Published in final edited form as:

Clin Immunol. 2015 July ; 159(1): 13–22. doi:10.1016/j.clim.2015.04.009.

17 β Estradiol Regulates Adhesion Molecule Expression in Mesangial Cells during Glomerulonephritis

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Abstract

We showed previously that 17 β Estradiol (E2) led to improved survival in nephrotoxic serum induced nephritis (NTN) in male mice. In this study we determined whether E2 regulates vascular cell adhesion molecule (VCAM)-1, an adhesion molecule that is upregulated in kidney during autoimmune nephritis, in mesangial cells (MC). We show that E2 inhibited VCAM-1 up-regulation in kidneys in vivo during NTN, and in MCs upon TNF α stimulation. VCAM-1 up-regulation in MCs was controlled by the transcription factor NF κ B. E2 inhibited RNA polymerase II recruitment to the VCAM-1 promoter, but not p65 recruitment. Interestingly E2 inhibited TNF α stimulated interaction between Poly (ADP-Ribose) Polymerase -1 (PARP-1) and p65. As PARP-1 is required for VCAM-1 upregulation in MCs, our data suggest that E2 may inhibit pre-initiation complex formation at VCAM-1 promoter by inhibiting PARP-1 recruitment to p65. We propose that E2 plays an important role in regulating renal inflammation locally.

Keywords

Mesangial cells; Estrogen; Nephritis; VCAM

1. Introduction

The immuno-modulatory roles of estrogens, although studied extensively, are not completely understood. The higher prevalence of autoimmunity in females is often linked to estrogens, on the other hand estrogens are proposed to protect from osteoporosis and kidney failure in diabetic females. The anti-inflammatory role of estrogens is well studied. 17 β estradiol (E2) at periovulatory to pregnancy levels inhibited pro-inflammatory cytokines from human peripheral blood mononuclear cells. Similarly in mouse primary macrophages

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Conflict of interest statement

The author(s) declare that there are no conflicts of interest.

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and macrophage cell lines E2 inhibited LPS stimulated TNF α and nitrite production [1; 2; 3] and decreased TNF α and IL-12 production in mature mouse dendritic cells (DC), with a shift toward cytokines such as IL-4 and IL-10 [4]. These data support the anti-inflammatory and protective effects of E2. Contrary to these reports, several groups have suggested that signaling through estrogen receptor alpha (ER α) promotes an inflammatory pathway of DC development, in addition to regulating myeloid hematopoietic progenitor numbers [5]. Evidence also suggests that E2 is able to stimulate antibody secretion [6; 7; 8], and therefore possibly contributes to the increased auto-antibody production in B cell driven autoimmune diseases such as systemic lupus erythematosus.

The role of estrogens in renal disease however, is not studied extensively. Pre-menopausal women have lower incidence of end stage renal disease (ESRD) in diabetic nephropathy, whereas post-menopausal women show an increase in ESRD. Male sex has been identified as a predictor of poor renal outcome [9; 10].

Estrogens reverse proteinuria and progressive renal injury in mice over-expressing TGF β presumably by inhibiting TGF β stimulated type IV collagen gene transcription [11; 12]. Estradiol also stimulates Matrix Metalloproteinase 2 (MMP2) secretion by mesangial cells [13], thereby preventing matrix accumulation and sclerosis in diabetic nephropathy.

A role for estrogens in modulating immune response during immune mediated renal disease has also been implied. Estrogens have been suggested to ameliorate hypertension-induced renal injury by reducing oxidative stress [14]. Using an induced model of immune-mediated nephritis, we showed previously that supplementing male mice with E2 results in reduced severity of disease and improved survival [15]. In the present study we further investigated the possible mechanism(s) by which E2 tames local inflammation and damage to renal tissue. To this extent we focused on the role of adhesion molecules on intrinsic renal cells.

