Klotho Gene Delivery Suppresses Nox2 Expression and Attenuates Oxidative Stress in Rat Aortic Smooth Muscle Cells *via* the cAMP-PKA Pathway

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Abstract

Background and Hypothesis—Klotho is a recently discovered anti-aging gene. The purpose of this study was to investigate if klotho gene transfer attenuates superoxide production and oxidative stress in rat aorta smooth muscle (RASM) cells.

Methods and Results—RASM cells were transfected with AAV plasmids carrying mouse klotho full-length cDNA (mKL) or LacZ as a control. Klotho gene transfer increased klotho expression in RASM cells. Notably, klotho gene expression decreased Nox2 NADPH oxidase protein expression but did not affect Nox2 mRNA expression, suggesting that the inhibition may occur at the post-transcriptional level. Klotho gene transfer decreased intracellular superoxide production and oxidative stress in RASM cells. Klotho gene expression also significantly attenuated the angiotensin II (AngII)-induced superoxide production, oxidative damage, and apoptosis. Interestingly, klotho gene delivery dose-dependently increased the intracellular cAMP level and PKA activity in RASM cells. Rp-cAMP, a competitive inhibitor of cAMP, abolished the klotho-induced increase in PKA activity, indicating that klotho activated PKA *via* cAMP. Notably, inhibition of cAMP-dependent PKA activity by RP-cAMP abolished klotho-induced inhibition of Nox2 protein expression, suggesting an important role of the cAMP-dependent PKA in this process.

Conclusions—The present finding revealed a previously unidentified role of klotho in regulating Nox2 protein expression in RASM cells. Klotho not only downregulated Nox2 protein expression and intracellular superoxide production but also attenuated AngII-induced superoxide production, oxidative damage, and apoptosis. The klotho-induced suppression of Nox2 protein expression may be mediated by the cAMP PKA pathway.

Keywords

klotho; Nox2; NADPH oxidase; superoxide; cAMP; PKA; smooth muscle cell

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Introduction

Klotho is a recently discovered anti-aging gene (Kuro-o *et al.* 1997). Mutation of klotho gene expedites the aging process and shortens the life-span in mice (Kuro-o *et al.* 1997; Wang & Sun 2009a). Interestingly, over-expression of klotho slows the aging process and extends the life-span in mice (Kurosu *et al.* 2005). Klotho has two transcripts: a single-pass transmembrane protein and a secreted protein (Matsumura *et al.* 1998; Shiraki-Iida *et al.* 1998). The transmembrane klotho can be released into the circulation when its short transmembrane domain is removed by ectodomain shedding (Kuro-o 2008). Although klotho is mainly expressed in distal convoluted tubules in the kidneys and choroid plexus in the brain (Kuro-o *et al.* 1997), but it functions as a circulating hormone (Imura *et al.* 2004; Wang & Sun 2009a) and gets access to vascular smooth muscle cells (VSMCs).

The NAD(P)H oxidase was originally found in the plasma membrane of phagocytes which generates superoxide to participate in host defense by killing or destroying invading microbes. Recent studies indicated that the NAD(P)H oxidase is the primary source of reactive oxygen species (ROS) in vasculature (Griendling *et al.* 2000; Touyz & Schiffrin 2004; Tain & Baylis 2006). Membrane NADPH oxidases could generate superoxide (O_2^-) by transferring the electron to O_2. Nox2 (gp91^{phox}) and its homologs (Nox1, Nox4, and Nox5) are catalytic subunits of the NAD(P)H oxidase. Superoxide or ROS modulates the downstream signaling molecules by altering the intracellular redox state and by oxidative modification of proteins (Touyz 2004). In physiological condition, the NAD(P)H oxidase-derived superoxide is generated in a controlled manner at low concentrations and functions as a signaling molecule regulating VSMC contraction relaxation and VSMC growth (Rao & Berk 1992; Cosentino *et al.* 1994; Zafari *et al.* 1998; Touyz & Schiffrin 1999). But excess superoxide production could lead to increased contractility, VSMC growth and apoptosis, which have been implicated in hypertension, atherosclerosis, and diabetes (Rao & Berk 1992; Harrison 1997; Pawlak *et al.* 2004; San Martin *et al.* 2007). Nox-derived ROS are involved in a variety of oxidative stress damage and vascular diseases (Bedard & Krause 2007). It was found Nox2 deficiency reduces vascular inflammation, cellular proliferation, and neointimal thickening after experimental angioplasty (Chen *et al.* 2004; Carlstrom *et al.* 2009).

