Loss-of-Function Deletion of the Steroid Receptor Coactivator-1 Gene in Mice Reduces Estrogen Effect on the Vascular Injury Response

Yuhui Yuan, Jianming Xu

Objective—The steroid receptor coactivator-1 (SRC-1) is a transcriptional coactivator for nuclear receptors including estrogen receptor (ER). SRC-1 can interact with ER in an estrogen binding-dependent manner to potentiate the transcriptional activity of ER. Previous studies showed that SRC-1 was required for the full function of ER in cultured cells and in the reproductive system. In this study, we have tested the hypothesis that SRC-1 is required for the inhibition of neointima formation by estrogen in a vascular wall.

Methods and Results—The expression of SRC-1 protein in the vascular wall was examined by immunoblotting and immunohistochemistry. Wild-type and SRC-1 null mice were ovariectomized, and then unilateral ligation of the carotid artery was performed to induce neointima growth in these mice. Mice were treated with placebo or estrogen. Neointima growth near the ligation site was examined and quantitatively analyzed. These experiments demonstrated that SRC-1 was expressed in the endothelial cells (ECs), vascular smooth muscle cells (VSMCs), and neointima cells. The neointima growth induced by the ligation of common carotid artery was almost completely inhibited by estrogen in wild-type mice, but was only partially inhibited in SRC-1-null mice. Further analysis revealed that the blunted inhibition of neointima formation by estrogen was attributed to a less inhibition of neointimal cell proliferation.

Conclusions—SRC-1 is expressed in ECs, VSMCs, and neointima cells. SRC-1 expression in these cells facilitates estrogen/ER-mediated vasoprotection through the inhibition of neointima formation after a vascular injury. (Arterioscler Thromb Vasc Biol. 2007;27:1521-1527.)

Key Words: SRC-1 ■ estrogen ■ estrogen receptor ■ carotid artery ■ neointima

It is well known that estrogen can protect women from cardiovascular diseases; however, the defensive molecular mechanisms are not fully understood.¹,² It is generally accepted that estrogen gives rise to vasoprotection through both systemic and direct effects on blood vessels by taking rapid nongenomic and longer-term genomic pathways.¹–⁴ Both estrogen receptor α (ERα) and β (ERβ) are expressed in blood vessel walls and reported to be involved in estrogen vasoprotection.⁵–¹⁰ The protective effects of ERα include the promotion of reendothelialization, the inhibition of vascular smooth muscle cell (VSMC) proliferation, and the attenuation of atherosclerotic plaque progression.¹,⁸ The protective effects of ERβ include the inhibition of VSMC proliferation, the reduction of neointima formation, and the improvement of vascular relaxation response.⁷,¹⁰,¹¹ Interestingly, a recent study has suggested that estrogen may contribute to the vascular healing process and to the prevention of restenosis by improving reendothelialization through ERα activation and by decreasing VSMC migration and proliferation through ERβ stimulation.¹² ERs, which do not act alone, require the recruitment of steroid receptor coactivators (SRCs) and general transcription factors to form an active transcriptional complex for enhancement of target gene expression.¹²,¹³

SRC-1 is the first identified member of the p160 SRC gene family that also includes SRC-2 (GRIP-1 or TIF2) and SRC-3 (p/CIP, RAC3, ACTR, or AIB1).¹²–¹⁴ These transcriptional coactivators interact with nuclear receptors in a ligand-binding dependent manner and recruit general coactivators such as cAMP-responsive element binding protein (CREB) binding protein (CBP) or p300 to the target gene promoter for activation of gene transcription.¹²,¹³ Because the cellular concentrations of these coactivators are usually limiting and these coactivators can serve as a platform to regulate gene expression by integrating signals from many signaling transduction pathways, changes in these coactivator levels and/or activities will lead to a significant alteration of gene expression.¹₂,¹³,¹⁵–¹⁷ Studies using knockout mouse models have shown that the p160 SRC coactivators play important pleiotropic roles in the regulation of development, somatic growth, reproduction, metabolic homeostasis, and breast cancer.¹³