Vascular cell adhesion molecule-1 (VCAM-1) is an adhesion molecule, which through its interaction with very late antigen-4 (VLA-4) present on leukocytes regulates recruitment of inflammatory cells. In a spontaneous mouse model of lupus, MRL/lpr, VCAM-1 expression was increased in the endothelium, glomeruli and tubules [16]. Increased VCAM-1 gene expression was also observed in microarray analysis of glomeruli from nephritic MRL/lpr mice when compared to control MRL/MpJ [17]. Soluble VCAM-1 (sVCAM-1) levels correlated with disease activity, and further more in patients with renal, hematological and vascular activity, significant differences in sVCAM-1 levels were reported [18; 19]. Urinary VCAM-1 levels were elevated in class IV LN [20]. Finally blocking VLA-4/VCAM-1 interactions ameliorated crescentic nephritis in rats [21].

In this study we show that estrogens inhibit VCAM-1 upregulation in vivo during nephritis and in vitro in mesangial cells upon TNF α stimulation. This upregulation was mediated through activation of transcription factor NF κ B. Moreover, E2 impaired NF κ B activation by inhibiting the formation of pre-initiation complex (PIC) at the VCAM-1 promoter. Our data suggest that E2 may block PIC formation by inhibiting the interaction between p65 and co-factor Poly (ADP-Ribose) Polymerase-1 (PARP-1). We therefore propose that during renal inflammation estrogens are protective locally.

2. Materials and Methods

2.1. Cell culture

Mesangial cells [15] were maintained in DMEM with 10% fetal bovine serum (FBS). To determine the effect of estrogens, cells were cultured in phenol red free medium with charcoal/dextran treated FBS (Hyclone) for 72h. Cells were then treated with 17 β -estradiol (E2 10⁻⁵ – 10⁻¹¹ M) for 48h prior to TNF α stimulation. 10⁻⁷ and 10⁻⁵ M E2 were used to determine effects on TNF α stimulated VCAM-1 mRNA levels, with similar results. For PARP-1 inhibition, cells were pretreated with the inhibitors 3-aminobenzamide (3AB, Sigma, 0.01–1mM) or CEP 8983 (CEP, Cephalon/Teva, 1–10 μ M) 30 min prior to TNF α stimulation. For NF κ B inhibition, cells were pretreated with selective inhibitor of I κ B kinase, IKK16 (Tocris Biosciences, 1 μ M) 30min prior to TNF α (1ng/ml) stimulation.

2.2. Mice

129sv mice were obtained from Jackson Laboratories. Breeding colonies were maintained at Temple University in accordance with the guidelines of the University Laboratory Animal Resource Office of Temple University. All experimental procedures were conducted according to the guidelines of the Institutional Animal Care and Use Committee. NTN was induced as described previously by a single injection of NTS [15]. For estrogen treatment, 17 β -estradiol (E2) pellets (Innovative Research of America) were implanted s.c. NTS (6mg/ml) was injected 5 days later. 30h following NTS injection, mice were euthanized, kidneys perfused, and frozen in OCT.

2.3. Confocal microscopy for p65

Mesangial cells were plated on chambered coverglass and stimulated with TNF α for various time points. Cells were fixed with 4% paraformaldehyde, permeabilized with saponin, blocked with 5% goat serum and incubated overnight with anti-p65 polyclonal antibody (Cell signaling). The cells were stained with Rhodamine conjugated goat anti-rabbit antibodies (Life Technologies) and DAPI and imaged using Zeiss META confocal microscope equipped with ZEN software. The data were analyzed by ImageJ software. Cells with nuclear p65 were counted with the help of 'counter' plug-in. A minimum 6 fields/condition were analyzed.

