Acyclic retinoid inhibits functional interaction of transcription factors Krüppel-like factor 5 and retinoic acid receptor-alpha

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Abstract  We show that transcription factor Krüppel-like factor 5 (KLF5), which is important in cardiovascular remodeling, interacts with retinoic acid receptor-alpha (RARα) to regulate downstream gene expression. Here, we investigated whether acyclic retinoid (ACR) regulates KLF5 and inhibits vascular remodeling. Co-immunoprecipitation and pull-down binding assay showed that ACR attenuates functional interaction of KLF5 and RARα. ACR affects KLF5 functions by regulating transcription of platelet-derived growth factor A (PDGF-A) chain. ACR may be a new vascular therapy to target KLF5 in cardiovascular pathology.

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Keywords: Vascular biology; Transcription

1. Introduction

We recently showed that Krüppel-like transcription factor 5 (KLF5), which is important in cardiovascular remodeling, interacts with retinoic acid receptor-alpha (RARα) to regulate downstream gene expression. Here, we investigated whether acyclic retinoid (ACR) regulates KLF5 and inhibits vascular remodeling. Co-immunoprecipitation and pull-down binding assay showed that ACR attenuates functional interaction of KLF5 and RARα. ACR affects KLF5 functions by regulating transcription of platelet-derived growth factor A (PDGF-A) chain. ACR may be a new vascular therapy to target KLF5 in cardiovascular pathology.

2. Materials and methods

2.1. Chemicals

ACR (3,7,11,15-tetramethyl-2,4,6,10,14-alltrans-hexadecapentaenoic acid) was provided by Nikken Chemical Co., and ATRA and dimethyl sulfoxide minimum (DMSO) were purchased from Sigma (Table 1). ATRA was dissolved in DMSO.

2.2. Cell culture

Bovine aortic cells (BAECs) and NIH3T3 cells were maintained in Dulbecco’s modified Eagle medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum with 100 μg/ml streptomycin and 100 U/ml penicillin G. KLF5-3T3 and mock-3T3 cells, which were constructed previously, were grown as described [6].

2.3. Co-immunoprecipitation assay

RARα and 3xFLAG-tagged KLF5 (3xFLAG-KLF5) expression vectors, which were previously constructed [7], were transfected into BAECs for 24 h and incubated with ACR for another 24 h. Then, BAECs were washed with lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton-X, 100 μM ZnSO4, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, and 1 μg/ml pepstatin). Ten microliters of anti-FLAG-M2 affinity gel (Sigma) was bound by rotatory for 2 h in lysis buffer containing 0.5% bovine serum albumin (BSA) (Sigma) at 4 °C, then washed with FLAG wash buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 100 μM ZnSO4, 0.5 mM...
phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, and 1 μg/ml pepstatin). Immunoprecipitate was subjected to SDS–PAGE and immuno-blotted using anti-RARα antibody (Santa Cruz).

2.4. In vitro ‘GST pull-down’ binding assay

The recombinant Glutathione S-transferase (GST)-tagged RARα (GST-RARα) construct was generated by PCR on pRS-RARα using primers (5'-GCCCGAATTCATGGCCAGCAACAGCAGC-3' and 5'-GCACACTCGAGTCACGGGGAGTGGGT-3') containing Xho1 and EcoRI restriction endonuclease sites. The recombinant GST-tagged RARα-DNA binding domain (DBD) (GST-RARα-DBD) construct was generated by PCR on pRS-RARα using primers (5'-TTGGAAATTCCGCCCATCTACAGCTTGCC-3' and 5'-TC TGCAGAATTCCTTCTTCTCTCTTGTTTCG-3') containing EcoRI restriction endonuclease sites. The PCR fragment was inserted in-frame into GST-expression vector, pGEX-4T1 (GE Healthcare), and

Table 1
Structure of ACR and ATRA

<table>
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<th>ACR</th>
<th>ATRA</th>
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<tr>
<td>ACR</td>
<td>(2E, 4E, 6E, 10E)-3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentanoic acid.</td>
<td>all-trans retinoic acid.</td>
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ACR: (2E, 4E, 6E, 10E)-3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentanoic acid.