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From the Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Tex.
Correspondence to Jianming Xu, PhD, Department of Molecular and Cellular Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. E-mail jxu@bcm.tmc.edu
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Genetic disruption of the SRC-1 gene in mice results in partial hormone resistance and developmental delay in the Purkinje cell development.18–20 Mice lacking SRC-2 exhibit severe defects in both male and female reproductive functions.21–23 Genetic deletion of the SRC-3 gene in mice causes growth retardation, delays pubertal development and mammary gland growth, reduces female reproductive function, and makes mice resistant to oncogene and carcinogen-induced mammary gland carcinogenesis.24–26 These distinct phenotypes observed in different SRC knockout mice indicate that each SRC family member possesses different tissue-specific functions.

Our previous study has investigated the expression and in vivo function of SRC-3 in the estrogen-modulated remodeling of vascular wall after a vascular injury.27 We found that SRC-3 is expressed in VSMCs and endothelial cells (ECs). The loss of SRC-3 function causes a decrease in sensitivity of estrogen-mediated neointimal growth inhibition, suggesting SRC-3 is an in vivo ER coactivator that enhances the vasoprotective role of estrogen. In this study, we further examined the expression pattern of SRC-1 in vascular walls and assessed the role of SRC-1 in the estrogen-dependent vasoprotection during vascular remodeling after injury by applying a carotid artery ligation model to SRC-1 knockout mice.

**Methods**

**Animals**

SRC-1–null mice were generated by gene targeting as described previously.18 The gene-targeting event resulted in a loss-of-function deletion of the SRC-1 gene. All mice were genotyped by polymerase chain reaction (PCR) using genomic DNA template and allele-specific primers as described previously.18 Four-month-old female wild-type (WT) and SRC-1–null (SRC-1–/–) mice were produced and used for all experiments. For all surgical procedures, mice were anesthetized by intraperitoneal injection of Avertin (2.5% in saline, 15 μL/g body weight). Animals were euthanized by overdose of Avertin. All animal protocols were approved by the Animal Care and Use Committee of Baylor College of Medicine.

**Carotid Ligation Model**

The carotid artery ligation model used in this study was described previously.27 Briefly, mice were anesthetized and their left common carotid arteries were exposed and ligated near the carotid bifurcation. All the animals recovered from surgery and showed no apparent symptoms of stroke and no signs of infection.

**Hormonal Treatment**

Female WT and SRC-1–/– mice were randomly divided into 4 groups. Each group contains 6 mice. On day 1, all mice were ovariectomized as described previously.27 Starting on day 7, only vehicle (sesame oil, 0.1 mL/mouse/d, s.c.) was injected into WT mice in the first group and SRC-1–/– mice in the second group, while 17β-estradiol in sesame oil (0.1 mg/0.1 mL/mouse/d, s.c.) was injected into WT mice in the third group and SRC-1–/– mice in the fourth group. We have previously demonstrated that this estrogen replacement protocol provides about 2 ng/mL of 17β-estradiol in the serum, which is sufficient to inhibit the carotid ligation-induced neointima formation in WT mice.27 On day 14, the procedure for ligation of the left common carotid arteries was performed on all mice. On day 28, mice were euthanized and transcardially perfused with 4% paraformaldehyde in phosphate-buffered saline under physiological pressure. About a 5-mm posterior fragment of the common carotid artery to the ligation site was excised for morphological and immunohistochemical analyses.

