C-Peptide Induces Vascular Smooth Muscle Cell Proliferation
Involvement of Src-Kinase, Phosphatidylinositol 3-Kinase, and Extracellular Signal-Regulated Kinase 1/2

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Abstract—Increased levels of C-peptide, a cleavage product of proinsulin, circulate in patients with insulin resistance and early type 2 diabetes mellitus. Recent data suggest a potential causal role of C-peptide in atherogenesis by promoting monocyte and T-lymphocyte recruitment into the vessel wall. The present study examined the effect of C-peptide on vascular smooth muscle cells (VSMCs) proliferation and evaluated intracellular signaling pathways involved. In early arteriosclerotic lesions of diabetic subjects, C-peptide colocalized with VSMCs in the media. In vitro, stimulation of human or rat VSMCs with C-peptide induced cell proliferation in a concentration-dependent manner with a maximal 2.6±0.8-fold induction at 10 nmol/L human C-peptide (P<0.05 compared with unstimulated cells; n=9) and a 1.8±0.2-fold induction at 0.5 nmol/L rat C-peptide (P<0.05 compared with unstimulated cells; n=7), respectively, as shown by [H3]-thymidine incorporation. The proliferative effect of C-peptide on VSMCs was inhibited by Src short interference RNA transfection, PP2, an inhibitor of Src-kinase, LY294002, an inhibitor of PI-3 kinase, and the ERK1/2 inhibitor PD98059. Moreover, C-peptide induced phosphorylation of Src, as well as activation of PI-3 kinase and ERK1/2, suggesting that these signaling molecules are involved in C-peptide--induced VSMC proliferation. Finally, C-peptide induced cyclin D1 expression as well as phosphorylation of Rb in VSMCs. Our results demonstrate that C-peptide induces VSMC proliferation through activation of Src- and PI-3 kinase as well as ERK1/2. These data suggest a novel mechanism how C-peptide may contribute to plaque development and restenosis formation in patients with insulin resistance and early type 2 diabetes mellitus. (Circ Res. 2006;99:1181-1187.)

Key Words: C-peptide ■ diabetes mellitus ■ restenosis ■ smooth muscle cells ■ proliferation

Patients with metabolic syndrome or type 2 diabetes mellitus exhibit an increased propensity for the development of a scattered and extensive pattern of arteriosclerosis. In addition, diabetic patients have a higher risk of restenosis after coronary intervention with or without stent implantation.1-3 Typically, these insulin resistant patients demonstrate increased serum levels of C-peptide, a cleavage product of proinsulin, released into the blood stream in amounts equimolar to those of insulin.4 For a long time, C-peptide has been considered to be biological inert, but recent data suggest that C-peptide binds to specific, yet unidentified cell surface receptors, thereby stimulating intracellular signaling processes such as activation of Na-K-ATPase or mitogen activated protein kinase (MAPK).5-9 Moreover, our group recently demonstrated significantly higher C-peptide deposition in early arteriosclerotic lesions of diabetic subjects compared with matched nondiabetic controls. In these lesions, C-peptide colocalized with monocytes/macrophages as well as CD4-positive lymphocytes and in vitro, C-peptide induced monocyte and T-cell chemotaxis,10,11 suggesting the involvement of C-peptide in inflammatory cell recruitment in early atherogenesis. C-peptide deposition in this study was mainly found in the intima, but in some subjects C-peptide also deposited in the media, potentially colocalizing there with vascular smooth muscle cells (VSMCs).

VSMCs play a crucial role in the development of arteriosclerotic plaques by proliferating and subsequently moving from the media into early lesions and fatty streaks.12 Furthermore, VSMCs are of critical importance in restenosis formation after coronary intervention. After vascular injury these cells start to proliferate and then migrate into the developing neointima, thus becoming the major cellular substrate of the restenotic tissue.13 Strategies to modulate VSMC proliferation after intervention, eg, by the implantation of drug-eluting stents, reduce neointima formation and restenosis,14 underscoring the pathophysiological relevance of VSMC proliferation in these processes. Several mechanisms like PDGF release from activated platelets or the secretion of cytokines...
and growth factors from inflammatory cells have been shown to induce VSMC proliferation during atherogenesis and restenosis formation. Previous work has shown that 3 days of treatment of rat VSMCs with human C-peptide at high glucose conditions decreases cell proliferation, but the direct mitogenic action of short-term C-peptide treatment on VSMC has not been examined yet.