ATRA: all-trans retinoic acid.

Fig. 1. (A) Co-immunoprecipitation assay showing binding of RARα and KLF5 treated with ACR or ATRA. Cell lysate with FLAG-tagged KLF5 overexpression and without overexpression was immunoprecipitated with anti-FLAG affinity gel. Lane 1 is input, confirming that applied protein amounts were similar. Note that ACR attenuated KLF5 and RARα interaction greater than ATRA. (B) RARα and KLF5 interaction as assessed by ‘GST pull-down’ binding assay. Immobilized GST-tagged RARα fusion protein was reacted with hexahistidine-tagged KLF5, separated by SDS–PAGE, and then analyzed by immunoblotting with anti-His antibody or anti-GST antibody. Lane 1 is input. (C) RARα-DBD and KLF5-DBD interaction as assessed by ‘GST pull-down’ binding assay. Immobilized GST-tagged RARα-DBD fusion protein was reacted with hexahistidine-tagged KLF5-DBD, separated by SDS–PAGE, and then analyzed by immunoblotting with anti-His antibody or anti-GST antibody. Lane 1 is input.
then expressed and purified essentially according to described methods [8]. Hexahistidine-tagged constructs for KLF5-DNA binding domain (DBD) (His-KLF5-DBD), the zinc finger peptides of KLF5, and protein expression and purification were described previously [6]. One micrograms of GST fusion protein was incubated with ACR or ATRA at room temperature. After that, 10 μl of Glutathione sepharose 4B resin (GE Healthcare) was added for 2 h at 4 °C in binding buffer (20 mM HEPES, pH 7.9, 40 mM KCl, 0.1 mM EDTA, pH 8.0). After washing with binding buffer, 500 ng of each His-KLF5-DBD protein was added and incubated for 2 h at 4 °C in the same buffer. The bound protein was washed three times in the same buffer. These proteins were resolved by SDS-PAGE analysis and then immunoblotted with anti-His probe (Santa Cruz) and anti-GST (Santa Cruz).

2.5. Co-transfection reporter assay

BAECs were transfected with reporter and expression plasmids by lipofectamine 2000 (Invitrogen). Cells were incubated for 24 h and were incubated with additive for 24 h then subjected to luciferase assay (Promega) by luminometry (Lumat LB9507, Berthold). β-Galactosidase activity was measured according to the manufacturer’s instructions (Promega). Shown are the results of a representative assay as done in duplicate and reproduced in at least two other occasions. Error bars denote S.D.

2.6. Cell count analysis

KLF5-3T3 and mock-3T3 cells were plated on 60-mm-plastic wells (1 x 10⁵ cells/well) at a cell density of 60–70% confluency. They were treated for 24 h in medium containing 1% FBS supplemented with 100 U/ml penicillin G and 100 μg/ml streptomycin with the indicated concentrations of ACR, ATRA or DMSO. Cell numbers were counted by the trypan blue dye exclusion method. To examine the growth-inhibitory effects of ACR, ATRA or DMSO, cells were treated for 24 h. Experiments were reproduced in at least three other occasions.

2.7. DNA fragmentation assay

3T3-KLF5 stable cells were treated with ACR, ATRA or DMSO (as vehicle control) for 24 h. The cells were harvested and then apoptosis was detected by DNA fragmentation assay using the Cell Death Detection ELISA™ Plus Kit (Roche) according to the manufacturer’s instructions. The assay was done in duplicate and reproduced in at least three other occasions.