2.4. Ex-vivo VCAM-1 expression

Frozen sections were fixed with acetone and stained with anti-VCAM-1 (BD biosciences, clone 429), followed by FITC conjugated anti-mouse IgG and DAPI for nuclear staining. The sections were mounted in Prolong gold (Life Technologies) and imaged using Zeiss META confocal microscope equipped with ZEN software. The data were analyzed by ImageJ software. The channels were separated (blue and green). The mean fluorescence intensity (MFI) was calculated for each field in the green channel. The MFI for the sections with secondary antibody only were used for background calculations and were subtracted from the MFI values obtained from the sections stained with primary antibody. A minimum of 5 fields for each section/mouse were analyzed.

2.5 Flow cytometry

For surface staining of VCAM-1, after stimulation, cells were incubated with biotin conjugated anti-VCAM-1 antibodies (eBioscience), followed by streptavidin conjugated APC (BD Biosciences), fixed in 1% paraformaldehyde and acquired on BD FACS Canto. 50,000 total events were acquired. For some experiments, cells were additionally stained with Annexin V FITC and 7AAD, and VCAM-1 up-regulation was measured in cells negative for both Annexin V and 7AAD (live cells). For intracellular PARP-1 staining, the cells were first stained for surface VCAM-1 as above. After fixation, the cells were permeabilized with saponin, blocked with 5% goat serum and incubated with either anti-PARP-1 monoclonal antibody (BD Biosciences) or mouse IgG1 isotype antibody (Biolegend). Cells were washed, stained with FITC conjugated goat anti-mouse antibody (Jackson ImmunoResearch) and acquired on FACS Canto. For intracellular Poly ADP-Ribose Polymer (PAR) staining we followed previously published method with some modifications [22]. Briefly, cells were fixed and permeabilized first with 70% ethanol and then with 0.1% Triton X 100. After blocking with 5% goat serum, cells were incubated with anti-PAR monoclonal antibody (Trevigen) or isotype IgG3 antibody (Biolegend) overnight at 4°C. Cells were washed, stained with anti-mouse FITC conjugated secondary antibodies and acquired on BD FACS Canto. The data were analyzed using FlowJo software.

2.6. Chromatin Immunoprecipitation (ChIP)

Following stimulation, chromatin was crosslinked by treating cell with 1% paraformaldehyde, and crosslinking was quenched with 0.125% glycine. Cells were then washed with PBS and lysed with 1% ChIP lysis buffer (1% SDS, 10mM EDTA, 50mM Tris) for 15 min on ice. The chromatin was sheared by sonication. Cleared lysates were diluted with ChIP dilution buffer (0.01% SDS, 1.2mM EDTA, 16.7mM Tris-HCl pH 8, 1.1% Triton X 100, 167mM NaCl) and incubated with either no antibody or rabbit anti-p65 (Millipore) or rabbit anti-Polymerase II (Millipore) antibodies overnight at 4°C. The lysates were then incubated with Protein A/DNA slurry (Millipore) for 2h. The beads were washed and immune-complexes were eluted using elution buffer (1% SDS, 0.1 M NaHCO₃). Crosslinks were reversed by incubating overnight at 65°C in presence of 200mM NaCl. The eluates were treated with Proteinase K (Sigma) for 1h. DNA was extracted using Qiagen PCR clean up kit and stored at -20°C. A SYBR green-based assay was used for quantitative PCR (qPCR). Primers from a region of mouse VCAM-1 promoter upstream of NFκB binding site were used for qPCR. Primer sequences used were: Forward: 5'-AGTGTCTGTGTTTCCCAGGAC-3', Reverse: 5'-GCCAGGGAAAAAGTTTAACTGA-3'. The Ct values were normalized to input and the fold changes were calculated by Ct method.

