Platelet-derived Growth Factor Regulates Vascular Smooth Muscle Cell Proliferation by Inducing Cationic Amino Acid Transporter Gene Expression*

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Since recent studies demonstrated that platelet-derived growth factor (PDGF) induces vascular smooth muscle cell (SMC) proliferation by stimulating polyamine synthesis, we examined whether the transcellular transport of L-ornithine, the cationic amino acid precursor of polyamines, could regulate the mitogenic response of PDGF. Treatment of SMC with PDGF stimulated DNA and putrescine synthesis, and this was enhanced further by increasing the extracellular concentration of L-ornithine. The potentiating effect of L-ornithine was reversed by the competitive inhibitor of cationic amino acid transport, methyl-L-arginine, or by preventing putrescine formation with α-difluoromethylornithine. Cationic amino acid uptake by SMC was Na⁺ independent and was mediated by both a high and low affinity carrier system. Treatment of SMC with PDGF initially (0–2 h) decreased basic amino acid transport, while longer exposures (6–24 h) progressively increased uptake. Kinetic studies indicated that PDGF-induced inhibition was associated with a decrease in affinity for cationic amino acids, while the stimulation was mediated by an increase in transport capacity. Endogenous PDGF released by collagen-activated platelets likewise up-regulated cationic amino acid transport in SMC. Reverse transcriptase-polymerase chain reaction detected the presence of mRNA encoding two distinct cationic amino acid transporter (CAT) proteins, CAT-1 and CAT-2B. Treatment of SMC with PDGF strongly induced the expression CAT-2B mRNA and modestly elevated the level of CAT-1 mRNA. These results demonstrate that PDGF-induced polyamine synthesis and SMC mitogenesis are dependent on the transcellular transport of L-ornithine. The capacity of PDGF to up-regulate the transport of L-ornithine by inducing the expression of the genes for CAT-1 and CAT-2B may modulate its mitogenic effect by providing SMC with the necessary intracellular precursor for polyamine biosynthesis.

Excessive proliferation of vascular SMC is an important contributing factor to a number of vascular disease states, including atherosclerosis, hypertension, and restenosis following angioplasty (1–3). One potent modulator of SMC replication is PDGF (1, 4). This cationic peptide is secreted by activated platelets and macrophages, and by vascular cells at sites of inflammation and vascular damage (4). PDGF has been implicated in the regulation of SMC proliferation both in vivo and in vitro, but the mechanism(s) by which PDGF induces SMC growth remains unclear (4, 5). Recent studies indicate that the synthesis of polyamines plays an integral role in the mitogenic response of arterial SMC to PDGF (6). PDGF-stimulated SMC proliferation is preceded by increases in cellular polyamine content, and inhibition of polyamine formation prevents cell growth. Furthermore, the exogenous addition of polyamines to SMC mimics PDGF in stimulating DNA synthesis.

The polyamines putrescine, spermidine, and spermine are naturally occurring aliphatic amines, which are present in all mammalian cells. Putrescine is synthesized from the cationic amino acid L-ornithine via a decarboxylation reaction catalyzed by the enzyme ornithine decarboxylase (7). Spermidine and spermine are synthesized from putrescine through the sequential addition of a propylamine moiety from S-adenosylmethionine (7). Early studies indicated that induction of ornithine decarboxylase and increases in polyamine biosynthesis are required for cell growth in response to hormones and growth factors (7–9). Although this highly regulated inducible enzyme is often believed to be rate-limiting in the polyamine biosynthetic pathway, the availability of L-ornithine also plays a crucial role in regulating polyamine biosynthesis. Previous studies demonstrate that the steady state level of L-ornithine markedly influences polyamine formation in neoplastic cells (10, 11). Moreover, exogenous L-ornithine administration further stimulates DNA synthesis in these cells, suggesting that the increase in DNA synthesis results from increased polyamine synthesis due to enhanced L-ornithine substrate availability (12).

