Dynamics of HSP60 expression and its regulatory role in atherogenic transformation of endothelial cells

Introduction

Endothelium stands as a single interface between the blood stream and the vessel wall, acting as a semi-permeable barrier protecting the wall. In response to various physiological stimuli, endothelium regulates cellular processes essential for vascular homeostasis (Godo and Shimokawa, 2017). Hence, compromise of the endothelial function is recognized to be of primary importance in pathogenesis of vascular diseases including atherosclerosis. Endothelial dysfunction (ED) marks the earliest pathogenic event in vascular wall that triggers the atherogenic initiation through multiple effects (Sitia et al., 2010). It is basically defined as the oxidative outburst together with reduced production of nitric oxide (NO) in response to endothelial stress. Reduction in NO bioavailability leads to inhibition of soluble guanylate cyclase mediated vascular smooth muscle cells (VSMC) activation culminating in reduced vasorelaxation. This in turn causes the vascular wall to lose its distensibility and hence contributing towards vascular stiffness (Chen et al., 2018). Further, ED induces the expression of chemokines such as MCP-1 and adhesion molecules such as VCAM-1, ICAM-1 and E-selectin that help in recruitment of mononuclear cells including macrophages (Liao, 2013). ED is also accompanied by loss of membrane barrier function of endothelium that facilitates the accumulation of low density lipoprotein (LDL) molecules in the sub-endothelial space where they get oxidized in presence of ROS to form oxidized LDL (Mundi et al., 2018). Macrophages tend to engulf these OxLDL molecules and consequently transform into foam cells that builds up over a period of time to form atheromatous plaques (de Villiers and Smart, 1999).

Classical atherogenic stressors including LPS (Amberger et al., 1997; Seitz et al., 1996), pro-inflammatory cytokines (Amberger et al., 1997), hypertension (Jakic et al., 2017), fluid shear stress (Hochleitner et al., 2000), cigarette smoke (Kreutmayer et al., 2011) and Chlamydial infection (Kreutmayer et al., 2013) have been reported to HSP60 upregulation and its subsequent translocation to plasma membrane. Simultaneous induction of adhesion molecules and HSP60 expression in stressed endothelium has also been widely reported (Amberger et al., 1997) and this co-expression serves as a pre-requisite for activating the antigen recognition by HSP60-reactive T-cells in the arterial bloodstream. In this regard, rabbits injected with LPS had shown sequential upregulation of ICAM-1 and HSP60 followed by increased attachment of monocytes and T-lymphocytes on the aortic endothelium (Seitz et al., 1996). Further, secretion of HSP60 has also been reported from stressed endothelium (Kreutmayer et al., 2011). Soluble HSP60 binds to CD14 co-receptor activating TLR-4 pro-inflammatory pathways in immune cells including macrophages, accelerating the progression of atherosclerosis (Kol et al., 2000). Thus, it can be said that HSP60 bridges ED with the subsequent immunological responses that are essential for atherogenic transformation of the arterial wall.

HSP60 is essentially a mitochondrial protein residing within the mitochondrial matrix and functions in concert with HSP10 as a molecular chaperone (Soltys and Gupta, 1996). Its presence in the extra-mitochondrial locations and its secretion is an unusual stress induced event that lacks clarity (Cappello et al., 2008). However, it is interesting to note that the surface expression and secretion of HSP60 is generally preceded by its stress induced overexpression, which is not observed in a resting cell (Kreutmayer et al., 2011). Besides, these alterations in endothelial HSP60 expression and localization define a common

endpoint for most of the atherogenic risk factors independently, making HSP60 overexpression in endothelial cells (ECs), a rather key atherogenic manifestation.

Cytosolic expression of HSP60 has been documented in various settings of its upregulation, wherein novel roles of HSP60 in intracellular signaling pathways have been reported. In cancer cells, cytosolic accumulation of HSP60 has been found to activate NF-κB by binding and regulating the inhibitor of κB kinase (IKK) complex (Chun et al., 2010). Further, HSP60 mediated regulation of apoptotic pathway has also been widely reported in various cell types (Chandra et al., 2007; Shan et al., 2003; Xanthoudakis et al., 1999). Also, cytosolic HSP60 overexpression has been found to induce smooth muscle cell proliferation (Deniset et al., 2018) that emphasizes the relevance of non-canonical functions of cytosolic HSP60 in vascular cells. Since stress induced HSP60 overexpression is a key event in ED, it can be believed to be involved in regulation of intracellular signaling pathways associated with the atherogenic changes.

In this context, HSP60 upregulation and surface localization in vascular endothelium is known, but its relevance in OxLDL induced atherogenic changes lacks clarity. HSP60 upregulation in response to OxLDL has been observed in monocytic cell lines (Frostegard et al., 1996) that supported our hypothesis of plausible cause-and-effect relationship between OxLDL and HSP60. In chapter 1, we had observed hyperlipidemia induced HSP60 upregulation in endothelium during vascular dysfunction. Based on these preliminary findings, we investigated the possible regulatory role of HSP60 in atherogenic transformation of ECs using human umbilical vein endothelial cells (HUVEC) as a model and OxLDL as the classical atherogenic stressor. The status of HSP60 was also studied to

obtain further evidence in support of our observation of hyperlipidemia induced HSP10 upregulation in chapter 1.

Materials and methods

Experimental model: Human umbilical vein endothelial cells (HUVEC).

Experimental protocol

(i) Study I:

Experimental groups:

1. Control: untreated cells
2. OxLDL: cells treated with 8, 80 and 200 µg/ml OxLDL for 24 h
3. Heat Shock: cells exposed to 42°C for 30 min followed by incubation at 37°C for 6 h (positive control for heat shock proteins)

Parameters tested:

1. Cell viability
2. Quantitative RT-PCR: HSP60, GAPDH

(ii) Study II:

Experimental conditions/groups:

1. Control: untreated cells
2. OxLDL: cells treated with 80 µg/ml OxLDL in serum-free media
3. Heat Shock: cells exposed to 42°C for 30 min followed by incubation at 37°C for 6 h (positive control for heat shock proteins)
4. Vector: cells transfected with either pCMV3-C-GFPSpark or pcDNA3.1-MycHis-A empty vectors for 24 h

5. HSP60-GFP: cells transfected with pCMV3-HSPD1-GFPSpark plasmid for 24 h (HSP60 overexpression)
6. pcDNA-HSP60: cells transfected with pcDNA3.1-Hsp60-MycHis plasmid for 24 h (HSP60 overexpression)
7. KD-Control: cells transfected with p*Silencer* 2.0-U6 Negative Control containing scrambled shRNA sequence for 48 h
8. HSP60 KD: cells transfected with pshRNA-609 plasmid for 48 h (HSP60 knockdown)
9. HSP60 KD + OxLDL: HSP60 KD cells treated with 80 µg/ml OxLDL in serum-free media

Parameters tested:

1. Immunoblotting: HSP60, HSP10, β-actin
2. Immunofluorescence staining for HSP60 (intracellular, mitochondrial, cell surface)
3. ELISA: HSP60
4. Quantitative RT-PCR: HSP60, eNOS, MCP-1, VCAM-1, ICAM-1, HSP10, HSF-1, GAPDH
5. NO assay
6. Monocyte endothelial adhesion assay

The detailed experimental protocol for the present study is depicted in Fig. 2.1. Detailed methodology for each parameter is described in materials and methods section.

