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Endothelin stimulates PDGF secretion in cultured human mesangial cells

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Endothelin stimulates PDGF secretion in cultured human mesangial cells. Endothelin, a 17-DKa peptide originally described as a potent vasoconstrictor, also stimulates the release of important regulators of glomerular hemodynamics such as atrial natriuretic factor and renin. In the present study we investigated the role of endothelin in the release of another potent vasoconstrictor and mitogen of human mesangial cells, the platelet-derived growth factor. Endothelin stimulated PDGF release at 12 hours and the effect was sustained for 36 hours. This effect was associated with the enhanced induction of mRNAs encoding PDGF A- and B-chain. Endothelin also induced mitogenesis in human mesangial cells which was accompanied by activation of phospholipase C with increased inositol phosphate turnover. These data suggest a mechanism by which endothelin may regulate mesangial cell function in disease states.

Endothelin, a novel vasoconstrictor, has recently been purified from medium conditioned by porcine aortic endothelial cells [1]. It is a potent vasoconstrictor of isolated blood vessels in vitro and of various vascular beds in vivo [2–5]. The most pronounced vasoconstrictor effects of endothelin so far reported are observed in the kidney [6]. Combined in vivo and in vitro studies in rats suggest that endothelin reduces glomerular filtration rate via its contractile effect on mesangial cells [7]. Evidence is rapidly accumulating to show that signals transduced in response to endothelin in both smooth muscle and rat mesangial cells include activation of phospholipases C and A_2, changes in cytosolic calcium, intracellular alkalinization and protooncogene activation [7–13]. We have recently demonstrated that human glomerular mesangial cells in culture express mRNAs that encode for both the A- and B-chain of PDGF and release PDGF [14]. Agents that activate phospholipase C and protein kinase C, including PDGF itself, thrombin and phorbol esters, enhance PDGF gene expression and release of PDGF from mesangial cells [15–18]. Mesangial cells also express receptors for PDGF that recognize at least two PDGF isoforms PDGF AB and PDGF BB [14, 15]. These observations suggest that PDGF genes and/or their released product(s) may be involved in the mitogenic response to a variety of growth factors. We studied the effect of endothelin on PDGF secretion and expression of PDGF mRNAs. In addition, we determined if endothelin activates phospholipase C, and if it is mitogenic to human mesangial cells as has recently been reported for rat mesangial cells.

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Methods

Mesangial cell cultures

Human mesangial cells were cultured from isolated glomeruli of normal human kidney tissue obtained from nephrectomy samples. Detailed characterization of the cells have been reported previously [14]. Cells were used between passages five and ten.

Measurement of released PDGF activity

Human mesangial cells grown to confluence in 100 mm Petri dishes, were washed twice with serum free medium, and exposed to endothelin for the time periods indicated. Conditioned media was then collected, centrifuged at 2000 rpm for 10 minutes to remove cell debris, and stored at 0° until assayed. For PDGF assay, human foreskin fibroblasts were placed on ice and washed with DVF-12 medium containing 25 mM HEPES and 2 mg/ml bovine serum albumin (BSA, binding medium). Binding medium alone or binding medium containing various aliquots of conditioned medium or standard concentrations of purified PDGF were added to duplicate wells. Fibroblasts were incubated for one hour at room temperature with constant, gentle rotary agitation. The media was aspirated, the cells were washed once with binding medium and ^{125}I-PDGF in binding medium was then placed on the cells for one hour at room temperature. Cells were then washed twice with ice-cold PBS containing 1 mM $CaCl_2$ and 2 mg/ml BSA and solubilized with 1% Triton X-100 containing 1 mg/ml BSA. The cell-bound radioactivity was measured. PDGF in the conditioned medium was quantitated from standard curves of competitor activity of purified PDGF as previously described [16].

RNA purification and Northern blotting

Cultured human mesangial cells were washed in phosphate buffered saline and lysed in guanidinium isothiocyanate and RNA prepared after centrifugation on cesium chloride gradients. Samples containing total RNA were fractionated on 1% agarose-formaldehyde gels and transferred to GeneScreen (NEN) overnight. Prehybridization and hybridization were performed overnight at 42°C in a hybridization buffer containing 50% formamide, 0.8 M NaCl, 0.02 M 1,4 piperazindiethanesulfonic acid pH 6.5, 20 mM EDTA, 100 μg/ml of denatured

salmon sperm DNA and 0.5% SDS. The probe used for the hybridization was an A-chain cDNA provided by C. Betsholtz and C.H. Heldin [19]. This cDNA was subcloned into M13 mp8 and a clone containing the sense strand was used as a template for cDNA synthesis with the Klenow fragment of DNA polymerase I and the M13 universal sequencing primer as described [15]. After hybridization, blots were washed successively in 2 × SSC, 0.5% SDS at 23°C, the same buffer at 65°C and 0.1 × SSC 0.5% SDS at 23°C.

