Endothelin Stimulates Phospholipase C, Na⁺/H⁺ Exchange, *c-fos* Expression, and Mitogenesis in Rat Mesangial Cells

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Abstract

A recently described peptide hormone, endothelin, is a potent vasoconstrictor, but it is unclear whether endothelin has other biological actions. These experiments extend the range of biological actions of endothelin to stimulation of mitogenesis. Endothelin at low concentrations (0.1–10 nM) induced mitogenesis by quiescent rat glomerular mesangial cells in culture. Mitogenesis induced by endothelin was accompanied by activation of phospholipase C with increased inositol phosphate turnover and increments of intracellular $[Ca^{2+}]$. Endothelin also activated Na⁺/H⁺ exchange, causing cytosolic alkalinization, and enhanced transcription of the *c-fos* protooncogene, additional biochemical signals closely linked to proliferation. In addition to being a vasoconstrictor, endothelin thus also functions as a mitogen, presumably through activation of phospholipase C.

Introduction

Paracrine factors, released by the endothelium, regulate vasoreactivity and metabolism of vascular smooth muscle cells (1–4). Eicosanoids, cyclic nucleotides, endothelium-derived relaxing factor, and angiotensin II, for example, are released by endothelial cells and regulate contraction and mitogenesis of vascular smooth muscle (1–4). Yanagisawa and co-workers recently purified and sequenced endothelin, a potent vasoconstrictor peptide released by porcine and human endothelial cells (5, 6). Endothelin-induced vasoconstriction was mediated by Ca^{2+} entry via plasma membrane Ca^{2+} channels (5). Because many constrictor peptides activate Ca^{2+} signaling through phospholipase C and induce mitogenesis (7–9), we tested the hypothesis that endothelin would stimulate cellular proliferation and activate polyphosphoinositide-phospholipase C signal transduction pathways.

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Methods

Mesangial cell culture and mitogenesis assay. Mesangial cells in passages 5–15, cultured and characterized as reported (10–11), were subcultured into 96-well plates (Costar Corp., Cambridge, MA) and made quiescent (G_0) by holding for 3 d in DME/F12 (1:1) with 0.5% fetal bovine serum (FBS).[1] Endothelin or 2.5% FBS were then added (time = 0) to DME/F12 + 0.5% FBS, and after a 23-h incubation, the cells were pulsed for 1 h with 5.0 μCi/ml [methyl-³H]thymidine (6.7 Ci/ mmol). Radiolabeled DNA was extracted by washing each well two times with 0.2 ml ice-cold Dulbecco's PBS, fixing in methanol/acetic acid/water (50:10:40, vol/vol) for 1 h at 4°C, and solubilizing in 1.0% SDS for 2 h at 37°C. Proliferation demonstrated by [³H]thymidine uptake was confirmed by counts of stained nuclei in 96-well plates (Costar Corp.) (12, 13) 48 h after the addition of endothelin. Porcine endothelin was obtained from two sources: Peptide Institute, Osaka, Japan (a generous gift from Dr. Keishi Abe, Tohoku University, Sendai, Japan) and from Peninsula Laboratories, Belmont, CA. Both preparations demonstrated similar activity.

Measurements of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was determined with the Ca^{2+}-sensitive dye fura-2 (14). As reported (10, 11), confluent monolayers on plastic coverslips (Aclar; Allied Engineering Plastics, Pottsville, PA) were loaded with 1 μM fura-2 acetoxymethylester (Molecular Probes, Eugene, OR) for 40 min at 37°C in RPMI 1640 and incubated again for 20 min in fura-2-free RPMI to allow for intracellular dye cleavage. Coverslips were mounted in a quartz cuvette with Krebs-Henseleit Hepes buffer, pH 7.4, maintained at 37°C with constant stirring. Fluorescence was measured with a University of Pennsylvania Biomedical Instruments Group spectrofluorometer (15) set at 339 nm excitation and 500 nm emission. Fluorescence measurements were converted to $[Ca^{2+}]$ by determining maximal fluorescence (F_{max}) with 60 μM ionomycin (Calbiochem-Behring Corp., La Jolla, CA), followed by minimal fluorescence (F_{min}) with 7.5 mM EGTA and 50 mM Tris, pH 10.5 (10). The following formula was then used: $[Ca^{2+}] = K_d(F - F_{min}/F_{max} - F)$; K_d for fura-2 = 224 nM (14).