Our laboratory and others have previously demonstrated that the transport of cationic amino acids, such as L-ornithine, by vascular SMC is mediated by the system y⁺ carrier (13, 14). This particular transport system is characterized by its recognition of cationic amino acids with high affinity, its Na⁺ independence, and the ability of substrate on the opposite (trans) side of the membrane to increase transport activity (15). Recently, the genes encoding the proteins responsible for the activity of the system y⁺ carrier have been cloned and designated as CAT-1, CAT-2A, and CAT-2B (16–19). CAT-1 was initially identified as an ectotropic retrovirus receptor in murine fibroblasts (16) and was subsequently shown to be a basic amino acid transporter in Xenopus oocytes (20, 21). CAT-2B derived growth factor (PDGF) induces vascular smooth muscle cell (SMC) proliferation by stimulating polyamine synthesis, we examined whether the transcellular transport of L-ornithine, the cationic amino acid precursor of polyamines, could regulate the mitogenic response of PDGF. Treatment of SMC with PDGF stimulated DNA and putrescine synthesis, and this was enhanced further by increasing the extracellular concentration of L-ornithine. The potentiating effect of L-ornithine was reversed by the competitive inhibitor of cationic amino acid transport, methyl-L-arginine, or by preventing putrescine formation with α-difluoromethylornithine. Cationic amino acid uptake by SMC was Na⁺ independent and was mediated by both a high and low affinity carrier system. Treatment of SMC with PDGF initially (0–2 h) decreased basic amino acid transport, while longer exposures (6–24 h) progressively increased uptake. Kinetic studies indicated that PDGF-induced inhibition was associated with a decrease in affinity for cationic amino acids, while the stimulation was mediated by an increase in transport capacity. Endogenous PDGF released by collagen-activated platelets likewise up-regulated cationic amino acid transport in SMC. Reverse transcriptase-polymerase chain reaction detected the presence of mRNA encoding two distinct cationic amino acid transporter (CAT) proteins, CAT-1 and CAT-2B. Treatment of SMC with PDGF strongly induced the expression CAT-2B mRNA and modestly elevated the level of CAT-1 mRNA. These results demonstrate that PDGF-induced polyamine synthesis and SMC mitogenesis are dependent on the transcellular transport of L-ornithine. The capacity of PDGF to up-regulate the transport of L-ornithine by inducing the expression of the genes for CAT-1 and CAT-2B may modulate its mitogenic effect by providing SMC with the necessary intracellular precursor for polyamine biosynthesis.

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was first detected in activated thymocytes and has recently been cloned from lipopolysaccharide-treated macrophages (17, 18). Both CAT-1 and CAT-2B are low capacity transporters which have a high affinity (K_m = 100 μM) for cationic amino acids. In contrast, CAT-2A is an alternate splice variant of CAT-2B that was cloned from murine liver that possesses low affinity (K_m = 2-5 mM) but high transport capacity (19).

Since polyamine generation is dependent on l-ornithine availability, the present study was designed to determine whether PDGF-induced polyamine synthesis and SMC mitogenesis are dependent on the transcellular transport of l-ornithine. We now report that PDGF up-regulates SMC cationic amino acid transport and induces the gene expression of specific CAT proteins. Furthermore, endogenous PDGF released by activated platelets likewise up-regulates cationic amino acid transport in vascular SMC.

**EXPERIMENTAL PROCEDURES**

**Materials—**Fatty acid-free albumin, Sepharose 2B-300, creatine phosphate, creatine phosphokinase, fetal calf serum, SDS, l-glutamine, l-arginine, l-ornithine, trichloroacetic acid, penicillin, streptomycin, elastase, collagenase, monoclonal antibody against smooth muscle cell α-actin, ninyhydrin spray, and TLC (silica gel-25) were purchased from Sigma; guanidine isothiocyanate and CsCl were from Life Technologies, L-arginine, L-ornithine, trichloroacetic acid, penicillin, streptomycin, GCCTCTTACTCACTCTTGCAA-3" (corresponding to sense bp 5-24 of CAT-2A and B) and reverse 5'GACCAGGATCTTGGATTGATG-3' (corresponding to antisense bp 777-798 of CAT-1) primers were used to detect the presence of 630-bp CAT-1 transcript while the forward 5'-CCACAGATGTCCTGAGA-3' (corresponding to sense bp 5-24 of CAT-2A and B) and reverse 5'-TGACT-GTCCCTGTGAGCC-3' (corresponding to antisense bp 777-798 of CAT-1) primers were used to detect the presence of 480-bp CAT-2B transcript.

