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Keratin-1 is a novel binding protein for C-reactive protein on the membrane of endothelial cells

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ABSTRACT

C-reactive protein (CRP) is a sensitive marker of systemic inflammation. Recent investigations have shown that CRP correlates with cardiovascular disease and endothelial dysfunction. CRP exerts its effects on endothelial cells through binding to its receptors, CD32 and the lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1). As an acute-phase protein, CRP is capable of binding to various molecules and may bind to the receptor other than CD32 and LOX-1. Here, we identified keratin-1 (KRT1) on endothelial cells as a novel protein for CRP binding by affinity chromatography and mass spectrometry. The interaction between KRT1 and CRP was also confirmed by the antibody blockade of KRT1 that decreased the CRP uptake and blocked CRP-mediated reduction in nitric oxide (NO) release in human aortic endothelial cells (HAECs). Additionally, a reduced KRT1 expression by KRT1 small hairpin RNAs increased NO release in the presence of CRP.

1. Introduction

C-reactive protein (CRP) is a marker for systemic inflammation [1]. High-sensitivity CRP (hsCRP) has recently been used as a predictor of cardiovascular disease. Levels of hsCRP < 1, 1–3, and > 3 mg/L in serum are respectively associated with low, moderate, and high risks of cardiovascular events [2– 3]. Studies have shown that administering CRP is harmful to endothelial cells [4–6]. Impaired endothelial cells show lower endothelial nitric oxide (NO) synthase (eNOS) activity [7–10], decreased prostacyclin release [9], and increased vascular cell adhesion molecule 1, intercellular adhesion molecule 1, E-selectin, and monocyte chemotactic protein 1 expression [11–13]. CD32, CD64 [14], and the lectin-like oxidized low-density lipoprotein (oxLDL) receptor-1 (LOX-1) [15] have been identified as CRP receptors on human endothelial cells. CRP is a pentameric molecule with two faces binding to various components [16]. The activity face of CRP binds to CD32 and CD64, whereas the binding face of CRP recognizes LOX-1.

Keratin 1 (KRT1), a member of the intermediate filaments, is present on the membrane of endothelial cells and has a high affinity for binding to high molecular weight kininogen (HK) [17,18]. The proteolytic cleavage of HK by kallikrein produces bradykinin, a potent vasoactive agent that has the potential to induce endothelial cells to release NO [19]. In physiological conditions, a small amount of bradykinin is produced, serving as an antithrombotic and proangiogenic peptide. The expression of KRT1 increases in response to cellular oxidative stress [20]. The recognition of KRT1 by human mannose-binding lectin activates the lectin complement pathway. KRT1 also binds and supports the internalization of myeloperoxidase (MPO) that is present at high levels in the serum of patients with inflammatory disease [21]. The internalized MPO causes oxidative damage and inactivates both HK and kallikrein, resulting in a decrease in bradykinin production.

CRP is an acute-phase protein that is capable of binding to various molecules. We tested whether human endothelial cells have CRP receptors other than CD32, CD64, and LOX-1 by affinity chromatography and mass spectrometry. We observed that a novel protein, KRT1, binds to CRP. The interaction between KRT1 and CRP was further verified by the cell uptake of CRP and the CRP effect on NO release in endothelial cells using an anti-KRT1 antibody and a KRT1-short hairpin RNA (shRNA) plasmid.

2. Materials and methods

2.1. Cell culture

Human aortic endothelial cells (HAECs) were purchased from Cascade Biologics (Portland, OR, USA) and cultured in an M199 medium supplemented with 1% low serum growth supplement (Life Technologies, Carlsbad, CA, USA), 10% cosmic calf serum (Hyclone Laboratories, Logan, UT, USA), 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B, 2 mM L-glutamine, 1 mM sodium pyruvate, and 50 μg/mL gentamicin (Sigma-Aldrich, Saint Louis, MO, USA) at 37 °C in a 5% CO_2 humidified atmosphere. The HAECs used in the experiments underwent 8–12 passages. For the antibody blockade

