Kallistatin is a plasma protein that exhibits pleiotropic effects in vasodilation, anti-angiogenesis, and anti-inflammation. To isolate a kallistatin-binding protein that mediates the vascular actions of kallistatin, we screened and identified a positive clone from a human heart cDNA expression library by using an alkaline phosphatase-kallistatin fusion protein binding assay. Sequence analysis revealed that kallistatin-binding protein is human Kruppel-like factor 4 (KLF4). KLF4 was localized on the plasma membrane of HEK-293 cells and endothelial cells over-expressing KLF4. KLF4 and kallistatin complex formation was identified in endothelial cells by immunoprecipitation followed by immunoblotting. We showed that kallistatin inhibits tumor necrosis factor-α-induced NF-κB activation, as well as vascular cell adhesion molecule-1 and monocyte chemoattractant protein-1 expression in endothelial cells, whereas knockdown of KLF4 by small interfering RNA oligonucleotide abolished the effect of kallistatin. Kallistatin increased endothelial nitric-oxide synthase (eNOS) expression and nitric oxide levels, and these effects were also blocked by KLF4 small interfering RNA small interfering RNA oligonucleotide. Moreover, inhibition of eNOS by RNA interference or by NOS inhibitor abolished the blocking effect of kallistatin on vascular cell adhesion molecule-1 and monocyte chemoattractant protein-1 expression. In summary, we identified KLF4 as a kallistatin-binding protein, which has a novel role in mediating the anti-inflammatory actions of kallistatin via increasing eNOS expression in endothelial cells. This study provides a new target for modulating endothelial function in vascular disease.

**EXPERIMENTAL PROCEDURES**

**Materials**—Rapid screen arrayed cDNA library panel LHT-1001 (human heart library DNA) and subplate LHT-10A were purchased from Origene Technologies (Rockville, MD). Alkaline phosphatase vector was obtained from GenHunter (Nashville, TN). TNF-α, Nω-nitro-l-arginine methyl ester, p-nitrophenyl phosphate and PKH26 red fluorescent cell membrane marker were purchased from Sigma. Scrambled, KLF4 and eNOS siRNA oligonucleotides and DharmaFECT1 transfect reagent were purchased from Dharmacon (Lafayette, CO). A human umbilical vein endothelial cell (HUVEC) nucleofector kit was purchased from Amaxis (Walkersville, MD). Luciferase assay system was from Promega (Madison, WI). TRIZol reagent

2 The abbreviations used are: TNF, tumor necrosis factor; KLF, Kruppel-like factor; eNOS, endothelial nitric-oxide synthase; VCAM, vascular cell adhesion molecule; siRNA, small interfering RNA; HUVEC, human umbilical vein endothelial cell; AP, alkaline phosphatase; KS, kallistatin; PBS, phosphate-buffered saline; MCP, monocyte chemoattractant protein.
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was purchased from Invitrogen. MCP-1 enzyme-linked immunosorbent assay kit was from R & D (Minneapolis, MN). eNOS polyclonal antibody was from Cell Signaling (Danvers, MA). VCAM-1 and KLF4 polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Human kallistatin monoclonal antibody was produced as previously described (13). Recombinant human kallistatin was purified from Escherichia coli as previously described (2).

Cell Culture—HUVECs were acquired from Cambrex BioScience and cultured in endothelial cell basal medium-2 supplemented with EGM-2 singleQuot kit (Clonetics) in a 5% CO2 incubator at 37 °C. HEK-293 cells were isolated from American Type Culture Collection and cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and 1% penicillin/streptomycin.

Expression of Alkaline Phosphatase-Kallistatin (AP-KS) Fusion Protein—Full-length cDNA of human kallistatin was inserted into the linearized AP vector. The AP-KS construct was verified by DNA sequencing. AP-KS vector was transfected into HEK-293 cells using Effectene transfection reagent and cultured with the serum-free Opti-MEM medium. The medium was collected 72 h later and buffered with 10 mM HEPES, pH 7.0. The expression of the fusion protein was confirmed by Western blotting with an anti-kallistatin monoclonal antibody (13) and by AP enzyme activity assay (14).

