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Klotho Protein Activates the PKC Pathway in the Kidney and Testis and Suppresses 25-Hydroxyvitamin D_3 1α-Hydroxylase Gene Expression

Michio Imai, Kazuhiko Ishikawa, Naomichi Matsukawa, Iwao Kida, Junsuke Ohta, Masashi Ikushima, Yukana Chihara, Xu Rui, Hiromi Rakugi, and Toshio Ogihara

Department of Geriatric Medicine, Osaka University Graduate School of Medicine, Osaka 565-0871, Japan

Homozygous Klotho mutant (*kl*$^{-}$/$^{-}$) mice exhibit a variety of phenotypes resembling human aging, including arteriosclerosis, infertility, skin atrophy, osteoporosis, and short life span. Calcium abnormality, one of the phenotypes in *kl*$^{-}$/$^{-}$ mice, is thought to be due to the elevated gene expression of 25-hydroxyvitamin D_3 1α-hydroxylase in the kidney. We studied 25-hydroxyvitamin D_3 1α-hydroxylase gene expression using a Klotho plasmid that we had previously constructed for Klotho protein production. It was found that Klotho protein medium upregulated cAMP and the PKC pathway, and suppressed 25-hydroxyvitamin D_3 1α-hydroxylase in kidney cells. However, both cAMP and PKC are known to elevate 25-hydroxyvitamin D_3 1α-hydroxylase gene expression, therefore, another unknown calcium regulation pathway using Klotho protein medium might exist. Furthermore, we found that activation of the PKC pathway by Klotho was observed only in the kidney and testis, where the Klotho gene is expressed, although activation of the cAMP pathway was observed in any kind of cell. These data suggest that calcium regulation through 25-hydroxyvitamin D_3 1α-hydroxylase by Klotho depends on non-cAMP and a non-PKC pathway and that the Klotho protein may have different signaling pathways, depending on the Klotho gene expression in different cells and organs.

Key Words: Klotho; PKC; 25-hydroxyvitamin D_3 1α-hydroxylase.

Introduction

Homozygous Klotho mutant mice (*kl*$^{-}$/$^{-}$), a useful model for human premature aging, exhibit a variety of phenotypes very similar to age-related diseases in humans, including, arteriosclerosis, pulmonary emphysema, ectopic calcification, infertility, skin atrophy, osteoporosis, and subcutaneous fat atrophy *(1,2)*. The Klotho gene, owing to alternative RNA splicing, produces two different types of proteins, a membrane and a secreted form *(3,4)*. Although the secreted form of the Klotho protein may be a reasonable candidate for the humoral regulating effect of Klotho *(5–7)*, a previous report suggested that the membrane form is also secreted by proteolytic cleavage *(7)*. Furthermore, the secreted form of the protein could not be detected using newly established antibodies for the secreted form of Klotho *(7)*. Klotho mRNA is predominantly expressed in the distal renal tubules, brain choroid plexus, and testis *(1,8–10)*.

It was previously reported that in the *kl*$^{-}$/$^{-}$ mouse, serum calcium, phosphate, and 1,25-$(OH)_2$D concentrations are discordantly elevated, which might be due to the elevated gene expression of 25-hydroxyvitamin D_3 1α-hydroxylase (1α-hydroxylase) in the kidney *(1,11,12)*. 1α-Hydroxylase plays an important role in calcium handling by converting the inactive to the active form of vitamin D, 1,25-$(OH)_2$D *(13)*. Interestingly, it was also demonstrated that most of the *kl*$^{-}$/$^{-}$ mouse phenotypes could be rescued with a vitamin D deficient diet, not a low calcium diet, showing that the main role of the Klotho gene is calcium handling *(12)*. Parathyroid hormone is the major hormone for calcium handling, and it regulates 1α-hydroxylase through both the PKA and PKC pathways in LLC-PK1 cells *(14,15)*. In addition, we previously reported that the cAMP pathway was upregulated in endothelial cells by incubation with the Klotho protein membrane form *(16)*. It was suggested that the Klotho protein mouse membrane form may act as a humoral factor through a cAMP-PKA dependent pathway *(16)*.

