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Activation of Thromboxane and Prostacyclin Receptors Elicits Opposing Effects on Vascular Smooth Muscle Cell Growth and Mitogen-Activated Protein Kinase Signaling Cascades

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SUMMARY

Thromboxane A_2 stimulation of smooth muscle cells contributes to the development of vascular lesions after percutaneous transluminal coronary angioplasty. In view of this, we examined the signaling pathways stimulated by a thromboxane receptor agonist, U-46619, in cultures of rat aortic smooth muscle cells. Treatment of rat aortic smooth muscle cells with U-46619 induced cellular hypertrophy ([^{14}C]leucine incorporation) without stimulating mitogenesis ([^{3}H]thymidine incorporation). Analysis of signaling pathways elicited by U-46619 revealed enhanced tyrosine phosphorylation and increased enzymatic activity of mitogen-activated protein (MAP) kinase (Erk2). U-46619 also activated signaling proteins upstream of p21-ras, inducing tyrosine phosphorylation on Shc and complex formation between Shc and growth factor receptor binding protein-2 (GRB2). Exposure of cells to a stable prostacyclin analogue, ciprostene calcium, attenuated U-46619-induced cellular hypertrophy and MAP kinase activity. Ciprostene treatment elevated cellular cAMP and inhibited U-46619-induced tyrosine phosphorylation on Shc and Shc/GRB2 complex formation. These results demonstrate that stimulation of thromboxane A_2 and prostacyclin receptors have opposing effects on smooth muscle cell hypertrophy and the signaling pathways associated with this process. We conclude that inhibition of Shc/GRB2 complex formation and MAP kinase activity by ciprostene may contribute to its ability to limit restenosis injury.

Production of thromboxane A_2 at sites of vascular injury contributes to lesion formation characteristic of restenosis and atherosclerosis (1). Although well known as a potent vasoconstrictor, thromboxane A_2 may also induce smooth muscle cell migration and growth that is typical of vascular occlusion. Several investigators have reported mitogenic or hypertrophic activities of thromboxane A_2/prostaglandin endoperoxide receptor agonists in studies of cultured vascular smooth muscle cells (2–6). Consistent with the growth-inducing properties of thromboxane A_2 is the apparent stimulation of mitogen-activated and S6 protein kinases in cultured smooth muscle cells exposed to a thromboxane A_2/prostaglandin endoperoxide receptor agonist, U-46619. Biochemical, pharmacological, and molecular cloning studies have placed the thromboxane A_2/prostaglandin endoperoxide receptor in the G protein-coupled, seven membrane-spanning receptor superfamily (7, 8). In the vasculature, the actions of thromboxane A_2 are opposed by prostacyclin to maintain normal hemostasis and vascular tone. Release of thromboxane by platelets is a potent signal for vasoconstriction and platelet aggregation. In contrast, prostacyclin is a potent vasodilator and antiaggregatory substance for platelets. Thromboxane actions are believed to occur primarily through activation of phospholipase C, whereas prostacyclin elicits responses through a G protein-coupled receptor linked to adenylate cyclase. Perturbation of the thromboxane A_2/prostacyclin balance may exacerbate vascular injury in restenosis and atherosclerosis by eliciting both vasoconstriction and smooth muscle cell growth. A stable analogue of prostacyclin has recently demonstrated efficacy in clinical trials for the prevention of vascular lesion formation after PTCA (9). Similarly, a prostacyclin analogue limited lesion formation in a rat model of restenosis (10).

The mechanism through which thromboxane and other G protein-coupled receptors transduce signals to MAP kinases is unclear. Induction of MAP kinase activity by platelet-derived and other growth factors appears subsequent to activation of p21-ras. Proteins upstream of p21-ras have recently been identified that detect growth factor receptor activation and stimulate guanine nucleotide exchange on

ABBREVIATIONS: PDGF, platelet-derived growth factor; EGF, epidermal growth factor; GRB2, growth factor receptor binding protein-2; SH2, src homology-2; SH3, src homology-3; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PVDF, polyvinyldifluoride; MAP, mitogen-activated protein; RASMC, rat aortic smooth muscle cells; PTCA, percutaneous transluminal coronary angioplasty; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; PBS-T, PBS containing 0.1% Tween 20; SOS, son-of-sevenless.

p21-ras (11–16). GRB2 senses activated receptors by binding specific, phosphorylated tyrosine residues via its SH2 domain. In addition to binding to phosphorylated tyrosine kinase receptors, GRB2 recognizes phosphorylated tyrosine residues on Shc proteins. A number of smooth muscle cell growth factors, including platelet-derived growth factor and angiotensin II, stimulate tyrosine phosphorylation on Shc (17–20). Shc can bind directly to PDGF β-receptors in stimulated cells and may, through recruitment of GRB2, relay receptor activation to downstream signaling proteins (18–19). The SH3 domains of GRB2 facilitate stable complex formation with the nucleotide exchange factor SOS. This Shc/GRB2/SOS complex thereby transmits receptor ligand binding to p21-ras. Shc/GRB2 complex formation appears to be necessary for activation of p21-ras by insulin and EGF and the transformation of fibroblasts by overexpression of Shc proteins (21–23).

