CHAPTER V

LOSS OF TIE1 PROMOTES ANTI-ATHEROGENIC eNOS SYNTHESIS AND DOWNREGULATES NF-κB ACTIVATION

Introduction

Previous studies have shown that activation of Tie1 inhibits the function of Tie2 to maintain endothelial quiescence (Kwak *et al.*, 1999; Papapetropoulos *et al.*, 2000; Harfouche *et al.*, 2002). Increased Tie1 expression has also been found in human tissue samples from patients with autoimmune inflammatory conditions such as rheumatoid arthritis and osteoarthritis (Shahrara *et al.*, 2002). Further, overexpression of Tie1 in human umbilical vein endothelial cells increased the transcriptional message of inflammatory markers such as VCAM-1, ICAM-1 and E-selectin in a manner that was blocked by a p85 inhibitor (Chan *et al.*, 2008). Suppression of Tie1 expression with siRNA downregulated expression of not just these markers, but also of TLR2 and IL-1β inflammatory molecules (Chan & Sukhatme, 2009), suggesting a new pro-inflammatory role for Tie1.

While Tie1 is pervasively expressed in the endothelia of developing embryos, post-natal expression is severely diminished in most organs except for the lungs (Taichman *et al.*, 2002). Pulmonary Tie1 expression was dramatically increased during the period correlating with increased angiogenic activity of the maturing lung. In partially denuded carotid rat arteries where activated endothelial cells are re-populating the injury sites, Tie1 expression was also found to be upregulated (Fujikawa *et al.*, 1999). Furthermore,

Tie1 expression is increased in epithelial tumor cells of various organs, consistent with a role in cellular activation (Cance *et al.*, 1993; Lin *et al.*, 1999; Tseng *et al.*, 2001; Yang *et al.*, 2003; Ito *et al.*, 2004; Uruno *et al.*, 2004). These studies suggest that Tie1 expression may promote endothelial cell activation. In the presence of either protein kinase C (PKC) (Yabkowitz *et al.*, 1997), vascular endothelial growth factor (VEGF) (Tsiamis *et al.*, 2002), phorbol 12-myristate 13 acetate (PMA) (Marron *et al.*, 2000) or tumor necrosis factor-α (TNF-α) (Yabkowitz *et al.*, 1999), Tie1 is cleaved releasing its extracellular domain and generating a membrane bound receptor fragment comprising the intracellular and transmembrane domains. The cleaved intracellular product persists in the cytosol for several hours (Marron *et al.*, 2000), and has been demonstrated to bind signaling partners such as Shp2 (Marron *et al.*, 2000). The prolonged existence of the cleaved Tie1 intracellular fragment in the cytosol and its association with secondary messengers suggest that this Tie1 endodomain may have further intracellular signaling functions (Marron *et al.*, 2000). However, the resultant effect of this proteolytic processing on Tie1 mediated signaling has not been studied.

One of the most important molecules investigated for its response to flow is nitric oxide. The intracellular level of this potent vasodilator is regulated by eNOS (Uematsu *et al.*, 1995). Levels of eNOS dramatically increases with onset of laminar flow and quantities remain high even after 24 hours (Kuchan & Frangos, 1994; Corson *et al.*, 1996). Nitric oxide has anti-inflammatory properties that regulate the nf-κB transcription factor (Hajra *et al.*, 2000). Disturbed flow conditions activate nf-κB resulting in downregulation of eNOS expression. In contrast, laminar flow with high shear stress increases expression of eNOS, which suppresses nf-κB activity (Davis *et al.*, 2001; Davis

et al., 2004). As many studies have shown eNOS production is a very sensitive assay of the effect of shear stress on endothelial cells (Kuchan & Frangos, 1994) and regulation of nitric oxide release is an important process in endothelial function.