Therefore, the present study assessed the effect of C-Peptide on VSMC proliferation and analyzed intracellular signaling pathways involved.

**Materials and Methods**

**Immunohistochemistry**

Immunohistochemical analyses of specimens from diabetic subjects and growth supplement, 10% fetal bovine serum, penicillin, and streptomycin (100 μg/mL) at 37°C with 5% CO₂. Cells were used at passages 2 to 7. Human C-peptide and rat C-peptide were purchased from Sigma Genosys and Thermo Hybaid (Germany), respectively. Scrambled C-peptide, containing similar amino acids in a random order, was also purchased from Thermo Hybaid (Germany).

**Proliferation Assays**

VSMCs were plated in 24-well plates and grown to 80% confluence. Cells were then starved in serum-free media for 12 hour and stimulated with C-peptide for 24 hours before 1 μCi/mL of [3H] thymidine was added to each well for 3 hours. Average [3H] thymidine incorporation was assessed using a scintillation counter (Wallac 1410, Pharmacia) and expressed as counts per well. In some experiments, inhibitors (the Src-kinase inhibitor PP2, the PI-3 kinase inhibitor LY294002, and the ERK1/2 inhibitors PD98059 or U0126) were added 30 minutes before C-peptide stimulation. As another technique to assess VSMC proliferation we used KI-67 immunofluorescence staining for proliferating cells. VSMCs were starved in serum-free media and then incubated with rat C-pentide for 24 hours. Cells were fixed with Methanol at −20°C for 2 minutes, then washed in phosphate buffer saline (PBS) and blocked in PBS containing 5% respective serum. The primary antibody (1:100, mouse anti-KI-67) diluted in PBS with 3% serum was added for 1 hour at room temperature. After washing with PBS, carboxymethylindocyanine 3- (Cy3) coupled goat anti-mouse immunoglobulin G was added as a secondary antibody (dilution 1:1000) for 45 minutes. Images were recorded with a confocal laser-scanning microscope (Leica).

**Western Blot Analysis**

Rat aortic smooth muscle cells (RASMCs) were cultured as described above and pretreated with various inhibitors for 30 minutes at 37°C. Cells were then incubated with 0.5 nmol/L rat C-peptide for times indicated and lysed in lysis buffer (Nonidet P40: 50 mmol/L Tris-HCl pH 8.0, 150 mmol/L NaCl, 1% NP-40) for 10 minutes on ice. Soluble extracts were prepared by centrifugation with 10,000g for 10 minutes at 4°C. Samples were separated by sodium dodecyl sulfate-polyacrylamide gels. After electrophoresis, proteins were electrotransferred to methanol treated polyvinylidene difluoride membranes. Blotted membranes were blocked with 5% nonfat dry milk in PBS buffer for 30 minutes at room temperature. For Western blot analysis of the expression/phosphorylation of Src-kinase, ERK1/2, AKT, cyclin D1 and phospho-Rb, membranes were probed with anti-pSrc, anti-Src (both from Cell Signaling Technologies), anti-ERK (Promega, Madison, USA), anti-phospho-ERK (Cell Signaling Technologies), anti-AKT, anti-phospho-AKT (both Cell Signaling Technologies), anti-cyclin D1 (BD), and anti-phospho-Rb (Scr807/811)(BD) followed by peroxidase-conjugated goat anti-rabbit/mouse immunoglobulin G and detected by enhanced chemiluminescence.

**Short Interference RNA Transfection**

Short interference RNA targeting Src and control siRNA were obtained from Upstate. VSMCs were transfected using Amaxa nucleofector kit (Amaxa, Cologne, Germany) according to the manufacturer instructions. After 36 hour cells were treated with C-peptide, and KI-67 immunofluorescence staining was performed.