AP-KS Binding to HUVECs—To detect AP-KS binding, HUVECs were washed with Hanks’ balanced salt solution containing 20 mM HEPES and 1 mg/ml bovine serum albumin. The plates were then incubated with AP-KS in Dulbecco’s modified Eagle’s medium containing 20 mM HEPES and 1 mg/ml bovine serum albumin for 2 h at 4 °C. The bound AP-KS was extracted, and AP activity was colorimetrically quantified by using p-nitrophenyl phosphate as substrate after heat inactivation of endogenous AP as described (14). Nonspecific binding was determined by measuring binding in the presence of a 100-fold molar excess of recombinant KS lacking the AP fusion protein. Specific binding was determined by subtracting the nonspecific binding from total binding. For determination of apparent $K_d$ the binding of AP-KS was measured in triplicate as described above, and the $K_d$ value was quantified by the Scatchard plot program of GraphPad prism using the ratio of bound ligand to free ligand as the $y$ axis and bound ligand (pM) as the $x$ axis.

Screening and Identification of Kallistatin-binding Protein—For screening kallistatin-binding protein, pools of 5,000 arrayed clones from a human heart CDNA library were transfected into HEK-293 cells, and AP-KS fusion protein was used for identifying positive clones. To detect AP-KS binding, transfected HEK-293 cells were washed with Hanks’ balanced salt solution and then incubated with AP-KS fusion protein in Dulbecco’s modified Eagle’s medium containing 20 mM HEPES and 1 mg/ml bovine serum albumin for 2 h at 4 °C. The bound AP-KS was detected using the blue substrate kit (15). The blue staining was examined by microscopy. Alternatively, the bound AP-KS was extracted, and AP activity was colorimetrically quantified using p-nitrophenyl phosphate described (14). The positive clone was identified by DNA sequencing analysis.

Immunoprecipitation and Western Blotting—Immunoprecipitation of kallistatin complex to its binding protein was performed as previously described (16). Briefly, protein concentrations were determined for the whole cell extract of HUVECs using the Lowry assay. Six hundred micrograms of protein were precleared with protein A-Sepharose beads (20 μl) for 1 h at 4 °C with rotation. Protein A-Sepharose was pelleted by centrifugation, and a fixed volume of lysate was added to a new microcentrifuge tube followed by the addition of 5 μg of anti-kallistatin monoclonal antibody to each tube. The next day, 50 μl of protein A beads was added to the tubes and allowed to incubate for 1 h at 4 °C. The immune complex was pelleted by centrifugation, and the pellets were then washed three times in phosphate-buffered saline. The cells were then eluted by boiling in SDS sample buffer and subjected to SDS-polyacrylamide gel electrophoresis. Interacting proteins were identified by Western blotting with anti-KLF4 antibody. The whole cell extract of HUVECs was also immunoprecipitated with anti-KLF4 antibody followed by immunoblotting with anti-kallistatin antibody.

Confocal Microscopy—HUVECs were incubated with TNF-α (10 ng/ml) for 16 h to up-regulate KLF4 expression as previously described (17). The cells were then incubated with blocking buffer (3% bovine serum albumin in PBS) for 30 min, washed twice with PBS, and incubated with KLF4 antibody (2 μg/ml) for 45 min on ice. The cells were washed twice with ice-cold PBS, and then incubated with fluorescein isothiocyanate-conjugated anti-rabbit IgG (1:100 dilution in blocking buffer) for 45 min. The cells were washed three times and then incubated with fluorescent dye PKH26 (4 μM) for 2 min to stain the plasma membrane. The cells were washed three more times in PBS and observed under a Carl Zeiss LSM5 Pascal confocal microscope.

siRNA Oligonucleotide Transfection—RNA interference was used to knock down KLF4 and eNOS expression in HUVECs. The siRNA oligonucleotides were transfected into cells using DharmaFECT1 transfection reagent according to the manufacturer’s instructions. As a control, the cells were also transfected with scrambled siRNA oligonucleotides. Successful knock-down of target gene was verified by quantitative PCR and Western blotting.

