Atherosclerotic Lesion Development in a Novel Ovary-Intact Mouse Model of Perimenopause

Loretta P. Mayer, Cheryl A. Dyer, Rebecca L. Eastgard, Patricia B. Hoyer, Carole L. Banka

Objective—Since the unexpected results from the Women’s Health Initiative, the possible protective role of estrogen in preventing heart disease in perimenopausal and postmenopausal women is uncertain. This study examined atherosclerotic lesion development in ovariectomized versus follicle-depleted ovary-intact cholesterol-fed female low-density lipoprotein (LDL) receptor–deficient mice.

Methods and Results—We studied lesion development in LDL receptor–deficient mice that were ovariectomized or follicle depleted with 4-vinylcyclohexene diepoxide (VCD) to induce ovarian failure, then treated with exogenous 17β-estradiol via pellet implant. At 120 days after start of cholesterol feeding, the extent of lesion in aorta and innominate artery was determined. Lesion area in both locations was similar in vehicle control, VCD-treated, and ovariectomized mice. Replacement with 17β-estradiol caused lesion reduction (P<0.05) in both arterial locations, but it was most efficacious in suppressing innominate lesion area in VCD-treated mice (12.9±5.2%) compared with ovariectomized mice (40.0±6.04%).

Conclusions—Endocrine status associated with the follicle-depleted ovary influences exogenous estradiol effects during the development of atherosclerotic lesions and, in particular, inhibits lesion progression in the innominate artery. (Arterioscler Thromb Vasc Biol. 2005;25:1910-1916.)

Key Words: atherosclerosis ■ estrogen supplementation ■ perimenopause model

Despite women being protected from cardiovascular disease (CVD) until well after menopause, CVD kills more women than men every year in the United States.1 It was presumed for decades that the atheroprotection enjoyed by women was primarily attributable to ovarian estradiol production. Once ovarian production of estradiol stopped because of surgical or spontaneous menopause, protection was lost, and CVD progressed to clinical complications.2–3 On the basis of this presumption, estrogen replacement therapy was initiated during the 1960s and was predicted to be atheroprotective until July 2002, with the announcement of the unexpected findings of increased CVD in the combined hormone therapy arm of the randomized, controlled Women’s Health Initiative (WHI) trial.4

The WHI results contradicted previous observational studies, such as the Nurses’ Health Study, that reported significant protection from CVD with hormone replacement5 but was consistent with the Heart and Estrogen/progestin Replacement Study (HERS) that reported estrogen replacement did not provide protection to women with existing CVD.6 Nonetheless, the WHI results were surprising in providing evidence that combined hormone replacement may be detrimental in healthy women and had a dramatic impact causing many women to stop hormone replacement therapy.7 In February 2004, the estrogen-only arm of WHI was stopped. Interestingly, unlike the findings in women receiving combined estrogen progestin therapy, there was no increase in cardiovascular events in women treated with conjugated equine estrogens alone.8

The putative atheroprotection of estrogen reported in observational human studies is supported by numerous studies demonstrating protection in a variety of animal models of CVD.9,10 Because only human females have extended postmenopausal survival (as much as one third of the life span is postreproductive), there are no appropriate age-dependent animal models of menopause, and studies rely on surgical removal of the ovaries to mimic the endocrine status of postmenopausal women.11 Surgical menopause (total hysterectomy) obviates the perimenopausal transition that can take 10 years and involves multiple ovarian hormonal changes with time.12 Surgical menopause occurs in <15% of postmenopausal women.13 Therefore, the majority of women experience menopause through a natural transition resulting from ovarian aging and follicle depletion and have residual ovarian tissue devoid of estrogen-producing follicles that can produce progesterone and androgen.3

