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Simultaneous Activation of Adenylyl Cyclase and Protein Kinase C Induces Production of Nitric Oxide by Vascular Smooth Muscle Cells

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SUMMARY

Rat aortic smooth muscle cells produced large quantities of nitric oxide (NO) after exposure to interleukin-1β, and this was depressed in the presence of the protein kinase C inhibitor bisindolylmaleimide. Intracellular cAMP levels were elevated mildly in cytokine-treated smooth muscle cells, and the presence of forskolin enhanced both the cAMP levels and NO production. Inhibition of GTP:cyclohydrolase I by 2,4-diamino-6-hydroxypyrimidine attenuated NO production by interleukin-1β-treated cells. GTP:cyclohydrolase is the regulatory enzyme for *de novo* tetrahydrobiopterin synthesis, and the latter is a required cofactor for NO synthase activity. Treatment of smooth muscle cells with forskolin induced GTP:cyclohydrolase mRNA expression, and simultaneous treatment of cells with forskolin and phorbol esters elicited NO production. Angiotensin II and arginine-vasopressin, acknowledged agonists for protein kinase C, elicited production of NO by forskolin-treated smooth muscle cells. These observations confirm the importance of GTP:cyclohydrolase activity for NO production by cultured smooth muscle cells and implicate both adenylyl cyclase and protein kinase C in this process.

Treatment of cultured smooth muscle cells with IL-1β induced NOS (EC 1.14.23) expression and the prolonged production of NO (1–4). This was accompanied by the activation of guanylyl cyclase and elevation of intracellular cGMP levels (1, 3). The possible physiological relevance of this metabolism in vascular homeostasis is unclear, and the process may impinge upon the hypotension associated with septic shock. In addition, little is known about the mechanisms of induction of NO production by IL-1β-treated smooth muscle cells (5).

The catalytic conversion of L-arginine to citrulline by inducible NOS and the concomitant production of NO is dependent on an adequate supply of tetrahydrobiopterin (6, 7). *De novo* synthesis of biopterin is regulated by the activity of GTP:CH (EC 3.5.4.16), and this in turn appears to be controlled by intracellular cAMP (8). This interaction could explain the robust effect of IL-1β on NO production by smooth muscle cells. The cytokine is capable of inducing the expression of GTP:CH (9), possibly via elevation of cAMP levels (10). Thus, the cytokine stimulates both the expression of NOS (by unknown mechanisms) and the pathway essential for cofactor (tetrahydrobiopterin) production (see Fig. 1).

The initial signal transduction events elicited by cytokines invariably involve activation of protein kinases (5, 11, 12). There are a number of inhibitors that have been utilized to gain insight into the role of these kinases in intracellular signal transduction (11, 12). Additionally, direct activation of PKC by phorbol esters has facilitated the identification of the role of this group of kinases in a number of signal cascades (12). In an analogous manner, forskolin and its biologically inactive analog 1,9-dideoxyforskolin have been used to study adenylyl cyclase and the role of cAMP in intracellular signaling (13, 14).

NO production in response to interferon-γ is attenuated by inhibition of PKC in murine macrophages (15). Similarly, down-regulation of PKC by prolonged treatment with phorbol esters results in diminished production of NO in the same cell system (15). These observations implicate PKC in the induction of NOS by cytokines. Experiments using bisindolylmaleimide, a specific PKC inhibitor (16), suggested a role for this group of kinases in the production of NO elicited by IL-1β treatment of smooth muscle cells. Additionally, an important role for the activation of adenylyl cyclase was indicated by the

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ABBREVIATIONS: IL-1β, interleukin-1β; DAHP, 2,4-diamino-6-hydroxypyrimidine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GTP:CH, GTP:cyclohydrolase I; MOPS, 3-(*N*-morpholino)propanesulfonate; NOS, nitric oxide synthase; 4αPDD, 4α-phorbol-12,13-didecanoate; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonate; MEM, minimal essential medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; RIA, radioimmunoassay; kb, kilobase(s); NO, nitric oxide.

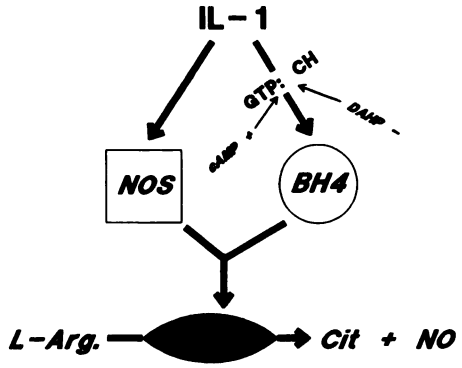


Fig. 1. Schematic diagram illustrating the two separate metabolic pathways required for NO production by inducible NOS. The induction of NOS expression by IL-1β is accompanied by expression of GTP:CH. The latter is the regulatory enzyme for the *de novo* pathway of tetrahydrobiopterin (*BH4*) synthesis in smooth muscle cells. NOS converts L-arginine (*L-Arg*) to citrulline (*Cit*) and NO in the presence of tetrahydrobiopterin. GTP:CH activity is inhibited by DAHP (*DAHP* −) but cAMP (*cAMP* +) is reputed to stimulate this enzyme.

elevation of intracellular cAMP levels in forskolin- and IL-1β-stimulated cells, which accompanied the induction of GTP:CH mRNA expression elicited by both agonists. These findings are reported here and add to our understanding of IL-1β-stimulated signal transduction in vascular smooth muscle cells.

Experimental Procedures

Materials

PMA, 4αPDD, DAHP, forskolin, 1,9-dideoxyforskolin, angiotensin II, arginine-vasopressin, 8-Br-cAMP, 8-(4-chlorophenylthio)-cAMP, *N*,2′-*O*-dibutyryl-cAMP, and all reagents for the determination of nitrite and nitrate levels in conditioned medium were from Sigma Chemical Co. (St. Louis MO). The chemicals used to supplement MEM used for the propagation of smooth muscle cells were also from Sigma. IL-1β was obtained from R&D Systems (Minneapolis, MN). Culture medium was obtained from ICN Flow Inc. (Irvine, CA). Nitro-L-arginine was from Aldrich Chemical Co. (Milwaukee, WI). Bisindolylmaleimide (GF109203X) was from Calbiochem Corp. (La Jolla, CA). Sepiapterin and tetrahydrobiopterin were purchased from Dr. Schirks (Dr. B. Schirks Laboratories, Jonas, Switzerland). PKC (a mixture of α, β, and γ isoforms) and PKC enzyme-linked immunosorbent assay kits were obtained from Upstate Biotechnology Incorporated. Reagents for random primed and T7 polymerase labeling were obtained from Stratagene Cloning Systems (La Jolla, CA). A 1.0-kb cDNA fragment of rat GAPDH was purchased from Clontech Laboratories (Palo Alto, CA). Radioisotopes used in these studies were [α-^{32}P]dCTP (6000 Ci/mmol), [α-^{32}P]UTP (3000 Ci/mmol), and L-[U-^{14}C]arginine (300 mCi/mmol) and were from Amersham Corp. (Arlington Heights, IL), as were Hybond nylon membranes and hybridization buffers for Northern analysis. Reagents for RNA extraction and analysis were obtained from GIBCO-BRL (Grand Island, NY) or Sigma.