The prevalence of hypertension and vascular dysfunction is increased in the aged population (Burt *et al.* 1995; Ong *et al.* 2007) whereas the level of klotho is decreased in the aged population (Xiao *et al.* 2004). An increase in superoxide production or oxidative stress plays a role in vascular dysfunction and aging. Therefore, it is important to know if klotho has any effect on NAD(P)H oxidase expression and superoxide production. The purpose of this study was to test our hypothesis that klotho gene transfer decreases NAD(P)H oxidase expression and superoxide production in rat aorta smooth muscle (RASM) cells.

Method

Plasmid construction

The pAAV-MCS plasmid vector was purchased from Stratagene (Stratagene, La Jolla, CA, USA). The plasmid pAAV-mKlotho was constructed by inserting the mouse klotho full-length cDNA into the EcoRI and Xba I site of the pAAV-MCS expression vector. Plasmids were amplified in Escherichia coli DH5α cells, extracted by the alkaline lyses method, and purified using a Qiagen Endo-free Plasmid Maxi Kit (Qiagen Science, Maryland, USA). The quantity and quality of the purified plasmid DNA were assessed by determining the absorbance at 260 and 280 nm, and also by electrophoresis in agarose gels. The plasmids were dissolved in TE buffer before use.

Cell culture and transfection with pAAV-mKlotho

Rat aortic smooth muscle (RASM) cells (ATCC, Manassas, VA, USA) were cultured in DMEM (Cell Signaling, Danvers, MA, USA) supplemented with 10% fetal bovine serum (ATCC), 100 μg/ml of streptomycin (Sigma-Aldrich, Atlanta, GA, USA) and 100 U/ml of penicillin (Sigma-Aldrich, Atlanta, GA, USA) at 37°C, 5% CO_2. The cells were then transfected with either pAAV-lacZ or pAAV-mKlotho at 0, 2, 4, 8, 12 μg/well of using OptifectTM Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, followed by 48–96 h of incubation in DMEM medium.

Quantitative Real-time RT-PCR (QRT-RT-PCR)

Mouse klotho mRNA and rat Nox2 mRNA expression were analyzed using QRT-RT-PCR as we described previously (Sun *et al.* 2003; Sun 2006). For details, refer to the Online Data Supplement.

Western blot

Western blot analysis was performed as described previously (Wang *et al.* 2005; de Oliveira 2006; Wang *et al.* 2006). For details, refer to the Online Data Supplement.

Measurement of intracellular superoxide production

Intracellular superoxide production was assessed by fluorescence microscopy using the oxidation sensitive dye dihydroethidium (DHE, Sigma-Aldrich, Atlanta, GA, USA) as we described recently (Dammanahalli & Sun 2008; Wang & Sun 2010b). For details, refer to the Online Data Supplement.

Immunocytochemical analysis of 4-HNE staining

The immunocytochemical procedure was described in our previous studies (Wang *et al.* 2005; Dammanahalli & Sun 2008). For details, refer to the Online Data Supplement.

Annexin V staining and cell counting

Apopotosis was assessed by an Annexin V staining kit (Invitrogen, Carlsbad, CA, USA), USA) after cells were transfected with the klotho plasmid for 24 h followed by incubation with or without angiotensin II (200 nM) for 72 hours. The cells were then harvested and resuspended with Annexin binding buffer at a concentration of 1.0×10^6 cells/mL and analyzed using an automated cell counter (TC 10™, Bio-RAD, CA, USA). One hundred microlitres of the suspension was mixed with 5 μL Annexin V conjugate and propidium iodide (PI) incubated for 15 min at room temperature in the dark. After incubation, 400 μL of binding buffer was added to each tube and fluorescence data for cells were acquired using flow cytometry(Sternfeld *et al.* 2009).

Intracellular cAMPimmunoassay

Intracellualr cAMP was measured using a kit from BioVision (BioVision, Mountain View, CA, USA) according to the manufacturer's instruction. For details, refer to the Online Data Supplement.

Protein kinase assay

Protein kinase A (PKA) activity was measured using a PKA activity assay kit from EMD Biosciences (EMD Biosciences, Inc., Darmstadt, Germany) according to the manufacturer's instruction. For details, refer to the Online Data Supplement.

Statistical analysis

Data were analyzed using a two-way ANOVA or an unpaired Student's t-test, as appropriate probability values of < 0.05 were considered to be statistically significant. A regression analysis was used to analyze the dose-dependent response. A correlation analysis was used to analyze the correlation of klotho expression and Nox2 expression.

