

# Vascular endothelial-cadherin tyrosine phosphorylation in angiogenic and quiescent adult tissues

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## Abstract

Vascular endothelial-cadherin (VE-cadherin) plays a key role in angiogenesis and in vascular permeability. The regulation of its biological activity may be a central mechanism in normal or pathological angiogenesis. VE-cadherin has been shown to be phosphorylated on tyrosine *in vitro* under various conditions, including stimulation by VEGF. In the present study, we addressed the question of the existence of a tyrosine phosphorylated form of VE-cadherin *in vivo*, in correlation with the quiescent versus angiogenic state of adult tissues. Phosphorylated VE-cadherin was detected in mouse lung, uterus and ovary but not in other tissues unless mice were injected with peroxovanadate to block protein phosphatases. Remarkably, VE-cadherin tyrosine phosphorylation was dramatically increased in uterus and ovary, and not in other organs, during PMSG/hCG-induced angiogenesis. In parallel, we observed an increased association of VE-cadherin with Flk1 (VEGF receptor 2) during hormonal angiogenesis. Additionally, Src kinase was constitutively associated with VE-cadherin in both quiescent and angiogenic tissues and increased phosphorylation of VE-cadherin-associated Src was detected in uterus and ovary after hormonal treatment. Src-VE-cadherin association was detected in cultured endothelial cells, independent of VE-cadherin phosphorylation state and Src activation level. In this model, Src inhibition impaired VEGF-induced VE-cadherin phosphorylation, indicating that VE-cadherin phosphorylation was dependent upon Src activation. We conclude that VE-cadherin is a substrate for tyrosine kinases *in vivo* and that its phosphorylation, together with that of associated Src, is increased by angiogenic stimulation. Physical association between Flk1, Src and VE-cadherin may thus provide an efficient mechanism for amplification and perpetuation of VEGF-stimulated angiogenic processes.

**MESH Keywords** Animals ; Cadherins ; chemistry ; metabolism ; Cell Extracts ; chemistry ; Cell Line ; Endothelial Cells ; chemistry ; metabolism ; Endothelium, Vascular ; chemistry ; cytology ; metabolism ; Female ; Gonadotropins, Equine ; pharmacology ; Humans ; Mice ; Mice, Inbred C57BL ; Neovascularization, Physiologic ; physiology ; Ovary ; drug effects ; metabolism ; Phosphorylation ; drug effects ; Phosphotyrosine ; metabolism ; Proteins ; chemistry ; metabolism ; Proto-Oncogene Proteins pp60(c-src) ; antagonists & inhibitors ; metabolism ; Umbilical Veins ; cytology ; Uterus ; drug effects ; metabolism ; Vanadates ; administration & dosage ; pharmacology ; Vascular Endothelial Growth Factor Receptor-2 ; metabolism

**Author Keywords** VE-cadherin ; tyrosine kinase ; endothelium ; angiogenesis

## INTRODUCTION

Angiogenesis, the sprouting of new vessels from the existing vasculature, is a tightly controlled process that plays its most obvious role in early development. Although the potential for angiogenesis is maintained throughout the lifetime of an organism, once the vasculature has been established, the endothelium remains extraordinarily quiescent in the adult.<sup>1, 2</sup> The hormonal control of the ovary and uterus in reproduction provides the only normal physiological exception to this rule.<sup>3</sup> All other activations of angiogenesis during adulthood occur in response to injury or pathological processes such as tumorigenesis and diabetic retinopathy.<sup>4</sup> During angiogenesis, an important role for endothelial receptor tyrosine kinases such as VEGF and FGF receptors and their cognate growth factors has been demonstrated.<sup>5</sup> Although the mechanisms that drive angiogenesis have not been fully elucidated, a large body of evidence has established important roles for tyrosine phosphorylation of the cadherin-catenin complex as a potent mechanism that regulates the stability of cell-cell junctions.<sup>6–8</sup> As an example, the increase of tyrosine phosphorylation of Flk-1 and VE-cadherin, an endothelial-specific cadherin, by VEGF has been correlated with endothelial cell migration as well as tubular formation.<sup>9</sup> The pivotal role of VE-cadherin in angiogenesis has been demonstrated by two *in vivo* experiments. First, VE-cadherin deficient embryos died from extensive angiogenesis defects.<sup>10</sup> Second, targeting VE-cadherin with blocking antibodies successfully inhibited tumor angiogenesis.<sup>11, 12</sup> Therefore, the regulation of VE-cadherin biological activity by tyrosine phosphorylation may strongly influence angiogenic processes.

In addition, several lines of evidence have demonstrated that VE-cadherin controls vascular permeability.<sup>13–18</sup> An array of endogenous inflammatory mediators liberated under disease conditions, including histamine,<sup>7, 19</sup> thrombin,<sup>20</sup> TNF- $\alpha$ ,<sup>21</sup> platelet-activating factor,<sup>22</sup> advanced glycation end-products<sup>23</sup> and activated leukocytes<sup>24, 25</sup> have the potential to increase microvascular permeability and to disrupt VE-cadherin from endothelial junctions. Tyrosine phosphorylation of VE cadherin has been described to accompany the increased endothelial cell permeability induced by histamine,<sup>19</sup> TNF- $\alpha$ ,<sup>8, 26</sup> and leukocytes.<sup>27</sup>

Although at present the biological function of VE-cadherin tyrosine phosphorylation is unclear, several lines of evidence suggest that it does indeed play a role in distinct signal transduction networks, most likely as a component of a signaling cascade initiated by receptor or membrane-associated tyrosine kinases. Several members of signaling proteins have been shown to interact with the VE-cadherin-catenin complex including the adapter protein Shc28 and the tyrosine phosphatase SHP2.<sup>29</sup> Therefore, modulation of the tyrosine phosphorylation status of VE-cadherin would be critical for regulating angiogenesis and permeability during inflammatory processes through binding with signaling molecules.