Methods

Cell culture. Smooth muscle cells isolated from rat thoracic aortae were propagated and characterized as described in detail previously (17, 18). Briefly, the thoracic aortae were rapidly removed from rats after sacrifice and were cleaned of adventitial tissue by dissection after a 10-min incubation in 0.3% collagenase. Sections of vessels were opened longitudinally and the luminal surface was scraped with a rounded spatula to remove the endothelium. Tissues were then minced finely with curved scissors and subjected to sequential digestion with trypsin (0.25%, for 30 min), elastase (0.05%, for 30 min), and finally collagenase (0.3%, for 3–5 hr). All digestive enzymes were dissolved in MEMTH tissue culture medium (MEM containing Earle's salts, 2 mM glutamine, 20 mM TES-NaOH, pH 7.3, 20 mM HEPES-NaOH, pH 7.3, 100 units/ml penicillin, and 100 units/ml streptomycin). At the end of the final incubation period, when all tissue was dissolved, cell suspensions were centrifuged (800 × *g* for 5 min) and cell pellets were resuspended in MEMTH containing 10% bovine fetal calf serum. Cell suspensions were plated into T-25 culture flasks and several wells of a 24-well multiwell plate for subsequent immunocytochemistry. Cells were grown to confluence and replated into T-75 culture flasks, and when confluence was attained the process was repeated. Experiments were performed using three different isolates of cells between passage 8 and passage 25, and experimental treatment procedures were initiated when cells reached a density of 2 × 10^5 cells/cm^2 after 48 hr of maintenance in serum-free medium (10% fetal calf serum replaced by 0.1% endotoxin-free bovine serum albumin). Immunocytochemical characterization of cultures was performed using specific monoclonal antibodies against α-smooth muscle actin (Sigma).

Nitrite levels in conditioned medium. The determination of NO production by cultured cells was performed by measurement of the nitrite anion content of conditioned medium (4). Nitrite concentrations were determined using Greiss reagent (a 1:1 mixture of 1% sulfanilamide and 0.1% naphthylethylenediamine in 2% phosphoric acid) (19). Because NO is converted to both nitrite and nitrate anions in aqueous solution, the levels of nitrite present were determined before and after incubation of samples with nitrate reductase (4, 20). Routinely, ±25% of the total NO released into conditioned medium was in the form of nitrate anions (4).

NOS activity. Cytosolic extracts were assayed for NOS activity essentially as described (21), by measuring the conversion of [U-^{14}C]-arginine to citrulline, with the following modifications. Incubation mixtures (final volume, 50 μl) contained 50 nM [^{14}C]arginine (specific activity, 3.8 × 10^5 dpm/nmol), 25 mM Tris·HCl, pH 7.4, 5 mM $CaCl_2$, 5 mM dithiothreitol, 100 μM NADPH, 10 μM FAD, 10 μM FMN, 10 μM tetrahydrobiopterin, 1 mM $MgCl_2$, 50 mM L-valine, 200 μM nitro-L-arginine when appropriate, and 25 μl of cytosolic extract (25–45 μg of protein). Incubations were terminated after 10 min by the addition of 50 μl of cold 80% ethanol containing 100 μM L-arginine and L-citrulline; after centrifugation (13,000 × *g* for 10 min), aliquots were applied to the origin of Whatman (concentration-zone) thin layer cellulose plates. Plates were developed using 77% ethanol as solvent; L-citrulline was located after reaction with ninhydrin reagent and was scraped into scintillation fluid for counting. Enzymatic production of L-citrulline was linear for 20 min and exhibited a concentration dependence with respect to protein levels of cytosolic extracts. In addition, conversion of L-arginine to citrulline was inhibited by the presence of nitro-L-arginine (see Results). Cytosolic extracts were prepared from cultures in 150-mm Petri dishes (3–5 × 10^7 cells) by scraping cells into a micro-Dounce glass homogenizer with 25 mM Tris·HCl buffer, pH 7.4, containing 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride HCl, 5 mM $CaCl_2$, 10 μM L-arginine, 1 μM tetrahydrobiopterin, and 5 mM dithiothreitol. After homogenization, extracts were centrifuged (13,000 × *g* for 15 min) in micro-centrifuge tubes, and aliquots of supernatants were collected and stored at −70° for subsequent assay. Enzyme activity was stable for at least three cycles of freezing and thawing; additionally, the presence of 10 μM L-arginine and 1 μM tetrahydrobiopterin during extraction appeared to stabilize enzymatic activity to freeze-thawing. NOS activity is defined as the amount of [^{14}C]citrulline (nanomoles) produced/10 min and was corrected for the protein concentration of cytosolic samples.

Intracellular cAMP levels. The intracellular levels of cAMP were determined by using a commercial RIA kit (Amersham), as described (4). After incubation, cell layers were washed (2 × 2 ml) with phosphate-buffered saline and extracted twice with 500 μl of acidified (10 mM HCl) ethanol, and combined extracts were dried under vacuum. Dried cell extracts were processed for RIA determination of cAMP.

PKC activity. The levels of PKC activity in control (quiescent) and agonist-stimulated smooth muscle cells were determined using a commercial enzyme-linked immunosorbent assay kit and purified

mixed isomers of the kinase as positive controls. Cell lysates were prepared using a solution of 20 mM HEPES, pH 7.4, containing 1 mM EDTA, 0.05% Triton X-100, 1 mM β-mecaptoethanol, 25% glycerol, and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride HCl as protease inhibitor. Lysates were clarified by centrifugation (100,000 × *g* for 20 min), and 12-μl aliquots were assayed for activity in reaction mixtures (final volume, 120 μl) containing 25 mM Tris·HCl, pH 7.0, 2 mM $MgCl_2$, 0.1 mM ATP, 0.8 mM $CaCl_2$, and 50 μg of phosphatidylserine. Assays were linear for lysate protein concentration, and a standard curve of enzyme concentration (nanograms of protein) versus activity was prepared using commercial pure PKC (mixed isomers) for each assay. The activity of cell lysates was expressed as the amount of PKC (determined from the standard curve)/milligram of lysate protein.