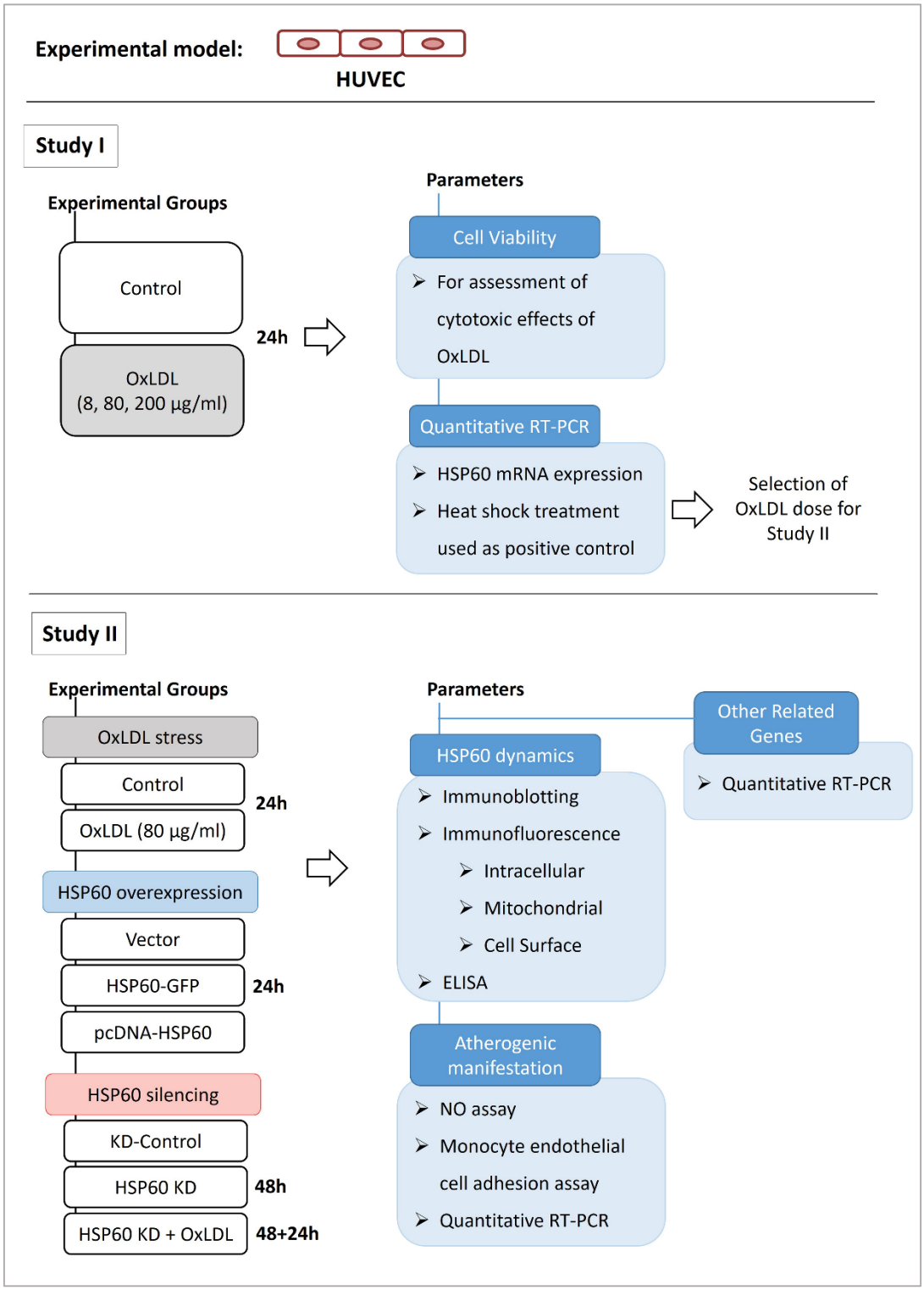

Figure 2.1: Schematic representation of experimental protocol followed for studying atherogenic changes in endothelial cells.

Results

OxLDL upregulates HSP60 in HUVEC

In a pilot experiment, HUVEC were treated with varying concentrations (8, 80, 200 µg/ml) OxLDL and the cell viability was assessed by MTT assay. OxLDL induced a significant cell death of ~35% at 200 µg/ml dose, whereas 80 µg/ml dose induced a non-significant cell death of ~26%. No significant change was recorded in cells treated with 8 µg/ml OxLDL as compared to control (Fig. 2.2). Consequently, 8 and 80 µg/ml concentrations were selected for assessment of HSP60 mRNA expression. Cells treated with 80 µg/ml OxLDL showed a significant upregulation of HSP60 mRNA expression. Also, this upregulation was significantly higher than that of the cells exposed to heat shock (positive control). HSP60 expression in cells treated with 8 µg/ml OxLDL was comparable to that of control (Fig. 2.3). All further experiments were conducted using 80 µg/ml OxLDL.

For further confirmation, HSP60 protein expression was checked in HUVEC treated with OxLDL wherein, a significant upregulation of HSP60 protein expression was observed in immunoblot (Fig. 2.4a & b). Further, OxLDL treated cells also showed prominent fluorescence of HSP60 staining that was significantly higher compared to control (Fig. 2.4c & d).

HSP60 overexpression induces its secretion in HUVEC

HSP60 overexpression was achieved by transfecting HUVEC with pCMV3.1-HSPD1-GFPSpark plasmid (HSP60-GFP) using liposome-mediated transfection protocol. Cells transfected with empty vector served as control (Vector). HSP60 mRNA and protein expression analysis confirmed the transient overexpression in HSP60-GFP cells (Fig. 2.5a) without inducing significant cell death (Fig. 2.5b). Further, HSP60 secretion was checked

by assessing its levels in conditioned media by ELISA. Levels of HSP60 were significantly elevated in OxLDL treated cells and HSP60-GFP cells as compared to their respective controls (Fig. 2.6a & b). At this stage, LDH assay was performed to rule out the possible contribution of cell lysis in HSP60 secretion. Conditioned media of both OxLDL treated cells and HSP60-GFP cells did not show any change in LDH activity compared to their respective controls confirming that the detected HSP60 in conditioned media is indeed a result of active secretion and not merely a consequence of cell lysis (data not shown).

Extra-mitochondrial localization of HSP60 in HUVEC

For this study, HSP60 overexpression was achieved by transfecting the cells with pcDNA3.1-Hsp60-MycHis (pcDNA-HSP60) and the same was confirmed by immunostaining using anti-HSP60 antibody (Fig. 2.7a). Further, the co-localization of HSP60 and mitochondria was checked by immunostaining of HSP60 in cells stained with MitoTracker Red CMXRos. Vector control cells showed mitochondria localized HSP60 as depicted by the overlapping of green and red fluorescence whereas, pcDNA-HSP60 cells showed a fraction of HSP60 expression in extra-mitochondrial sites represented by green fluorescence in addition to mitochondrial expression (Fig. 2.7b).

Surface localization of HSP60 in HUVEC

Immunocytochemical staining of plasma membrane localized-HSP60 was standardized by co-staining of HSP60 and β-actin as per the protocol described by Jakic et al. (2017), wherein β-actin served as an internal positive and negative control for intracellular and surface staining, respectively. Fixation of cells with 1% PFA ensured plasma membrane integrity that allowed surface detection of HSP60 with β-actin remaining unstained (Fig. 2.8a- upper lane) whereas, fixation with 2% PFA+MeOH led to cell membrane

permeabilization resulting in intracellular dual staining of HSP60 and β-actin (Fig. 2.8b-lower lane). Further, surface expression of HSP60 was checked in OxLDL treated HUVEC and pcDNA-HSP60 cells. Both the groups showed prominent fluorescence of HSP60 on cell membrane that was significantly elevated as compared to control (Fig. 2.8c & d).