In order to gain insights into the potential *in vivo* tyrosine phosphorylation of VE cadherin, we examined whether the phosphorylated form of VE-cadherin was expressed in the endothelium of the quiescent adult vasculature and whether it was regulated during angiogenesis. We also examined VE-cadherin binding partners in these two situations.

## **MATERIALS AND METHODS**

### **Animals**

All protocols in this study were conducted in strict accordance with the "Ministère de l'Éducation Nationale, de la Recherche et de la Technologie" Guidelines for the Care and Use of Laboratory Animals. C57/Bl6 mice were purchased from Charles Liver Laboratories (Les Oncins, France). Females between 8 and 12 weeks of age were used in all experiments.

### **Peroxovanadate treatment of mice**

Peroxovanadate administration was performed as previously described.<sup>30</sup> Peroxovanadate was diluted to 50 mmol/L in PBS containing 0.5 mg/mL Evans blue to monitor correct systemic injection. Mice were anesthetized by intraperitoneal injection of xylazine (10 mg/kg)/ketamine (80 mg/kg). Peroxovanadate solution or vehicle alone (200 µL) were administered by intracaudal vein injection. Mice were sacrificed by cervical dislocation 5 min later and the tissues were removed.

### **Superovulation**

Mice were given an intraperitoneal injection of 10 IU of PMSG in 0.75 mL of 0.9% NaCl on day 1, followed by 5 IU of hCG (both from Sigma-Aldrich) in 0.4 mL of 0.9% NaCl, 48 h later. Animals were sacrificed 6 h after second injection by cervical dislocation after peroxovanadate administration.

### **Antibodies**

Commercially available antibodies used were as follows : for immunoprecipitation, the rabbit polyclonal anti-Flk1 SC 504 (Santa Cruz), the rabbit polyclonal anti-Src (Upstate Biotechnology), the goat polyclonal anti-human VE-cadherin SC6458 (Santa Cruz); for western blotting, the mouse monoclonal anti-phosphotyrosine 4G10 (Upstate Biotechnology), the rat monoclonal anti-Flk1 12B11 (BD Biosciences), the horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma-Aldrich), goat anti-rabbit IgG, rabbit anti-rat IgG (both from Bio-Rad Laboratories); for immunofluorescence, the alexa 488-conjugated anti-rabbit IgG (Molecular Probes), the cyanine 3-conjugated anti-goat IgG (Jackson Laboratories). The rabbit polyclonal anti VE-cadherin antibody was previously described.<sup>16</sup>

### **Chemicals**

PMSG, hCG and sodium ortho-vanadate were purchased from Sigma-Aldrich. Human recombinant VEGF 165 was from Peprotech. Fluorsave mounting medium, PP2 and SU6656 were from Calbiochem.

### **Preparation of tissue extracts, immunoprecipitation, SDS/PAGE and Western blotting**

Tissue lysates and immunoprecipitates were prepared and analyzed as previously described.<sup>31, 32</sup>

### **Cell culture**

Human umbilical vein endothelial cells (HUVEC) were isolated as previously described.<sup>33</sup> Only first to third passage HUVEC were used in experiments. Ten minutes before VEGF stimulation, endothelial cells were pre-treated with 10 µmol/L sodium pervanadate. VEGF stimulation was then performed at 37°C for the concentrations and durations indicated in text and figure legends. Src inhibitors were added 4 h before VEGF treatment. The cells were homogenized in the same lysis buffer as described<sup>31</sup> supplemented with 0.5% SDS.

### **Immunofluorescence staining**

Confluent HUVEC were fixed in ethanol : acetone (1 :1) for 10 min at -20°C and double stained with anti-VE-cadherin (1 µg/mL) and anti-Src (20 µg/mL) antibodies. After three washes in PBS, slides were incubated with cyanine 3-conjugated anti-goat IgG (1:500) and alexa 488-conjugated anti-rabbit IgG (1:500) antibodies. Slides were rinsed and mounted in Fluorsave.

### **Data analysis**

Each experiment has been reproduced at least three times in identical or similar configuration with similar results. Densitometric analysis was performed with Kodak 1D software.

## RESULTS

### Tyrosine phosphorylation of VE-cadherin in adult quiescent tissues

We first analyzed by Western blot VE-cadherin expression levels in different adult tissues using an antibody directed against the extracellular domain of mouse VE-cadherin (Fig. 1A). Although VE-cadherin could be detected in all tissues examined, the data showed a marked difference in signal intensity between lung and other tissues, as indicated by band quantification (Fig 1B).

To examine the presence of P-tyr-VE-cadherin *in vivo*, the protein was immunoprecipitated from different tissue lysates and immunoblotted with an anti-phosphotyrosine antibody. After long exposure time, signals of P-tyr-VE-cadherin were obtained for lung and uterus extracts (Fig. 1C). A faint band was also visible for ovary. In contrast, no signal could be detected for heart, kidney and liver, whereas VE-cadherin was present in each immunoprecipitate (Fig. 1C). We thus conclude that P-tyr-VE-cadherin is present, however in small amounts, in lung, uterus, and ovary of adult mice. Attempts to immunoprecipitate larger amounts of VE-cadherin from heart, kidney and liver did not yield signals with the anti-phosphotyrosine antibody, suggesting that P-tyr-VE-cadherin was not present in these organs.