Northern analysis. Total RNA (20 μg), extracted from smooth muscle cells by standard procedures using a 4 M guanidinium thiocyanate/citrate buffer system (22), was electrophoresed through 1.0% agarose containing 2.20 M formaldehyde. Gels were run for 16–18 hr at 25 V using recycled 20 mM MOPS buffer, pH 7.0, containing 5 mM sodium acetate and 1 mM EDTA, and were blotted to Hybond nylon membranes with 20× standard saline citrate as blotting buffer. After transfer, RNA was cross-linked to membranes by UV irradiation. Blots were hybridized either at 65° for random-primed cDNA probes or at 70° for T7-generated riboprobes, using commercial buffers. Probes used in these studies were a 0.9-kb *Nco*I-*Hind*III cDNA fragment of rat liver GTP:CH kindly supplied by Dr. Hatakeyama (Osaka Medical College, Osaka, Japan) (23), a 1.0-kb fragment of rat GAPDH obtained commercially (see Materials), and *Hind*III-linearized pGEM-3 containing a 4-kb fragment of murine macrophage inducible NOS, kindly provided by Dr. Cunningham (Harvard Medical School, Brigham and Women's Hospital, Boston, Massachusetts) (24). The latter was labeled using T7 polymerase, and random priming of the other probes was performed as described (21). Washed blots (100 ml of 0.5–0.1× standard saline citrate, 0.1% sodium dodecyl sulfate, at 65° for 1 hr) were exposed to Kodak X-Omat film for 1–2 days. After stripping (22), blots were rehybridized to labeled GAPDH probes.

Protein concentration. The protein concentration of cytosolic extracts was determined using a modified Bradford procedure (25).

Statistical analysis. Results are expressed as means ± standard deviations. In some instances the symbol size in the figures exceeds the range of the error bars. The number of experiments is denoted by *n*, and experiments were performed using at least two different isolates of smooth muscle cells prepared as described above. Statistical comparisons were made between control and agonist-stimulated values as appropriate, using Student's *t* test; *p* values of <0.05 were considered statistically significant and are indicated in the text.

Results

Induction of NOS by IL-1β and the effect of bisindolylmaleimide. Treatment of rat smooth muscle cells with IL-1β resulted in the induction of NOS, as assessed by both enzymatic activity and Northern analysis (Fig. 2). Inclusion of nitro-L-arginine (200 μM) in enzyme assay incubates significantly attenuates the conversion of L-arginine to L-citrulline. NO production (assessed by nitrite anion levels in conditioned medium) by cytokine-treated cells was significantly attenuated by the presence of the PKC inhibitor bisindolylmaleimide (16), in a concentration-dependent manner ($IC_{50} \sim 5$ μM) (Fig. 3). Bisindolylmaleimide did not modulate basal levels of nitrite anions in conditioned culture medium, and maximum inhibition of IL-1β-elicited NO production was observed at 20 μM.

PKC activity was rapidly elevated in cytosolic extracts of smooth muscle cells exposed to IL-1β (Fig. 4A), and bisindolylmaleimide inhibited this activity significantly, in a concentration-dependent manner (Fig. 4B). Cultures exposed to PMA exhibited a similar response in terms of cytosolic PKC activity,

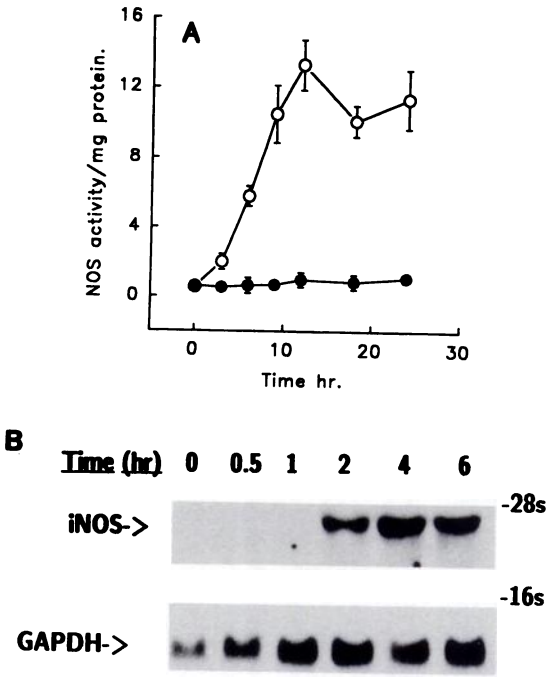


Fig. 2. Induction of NOS by IL-1β in cultured smooth muscle cells. Cells were exposed to IL-1β (2 ng/ml) for the indicated times and the cytosolic extracts (A) or total RNA samples (B) were prepared. Cytosolic extracts were used to assay the conversion of [^{14}C]arginine to citrulline in the presence (●) or absence (○) of 200 μM nitro-L-arginine. Total RNA samples (20 μg) were blotted to nylon membranes after electrophoresis through 1.0% agarose gels (see Experimental Procedures). The size of the inducible NOS (*iNOS*) transcript, estimated from the positions of migration of 28 S, 23 S, 18 S, and 16 S rRNA, was 4.60 kb. Transcripts indicated are inducible NOS and GAPDH. Data shown for assays of enzymatic activity are from experiments ($n = 4$) performed with two different isolates of smooth muscle cells. Northern analysis shown was with RNA from one cell isolate, and similar results were obtained with total RNA extracted from one other cell isolate.

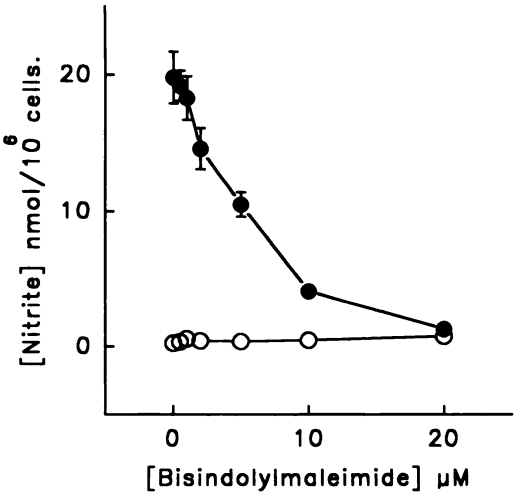


Fig. 3. Effects of bisindolylmaleimide on NO (nitrite) production by IL-1β-treated smooth muscle cells. Cells were treated with IL-1β (2 ng/ml) in the presence of the indicated levels of the PKC inhibitor (●). Additionally, cultures were treated with inhibitor alone at the levels shown (○). Conditioned medium was collected after 20 hr for nitrite anion determination and data shown are from one of three experiments. Similar data were obtained in the other two determinations.