Measurements of inositol phosphates. As previously reported (10, 11), total inositol phosphates were measured in cells prelabeled for 36 h with 4 μCi/ml of *myo*-[2-³H(*N*)]inositol (15 Ci/mmol; American Radiolabeled Chemicals, St. Louis, MO). Neutralized perchloric acid extracts of cells exposed to endothelin (15 min in the presence of 10 mM LiCl at 37°C) were applied to 0.8 ml AG 1 × 8 (200–400 mesh) anion-exchange columns, and total inositol phosphates were eluted with 12 ml of 1 M NH₄ formate-0.1 M formic acid. Each 12-ml eluate was evaporated and desalted by vacuum centrifugation before being counted. Inositol phosphate turnover was corrected for protein measured in concurrent wells. For analysis of inositol phosphates by

1. *Abbreviations used in this paper:* BCECF, 2',7'-bis (carboxymethyl)-5(6))-carboxyfluorescein; $[Ca^{2+}]_i$, intracellular Ca^{2+}; FBS, fetal bovine serum; pH$_i$, intracellular pH.

HPLC, mesangial cells in six-well plates were labeled for 36 h with 4 μCi/ml myo[2-^{3}H(N)] inositol. After incubating with endothelin for the indicated times, the water soluble inositol phosphates were extracted in perchloric acid, neutralized with 1:1 (vol/vol) freon (1,1,2-trichlorotrifluoroethane) and tri-n-octylamine, and separated by HPLC using a previously described ammonium formate (pH adjusted to 3.7 with phosphoric acid) gradient (16). Equivalent amounts of water soluble material were injected into each column. Also, we confirmed in parallel experiments that [Ca^{2+}]$_i$ transients, in response to endothelin, were unaffected by 36 h labeling in inositol-free medium.

Measurements of intracellular pH (pH$_i$). As previously reported for cultured mesangial cells (17), pH$_i$ was determined by dual-wavelength fluorescence excitation of confluent monolayers on plastic coverslips loaded with the pH-sensitive probe, 2′,7′-bis(carboxyethyl-5(6))-carboxyfluorescein (BCECF; Molecular Probes) (18). Monolayers were loaded, after a 24-h incubation in serum-free medium, with 3 μM BCECF pentaacetoxymethyl ester for 30 min at 37°C in serum-free RPMI 1640 medium. The cells were then incubated in BCECF-free medium for 20 min at 37°C and kept on ice until assayed. Fluorescence measurements were made using a University of Pennsylvania Biomedical Group fluorometer (19), equipped with an air-driven rotary shutter for alternate 440- and 500-nm excitation wavelengths and emission at 540 nm. Cells were incubated at 37°C under continuous stirring in nominally HCO$_3^-$-free KHH buffered with 20 mM Hepes containing 0.2% fatty acid–free BSA (Calbiochem-Behring Corp.). The ratio 500/440 nm was automatically computed and plotted together with a tracing of either wavelength, and calibration with the K$^+$/H$^+$ ionophore, nigericin (5 μg/ml), was performed exactly as described (17).

Northern analysis of c-fos expression. Total mesangial cell RNA from four confluent 100-cm dishes was extracted using 4 M guanidinium thiocyanate and purified by ultracentrifugation through a cesium chloride cushion as previously described (20, 21). The 2.0-kb Eco RI-Sal 1 fragment of pc-fos (mouse)-3 (American Type Culture Collection 41041) was nick translated using a kit (Bethesda Research Laboratories, Gaithersburg, MD) with [α-^{32}P]deoxycytidine 5′-triphosphate (New England Nuclear, Wilmington, DE) to a specific activity of $> 10^8$ cpm/μg DNA. Total cellular RNA (20 μg) was fractionated on a 1% agarose, 2.2 M formaldehyde denaturing gel and transferred by capillary blotting with 25 mM KPO$_4$ (pH 6.5) to Genescreen (New England Nuclear) and the filters were hybridized as previously described (21). A RNA ladder (Bethesda Research Laboratory) was used for molecular weight markers.

