Parathyroid hormone decreases endothelial osteoprotegerin secretion: role of protein kinase A and C

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Submitted 31 December 2007; accepted in final form 14 October 2008

Parathyroid hormone decreases endothelial osteoprotegerin secretion: role of protein kinase A and C. Am J Physiol Renal Physiol 296: F60–F66, 2009. First published October 22, 2008; doi:10.1152/ajprenal.00622.2007.—Parathyroid hormone (PTH), which is elevated in patients with chronic renal failure, has been shown to participate in the development of vascular calcification. Previous studies have demonstrated that PTH may promote endothelial expressions of proinflammatory parameters. On the basis of these data, we evaluated whether PTH may have an impact on endothelial osteoprotegerin (OPG), a vascular-protective factor which may control vascular calcification. Endothelial cells were stimulated with 10−12 to 10−10 mol/l PTH. PKC and PKA are the main cellular pathways of PTH. Inhibitors and activators of PKC or PKA were used to determine whether these signaling pathways are involved in the control of endothelial OPG. PTH induced a decrease in OPG secretion and mRNA expression. Treatment of PTH-stimulated cells by calphostin C (PKC inhibitor) induced a further decrease in OPG secretion, while Rp-cAMP (PKA inhibitor) had no additional effect. In nonstimulated cells, a PKC activator significantly stimulated OPG secretion, while a PKA activator was associated with a decline. These effects were blunted in the presence of calphostin C and Rp-cAMP, respectively. An increase in OPG secretion induced by a PKC activator indicates that the basal OPG secretion is mediated through PKC. The decrease induced by a PKA activator, which is similar to the decrease observed with PTH, suggests that the action of PTH on OPG secretion and mRNA expression may be due to the PKA pathway.

cardiovascular (CV) diseases are the leading cause of death in dialyzed patients (11), the majority of whom have an increased severity of coronary artery and peripheral vascular calcifications and more frequent CV morbidity and mortality (19, 22, 37).

Chronic excess of parathyroid hormone (PTH) has been implicated in the development of vascular calcification (24), hypertension, and uremic vasculopathy (with diffuse atherosclerosis), particularly in patients with end-stage renal failure (30). Basically, the main role of PTH is to maintain an adequate calcium-phosphorus homeostasis through its effect on bone and kidney (3), but it may also affect the function of other target organs and cells (2).

Osteoprotegerin (OPG), a member of the TNF receptor superfamily, is considered to function as a soluble decoy receptor by binding the receptor activator of nuclear factor-κB ligand (RANKL), competitively inhibiting the RANKL-receptor interaction (10, 14, 41, 44). OPG therefore acts as a secreted decoy receptor to negatively regulate osteoclasts differentiation, activity, and survival in vivo and in vitro (39, 45). OPG has been found not only to regulate bone resorption but also to serve as a suppressive signal of local vascular calcification (5). It has been shown that OPG-deficient mice develop severe osteoporosis and medial arterial calcification of the aorta and renal arteries (5) and that the development of osteoporosis and arterial calcification was completely prevented by restoration of the gene (21). Recent reports have shown that OPG administration prevents vascular calcification induced by warfarin and vitamin D (26) and in LDL−/− mice receiving an atherogenic diet (23). OPG also protects endothelial cells from apoptosis (20) and represents as a protective factor for the vascular system (35). These findings suggest that OPG may have a protective role in the vasculature. In hemodialyzed patients with vascular calcification and more severe coronary artery disease, the blood levels of OPG are elevated (13, 15, 25, 36), a finding which may be seen as a paradox as the increase in OPG can be considered to be a vascular-protective agent in blunting calcification (5, 10). Such a paradox may be strengthened by the fact that an elevated risk of uremic CV disease and CV morbidity and mortality is recognized in patients with high OPG (16, 43) and that OPG is associated with a higher incidence of arterial calcification, a component of atherosclerotic lesions (4, 8). The picture emerging from these previous studies is quite complex: although basal production of OPG by vascular smooth muscle and endothelial cells is essential to protect the wall against calcification and may act as a protective factor for calcification-related vascular diseases, an increased release of OPG correlates with an increase in cardiovascular morbidity and mortality, such as in diabetes mellitus or atherosclerosis (15, 38). As inflammatory mediators stimulate the production of OPG (6) and uremia is a state of chronic inflammation (40), an increase in OPG blood levels as found in patients with chronic renal failure (CRF) (13, 25) may be related to the prolonged inflammatory situation in association with more severe atherosclerosis. In addition, the high blood levels of PTH found in CRF are considered to play a role in the development of vascular calcification (24). Can we expect that PTH will affect OPG production? Considering that in vitro studies have shown that PTH stimulates the expressions of proatherosclerotic and proinflammatory markers such as the receptor of advanced glycation end products (RAGE) and IL-6 in endothelial cells (28) and may affect in vivo the vascular structures, the purpose of the present study was to determine
whether PTH affects the endothelial secretion and mRNA expression of a putative protective agent such as OPG. In addition, we investigated the signaling pathways involved in PTH-induced cellular activity (PKC and/or PKA pathways) (28, 29, 34).

