Characterization of \textit{Ath29}, a major mouse atherosclerosis susceptibility locus, and identification of \textit{Rcn2} as a novel regulator of cytokine expression

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\textsuperscript{1}Center for Public Health Genomics and Departments of \textsuperscript{2}Public Health Sciences, \textsuperscript{3}Radiology and Medical Imaging, \textsuperscript{4}Pharmacology, and \textsuperscript{5}Biochemistry and Molecular Genetics, University of Virginia, Charlottesville, Virginia

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Manichaikul A, Wang Q, Shi YL, Zhang Z, Leitinger N, Shi W. Characterization of \textit{Ath29}, a major mouse atherosclerosis susceptibility locus, and identification of \textit{Rcn2} as a novel regulator of cytokine expression. \textit{Am J Physiol Heart Circ Physiol} 301: H1056–H1061, 2011. First published June 10, 2011; doi:10.1152/ajpheart.00366.2011.---\textit{Ath29} is an atherosclerosis susceptibility locus on chromosome 9 identified in an intercross between C57BL/6 (B6) and C3H/HeJ (C3H) apolipoprotein E-deficient (apoE\textsuperscript{−/−}) mice. This locus was subsequently replicated in two separate intercrosses that developed early or advanced atherosclerotic lesions. The objective of this study was to characterize \textit{Ath29} through construction and analysis of a congenic strain and identify underlying candidate genes. A congenic line was constructed by introgressing the chromosomal segment harboring \textit{Ath29} from C3H.apoE\textsuperscript{−/−} into B6.apoE\textsuperscript{−/−} mice. Congenic mice developed significantly smaller early and advance atherosclerotic lesions than B6/apoE\textsuperscript{−/−} mice. Microarray analysis revealed 317 genes to be differentially expressed in the aorta of congenic mice compared with B6.apoE\textsuperscript{−/−} mice. Pathway analysis of these genes suggested the Ca\textsuperscript{2+} signaling pathway to be implicated in regulating atherosclerosis susceptibility. \textit{Rcn2} is located underneath the linkage peak of \textit{Ath29} and involved in Ca\textsuperscript{2+} signaling. Multiple single-nucleotide polymorphisms between B6 and C3H mice were detected within and surrounding \textit{Rcn2} with one single-nucleotide polymorphism falling within an upstream cAMP response element. Immunostaining demonstrated its expression in atherosclerotic lesions. Knockdown of \textit{Rcn2} with small interfering RNAs resulted in significant reductions in both baseline and oxidized phospholipid-induced VCAM-1 and monocyte chemoattractant protein-1 expression by endothelial cells. \textit{Ath29} is confirmed to be a major atherosclerosis susceptibility locus affecting both early and advanced lesion formation in mice, and \textit{Rcn2} is identified as a novel regulator of cytokine expression.

Atherosclerosis, which is the primary cause of coronary heart disease, ischemic stroke, and peripheral arterial disease, constitutes one of the most important public health problems in the United States and worldwide. Although environmental factors, such as high-fat diet, lack of exercise, and smoking, play a role in atherosclerosis, genetic factors are a major determinant for the development of the disease (27, 26). Only a small portion of atherosclerosis cases are caused by rare genetic mutations that result in Mendelian traits segregating in families. The common forms of atherosclerosis involve multiple genes and exhibit significant gene-environment interactions. The identification of susceptibility genes involved has not been readily achieved in humans. To date, robust and replicable associations in nonisolated populations are limited to variants in only two genes: \textit{APOE} (16) and \textit{ALOX5} (2). The recent advent of genome-wide association studies (GWAS) has led to the revelation of a dozen genes that may confer an increased risk to coronary heart disease, including \textit{PSRC1, MTHFD1L, CXL12, SMAD3, MIA3, MRAS, HNF1A, C12orf43, the SLC22A3-LPA2-LPA gene cluster, CDKN2A/CDKN2B, and KIAA1462} (15, 28, 3, 23, 4), but it is challenging to establish causality between a genetic variant and disease in humans due to relative small gene effect, complex genetic structure, and environmental influences.