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experiment, the cells were pretreated with 4 μg/mL of the rabbit anti-KRT1 polyclonal antibody (Proteintech Group, Chicago, IL, USA) or the mouse anti-CD32 monoclonal antibody (Thermo Fisher Scientific Pierce Biotechnology, Rockford, IL, USA) for 30 min before the CRP challenge. To reduce the intracellular level of the KRT1 protein, the HAECs were transfected with the KRT1-shRNA plasmids. One day before transfection, 2×10^5 HAECs were passaged onto 12-well plates after trypsinization. HAECs reaching 50–80% confluency were transfected with 2.5 μg of plasmid DNA in an M199 medium using an Xfect transfection reagent (Clontech Laboratories, Mountain View, CA, USA) at 37 °C for 4 h. The cells were incubated in 500 μL of a fresh M199 growth medium for 48 h. To measure the NO release, the medium was replaced by 500 μL of fresh M199 medium without serum and the concentrations of NO in the samples were recorded at 0 and 1 h. The HAECs were lysed in a lysis buffer (pH 7.4, 20 mM Tris buffer, 0.1 μM phenylmethanesulfonyl fluoride, 1% Triton X-100) and the protein concentration was determined using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific Pierce Biotechnology).

2.2. Preparation of the CRP protein

Recombinant human CRP was purchased from Merck Millipore (Billerica, MA, USA). To prevent endotoxin and NaN_3 interference in the experiments, endotoxin was first removed by passing the CRP solution through the Detoxi-gel endotoxin-removing columns (Thermo Fisher Scientific Pierce Biotechnology). The endotoxin levels in the solution were measured using a *Limulus* amebocyte lysate assay, and were < 0.3 EU/mL (Associates of Cape Cod, East Falmouth, MA, USA). NaN_3 was removed by conducting dialysis 3 times against Tris–HCl buffer (20 mM Tris, 140 mM NaCl and 2 mM $CaCl_2$, pH 7.5) at 4 °C. For the CRP uptake experiment, CRP was labeled with fluorescein isothiocyanate (FITC) using a Pierce™ FITC antibody labeling kit (Thermo Fisher Scientific Pierce Biotechnology).

2.3. Plasma membrane isolation

After 2 washes with ice-cold phosphate-buffered saline (PBS), four 100-mm culture dishes of cells were scraped into 2 mL of the packing buffer (20 mM Tris-base, 125 mM NaCl, 1 mM $CaCl_2$, 0.1 mM PMSF, pH 6) and then homogenized in a dounce homogenizer (Kontes Glass, Vineland, NJ, USA). The unbroken cells were removed from the homogenate by performing centrifugation at 1000 × g for 15 min at 4 °C. The cell membrane pellet, collected by performing centrifugation of the supernatant at 10,000 × g for 15 min, was resuspended in 0.5 mL of the packing buffer containing 1% NP-40 and further incubated at 4 °C for 2 h. The resulting membrane suspension was ready for interaction experiments.

2.4. Characterization of CRP binding proteins

To identify the membrane proteins that interact with CRP, 1 mg/mL of a rabbit anti-CRP antibody (EMD Biosciences, San Diego, CA, USA) in the coupling solution (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) was coupled to CNBr-activated Sepharose (volume ratio 0.5:1; GE Healthcare, Piscataway, NJ, USA) according to the manufacturer's instructions. The cell membrane pellet was incubated with 1.5 mg/mL of CRP at 4 °C for 6 h and passed through the anti-CRP affinity column. The bound proteins were eluted using 100 mM sodium citrate (pH 3), resolved using 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), and stained with the ammoniacal silver solution. The proteins were identified using liquid chromatography tandem mass spectrometry (LC–MS–MS) according to the aforementioned methods [22].

2.5. Immunofluorescence assays

To determine the CRP uptake by HAECs, the cells were cultured on a 0.1% gelatin-coated glass coverslip. Following various treatments, HAECs were incubated for 15 min with 10 μg/mL of FITC labeled CRP and the cells were fixed with 4% formaldehyde in Dulbecco's phosphate-buffered saline (DPBS) at room temperature for 10 min and permeabilized with 0.5% triton X-100 in DPBS for 10 min. Cell nuclei were stained with DAPI (Sigma) and images were captured by Leica SP5 II (Heidelberg, Germany). Images were processed using Image-Pro Plus 5.0 (Media Cybernetics, Rockville, MD, USA). For determining the intracellular KRT1 level, the rabbit anti-KRT1 polyclonal antibody (Proteintech) at a 1:500 dilution and an FITC-conjugated goat anti-rabbit IgG (Millipore) were used.

