LABORATORY INVESTIGATION

NITRATES AND EICOSANOIDs

Nitrates and endothelial prostacyclin production: studies in vitro

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ABSTRACT The hypothesis that nitrates evoke prostacyclin production by vascular endothelium has been reevaluated on cultured umbilical vein endothelial cells and in vascular fragments, both obtained from humans. Endothelial cell monolayers (passages 1 and 2) were washed free of culture medium and exposed for 3 to 5 min to buffer or nitroglycerin (NTG), isosorbide dinitrate (ISDN), or isosorbide-5-mononitrate (ISMN) over a range of concentrations (10^-8M to 10^-4M) encompassing those usually attained in vivo, with or without 25 μM sodium arachidonate. Basal prostacyclin production, measured by radioimmunoassay of the stable metabolite 6-keto-PGF_1α, depended on cell density in the endothelial monolayer (being higher in preconfluent cultures) and on incubation time. Basal prostacyclin, however, was not altered by incubation with NTG (3.3 ± 2.0 pg/1000 cells without drug vs 3.9 ± 3.8 pg/1000 cells with drug, mean ± SD), ISDN (3.1 ± 1.9 vs 3.1 ± 2.2), or ISMN (2.0 ± 0.9 vs 2.3 ± 1.5) at 10^-7M (all differences NS). Also, long-term incubation (2, 6, and 24 hr) with ISDN and ISMN did not alter prostacyclin production over control. Over a 30-fold increase (p < .001) in prostacyclin production was obtained with arachidonate stimulation, but incubation with nitrates did not significantly modify the stimulated production. Saphenous vein, mesenteric artery, and atrial appendage fragments incubated at 37° C for 20 min in a shaking water bath with a control buffer produced 27.8 ± 13.9, 189.7 ± 75.2, and 662.3 ± 390.6 pg 6-keto-PGF_1α/mg tissue, respectively. Arachidonate (25 μM) increased prostacyclin production by these tissues to 158.8 ± 120.6 (p < .001), 381.0 ± 48.2 (p < .01), and 885.6 ± 291.4 (NS), respectively. However, no significant variation from control prostacyclin was obtained with 20 min exposure to NTG, ISDN, or ISMN in a range from 10^-8M to 10^-4M. No dose-effect relationship for these drugs on prostacyclin production was found. These data suggest that nitrates do not increase endothelial prostacyclin production.

preparations (Nitrostat IV, Parke-Davis, Morris Plains, NJ; and Tridil, American Critical Care, McGaw Park, IL), isosorbide dinitrate (ISDN) (Isoket, Pharma-Schwarz, GMBH, Monheim, Federal Republic of Germany), and isosorbide-5-mononitrate (ISMN) (Elantan, Sanol Schwarz, GMBH, Monheim). ISMN is the product of liver biotransformation of ISDN and is thought to be mainly responsible for the sustained effects of the parent drug in vivo. All drugs, representing several different lots of clinical preparations, were used before expiration date. Since NTG may be adsorbed to plastic, dilutions of all drugs were made in glass vials. A new ampule of the drug was used in each experiment. Dilutions of each drug were made in a buffer containing 10 mM sodium N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate (HEPES, Sigma, Chemical Co., St. Louis), 137 mM NaCl, 5 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, and 5.5 mM glucose, pH 7.35 at 37°C (referred to as HEPES buffer). Preliminary specific experiments demonstrated that each of the vehicles of the various drugs tested, over the full range of concentrations used, induced prostacyclin production comparable to that induced with the HEPES buffer alone, thus ruling out any nonspecific effect of the excipient contained in any of the drug preparations. These vehicles were 5% (volume to volume) absolute ethanol in water for Nitrostat IV, 30% ethanol and 30% propylene glycol in water for Tridil, and isotonical saline for both Isoket and Elantan. This last vehicle was therefore used in experiments involving multiple comparisons of different drugs. Dilutions of vehicles, as for drugs, were made in HEPES buffer. Final concentrations from 10^{-6} M to 10^{-3} M of each drug were prepared shortly before use. These concentrations encompass the maximum clinically attainable venous concentration, which is about 10^{-3} M for both NTG and the long-acting nitrates. Only the selected dose range also spans arterial levels of these drugs, reported as higher than the venous ones during intravenous infusions in patients.