**Figure 1.** C-peptide induces VSMC proliferation A, HASMCs were stimulated with different concentrations of human C-peptide (0.1 to 10 nmol/L) for 24 hour before cell proliferation was assessed by [3H] thymidine incorporation. Scrambled C-peptide (Scr) (10 nmol/L) was used as a negative and PDGF (10 ng/mL) as a positive control. Data are expressed as fold induction of unstimulated cells. Bars represent the mean±SD of 9 independent experiments. *P<0.05 compared with control. B, RASMCs were stimulated with different concentrations of rat C-peptide (0.1 to 1 nmol/L) for 24 hour before cell proliferation was assessed by [3H] thymidine incorporation. PDGF (10 ng/mL) was used as a positive control. Data are expressed as fold induction of unstimulated cells. Bars represent the mean±SD of 7 independent experiments. *P<0.05 compared with control. C, RASMCs were stimulated with rat C-peptide (0.5 nmol/L) or PDGF (10 ng/mL) in the absence or presence of high glucose concentrations (20 mmol/L) for 24 hour before cell proliferation was assessed by [3H] thymidine incorporation. Bars represent the mean±SD of 4 independent experiments. *P<0.05 compared with control.
Phosphatidylinositol Kinase Assay

Cells were stimulated with 0.5 nmol/L of rat C-peptide for different time points. PDGF was used as a positive control and heat-inactivated C-peptide was used to exclude endotoxin effect. Standard PI-3 kinase activity assays were performed using rabbit anti-P85 (Upstate, Lake Placid, NY) antibody.

Statistical Analysis

Results of the experimental studies are reported as mean ± standard error of the mean (SEM). Differences were analyzed by 1-way ANOVA followed by the appropriate post-hoc test. A probability value <0.05 was regarded as significant.

Results

C-Peptide Colocalizes With VSMCs in Early Arteriosclerotic Lesions of Diabetic Subjects

To examine colocalization of C-peptide with medial VSMCs in diabetic subjects, we used postmortem thoracic artery specimens from the PDAY study. As previously described, C-peptide deposition was detectable in the intima of all diabetic subjects included in this recent study. In addition, 8 of these 21 individuals with diabetes exhibited C-peptide deposition in the media, where C-peptide colocalized with VSMCs, as assessed by α-actin staining (supplemental Figure I in the online data supplement available at http://circres.ahajournals.org).

C-Peptide Induces VSMC Proliferation

To assess the effect of C-peptide on VSMC proliferation, [3H] thymidine incorporation assays were performed. Twenty-four hour stimulation of human aortic smooth muscle cells (HASMCs) with human C-peptide increased cell proliferation in a concentration-dependent manner with a maximal 1.8 ± 0.2-fold increase at 0.5 nmol/L C-peptide (P < 0.05 compared with unstimulated cells; n = 7) (Figure 1B). Higher concentrations of C-peptide did not further enhance cell proliferation. Moreover, scrambled C-peptide did not significantly affect RASMC proliferation (data not shown). Stimulation of RASMCs with C-peptide in the presence of high-glucose concentrations (20 mmol/L) (Figure 1C) or treatment of cells for 48 or 72 hour (data not shown) did not alter the mitogenic effect of C-peptide on VSMCs.

In another set of experiments, the effect of C-peptide on RASMC proliferation was evaluated by assessment of Ki-67 expression, an established indicator of cell proliferation. As shown in Figure 2, stimulation of cells with C-peptide for 24 hour significantly enhanced Ki-67 cell positivity, whereas both, heat-inactivated (A) as well as scrambled C-peptide (B) had no such effect. These results in cells from different species as well the different techniques used, support the conclusion that C-peptide exhibits mitogenic activity in VSMCs.

