Steroid Receptor Coactivator-3 Is Required for Inhibition of Neointima Formation by Estrogen

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Background—The vasoprotective effects of estrogen are mediated by estrogen receptors (ERs). ERs are transcription factors that require coactivators to exert transcriptional activity. The steroid receptor coactivator-3 (SRC-3, also known as pCIP, AIB1, ACTR, and TRAM-1) interacts with estrogen-bound ERs and strongly coactivates the transcription of target genes in cultured cells. This study has characterized the expression of SRC-3 in cardiovascular tissue and the role of SRC-3 in estrogen-dependent vasoprotection from vascular injury.

Methods and Results—Phenotypically normal SRC-3+/− mice with a knock-in LacZ reporter were used to characterize SRC-3 expression by X-gal staining within the cardiovascular system. Staining signals were specifically detected in vascular smooth muscle cells and endothelial cells but not in myocardial cells. The role of SRC-3 during vascular remodeling was analyzed using a unilateral carotid ligation model. The extent of neointima formation in SRC-3−/− mice was significantly higher than in wild-type mice, and this difference was diminished after depletion of estrogen by ovariectomy. After ovariectomy, neointimal growth in wild-type mice was almost completely inhibited by estrogen treatment but only partially inhibited in SRC-3−/− mice. Furthermore, estrogen treatment resulted in reduced inhibition of intimal cell proliferation in SRC-3−/− mice.

Conclusions—SRC-3 is highly expressed in vascular smooth muscle cells and endothelial cells. The loss of SRC-3 function causes a decrease in sensitivity of estrogen-mediated inhibition of neointimal growth, which may be attributable to an insufficient suppression of vascular cell proliferation. These results indicate that SRC-3 largely facilitates ER-dependent vasoprotective effects under conditions of vascular trauma. (Circulation. 2002;105:2653-2659.)

Key Words: hormones ▪ receptors ▪ arteries

The vasoprotective effects of estrogen are well recognized. The incidence of cardiovascular disease among premenopausal women is significantly less than that among age-matched men and postmenopausal women.1 Estrogen replacement therapy for postmenopausal women significantly reduces the incidence of atherosclerotic diseases.2 Additionally, estrogen increases vasodilatation and inhibits the response to vascular injury.1 The vasoprotective effects of estrogen are attributed both to systemic effects, such as decreased serum total cholesterol and alteration of serum lipoproteins, and to direct effects on blood vessels through rapid nongenomic and longer-term genomic pathways.1,2 Both the nongenomic and the genomic cardiovasculocortic effects of estrogen are mediated by estrogen receptor α (ERα) and ERβ.3,4 Both ERα and ERβ are expressed in vascular smooth muscle cells (VSMCs) and endothelial cells (ECs).5,6 Both receptors are responsible for the inhibition of the vascular injury response by estrogen in animal models.7,8 ERs are hormone-inducible transcription factors requiring coactivators to modulate the transcription of their target genes.9 The steroid receptor coactivator-3 (SRC-3, also known as pCIP, RAC3, AIB1, ACTR, and TRAM-1) belongs to the SRC family that also contains the other 2 homologous coactivators, SRC-1 and the transcriptional intermediary factor 2 (TIF2, also known as GRIP-1).9 These coactivators interact with ERs and other steroid receptors (SRs) in a ligand-dependent manner and strongly coactivate the transcription of cellular target genes by SRs.9 Interestingly, both genomic amplification and mRNA overexpression of the SRC-3 gene are observed in many ER-positive breast and ovarian tumors.10 SRC-3 was recently shown to be associated with ERα bound to the estrogen-responsive promoters of target genes after estrogen treatment.11 The levels of SRC-3 are also related to the estrogen-dependent growth of certain breast cancer cells.12 These findings indicate that SRC-3 plays an important role in mediating ER function.