Two tissue systems were used: cultured endothelial cells and vascular fragments, both derived from human sources. In the first system, prostacyclin production in the presence of nitrates was studied in human umbilical vein endothelial cell monolayers. These were cultured in 24-well (16 mm diameter) cluster plates (Costar, Cambridge, MA) as previously described. After each experiment, cell counts of each well were performed as previously described. Since shear rate stress has been shown to induce prostacyclin production by endothelial cells, a nonvibrating thermostatic bath (Meyer N-Evap R, Organoma- tion Associates Inc., South Berlin, MA) was used at 37°C. Each monolayer of endothelial cells was washed twice with HEPES buffer to remove the culture medium and then incubated with the drug to be tested at the appropriate dilution or with the control buffer. Special care was taken to standardize the washing procedure and the timing of the washing steps to avoid pH changes in culture wells. For this purpose, control experiments were performed limiting the number of wells used in a single experiment to 18, 12, or six wells per plate. A total of 474 monolayers of human umbilical vein endothelial cells were used. The possibilities that nitrate effects vary between early and late cell passages, depend on the degree of cell confluence, or are detected in either basal or stimulated conditions were all explored. For this purpose we tested passage 1 and 2 cells (first and second passage after primary cultures) at confluence and preconfluent cell densities (<100,000 cells/well), unstimulated or stimulated with 25 μM sodium arachidonate (AA) (NuChek, Elysian, MN). Incubation times were 3 and 5 min for all drugs. For long-acting nitrates, prolonged incubations (3, 6, and 24 hr at 37°C under 5% CO₂) were also used, diluting the drugs or the control vehicle directly in serum-containing tissue culture medium. At the end of the incubation times, supernatants were directly collected and stored frozen until the assay (see below).

In the second system, prostacyclin production in the presence of nitrates was assessed on human vascular fragments removed at surgery. Fragments were obtained from patients undergoing coronary or abdominal surgery. In most of these patients, platelet aggregation in response to AA, ADP, adrenaline, and collagen was performed in a four-channel aggregation module (Platelet Aggregation Profiler II, Bio-Data Corp., Hatboro, PA), with fixed concentrations of 250 μM, 1 μM, and 0.8 μg/ml, respectively, of these agents, according to previously described techniques. If no aggregation occurred with AA, a fresh sample of platelet-rich plasma was challenged with an endoperoxide analogue (U-44069, 3μM, a gift of Dr. John Pike, Upjohn Co., Kalamazoo, MI), which bypasses aspirin inhibition of platelet cyclooxygenase. Aggregation with U-44069 confirmed an aspirin-induced or aspirin-like aggregation defect. Also, samples of freshly drawn whole venous blood were placed in plain glass tubes for measurement of serum thromboxane (TX) B₂ by radioimmunoassay. Recording of platelet aggregation and/or measurement of serum TXB₂ generation served to identify patients treated with nonsteroidal anti-inflammatory agents. The existence of an aspirin-like effect was defined as less than 10% aggregation with AA with a positive response to U-44069 or a reduction in serum TXB₂ levels to less than 100 ng/ml of serum. Aggregation with other agents was confirmatory. In the few (<10%) patients for whom blood sampling was unavailable, history of recent (<10 days before the operation) exposure to nonsteroidal anti-inflammatory agents was carefully checked. Only patients without evidence of such exposure were included in the study.

The vascular fragments were derived from three different sources: saphenous vein, mesenteric artery, and atrial appendage (113, 48, and 111 fragments, respectively). These were selected to represent venous, arterial, and capillary types of vascular endothelium, respectively. Most fragments (211/272) were processed within 4 hr of surgery, and all within 18 hr. The technique used for handling and treating the fragments was similar to that previously described. Segments of the vessels or fragments of atrial appendages were cut into grossly uniform pieces weighing a minimum of 10 mg (weight), placed into polypropylene tubes containing 1 ml of a solution of the test drug at the selected concentration or into the control buffer, and prewarmed at 37°C for 5 min. Tubes were subsequently shaken in a water bath shaker at 37°C and 20 oscillations/min for 15 min, with or without the addition of AA at 25 μM final concentration. Some experiments were also performed without shaking, in unstimulated conditions. At the end of the timed procedure, buffer was removed and centrifuged at 15,600 g for 3 min to sediment cellular elements, and the supernatant was rapidly frozen for later measurement of prostacyclin.

Measurement of thromboxane and prostacyclin. TXB₂ was measured in appropriately diluted serum by a previously detailed radioimmunoassay, with a double-antibody separation using Immunobeads (Bio-Rad Laboratories, Richmond, CA). Samples for prostacyclin measurement were incubated at 37°C for 1 hr to convert all prostacyclin to 6-keto-PGF₁α, and the latter compound was measured by radioimmunoassay. A double-antibody separation (Immunobeads) was used in experiments in which prostacyclin was assayed in a serum-containing medium (long-term experiments with ISDN and ISMN). Levels of prostacyclin in the supernatant medium from cultured endothelial cells were expressed as picograms of 6-keto-PGF₁α per 1000 cells and levels of prostacyclin in the incubation medium of vascular fragments as picogram per milligram of tissue wet weight.

