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# Nitrovasodilators Inhibit Platelet-Derived Growth Factor–Induced Proliferation and Migration of Activated Human Hepatic Stellate Cells

PAOLA FAILLI,* RAFFAELLA M. S. DEFRANCO,† ALESSANDRA CALIGIURI,† ALESSANDRA GENTILINI,† ROBERTO GIULIO ROMANELLI,† FABIO MARRA,† GIACOMO BATIGNANI,§ CRISTINA TOSTI GUERRA,† GIACOMO LAFFI,† PAOLO GENTILINI,† and MASSIMO PINZANI†

*Dipartimento di Farmacologia Preclinica e Clinica, †Dipartimento di Medicina Interna, Firenze; and §Dipartimento di Fisiopatologia Clinica-Unità di Chirurgia, Università di Firenze, Firenze, Italy

***Background & Aims:*** **Nitrovasodilators have been proposed for the treatment of portal hypertension alone or in combination with β-blockers. In addition to their vasodilatory properties, nitric oxide (NO) donors may exert direct antifibrogenic properties. We evaluated the effect of nitroglycerin (NTG) and *S*-nitroso-*N*-acetyl penicillamine (SNAP) on the mitogenic and chemotactic properties of platelet-derived growth factor (PDGF)-BB and the modulation of the relative intracellular signaling pathways in fully activated human hepatic stellate cells (HSCs), a cell type that plays an active role in liver fibrogenesis and portal hypertension.** ***Methods & Results:*** **Both NTG and SNAP induced a dose-dependent decrease in PDGF-induced DNA synthesis and cell migration, which was associated with a decrease in PDGF-induced intracellular $Ca^{2+}$ increase and extracellular signal-regulated kinase (ERK) activity. These effects were not related to activation of the classic soluble guanylate cyclase (sGC)/guanosine 3′,5′-cyclic monophosphate pathway; accordingly, Western blot analysis of HSC lysates revealed the absence of the $\alpha_1\beta_1$ ubiquitous subunits of sGC, whereas they were detectable in quiescent HSCs, freshly isolated from normal human liver. Conversely, both NTG and SNAP induced a more than 10–20-fold increase in prostaglandin $E_2$ in cell supernatants within 1 minute, associated with an increase in intracellular adenosine 3′,5′-cyclic monophosphate levels. Accordingly, the inhibitory effects of NO donors on PDGF action and signaling were eliminated after preincubation with ibuprofen.** ***Conclusions:*** **These results suggest that NO donors may exert a direct antifibrogenic action by inhibiting proliferation, motility, and contractility of HSCs in addition to a reduction of fibrillar extracellular matrix accumulation.**

Experimental and clinical studies have indicated that short-term and long-term administration of different nitrovasodilators may reduce portal pressure.[1–3] Accordingly, these compounds, particularly isosorbide-5-mononitrate, have been proposed for the treatment of portal hypertension alone or in combination with β-blockers,[4,5] although their use may be contraindicated in patients with cirrhosis with ascites.[6]

The direct action of organic nitrates on the intrahepatic circulation, although well conceivable according to the constitutive vasodilatory properties of these compounds, has not been substantiated in cellular and molecular terms.

A close relationship occurs between the progression of liver fibrosis and development of portal hypertension. Particularly, development of portal-central anastomoses and arterialization/capillarization of sinusoids represent hallmarks of the fibrogenic process as well as key determinants for the increase in intrahepatic vascular resistance.[7] Indeed, portal-central anastomoses, although representing direct connections between the portal and systemic circulation, follow irregular patterns and are embedded in developing scar tissue where a complex interplay between several cell types and soluble mediators occurs. In this context, several in vitro and in vivo studies have highlighted the role of activated hepatic stellate cells (HSCs),[8–10] a cell type primarily involved in the development of liver fibrosis.[11,12] In their activated phenotype, stellate cells are characterized by remarkable proliferative, chemotactic, and contractile properties. Particularly, contraction of activated HSCs in response to

*Abbreviations used in this paper:* COX, cyclooxygenase; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; HSC, hepatic stellate cell; IBMX, 3-isobutyl-1-methyl-xanthine; NO, nitric oxide; NTG, nitroglycerin; PDGF, platelet-derived growth factor; PK, protein kinase; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SFIF, serum-free/insulin-free; sGC, soluble guanylate cyclase; SNAP, *S*-nitroso-*N*-acetyl penicillamine.

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different vasoconstrictors, including endothelin 1 and thrombin,[8,13,14] has been suggested to play an important role in the contraction of developing scar tissue, and, consequently, in the progression of portal hypertension. HSC contraction is counterbalanced by vasorelaxing agents such as nitric oxide (NO),[15] which has been proposed as a regulator of sinusoidal blood flow in the normal liver.[16] Along these lines, in vitro and in vivo evidence indicates that sinusoidal endothelial cells express constitutive NO synthase and produce NO, and increase their production in response to flow.[17] However, an endothelial dysfunction associated with decreased production of NO in the intrahepatic microcirculation has been extensively documented in the cirrhotic liver,[18,19] and these defects could directly contribute to the increased intrahepatic resistance typical of portal hypertension.[20] These observations provide per se a sound rationale for the use of nitrovasodilators in the treatment of portal hypertension.

Expression of platelet-derived growth factor (PDGF), a key mitogen and chemoattractant for HSCs[21,22] and of the relative receptor subunits, is markedly up-regulated in human fibrotic liver and correlates with the extent of necroinflammatory and fibrogenic activity.[23] Furthermore, expression of PDGF receptors is a consistent feature of the process of HSC activation in vitro and in vivo.[21,23] NO donors have been shown to reduce collagen type I deposition in vascular smooth muscle cells and in human HSCs,[24,25] and NO release induced by proinflammatory cytokines has been proven responsible for the inhibition of α–smooth muscle actin expression, a marker of HSC activation.[26] In addition, a possible direct antifibrogenic effect of NO donors is suggested by in vitro studies showing antiproliferative and antichemotactic actions of these compounds in vascular smooth muscle cells and glomerular mesangial cells.[27–30] However, the possible cellular and molecular mechanisms responsible for the action of NO donors in this setting have not been clarified.

Because of the close link between the progression of liver fibrosis and the development of portal hypertension, we evaluated the effects of two NO donors, nitroglycerin (NTG) and *S*-nitroso-*N*-acetyl penicillamine (SNAP), on the biological actions and the intracellular signaling of PDGF in activated human HSCs.

## Materials and Methods

### Reagents

Monoclonal antiphosphotyrosine antibodies for Western blotting were purchased from UBI (Lake Placid, NY). Polyclonal antibodies against extracellular signal–regulated kinase (ERK)-1 and phospholipase Cγ1 (PLCγ) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies against soluble guanylate cyclase (sGC) ($\alpha_1\beta_1$ heterodimeric form) were purchased from Alexis Corp. (San Diego, CA). Myelin basic protein was purchased from Sigma Chemical Co. (St. Louis, MO), and protein A–Sepharose from Pharmacia (Uppsala, Sweden). [γ-$^{32}$P]adenosine triphosphate (ATP) (3000 Ci/mmol), [α-$^{32}$P]deoxycytidine triphosphate (3000 Ci/mmol), and [*methyl*-$^{3}$H]thymidine (6.7 Ci/mmol) were from New England Nuclear (Milan, Italy). Human recombinant PDGF-BB was purchased from Peprotech (Rock Hill, NJ), and human thrombin from Boehringer Mannheim GmbH (Mannheim, Germany). SNAP was purchased from Tocris Cookson Ltd. (Bristol, England); LY-83583 and KT5823 from Calbiochem Corp. (San Diego, CA); and Fura-2-acetyloxymethylester purchased from Molecular Probes (Eugene, OR). A working solution of NTG was obtained by diluting a commercially available preparation for intravenous infusion (Venitrin; Astra, Milan, Italy) with sterile water. All other reagents were of analytical grade.