Results

Klotho gene transfer resulted in dose-dependent expression of mKL in the RASM cells

Transfer of mKL gene resulted in strong expression of transmembrane form klotho protein (130 kDa) and mRNA (710 bp) in RASM cells (Fig. 1A&B, Supplemental Figure S1 A&B, Fig. 2A). A quantitative and regression analysis showed that klotho protein was expressed in a dose-dependent manner following transfer of mKL plasmid (2–8 μg) (Figs. 1A&B). mKL at 8 μg resulted in a maximal expression of klotho protein while 12 μg of mKL did not further increase klotho expression. Klotho protein expression was not detectable in RASM cells transfected with pAAV-LacZ or in RASM cells without transfection (control) (Fig. 1A, S1A), indicating that the rat klotho protein may not be expressed in the RASM cells. The klotho antibody used can recognize both rat and mouse klotho protein (Wang & Sun 2009b).

Klotho gene transfer decreased Nox2 protein expression

To determine the effect of klotho expression on NADPH oxidases, we measured protein expression of Nox family members using western blot analysis. Klotho gene transfer selectively decreased Nox2 protein expression (Fig 1A&C, S2A), the major membrane-bound NADPH oxidase. A correlation analysis showed that klotho gene transfer decreased Nox2 protein expression in a dose-dependent manner. However, Nox2 mRNA expression was not altered significantly by klotho gene transfer (Fig. 2B), suggesting that klotho down-regulated Nox2 protein expression at the post-transcriptional level. In contrast, protein expression of Nox1 was not altered significantly by the klotho gene transfer (Fig. 2C&D). Nox4 protein expression was not detectable in RASM cells (S2B).

Klotho gene transfer attenuated intracellular superoxide production in RASM cells

The intracellular superoxide production in RASM cells were assessed by the oxidation of DHE. Klotho gene transfer significantly decreased the basal level of superoxide production (Fig. 3A&B). AngII increased superoxide production in RASM cells (Fig. 3A&B). Interestingly, klotho gene expression significantly attenuated AngII-induced superoxide production (Fig. 3A&B).

Klotho gene transfer decreased angiotensin II-induced oxidative stress and apoptosis

4 Hydroxynonenal (4-HNE) is a by-product of lipid peroxidation and serves as a marker of oxidativestress.(Wei *et al.* 2008) Klotho gene transfer decreased the basal level of 4-HNE protein level (Fig. 4A&B). AngII significantly increased the 4-HNE expression. Klotho gene transfer abolished the AngII-induced increase in 4-HNE expression (Fig. 4A&B), suggesting that klotho decreased oxidative stress-related cell damage. The immuncytochemical analysis showed that klotho gene transfer decreased the basal level of 4-HNE and prevented the AngII-induced oxidative damage (Fig. 4C&D).

Klotho gene transfer significantly decreased the number of apoptotic cells (Fig. 4E). AngII significantly increased apoptosis (Fig. 4E). Klotho gene transfer significantly attenuated AngII-induced apoptosis (Fig. 4E)

Klotho gene transfer increased intracellular cAMP production and PKA activity

To assess the intracellular mechanism that mediates the inhibitory effect of klotho on Nox2 protein expression, we measured cAMP and PKA activity in RASM cells. Klotho gene transfer dose-dependently increased cAMP production and PKA activity (Fig. 5A&B). The intracellular cAMP level and PKA activity were doubled by klotho gene transfer (8 μg mKL) (Fig. 5A&B). The increase in intracellular cAMP parallels with the increase in PKA activity. Interestingly, pretreatment with Rp-cAMP, a competitive inhibitor of cAMP, prevented the klotho-induced increase in PKA activity, suggesting that the increase in PKA activity is cAMP-dependent.

Rp-cAMP abolished klotho-induced inhibition of Nox2 protein expression

Klotho gene transfer dose-dependently increased klotho expression which, however, failed to decrease Nox2 protein expression in RASM cells pretreated with Rp-cAMP (Fig. 6A,B,C), an inhibitor of cAMP-dependent PKA. This result suggested that klotho-induced inhibition of Nox2 protein expression may be mediated by the cAMP-PKA pathway (Fig. 6).

Discussion

The present study reveals that mouse klotho gene transfer significantly decreased superoxide production in rat aortic smooth muscle (RASM) cells (Fig. 3). The suppressing effect of klotho gene transfer on superoxide production may be attributed, at least in part, to the downregulation of Nox2 protein expression. Indeed, klotho gene transfer dose-dependently decreased Nox2 protein expression in RASM cells (Fig. 1). The finding that klotho suppressed Nox2 protein expression in RASM cells is interesting, which suggests, for the first time, that klotho may regulate superoxide generation in vascular smooth muscle cells (SMCs). It is noted that klotho expression selectively downregulated Nox2 protein expression while Nox1 was not affected (Fig. 2C&D). Although the detailed molecular mechanism of the selective inhibition requires an additional study, it seems that klotho may downregulate Nox2 protein expression at the post-transcriptional level because klotho gene expression did not alter Nox2 mRNA expression (Fig. 2).

