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ORIGINAL PAPER

Anti-oxidative effect of Klotho on endothelial cells through cAMP activation

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Abstract Klotho, a regulatory factor implicated in countering the aging process, has been reported to ameliorate endothelial dysfunction in vivo. To clarify whether Klotho protein directly affects endothelial cell function, we studied the effects of membrane-form Klotho on manganese superoxide dismutase (Mn-SOD) expression and nitric oxide production in human umbilical vein endothelial cells (HUVEC). We incubated HUVEC with conditioned medium from COS-1 cells transfected with expression vector, pCAGGS-klotho (Klotho-CM) or a recombinant, purified 6His-tagged Klotho protein. Both Klotho-CM and 6His-tagged Klotho protein enhanced Mn-SOD expression by approximately two-fold, partially via activation of the cAMP signaling pathway. Furthermore, Klotho-CM increased nitric oxide production, which also contributed to the up-regulation of Mn-SOD. Using the oxidation-sensitive dye dihydroethidium, we found that Klotho inhibited angiotensin II-induced reactive oxygen species production in HUVEC. These findings provide new insights into the mechanisms of Klotho action and support the therapeutic potential of membrane-form Klotho to regulate endothelial function.

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Introduction

Mice deficient in Klotho gene expression exhibit a syndrome resembling premature human aging [1]. Importantly, a recent study showed an association between human longevity and a functional variant of Klotho [2]. A recent report showed that Klotho over-expression in mice extends lifespan through repression of insulin or insulin-like growth factor-1 (IGF-1) signaling, an evolutionarily conserved mechanism for lifespan extension [3]. Yamamoto et al. reported that Klotho induced Mn SOD protein in COS, HELA, and CHO cells in association with its inhibition of insulin/IGF-1 signaling and indicated that anti-oxidative effect of Klotho potentially contributes to the anti-aging properties of Klotho [4]. Notably, it was reported that Klotho gene delivery ameliorates endothelial dysfunction by increasing endothelial nitric oxide (NO) production [5]. Atherosclerosis induced by hypertension, diabetes mellitus, and dyslipidemia is associated with endothelial dysfunction, which causes reduction of NO production. NO plays an important role in maintaining of vascular expansion and scavenging of oxidative stress.

The Klotho gene, owing to alternative RNA splicing, produces two different types of proteins, a membrane and a secreted form [6, 7]. Although the secreted form of the Klotho protein may be reasonable candidate for the humoral regulating effect of Klotho [8–10], a previous report suggested that the membrane form is also secreted by proteolytic cleavage [8]. Furthermore, the secreted form of the protein could not be detected using newly

established antibodies for the secreted form of Klotho [8]. Klotho mRNA is predominantly expressed in the distal renal tubules, brain choroids plexus, and testis [1, 11–13].

We have previously reported using a conditioned-medium (CM) containing Klotho experimental system that mouse membrane-form Klotho protein expressed in COS-1 cells might function as a humoral factor to up-regulate angiotensin-converting enzyme activity in HUVEC through a cAMP-dependent pathway [14, 15]. Cyclic AMP may affect endothelial function via various pathways such as Mn-SOD activation and NO production.

In the present study, we examined the effects of mouse membrane-form Klotho protein on Mn-SOD and NO production and on cellular signal transduction in HUVEC. We also examined potential usefulness of Klotho as an anti-oxidative agent against angiotensin II-induced reactive oxygen species (ROS) in endothelial cells.

Results

pCAGGS-klotho-conditioned medium up-regulates Mn-SOD and NO production in HUVEC

Due to membrane-form Klotho protein can be released into the culture medium [3], we investigated whether conditioned medium could up-regulate the production of Mn-SOD and NO in HUVEC. We found that pCAGGS-klotho-CM increased Mn-SOD expression and total SOD activity in HUVEC (Fig. 1a). Maximal Mn-SOD expression in HUVEC cultured with pCAGGS-Klotho-CM was significantly 2.01 ± 0.21 times greater than in control HUVEC. Total SOD activity in HUVEC cultured with pCAGGS-Klotho-CM was significantly increased, compared to that in cells cultured with pCAGGS-CM (Klotho: 3.97 ± 0.31, control: 1.92 ± 0.28 units/mg protein) (Fig. 1a). Up-regulation of Mn-SOD and total SOD activity by Klotho was confirmed by adding purified His-Klotho protein (2 nM, 48 h) to endothelial cell culture (Fig. 1b).

