Preferential Inhibition of Platelet-derived Growth Factor-stimulated DNA Synthesis and Protein Tyrosine Phosphorylation by Nordihydroguaiaretic Acid*

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Nordihydroguaiaretic acid (NDGA), a reportedly specific lipoxigenase inhibitor, was found to selectively inhibit platelet-derived growth factor (PDGF)-stimulated DNA synthesis in Swiss 3T3 cells. Maximal inhibition of PDGF-induced [3H]thymidine incorporation (96%) was observed using 4 μM NDGA (IC₅₀ = 1.5 μM). No effect of NDGA was observed upon DNA synthesis stimulated with either fetal bovine serum, bombesin, or epidermal growth factor (EGF) in the presence of insulin, or with the potent mitogen Pasteurella multocida toxin. The selective inhibition of PDGF-stimulated DNA synthesis by NDGA was also observed in diploid murine cells, rat, and human fibroblasts. Furthermore, 4 μM NDGA also inhibited PDGF-stimulated anchorage-independent colony growth of rat-1 cells by 76%. Using Swiss 3T3 cells, we found that PDGF-stimulated arachidonic acid mobilization and prostaglandin E₂ production was abolished by NDGA in a dose-dependent manner. Inhibition of PDGF-stimulated arachidonic acid mobilization by NDGA could not, however, explain its potent inhibitory effect upon PDGF-stimulated DNA synthesis.

Our results showed that NDGA also selectively inhibited PDGF receptor tyrosine phosphorylation in a dose-dependent manner in intact cells. Protein tyrosine phosphorylation stimulated by EGF or bombesin was not altered by NDGA treatment. Crucially, NDGA inhibited in vitro the tyrosine kinase activity of anti-phosphotyrosine and anti-PDGF receptor immunoprecipitates prepared from cultures stimulated with PDGF. This inhibition of receptor tyrosine phosphorylation in a cell-free system confirmed that NDGA acts directly at the level of the PDGF receptor tyrosine kinase domain. These results suggest that the potent and selective inhibitory effect of NDGA on PDGF-stimulated DNA synthesis results from its inhibitory action on tyrosine phosphorylation.

PDGF* is a potent mitogen for cells of mesenchymal origin and has been implicated in wound healing, development, and inflammation in addition to the etiology of many disease processes including atherosclerosis, rheumatoid arthritis, and oncogenesis (1, 2). PDGF is a disulfide-linked dimer of two related polypeptide chains which are assembled either as homodimers or as a heterodimer. Binding of PDGF to its cell surface receptor causes receptor dimerization and transphosphorylation of specific residues along the PDGF receptor polypeptide chain (1, 3, 4). These phosphorylated tyrosine residues serve as attachment sites for intracellular proteins, an interaction mediated through SH2 domains present in the effector proteins (5). Substrates for the PDGF receptor include phospholipase Cγ, the ras GTPase activating protein, the p85 regulatory subunit of phosphatidylinositol 3-kinase, members of the pp60⁶ family of protein tyrosine kinases, Nck, GRB2, and the phosphotyrosine phosphatase Syp (6–9). Once bound to the receptor, many of these substrates are phosphorylated on tyrosine residues by the receptor kinase activity. PDGF thus triggers a diverse array of downstream early signaling events (10–14). Despite the role of PDGF in a variety of disorders characterized by excessive cell proliferation and the increasing understanding of the molecular events involved in PDGF-stimulated signal transduction, few pharmacological agents exist which selectively inhibit the ability of PDGF to induce DNA synthesis.

The proliferation of Gₛ-arrested cells can be triggered by multiple signal transduction pathways that act in a combinatorial and synergistic fashion (11, 15). The release of arachidonic acid from the sn-2 position of membrane phospholipids is increasingly implicated as one of the signals involved in this process (16–18). PDGF stimulates a striking biphasic mobilization of arachidonic acid in Swiss 3T3 cells (19). The mechanisms by which arachidonic acid participates in mitogenic signal transduction, however, remain unclear. Arachidonic acid could either act directly as a second messenger (20–23) or alternatively serve as the substrate for the production of a variety of biologically active eicosanoids (24). To evaluate the action of eicosanoids in many biological systems, specific inhibitors of these substrates are themselves phosphorylated on specific residues along the PDGF receptor polypeptide chain (1, 2, 4). The phosphorylated tyrosine residues serve as attachment sites for intracellular proteins, an interaction mediated through SH2 domains present in the effector proteins (5). Substrates for the PDGF receptor include phospholipase Cγ, the ras GTPase activating protein, the p85 regulatory subunit of phosphatidylinositol 3-kinase, members of the pp60⁶ family of protein tyrosine kinases, Nck, GRB2, and the phosphotyrosine phosphatase Syp (6–9). Once bound to the receptor, many of these substrates are phosphorylated on tyrosine residues by the receptor kinase activity. PDGF thus triggers a diverse array of downstream early signaling events (10–14). Despite the role of PDGF in a variety of disorders characterized by excessive cell proliferation and the increasing understanding of the molecular events involved in PDGF-stimulated signal transduction, few pharmacological agents exist which selectively inhibit the ability of PDGF to induce DNA synthesis.