**Platelets—**Platelets were isolated from blood obtained from drug-free donors and platelet releasates prepared from collagen-activated platelets, as described previously (28). In some experiments, active PDGF present in the releasates was neutralized by incubating the platelet releasate with the IgG fraction (100 μg/ml) of PDGF neutralizing polyclonal antibody for 90 min at 22°C.

**Preparation of Platelet Releasates—**Platelets were isolated from blood obtained from drug-free donors and platelet releasates prepared from collagen-activated platelets, as described previously (28). In some experiments, active PDGF present in the releasates was neutralized by incubating the platelet releasate with the IgG fraction (100 μg/ml) of PDGF neutralizing polyclonal antibody for 90 min at 22°C.
Increased both the vascular SMC with PDGF (30 ng/ml) for 24 h significantly increased the incorporation of radioactive thymidine into DNA (Fig. 1). The simultaneous addition of L-ornithine (0.1–1.0 mM) to the culture media further increased PDGF-induced [3H]thymidine incorporation in a concentration-dependent manner (Fig. 1A). In the absence of PDGF, however, L-ornithine failed to stimulate thymidine incorporation (data not shown). The ability of L-ornithine to increase [3H]thymidine incorporation in PDGF-treated cells was inhibited by L-NMA (10 mM) and by DFMO (2 mM) (Fig. 1B). The PDGF-induced increase in DNA synthesis was associated with an increase in the capacity of SMC to generate the polyamine putrescine from extracellular L-ornithine (Fig. 2). Increasing the extracellular L-ornithine concentration from 50 μM to 1 mM further augmented the capacity of PDGF to stimulate putrescine formation (Fig. 2). Both L-NMA (10 mM) and DFMO (2 mM) inhibited PDGF-induced putrescine formation (Fig. 2).

Treatment of vascular SMC with PDGF regulated the capacity of these cells to transport L-ornithine and other cationic amino acids. Time course studies demonstrated that PDGF (30 ng/ml) had a biphasic effect on cationic amino acid transport (Fig. 3). Initially, PDGF exposure inhibited the transport of cationic amino acids, but by 6 h of PDGF treatment a significant rise in cationic amino acid transport was observed, and this was further increased following 24 h of treatment (Fig. 3). The increase in basic amino acid uptake at 24 h was dependent on the concentration of PDGF (Fig. 4).

In subsequent kinetic studies, saturable uptake of radiolabeled L-arginine (0.005–10 mM) was measured. As evident from the Eadie-Hofstee plots, uptake of L-arginine by SMC was biphasic, with two clearly separated affinity states (Fig. 5). Similar results were obtained for L-ornithine uptake. A high affinity transporter having a Michaelis constant ($K_m$) of 62 ± 13 μM and a maximum transport velocity ($V_{max}$) of 1.24 ± 0.23 nmol/mg of protein/45 s was apparent. A second low affinity system was also demonstrated with a $K_m$ of 2.48 ± 0.3 mM and a $V_{max}$ of 8.53 ± 1.41 nmol/mg of protein/45 s. Pretreatment of vascular SMC with PDGF (30 ng/ml) for 24 h significantly increased both the $K_m$ (107 ± 12 μM; p < 0.01) and $V_{max}$ (2.16 ± 0.06 nmol/mg of protein/45 s; p < 0.01) of the high affinity transporter (Fig. 5). Similarly, PDGF pretreatment significantly elevated the $V_{max}$ (14.24 ± 1.65 nmol/mg of protein/45 s; p < 0.05) of the low affinity transporter but did not affect the $K_m$ (2.40 ± 0.4 mM) of this carrier (Fig. 5). The increase in the $V_{max}$ of cationic amino acid uptake by both carrier systems was completely abolished with cycloheximide (5 μg/ml) (data not shown).