A further mechanistic investigation revealed that the klotho-induced suppression of Nox2 protein expression may be mediated by the cAMP-PKA pathway. This interesting observation is based on the following new findings: (1) Klotho gene transfer dose-dependently increased the intracellular cAMP level and the PKAactivity in RASM cells; (2) The activation of PKA is cAMP-dependent, i.e., cAMP-PKA; (3) Inhibition of the cAMP-PKA pathway by the Rp-cAMP abolished the klotho-induced decrease in Nox2 protein expression. Therefore, the present results suggest a new pathway that may mediate klotho-induced inhibition of Nox2 expression, i.e., klotho ↑ → cAMP ↑ → PKA ↑ → Nox2 protein expression ↓. PKA is normally inactive, but becomes activated upon binding to cAMP. The activated PKA phosphorylates a number of other proteins, many of which are also enzymes that are either activated or suppressed upon phosphorylation. It seems that the phosphorylated proteins may inhibit the translation of Nox2 or increase the degradation of Nox2 protein. This hypothesis, however, needs to be tested.

Nox2 is widely expressed in the vasculature (Bedard & Krause 2007). The Nox2 NAD(P)H oxidase is the major source of ROS in the vasculature (Dworakowski *et al.* 2008). Growing evidence suggests that AngII acts as one of the most important vasoactive factors in regulating vascular NAD(P)H oxidase (Berry *et al.* 2000; Griendling *et al.* 2000; Touyz *et al.* 2002). Nox2 can be activated by AngII and may play an important role in AngII-induced hypertension (Li & Shah 2003). Excess superoxide or ROS leads to oxidative stress damage which is involved in various cardiovascular diseases (Landmesser & Harrison 2001; Zalba *et*

al. 2001). Our recent study indicates that the *in vivo* klotho gene delivery may attenuate the progression of spontaneous hypertension (Wang & Sun 2009b).

Klotho is mainly expressed in kidneys and brain choroid plexus (Wang & Sun 2009a; Wang & Sun 2010a). However, the kidney-derived klotho circulates and may regulate vascular function (Wang & Sun 2009a). Although klotho was reported to protect against endothelial function (Saito *et al.* 1998; Saito *et al.* 2000; Wang & Sun 2009a), this is the first study demonstrating that klotho may have beneficial effect in the SMCs. Interestingly, klotho not only decreased the basal superoxide level but also attenuated AngII-induced superoxide production in RASM cells (Fig. 3). Further, klotho gene transfer decreased oxidative damage as evidenced by a significant decrease in the level of 4-HNE (Fig. 4), a by-product of lipid peroxidation of cell membrane. Importantly, klotho gene expression prevented AngII-induced apoptosis (Fig. 4), suggesting that klotho may protect RASM cells from oxidative damage. The present finding points to a new direction for understanding how klotho may protect vascular SMCs. Klotho was discovered to have anti-aging effect (Kuro-o *et al.* 1997; Kurosu *et al.* 2005; Wang & Sun 2009a). In humans, the level of circulating klotho decreases after 40 years of age (Xiao *et al.* 2004). Aging is associated with increased prevalence of vascular disorders such as endothelial dysfunction, arterial stiffening, and hypertension (Burt *et al.* 1995; Ong *et al.* 2007). The suppressing action of klotho on oxidative stress in SMCs could contribute to its anti-aging effect.

Activation of the cAMP-PKA pathway by klotho was reported in HUVECs (Yang *et al.* 2003; Rakugi *et al.* 2007), which leads to up-regulation of angiotensin-I converting enzyme (ACE) and potentially increases AngII production (Yang *et al.* 2003). The ability of klotho to suppress AngII-induced oxidative stress in SMCs may counteract the increased AngII release from endothelial cells and contribute to prevention of vascular damage. Because klotho may increase the cAMP-dependent PKA activity in endothelial and smooth muscle cells, a further study is warranted to assess if this effect is due to activation of adenylate cyclase, a key enzyme for generating cAMP.