To study the physiological role of SRC-3 during development and in hormonal actions, we generated SRC-3 knockout (SRC-3−/−) mice through gene targeting.13 Our previous results indicated that genetic disruption of SRC-3 in mice...
causes growth retardation, delays puberty, reduces female reproductive function, and attenuates mammary gland development. In the present study, we demonstrate that SRC-3 is expressed in both VSMCs and ECs, and removal of SRC-3 from these vascular cells attenuates estrogen-induced inhibition of neointima formation.

Methods

Animals
The Animal Research Committee of Baylor College of Medicine approved procedures involving experimental mice. For all surgical procedures, mice were anesthetized by intraperitoneal injection of Avertin (2.5% in saline, 15 μL/g body weight). At specific times, animals were euthanized by overdose of Avertin.

SRC-3 mutant mice were generated in our laboratory by replacing a portion of the SRC-3 genomic DNA with the promoterless LacZ sequence as previously described. The SRC-3 mutant colony was maintained by breeding heterozygous pairs with mixed genetic background of 129/SvEv and C57BL/6J. All offspring were genotyped by polymerase chain reaction, as previously described. Four-month-old female wild-type (WT) and SRC-3 mutant littermates were used for all experiments.

X-Gal Staining
After perfuse fixation with PBS and 2% paraformaldehyde, blood vessels were removed and postfixed in paraformaldehyde for 15 minutes. Samples were stained for β-galactosidase activity with X-gal as previously described. After additionally fixed in 4% paraformaldehyde overnight, samples were paraffin-embedded, sectioned, and counterstained with nuclear fast red. Positive signal from X-gal staining was indicated by blue precipitation in cell nuclei.

Carotid Ligation Model
The carotid artery ligation model used in these experiments has been previously described. Briefly, 3-month-old female WT and SRC-3 mutant mice were anesthetized, and the left common carotid artery was exposed and ligated near the carotid bifurcation. All animals recovered from surgery and showed no apparent symptoms of stroke and no signs of infection. Success of the model was verified in nonmutant female mice by morphological analysis of the carotid arteries 24 or 28 days after ligation.

Animal Treatment
Female WT and SRC-3 mutant mice were randomly divided into 6 groups with 5 to 8 mice per group. Mice in group 1 (WT) and group 2 (SRC-3/H11002) served as untreated intact controls. On day 1, mice in groups 3 and 5 (WT) and in groups 4 and 6 (SRC-3/H11002) were ovariectomized as previously described. Starting on day 7, sesame oil (vehicle) was injected (0.1 mL/mouse per day, SC) in groups 3 and 4, whereas 17β-estradiol (E2) in vehicle was used in groups 5 and 6 (0.1 mg/0.1 mL per mouse per day, SC). On day 14, ligation of left common carotid artery was performed on all mice. On days 21, 28, (groups 3 to 6), or 42 (groups 1 and 2), mice were euthanized and transcardially perfused with 4% paraformaldehyde in PBS under physiological pressure. Both left ligated and right unligated common carotid arteries were excised for morphological and immunohistochemical analyses.

Morphological Analysis
Fixed vessels were embedded in paraffin, and 5-μm serial cross-sections were prepared. Sections were stained with Verhoeff–van Geison solution and counterstained with hematoxylin and eosin. For each carotid, digitized images were taken from 3 adjacent serial sections, and morphometric analysis of digitized images was performed as routine using NIH Image 1.61 software.

Immunohistochemical Staining and Western Blot Analysis
Immunohistochemistry was performed to detect the proliferating cell nuclear antigen (PCNA) on vascular tissues, as previously described. Detection of apoptotic cells was performed using terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) reaction, as previously described. 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). Samples of 70 μg were separated by SDS-PAGE and blotted to a nitrocellulose membrane. The blots were probed with an ERα polyconal antibody (1:100, Zymed Laboratories) or with an ERβ monoclonal antibody CWK-F12 (1:50) and developed by using appropriate secondary antibodies and enhanced chemiluminescence reagents (Amersham).

Estrogen Assay
Blood samples were collected by venipuncture at the time of euthanasia. Serum samples were prepared and stored at −80°C. The serum concentration of E2 was measured using an EIA kit from Diagnostic Systems Laboratories with a sensitivity of 8 pg/mL, as we previously reported.