### Systemic injection of peroxovanadate induces extensive tyrosine phosphorylation of proteins and VE-cadherin in mouse tissues

Tyrosine phosphorylation is a well regulated process<sup>34</sup> and phosphotyrosine containing proteins are quantitatively rare in quiescent cells until a specific event, such as growth factor binding or oncogenic transformation, activates tyrosine kinases.<sup>35</sup> Thus, to examine VE-cadherin phosphorylation levels in absence of protein tyrosine phosphatase (PTP) activity, we injected peroxovanadate or vehicle alone, in the caudal vein, 5 min before mouse sacrifice. The administration of peroxovanadate resulted in a dramatic increase in the phosphorylation level of numerous proteins in all organs (Fig. 2A, right panel compared to left panel). In particular, lanes for heart, kidney and spleen that were devoid of signals in absence of treatment exhibited multiple bands in treated mice, for same exposure time. Thus, as previously reported,<sup>30</sup> peroxovanadate systemic injection allowed a better detection of tyrosine kinase substrate proteins.

After peroxovanadate treatment, P-tyr-VE-cadherin was detected in heart, kidney and spleen immunoprecipitates, whereas VE-cadherin phosphorylation was undetectable without the PTP blocker (Fig. 2B). VE-cadherin phosphorylation was also dramatically induced in lung, while the treatment was less effective in uterus and ovary (Fig. 2B). We conclude that VE-cadherin is a substrate for intracellular tyrosine kinases in all tissues examined. Furthermore, these results suggest that VE-cadherin phosphorylation levels are differentially regulated by PTP activity. All following experiments were performed after PTP blockade.

### VE-cadherin tyrosine phosphorylation is enhanced in tissues submitted to angiogenic stimulation

Since VE-cadherin was found to be tyrosine phosphorylated in quiescent tissues of the mouse, we examined whether its phosphorylation was regulated in tissues submitted to angiogenic stimulation. Adult mammalian angiogenesis occurs predominantly in female reproductive organs, namely the ovary and the uterus. Several studies demonstrated that ovarian angiogenesis was VEGF/Flk1-dependent.<sup>36–38</sup> Hence, we investigated VE-cadherin phosphorylation levels in ovary and uterus after hormonal induction obtained by the administration of PMSG and hCG. The hormonal treatment dramatically increased tyrosine phosphorylation levels of numerous proteins ranging from 50 to 250 kDa, in both ovary and uterus (Fig. 3A). This effect was specific to the genital tract since the phosphorylated protein levels in heart and lung were not affected by hormonal treatment (Fig. 3A).

The amount of P-tyr-VE-cadherin was also strongly increased in both ovary and uterus by the treatment (Fig. 3B, upper panel), while it remained unchanged in lung (Fig. 3B, upper panel). Control Western blot showed that VE-cadherin was present in each immunoprecipitate (Fig. 3B, lower panel). Interestingly, the amount of VE-cadherin was slightly higher in treated ovary and uterus compared to untreated organs (see also Fig. 4A), probably reflecting the increase in vessel density after hormonal induction.<sup>39</sup> Signal quantification showed that P-tyr over total VE-cadherin ratios were increased 3-fold in ovary and 4-fold in uterus by the hormonal treatment (Fig. 3C).

### VE-cadherin is associated to Flk1 in hormonally –stimulated ovary and uterus

Flk1 was previously found to be associated with the VE-cadherin-catenins complex *in vitro*, upon VEGF induction<sup>28, 40, 41</sup> This complex was also detected in endothelial cells cultured under flow condition.<sup>42</sup> Therefore, we wondered whether such an association could be observed *in vivo* and whether it was regulated by hormonal stimulation in ovary and uterus. Lysates from ovary and uterus of mice injected by PMSG/hCG or PBS alone were subjected to immunoprecipitation with anti-VE-cadherin antibody and immunoblotted with anti-Flk1 antibody. Flk1 signals were prominent in VE-cadherin immunoprecipitates from ovary and uterus of treated mice and not

visible in untreated mice (Fig. 4A). In lung and heart immunoprecipitates, the Flk1 band was faint but detectable (Fig. 4A). Flk1 could not be detected in VE-cadherin immunoprecipitates in kidney and liver (Fig. 4A). Similar results were obtained in mouse tissues not treated by peroxovanadate or hormones, indicating that association was independent of VE-cadherin phosphorylation level (data not shown).

Conversely, VE-cadherin was detected in Flk1 immunoprecipitate from hormonally-treated uterus extracts (Fig. 4B), thereby confirming Flk1-VE-cadherin association.

Altogether, our data indicate that hormonal stimulation promoted the association of Flk1 and VE-cadherin, possibly through a VEGF-dependent mechanism.