In addition, pCAGGS-klotho-CM increased NO production in HUVEC (Fig. 2). NO production in HUVEC cultured in pCAGGS-klotho-CM was significantly 1.97 ± 0.18 times greater than in control HUVEC.

Next we checked whether Klotho protein directly mediated the up-regulation of Mn-SOD and NO production in HUVEC. We have found that an antibody to the Klotho protein (KM2076) can be used as a neutralizing antibody against mouse membrane-form Klotho [14]. KM2076 completely inhibited the stimulatory effects of pCAGGS-klotho-CM on Mn-SOD expression, total SOD activity and NO production in HUVEC. These data indicate that mouse membrane-form Klotho protein can act as an humoral factor to directly induce enhanced Mn-SOD and NO production in HUVEC.

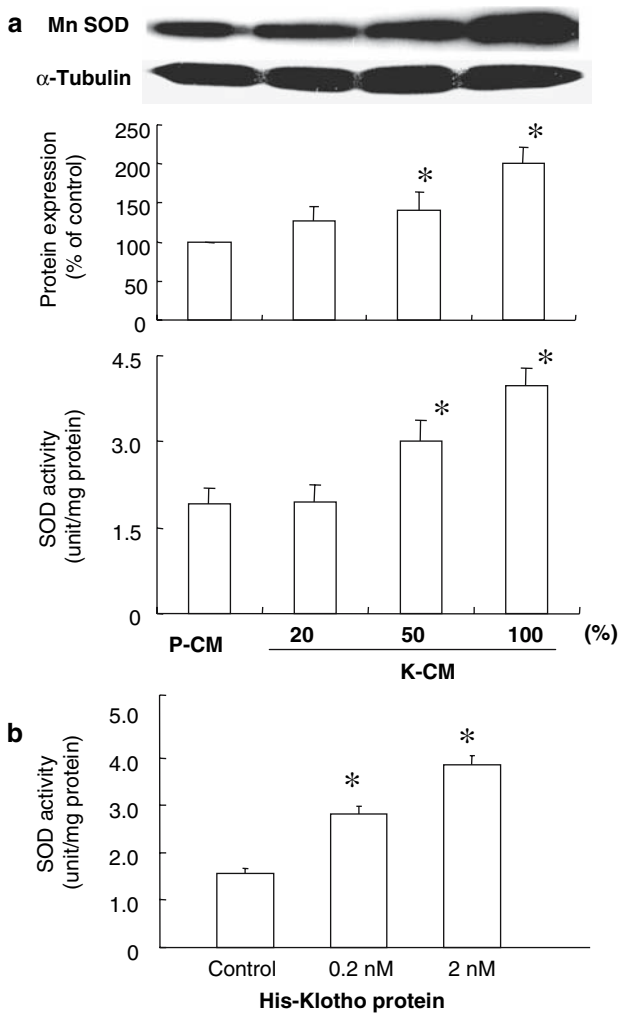

Fig. 1 Up-regulation of Mn-SOD expression and total SOD activity by pCAGGS-Klotho-CM and His-Klotho recombinant protein. (**a**) pCAGGS-klotho-CM enhanced Mn-SOD protein expression and total SOD activity in HUVEC. SOD expression was determined by Western blot analysis. The blot was reprobed with α-tubulin to confirm equal loading of protein in each well. SOD expression was quantified by densitometric analysis of Western blots from five independent experiments (data are normalized against α-tubulin and expressed as mean ± s.e.m.; $n = 5$). *$P < 0.05$, significantly different from individual controls. (**b**) His-Klotho recombinant protein increased total SOD activity in HUVEC. SOD activity was determined as described in the text. Values are mean ± s.e.m. of four individual experiments, each containing two replicates. *$P < 0.05$, significantly different from control

Mechanism of Klotho-induced up-regulation of Mn-SOD and NO production

In order to establish the mechanism by which Klotho protein up-regulates Mn-SOD and NO production in vitro, we evaluated the effects of Klotho protein on endothelial cell signaling pathways.