For immunoprecipitations, nuclear proteins were enriched by first lysing cells in hypotonic lysis buffer (10mM Hepes, pH 7.9, 1mM EDTA, 10mM KCl, 0.5%NP40, protease inhibitor cocktail). The pellets were then lysed in high salt buffer (20mM Hepes, pH 7.9, 0.5M NaCl, 1mM EDTA, 1mM EGTA, 1mM DTT, 0.5%NP40) by rigorous vortexing and rotating for 20min at 4°C. The lysates were cleared and diluted with immunoprecipitation wash buffer (10mM Tris, 100mM NaCl, 0.5% NP40), and incubated overnight with Protein A/G agarose beads covalently linked to p65 antibodies. The covalent linking of antibodies to agarose

beads was performed with dimethyl pimelimidate. The beads were washed with wash buffer and protein complexes eluted by boiling in presence of 2X Laemmli buffer. The proteins were separated on a 10% SDS PAGE and transferred to nitrocellulose membrane. Anti-PARP-1 monoclonal and rabbit anti-p65 (Cell Signaling technology) antibodies were used to detect proteins on membranes. To determine nuclear translocation of p65, equal amounts of protein from nuclear and cytoplasmic extracts were separated on 10% SDS PAGE and transferred to nitrocellulose membrane. Anti-LaminA/C (Cell Signaling technology) and anti-GAPDH (ThermoFisher-pierce) were used as loading controls for nuclear and cytoplasmic extracts respectively.

2.7. Real time PCR

Total RNA was isolated using Qiagen RNeasy kit. RNA (400ng) was reverse transcribed using Applied Biosystems high capacity reverse transcription kit. VCAM-1, normalized to GAPDH, was detected by real-time PCR using QuantiTect primer assays (Qiagen) and Applied Biosystems Step2 plus thermal cycler. Relative gene expression was calculated by Ct method.

2.8. Statistical analysis

ANOVA and t-tests were performed using GraphPad Prism 4.0c software for Mac (GraphPad). Differences among groups were determined using the Tukey-Kramer post hoc test. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Estrogens inhibit VCAM-1 up-regulation

To determine whether estrogens protect mice from nephritis by down-regulating of adhesion molecules, we investigated the effect of E2 on VCAM-1 expression *in vivo*. Mice were treated with E2 by implanting E2 pellets s.c., as we described previously [15; 23]. Direct renal injury was induced by injecting nephrotoxic serum 5 days after E2 implantation [15; 23] and kidneys were harvested 30h later. We observed that E2 treatment inhibited VCAM-1 up-regulation in kidneys *in vivo* during nephrotoxic serum induced nephritis (NTN) in both male and female mice (Figure 1A, B). To determine whether the reduction in VCAM-1 expression in the kidneys was due to inhibition of VCAM-1 up-regulation in mesangial cells, we determined the direct effect of E2 on TNF α stimulated VCAM-1 up-regulation in mouse mesangial cells. Our data show that E2 pre-treatment of mesangial cells inhibited TNF α stimulated VCAM-1 at both protein and mRNA levels (Figure 1C, D).

NF κ B regulates VCAM-1 transcription in endothelial cells and is widely accepted as a transcription factor that regulates VCAM-1 [24; 25; 26; 27]. However, the role of NF κ B in regulating VCAM-1 expression in mesangial cells has not been previously reported. We therefore determined whether NF κ B is responsible for VCAM-1 up-regulation in mesangial cells as well. To this end we used an inhibitor of I κ B kinase activity to inhibit NF κ B activation. Figure 2 shows that inhibition of IKK inhibited VCAM-1 upregulation and therefore our data show that NF κ B regulates TNF α stimulated VCAM-1 up-regulation in mesangial cells.