### **Constitutive VE-cadherin-Src association, and Src phosphorylation upon hormonal stimulation**

The cytoplasmic tyrosine kinase Src has been found to play a crucial role in VEGF-induced angiogenesis and vascular permeability.<sup>43</sup> Furthermore, Liu and Senger<sup>45</sup> showed that VE-cadherin was disrupted from intercellular junctions through a Src-dependent mechanism in collagen I-induced angiogenesis. We thus examined whether VE-cadherin was associated with Src *in vivo*. VE-cadherin immunoprecipitates from uterus and ovary of treated or untreated mice were immunoblotted with an anti-Src antibody, as shown Fig. 5A (upper panel). The presence of Src was detected in each immunoprecipitate, independent of hormone treatment, suggesting a permanent association of Src with VE-cadherin. Such an association was also found in lung (Fig. 5A, upper panel) and heart (not shown), as well as in tissues not treated by peroxovanadate (not shown). Analysis of Src phosphorylation state with anti-phosphotyrosine antibody, revealed that Src was strongly phosphorylated upon hormonal treatment (Fig. 5A, lower panel). Densitometric analysis showed 2- and 4-fold increases in phospho-Src upon treatment in ovary and uterus, respectively (not shown). These experiments established that the phosphorylation state of VE-cadherin-associated Src in the female reproductive system is markedly increased during hormone-induced angiogenesis.

A prominent VE-cadherin band was observed in Src immunoprecipitate from hormone-treated uterus extracts (Fig. 5B). These data confirm the existence of a robust association between Src and VE-cadherin *in vivo*.

### **Src inhibitors impaired VEGF-induced VE-cadherin phosphorylation but preserved Src-VE-cadherin association**

To examine whether Src activation is a necessary step for VE-cadherin phosphorylation, we used confluent primary endothelial cells (HUVEC) stimulated by VEGF together with Src inhibitors. In this system, VEGF rapidly induced VE-cadherin phosphorylation with a maximum at 15 min (Fig. 6A). When HUVEC were treated with Src inhibitors, either SU6656 or PP2, VEGF-induced VE-cadherin phosphorylation was inhibited in a dose-dependent manner (Fig. 6B). We conclude that Src is required for VEGF-induced VE-cadherin phosphorylation. Furthermore, we show that Src inhibition does not interfere with Src-VE-cadherin association (Fig. 6C). In agreement with these data, Src immunofluorescence staining of untreated confluent HUVEC showed that a significant subset of Src was located at cell-cell junctions, where it colocalized with VE-cadherin (Fig. 6D). VE-cadherin-Src colocalization was not altered by VEGF activation of cells (data not shown). Altogether, these *in vitro* data confirmed the VE-cadherin-Src association observed *in vivo*. We further show that this association is independent of VE-cadherin tyrosine-phosphorylation state and Src activation level.

## **DISCUSSION**

### **VE-cadherin tyrosine phosphorylation in the mature vasculature**

The tyrosine phosphorylation of VE-cadherin in endothelial cells upon stimulation<sup>7, 8, 19, 45, 46</sup> or in sparse cell culture<sup>6</sup> has been established, but its existence in the adult vasculature has been relatively unexplored. Of interest, we show in the present study, basal P-tyr-VE-cadherin levels in lung and uterus, and to a lesser extent in ovary, indicating that VE-cadherin is indeed a substrate of tyrosine kinase *in vivo*. VE-cadherin tyrosine phosphorylation in the female reproductive system is in agreement with our data on estrogen-induced VE-cadherin phosphorylation (see below). One possible explanation for the presence of P-tyr-VE-cadherin in lung is that it is a significant site for macrophage-endothelium interaction.<sup>47</sup> Macrophages may influence the pulmonary endothelium through the release of inflammatory mediators, which may induce VE-cadherin phosphorylation.

In contrast, VE-cadherin tyrosine phosphorylation was undetectable in other organs unless mice were injected with a potent tyrosine phosphatase inhibitor. This is usually the case for other cellular proteins that are phosphorylated on tyrosine in response to extracellular activating ligands for which the identification has been hampered by their low abundance and the ubiquitous presence of tyrosine phosphatases. Thus, our results suggest that VE-cadherin tyrosine phosphorylation might be regulated in adult quiescent endothelium through tyrosine phosphatase activities leading to a dephosphorylated form of VE-cadherin in resting endothelium. This is consistent with *in vitro* data showing that when endothelial cells reach confluence, they undergo contact inhibition of proliferation and stabilization of cell-cell junctions, together with downregulation of tyrosine phosphorylation of VE-cadherin and associated catenins.<sup>6</sup> This is also in agreement with previous data showing the density-dependent increase in PTP activity and expression at cell-cell junctions where they associate with the cadherin-catenin complex proteins and platelet endothelial cell adhesion molecule.<sup>48, 49</sup> Recently a tyrosine phosphatase, VE-PTP, has been shown to interact with VE-cadherin, however, this molecule does not directly dephosphorylate

VE-cadherin.50 Other PTP, such as SHP1, SHP2, PTP $\kappa$ , PTP $\mu$ , PTP-LAR and PTP-1B, have been shown to be indirectly associated to cadherins through catenin binding.<sup>29, 51–55</sup> These PTP may potentially dephosphorylate VE-cadherin. Our data show that VE-cadherin is only weakly phosphorylated in resting vasculature, suggesting that PTP activity overcomes that of protein kinases for VE-cadherin phosphorylation.