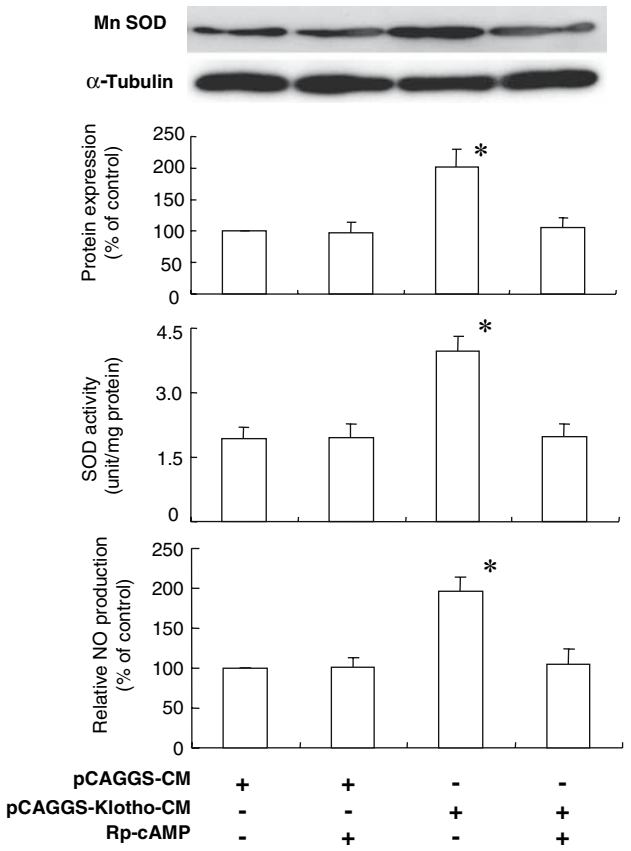

Fig. 2 Contribution of cAMP-dependent pathway to Klotho protein effects on Mn-SOD expression, total SOD activity, and NO production. Rp-cAMP, a cAMP antagonist, completely inhibits the up-regulation of Mn-SOD expression, total SOD activity, and NO production induced by Klotho protein (pCAGGS-Klotho-CM) in HUVEC. Values are mean ± s.e.m. of four individual experiments, each containing two replicates. *$P < 0.05$, significantly different from control treatment (pCAGGS-CM)