### Isolation and Culture of Human HSCs

Human HSCs were isolated from wedge sections of normal human liver unsuitable for transplantation, as reported previously.[8,31] Briefly, after a combined digestion with collagenase/pronase, HSCs were separated from other liver nonparenchymal cells by ultracentrifugation over gradients of stractan (Cellsep isotonic solution; Larex Inc., St. Paul, MN). Extensive characterization was performed as described previously.[31] Cells were cultured on plastic culture dishes (Falcon; Becton Dickinson, Lincoln Park, NJ) in Iscove's modified Dulbecco's medium supplemented with 0.6 U/mL insulin, 2.0 mmol/L glutamine, 0.1 mmol/L nonessential amino acids, 1.0 mmol/L sodium pyruvate, antibiotic antifungal solution (all provided by GIBCO Laboratories, Grand Island, NY), and 20% fetal bovine serum (FBS) (Imperial Laboratories, Andover, England). Experiments described in this study were performed on cells between the third and fifth serial passages (1:3 split ratio) using 3 independent cell lines. As already reported,[31] at these stages of culture, human HSCs showed transmission electron-microscopic features of myofibroblast-like cells, thus indicating complete transition to their activated phenotype. In experiments using freshly isolated human HSCs, an aliquot of the cell isolate was immediately used for guanosine 3′,5′-cyclic monophosphate (cGMP) determinations or frozen for protein analysis, whereas the remaining aliquot was cultured as specified earlier.

### DNA Synthesis

DNA synthesis was measured as the amount of [*methyl*-$^{3}$H]thymidine ([$^{3}$H]TdR) incorporated into trichloroacetic acid–precipitable material. Cells were plated in 24-well dishes at a density of $2 \times 10^4$ cells/well in complete culture medium containing 20% FBS. Confluent cells (approximately $1 \times 10^5$ cells/well) were made quiescent by incubation in serum-free/insulin-free (SFIF) medium for 48 hours. Unless otherwise

specified, cells were incubated with or without agonists at the indicated doses and modalities for 20 hours and then pulsed for an additional 4 hours with 1.0 μCi/mL [$^3$H]TdR. At the end of the pulsing period, [$^3$H]TdR incorporation into cellular DNA was measured as previously reported.[21] Cell number was determined in 3 separate wells from each dish; results are expressed as counts per minute per $10^5$ cells.

### Cell Growth Assay

Human HSCs were plated in 12-well dishes at a density of $6 \times 10^4$ cells/well in complete culture medium. After 24 hours cells were washed twice with SFIF medium and placed in the same medium containing PDGF-BB (10 ng/mL) with or without NO donors (day 0). Cell counts were performed on triplicate wells at day 0, 2, and 4 by trypsinizing the cells and using a Coulter counter (Coulter Electronics Inc., Hialeah, FL). Fresh SFIF medium containing PDGF-BB with or without NO donors at the same concentrations was added to the remaining wells at day 2. Results are expressed as the absolute cell number at each day of observation.

### Chemotactic Assay

Cell migration was performed as described previously.[22,32] Briefly, the experiments were performed using a modified Boyden chamber technique equipped with 8-μm-pore polyvinylpyrrolidone-free polycarbonate filters (13-mm diameter). Polycarbonate filters were precoated with 20 μg/mL of human type I collagen for 30 minutes at 37°C and placed between the upper and the bottom chamber. Confluent HSCs in 6-well dishes were incubated in SFIF medium for 48 hours and then treated with increasing concentrations of NO donors for 10 minutes. They were then suspended by mild trypsinization (0.05% trypsin/EDTA), and an aliquot (210 μL) of the obtained cell suspension, corresponding to $4 \times 10^4$ cells, was added to the top well and incubated at 37°C for 6 hours. The lower chamber was filled with SFIF medium (control) or PDGF-BB (10 ng/mL) in the presence or absence of the same concentration of NO donors used in the preincubation. After fixing in 96% methanol and staining with Harris' hematoxylin solution, cells migrated to the underside of the filters and were quantified as the mean number of cells in 10 high-power fields. All experiments were performed in triplicate. Each triplicate assay was repeated 2 times on separate occasions with different HSC preparations. Possible cytotoxic effects were monitored by the trypan blue exclusion test.

### ERK Assay

ERK was measured as the myelin basic protein kinase activity of ERK immunoprecipitates. The cell monolayer was lysed in RIPA (radioimmunoprecipitation assay) buffer (20 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 5 mmol/L EDTA, 1% Nonidet P-40, 1 mmol/L $Na_3VO_4$, 1 mmol/L phenylmethylsulfonyl fluoride, and 0.05% [wt/vol] aprotinin). Insoluble proteins were discarded by high-speed centrifugation at 4°C. Protein concentration in the supernatant was measured in triplicate using a commercially available assay (Bio-Rad, Hercules, CA). Fifty micrograms of total cell lysate was immunoprecipitated with anti–ERK-1 antibodies and protein A–Sepharose. Measurement of ERK activity was performed as described previously.[33] Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were performed using previously described techniques.[22]

### Digital Video Imaging of Intracellular-Free Calcium Concentration in Individual Human HSCs

Digital video imaging of intracellular-free calcium concentration ($[Ca^{2+}]_i$) in individual human HSCs was performed as described previously.[8,34] Human HSCs were grown to subconfluence in complete culture medium on round glass cover slips (25-mm diameter, 0.2-mm thick) for 72 hours, and then incubated for 48 hours in SFIF medium. Cells were then loaded with 10 μmol/L Fura-2AM and 15% Pluronic F-127 for 30 minutes at 22°C. $[Ca^{2+}]_i$ was measured in Fura-2–loaded cells in HEPES-$NaHCO_3$ buffer containing 140 mmol/L NaCl, 3 mmol/L KCl, 0.5 mmol/L $NaH_2PO_4$, 12 mmol/L $NaHCO_3$, 1.2 mmol/L $MgCl_2$, 1.0 mmol/L $CaCl_2$, 10 mmol/L HEPES, and 10 mmol/L glucose, pH 7.4. Ratio images (340/380 nm) were collected every 3 seconds, and calibration curves were obtained for each cell preparation.[34] PDGF-BB (10 ng/mL) or thrombin (0.3 U/mL) was added directly to the perfusion chamber immediately after recording the $[Ca^{2+}]_i$ basal value. In parallel experiments, cells were preincubated with NO donors 10 minutes before the addition of PDGF-BB or thrombin. In experiments evaluating changes in cell area induced by thrombin, spatial calibration was performed by measuring division on a graticule under the same optical conditions as the rest of the experiments, as reported.[8]

### Northern Blot Analysis

RNA was isolated according to Chomczynski and Sacchi.[35] Total RNA (10 μg) was fractionated by 1% agarose-formaldehyde gel electrophoresis and blotted on a nylon membrane. Procedures for DNA radiolabeling and filter hybridization have been described.[36]

### Assay of Intracellular cGMP Levels

Confluent HSCs in 35-mm well dishes were incubated in SFIF medium for 48 hours. This medium was replaced with SFIF containing 100 μmol/L 3-isobutyl-1-methyl-xanthine (IBMX). Cells were incubated for 10 minutes with 10 ng/mL PDGF-BB, 10 and 100 μmol/L NTG, or 10 or 100 μmol/L SNAP. The reaction was stopped by the addition of 500 μL of 20% ice-cold $HClO_4$, and dishes were placed on a shaker at 4°C for 20 minutes. Samples were collected, neutralized at pH 7.5 by adding 1.08 mol/L $K_3PO_4$, and kept at 4°C overnight. After centrifugation at 6000 rpm at 4°C for 10 minutes, supernatants were collected and lyophilized. In experiments using freshly isolated human HSCs, cells were divided in

aliquots of $5 \times 10^5$/tube and resuspended in 1 mL of SFIF medium containing 100 μmol/L IBMX. After 10 minutes of incubation at 37°C, cells were stimulated with NTG or SNAP (both at a concentration of 100 μmol/L) for 10 minutes. The reaction was then terminated as indicated earlier. cGMP levels were measured by Cyclic GMP [$^3$H] Assay System (Amersham Pharmacia Biotech UK Ltd., Little Chalfont, England) according to the manufacturer's instructions. Cell number was determined in 3 separate wells from each dish after trypsinization and counting. Time course experiments were performed to monitor cGMP synthesis at 5, 10, 15, and 30 minutes after addition of the agonists.