Tie1 expression and activity is also mediated by shear stress. Laminar flow with high shear stress decreased Tie1 protein levels *in vitro* (Chen-Konak *et al.*, 2003). Interestingly, Chen-Konak et al showed that following 6 hours of pre-conditioning, alterations in shear stress magnitude resulted in a decrease of Tie1 levels. Conversely, Porat et al showed that disturbed flow conditions *in vitro* upregulates Tie1 promoter activity (Porat *et al.*, 2004). Also, bovine aortic endothelial cells exposed to shear stress generated higher levels of the cleaved Tie1 intracellular fragment when compared to static flow controls (Chen-Konak *et al.*, 2003). Taken together, these studies suggest that Tie1 modulates endothelial cell activation, and Tie1 expression and activity is regulated by shear stress. However, an effect of shear stress on Tie1 mediated endothelial cell activation has not been studied.

Experimental Procedures

Genotyping

At three weeks of age, tail samples from offspring were digested in 100 mM Tris pH 8.5, 5 mM EDTA, 0.2% sodium dodecyl sulfate, 200 mM NaCl, 100 μg/ml of Proteinase K overnight at 55^{o}C. Mice were genotyped by polymerase chain reaction with REDExtract-N-Amp PCR Reaction Mix (Sigma) using the following primers,

cre: 5'- TCC GGG CTG CCA CGA CCA A -3' and 5'- GGC GCG GCA ACA CCA TTT TT -3';

tie1: 5'- TGC CCC CCC TTC CAG AGA CTT CC -3' and 5'- GCA AAG AGG ATC CCC ACC AGA CCA TAC T –3' and 5'- GGG GAT GTG CTG CAA GGC GAT TAA G -3';

tie-flox: 5'- TCG GGC GCG TTC AGA GTG GTA T -3' and 5'- ATG CCT GTT CTA TTT ATT TTT CCA G -3'

tsA58: 5'- CCT CTG AGC TAT TCC AGA AGT AGT G -3' and 5'- TTA GAG CTT TAA ATC TCT GTA GGT AG -3'

Animal Breeding and Tamoxifen Injections

SCL-ER^{T}-Cre mice (Gothert *et al.*, 2004) were a kind gift from Dr. Gothert. Tie1$^{flox/flox}$:SCL-ER^{T}-Cre mice were bred in our laboratory. All mice used in this study were bred onto a pure C57BL/6 background and were maintained in microisolator cages on a rodent chow diet (Purina Mills Inc) and autoclaved water ad libitum. Animal care and experimental procedures were performed according to the regulations of Vanderbilt University's Animal Care and Use Committee.

Generation of Tie1 mutant alleles

To generate Tie1$^{flox/flox}$ mice a loxP site and a neomycin resistance cassette were introduced within the first intron of Tie1, and another loxP site just upstream of the minimal promoter region. This strategic placement of loxP sites would allow excision of the Tie1 minimal promoter and exon 1 upon Cre activation (Qu *et al.*, 2010).

Protein Isolation and Western Blotting

Tissue samples or cells were lysed on ice using a lysis buffer (50mM Tris, 50mM NaCl, 1% Triton X-100, 1mM EGTA, 1mM Na_3VO_4, 5mM NaF, 1x β-glycerophosphate) supplemented with Complete protease inhibitor cocktail (Roche). After centrifugation at 12000 rpm at 4^{o}C, the supernatant was collected. Protein concentrations were determined with Protein Assay Reagent (Bio-Rad Laboratories). The nuclear pellet was rinsed three times in lysis buffer and incubated at 100^{o}C with 2x sample buffer to extract nuclear proteins. Lysates were resolved by SDS-PAGE electrophoresis and transferred onto Hybond ECL nitrocellulose membranes (GE Healthcare). Blots were blocked with 5% non-fat milk and probed with rabbit antibodies to Tie1 (sc342), NOS3 (sc654), p50 (sc114), sp3 (sc644) (Santa Cruz Biotechnology) and mouse antibody to β-actin (Sigma cat# A5316) overnight at 4^{o}C. After washing with 0.1% Tween-20 supplemented phosphate buffered saline (PBST), blots were incubated with secondary antibodies IRDye 800CW goat anti-rabbit IgG and IRDye 680CW goat anti-mouse IgG for 1 hour at room temperature with gentle agitation. Blots were washed and scanned on the Odyssey Infrared Imaging System (LI-COR Biosciences) and densitometry analyses were performed using Odyssey Software.