3.2 Estrogens inhibit RNA polymerase II recruitment to the VCAM-1 promoter

We then determined the molecular mechanism of regulation of VCAM-1 by E2. Inhibition of NF κ B activation and inhibition of transcription of genes regulated by NF κ B can be inhibited at different stages: 1) I κ B kinase (IKK) activation and/or I κ B degradation, either of which would inhibit nuclear translocation of p65/p50 heterodimer, 2) p65 binding to the promoter region, or 3) recruitment of co-activators at the promoter site. We first determined whether E2 inhibits IKK activation or I κ B degradation by measuring nuclear p65 upon TNF α stimulation. Figure 3 shows that there is peak of nuclear p65 within 30 minutes following TNF α stimulation. However, the cells that were pretreated with E2 did not show any impairment in p65 nuclear translocation. These data suggest that estrogens do not regulate NF κ B activation by inhibiting either IKK activation or I κ B degradation. To further determine whether estrogens inhibit p65 DNA binding, we performed ChIP assays. Figure 4A shows that, as expected, there was an increase in p65 binding to VCAM-1 promoter upon TNF α stimulation. E2 pretreatment, however, did not inhibit p65 binding to the VCAM-1 promoter. Surprisingly we saw that E2 inhibited RNA polymerase II recruitment to the VCAM-1 promoter (Figure 4B), suggesting that E2 may inhibit the recruitment of co-activators and the formation of pre-initiation complex (PIC) at the VCAM-1 promoter.

3.3. Estrogens inhibit the interaction between p65 and PARP-1

We showed previously that absence of Poly (ADP-Ribose) Polymerase-1 (PARP-1) inhibited TNF α stimulated VCAM-1 up-regulation in mouse mesangial cells [15]. PARP-1 has been shown to interact with estrogen receptor [28] and has been also proposed as a co-factor for NF κ B activation [29]. We therefore determined whether E2 regulates VCAM-1 up-regulation through PARP-1. Figure 4C shows that PARP-1 interacts with p65 upon TNF α stimulation and this interaction is inhibited in the presence of E2. These data suggest that inhibition of PARP-1 recruitment to VCAM-1 promoter by E2 may be responsible for inhibition of recruitment of RNA Polymerase II to the promoter and therefore, inhibition of VCAM-1 transcription.

3.4. p65/PARP-1 interaction is not mediated through PARP-1 activity

PARP-1 is known to interact with transcription factors through direct interaction or through Poly (ADP-Ribose) Polymers (PARs) generated as a result of PARP-1 activation. We showed previously that E2 inhibits PARP-1 activity in bone marrow derived macrophages [23]. We therefore determined whether PARP-1 activity regulates VCAM-1 up-regulation stimulated by TNF α . Figure 5A–D show that pretreatment with PARP-1 inhibitors did not inhibit VCAM-1 up-regulation. We then determined whether TNF α stimulation activates PARP-1 in mesangial cells. PARP-1 activity was measured as PAR accumulation by flow cytometry. Figure 5E shows that although E2 inhibited basal PARP-1 activity, TNF α did not induce PARP activity, further supporting our data that PARP-1 activity is not essential for TNF α stimulated VCAM-1 upregulation in mesangial cells.

Another mechanism by which E2 can inhibit PARP-1/p65 interaction is by lowering the levels of PARP-1 protein through transcriptional regulation, thereby making PARP-1 unavailable to bind to p65. We therefore determined intracellular PARP-1 levels after stimulation with TNF α in cells pretreated with E2. Figure 6A and 6B show that although E2

inhibited VCAM-1 upregulation, E2 or TNF α treatment did not affect PARP-1 levels in mesangial cells.

4. Discussion

There is increasing evidence that estrogens regulate the functional responses of immune cells and thereby influence the immune response. We therefore sought to determine whether estrogens may also regulate the immune response in the kidney during renal inflammation. In addition to the established role of estrogens in reducing renal sclerosis, our data also support the notion that estrogens regulate the inflammatory response in the kidney, and therefore they exert a protective effect on renal injury through amelioration of inflammation.

Our data show that estrogens inhibit up-regulation of the adhesion molecule VCAM-1 in nephritic kidneys. Using mesangial cells we further show that estrogens inhibit VCAM-1 up-regulation by inhibiting the formation of pre-initiation complex at the VCAM-1 promoter, thereby inhibiting the recruitment of RNA polymerase II and transcription at the VCAM-1 promoter. Our data suggest that the inhibition of RNA Polymerase II recruitment may be due the inhibition of PARP-1/p65 interaction by estrogens (Figure 7). We propose that estrogens play an important role in regulating renal inflammation locally.