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In the present study, we have examined the ability of NDGA to attenuate PDGF-stimulated DNA synthesis in an effort to determine the contribution of leukotriene biosynthesis to this process. We found that this phenolic plant lignan dramatically inhibited PDGF-stimulated DNA synthesis in a selective manner. Surprisingly, the mechanism by which this effect is achieved involves inhibition of PDGF receptor protein tyrosine kinase activity.

EXPERIMENTAL PROCEDURES

Cell Culture—Stock cultures of Swiss 3T3 cells were propagated as previously described (32). For experimental purposes, cells were sub-
cultured in 35- or 90-mm Nunc dishes with DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were incubated in a humidified atmosphere of 10% CO2, 30% air at 37 °C. Cells were rendered quiescent by incubation under these conditions for 6-8 days prior to use.

Tertiary passage mouse embryo fibroblasts (TMEF), rat-1 cells, and human foreskin fibroblasts used at the 18th passage were seeded at 105 cells per 33-mm dish in DMEM supplemented with 10% FBS. After 3 days, the cultures were switched to DMEM containing 0.5% FBS. The cells were confluent and quiescent 4-6 days later.

**[H]Thymidine Incorporation Assay**—Determination of DNA synthesis was performed as previously described (33). Briefly, cultures were washed twice with Hanks' balanced salt solution and incubated with DMEM-Wyman's medium (1:1 v/v) containing [3H]thymidine (1 µCi/ml) and various additions as described. After 40 h, the cultures were washed twice with phosphate-buffered saline and incubated in 5% trichloroacetic acid for 30 min at 4 °C. Trichloroacetic acid was then removed, and the cultures were washed twice with ethanol and extracted in 1 ml of 2N NaOH, 0.1M NaOH, 1% SDS. Incorporation was determined by scintillation counting in 10 ml of Picofluor.

**Autoradiography of Labeled Nuclei**—Labeled nuclei were determined by autoradiography as described previously (34). Cultures were washed twice with DMEM and incubated in 2 ml of DMEM/Wyman's medium (1:1 v/v) containing [3H]thymidine (5 µCi/ml) and various additions. After 40 h, the medium was removed, and the cultures were washed twice with Triage saline, pH 7.4, at 4 °C, fixed (for 5 and 2 min) with 5% trichloroacetic acid at 4 °C, and washed three times with 70% ethanol. Autoradiographic film was put on the cultures and developed 6 days later. After staining with Giemsa, the labeled nuclei were counted.

**Clonogenic Assay**—Stock cultures of rat-1 cells, 3-4 days after passage, were trypsinized and gently dispersed to ensure a single-cell suspension. Cells were added to DMEM supplemented with 2.5% FBS and other factors, as described for each experiment, to give 5 x 103 cells per ml. Agarose was added at a dilution of 0.3%. An aliquot (1 ml) of this mixture was plated onto 35-mm plastic dishes containing a 2-ml base medium. Stock cultures of rat-1 cells, 50 µl were removed and processed as described above. The volume of medium removed was replaced with fresh medium at 37 °C containing the corresponding factors.

**Production of PGE2**—Cultures pretreated for 1 h in the absence or presence of NDGA were washed twice with DMEM and incubated with PDGF and NDGA at 37 °C in 1 ml of DMEM. After 1 h, the medium was removed, and the PGE2 released into the medium was determined by radioimmunoassay as follows. Samples were diluted in assay buffer consisting of 0.15 M NaCl, 5 mM Na2HPO4, 0.15 M NaH2PO4, 0.1% bovine y-globulin, 0.005% Triton X-100, 0.05% sodium azide, and 25 µm phosphate buffer, pH 6.8. Rabbit anti-PGE2 antibody, 152-gPGE2, and the samples were incubated for 24 h at 4 °C. Antibody-bound tracer was precipitated by the addition of 16% polyethylene glycol, 0.05% sodium azide, and 50 µl 100 units/ml of EGF. After 30 min at 4 °C, the precipitates were centrifuged at 4 °C (5,000 g, 30 min), and the supernatants were aspirated. The radioactivity in the pellets was determined by scintillation counting.

**Immunoprecipitations**—Quiescent cultures of Swiss 3T3 cells were incubated in the absence or presence of NDGA for 1 h. After this time, the cells were washed twice with DMEM and treated with factors for 10 min at 37 °C in the absence or presence of NDGA as indicated. The cells were then lysed at 4 °C in 1 ml of 100 µM Tria/HCl, pH 7.6, 5 µm EDTA, 50 µm NaCl, 30 µm sodium pyrophosphate, 50 µm Na3, 10 µm Na3VO4, 1% Triton X-100, and 1 µm phenylmethylsulfonyl fluoride (lysine buffer). Lysates were clarified by centrifugation at 13,000 g and preceeded by incubation with albumin/agarose for 1 h at 4 °C. After removal of albumin/agarose by a brief (10 s) centrifugation, the supernatants were transferred to a fresh tube, and phosphotyrosyl proteins were immunoprecipitated for 16 h at 4 °C with argoside-linked antiblastic phosphotyroline monoclonal antibody (Ab 1-1). For the immunoprecipitation of p120, the 4B12 monoclonal antibody was precoupled to goat anti-mouse IgG-agarose. Lysates were also incubated with anti-GTPase activating protein, anti-phospholipase C-γ1, and anti-α-pDGFR-protecting antibody (PDGF-R7) anti-serum (35) for 16 h at 4 °C. At the end of this time, the immune complex was washed twice with ice-cold 1% non-fat milk in phosphate-buffered saline, and the immune precipitates were then transferred to Immobilon membranes. These membranes were blocked using 5% non-fat dried milk in phosphate-buffered saline, pH 7.2, and incubated for 3-5 h with the anti-phosphotyrosine monoclonal antibody mix (Py20 and 4G10, 1 µg/ml anti-body) in PBS containing 3% non-fat milk as described (36). Immune reactive bands were visualized using [125I]-labeled sheep anti-mouse iγG followed by autoradiography.