In summary, this present findings demonstrated a previously unidentified role of klotho in regulating Nox2 protein expression in RASM cells. This study further revealed that klotho decreased Nox2 protein expression through the cAMP-PKA-dependent pathway. In addition, klotho inhibited AngII-induced superoxide production and oxidative stress thereby attenuating apoptosis. Therefore, klotho gene transfer may offer a protection in smooth muscle cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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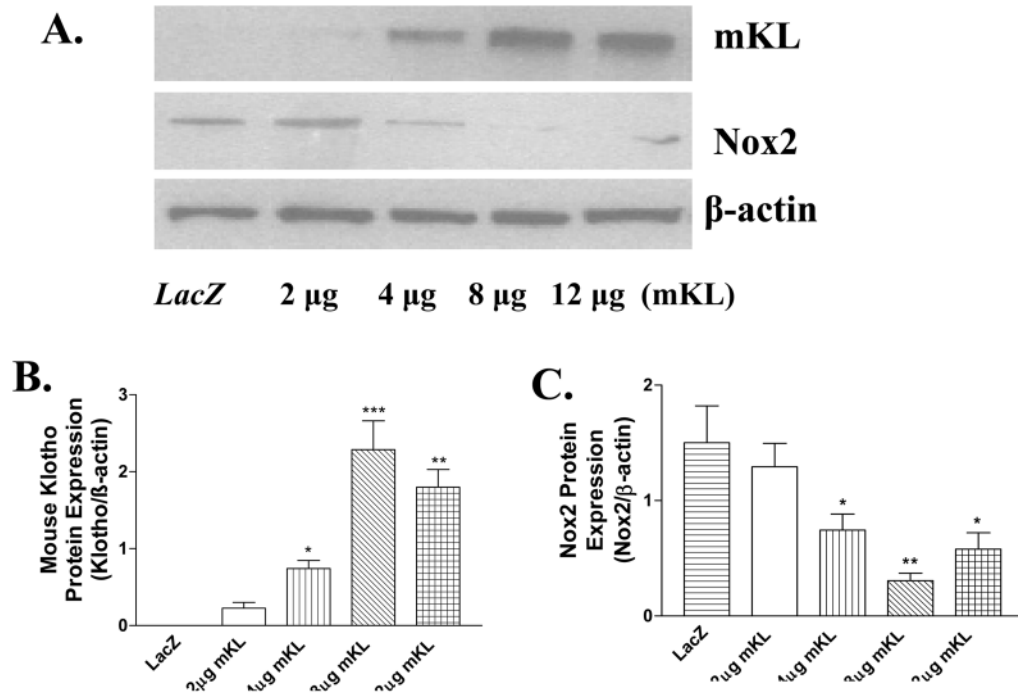
Figure 1.
Mouse klotho (mKL) gene transfer decreased Nox2 protein expression in RASM cells. **A,** western blot bands of mouse klotho protein (transmembrane form) and Nox2 protein expression. **B,** Quantitative analysis of mouse klotho (mKL) protein expression. **C,** Quantitative analysis of Nox2 protein expression. Klotho protein and Nox2 protein were measured 48 hours following mKL klotho gene transfer. Values=means±SEM. *p<0.05, **p<0.01, ***p<0.001 *vs* the LacZ group. N=3 independent experiments with 3 measurements each.
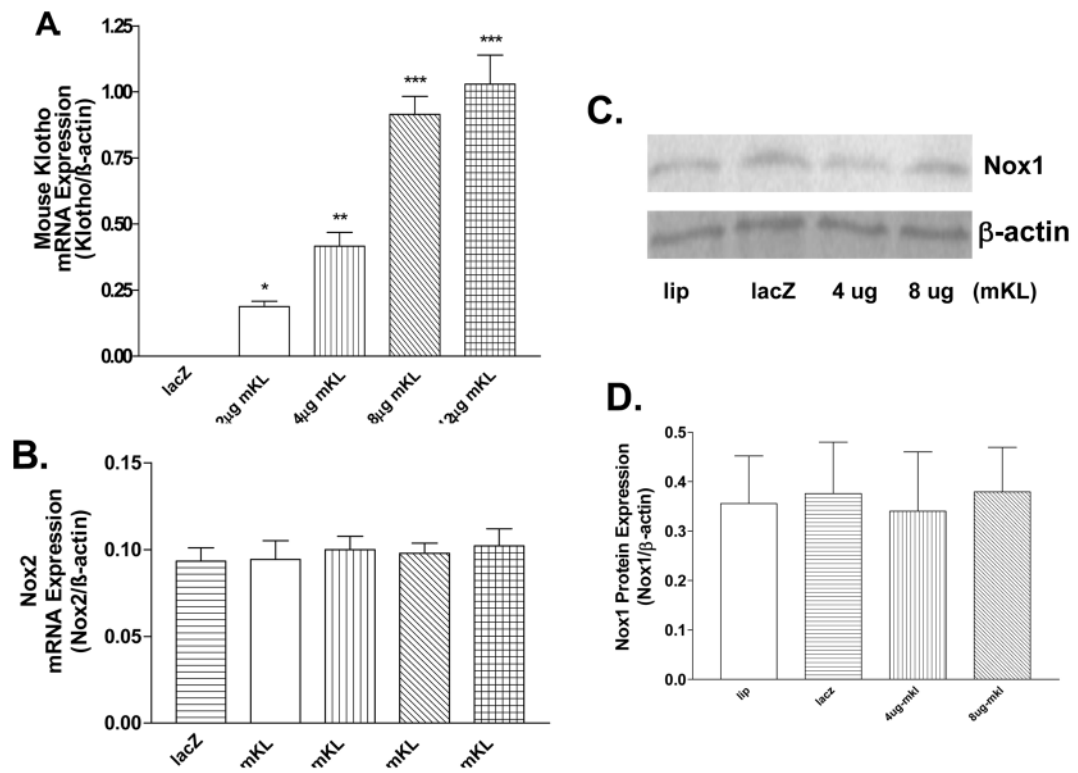
Figure 2.
mKL gene transfer did not alter Nox2 mRNA expression or Nox1 protein expression in
RASM cells. **A,** Quantitative real-time RT-PCR (QRT-RT-PCR) analysis of mKL mRNA
expression. **B,** QRT-RT-PCR analysis of rat Nox2 mRNA expression. mKL mRNA and rat
Nox2 mRNA expression were measured 48 hours following mKL gene transfer. **C,** Nox1
protein expression. **D,** Quantification of Nox1 protein expression. Values=means±SEM.
*p<0.05, **p<0.01, ***p<0.001 *vs* the LacZ group. N= 3 independent experiments with 3
measurements each.