Results and Discussion

When endothelin was added to quiescent (G$_0$) mesangial cells, a smooth muscle-like perivascular cell from the kidney glomerulus (22–24), the mesangial cells reentered G$_1$ and progressed to S phase 12–18 h later (Fig. 1 A). A similar time course was seen with 2.5% FBS. [^{3}H]Thymidine uptake in response to endothelin was dose dependent, with a threshold at 0.1 nM, 50% effective concentration (EC$_{50}$) at 0.9 nM, and maximal stimulation $\geq$ 10.0 nM (Fig. 1 B). 10 nM endothelin increased [^{3}H]thymidine uptake 5.8-fold, and the potency of endothelin was similar to 2.5% FBS (Fig. 1 A). Endothelin-induced mitogenesis was confirmed by cell counts 48 h after addition (Results: time 0, 48 $\times$ 10^3±5 cells/well; 48 h control, 51±3; 48 h with 0.1 μM endothelin, 75±6). In contrast to its effect on mesangial cells, endothelin (1.0 pM–0.1 μM) failed to stimulate [^{3}H]thymidine uptake in quiescent Swiss 3T3 fibroblasts (data not shown). These data demonstrate that endothelin is a potent mitogen for glomerular mesangial cells in culture, and mesangial cells proliferate in response to low concentrations (EC$_{50}$ = 0.9 nM) of this vasoconstrictor peptide.

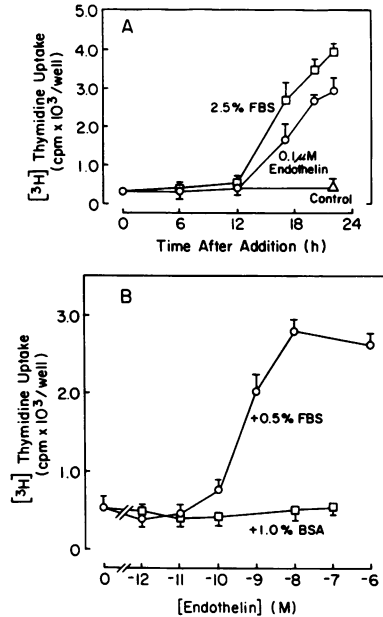*Figure 1.* Endothelin stimulates time- and dose-dependent uptake of [^{3}H]thymidine in quiescent rat mesangial cells. (A) Time course of [^{3}H]thymidine uptake in the presence of 0.1 μM endothelin or 2.5% FBS, both added to DME/F12 + 0.5% FBS (i.e., control). Cells were pulsed for 1 h at the times indicated. Data are mean±SEM for two experiments in triplicate. (B) Dose-response curve and cofactor requirements for endothelin-stimulated [^{3}H]thymidine uptake. Quiescent mesangial cells were incubated with the indicated concentrations of endothelin for 23 h, at which time the cells were pulsed for 1 h and extracted as described in Methods. Cells were made quiescent and reactivated in DME/F12 containing either 0.5% FBS or 1.0% BSA. Data are mean±SEM for three to five experiments in triplicate. Endothelin-induced mitogenesis was significant ($P < 0.01$ by ANOVA) compared with control.

The failure of endothelin to stimulate proliferation by Swiss 3T3 fibroblasts suggests that the mitogenic effect was not a ubiquitous response.

We further characterized endothelin-induced mitogenesis by testing cofactor requirements. In mesangial cells made quiescent and reactivated with DME/F12 + 1.0% BSA (i.e., without 0.5% FBS), endothelin was inactive (Fig. 1 B). Endothelin synergistically stimulated [^{3}H]thymidine uptake in mesangial cells when coincubated with 0.1 μg/ml insulin (control, 465±32 cpm/well; 0.1 μM endothelin, 503±76; 0.1 μg/ml insulin, 679±112; endothelin + insulin 1,663±157; all incubations in DME/F12 + 1.0% BSA, mean±SEM for three experiments in triplicate). We conclude, therefore, that endothelin is a comitogen and requires either a cofactor present in low concentrations of FBS, or the presence of a competence factor, such as insulin, to express mitogenic activity in mesangial cells.