2.6. Construction of the KRT1-shRNA plasmids

The sequences for targeting the sense mRNA sequence of KRT1 were determined to be 5'-GACTCAAATCAGTGAAACTAA-3' (shRNA) by using the RNAi database from the National RNAi Core Facility in Taiwan. To create pSIREN-KRT1, the shRNA sequences designed using the shRNA Sequence Designer (Clontech) were ligated through BamHI/EcoRI into the pSIREN vector (Clontech). pSIREN-KRT1 was amplified in *E. coli* DH5α and extracted using a PureLink™ HiPure Plasmid DNA Purification Kit (Life Technologies).

2.7. NO measurement

The production of NO by HAECs was determined using the inNO-T system with the amiNO-700 probe (Innovative Instruments, Tampa, FL, USA). The standard curve was obtained using the $NaNO_2$ concentrations ranging from 24 to 100 nM. Fifty microliters of the sample was injected into a KI buffer (2.4 mM), and the signal was recorded in real time.

2.8. Statistical analysis

Statistical comparisons were performed using the Student's *t* test, and $P < 0.05$ was considered statistically significant.

3. Results

3.1. KRT1 binds to CRP

CRP binds to various receptors including CD32, CD64, and LOX-1. To determine whether CRP binds to other membrane proteins, an affinity column was coupled to the anti-CRP antibody to bind CRP and its interacting proteins. The bound proteins were then resolved using gel electrophoresis. Fig. 1a shows that the anti-CRP antibody affinity column mainly bound two proteins, CRP and its interacting protein. The size of the protein in the gel for CRP is ~23 kDa, consistent with the theoretical molecular weight for the monomeric CRP. The CRP-binding protein was trypsinized into peptide fragments that were characterized using LC–MS–MS. Eleven tryptic peptide fragments were identified in the chromatographic fractions, corresponding the sequences of KRT1 (Fig. 1b). Notably, the size of the protein for KRT1 (Fig. 1a) is greater than 55 kDa, consistent with the molecular weight of 67 kDa for KRT1 [23].

3.2. Antibody to KRT1 inhibits the CRP uptake by HAECs and blocks the CRP-mediated reduction in NO

If KRT1 is one of the CRP receptors, we should be able to inhibit the CRP effects on cells using an anti-KRT1 antibody that blocks CRP binding to KRT1. However, the extracellular regions of KRT1 are not completely known. Shariat-Madar et al. demonstrated that