Solution hybridization assay

The levels of c-sis mRNA were determined using a ribonuclease protection (solution hybridization) assay. The probe used was a genomic fragment of the c-sis gene subcloned into the sma I site of pT7-2 (US Biochemical). The RNA probe was synthesized from the DNA template using T7 polymerase. For hybridization, 50 μg aliquots of total RNA prepared from mesangial cells were distributed in Eppendorf tubes, and dried in a speed vac. Hybridization was carried out by adding the labeled RNA probe (1×10^6 cpm) to the RNA samples in the presence of 80% formamide, 10 mM Pipes pH 6.4, 400 mM NaCl and 1 mM EDTA, followed by overnight incubation at 50°C in a water bath. After hybridization, samples were digested with RNase A (50 μg/ml) and RNase T_1 (2 μg/ml) followed by the addition of proteinase K to inactivate the remaining RNase. After extraction and the addition of t-RNA, samples were precipitated and redissolved in loading buffer containing 90% formamide, and separated on 6% polyacrylamide/urea gels. Autoradiograms of the gels were exposed after 48 hours using enhancing screens. The sis bands were identified by their position which corresponds to the sixth and seventh exons of the undigested probe.

[^{3}H]-Thymidine incorporation

DNA synthesis was measured as the amount of [^{3}H]-thymidine incorporated into trichloroacetic acid insoluble material [14]. Cells were plated in 24 well dishes in Waymouth's medium containing 17% serum until they became confluent. Cells were then made quiescent by incubation in Waymouth's medium containing 1% Zeta serum but free of insulin or FCS for three days. Endothelin was added and after 24 hours the cells were pulsed with [^{3}H]-thymidine for four hours at 37°C. At the end of the four hour pulse period, medium was removed and ice-cold 5% trichloroacetic acid was added for 15 minutes and dishes were kept on ice. After two more washes with the acid, cells were solubilized by adding 0.25 N NaOH and 0.1% SDS. One half ml of this solubilized cell solution was then neutralized and counted in a scintillation counter.

Cell proliferation assay

Subconfluent human mesangial cells were plated in Waymouth's medium with 17% FCS. After 24 hours, the cells were washed and placed in SFIF in the presence or absence of endothelin. Cell counts were performed on quaduplicate wells every two or three days by trypsinizing the cells and counting in Coulter counter.

Analysis of inositol phosphates

Confluent mesangial cells were washed and placed in serum-free media three days prior to the assay. The media was switched to inositol-free RPMI 1640 media (Gibco, Grand Island, New York, USA) containing 3 μCi/ml [^{3}H]-myoinositol (New England Nuclear, Boston, Massachusetts, USA) 36 hours before the assay. Medium was removed and cells were washed twice with Hepes buffer, containing 1.3 mM Ca^{++} to remove unincorporated label. In experiments in which the length of the incubation was five minutes or greater, 7.5 mM lithium chloride was included in the buffer, and the second wash was left on the cells for 10 minutes. Test substances, diluted in Hepes buffer, were added to duplicate or triplicate wells and incubated for the times indicated at 37°C. The incubation was terminated by aspirating the buffer and adding 5% perchloric acid to each well. The dishes were refrigerated for two hours, to allow complete extraction of the water soluble inositols. The solution was then removed and 10 N KOH was added to precipitate the perchlorate. The samples were centrifuged, the supernatant removed and neutralized with 5 mM sodium tetraborate (pH 8.8). Each sample was separated on 1 ml AG 1-X 8 anion-exchange columns (Bio-Rad) by loading the samples, washing with 20 ml distilled H_2O, and then eluting with 8 to 10 ml of increasing concentration of NH_4 formate in 0.1 M formic acid. Using this technique, inositol monophosphate elutes with 0.2 M NH_4 formate, inositol bis-phosphate with 0.4 M NH_4 formate, and inositol tris-phosphate with 1 M NH_4 formate. Two ml of each collected fraction was mixed with Scintiverse E scintillation fluid and counted in a beta counter. The acid-insoluble protein remaining in the tissue culture wells was solubilized with NaOH and assayed for protein. Results are expressed as cpm [^{3}H]-inositol/μg protein.

