Possible Role of Parathyroid Hormone–Related Protein as a Proinflammatory Cytokine in Atherosclerosis

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Background and Purpose—Parathyroid hormone–related protein (PTHrP) is a vasodilator peptide. In addition, PTHrP appears to affect vascular growth and to be a mediator of inflammation in rheumatic and brain disorders. We examined the possible role of PTHrP in the inflammatory process in atherosclerosis.

Methods—We immunohistochemically analyzed the cellular localization of PTHrP, the type 1 PTH/PTHrP receptor (PTH1R), and monocyte chemoattractant protein-1 (MCP-1) in 26 human carotid atherosclerotic plaques.

Results—The inflammatory region of plaques was characterized by high PTHrP, PTH1R, and MCP-1 immunostaining in relation to the cap (0.75 ± 0.1 versus 0.29 ± 0.04, 0.5 ± 0.1 versus 0.25 ± 0.05, 0.72 ± 0.2 versus 0.29 ± 0.05, respectively; P < 0.05). PTHrP and MCP-1 were colocalized in both resident and inflammatory cells in the plaque. Moreover, in cultured vascular smooth muscle cells (VSMC), PTHrP(1–36) increased MCP-1 mRNA (3-fold at 6 hours) and MCP-1 protein (2.5-fold at 24 hours). This effect was inhibited by either PTHrP(7–34) or various protein kinase A inhibitors and by the nuclear factor-κB (NF-κB) inhibitor parthenolide. Furthermore, PTHrP(1–36) elicited an increase in NF-κB activation in VSMC. The 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor simvastatin inhibited the PTHrP(1–36) induction of both NF-κB activity and MCP-1 overexpression, and this was reversed by mevalonate.

Conclusions—PTHrP appears to be a novel proinflammatory mediator in the atheroma lesion and may contribute to the instability of carotid atherosclerotic plaques. Our data provide a new rationale to understand the mechanisms involved in the beneficial effects of statins in atherosclerosis. (Stroke. 2003;34:1783-1789.)

Key Words: atherosclerosis ■ carotid arteries ■ inflammation ■ monocyte chemoattractant protein-1 ■ parathyroid hormone–related protein

The pathophysiological aspects of atherosclerosis include an inflammatory process and increased vascular smooth muscle cell (VSMC) growth. While the latter is a key event for vascular occlusion, the inflammatory process has been related to plaque disruption. In this sense, studies on coronary arteries of patients suffering myocardial infarction demonstrated that the rupture of atheroma usually takes place in the shoulder region, an area characterized by a high inflammatory content. A possible explanation was the increased collagenolysis mediated by metalloproteinases (MMPs), whose expression was mostly confined to the shoulder region of plaques. In contrast, the mechanisms by which macrophages accumulate in this region still remain undefined.

Recent clinical trials have established that lipid lowering with 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) reduces the incidence of cardiovascular disease. Some of the beneficial effects of these drugs may involve nonlipid mechanisms because they have been shown to reduce blood thrombogenicity and inflammation in humans. Moreover, C-reactive protein levels decrease after treatment with statins. In this regard, in a rabbit model of atherosclerosis, atorvastatin inhibited the nuclear factor-κB (NF-κB)–dependent increase of monocyte chemoattractant protein-1 (MCP-1) expression, and this effect was associated with a decrease in both macrophage infiltration and neointima formation.

Both parathyroid hormone (PTH)–related protein (PTHrP) and the type 1 PTH/PTHrP receptor (PTH1R) are abundant in the vascular system. Different vasoconstrictors, such as angiotensin II, stimulate PTHrP and the PTH1R expression in rat aortic VSMC. The N-terminal fragment of PTHrP is a potent vasodilator and can inhibit VSMC growth when acting in an autocrine/paracrine fashion. However, PTHrP can also be internalized into the nucleus of VSMC and thus increase their growth.