HSP60 regulates endothelial dysfunction in HUVEC

The regulatory role of HSP60 in ED was assessed in HUVEC with experimentally altered HSP60 expression. Transfection of HUVEC with pshRNA-609 (HSP60 KD cells) led to significant decrement in HSP60 mRNA and protein expression as compared to KD-Control confirming HSP60 knockdown (Fig. 2.9a & b). Further, HSP60 KD cells recorded a significant cell death of ~21% that was increased to overall mortality of ~42% on exposure to 80 µg/ml OxLDL (Fig. 2.9c).

ED was assessed in OxLDL, HSP60-GFP, HSP60 KD and HSP60 KD + OxLDL groups by checking the NO production and eNOS mRNA expression. Griess' reagent was used to quantify the NO levels in conditioned media of cells and the results showed a significant decrement in nitrite content in OxLDL treated cells and HSP60-GFP cells. However, HSP60 knockdown prevented this decrement with both HSP60 KD and HSP60 KD + OxLDL groups showed nitrite levels comparable to that of control (Fig. 2.10a). Similarly, eNOS mRNA expression was significantly downregulated in OxLDL treated cells and HSP60-GFP cells. However, HSP60 KD and HSP60 KD + OxLDL groups showed slightly higher eNOS mRNA expression (non-significant) as compared to control (Fig. 2.10b), indicating failure of OxLDL induced lowering of eNOS in conditions of HSP60 knockdown.

HSP60 regulates endothelial activation in OxLDL treated HUVEC

Monocyte adhesion to ECs was assessed in OxLDL, HSP60-GFP, HSP60 KD and HSP60 KD + OxLDL HUVEC using THP-1 monocytes. Results showed significantly higher number of monocytes adhering to OxLDL treated HUVEC as compared to control. Also, HSP60-GFP group recorded significant increment in % monocyte adhesion compared to control as well as OxLDL group. HSP60 knockdown caused a significant reduction in the number of adhering monocytes in HSP60 KD and HSP60 KD + OxLDL groups but, the values were higher compared to control (Fig. 2.11a & b). Further, the expression of MCP-1 and adhesion molecules (VCAM-1, ICAM-1) were assessed as markers of endothelial activation. As compared to the control cells, OxLDL treated and HSP60-GFP cells recorded a significant upregulation of MCP-1 mRNA expression whereas, HSP60 KD cells showed a non-significant decrement. But, OxLDL treatment to HSP60 KD cells recorded significant decrement in MCP-1 levels. Further, mRNA expression of VCAM-1 was found to increase significantly in OxLDL treated and HSP60-GFP cells. HSP60 KD cells too recorded a significant increment in VCAM-1 expression as compared to the control but, its value was significantly lower than HSP60-GFP and OxLDL treated cells. Also, OxLDL treatment to HSP60 KD cells failed to prevent VCAM-1 upregulation with the indices as high as OxLDL treated or HSP60-GFP cells. A significant upregulation in ICAM-1 expression was recorded in OxLDL treated and HSP60- GFP cells but, the increment was more prominent in the latter group. However, HSP60 KD cells showed a non-significant downregulation of ICAM-1 mRNA expression wherein; OxLDL treatment failed to induce any further changes in mRNA levels (Fig. 2.12).

OxLDL upregulates HSP10 expression in HUVEC.

The expression of HSP10 mRNA was evaluated in OxLDL treated cells, wherein it was observed to be significantly upregulated as compared to control. Also, the observed upregulation was significantly higher as compared to that in cells exposed to heat shock (Fig. 2.13)

OxLDL induced HSP60 upregulation is independent of HSF-1 regulation

Heat shock factor-1 (HSF-1) is the major transcriptional factor that regulates the expression of both HSP60 and HSP10 and hence, we checked its expression in OxLDL treated cells. Herein, a significant decrement in HSF-1 mRNA levels was observed in OxLDL treated cells whereas the levels were significantly elevated in cells exposed to heat shock treatment as compared to control (Fig. 2.14).

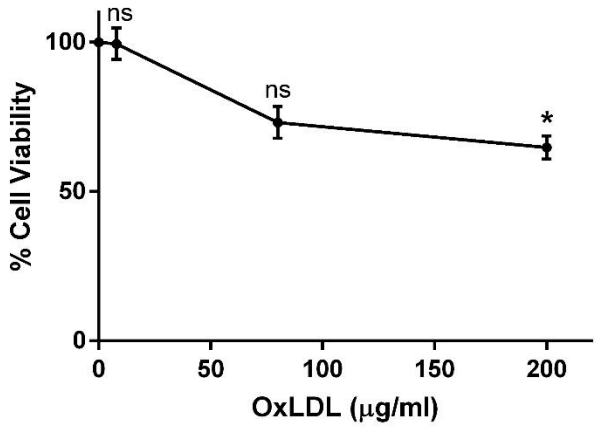

Figure 2.2: OxLDL induced cytotoxicity in HUVEC. Cells were treated with 8, 80 and 200 µg/ml OxLDL for 24 h and cell viability was assessed by MTT assay. Data were represented as Mean ± SEM (n=3). *p<0.05.

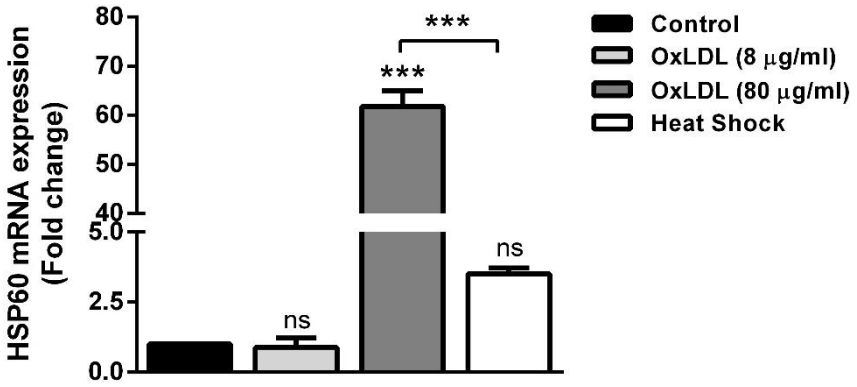

Figure 2.3: HSP60 mRNA expression in OxLDL treated HUVEC. mRNA levels of HSP60 was assessed in cells treated with 8 and 80 µg/ml OxLDL and heat shock (42°C) by quantitative RT-PCR. Data were represented as Mean ± SEM (n=3). ***p<0.001, ns-non-significant.