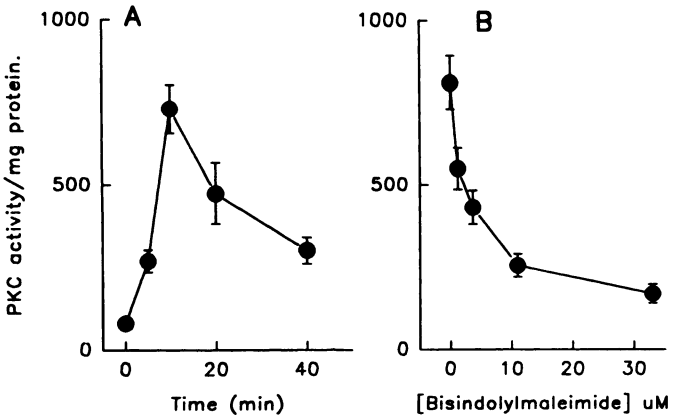


Fig. 4. PKC activity in cytokine-stimulated smooth muscle cells. Quiescent cultures of smooth muscle cells were treated for the indicated times (A) or for 10 min (B) with 2 ng/ml IL-1β. Cell lysates were prepared and assayed for PKC activity as described in Experimental Procedures. Also, some assays were performed in the presence of the indicated levels of bisindolylmaleimide. PKC activity is defined as the amount of PKC (determined from a standard curve using purified mixed isomers of PKC) present in cell lysates and is expressed per mg of total lysate protein. Experiments (n = 4) were performed with two different isolates of smooth muscle cells.

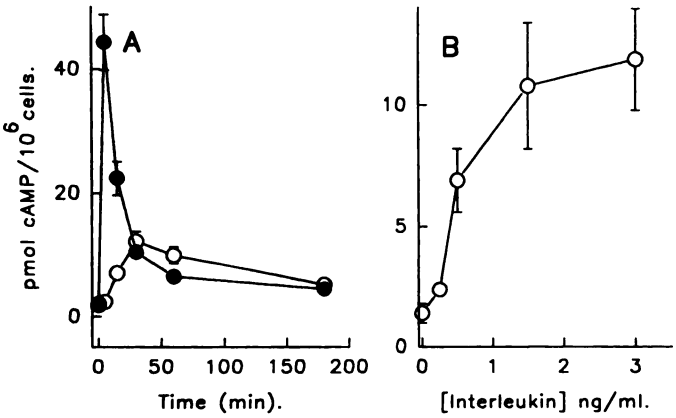


Fig. 5. Stimulation of adenylyl cyclase by IL-1β or forskolin in smooth muscle cells. Cells were exposed to either IL-1β (2 ng/ml) (○) or 10 μM forskolin (●) for the times indicated (A) or to the indicated levels of cytokine for 30 min (B). Cell layers were extracted with acidified ethanol and intracellular cAMP levels were determined using a commercial RIA kit. The data are from one representative experiment (n = 4) with one cell isolate, and similar results were obtained using a second isolate.

and this was subject to inhibition by bisindolylmaleimide (data not shown).

Activation of adenylyl cyclase by IL-1β and forskolin and NO production. When quiescent smooth muscle cells were treated with IL-1β, there was a significant time- and concentration-dependent increase in intracellular cAMP levels. However, the time course for cAMP elevation was slower and significantly less pronounced at 5 min, compared with that observed when cells were treated with 10 μM forskolin (Fig. 5A). Maximum elevation of cAMP levels was obtained in forskolin-treated cells by 5 min, compared with 30 min in cells treated with IL-1β (Fig. 5).

Stimulation of smooth muscle cells with IL-1β elicited a significant concentration-dependent increase in NO production (Fig. 6), which was paralleled by increased levels of intracellular cAMP measured at 30 min after stimulation (Fig. 5B). In the

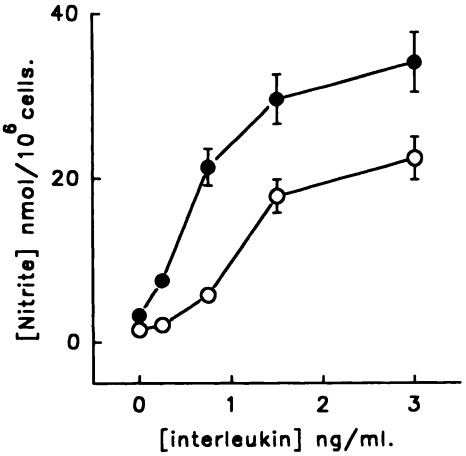


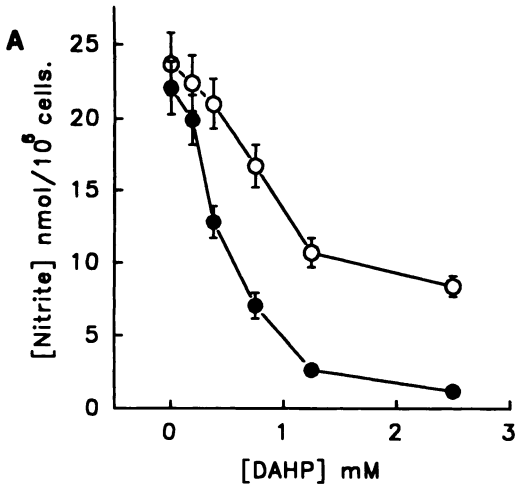
Fig. 6. Effect of forskolin on NO production by IL-1β-treated smooth muscle cells. Smooth muscle cells were exposed to the indicated levels of IL-1β in the absence (○) and presence (●) of 10 μM forskolin. Conditioned medium was collected after 20 hr and levels of nitrite anions were measured. The data are from experiments (n = 4) performed with one of two isolates of smooth muscle cells, and similar results were obtained with the other isolate.

presence of 10 μM forskolin the levels of NO produced in IL-1β-treated cells were significantly enhanced (Fig. 6).

