streptomycin were purchased from Gibco, Eggenstein, FRG. NO-aspirin (2-acetoxybenzoate-2-[1-nitroxy-methyl]-phenyl-ester, NCX-4016) was kindly provided by Nicox, Nice, France. GTN was a gift from Schwarz Pharma AG, Monheim, FRG. SIN-1 (linsidomine, 3-morpholinosydnonimine) was provided by Aventis, Frankfurt, FRG. 2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) was obtained from Alexis Deutschland, Grünberg, FRG.

Cell culture. LLC-PK1 cells were maintained and subcultured in Ham's F12 medium supplemented with 20% Dulbecco's modified Eagle medium, 15% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were grown in a humidified incubator at 37°C and 5% CO2.

Incubation procedure and cGMP determination. Cells grown to confluence in 35-mm culture dishes were washed twice with phosphate-buffered saline. Cells were preincubated for 5 h with Ham's F12 medium in the presence or absence of NO donors or NO-aspirin. After the preincubation period, cells were washed twice with phosphate-buffered saline and incubated with Ham's F12 medium containing 0.5 mM isobutylmethylxanthine. After 10 min, NO-aspirin or NO donors were added and the incubation was continued for another 10 min. The NO-scavenger PTIO was added 10 min prior to the NO donors. The final assay volume was 1 ml. Supernatants were aspirated and cGMP levels were determined by radioimmunoassay after addition of ethanol to the cells and subsequent evaporation as described previously (13).

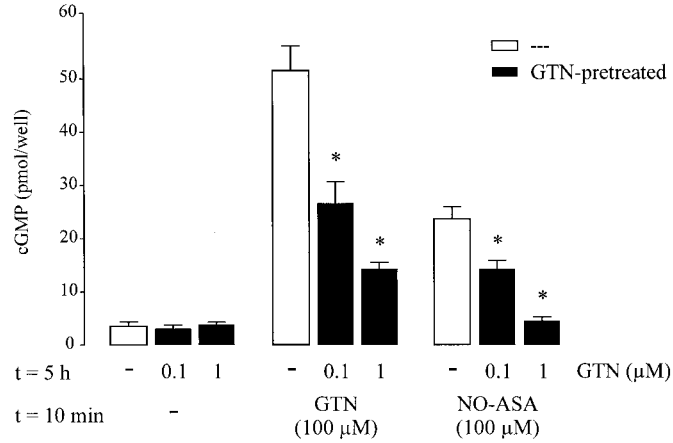

FIG. 2. Effect of a 5-h pretreatment with GTN on subsequent cGMP accumulation (10 min) by GTN or NO-aspirin (NO-ASA) in LLC-PK1 cells. Values are means ± S.E.M. of $n = 6$ observations. *$P < 0.05$, GTN-pretreated (filled columns) vs untreated (open columns) cells (one-way ANOVA plus Bonferroni test).

RESULTS

In LLC-PK1 cells, NO-aspirin (1–500 μM) led to a concentration-dependent increase in cGMP up to 9-fold over basal (Fig. 1). The NO-scavenger PTIO (100 μM) completely abolished cGMP stimulation by NO-aspirin (Fig. 1). PTIO alone was without effect on basal cGMP accumulation (not shown). A 5-h pretreatment with GTN (0.1–1 μM) significantly attenuated the cGMP response to a subsequent 10-min challenge with both NO-aspirin or GTN (Fig. 2). Using the same incubation protocol, NO-aspirin (10–100 μM) was found to induce tolerance to its own cGMP stimulatory action and to that of GTN (Fig. 3). In contrast and as a control, cGMP stimulation by the spontaneous NO donor SIN-1 (10 μM), which releases NO independently of enzymatic catalysis, remained unimpaired in cells pretreated

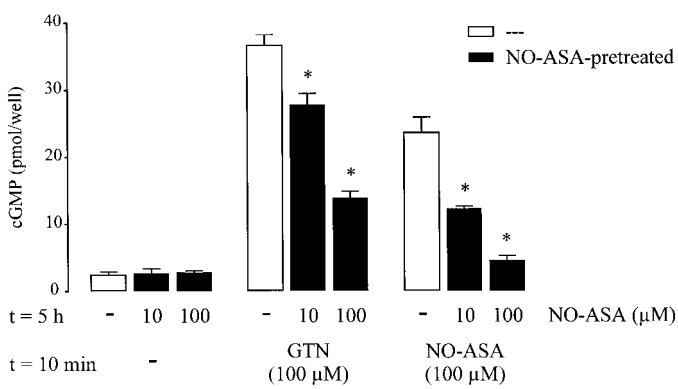

FIG. 3. Effect of a 5-h pretreatment with NO-aspirin (NO-ASA) on subsequent cGMP accumulation (10 min) by GTN or NO-ASA in LLC-PK1 cells. Values are means ± S.E.M. of $n = 6$ observations. *$P < 0.05$, NO-ASA-pretreated (filled columns) vs untreated (open columns) cells (one-way ANOVA plus Bonferroni test).