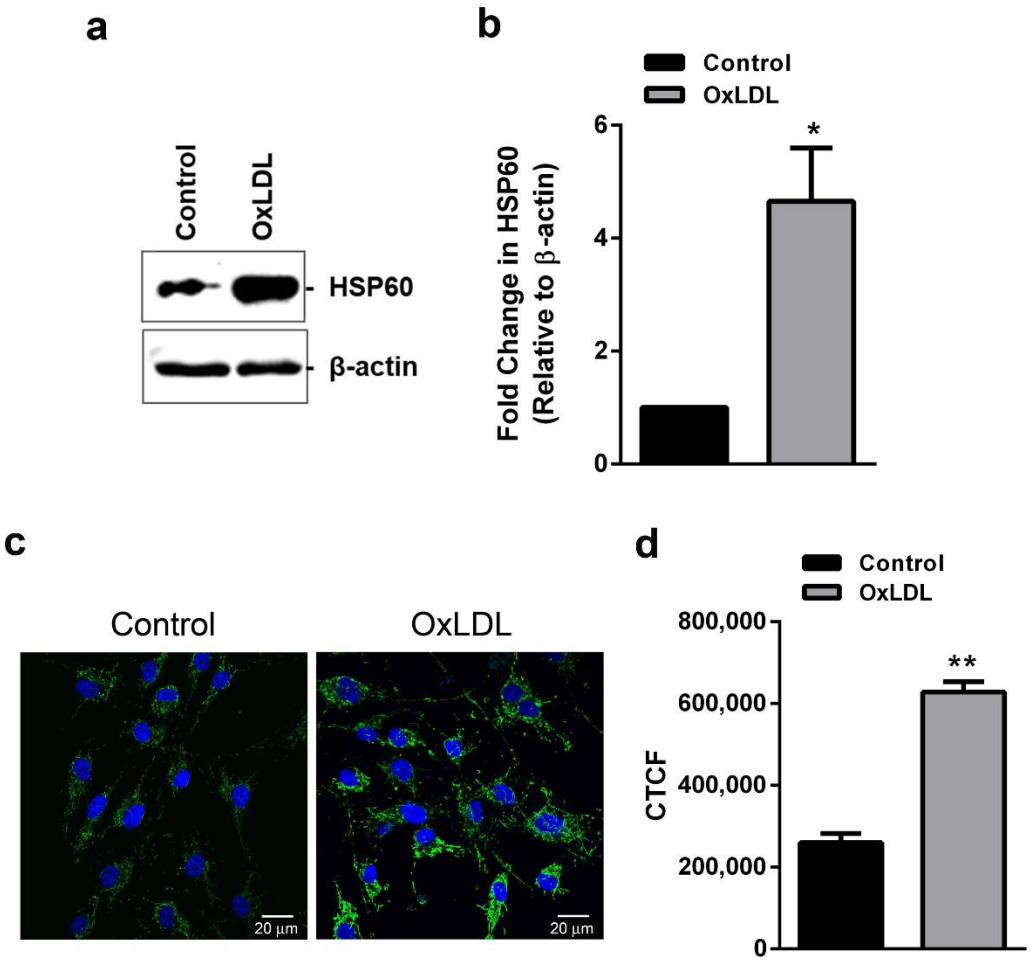

Figure 2.4: HSP60 protein expression in OxLDL treated HUVEC. Cells were treated with OxLDL (80 µg/ml) and HSP60 expression was checked by (a) immunoblotting followed by its (b) densitometry and (c) immunostaining followed by its (d) quantification using ImageJ. Scale bar= 20µm. HSP60-green, nucleus-blue. CTCF = corrected total cell fluorescence. Data were expressed as Mean ± SEM (n=3). *p<0.05, **p<0.01.

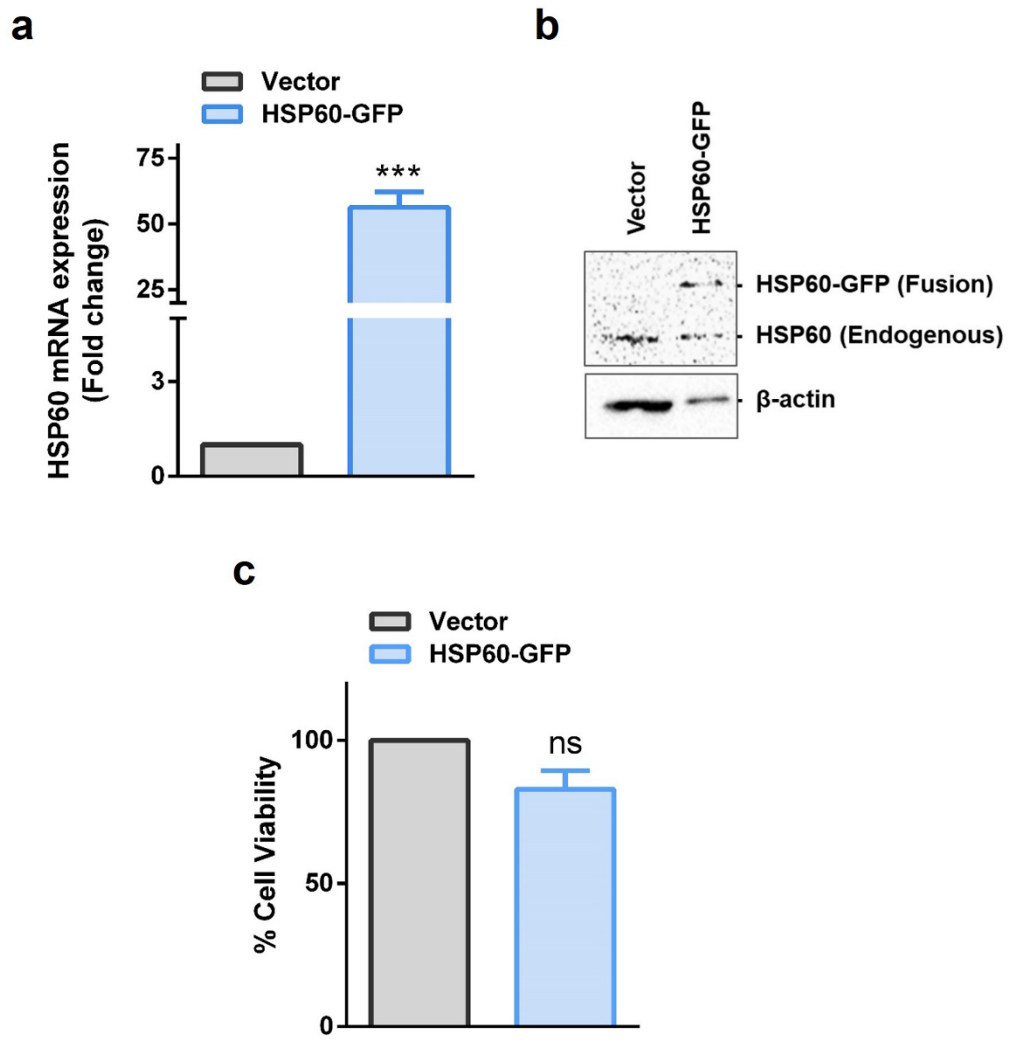

Figure 2.5: HSP60 overexpression in HUVEC. Cells were transfected with pCMV3.1-HSPD1-GFPSpark plasmid (HSP60-GFP) or empty vector (Vector) for 24 h and the transfection was confirmed by (a) quantitative RT-PCR and (b) immunoblotting. (c) the cell viability was assessed by MTT assay. Data were expressed as Mean ± SEM (n=3). ***p<0.001, ns- non-significant.