GTP:CH-dependent production of NO and mRNA expression of the enzyme. Treatment of cultures with DAHP, an inhibitor of GTP:CH, significantly attenuated the IL-1β-induced production of NO (nitrite accumulation) (Fig. 7A). Inclusion of sepiapterin, a precursor molecule for an alternative pathway of tetrahydrobiopterin production in many cells (7), partially restored the production of NO by cells treated simultaneously with cytokine and DAHP (Fig. 7A). Rapid expression of GTP:CH mRNA in smooth muscle cells exposed to IL-1β has been reported (9), and the presence of DAHP did not adversely affect induction of the enzyme by the cytokine (Fig. 7B).

Because IL-1β induced GTP:CH mRNA expression and stimulated adenylyl cyclase, which has been shown to increase GTP:CH activity (8), cells were treated with forskolin to examine GTP:CH transcript levels. Forskolin (10 μM) elicited a time-dependent induction of GTP:CH mRNA expression (Fig. 8). Two mRNA transcript sizes (~3.4 kb and 1.2 kb) were detected by Northern analysis (Figs. 7 and 8). The smaller mRNA species has been described by Hatakeyama *et al.* (23) in rat liver. When total RNA from rat liver was analyzed by Northern analysis, using the same cDNA probe (23) used for smooth muscle cell RNA, two transcript sizes were identified; however, the larger species was less abundant in hepatic tissue (Fig. 8). Smooth muscle cells exposed to IL-1β appear to express the larger transcript predominantly.

Induction of NO production by simultaneous activation of adenylyl cyclase and PKC. The data obtained in the preceding experiments suggested that activation of adenylyl cyclase in conjunction with the stimulation of PKC could lead to the expression of NOS and production of NO. Therefore, smooth muscle cells were stimulated either with increasing concentrations of forskolin in the presence or absence of a fixed concentration (1 μM) of PMA or with different levels of the latter in the presence of a fixed concentration (10 μM) of forskolin. The data (Fig. 9) indicate that combinations of forskolin and PMA elicited significant production of NO (ni-



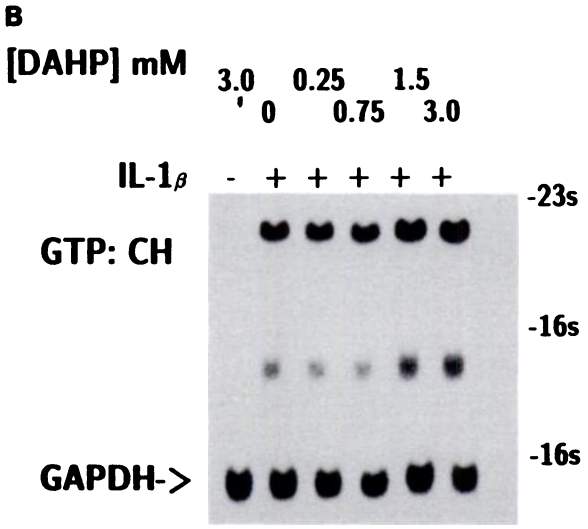


Fig. 7. Evidence that inhibition of GTP:CH enzymatic activity attenuates NO production by cytokine-stimulated cells. A, Smooth muscle cells were exposed to IL-1β (2 ng/ml) in the presence of the indicated levels of DAHP (●). Additionally, some cultures were exposed to cytokine and increasing levels of DAHP in the presence of 1 mM sepiapterin (○). Treatments were for 20 hr and conditioned medium was collected for nitrite anion determination. Data are from experiments (n = 6) performed with one of three different cell isolates, and similar data were obtained with the other two preparations. B, Induction of GTP:CH gene expression in smooth muscle cells exposed to IL-1β, in the presence or absence of the indicated concentrations of DAHP, for 4 hr. Data are from an experiment with one of two cell isolates, and similar data were obtained with the second isolate. The two transcript sizes detected for GTP:CH were estimated to be 3.4 and 1.2 kb using rRNA markers (28 S, 23 S, 18 S, and 16 S).

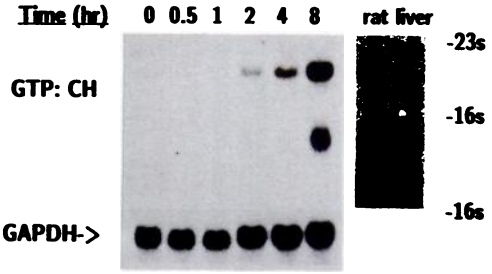


Fig. 8. Induction of GTP:CH gene expression by forskolin. Smooth muscle cells were exposed to 10 μM forskolin for the times shown and GTP:CH mRNA expression was assayed by Northern analysis. The sizes of the two transcripts detected were estimated to be 3.40 and 1.20 kb by their positions of migration, relative to those of 28 S, 23 S, 18 S, and 16 S rRNA. Two different samples of rat liver total RNA (isolated as described in Ref. 22) were analyzed at the same time and both transcript sizes are evident. Data are from one experiment, which was repeated twice using different isolates of cells.

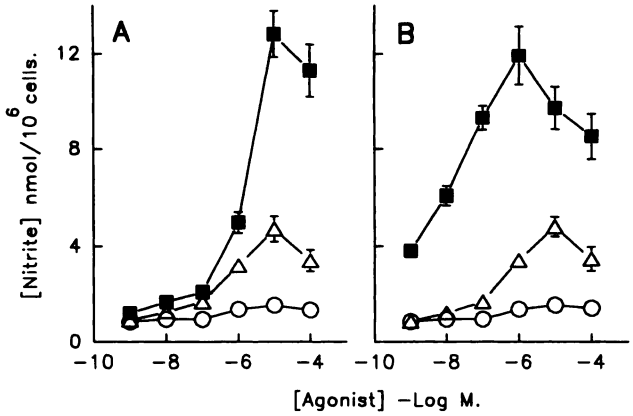


Fig. 9. Induction of NO production in cells treated with phorbol ester and forskolin. Smooth muscle cells were exposed to the indicated levels of forskolin (Δ) or phorbol ester (○) for 20 hr and then conditioned media were analyzed for nitrite anion levels. In addition, some cultures were treated with increasing concentrations of forskolin in the presence of 1 μM phorbol ester (■) (A) or with increasing levels of phorbol ester in the presence of 10 μM forskolin (■) (B). Data are from one typical experiment (n = 3), and similar results were obtained with two other cell isolates.

trite in conditioned medium) by smooth muscle cells. In cells exposed to either 1,9-dideoxyforskolin and PMA (Fig. 10) or the inactive phorbol derivative 4αPDD and forskolin, production of NO was significantly reduced, compared with cells exposed to combinations of forskolin and PMA (Figs. 9 and 10). Also, when cells were treated with 10 μM bisindolylmaleimide for 30 min before exposure to phorbol ester and forskolin, production of NO (nitrite) was significantly attenuated (Fig. 10B).