We investigated the signaling mechanisms through which U-46619 induces MAP kinase activity and the effects of ciprostene on this signaling cascade in RASMC. Our data show that U-46619 stimulates tyrosine phosphorylation on Shc and Shc/GRB2 complex formation in parallel with stimulation of MAP kinases. Consistent with the abilities of prostacyclin to oppose thromboxane in other biological systems, ciprostene attenuated U-46619-induced Shc/GRB2 complex formation and subsequent activation of MAP kinases. This observation suggests a mechanism for the ability of ciprostene to reduce lesion formation in patients after PTCA.

Experimental Procedures

Materials. All reagents were from Sigma Chemical Co. unless indicated otherwise. U-46619 and ciprostene were from The Upjohn Company. Antibodies specific for phosphotyrosine, Shc, Erk2, and GRB2 were from Upstate Biotechnology, Transduction Laboratories, or Santa Cruz Biotechnology. Cell culture reagents and protein G-agarose were from GIBCO. Secondary antibodies were purchased from Jackson ImmunoResearch. ECL reagents were from Amersham Chemical Co.

Cell culture. RASMC were isolated as described (24) and maintained in Dulbecco's modified Eagle's medium containing 15% fetal calf serum. Cells were split at confluence and fed every third day. Cultures were used between passages 5 and 10.

Immunoprecipitations. Cells were grown to confluence and serum-starved in Dulbecco's modified Eagle's medium lacking fetal calf serum for 18 hr. After stimulation, cell medium was removed, and cells were rinsed twice with ice-cold PBS (Ca^{2+} and Mg^{2+} free). Cells were scraped into lysis buffer (20 mM HEPES, pH 7.4, 1% Triton X-100, 50 mM sodium chloride, 1 mM EGTA, 5 mM β-glycerophosphate, 30 mM sodium pyrophosphate, 100 μM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) (25). Cell debris was removed by centrifugation at 12,000 × *g* for 10 min. Supernatants (350 μg of protein) were transferred to new tubes containing 2 μg of indicated antibody and incubated at 4° for 2.5 hr. Protein G-agarose was added, and lysates were incubated for an additional 30 min. Precipitates were washed three times in ice-cold lysis buffer and resuspended finally in SDS-PAGE (26) sample buffer. Complexes were boiled for 5 min and electrophoresed through 10% SDS-PAGE gels. Proteins were transferred to PVDF membranes (Immobilon, Millipore Corp) and processed for immunoblot analysis.

Immunoblot analysis. Nonspecific binding sites were blocked in PBS-T and 1% bovine serum albumin for 1 hr at 20°. Primary antibodies were diluted in blocking solution and incubated with the membranes for 1 hr at room temperature. Excess primary antibody was removed by washing the membranes four times in PBS-T. The blots were incubated with appropriate secondary antibodies in PBS-T containing 5% milk diluent (Kirkegaard and Perry Laboratories) for 1 hr. Membranes were washed as before and processed for ECL. In certain experiments, the filters were reprobed after stripping in 0.1 M Tris·HCl, pH 8.0, 2% SDS, and 100 mM β-mercaptoethanol for 30 min at 52°.

MAP kinase enzyme assays. After stimulation, RASMC were harvested in lysis buffer as described. Supernatants containing ~50 μg of protein were analyzed using a p42/p44 MAP kinase enzyme assay kit from Amersham. Assays were performed as directed, and ^{32}P-phosphate incorporated into the substrate peptide was determined by liquid scintillation spectroscopy.

Determination of cAMP levels. After stimulation, RASMC were harvested in 100 μl of 4 mM EDTA, boiled to denature protein, and centrifuged to remove cellular debris. Supernatants (50 μl) were analyzed for cAMP using a radioimmunoassay kit from Amersham. Assays were performed as directed, and cAMP levels were determined from a standard curve.

Measurement of RASMC mitogenesis and hypertrophy. Serum-starved RASMC were treated for 24 hr with U-46619 in the presence of 0.5 μCi [^{14}C]leucine and 0.5 μCi [^{3}H]thymidine per 16-mm well. Unincorporated label was removed by washing with ice-cold 10% trichloroacetic acid. Acid-insoluble material was hydrolyzed by the addition of 0.25 N NaOH, and label incorporation was determined by liquid scintillation spectroscopy. Before U-46619 treatment, some cells received either vehicle or 10 μM ciprostene. U-46619 was then added directly to these cultures for 24 hr, and label incorporation was determined as described. All statistical comparisons were made using one-way analysis of variance. Statistical significance was assessed using an unpaired Student's *t* test.