### **VE-cadherin tyrosine phosphorylation in angiogenic tissues**

To further explore the physiological existence of the VE-cadherin phosphorylation *in vivo*, we next examined the endothelium of the developing vasculature in the hormone-stimulated female reproductive system. Indeed, the female reproductive organs (ovary, uterus) are some of the few adult tissues that exhibit rapid growth accompanied by extensive modifications of vascularization and vascular permeability.<sup>3, 56</sup> Angiogenesis is thus an important component of the growth and function of these tissues. Our data show a higher phosphorylation state of VE-cadherin, indicating activation of downstream signaling during hormonally-induced angiogenesis. VEGF has a crucial role in the control of angiogenesis in the ovary<sup>36</sup> and neovascularization is essential in preparing the uterine endometrium for implantation.<sup>36, 57</sup> Previous data showed an increase in P-tyr-VE-cadherin in endothelial cells activated by VEGF.<sup>46</sup> Therefore, it is likely that induction of VE-cadherin phosphorylation be mediated by VEGF in hormone-stimulated organs. Others reported that VE-cadherin phosphorylation was upregulated in heart lysates of mice submitted to VEGF administration.<sup>44</sup> Our results on VE-cadherin phosphorylation in ovary and uterus are consistent with their data in heart.

The exact role of VE-cadherin phosphorylation remains to be clarified. Several disease processes are known to be driven by angiogenesis, including cancer, atherosclerosis, diabetic retinopathy and arthritis.<sup>58</sup> If VE-cadherin phosphorylation is a necessary step for the angiogenic process, inhibition of VE-cadherin phosphorylation may provide a useful therapeutic approach to limit such “angiogenic” diseases.

### **VE-cadherin-Flk1 association**

An association between VE-cadherin and Flk1 has been observed *in vitro* upon VEGF stimulation<sup>28, 40, 41</sup> or when cells were grown under flow conditions.<sup>42</sup> In the present study, we have demonstrated the presence of such an association in ovary and uterus following hormone stimulation. It is very likely that VEGF be the mediator triggering also the association with Flk1. VE-cadherin-Flk1 association was weak in lung and heart, and absent in kidney and liver. In contrast with these data, Weis and collaborators<sup>44</sup> found that both proteins were associated in heart lysates in resting conditions, whereas the complex was rapidly dissociated when mice were injected with VEGF. We do not know the reason of this difference. Endothelial subtype-specific mechanisms may explain this discrepancy. Alternatively, heart endothelial cells may be continuously activated by high flow rates, thereby promoting VE-cadherin-Flk1 association.

### **VE-cadherin-Src association**

We demonstrate for the first time that Src had a permanent association with VE-cadherin, *in vivo* and *in vitro*, independent of VE-cadherin phosphorylation state and Src activation level. VE-cadherin may serve as an anchor to maintain Src at the endothelial cell junction, where it could exert its activity on junctional components. We show that VE-cadherin-associated Src was tyrosine-phosphorylated upon angiogenic stimulation *in vivo*. Furthermore, inhibition of Src impaired VE-cadherin phosphorylation in VEGF-stimulated HUVEC, indicating that VE-cadherin phosphorylation is dependent upon Src activation in this model. Src kinases are considered to play a general role in regulating cadherin function in a wide variety of cell types.<sup>59</sup> In fact, Src can phosphorylate E-cadherin, causing epithelial cells to dissociate from one another.<sup>59</sup> It is tempting to speculate that Src also phosphorylates VE-cadherin. Src was shown to associate with Flk1 upon VEGF stimulation.<sup>60</sup> It is likely that VE-cadherin, Flk1 and Src form a multimeric complex upon VEGF activation and not separate associations. VEGF-dependent angiogenesis requires Src kinase activity.<sup>43</sup> Furthermore, it has been recently demonstrated that VEGF disrupted VE-cadherin-dependent junctions *in vivo*, through a Src-mediated mechanism.<sup>61</sup> It is conceivable that complex formation allows Flk1-activation of Src, which in turn phosphorylates VE-cadherin. Collagen I was shown to promote capillary morphogenesis together with VE-cadherin disruption from cell-cell contacts through a Src-dependent mechanism.<sup>45</sup> In these experiments, inhibition of Src prevented capillary morphogenesis and preserved VE-cadherin at cell junctions. Altogether, these data suggest that VE-cadherin plays a central role in the angiogenic process, in agreement with our observations on VE-cadherin-deficient mice.<sup>10</sup>

### **Conclusions**

In this report, we have shown that VE-cadherin phosphorylation levels are weak in quiescent tissues and markedly increased in angiogenic tissues. Importantly, a VE-cadherin-Flk1 association was prominent in angiogenic tissues, while a permanent association with Src was present in all tissues. Furthermore, the phosphorylation state of VE-cadherin-associated Src was increased in angiogenic tissues. Continued progress in the study of VE-cadherin tyrosine phosphorylation function will require further dissection of its downstream signaling pathways and relating these pathways to specific cellular responses.