Results

Expression of SRC-3 in VSMCs and ECs
In SRC-3 mutant mice, a promoterless LacZ reporter was engineered to be regulated by the endogenous SRC-3 promoter (Figure 1A). To characterize the cell type–specific expression of SRC-3 in cardiovascular tissues, X-gal staining was performed on heart and blood vessels isolated from phenotypically normal SRC-3/H11002 mice. SRC-3 expression was undetectable in the nuclei of myocardial cells, whereas clear signals were observed in the nuclei of VSMCs in small blood vessels inside of the heart (Figure 1B, a). The aorta, vena cava, renal artery and vein, pulmonary artery, mesenteric vessels, and small arteries and veins of various other tissues showed strong SRC-3 expression. Additional analysis revealed that the staining signals for SRC-3 expression are located in the nuclei of both VSMCs and ECs (Figure 1B, b and d through f). Furthermore, positive staining was observed in the nuclei of medial smooth muscle cells and in the neointimal cells after carotid ligation (Figure 1B, g). Blood vessels and heart tissues from WT mice without LacZ served as negative controls and showed no positive signals after X-gal staining (Figure 1B, c), validating that the staining signals in tissues of SRC-3/H11002 mice were specific to the SRC-3 promoter-regulated expression of LacZ. These results indicate that SRC-3 is highly expressed in VSMCs and ECs but may not be present in cells of the myocardium.

Neointima Growth in Intact SRC-3−/− Mice
To investigate the role of SRC-3 in vascular remodeling after vessel injury, we used a carotid artery ligation model whereby neointima formation is consistently induced after cessation of blood flow. In WT mice, significant growth of a stenotic neointima was observed 4 weeks after ligation. Much higher levels of arterial neointima formation were observed in SRC-3−/− mice compared with WT mice (Figure 2A). Quantitative measurements revealed that the average intimal area in SRC-3−/− mice was 2-fold larger than that in the age-matched WT mice. No differences in medial wall growth were observed between WT and SRC-3−/− mice (Figure 2B);
therefore, the ratio of intimal to medial area in SRC-3−/− mice was ~2 fold higher than that in WT mice (Figure 2C). These observations clearly demonstrate that disruption of the SRC-3 gene facilitates neointimal growth during injury-induced vascular remodeling. However, no differences in circumferential lengths of both IEL and EEL were observed between WT and SRC-3−/− mice after carotid ligation.

Figure 1. A, SRC-3−/− mice harbor both WT and mutant (Mut) alleles. At the Mut allele, the promoterless LacZ sequence is connected to the second exon of SRC-3. The 5’ transcriptional regulatory region (empty box), all exons (shaded and black boxes), and amino acid coding regions (black boxes) are diagrammed. B, Characterization of SRC-3 expression by X-gal staining. a, Nuclei of VSMCs (arrow) in a small coronary artery are positively stained cells (blue). Little signal is seen in the surrounding myocardial cells (*). b and c, Whole mounts of mesenteric vasculature, including arterial arcades (A) and their accompanied veins (V) attached to the small intestine (SI) of SRC-3−/− (b) and WT (c, negative control) mice. d, Cross-section of the aorta. Both VSMCs and ECs are positively stained. e, Longitudinal section of the renal artery (A) and vein (V). f, Cross-section of the common carotid artery. g, Cross-section of the ligated carotid artery from SRC-3−/− mice, showing positive blue signals in the medial and neointimal (*) cells. I indicates intima; M, media; and L, lumen.

Attenuated Inhibition of Neointima Formation in SRC-3−/− Mice by Estrogen

Considering the contributing factors involved in estrogen-induced inhibition of neointima formation, we examined the levels of ERα and ERβ in SRC-3−/− mice. Western blot analyses indicate that both WT and SRC-3−/− mice express comparable amounts of ERα and ERβ in the aorta and uterus.