### Assay of Intracellular cAMP Levels

Confluent HSCs in 22-mm well dishes were incubated in SFIF medium for 48 hours. This medium was then removed and replaced with 200 μL/well of a buffer containing 0.025 mol/L Tris-HCl, 0.25 mol/L sucrose, 0.5% bovine serum albumin, and 100 μmol/L IBMX, pH 7.4. Agonists were added to the wells, and the cells were incubated at 37°C for 10 minutes. The reaction was stopped by the addition of 500 μL of 100% ice-cold ethanol, and dishes were placed at −20°C for at least 24 hours. Samples were then centrifuged at 2500*g* at 4°C for 20 minutes, and supernatants were collected, lyophilized, and reconstituted with 50 mmol/L sodium acetate buffer, pH 6.2. Intracellular adenosine 3′,5′-cyclic monophosphate (cAMP) levels were measured by the cAMP [$^{125}$I] assay system (Amersham Pharmacia Biotech UK Ltd.) according to the manufacturer's instructions. Cell number was determined in 3 separate wells from each dish after trypsinization and counting.

### Preparation of Human Platelets

Blood from healthy volunteers who had not taken drugs for at least 10 days was collected by venipuncture and immediately diluted 1/5 with citric acid/trisodium citrate/glucose (1.5%:2.5%:2%, wt/vol). Platelet-rich plasma was prepared by centrifugation at 180*g* at 20°C for 15 minutes. Platelets were washed twice and collected by centrifugation as described previously.[37]

### Immunoprecipitation and Immunoblotting

Cells or platelets were lysed in 50 mmol/L HEPES, pH 7.5, 150 mmol/L NaCl, 1.0% Triton X-100, 5.0 mmol/L $MgCl_2$, 1.0 mmol/L EGTA, 25 μg/mL leupeptin, 2.0 mmol/L sodium orthovanadate ($Na_3VO_4$), 10 μg/mL pepstatin A, and 2.0 mmol/L phenylmethylsulfonyl fluoride. The lysates were then cleared by centrifugation (14,000 rpm, 10 minutes). Immunoprecipitation, SDS-PAGE, and immunoblotting analysis were performed as previously described.[38] Antibodies bound to polyvinylidene fluoride membranes were detected by chemiluminescence with enhanced chemiluminescence (ECL; Amersham Life Sciences, Little Chalfont, England).

### Determination of Prostaglandin $E_2$ Levels in Cell Supernatants

Prostaglandin (PG) $E_2$ levels in cell supernatants were determined by a specific radioimmunoassay.[39] $PGE_2$ values were expressed as picograms per $10^5$ cells. All determinations were performed in triplicate.

### Statistical Analysis

Results, relative to the number of experiments indicated, are expressed as mean ± SD. Statistical analysis was performed by one-way analysis of variance, and, when the F value was significant, by Duncan's test. Unless otherwise specified, $P$ values of $<0.05$ were considered statistically significant.

## Results

### NO Donors Reduce PDGF-Induced Cell Proliferation and Chemotaxis

The effects of increasing concentrations of NTG and SNAP on PDGF-BB–induced DNA synthesis, measured as [$^3$H]thymidine incorporation into DNA, were first evaluated. The 2 NO donors used for this study are characterized by different intracellular pharmacologic dynamics leading to NO release. NO donors such as SNAP release NO spontaneously, whereas NTG, a classic organic nitrate, releases NO after enzymatic metabolism involving conjugation with cysteine-rich residues in various cells including fibroblasts and smooth muscle cells.[40]

As shown in Figure 1*A*, preincubation with both NO donors for 10 minutes induced a dose-dependent inhibition of PDGF-induced mitogenesis. This inhibitory effect was statistically significant starting at 1 μmol/L. The difference between the 2 compounds was statistically significant only at 100 μmol/L, although SNAP tended to be slightly more potent than NTG at any concentration tested. Growth curve experiments for activated human HSCs in response to PDGF-BB with or without NO donors were performed to show whether the decrease in PDGF-induced mitogenesis is associated with an actual decrease in cell growth. As shown in Figure 1*B*, PDGF-BB at a dose of 10 ng/mL significantly increased HSC growth after 2 and 4 days of incubation compared with unstimulated control cells ($P < 0.001$). This effect was clearly reduced by pretreatment with both NTG and SNAP, and this reduction was already statistically significant after 2 days of culture. The effects of NO donors were not associated with any cytotoxic effects in the dose range used.

To provide evidence that the inhibitory effects of NO donors on PDGF mitogenic potential were indeed caused

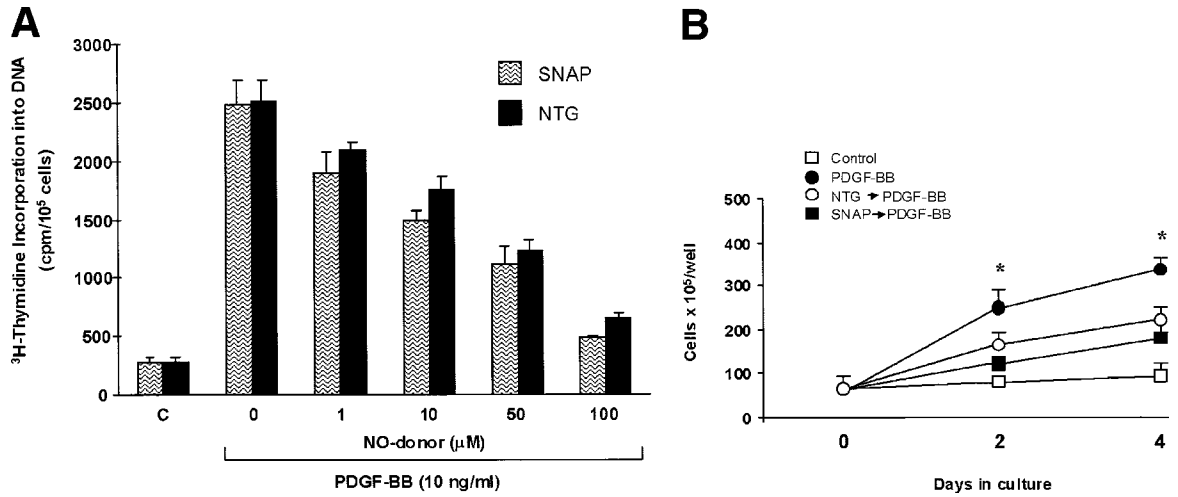


**Figure 1.** Effect of NO donors on PDGF-induced cell proliferation in activated human HSCs. (*A*) Dose response for the effect of increasing concentrations of NO donors on DNA synthesis, evaluated as [$^3$H]thymidine incorporation into DNA, induced by PDGF-BB. Confluent cells in 24-well dishes were incubated for 48 hours in SFIF medium, then pretreated for 10 minutes with increasing concentrations of NO donors before starting an incubation with 10 ng/mL PDGF-BB for a total of 24 hours. Cells were pulsed with [$^3$H]thymidine during the last 4 hours of incubation. Data are mean ± SD for 4 experiments performed in triplicate. Compared with the effect of PDGF-BB alone, changes were statistically significant ($P < 0.05$ or higher degree of significance) starting at 1.0 μmol/L for both NO donors. (*B*) Effect of NO donors on PDGF-BB–induced HSC proliferation. Cells were plated in 12-well dishes at a density of $6 \times 10^4$ cells/well in complete culture medium. After 24 hours (day 0), cells were washed twice with SFIF medium and then incubated in fresh SFIF medium alone (control) or containing 10 ng/mL PDGF-BB with or without preincubation with NTG (50 μmol/L) or SNAP (10 μmol/L). At each time point (day 2 and day 4), cells were trypsinized and counted with a Coulter cell counter. Fresh medium containing the same test conditions was added to the remaining wells at each time point. Data (mean ± SD), expressed as the absolute cell number, are from 2 experiments performed in triplicate. *$P < 0.005$ or higher degree of significance compared with cells treated with NO donors.

by NO release, we performed [$^3$H]thymidine incorporation experiments by incubating HSCs with $10^{-6}$ mol/L methylene blue, a well-known NO oxidizing agent,[41] before the addition of NO donors. This treatment resulted in a statistically significant reduction of the inhibitory effect of both NTG and SNAP, thus confirming that their effects are actually caused by NO release (data not shown).