Statistical analysis. Student’s t test for paired or unpaired samples was used in experiments involving single comparisons of treatments with one drug concentration vs controls. When
multiple comparisons were made within a single experiment, one-way (for one drug, in different concentrations, vs control) or two-way (for more than one drug, with different concentrations) analysis of variance was used. An HP 9815 A computer (Hewlett-Packard Co., Palo Alto, CA) with prepared programs contained in volumes I and II of the HP statistic library (09815-15001) was used. Unpaired t tests in single comparisons and analysis of variance in multiple comparisons were also used when data were pooled from different experiments. Relationships between the logarithm of the doses and prostacyclin production were analyzed by linear regression. A minimum level of significance was set at p < .05. All results are expressed as mean ± SD.

Results

Experiments with cultured endothelial cells. Basal production of prostacyclin by endothelial cells was examined in different culture passages (1 and 2), at confluent and preconfluent cell densities, and with different exposure times (3 and 5 min, 2, 6, and 24 hr). When these individual variables were examined, the cell density of the monolayer was the most important factor for prostacyclin production. First-passage cells incubated for 5 min with control buffer produced (mean ± SD) 2.8 ± 2.1 pg/1000 cells at confluence and 6.7 ± 2.6 pg/1000 cells at preconfluence (p < .001). Production also appeared to be affected by increasing the incubation time from 3 to 5 min (0.9 ± 0.4 vs 3.3 ± 1.4 pg/1000 cells at confluence, respectively). A slow increase of prostacyclin production was observed in long-term experiments: basal production was 18.3 ± 1.7 pg/1000 cells after 2 hr, 20.0 ± 2.1 pg/1000 cells after 6 hr, and 31.2 ± 7.2 pg/1000 cells after 24 hr incubation of the cells in medium containing 20% human serum (p < 0.001 by analysis of variance). However, direct comparison with short-term experiments is not possible because culture medium, instead of HEPES buffer, was used in these last experiments. Passage 1 and 2 cells, when the other variables were held fixed, produced comparable amounts of prostacyclin: at confluence, with 5 min incubation, prostacyclin production was 2.8 ± 2.1 and 3.2 ± 1.5 pg/1000 cells, respectively (p = NS). The results of incubation of endothelial cells with NTG on prostacyclin production are shown in figure 1. Over a 30-fold increase of prostacyclin production was obtained with AA stimulation. In control conditions, with passage 1 cells at confluence and 5 min incubation, prostacyclin production was 3.2 ± 1.9 pg/1000 cells in the basal and 94.1 ± 25.3 in the AA-stimulated setting (p < .001). However, no clear stimulatory effect of the NTG incubation was evident, and no trend appeared as a function of the dose (figure 1).

The results of short-term incubation of ISDN and ISMN on basal prostacyclin production by cultured endothelial cells are represented in figure 2. Here too, neither drug modified basal production of prostacyclin. The possibility that a dose-effect relationship might appear at doses below 10^-8M was ruled out with ISDN and ISMN in a lower range of doses reaching 5 × 10^-10M (data not shown). In no case was any significant correlation between doses of the drug and prostacyclin production found. Negative results were also obtained for any effect of these drugs on AA-stimulated production of prostacyclin. Furthermore, the long-term (2, 6, and 24 hr) exposure of endothelial cells to these drugs did not change their spontaneous production (with 2 hr incubation — control, 18.3 ± 1.7; ISDN 10^-7M, 20.5 ± 0.4; ISMN 10^-7M, 18.2 ±

**FIGURE 1.** Nitroglycerin and prostacyclin production by cultured human endothelial cells (passage 1, confluent, 5 min incubation). *Left.* Basal production of prostacyclin. *Right.* AA-induced production of prostacyclin. The control vehicle and three increasing concentrations of NTG are represented on the abscissa; prostacyclin production, measured as concentration of 6-keto-PGF_1α in the supernatant, is shown on the ordinate. The total number of experiments used to compare control vs drug-exposed cells is shown within each bar, with the total number of monolayers treated for each experimental subset in parentheses. Differences in prostacyclin production between control vs drug-exposed monolayers are all nonsignificant.
1.6; with 6 hr incubation — control, 20.0 ± 2.1; ISDN 10^{-7}M, 18.1 ± 0.6; ISMN 10^{-7}M, 22.4 ± 0.3; with 24 hr incubation — control, 31.2 ± 7.2; ISDN 10^{-7}M, 30.7 ± 10.4; ISMN 10^{-7}M, 32.5 ± 6.0; all differences drug vs control are nonsignificant.

