Parathyroid hormone stimulates endothelial expression of atherosclerotic parameters through protein kinase pathways

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Rashid G, Bernheim J, Green J, Benchetrit S. Parathyroid hormone stimulates endothelial expression of atherosclerotic parameters through protein kinase pathways. Am J Physiol Renal Physiol 292: F1215–F1218, 2007. First published December 26, 2006; doi:10.1152/ajprenal.00406.2006.—Parathyroid hormone (PTH), the major systemic calcium-regulating hormone, has been linked to uremic vascular changes. Considering the possible deleterious action of PTH on vascular structures, it seemed logical to evaluate the impact of PTH on the receptor of advanced glycation end products (RAGE) and interleukin 6 (IL-6) mRNA and protein expression, taking into account that such parameters might be involved in the pathogenesis of vascular calcification, atherosclerosis, and/or arteriolosclerosis. Human umbilical vein cord endothelial cells (HUVEC) were stimulated for 24 h with 10⁻¹¹–10⁻¹⁰ mol/l PTH. The mRNA expression of RAGE and IL-6 was established by reverse transcriptase/PCR techniques. RAGE protein levels were determined by Western blot and IL-6 secretion was measured by ELISA. The pathways by which PTH may have an effect on HUVEC functions were evaluated. PTH (10⁻¹¹–10⁻¹⁰ mol/l) significantly increased RAGE mRNA and protein expression. PTH also significantly increased IL-6 mRNA expression without changes at protein levels. The addition of protein kinase (PKC or PKA) inhibitors or nitric oxide (NO) synthase inhibitors significantly reduced the RAGE and IL-6 mRNA expression and the RAGE protein expression. PTH stimulates the mRNA expressions of RAGE and IL-6 and the protein expression of RAGE. These stimulatory effects are probably through PKC and PKA pathways and are also NO dependent. Such data may explain the possible impact of PTH on the atherosclerotic and arteriosclerotic progression.

Advanced glycation end products (AGEs) are involved in the development of atherosclerosis and in the occurrence of uremic, ageing, and diabetic vascular disease (11, 15, 27). In uremia, the blood levels of AGEs are elevated (10) and endothelial RAGE is overexpressed (4). RAGE mediates the binding of AGEs to endothelial and mononuclear phagocytes and this stimulates the cell activities (20, 21).

IL-6 is considered to be one of the main mediators of inflammation as reflected by an enhanced production of fibrinogen and C-reactive protein in the liver and strongly affects the inflammatory process involved in the development of atherosclerosis through the stimulation of acute phase protein synthesis (3, 5, 25). On the basis of these data, we evaluated the possible action of PTH on gene and protein expression of RAGE and IL-6.

MATERIALS AND METHODS

Endothelial cell culture and incubation. Endothelial cell cultures were obtained from umbilical cords as previously described (16). Ethics Review Committee approved the study and the parturient gave written informed consent. Only umbilical cords from women who had a normal pregnancy and birth were used. Cultured cells were identified as endothelial by their morphology and the presence of von Willebrand factor. Confluent cultures of human umbilical vein cord endothelial cells (HUVEC) used for experiments at passages 2–4 were incubated with different concentrations of PTH (fragment 1-34, 10⁻¹²–10⁻¹⁰ mol/l, equivalent to 4.1–410 pg/ml, respectively, Sigma) for 24–72 h. Each experiment included all the controls and experiment groups that were investigated.

The pathways by which PTH may have an effect on HUVEC functions were evaluated on cells pretreated for 30 min with protein kinase C (PKC) inhibitor (calphostin C, 50 nmol/l, Sigma) and/or cAMP antagonist (Rp-cAMP, 10 μmol/l, Sigma). Calphostin C inhibits PKC activity by binding to the regulatory domain of PKC (8). Rp-cAMP is a diastereomer of cAMP that competitively binds to the regulatory subunit of PKA to prevent cAMP-induced dissociation and activation of the enzyme (19). A possible involvement of nitric oxide (NO) in the PTH-induced gene expression of HUVEC was evaluated by a pretreatment with 200 μmol Nω-nitro-l-arginine methyl ester (l-NAME; NOS inhibitor).