In many cells growth factors elevate [Ca^{2+}]$_i$, and the increase in [Ca^{2+}]$_i$ is thought to partially mediate the mitogenic response (7–9). We thus tested whether endothelin would elevate [Ca^{2+}]$_i$ in fura-2-loaded mesangial cells. Endothelin increased [Ca^{2+}]$_i$ with two distinct kinetic patterns that varied with the concentration (Fig. 2 A–F). At concentrations between 0.1 and 10.0 pM, endothelin stimulated a slow but sustained increase in [Ca^{2+}]$_i$. The increment in [Ca^{2+}]$_i$ persisted for at least 20 min. This slow increase in [Ca^{2+}]$_i$ was unaffected by calcium channel blockade (10 μM nifedipine, 10 μM verapamil, or 20 μM LaCl$_3$) but was abolished by pretreatment with 3 mM EGTA to chelate extracellular Ca^{2+}. Although these results suggest a dihydropyridine- and phenylalkylamine-insensitive entry of Ca^{2+} from the extracellular space, the outward Ca^{2+} gradient created by EGTA could obscure low level release of Ca^{2+} from intracellular stores. At higher con-
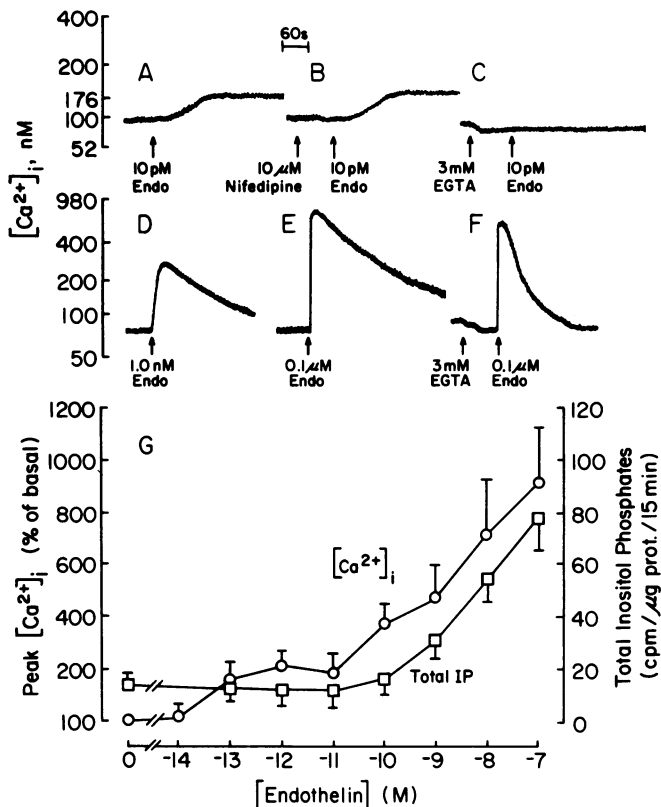
Figure 2. Endothelin elevates $[Ca^{2+}]_i$ and inositol phosphate turnover in mesangial cells. (*A*) Slow but sustained increase in $[Ca^{2+}]_i$ at 10 pM endothelin. (*B*) Effect of 10 μM nifedipine and (*C*) 3.0 mM EGTA pretreatment in Ca^{2+}-free buffer at 10 pM endothelin. (*D* and *E*) Transient increase in $[Ca^{2+}]_i$ observed at $\geq$ 0.1 nM endothelin. $[Ca^{2+}]_i$ returned to baseline after 10–12 min. (*F*) Effect of 3.0 mM EGTA pretreatment at 0.1 μM endothelin. $[Ca^{2+}]_i$ returned to baseline after 3.5–4 min. Nifedipine and 20 μM $LaCl_3$ had no effect on the $[Ca^{2+}]_i$ waveform (data not shown). (*G*) Dose dependence of peak $[Ca^{2+}]_i$ and turnover of total inositol phosphates. Data are mean±SEM for three to seven determinations of $[Ca^{2+}]_i$ and three experiments in duplicate for inositol phosphates.