**Assay of Protein Kinase Activity**—Immunoprecipitates prepared from 1 x 105 cells as described above were washed three times with lysis buffer and twice with 50 µl HEPES, pH 7.4, 0.1 µl EDTA, 0.01% Brij, 75 µm NaCl, and 100 µm Na3VO4 (kinase assay buffer, NDGA). NDGA was added to the immunoprecipitates in 1 ml α'kinase assay buffer, and the mixture was incubated for 15 min at 4 °C. At this time, the immunoprecipitates were resuspended in 20 µl of kinase buffer, and the reaction was initiated by the addition of 10 µm MgCl2 and 100 µM [γ-32P]ATP (10 Ci/ml) in a total volume of 30 µl at 30 °C. After a 10-min incubation, immunoprecipitates were washed twice with lysis buffer and analyzed by SDS-PAGE followed by autoradiography.

**Materials—**PDGF (c-iso homodimer), (5,6,8,9,11,12,14,15-[H]lArachidonic acid (211 C/mmol), [γ-32P]ATP (5000 Ci/mmol), [125I]-labeled sheep anti-mouse IgG (15 µCi/pg), and [3H]thymidine were obtained from Amersham International. NDGA, bombesin, vasopressin, phorbol 12,13-dibutyrate, EGF, and goat anti-mouse IgG-agarose were from Sigma. Agarose-linked anti-phosphotyrosine monoclonal antibody (Ab-1) was purchased from Oncogene Science (Mineola, NY), and Py20 anti-phosphotyrosine antibody was from ICN. The 4G10 anti-phosphotyrosine monoclonal antibody was from Tissue Culture Supplies, Buckingham, UK. The monoclonal antibody directed against p120 (2B12) was a generous gift of Dr. Thomas Persons, University of Virginia, Charlottesville, Virginia. Rabbit antiseraum raised against the α-PDGFR receptor chain (PDGR-F7) was kindly provided by Dr Carl-Henrik Hel-din, Ludwig Institute for Cancer Research, Uppsala, Sweden (35). Anti-Factor Xa reactivity against protein A-agarose was 0.15 µg/ml, and anti-phosphotyrosine antibody was from Sigma. NDGA was obtained from BDH (Poole, England). Anti-PLC-γ1 antiseraum was a generous gift of Dr. Peter Parker, ICRF, UK. All other reagents used were of analytical grade.

**RESULTS**

**NDGA Selectively Inhibits PDGF-stimulated DNA Synthesis**—Confluent and quiescent cultures of Swiss 3T3 cells were stimulated with PDGF in the absence or presence of increasing concentrations of NDGA. At the end of 40 h, the incorporation of [3H]thymidine into cellular DNA was determined. Fig. 1 shows that NDGA caused a dramatic inhibitory effect upon PDGF-mediated mitogenesis. Maximal inhibition of PDGF [3H]thymidine incorporation (96%) was observed at 4 µM NDGA. Parallel cultures were stimulated with PDGF in the presence of insulin or with the potent mitogen Pasteurella multocida toxin (37). No effect of NDGA was observed upon DNA synthesis stimulated by these mitogens over the same concentration range of NDGA which completely blocked the stimulation by PDGF. In addition, no effect of NDGA at 4 µM was observed upon FBS- and insulin-stimulated DNA synthesis while the response to vasopressin and insulin and phorbol 12,13-dibutyrate and insulin was attenuated by 22% and 34%, respectively.

On the basis of these results, we proceeded to examine the
NDGA Inhibits PDGF-stimulated DNA Synthesis