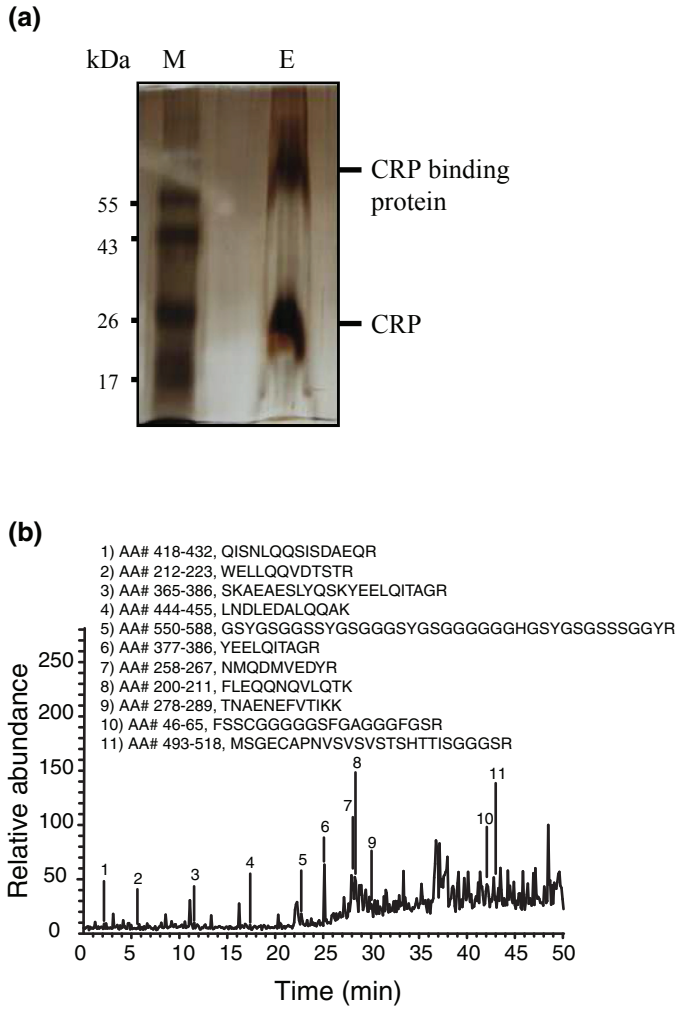

Fig. 1. KRT1 binding to CRP. (a) Silver staining of the SDS–PAGE of eluted proteins from the anti-CRP antibody affinity column. The mixture of CRP and cell membrane was loaded onto the anti-CRP antibody affinity column. The bound proteins were eluted and resolved by 8% SDS–PAGE. (b) Liquid chromatographic profiles of the tryptic peptide fragments of the CRP binding protein. The CRP binding protein in gel was subjected to trypsin digestion and analyzed using LC–MS–MS. The sequences of peptide fragments were determined based on the observed mass-to-charge ratios of the *b*- and *y*-ions of LC–MS–MS spectra. M and E stand for protein marker and eluent, respectively. The CRP binding protein was identified as KRT-1.

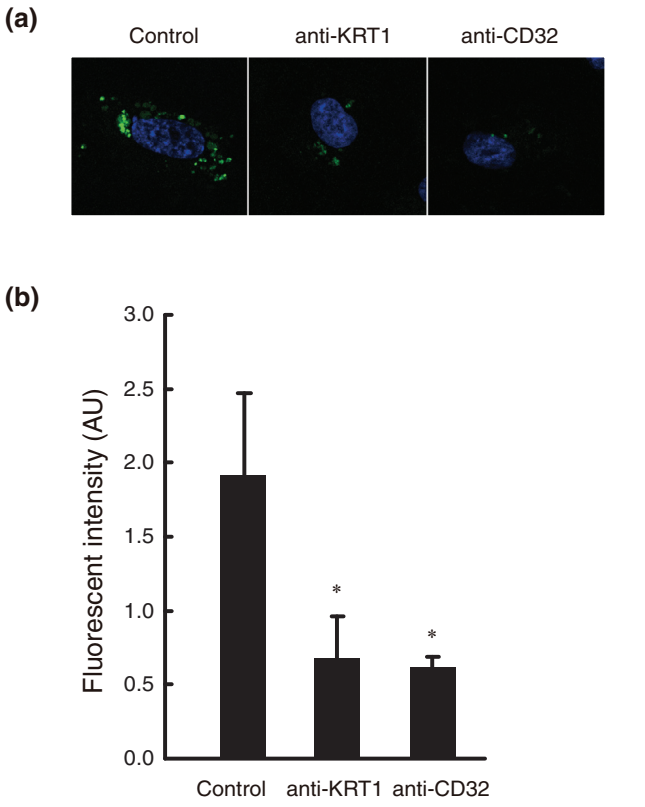

Fig. 2. Inhibition of the endothelial CRP uptake by antibody blockade of KRT1. HAECs were incubated with 4 μg/mL of the anti-KRT1 or anti-CD32 antibody for 30 min before treatment of CRP. The cellular uptake of the FITC-labeled CRP was performed for 15 min and was visualized using an Leica SP5 II confocal microscope. $^{*}P < 0.05$ *vs.* control. The error bars stand for standard error of mean, $n = 4$.

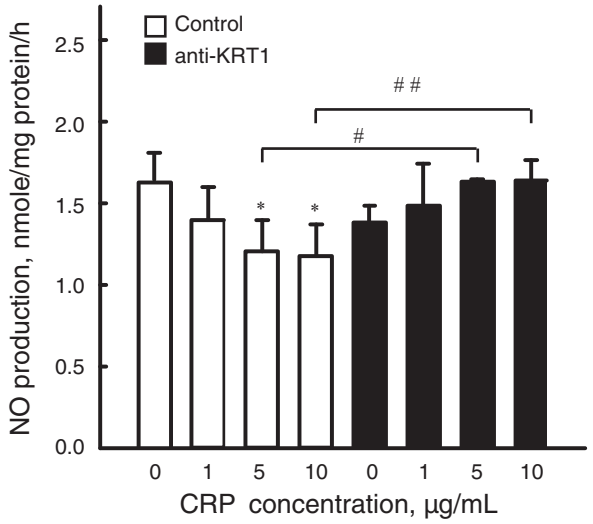

Fig. 3. Abolishment of the CRP antagonistic effect on endothelial NO release by antibody blockade of KRT1. HAECs were incubated with 4 μg/mL of the anti-KRT1 antibody for 30 min before treatment of CRP. The release of NO from HAECs was measured using the amiNO-700 probe. $^{*}P < 0.05$ *vs.* control; $^{\#}P < 0.05$ *vs.* 5 μg/mL CRP + antiKRT1; $^{\#\#}P < 0.05$ *vs.* 10 mg/mL CRP + antiKRT1. The error bars stand for standard error of mean, $n = 3$.