RNA Extraction and Quantitative PCR—Total RNA was isolated from cultured cells with TRIzol following the manufacturer’s protocol. Total RNA was reverse-transcribed using the high capacity cDNA reverse transcription kit. Real time quantitative reverse transcription-PCR was performed with the TaqMan gene expression assay for genes of interest and were normalized against 18 S RNA using an ABI 7300 real time PCR system. The gene expression assays used were as follows: Hs00358836_ml for KLF4, Hs0167166_ml for eNOS, Hs00365486_ml for VCAM-1, and Hs00234140_ml for MCP-1.

Nitrate/Nitrite Detection in the Medium—Endothelial NO production was determined in culture medium as previously described (18). After 24 h of incubation with different doses of kallistatin (0.1–0.4 μM), the culture medium was collected and measured by a fluorometric assay for nitrate/nitrite, the stable breakdown products of NO. The quantity of nitrite production was normalized to total protein in the medium.

Enzyme-linked Immunosorbent Assay of MCP-1—HUVECs transfected with KLF4 or scrambled siRNA oligonucleotide
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To screen for the binding protein, we examined the binding of AP-KS fusion protein to the surface of endothelial cells. Saturation binding of AP-KS to HUVECs is shown in Fig. 1. Scatchard plot analysis of the binding data revealed the presence of high affinity binding sites on endothelial cells for kallistatin with an estimated $K_d$ of 7.6 nM (Fig. 1C). 

Interaction of Kallistatin with HUVEC Surface Protein—To identify the specific binding protein for kallistatin, we used the recombinant AP-KS fusion protein to screen a full-length cDNA expression library from human heart. HEK-293 cells were transfected with cDNA pools each consisting of 5,000 clones. The positive clone expressing kallistatin-binding protein on the surface of HEK-293 cells was identified by binding to AP-KS fusion protein and subsequent AP activity assay. HEK-293 cells did not bind AP-KS (Fig. 2A). After several rounds of screening, a single clone was isolated. Sequence analysis identified the kallistatin-binding protein as human KLF4. HEK-293 cells transfected with the cDNA encoding KLF4 selectively bound to AP-KS, but not AP (Fig. 2A). KLF4 was localized on the cell surface in HEK-293 cells transfected with KLF4 plasmid (data not shown). Moreover, localization of KLF4 on HUVECs was examined under confocal microscope. HUVECs were stimulated with inflammatory cytokine to up-regulate KLF4 expression. KLF4 expression on endothelial cell surface was identified by immunocytochemistry. Representative images showed co-localization of KLF4 immunostaining and PKH26 membrane fluorescence dye in nonpermeabilized endothelial cells (Fig. 2B). These results indicate that KLF4 can distribute to the plasma membranes of cells overexpressing KLF4.

Identification of KLF4-Kallistatin Complex Formation in Endothelial Cells—Immunostaining and flow cytometry analyses confirmed the expression of kallistatin in endothelial cells (data not shown). Kallistatin and KLF4 complex formation in endothelial cells was identified by immunoprecipitation and Western blot. The cell lysate was subjected to immunoprecipitation using an anti-KLF4 antibody, followed by immunoblotting with monoclonal anti-kallistatin antibody (Fig. 2C). Kallistatin formed a complex with KLF4, which was pulled down by the anti-KLF4 antibody but not by the control antibody. Complex formation was also detected when the lysate was immuno-

were stimulated with TNF-α (10 ng/ml) in serum-free medium with or without kallistatin pretreatment (0.4 μM). Twenty-four hours later, secreted MCP-1 in the culture medium was determined by enzyme-linked immunosorbent assay (19).