METHODS

Endothelial cell culture and incubation. Cultures of human umbilical vein endothelial cells (HUVEC) were obtained from umbilical cords as previously described (28). The Meir Medical Center Ethics Review Committee approved the protocol of the study, and the parturients gave written informed consent. Only umbilical cords from women who had a normal pregnancy and birth were used. Endothelial cells were identified by their cobblestone appearance and by the presence of von Willebrand factor and CD31. Confluent cultures of HUVEC used for experiments at passages 2–4 were incubated for 24 h with three different concentrations of human PTH [fragment 1–34 (10^{-12} to 10^{-10} mol/l, corresponding to 4.1, 41, and 410 pg/ml, physiological and pathophysiological concentrations encountered in clinical medicine), Sigma]. Each experiment included all controls and experimental groups investigated. PKC and/or PKA activators and inhibitors were used to examine the signaling pathways involved in OPG secretion. HUVEC were pretreated for 30 min with a PKC inhibitor (calphostin C, 50 nmol/l, Sigma) or PKA-cAMP antagonist (Rp-cAMP, 10 μmol/l, Sigma). The cells were then treated for 6–24 h with different concentrations of PMA (5–50 nmol/l, Sigma), a potent activator of PKC (18) or forskolin (10^{-7} to 10^{-4} mol/l, Sigma), an activator of cAMP (7). Calphostin C inhibits PKC activity by binding to the regulatory domain of PKC (17). Rp-cAMP is a diasteromer of cAMP that competitively binds to the regulatory subunit of PKA to prevent cAMP-induced dissociation and activation of the enzyme (31).

OPG immunoassay. OPG in HUVEC supernatants was measured using a DuoSet ELISA development kit (R&D Systems).

RT-PCR. Expressions of the OPG and β-actin genes were 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-stranded DNA with 200 units 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.

Conventional RT-PCR. Semiquantitative multiplex RT-PCR amplification was performed on one-tenth of the cDNA solution with 0.5 units of Taq DNA polymerase (Sigma) at a final volume of 50 μl. The PCR conditions and primer sequences were as follows: for OPG mRNA amplification, forward 5’-GCTGTTCCTACAAAGTTTACG-3’.

Fig. 1. Effect of parathyroid hormone (PTH) on osteoprotegerin (OPG) protein secretion. Human umbilical vein endothelial cells (HUVEC) were incubated for 24 h with increasing concentrations of PTH (10^{-12} to 10^{-10} mol/l). OPG in supernatants was measured by ELISA. Values are means ± SE of 8 independent experiments and are expressed as percentage of control (without treatment: 100% = 325 ± 90 pg/ml). *P < 0.05 vs. control. #P < 0.02 vs. 10^{-10} mol/l PTH.