Mouse klotho gene delivery attenuated intracellular superoxide production
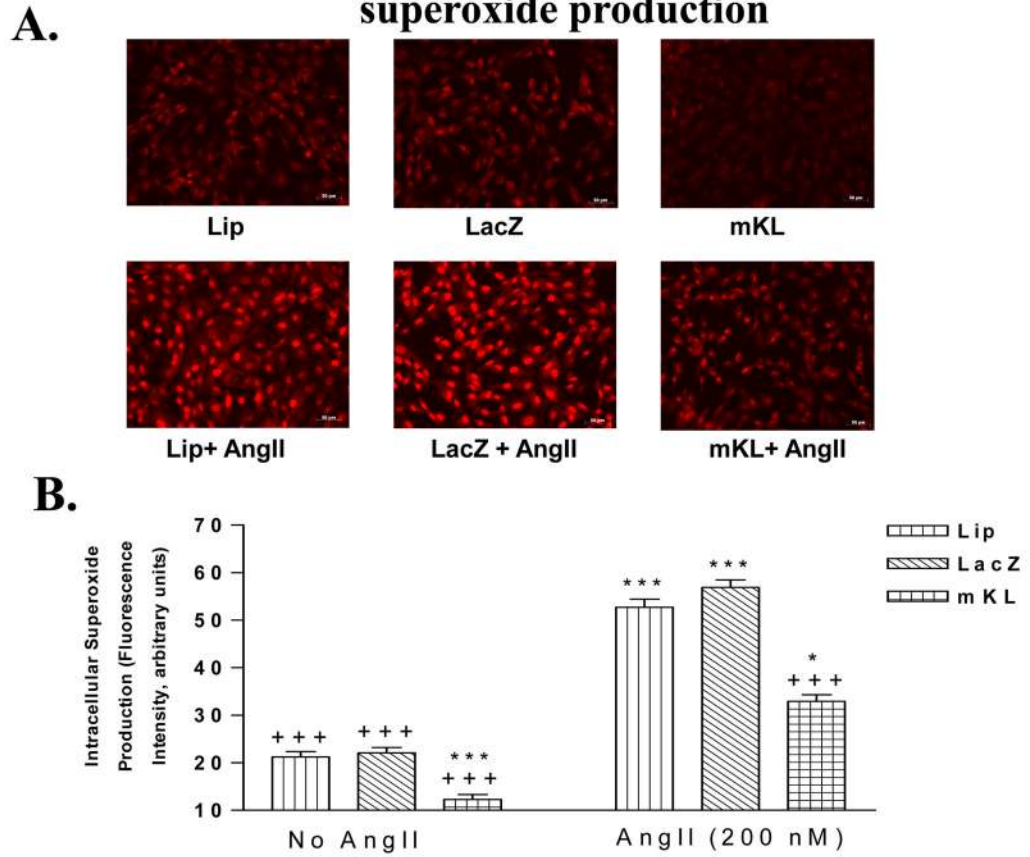