Incubation of SMC with the releasate from collagen-activated platelets for 24 h also stimulated the transport of cationic amino acids (Fig. 6). The addition of a PDGF neutralizing antibody to untreated SMC had no effect on cationic amino acid transport but prevented the increase in uptake evoked by the exogenous addition of PDGF (30 ng/ml), confirming the efficacy of the antibody (Fig. 6). Nonimmune IgG failed to modulate basic amino acid uptake in untreated cells and also had no effect on PDGF-induced increases in transport (Fig. 6). Treatment of the platelet releasate with a PDGF neutralizing antibody significantly reduced the ability of the platelet releasate to augment cationic amino acid uptake, although it did not
completely reverse the stimulatory effect of the platelet releasate (Fig. 6). In contrast, nonimmune IgG did not modify the stimulatory effect of the releasate (Fig. 6).

Reverse transcription coupled PCR identified cDNA encoding CAT-1 (630 bp) and CAT-2B (1150 bp) in untreated or PDGF-stimulated (30 ng/ml for 24 h) SMC, but in four out of five experiments failed to detect cDNA encoding CAT-2A (Fig. 7). In one experiment, an extremely faint CAT-2A band was detected (data not shown). No RT-PCR products were obtained with RNA samples in the absence of reverse transcriptase or when cDNA was omitted from the PCR reaction (data not shown). Ribonuclease protection assays demonstrated that PDGF treatment (3–50 ng/ml for 6 h) resulted in a concentration-dependent increase in the expression of both CAT-1 and CAT-2B; however, the PDGF-induced increase in CAT-2B message was much greater and was observed at lower PDGF concentrations (Fig. 8).

**DISCUSSION**

The present study demonstrates that PDGF-induced SMC mitogenesis is dependent on the transcellular transport of L-ornithine and its subsequent metabolism to polyamines. In addition, we show that PDGF increases the transport of cationic amino acids, such as L-ornithine, in vascular SMC by inducing the expression of the genes encoding the system y\(^+\) transporter.

Recent studies indicate that SMC proliferation following PDGF treatment is dependent on polyamine synthesis (6). Consistent with this, our data demonstrate that PDGF-stimulated DNA synthesis is associated with a prominent increase in the capacity of SMC to convert L-ornithine to putrescine. Further-
more, the selective ornithine decarboxylase inhibitor, DFMO (9), blocks putrescine formation and inhibits thymidine incorporation into DNA. The exogenous addition of L-ornithine potentiates PDGF-induced putrescine formation and DNA synthesis in SMC. Both these effects of L-ornithine are reversed by treating SMC with a cationic amino acid transport inhibitor, L-NMA (13), or by preventing the conversion of L-ornithine to putrescine with DFMO. These results suggest that the availability of L-ornithine is a limiting factor, and its metabolism to polyamines regulates the mitogenic response of PDGF in vascular SMC.

SMC can obtain L-ornithine from extracellular sources via specific plasma membrane transport proteins or from intracellular sources by protein degradation or by endogenous synthesis. We have previously demonstrated that transport of physiological levels of cationic amino acids (50–100 μM) by SMC is mediated by the Na"+-independent system y" carrier (13). Our current study demonstrates that PDGF can regulate the transport of cationic amino acid transport by this carrier system in both a time- and concentration-dependent manner. Our finding of a delayed increase in L-ornithine uptake complements an earlier study, which demonstrated a similar time-dependent increase in the activity of ornithine decarboxylase in SMC following PDGF treatment (6). These observations suggest that PDGF-mediated increases in both L-ornithine uptake and metabolism are coordinated to maximize the cellular capacity for polyamine biosynthesis.