Fig. 2. Effect of PTH on OPG mRNA expression. HUVEC were incubated for 24 h with increasing concentrations of PTH (10^{-12} to 10^{-10} mol/l). Total RNA was extracted, and the level of OPG and β-actin mRNA expression was assessed by semiquantitative and real-time PCR. A: results of representative RT-PCR. Similar results were obtained in 4 independent experiments. B: OPG mRNA levels normalized to the levels of β-actin mRNA expression and relative mRNA content was expressed as percentage of control (0). C: real-time PCR. Values are means ± SE of 4 independent experiments. *P ≤ 0.02 vs. control (0). #P ≤ 0.02 vs. 10^{-11} or 10^{-12} mol/l PTH.
3’, reverse 5’-CTTTGAGTGCTTTAGTGCGTG-3’, generating a 432-bp PCR product; for β-actin, primer sequences were forward 5’-GACCACACCTTCTACAATGAG-3’ and reverse 5’-GCATAACCCCTCGTAGATGGG-3’, generating a 274-bp PCR product. The PCR program for OPG was 30 cycles of 94°C, for 30 s, 60.6°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 OPG mRNA expression in endothelial cells, real-time RT-PCR was performed with a Light Cycler instrument (Roche Diagnostics, Mannheim, Germany) in glass capillary tubes. The Light Cycler Fast Start DNA Master SYBR Green I reaction mix (Roche Diagnostics) and primers were added to cDNA dilutions. Primers for human OPG and β-actin were the same as for conventional PCR. The thermal profile for SYBER Green PCRs was 95°C for 10 min, followed by 35 cycles of 95°C for 10 s, 58°C for 7 s, 72°C for 16 s, and 83°C for 5 s. To prove the specificity of the PCR product, a melting curve analysis was performed by 95°C for 5 s, 70°C for 20 s. A dilution series of a standard sample was run with the unknown samples. Gene expression was determined by normalization against β-actin expression.

**Measurement of PKA and PKC activity.** The activities of PKA and PKC were measured by the MESACUP protein kinase assay kit (MBL, Naka-ku Nagoya, Japan). The kit is based on ELISA that utilizes a synthetic peptide and a monoclonal antibody recognizing phosphorylated form of the peptide.

**Statistical analysis.** The results are expressed as means ± SE. Student’s paired t-test was used for data analysis. P values of <0.05 were considered significant.

**RESULTS**

**Effect of PTH on OPG secretion.** PTH induced a significant dose-dependent suppression of OPG secretion, even at low concentrations [10^{-12} mol/l: 82.8 ± 6.4%, P = 0.05; 10^{-11} mol/l: 71.6 ± 6.5%, P = 0.002; 10^{-10} mol/l: 56.6 ± 9.8%, P = 0.001 vs. control (0: without treatment)] (Fig. 1). At the highest concentration of PTH (10^{-10} mol/l equivalent to 410 pg/ml) OPG secretion was significantly diminished compared with the other concentrations of PTH (10^{-11} mol/l: P = 0.014 and 10^{-12} mol/l: P = 0.019).

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Effect of PTH on OPG mRNA expression in HUVEC. PTH at a normal physiological concentration (10^{-11} mol/l) did not affect OPG mRNA expression, while at a pathophysiological concentration (10^{-10} mol/l) its expression was depressed as measured by semiquantitative RT-PCR (Fig. 2A). The relative fold of OPG signal was plotted against 0 (Fig. 2B). A high concentration of PTH (10^{-10} mol/l) induced a significant decrease (50.8 ± 13.1%) in OPG mRNA expression vs. control (P = 0.02) as well as vs. the results obtained after incubation with 10^{-12} and 10^{-11} mol/l PTH (111.3 ± 22.8%, P = 0.01 and 112 ± 19.6%, P = 0.02, respectively). This inhibitory effect of PTH on OPG mRNA expression was also observed by the real-time PCR technique [10^{-11} mol/l: 31.0 ± 12.6%, P = 0.006; 10^{-10} mol/l: 39.6 ± 13.7%, P = 0.0003 vs. control (0)] (Fig. 2C).

Effect of PTH on PKA and PKC activities. Incubation of endothelial cells during 2–4 h with PTH (10^{-10} mol/l) induced a significant increase in PKA activity (20% elevation, P = 0.006 vs. control, nonstimulated cells). No changes were detected concerning the effect on PKC activity.

Activation of PKC stimulates secretion and mRNA expression of OPG, and activation of PKA inhibits its secretion and expression in nonstimulated HUVEC. To determine the possible involvement of the two main signaling pathways recognized to regulate the endothelial-stimulating effect of PTH (28, 29) on OPG secretion, we evaluated whether activation or inhibition of PKC or PKA in nonstimulated HUVEC may mimic the effect of PTH.