Figure 2. A, Representative photomicrographs of neointima formation 4 weeks after carotid ligation in intact WT and SRC-3−/− (KO) mice. Sections were stained with H&E. B, Average intimal and medial wall areas. C, Ratios of intimal area to medial area for ligated carotid arteries of WT and SRC-3−/− mice. Number of mice is indicated in each bar. **P<0.01; ***P<0.001 by t test. P<0.05 is considered significant.
respectively (Figure 3). These results suggest that the elevated neointima formation after carotid ligation in SRC-3−/− mice is not related to a change of vascular ER concentrations.

Variations in physiological estrogen levels may be another contributing factor. To evaluate the role of SRC-3 in vascular remodeling under controlled endogenous estrogen levels, mice were ovariectomized (OVX) and treated with vehicle or E2. Treatments started 1 week after OVX and 1 week before carotid ligation and continued through the end of the experiment. The serum E2 levels after OVX were barely detectable in both WT and SRC-3−/− mice. E2 treatment significantly increased serum E2 to a level approximately the same in both WT and SRC-3−/− mice (Figure 4A). In addition, E2 treatment almost equally stimulated uterine growth in both WT and SRC-3−/− mice (Figure 4B). These results indicate that depletion of E2 by OVX and maintenance of equal E2 levels in WT and SRC-3−/− mice by hormonal therapy were successful before and after carotid ligation.

After OVX, the right unligated carotid arteries in both WT and SRC-3−/− mice exhibited normal morphology (Figure 5A, a and b). Without E2 treatment, the ligated carotid arteries of both WT and SRC-3−/− mice exhibited robust neointimal growth at day 14 postligation (Figure 5A, c and d). However, there were no statistical differences between the average intimal areas of vehicle-treated WT and SRC-3−/− mice at both day 7 and day 14 (Figure 5B). E2 treatment almost completely inhibited the neointima formation after the carotid ligation in WT mice (Figure 5, A [e] and B). In contrast, the parallel treatment with equal amounts of E2 only partially inhibited the neointima formation in the age-matched SRC-3−/− mice at days 7 and day 14 after ligation (Figure 5, A [f] and B). Statistics reveal a highly significant difference between the average intimal areas of WT and SRC-3−/− mice at both days 7 and 14 after E2 treatment (Figure 5B). In addition, minimal changes in medial wall area were observed in vehicle- or E2-treated WT and SRC-3−/− mice. (Figure 5B). The ratios of intimal to medial wall area were identical for vehicle-treated WT and SRC-3−/− mice. However, the ratios of intima to media for WT mice were significantly lower than those for SRC-3−/− mice after E2 treatment at both days 7 and 14 after injury (Figure 5C). These results strongly suggest that the increased neointima formation in the intact SRC-3−/− mice is attributable to an attenuated inhibitory function of ovarian hormones such as estrogen, because the neointimal growth became similar in WT and SRC-3−/− mice after OVX. Because E2 treatment only caused partial inhibition of neointima formation in SRC-3−/− mice, we conclude that the loss of SRC-3 in VSMCs or ECs results in a loss of sensitivity to estrogen-induced inhibition of neointima formation.

To elucidate potential cellular mechanisms responsible for the reduced inhibition of neointima by estrogen in SRC-3−/− mice, cell proliferation and programmed cell death were examined by PCNA staining and TUNEL assay. The total number of PCNA-positive cells in the neointimal area of SRC-3−/− mice after OVX was similar to that of WT mice. However, SRC-3−/− mice exhibited 4.4- and 6-fold higher PCNA-positive cells than WT mice at days 7 and day 14 after ligation, respectively, after E2 treatment (Figure 6). No differences were detected in adventitial and medial wall PCNA labeling at both time points (data not shown). After E2 treatment, only a few TUNEL-positive cells were identified in the intimal area, and no differences were observed between WT and SRC-3−/− mice either with or without E2 treatment (data not shown).