Results

Data describing the role of thromboxane as a mitogenic or hypertrophic stimulus are inconsistent and may be cell type specific (2–6). We first examined the ability of thromboxane to induce [^{3}H]thymidine and [^{14}C]leucine uptake in cultures of quiescent RASMC. Cells were stimulated with various concentrations of U-46619 in the presence of [^{3}H]thymidine and [^{14}C]leucine for 24 hr. After stimulation, acid-insoluble fractions of the cells were harvested, and radiolabel incorporation was determined. Fig. 1 illustrates a concentration-dependent increase in [^{14}C]leucine incorporation after a 24-hr incubation with U-46619. Stimulation of [^{14}C]leucine uptake was significantly different from control with as little as 0.01 μM and maximal with 1 μM U-46619. In the same experiments, measurement of [^{3}H]thymidine uptake failed to demonstrate significant increases in DNA synthesis by RASMC at any U-46619 concentration examined (data not shown). Confirmation that the hypertrophic actions of U-46619 are facilitated through direct binding to the thromboxane receptor was provided by preincubation of RASMC cultures with the thromboxane receptor antagonist U-77433. Preincubation of RASMC with 100 μM U-77433 resulted in an 81 ± 6% (eight experiments) inhibition of [^{3}H]leucine incorporation in cells stimulated with 10 μM U-46619.

I-BOP, a thromboxane receptor agonist, stimulates MAP kinases in cultures of guinea pig coronary smooth muscle cells (5). Therefore, we evaluated whether activation of these kinases preceded the hypertrophic phenotype elicited by U-46619 in RASMC. Quiescent cultures of RASMC were stimulated with 10 μM U-46619 for various times. After stimulation, cell lysates were prepared and analyzed for tyrosine phosphorylation on MAP kinases and MAP kinase enzyme

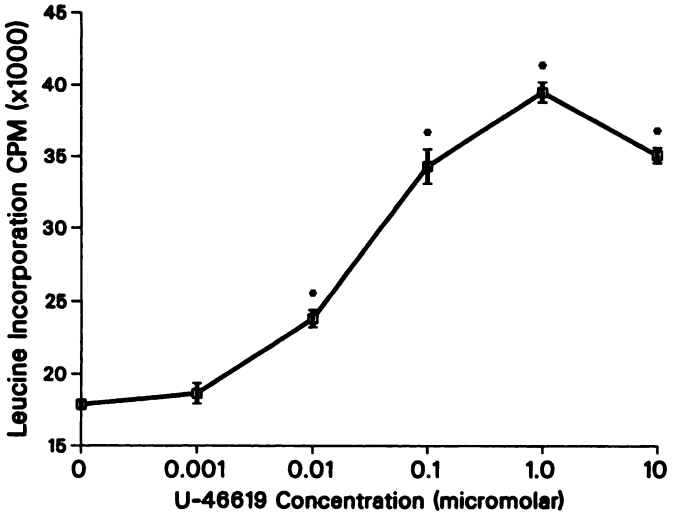

Fig. 1. U-46619 induces hypertrophy in cultures of RASMC. Quiescent cultures of RASMC were stimulated with various concentrations of U-46619 in the presence of [^{14}C]leucine for 24 hr. After incubation, acid-insoluble fractions of the cells were harvested, and [^{14}C]leucine incorporation was determined by scintillation spectroscopy. Results shown were performed in quadruplicate and are expressed as the mean ± standard error. *, Significantly different ($p < 0.05$) from vehicle-treated cells.

activity. Analysis of lysates from cells exposed to U-46619 demonstrated a time-dependent increase in tyrosine phosphorylation on a 42-kDa protein, consistent with the erk 2 isoform of the MAP kinases (Fig. 2A). Phosphorylation on the 42-kDa protein was rapid and transient. Maximal tyrosine phosphorylation occurred within 5 min, and the signal decayed to near-resting levels by 30 min. Confirmation of the p42 tyrosine phosphorylated protein as a MAP kinase was provided by analyzing the membrane with an antibody specific for Erk2 (Fig. 2B). Levels of Erk2 detected in the lysates remained constant throughout the time course studied, indicating that changes in tyrosine phosphorylation, not protein amounts, accounted for the results observed in Fig. 2A. Similar experiments demonstrated concentration-dependent phosphorylation on Erk2 in response to U-46619 that corresponded with the hypertrophic effects observed in Fig. 1. Phosphorylation on Erk2 was detectable with 0.01 μM U-46619 and plateaued at concentrations of U-46619 between 1 and 10 μM (data not shown). In parallel cultures, we used a specific assay for quantifying MAP kinase enzyme activity. MAP kinase activity increased rapidly and corresponded closely with tyrosine phosphorylation on Erk2. Kinase activity was significantly elevated compared with control cells at 5 and 15 min after the addition of 10 μM U-46619 and was indistinguishable from vehicle-treated cultures by 30 min (Fig. 3).