Inhibition of Src-Kinase, PI-3 Kinase, and the MAP-Kinase ERK1/2 Reduce C-Peptide–Induced RASMC Proliferation

To further examine the molecular mechanisms involved in C-peptide–induced RASMC proliferation, we employed inhibitors of Src-kinase, PI-3 kinase, and the MAP-kinase ERK1/2 to block crucial signaling molecules involved in cell proliferation. 30 minutes pretreatment of cells with PP2 or LY294002 (both at 5 μmol/L) significantly reduced C-peptide–induced RASMC proliferation, suggesting the involvement of Src-kinase as well as PI-3 kinase in these signaling pathways. Moreover, 30 minutes pretreatment of cells with PD98059 (5 μmol/L) or U0126 (10 μmol/L), 2 inhibitors of ERK1/2, also significantly diminished RASMC proliferation induced by C-peptide (Figure 3). Similar data were obtained in HASMCs (data not shown). Cell viability was above 95% in all conditions.

Because Src-kinase is the most upstream signaling molecule involved, we performed Src siRNA transfection and assessed Ki-67 positivity after C-peptide treatment. C-peptide stimulation of mock transfected cells significantly increased

![Figure 2](http://circres.ahajournals.org/)
KI-67 positivity whereas transfection of cells with Src siRNA abolished this induction, underscoring the importance of Src in C-peptide induced SMC proliferation (Figure 4).

**C-Peptide Activates Src- and PI-3 Kinase**

Given the inhibitory effect of PP2 on C-peptide–induced VSMC proliferation, we next investigated whether C-peptide activates Src-kinase in VSMCs. As shown in Figure 5, C-peptide induced Src phosphorylation in RASMCs significantly within 1 minute, suggesting that Src-kinase activation is involved in C-peptide–mediated VSMC proliferation.

Previous data have shown that activation of PI-3 kinase is a critical step in VSMC proliferation downstream of Src.17,18 Because treatment of cells with the PI-3 kinase inhibitor LY294002 abolished C-peptide–induced VSMC proliferation, the effect of C-peptide on PI-3 kinase activity was assessed. In RASMCs, C-peptide significantly increased PI-3 kinase activity already after 1 minute (Figure 6A). Heat-inactivated C-peptide had no such effect (data not shown). Pretreatment of cells with the Src kinase inhibitor PP2 significantly reduced C-peptide–induced PI-3 kinase activity, suggesting that Src-kinase acts upstream of PI-3 kinase (Figure 6B).

**C-Peptide Activates ERK1/2 and AKT in VSMCs**

Downstream of PI-3 kinase, ERK1/2 is the major signaling molecule activated eg, in PDGF-induced VSMC proliferation. To investigate whether C-peptide–mediated VSMC proliferation involves similar signaling pathways, we investigated the effect of C-peptide on ERK1/2 phosphorylation. Western blot analysis revealed that C-peptide increased ERK1/2 phosphorylation in RASMCs significantly in a time- and concentration-dependent manner with a maximal effect after 10 minutes at 0.5 nmol/L C-peptide (Figure 7A). To determine whether phosphorylation of Src and activation of PI-3 kinase are crucial upstream signaling pathways associated with C-peptide–induced ERK1/2 phosphorylation, we next examined the effect of Src-kinase inhibitor PP2 and the PI-3 kinase inhibitor LY294002 on ERK1/2 activation after C-peptide–stimulation. Both inhibitors abolished C-peptide–mediated ERK1/2 phosphorylation, suggesting that Src- and PI-3 kinase act upstream of the MAPK ERK1/2 (Figure 7B).

Previous data suggest that ERK1/2 activation may not only occur directly downstream of PI-3 kinase but may also be mediated via AKT.19 Therefore, we examined the effect of C-peptide on AKT activation. C-peptide significantly enhanced AKT phosphorylation in a time- and concentration-dependent manner in RASMCs (supplemental Figure II in the online data supplement).

**C-Peptide Increases Cyclin D1 Expression and Retinoblastoma Protein Phosphorylation**

Cell cycle progression from G1- to S-phase is a critical downstream step in cell proliferation. D-type cyclins, like ERK1/2 phosphorylation in RASMCs significantly in a time-and concentration-dependent manner with a maximal effect after 10 minutes at 0.5 nmol/L C-peptide (Figure 7A). To determine whether phosphorylation of Src and activation of PI-3 kinase are crucial upstream signaling pathways associated with C-peptide–induced ERK1/2 phosphorylation, we next examined the effect of Src-kinase inhibitor PP2 and the PI-3 kinase inhibitor LY294002 on ERK1/2 activation after C-peptide–stimulation. Both inhibitors abolished C-peptide–mediated ERK1/2 phosphorylation, suggesting that Src- and PI-3 kinase act upstream of the MAPK ERK1/2 (Figure 7B).