When cells were exposed to IL-1β or combinations of forskolin and PMA, used at the optimal concentrations (see Fig. 9), the kinetics of NO (nitrite) production were similar (Fig. 11). However, the amounts of NO produced by cells in response to the cytokine were significantly greater after 10 hr of agonist exposure, compared with combinations of forskolin and PMA (Fig. 11).

Stimulation of smooth muscle cells with other agonists that either activate PKC (angiotensin II or arginine-vasopressin, both at 100 nM) or are capable of activation of adenylyl cyclase [isoproterenol, 8-Br-cAMP, *N*,2′-*O*-dibutyryl-cAMP, and 8-(4-chlorophenylthio)-cAMP] resulted in production of significant levels of NO (Fig. 12; Table 1). Thus, both angiotensin II and arginine-vasopressin, acknowledged activators of PKC, elicited NO production in the presence of forskolin or isoproterenol (Fig. 12). However, neither vasoconstrictor peptide induced an elevation in the nitrite anion levels of conditioned medium in the absence of agonists that activate adenylyl cyclase (data not shown). Derivatives of cAMP in combination with PMA all stimulated NO production significantly when used at 10^{-4} μM; however, at lower concentrations none of these agonists were as efficacious as forskolin or isoproterenol (data not shown).

NOS activity and induction of NOS and GTP:CH gene expression. Cytosolic extracts from cells exposed to phorbol

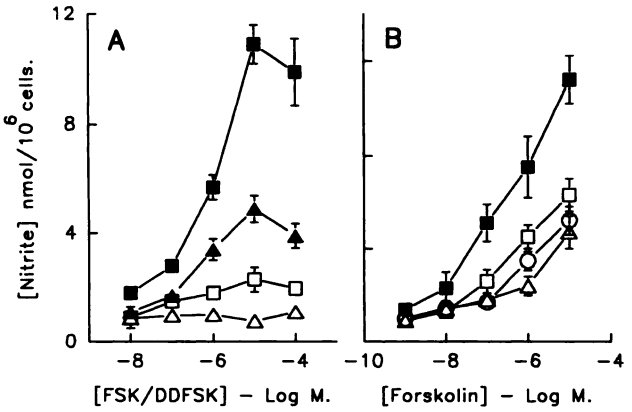


Fig. 10. Stimulation of NO production in smooth muscle cells with 1,9-dideoxyforskolin or 4αPDD. A, Cells were exposed to the indicated levels of forskolin (*FSK*) (▲, ■) or 1,9-dideoxyforskolin (*DDFSK*) (△, □) in the presence (■, □) or absence (▲, △) of 1 μM phorbol ester. B, Cells were exposed to the indicated levels of forskolin in the absence (○) or in the presence of either of 1 μM phorbol ester (■), 1 μM 4αPDD (□), or 1 μM phorbol ester and 10 μM bisindolylmaleimide (△). Exposure to agonists was for 20 hr in all experiments and then conditioned media were collected for assay of nitrite anion concentrations. Data are from one typical experiment (n = 4) performed using two different isolates of smooth muscle cells.

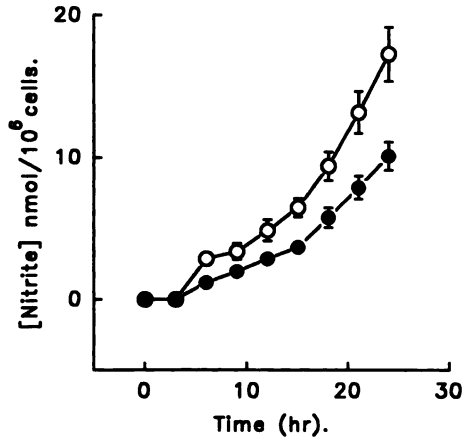


Fig. 11. Kinetics of NO (nitrite anion) production by smooth cells treated with forskolin and PMA. Cells were exposed to a combination of 10 μM forskolin and 1 μM PMA for the indicated times and conditioned medium was collected for nitrite anion analysis (●). A parallel set of cultures were treated with 2 ng/ml IL-1β for the same time periods and conditioned medium was analyzed (○). Experiments (n = 3) were performed in quadruplicate using a single isolate of cells.

ester or angiotensin II exhibited NOS activity, based on the criterion of conversion of [^{14}C]arginine to radiolabeled citrulline in the presence of added cofactors. This activity was inhibited in the presence of nitro-L-arginine, an acknowledged inhibitor of NOS (Fig. 13). Cells treated with combinations of PMA or angiotensin II and forskolin also exhibited cytosol-associated NOS enzymatic activity, which was marginally higher that that observed in cells treated with PKC agonists in the absence of forskolin (Fig. 13).

Northern analysis of RNA from cells treated with PMA or angiotensin II indicated that both agonists induced NOS gene expression but did not appear to have a similar action on GTP:CH transcript expression (Fig. 14B). In contrast, forskolin induced strong expression of GTP:CH mRNA, as observed in

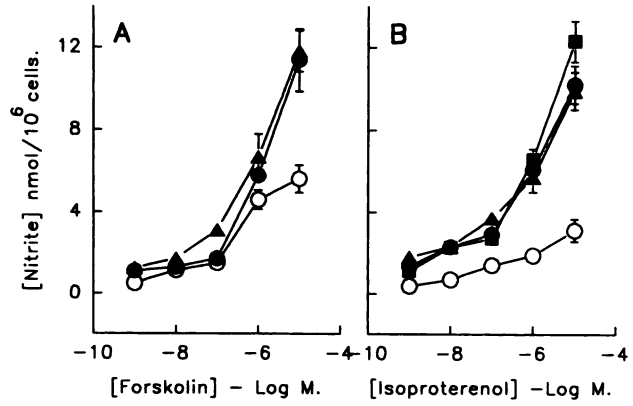


Fig. 12. Stimulation of NO production in smooth muscle cells with agonists of PKC or adenylyl cyclase. Cells were exposed to the indicated levels of forskolin (○) (A) or isoproterenol (○) (B) alone or in the presence of 100 nM angiotensin II (●) or arginine-vasopressin (▲). Additionally, some cultures were exposed to the indicated levels of isoproterenol and 1 μM phorbol ester (■) (B). Data are from one typical experiment (n = 4) performed using two different isolates of smooth muscle cells.