In addition to their effects on PDGF-induced mitogenesis, both NTG and SNAP caused a dose-dependent reduction of PDGF-BB–induced chemotaxis in a modified Boyden chamber system (Figure 2). The inhibitory effect of SNAP was more pronounced than that of NTG, with statistically different potency at any concentration tested.

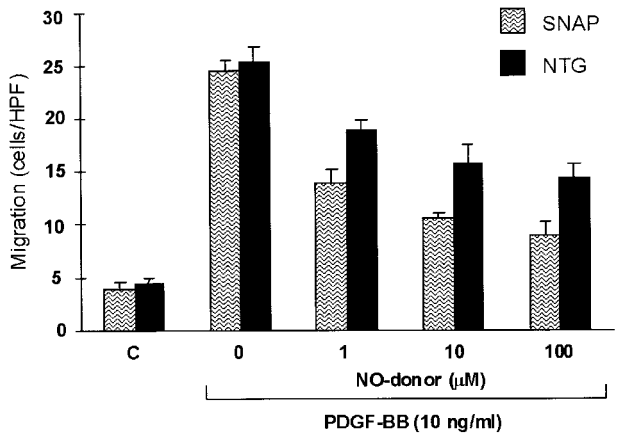


**Figure 2.** Effect of NO donors on PDGF-induced cell migration in activated human HSCs. Dose response for the effect of increasing concentrations of NO donors on PDGF-induced cell migration. Cell migration was measured by the Boyden chamber assay (see Materials and Methods). Cells were seeded in SFIF medium in the upper compartment of the Boyden chamber and tested for migration in the lower chamber through a filter precoated with collagen type I. Cells that had migrated to the lower surface of the filter after 6 hours were stained and visually quantitated by cell counting. The values (cells migrated per high-power fields) are expressed as mean ± SD and are from 8 separate fields counted in 3 different experiments. Compared with the effect of PDGF-BB alone, changes were statistically significant ($P <$ 0.05 or higher degree of significance) starting at 1.0 μmol/L for both NO donors.

## NO Donors Affect PDGF-Induced Intracellular Signaling

The effects of NO donors on PDGF-induced signaling were then evaluated. As shown in Figure 3, in individual Fura-2–loaded human HSCs, stimulation with PDGF (control) caused the occurrence of an earlier synchronous $Ca^{2+}$ spike over an average basal $[Ca^{2+}]_i$ of 90–100 nmol/L, followed by a long-lasting plateau, as

reported previously.[34] Ten-minute preincubation with either NTG (100 μmol/L) or SNAP (100 μmol/L) caused a marked reduction of both the peak and the plateau phase. Similar effects, although less marked, were observed with a lower concentration of both NO donors (10 μmol/L; data not shown).

Exposure of serum-deprived human HSCs to PDGF-BB resulted in ERK activation after 5 minutes of incubation with a peak effect at 10 minutes (Figure 4*A*, lanes 1 and 2). Preincubation with both NTG and SNAP before the addition of PDGF-BB caused a dose-dependent inhibition of ERK activity (Figure 4*A*, lanes 3–6). Again, SNAP appeared more potent than NTG, with complete abrogation of the PDGF-induced increase of ERK activity at a dose of 10 μmol/L.

The effects of NO donors on PDGF signaling were further detailed by evaluating their action on PDGF-induced increase in c-*fos* gene expression as a downstream target of ERK activation. As shown in Figure 4*B*, c-*fos* messenger RNA (mRNA) expression, not detectable in baseline conditions (lane 1), became clearly evident 30 minutes after the addition of PDGF (lane 2), and was clearly reduced by cell preincubation with both SNAP and NTG (lanes 3 and 4, respectively).

### Effect of NO Donors on the Guanylate Cyclase/cGMP System

The action of NO donors is known to be largely dependent on the NO-induced activation of sGC, particularly of the heterodimeric form $\alpha_1\beta_1$.[42,43] Activation of sGC leads to cGMP generation and to other downstream events including activation of protein kinase (PK) G.[44–47] To verify this hypothesis, the effects of NTG and SNAP on the sGC/cGMP system were investigated. The inhibitory effect of NTG (100 μmol/L) on PDGF-induced DNA synthesis was evaluated in the presence of LY-83583, an inhibitor of guanylyl cyclase activity, and

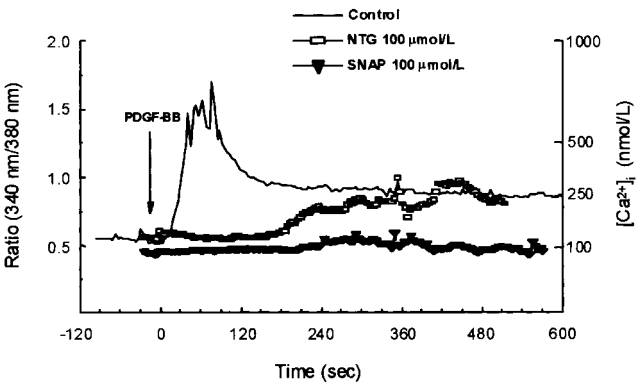


**Figure 3.** Effect of NO donors on PDGF-induced intracellular $Ca^{2+}$ increase in individual Fura-2–loaded activated human HSCs. Subconfluent (70%–80% confluent) cells were maintained in SFIF medium for 48 hours and were then loaded with Fura-2–AM (see Materials and Methods). PDGF-BB (10 ng/mL) was added (*arrow*, time 0). The tracings show changes in $[Ca^{2+}]_i$ induced by PDGF without (control) or with a 10-minute preincubation with 100 μmol/L NTG or 100 μmol/L SNAP. Data are the mean of 25 determinations in individual cells stimulated with PDGF compared with an identical number of cells pretreated with NO donors. For each single point, SD did not exceed 15% of the mean value. Measurements were obtained at 3-second time intervals.

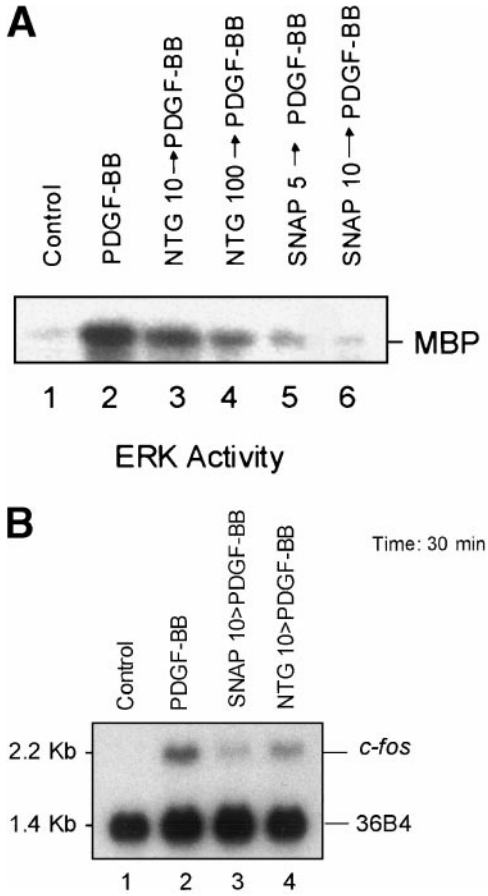


**Figure 4.** Effect of NO donors on PDGF-induced ERK activity and c-*fos* mRNA expression in activated human HSCs. (*A*) Confluent cells in 100-mm dishes were maintained for 48 hours in SFIF medium and then pretreated with NTG (10 and 100 μmol/L; *lanes 3* and *4*, respectively) or SNAP (5 and 10 μmol/L; *lanes 5* and *6*, respectively) for 10 minutes before stimulation with PDGF-BB (10 ng/mL; *lanes 2–6*) for 10 minutes. ERK activity was analyzed as the ability of ERK immunoprecipitates to phosphorylate myelin basic protein (MBP) (see Materials and Methods). Control refers to cells incubated for 48 hours in SFIF medium alone. Representative experiment of 3. (*B*) Confluent cells in 100-mm dishes were maintained for 48 hours in SFIF medium and then pretreated with SNAP (10 μmol/L; *lane 3*) or NTG (10 μmol/L; *lane 4*) for 10 minutes before stimulation with PDGF-BB (10 ng/mL; *lanes 2–4*) for 30 minutes. Control refers to RNA isolated from cells incubated for 48 hours in SFIF medium alone. RNA purification and hybridization were performed as detailed in Materials and Methods. c-*fos* complementary DNA probe was then removed by boiling, and the same blot was hybridized to a complementary DNA encoding for the ribosomal protein 36B4 (control gene). Representative experiment of two.