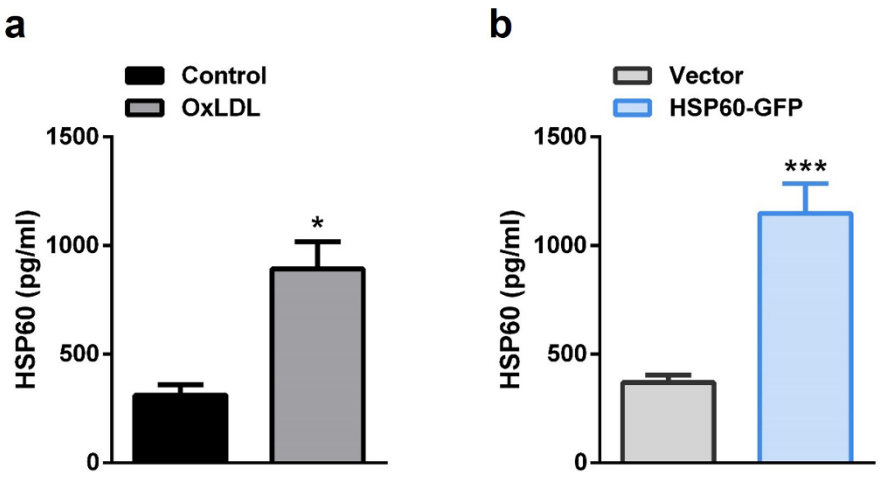

Figure 2.6: HSP60 secretion in HUVEC. Levels of HSP60 in conditioned media was assessed by ELISA in (a) OxLDL (80 µg/ml) treated cells and (b) HSP60-GFP cells. Data were expressed as Mean ± SEM (n=3). *p<0.05, ***p<0.001.

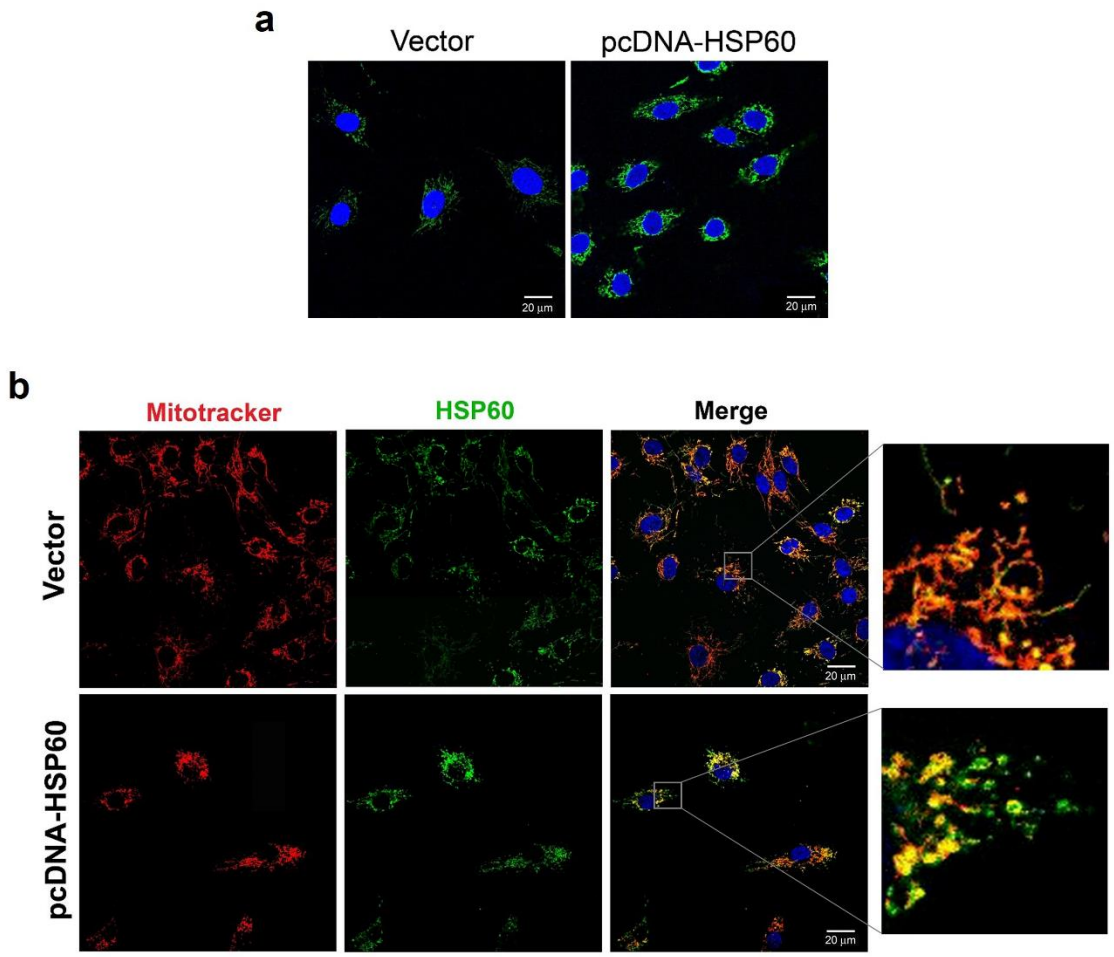

Figure 2.7: Extra-mitochondrial localization of HSP60 in HUVEC. Cells were transfected with of pcDNA3.1-Hsp60-MycHis and (a) HSP60 overexpression was confirmed by immunostaining. Cells transfected with empty vector were used as control (Vector). Further, (b) mitochondria were stained with MitoTracker Red CMXRos and HSP60 expression was assessed by immunostaining (A488). Scale bar= 20µm. HSP60-green, mitochondria-red, nucleus-blue. Yellow indicates extensive co-localization of HSP60 and mitochondria.