A complementary approach to the identification of human disease genes is to use model organisms. The mouse is the leading mammalian model organism for basic genetic research and for studying human diseases, including atherosclerosis (1). Inbred mouse strains exhibit a wide spectrum of variations in atherosclerosis and associated traits (11, 10). Among them, C57BL/6 (B6) and C3H mice are the most phenotypically divergent strains in terms of variations in atherosclerotic lesion size (12, 14). B6 mice develop much larger atherosclerotic lesions than C3H mice when fed an atherogenic diet or deficient in apolipoprotein E (apoE\textsuperscript{−/−}) (12, 18). Using an intercross derived from B6.apoE\textsuperscript{−/−} and C3H.apoE\textsuperscript{−/−} mice, we identified a significant quantitative trait locus (QTL), \textit{Ath29}, on chromosome 9 that affects atherosclerotic lesion size. This QTL was subsequently replicated in two separate intercrosses that developed early or advanced atherosclerotic lesions (24, 25).

In this study, we confirmed \textit{Ath29} through construction and analysis of a congenic strain carrying the resistant C3H allele on the B6.apoE\textsuperscript{−/−} background. We then performed microarray analysis of gene expression in the aorta of congenic and B6.apoE\textsuperscript{−/−} control mice to prioritize candidate genes in the linkage region of \textit{Ath29}. \textit{Rcn2}, encoding reticulocalbin 2, was identified as the most promising candidate gene for \textit{Ath29}. A functional analysis using small interfering RNAs (siRNAs) to knockdown the gene led to the revelation of a critical role for \textit{Rcn2} in regulating cytokine production.

METHODS

Mice. B6.apoE\textsuperscript{−/−} mice were purchased from Jackson Laboratory, and C3H.apoE\textsuperscript{−/−} mice were generated in our laboratory (22). Chromosome 9 congenic mice (B6.C3H-Chr9 apoE\textsuperscript{−/−}) were generated by introgressing the chromosomal segment harboring \textit{Ath29} from the donor strain C3H.apoE\textsuperscript{−/−} into B6.apoE\textsuperscript{−/−} recipient mice using the standard congenic breeding strategy (20). Microsatellite markers, \textit{D9Mit84} (13.7 Mb), \textit{D9Mit247} (36.9 Mb), \textit{D9Mit208} (62.1 Mb), \textit{D9Mit196} (85.8 Mb), and \textit{D9Mit117} (115.5 Mb), were used to evaluate introgression of the congenic segment. The mice were weaned onto a chow diet at 3 wk of age. At 6 wk of age, the mice either continued with the chow diet or switched onto a Western diet...
containing 21% fat, 34.1% sucrose, 0.15% cholest erol, and 19.5% casein (TD-88137, Harlan) and then maintained on the diet for 12 wk. All procedures were carried out in accordance with current National Institutes of Health's guidelines and approved by the Institutional Animal Care and Use Committee.

**Microarray assays.** Total RNA was isolated with a QIAGEN RNeasy kit from the thoracic aorta of 8-wk-old female congenic and B6.apoE−/− mice that had been fed a chow or 2 wk of the Western diet, as we previously reported (29). At these stages, the aorta had no detectable atherosclerotic lesions. The quality of RNA was evaluated by an Agilent Bioanalyzer. Because the yield of RNA from a single aorta was low, each RNA sample used for microarray assays was pooled in an equal amount from three individual mice for each group. Microarray analysis with Affimetrix GeneChip Mouse Genome 430 2.0 arrays was conducted at our GeneChip/Microarray Bioinformatics facility, according to Affymetrix's instructions. The microarray data have been deposited in the NCBI Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/), and the GEO series accession number is GSE29149.

Background correction and normalization of raw gene expression data were performed using the R/limma package. We then ranked genes for differential expression across the two strains using a widely used empirical Bayes approach implemented in the R package EBarrays posterior probability of differential expression (6).