Experiments with vascular fragments. Basal production of prostacyclin was 27.8 ± 13.9 pg/mg for saphenous veins, 189.7 ± 75.2 pg/mg for mesenteric arteries, and 662.3 ± 390.6 pg/mg for atrial appendages. Each of these values is significantly different from the others (p < .001, Scheffe’s multiple contrasts after one-way analysis of variance). The highest value observed with atrial appendages was compatible with the histologic appearance of the samples, which consisted of a sponglike structure with a large endothelial surface area per unit of tissue wet weight. In the system used, tissue responded well to stimulation with AA. After 15 min incubation with 25 μM AA, prostacyclin production increased to 158.8 ± 120.6 pg/mg for saphenous veins (p < .001), 381 ± 48.7 pg/mg for mesenteric arteries (p < 0.01), and 885.6 ± 291.4 pg/mg for atrial appendages (p = NS). However, none of the concentrations of NTG, ISDN, or ISMN used (10^{-7}M to 10^{-6}M) modified prostacyclin production, either in basal or in AA-stimulated conditions. Results for saphenous vein at the 10^{-7}M concentration are shown in figure 3, and are superimposable to those obtained at the other drug concentrations. The results

FIGURE 3. Nitrates and prostacyclin production by human saphenous vein fragments. Left. Effects on basal prostacyclin production of one concentration (10^{-7}M) of nitrates. Right. Effects on AA-stimulated prostacyclin production of a similar concentration of each drug. The control vehicle, NTG, ISDN, and ISMN are represented on the abscissa, and prostacyclin production, measured by concentrations of 6-keto-PGF_{1α} in the incubation medium, is on the ordinate. The total number of experiments used to compare control vs drug-exposed fragments is reported within the bars, with the number of fragments in parentheses. Differences in prostacyclin production between control vs drug-exposed fragments are all nonsignificant.
in the two other systems, atrial appendages and mesenteric arteries, are shown in figure 4, representing data pooled for concentrations from $10^{-8}M$ to $10^{-6}M$. This pooling of data is possible since (1) all these concentrations are within or just above the range reached in clinical situations and (2) no dose-related difference was observed in prostacyclin production in single-dose experiments. Indeed, prostacyclin production by atrial appendages and mesenteric arteries showed a trend toward decreased production in the presence of nitroderivatives as compared with control values (in figure 4 most points are below the identity line).

Discussion

The mechanism by which nitrates relieve myocardial ischemia remains a matter of current debate, both at hemodynamic and cellular levels of action. Dilatation of peripheral veins and arteries, increase in collateral blood flow to areas supplied by diseased coronary arteries, intramyocardial redistribution of flow from nonischemic to ischemic areas, and specific dilatation of coronary arteries including stenotic segments are the most commonly postulated mechanisms. Beyond these vascular effects, nitrates also possess other properties potentially useful in therapy of patients with coronary artery disease. Nitrates are able to inhibit platelet function at concentrations in vivo that are ineffective in vitro, thereby suggesting an indirect mechanism of action. Thus reports that NTG stimulates the production of vasodilator and antiaggregatory prostacyclin by endothelial cells and by vascular fragments have received considerable attention. Since prostacyclin synthesis can be depressed by commonly used nonsteroidal anti-inflammatory agents like aspirin, it has even been advised that "physicians should be cautious in giving the drug [aspirin] to patients receiving nitroglycerin for relief of myocardial ischemia or infarction in whom increased vasomotor tone is suspected." Contradictory reports, however, have also appeared, suggesting the need for further validation of the hypothesis that nitrates stimulate prostacyclin synthesis. The findings of our study do not support the idea that nitrates increase prostacyclin production by cultured human endothelial cells or by human vascular fragments. NTG and two long-acting nitrates, ISDN and ISMN, were used in this study with virtually identical results.

Our work first reproduced the specific conditions previously used for endothelial cell cultures. Additional studies addressed possible sources of variation for prostacyclin production by cultured endothelial cells, such as cell passage number, cell density, and incubation time. Cell density and growth state affect prostacyclin synthesis, which, in human endothelial cells, is higher in preconfluent than confluent monolayer cultures. Prostacyclin production also increases with incubation time both over the short range (1 to 5 min) and over a 24 hr period. Early passage number (primary or secondary passage), on the other hand, does not significantly influence prostacyclin production.