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Recently, we reported a method to chemically induce follicle depletion in mice by accelerating the natural process of atresia.\textsuperscript{14} The industrial chemical 4-vinylcyclohexene diepoxide (VCD) selectively targets primordial and primary ovarian follicles.\textsuperscript{14} Because larger-growing follicles, the source of ovarian estrogen, are not targeted by VCD, their depletion is only manifest after all nonrenewing primordial follicles are depleted and can no longer serve as a pool for recruitment to larger follicles. Once ovarian estrogen and inhibin production ceases as a result of follicle depletion, the negative feedback to the pituitary is gone, and plasma follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels rise.\textsuperscript{14,15} In response to elevated LH, cells in the residual ovarian tissue, likely interstitial in origin, continue to produce steroids: progesterone and androgen, primarily.\textsuperscript{14} Because ovarian follicles are lost gradually and the animal retains residual steroidogenic ovarian tissue, the VCD-treated mouse has an endocrine profile more closely parallel to that in human perimenopause and postmenopause than that in ovariecctomized mouse.\textsuperscript{14}

We reported previously that 17\textbeta-estradiol replacement resulted in a significant reduction of atherosclerotic lesions in ovariecctomized low-density lipoprotein receptor--deficient (LDLR\textsuperscript{−/−}) mice fed a high-fat, high-cholesterol diet, and the effect was unrelated to plasma cholesterol levels.\textsuperscript{16} The present study was designed to compare the effect of 17\textbeta-estradiol treatment on lesion formation in VCD-induced follicle-depleted, ovary-intact mice and ovariecctomized mice to determine whether the differences in endocrine milieu altered the extent of lesions formed in the aortic and innominate arteries.

Methods

Animals

C57BL/6J LDLR\textsuperscript{−/−} mice were purchased from The Jackson Laboratory (Bar Harbor, Maine) and bred in the Banka laboratory. All studies were approved by the La Jolla Institute for Molecular Medicine institutional animal care and use committee and comply with National Institutes of Health guidelines. Mice (75 total) were weaned at 28 days (d), initially fed ad libitum a standard mouse chow diet (Diet No. 5015; Harlan Teklad), housed 3 per cage in autoclaved commercially available through The Jackson Laboratory (Bar Harbor, Maine) and bred in the Banka laboratory. The protocol for VCD induction of follicular depletion has a patent (The University of Arizona). VCD-treated mice are compared endocrine and cardiovascular attributes in normal cycling, VCD-treated, and ovariecctomized LDLR\textsuperscript{−/−} mice on a high-fat diet. Treatments and sample collections were performed as indicated.

Tissues

Heparinized venous blood was collected from the retro-orbital sinus under anesthesia after an 8-hour fast unless otherwise specified. Blood samples were collected from animals on a chow diet to correspond to animals on high-fat diet collected at 25, 53, 81, and 120 d after the onset of VCD treatment (Figure 1). The only nonfasting samples were collected at killing (90 d after surgery and 120 d after the onset of VCD treatment). Plasma was obtained by centrifugation and stored (−20°C) before analysis. Mice were weighed while anesthetized. A cumulative plasma pool was generated for steroid hormone measurements by pooling 125 μL of plasma from each blood sample collection time point on day 25, 53, 81, 109, and 120 after the onset of VCD treatment. On day 120 after VCD treatment, animals were weighed, bled, and perfused under anesthesia. An incision was made in the inferior vena cava, and a cannula was inserted into the left ventricle for perfusion with PBS followed by formal sucrose (4% parafomaldehyde and 5% sucrose in PBS; Sigma-Aldrich). The adrenals, spleen, uteri, livers, kidneys, and ovaries were collected.

All organs were trimmed free of fat and wet weights recorded. All organ weights were normalized to body weight to correct for differences in total body weight. Uteri were excised from the oviduct at the uterotubal junction bilaterally and transected at the junction of the uterine body and cervix. After removal of luminal fluid, the entire uterus was weighed on a microbalance. One ovary from each treatment group was prepared for histological evaluation (n=6 animals per group). The ovary was placed in 4% paraformaldehyde fixative, transferred to 70% ethanol, paraffin embedded and serially sectioned (4 to 5 μm), mounted, and stained with hematoxylin and eosin. Follicles were counted in every twentieth section and classified as described previously.\textsuperscript{14}

Innominate arteries were isolated, cleaned, and photographed in situ under the dissecting microscope using an Olympus DP11 digital camera. Photographic images were subsequently analyzed morphometrically by drawing a line across the origin from the aorta and a parallel line intersecting the bifurcation to the subclavian and common carotid arteries (Figure 2, red lines). This defined the total area of the innominate artery. Lesion area was outlined and the percentage of the total area covered by lesion calculated using NIH Image software. Using a dissecting microscope, the entire mouse aorta was dissected, removed, and prepared as described previously.\textsuperscript{16} The Sudan IV--stained aortae were photographed at a fixed magnification using an Olympus DP11 and imported into a computer for morphometric analysis (NIH Image software) as described previously.\textsuperscript{16}

Biochemical Analyses

Hepatocellular histopathology and enzymatic activity of circulating aspartate aminotransferase (AST) and alanine aminotransferase...
performed to ensure that the assumptions of the ANOVA were met. Variance (Bartlett) and normality (Shapiro–Wilk) were routinely assessed. 