Results

Release of PDGF activity in response to endothelin

Conditioned medium was collected from confluent quiescent mesangial cells treated with endothelin and analyzed for the presence of PDGF by a radioreceptor assay. In the absence of endothelin, mesangial cells constitutively released PDGF activity. The cumulative PDGF activity in ng PDGF equivalent is shown in Table 1. Cells exposed to 10^{-8} M endothelin continued to release more PDGF activity than did untreated cells.

Regulation of the PDGF A-chain and c-sis mRNAs by endothelin

To determine if the release of PDGF activity is associated with secretion of newly synthesized rather than preformed PDGF, expression of the genes for the A-chain and B-(c-sis) chains of PDGF was assessed in cells treated with endothelin. Figure 1 shows a representative Northern blot demonstrating the expression of A-chain mRNA in human mesangial cells treated with endothelin 10^{-8} M for various time points. Three A-chain transcripts 2.3, 1.9 and 1.5 kilobases, are detected in mesangial cell mRNA as previously reported [15]. In the presence of endothelin there was 1.9-fold increase in the amount of message by four hours that remained elevated for up to 24 hours. When the same blot was hybridized to an alpha-tubulin probe, there were no time dependent alterations in the expression of this gene, demonstrating that the response in A-chain mRNA does not simply reflect a generalized nonspecific change in total RNA expression.

Table 1. Effect of endothelin on PDGF secretion

	12 hrs		24 hrs		36 hrs	
	Control	Endoth.	Control	Endoth.	Control	Endoth.
Exp 1	0.4 ± 0*	0.75 ± 0.20	1.7 ± 0	2.15 ± 0.1	4.0 ± 0.7	6.4 ± 0
Exp 2	—	—	0.25 ± 0	0.35 ± 0.03	1.65 ± 0.1	2.05 ± 0.1
Exp 3	0.06 ± 0.02	0.16 ± 0.01	0.07 ± 0.01	0.17 ± 0.02	0.29 ± 0.01	0.43 ± 0.01
Mean ± SEM	0.303 ± 0.12	0.45 ± 0.17	0.67 ± 0.5	0.89 ± 0.6	1.98 ± 1.0	2.96 ± 1.7

Confluent mesangial cells in 100 mm Petri dishes were incubated in serum free medium for 3 days. Endothelin at a concentration of 10^{-8} M was added at the end of 3 day incubation. Medium collected was centrifuged, and assayed for PDGF as described in **Methods**. Values represent cumulative levels of PDGF that is secreted during times indicated. Values are in ng PDGF/10^6 cells and are average ± range of duplicate samples.

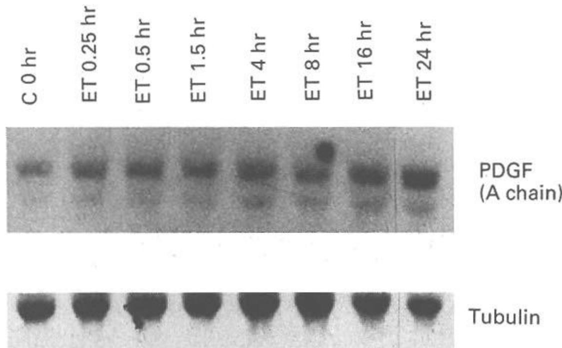

Fig. 1. *Northern blot of induction of PDGF-A chain mRNA in mesangial cells in response to endothelin.* Cells were made quiescent in serum-free media for 3 days prior to exposure to endothelin (10^{-8} M). At the time points indicated, the cells were harvested and RNA was isolated. Control (C) represents RNA isolated from cells incubated without endothelin. Each lane contains 15 μg of total RNA. After hybridization with a single stranded A-chain cDNA, the probe was removed by boiling and the blot was rehybridized to a single stranded alpha-tubulin cDNA probe. Blots were exposed to Kodak XAR film with intensifying screens overnight at −70°C (representative of three experiments).

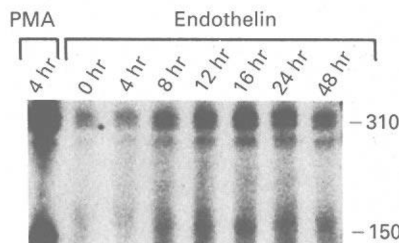

Fig. 2. *Induction of PDGF-B chain mRNA (c-sis) in mesangial cells in response to endothelin.* Confluent cells were placed in serum-free media for 3 days prior to exposure to endothelin or PMA. At the time points indicated the cells were harvested and RNA was isolated. A total of 50 μg samples of total RNA from mesangial cells were hybridized with a uniformly labeled c-sis RNA probe. Following digestion with RNAases A and T_1, protected fragments were electrophoresed on a denaturing polyacrylamide gel and detected by autoradiography (representative of three experiments).