TABLE 1

NO production by smooth muscle cells elicited by analogs of cAMP

Quiescent cultures of smooth muscle cells were exposed to the indicated analogs of cAMP in the presence or absence of 1 μM PMA for 20 hr. Conditioned medium was then collected and analyzed for levels of nitrite accumulation. All cAMP analogs were used at a final concentration of 100 μM, and experiments (n = 3) were performed using quadruplicate determinations for each experiment.

Treatment	Nitrite concentration	
	+PMA	–PMA
	nmol/10^6 cells	
Control[a]	0.92 ± 0.21	1.12 ± 0.30
Dibutyryl-cAMP	9.65 ± 1.03	1.05 ± 0.23
8-Br-cAMP	8.87 ± 1.16	1.32 ± 0.30
8-(4-Chlorophenylthio)-cAMP	8.49 ± 0.96	1.29 ± 0.18

[a] Vehicle or 1 μM PMA alone.

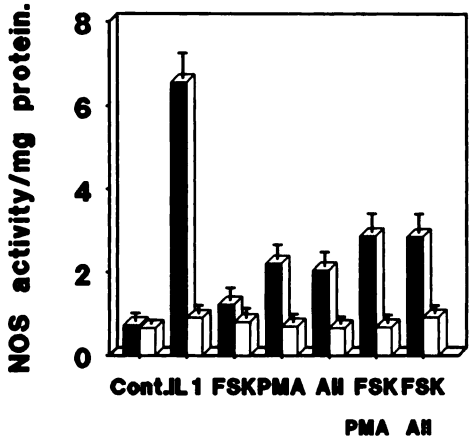


Fig. 13. NOS activity in cytosolic extracts of stimulated smooth muscle cells. Cytosolic extracts were prepared from cells that had been exposed to agonists for 20 hr and were assayed for NOS activity as described (see Experimental Procedures). Assays were performed in the presence (□) or absence (■) of 200 μM nitro-L-arginine, and NOS activity is defined as nmol of citrulline produced/10 min. Data are from a typical experiment (n = 3) performed using three different isolates of smooth muscle cells. *Cont.*, control; *FSK*, forskolin; *All*, angiotensin II.

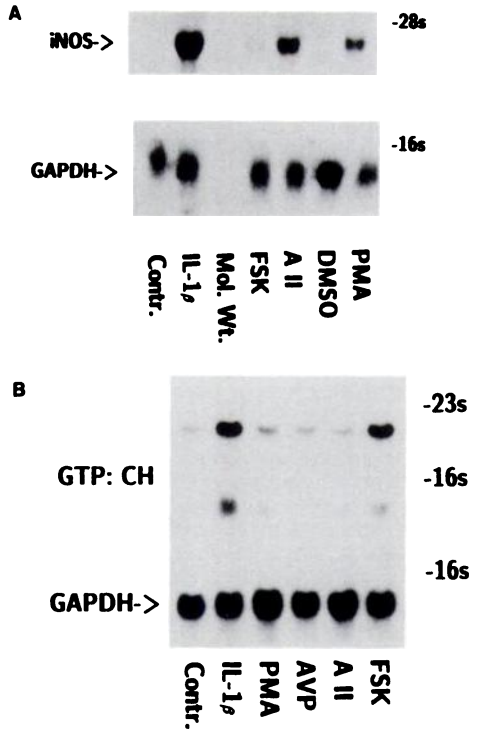


Fig. 14. Induction of NOS and GTP:CH gene expression by agonists that stimulate PKC or adenylyl cyclase activity. Quiescent smooth muscle cell cultures were stimulated with the indicated agonists for 8 hr and then total RNA was extracted and analyzed by Northern procedures. Angiotensin II (*A II*) and arginine-vasopressin (*AVP*) were used at concentrations of 100 nM, PMA at 1 μM, forskolin (*FSK*) at 10 μM, and IL-1β at 2 ng/ml. *Mol. Wt.*, rRNA standards (28 S, 23 S, 18 S, and 16 S) (A). For NOS induction (A) cells were either untreated (contr.) or exposed to the agonists indicated, including dimethyl sulfoxide (DMSO), whereas for induction of GTP:CH (B) control cells were exposed to DMSO at a concentration equal to that present as solvent for PMA or forskolin (B). Data are from one of two different cell isolates, and similar data were obtained for all treatments using total RNA extracted from the second cell isolate.

previous experiments (Fig. 8), but had no positive action on NOS gene expression (Fig. 14A).

Discussion

Importance of GTP:CH activity for NO production. The production of NO from the guanidino nitrogens of L-arginine takes place by oxidation involving five electrons, two of which are derived from tetrahydrobiopterin (26). The latter is particularly important because it is utilized by a limited number of enzymes and appears to be in short supply in cultured smooth muscle cells (7). Thus, the production of NO is limited by the amount of tetrahydrobiopterin available. This was clearly demonstrated by the experiments using DAHP to inhibit GTP:CH. Therefore, induction of GTP:CH activity by IL-1β contributes to the efficiency of NO production elicited by the cytokine. The induction by cytokines of NO production in smooth muscle cells has been shown to be dependent on the cytokine used (27). Studies with rat smooth muscle cells have demonstrated that combinations of interferon-γ and tumor necrosis factor-α are required for production of NO (27). A possible explanation for this observation may be that one of the cytokines is responsible for the induction of NOS expression and the other induces production of tetrahydrobiopterin. Direct activation of adenylyl cyclase by forskolin induced expression of GTP:CH (Fig. 8); this suggests that only cytokines that enhance cAMP levels (either directly or by induction of other factors) may be capable of eliciting NO production. The actions of forskolin on GTP:CH expression support previous observations regarding the sensitivity of GTP:CH activity to levels of cAMP (8).

NO production and activation of adenylyl cyclase. The elevation of cAMP levels by IL-1β was delayed, compared with that observed with forskolin, and the levels attained with the cytokine were lower. Thus, it is possible that IL-1β does not activate adenylyl cyclase directly; furthermore, GTP:CH stimulation may be submaximal at the low levels of cAMP elicited by the cytokine. This was supported by the enhancement of NO production by cytokine-treated cells exposed simultaneously to forskolin, especially at low levels of IL-1β (Fig. 6).