Figure 3.
mKL gene transfer attenuated the basal intracellular superoxide level and angiotensin II-induced superoxide production in RASM cells. **A,** Photomicrograph of DHE staining of superoxide production in RASM cells transfected with klotho for 24 h followed by incubation in the presence or absence of angiotensin II (200 nM) for 24 h. **B,** Quantitative analysis of superoxide production. Lip, lipofectamine. Values=means±SEM. ***p<0.001 *vs* the LacZ group (No AngII); +p<0.05, +++p<0.001 *vs* the LacZ+AngII group. N=3 independent experiments with 3 measurements each.
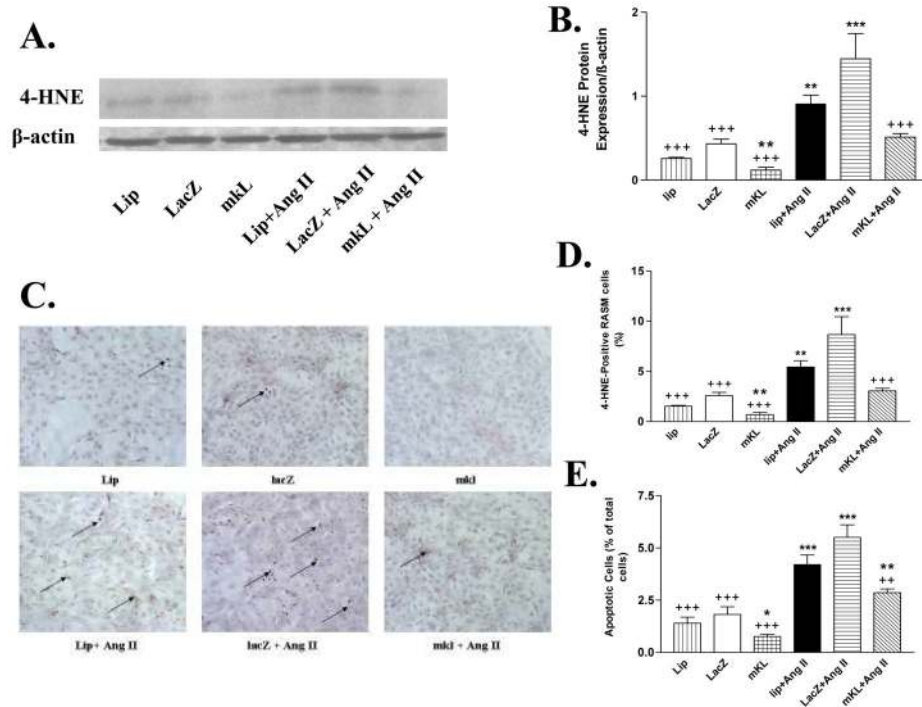
Figure 4.
mKL gene transfer attenuated AngII-induced oxidative stress and apoptosis in RASM cells.
A, Western blot bands of 4-HNE protein (lipid peroxidation product, oxidative stress
marker) in RASM cells transfected with the klotho plasmid for 24 h followed by incubation
with or without angiotensin II (200 nM) for 72 hours. **B,** Quantitative analysis of 4-HNE
protein expression. **C,** Photomicrograph of 4-HNE staining in RASM cells. Arrows indicate
4-HNE-positive cells. **D,** Quantitative analysis of 4-HNE-positive cells. **E,** Quantitative
analysis of apoptotic cells (Annexin V). Values=means±SEM. *p<0.05, **p<0.01,
***p<0.001 *vs* the LacZ group; ++p<0.01, +++p<0.001 *vs* the LacZ+AngII group. N=3
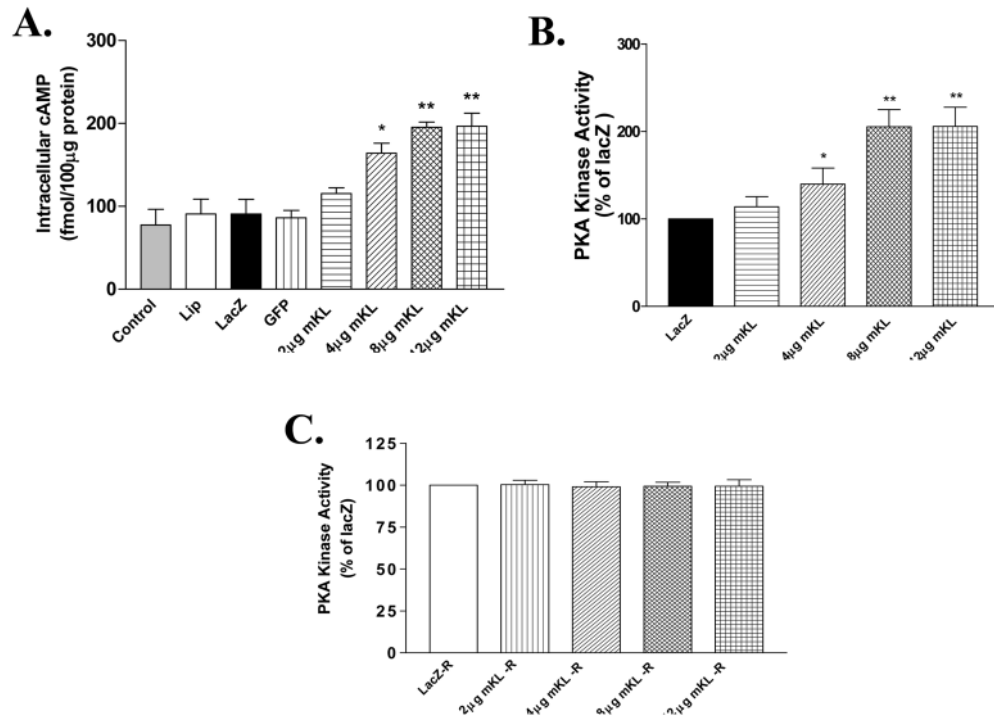independent experiments with 3 measurements each.