the region encompassing residues 153–267 of KRT1 protein binds to HK on human umbilical vein endothelial cells [18], suggesting that these KRT1 residues, at least in part, are present on the extracellular face of the plasma membrane. We therefore selected the available anti-KRT1 antibody that was raised against the KRT1 residues ^{241}RVDQLKSDQSRLD^{253} trying to block CRP binding to KRT1. Pretreating HAECs with 4 μg/mL of the anit-KRT1 antibody as well as the anti-CD32 antibody both significantly decreased the uptake of CRP by HAECs (Fig. 2). Additionally, CRP reduced NO production from HAECs in a dose-dependent manner (Fig. 3). Treatment of HAECs with 4 μg/mL of the anti-KRT1 antibody for 4.5 h abolished the decrease of NO by CRP.

3.3. KRT1 shRNA increases NO release in the presence of CRP

To further confirm involvement of KRT1 proteins in the CRP effect on NO release, the KRT1 protein level was reduced by transfecting the HAECs with a KRT1-shRNA plasmid. The result shows that transfecting the cells with the KRT1-shRNA plasmid for 48 h decreased the KRT1 protein level by 85% as compared with the vector-only controls (Fig. 4a). The introduction of the KRT1-shRNA into HAECs did not affect the basal NO release as compared with the vector-only control. The NO release in the presence of CRP was significantly higher in the KRT1-shRNA-transfected sample than in the vector-only control. (Fig. 4b).

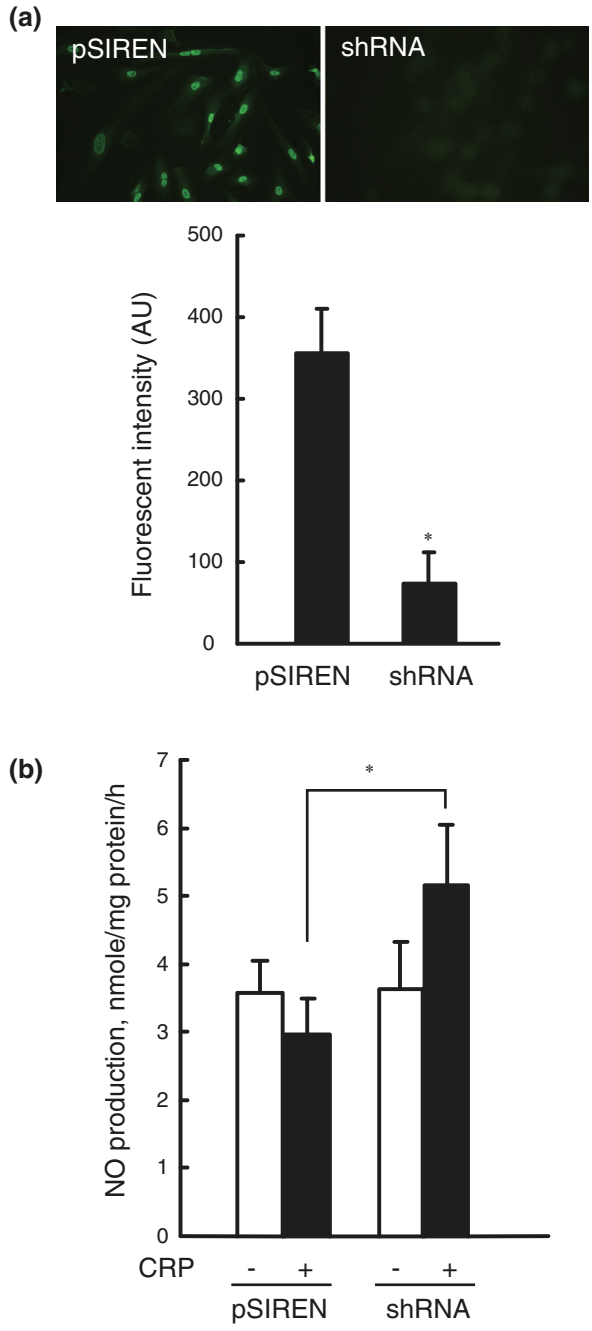

Fig. 4. Inhibition of the CRP antagonistic effect on endothelial NO release by a KRT1-shRNA. HAECs were transfected with pSIREN-KRT1. Endothelial KRT1 proteins were visualized using fluorescence microscopy after treating the cells with the rabbit anti-KRT1 polyclonal antibody and FITC-conjugated goat anti-rabbit IgG (a). The release of NO from HAECs was measured using the amiNO-700 probe (b). $^{*}P < 0.05$ *vs.* shRNA + CRP. The error bars stand for standard error of mean, $n = 3$.