Body weights, tissue weights, and plasma hormone concentrations were determined by the diagnostic laboratory at the Arizona Health Sciences Center. Total cholesterol and triglycerides were quantified using Sigma reagents 401-25P and 344-20, respectively. High-density lipoprotein (HDL) cholesterol was measured using the Sigma 401-25P reagent after precipitation of the apolipoprotein B–containing lipoproteins (reagent 543004; Boehringer Mannheim). Plasma levels of 17β-estradiol and androstenedione were determined by Coat-A-Count radioimmunossay (RIA; Diagnostic Products Inc.). Sensitivity and interassay and intra-assay coefficients of variation were 17β-estradiol, 1.4 pg/mL, 4.6% and 14%; androstenedione, 0.04 ng/mL, 8.4% and 5.7%, respectively. Plasma FSH was measured by RIA according to instructions with the murine kit from the National Hormone and Pituitary Distribution Program. All samples were assayed in duplicate. Sensitivity and interassay and intra-assay coefficients of variation were 200 pg/mL, 2.7%, and 6.7%. The results of all RIAs were calculated by 4-parameter logistic analysis using the software Assay Zap (BioSoft).

Data Analysis

Body weights, tissue weights, and plasma hormone concentrations were determined from individual animals and averaged for each treatment, and the means (±SEM) in control versus treated animals were analyzed for significant differences by 1-way ANOVA with Dunnett’s used to determine differences from control and Student t test to compare each pair. Tests for homogeneity of variance (Bartlett) and normality (Shapiro–Wilk) were routinely performed to ensure that the assumptions of the ANOVA were met. Lipid measurements were analyzed using a Friedman repeated-measures ANOVA on ranks. The Student-Newman–Keuls method was used for pairwise multiple comparisons of groups as a post hoc test.

Results

Innominate Atherosclerotic Lesion Area

The extent of innominate lesion area was similar and not significantly different among control (47.3±4.8%), VCD (45.04±5.7%), and OVX (57.0±7.3%) treatment groups ranging from 40% to 60% (Figure 2A). However, E2 supplementation in VCD-treated mice resulted in significant differences in lesion area compared with that in OVX mice. The VCD+E2 (12.9±5.2%) treatment group had less (P<0.05) lesion area than the OVX+E2 (40.0±6.0%) treatment group (n=6 to 12).

Aortic Atherosclerotic Lesion Area

The extent of aortic lesion area was similar and not significantly different among the control (6.4±0.7%), VCD (6.8±0.7%), and OVX (8.3±1.5%) groups, ranging from 5% to 8% (Figure 2B). In VCD+E2 (1.3±0.9%) and OVX+E2 (1.7±0.7%), there was a reduction (P<0.05) in aortic lesion area; however, the extent of E2 effects in these 2 treatment groups was not different (n=6 to 12).

Systemic and Liver Function

Body weights at day 120 did not differ significantly among the 5 groups: vehicle, VCD, VCD+17β-estradiol (E2), OVX, OVX+E2 (data not shown). There was no effect of either VCD+E2 or OVX+E2 on adrenal, kidney, or spleen weights normalized to body weight (data not shown). Levels of liver enzymes AST and ALT were within the normal range for VCD-treated and control animals (AST; control, 157.85±56.55 U/L; VCD, 57.30±2.30 U/L; normal range 54 to 298 U/L; ALT; control, 34.53±10.31 U/L; VCD, 35.84±12.40 U/L; normal range 17 to 77 U/L). ALT and AST circulating levels were not analyzed in OVX+E2 mice. There was no histological evidence of hepatocellular pathology in livers from VCD+E2 or OVX+E2 mice (data not shown).