Mouse Aortic Endothelial Cell Isolation (MAEC)

The Immorto mouse is a transgenic mouse generated by the introduction of thermolabile SV40 T Ag, tsA58(Jat *et al.*, 1991). In this model, the simian virus 40 (SV40) large tumor antigen of a temperature-sensitive strain (tsA58) is fused with the

major histocompatibility complex promoter H-2K^{b}. Expression of the promoter and the large T antigen protein is only evident when immorto mouse-derived cells are cultured at a permissive temperature (33^{o}C). The addition of IFN-γ was also used to further enhance promoter activity(Jat *et al.*, 1991). A 4 to 8 week-old immorto mouse was anesthetized with isoflurane and sacrificed by cervical dislocation. The lumbar aorta was punctured and 5ml of Hanks Balanced Salt Solution (HBSS) was perfused through the left ventricle to flush out blood. The heart, lung and aorta bloc was excised and transferred to a dish containing HBSS and antibiotics. The heart and lungs were removed. The rest of the aorta was incubated at 37^{o}C for 15 minutes with filtered 10mg/ml collagenase Type II (Worthington Chemicals) and 1x antibiotics dissolved in dispase (Roche). The adventitia was gently removed and the remaining intima layer comprising the endothelium and internal elastic lamina was cut open longitudinally and incubated at 37^{o}C for 30 minutes in filtered 20mg/ml collagenase Type II solution and 1x antibiotics dissolved in dispase. The aorta was dissociated by pipetting several times in the dissolving solution and filtered through a 100μm sterile cell strainer (Fisher Scientific). Culture media (MCDB 131 [Gibco], 10% FBS [Hyclone], 10U/ml heparin [Sigma], 2.75nM hydrocortisone [Sigma] and 0.2% Bovine Brain Extract [Hammond Celltech], 10U/ml recombinant murine IFN-γ [Peprotech], 1x antibiotics [Gibco]) was added to the cell suspension and centrifuged at 375xg for 10 minutes. The cell mixture was then plated onto a fibronectin coated culture dish and propagated at 33^{o}C in a mixture of 5% carbon dioxide and 95% oxygen. MAECs were isolated from wild-type and Tie1$^{flox/flox}$:SCL-ER^{T}-Cre immorto mice.

4-hydroxytamoxifen Induction of Cre Activation and Immunocytochemistry

Mouse aortic endothelial cells from Tie1$^{flox/flox}$:SCL-ER^{T}-Cre mice were cultured at 37^{o}C in complete media (as described above) without IFN-γ and treated with 5μM of 4-hydroxytamoxifen (Sigma) every two days for six days. To ascertain ER^{T}-Cre nuclear localization, 4-hydroxytamoxifen treated cells were rinsed once with HBSS, fixed with cold 100% methanol for 3 minutes and rinsed twice with HBSS. Cells were blocked with 5% goat serum, incubated overnight at 4^{o}C with Cre antibody (Abcam, AB24608), rinsed and immunostained with Alexa 488 conjugated goat anti-rabbit secondary antibody (BD Biosciences).

Shear Stress Experiments

Shear stress experiments were performed using a custom cone-and-plate shear stress viscometer design. An inverted servo motor (ElectroCraft, USA) is attached to a plexiglass cone with 0.5^{o} angle. A Motomatic II motor controller (Reliance Electric, ElectroCraft, USA) regulates velocity in laminar flow, and a digital function generator (Instek, San Diego, CA, USA) is used to produce a sinusoidal waveform modulating oscillatory flow. A digital oscilloscope (Tektronix, USA) is used in parallel to monitor the output magnitude and waveform. The servomotor and cone are lowered onto the culture dish by a step controlled base stand. Laminar shear is attained with unidirectional motion of the cone while oscillatory shear is achieved by bidirectional motion at ± 5 dynes/cm^{2}. Shear stress experiments are performed for 24 hours in complete media in a sterile, 5% CO_2 incubator at 37^{o}C.