Therefore, the true role of PTHrP in the vascular system has not yet been established. Recent studies suggest that PTHrP may also act as a proinflammatory cytokine in some clinical settings. PTHrP overexpression occurs in human and experimental atherosclerotic lesions related to the sever-
ity of the disease. However, the putative role of PTHrP in the pathogenesis of atherosclerosis remains unclear. In the present study we examined whether PTHrP might be involved in the inflammatory process associated with atherosclerosis.

Methods

In Vivo Studies

Tissue Sampling

Twenty-six consecutive patients undergoing carotid endarterectomy at our institution were included in the study, and informed consent was obtained before enrollment (Table, available online at http://stroke.ahajournals.org). The study was approved by the local ethical committee in accordance with the institutional guidelines. For analysis, we selected the carotid artery with its bifurcation, the predilection site for plaque formation. We studied carotid atherosclerotic plaques in 2 different areas (shoulder and cap). The shoulder region was composed of the plaque area at both sides of the lipid core, and the fibrous cap was the rim over atheroma. Specimens were collected and stored in 4\% formaldehyde for 24 hours and then in ethanol until paraffin embedding.

Immunohistochemistry

Paraffin-embedded carotid arteries were cross-sectioned into 4-μm-thick pieces at 5-mm intervals and then were dewaxed and rehydrated. Tissue samples were incubated with trypsin (0.01\%) and then incubated with 6\% swine (or goat) serum/4\% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 hour to block nonspecific staining. The following primary antibodies were used: monoclonal anti-human macrophage antibody HAM-56 (Dako), monoclonal anti-α-smooth muscle actin antibody HHF-35 (Sigma), rabbit anti-human CD3 antibody (Dako), and a polyclonal rabbit anti-human MCP-1 (Immugenex), at 1:100 dilution in BSA/PBS. PTHrP and PTH1R staining was performed with either the rabbit polyclonal anti-PTHrP antiserum C13 recognizing the (24–35) epitope in the PTHrP molecule or affinity-purified antibody Ab-VII (Babco) at 1:200 dilution in BSA/PBS. After overnight incubations, biotinylated swine or goat anti-rabbit IgG, at 1:200 dilution, was added for 1 hour. The avidin-biotin-peroxidase complex (Dako) was added for an additional 30-minute period. Sections were then stained for 10 minutes with 3,3′-diaminobenzidine (Dako), counterstained with hematoxylin, and mounted in Pertex (Medite). For colocalization studies, after immunohistochemistry was performed for macrophages and VSMC, immunofluorescence for PTHrP was performed on the same tissue sections. As secondary antibody, fluorescein isothiocyanate–conjugated goat anti-rabbit IgG was used, and slides were mounted in 90\% glycerol in PBS. In each experiment, negative controls either without the primary antibody or with the corresponding IgG were included to check for nonspecific staining. In addition, for PTHrP, we also used preincubation of the primary antibody C13 with [Cys\textsuperscript{23}]human PTHrP(24–25) amide, the immunogen used to raise this antibody.

Figure 1. PTHrP, PTH1R, and MCP-1 immunodetection in human atherosclerotic plaques. Positivity for PTHrP and PTH1R is increased in the shoulder region (B, D), an area in the plaque with a high inflammatory content, related to the cap (A, C). MCP-1 positivity was also increased in the shoulder region (F) compared with the cap (E). G, Schematic representation of a human carotid plaque, showing the atheroma region by 1 asterisk and the cap region by 2 asterisks. The shoulder region is shown by 2 triangles.
Immunoprecipitation and Detection of Rat MCP-1