NF-κB Luciferase Assay—One microgram of NF-κB-driven luciferase plasmid was transfected into HUVECs with a HUVEC nucleofector kit according to the manufacturer’s instructions. All of the transfections were performed in triplicate. Luciferase activity was measured using the luciferase assay system according to the manufacturer’s instructions. The light produced was measured using a luminometer. Luciferase activity was normalized to total cell proteins (7).

Statistical Analysis—All of the data are presented as the means ± S.E. Comparison between groups was made using one-way analysis of variance with the Fisher multiple comparison test. A probability value of $p < 0.05$ was considered statistically significant. All of the experiments were performed in triplicate on at least two separate occasions.

RESULTS

Construction of AP-KS Vector and Generation of AP-KS Fusion Protein—AP-KS vector was generated by inserting full-length cDNA of human kallistatin into AP vector. The orientation and validity of the clone was determined by DNA sequencing. Kallistatin was fused at the 5′ end with alkaline phosphatase sequence in the correct reading frame. Secretion of AP-KS fusion protein into the medium of HEK-293 cells transfected with the plasmid DNA was identified by Coomassie Blue staining and Western blotting (Fig. 1A).

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Screening and Identification of KLF4 as Kallistatin-binding Protein from a Human Heart cDNA Library—To identify the specific binding protein for kallistatin, we used the recombinant AP-KS fusion protein to screen a full-length cDNA expression library from human heart. HEK-293 cells were transfected with cDNA pools each consisting of 5,000 clones. The positive clone expressing kallistatin-binding protein on the surface of HEK-293 cells was identified by binding to AP-KS fusion protein and subsequent AP activity assay. HEK-293 cells did not bind AP-KS (Fig. 2A). After several rounds of screening, a single clone was isolated. Sequence analysis identified the kallistatin-binding protein as human KLF4. HEK-293 cells transfected with the cDNA encoding KLF4 selectively bound to AP-KS, but not AP (Fig. 2A). KLF4 was localized on the cell surface in HEK-293 cells transfected with KLF4 plasmid (data not shown). Moreover, localization of KLF4 on HUVECs was examined under confocal microscope. HUVECs were stimulated with inflammatory cytokine to up-regulate KLF4 expression. KLF4 expression on endothelial cell surface was identified by immunocytochemistry. Representative images showed co-localization of KLF4 immunostaining and PKH26 membrane fluorescence dye in nonpermeabilized endothelial cells (Fig. 2B). These results indicate that KLF4 can distribute to the plasma membranes of cells overexpressing KLF4.

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precipitated with an anti-kallistatin antibody and immunoblotted with an anti-KLF4 antibody (Fig. 2C).

KLF4 Mediates Kallistatin Inhibition of NF-κB Activation and Inflammatory Mediator Expression—To assess the role of KLF4 on the anti-inflammatory effect of kallistatin, RNA interference was used to knock down KLF4 expression in cultured endothelial cells. KLF4 siRNA oligonucleotide effectively inhibited KLF4 mRNA and protein expression (Fig. 3, A and B). TNF-α-induced NF-κB activation was significantly inhibited upon kallistatin treatment, but KLF4 siRNA oligonucleotide abolished the effect of kallistatin (Fig. 3C). Moreover, VCAM-1 expression was significantly elevated after TNF-α treatment, whereas kallistatin pretreatment prevented up-regulation of these pro-inflammatory genes. However, the effects of kallistatin were blocked by KLF4 siRNA oligonucleotide (Fig. 4, A and B). Similarly, the effect of kallistatin on inhibiting MCP-1 expression was blocked by KLF4 siRNA (Fig. 4, C and D). These data suggest that kallistatin inhibits endothelial inflammatory mediator expression through interaction with KLF4.

