Influence of Race and High Laminar Shear Stress on TNFR1 Signaling in Endothelial Cells

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Research Article

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Abstract

Background: In the endothelium, tumor necrosis factor (TNF) binding to TNF receptor-I (TNFR-I) facilitates monocyte recruitment and consequently the development of atherosclerosis. The prevalence of inflammation, endothelial dysfunction, and subclinical atherosclerosis is higher in the African American (AA) population. This is supported by in vitro evidence demonstrating heightened inflammatory response and atherogenic potential in endothelial cells (ECs) from AA donors. Evidence suggests that high unidirectional laminar shear stress (HSS), as an exercise mimetic, can mitigate some aspects of racial differences in endothelial function at the cellular level. Therefore, we hypothesized that TNF-induced monocyte adhesion as well as TNFR-I signaling complex expression/activity would be exacerbated in AA derived ECs. Further, we hypothesized that HSS would attenuate potential racial differences.

Methods: THP-1 monocytes and human umbilical vein ECs (HUVECs) from Caucasian American (CA) and AA donors were used in a co-culture system to examine racial differences in monocyte adhesion. An in vitro exercise mimetic model was applied to investigate the potential modulatory effect of HSS.

Results: THP-1 adherence appeared elevated in untreated and TNF-treated AA ECs compared to CA ECs, but not statistically significant. Additionally, we report no significant racial differences in the expression of TNFR-I signaling complex or TNF-induced activation of NF-κB. Application of HSS significantly increased the expression and shedding of TNFR-I in both racial groups.

Conclusion: Our data do not support TNF-induced NF-κB activation as a potential mediator of racial disparity or HSS atheroprotective effect. Future research should investigate the role of other pathways activated by TNFR-I signaling complex.

Introduction

Tumor necrosis factor (TNF) is a potent, pro-inflammatory cytokine produced predominately by activated leukocytes. One of the main targets of TNF is the endothelium where it initiates a cascade of events leading to endothelial dysfunction [1–3]. The diverse effects of TNF are mediated primarily by the cell surface receptor TNF Receptor1 (TNFR1) [4]. Once activated by TNF binding, TNFR1 forms a signaling complex with adaptor proteins and TNFR associated factors (TRAFs); and activate multiple signaling pathways like nuclear factor kappa B (NF-κB) and mitogen-activated protein (MAP) kinase [5–8]. This induces the expression of adhesion molecules and chemokines that cause monocytes recruitment and activation, consequently amplifying inflammation and initiating atherogenesis [9–11]. Atherosclerotic arteries exhibit greater levels of all TRAFs compared to control arteries, as TRAFs have a role in plaque progression [12]. Additionally, TNF has a detrimental effect on endothelial nitric oxide synthase (eNOS) promoter activity compromising nitric oxide (NO) bioavailability, a hallmark of endothelial dysfunction [13].

Efficacy of exercise training in improving endothelial function, by increasing NO bioavailability and reducing inflammation, is well established [14–16]. Further, soluble TNFR1 (sTNFR1) levels improve with
aerobic training [17, 18]. TNFα converting enzyme (TACE) mediates the shedding of TNFR1 extracellular domain forming sTNFR1 in response to inflammatory stimuli, thereby decreasing the expression of active membrane-receptors [1, 4, 19]. sTNFR1 binds to circulating TNF and neutralizes its activity [19]. Clinically, administering sTNFR1 is an effective treatment strategy to control inflammatory conditions [4].

High laminar shear stress (HSS) mediates functional and structural vascular adaptations associated with exercise training [20]. In vitro, application of HSS is a validated exercise mimetic model. Evidence suggests that HSS mitigates inflammation and induces atheroprotective effects [21]. Specifically, HSS has been shown to inhibit some of the TNF-induced downstream effects [21–23]. Moreover, HSS induces a greater reduction in oxidative stress and inflammatory markers in African American (AA) endothelial cells (ECs) compared to Caucasian American (CA) ECs [24].

AA have the highest prevalence of cardiovascular disease (CVD) [25]. This may be partially attributed to endothelial dysfunction commonly seen in this population. Even young and healthy AA have lower flow-mediated dilation (FMD) levels, a measure of endothelial function, than CA [26, 27]. Additionally, our previous in vitro data showed that AA ECs exhibit greater oxidative stress and heightened inflammation compared to CA ECs [24]. AA human umbilical vein ECs (HUVECs) express higher levels of NADPH oxidase subunits and lower superoxide dismutase 1 (SOD1) activity than CA HUVECs [28]. Moreover, we showed that AA HUVECs produce higher basal levels of interleukin (IL)-6, an inflammatory cytokine, than CA HUVECs in response to TNF stimulation [29].