It remains unknown how the Klotho protein regulates 1α-hydroxylase gene regulation, and how the Klotho signaling pathway functions. In the present study, we attempt to clarify the signaling pathway of the Klotho protein in kidney and other cells, using Klotho gene transfection, and to explain the association between the Klotho protein and 1α-hydroxylase.

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Author to whom all correspondence and reprint requests should be addressed: Hiromi Rakugi, MD, PhD, Associate Professor of Medicine, Department of Geriatric Medicine, Osaka University Graduate School of Medicine, 2-2, Yamadaoka, #B6, Suita, Osaka 565-0871, Japan. E-mail: rakugi@geriat.med.osaka-u.ac.jp

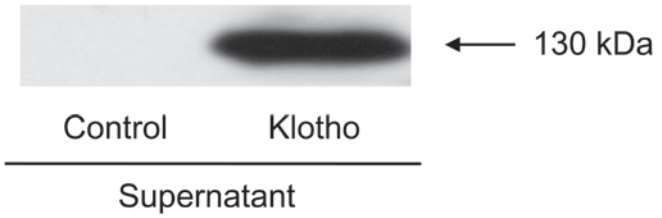

Fig. 1. Klotho protein expression in culture medium. For culture supernatant, COS-1 cells were transfected with pCAGGS or pCAGGS-Klotho for 6 h, incubated in Medium-41 for 36 h, followed by incubation in serum-free Medium-41 for an additional 36 h. The Klotho protein expression was determined using Western blot analysis for four individual experiments. Klotho, conditioned medium obtained by incubation with pCAGGS-Klotho plasmid; Control and conditioned medium obtained by incubation with pCAGGS plasmid.

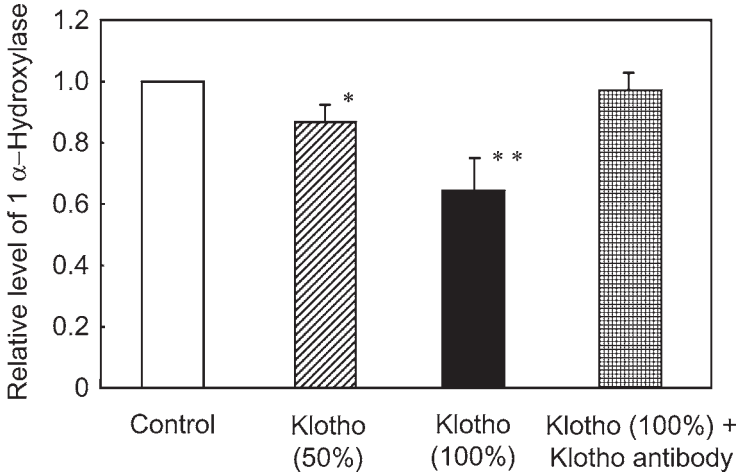

Fig. 2. Effect of the Klotho protein medium on 1α-hydroxylase gene expression in LLC-PK1 cells. The relative level of 1α-hydroxylase mRNA expression in LLC-PK1 cells incubated with conditioned medium (control □, 50% Klotho ▨, 100% Klotho ■, 100% Klotho+antibody ▦) was measured as described in Materials and Methods. Control: conditioned medium (CM) obtained by incubation with pCAGGS plasmid; Klotho (50%): mixture of the same amount of CM obtained by incubation with pCAGGS and pCAGGS-Klotho plasmid; Klotho (100%): CM obtained by incubation with pCAGGS-Klotho plasmid; Klotho antibody (KM2076, 10 μg/mL): anti-Klotho rat monoclonal antibody (Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan). Values are means ± SE for four individual experiments, each containing two replicates. *$p < 0.05$, significantly different from control. **$p < 0.05$, significantly different from Klotho (50%).

Results

Western Blot Analysis

To confirm the successful production of the Klotho protein membrane form, the Klotho protein was measured in conditioned medium (CM) using Western blot analysis with the anti-Klotho rat monoclonal antibody, KM2076 *(17)*. A 130 kDa band, which is the same size as the Klotho protein membrane form, was detected (Fig. 1) *(16)*.