Kinetic experiments reveal the presence of both a high (K_m \approx 60 μM) and low affinity (K_m \approx 2.5 mM) carrier system in SMC. Treatment of SMC with PDGF for 24 h increases the V_{max} for both the high and low affinity transporter, while the K_m of the high affinity carrier is elevated. These kinetic data suggest that the PDGF-induced increase in uptake observed at later time points results from the de novo expression of additional transport proteins. The capacity of cycloheximide to abolish the PDGF-mediated increase in V_{max} for both high and low affinity transport systems is consistent with this notion. In contrast, the PDGF-induced inhibition of uptake observed at early time points likely arises from a PDGF-mediated decrease in affinity of the high affinity transporter.

We have found that vascular SMC express mRNA for CAT-1 and CAT-2B. The co-expression of both CAT proteins is also observed in several murine organs including stomach, skin, lung, and uterus, but contrasts with the selective expression of CAT-2 in mouse liver (29–31). CAT-1 and CAT-2B possess similar kinetic properties and are consistent with our kinetic data for the high affinity carrier system, which, under physiological conditions, would mediate most of the transport of cationic amino acids by SMC. Treatment of SMC with PDGF stimulates both CAT-1 and CAT-2B mRNA expression. The concentration-dependent induction of CAT-2B message by PDGF more closely parallels the PDGF-mediated elevation in cationic amino acid transport; however, the relative contribution of CAT-1 and CAT-2B to the overall activity of the system y" carrier is difficult to ascertain, owing to their similar uptake kinetics. The recent finding that angiotensin II can also up-regulate CAT-1 and CAT-2 in vascular SMC suggests a general mechanism by which growth factors can increase L-ornithine supply leading to elevated polyamine production (32). Thus, the capacity of vascular mitogens to augment the transport of cationic amino acids via the induction of CAT proteins provides not only the necessary amino acids required for the synthesis of new proteins during cell growth, more importantly, it plays an integral role in mediating the mitogenic response of vascular SMC. The uptake of cationic amino acids by vascular SMC is also mediated by a low affinity carrier system whose transport kinetics resemble CAT-2A. However, we could not consistently identify any CAT-2A transcripts in SMC using a variety of primers based on the murine sequence (19). In addition, treatment of SMC with PDGF did not induce CAT-2A expression. Whether this low affinity carrier system represents a species variant of CAT-2A or a new transport protein is not known. However, given its high K_m \approx 2.5 mM for cationic amino acids, it is unlikely to play an important physiological role. Our inability to detect CAT-2A message is consistent with the suggestion by Closs et al. (18) that the alternate splice products of CAT-2 are expressed in a tissue-specific manner.

Finally, the physiological significance of our findings is suggested by the observation that the release of collagen-activated platelets stimulates the transport of cationic amino acids. This stimulatory effect of the platelet releasate is largely mediated by the release of PDGF. However, other platelet-derived factors are also likely to be involved since a neutralizing antibody directed against PDGF can not fully reverse the platelet-mediated increase in basic amino acid transport. The capacity of activated platelets to induce the transport of L-ornithine by vascular SMC may be of pathophysiological importance. Following local injury of the vessel wall, platelets are recruited and subsequently activated by interacting with subendothelial collagen, releasing various mitogens, including PDGF, from their α granules. The ability of growth factors to augment L-ornithine uptake would increase cellular polyamine production and thereby potentiate SMC proliferation. Furthermore, our earlier observations of the capacity of platelets to inhibit the expression of inducible nitric oxide synthase in vascular SMC (28) would further promote intimal proliferation by inhibiting the synthesis of the platelet inhibitory and anti-proliferative molecule, nitric oxide. Thus, the combined ability of platelet-derived mitogens to up-regulate L-ornithine transport and metabolism and inhibit inducible nitric oxide synthase expression may contribute to their SMC proliferative actions at sites of vessel wall injury.

In conclusion, the present study demonstrates that PDGF-induced polyamine synthesis and SMC mitogenesis are dependent on the transcellular transport of L-ornithine. In addition, we show that PDGF can stimulate the transport of cationic amino acids in vascular SMC by inducing the expression of the genes for CAT-1 and CAT-2B. The capacity of PDGF to up-regulate the transport of L-ornithine may modulate its mitogenic effect by providing SMC with the necessary intracellular precursors for polyamine biosynthesis.

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Regulation of CAT Proteins by PDGF

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