Racial differences in EC responses to stimuli, particularly TNF, could play an important role in the promotion of endothelial dysfunction, plaque development, and consequent CVD. Despite the compelling data supporting higher prevalence of endothelial dysfunction and CVD in AA, it is predominantly of observational nature [26, 27, 30–32]. Evidence explaining the mechanism(s) prompting this disparity is still lacking. Therefore, research exploring the underlying cellular mechanism(s) that can be targeted for treatment/prevention is needed and of high importance.

In this in vitro study, we hypothesized that AA derived ECs would exhibit greater THP-1 monocyte adhesion and higher expression/activity of the TNFR1 signaling complex that can be attenuated by HSS. Using naïve ECs such as HUVECs, a valid model to mechanistically investigate EC function, minimizes the influence of pre-existing factors and enables race/ethnicity-related EC profiling. The aims of our study were to examine the following: 1) possible racial differences in TNF-induced monocyte adhesion, 2) possible racial differences in TNFR1 signaling complex expression/activity, negative regulators, and downstream effects, and 3) the effects of HSS on mitigating possible racial differences.

**Methods**

**Cell culture**

**HUVECs**
HUVECs were purchased from Lonza (Morristown, NJ) and cultured in the endothelial basal medium-2 medium containing 2% fetal bovine serum and growth supplements and used between passages 6 and 7. Cells from three AA and three CA donors were grown in parallel and the experiment was repeated three times. This experimental design has been validated in our previous work [28, 29, 33]. Cells were maintained at 37°C in a 5% CO₂ atmosphere, and cultured in gelatin coated 100 mm tissue culture dishes. When cells reached 80–90% confluence, they were washed with Hank's Balanced Salt Solution (HBSS) buffer and serum starved for 2 hrs before stimulation or application of shear stress. A concentration of 30 ng/mL of TNF (cat. No. 210-TA-020/CF) from R&D Systems (Minneapolis, MN) was used and incubated for 6 hrs. This dose was chosen based on preliminary experiments.

**THP-1**

THP-1 monocytes (ATCC® TIB202™, Manassas, VA) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco, Waltham, MA), 1% penicillin/streptomycin (VWR, Radnor, PA), and 2mM/L GlutaMAX (Gibco). Cells were cultured in T-75 flasks and maintained at 37°C in a 5% CO₂ atmosphere.

**Cell adhesion assay**

HUVECs were seeded in gelatin coated 24-well plates at a density of 5x10⁴ cells per well. Once confluent, HUVECs were washed with HBSS buffer, serum starved for 2 hrs, and then activated with TNF (30 ng/mL, 6 hrs) or left untreated. THP-1 monocytes were labeled with 5 µM of Calcein-AM (cat. no. 354216) from Corning (Corning, NY) and suspended in serum-free RPMI 1640 medium. After activation of HUVECs, labeled THP-1 cells were added to the HUVECs monolayer (5x10⁵ per well) and incubated at 37°C with 5% CO₂ for 1 hr. Labeled THP-1 cells were added to empty wells as a background control. Nonadherent THP-1 monocytes were removed and washed with 1X phosphate buffer saline (PBS) three times. Adherent cells were then lysed with 200 µl of RIPA lysis buffer. To quantify cell adhesion, fluorescence intensity was measured with SpectraMax M2 plate reader (Molecular Devices, San Jose, CA) at excitation wavelength set at 494 nm and emission wavelength at 517 nm. Cell adhesion was determined as a percentage of the control (untreated HUVECs).

**Laminar shear stress**

After 2 hrs of serum deprivation, confluent monolayers were exposed to unidirectional shear stress for 24 hrs with a rotating cone-in-plate instrument at a 0.5° angle, designed for 100 mm tissue culture dishes, as we have used previously [33]. Cone-and-plate experimental models have the advantage of inducing flow with a moving upper conical boundary and thus, do not generate any pressure gradients that could alter cell function [34]. Two conditions were applied: HSS (20 dyne/cm²), and HSS (20 dyne/cm²) followed by TNF (30 ng/mL, 6 hrs) incubation.