Because MAP kinase activation occurs subsequent to activation of p21-ras, we next investigated whether U-46619 activated signaling proteins upstream of p21-ras in RASMC. Activation of Shc/GRB2 complexes was measured in cells treated with 10 μM U-46619 for various times. After incubation, cell lysates were precipitated with an antibody specific for Shc. Shc signaling complexes were resolved by PAGE and analyzed for phosphotyrosine-containing proteins and GRB2 by immunoblot analysis. Fig. 4A shows the time-dependent phosphorylation of 46-, 52-, and 66-kDa Shc isoforms. Phosphorylation of these proteins paralleled activation of MAP kinases both temporally and with respect to U-46619 concentration. Phosphorylation on Shc was stimulated within 5 min, increased until 15 min, and remained elevated for up to 120 min. Coincident with Shc phosphorylation was the recruitment of GRB2 to Shc immune complexes. GRB2 observed in Shc immune precipitations increased at 5 min and remained elevated throughout the time course evaluated

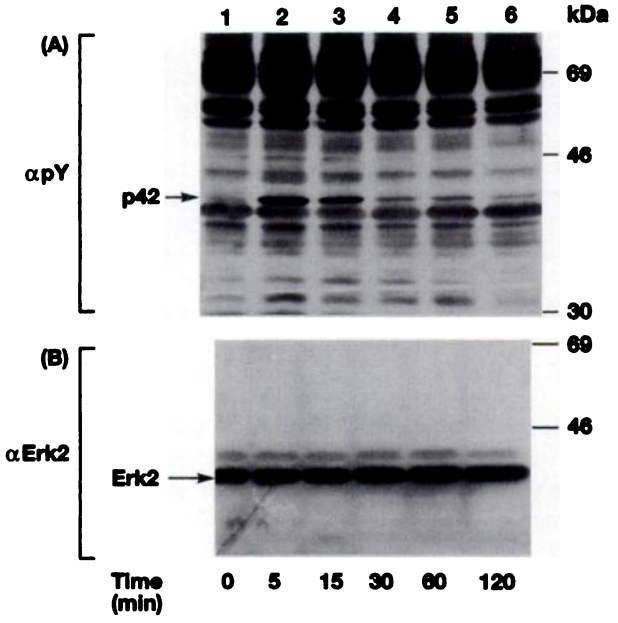

Fig. 2. Stimulation of MAP kinase phosphorylation in RASMC exposed to U-46619. Serum-deprived cultures of RASMC were stimulated with 10 μM U-46619 for various times. Cell lysates were then analyzed for activation of MAP kinases. A, Phosphotyrosine-containing proteins were visualized by immunoblotting with an antibody specific for phosphotyrosine (*αpY*). B, The membrane in A was stripped and reprobed with an antibody specific for Erk2. Results shown are typical of five individual experiments.

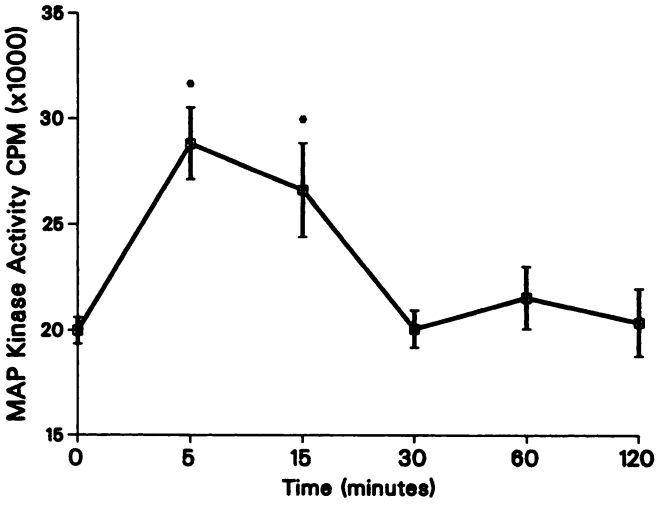

Fig. 3. Stimulation of MAP kinase enzyme activity in RASMC exposed to U-46619. Serum-deprived cultures of RASMC were stimulated with 10 μM U-46619 for various times. MAP kinase enzymatic activity present in cell lysates was determined by measuring phosphate incorporation into a synthetic MAP kinase substrate peptide. Results shown represent the mean ± standard error of seven or eight determinations. *, Significantly different ($p < 0.05$) from vehicle-treated cells.