RNA Extraction

RNA from tissue or cells was isolated using TriReagent (Molecular Research Center, Cincinnati, OH, USA) following the manufacturer's instructions. RNA concentration and purity were analyzed on a NanoVue spectrophotometer (Thermo Scientific). Samples with 260/280 ratio > 1.8 (1.9-2.1 preferrably) and 260/230 ratio > 1.5 were deemed of sufficient quality for qRT-PCR. 2 μg of total RNA was reverse-transcribed into cDNA using SuperScript III First-Strand System (Invitrogen) in 20 μl as per manufacturer's instructions.

Quantitative real-time Polymerase Chain Reaction (qRT-PCR)

All qRT-PCR was performed using iQ SYBR Green Supermix (Bio-Rad) on the iQ5 Real Time PCR Detection System (Bio-Rad). Experiments were performed in triplicate using glyceraldehyde-3-phosphate dehydrogenase as the housekeeping gene to normalize the data. The relative change in gene expression was determined using the critical threshold (C_t) and the equation Fold Induction = $2^{(-\Delta\Delta C_t)}$ (Schmittgen & Livak, 2008). Primers used were as follows, Tie1: 5'-GTGCCACCATTTTGACACTG-3' and 5'-CAGGCACAGCAGGTTGTAGA-3'; glyceraldehyde-3-phosphate dehydrogenase: 5'-CACTGGCATGGCCTTCCGTG-3' and 5'-AGGAAATGAGCTTGACAAAG-3

Statistical Analyses

All statistical differences in this study were determined by a 2-tailed, unpaired, student's t-test with 95% confidence intervals, using Prism 4.0 software (Graphpad Inc).

Results

4-hydroxytamoxifen treatment activates Cre-mediated Tie1 deletion

To determine the effect of Tie1 deletion *in vitro*, we isolated endothelial cells from homozygous Tie1$^{flox/flox}$:SCL-ER^{T}-Cre immorto mice, allowing for the use of 4-hydroxytamoxifen (4OHT) to activate Cre transcription and hence induce Tie1 deletion. To ascertain activation of Cre *in vitro* we immunostained 4OHT treated Tie1$^{flox/flox}$:SCL-ER^{T}-Cre MAECs using Cre antibody. Increased nuclear staining indicates successful translocation of Cre (Figure 5A, B). qRT-PCR and western blot analyses of full-length Tie1 indicate 4OHT administration resulted in 41% and 45% reduction of Tie1 expression respectively (Figure 5C, D). Additionally, the cleaved Tie1 intracellular fragment (McCarthy *et al.*, 1999; Marron *et al.*, 2000; Marron *et al.*, 2007) was reduced by 72% (Figure 5E).

Tie1 attenuation augments eNOS expression and decreases p50 nuclear translocation

We exposed the MAECs to laminar or oscillatory flow for 24 hours to investigate the effect of shear stress on Tie1 *in vitro*. In these MAECs, laminar flow at 20 dynes/cm^2 decreased Tie1 receptor expression while oscillatory flow had no effect on Tie1 expression (Figure 5F). Laminar flow also increased eNOS protein levels in Tie1$^{flox/flox}$:SCL-ER^{T}-Cre MAECs as expected (Kuchan & Frangos, 1994). Interestingly, after tamoxifen-induced deletion of Tie1, laminar flow further augmented eNOS expression (Figure 5G). Also, oscillatory flow now increased eNOS levels when compared either with cells grown under static conditions or with cells not exposed to

tamoxifen (Figure 5H). Laminar flow with Tie1 deletion had no significant effect on p50 nuclear localization (Figure 5I). Under oscillatory flow conditions, however, we found that Tie1 deletion led to a 56% reduction of p50 nuclear translocation ($p<0.01$) (Figure 5J). In conclusion, these results suggest that Tie1 expression may be regulated by shear stress and Tie1 may play a pro-inflammatory role acting via nf-κB signaling in promoting the progression of atherosclerosis.