Because of the low abundance of the c-sis mRNA expression in mesangial cells, we used a solution hybridization technique to determine if endothelin also regulates the induction of PDGF B-chain mRNA. As shown in Figure 2, endothelin increased the level of B-chain mRNA over that in control cultures, with similar time course kinetics to those for A-chain induction. The

Table 2. Effect of endothelin on DNA synthesis

	[³H]-Thymidine incorporation *cpm*	
	Serum free	1% Z Serum
Control	2652 ± 156	2731 ± 331
EN 10^{-12}	2450 ± 179	3037 ± 446
EN 10^{-11}	2596 ± 192	3159 ± 460
EN 10^{-10}	3206 ± 367	4308 ± 789
EN 10^{-9}	3406 ± 364[a]	4968 ± 752[a]
EN 10^{-8}	3469 ± 209[a]	5392 ± 925[a]

Cells were plated in 24 well dishes at a density of 2 × 10^4/well and maintained in Waymouth medium with FCS until confluent. Cells were then made quiescent and incubated with various concentrations of endothelin for 24 hours followed by a 4 hour pulse with [^{3}H]-thymidine. Cells were then harvested as described in **Methods**. Data represent average ± range of three experiments, each assayed in triplicate wells.
[a] $P < 0.05$.

effect on both A-chain and c-sis mRNAs is not transient, but progressive and prolonged, unlike the transient induction we have observed in response to other peptide mitogens [15]. This prolonged effect most likely reflects a cumulative effect of endothelin by irreversible binding to its putative receptor site [20–23]. Also shown in Figure 2 is the stimulatory effect of phorbol myristate acetate (PMA) on PDGF B-chain mRNA. PMA markedly increase the steady state levels of PDGF B-chain mRNA. The modest accumulation of PDGF A- and B-chain mRNAs is associated with an increase in the PDGF activity released by the cells in response to endothelin as shown in Table 1.

Effect of endothelin on DNA synthesis

Endothelin stimulates DNA synthesis in mesangial cells as assessed by [^{3}H]-thymidine incorporation into DNA in a dose dependent manner, as shown in Table 2. Maximal stimulation of DNA synthesis was observed at a dose of 10^{-8} M and the half-maximal stimulation at 7 × 10^{-11} M. This is compatible with the potency of the peptide for stimulating smooth muscle contraction. The addition of endothelin in the presence of 1% Zeta Serum caused a further dose dependent increase in [^{3}H]-TdR incorporation. The mitogenic effect of endothelin was further demonstrated by cell counting, as shown in Table 3.

Effect of endothelin on inositol phosphates

We next investigated if DNA synthesis in response to endothelin is associated with activation of phospholipase C. As shown in Figure 3A, endothelin increased turnover of total inositol phosphates measured at 30 minutes. Inositol phosphate turnover was significantly elevated in response to 10^{-8} M and

Table 3. Effect of endothelin on mesangial cell growth

Days	Cell number × 10^4	
	Serum free	Endothelin 10^{-8} M
0	3.2 ± 0.1	3.2 ± 0.1
3	12.5 ± 0.8	15.4 ± 0.2[a]
5	20.8 ± 0.1	27.0 ± 0.3[a]

Mesangial cells were plated in 24 well dishes in Waymouth medium containing FCS. Following cell attachment overnight, the subconfluent cells were washed with serum free medium and placed in SFIF medium with or without 10^{-8} M endothelin. At indicated time points, cells were trypsinized and counted in a Coulter counter. Counts are mean ± SEM from quadruplicate wells.

[a] Significant difference from control wells, $P < 0.05$

10^{-7} M endothelin. As shown in Figure 3B endothelin (10^{-8} M) induces an increase in all three inositol phosphates. Inositol bis- and tris-phosphate increase promptly, peak within 60 seconds and then reach new steady state levels at two minutes. In contrast to these two inositol phosphates, inositol monophosphate continues to increase at two minutes. These results indicate that endothelin activates phospholipase C which catalyzes the breakdown of polyphosphinositides.