Estrogens were shown to inhibit Matrix Metalloproteinases 2, MMP2, which possibly inhibits accumulation of extracellular matrix and reduce sclerosis [13]. Estrogens also inhibit TGF β -dependent collagen IV synthesis in mesangial cells by limiting the availability of the transcription factor Sp1. This inhibition by estrogens is mediated through down-regulation of Casein Kinase 2 (CK2) [12]. However, the role of estrogens in regulating immune response in renal inflammation by influencing the activation of intrinsic renal cells was not studied previously. Mesangial cells that constitute the mesangium are embedded in extracellular matrix and have a unique location in the glomerulus where they can communicate with both the vasculature and the interstitium. Due to their presence in this unique environment, apart from the contractile function of mesangial cells, these cells can play a major role in facilitating the infiltration of cells from circulation. Indeed, mesangial cells have been proposed to regulate neutrophil and monocyte infiltration in the kidney by up-regulating adhesion molecules [30; 31]. The role of estrogens in regulating renal inflammatory cell infiltration has not been studied previously. We show that estrogens inhibit VCAM-1 upregulation in the kidney, and therefore may ameliorate renal inflammation. These data are in line with our previous published report that showed protection of mice from nephritis by estrogen treatment [15].

The role of estrogens in regulating NF κ B activation has been studied previously. Estrogens inhibit IKK and reduce I κ Ba phosphorylation in HeLa cells [32; 33]. In vitro studies show that estrogen-induced activation of ER inhibits expression of IL6 in osteoblasts and thus underscore the importance of estrogens in osteoporosis [34]. On similar lines, ER was suggested to inhibit NF κ B DNA binding in several cell types namely breast cancer cell line MCF7 [35], human umbilical vein endothelial cells [36] and vascular smooth muscle cells [37]. However, these in vitro studies did not differentiate between ability of estrogens to inhibit NF κ B nuclear translocation and to inhibit DNA binding. To overcome this problem,

we employed a different strategy. We determined the nuclear translocation of p65 and also determined the ability of NF κ B to bind VCAM-1 promoter in vivo in the presence of estrogens using chromatin immunoprecipitation. However, we found that in mesangial cells, estrogens did not inhibit p65 nuclear translocation or binding to VCAM-1 promoter. We showed previously that the absence of Poly (ADP-Ribose) Polymerase-1 (PARP-1) inhibited the up-regulation of VCAM-1 in mesangial cells. We now show that estrogens inhibit the interaction of PARP-1 with p65-subunit of NF κ B, the transcription factor that regulates VCAM-1 up-regulation in mesangial cells. These data suggest that estrogens inhibit VCAM-1 up-regulation in mesangial cells by inhibiting p65 and PARP-1 interaction (Figure 7).

PARP-1 dependent NF κ B activation was shown to be dependent on PARylation of p65-binding proteins in *Trypanosoma cruzi* infected cardiomyocytes, and inhibition of PARP-1 by pharmacological inhibitors inhibited cytokine gene expression in infected cardiomyocytes. Our results show that in mesangial cells, PARP-1 was not activated upon TNF α stimulation. However, as we published previously in bone marrow derived macrophages [23], estrogens inhibited basal PARP-1 activity. Similarly, PARP-1 inhibitors did not inhibit VCAM-1 up-regulation. These data suggest that in mesangial cells, although PARP-1 interacts with p65 upon TNF α stimulation, PARP-1 activity is not required for NF κ B activation. Our data, however, are in line with previous report that enzymatic activity and the DNA binding activity of PARP-1 are not required for NF κ B activation in mouse fibroblasts [38]. We also show that E2 does not affect the levels of PARP-1 protein in the cells therefore excluding the possibility that inhibition of PARP-1 interaction with p65 is due to unavailability of PARP-1. A possible reason for this inhibition of interaction may be due to sequestration of PARP-1 by E2/ER complex [28]. It remains to be determined how inhibition of PARP-1/p65 interaction inhibits RNA polymerase II recruitment. Other post-translation modifications of PARP-1 that may facilitate its binding to p65 that are disrupted by estrogens may be involved and understanding those modifications require further studies.