KLF4 Mediates the Anti-inflammatory Effect of Kallistatin through Promotion of eNOS Expression—In cultured endothelial cells, kallistatin significantly increased eNOS levels and NO formation in a dose-dependent manner (Fig. 5, A–C). KLF4 knockdown abolished the effect of kallistatin on eNOS expression (Fig. 5D). Moreover, N'-nitro-L-arginine methyl ester, an inhibitor of NOS, abolished the inhibitory effect of kallistatin on TNF-α-induced VCAM-1 and MCP-1 expression (Fig. 6). Furthermore, the effect of eNOS on pro-inflammatory protein expression was assessed by eNOS knockdown in endothelial cells under basal conditions and TNF-α stimulation. eNOS siRNA oligonucleotides effectively inhibited eNOS mRNA and protein expression (Fig. 7A). Knockdown of eNOS expression abolished the inhibitory effect of kallistatin on TNF-α-induced MCP-1 and VCAM-1 expression (Fig. 7, B and C). Taken together, these findings indicate that KLF4 mediates the anti-inflammatory effect of kallistatin through increased eNOS expression and NO production in endothelial cells.
We have demonstrated that KLF4 is a novel mediator of the anti-inflammatory action of kallistatin in endothelial cells. Among more than 500,000 cDNA clones in a human heart cDNA library, we have screened and identified KLF4 to be a kallistatin-binding protein. The complex formation between kallistatin and KLF4 was further confirmed by immunoprecipitation and Western blot in endothelial cells. Moreover, we have shown that kallistatin inhibited TNF-α (10 ng/ml) -induced NF-κB activation and limited the expression of pro-inflammatory mediator expression in endothelial cells. Kallistatin stimulated eNOS expression and NO formation, whereas inhibition of KLF4 by siRNA oligonucleotides abolished the effects of kallistatin, indicating a role for KLF4 in mediating the anti-inflammatory action of kallistatin. These combined results indicate that kallistatin is a new anti-inflammatory agent and that the action of kallistatin is mediated by KLF4 through eNOS and NO production.

KLF4 was initially characterized as an epithelially specific transcription factor (20–22). Subsequent studies have shown that KLF4 is expressed in a variety of arterial and venous endothelial cells and plays a role in regulating vascular biology (13, 23). As a transcription factor, KLF4 is predominately localized to the cytoplasm and nucleus (21, 23). We employed an AP-KS fusion protein-based enzymatic activity assay to screen a human heart expression library to show that KLF4 is a kallistatin-binding protein. We demonstrated that KLF4 was localized on the plasma membrane of HEK-293 cells and endothelial cells overexpressing KLF4. Structure analysis indicated that KLF4 possesses potent nuclear localization signals. However, it is also possible that KLF4 may be migrated to cell surface under overexpression conditions. Recent studies showed that nucleolin, a nuclear protein, was present on the surface of various cell types, despite lacking a transmembrane domain or a signal sequence (24, 25). Therefore, nucleolin acts as a receptor on plasma membrane and shuttles the ligand from the plasma membrane to the cytoplasm and nucleus (24, 26). Likewise, KLF4 may also migrate to the cell surface and act as a kallistatin-binding protein.

Accumulating evidence has shown that kallistatin inhibits inflammatory responses in animal models of arthritis, acute cardiac infarction, and salt-induced renal injury (6, 11, 12). Expression of pro-inflammatory mediators, such as VCAM-1 and MCP-1, is very low or absent under physiologic conditions but can be rapidly elevated by cytokines (27). Up-regulation of adhesion molecules at the endothelial cell surface initiates...
pathological leukocyte-endothelial cell interaction, which ultimately exposes the vascular wall and surrounding tissues to the damaging action of activated leukocytes and causes the subsequent development of endothelial dysfunction/atherosclerosis (28–30). KLF4 has been shown to be an important regulator of endothelial activation in response to pro-inflammatory stimuli, because it suppresses the secretion of several cytokines and chemokines (17). Our results showed that kallistatin attenuated TNF-α-induced NF-κB activation and MCP-1 and VCAM-1 expression. Moreover, inhibition of KLF4 by RNA interference abolished the effects of kallistatin. Taken together, these data indicate that KLF4 may