Discussion

We have demonstrated that endothelin stimulates PDGF release and induces the steady state levels of the PDGF A-chain and B-chain (c-sis) mRNAs in human mesangial cells. In addition, endothelin activates phospholipase C and stimulates DNA synthesis and proliferation of human mesangial cells. Our finding that endothelin stimulates release of PDGF by smooth muscle-like mesangial cells expands the potential role of endothelin in modulating the biology of the microvascular wall. If glomerular endothelial cells release endothelin analogous to other endothelial cells [20, 21], endothelin should be readily accessible to mesangial cells in both physiologic and pathophysiologic states. In an analogous matter, it is likely that endothelin released by endothelial cells affect adjacent smooth muscle cells in other vascular beds [22]. Released PDGF in response to endothelin may regulate growth of mesangial and perhaps smooth muscle cells, since both cell types express PDGF receptors. The observation that the enhanced PDGF activity in the medium in response to endothelin is associated with increased expression of PDGF mRNAs suggests that at least a portion of this activity results from de novo protein synthesis. It should be emphasized that the amount of PDGF activity measured in the conditioned medium may underestimate the actual amounts synthesized by the cells, since our radioreceptor assay is not optimal for measuring the various PDGF isoforms. In addition, a portion of PDGF may bind to PDGF receptors on the cells without being secreted. It remains to be determined what form of PDGF is released by human mesangial cells after stimulation by endothelin. Whether the secreted protein is a homodimer (A-A or B-B) or heterodimer (AB) or a combination of three isoforms needs to be explored. However, the levels of PDGF B-chain mRNA expression either constitutive or induced are very low, as they cannot be detected with Northern blot analysis. While PDGF AA is likely to be the major isoform secreted by mesangial cells, other factors besides the relative