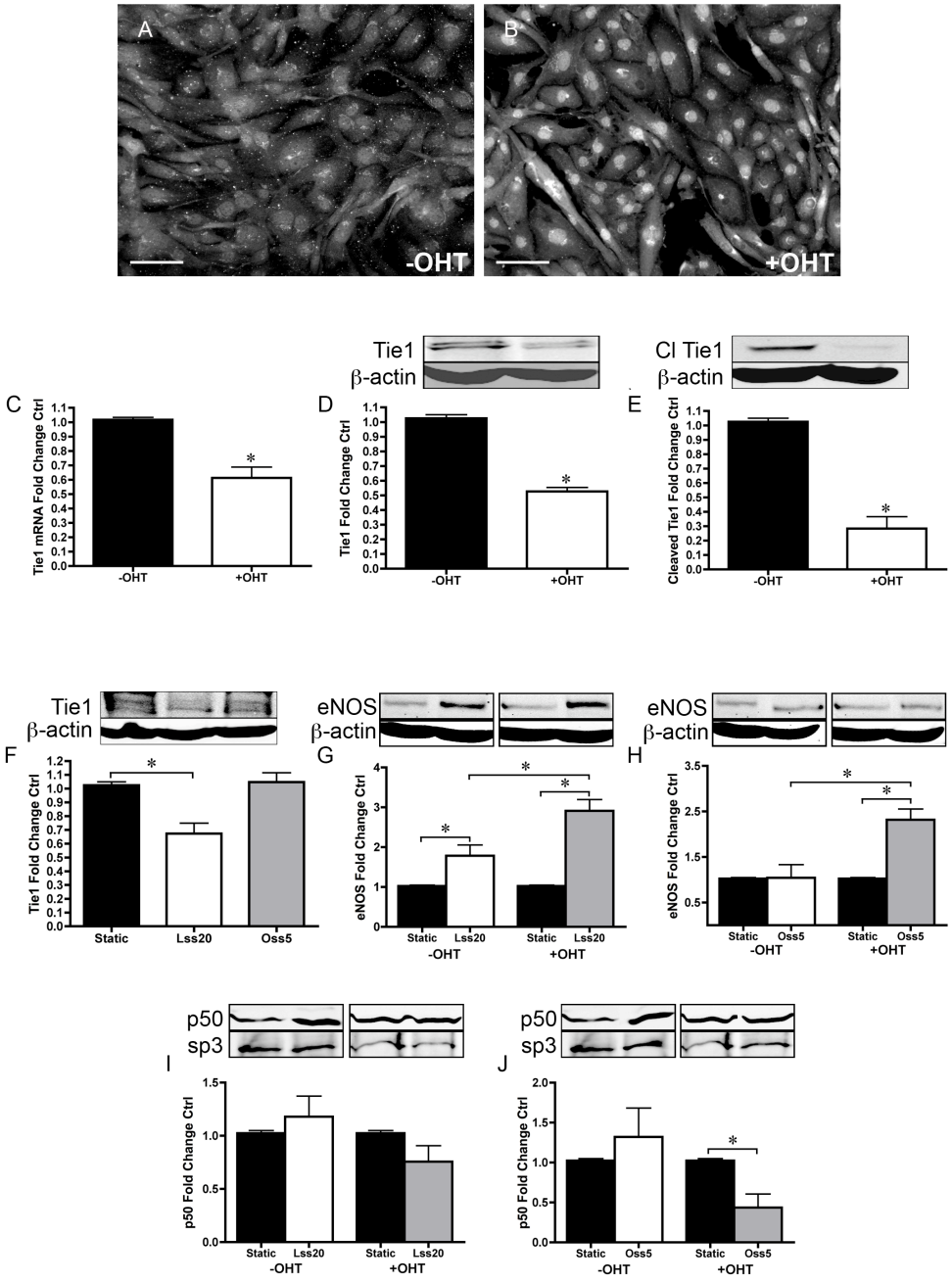

Figure 5.1 In vitro deletion of Tie1 and endothelial cell response to shear stress
A-E. Treatment of Tie1$^{flox/flox}$:SCL-ER^{T}-Cre mouse aortic endothelial cells with 4-hydroxytamoxifen (4OHT) activated Cre and reduced Tie1 expression. Representative immunofluorescent images showing Cre nuclear localization (**B**) following 4OHT treatment compared to (**A**) untreated controls. (**C**) RT-PCR analysis of Tie1 mRNA levels. (**D**) Western blot analyses of full length protein levels and (**E**) cleaved Tie1 45kDa endodomain levels. (**F**) Western blot analysis of full length Tie1 receptor after twenty-four hours of shear stress showing laminar flow at 20 dynes/cm^2 decreased Tie1 expression. Western blot analyses of Tie1 deleted (+OHT) versus control (-OHT) cells

comparing (**G**) laminar shear and (**H**) oscillatory shear induced eNOS expression. Western blot analyses of Tie1 deleted (+OHT) versus control (-OHT) cells comparing (**I**) laminar shear and (**J**) oscillatory shear induced p50 nuclear localization (normalized to sp3). (*$p<0.05$; White bar represents 100μm scale; Cl Tie1, cleaved Tie1; Lss20, laminar shear 20 dynes/cm^2; Oss5, oscillatory shear 1Hz 5 dynes/cm^2; OHT, 4-hydroxytamoxifen)

Discussion

We achieved a 50% deletion of Tie1 *in vitro*, concordant with *in vivo* tamoxifen induced deletion in Tie1$^{flox/flox}$:SCL-ER^{T}-Cre mice. We surmise that Cre expression under the SCL promoter may not be sufficient for extensive deletion of bi-allelic floxed-Tie1. Marron et al previously reported cleavage of Tie1 with phorbol ester or VEGF (Marron *et al.*, 2007) while Chen-Konak and colleagues reported changes in levels of Tie1 intracellular fragment after brief alterations in shear stress (Chen-Konak *et al.*, 2003). Upon administration of 4OHT, we also noted a concomitant decrease in levels of cleaved Tie1 intracellular fragment. While deletion of Tie1 resulted in a 50% reduction in full-length Tie1 receptor, the cleaved Tie1 fragment was decreased by 74%.