Previous data suggest that ERK1/2 activation may not only occur directly downstream of PI-3 kinase but may also be mediated via AKT.19 Therefore, we examined the effect of C-peptide on AKT activation. C-peptide significantly enhanced AKT phosphorylation in a time- and concentration-dependent manner in RASMCs (supplemental Figure II in the online data supplement).
C-peptide stimulation of VSMC proliferation in vitro, and the present study demonstrates that C-peptide, a cleavage product of proinsulin, exhibits mitogenic activity on VSMCs by involving the ERK1/2 MAPK pathway downstream of Src- and PI-3 kinase activation. This leads to an increase in cyclin D1 expression and Rb phosphorylation thus dedicating cells into the S-phase of the cell cycle. These data suggest that C-peptide could play a role in VSMC proliferation in atherosclerosis and neo-intima formation after coronary intervention.

For a long time C-peptide was considered to be biologically inert, but increasing data suggest that it may exhibit biological activity in different cell types. As such, C-peptide has been shown to stimulate Na-K-ATPase in renal tubular cells as well as MAPK in Swiss 3T3 fibroblasts, a process requiring protein kinase C and PI-3 kinase activation.8,9 In addition, C-peptide induces monocyte and CD4-positive lymphocyte chemotaxis by activating PI-3 kinase.10,11 The present study extends our knowledge on cellular effects of C-peptide by suggesting a mitogenic effect on VSMCs with activation of various signaling molecules. The effect of C-peptide, inducing HASMC proliferation by 2.5 to 3-fold, is modest in vitro, but the extent of C-peptide’s mitogenic activity is similar to PDGF, an established inducer of VSMC proliferation. In addition, the C-peptide concentrations used are within the range of C-peptide levels found in insulin resistant patients, suggesting that these concentrations may resemble the in vivo situation. Rats exhibit lower C-peptide serum levels, explaining the maximal effects of C-peptide on RASMC proliferation at lower concentrations.

A prior report by Kobayashi et al, demonstrating an inhibition of RASMC proliferation after 3 days of treatment with human C-peptide under high glucose concentrations,15 varied in design and results from ours in important ways. Firstly, our study found the direct mitogenic effect of C-peptide treatment on VSMC proliferation under normal glucose conditions after 24 hour treatment and in our experimental setting, neither treatment of cells with C-peptide in the presence of high glucose concentrations nor stimulation of cells with C-peptide for 48 or 72 hour affected its mitogenic activity on VSMCs. Secondly, Kobayashi and colleagues used human C-peptide in RASMCs whereas we did not stimulate cells with C-peptide across species. Finally, the C-peptide concentrations employed in their study were much higher (up to 100 nmol/L) compared with the concentrations used here. Such different experimental conditions may account for the discrepant results between the 2 studies varied in design and results from ours in important ways.

Our experiments demonstrate that C-peptide stimulation of VSMCs activates Src- and PI-3 kinase and leads to downstream phosphorylation of the MAPK ERK1/2. Previous work has shown that Src-kinase is involved in LDL induced VSMC-proliferation.22 A specific inhibitor of Src-kinase, PP2, as well as transfection of Src siRNA abolished C-peptide induced VSMC proliferation in our experiments, suggesting that C-peptide also signals through this pathway. Similarly, inhibition of PI-3 kinase reduced C-peptide–activated VSMC proliferation. Activation of PI-3 kinase by C-peptide has recently been demonstrated in various cell types including vascular cells like monocytes and CD4-positive lymphocytes, suggesting a critical role of this signaling molecule in C-peptide induced cell activation in different cell types.