Lipids

Over the time course of the experiment, once fat feeding had commenced, plasma total cholesterol increased 3- to 4-fold from day 25 to day 53 in all experimental groups: vehicle, VCD, VCD+E2, OVX, and OVX+E2 (Figure 3). On day 53, total cholesterol was significantly less in VCD+E2, OVX, and OVX+E2 compared with vehicle control mice. On day 81, plasma total cholesterol was elevated in VCD and OVX mice compared with vehicle control. At day 120, OVX mice had significantly elevated cholesterol compared with the other treatment groups, and there was a significant reduction in plasma cholesterol in OVX+E2 mice. On day 120, neither HDL nor triglycerides differed among any of the groups, suggesting that the significant decrease observed in plasma total cholesterol in OVX+E2 mice was attributable to reduction in LDL cholesterol because there were no significant reductions in plasma triglycerides, indicating that the affected fraction was LDL and not very–low-density lipoprotein (Table 1).
were ovariectomized and received 17β-estradiol supplementation as indicated. Plasma samples were collected as described in Methods and total cholesterol levels determined (*P<0.05).

**Endocrine Effects**

On day 120 after the onset of VCD treatment, a reduction (P<0.05) in ovarian weight was observed in VCD and VCD+E2-treated mice compared with vehicle control (Figure 4A). The weight of ovaries from VCD-treated mice was 34% of control, whereas ovaries from VCD+E2 were 23% of control (control 0.000235±0.0001; VCD 0.00080±0.0001; VCD+E2 0.000053±0.00001 gm/gm body weight; Figure 4A). Furthermore, on histological examination, VCD-treated ovaries had no ovarian follicles compared with ovaries from vehicle control cycling animals, which contained follicles at all stages of development and corpora lutea (data not shown).

Uterine weight in OVX mice was reduced (P<0.05) compared with vehicle control (Figure 4B). Uterine weight in VCD-treated mice was not different from vehicle control mice (Figure 4B). VCD+E2 and OVX+E2 mice had increased (P<0.05) uterine weights compared with VCD and OVX uterine weights.

On day 53 after the onset of VCD dosing, plasma FSH levels were greater (P<0.05) in OVX and VCD-treated mice relative to vehicle-treated cycling controls (Table 2). Plasma FSH levels in OVX mice were not significantly different from VCD-treated mice. Estradiol supplementation reduced (P<0.05) plasma FSH in VCD+E2 and OVX+E2 mice, but plasma FSH levels were not as low as the level observed in the cycling vehicle control mice (Table 2).

Plasma levels of 17β-estradiol, measured in a cumulative plasma pool as described in Methods, were nondetectable in vehicle control, OVX, and VCD-treated animals. Whereas plasma levels of estradiol in the OVX+E2 mice were increased above physiological levels, the estradiol level in VCD+E2-treated mice was within the physiological range during the estrus cycle. Plasma levels of E2 in various stages of cycling LDLR−/− mice in a baseline cohort were 82.6±48.0 pg/mL (n=6). The plasma estradiol levels were lower (P<0.05) in VCD+E2 mice compared with OVX+E2 mice.

Plasma levels of androstenedione were similar in vehicle and VCD-treated mice measured in a cumulative plasma pool, were reduced (P<0.05) in OVX mice compared with vehicle control mice, and reduced (P<0.05) in VCD+E2 mice compared with mice treated with only VCD (Table 2).

**Discussion**

The aim of this study was to compare development of atherosclerotic lesions in VCD-treated and OVX+E2 mice. The rationale was based on comparing lifetime hormone profiles and temporal patterns of ovarian failure between individuals who undergo surgical (OVX) versus natural (VCD-treated) menopause. Here, in LDLR−/− mice, protection against lesion development was provided by E2 supplementation in animals given hormone before complete ovarian...