**Discussion**

Estrogen and its receptors regulate gene expression in vascular cells and contribute to longer-term vascular protection. Coactivators of the SRC family interact with steroid receptors in a hormone-dependent manner and coactivate the transcription of target genes by multiple means, including recruitment of histone acetyltransferases (ie, CBP, p300, and pCAF) and methyltransferases (ie, CARM-1 and PRMT1) for chromatin remodeling and interaction with general transcription factors (ie, TFIIB and TBP). Although multiple coregulators for ERs have been identified, their expression and function in cardiovascular tissues remain to be characterized. The generation of SRC-3 mutant mice provides a unique animal model that permits investigation into the role of this newly identified coactivator in estrogen-dependent vasoprotective function.

To characterize SRC-3 expression in cardiovascular tissues, we used SRC-3−/− mice harboring a LacZ indicator to report the activity of the SRC-3 promoter. The X-gal staining reveals that SRC-3 is highly expressed in VSMCs and ECs within the cardiovascular system. The spatial coexpression of SRC-3 with ERs in these vascular cells sheds light on their functional partnerships.

In this study, a well-established carotid ligation model was used to investigate the role of SRC-3 in the vasoprotective mechanisms of estrogen. A major advantage of this model
over other vascular injury models is that an extensive and reproducible neointima is developed in the presence of an intact endothelial lining. Using this model, a significant and robust neointimal lesion was observed in SRC-3\(^{-/-}\) mice compared with WT mice. This enhanced neointima formation in SRC-3\(^{-/-}\) mice is not attributable to any change of ER levels, because ER\(^{\alpha}\) and ER\(^{\beta}\) proteins were similar in WT and SRC-3\(^{-/-}\) mice.

In intact SRC-3\(^{-/-}\) mice, it is difficult to determine whether the more severe intimal lesion was caused by the loss of SRC-3 or the change of circulating estrogen, because SRC-3\(^{-/-}\) mice also exhibit lower levels of E2.\(^{13}\) Therefore, we manipulated endogenous estrogen to achieve comparable levels in WT and SRC-3\(^{-/-}\) mice by OVX and E2 treatment. After OVX, no significant differences in neointima formation were observed in WT and SRC-3\(^{-/-}\) mice, indicating that the enhanced neointimal growth in intact SRC-3\(^{-/-}\) mice is attributable to an attenuated inhibition by E2. After E2 treatment, comparable E2 levels equally stimulated uterine growth in WT and SRC-3\(^{-/-}\) mice. This is an expected result, because SRC-3 is not expressed in the endometrium.\(^{13}\) Importantly, the equal E2 treatment caused significantly less inhibition of neointima in SRC-3\(^{-/-}\) mice than in WT mice at both examined time points, days 7 and 14, after carotid ligation, indicating that the absence of SRC-3 in vascular cells results in a decrease of ER function necessary for vasoprotection. Correlated with the insensitive neointimal inhibition by E2, the neointima in SRC-3\(^{-/-}\) mice also exhibits a higher degree of cell proliferation at the 2 examined time points. However, the proliferation of both adventitial fibroblasts and medial VSMCs in WT and SRC-3\(^{-/-}\) mice at days 7 and 14 after carotid ligation were equally affected by E2 treatment, which were inconsistent with previous observations.\(^{18}\) In addition, apoptotic cells were few and not different between WT and SRC-3\(^{-/-}\) mice. These results suggest that the insensitive inhibition of neointimal growth by estrogen in SRC-3\(^{-/-}\) mice may be caused by an impaired suppression of intimal cell proliferation during vascular remodeling through a direct or indirect estrogen-regulated pathway.

The origin of neointima cells after vascular injury is not fully understood. Although the proliferation and migration of medial VSMCs may directly contribute to neointima, opposite observations have been made for contributions of adventitial fibroblasts to neointimal cells.\(^{19,20}\) Because both wild-type and SRC-3\(^{-/-}\) mice showed similar activity of cellular proliferation in adventitia and media at days 7 and 14 after injury, the inhibition of cellular migration by E2 could be affected in SRC-3\(^{-/-}\) vessel wall. The plasminogen activator inhibitor-1 (PAI-1) competes with the cell adhesion molecule uPAR and facilitates cell migration.\(^{21}\) Overexpression of PAI-1 promotes neointima growth,\(^{22}\) but disruption of the PAI-1 gene inhibits neointima formation.\(^{23}\) To examine whether cellular migration is altered by PAI-1 in SRC-3\(^{-/-}\) mice, we compared PAI-1 protein levels using aorta extracts by Western blot and found no obvious differences between WT and SRC-3\(^{-/-}\) mice (data not shown), suggesting that the insensitive inhibition of neointima by E2 in SRC-3 mice is not attributable to any change of PAI-1 expression.