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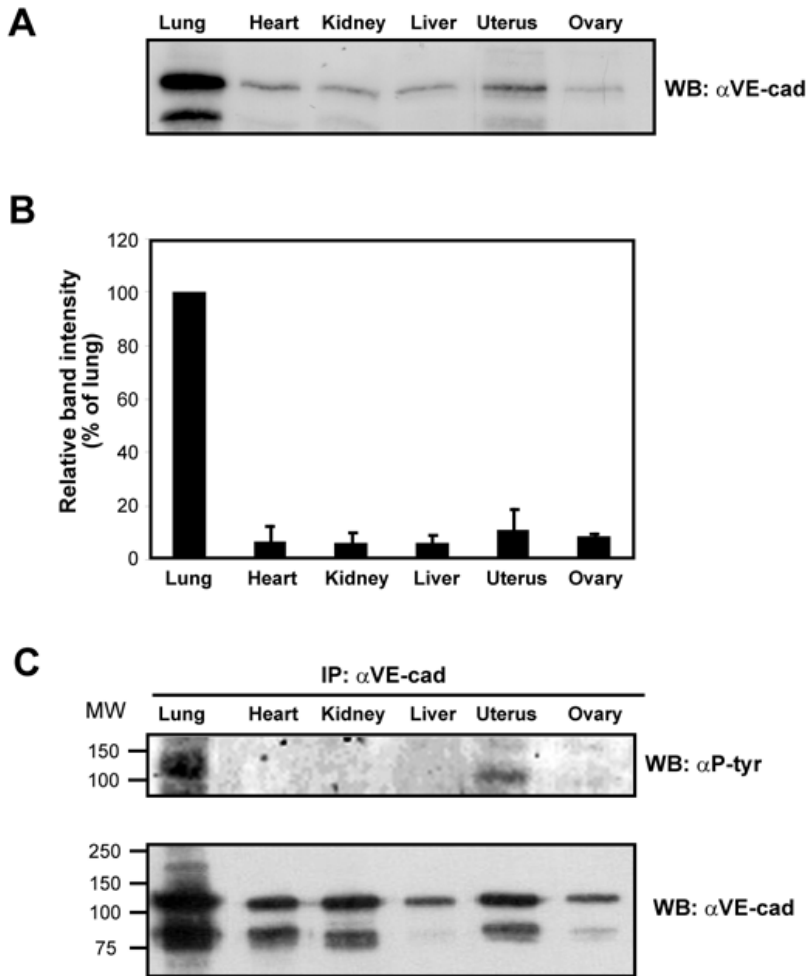
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**FIGURE 1**

VE-cadherin protein expression in mouse adult tissues

**Panel A:** Twenty  $\mu\text{g}$  of tissue lysate proteins were analyzed by SDS-PAGE and Western blotted with the anti VE-cadherin antibody. **Panel B:** Films from three Western blots were semiquantified by densitometry for evaluation of variations in VE-cadherin content among tissues. Results are expressed as mean percentages ( $\pm$  SEM) of lung VE-cadherin content. **Panel C:** VE-cadherin immunoprecipitates (1 mg of tissue proteins) were analyzed by Western blot first with the anti-phosphotyrosine (P-tyr) antibody and second with the anti-VE-cadherin antibody after stripping.

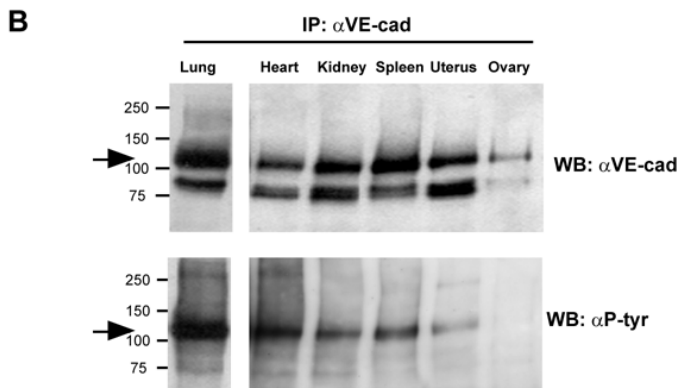
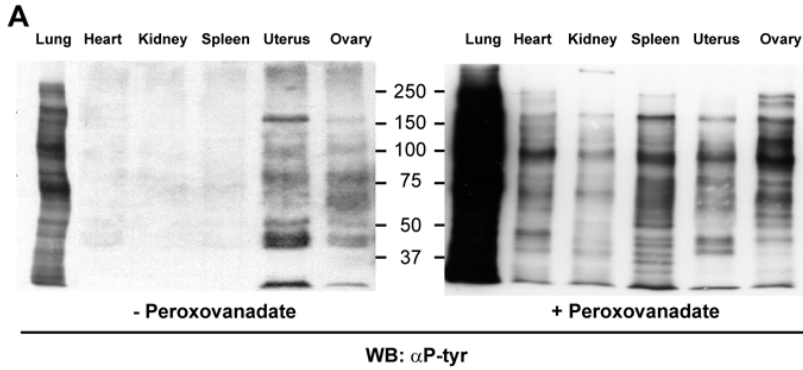




**FIGURE 2**

Effect of peroxovanadate treatment on tyrosine phosphorylation pattern in adult tissues