Fig. 1. Effect of NDGA on mitogen-stimulated DNA synthesis in Swiss 3T3 cells. Upper panel, cultures of Swiss 3T3 cells were washed twice with DMEM and incubated at 37 °C in 2 ml of DMEM/Waymouth’s medium containing 1 μCi/ml [3H]thymidine and various mitogens: 10% FBS, 50 ng/ml PDGF (closed circles), 10 ng/ml P. multocida toxin (open circles), 6 ng bombesin, and 1 μg/ml insulin (open squares), and 5 ng/ml EGF and 1 μg/ml insulin (closed squares) in the presence of various concentrations of NDGA as indicated. Lower panel, confluent and quiescent cultures were washed and incubated at 37 °C in 2 ml of DMEM/Waymouth’s medium containing 1 μCi/ml [3H]thymidine and various mitogens: 10% FBS, 25 ng/ml PDGF, 1 μg/ml insulin (INS), 20 nm vasopressin and 1 μg/ml insulin (VP+INS), and 200 nm phorbol 12,13-dibutyrate and 1 μg/ml insulin (PDB+INS), in the absence (open bars) or presence (closed bars) of 4 μM NDGA. After 40 h, DNA synthesis was assessed by measuring the [3H]thymidine incorporated into acid-precipitable material. Each point is expressed as a percentage of the incorporation induced by 10% FBS, and data are shown as mean ± S.E. (n = 6-41).

effect of NDGA upon PDGF-induced DNA synthesis in greater detail. Fig. 2A shows the dose-dependent stimulation of DNA synthesis by PDGF in the absence and presence of NDGA. In control cultures, maximal stimulation of DNA synthesis by PDGF was obtained using 7.5 ng/ml (EC50 = 3.5 ng/ml). This response was dramatically inhibited (≤97%) by 4 μM NDGA. At PDGF concentrations greater than 5 ng/ml, however, some stimulation was still retained. It is known that insulin markedly increases the potency of PDGF for inducing DNA synthesis (38). Fig. 2B shows the synergistic stimulation of DNA synthesis by PDGF and insulin. In the presence of 1 μg/ml insulin, the PDGF mitogenic dose response is shifted to the left. Under these conditions, maximum [3H]thymidine incorporation was observed at 2.5 ng/ml PDGF (EC50 = 0.5 ng/ml). NDGA caused a 15-fold displacement of this response (EC50 = 7.5 ng/ml). Stimulation of DNA synthesis by 10 ng/ml PDGF together with insulin in the absence and presence of 4 μM NDGA was 101% and 85% of FBS, respectively.

EGF in the presence of a fixed concentration of insulin (0.5 μg/ml) also produced a dose-dependent increase in [3H]thymidine incorporation (Fig. 2C). Maximal stimulation of DNA synthesis was obtained by 1.25 ng/ml EGF (EC50 = 0.15 ng/ml). In the presence of NDGA (4 μM), a slight stimulation of [3H]thymidine incorporation was observed with insulin alone, and, at maximal EGF concentrations, only a slight inhibitory effect was observed (18%).

To confirm that NDGA was inhibiting PDGF-stimulated DNA replication rather than changing the specific activity of the [3H]thymidine precursor pool, quiescent cultures of Swiss 3T3 cells were treated in the absence or presence of NDGA together with various factors, and the incorporation of [3H]thymidine into DNA subsequently was quantified by autoradiography of labeled nuclei. Fig. 3A shows the dose-dependent enhancement of the labeling index by PDGF. Maximum stimulation by PDGF was observed using 10 ng/ml (EC50 = 3.5 ng/ml). These values are in good agreement with the measurement of [3H]thymidine incorporation. Stimulation of PDGF-induced DNA synthesis was severely inhibited (>98%) by 4 μM NDGA. The selective nature of NDGA inhibition as assessed by autoradiography of labeled nuclei is shown in Fig. 3B. A slight synergistic effect of NDGA with insulin was observed (control 2.3%, NDGA 15.4%) while NDGA inhibited the effect of P. multocida toxin, bombesin and insulin, EGF and insulin, and vasopressin and insulin by 13%, 28%, 34%, and 14%, respectively. Such inhibitions are similar to those observed when the incorporation of [3H]thymidine stimulated by these mitogens was measured (Fig. 1). The results of these experiments confirm a marked inhibitory effect of NDGA upon PDGF-stimulated DNA synthesis in Swiss 3T3 cells.

In view of the selective effects of NDGA in preventing PDGF-stimulated DNA synthesis in Swiss 3T3 cells, we determined whether similar effects of NDGA could be observed in diploid mouse fibroblasts as well as in cells from other species. Quiescent cultures of TMEF cells, rat-1 cells, and human foreskin fibroblasts were stimulated with PDGF and other growth-promoting factors either in the absence or presence of 2 μM NDGA. Cumulative [3H]thymidine incorporation was then measured after 24 h of incubation. NDGA abolished the PDGF stimulation of DNA synthesis in each cell type (Fig. 4). No significant effect of NDGA was observed upon the DNA synthesis stimulated by a range of other mitogens in these cells with the exception of an inhibition (36%) of the mitogenic effect of EGF and insulin in TMEF cells.

We also examined the effect of NDGA upon PDGF-stimulated anchorage-independent growth of rat-1 cells. As shown in Table 1, PDGF and EGF both induced colony formation of this cell type causing a 9-fold and 6-fold increase in the number of colonies per dish, respectively. When both factors were added...
together, a synergistic stimulation was observed (39-fold). Addition of 4 \( \mu \)M NDGA markedly inhibited the PDGF effect (78% inhibition) while no inhibition of EGF-stimulated colony formation was seen. NDGA also inhibited the synergistic effect of PDGF and EGF (79% inhibition) to that observed in the presence of EGF alone. The results of these experiments demonstrated that the marked inhibitory effect of NDGA upon PDGF-stimulated DNA synthesis is not restricted to the Swiss 3T3 cell line but is also observed in murine diploid cells (TMEF), rat-1 cells, and human fibroblasts.