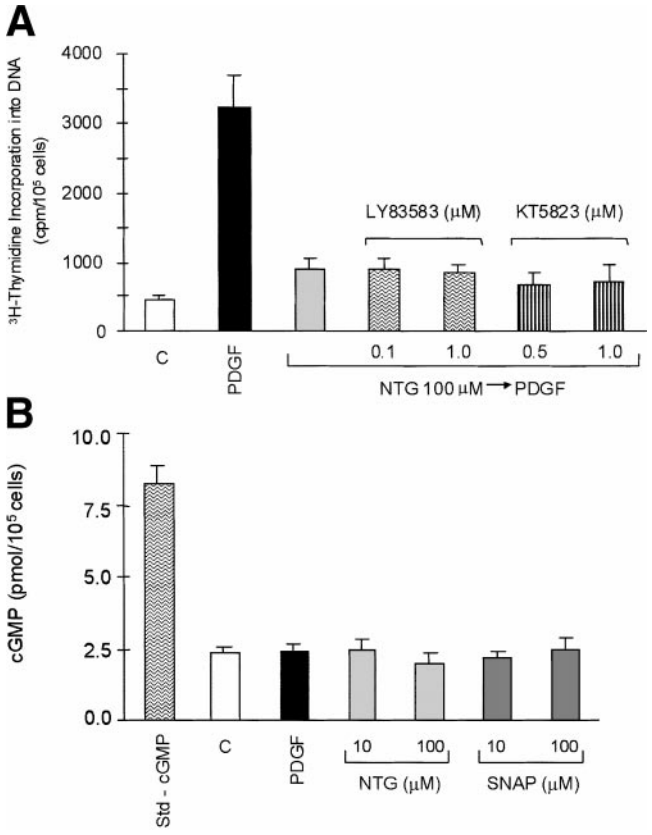


**Figure 5.** Effect of NO donors on the guanylate cyclase/cGMP system in activated human HSCs. (*A*) Effect of LY-83583, an inhibitor of sGC activity, and KT5823, an inhibitor of PKG, on the inhibitory action exerted by NTG on PDGF-induced DNA synthesis, evaluated as [$^3$H]thymidine incorporation into DNA. Experiments were conducted as indicated in Figure 1*A* and in Materials and Methods. LY-83583 and KT5823, at the concentrations indicated, were added 10 minutes before starting the preincubation with NTG. (*B*) Effect of NO donors on intracellular cGMP levels. Confluent cells were incubated in SFIF medium for 48 hours and then stimulated with PDGF-BB (10 ng/mL) or the indicated doses of NTG or SNAP for 10 minutes. Std-cGMP, data relative to a standard concentration of cGMP (8 pmol), included as a positive control for the assay. Radioimmunoassay procedures were as described in Materials and Methods. Data, expressed as picomoles per $10^5$ cells, are the mean ± SD of 4 experiments performed in triplicate.

KT5823, an inhibitor of PKG activity. At the concentrations tested, both compounds are rather specific in their action.[48,49] However, as shown in Figure 5*A*, inhibition of either guanylyl cyclase or PKG did not affect the inhibitory effect of NTG. Identical results were obtained when the action of both compounds was tested on the inhibitory effect of SNAP and when a wider range of concentrations was used in the same experiment (LY-83583, $10^{-11}$ to $10^{-6}$ mol/L; KT5823, 0.05–2.0 μmol/L) (data not shown). At concentrations higher than those indicated, both LY-83583 and KT5823 induced further inhibition of DNA synthesis caused by cytotoxicity. We next examined cGMP synthesis in response to NO donors in our cell system. As shown in Figure 5*B*, incubation of human HSCs with NTG and SNAP for 10 minutes, even at the maximal doses used, did not result in cGMP synthesis over control values. Identical results were obtained with shorter (1- and 5-minute) and longer (15- and 30-minute) periods of incubation with NO donors (data not shown). Because of these results, we examined the expression of the $\alpha_1\beta_1$ heterodimeric form of sGC in protein extracts obtained from different lines of activated human HSCs, including the 3 cell lines used in our present experiments (HFSC3, HFSC6, and HFSC7) by Western blot analysis. As shown in Figure 6*A*, a clear band corresponding to the $\alpha_1\beta_1$ heterodimeric form of sGC was detected in protein extracts obtained from human platelets (lane 1) included as a positive control, whereas it was absent in all the preparations obtained from activated human HSCs. To establish whether the lack of expression of the $\alpha_1\beta_1$ heterodimeric form of sGC constitutes a peculiar feature on human HSCs in their activated state, the expression of this protein was investigated in HSCs freshly isolated from normal human liver and followed during their activation caused by serial subculture on plastic. As shown in Figure 6*B*, in freshly isolated HSCs (lane 2), expression of the $\alpha_1\beta_1$ heterodimeric form of sGC was clearly evident, but became undetectable after activation in culture (serial passages 1–3, split ratio 1:3, lanes 3–5, respectively). Accordingly, NO donors, particularly SNAP, were able to induce cGMP synthesis in freshly isolated HSCs (Figure 6*C*), whereas no increase was observed after activation in culture (serial passage 1). Together, the results of these experiments suggest that, in activated human HSCs, the sGC/cGMP system does not represent a major effector of the action of NO donors, at least in the dose range used. In addition, the lack of expression of the ubiquitous $\alpha_1\beta_1$ heterodimeric form of sGC may represent a distinct feature of the process of human HSC activation and phenotypical modulation.

### Effect of NO Donors on $PGE_2$ Synthesis

NO, either as a gaseous solution or as released by an NO donor, has been shown to promote cyclooxygenase (COX) activity and, consequently, PG synthesis.[50] Therefore, we evaluated $PGE_2$ release in HSC supernatants in response to NTG and SNAP. As shown in Figure 7, compared with a representative time-course experiment performed using maximal doses of NO donors, both compounds elicited a significant increase in $PGE_2$ synthesis over control starting as soon as 2.5 minutes after stimulation, and still present after 30 minutes. At each time point tested, NTG was always remarkably