2.8. Mouse ischemic hind-limb model

Unilateral hind-limb ischemia was induced in 30-week-old male C57BL/6/N mice as described [9]. ACR and soybean oil (control) were administered from 1 week before the procedure up to 7 days after the procedure. Agents were administered orally with a stomach tube (n = 10, five from the control group and five from the ACR 200 mg/kg group). All experiments were approved by the University of Tokyo. Hind-limb blood perfusion was measured with a laser Doppler perfusion imager (LDPI) system (Moor Instruments Ltd.) as described previously [9]. In the models, LDPI was performed after surgery and then on days 2, 4 and 7.

2.9. Statistical analysis

All data are expressed as mean values ± S.D. Statistical analysis of the co-transfection reporter assays and DNA fragmentation assays were analyzed by Student’s t-test, and of the mouse ischemic hind-limb model experiments by ANOVA followed by Student’s t-test. P < 0.05 was considered statistically significant.

3. Results

3.1. ACR inhibits interaction of KLF5 and RARα

We first examined whether ACR affects the interaction of KLF5 and RARα by co-immunoprecipitation assay. Under conditions which KLF5 and RARα interacted in endothelial cells, ACR and ATRA (used as control) inhibited interaction in a dose-dependent manner. Effects of ACR were greater than that for ATRA (Fig. 1A).

As KLF5 and RARα are both zinc-finger proteins which often interact through their zinc fingers, we next examined whether the zinc-finger region of KLF5 mediates this interaction and to mediate the effects of ACR using GST pull-down assay. GST pull-down assay showed that the KLF5 zinc-finger region was sufficient for interaction with full-length RARα, and that ACR attenuated interaction greater than ATRA (Fig. 1B). Additional domain mapping showed that the zinc finger DBDs of KLF5 and RARα were sufficient for interaction, but that
ACR and ATRA did not influence their interaction on GST pull-down assay (Fig. 1C).

ACR thus attenuates interaction of KLF5 and RARα as mediated by the former’s zinc finger region in a dose-dependent manner. The effects of ACR were greater than for ATRA when both were used at the same concentration.

3.2. ACR attenuates the transcriptional effects of KLF5 and RARα on KLF5-downstream PDGF-A chain promoter activity

We next examined effects of ACR on transcription of a KLF5-downstream gene, PDGF-A chain, which is regulated by KLF5 and RARα. Under conditions in which co-transfection of both expression vectors of KLF5 and RARα transactivated PDGF-A chain promoter activity in endothelial cells in a cooperative manner and under which RARα alone did not transactivate the promoter and KLF5 only marginally, ACR attenuated PDGF-A chain promoter activity in a dose-dependent manner (Fig. 2A). These repressive effects of ACR were greater than that for ATRA (Fig. 2A). Co-transfection analysis of RARβ or RARγ with KLF5 showed no effects (Fig. 2B and C). Thus, ACR attenuated the transactivation of KLF5 and RARα on PDGF-A chain promoter activity as regulated by KLF5 and RARα in a selective manner.

3.3. ACR inhibits the growth-stimulatory effect of KLF5

To further understand effects of ACR on KLF5 functions in the cell, we next examined whether ACR might affect cell growth as regulated by KLF5. Using a stable transfectant expressing KLF5 which we previously reported to show increased cell proliferation due to effects of KLF5 [6], ACR inhibited growth in a dose-dependent manner, with the effect on stable transfectants being larger than on mock cells suggesting involvement of selective cell growth inhibition through effects on KLF5 (Fig. 3A and B). Further, ACR inhibited growth of the KLF5-stable transfectant in a manner that was both dose-dependent and greater than that for ATRA (Fig. 3C). Moreover, we investigated the effects of ACR on cell apoptosis as we have recently shown that ACR induces apoptosis [5], which showed that ACR markedly induces apoptosis of 3T3-KLF5 stable cells in a dose-dependent manner as compared to the dose-dependent yet very marginal increases as induced by ATRA (Fig. 3D). Taken together, ACR inhibits the growth proliferative effects of KLF5 at least in part through apoptosis.