Cells were washed with PBS buffer containing 400 mmol/L sodium orthovanadate (Na$_3$VO$_4$) and 10 mmol/L NaF. This reaction was stopped by the addition of 200 μL of lysis buffer (1% Igepal; 50 mmol/L HEPES, pH 7.5; 100 mmol/L NaCl; 2 mmol/L EDTA; 1 mmol/L pyrophosphate; 10 mmol/L Na$_3$VO$_4$; 1 mmol/L phenylmethylsulfonyl fluoride; and 100 mmol/L NaF). For MCP-1 immunoprecipitation, cell lysates (400 μg of whole protein) were incubated with 1 μg of rabbit polyclonal anti-rat MCP-1 (Ab7202, Abcam) overnight at 4°C in lysis buffer. Then 50 μL of protein A Sepharose beads (Pharmacia Biotech) were added to the lysate for 4 hours at 4°C. After they were washed with lysis buffer (×3) and with kinase buffer (20 mmol/L HEPES, pH 7.6; 20 mmol/L MgCl$_2$; 20 mmol/L β-glycerophosphate; 10 mmol/L NaF; 0.2 mmol/L Na$_3$VO$_4$; 0.2 mmol/L dithiothreitol) (×2), Sepharose beads were resuspended in sodium dodecyl sulfate–polyacrylamide gel electrophoresis sample buffer, boiled for 5 minutes, and subjected to electrophoresis. The detection was made with the use of the anti-rat MCP-1 antibody and enhanced chemiluminescence (ECL, Amersham), as described.$^{15}$

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assay for NF-κB binding activity was performed with protein extracts from VSMC as described.$^{19}$ The specificity of the assay was tested with a 100-fold excess of unlabeled NF-κB consensus oligonucleotide added to the $^{32}$P-labeled probe-binding reaction.

Statistical Analysis

Statistical analysis was performed with GraphPAD InStat software. Immunohistochemistry and Northern blot analysis data are mean±SEM and were analyzed by either Mann-Whitney or ANOVA test when appropriate. Significant differences were considered for $P<0.05$.

Results

PTHRP, PTH1R, and MCP-1 Immunostaining in Human Atherosclerotic Plaques

We found that human atherosclerotic plaques contain higher macrophage and T-cell infiltration and lower VSMC in the shoulder region than in the cap (not shown). This is consistent with the presence of a high inflammatory content in the former region.$^2$ However, there were no significant differences in total cell positive staining for both macrophages and VSMC when these 2 regions were compared. Immunostaining for PTHrP, PTH1R, and MCP-1 was significantly higher in the shoulder region than in the cap (0.75±0.1 versus 0.29±0.04, 0.5±0.1 versus 0.25±0.05, 0.72±0.2 versus 0.29±0.05, respectively;
To ensure the specificity of the technique, we performed negative controls by omitting the corresponding primary antibodies or using the corresponding IgG. In addition, for PTHrP, we also used preincubation of the primary antibody C13 with [Cys23]human PTHrP(24-25) amide, the immunogen used to raise this antibody, and there was no staining in any of the cases (not shown).

In addition, we performed a double-staining procedure, using immunoperoxidase/immunofluorescence, to determine the cell type(s) contributing to PTHrP overexpression in human atherosclerotic plaques. By this manner, PTHrP staining was detected in both VSMC (Figure 2A and 2B) and macrophages (Figure 2C and 2D). Moreover, immunostaining for PTHrP and MCP-1 in serial tissue sections showed the presence of both proteins in the same cells (Figure 2E and 2F). Taken together, these results suggest that both PTHrP and MCP-1 are likely to be involved in the inflammatory process in the vulnerable region of human atheroma.

In Vitro Studies

**PTHrP(1–36) Stimulates MCP-1 Expression in VSMC**

Since MCP-1 and PTHrP were colocalized in human atherosclerotic plaques, we explored the potential proinflammatory effect of PTHrP in cultured VSMC. PTHrP(1–36) at 10^{-8} mol/L increased MCP-1 mRNA (with a maximal stimulation at 6 hours, representing 3-fold over control) and MCP-1 protein (approximately 2.5-fold over control at 24 hours) (Figure 3). These results suggest that PTHrP may be a novel mediator involved in the recruitment of mononuclear cells into the atheroma lesion through the induction of MCP-1.