Effect of Klotho Protein Medium on 1α-Hydroxylase Gene Expression

LLC-PK1 cells were incubated with CM and the measured 1α-hydroxylase gene expression was significantly lower than the control (Fig. 2). The reduction of 1α-hydroxylase gene expression was dose-dependent, and the anti-Klotho rat monoclonal antibody caused it to disappear (Fig. 2).

Effect of cAMP Pathway on the Regulation of 1α-Hydroxylase Gene Expression by the Klotho Protein Medium

To examine the effect of Klotho protein medium on cAMP in kidney cells, we measured the cAMP concentration in LLC-PK1 cells. The cAMP concentration was elevated and dependent on the levels of Klotho protein when compared to the control. The same results were obtained using IBMX, which can inhibit cAMP degeneration (Fig. 3). cAMP is known to increase 1α-hydroxylase gene expression *(14,15)*. To understand the effect of Klotho regulation on 1α-hydroxylase, we incubated LLC-PK1 cells with both CM and forskolin, an activator of cAMP. 1α-Hydroxylase gene expression was not significantly different from those cells with CM only, even though the cAMP concentration was threefold higher than the control (Fig. 4). Furthermore, we used Rp-cAMPS, an inhibitor of cAMP, with CM in

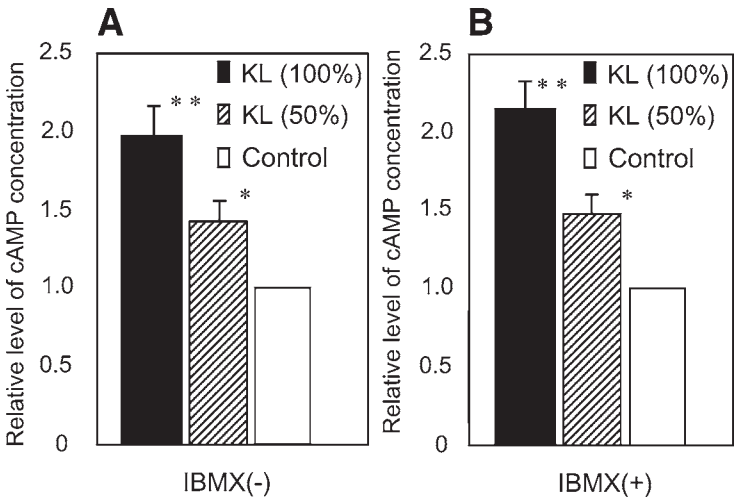

Fig. 3. (**A**) Effects of the Klotho protein medium on cAMP concentration in LLC-PK1. (**B**) IBMX was added to inhibit cAMP degeneration. (**A**) The relative level of cAMP concentration in LLC-PK1 cells incubated with conditioned medium (control □, 50% Klotho ▨, 100% Klotho ■) was measured as described in Materials and Methods. (**B**) IBMX was added to the conditioned medium. Control: conditioned medium (CM) obtained by incubation with pCAGGS plasmid; Klotho (50%): mixture of the same amount of CM obtained by incubation with pCAGGS and pCAGGS-Klotho plasmid; Klotho (100%): CM obtained by incubation with pCAGGS-Klotho plasmid; LLC-PK1: proximal tubule cells, kidney, porcine; IBMX: an inhibitor of PDE. Values are means ± SE for four individual experiments, each containing two replicates. *$p < 0.05$, significantly differently different from control. **$p < 0.05$, significantly different from Klotho (50%).