RT-PCR. Expression of the RAGE and IL-6 genes was performed by semiquantitative multiplex RT-PCR and real-time PCR techniques. Total RNA was extracted from endothelial cells using the PUREscript RNA isolation kit (Gentra Systems), according to the manufacturer’s instructions. RNA (1 μg) was then reverse transcribed into single-strand DNA with 200 U of SUPERSCRIPT II RNase Reverse Transcriptase (Invitrogen) and oligo (dT)₁₅ primer (Promega, Madison, WI) at 37°C for 45 min, 42°C for 15 min, and 99°C for 5 min.

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Conventional RT-PCR. Semiquantitative multiplex RT-PCR amplification was performed on 1/10th of the cDNA solution with 0.5 U of Taq DNA polymerase (Sigma) at a final volume of 50 μl. The PCR conditions and primers sequence were as follows: for RAGE mRNA amplification: forward primer: 5'-CACCTTTCTCTGTAGCTTCA-3', reverse primer: 5'-TGGAACCGTGACTCTC-3'; generating a 480 bp PCR product. For IL-6 mRNA amplification: forward primer: 5'-GGTACATCCTCGACCGATCTC-3', reverse primer: 5'-GGTACATCCTCGACCGATCTC-3'; generating a 334-bp PCR product. β-Actin primers sequence (for semiquantitative RT-PCR of RAGE): forward primer: 5'-GAGACCTTCAACACCCCAGC-3', reverse primer: 5'-GAGACCTTCAACACCCCAGC-3', generating a 388-bp PCR product. β-Actin primers sequence (for semiquantitative RT-PCR of IL-6): forward primer: 5'-GGTTACATCCTCGACCGATCTC-3', reverse primer: 5'-GGTTACATCCTCGACCGATCTC-3', generating a 274-bp PCR product. PCR program for IL-6: 30 cycles of 94°C for 30 s, 58°C for 40 s, and 72°C for 30 s. PCR program for RAGE: 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s. All primers were chosen to be complementary to domains in different exons to avoid false-positives caused by DNA contamination of the RNA preparations. RT PCR products were separated on 1.5% agarose (Sigma).

Real-time RT-PCR. To quantify the amounts of RAGE and IL-6 mRNA expression in endothelial cells, real-time RT-PCR was performed with a Light Cycler instrument (Roche Diagnostics GmbH, Mannheim, Germany) in glass capillary tubes. The Light Cycler Fast Start DNA Master SYBR Green I reaction mix (Roche Diagnostics GmbH) and primers were added to cDNA dilutions. Primers for human IL-6 and β-actin were the same as conventional PCR. RAGE primers were: forward primer: 5'-GGTAACGCCATACCCGCTC-3', reverse primer: 5'-GGGAACCTTCAACACCCCAGC-3', generating a 334-bp PCR product. Total RNA was extracted and the levels of the genes mRNA expression were assessed by real-time PCR. RAGE mRNA expression were assessed by real-time PCR. RAGE mRNA expression was determined with a Light Cycler instrument (Roche Diagnostics GmbH, Mannheim, Germany) in glass capillary tubes. The Light Cycler Fast Start DNA Master SYBR Green I reaction mix (Roche Diagnostics GmbH) and primers were added to cDNA dilutions. Primers for human IL-6 and β-actin were the same as conventional PCR. RAGE primers were: forward primer: 5'-GGGAACCTTCAACACCCCAGC-3', reverse primer: 5'-GGGAACCTTCAACACCCCAGC-3', generating a 334-bp PCR product.

Table 1. Effect of PTH on the mRNA expression of RAGE and IL-6 by HUVEC: densitometric analysis

<table>
<thead>
<tr>
<th>PTH (mol/l)</th>
<th>Conventional PCR</th>
<th>Real-time PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RAGE</td>
<td>IL-6</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>10^-12</td>
<td>1.32±0.06*</td>
<td>1.1±0.06</td>
</tr>
<tr>
<td>10^-11</td>
<td>1.75±0.11*</td>
<td>1.3±0.07*</td>
</tr>
<tr>
<td>10^-10</td>
<td>1.78±0.25*</td>
<td>1.45±0.01*</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE of 6 independent experiments. Two-tailed Student’s paired t-test was used for data analysis. P values of <0.05 were considered significant.