**Morphological Analysis**

Paraformaldehyde-fixed vessel fragments were embedded in paraffin. Serial cross-sections were cut at 5 μm in thickness for morphological and immunohistochemical analysis. Because the neointima growth at a given location was inversely correlated with the distance from the ligation site in the carotid artery, the following procedure was performed to ensure that the vascular morphology in different mice was examined at comparable location. The embedded vessels were cut from the ligation site and the distance from the ligation site was calculated from the number of sections and the thickness of each section. Three adjacent sections at the distance of 2 mm from the ligation site were used for morphological analysis of neointima growth. Sections were stained with Verhoeff-van Geison solution and counterstained with hematoxylin and eosin. Digitized images were taken under a Zeiss Axioskop 2 equipped with a Spot charge-coupled device (CCD) camera digital image system. Morphometric analysis of digitized images was performed using the NIH Image 1.61 software as previously described.27

**Immunohistochemistry (IHC) and Western Blot Analysis**

IHC and western blot analysis were performed as previously described.27 Antibodies against the proliferating cell nuclear antigen (PCNA), ERα, and SRC-2 were purchased from Santa Cruz. Antibodies against SRC-1 and ERβ were purchased from Abcam. The SRC-3 antibody was a gift from Dr J. Wong at Baylor College of Medicine. The β-actin antibody was obtained from Sigma. For Western blot analysis, fresh tissues were homogenized in cold lysis buffer containing 0.1% SDS, 2 μmol/L PMSF, 10 μg/mL leupeptin and aprotinin, and 50 mmol/L Tris-HCl (pH 7.4), and clear tissue lysates were collected after centrifugation. Samples were separated by SDS-PAGE and blotted onto a nitrocellulose membrane. The blots were probed with primary antibodies against SRC-1, ERα, ERβ, SRC-2, SRC-3, or β-actin and developed by using appropriate secondary antibodies and enhanced chemiluminescence reagents (Amersham).

**Results**

**Detection of SRC-1 Protein in VSMCs and ECs**

To determine whether SRC-1 protein was present in the blood vessels, we extracted total proteins from aortas and carotid arteries of WT and SRC-1–/– mice and analyzed SRC-1 protein by Western blot. The SRC-1 protein band was clearly detected from the WT tissue extracts, but not from the SRC-1–/– tissue extracts (Figure 1A). To identify the cell types in which SRC-1 might have a physiological function in the blood vessel, IHC was performed with a SRC-1 antibody. The specificity of this antibody was validated by using vascular tissue sections prepared from SRC-1–/– aorta as the negative control (Figure 1B). In the WT vascular tissue sections, SRC-1 immunoreactive signals were detected in the nuclei of both VSMCs and ECs (Figure 1C). In addition, positive SRC-1 staining signals were also found in most of the cells located in the intima region of the carotid arteries where the neointima growth was induced by the ligation injury in the ovariectomized mice (Figure 1E). As expected, SRC-1 was undetectable in the intima of carotid arteries in SRC-1–/– mouse tissue sections (Figure 1E). These results indicate that SRC-1 is expressed in VSMCs, ECs, and in intimal cells of normal or injured vessels.
Decreased Sensitivity of Estrogen-Mediated Inhibition of Neointima Formation

To explore the role of SRC-1 in vascular remodeling after vessel injury, we used a well-established carotid artery ligation model whereby neointima formation is steadily induced after cessation of blood flow. In both ovariec-tomized WT and SRC-1−/− mice with depleted estrogen, robust neointimal growth near the ligation site was induced at day 14 after the surgery of carotid artery ligation (Figure 2C). In contrast, the identical estrogen treatment only partially inhibited the neointima formation in the ligated carotid arteries of SRC-1−/− mice at day 14 after ligation (Figure 2D). Quantitative measurements showed that the average intimal area of ligated carotid arteries was similar in WT and SRC-1−/− mice in the absence of estrogen treatment (Figure 3A). The average intimal area of the ligated carotid arteries in WT mice with estrogen treatment was drastically reduced to 10% of that in WT mice with vehicle treatment. However, the average intimal area of the ligated carotid arteries in SRC-1−/− mice with estrogen treatment was only reduced to 69% of that in SRC-1−/− mice with vehicle treatment (Figure 3A). Accordingly, the average intimal area in SRC-1−/− mice was significantly larger than that in WT mice in the presence of estrogen treatment (Figure 3A). Nevertheless, there were no obvious changes in the medial area of the ligated carotid arteries in all mice regardless of hormonal treatment (Figure 3A). Consequently, the ratios of intima to media in the ligated carotid arterial wall were comparable between vehicle-treated WT and SRC-1−/− mice, but the ratio of intima to media in the ligated carotid arterial wall of estrogen-treated SRC-1−/− mice was significantly higher than that in the estrogen-treated WT mice (Figure 3B).