Our data show that estrogens regulate VCAM-1 up-regulation in the kidney during renal inflammation by interfering with NF κ B signaling. Our data also suggest that PARP-1 may be the co-activator that is responsible for NF κ B activation required for VCAM-1 up-regulation in mesangial cells. Based on our data and published reports we propose that despite of the known autoimmune enhancing effects of estrogens in B cell driven autoimmune diseases, estrogens are protective locally in the kidney. The protection in the kidney is through 1) reducing the inflammatory response, and 2) reducing sclerosis thereby inhibiting and possibly delaying end stage renal disease. These data might explain why autoimmune renal disease is worse in males and could lead to a sex-based anti-inflammatory therapy.

Acknowledgments

This work was supported by NIH/NIAMS R01AR061569 to RC

Abbreviations

E2	17 β Estradiol
ER	estrogen receptor
MC	Mesangial cells
NTS	Nephrotoxic serum
NTN	Nephrotoxic Serum Induced Nephritis
PARP-1	Poly (ADP-Ribose) Polymerase-1
PIC	Pre-Initiation Complex
VCAM-1	Vascular Cell Adhesion Molecule-1

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Highlights

- E2 inhibits VCAM-1 expression in kidney and in mesangial cells
- E2 inhibited RNA polymerase II, but not p65, recruitment to the VCAM-1 promoter
- E2 inhibited PARP-1 and p65 interaction
- E2 may impair VCAM-1 upregulation by inhibiting pre-initiation complex formation

Figure 1. High levels of 17 β estradiol inhibit VCAM-1 up-regulation in the kidneys following nephritic insult

Female (A) or Male (B) 129sv/J mice were treated with estrogens by implanting E2 pellets s.c. NTS (6 ml/kg) was injected 5 days following pellet implantation, and the kidneys were collected after 30 h. Frozen sections were stained for VCAM-1 (green) and DAPI (blue), and visualized by confocal microscopy. Fluorescence intensities of green channel were measured using ImageJ software (lower panels). Fluorescence intensities of the secondary controls were subtracted from the stained sections. An average of 7–10 fields was calculated for each mouse. Data are mean $\pm$ S.E.M., representative of eight mice. The data show that E2 treatment inhibits VCAM-1 up-regulation. *** $p < 0.001$. C and D. 17 β Estradiol inhibits

VCAM-1 up-regulation in mesangial cells. MCs were cultured in phenol red free DMEM supplemented with charcoal/dextran treated fetal bovine serum for 72h. Cells were then treated with increasing concentrations of E2 for 48h. C) Cells were stimulated with TNF α (1ng/ml) for 16h and protein levels of VCAM-1 were determined by flow cytometry. $*p<0.05$, $n=6$. D) Cells were stimulated with TNF α for 3h and mRNA levels of VCAM-1 were determined by qPCR. $*p<0.05$, $n=4$. E. Cells were treated with different concentrations of E2 and then stimulated with TNF α . Cells were then stained with FITC conjugated Annexin V and 7AAD, and acquired on flow cytometer. Cells negative for both Annexin V and 7AAD were termed as live cells. The figure shows that the percent of live cells in the different experimental conditions were similar. The data are from 4 independent experiments.

Figure 2. NF κ B regulates TNF α stimulated VCAM-1 up-regulation

To determine the role of NF κ B in VCAM-1 up-regulation in MCs, cells were treated with IKK inhibitor, IKK16 (1 μ M) for 1h prior to stimulation with TNF α (1ng/ml) for 1 or 3h. Cells were harvested and mRNA levels of VCAM-1 were determined by qPCR. * $p < 0.05$; ** $p < 0.01$.