centrations ($\geq$ 0.1 nM), endothelin evoked a rapid (3–8 s), transient increase in $[Ca^{2+}]_i$ that slowly (10–12 min) fell to the original baseline. The peak transient $[Ca^{2+}]_i$ was dose dependent (Fig. 2 *G*), and the threshold (0.1 nM) for the occurrence of the transient increase in $[Ca^{2+}]_i$ corresponded to that for endothelin-induced mitogenesis (Fig. 1), suggesting that the two events might be linked. Nifedipine and $LaCl_3$ had no effect on the $[Ca^{2+}]_i$ waveform at 0.1 μM endothelin (legend to Fig. 2 *F*), and pretreatment with EGTA had no effect on the transient phase of $[Ca^{2+}]_i$ but markedly attenuated the sustained phase (Fig. 2 *F*). These results demonstrate that release of Ca^{2+} from intracellular stores accounts for the transient increase in $[Ca^{2+}]_i$ whereas entry of Ca^{2+} contributes to the tonic phase. Endothelin causes a greater sustained elevation of $[Ca^{2+}]_i$ than any agonist studied thus far in mesangial cells (10, 11, 25, 26).

We next investigated if endothelin elevates $[Ca^{2+}]_i$ by activating phospholipase C-catalyzed phosphoinositide turnover to release inositol trisphosphate (($(1,4,5)IP_3$) (27, 28). Endothelin increased turnover of total inositol phosphates measured at 15 min in the presence of 10 mM LiCl (Fig. 2 *G*). Inositol phosphate turnover was elevated 5.2-fold by 0.1 μM endothe-

lin, and the dose response curve closely paralleled endothelin-induced transient increases in $[Ca^{2+}]_i$ (Fig. 2 *G*) and mitogenesis (Fig. 1). In concurrent experiments, endothelin stimulated greater inositol phosphate turnover than 0.1 μM arginine vasopressin (i.e., 42±7 cpm/μg protein/15 min, n = 3), a potent phospholipase C-linked agonist in mesangial cells (25). The inability to show higher inositol phosphate turnover at the mitogenic threshold of endothelin (0.1 nM) probably reflects the relative insensitivity of inositol phosphate measurements and the high positive cooperativity of intracellular $(1,4,5)IP_3$ receptors (29), which could respond to minute changes in $(1,4,5)IP_3$ concentration that are undetectable by ion-exchange chromatography. The presence of $(1,4,5)IP_3$ was confirmed by HPLC 10 and 30 s after the addition of endothelin (Table I). Levels of $(1,4,5)IP_3$ increased at 10 and 30 s whereas the $(1,3,4)IP_3$ isomer increased more slowly. Consistent with the slower rate of degradation of $(1,3,4)IP_3$ (30, 31), levels of this isomer exceeded that of $(1,4,5)IP_3$ at 30 s (Table I). The increase in $(1,3,4,5)IP_4$ at 30 s suggests that in addition to degradation via $(1,4)IP_2$, $(1,4,5)IP_3$ metabolism in mesangial cells also involves sequential phosphorylation and dephosphorylation to produce $(1,3,4,5)IP_4$ and $(1,3,4)IP_3$ (30, 31).