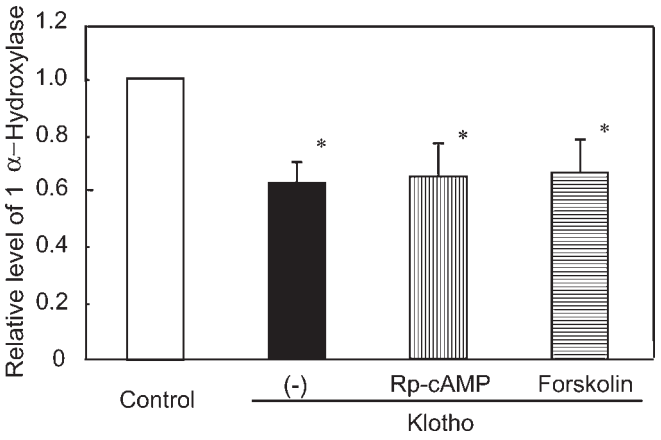

Fig. 4. Effect of the cAMP pathway on the regulation of 1α-hydroxylase gene expression by the Klotho protein medium. The relative level of 1α-hydroxylase mRNA expression in LLC-PK1 cells incubated with conditioned medium (control □, Klotho ■, Klotho+Rp-cAMP ▥, Klotho+Forskolin ▤) was measured as described in Materials and Methods. Control: conditioned medium (CM) obtained by incubation with pCAGGS plasmid; Klotho: CM obtained by incubation with pCAGGS-Klotho plasmid; Rp-cAMP: Rp-cAMP (10 μ*M*), inhibitor of cAMP, was added into CM in Klotho; forskolin: forskolin (50 μ*M*), activator of cAMP, was added into CM in Klotho. Values are means ± SE for four individual experiments, each containing two replicates. *$p < 0.05$, significantly different from control.

LLC-PK1 cells, but no difference was found in 1α-hydroxylase gene expression between those cells with and without Rp-cAMPS (Fig. 4). These results suggested that Klotho protein medium regulation of 1α-hydroxylase might not be via the cAMP pathway.

Effect of the PKC Pathway on the Regulation of 1α-Hydroxylase Gene Expression by the Klotho Protein Medium

To investigate the possible pathway between the Klotho protein and 1α-hydroxylase gene expression, we studied whether the Klotho protein medium has some effect on PKC activity. We incubated kidney cells (LLC-PK1) with CM to measure the PKC activity, and found that the Klotho protein medium increased PKC activity dose-dependently compared to the control (Fig. 5). The Klotho antibody restored 1α-hydroxylase gene expression to the control level. To confirm our results, we studied this expression using PMA, an activator of PKC; 1α-hydroxylase gene expression was not significantly different from the control (Fig. 6). Furthermore, we used staurosporine, an inhibitor of PKC, with CM in LLC-PK1 cells, but no difference was found in 1α-hydroxylase gene expression between cells with and without staurosporine (Fig. 6).

Effect of the Klotho Protein Medium on cAMP and PKC Activity in Different Kinds of Cells

The effect of the Klotho protein medium on cAMP and PKC activities was measured in different kinds of cells, and the cAMP level was increased in HUVEC, LLC-PK1, COS-1, 3T3L1, and HeLa cells (Fig. 7). However, PKC was activated only in kidney and testis cells, such as LLC-PK1, COS-1, RPTEC, and TM3 cells (Fig. 5). Klotho protein medium did not activate PKC in HUVEC, 3T3L1, and HeLa cells (Fig. 5). These results show that PKC activity was upregulated by the Klotho protein medium only in kidney and testis cells, where the Klotho gene is expressed.

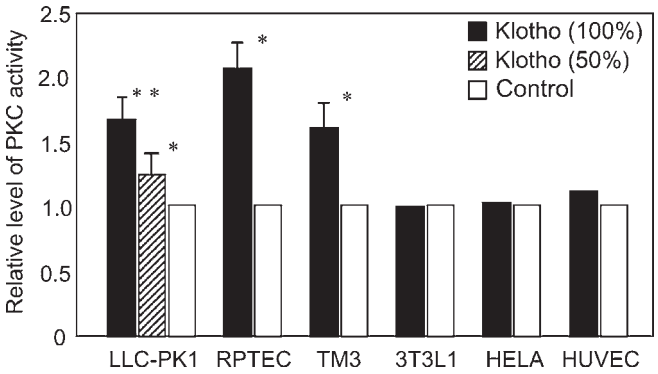

Fig. 5. Effects of the Klotho on PKC activity in different kind of cells. The relative level of PKC activity in each cells incubated with conditioned medium (control □, 50% Klotho ▨, 100% Klotho ■) was measured as described in Materials and Methods. Control: conditioned medium (CM) obtained by incubation with pCAGGS plasmid; Klotho (50%): mixture of the same amount of CM obtained by incubation with pCAGGS and pCAGGS-Klotho plasmid; Klotho (100%): CM obtained by incubation with pCAGGS-Klotho plasmid; LLC-PK1: proximal tubule cells, kidney, porcine; RPTEC: proximal tubule cells, kidney, human; TM3: Leydig cells, testis, mouse; 3T3L1: fibroblast, mouse; HeLa: cervical cancer cell, human; HUVEC: endothelial cells, human. Values are means ± SE for four individual experiments, each containing two replicates. *$p < 0.05$, significantly different from control. **$p < 0.05$, significantly different from Klotho (50%).