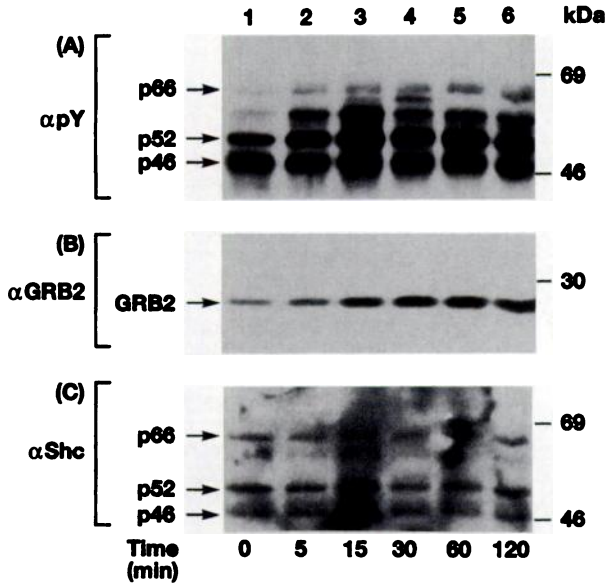

Fig. 4. U-46619 induces tyrosine phosphorylation on Shc and complex formation with GRB2. Quiescent RASMC monolayers were treated with 10 μM U-46619 for varying lengths of time. Cells were lysed, and Shc signaling complexes were immunoprecipitated. Proteins were resolved by SDS-PAGE and transferred to PVDF membranes. The membranes were cut at 30 kDa. A, The top portion (*30–200 kDa*) was probed with an antibody against phosphotyrosine (*αpY*). B, The bottom portion (*21.5–30 kDa*) was probed with an antibody specific for GRB2. C, The blot in A was stripped and reprobed with an antibody directed against Shc. Similar responses were observed in five individual experiments.

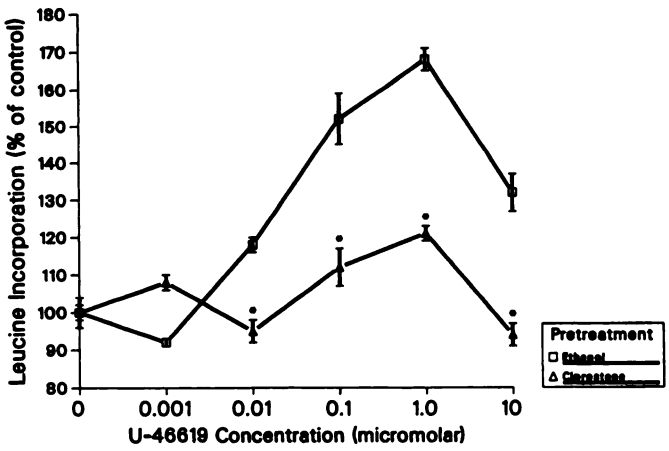

Fig. 5. Ciprostene calcium inhibits RASMC hypertrophy induced by U-46619. Serum-deprived RASMC were preincubated with 10 μM ciprostene calcium for 60 min before the addition of U-46619 at various concentrations. Cells were incubated for 24 hr in media supplemented with [^{14}C]leucine. After incubation, acid-insoluble material from the cells was harvested, and [^{14}C]leucine incorporation was determined by scintillation spectroscopy. Results shown were obtained from quadruplicate samples and are expressed as mean ± standard error. *, Significantly different ($p < 0.05$) from vehicle-treated cells.

(Fig. 4B). Analysis of the blot with an antibody directed against Shc confirmed the identity of the phosphorylated proteins and ensured equivalent Shc precipitation (Fig. 4C).

Prostacyclin balances the actions of thromboxane in maintaining vascular hemostasis and tone. This prompted us to examine whether a stable analogue of prostacyclin, ciprostene calcium, would interfere with U-46619 induction of hypertrophy and related signaling pathways. Serum-deprived confluent monolayers of RASMC were pretreated with 10 μM ciprostene for 60 min and then stimulated with various concentrations of U-46619 for 24 hr. [^{14}C]Leucine present in extracted proteins was then quantified by scintillation spectroscopy. Fig. 5 illustrates a typical profile of ciprostene effects on [^{14}C]leucine incorporation stimulated by U-46619. Ciprostene attenuated [^{14}C]leucine incorporation at all concentrations of U-46619, consistent with the opposing actions of these compounds in other systems.

Because ciprostene blocked U-46619-induced smooth muscle cell hypertrophy, we investigated whether this inhibition was accomplished through blockade of the MAP kinase signaling pathway. RASMC were pretreated with ciprostene for various times before the addition of 10 μM U-46619. After incubation for 5 min, cell lysates were examined for tyrosine phosphorylation on Erk2 and corresponding enzyme activity. Ciprostene alone evoked little change in Erk2 phosphorylation (Fig. 6A). Ciprostene did, however, demonstrate time-dependent inhibition of U-46619-induced Erk2 phosphorylation. Inhibition of MAP kinase phosphorylation was evident after 15 min of pretreatment with ciprostene and persisted for at least 120 min. Analysis of the blot with an Erk2-specific antibody demonstrated that expression of Erk2 protein was unaffected by ciprostene throughout the time course (Fig. 6B). Consistent with the observed inhibition of Erk2 phosphorylation was a ciprostene-dependent decline in MAP kinase enzyme activity elicited by U-46619 (Fig. 7). Inhibition of MAP kinase activity correlated closely with inhibition of Erk2 tyrosine phosphorylation. Activity levels declined after 15 min of pretreatment with ciprostene and were significantly inhibited after 30 min of preincubation. MAP kinase activity remained significantly below that seen in control cells for the duration of the experiment.