**Western blotting**
Cell lysate was fractionated using the Nuclear Extraction Kit (cat. No. ab113474, Abcam, Cambridge, MA) following manufacturer's instructions with some modifications. Cytosolic extract was used for western blotting. For each experimental condition, 10–15 µg protein was loaded into wells with the amount kept constant within each gel. All samples were run on duplicate gels. Proteins were separated using SDS-PAGE (4–15% TGX gels, Bio-Rad, Hercules, CA) according to molecular weight, and transferred to PVDF membranes which were blocked with 5% non-fat dry milk (NFDM) for 1 h at room temperature. This was followed by incubation with primary antibodies of interest with gentle agitation overnight at 4–8°C. Primary antibodies used were: TNFR1 (cat. no. 3736), TACE (cat. no. 6978), TRAF2 (cat. no. 4724), TRAF3 (cat. no. 4729), and TRAF5 (cat. no. 41658) from Cell Signaling Technology (Beverly, MA). β-actin (cat. no. 3700) from Cell Signaling Technology was used as loading control. Protein was visualized by chemiluminescent detection using an HRP illumination substrate Luminata Forte (EMD Millipore, Billerica, MA). The UVP ChemiDoc-It2 imaging system (UVP, Upland, CA) was used to image each PVDF membrane and Vision Woks software was used to quantify protein expression by band densitometry analysis. Densities of selected proteins were expressed relative to a loading control for normalization.

Assays

Cell culture supernatant was collected, centrifuged, aliquoted, and immediately stored at −80°C until analysis. sTNFR1 assay kit (cat. no. DRT100) from R&D Systems (Minneapolis, MN) was used to detect the sTNFR1 production in HUVECs cell culture supernatant. NF-κB factor assay kit (cat. no. 43296) from Active Motif (Carlsbad, CA) was used to quantify NF-κB DNA binding activity in nuclear extracts.

Statistical analysis

A two-way ANOVA was performed to examine potential race by condition interactions. Violations of assumptions were examined. Non-normally distributed data were natural log-transformed. Post hoc adjustment for multiple comparisons was done using the Bonferroni’s test. Analysis was performed using SPSS version 26 (SPSS Inc., Chicago, IL). Data are expressed as mean ± SE and the level of significance was set at \( p \leq 0.05 \).

Results

THP-1 monocytes adhesion

TNF treatment increased THP-1 monocyte adhesion in both racial groups \( (p < 0.001, \text{Fig. 1}) \). THP-1 monocyte adhesion to untreated HUVECs appeared higher in AA HUVECs compared to CA HUVECs, but no racial \( (p = 0.77, \text{Fig. 1}) \) or interaction effects \( (p = 0.12, \text{Fig. 1}) \) were detected.

TNFR1 signaling complex and HSS

TNFR1 and TRAF3 were significantly upregulated in response to HSS compared to all other conditions \( (p < 0.001, \text{Fig. 2} \) and \( p = 0.02, \text{Fig. 3 (b)}) \), whereas TRAF5 was downregulated in response to HSS, an effect that was not altered by TNF treatment \( (p = 0.003, \text{Fig. 3 (c)}) \). TRAF2 levels were not significantly affected.
by any of the experimental conditions ($p = 0.06$, Fig. 3 (a)). No racial or interaction effects in the expression of TNFR1 or TRAFs were detected.

**TNFR1 shedding and HSS**

HSS induced an upregulation in TACE expression in both racial groups. TACE expression remained significantly elevated after the follow-up TNF treatment, but it was numerically lower compared to the HSS group ($p < 0.001$, Fig. 4). Further, HSS significantly increased sTNFR1 levels in CA and AA HUVECs, an increase that was diminished by the follow-up TNF treatment ($p < 0.001$, Fig. 5). Shedding of TNFR1 was lower in AA HUVECs compared to CA HUVECs; however, this difference was not statistically significant ($p = 0.07$, Fig. 5). No interaction effect in the expression of TACE or TNFR1 shedding was detected.

**NF-κB binding activity**

TNF treatment significantly increased NF-κB DNA binding activity in both racial groups ($p < 0.001$, Fig. 6). NF-κB DNA binding activity was numerically higher in AA HUVECs in response to TNF treatment, nonetheless, it was not significant ($p = 0.67$, Fig. 6). Additionally, HSS had no protective effect against TNF-induced NF-κB activation ($p < 0.001$, Fig. 6); and there was no interaction effect in NF-κB activation across all conditions ($p = 0.65$, Fig. 6).