Studies using animal models have clearly demonstrated the vasoprotective effects of estrogen. However, the role of...
known ERs in neointima formation has not been fully characterized. The significant increase in neointima formation in mice treated with specific estrogen antagonist ICI suggests that the vasoprotective effects of estrogen are mediated by ERs. However, the full maintenance of vasoprotective effects of estrogen on VSMC proliferation and medial expansion in either ERα or ERβ knockout mice after injury indicates that neither of the ERs is essential for mediating these estrogen effects. In mice with disruption of both ER genes, estrogen still inhibits VSMC proliferation but does not inhibit media growth after injury, indicating that both ERs are involved in mediating at least 1 component of the estrogen effects during vessel remodeling. Unfortunately, the contribution of ERs to neointima in these studies using ER mutant mice was not quantitatively analyzed because of the limitation of the model. Therefore, it is difficult to compare the insensitive inhibition of neointima by E2 in SRC-3−/− mice with that in ER mutant mice. At the present time, it is also unclear whether SRC-3 is involved in ERα- or ERβ-mediated or both ERα- and ERβ-mediated inhibition of neointima growth. One previous study reported that SRC-3 preferentially interacts and coactivates ERα. Another study used a perivascular electric injury model and demonstrated that only ERα is required for estrogen-accelerated reendothelialization. In the model used here, although the endothelium is present at all times, focal detachment of ECs from the underlying IEL has been reported to occur. The ERα-mediated reendothelialization may play a critical role in protecting the endothelium from the focal detachment, thus inhibiting neointima formation. Therefore, SRC-3 may mainly facilitate this ERα-mediated inhibition of neointima formation. On the other hand, a recent study reported that ERβ-deficient mice exhibit higher vasoconstriction and blood pressure than WT mice. Because vasoconstriction may contribute to the neointima formation in the carotid ligation model, a potential decrease in ERβ function in SRC-3−/− mice may provide an additional mechanism. However, additional studies are required to address whether SRC-3 is involved in regulation of basal NO production, vasoconstriction, and blood pressure.

The neointimal reduction by E2 at day 14 after injury is 98% in WT and 73% in SRC-3−/− mice, suggesting that SRC-3 is responsible for 25% of E2 effects and not the only coactivator involved in the ER-dependent vasoprotection. In addition to the possible compensation from other members of the SRC family, many other types of coactivators also interact with and coactivate ERs. Furthermore, SRC-3 also interacts and coactivates other transcription factors such as CREB and STATs and, thus, participates in multiple regulatory pathways involving cytokines and growth factors. Given the significant redundancy among multiple coactivators, the 25% contribution to the neointimal inhibition by E2 can be considered a major share for a single coactivator. Obviously, we cannot exclude the possibility that a portion of the SRC-3 contribution might be through an unknown ER or an ER-independent pathway.

The clinical trial of the Heart Estrogen-Progestin Replacement Study (HERS) was unable to prove therapeutic effects of estrogen on preexisting coronary artery diseases. The reasons for the protective failure of diseased vessels by estrogen are unclear. Probably estrogen is required in early stages of vascular injury to inhibit neointima formation, as demonstrated in animal models. In addition, the largely reduced ER levels in diseased human coronary arteries may render the vessels resistant to estrogen. This study showed that without SRC-3, the inhibition of neointima by estrogen is attenuated, suggesting that possible alteration of ER coactivator levels and functions in the diseased arteries may also result in estrogen resistance.

In summary, this study demonstrates that SRC-3, an ER coactivator, is highly expressed in VSMCs and ECs and is required for efficient inhibition of neointima formation by estrogen.

References


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