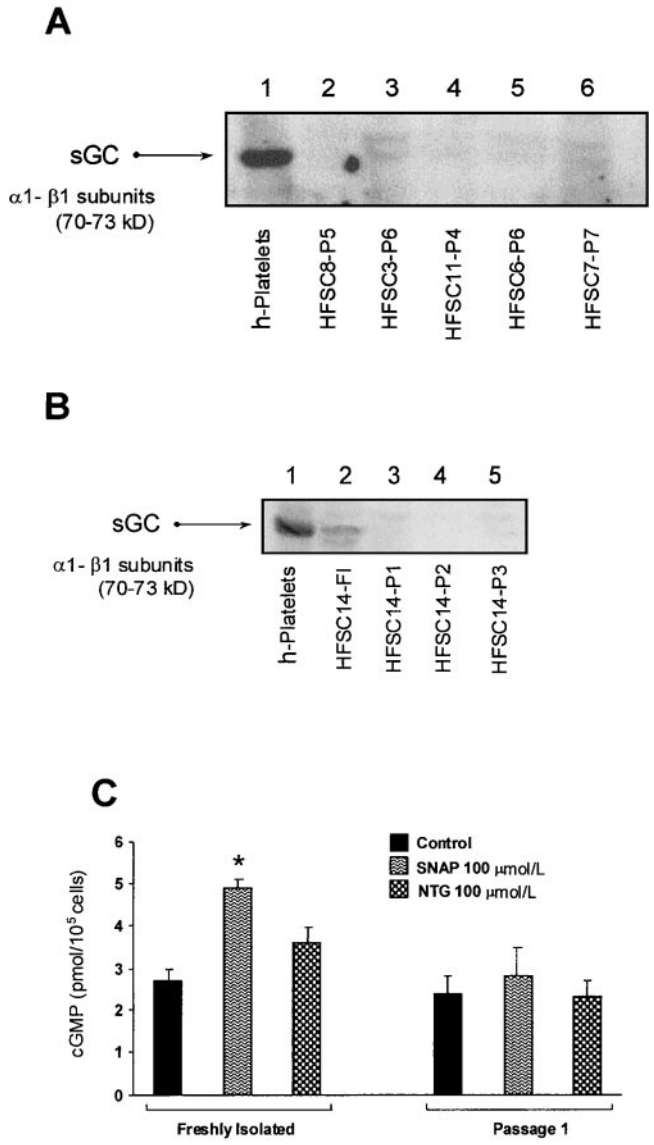


**Figure 6.** Activation of human HSCs is associated with down-regulation of the $\alpha_1\beta_1$ heterodimeric form of sGC. (*A*) Expression of sGC $\alpha_1$–$\beta_1$ subunits in different lines of activated human HSCs. Western blot analysis of 50 μg of protein obtained from cell lysates separated by 7.5% SDS-PAGE. Cell lysates were obtained from human platelets (positive control; *lane 1*) and from 5 independent lines of human HSCs (cell lines 8, 3, 11, 6, and 7; *lanes 2–6*) maintained in standard culture conditions. −P(number) refers to the serial passage of culture. Representative blot of 2. (*B*) Expression of sGC $\alpha_1\beta_1$ subunits during the process of human HSC activation. Western blot analysis of 50 μg of protein obtained from cell lysates separated by 7.5% SDS-PAGE. Cell lysates were obtained from human platelets (positive control; *lane 1*), from HSCs freshly isolated from normal liver (*lane 2*), and from the same cell preparation after 3 serial passages in culture (P1–P3; *lanes 3–5*, respectively). (*C*) Effect of NO donors on intracellular cGMP levels in freshly isolated human HSCs and after the first serial passage (passage 1). Freshly isolated cells were divided into aliquots of $5 \times 10^5$/tube and resuspended in 1 mL of SFIF medium containing 100 μmol/L IBMX. After 10 minutes of incubation at 37°C, cells were stimulated with NTG or SNAP for 10 minutes. Experiments on passage 1 cells were performed as described in Figure 5. Radioimmunoassay procedures were as described in Materials and Methods. Data (pmol/$10^5$ cells) are the mean ± SD of 3 experiments. *$P < 0.05$ (or higher degree of significance) vs. unstimulated cells.

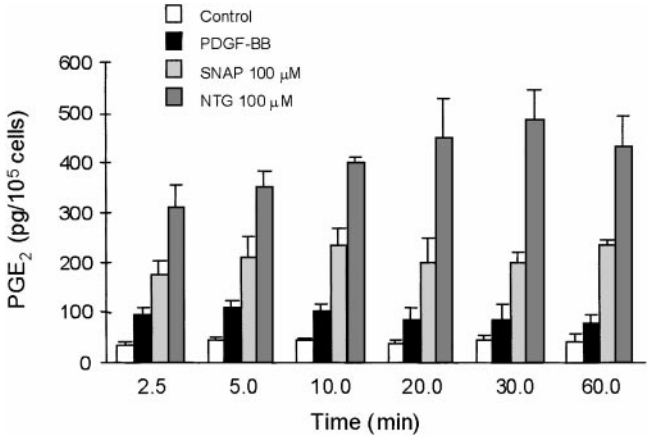


**Figure 7.** Effect of PDGF-BB and NO donors on $PGE_2$ synthesis in activated human HSCs. Determination of $PGE_2$ immunoreactivity in cell culture supernatants. Confluent cells were incubated for 48 hours in SFIF medium. Cells were then washed twice with the same medium and incubated without (control) or with test conditions for the indicated times. $PGE_2$ was extracted from conditioned medium, and $PGE_2$ immunoreactivity was determined as detailed in Materials and Methods. Data (pg/$10^5$ cells) are the mean ± SD of 4 experiments performed in triplicate. Compared with control conditions, all agonists induced statistically significant changes ($P < 0.05$ or higher degree of significance) in $PGE_2$ synthesis starting at 2.5 minutes of incubation.

more potent than SNAP in inducing $PGE_2$ synthesis. In addition, a 2-fold increase in $PGE_2$ synthesis was also observed after stimulation with PDGF-BB, thus confirming recently reported observations in this cell type.[51]

### $PGE_2$ Synthesis Blockade Abrogates the Inhibitory Effect of NO Donors on PDGF Action

Because the increase in $PGE_2$ synthesis elicited by NO donors could activate the adenylate cyclase/cAMP pathway through specific receptors[52] and cAMP affects PDGF biological effects and intracellular signaling,[52,53] experiments were repeated by evaluating DNA synthesis and ERK activity in the presence of the COX inhibitor ibuprofen. As shown in Figure 8*A*, pretreatment with ibuprofen exerted a dose-related abrogation of the inhibitory effect of NO donors on PDGF-induced DNA synthesis. In addition, pretreatment with ibuprofen significantly enhanced the mitogenic effect of PDGF, similar to the data of Mallat et al.[51] Accordingly, the observed inhibition of PDGF-induced ERK activity exerted by NTG and SNAP was completely abolished when cells were preincubated with ibuprofen (Figure 8*B*). Finally, to characterize the involvement of the adenylate cyclase/cAMP pathway, we examined intracellular cAMP synthesis in response to NO donors in the absence and presence of ibuprofen. In these experiments, pentoxifylline, a phosphodiesterase inhibitor, was included as a positive control.[53] As shown in Figure 8*C*, both NTG

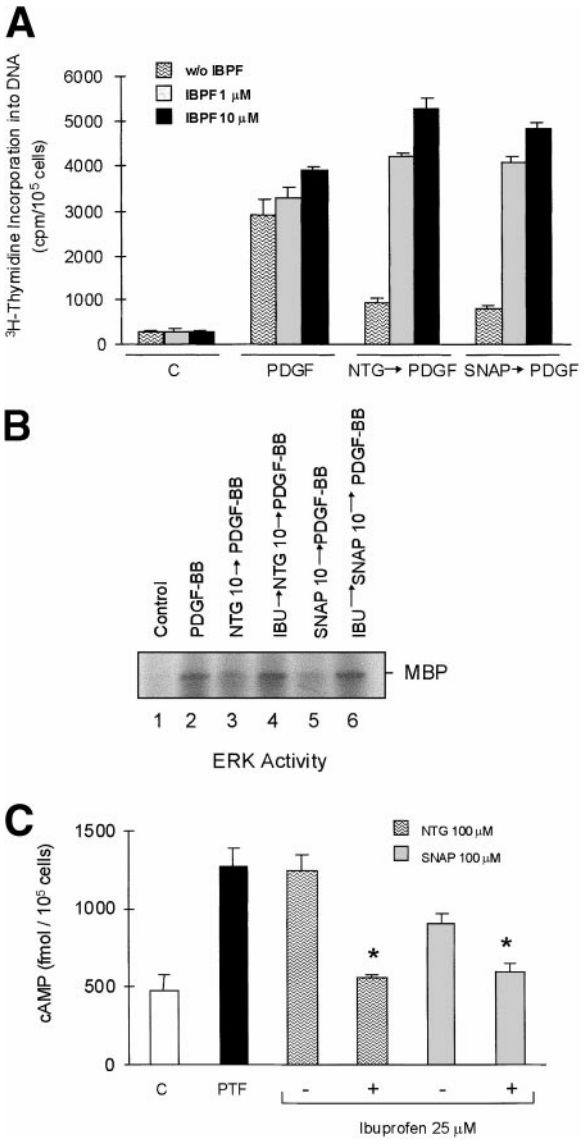


**Figure 8.** Blockade of $PGE_2$ synthesis eliminates inhibitory effects of NO donors. (*A*) Effect of ibuprofen (IBPF), an inhibitor of COX activity, on the inhibitory action exerted by NTG and SNAP on PDGF-induced DNA synthesis, evaluated as [$^3$H]thymidine incorporation into DNA. Experiments were conducted as in Figure 1*A* and in Materials and Methods. Ibuprofen, at the concentrations indicated, was added 10 minutes before starting the preincubation with NO donors. (*B*) Effect of ibuprofen (IBU) on the inhibitory action exerted by NTG and SNAP on PDGF-induced ERK activity. Experiments were conducted as in Figure 3 and in Materials and Methods. Ibuprofen ($10^{-6}$ mol/L) was added 10 minutes before starting the preincubation with NO donors. Control, cells incubated for 48 hours in SFIF medium alone. MBP, myelin basic protein. Representative experiment of 2. (*C*) Effect of NO donors on intracellular cAMP levels in the presence or absence of ibuprofen. Confluent cells were incubated in SFIF medium for 48 hours and then stimulated with NTG or SNAP for 10 minutes. Ibuprofen ($25 \times 10^{-6}$ mol/L) was added 10 minutes before stimulation with NO donors. Control, cells incubated for 48 hours in SFIF medium alone. Stimulation with pentoxifylline (170 μmol/L) was included as a positive experimental control. Data (fmol/$10^5$ cells) are the mean ± SD of 2 experiments performed in triplicate. $*P < 0.05$ (or higher degree of significance) vs. the same condition without ibuprofen.