**Pathway analysis.** EBarrays analysis revealed that 317 genes were differentially expressed across congenic and B6.apoE−/− strains in a pooled analysis of mice on both chow and Western diets, using a cutoff of EBarrays posterior probability > 0.5. These genes were used to detect biological pathways enriched using Ingenuity (Ingenuity Systems, Mountain View, CA), as we previously reported (29).

**Real-time PCR analysis.** The mRNA expression level of Rcn2 relative to GAPDH mRNA in the aortic wall of congenic and B6.apoE−/− mice was determined by real-time PCR. Total RNA prepared from the aorta was treated with DNase I and reverse transcribed to cDNA using ThermoScript RT-PCR system (Invitrogen). cDNA was added to each PCR reaction that contained SYBR Green supermix reagent (Bio-Rad) and 0.2 μM forward and reverse primers (Rcn2: 5′-ACCAGGGCATTGCACAAAGAG-3′/5′-GGCTTC-GCTGGTAAAGGAACA-3′; GAPDH: 5′-GAGGCCGTTGCTGAG-TATGT-3′/5′-AAGGGTGAGCCAAAAGGGTCATC-3′). All reactions were run in triplicate on an iCycler iQ5 machine (Bio-Rad) under the condition of 15 s at 95°C, 30 s at 55°C, and 30 s at 72°C for 40 cycles. The cycle threshold was determined for each sample, and the ratios of Rcn2 to GAPDH in cycle threshold values were calculated.

**Immunohistochemical analysis.** Immunostaining of acetone-fixed cryosections of mouse aorta was performed as previously described (19), using a rabbit anti-mouse Rcn2 antibody (ProteinTech Group).

**In vitro functional assays.** The role of Rcn2 in cytokine production was tested by transfecting endothelial cells with specific siRNAs.

Endothelial cells were isolated from the aorta of B6.apoE−/− mice using an established explant technique as we previously reported (17). Three Rcn2-specific siRNAs and a control siRNA were purchased from Qiagen. Each siRNA at the final concentration of 0.2 μM was incubated at room temperature with 1% lipofectamine 2000 (Invitrogen) in DMEM supplemented with 1% FBS and 1% penicillin-streptomycin for 30 min. The transfection mix was used to treat endothelial cells at ~70% confluence. After a 24-h incubation with endothelial cells at 37°C in a 95% air-5% CO2 incubator, the transfection medium was collected and reserved for the determination of baseline VCAM-1 and monocyte chemoattractant protein-1 (MCP-1) levels by ELISA. The cells were then treated with 100 μg/ml oxidized 1-palmitoyl-2- arachidonoyl-sn-glycéro-3-phosphocholine (oxPAPC) in DMEM supplemented with 1% FBS and 1% penicillin-streptomycin for 4 h. At the end of treatment, the medium was again collected and reserved for determination of VCAM-1 and MCP-1, and the cells were harvested for extraction of total RNA and protein with TRIzol reagents (Invitrogen).

**Quantification of VCAM-1 and MCP-1.** Soluble VCAM-1 and MCP-1 were quantified with ELISA kits from R&D Systems according to the manufacturer's instructions.

**Statistical analysis.** Data were expressed as means ± SE. Student's t-test was used to determine the statistical significance of differences in measurements between congenic and B6 mice with the same treatment. Differences were considered statistically significant at P < 0.05.

**RESULTS**

**Atherosclerotic lesion formation in congenic mice.** Female B6.C3H-Chr9.apoE−/− congenic mice were compared with their background B6.apoE−/− mice for both early and advanced atherosclerotic lesion formation in the aortic root. At 12 wk of age on the chow diet, congenic mice had a mean lesion area of 6,029 ± 1,023 μm²/section (n = 9), which was threelfold smaller than the lesion area of 20,733 ± 4,209 μm²/section in B6.apoE−/− mice (n = 16; P = 0.0034) (Fig. 1). When fed the Western diet for 12 wk, which started at 6 wk of age, congenic mice had a mean lesion area of 296,950 ± 30,742 μm²/section (n = 12), significantly smaller than the lesion area of 512,309 ± 20,760 in B6.apoE−/− mice (n = 24; P < 0.0001).