Role of PKC in the stimulation of NO production by smooth muscle cells. PKC plays a central role in intracellular signal transduction (see Ref. 12 for review). Thus, the actions of bisindolylmaleimide on the induction of NOS may be relevant to the understanding of how cytokines exert their individual actions on this process. Several distinct isoenzyme forms of PKC have been identified (12), and there is no clear indication which or how many of these are expressed by the cultured smooth muscle cells used in the present studies. However, bisindolylmaleimide inhibited the isoforms responsive to IL-1β in the culture system studied here.

The effects of PKC on the activity of isolated constitutive NOS have been studied, and putative phosphorylation sites have been identified from the amino acid sequence data (28, 29). Treatment of the purified enzyme with PKC resulted in a reduction of enzymatic activity (28), whereas in a second comparative study a moderate increase in activity was observed (29). One explanation for these contrasting observations may reside in the purity of the NOS preparations used and their stability during treatment. The data presented here suggested that inhibition of PKC attenuates NO production by inducible NOS in smooth muscle, an observation supported by the actions of another PKC inhibitor, RO31–8220, on inducible NOS in murine macrophages (15). Additionally, prolonged exposure of cells to phorbol esters (to down-regulate PKC activity) before cytokine treatment reduced NO production (15). Also, acute phorbol ester treatment of rat peritoneal macrophages stimulated their production of nitrite, and this was attenuated by the PKC inhibitor calphostine but not by staurosporine (30). In other studies the apparent lack of an inhibitory effect of staurosporine on IL-1β-induced NO production has been interpreted as implying that PKC activation plays no role in the induction of NOS in cultured rat smooth muscle cells (31). Careful perusal of the data (31) suggests that staurosporine may have even enhanced expression of NOS mRNA. Similar results were obtained in this laboratory (32). A detailed comparative study on the specificity of bisindolylmaleimide versus staurosporine for the inhibition of PKC concluded that the former demonstrated significantly better specific inhibition of the kinase in a number of cell types (16). Staurosporine appeared to have a broad range of activities with a number of different kinases, whereas bisindolylmaleimde was a specific inhibitor of PKC at concentrations similar to those used in the studies reported here (16). Thus, the data presented here and

those from other sources (15, 30) strongly suggest an important role for PKC in the induction of NOS gene expression.

Because full production of NO by inducible NOS involves the simultaneous induction of GTP:CH, PKC activation may influence either enzyme. Northern analysis of cells treated with phorbol ester suggests, however, that PKC is instrumental in the induction of NOS but not GTP:CH. Basal expression of GTP:CH mRNA transcripts was not enhanced in cells exposed to agonists of PKC.

Cytosolic extracts from cells exposed to PKC agonists exhibited NOS activity (citrulline production) only in the presence of added tetrahydrobiopterin (data not shown). The NOS activity of cytosolic extracts from cells treated with activators of PKC (PMA and angiotensin II) was enhanced when cells had also been exposed to forskolin. Because enzyme assays were normally performed in the presence of added cofactors (including tetrahydrobiopterin), the increased activity of extracts from forskolin-exposed cells could not be explained readily by the presence of an elevated amount of tetrahydrobiopterin. The explanation for these observations is uncertain but may relate to the stability of the enzyme during isolation. The activity of extracts from cells exposed only to PKC agonists appeared more labile than that from cells exposed to PKC agonists and forskolin. Inducible NOS exists as a polypeptide dimer, and dimerization is stabilized by the presence of tetrahydrobiopterin (33). Alternatively, forskolin may regulate NOS activity via activation of protein kinase A. No direct effect of forskolin on enzyme activity was observed when it was included in assay incubates (data not shown).

cAMP and NOS activity in smooth muscle cells. A consensus sequence for protein kinase A has been identified in all of the NOS sequences determined to date (28). Phosphorylation of purified constitutive NOS by protein kinase A, presumably at the consensus site, did not appear to alter catalytic activity (34). Similar experiments with inducible NOS have not been reported. Thus, a role for protein kinase A in the regulation of either enzyme induction or activity remains to be demonstrated. In the studies reported here a low level of induction of NO production (nitrite anions) was observed in cells treated with maximal levels of forskolin. However, under similar conditions there was no elevation of NOS transcript levels. Also, the enzymatic activity of cytosolic fractions from cells exposed to forskolin alone was not significantly elevated above background levels. Recently published data complement the findings reported here, namely that agents that elevate intracellular cAMP levels enhance cytokine-elicited induction of NO production in smooth muscle cells (35). However, those authors reported low levels of NOS mRNA and protein expression in smooth muscle cells exposed to forskolin alone (35). This was accompanied by minimal increases in nitrite anion production after 30 hr of exposure to the agonist (35).

Stimulation of adenylyl cyclase by forskolin resulted in a strong induction of GTP:CH and, in combination with angiotensin II, the production of NO. One consequence of angiotensin II-receptor (AT_1) interaction appears to be inhibition of adenylyl cyclase (36), and this presumably impairs the induction of GTP:CH. The levels of GTP:CH transcripts in cells treated for short periods (2–4 hr) with combinations of angiotensin II and forskolin were depressed, compared with levels in cells treated with forskolin alone (data not shown). Thus, angiotensin II may reduce adenylyl cyclase activity initially but this is countered by the presence of forskolin; therefore, overall GTP:CH induction leads to adequate tetrahydrobiopterin production. Also, because the vasoconstrictor peptide induces strong expression of NOS, the production of tetrahydrobiopterin that ensues from the presence of forskolin results in a significant production of NO. Experiments with a variety of cell types have demonstrated "cross-talk" between signal transduction pathways coupled to the cAMP and inositol phosphate systems (37, 38). In many instances this leads to an enhanced elevation of cAMP levels, and this may account for the positive stimulatory action of forskolin on GTP:CH activity in the presence of angiotensin II after prolonged exposure of cultures to both agonists.

Peptide growth factors and proteases associated with events occurring during vascular injury differentially modify NOS production by IL-1β-treated smooth muscle cells (2, 4, 39, 40). The initiation by IL-1β of two distinct signal transduction cascades that lead to NO production by smooth muscle cells identifies points at which peptide growth factors and proteases may intervene in the process. In addition, the data provide additional evidence for an important regulatory role of GTP:CH activity in the production of NO. The possibility that peptide growth factors, plasmin, and thrombin may regulate NO production by modulating the activity of GTP:CH, rather than by having a direct action on NOS expression, is ripe for investigation (9). However, the consequences of NOS enzymatic activity in the absence of tetrahydrobiopterin also remain to be studied. The free radical production that occurs under such conditions may have serious pathological consequences for blood vessel wall cells (41).

Acknowledgments

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