Our *in vitro* experiments in murine aortic endothelial cells showed that twenty-four hours of laminar flow at 20 dynes/cm^2 suppressed Tie1 expression and increased eNOS levels. Using bovine aortic endothelial cells, Chen-Konak et al found that brief *in vitro* application of 10 dyne/cm^2 shear stress temporarily decreased Tie1 but expression levels returned to baseline after 2 hours (Chen-Konak *et al.*, 2003). An octameric negative shear stress response element (nSSRE) was also found that downregulates Tie1 expression (Chen-Konak *et al.*, 2003). Shear stress response elements are targets of the nf-κB transcription factor, which in turn are regulated by shear stress (Gimbrone *et al.*, 1999). Tie1 was upregulated with a step increase (15 dyne/cm^2) and downregulated with a step decrease (5 dyne/cm^2) in shear stress. Tie1 promoter activity in HUVECs was found augmented by non-laminar flow in an *in vitro* step flow system(Porat *et al.*, 2004). Similar to the effect of adding PMA, VEGF or TNF-α (Yabkowitz *et al.*, 1999), and also mirroring our results, Chen-Konak found that shear stress induced cleavage of Tie1.

We also showed that genetic deletion of Tie1 *in vitro* augmented shear stress induced eNOS expression and reduced p50 levels. Recent studies have shown that siRNA silencing of Tie1 *in vitro* reduced expression of inflammatory markers (Chan & Sukhatme, 2009) while overexpression studies demonstrated complementary findings (Chan *et al.*, 2008), suggesting a pro-inflammatory for Tie1. These findings together support our *in vivo* data and together suggest a novel pro-inflammatory role for Tie1 and a potential mechanism for Tie1 in atherosclerosis progression via the nf-κB pathway.