The simplest interpretation of these data is that endothelin stimulates the phosphoinositide cascade by activating phospholipase C to generate $(1,4,5)IP_3$, which subsequently releases Ca^{2+} from intracellular stores (27, 28). The diacylglycerol released would presumably activate protein kinase C. The slow increase in $[Ca^{2+}]_i$ below 0.1 nM endothelin could simply reflect weak activation of phospholipase C. It seems possible, however, that endothelin might be coupled to two classes of receptors, one linked to phospholipase C and mitogenesis, the other to activation of Ca^{2+} channels independent of phospholipase C. Alternatively, the same receptor could exist in multiple-affinity states. Our experiments fail to resolve the issue of different pathways of Ca^{2+} signaling by endothelin, but that mitogenesis corresponds with the occurrence of a transient spike of $[Ca^{2+}]_i$ (Fig. 2), and the turnover of inositol phosphates (Fig. 2 *G*), implicates activation of phospholipase C as a proximal event leading to endothelin-induced proliferation.

Alkalinization of pH_i, brought about by activation of Na^+/H^+ exchange by growth factors, is thought to be a neces-

Table I. HPLC Profile of Inositol Phosphates Synthesized in Response to Endothelin

		Inositol phosphates			
Condition	Time	$(1, 4, 5)IP_3$	$(1, 3, 4)IP_3$	$(1, 3, 4, 5)IP_4$	$(1, 4)IP_2$
	s	*(cpm/well)*			
Control	10	387	211	277	ND
Endothelin					
(0.1 μM)	10	458	232	265	ND
Control	30	427	418	260	2,429
Endothelin					
(0.1 μM)	30	696	1,082	488	7,674

Mesangial cells were prelabeled with 4 μCi/ml myo-[2-^{3}H(N)]inositol, incubated for 10 or 30 s with 0.1 μM endothelin, extracted in perchloric acid, and the water-soluble inositol phosphates separated by HPLC as described in Methods. Data are from two experiments in the same cell line.
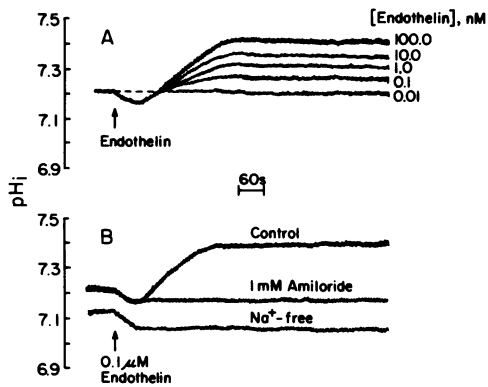
Figure 3. Endothelin activates Na^+/H^+ exchange resulting in cytosolic alkalinization. (A) Changes in pH_i induced by increasing concentrations of endothelin. pH_i was unaffected below 0.1 nM endothelin; moreover, transient acidification was observed only at 10.0 and 100.0 nM endothelin, but significant alkalinization occurred at all doses. Tracings were superimposed to facilitate comparison of the transients. Broken line indicates lack of acidification at < 10 nM. Similar results were seen in three experiments using two different cell lines. (B) Composite of separate experiments showing the effects of sequential addition of 0.1 μM endothelin in the absence and presence of 1 mM amiloride and in media in which Na^+ was isoosmotically replaced by choline.
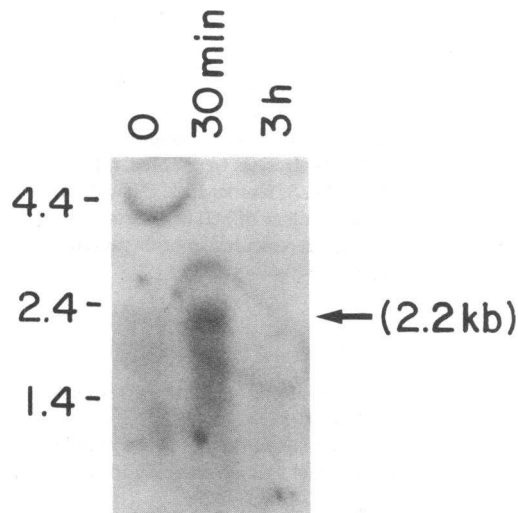

Figure 4. Endothelin activates transcription of the c-fos protooncogene in quiescent mesangial cells. Endothelin was added to quiescent mesangial cells at time = 0, and as described in Methods, total RNA was extracted 30 min and 3 h later. Northern analysis of fractionated RNA depicts a 2.2-kb c-fos transcript in endothelin-stimulated mesangial cells.