Figure 3. 17 β Estradiol does not inhibit TNF α stimulated NF κ B nuclear translocation

To determine whether E2 regulates IKK activation or I κ B degradation and therefore, NF κ B activation, we determined p65 nuclear translocation. MCs were cultured in E2 depleted medium were treated with E2 for 48h in chambered coverglass and stimulated with TNF α for various time points. A) Cells were fixed, permeabilized, and stained for p65 (red) and DAPI (blue). B) Number of cells with nuclear p65 were counted using ImageJ. C) Cells were stimulated with TNF α for 30 and 60min. Cytoplasmic and nuclear extracts were subjected to SDS PAGE. Lamin A/C and GAPDH were used as loading controls. The data show that E2 does not inhibit p65 nuclear translocation. Experiments were repeated twice.

Figure 4. NFκB p65 is recruited to VCAM-1 promoter following TNFα stimulation

To determine whether E2 inhibits binding of p65 to VCAM-1 promoter, ChIP assays were performed. Sheared chromatin was immunoprecipitated with either (A) anti-p65 or (B) anti-RNA polymerase II. The amount of immunoprecipitated VCAM-1 promoter was determined by qPCR as fold difference over basal unstimulated condition after normalizing to input. Data show that although E2 did not inhibit p65 binding, E2 treatment inhibited recruitment of RNA polymerase II to the VCAM-1 promoter. One representative of three independent experiments is shown. C. MCs were treated as in Figure 1. Following 30 min stimulation, cells were subjected to immunoprecipitation as described in methods. The data show that PARP-1 associates with p65 in MCs upon TNFα stimulation and this interaction is inhibited by E2. One representative of 5 independent experiments is shown.

Figure 5. PARP-1 activity is not required for VCAM-1 up-regulation

MCs pre-treated with PARP-1 inhibitors 3AB (A, 1mM and C, 0.01–1mM) or CEP8983 (B, 10μM, D, 1–10μM) for 30min. Cells were stimulated with TNFα for 1h or 3h (A and B) or 16h (C and D). VCAM-1 up-regulation was determined by qPCR (A and B) or flow cytometry (C and D). Intracellular Poly ADP-Ribose polymers (PARs) generated in MCs upon TNFα stimulation were determined as a measure of PARP-1 activity by flow cytometry (E). The data show that PARP-1 activity is dispensable for VCAM-1 up-regulation in MCs. Experiments were repeated with 2–3 times.

Figure 6. PARP-1 expression is not altered by TNF α stimulation

To determine whether E2 reduces PARP-1 levels in MCs, estrogen treated cells were stimulated with 16h with TNF α . Surface VCAM-1 and intracellular PARP-1 was quantified by flow cytometry. Data show that although E2 inhibited VCAM-1 upregulation (A), E2 did not affect the PARP-1 protein levels in MCs (B). * $p=0.0129$

Figure 7. Molecular Basis of VCAM-1 Regulation by Estradiol and PARP-1

TNF α and TNF receptor ligation leads to activation of I κ B kinase complex (IKK). IKK phosphorylates the inhibitor proteins I κ B and targets for proteasomal degradation. The NF κ B p65 subunit then translocates to the nucleus and binds to VCAM-1 promoter. PARP-1 and RNA polymerase II are recruited to VCAM-1 promoter and initiated transcription. In presence of E2, PARP-1 is unable to bind to p65 and RNA polymerase II is not recruited to the promoter, thereby inhibited transcription from VCAM-1 promoter. We propose that E2 inhibits VCAM-1 up-regulation in MCs stimulated by inflammatory cytokines by inhibiting the interaction between PARP-1 and p65, which then inhibits the formation of pre-initiation complex at the VCAM-1 promoter, consequently inhibiting transcription of VCAM-1.