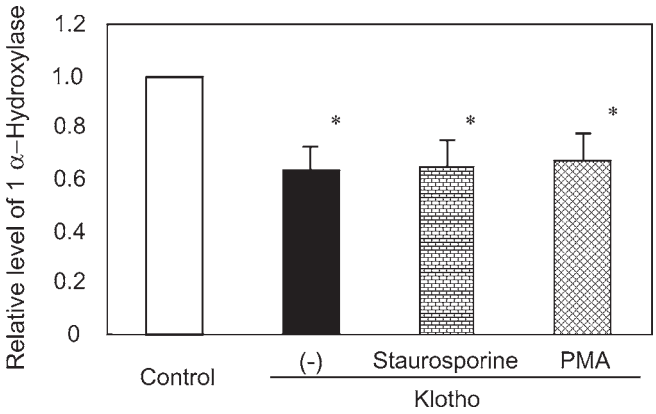

Fig. 6. Effect of the PKC pathway on the regulation of 1α-hydroxylase gene expression by the Klotho protein medium. The relative level of 1α-hydroxylase mRNA expression in LLC-PK1 cells incubated with conditioned medium (control □, Klotho ■, Klotho+Staurosporine ▦, Klotho+PMA ▩) was measured as described in Materials and Methods. Control: conditioned medium (CM) obtained by incubation with pCAGGS plasmid; Klotho: CM obtained by incubation with pCAGGS-Klotho plasmid; staurosporine: staurosporine (100 n*M*), inhibitor of PKC, was added into CM in Klotho; PMA: PMA (100 n*M*), activation of PKC, was added into CM in Klotho. Values are means ± SE for four individual experiments, each containing two replicates. *$p < 0.05$, significantly different from control.

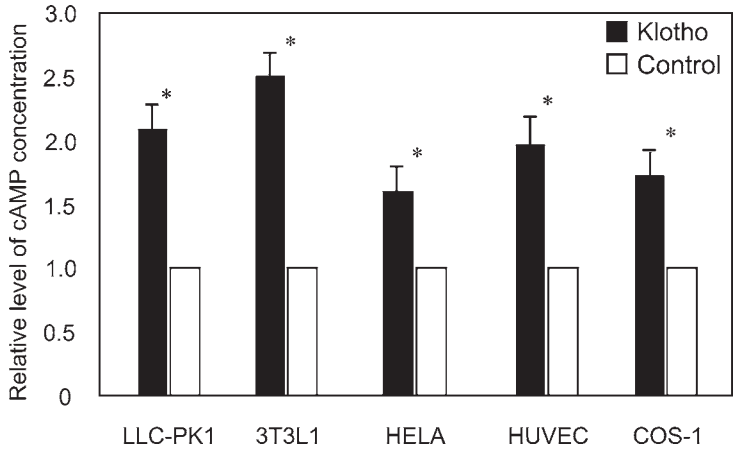

Fig. 7. Effects of the Klotho protein medium on cAMP concentration in different kind of cells. The relative level of cAMP concentration in each type of cells incubated with conditioned medium (control □, Klotho ■), was measured as described in Materials and Methods. Control: conditioned medium (CM) obtained by incubation with pCAGGS plasmid; KL: CM obtained by incubation with pCAGGS-Klotho plasmid; LLC-PK1: proximal tubule cells, kidney, porcine; 3T3L1: fibroblast, mouse; HeLa: cervical cancer cell, human; HUVEC: endothelial cells, human; COS-1: proximal tubule cells, kidney, monkey. Values are means ± SE for four individual experiments, each containing two replicates. *$p < 0.05$, significantly different from control.