RESULTS

PTH and RAGE mRNA expression. PTH (10^-12–10^-10 mol/l) significantly increased the RAGE mRNA expression after 24-h incubation (Fig. 1 and Table 1). HUVEC, pretreated with calphostin C (50 μmol/l) and/or Rp-cAMP (10 μmol/l) before PTH stimulation, significantly reduced the expression of RAGE mRNA expression [calphostin C: 30.7 ± 7.8%, P = 0.0001; Rp-cAMP: 45 ± 11.1%, P = 0.003 vs. control (PTH)] (Fig. 2). The combined inhibition with calphostin C and Rp-cAMP did not further modify the RAGE mRNA expression (36 ± 10.6%). Interestingly, the PTH-induced RAGE mRNA expression in the cells pretreated with 200 μmol l-NAME was inhibited [l-NAME: 46 ± 14.4% vs. control (PTH), P = 0.03; Fig. 2]. This interaction between NO and RAGE presently confirmed in endothelial cells has not been previously recorded in the literature.

![Fig. 1. Effect of parathyroid hormone (PTH) on receptor of advanced glycation end products (RAGE) mRNA expression by human umbilical vein cord endothelial cells (HUVEC). HUVEC were incubated for 24 h with increasing concentrations of PTH (10^-12–10^-10 mol/l). Total RNA was extracted and the level of RAGE and β-actin mRNA expression was assessed by semiquantitative PCR. Results of representative RT-PCR; similar results were obtained in 6 independent experiments.](http://ajprenal.physiology.org/)
**DISCUSSION**

The present data demonstrate that PTH affects the endothelial gene expression of RAGE and IL-6 and protein levels of RAGE and that both PKA and PKC pathways are involved.

The development of vascular atherosclerosis, arteriosclerosis, and/or calcification in the presence of elevated PTH has been related partially to an increased production (and reorganization) of collagen by VSMC (12). We found that PTH could stimulate the mRNA expression of RAGE and IL-6 and the protein expression of RAGE. PTH did not affect IL-6 secretion in HUVEC. Its impact on RAGE, which is upregulated in diabetes and uremia and is associated with higher risk of vasculopathy and atherosclerosis (21, 26, 28) and on IL-6, which is one of the main inflammatory mediators involved in the atherosclerotic disease (25), fits well with the concept that PTH may be considered an active actor in accelerating vascular atherosclerotic processes. Incubation with PKC or PKA inhibitors in the presence of PTH was associated with a reduction of RAGE mRNA and protein expression and IL-6 mRNA expression to a level equivalent to that found in nontreated cells showing that both PKC and PKA pathways may be implicated in this expression. The possible role of NO in the stimulation of RAGE and IL-6 expression induced by PTH was also estimated. Pretreatment with L-NAME inhibited RAGE and IL-6 mRNA expression and RAGE protein levels, suggesting that the elevation of RAGE and IL-6 mRNA expression could be NO dependent. As we recently demonstrated that PTH activates the eNOS system (17), it is conceivable that the
parallel effects of PTH on the eNOS and RAGE and IL-6 may be interrelated.

It is well known that PTH may activate either adenylate cyclase, and subsequently PKA, or phospholipase C/PKC pathways (14, 24). In classical target cells (chondrocytes, osteoblasts, osteoclasts, and kidney-derived cells), PTH activates both pathways. In smooth muscle cells, PTH activates only the adenylate cyclase pathway (22) and may exert its vasorelaxant action via cAMP-dependent inhibition of VSMC L-type Ca$^{2+}$ channel (13, 23). Our results could demonstrate that both pathways seem to be involved in PTH-related endothelial cell activation. In addition, PTH may be considered as a relevant actor in vascular remodeling processes by its stimulating action on NO release, which affects RAGE or IL-6 endothelial expression.

**GRANTS**

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**REFERENCES**