To delineate the point at which ciprostene inhibits the MAP kinase signaling pathway, we evaluated whether ciprostene affected Shc/GRB2 complex formation in response to U-46619. Again, cells were pretreated with ciprostene at 10 μM for 0–120 min. Cells were then stimulated with 10 μM U-46619 for 15 min. Shc immune complexes were isolated from cell lysates and analyzed for phosphotyrosine incorporation and recruitment of GRB2. Fig. 8A demonstrates a decline in phosphotyrosine incorporation into Shc and a corresponding decrease in GRB2 recruitment (Fig. 8B) when Shc was precipitated from cells pretreated with 10 μM ciprostene. In agreement with inhibition of MAP kinase tyrosine phosphorylation and enzyme activity, Shc activation and complex formation with GRB2 were maximally inhibited after ciprostene pretreatment for 30–120 min.

Prostacyclin exerts its biological actions through elevation of cAMP and subsequent activation of protein kinase A. Elevation of cAMP can inhibit smooth muscle cell mitogenesis (27–29). Furthermore, activation of protein kinase A inhibits MAP kinase activation in response to several growth factors (30–32). We therefore measured cAMP levels in RASMC responding to ciprostene. Ciprostene stimulated a significant increase in cAMP content of RASMC within 5 min that remained elevated for 120 min (Fig. 9). This rapid increase in cAMP preceded inhibition of Shc/GRB2 complex formation and MAP kinase activation and suggests a cAMP-independent component to the inhibitory action of ciprostene.

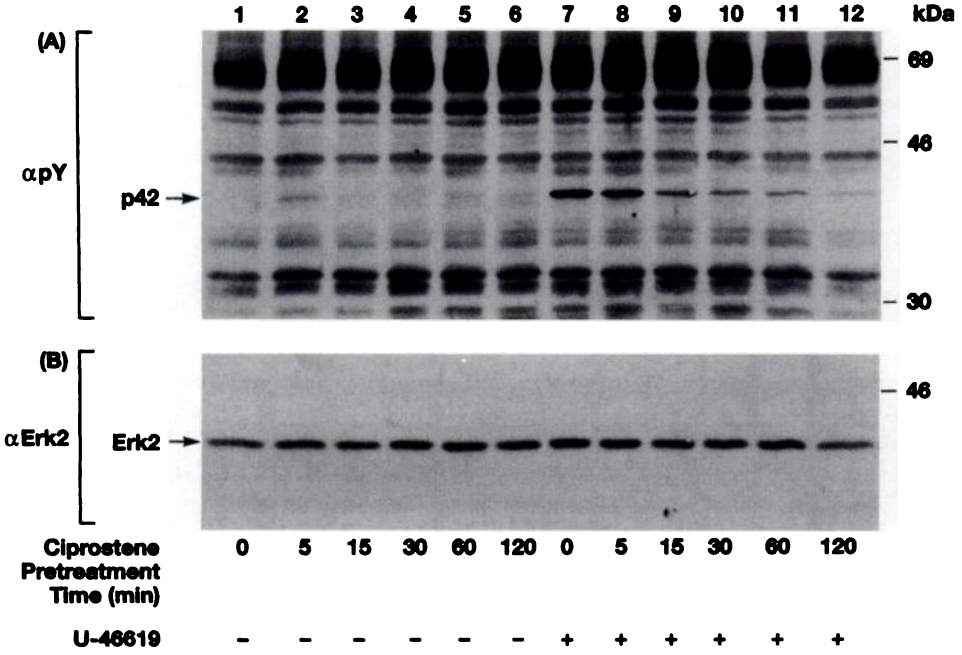

Fig. 6. Time-dependent blockade of MAP kinase phosphorylation by ciprostene calcium. Serum-deprived RASMC were preincubated with 10 μM ciprostene calcium for various times before the addition of 10 μM U-46619 for an additional 5 min. Cell lysates were then analyzed for activation of MAP kinases. A, Phosphotyrosine-containing proteins were visualized by immunoblotting with an antibody specific for phosphotyrosine (*αpY*). B, The membrane in A was stripped and reprobed with an antibody specific for Erk2. Similar inhibition profiles were observed in four separate experiments.

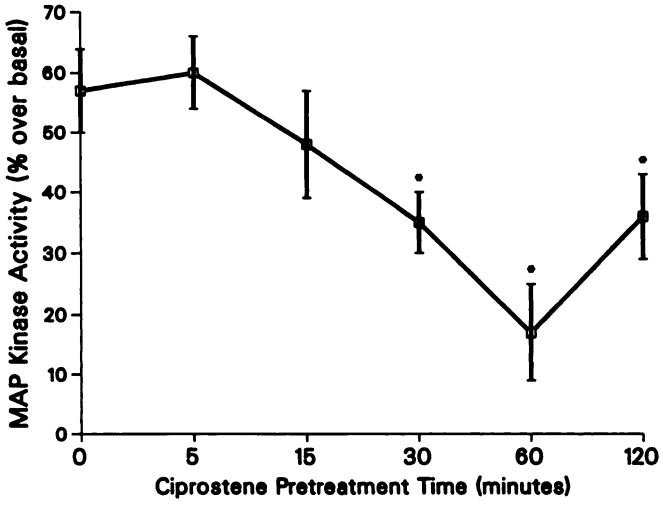

Fig. 7. Time-dependent blockade of MAP kinase enzyme activity by ciprostene calcium. Serum-deprived RASMC were preincubated with 10 μM ciprostene calcium for various times before the addition of 10 μM U-46619 for an additional 5 min. Cell lysates were then analyzed for activation of MAP kinases. MAP kinase enzymatic activity present in cell lysates was determined by measuring phosphate incorporation into a synthetic MAP kinase substrate peptide. Results shown represent mean ± standard error from triplicate samples. *, Significantly different ($p < 0.05$) from vehicle-treated cells.