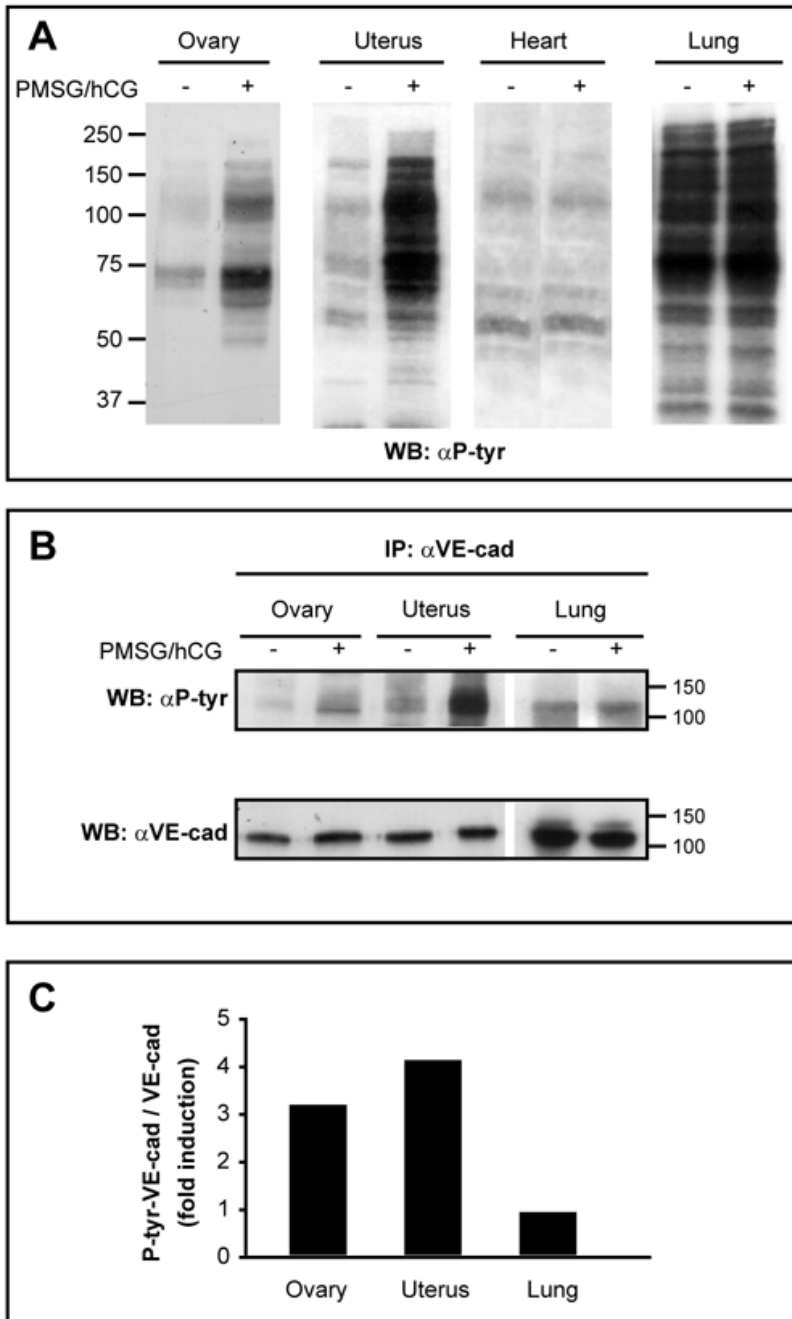
**Panel A:** The pattern of tyrosine phosphorylated proteins were analyzed in mice treated with peroxovanadate or vehicle alone. After 5 min of treatment, the animals were sacrificed and tissue proteins were extracted. Samples containing 10  $\mu$ g of total proteins were analyzed by Western blot with the anti-phosphotyrosine antibody. This analysis was performed for each treated mouse to check that the treatment was successful. **Panel B:** Evaluation of VE-cadherin phosphorylation in peroxovanadate-treated adult tissues. VE-cadherin immunoprecipitates were Western blotted with the anti VE-cadherin antibody and the anti-phosphotyrosine antibody, sequentially. Lung extracts were analyzed separately because of the much higher VE-cadherin content of this organ. The anti-phosphotyrosine antibody only labeled the full-length VE-cadherin (arrow) and not the truncated form below, devoid of the cytoplasmic domain.



**FIGURE 3**

VE-cadherin tyrosine phosphorylation in hormone-stimulated ovary and uterus

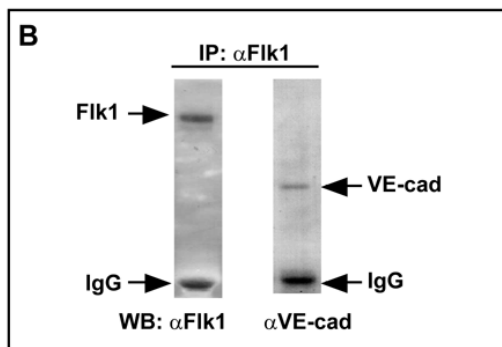
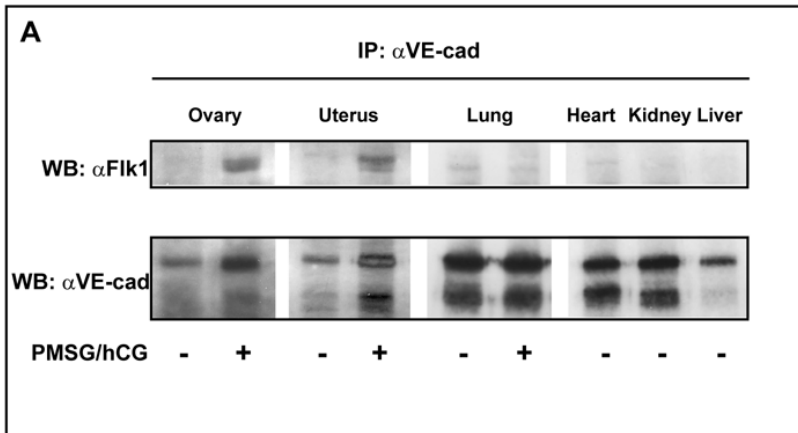
**Panel A:** Female mice were stimulated with PMSG/hCG and treated with peroxovanadate (see "Materials and Methods"). Tyrosine phosphorylated proteins (10  $\mu$ g) were analyzed by Western blot with the anti-phosphotyrosine antibody. **Panel B:** VE-cadherin was immunoprecipitated from 1 mg of proteins from ovary, uterus and lung extracts. Immunoprecipitates were immunoblotted with the anti-phosphotyrosine antibody, stripped and re-probed with anti-VE-cadherin antibody. **Panel C:** Western blots were semiquantified by densitometry for calculation of the ratio of the phosphorylated versus total VE-cadherin in the immunoprecipitates. Data are presented as fold induction by hormone treatment. This experiment is representative of two additional experiments.