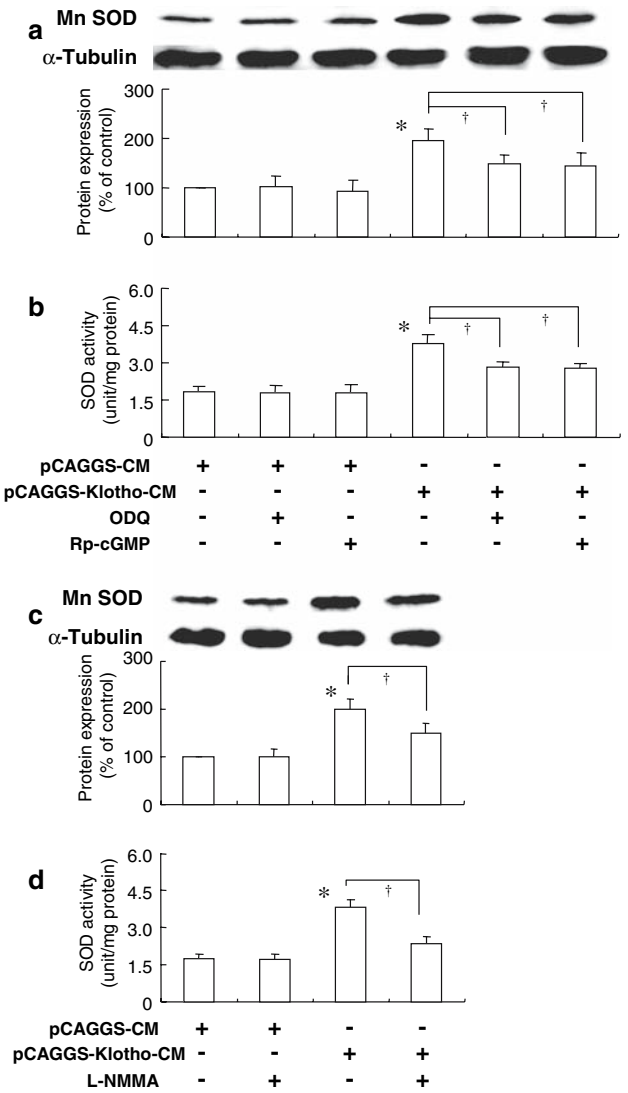

Fig. 3 Partial contribution of NO-cGMP-PKG signaling pathway to effects of Klotho protein on Mn-SOD expression and total SOD activity. (**a**, **b**) Both ODQ (NO-sensitive soluble guanylate cyclase inhibitor) and Rp-cGMP (cGMP-dependent PKG inhibitor) partly inhibited Klotho-induced increases in Mn-SOD expression (**a**) and total SOD activity, (**b**) in HUVEC. (**c**, **d**) L-NMMA (an inhibitor of NO synthase) partly inhibited Klotho-induced increases in Mn-SOD expression, (**c**) and total SOD activity, (**d**) in HUVEC. Values are mean ± s.e.m. of four individual experiments, each containing two replicates. *$P < 0.05$, significantly different from control treatment (pCAGGS-CM). †$P < 0.05$, significantly different between the two treatments. ODQ, 1H-(1,2,4) oxadiazolo (4,3-a) quinoxalin-1-one; L-NMMA, NG-monomethy-L arginine

We first studied whether up-regulation of Mn-SOD and NO by Klotho protein is cAMP-dependent because the Klotho protein up-regulated cAMP production in HUVEC [14] and that anti-Klotho monoclonal antibody, KM2076, completely inhibited cAMP up-regulation by Klotho (data not shown). The up-regulation of Mn-SOD expression, total SOD activity, and NO production in HUVEC was completely inhibited by Rp-cAMP, an antagonist of cAMP (Fig. 2).

The NO-sensitive cGMP inhibitor ODQ and cGMP-dependent PKG inhibitor Rp-cGMP both partly inhibited Mn-SOD protein expression (Fig. 3a) and SOD activity (Fig. 3b) in HUVEC. In addition, L-NMMA, an inhibitor of NO synthase, partly inhibited the increase in Mn-SOD expression (Fig. 3c) and SOD activity (Fig. 3d) induced by Klotho in HUVEC.

These findings indicate that not only cAMP but also the NO-cGMP pathway contribute to the up-regulation of Mn-SOD by Klotho.

Klotho protein decreased angiotensin II-induced ROS production in HUVEC

We found that pCAGGS-klotho-CM had no effect on ROS production in HUVEC (Fig. 4). However, pretreatment of HUVEC with pCAGGS-klotho-CM had a protective effect against angiotensin II-induced ROS production (Fig. 4).