**Mechanisms Involved in PTHrP(1–36)-Induced MCP-1 Expression in VSMC**

In the next set of experiments, we analyzed the possible mechanisms involved in MCP-1 mRNA induction by PTHrP(1–36). Since this peptide can stimulate cAMP in VSMC, we initially tested the effect of protein kinase A (PKA) inhibitors. Both RpcAMPS and H89, at 5×10^{-5} and 10^{-7} mol/L, respectively, prevented the PTHrP-induced MCP-1 gene expression at 6 hours in these cells (Figure 4). Moreover, pretreatment with PTHrP(7–34), at 10^{-6} mol/L, which stimulates protein kinase C but not PKA by interacting with the PTH1R, abrogated the PTHrP(1–36)-induced MCP-1 mRNA, while it was inefficient by itself (Figure 4).

NF-κB is a key regulatory factor of MCP-1 gene expression. We found that the NF-κB inhibitor parthenolide (10^{-5} mol/L) abolished the increase of MCP-1 mRNA induced by PTHrP(1–36) (10^{-5} mol/L) in VSMC (Figure 4). Thus, we...
examined whether PTHrP would have a direct effect on NF-κB activation in VSMC, as occurs in osteoblastic cells. PTHrP(1–36) (10⁻⁸ mol/L) was shown to induce an increase in NF-κB activation in a time-dependent manner (Figure 5A). This effect was specific since a 100-fold excess of unlabeled NF-κB oligonucleotide probe abolished such effect.

**Figure 5.** Effect of PTHrP on NF-κB activation in VSMC. A, VSMC stimulated with PTHrP(1–36) (10⁻⁸ mol/L) for different time periods induced NF-κB activation with a maximal effect at 90 minutes. The 100-fold excess of cold NF-κB oligonucleotide abolished such effect. B, Pretreatment with simvastatin (SV) (10⁻⁶ mol/L) diminished NF-κB activation at 90 minutes, and this effect was reversed by mevalonate (MVA) (10⁻⁴ mol/L). A representative electrophoretic mobility shift assay from 3 independent experiments is shown.

Effect of the HMG-CoA Reductase Inhibitor Simvastatin on PTHrP(1-36)-Induced MCP-1 mRNA

Since statins can decrease both MCP-1 upregulation and NF-κB activation both in vitro and in vivo, we explored whether simvastatin could also downregulate the effect of PTHrP(1–36) on MCP-1 mRNA and NF-κB activation in VSMC. We found that pretreatment with simvastatin, within the therapeutic range (10⁻⁶ to 10⁻⁷ mol/L), inhibited the PTHrP-induced MCP-1 mRNA overexpression. When VSMC were treated with PTHrP and simvastatin in the presence of increasing concentrations of simvastatin (10⁻⁶ to 10⁻⁷ mol/L), with or without mevalonate (MVA) (10⁻⁴ mol/L), Northern blots corresponding to MCP-1 and GADPH mRNA and relative densitometric values from 3 independent experiments are shown. *P<0.05 vs PTHrP-stimulated value.

**Figure 6.** Effect of simvastatin (SV) on the PTHrP-induced increase of MCP-1 mRNA in VSMC. VSMC were incubated with PTHrP(1-36) (10⁻⁸ mol/L) for 6 hours in the presence of increasing concentrations of simvastatin (10⁻⁶ to 10⁻⁷ mol/L), with or without mevalonate (MVA) (10⁻⁴ mol/L). Northern blots corresponding to MCP-1 and GADPH mRNA and relative densitometric values from 3 independent experiments are shown. *P<0.05 vs PTHrP-stimulated value. LPS indicates lipopolysaccharide.
presence of mevalonate (10^{-6} mol/L), the metabolite that is directly synthesized by the HMG-CoA reductase, these effects were reversed (Figure 6). Moreover, simvastatin (10^{-8} mol/L) diminished NF-κB activation, and this effect was also reversed by mevalonate (Figure 5B). These results suggest that statins could downregulate MCP-1 expression, at least in part, by interfering with the effect of PTHrP in VSMC.