**Discussion**

The current data suggest no racial differences exist in: 1) TNF-induced monocyte adhesion, 2) TNF-induced activation of TNFR1 signaling complex, or 3) the modulatory effects of HSS on TNFR1 signaling. Additionally, pre-shearing was not sufficient to mitigate TNF detrimental effects as evident by NF-κB binding activity and diminished shedding of TNFR1. Although our results do not support our proposed hypothesis of higher TNFR1 signaling complex expression/activity and downstream effects in AA derived ECs, HSS-induced atheroprotective effect mitigated TNFR1 signaling in both racial groups.

TNFR1 activation by TNF binding induces endothelial dysfunction manifested by suppressed eNOS activity, higher oxidative stress levels and amplified expression of adhesion molecules. Adhesion molecules (such as VCAM-1, ICAM-1, MCP-1 and E-selectin) play a key role in recruiting monocytes and initiating atherogenesis [2, 10]. Plasma levels of TNF and adhesion molecules are associated with subclinical atherosclerosis [35]. Additionally, data from the Atherosclerosis Risk in Communities (ARIC) study highlighted adhesion molecules as independent molecular markers of coronary heart disease [36]. Previous research from our lab suggests a heightened inflammatory response to TNF in ECs from AA donors [29]. Further, basal and induced ICAM-1 and VCAM-1 levels are higher in AA HUVECs compared to CA HUVECs [37]. Therefore, we hypothesized TNF-induced monocytes adhesion to be higher in AA HUVECs; however, our results failed to support this hypothesis.
Research implementing co-culture systems to investigate molecular mechanisms prompting racial differences in CVD is scarce. In our experiments, we used THP-1 cells, a validated monocyte cell model to investigate atherogenesis and other vascular inflammatory conditions [38]. Nonetheless, we acknowledge the differences between THP-1 cells and primary monocytes and the limitations of THP-1 cells as a monocytes cell line model. Interestingly, recent evidence has shown that peripheral blood mononuclear cells (PBMCs) from young-normotensive AA exhibit higher resting oxidative stress levels [39]. Thus, examining the interactions between AA derived PBMCs and AA derived ECs would also be relevant.

Contrary to our hypotheses, our results do not support differential expression/activity of TNFR1 signaling between races. Both CA and AA HUVECs responded similarly to all experimental conditions. NF-κB activation was numerically higher in AA HUVECs in response to TNF; however, it was not statistically significant. This may be explained by two factors. First, we did not measure the expression or activity of MAP kinases nor other transcription factors such as AP-1 that may play a bigger role in prompting racial differences than NF-κB signaling. Second, in addition to mediators of inflammation, NF-κB induces transcription of TNFR1 signaling negative regulators that may be differentially expressed such as IκBα and A20. It has been shown that, despite the well-established suppressive effect of A20 on TNF-induced genes regulated by NF-κB, it does not interfere with the nuclear translocation of the NF-κB heterodimers or DNA binding [40]. If such is the case, we speculate lower expression/activity of negative regulators in AA ECs. Furthermore, HSS-induced shedding of TNFR1 tended to be lower in AA HUVECs; however, it was not statistically significant. Most available assays, including the one we have used, are not sensitive enough to distinguish between free form of sTNFR1 and that bound to TNF. Additionally, given that we did not measure TNF levels, we cannot speculate if sTNFR1 levels in AA HUVECs that is comparable to CA are sufficient to neutralize the potentially higher TNF levels in AA HUVECs.

Several clinical reports have demonstrated higher shedding of TNFR1 with aerobic exercise and its association with better vasodilation and functional outcomes [17, 18, 41]. In the present study, we used a cone and plate viscometer that mimics the shear stress patterns the endothelium is exposed to in vivo during aerobic exercise. Our results support clinical data in that HSS induced higher shedding of TNFR1. Unexpectedly, the application of HSS prior to TNF treatment did not lessen the unfavorable effects of TNF. Yamawki et al. [23] have suggested that shear stress atheroprotective effects are mediated by inhibiting MAP kinase signaling as well as TRAF2-TNFR1 interaction. They have shown that pre-exposing rabbit aortas to HSS significantly attenuated TNF-induced VCAM-1 expression by inhibiting MAP Kinase signaling while TNF-induced NF-κB activation remained unaffected. Collectively, these results suggest signaling pathway specificity in terms of atheroprotective effects of HSS.