These results demonstrate that the loss-of-function deletion of the SRC-1 gene reduces the sensitivity of the estrogen-dependent inhibition of neointima formation.

SRC-1 Deficiency Attenuates the Inhibitory Effect of Estrogen on Neointimal Cell Proliferation

Estrogen, mainly through binding and activating the ERα and ERβ receptors, regulates vascular wall remodeling by inhibition of cell proliferation induced by vascular injury. Therefore, Western blot analyses of tissue lysates prepared from individual aortas and pooled carotid arteries of WT and SRC-1−/− mice were performed first to examine whether SRC-1 deficiency would alter ERα and ERβ expression levels. Our analyses showed that although the levels of both ERα and ERβ proteins were somewhat variable between individual WT and SRC-1−/− mice (Figure 4A and 4C and data not shown), but the average protein levels of ERα and ERβ were similar in the aortic and carotid arterial tissues of WT and SRC-1−/− mice when normalized to β-actin (Figure 4B and 4D). This indicates that SRC-1 deficiency does not affect ERα and ERβ expression. In addition, Western blot analyses of the same sets of samples were also performed to examine whether SRC-1 deficiency would change the expression levels of SRC-2 and SRC-3, the other two members of the SRC family. The SRC-2 and SRC-3 protein levels also showed certain variability between individual mice in both WT and SRC-1−/− mice (Figure 4A and 4C and data not shown). Overall, the average levels of SRC-2 and SRC-3 in the aorta and carotid arteries of WT and SRC-1−/− mice remained comparable when normalized to β-actin levels (Figure 4B and 4D). These results suggest that the decreased estrogen-mediated inhibition of neointima formation in SRC-1−/− mice is not attributable to the differences in the expression level of estrogen receptors and other SRC family members.

Next, we analyzed the contribution of SRC-1 to the inhibition of vascular cell proliferation induced by the vascular ligation injury. In both ovariec-tomized WT and SRC-1−/− mice treated with vehicle, the carotid ligation injury induced an extremely high rate of cell proliferation in the neointimal tissue as indicated by PCNA immunostaining (Figure 5A). The result was consistent with the robust
neointimal growth shown in Figure 2A and 2B. In WT mice treated with estrogen, the cell proliferation in the neointima was strikingly inhibited. However, the number of proliferative cells in the neointima of SRC-1−/− mice with estrogen treatment was only partially reduced (Figure 5A). To obtain quantitative data, we counted PCNA-positive cells and total cells located in the neointima and media on sections prepared from 4 to 5 mice for each group and calculated the ratios of PCNA-positive cells to total cells. About 27% PCNA-positive cells were observed in the intimal area of the ligated carotid arteries in both WT and SRC-1−/− mice treated with vehicle. The estrogen treatment reduced the average cell proliferation rate in the intima of WT mice to about 5%, but the same treatment only reduced the proliferation rate in the intima of SRC-1−/− mice to about 17.5% (Figure 5B). The cell proliferation rate in the intima of SRC-1−/− mice was significantly higher than that in WT mice in the presence of estrogen treatment. Nevertheless, the average cell proliferation rate detected by PCNA immunostaining remained the same in the media of WT and SRC-1−/− mice treated with either vehicle or estrogen, which was about 13% (Figure 5B). These results suggest that SRC-1 is required for estrogen and its receptor-mediated inhibition of neointimal cell proliferation during vascular wall remodeling after injury.