**TABLE 1. Lipid Profiles**

<table>
<thead>
<tr>
<th>Circulating Plasma Lipid Levels*</th>
<th>Vehicle</th>
<th>VCD</th>
<th>VCD+E2</th>
<th>OVX</th>
<th>OVX+E2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>1151.46±62.98</td>
<td>1193.75±76.99</td>
<td>1048.22±104.21</td>
<td>1406.6±102.46</td>
<td>1000.17±138.87</td>
</tr>
<tr>
<td>HDL, mg/dL</td>
<td>42.07±2.36</td>
<td>42.75±2.28</td>
<td>51.46±3.00</td>
<td>55.45±7.91</td>
<td>42.42±4.85</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>85.49±8.47</td>
<td>77.08±10.64</td>
<td>66.30±5.11</td>
<td>68.78±13.91</td>
<td>62.88±10.28</td>
</tr>
</tbody>
</table>

*Plasma collected for analysis at the conclusion of the study, day 120 after commencement of dosing. Values represent mean±SEM.
*Different from vehicle control; and letters represent groups that are different; n=5 to 13; *P<0.05.
failure induced by VCD. In fact, E2 supplementation before or during onset of follicle depletion appeared to be more beneficial because innominate lesion area was significantly less in VCD+E2 compared with OVX+E2. This result is consistent with observations in monkeys in which E2 supplementation at the time of OVX is protective against CVD development, whereas it is detrimental if given after cardiovascular lesion progression is under way.\textsuperscript{17} Our findings suggest the timing of estrogen replacement may be critical to the subsequent influence on atherosclerosis progression, as suggested recently by Dubey et al,\textsuperscript{18} and may reflect tissues that continue to respond because they have not been estrogen deficient for long periods of time.\textsuperscript{19}

The data presented here are consistent with previous findings of E2-mediated lesion area reduction independent of alterations in lipid levels.\textsuperscript{16} Here, aortae lesion development was significantly less in E2-supplemented OVX and VCD-treated groups; however, in the innominate artery, there was a significant difference in atherosclerotic lesion area. Recent studies suggest the innominate artery may be more relevant for investigating the rate of lesion progression and features that contribute to clinically significant disease.\textsuperscript{20–22} Striking was the effect of E2 supplementation on reducing innominate lesion in the VCD-treated mice compared with OVX mice. Perhaps gradual loss of estrogen, which could include spiking of estrogen to levels higher than in normal estrus cycling in the VCD-treated mouse, added to the atheroprotective effect of the E2 supplementation when compared with the abrupt changes observed in the OVX mice. Circulating androgen in the VCD-treated animal could be aromatized in the vessel wall to exert a localized protective effect of estrogen.\textsuperscript{23} Nor can we rule out the possibility of a direct effect of circulating androgen on reducing the carotid intimal-medial thickness, which has been reported for women.\textsuperscript{24}

VCD-induced premature ovarian failure in mice leads to the development of a follicle-depleted, ovary-intact animal that resembles the dynamic endocrine state in perimenopausal and postmenopausal women.\textsuperscript{14} Perimenopause is defined as the period of ≈1 to 10 years before the final menstrual period, when symptoms of menopause begin and many disease risks increase.\textsuperscript{25,26} The VCD-induced follicle-depletion protocol was used in the LDLR\textsuperscript{−/−} atherosclerosis model to mimic conditions observed in a healthy, perimenopausal population. This made it possible to determine the effects of estrogen supplementation on the development of atherosclerosis in mice undergoing gradual loss of ovarian-derived 17β-estradiol (VCD-treated) versus ovariectomized mice, with an abrupt withdrawal of ovarian function coincident with atherosclerotic lesion development.

There was no evidence of any generalized toxic effect of VCD treatment on the LDLR\textsuperscript{−/−} C57BL/6J mice. This finding is consistent with previous findings in the hybrid B6C3F\textsuperscript{14} and inbred C57BL/6J and C3H strains (Kitten AM, unpublished data, 2003). Nor were there significant differences in plasma total cholesterol, HDL cholesterol, or triglycerides with VCD treatment, another indication that liver function was not compromised by VCD treatment. At the end of the experiment on day 120, there was no change in plasma total cholesterol of VCD-treated mice relative to OVX mice, indicating that differences in innominate lesion area were not attributable to plasma lipids.