and SNAP increased intracellular cAMP synthesis over control values. NTG appeared to be significantly more potent than SNAP. Preincubation with ibuprofen completely abrogated this effect, thus confirming that this effect of NO donors was probably mediated by the increase in $PGE_2$ synthesis.

### Effect of NO Donors on PDGF-Induced PLC-γ Phosphorylation

The effect of NO donors on PDGF-induced phosphorylation of PLC-γ, a signaling molecule physically associated with the PDGF receptor, was also evaluated. This experimental approach was introduced to attempt to explain the observed different order of potency of SNAP vs. NTG on PDGF-induced biological effects and signaling (i.e., SNAP always more potent than NTG) and their effect on $PGE_2$ and cAMP synthesis (i.e., NTG always more potent than SNAP). Indeed, this discrepancy argues against the assumption that the effect of both NO donors is largely, if not exclusively, caused by increased $PGE_2$ synthesis. As shown in Figure 9*A*, NTG did not modify the level of PLC-γ phosphorylation after stimulation with PDGF, whereas SNAP produced a marked inhibition with complete abrogation of this effect at 10 μmol/L. Pretreatment with either NTG or SNAP did not affect the levels of PDGF receptor phosphorylation after stimulation with the growth factor (Figure 9*B*). These results suggest that, compared with NTG, SNAP exerts at least this additional effect onPDGF signaling that is independent from $PGE_2$ synthesis.

### Effect of NO Donors on Thrombin-Induced $[Ca^{2+}]_i$ Increase and Cell Contraction

To provide further evidence supporting the action on NO donors in the treatment of portal hypertension, the effects of NTG and SNAP on thrombin-induced $[Ca^{2+}]_i$ increase and contraction in activated human HSCs were evaluated (Figure 10). As previously reported,[8] stimulation of HSCs with 0.3 U/mL of human thrombin resulted in increased $[Ca^{2+}]_i$ coupled with a simultaneous and transient reduction of cell area, indicating reversible cell contraction. Preincubation with 100 μmol/L of NTG resulted in a very marked reduction of the $[Ca^{2+}]_i$ increase and in a complete abrogation of cell contraction. Identical results were obtained by preincubating the cells with 100 μmol/L of SNAP (data not shown).

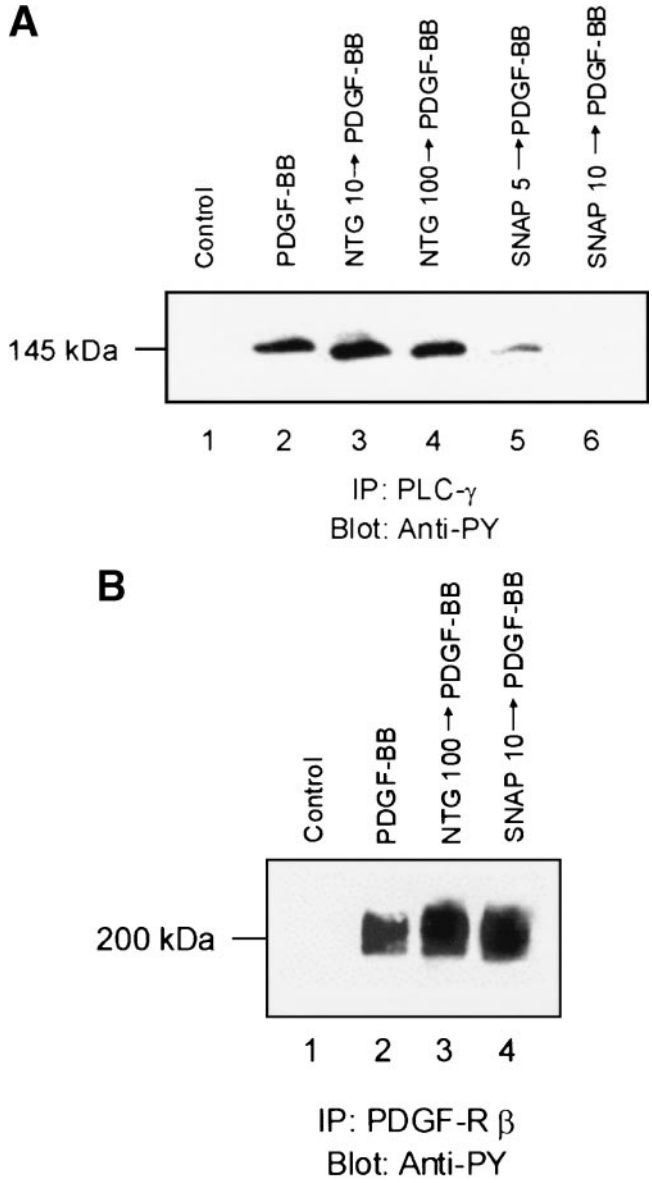


**Figure 9.** Effect of NO donors on PLCγ phosphorylation in activated human HSCs. (*A*) Confluent cells were incubated for 48 hours in SFIF medium. Cells were then pretreated with the indicated doses of NO donors for 10 minutes before stimulation with PDGF-BB (10 ng/mL) for 10 minutes. Equal amounts of proteins were immunoprecipitated using an anti-PLCγ antibody. The immunoprecipitates were analyzed by SDS-PAGE (7.5%), followed by immunoblotting with antiphosphotyrosine (PY) antibody (See Materials and Methods). Control, cells incubated for 48 hours in SFIF medium alone. Representative experiment of 3. (*B*) Confluent cells were incubated for 48 hours in SFIF medium. Cells were then pretreated with the indicated doses of NO donors for 10 minutes before stimulation with PDGF-BB (10 ng/mL) for 10 minutes. Equal amounts of proteins were immunoprecipitated using an anti–PDGF receptor (R) β subunit antibody. The immunoprecipitates were analyzed by SDS-PAGE (7.5%), followed by immunoblotting with antiphosphotyrosine (PY) antibody (see Materials and Methods). Representative experiment of 2. In *A* and *B*, control refers to cells incubated for 48 hours in SFIF medium alone.

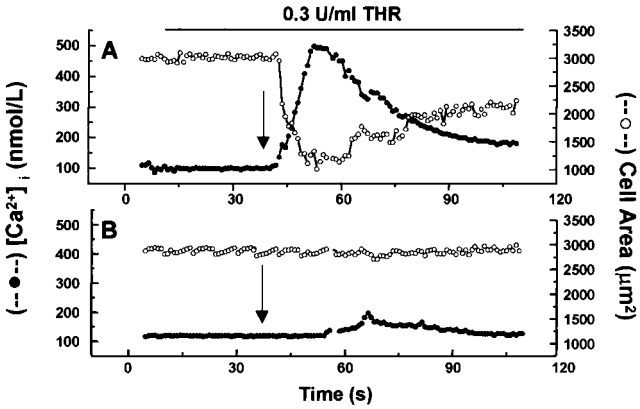


**Figure 10.** Effect of NTG on thrombin-induced $[Ca^{2+}]_i$ increase and cell contraction. Effect of thrombin (THR, 0.3 U/mL, *arrow*) on $[Ca^{2+}]_i$ (nmol/L, *closed symbols*, left vertical axis) and cell area (μm², *open symbols*, right vertical area) in a single Fura-2–loaded human HSC in (*A*) control condition and (*B*) after preincubation with 100 μmol/L NTG. Time courses represent typical examples of the thrombin effect. Horizontal axis indicates time in seconds.