**FIGURE 4**

Flk1/VE-cadherin protein association in mouse ovary and uterus stimulated by hormones

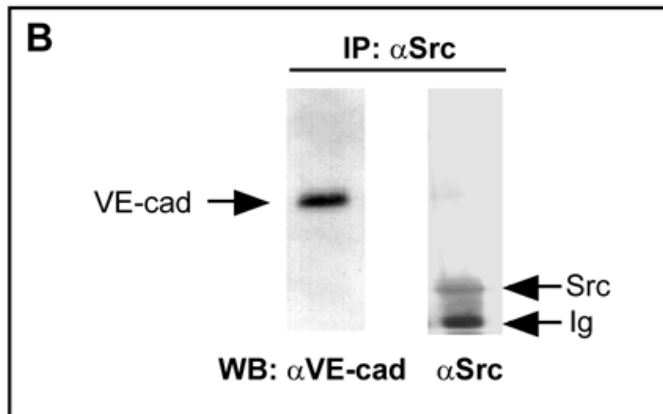
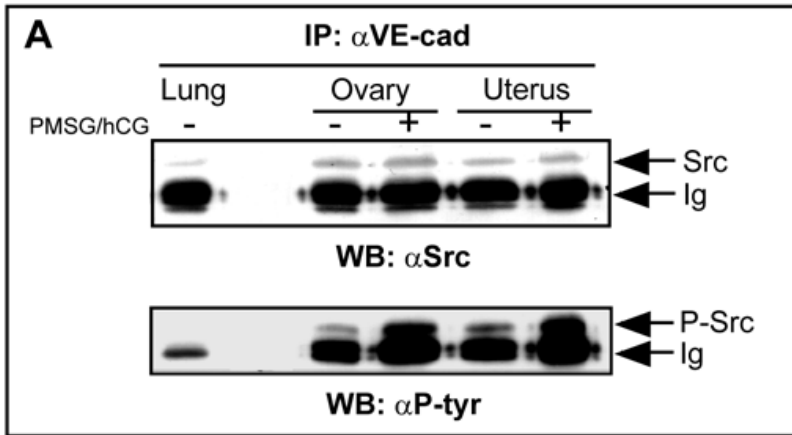
**Panel A:** Mice were treated with PMSG/hCG or vehicle alone, and peroxovanadate. Flk1/VE-cadherin association was revealed by immunoprecipitation of VE-cadherin from 1 mg of tissue protein lysates, immunoblotting with the anti-Flk1 and the anti-VE-cadherin antibodies. This experiment is representative of two additional experiments. **Panel B:** Flk1-VE-cadherin association was confirmed by immunoprecipitation of uterus protein extracts (1 mg) with the anti-Flk1 antibody and immunoblotting with the anti-Flk1 and the anti-VE-cadherin antibodies. The lower band (50 kDa) is the Ig-heavy chain.



**FIGURE 5**

Src/VE-cadherin association and Src phosphorylation state in mouse ovary and uterus

**Panel A:** Mice were treated by PMSG/hCG or vehicle alone, and peroxovanadate. Src/VE-cadherin association was analyzed in VE-cadherin immunoprecipitates from protein lysates (0.5 mg for lung and 1 mg for ovary and uterus) and immunoblotting with the anti-Src antibody. The membrane was stripped and reprobed with the anti-phosphotyrosine antibody. The lower band (50 kDa) is the Ig-heavy chain. **Panel B:** To confirm Src/VE-cadherin association, uterus extracts (1 mg) were immunoprecipitated with anti-Src antibody. Proteins were then Western blotted with the anti-VE-cadherin and the anti-Src antibodies. This experiment is representative of three additional experiments.



**Figure 6**

Src inhibitors impaired VEGF-induced VE-cadherin phosphorylation in endothelial cells but not VE-cadherin-Src association

**Panel A:** Confluent cultures of HUVEC were serum starved (1% serum) for 6 h and incubated with 50 ng/ml VEGF for 0 to 30 min. Proteins were immunoprecipitated with anti-VE-cadherin and Western blotted with anti-P-tyr and anti-VE-cadherin antibodies. **Panel B:** Src inhibitors prevented VEGF-induced VE-cadherin phosphorylation. VE-cadherin was immunoprecipitated from VEGF-stimulated HUVEC treated with increasing concentrations of SU6656 or PP2, as indicated. The presence of P-tyr- VE-cadherin was detected as above. **Panel C:** Src inhibitor did not disrupt VE-cadherin-Src association. VE-cadherin was immunoprecipitated from HUVEC treated with VEGF and increasing concentrations of SU6656. VE-cadherin and Src were revealed by Western blotting. **Panel D:** Immunolocalization of Src and VE-cadherin in confluent HUVEC. Double immunofluorescence staining (Src, green; VE-cadherin, red) showed protein colocalization at cell-cell junctions (yellow staining in merged images).