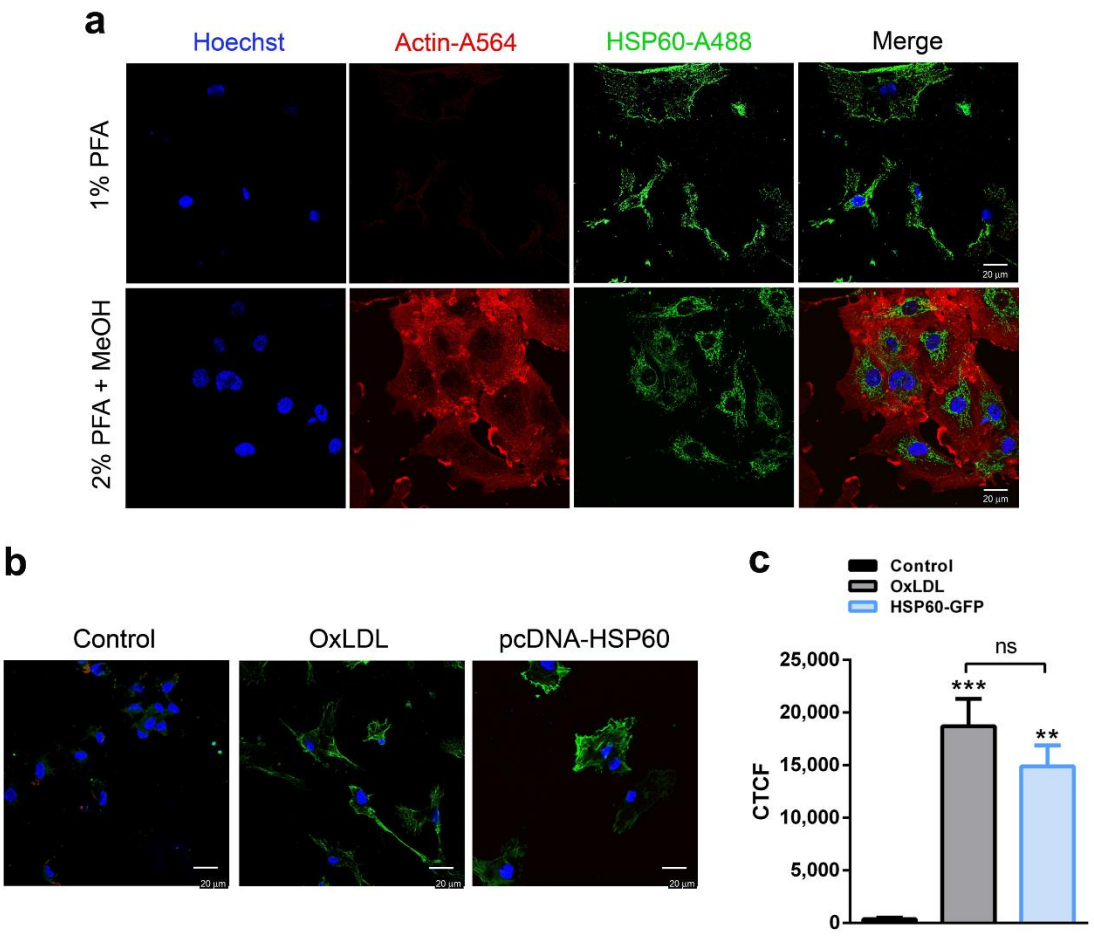

Figure 2.8: Surface localization of HSP60 in HUVEC. (a) Surface staining protocol was standardized using HUVEC treated with OxLDL (80 µg/ml). Cells were fixed with either 1% PFA for surface staining (upper lane) or 2% PFA followed by permeabilization with methanol (2% PFA + MeOH) for intracellular staining (lower lane). Representative images of cells immunostained for HSP60 (A488) and β-actin (A564) are shown. (b) Surface expression of HSP60 was checked in OxLDL treated cells and pcDNA-HSP60 cells using 1% PFA fixation protocol. Representative images showed HSP60-green and nucleus-blue. (c) Graph represents quantitative analysis of HSP60 fluorescence measured using ImageJ. Scale bar= 20µm. CTCF = corrected total cell fluorescence. Data were represented as Mean ± SEM (n=4). **p<0.01, ***p<0.001, ns- non-significant.

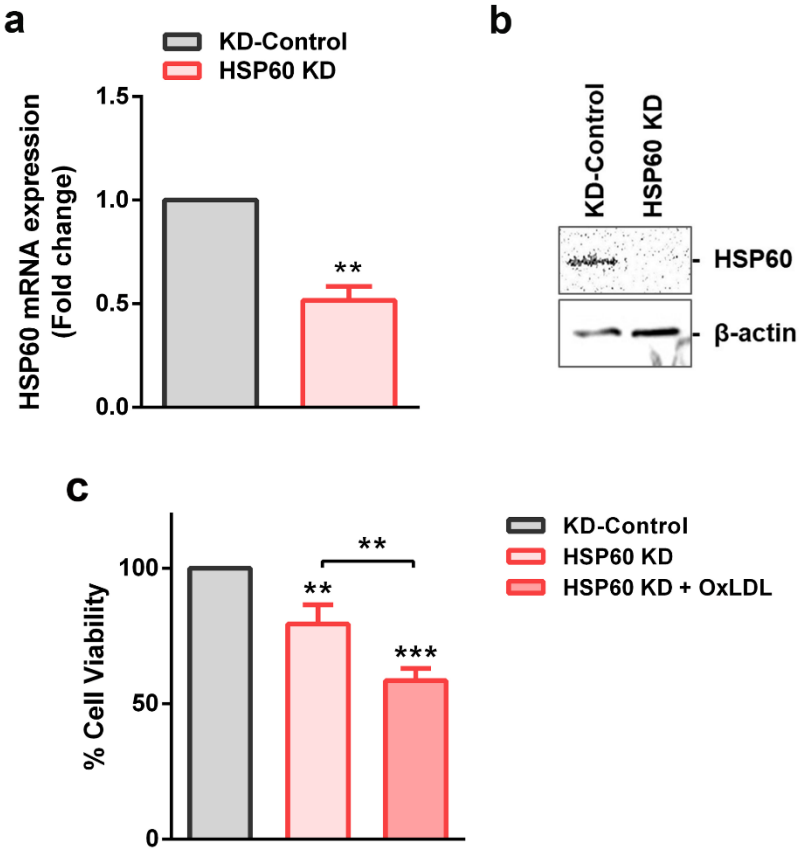

Figure 2.9: HSP60 knockdown in HUVEC. Cells were transfected with pshRNA-609 to knockdown HSP60 (HSP60 KD) that was confirmed by (a) quantitative RT-PCR and (b) western blotting. (c) HSP60 KD cells were further treated with 80 µg/ml OxLDL and cell viability of HSP60 KD and HSP60 KD + OxLDL groups were measured by MTT assay. KD-Control refers to the cells transfected with negative control plasmid contained scrambled shRNA. Data were represented as Mean ± SEM (n=3). **p<0.01.

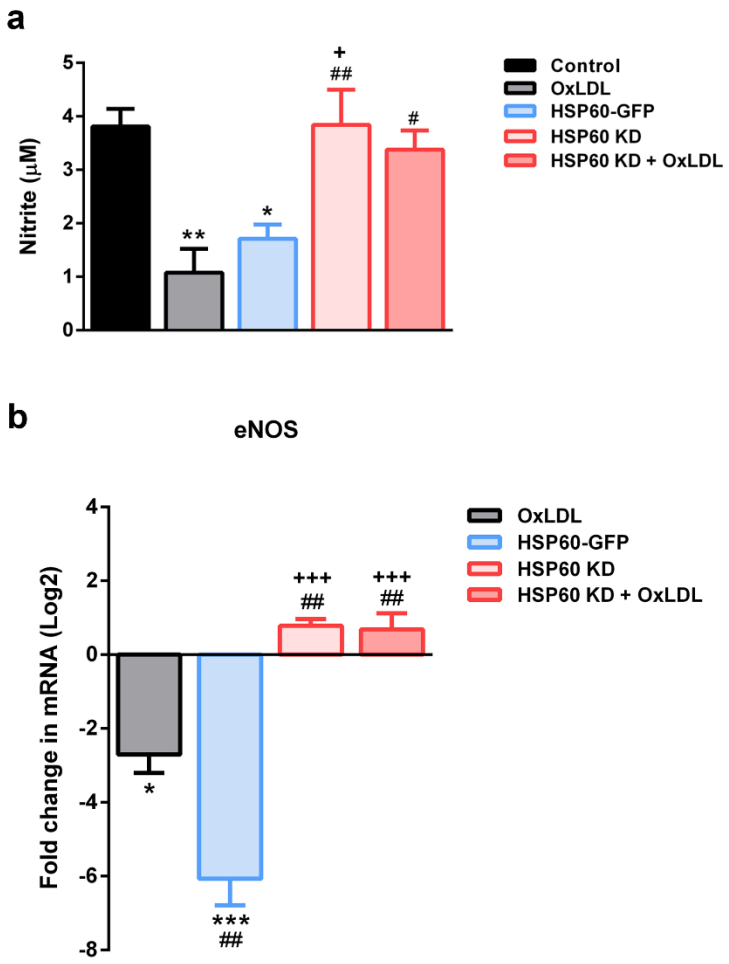

Figure 2.10: HSP60 mediated endothelial dysfunction in HUVEC. (a) NO production was assessed by quantifying nitrites in conditioned media of cells using Griess' reagent. Also, (b) eNOS mRNA expression was analyzed by quantitative RT-PCR. Data were expressed as Mean ± SEM (n=3). *p<0.05, **p<0.01, ***p<0.001 vs untreated control, ##p<0.01, ###p<0.001 vs OxLDL group, and +p<0.05, +++p<0.001 vs HSP60-GFP group.