The drugs used to test for nitrate effects on prostacyclin production by human atrial appendages (left) and mesenteric arteries (right). The abscissa represents NTG, ISDN, and ISMN, summing data with concentrations from $10^{-8}M$ to $10^{-6}M$. The ordinate represents the percent change from control 6-keto-PGF$_{1\alpha}$ measurement, as a reflection of changes in prostacyclin production.
Clin production were all clinical preparations. Since stimulation of prostacyclin production was previously reported with a preparation of NTG no longer manufactured,5 two other preparations were used and compared. Neither of the two elicited a significant increase in basal or AA-stimulated production. Similar negative findings were obtained with the two long-acting nitrates tested, which act by similar, if not identical, chemical mechanisms.32 The possibility of a statistical type-B error was minimized by the large number of experiments performed and by the use of several similarly acting drugs in different doses. Although individual experiments varied in terms of prostacyclin production, such variation was not unidirectional, consistent, or generalizable. The baseline values of prostacyclin synthesis by different cultures demonstrated a large standard deviation, exceeding 50% of the mean value. However, both the mean values of basal prostacyclin synthesis and the standard deviations were similar to those usually obtained in our laboratory, and the basal production found in these studies is virtually the same as that previously reported.5 We therefore consider this baseline variability as inherent in the method used. The extent of this variability may prevent the detection of very subtle drug effects, but any biological significance of more subtle changes in prostacyclin production is doubtful. The conclusion that nitrates do not elicit prostacyclin production by cultured human endothelial cells is further supported by the lack of a dose-effect relationship over the wide range of doses tested (more than four orders of magnitude), which spanned the range of clinically attainable concentrations of these drugs. A dose-effect relationship does not exist in our experimental results either for each drug alone or for combined data on all drugs.

Our results with vascular fragments support the tissue culture data. Human vascular fragments respond to stimulus with AA with a clear increase in prostacyclin production; however, the vascular tissues do not show increases in prostacyclin after incubation with any of the three different nitrates used.

Reports that vascular fragments produce more prostacyclin when treated with NTG have appeared.6–7 In one study, stimulation of basal production of prostacyclin by NTG from slices of bovine coronary arteries and rabbit aortas was found.6 In the other, human saphenous vein rings increased prostacyclin synthesis only when stimulated with AA.7 Our system of treating vascular fragments differs from the one used by Schror et al.5 in duration of incubation and in type and species of vessels assayed. However, the prolonged incubation times used by these authors for NTG are much greater than is needed for the drug to evoke its vascular effects. Our incubation times are more pertinent to clinical use.

Prostacyclin release in the presence of NTG has also been studied in the Langendorff rabbit and guinea pig heart preparations, in isolated rabbit aortic rings after addition of cyclic endoperoxides, and in pig aortic microsomes.9 No clear effect of NTG was demonstrated. We similarly found no effect with ISDN in the rat Langendorff heart preparation (De Caterina et al., unpublished observations). Neichi et al.10 also reported a lack of effect of NTG in the coupled system of rat platelets and pig aortic microsomes. Studies in vivo also suggest that a prostaglandin-mediated mechanism is not involved in the biological action of nitrates; these studies usually involve cyclooxygenase blockade. Thus canine renal artery vasodilatation induced by bolus injection of high doses of NTG was not altered after pretreatment of the animal with indomethacin or meclofenamate,33 and the increases in canine coronary blood flow or decreases in coronary resistance to infusion of either NTG or nitroprusside were not changed after blockade of prostaglandin synthesis with indomethacin.34 In human subjects, high-dose aspirin pretreatment, which causes a decrease in vascular prostacyclin production,19,35 failed to modify to any extent large coronary artery vasodilatation induced by ISDN, as measured by quantitative angiography.36 Similar results were recently reported with NTG.37 Systemic hemodynamic changes by NTG were similarly unaffected by indomethacin38 and, after indomethacin, NTG-induced increase in workload exercise testing was not significantly altered in patients with stable angina on effort.39 In addition, the hemodynamic effects produced by prostacyclin infusion (mainly arteriolar vasodilation) are clearly different from those produced by nitrates.40,41 In contrast to prostacyclin,42 nitrates consistently relieve ischemic episodes, and no report has yet suggested that their efficacy is blunted by drugs interfering with prostaglandin synthesis. Finally, a reliable estimation of prostacyclin production in humans during nitrate administration in vivo, by the assay of the main urinary metabolite 2,3-dinor-6-keto-PGF1α, has been recently made by Fitzgerald et al.43 without showing any significant change as compared to control conditions.

Our present findings with cultured endothelial cells and different types of human vascular fragments in vitro are consistent with the animal and clinical data available and indicate that nitrates do not act via stimulation of endothelial prostacyclin.
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