**Discussion**

In this study, we have demonstrated that SRC-1, an ER coactivator, is expressed in VSMCs and ECs of the normal arterial wall as well as in the cells of neointima induced by vascular injury. After the endogenous estrogen was depleted by ovariectomy, both WT and SRC-1−/− mice developed a robust neointimal growth in their common carotid arteries after the ligation injury. However, after these mice were treated with estrogen, WT and SRC-1−/− mice exhibited different sensitivities to estrogen-induced inhibition of neointimal growth. After estrogen treatment, the neointima formation in the ligated common carotid artery of WT mice was almost completely suppressed, whereas the same surgery-induced neointima formation in SRC-1−/− mice was only partially reduced. In agreement with the respective 90% and 31% reductions of neointima growth in estrogen-treated WT and SRC-1−/− mice, estrogen treatment also caused significantly less inhibition of cell proliferation in the vascular walls of SRC-1−/− mice (45% inhibition) compared with that in WT mice (92% inhibition) after the ligation injury. These results indicate that SRC-1 serves as an in vivo coactivator for ER in the blood vessel wall to mediate the effect of estrogen on vasoprotection during vascular wall remodeling after an injury. Conversely, SRC-1 downregulation or loss-of-function mutation will reduce or impair the ER function in the vascular wall and thereby enhance the neointima formation in response to a vascular injury.

Similar to the expression pattern of SRC-1 in the vascular wall, estrogen receptors are also expressed in VSMCs, ECs, and intimal cells. The colocalization of SRC-1 with estrogen receptors in these cells is consistent with their functional partnership in the estrogen-induced vasoprotective effect. Interestingly, our previous study showed that SRC-3, another member of the p160 SRC gene family, is also expressed in VSMCs and ECs and the estrogen-induced
inhibition of neointima formation is partially reduced in SRC-3 knockout mice. These findings suggest that the expression of SRC-1 and SRC-3 in the blood vessel wall is overlapping and the roles of SRC-1 and SRC-3 in estrogen-induced vasoprotection after a vascular injury are partially redundant. This partially redundant function of SRC-1 and SRC-3 in the vascular wall may provide an explanation that either SRC-1 or SRC-3 deficiency can only partially impair, but not completely diminish, the inhibitory effect of estrogen on neointima formation induced by a vascular injury. In the future, it would be interesting to know whether SRC-2 also plays a role in the estrogen-dependent vasoprotection and whether inactivation of 2 or all 3 of the SRC family members will completely abolish the inhibitory effect of estrogen on neointima development. It would be also very interesting to discern the contribution of individual SRC family members specific to ERα or ERβ-mediated vasoprotection in additional studies using combinatorial mouse models or selective ER modulators.

Recent studies have demonstrated that both SRC-1 and SRC-3 are overproduced in certain human breast and prostate tumors as well as in the cell lines of these tumor types.21–26 Overexpression of SRC-1 enhances the transcriptional activities of ER and androgen receptor and the proliferation and motility of prostate cancer cells.23,24,27 Overexpression of SRC-3 promotes breast and prostate cancer cell growth in culture and causes mammary tumors in transgenic mice.25,26 Conversely, SRC-1 deficiency results in partial hormone resistance and reduces the growth of steroid target tissues and prostate cancer cells.18,27 SRC-3 deficiency suppresses oncogene- and chemical carcinogen-induced mammary tumors in mice.24,26 These findings indicate that SRC-1 and SRC-3 play crucial roles in hormonal promotion of tumorigenesis and cancer progression. Therefore, future strategies to target these SRC molecules for cancer therapy may be developed. On the other hand, our former and present studies demonstrated that SRC-1 and SRC-3 play important roles in estrogen-induced vasoprotection during vascular wall remodeling after vessel injury. Therefore, the beneficial function of SRC-1 and SRC-3 in the vascular wall should be preserved when the inhibition of these coactivators is considered as a future strategy for cancer treatment.