Nonstimulated resting HUVEC were incubated with increasing concentrations of PMA, to activate PKC, or forskolin, to activate PKA, for 6 or 24 h (Figs. 3 and 4). PMA significantly stimulated OPG secretion after 6 h [5 nmol/l: 178.3 ± 21.3%, P = 0.01; 10 nmol/l: 218.2 ± 37.5%, P = 0.02; 25 nmol/l: 271.4 ± 53.8%, P = 0.01; 50 nmol/l: 271 ± 57.3%, P = 0.01 vs. control (0)] (Fig. 3A). Preincubation of the cells with calphostin C, a PKC inhibitor, depressed this effect (Fig. 3A). The increase in OPG secretion after PMA activation was also observed after 24 h [5 nmol/l: 146.1 ± 26.53%, not significant (NS); 10 nmol/l: 195.2 ± 25.5%, NS; 25 nmol/l: 287.4 ± 76%, P = 0.04; 50 nmol/l: 283.7 ± 84.1%, P = 0.04 vs. control] (Fig. 3B). Confirming these results, a decrease in OPG secretion was found in nonstimulated HUVEC when calphostin C (50 nmol/l), the PKC inhibitor, was added, indicating that the PKC pathway is involved in maintaining the basal OPG secretion [26.7 ± 14.1% vs. control (0), P = 0.009, results not shown as figure]. Incubation with DMSO, used as the diluent for calphostin C did not change the secretion of OPG (results not shown).

In contrast, forskolin, which activates PKA, was found to significantly inhibit the OPG secretion after 6 h of incubation [10^{-5} mol/l: 87 ± 3.4%, P = 0.0006; 10^{-4} mol/l: 89 ± 3.3%, P = 0.01 vs. control (0)], a phenomenon that is similar to that found with PTH (Fig. 4A). Preincubation of the cells with Rp-cAMP, a cAMP antagonist, significantly blunted this action (Fig. 4A). This decrease in OPG secretion was also present after 24 h [10^{-4} mol/l: 69.6 ± 11.5%, P = 0.016 vs. control] (Fig. 4B) and was found to be similar to that observed with PTH (Fig. 4B). No significant difference was observed between them.

The effects of PKC and PKA activators on OPG mRNA levels were also determined. PMA increased and forskolin decreased OPG mRNA expression (Fig. 5, Tables 1 and 2). Knowing that the nitric oxide synthase (NOS) system may be involved in some of the PTH-related endothelial cell activities (33, 34), N-nitro-L-arginine methyl ester, a NOS inhibitor, was used to evaluate the possible impact of NO on OPG secretion, and no significant effect was observed (88 ± 4.1% vs. control; results not shown).

Involvement of PKC and PKA pathways in PTH regulation of OPG secretion. In view of the present results, it appears that activation of PKA may be involved in OPG reduction and activation of PKC may be involved in OPG stimulation in HUVEC (resting cells without PTH stimulation). Therefore, to determine whether the PTH-related reduction in OPG is PKC or PKA dependent, the effects of calphostin C (PKC inhibitor) or Rp-cAMP (PKA inhibitor) were evaluated in PTH-stimulated HUVEC (Fig. 6), focusing our study on the pathophysiological concentration of PTH, 10^{-10} mol/l (a value found in normal physiological concentration of PTH, 10^{-10} mol/l). Total RNA was extracted, and the levels of OPG and β-actin mRNA expression were assessed by conventional semiquantitative and real-time PCR. OPG mRNA levels were normalized to the levels of β-actin mRNA expression, and relative mRNA content is expressed as fold of control (0) values.

| Table 1. Effect of PMA (PKC activator) on OPG mRNA expression: densitometric analysis |
|-------------------|------------------|-----------------|-----------------|------------------|
| PMA, nmol/l | Conventional PCR | P vs. 0 | Real-Time PCR | P vs. 0 |
| 0 | 1±0 | 0.006 | 1±0 | 0.035 |
| 5 | 2.33±0.21 | 0.016 | 8.94±2.87 | 0.035 |
| 10 | 2.53±0.27 | 0.020 | 9.66±5.29 | 0.022 |
| 25 | 2.62±0.30 | 0.007 | 8.78±1.95 | 0.014 |
| 50 | 2.69±0.49 | 0.021 | 9.13±2.39 | 0.021 |