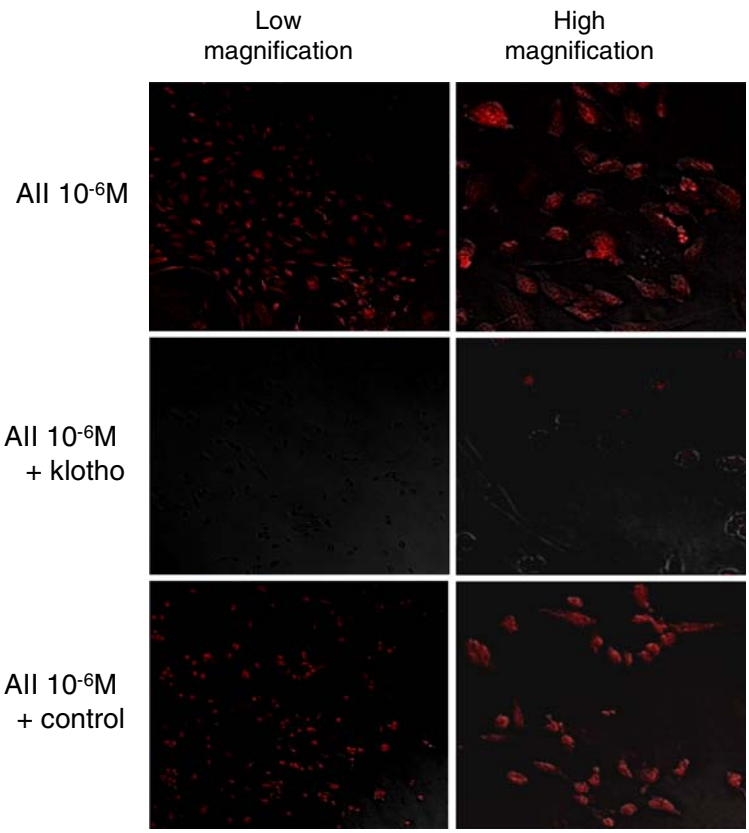

Fig. 4 Inhibitory effect of Klotho protein on angiotensin II-induced ROS production in HUVEC. Representative images in four upper-panels display no effect of pCAGGS-klotho-CM (Klotho) on ROS production in endothelial cells. Representative images in the four lower-panels display that angiotensin II (10^{-6} M, 48 h) induced ROS, and that Klotho-CM but not control-CM inhibited angiotensin II-induced ROS production. Lower and higher magnifications indicate 10× and 50×, respectively

These results revealed that enhanced Mn-SOD and NO productions were not induced by Klotho-mediated ROS activation, and that Klotho functions in reducing ROS in HUVEC stimulated with angiotensin II.

Discussion

In the present study, we showed that mouse membrane-form Klotho functions as a humoral factor to up-regulate Mn-SOD and NO production in endothelial cells using two types of Klotho protein, a purified recombinant protein and Klotho containing CM. As anti-Klotho antibody completely inhibited the effects of pCAGGS-klotho-CM in HUVEC, it is unlikely that Klotho protein acts as a humoral enzyme by converting a circulating inactive precursor to a biologically active substance to exert its effects. If Klotho acted as a humoral enzyme, active substrates cleaved by Klotho should be present in the CM collected 36 h after gene transfection into COS-1 cells, and KM-2076 should not have a strong inhibitory effect.

Regarding the up-regulation of Mn-SOD by Klotho, multiple mechanisms may contribute including protein kinase C, PI3K, and MAP kinase-mediated pathways. The present study clarified that Klotho-induced cAMP up-regulate Mn-SOD in HUVEC. It was reported that the human Mn-SOD gene promoter contains a cAMP-responsive element-like sequence [16], and increased cAMP can up-regulate Mn-SOD production through a PKA-dependent pathway [17]. Therefore, the possibility exists that phosphorylation of CREB by a cAMP-PKA-dependent pathway directly mediates the up-regulation of Mn-SOD.

Furthermore, our data that the up-regulation of Mn-SOD was partly inhibited by L-NMMA, ODQ, and Rp-cGMP indicates that the NO-cGMP-PKG signaling pathway contributes to the Klotho-induced up-regulation of Mn-SOD in HUVEC. This possible mechanism of Mn-SOD upregulation is consistent with the previous reports that NO increases extra cellular SOD in vasculature [18], Cu/Zn SOD in rat glomerular mesangial cells [19], and Mn-SOD in neurons [20, 21].

It is interest that Klotho up-regulated NO production in HUVEC via cAMP-dependent pathway. Regarding the mechanisms of cAMP dependent up-regulation of NO production, it was reported that cAMP-PKA-pathway contributes to phosphorylation of endothelial NO synthase (eNOS) at Ser-1179 and Ser-635, and to dephosphorylation at Thr-495 [22, 23]. In addition, the cAMP signal transduction cascade was reported to increase endothelial NO production in coronary arteries [24] and NO production in murine macrophages [25]. Taken together, these findings indicate that up-regulation of eNOS activity in a cAMP-PKA-dependent

manner may contribute to the enhanced NO production induced by Klotho protein in HUVEC.

The mechanisms by which Klotho ameliorates endothelial dysfunction have not been well characterized. Previous reports have suggested that Klotho gene delivery improves endothelial dysfunction by increasing endothelial-derived NO production in vivo [5, 9, 26]. Our results showed that mouse membrane-form Klotho upregulated Mn-SOD and NO production and inhibited angiotensin II-induced ROS production in endothelial cells. It was demonstrated that angiotensin plays an important role in endothelial dysfunction associated with aging via activation of superoxide anions [27]. These findings indicate that Klotho protein may improve endothelial dysfunction by regulating the homeostatic balance between reactive oxygen and antioxidant agents. In support of this hypothesis, our recent study demonstrated that Klotho protein reduced hydrogen peroxide-induced apoptosis and cellular senescence in cultured endothelial cells [28].