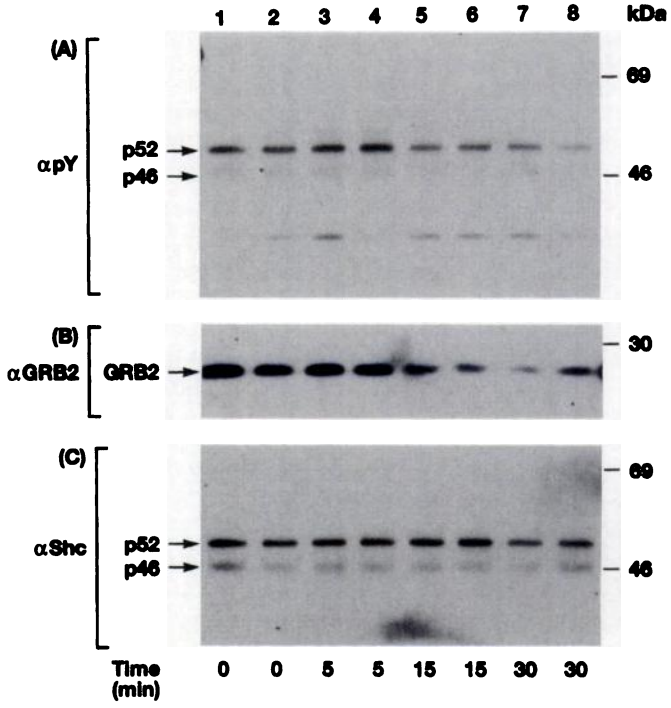

Fig. 8. Ciprostene calcium attenuates Shc phosphorylation and complex formation with GRB2 in RASMC responding to U-46619. Serum-deprived RASMC were preincubated with 10 μM ciprostene calcium for various times before the addition of 10 μM U-46619 for an additional 15 min. Cells were lysed, and Shc signaling complexes were immunoprecipitated. Proteins were resolved by SDS-PAGE and transferred to PVDF membranes. The membranes were cut at 30 kDa. A, The top portion (*30–200 kDa*) was probed with an antibody against phosphotyrosine (*αpY*). B, The bottom portion (*21.5–30 kDa*) was probed with an antibody specific for GRB2. Similar patterns were observed in three separate experiments.

Discussion

Thromboxane A_2 and prostacyclin have important effects on vascular smooth muscle cell contractility and growth. In this report, we demonstrate that G protein-coupled thromboxane A_2/prostaglandin endoperoxide and prostacyclin receptors converge on the MAP kinase signal transduction cascade with opposing actions. Data describing U-46619 as a mitogenic or hypertrophic agonist for smooth muscle cells are contradictory and inconsistent (2–6). Our results demonstrate little effect of U-46619 on DNA synthesis by RASMC but dramatic increases in protein synthesis. This supports the hypothesis that U-46619 elicits hypertrophy rather than mitogenesis in cultured RASMC. Consistent with the hypertrophic nature of thromboxane, U-46619 stimulated MAP kinase phosphorylation and enzymatic activity with a time course similar to that elicited by known growth factors for

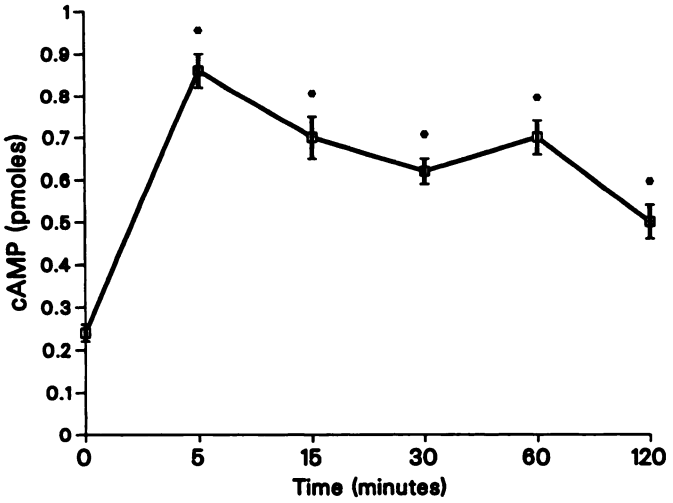

Fig. 9. Ciprostene calcium stimulates a rapid and persistent elevation of cAMP in RASMC. Serum-deprived RASMC were incubated with 10 μM ciprostene calcium for various times. After incubation, cells were harvested, and cAMP content was measured using a sensitive radioimmunoassay specific for cAMP. Results represent the mean ± standard error for six determinations. *, Significantly different ($p < 0.05$) from vehicle-treated cells.