Values are means ± SE of 4 independent experiments. OPG, osteoprotegerin. Human umbilical vein endothelial cells (HUVEC) were incubated for 6 h with increasing concentrations of PMA. Total RNA was extracted, and the levels of the OPG and β-actin mRNA expression were assessed by conventional semiquantitative and real-time PCR. OPG mRNA levels were normalized to the levels of β-actin mRNA expression, and relative mRNA content is expressed as fold of control (0) values.
Table 2. Effect of forskolin (PKA activator) on OPG mRNA expression: densitometric analysis

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<thead>
<tr>
<th>Forskolin, mol/l</th>
<th>Conventional PCR</th>
<th>P vs. 0</th>
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<tr>
<td>10⁻⁷</td>
<td>88.8±6.9</td>
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<tr>
<td>10⁻⁶</td>
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<tr>
<td>10⁻⁵</td>
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<td>10⁻⁴</td>
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<td>13.2±11.4</td>
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Values are means ± SE of 4 independent experiments. NS, not significant; ND, not done. HUVEC were incubated for 6 h with increasing concentrations of forskolin. Total RNA was extracted, and the levels of the OPG and β-actin mRNA expression were assessed by conventional semiquantitative and real-time PCR. OPG mRNA levels were normalized to the levels of β-actin mRNA expression, and relative mRNA content is expressed as percentage of control (0) values.

DISCUSSION

PTH significantly reduced the secretion of OPG in a dose dependent manner. This effect of PTH on endothelial secretion of OPG may eventually have a clinical significance knowing that in situations characterized by chronic PTH excess, the risks of CV complications as well as the frequency of morbidity and mortality are markedly increased (30). On the basis of previous data, which have shown that OPG may suppress signals of local vascular calcifications (5, 10), a proportional lower OPG secretion may lead to more severe vascular calcification. As mentioned above elevated blood levels of OPG have been found in dialyzed patients particularly those with marked vascular calcification (13, 15, 25, 36). In this situation, OPG has been found to predict an increased risk of heart failure and acute coronary disease (16, 43). No correlation was found between blood levels of OPG and PTH (1, 9, 32, 33, 35). However, until now the most accepted concept is that high levels of OPG may represent a condition of vascular defense to limit the direct deleterious action of other vascular-calcifying factors such as elevated PTH, high ionized calcium, hyperphosphatemia, and/or elevated calcium-phosphate products. Therefore, the significant decrease in OPG secretion induced by PTH may be interpreted as a deleterious phenomenon, which may facilitate vascular calcification. In consequence, it is conceivable that in uremic patients with secondary hyperparathyroidism and chronically high blood PTH levels, the endothelial release of OPG may be partly blunted, leading to more risks of vascular calcifications.

In our study, we confirmed the expression of OPG mRNA and the secretion of OPG in nonstimulated HUVEC (46). Others have shown a similar phenomenon in human microvascular endothelial cells (HMVEC) (6). In addition, it has been reported that OPG serves as a survival factor for cultured endothelial cells (20, 27) and that proinflammatory cytokines (IL-1 and/or TNF-α) may stimulate its expression and secretion (6). The endothelial cell’s ability to continuously produce OPG may be seen as a defense mechanism. When the main cellular pathway systems were evaluated in our study protocol, we took into account the fact that PTH activates both PKA and PKC in most classic target cells (such as chondrocytes, osteoblasts, osteoclasts, and kidney-derived cells), while in nonclassic target cells (smooth muscle cells) only the adenylate cy-