**Global gene expression analysis.** Three hundred seventeen genes and transcribed sequences were found to be differentially expressed in the aortic wall of female congenic and B6.apoE−/− mice in pooled analysis of mice on both chow and Western diets (supplemental Table 1). Twenty-two genes were mapped

![Fig. 1. Atherosclerotic lesion areas (in μm²/section) in the aortic root of female chromosome 9 congenic mice and B6.apoE−/− control mice. Mice were maintained on a rodent chow diet and euthanized at 12 wk of age (left) or switched onto a Western diet at 6 wk of age and fed the diet for 12 wk (right). Values are means ± SE for 9 to 24 mice. *P < 0.005 vs. B6.apoE−/−.](http://ajpheart.physiology.org/)

1 Supplemental material for this article is available at the American Journal of Physiology-Heart and Circulatory Physiology website.
to the confidence interval of Ath29 (30–80 Mb). Ets1, Cbl, Tnem25, Cadm1, Calml4, Dis3l, Tmod3, and Gsta4 were upregulated in the congenics, and Fez1, Assam, Hspa8, Sc5dl, Fam55d, Nnut, Slh, Rcn2, Hmg20a, Tipin, Cilp, Tpm1, C9200006011RIK, and Fam55d were downregulated.

Those genes that were differentially expressed between congenic and background control mice were analyzed using the Ingenuity Systems to identify underlying biological pathways. The most significantly enriched pathway was the Ca2+ signaling pathway (Fig. 2). Other significantly enriched pathways include cellular effects of sildenafil (Viagra), integrin-linked kinase signaling, actin cytoskeleton signaling, hepatic fibrosis/hepatic stellate cell activation, tight junction signaling, cardiac hypertrophy signaling, and germ cell-sertoli cell junction signaling (supplemental Table 2).

Rcn2 and Calml4 are the two genes that are located within the confidence interval of Ath29 and are potentially involved in Ca2+ signaling. Multiple single-nucleotide polymorphisms (SNPs) between B6 and C3H were detected within and upstream the Rcn2 gene, including 1039T/C, 1700A/G, 1988G/A, 2486C/T, 3700G/A, 5623G/A, 6832G/T, 7562A/G, −1877T/C, and −1510A/G. None of the SNPs led to amino acid substitutions. The T→C SNP at −1,877 bp upstream of Rcn2 in C3H creates a DNA sequence of GTGACAA, which is a binding site for the cAMP-responsive transcription factor. In contrast, no polymorphisms between the B6 and C3H strains were found within or close to Calml4.

Biological relevance of Rcn2 to atherosclerosis. Quantitative real-time PCR was performed to confirm the difference between congenic and B6.apoE−/− mice in Rcn2 mRNA expression levels in the aortic wall. The ratio of Rcn2 to GAPDH in real-time PCR cycle threshold values was 0.075 ± 0.014 in the congenics (n = 7), significantly lower than that of 0.130 ± 0.009 in B6.apoE−/− mice (n = 5, P = 0.0096) (Fig. 3). Immunohistochemical analysis revealed the expression of Rcn2 in atherosclerotic lesions (Fig. 4). It was more abundantly expressed in the endothelial layer and adjacent atherosclerotic lesions. To determine the role of Rcn2 in cytokine production, endothelial cells isolated from the aorta of B6 mice were transfected with either siRNAs targeting Rcn2 or control siRNA for 24 h before they were treated with oxPAPC in a separate medium for 4 h. oxPAPC is an active component of minimally modified LDL. When compared with the control siRNA, all three siRNAs resulted in a reduced expression of Rcn2 protein (Fig. 5). In contrast, the expression level of GAPDH in endothelial cells was not reduced by these siRNAs. Soluble VCAM-1 and MCP-1 levels in the medium that had been incubated with endothelial cells were measured by ELISA. When compared with control siRNA, all three siRNAs targeting Rcn2 resulted in a 40% reduction in VCAM-1 levels and a 70% reduction in MCP-1 levels before the addition of oxPAPC. After treatment with oxPAPC, the cells transfected with specific siRNAs showed a 70% reduction in VCAM-1 levels and a 90% reduction in MCP-1 levels in the medium compared with the cells transfected with control siRNA. Because the medium at the baseline had been incubated with endothelial cells for 24 h, whereas the incubation time for oxPAPC was only 4 h, the longer incubation time probably explains the comparable and even higher sVCAM-1 or MCP-1 levels observed at the baseline.