PDGF-stimulated Arachidonic Acid Mobilization and PGE\(_2\) Release Is Also Inhibited by NDGA—PDGF, unlike EGF, stimulates a sustained mobilization of arachidonic acid in Swiss 3T3 cells (19). Once mobilized, the metabolism of this fatty acid in these cells is directed toward PGE\(_2\) biosynthesis (10, 14, 19). If

NDGA inhibits eicosanoid production by acting as a potent lipoygenase inhibitor (25-27), it should not interfere with either arachidonic acid mobilization or PGE\(_2\) production in response to PDGF stimulation. To test this, cultures were labeled with \(^{3}H\)arachidonic acid for 18 h followed by pretreatment for 1 h with or without NDGA as indicated. After this time, the cells were incubated for 1 h in the absence or presence of PDGF and NDGA. Fig. 5A shows that PDGF causes a 34-fold increase in arachidonic acid release after 1 h of stimulation. Suprisingly, NDGA produced a dose-dependent inhibition of this effect \((IC_{50} = 1.4 \, \mu M)\). Maximum inhibition (94%) was achieved by 5 \( \mu \)M NDGA. PDGF stimulated arachidonic acid mobilization in a dose-dependent manner (Fig. 5B). Maximal arachidonic acid mobilization was achieved by 12 ng/ml PDGF \((EC_{50} = 7.5 \, ng/ml)\). NDGA (4 \( \mu \)M) potently inhibited PDGF-mediated arachidonic acid release over the entire dose-response curve.

The inhibition of PDGF-stimulated arachidonic acid mobilization by NDGA was not due to a shift in the kinetics of the response. Fig. 5C shows the biphasic pattern of PDGF-mediated arachidonic acid release previously described in Swiss 3T3 cells (19). NDGA (4 \( \mu \)M) inhibited the arachidonic acid release stimulated by PDGF (25 ng/ml) over a 1-h period. In Swiss 3T3 cells, the predominant arachidonic acid metabolite is PGE\(_2\). PDGF (25 ng/ml) produced a 36-fold increase in PGE\(_2\) production after 1 h of stimulation (Fig. 5D). This effect was inhibited by NDGA in a dose-dependent manner. Half-maximal inhibition was achieved with 0.9 \( \mu \)M NDGA, and maximal inhibition (97%) was obtained by 5 \( \mu \)M NDGA.

The data shown in Fig. 5 demonstrate that NDGA severely inhibits PDGF-stimulated arachidonic acid mobilization, and, consequently, PGE\(_2\) production in a dose-dependent manner in Swiss 3T3 cells.

PDGF-stimulated Tyrosine Phosphorylation in Intact Cells Is Inhibited by NDGA—Inhibition of arachidonic acid mobilization by NDGA could not account for the dramatic effect of NDGA on PDGF-stimulated DNA synthesis over its entire dose response (14). We reasoned that other signaling events were also affected. Since stimulation of PDGF receptor tyrosine kinase activity is a prerequisite for the induction of DNA synthesis by this ligand (1, 3, 4), we examined whether exposure of Swiss 3T3 fibroblasts to NDGA inhibited PDGF-stimulated tyrosine phosphorylation. Quiescent cultures were pretreated for 1 h with or without NDGA and then stimulated with PDGF, EGF, or bombesin in the absence or presence of NDGA as in-
NDGA Inhibits PDGF-stimulated DNA Synthesis

![Graph](image_url)

**Figure 5.** NDGA inhibits PDGF-stimulated arachidonic acid mobilization and PGE₂ production in Swiss 3T3 cells. Panel A, confluent and quiescent cultures were labeled for 1 hr with [³H]arachidonic acid as described under “Experimental Procedures.” NDGA was added to the cultures 1 hr prior to the start of the experiment. The duration of NDGA treatment is indicated by the closed circles. Panel B, cultures labeled with [³H]arachidonic acid were stimulated with various concentrations of PDGF as indicated in the absence (open circles) or presence (closed circles) of 25 ng/ml PDGF together with NDGA as indicated. Panel C, cultures of Swiss 3T3 cells were labeled with [³H]arachidonic acid for 1 hr. Some dishes received NDGA (4 μM) 1 hr prior to stimulation. At the end of the time indicated, aliquots (50 μl) of the extracellular medium were removed and the radioactivity released was determined. Where appropriate, cultures received NDGA 1 hr prior to PDGF stimulation in addition to its presence throughout the experiment. Panel D, cultures of Swiss 3T3 cells were labeled with [³H]arachidonic acid for 1 hr. Some dishes received NDGA (4 μM) 1 hr prior to stimulation. At the end of the time indicated, the cultures were washed twice with DMEM and incubated in the absence (open symbols) or presence (closed symbols) of PDGF and absence (circles) or presence (squares) of 4 μM NDGA. At the times indicated, aliquots (50 μl) of the extracellular medium were removed, and the radioactivity present was determined by β counting. The volume of medium removed was replaced together with the corresponding factors. Values obtained in the absence of PDGF (open symbols) overlap with the values obtained in cultures treated with PDGF and NDGA (closed squares). Panel D, confluent and quiescent cultures were washed twice with DMEM and incubated in the absence (open circles) or presence (closed circles) of 25 ng/ml PDGF together with NDGA as indicated for 1 hr at 37°C. At the end of the time, the extracellular medium was removed and centrifuged at 15,000 x g for 5 min, and the supernatant was subjected to a specific radioimmunoassay for PGE₂ as described under “Experimental Procedures.” Where used, NDGA was added to the cultures 1 hr prior to the start of the experiment and was present throughout.