FIGURE 5. Up-regulation of eNOS by kallistatin was abolished by KLF4 knockdown in human endothelial cells. A and B, eNOS immunostaining (A) and Western blot analysis (B) of eNOS levels in endothelial cells with kallistatin treatment. C, nitrate/nitrite levels in the culture medium of endothelial cells with kallistatin treatment. HUVECs were treated with recombinant human kallistatin (0.4 μM) for 24 h and then fixed for eNOS immunostaining. Parallel plate cells were treated with recombinant human kallistatin (0.1–0.4 μM) for 24 h, and total protein was collected for Western blot analysis of eNOS levels. The medium was collected, and NO released into the medium was measured by a fluorometric assay for nitrate/nitrite. D, quantitative PCR analysis for eNOS expression in the presence of KLF4 siRNA. HUVECs with/without KLF4 depletion were treated with recombinant human kallistatin (0.4 μM) for 16 h. The cells were then harvested, and total RNA was processed for eNOS analysis by quantitative PCR. All of the experiments were performed twice, with n = 3/experiment.

FIGURE 6. Kallistatin inhibits TNF-α-induced inflammatory mediator expression in endothelial cells via NO formation. Shown are quantitative PCR analyses for VCAM-1 (A) and MCP-1 expression (B) in endothelial cells. HUVECs were pretreated with Nω-nitro-L-arginine methyl ester (l-NAME) with/without recombinant human kallistatin (0.4 μM) for 30 min and then exposed to TNF-α (10 ng/ml). After 16 h of treatment, the cells were harvested, and total RNA was analyzed by quantitative PCR. All of the experiments were performed twice, with n = 3/experiment.
function as a mediator in the anti-inflammatory actions of kallistatin in endothelial cells.

It is well known that oxidative stress and associated oxidative damage are mediators of vascular injury and inflammation. TNF-α has been reported to target vascular endothelial cells and stimulate ROS production via activation of NAD(P)H oxidase or xanthine oxidase (31–33). Many studies suggest that increased ROS production accounts for a significant proportion of the NO deficit in inflammatory vessels. NO functions as an endothelial protective agent by inhibition of NADPH oxidase activity or through neutralization of toxic oxygen-derived radicals (34, 35). The anti-inflammatory action of NO is also largely based on its inhibitory effect on leukocyte-endothelium adhesion (36, 37). At the molecular level, NO suppresses leukocyte-endothelium adhesion by modulating the activity of NF-κB (36). Recent evidence has linked increased leukocyte-endothelium interaction in the microcirculation with inflammatory signal activation and the vascular complications of diabetes, a metabolic condition associated with reduced levels of kallistatin (38–40). Indeed, *in vitro* studies demonstrated that kallistatin exhibits anti-inflammatory effects through increasing NO formation and suppressing inflammatory cell infiltration (6, 7, 12). Therefore, increased NO production may account for the protective role of kallistatin after oxidative injury.

The observation that kallistatin induces eNOS expression and promotes NO production may have important functional consequences in vascular function. The concentration of NO is controlled by constitutively expressed eNOS (41). Because eNOS is an endothelial protective agent, mice deficient in eNOS are hypertensive, lack endothelium-dependent vasodilation, respond poorly to inflammatory challenge, and exhibit enhanced atherosclerotic lesion formation (42). A recent study showed that KLF4 overexpression induced eNOS expression (17). Consistent with this finding, we showed that kallistatin-induced eNOS expression is mediated by KLF4. Moreover, we found that pharmacological inhibition of eNOS activity or knockdown of eNOS expression markedly impaired the inhibitory...
KLF4 Mediates Anti-inflammatory Effect of Kallistatin

Kallistatin, a natural proteinase inhibitor, has been shown to modulate inflammation through its interaction with KLF4 via increased eNOS synthesis and NO formation. This study demonstrates kallistatin to be a novel anti-inflammatory agent likely that KLF4 may act as a cell surface protein and shuttle kallistatin into the cell to exert its actions. Future studies will be conducted to investigate other potential kallistatin-binding proteins.