DISCUSSION

The genetics of atherosclerotic vascular disease is poorly understood outside of the known risk factors, such as blood cholesterol levels, hypertension, and hyperhomocysteinemia. Using the B6 and C3H mouse model, we have demonstrated that genetic factors affect atherosclerosis susceptibility through pathways different from the traditional risks. When transplanted into identical F1 recipients, the donor aortic segments of B6.apoE−/− mice developed much larger atherosclerotic lesions than those of C3H.apoE−/− mice (13), indicating that...
the arterial wall is a major source of variation in atherosclerosis susceptibility. Reciprocal bone marrow transplantation between wild-type B6 and C3H mice had little influence on atherosclerotic lesion size of recipient mice (18), indicating that variations in monocyte function are not responsible for differential atherosclerosis susceptibility. Moreover, even though C3H. apoE<sup>−/−</sup> mice had higher plasma cholesterol levels, they developed much smaller atherosclerotic lesions than B6. apoE<sup>−/−</sup> mice on a chow diet (18), indicating that major atherosclerosis susceptibility genes exert effects beyond plasma cholesterol.

In the present study, we confirmed the existence of Ath29 through an analysis of congenic mice that carried the resistant C3H allele on the B6. apoE<sup>−/−</sup> genetic background. Congenic mice exhibited a threefold reduction in atherosclerotic lesion size on the chow diet and an approximately twofold reduction on the Western diet. However, the reductions were much smaller compared with a 100-fold difference in lesion size on the chow diet and a 10-fold difference on the Western diet between the two parental strains (17), indicating that other QTLs also contribute to the differential atherosclerosis susceptibility of the two parental strains.

Since the arterial wall is a major source of variation in atherosclerosis susceptibility, we examined the gene expression in the aorta of congeneric and B6. apoE<sup>−/−</sup> mice by microarrays. Much fewer genes were found to be differentially expressed relative to the number detected in the two parental strains in the previous study (29). Because congenics differed from B6. apoE<sup>−/−</sup> mice only in the congeneric region, the genes that differed in expression were probably those in the congeneric region or secondary consequences of gene differences in the congeneric region. Using combined congeneric and gene expression profiling strategies, we have pinpointed Rcn2 as the most probable candidate gene of Ath29. Rcn2 was downregulated in the arterial wall of congenic mice compared with their background strain as detected by the microarrays, and this result was confirmed by real-time PCR. There were multiple SNPs between the B6 and C3H strains within and surrounding Rcn2, and these SNPs should be responsible for changes in Rcn2 mRNA expression levels. Immunostaining demonstrated the...

**Fig. 4.** Representative cryosections showing the expression of Rcn2 protein in atherosclerotic lesions. Cross sections of the aortic root of a B6. apoE<sup>−/−</sup> mouse were stained with (right) or without (left) the presence of an anti-Rcn2 antibody.