VCD treatment caused a complete loss of all stages of ovarian follicles by day 120 after the onset of treatment, confirming premature ovarian failure had been induced. VCD-induced follicle loss and ovarian weight were not affected by E2 supplementation. Conversely, uterine weight, a physiological measure of uterotrophic effects of E2, was significantly greater in response to estrogen supplementation in OVX and VCD-treated animals. Uterine weight in VCD-treated mice was equivalent to that in intact cycling mice and significantly greater than OVX mice, which was apparently because of androgen promotion of uterine weight increase.\textsuperscript{27,28} The lack of differences in uterine weights between VCD-treated animals and vehicle cycling controls may be attributable to a strain difference or variation in collection methods from our previous report.\textsuperscript{14}

FSH levels increased in LDLR\textsuperscript{−/−} transgenic mice similar to VCD-induced follicle loss in other strains of mice.\textsuperscript{14,15} E2 supplementation of OVX and VCD mice caused FSH to drop, but not to levels observed in cycling mice. This supports that exogenous E2 was only partially effective at invoking negative feedback on pituitary FSH secretion. FSH release by the pituitary is also inhibited by ovarian-derived inhibin, a member of the transforming growth factor-β family of peptides.\textsuperscript{29} Inhibin is produced by ovarian granulosa cells,\textsuperscript{30} and, without granulosa cells, whether in OVX mice or follicle-depleted ovaries of VCD mice, the absence of inhibin was reflected in the FSH levels being reduced by half with E2 treatment but still significantly greater than in the cycling, intact mice.

The cumulative steroid pool collected throughout the study was designed to mimic the cumulative hormone exposure a woman would experience during the transition from reproductive years into the perimenopause and postmenopause periods.\textsuperscript{25,26} It has been reported that the rodent adrenal does
not produce androgen.\textsuperscript{29,31} However, our results in the OVX mice, as well as those of Villablanca in castrated male mice,\textsuperscript{32} suggest an extragonadal source of androgen in mice. In our previous study, plasma androstenedione measured at a single time point on day 120 after initiation of VCD treatment was one-third the concentration in a cycling control animal.\textsuperscript{14} However, in the study reported here, androstenedione levels were measured in a pooled cumulative sample, which may explain why the level was not different between vehicle control and VCD-treated mice. E2 supplementation significantly reduced androstenedione in the VCD+E2 follicle-depleted mice compared with an increase in androstenedione seen in OVX+E2 mice. Interstitial cells of the follicle-depleted ovarian tissue produce androstenedione in culture, which is blocked by E2.\textsuperscript{33} We conclude that E2 supplementation in VCD-treated mice reduced ovarian interstitial cell production of androstenedione.

An additional endocrine difference was decreased, circulating estradiol in VCD+E2-treated mice compared with OVX+E2 mice. Estradiol is inactivated and removed by sulfoconjugation catalyzed by estrogen sulfotransferase, the expression of which in the ovary is stimulated by LH, the gonadotropin that stimulates interstitial cells.\textsuperscript{34,35} The follicle-depleted ovary enriched in interstitial cells may metabolize estradiol via estrogen sulfotransferase, resulting in less estradiol in the VCD+E2 mice compared with the OVX+E2 mice.

Together, our findings suggest that studies using the VCD-treated mouse as a model for CVD and menopause may provide an endocrine milieu more like that in perimenopausal and postmenopausal women. The VCD model would be better to assess the potential impact of therapeutic interventions in primary or secondary CVD prevention, for instance, to investigate the role of cyclooxygenase 2 inhibitors,\textsuperscript{36} a subject of recent investigations. Furthermore, using a more endocrine-relevant model will enable the performance of animal studies designed to reconcile the differences reported in clinical investigations regarding the risks or benefits of hormone supplementation.\textsuperscript{2–6,18} Another uncertainty that could be addressed using the VCD model of ovarian failure is the role of androgens in CVD. It has been assumed for decades that androgen is atherogenic and estrogen atheroprotective, but, careful re-examination suggests that androgen and estrogen are atheroprotective.\textsuperscript{37–39} The VCD mouse model with sustained androgen and progesterone production from the follicle-depleted ovary provides a physiological setting in which to identify the roles of these steroids in atherosclerotic lesion development. Finally, the potential utility of this model for screening therapeutic approaches will likely facilitate drug discovery in women’s health and, more importantly, provide an early warning for contraindications of drugs prescribed for women in perimenopausal years.

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References

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