**Table I**

| Addition(s) | None | PDGF | EGF | PDGF + EGF |
|------------|------|------|-----|------------|
| None       | 4 ± 0.7 | 37 ± 2.9 | 25 ± 3.6 | 156 ± 10.2 |
| NDGA       | 3 ± 0.1 | 9 ± 0.7  | 28 ± 3.7  | 33 ± 3.9   |

**Legend:**

**TABLE I** The effect of NDGA on PDGF- and EGF-stimulated colony formation by rat-1 cells

NDGA was used at concentration of 4 μM. PDGF and EGF were both used at 25 ng/ml. Colony formation (mean ± S.E., n = 5) of rat-1 cells was determined in 0.2% agarose containing DMEM and 2.5% FBS in the absence or presence of growth factors as indicated.

Dedicated. Bombesin was used as an example of tyrosine phosphorylation induced through a G-protein-linked signal transduction pathway (36, 39–41). After 10 min, the cultures were lysed, and anti-phosphotyrosine immunoprecipitates were prepared. The immunoprecipitates were analyzed by Western blotting with a specific anti-phosphotyrosine monoclonal antibody.

Stimulation of cultures with PDGF resulted in the increased labeling of a major band migrating with an apparent M₆ of 180,000–190,000 comprising both α- and β-PDGF receptors (Fig. 6, upper panel). The labeling of additional components including bands of M₆ = 110,000–120,000 and 70,000–80,000 was also observed. NDGA (10 μM) abolished receptor autophosphorylation using 10 ng/ml PDGF and severely inhibited receptor autophosphorylation by 25 ng/ml PDGF (60% inhibition). Stimulation of Swiss 3T3 cells with EGF resulted in the tyrosine phosphorylation of substrates, migrating as two major bands upon SDS/PAGE. The first of M₆ = 170,000–180,000 includes the EGF receptor (42), the second has a M₆ = 100,000–130,000. Bombesin stimulation resulted in the tyrosine phosphorylation of substrates migrating as two major bands upon SDS/PAGE. The first of M₆ = 110,000–130,000 and 70,000–80,000. No inhibitory effect of NDGA was observed upon the tyrosine phosphorylation stimulated in intact cells by either EGF or bombesin.

NDGA-mediated inhibition of PDGF receptor phosphorylation was dose-dependent (Fig. 6A). Total inhibition of receptor autophosphorylation stimulated by 10 ng/ml PDGF was achieved using 3 μM NDGA (IC₅₀ = 0.85 μM). When the concentration of PDGF was increased to 25 ng/ml, maximal inhibition of receptor phosphorylation by NDGA was 60%.

To complement the Western blot analysis, protein tyrosine kinase activity of anti-phosphotyrosine immunoprecipitates obtained from cells stimulated in the absence and presence of PDGF and NDGA was also determined. Cultures incubated with or without NDGA for 1 hr were stimulated in the presence and absence of PDGF and NDGA for 10 min. After cell lysis and immunoprecipitation with anti-phosphotyrosine monoclonal antibodies, the resulting immune complexes were incubated with [γ-³²P]ATP for 10 min in the absence or presence of NDGA as indicated, and the products were analyzed by SDS-PAGE. Immunoprecipitates prepared from cultures stimulated with 10 ng/ml and 25 ng/ml PDGF produced a marked phosphorylation of the PDGF receptor migrating with an M₆ of 180,000–190,000 (Fig. 6B). Addition of NDGA caused a dose-dependent inhibition of receptor phosphorylation stimulated by both concentrations of PDGF with maximal inhibition achieved at 4 μM and 10 μM NDGA, respectively.

**NDGA Inhibits PDGF-stimulated Tyrosine Phosphorylation of Distinct Receptor Substrates in Intact Cells—Ligand activation and autophosphorylation of the PDGF receptor is known to result in the tyrosine phosphorylation of multiple receptor substrates including GTPase activating protein, p120, and phospholipase Cγ₁ (6). We examined the degree to which tyrosine phosphorylation of these substrates was inhibited by NDGA. Quiescent cultures of Swiss 3T3 cells were incubated with PDGF in the absence or presence of NDGA which was added 1 hr prior to stimulation. At the end of this time, cells were lysed, and proteins were immunoprecipitated with either anti-phosphotyrosine-agarose monoclonal antibodies, agarose-linked anti-p120 monoclonal antibody, anti-GTPase activating protein, or anti-phospholipase Cγ₁ antisera. The immunoprecipitates were analyzed by SDS-PAGE followed by immunoblotting with anti-phosphotyrosine monoclonal antibodies. Fig. 7 shows that 6 μM NDGA markedly inhibits the PDGF-stimulated tyrosine phosphorylation of GTPase activating protein (60%), p120 (88%), and phospholipase Cγ₁ (40%). Each antiserum also immunoprecipitated a band migrating with a M₆ = 180,000–190,000 which is most likely associated PDGF receptor. The anti-GTPase activating protein antiserum constitutively immunoprecipitated a band of M₆ = 180,000–185,000.