In summary, we made several important new findings in this study. First, we identified the transcription factor KLF4 as a novel kallistatin-binding protein. Second, this is the first study to identify localization of KLF4 on the surface of cultured HEK-293 cells. Third, we demonstrated that KLF4 regulates cytokine-mediated induction of VCAM-1 and MCP-1 expression in endothelial cells. These findings indicate that kallistatin protects against endothelial inflammation through KLF4-dependent eNOS expression.

REFERENCES

1. Chao, J., Tillman, D. M., Wang, M. Y., Margolius, H. S., and Chao, L. (1986) J. Biol. Chem. 261, 325–331
2. Chai, K. X., Chao, L. M., and Chao, J. (1992) Agents and Actions 38, 174–181
3. Chen, L. M., Chao, L., and Chao, J. (1997) Life Sci. 60, 1431–1435
4. Davenpeck, K. L., Gauthier, T. W., and Cao, L. M. (1994) Blood 83, 2449–24505
5. Chen, M. W., and Chao, L. (1995) J. Biol. Chem. 270, 13,197–13,204
6. Chao, J., Stallone, J. N., Liang, Y. M., Wang, D. Z., and Chao, L. (1997) J. Clin. Invest. 100, 11–17
7. Chao, J., Stallone, J. N., Liang, Y. M., Chen, L. M., Wang, D. Z., and Chao, L. (1997) J. Biol. Chem. 272, 198–210
8. Chao, J., Stallone, J. N., Liang, Y. M., Wang, D. Z., and Chao, L. (1997) J. Biol. Chem. 272, 198–210
9. Chen, J., Chao, L., and Chao, J. (1992) Agents and Actions 38, 174–181
10. Chao, J., Tillman, D. M., Wang, M. Y., Margolius, H. S., and Chao, L. (1986) J. Biol. Chem. 261, 325–331
11. Chen, L. M., Chao, L., and Chao, J. (1997) Life Sci. 60, 1431–1435
12. Wang, C. R., Chen, S. Y., Wu, C. L., Liu, M. F., Jin, Y. T., Chao, L., and Chao, J. (2005) Arthritis Rheum. 52, 1319–1324
13. Shen, B., Hagiwara, M., Yao, Y. Y., and Chao, J. (2008) Hypertension 51, 1358–1365
14. Flanagan, J. G., and Cheng, H. J. (2000) Methods Enzymol. 327, 198–210
15. Miao, R. Q., Gao, Y., Harrison, K. D., Prendergast, J., Acevedo, L. M., Yu, J., Hu, F., Strittmatter, S. M., and Sessa, W. C. (2006) Proc. Natl. Acad. Sci. U.S.A. 103, 10997–11002
16. Kim, J. Y., Omori, E., Matsumoto, K., Núñez, G., and Ninomiya-Tsuji, J. (2008) J. Biol. Chem. 283, 137–144
17. Hamik, A., Lin, Z., Kumar, A., Balcells, M., Sinha, S., Katz, J., Feinberg, M. W., Gerzsten, R. E., Edelman, E. R., and Jain, M. K. (2007) J. Biol. Chem. 282, 13769–13779
18. Fontana, J., Jovan, D., Chen, Y., Fairchild, T. A., McCabe, T. J., Fujita, N., Tsurow, T., and Sessa, W. C. (2002) Circ. Res. 90, 866–873
19. Goebeler, M., Schnarr, B., Toksoy, A., Kunz, M., Bröcker, E. B., Duschl, A., and Gillitzer, R. (1997) Immunology 91, 450–457
20. Shields, J. A., Christy, R. J., and Yang, V. W. (1996) J. Biol. Chem. 271, 20009–20017
21. Brembeck, F. H., and Rustgi, A. K. (2000) J. Biol. Chem. 275, 28230–28239