4. Discussion

The identification of KRT1 as a CRP binding protein, a putative receptor, on endothelial cells has extended the roles of KRT1 in modulating endothelial function. Independent of the affinity isolation, we confirmed the involvement of KRT1 in the biological functions of CRP on endothelial cells. First, pretreating HAECs with an anti-KRT1 antibody decreased the CRP uptake by HAECs (Fig. 2) and blocked the CRP antagonistic effect on NO release (Fig. 3). Second, a reduced KRT1 expression by KRT1-shRNAs abolished the CRP-induced declines in NO release (Fig. 4).

KRT1, which forms a multiprotein receptor complex with gC1qR and the urokinase plasminogen activator receptor, involves in kinin production activity on endothelial cells through binding of HK and factor XII [17,24]. Bradykinin released from proteolytic cleavage of HK by kallikrein induces a hypotensive and anti-thrombotic condition through increasing endothelial NO synthesis, prostacyclin production, and tissue plasminogen activator release [25–27]. In contrast, KRT1 can also bind and support the internalization of MPO that causes oxidative damage of HK and kallikrein, resulting in a decrease in bradykinin production. It remains unknown whether KRT1 is able to modulate endothelial function by binding ligands other than HK and MPO. In the present study, we demonstrate for the first time in HAECs that KRT1 binds to CRP and the antibody blockade of KRT1 is able to blunt the CRP effect on NO release.

The expression of KRT1 is increased by cellular oxidative stress when endothelial cells are exposed to the hypoxia/reoxygenation injury [20]. CRP is also reported to induce oxidative stress in endothelial cells through an NAD(P)H-oxidase-dependent pathway [28,29]. It is reasonable to expect that the CRP-induced oxidative stress may increase the KRT1 expression. Indeed, we found that 1 μg/mL of CRP transiently enhanced KRT1 protein expression in HAECs for a 24-h period (Fig. S1). Similarly, the expression of CD32 and LOX-1, the other found CRP receptors, are also induced by CRP on endothelial cells [30,31]. The resulting high-level expression of the CRP receptors may exaggerate the deleterious effect of CRP on endothelial cells.

How KRT1 folds into a transmembrane protein is unclear. KRT1 consists of 3 domains, the head domain (residues 2–179) containing the Gly/Phe/Ser-rich region (residues 2–151) at the *N*-terminus, the central rod domain (residues 180–489), and the tail domain (residues 490–644) containing the Gly/Ser-rich region at the *C*-terminus [32]. The head domain consists of 3 subdomains, the end subdomain (E1), the variable subdomain (V1), and the homologous subdomain (H1). Part of the Gly/Phe/Ser-rich domain may be localized intracellularly, where two serine sites (residues 21 and 66) can be phosphorylated [33] and may involve in the regulation or signaling of KRT1. The KRT1 protein residues ^{153}VTINQ^{157} block the HK binding to KRT1 [18] and the antibody raised to against the KRT1 protein residues ^{241}RVDQLKSDQSRLD^{253} inhibits the binding of CRP to HAECs and the CRP-mediated NO declines (Figs. 2 and 3), suggesting these two residues are exposed to the extracellular environment. Certainly, additional research is warranted to further characterize the KRT1 binding site to CRP.

It is not clear why addition of the anti-KRT1 antibody for 4.5 h decreases the NO production in the absence of CRP (Fig. 3). This suggests that the anti-KRT1 antibody itself may have effect on reducing NO production upon binding to KRT1. When KRT1 was blocked by the anti-KRT1 antibody, CRP dose-dependently increases the NO production (although not significantly), suggesting that CRP may induce inflammatory response through KRT1 and also generate an anti-inflammatory response through an unknown mechanism. A same trend was also found in the shRNA-knockdown experiment (Fig. 4).

In summary, we demonstrated that KRT1 binds to CRP and mediates, at least in part, the effect of CRP on endothelial cells. The finding of interaction between KRT1 and CRP may help us better understand the multiple receptors for CRP and develop a novel therapeutic strategy for atherosclerosis

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jtice.2015.04.019.

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