smooth muscle cells. Activation of MAP kinases is a common feature of hypertrophic agents on muscle cells derived from various tissues. Angiotensin II, for example, elicits hypertrophic responses from vascular smooth muscle cells and cardiac myocytes (33–35). In each cell type, the hypertrophic response is preceded by activation of MAP kinases and expression of immediate early genes. Activation of MAP kinases by U-46619 in RASMC concurs with results presented by Morinelli *et al.* demonstrating activation of S6 and MAP kinases in guinea pig coronary smooth muscle cells (5). These investigators, however, found that U-46619 induced mitogenesis. Although they did not evaluate the hypertrophic effects of U-46619, this may suggest a species- or tissue-specific response. Rapid and transient activation of the MAP kinases by U-46619 in RASMC suggests direct activation of the hypertrophic signaling cascade and is in agreement with studies demonstrating that transient activation of these kinases precedes smooth muscle cell hypertrophy in response to angiotensin II (33, 34).

We also observed that U-46619 activates signaling proteins upstream of MAP kinases and p21-ras. Activation of Shc/GRB2 complexes is a previously undefined mechanism linking the G protein-coupled thromboxane receptor to growth factor signaling pathways. Shc plays a critical role in the transduction of growth factor receptor tyrosine kinase activation of the p21-ras signaling cascade. Complex formation between Shc and GRB2 appears to be required for p21-ras activation in cells exposed to insulin or EGF and in oncogenic transformation of rat fibroblasts by introduction of Shc (21–23). Activation of Shc in response to U-46619, therefore, is consistent with the hypothesis that thromboxane A_2/prostaglandin endoperoxide receptor activation stimulates cell growth. Activation of Shc/GRB2 complexes is also involved in the signal transduction cascade elicited by other G protein-coupled receptors. Angiotensin II stimulates Shc phosphorylation and GRB2 recruitment in cardiac fibroblasts (20). It is interesting that agonists using Shc/GRB2 complex formation for signal transduction elicit both vasoconstrictor and growth promoting responses in vascular smooth muscle cells, regardless of the receptor class eliciting the response. This expands the role of Shc/GRB2 complex formation from a growth regulating context and implicates it in contractility responses. Hollenberg recently implicated Src family tyrosine kinases in smooth muscle contractility responses (36). The action of U-46619 fits with this hypothesis in that Src is a putative activator of Shc. Furthermore, coprecipitation of Src with Shc/GRB2 complexes from smooth muscle cells responding to PDGF has been demonstrated (19).

Our data also point to a mechanism through which prostacyclin inhibits smooth muscle cell growth in response to U-46619. We demonstrate that the prostacyclin analogue ciprostene attenuates MAP kinase phosphorylation and enzymatic activity induced by U-46619. Inhibition of this process correlated with inhibition of cellular hypertrophy. Recently, elevation of intracellular cAMP has been shown to inhibit MAP kinase activation in cells responding to EGF and PDGF (30–32). These investigators identified the blockade as far upstream as raf-1. Although ciprostene elevates cAMP in RASMC (Fig. 9), the time course of elevation diverges from that for inhibition of MAP kinase activity and phosphorylation. Inhibition of MAP kinase activation in RASMC required at least a 15-min preincubation with ciprostene. In contrast, cAMP levels were maximal within 5 min. The delayed inhibition of MAP kinase is unusual for a direct effect of protein kinase A activation on c-raf as observed previously (30–32). Convergence at a point distinct for c-raf is consistent with our observation that Shc/GRB2 complex formation was inhibited by ciprostene with a time course that paralleled MAP kinase inhibition. The mechanism of this upstream blockade requires further investigation but could stem from desensitization of the thromboxane receptor (37) or the kinase that links the thromboxane receptor to Shc. The ability of ciprostene to inhibit the MAP kinase signaling pathway warrants attention in that ciprostene limits lesion formation in patients after PTCA (9). The mechanism through which ciprostene affords this protection *in vivo* is unknown but may be due to a combination of vasodilator actions and antigrowth-promoting properties. The present study and previous findings suggest that blockade of the MAP kinase signaling cascade could facilitate these activities. The role of thromboxane in this process should be viewed with some caution. Aspirin effectively reduces recurrence of myocardial and cerebral infarction by limiting platelet thromboxane synthesis (38). This strategy, however, has proved to be disappointing in the prevention of vascular injury after PTCA and suggests that factors in addition to thromboxane contribute to lesion development (39, 40). The efficacy of ciprostene may stem from the blockade of multiple stimuli. In this regard, PDGF and angiotensin II are also released at sites of vascular injury. Because U-46619 shares upstream signaling pathways with PDGF and angiotensin II, it will be of interest to examine whether ciprostene prevents activation of this signaling pathway in response to other growth factors.

Acknowledgments

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