22. Yet, S. F., Mca’Nulty, M. M., Foita, S. C., Yen, H. W., Yoshizumi, M., Hsieh, C. M., Layne, M. D., Chen, M. T., Wang, H., Perrella, M. A., Jain, M. K., and Lee, M. E. (1998) J. Biol. Chem. 273, 1026–1031
23. McCormick, S. M., Eskin, S. G., McIntire, L. V., Teng, C. L., Lu, C. M., Russell, C. G., and Chittur, K. K. (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 8955–8960
24. Chen, X., Kube, D. M., Cooper, M. J., and Davis, P. B. (2008) Mol. Ther. 16, 333–342
25. Soundararajan, S., Chen, W., Spencer, E. K., Courtenay-Luck, N., and Fernandez, D. J. (2008) Cancer Res. 68, 2358–2365
26. Soundararajan, S., Wang, L., Sridharan, V., Chen, W., Courtenay-Luck, N., Jones, D., Spencer, E. K., and Fernandez, D. J. (2009) Mol. Pharmacol. 76, 984–991
27. Carlos, T. M., and Harlan, J. M. (1994) Blood 84, 2068–2101
28. Davenpeck, K. L., Gauthier, T. W., and Lefer, A. M. (1994) Gastroenterology 107, 1050–1058
29. Martin, J., Collot-Texeira, S., McGregor, L., and McGregor, J. L. (2007) Curr. Pharm. Des. 13, 1751–1759
30. Nakashima, Y., Raines, E. W., Plump, A. S., Breslow, J. L., and Ross, R. (1998) Arterioscler. Thromb. Vasc. Biol. 18, 842–851
31. Brenner, L. A., Steinhorn, R. H., Wedgwood, S., Mata-Greenwood, E., Roark, E. A., Russell, J. A., and Black, S. M. (2003) Circ. Res. 92, 683–691
32. Basu, S., Bhattacharya, S., Leffler, C. W., and Parfenova, H. (2009) Am. J. Physiol. Cell Physiol. 296, C422–C432
33. Zhang, C., Xue, P., Potter, B. J., Wang, W., Kuo, L., Michael, L., Bagby, G. J., and Chilian, W. M. (2006) Arterioscler. Thromb. Vasc. Biol. 26, 475–480
34. Bautista, A. P., and Spitzer, J. J. (1994) Am. J. Physiol. Gastroenterol. Liver Physiol. 266, G783–G788
35. Clancy, R. M., Leszczynska-Piziak, J., and Abramson, S. B. (1992) J. Clin. Invest. 90, 1116–1121
36. DeCaterina, R., Libby, P., Peng, H. B., Thannickal, V. J., Ravajavishth, T. B., Gimbrone, M. A., Jr., Shin, W. S., and Liao, J. K. (1995) J. Clin. Invest. 96, 60–68
37. Matsushita, K., Morrell, C. N., Cambien, B., Yang, S. X., Yamakuchi, M., Bao, C., Hara, M. R., Quick, R. A., Cao, W., O’Rourke, B., Lowenstein, J. M., Pevsner, J., Wagner, D. D., and Lowenstein, C. J. (2003) Cell 115, 139–150
38. Joussen, A. M., Poulaki, V., Le, M. L., Koizumi, K., Esser, C., Janicki, H., Schaerumeyer, U., Kociok, N., Fauser, S., Kirchhof, B., kern, T. S., and Adanis, A. P. (2004) FASEB J. 18, 1450–1452
39. Ma, J. X., Li, X., Yang, Z., Crouch, R. K., Chao, L., and Chao, J. (1996) Curr. Eye Res. 15, 1117–1123
40. Hatcher, H. C., Ma, J. X., Chao, J., Chao, L., and Oltzec, A. (1997) Invest. Ophthalmol. Vis. Sci. 38, 658–664
41. Tai, S. C., Robb, G. B., and Marsden, P. A. (2004) Arterioscler. Thromb. Vasc. Biol. 24, 405–412
42. Ortiz, P. A., and Garvin, J. L. (2003) Am. J. Physiol. Regul. Integr. Comp. Physiol. 284, R628–R638