![Fig. 6. Involvement of PKC and PKA pathways in PTH regulation of OPG secretion.](http://ajprenal.physiology.org/)
class (PKA) pathway is activated (34). In recent studies, we demonstrated that PTH receptor-1 (PTH1R) is present on the membrane of cultured HUVEC and that by binding to PTH1R, PTH (fragment 1–34 as used presently) is able to stimulate the expression of eNOS, RAGE, and IL-6 as well as eNOS activity through either PKC or PKA pathways (28, 29). Previous studies have reported that the PTH-related inhibition of OPG in stromal/osteoblastic cells involves PTKA (12) or calcium/PKC (42) pathways. On this basis, in the second step of experiments, we determined the impact of PKC and PKA activators and inhibitors on basal OPG secretion and mRNA expression in nonstimulated HUVEC. PMA, a potent activator of PKC (18) used in resting nonstimulated HUVEC, increased OPG secretion, an effect which was blunted by calphostin C (a recognized PKC inhibitor) (17). These results indicate that the basal endothelial OPG secretion is regulated through a PKC pathway. Forskolin, an activator of cAMP signals (7), induced a decrease in OPG secretion in resting HUVEC nonstimulated by PTH. As expected, preincubation with Rp-cAMP, a cAMP antagonist (31), significantly prevented the inhibitory action of forskolin. Similar findings were noted in the expression of OPG mRNA. These results suggest that the inhibitory action of PTH on OPG secretion and mRNA expression may eventually be related to PKA activation. As we found that PTH after 2–4 h has a stimulating effect on endothelial PKA activity but not on PKC activity, the concept that the blunting action of PTH on OPG is through the PKA pathway may be considered to be supported by the present findings.

In the third set of experiments dealing with PTH-stimulated HUVEC, we found a superimposed action of calphostin C (PKC inhibitor) to further diminish OPG secretion already depressed by PTH, leading to an almost complete blockade of OPG release at highest concentrations, suggesting that the PTH-inhibitory effect might not be due to PKC activation. We expected that Rp-cAMP (the PKA inhibitor) would reduce the PTH suppression of OPG, but such PKA inhibition did not change the first inhibitory action of PTH. This leads us to hypothesize that PKA inhibition by Rp-cAMP was not strong enough to inhibit the PTH-related OPG reduction, or it may be that other pathways are involved in this PTH-dependent OPG reduction. To strengthen this assertion, experiments where cells were pretreated with forskolin, the PKA activator, and PMA, the PKC activator, in addition to PTH were performed. Addition of PTH to PMA-stimulated cells did not change the stimulatory effect of PMA on OPG secretion. These results indicate that the stimulatory effect of PMA, the PKC activator, is more powerful than the inhibitory effect of PTH. Pretreatment of the cells with forskolin and then treatment with PTH had no additive effect on OPG reduction. From these results, it can be concluded that the OPG stimulation by PKC activators is stronger than OPG reduction by PTH. We also examined the effect of the PKA activator in presence of the PKC inhibitor (results not shown). As expected forskolin, the PKA activator induced a decrease in OPG secretion and the addition of calphostin C, the PKC inhibitor, to these forskolin-treated cells decreased the OPG reduction (results not shown). The PKC activator (PMA) strongly stimulated OPG secretion and, in the presence of the PKA inhibitor, no difference in OPG levels was noted, showing the dominant action of the PKC activator.

As OPG is considered to function as a soluble decoy receptor by binding RANKL in bone (10, 14, 41, 44), the OPG/ RANKL ratio could be considered to be important. However, recent studies have shown clearly that HUVEC do not release or express protein and mRNA of RANKL (38, 46, 47). To be noted, Collin-Osdoby et al. (30) showed that HMVEC may express RANKL mRNA. HUVEC, unlike HMVEC, express mRNA transcripts for OPG but not for RANKL. According to these studies, as the amounts of RANKL are undetectable in HUVEC, measuring the expression of RANKL in HUVEC was considered to be irrelevant in this set of experiments.

Finally to be noted, we also evaluated the possible impact of NO on OPG secretion and it appeared that NO is not involved. These data have shown that PTH has a direct impact on endothelial OPG metabolism. This effect may be PKA dependent and strengthens the concept that PTH may also affect the cardiovascular system through its effect on OPG.

REFERENCES


AJP-Renal Physiol • VOL 296 • JANUARY 2009 • www.ajprenal.org
PARATHYROID HORMONE DECREASES OSTEOPROTEGERIN