**NDGA Inhibits PDGF-stimulated Tyrosine Phosphorylation in a Cell-free System—**To determine whether NDGA inhibits receptor kinase activity directly, cultures of Swiss 3T3 cells were stimulated in the absence or presence of 25 ng/ml PDGF,
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Fig. 6. NDGA inhibits PDGF stimulation of tyrosine phosphorylation in intact cells. Upper panel, quiescent and confluent cultures of Swiss 3T3 cells were incubated for 1 h in the absence or presence of 10 μM NDGA as indicated. The cultures were then washed twice with DMEM and incubated for 10 min with either 10 ng/ml or 25 ng/ml PDGF, 10 mM bongkrekic acid, or 5 ng/ml EGF in the absence or presence of 10 μM NDGA. Cells were lysed, and anti-phosphotyrosine immunoprecipitates were prepared as described under “Experimental Procedures.” Tyrosine-phosphorylated proteins were detected by Western blot analysis using anti-phosphotyrosine monoclonal antibodies. Panel A, in intact cells, NDGA inhibited PDGF tyrosine phosphorylation in a dose-dependent manner. Quantitation of the NDGA-induced inhibition of PDGF receptor phosphorylation by 10 ng/ml PDGF (open circles) was performed by scanning densitometry. Panel B, confluent and quiescent cultures were incubated for 1 h in the absence or presence of NDGA as shown. The cultures were washed with DMEM and incubated for 10 min with either 10 ng/ml PDGF (closed circles) or 25 ng/ml PDGF (open circles) in the presence or absence of NDGA. Following cell lysis, anti-phosphotyrosine immunoprecipitates were prepared as described under “Experimental Procedures.” Protein kinase activity of the immunoprecipitates was determined by Western blotting with anti-phosphotyrosine monoclonal antibodies and autoradiography. Phosphorylation of the PDGF receptor was quantified by scanning densitometry.

and immunoprecipitates from lysates of these cells were prepared using anti-phosphotyrosine monoclonal antibodies. These immunoprecipitates were then preincubated in the absence or presence of NDGA for 15 min at 4 °C. After this time, the kinase activity of the immunoprecipitates was determined. Fig. 8 (left and center panels) shows that NDGA produces a dose-dependent inhibition of PDGF-stimulated kinase activity in the immunoprecipitates. Maximal inhibition was achieved using 10 μM NDGA (IC50 = 3.8 μM).

The effect of NDGA in vitro upon the protein tyrosine kinase activity of immunoprecipitates prepared using an α-PDGF receptor monoclonal antibody was also examined. Confluent and quiescent cultures of Swiss 3T3 cells were stimulated with 10 ng/ml PDGF for 10 min at 37 °C. The monolayer was lysed, and the extract was incubated with an α-PDGF receptor antibody (35) for 16 h at 4 °C. The immunoprecipitates were incubated in the absence or presence of 6 μM NDGA for 15 min at 4 °C, and their protein tyrosine kinase activity was determined as described under “Experimental Procedures.” Fig. 8 (right panel) shows the phospho-proteins obtained. The phosphorylation of the major band (M r = 170,000–190,000) representing the PDGF receptor was not dependent upon the exposure of PDGF prior to cell lysis. This finding confirms the deregulation of receptor kinase activity in solubilized preparations as previously described (43). Tyrosine phosphorylation of additional bands of M r = 90,000–100,000, 60,000–80,000, and 40,000–50,000 was also observed (Fig. 8, arrowheads). NDGA (6 μM) markedly inhibited the phosphorylation in vitro of the M r = 90,000–100,000 band (73%) and the 60,000–70,000 band (70%) and abolished the phosphorylation of the M r = 43,000–50,000 band. Treatment of intact cells with PDGF resulted in the association and subsequent tyrosine phosphorylation of a set of proteins of M r = 70,000–80,000 (Fig. 8, vertical bars). NDGA abolished the phosphorylation of these PDGF receptor substrates.

The result of this series of experiments (Figs. 6–8) demonstrates that NDGA selectively inhibits PDGF-stimulated tyrosine phosphorylation in intact cells and cell-free preparations.