Figure 5.
mKL gene transfer increased intracellular cAMP and PKA activity in RASM cells. **A,** Intracellular cAMP level measured at 48 hours after mKL gene transfer. **B,** PKA activity. **C,** PKA activity measured in RASM cells pre-treated with Rp-cAMP (10^{-3} mol/L) followed by klotho gene transfer. Values=means±SEM. *p<0.05, **p<0.01 *vs* the LacZ group. N=3 independent experiments with 3 measurements each.
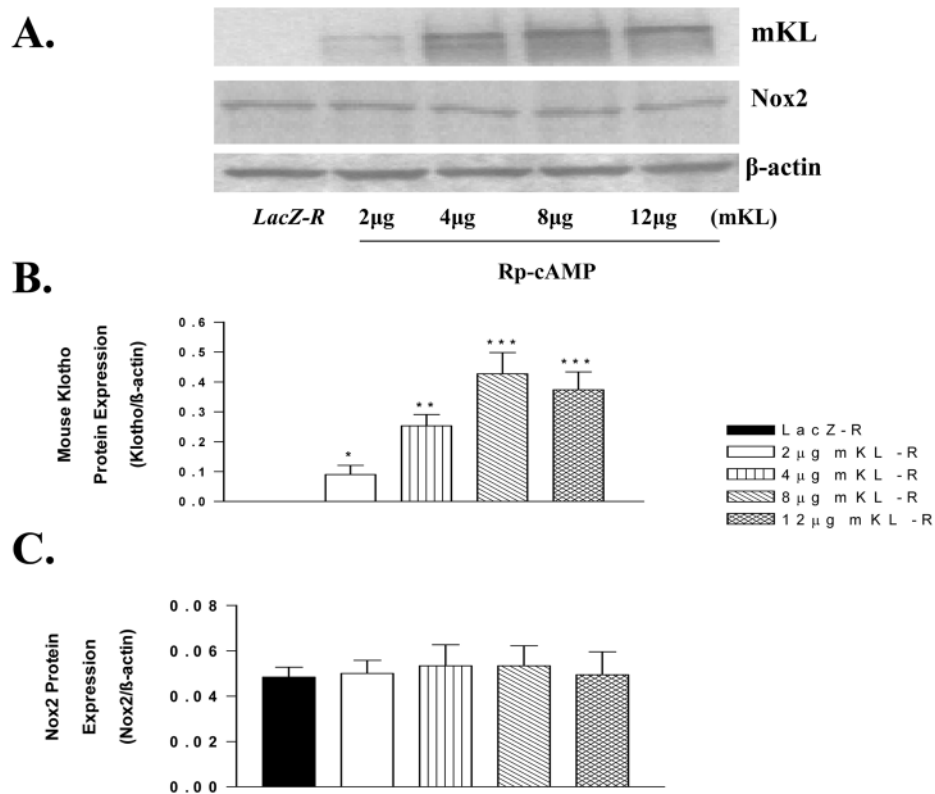
Figure 6.
Inhibition of PKA activity by Rp-cAMP abolished mKL-induced inhibition of Nox2
expression. **A,** Western blot bands of mKL and Nox2 protein expression at 48 hours
following treatment with Rp-cAMP (10 μM) and mKL gene transfer. **B,** Quantitative
analysis of mKL protein expression. **C,** Quantitative analysis of Nox2 protein expression.
Rp-cAMP (10 μM) is a common concentration used to inhibit protein kinase A.
Values=means±SEM. *p<0.05, **p<0.01, ***p<0.001 *vs* the LacZ group. N=3 independent
experiments with 3 measurements each.