**Fig. 5.** Rcn2 mediates both baseline and oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (oxPAPC)-induced VCAM-1 and monocyte chemotactic protein-1 (MCP-1) production. Endothelial cells isolated from the aorta of B6. apoE<sup>−/−</sup> mice were incubated with a transfection medium containing a specific small interfering RNA (siRNA) or a control siRNA for 24 h and then treated with a fresh medium containing oxPAPC for additional 4 h. The medium that had been incubated with endothelial cells was collected for VCAM-1 and MCP-1 measurements, and cells were harvested for Western blot analysis of Rcn2 and GAPDH expression. A: Western blot analysis of Rcn2 and GAPDH expression in endothelial cells. B: VCAM-1 concentrations (in ng/ml) in the medium before and after addition of oxPAPC. C: MCP-1 concentrations (in pg/ml) in the medium before and after addition of oxPAPC. For the baseline, the medium had been incubated with endothelial cells for 24 h, whereas the incubation time for oxPAPC was only 4 h. The longer incubation time may explain a comparable and even higher sVCAM-1 or MCP-1 level at the baseline.
expression of Rcn2 in atherosclerotic lesions. Furthermore, functional studies uncovered a novel role for Rcn2 in regulating both basal and oxidized lipid-induced MCP-1 and VCAM-1 production. The expression of Rcn2 in endothelial cells was only partially downregulated rather than was completely abolished by the siRNAs. Despite this partial inhibition, the impact on the expression of VCAM-1 and MCP-1 in endothelial cells was dramatic, suggesting a critical role for Rcn2 in regulating cytokine production. It is noteworthy that the protein used for Western blot analysis was prepared from endothelial cells that were transfected with siRNAs for 24 h and then treated with oxPAPC for 4 h. Since siRNAs were not present during the incubation with oxPAPC, Rcn2 expression could partially be recovered during this period. MCP-1 and VCAM-1 are primarily associated with the recruitment of monocytes into the arterial walls, the key process in the pathogenesis of atherosclerosis. A greater expression of these molecules by vascular wall cells is expected to lead to more monocytes recruited to the subendothelial space where they differentiate, take up lipids, and form foam cells.

Rcn2 encodes a 55-kDa Ca\(^{2+}\)-binding protein with a signal sequence, six EF-hands and an endoplasmic reticulum (ER)-retention sequence leading to the protein being localized strictly in the ER (5). Its localization in the ER has been nearly completely abolished by the siRNAs. Despite this partial inhibition, the impact on the expression of VCAM-1 and MCP-1 in endothelial cells was dramatic, suggesting a critical role for Rcn2 in regulating cytokine production. It is noteworthy that the protein used for Western blot analysis was prepared from endothelial cells that were transfected with siRNAs for 24 h and then treated with oxPAPC for 4 h. Since siRNAs were not present during the incubation with oxPAPC, Rcn2 expression could partially be recovered during this period. MCP-1 and VCAM-1 are primarily associated with the recruitment of monocytes into the arterial walls, the key process in the pathogenesis of atherosclerosis. A greater expression of these molecules by vascular wall cells is expected to lead to more monocytes recruited to the subendothelial space where they differentiate, take up lipids, and form foam cells.

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The present finding on the role of Rcn2 in regulating cytokine production provides an explanation to the genetic links among responsiveness to oxidized LDL, inflammation, and atherosclerosis. We have observed marked differences between the B6 and C3H strains in response to oxidized LDL. Endothelial cells and vascular smooth muscle cells isolated from atherosclerosis-susceptible B6 mice exhibit a dramatic induc tion of MCP-1, macrophage colony-stimulating factor (MCSF), VCAM-1, and heme oxygenase-1 after treatment with oxidized LDL, whereas cells from resistant C3H mice showed little or no induction (9, 17). Moreover, endothelial responses to oxidized LDL cosegregated with atherosclerotic lesion size in a set of recombinant inbred strains derived from B6 and C3H mice. As inflammatory gene products, such as MCP-1, MCSF, and VCAM-1, are associated with the recruitment or differentiation of monocytes, the magnitude of the activation of these genes by oxidized LDL would influence the number of monocytes recruited and, consequently, affect atherosclerotic lesion size.

The present pathway analysis of differentially expressed genes in the congeneric strain indicated that the Ca\(^{2+}\) signaling pathway was the most enriched pathway. This result is consistent with our previous observations made in the two parental strains (29). A number of other genes showed differential expression on the microarrays, and some of them might be directly or indirectly affected by Rcn2.

In summary, we have confirmed the effect of Ath29 on both early and advanced lesion formation and discovered a new role for Rcn2 in regulating cytokine production in mice. As the likely key regulator in the pathway of oxidized LDL-induced cytokine production, Rcn2 appears to be an appropriate target for pharmacological interventions of atherosclerosis.