Discussion

In the present study, we determined that how 1α-hydroxylase gene expression is regulated by the Klotho protein medium, and attempted to clarify the Klotho protein signaling pathway.

1α-Hydroxylase gene expression is discordantly increased in the *kl*$^{-/-}$ mouse *(11,12)*, showing that 1α-hydroxylase gene expression may be the key to explaining calcium handling by the Klotho protein. In this study, Klotho protein medium suppressed 1α-hydroxylase gene expression, which is consistent with the result of *kl*$^{-/-}$ mouse. This report demonstrates the regulation of 1α-hydroxylase in kidney cells by the Klotho protein medium. It may be possible that suppression of 1α-hydroxylase by Klotho protein medium is

mirrored by a diminishment in the conversion of substrate 25-hydroxyvitamin D_3 to 1,25-dihydroxyvitamin D_3. However, extremely high levels of 1α-hydroxylase expression in *kl*$^{-}$/$^{-}$ mouse indicate that changes in Klotho expression are the cause of changes in 1α-hydroxylase expression.

We previously reported that cAMP concentration was increased by the Klotho protein medium in HUVEC cells *(16)*, but its effect is not known in other cells. Calcium handling is thought to be a main function of the Klotho protein, so it was necessary to examine the cAMP activity induced by the Klotho protein in kidney and other cells. In our study, cAMP concentration was increased in most of the cells studied, including kidney cells. This provides evidence that cAMP may be involved in the Klotho protein's function as a circulating factor.

1α-Hydroxylase converts 25-hydroxyvitamin D_3 substrate to 1,25-dihydroxyvitamin D_3. 1,25-Dihydroxyvitamin D_3 has other roles to promote cell differentiation and inhibit cell proliferation except for calcium regulation *(18)*. Klotho protein medium may inhibit cell differentiation and promote cell proliferation through the suppression of 1α-hydroxylase, and may lead to have an anti-senescence effect on various cells.

If we assume the Klotho protein regulates the 1α-hydroxylase gene through the cAMP pathway, there might be a contradiction. The Klotho protein medium increased cAMP, and cAMP is known to increase 1α-hydroxylase gene expression *(14,15)*, but Klotho reduced 1α-hydroxylase gene expression. We hypothesized that another pathway exists for the transduction between Klotho and 1α-hydroxylase, which led us to investigate Klotho protein–induced PKC activity. As a result, the Klotho protein medium increased PKC activity in kidney cells, which is a new Klotho protein signaling pathway. However, PKC activity could not account for the transduction between Klotho and 1α-hydroxylase. Interestingly, PKC activity was increased only in kidney and testis cells, where the Klotho gene was expressed, but not in fibroblast, HeLa, or endothelial cells. These results suggest that the PKC pathway may be involved in the main function of Klotho, which is specific to organs where the Klotho gene is expressed, and acts spontaneously in the kidney and testis.

Klotho gene product is expressed principally in the distal nephron. COS-1 cells and LLC-PK1 cells used in the present study were derived from the proximal nephron, in which 1α-hydroxylase gene is predominantly expressed. Microvasculars closely linked with nephron are well connected surrounding both proximal and distal tubules. Therefore, it might be possible that Klotho protein secreted from the distal epithelial cells acts on proximal cells via the closely linked microvascular system.

In the present study, we used conditioned medium containing the Klotho protein, to stimulate kidney and other cells. We were not able to synthesize or extract Klotho peptides for this experiment, which is why we used CM. This might be a limitation to the present study. However, the amount of Klotho mRNA in the kidney cells, into which the Klotho plasmid was transfected, was 500–1000 times more than in the control. Furthermore, the Klotho protein was confirmed to exist sufficiently in Klotho CM, while it does not exist in the control; therefore, we decided to use CM as representative of the Klotho protein.

In conclusion, our study revealed that the Klotho protein medium reduces 1α-hydroxylase gene expression, and activates the cAMP pathway in most cells, while it activates the PKC pathway only in cells of those organs where the Klotho gene is expressed. It was also suggested that calcium regulation through 25-hydroxyvitamin D_3 1α-hydroxylase by Klotho depends on non-cAMP and non-PKC pathway. The Klotho protein may have different functions depending on the signaling pathway and its presence in Klotho-expressing organs.