In regards to TRAFs, our results are in agreement with Urbich et al. [42]. HSS induced an upregulation of TRAF3 while TRAF2 levels remained unaffected. TRAF3 is an important negative regulator of NIK [43–45]. Consistent with the anti-inflammatory effects of HSS, TRAF3 upregulation, brought about by HSS, is associated with inhibition of CD40-induced endothelial cells activation [42]. On the other hand, while Urbich et al. [42] showed no effect of HSS on TRAF5 levels in HUVECs, our results demonstrated a significant decrease in the expression of TRAF5 following the application of HSS. This finding could be
due to differences in the doses of shear stress. Whereas they exposed HUVECs to 15 dyne/cm$^2$ of shear for 18 hrs, we used 20 dyne/cm$^2$ for 24 hrs. *In vivo* data suggest a negative regulatory role for TRAF5, as knocking out TRAF5 accelerated atherosclerosis in animal models. Additionally, higher systemic levels of TRAF5 are associated with recovery in patients with coronary heart disease [46]. Therefore, as one of the atheroprotective effects of laminar shear, we expected TRAF5 levels to increase with HSS. Yet, TRAF5 levels decreased. Hence, it is plausible that the role of TRAF5 as a pro- or anti-inflammatory mediator is cell specific.

**Limitations**

Different concentrations of serum were used in HUVECs culture media for different conditions. Serum deprivation is recommended to prime cells when examining proinflammatory cytokines signaling. Therefore, we used serum free culture medium for the static conditions; however, we had to use full serum culture medium for the shear stress conditions. This is mainly because our cone and plate shear stress model is optimized for a limited range of medium viscosity. Nonetheless, we used only 2% fetal bovine serum which is considered on the lower range [47]. Additionally, we used HUVECs as our endothelial cell model which is a validated cell model in the field of vascular physiology. It has been shown that TNF-induced inflammatory cell adhesion in HUVECs is comparable to human umbilical arterial endothelial cells (HUAEC) and primary human coronary artery endothelial cells (HCAEC) [48]. HUVECs are often used by our group and others to investigate molecular mechanisms of vascular disease and, in particular, racial disparity in endothelial (dys)function [24, 28, 33]. Further, utilizing HUVECs, naïve cells, can facilitate the identification of endothelial phenotypes and activities that may predispose some racial groups such as AA to adverse vascular outcomes. However, we acknowledge its drawbacks; hence our results should be interpreted with caution. We also agree with the remarks of Cook et al. and Robinson et al. [37, 49] that shed light on the disadvantages of using commercially available HUVECs and how vendors offer limited access to information about the health and lifestyle of the mother that can lead to epigenetic and phenotypic changes of ECs.

**Conclusion**

Our results do not support racial differences in the expression of TNFR1 complex or its signaling activity, particularly NF-κB activation. Additionally, while HSS can mitigate some aspects of TNFR1 signaling similarly in ECs from both racial groups, this modulatory effect was not long-lasting. Given the higher burden of subclinical atherosclerosis in AA and the central role that TNF plays in atherogenesis, we still maintain that the TNFR1 signaling complex takes part in prompting racial differences in this context. Therefore, we propose other aspects of TNFR1 signaling such as MAP kinase signaling and their associated down-stream effects as recommended targets for future research.

**Abbreviations**

TNF Tumor necrosis factor
TNFR I TNF receptor I
TRAF TNFR associated factor
NF-κB Nuclear factor of kappa B
MAP Kinase Mitogen-activated protein kinase
eNOS Endothelial nitric oxide synthase
NO Nitric oxide
sTNFR Soluble TNF receptor
HSS High laminar shear stress
AA African Americans
ECs Endothelial cells
CA Caucasian Americans
CVD Cardiovascular diseases
FMD Flow-mediated dilation
HUVECs Human umbilical vein endothelial cells
SOD1 Superoxide dismutase 1
IL-6 Interleukin-6
TACE TNFα converting enzyme
NIK NF-κB inducing kinase
AP-1 Activator protein 1
IL-1 Interleukin-1
VCAM-1 Vascular cell adhesion molecule 1
ICAM-1 Intracellular adhesion molecule 1
MCP-1 Monocyte chemoattractant protein 1
PBMCs Peripheral blood mononuclear cells
Declarations

Data availability

All data is provided in the manuscript and further inquiries can be directed to the corresponding author.