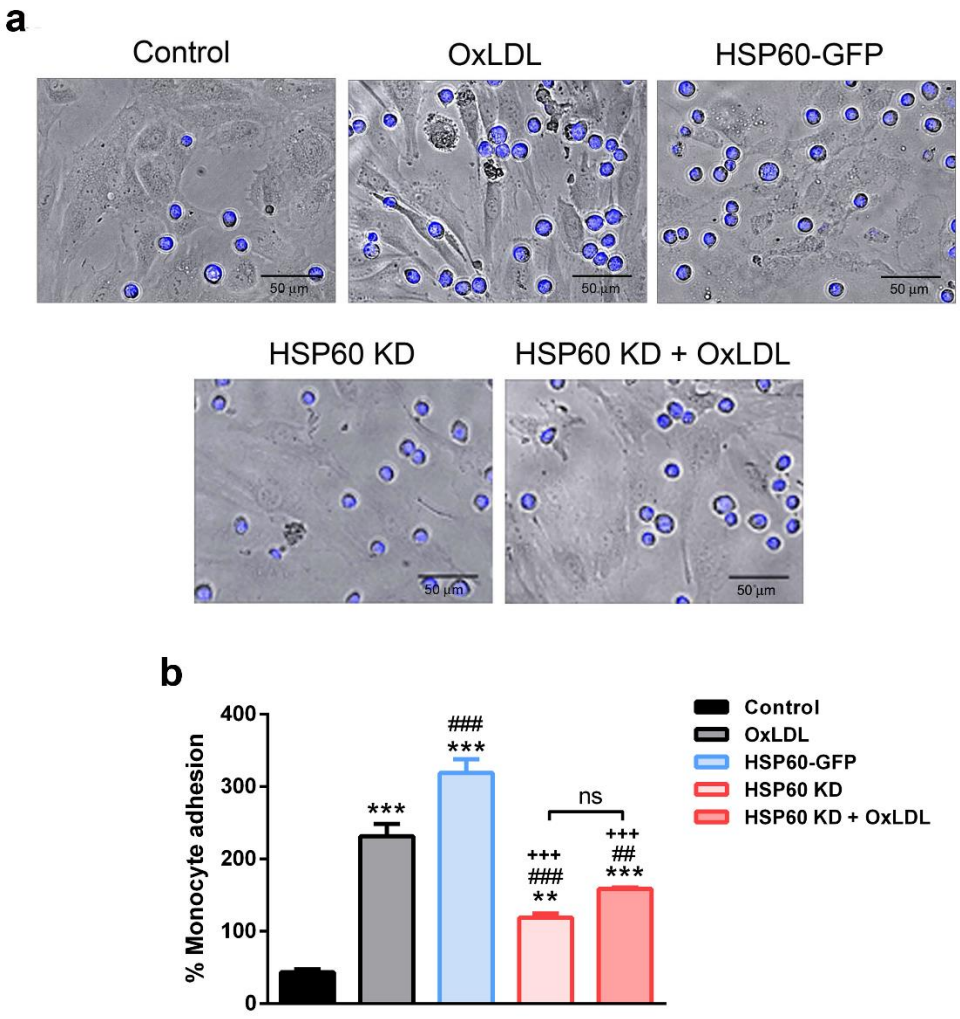

Figure 2.11: THP-1 cell adhesion to HUVEC. (a) Representative images of THP-1 (blue) adhered to HUVEC subjected to various experimental conditions are shown. (b) The graph represents % monocyte adhesion relative to the number HUVECs. Scale bar= 50µm. Data were expressed as Mean ± SEM (n=5). **p<0.01, ***p<0.001 vs untreated control, ##p<0.01, ###p<0.001 vs OxLDL group, and +++p<0.001 vs HSP60-GFP group.

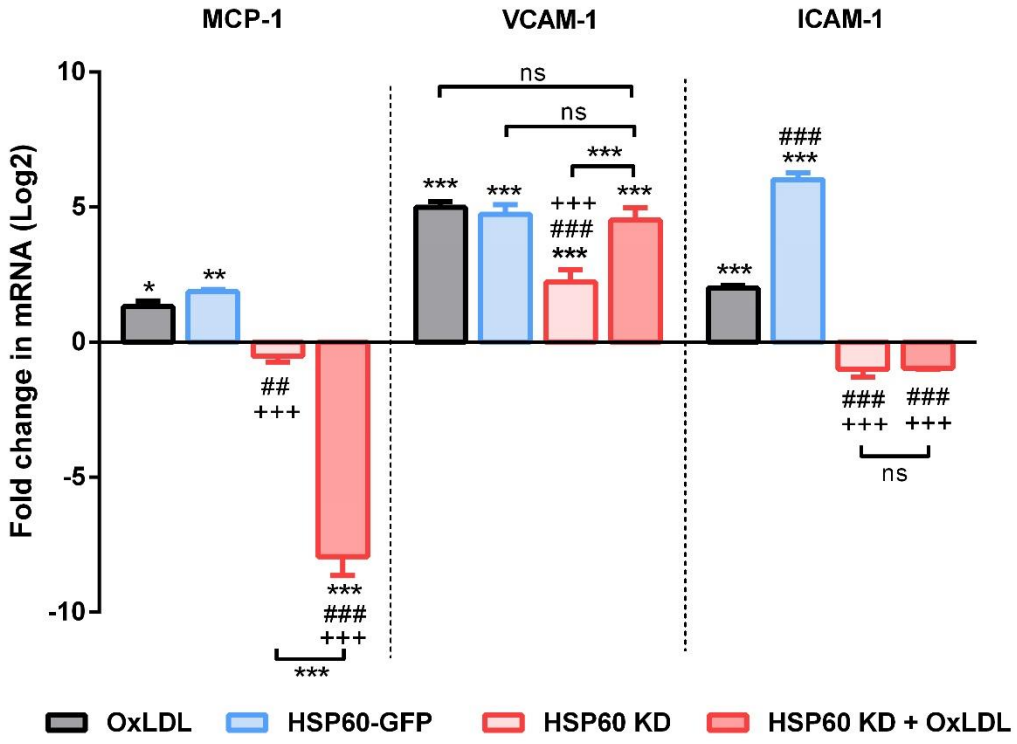

Figure 2.12: HSP60 mediated expression of adhesion molecules in HUVEC. mRNA expression of MCP-1, VCAM-1 and ICAM-1 was assessed in HUVEC subjected to various experimental conditions by quantitative RT-PCR. Data were expressed as Mean ± SEM (n=3). *p<0.05, **p<0.01, ***p<0.001 vs untreated control, ##p<0.01, ###p<0.001 vs OxLDL group, and +++p<0.001 vs HSP60-GFP group.

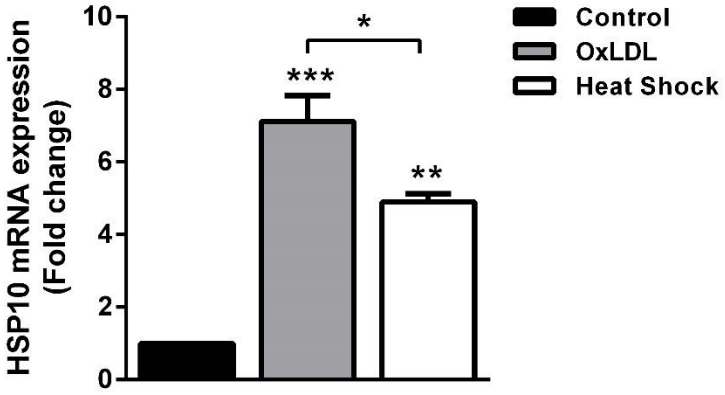

Figure 2.13: HSP10 expression in HUVEC. mRNA expression of HSP10 was analyzed in cells stimulated with OxLDL (80 µg/ml) and heat shock (42°C). Data represents Mean ± SEM (n=3). *p<0.05, **p<0.01, ***p<0.001.