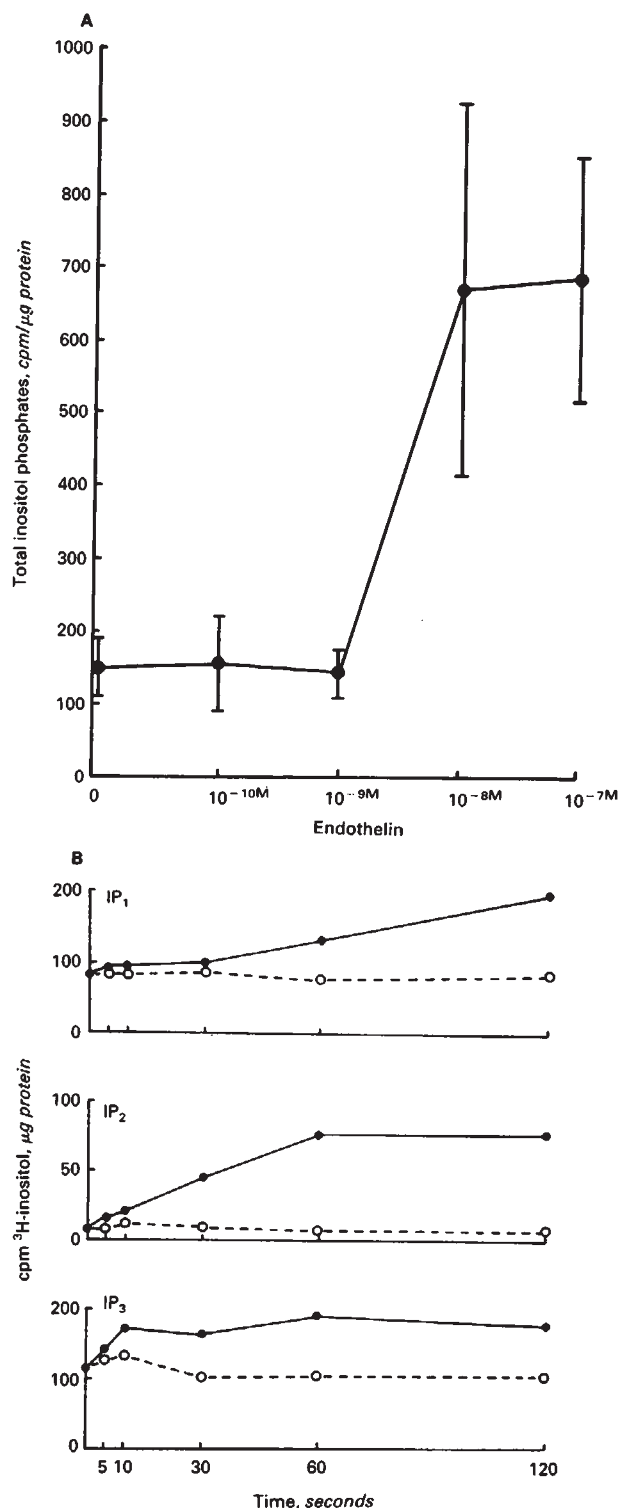

Fig. 3. *Endothelin-induced stimulation of inositol phosphates.* Inositol phosphates were extracted and separated on AG 1-X8 column as described in **Methods**. **A.** Effect of increasing concentrations of endothelin on total inositol phosphate levels at 30 minutes. Each bar represents the average ± range of 2 experiments, each assayed in duplicate wells. Endothelin at a concentration of 10^{-8} M and 10^{-7} M significantly stimulated total inositol phosphates, $*P < 0.001$. **B.** Time course for the increase in inositol monophosphate (IP_1) inositol bisphosphate (IP_2), and inositol trisphosphate (IP_3) in response to 10^{-8} M endothelin, each point is the mean from 4 dishes assayed in duplicate. The SE at all time points is 20% or less of the mean.

amounts of A or B chain mRNA may influence PDGF dimer assembly and secretion in a cell specific manner.

Of major interest is our finding that the time course of induction of PDGF A-chain and c-sis mRNAs in response to endothelin differs from that in response to other peptide growth factors. Endothelin elicits a sustained increase in the expression of both mRNAs, unlike most other peptide growth factors which stimulate the transient expression of PDGF mRNAs with a peak effect after four hours. The mechanism that dissociates this vasoconstrictor from other peptide mitogens is not known, although one possibility is that endothelin appears to bind irreversibly to its receptors and elicits a sustained effect, as demonstrated by Yanigasawa et al and Badr et al [1, 23]. Since endothelin stimulates phospholipase C, as evidenced by the increased turnover of phosphoinositides and release of inositol trisphosphate, it is possible that diacylglycerol-induced activation of protein kinase C is involved in mediating the release of PDGF and the increased expression of PDGF mRNAs. This interpretation is supported by our finding that the protein kinase C activator phorbol ester, phorbol myristate acetate (PMA) also increases mesangial cell steady-state PDGF mRNA levels [18]. It has been shown that endothelin stimulates DNA synthesis in variety of cell lines of mesenchymal origin such as vascular smooth muscle cells, Swiss 3T3 fibroblasts and rat mesangial cells [8, 9, 12]. We have recently shown that induction of PDGF mRNAs in mesangial cells in response to several peptide growth factors is associated with DNA synthesis [15]. Unlike recent observations made in rat mesangial cells demonstrating that endothelin is a comitogen and requires either a cofactor present in serum, or insulin to express mitogenic activity [12], endothelin appears to act as a complete mitogen in human mesangial cells, since DNA synthesis occurred when the cells were exposed to endothelin alone. However, we cannot exclude that endothelin acts in concert with other growth factors released by the cells, including PDGF itself, to stimulate DNA synthesis. Of note is that the mitogenic effect of endothelin was potentiated in the presence of low concentrations of Zeta serum. This finding is consistent with the study of Simenson et al [12] in rat mesangial cells and Takawa et al [9] in smooth muscle cells, demonstrating that the mitogenic effect of endothelin is potentiated in the presence of serum or PDGF.

Vasoconstrictors have recently been reported to initiate several cellular processes that are activated by classic mitogens, such as phospholipase C cleavage or phosphoinositides, mobilization of intracellular calcium and Na^+/H^+ exchange [24, 25]. More recently, protooncogene activation have been recognized as an early cellular response to vasoconstrictors [26]. Induction of c-fos and c-myc in response to angiotensin II (Ang II) have been reported [25, 26]. Clear evidence for a definite relationship between these signals and DNA synthesis in mesangial cells or other cell types is still lacking. A role for phospholipase C in mediating the mitogenic effect of certain growth factors is suggested by the observation that microinjection of an antibody that neutralizes phosphatidylinositol 4,5-bisphosphate (PIP_2) inhibits DNA synthesis [27]. However additional pathways involving novel inositol lipids have recently been proposed [28]. More recently, the role of phospholipase C gamma in DNA synthesis have been questioned [29]. Of interest also is the recent description of constriction as an additional biologic activity for certain peptide growth factors such as PDGF and EGF [30, 31]. The association of activation of many of these signals with endothelin suggest their involvement in mediating some of its biologic effects. It is also likely that released PDGF and increased expression of PDGF mRNA may mediate or modulate the mitogenic effect of endothelin in human mesangial cells in a positive fashion.

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