Disclosures

The findings from this project are not being considered for publication by another journal at this time. All authors have given consent for publication of this work.

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Ethical Guidelines

Ethical concerns pertaining to human cell culture work are of high importance and not overlooked. According to Auburn University Protection of Human Subjects in Research Compliance Program, this work does not meet the definition of human subject research as defined by 45 CFR 46.102. Further, Lonza Walkersville, Inc. accepts tissue only if consent for research has been obtained. Audits are frequently conducted to ensure appropriate operational procedures; and compliance with the Protection of Human Subjects regulations of consent processes and receipt of any de-identified demographic or medical history information from donors.

Conflicts of interest

The authors declare that there is no conflict of interest.

Authors’ contributions

Concept and design: Maitha Aldokhayyil, Andreas N. Kavazis, Michael D. Roberts, Thangiah Geetha and Michael D. Brown. Data collection: Maitha Aldokhayyil and Dulce H. Gomez. Data analysis and interpretation: Maitha Aldokhayyil, Dulce H. Gomez, and Michael D. Brown. Manuscript writing: Maitha Aldokhayyil and Marc Cook. All authors have reviewed and approved the final manuscript.

References


Figures

Figure 1

THP-1 monocyte adhesion on HUVECs.
(a) Adhesion of labeled THP-1 on TNF-treated HUVECs was quantified by fluorescence microplate reader. Net fluorescence is expressed relative to CA untreated HUVECs. (B) Representative images of labeled THP-1 adhesion on untreated and TNF-treated HUVECs. Data are represented as mean ± SE from 3 independent experiments.

* p< 0.001 compared to untreated.

Figure 2

HSS upregulates TNFR1 expression in CA and AA HUVECs.

HUVECs from CA and AA were incubated with TNF (30ng/mL, 6hrs), exposed to HSS (20dyne/cm², 24hrs), or HSS (20dyne/cm², 24hrs) followed by TNF (30ng/mL, 6hrs). HSS significantly increased TNFR1 expression in both groups. Densitometric quantification was normalized to housekeeping protein (β-Actin). Data are represented as mean ± SE from 3 independent experiments.

* p< 0.001 compared to other conditions.

Figure 3

TRAFs expression under different experimental conditions.

HUVECs from CA and AA were incubated with TNF (30ng/mL, 6hrs), exposed to HSS (20dyne/cm², 24hrs), or HSS (20dyne/cm², 24hrs) followed by TNF (30ng/mL, 6hrs). HSS significantly upregulated TRAF3 expression (b) and downregulated TRAF5 (c). However, TRAF2 expression did not change across all conditions. Data are represented as mean ± SE from 3 independent experiments.

* p= 0.02 compared to other conditions.
# p= 0.003 compared to control and TNF.

Figure 4

HSS upregulates TACE expression in CA and AA HUVECs.

HUVECs from CA and AA were incubated with TNF (30ng/mL, 6hrs), exposed to HSS (20dyne/cm², 24hrs), or HSS (20dyne/cm², 24hrs) followed by TNF (30ng/mL, 6hrs). Densitometric quantification was
normalized to housekeeping protein (β-Actin). Data are represented as mean ± SE from 3 independent experiments.

* p< 0.001 compared to control and TNF.

**Figure 5**

**TNFR1 shedding under different experimental conditions.**

HUVECs from CA and AA were incubated with TNF (30ng/mL, 6hrs), exposed to HSS (20dyne/cm², 24hrs), or HSS (20dyne/cm², 24hrs) followed by TNF (30ng/mL, 6hrs). Shedding of TNFR1 was quantified in cell culture supernatant. High shear stress-induced upregulation of sTNFR1 was significantly suppressed by TNF treatment. Data are represented as mean ± SE from 3 independent experiments.

* p< 0.001 compared to control.

# p< 0.001 compared to TNF.

Φ p< 0.001 compared to HSS.

$ p< 0.001 compared to HSS+TNF.

**Figure 6**

**NF-κB binding activity under different experimental conditions.**

HUVECs from CA and AA were incubated with TNF (30ng/mL, 6hrs), exposed to HSS (20dyne/cm², 24hrs), or HSS (20dyne/cm², 24hrs) followed by TNF (30ng/mL, 6hrs). NF-κB binding activity was quantified in nuclear extract. TNF-induced NF-κB activation was not altered by HSS in both groups. Data are represented as mean ± SE from 3 independent experiments.

* p< 0.001 compared to control and HSS.