Materials and Methods

Plasmid Construction

Plasmid pCAGGS-Klotho was constructed by inserting the complete mouse membrane form Klotho cDNA into the *Eco*RI site between the cytomegalovirus immediate-early enhancer-chicken β-actin hybrid promoter and the 3'-flanking sequence of the rabbit β-globin gene of the pCAGGS expression vector *(1,19)*.

Cell Culture

HUVEC cells were cultured in EBM-2 supplemented with 2% FBS, COS-1, and LLC-PK1 in Medium-41 with 10% FBS, RPTEC in REBM with 5% FBS, TM3 in DMEM/F-12 with 2.5% FBS and 5% HS, and 3T3L1 and HeLa cells in DMEM with 2% FBS. For the conditioned medium (CM) experiments, pCAGGS or pCAGGS-Klotho was pretransfected into COS-1 cells for 6 h, incubated with Lipofectamine plus 2000 (GIBCO-BRL) and Medium-41 for 36 h, and then cells were incubated in serum-free medium, specific for each of the cell types, for another 36 h *(16)*. Conditioned medium, which contained Klotho protein, was obtained to stimulate the cultured subconfluent. pCAGGS-CM was used for the control.

Western Blot Analysis for the Klotho Protein Medium

After centrifugation at 100*g*, 10 μL of CM was treated with 10 μL/10^6 cells of lysis buffer (1% SDS; 100 m*M* NaCl; 50 m*M* Tris-HCl, pH 8.0; 20 m*M* EDTA). All the samples were boiled for 3 min. Twenty microliters of CM mixture, as described above, was loaded onto a 12.5% SDS PAGE gel, and electroblotted onto nitrocellulose filters. Blots were blocked in 5% skimmed milk in PBS for 1 h, treated for 1 h with anti-Klotho rat monoclonal antibody, KM2076, and incubated with peroxidase-conjugated second antibodies for 1 h. Immunoblots were developed using an ECL Western blotting detection system (Amersham Pharmacia Biotech).

Quantification of 1α-Hydroxylase Gene Expression

Total RNA was extracted from the cells using a SV Total RNA Isolation kit (Promega). To quantify the 1α-hydroxylase gene expression, the TaqMan-PCR (Applied Biosystems) method was conducted using a PRISM 7900 HT (Applied Biosystems), which can detect RNA amounts in real time using a fluorescent probe complementary to the RNA sequence. 1α-Hydroxylase gene expression was calculated by dividing by the control gene expression (β-actin gene). Furthermore, 1α-hydroxylase expression of pCAGGS-Klotho was presented as the relative level of 1α-hydroxylase after being devided by control levels of 1α-hydroxylase expression.

Measurement of Intracellular cAMP

Subconfluent cells (1.5×10^4 cells/well) were cultured with CM for 6 h in a 96-well plate with Medium-41 under the indicated conditions. Subconfluent cells were cultured with CM for 6 h in a 96-well plate, and cAMP was measured with an Enzyme Immunoassay Biotrak system (Amersham Pharmacia Biotech) using a non-acetylation procedure. When IBMX was used, 0.5 m*M* IBMX was added and incubated for 1 h with serum-free Medium-41, after 23 h of incubation with serum-free Medium-41, and treated with the CM containing 0.5 m*M* IBMX for 1 h. Samples were standardized for total cellular protein using the method described by Bradford *(20)*.

cAMP levels stimulated by the Klotho protein medium were presented as a relative level, divided by the control cAMP concentration levels.

Measurement of PKC Activity

Subconfluent cells were cultured with CM for 24 h in a six-well plate, PKC homogenization buffer was added, and PKC activity was measured with a PepTag Non-Radioactive Protein Kinase C Assay System (Promega) *(21–23)*. Samples were standardized for total cellular protein using the method described by Bradford *(20)*. The PKC activity level was presented the same as cAMP.

Statistical Analysis

Statistical analysis was performed using a Student *t* analysis. Results were expressed as means ± SE. A value of $p < 0.05$ was considered significant.

Acknowledgments

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