In conclusion, this study demonstrates that mouse membrane-form Klotho functions as a humoral factor, in part through a cAMP-dependent signaling pathway, to upregulate Mn-SOD and NO production. These findings provide new insights into the mechanism by which Klotho protein improves endothelial dysfunction and support the therapeutic potential of membrane-form klotho to regulate unfavorable effects of oxidative stress.

Methods

Preparation of Klotho protein

Plasmid pCAGGS-klotho was constructed by inserting the complete mouse membrane-form Klotho cDNA [1] (donation from Professor Yo-ichi Nabeshima, Kyoto University Graduate School of Medicine) into the EcoRI site just downstream of the hybrid promoter of the pCAGGS expression vector [29]. CM containing Klotho protein (pCAGGS-klotho-CM) was prepared from pCAGGS-klotho transfected COS-1 cells (Sanko Junyaku, Tokyo, Japan) [14]. A recombinant, purified, 6His-tagged Klotho (His-Klotho) protein was also purified as previously described [28].

Cell culture

We cultured sub-confluent HUVEC (Sanko Junyaku) for 36 h with pCAGGS-klotho-CM, control-CM, and His-Klotho protein in the absence or presence of the following agents; Rp-cAMP (10 μM), Rp-cGMP (10 μM), ODQ (1H-(1,2,4) oxadiazolo (4,3-a) quinoxalin-1-one; 1 μM), L-NMMA (L-NMMA, NG-monomethy-L arginine; 300 mM) (Sigma Chemical Co, St Louis, MO), and KM2076 (10 μg/ml), an anti-Klotho rat monoclonal antibody (donation from Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan) [30]. We used EGM-2 (Cambrex Bio Science Walkersville Inc) medium for the culture of HUVEC. To investigate the effect of pCAGGS-klotho-CM, we prepared dilutions of 0, 20, 50, and 100% pCAGGS-klotho-CM by mixing with pCAGGS-CM.

Western blot analysis

We mixed harvested cells with lysis buffer (1% SDS; 100 mM NaCl; 50 mM Tris-HCl, pH 8.0; 20 mM EDTA) at 10 μl/10^6 cells. Western blot analysis was performed as described previously using KM2076 and anti-Mn-SOD rabbit polyclonal antibody (Funakoshi Chemical Co., Tokyo, Japan) [28].

Determination of SOD activity

We measured total SOD activity essentially as described previously [31]. We lysed cultured cells in water by freezing/thawing three times, and cell lysates were centrifuged at 1,000*g* at 4°C for 5 min. The resultant supernatant was used for determination of total SOD activity by monitoring the inhibition of xanthine oxidase-mediated cytochrome c reduction. Absorbance at 550 nm was measured over 3 min. Values were normalized to wet tissue weight or total cellular protein in the homogenate measured by the method of Bradford [32].

Measurement of NO production in cells

In order to determine the level of NO released from cells into the medium, we incubated cells for 2 h at 37°C with the NO-reactive dye 4,5-diaminofluorescein (DAF-2, Daiichi Pure Chemicals Co., Tokyo, Japan) at a concentration of 10 μmol/L in HEPES-buffer containing 0.1 mmol/L L-arginine. In the presence of NO, DAF-2 forms a triazole derivative that emits light at 515 nm upon excitation at 489 nm in proportion to the amount of NO present [33].

Measurement of intracellular ROS production

We measured ROS (including superoxide and/or hydroxyl radical) production by fluorescence microscopy using the oxidation sensitive dye dihydroethidium (DHE, Merck, USA) as described previously [34]. Endothelial cells were incubated with serum-free EBM-2 for 24 h in the presence or absence of angiotensin II (10^{-6} M), then incubated with DHE (10^{-3} M) solution for 30 min at 37°C. After washing the cells twice with cold PBS, fluorescence was recorded

using a Carl Zeiss LSM510/UV laser scanning confocal imaging system (Carl Zeiss Co. Ltd. Germany).

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