**Discussion**

In recent years, PTHrP has gained increasing interest because of its diverse actions in the cardiovascular system. PTHrP gene is overexpressed in rat and human vessels during neointimal formation, and intensity of PTHrP staining in VSMC has been shown to correlate with the severity of coronary atherosclerosis. These findings raise the possibility that PTHrP may function, in a stimulatory or contributory manner, in the pathogenesis of arterial sclerosis and restenosis. The N-terminal region of PTHrP has been shown to inhibit migration and proliferation of VSMC both in vitro and in vivo in atherosclerotic lesions. Consistent with these findings, in the present study both PTHrP and the PTH1R staining were increased in the more vulnerable area in the human plaque, containing a lower VSMC number. In marked contrast, PTHrP(+/-) stably transfected into A10 rat VSMC induced marked cell growth, and fetal aortic VSMC from PTHrP(-/-) mice showed a decreased proliferation rate. The mechanism responsible for this proliferative effect involves PTHrP targeting to the nucleus. Interestingly, a recent study has shown that these opposite effects of PTHrP on VSMC proliferation are reversed in spontaneously hypertensive rats. Collectively, these data strongly suggest that PTHrP may participate in the altered mechanisms of VSMC growth in vascular pathology. However, the specific impact of this suggested role of PTHrP in the atherosclerotic process remains to be elucidated.

Previous studies suggest the view of PTHrP as a member of the cytokine network involved in the inflammatory response in rheumatic and brain disorders. Inflammation is involved in the genesis, rupture, and thrombosis of atherosclerotic plaques. The breakdown of the plaque occurs more frequently at points where the fibrous cap is thinner and where there is a great amount of inflammatory cells such as macrophages and T lymphocytes. A possible explanation was the increased collagenolysis mediated by MMP, whose effects were reversed (Figure 6). Moreover, simvastatin (10^{-8} mol/L) diminished NF-κB activation, and this effect was also reversed by mevalonate (Figure 5B). These results suggest that statins could downregulate MCP-1 expression, at least in part, by interfering with the effect of PTHrP in VSMC.

The N-terminal fragment of PTHrP signals by increasing the production of cAMP in VSMC. We showed herein that PKA inhibitors as well as PTHrP(7–34), which lacks cAMP-inducing activity, blocked the effect of PTHrP(1–36) on MCP-1 overexpression in VSMC. In this regard, MCP-1 upregulation by leptin in aortic endothelial cells has recently been reported to depend on PKA activation. In addition, our findings indicate that NF-κB activation also seems to be involved in the PTHrP(1–36)-induced increase of MCP-1 mRNA in VSMC. In fact, in a preliminary report, it was shown that the shoulder region of human carotid atherosclerotic plaques, displaying an intense PTHrP staining as shown herein, has augmented NF-κB activity. Collectively, these findings support the involvement of both PKA and NF-κB in the signaling mechanism of MCP-1 upregulation by PTHrP(1–36) in VSMC.

HMG-CoA reductase inhibitors decrease the incidence of acute coronary events. Previous studies have demonstrated that these drugs decrease the extent of atherosclerosis in experimental models without hyperlipidemia and in the absence of lipid reduction. In a rabbit model of atherosclerosis, atorvastatin decreased NF-κB activity in femoral arteries, coinciding with a decrease in MCP-1 expression, macrophage content, and lesion size. Moreover, atorvastatin inhibited NF-κB activity and MCP-1 mRNA elicited by tumor necrosis factor-α and angiotensin II in VSMC and mononuclear cells in vitro. In the present study simvastatin prevented the effect of PTHrP(1–36) on both NF-κB activation and MCP-1 mRNA overexpression in cultured VSMC. These results provide a new potential mechanism by which statins could exert their known anti-inflammatory properties and then contribute to plaque stabilization. In conclusion, our findings suggest that PTHrP may be an important regulator of some events involved in atherosclerotic plaque formation, such as macrophage accumulation. Our data provide a new rationale to understand the mechanisms involved in the beneficial effects of statins in atherosclerosis.

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