## Discussion

The results of this study indicate that NO donors belonging to different pharmacologic classes (organic nitrates and *S*-nitrosothiols) are able to negatively modulate two key biological effects of PDGF in fully activated human HSCs (cell proliferation and chemotaxis), as well as the activation of PDGF-induced intracellular signaling pathways. These effects of NO donors are predominantly exerted through mechanisms that are alternative to the classic sGC/cGMP pathway caused by the lack of sGC expression, occurring during the process of HSC activation and phenotypical modulation.

The action of NO donors has been shown to be largely dependent on activation of the sGC/cGMP pathway in most cell types,[41,42] including rat stellate cells.[54] Therefore, the involvement of this system in the inhibitory effect of NO donors on PDGF-induced effects and signaling in human HSCs was first evaluated. NO causes activation of sGC, particularly of the heterodimeric form $\alpha_1\beta_1$. cGMP generation then leads to other signaling events, including activation of PKG.[44–47] Several lines of evidence, obtained in vascular smooth muscle cells, indicate that the activation of this pathway could directly affect PDGF intracellular signaling at different levels.[55–58] According to this working hypothesis, inhibition of cGMP synthesis and/or of PKG activity should cause a reduction, if not the abrogation, of the inhibitory effect exerted by NO donors on PDGF-induced DNA synthesis. This dual strategy was motivated by recent experimental evidence indicating that inhibition of PKG activity may not be sufficient to abrogate cGMP-depen-

dent effects of NO.[29,59] Both procedures did not result in evident changes in the inhibitory effects exerted by both NTG and SNAP. This discrepancy with the working hypothesis was initially ascribed to a possible lack of specificity of both LY-83583 and KT5823, although they were used according to the modalities and dosages reported in the literature.[48,49] However, showing that stimulation with NO donors does not result in increased cGMP levels confirmed the possibility of an impairment of this system in activated human HSCs. To gain further insight, the expression of the heterodimeric form $\alpha_1\beta_1$ of sGC was analyzed in different cell lines of activated human HSCs. The heterodimeric form $\alpha_1\beta_1$ is referred to as ubiquitous and represents a specific target for NO: the enzyme contains heme as a prosthetic group, and NO, in binding to the heme moiety, causes a marked activation of the enzyme.[60,61] The observation that the expression of the heterodimeric form $\alpha_1\beta_1$ of sGC is not detected in activated human HSCs raises the possibility of a down-regulation of this enzyme, as suggested for other pathophysiologic conditions.[42] At the cellular level, prolonged pretreatment with mediators such as interleukin 1β and lipopolysaccharide causes a marked inhibition of sodium nitroprusside–induced activation of sGC.[62] In addition, an endogenous inhibitor of sGC has been identified and partially purified from bovine lung.[63] Because the lack of detectable expression of the heterodimeric form $\alpha1\beta1$ of sGC is common to HSC lines isolated from normal liver tissue obtained from different individuals and activated by culture on plastic, it is tempting to speculate that this represents a general feature related to the process of HSC activation in which several structural and functional changes occur, including the establishment of autocrine loops for growth factors, cytokines, and other soluble mediators.[11,12] This possibility is consistent with the finding that expression of the heterodimeric form $\alpha1\beta1$ of sGC and NO donor–induced cGMP synthesis are evident in quiescent HSCs, freshly isolated from normal human liver, whereas they become undetectable during serial subculture on plastic.

The inability of NO donors to induce cGMP accumulation in fully activated human HSCs is in contrast with previous reports in stellate cells isolated from rat liver,[54] in which sodium nitroprusside at rather high doses (500 μmol/L) was shown to increase intracellular cGMP levels. This discrepancy could be caused by the fact that rat stellate cells were used in an earlier stage of activation, although species differences between rat and human cells cannot be ruled out.

The role of NO in the regulation of COX activity and its importance in physiology, pathology, and therapy has been highlighted in recent years.[50,64–66] Evidence from several studies suggests that COX enzymes are targets for the pathophysiologic roles of NO and that, once activated in the presence of NO, they represent important transduction mechanisms for its multifaceted actions. The pathway leading to COX activation by NO is still poorly elucidated but, analogously to sGC, may involve an interaction with the iron-heme center of the enzyme.[50] The data in our present study suggest that incubation with NO donors results in a rapid and sustained increase in $PGE_2$ synthesis in activated human HSCs. In agreement with this observation, it is possible that the inhibitory action of NO donors on PDGF action and signaling is mediated through an autocrine loop involving $PGE_2$: these autacoids, by interacting with the specific membrane receptor, activate the adenylate cyclase/cAMP/PKA. Along these lines, established experimental evidence obtained in activated human HSCs has clearly shown that activation of this signaling pathway is associated with effects on PDGF action and signaling similar to those observed in the presence of NO donors.[51–53] In agreement with this alternative working hypothesis, $PGE_2$ synthesis blockade obtained by using the COX inhibitor ibuprofen results in a decrease in NO donor–induced cAMP synthesis and in abrogation of the inhibitory effect of NO donors on both PDGF action and intracellular signaling.

These results suggest that the pharmacologic effects of NO donors in activated human HSCs are largely dependent on the activation of the adenylate cyclase/cAMP/PKA pathway. However, in comparing the effects of the compounds used as tools to deliver intracellular NO in human HSCs, NTG, and SNAP, it is clear that the relative mechanisms of action may be, at least in part, different. Although SNAP always appears more potent than NTG in inhibiting PDGF biological effects and intracellular signaling, its effect on $PGE_2$ and cAMP synthesis is significantly lower in comparison with NTG. It is therefore conceivable that the action of SNAP is partially independent of the activation of the adenylate cyclase/cAMP/PKA pathway. In particular, considering the effect of NO donors on PDGF-induced $[Ca^{2+}]_i$ increase, it is evident that SNAP exerts a more pronounced inhibitory action when compared with NTG. According to the available knowledge, activation of the cAMP/PKA pathway may cause phosphorylation of both membrane and sarcoendoplasmic reticulum calcium ATPases in several cell types, including smooth muscle cells. Phosphor-

ylation is associated with increased activity of these calcium pumps with a consequent increase in the velocity of cytosolic calcium resetting to prestimulatory values after calcium transient.[67] The results of the present study suggest that, in addition to this mechanism, pretreatment with SNAP may influence PDGF-induced $[Ca^{2+}]_i$ increase through a dose-dependent inhibition of PDGF-induced PLCγ phosphorylation, and possibly its activation with reduced synthesis of downstream effectors, such as $IP_3$, is able to affect $[Ca^{2+}]_i$ dynamics. This observation is similar to previous findings in fibroblasts where inhibition of PLCγ phosphorylation was shown to be a possible molecular target for the action of NO.[55]

Demonstration of an inhibitory effect of NO donors on PDGF-induced biological effects in human HSCs suggests that these therapeutic agents may exert a direct antifibrogenic action by inhibiting proliferation, motility, and contractility of this key cell type, in addition to a reduction of fibrillar extracellular matrix accumulation. Accordingly, prolonged administration of orally active NO donors may reduce the contractility of HSCs present in fibrotic septa as well as their proliferation, migration, and extracellular matrix accumulation, thus affecting several mechanisms responsible for the increased intrahepatic resistance to portal flow. Although oral administration of nitrovasodilators, NTG in particular, may result in active concentrations within the portal circulation fairly close to those used in the present study,[68] the introduction of orally active NO donors, reaching higher concentrations within the portal circulation and provided with better intrahepatic bioavailability, may further improve the treatment of portal hypertension caused by fibrogenic chronic liver diseases.

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**Address requests for reprints to: Massimo Pinzani, M.D., Ph.D., Dipartimento di Medicina Interna, Università degli Studi di Firenze, Viale G.B. Morgagni, 85, I-50134 Firenze, Italy. e-mail: m.pinzani@dfc.unifi.it; fax: (39) 055-417123.**

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