3.4. ACR inhibits angiogenesis in the acute phase of ischemia

To test functional effects of ACR on angiogenesis, hind-limb ischemia experiments in mice were done with ACR or soybean oil (control). Blood flow of the ischemic muscle treated with

![Fig. 3](image-url)
ACR showed mild recovery in comparison with those treated with control up to 7 days (Fig. 4A and B) thus indicating that ACR impairs angiogenesis-dependent recovery from ischemia consistent with its role to inhibit tumor angiogenesis [10].

4. Discussion

Retinoids have been shown to indirectly modulate the effects of transcription factor KLF5 on vascular remodeling through RARα [1]. Agonists and antagonists affect KLF5 functions in opposing manners and thus may be of use to differentially modulate KLF5 actions. In addition to ATRA, the synthetic retinoids Am80 and LE135 have been pursued to date. In the present study, we further investigated whether ACR, which has a high safety profile and is being presently pursued for clinical use [3,4] and that we have recently shown to modulate car

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References

[1] Shindo, T., Manabe, I., Fukushima, Y., Tobe, K., Aizawa, K., Miyamoto, S., Kowase, K., Moriyama, N., Imai, Y., Kawakami, H., Nishimatsu, H., Ishikawa, T., Suzuki, T., Morita, H., Maemura, K., Sata, M., Hirata, Y., Komukai, M., Kagechika, H., Kadowaki, T., Kurabayashi, M. and Nagai, R. (2002) Krüppel-like transcription factor 9 (KLF9), has further been shown to interact with progesterone receptor-A (PR-A) [18]. Our present knowledge of interaction pathways is still relatively limited, but once we have a comprehensive understanding of protein–protein interactions and their regulation, especially by ligand and other pathophysiologic conditions, we should be able to better target specific pathways in a selective manner. Further crystallographic studies will also help us better understand the mechanistic basis underlying interaction and their regulation.

ACR attenuated growth stimulatory effects of KLF5 at least in part through apoptosis. Therefore, ACR modulated KLF5 activities in a functional manner.

Modulation of transcription factor function through indirect modulation of interacting partners or complex formation is relatively new both as a molecular mechanism but even more as a therapeutic pathway. Zinc-finger proteins are known to interact often through their zinc-finger regions, and as expected, KLF5 and RARα interacted through these regions [11]. As the ligand-binding domain of RARα is separate from the zinc-finger region of RARα [12], and as the interaction of the DNA-binding domain of RARα lacking its ligand binding domain with KLF5 was not affected by ligand, we believe that ACR by binding the ligand-binding domain induces an allosteric effect on the zinc-finger domain thus affecting interaction with its interactor, in this case being KLF5. Modulation of KLF5 functions or any other transcription factor using small molecules is difficult given that they must penetrate the cell and nucleus to exert functions on transcription. Regulation of expression by gene silencing (e.g. RNA interference) has proven to be the most potent means to regulate molecular function which is a commonly used method [13], but RNA interference has limitations as it is still not readily applicable at the tissue and animal level as compared to successful experimental use at the cellular level. Translation of RNA interference technology to the clinic has therefore been limited to use in ocular disease (e.g. macular degeneration) and cancer requiring direct injection into cells [14,15]. Thus, indirect modulation through regulation of interactor is a viable means for therapeutic intervention at present. Although we have shown that KLF5 actions can be modulated by retinoids, other combinations through different interactors will surely be possible.

Mechanistically, nuclear receptors have been well documented to show regulation of transcriptional complex formation by ligand with interchange of co-repressors by co-activator proteins and complexes [16]. Interaction between nuclear receptor and zinc-finger transcription factor has also been documented as described for interaction of Sp1 with nuclear receptor [17]. A related protein, Krüppel-like transcription factor 9 (KLF9), has further been shown to interact with progesterone receptor-A (PR-A) [18]. Our present knowledge of interaction pathways is still relatively limited, but once we have a comprehensive understanding of protein–protein interactions and their regulation, especially by ligand and other pathophysiologic conditions, we should be able to better target specific pathways in a selective manner. Further crystallographic studies will also help us better understand the mechanistic basis underlying interaction and their regulation.