DISCUSSION

The results of the present study demonstrate that NDGA produces a potent inhibition of PDGF-mediated DNA synthesis in a variety of cell types. NDGA, however, exerted little or no consistent effect upon the mitogenic response to a wide range of other growth factors including EGF, a ligand whose receptor also possesses intrinsic tyrosine kinase activity. Indeed, a small synergistic stimulation of DNA synthesis was observed when NDGA was added together with insulin. NDGA maintained its potent inhibition of PDGF-mediated DNA synthesis in the presence of insulin. This selective inhibition of PDGF-stimulated DNA synthesis was not confined to Swiss 3T3. Our results demonstrate a selective inhibition of PDGF-stimulated DNA synthesis in diploid murine cells, rat, and human fibroblasts. We have also shown that NDGA inhibits PDGF stimulation of
NDGA Inhibits PDGF-stimulated DNA Synthesis

Fig. 8. NDGA inhibits PDGF-stimulated tyrosine kinase activity in a cell-free system. Left panel, quiescent and confluent cultures were stimulated with 25 ng/ml PDGF for 10 min at 37 °C. Following cell lysis, anti-phosphotyrosine immunoprecipitates were prepared as described under "Experimental Procedures." The immunoprecipitates were then incubated in the absence or presence of various concentrations of NDGA for 15 min at 4 °C. After this time, protein kinase activity was determined by incubation with [γ-32P]ATP at 30 °C for 10 min in the absence or presence of NDGA. The products of the reaction were analyzed by SDS/PAGE and autoradiography. Center panel, in the cell-free system, NDGA inhibited PDGF tyrosine phosphorylation in a dose-dependent manner. Quantitation of the NDGA-induced inhibition of PDGF receptor phosphorylation by 25 ng/ml PDGF (open circles) was achieved by scanning densitometry. Right panel, cultures of Swiss 3T3 cells were incubated in the presence or absence of 10 ng/ml PDGF for 10 min at 37 °C. After cell lysis, immunoprecipitates were prepared using anti-α-PDGF receptor antisera (PDGFR-7). The immunoprecipitates were then incubated in the absence or presence of 6 μM NDGA for 15 min at 4 °C. After this time, protein kinase activity was determined by incubation with [γ-32P]ATP at 30 °C for 10 min in the absence or presence of 6 μM NDGA. The products of the reaction were analyzed by SDS/PAGE and autoradiography.

The results of our studies produced several lines of evidence indicating that NDGA potently inhibits PDGF receptor tyrosine kinase activity. Western blot analysis demonstrated that NDGA specifically inhibits PDGF-stimulated tyrosine phosphorylation in intact cells in a dose-dependent manner. Since the PDGF receptor is the major substrate of its own tyrosine kinase activity, this was taken as the reference for the inhibitory effect of NDGA. Analysis of protein tyrosine kinase activity of anti-phosphotyrosine immunoprecipitates prepared from cells stimulated with PDGF in the presence or absence of NDGA also revealed an inhibitory effect of this compound. Western blot analysis for specific PDGF receptor substrates demonstrated that NDGA inhibited the tyrosine phosphorylation of GTPase activating protein, phospholipase Cγ, and p120. Since the inhibition of p120 was quantitatively the most striking of the three substrates examined, these results suggest a differential inhibition of PDGF receptor substrate tyrosine phosphorylation by NDGA. Protein tyrosine phosphorylation stimulated by either EGF or bombesin was unaltered by NDGA treatment. Crucially, NDGA inhibited the ability of anti-phosphotyrosine and anti-α-PDGF receptor immunoprecipitates prepared from cultures treated with PDGF to stimulate tyrosine phosphorylation in vitro. These results suggest that the potent and selective inhibitory effect of NDGA on PDGF-stimulated DNA synthesis results from its inhibitory action on tyrosine phosphorylation.

In the search for antagonists of PDGF action, a number of low molecular weight compounds have emerged as promising candidates. One of the earliest examples was suramin (48). This compound was, however, subsequently shown to act in a nonspecific manner (49). The aminoglycoside neomycin was also found to affect certain PDGF responses (50). Its use is limited, however, because of the high concentrations required to achieve the effect on receptor binding. Peptides derived from the primary sequence of either PDGF or the extracellular portion of the PDGF receptor have also been shown to act as receptor antagonists (51, 52) as have antibodies to PDGF which inhibited autocrine stimulation of Simian Sarcoma virus-transformed cells (53). Since stimulation of receptor-mediated phosphorylation is the primary event following binding of many polypeptide growth factors to their receptors, one approach in the development of selective antagonists has been directed against this protein tyrosine kinase activity. The tryphostins are a group of synthetic compounds which exhibit potent inhibitory effects upon both membrane-bound and cytosolic protein tyrosine kinases (54). These compounds are themselves modelled upon natural compounds shown to inhibit phosphorylation including erbastatin, the flavone quercetin, the isoflavone genistein, and herbimycin A (55).
Our findings suggest that NDGA may provide a novel structural motif to generate tyrosine kinase inhibitors with selectivity for PDGF. In contrast to other PDGF receptor antagonists or inhibitors of tyrosine phosphorylation, NDGA has already been shown to possess low toxicity in animal models (56). Identification of such preferential inhibitory effects, novel mechanism of action, and low toxicity raises the possibility that NDGA or its derivatives may have important pharmaceutical potential. NDGA could play an important role in the treatment of various disease states which implicate PDGF as a causative proliferative agent including atherosclerosis, fibrotic conditions, and cancer.

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