Intriguingly, despite the fact that numerous epidemiological and basic studies have demonstrated that estrogen plays an important protective role in the cardiovascular system in women,1,2,5 the recent “Women’s Health Initiative Randomized Controlled Trial (WHIRCT)” have been unable to demonstrate a cardiovascular benefit of hormonal replacement with either estrogen alone or estrogen plus progesterone.
SRC-1 deficiency reduces the inhibitory effect of estrogen on the neointimal cell proliferation. A, Detection of proliferating cells by PCNA immunostaining. The cross sections of the carotid common arteries were prepared from ovariectomized WT and KO (SRC-1−/−) mice treated with vehicle or estrogen (E2) after the ligation injury. The sections were immunostained with the PCNA antibody for detection of proliferating cells (brown color). The dotted lines mark the boundaries between intima and media. B, Quantitative analysis of the PCNA-positive cells in the intima and media of the ligated carotid common arteries of WT and SRC-1−/− mice treated with vehicle or E2. Two to 3 sections per mouse and 4 to 5 mice per group were immunostained with PCNA antibody. Cells with strong nuclear staining of PCNA were counted as positive cells. The number of immunostained with PCNA antibody. Cells with strong nuclear staining of PCNA were counted as positive cells. The number of PCNA-positive cells and the number of total cells in the intima and media of each section were separately counted. The percentage of PCNA-positive cells to total cells was calculated for each section. Data are presented as mean ± SD (%). The intima area per section contains about 200 to 230 cells in WT and each section. Data are presented as mean ± H11006 SD (%). The intima percentage of PCNA-positive cells to total cells was calculated for and media of each section were separately counted. The per-
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area per section contains about 200 to 230 cells in WT and
SRC-1−/− mice treated with vehicle, about 35 cells in WT mice
treated with E2 and about 140 cells in SRC-1−/− mice treated
with E2. The media area on each section contains an average of
120 to 130 cells for all groups. *P<0.05; **P<0.01; ***P<0.001;
NS, not statistically significant.

Figure 5. SRC-1 deficiency reduces the inhibitory effect of estrogen on the neointimal cell proliferation. A, Detection of proliferating cells by PCNA immunostaining. The cross sections of the carotid common arteries were prepared from ovariectomized WT and KO (SRC-1−/−) mice treated with vehicle or estrogen (E2) after the ligation injury. The sections were immunostained with the PCNA antibody for detection of proliferating cells (brown color). The dotted lines mark the boundaries between intima and media. B, Quantitative analysis of the PCNA-positive cells in the intima and media of the ligated carotid common arteries of WT and SRC-1−/− mice treated with vehicle or E2. Two to 3 sections per mouse and 4 to 5 mice per group were immunostained with PCNA antibody. Cells with strong nuclear staining of PCNA were counted as positive cells. The number of PCNA-positive cells and the number of total cells in the intima and media of each section were separately counted. The percentage of PCNA-positive cells to total cells was calculated for each section. Data are presented as mean ± SD (%). The intima area per section contains about 200 to 230 cells in WT and SRC-1−/− mice treated with vehicle, about 35 cells in WT mice treated with E2 and about 140 cells in SRC-1−/− mice treated with E2. The media area on each section contains an average of 120 to 130 cells for all groups. *P<0.05; **P<0.01; ***P<0.001; NS, not statistically significant.

in postmenopausal women.39,40 Analysis of the estrogen signaling components may provide an explanation to this controversy. In the animal model used in this study, estrogen treatment strongly inhibits vascular injury-induced neointima formation in WT mice. Under this circumstance, the estrogen is present and the ER and SRCs are normally expressed at the time of the vascular injury. However, after SRC-1 or SRC-3 is inactivated in knockout mice, the beneficiary effect of estrogen on vasoprotection during vascular remodeling is reduced. Our findings raise a possibility that some postmeno-
pauper women might have a decrease in SRC expression or function. It would be interesting to look into this matter in future studies. In addition, possibilities of reduced ER levels in diseased human coronary arteries and inappropriate timing of estrogen treatment have been suggested to explain the controversial WHIRCT outcome.41,42

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Disclosures
None.

References
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