It is generally accepted that ERK1/2 activation plays a major role in cell proliferation and differentiation23 with...
particular importance in VSMC proliferation. As such, several in vivo studies have demonstrated a marked increase in ERK1/2 activation after balloon catheter injury in animal arteries and inactivation of this pathway has been shown to limit intimal hyperplasia after vascular intervention.24,25 Like other mitogens, C-peptide-mediated VSMC proliferation also depends on ERK1/2 activation because inhibition of this MAPK pathway with PD98059 or U0126 significantly reduced C-peptide’s effect. Activation of ERK1/2 has been demonstrated to be a direct result of PI-3 kinase activity, but recent data from Stabile et al suggest that ERK1/2 phosphorylation downstream of PI-3 kinase may also occur via AKT.19 In our study, C-peptide also leads to phosphorylation of AKT. Because there is no specific inhibitor of AKT, it remains unclear whether C-peptide-induced ERK activation is mediated via AKT or occurs directly downstream of PI-3 kinase.

The downstream control of VSMC proliferation by extracellular stimuli takes place in mid- to late G1 phase of the cell cycle, where D-type cyclins promote G1 to S-phase transition by leading to Rb phosphorylation.20,21 Our data, showing an increase in cyclin D1 expression as well as Rb phosphorylation suggest that C-peptide acts via similar signaling pathways.

Previous work has shown that C-peptide binds to a cell surface receptor and recent data in renal cells as well as in CD4-positive lymphocytes implicates a pertussis toxin-sensitive G-protein-coupled receptor in mediating C-peptide’s effects.5,6 but hitherto this receptor remains unidentified. Future studies have to identify this receptor and evaluate its role in C-peptide’s mitogenic action on VSMCs as well as the molecular mechanism involved in Scr/PI3K activation by C-peptide.

VSMC proliferation is a crucial process in the development of arteriosclerotic lesions as well as the formation of restenosis after coronary intervention. In a serial intravascular ultrasound study, Kornowski et al have shown that the increased restenosis rate in patients with diabetes mellitus after coronary intervention is because of exaggerated intimal hyperplasia.1 In the intact artery, VSMCs exhibit a low baseline proliferation but they are stimulated to divide after endothelial denudation, leading to the accumulation of growth factors and proinflammatory mediators.26 Among them, C-peptide may contribute to VSMC proliferation in
patients with insulin resistance and early type 2 diabetes mellitus. These patients temporarily present high serum levels of C-peptide and immunohistochemical data have shown that C-peptide deposits in the vessel wall of diabetic patients. In addition to its chemotactic effect on monocytes and CD4-positive lymphocytes, C-peptide could enhance VSMC proliferation in the vasculature, thus promoting both, the development of arteriosclerotic lesions as well as neointima formation after coronary intervention. Still, future studies in animal models have to evaluate the pathophysiological relevance of C-peptide–induced VSMC proliferation in atherogenesis and neointima formation in vivo.

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Disclosures
None.

References
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**Online Figure I:** C-peptide colocalizes with SMCs in early arteriosclerotic lesions of diabetic subjects. A. Representative human thoracic artery section of a diabetic subject shows immunoreactive C-peptide in the intima (stained in red; indicated by arrows) as well as in the media (indicated by arrowheads). C: Adjacent section stained with anti-α-actin antibodies demonstrates SMCs (stained in red; indicated by arrowheads) in the medial area containing C-peptide. B. and D: Adjacent sections to A and C stained with similar concentrations of type and class matched IgG. Magnification 200 x. Specimens from 8 of 21 diabetic subjects yielded similar results.

**Online Figure II:** C-peptide activates AKT in SMCs. RASMCs were stimulated with C-peptide for times (C-peptide at 0.5 nmol/L) and concentrations (5 min stimulation) indicated and phosphorylation of AKT was determined by western blot analysis using specific anti-phospho-AKT antibodies. PDGF (10 ng/mL) was used as a positive control. For loading control membranes were reprobed with antibodies against the non-phosphorylated forms of proteins. Lower panels: Densitometric analyses of p-AKT western blots. Data are expressed as p-AKT normalized to AKT. Bars represent mean±SD. *p<0.05 compared to unstimulated cells; n=6 (time); n=4 (concentration).
Online Figure II

**p-AKT/AKT (fold induction)**

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<th>Co</th>
<th>1</th>
<th>5</th>
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*Significant difference