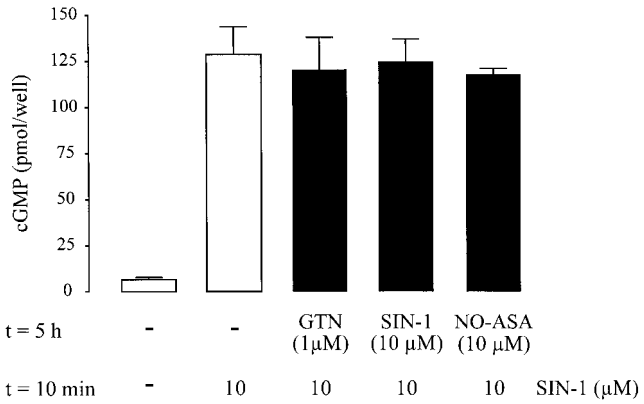

FIG. 4. Effect of a 5-h pretreatment with GTN, SIN-1, or NO-aspirin (NO-ASA) on subsequent cGMP accumulation (10 min) by SIN-1 in LLC-PK1 cells. Values are means ± S.E.M. of $n = 6$ observations.

with GTN (1 μM) or NO-aspirin (10 μM) (Fig. 4). Basal cGMP accumulation was not significantly altered in cells pretreated with GTN, SIN-1 or NO-aspirin.

DISCUSSION

The present study demonstrates that NO-aspirin, a newly developed NSAID with an NO moiety, is bioactivated through pathways that are also responsible for NO formation from nitric acid esters such as GTN. Using LLC-PK1 cells, an established cell culture model for studying organic nitrate-dependent activation of the guanylyl cyclase/cGMP system (19, 20), the present study compares NO-aspirin to the clinically used NO donors GTN and SIN-1. NO-aspirin produced a concentration-dependent increase in cGMP that was abrogated in the presence of the NO scavenger PTIO (21) and is thus fully attributable to released NO. Cells pretreated with GTN at nanomolar and low micromolar concentrations showed a reduced cGMP response to a subsequent challenge with both GTN and NO-aspirin. This cross-tolerance to NO-aspirin in GTN-tolerant cells suggests that mechanisms underlying bioactivation of GTN also play a pivotal role in mediating NO release from NO-aspirin. According to earlier reports, bioconversion of GTN to NO is dependent on redox-active enzymes such as cytochrome P450, which are down-regulated in tolerant vascular tissue (12–18). Thus, it is reasonable to assume that the same enzymes assume a crucial function in catalyzing NO generation from NO-aspirin. This is further corroborated by our observation that NO-aspirin after several hours of pretreatment induced tolerance to its own cGMP stimulatory action and to that of GTN. These findings clearly demonstrate bidirectional cross-tolerance between GTN and NO-aspirin and argue strongly for a common pathway of NO generation residing in the same down-regulatable protein. Moreover, cells made tolerant to glyceryl tinitrate or NO-aspirin remained fully responsive to SIN-1. Data from a number of sources including ours have clearly demonstrated that SIN-1 in intact cells, i.e., in the presence of electron acceptors other than oxygen, functions as a true and spontaneous donor of NO without requiring enzymatic catalysis (7, 8, 22, 23). Therefore, the unimpaired cGMP stimulation by SIN-1 in NO-aspirin-treated cells precludes desensitization of soluble guanylyl cyclase or a decrease in NO half-life under these circumstances and indicates that tolerance induction rather occurs at a site upstream of NO-dependent guanylyl cyclase activation. It can be concluded that this site is a protein responsible for the bioconversion of both NO-aspirin and GTN to NO.

In the present study prolonged exposure to NO-aspirin at concentrations as low as 10 μM resulted in significant attenuation of the cGMP response. Since analgesic dosing regimens for NO-NSAIDs correspond to those of the NO-free parent compounds, concentrations of 10–100 μM are at the lower end of expected tissue or plasma levels and therefore of clinical relevance (10, 11). Thus, future studies will have to address the question of whether patients treated with NO-aspirin and other NO-NSAIDs will develop tolerance to the NO-mediated actions of these drugs and other nitric acid esters.

Together we have shown that bidirectional cross-tolerance exists between the organic nitrate GTN and NO-aspirin at the level of cGMP stimulation suggesting that a common pathway mediates bioactivation of, and NO release from, these compounds. Our data suggest that tolerance induction at least to the NO-dependent actions may occur under therapy with NO-NSAIDs and may require eccentric dosing and drug-free intervals as with long-acting organic nitrates.

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