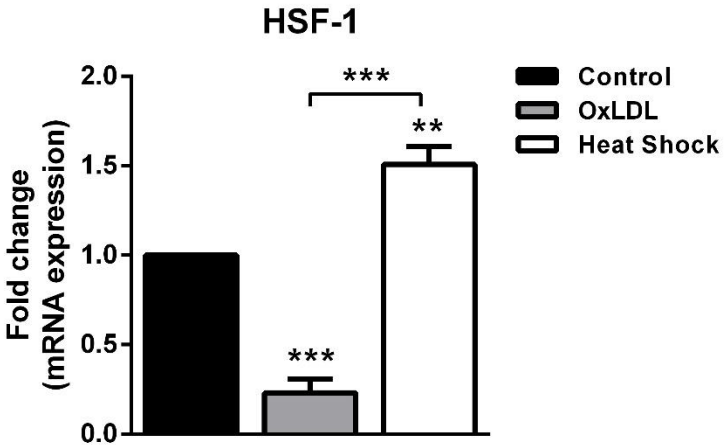

Figure 2.14: HSF-1 expression in HUVEC. mRNA expression of HSF-1 was analyzed in cells stimulated with OxLDL (80 µg/ml) and heat shock (42°C). Data represents Mean ± SEM (n=3). **p<0.01, ***p<0.001.

Discussion

Elevated levels of HSP60 in the endothelium is one of the earliest event in the development of atherosclerotic lesion wherein, its role as an auto-antigen is supported by evidences showing the presence of HSP60-reactive T-lymphocytes in the vascular wall (Almanzar et al., 2012; Xu et al., 1993). In this regard, expression of adhesion molecules in ECs overexpressing HSP60 has been acknowledged as a crucial event in recruiting the auto-reactive T-cells (Amberger et al., 1997; Seitz et al., 1996). Since the co-expression of HSP60 and adhesion molecules is common endpoint for various risk factors, it raise the possibility of an unexplained functional relationship between HSP60 and endothelial activation. In chapter-1, we had observed upregulation of HSP60 during ED and activation in high fat high fructose (HFHF) diet fed mice (chapter 1). In the present study, the regulatory role of HSP60 in atherogenic transformation of vascular endothelium was investigated using OxLDL treated HUVEC as a model.

OxLDL is a well-established atherogenic stressor that is known to induce ED and activation (Takei et al., 2001; Valente et al., 2014) but, its association with HSP60 lacks clarity. Herein, we observed a marked upregulation of HSP60 in cells exposed OxLDL at a concentration of 80 µg/ml, a dose that represent average in vivo concentration in humans that can induce expression of adhesion molecules. 8 µg/ml corresponds to the lower limit of serum levels in atherosclerosis patients but, this dose failed to induce HSP60 expression. These observations supports our claim of functional association between HSP60 and adhesion molecules, as their simultaneous expression is a pre-requisite for their cross-talk.

Pro-atherogenic role of soluble HSP60 is well-documented with activation of immune cells especially pro-inflammatory pathways being a major consequence (Matsuura et al., 2009).

Soluble HSP60 has also been reported to induce VSMC proliferation and migration that contribute to vascular wall hypertrophy and atheromatous plaque formation (Zhao et al., 2015). Our results of OxLDL induced HSP60 secretion in HUVEC might explain the hypertrophy observed in HFHF diet fed mice. Also, secretion observed in HSP60-GFP cells indicate that HSP60 overexpression itself triggers its secretion. Further, extra-mitochondrial accumulation and prominent cell surface expression of HSP60 following its overexpression explains the secretory route. The surface expression of HSP60 also has its own merits as a stimulus for eliciting autoimmune response that precedes lesion formation (Matsuura et al., 2009).

Depending upon the hemodynamic stress and biochemical stimuli, eNOS catalyzes the production of NO in ECs that further diffuses into the sub-intimal layers and activate VSMCs to regulate vasodilation (Godo and Shimokawa, 2017). The decrement in NO levels and eNOS expression confirmed OxLDL induced ED. Further, the regulatory role of HSP60 in ED was studied by overexpressing or silencing HSP60 in HUVEC. Overexpression of HSP60 was capable of reducing both NO production and eNOS expression, similar to OxLDL, whereas silencing the same led to prevention of OxLDL induced changes in the said markers emphasizing the role of endogenous HSP60 as a regulator of eNOS expression.

ED is often followed by its activation wherein, chemokine (MCP-1) and adhesion molecules (VCAM-1, ICAM-1) mediate the infiltration of monocytes, which in later stages, form key component of atheromatous plaques (Sitia et al., 2010). Herein, HSP60 overexpression induced ED as demonstrated by increased monocyte adhesion. Further, the decrement in OxLDL induced monocyte adhesion in conditions of HSP60 indicated

towards the dependency of OxLDL on HSP60 for endothelial activation. Also, HSP60 knockdown prevented OxLDL induced ICAM-1 expression whereas the response in case of VCAM-1 was weak indicating towards distinct regulatory mechanisms that is oblivious to the HSP60 status. Further, the observed reduction in OxLDL induced MCP-1 expression in HSP60 KD cells is also a key finding that underlines the importance of HSP60 protein in governing the synchrony of expression of atherogenic markers essential for monocyte infiltration.

In our preliminary study with HFHF diet fed mice, we had observed an upregulation of HSP10 in thoracic aorta in addition to HSP60 (chapter 1). Based on these findings, we evaluated HSP10 mRNA expression in OxLDL treated HUVEC and recorded a marked upregulation. This is an interesting finding as there are no reports in this regard, to the best of our knowledge. However, the implications of HSP10 upregulation in atherogenic processes remains to be explored.

The atherogenic upregulation of HSP60 has been widely reported with respect to various atherogenic stressors, however, the molecular mechanisms regulating atherogenic HSP60 expression have not been studied. HSF-1 is the main transcriptional factor regulating the transcription of both HSP10 and HSP60 and is upregulated in response to heat shock (Pirkkala et al., 2001). In this context, we checked the expression of HSF-1 in OxLDL treated cells and found a decrement in the same, implying towards HSF-1 independent mechanism of HSP10 and HSP60 upregulation induced by OxLDL. The genes for HSP10 and HSP60 are arranged head-to-head joined by a common bidirectional promoter that carries sequences recognized by various transcriptional factors, one of more of which might be involved in atherogenic regulation of these proteins (Hansen et al., 2003; Kleinridders

et al., 2013). However, at present it can be certainly said that HSF-1 is not a mediator of OxLDL induced HSP60 and HSP10 upregulation in HUVEC and the regulatory events warrants detailed scrutiny.

Taken together, our study provides an evidence on OxLDL mediated upregulation of HSP60, its extra-mitochondrial accumulation, surface localization and secretion. Valuable insights on the role of HSP60 in ED were obtained via the status of NO and eNOS. Further, our work reports endothelial activation and subsequent monocyte adhesion wherein, HSP60 overexpression was found to be a key link. Also, OxLDL induced upregulation of HSP10 is a novel observation and its atherogenic relevance needs investigation. Further studies on the missing links explaining the non-canonical functions of HSP60 and the regulatory mechanisms thereof are needed.