sary response for the G_0/G_1 transition and mitogenesis (32–34). In mesangial cells loaded with the pH-sensitive probe, BCECF, mitogenic concentrations of endothelin also elevated pH_i (Fig. 3). Endothelin (10–100 nM) induced a rapid, transient acidification that reversed beyond baseline, resulting in net alkalinization at 5 min that was sustained for at least 15 min. Transient acidification was seen only with 10 and 100 nM endothelin, but dose-dependent alkalinization was always observed > 0.01 nM (Fig. 3 A). 1 mM amiloride completely inhibited alkalinization but did not affect the initial acidification in response to 0.1 μM endothelin (Fig. 3 B). Alkalinization was similarly prevented by incubating the cells in media in which Na^+ was isoosmotically replaced by choline (Fig. 3 B), consistent with stimulation of amiloride-inhibitable Na^+/H^+ exchange. Activation of the Na^+/H^+ exchanger by endothelin was greater than any agonist tested thus far in mesangial cells (17).

We extended our analysis of endothelin-induced mitogenesis by studying c-fos protooncogene expression, which normally precedes cell proliferation (35, 36). c-fos is the protooncogene homologue of the Finkel-Biskis-Jinkins osteosarcoma virus and encodes a 55-kD nuclear protein (see reference 35). When endothelin (0.1 μM) was added to mesangial cells in G_0, c-fos mRNA increased markedly 30 min after addition and returned to basal levels at 3 h (Fig. 4). FBS (5%) induced less c-fos expression but followed a similar time course (data not shown). A similar time course for c-fos induction has been observed in quiescent cells stimulated to divide by growth factors and phospholipase C agonists (7, 9, 35, 36).

These experiments support the hypothesis that endothelin is a mitogen for quiescent (G_0) mesangial cells. The dose dependence for mitogenesis closely corresponded to activation of the phosphoinositide cascade and Na^+/H^+ exchange, which supports, but does not prove, our hypothesis that these signaling mechanisms are responsible for endothelin-induced prolif-

eration. Moreover the finding that endothelin stimulated c-fos transcription further supports a mitogenic function. Although the exact role of phospholipase C in mitogenesis remains unclear (7–9), Na^+/H^+ exchange appears to be a necessary step to activate reentry of cells from G_0 to G_1 (33, 34).

Yanigisawa et al. (5) proposed that endothelin acts as an endogenous agonist of dihydropyridine-sensitive, voltage-gated Ca^{2+} channels. Indeed, the conformation and amino acid sequence of endothelin is homologous with several neurotoxins (5, 6), such as α scorpion toxin (37, 38), which act directly on tetrodotoxin-sensitive, voltage-gated Na^+ channels (39). In mesangial cells, however, endothelin stimulated accumulation of inositol phosphates and mobilization of intracellular Ca^{2+} stores rather than activation of dihydropyridine-sensitive Ca^{2+} channels. Ca^{2+} entry through other types of channels apparently accounted for endothelin-mediated increments of $[Ca^{2+}]_i$ at low concentrations of endothelin as well as the sustained elevations of $[Ca^{2+}]_i$ at high concentrations of endothelin. Thus it appears that endothelin evokes multiple pathways of transmembrane signaling that might be coupled to different physiologic events in various target cells. Mesangial responses to endothelin point to the possibility of paracrine control, by endothelial cells, of these contiguous pericytes within the glomerulus.

Acknowledgments

Addendum

Hirata et al. have described endothelin-induced calcium transients in rat vascular smooth muscle cells. The sustained phase, but not the spike increases of $[Ca^{2+}]_i$, were reduced by zero calcium solutions or nicardipine but no changes of PIP_2 hydrolysis were noted. (Hirata, Y., H. Yoshimi, S. Takata, T. Watanabe, S. Kumagai, K. Nakajima, and S. Sakakibara. 1988. Cellular mechanism of action by a novel vasoconstrictor endothelin in cultured rat vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* 154:868-875.)

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