Alterations in Intercellular Adhesion Molecule 1 (ICAM-1) and Vascular Cell Adhesion Molecule 1 (VCAM-1) in Human Endothelial Cells

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Abstract

Alterations of Endothelial cells (ECs) play a critical role in different pathogenesis of many serious human diseases, and dysfunction of the vascular endothelium is an indicator for human disorders. Endothelial dysfunction is considered to be an early indicator for atherosclerosis, which is characterised by overexpression of adhesion molecules, including intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). Hydrogen peroxide (H_2O_2) released via neutrophils is an important mediator of endothelial cell function. Ambient production of superoxide anion (O_2^-) and subsequently H_2O_2 at low levels is critical for regulating endothelial cell functions and proliferation. In this study, we investigated the effects of H_2O_2 on the expression of adhesion molecules VCAM-1 and ICAM-1 in cultured human umbilical vein endothelial cells (HUVECs). Intracellular superoxide anion production was detected by using p-Nitro Blue Tetrazolium (NBT) assay. Our results showed that administration of 100μM of H_2O_2 on HUVECs for 2, 6, 12 and 24 hr induced a time-dependent increase in ICAM-1 and VCAM-1 mRNA and protein expression levels with a significant increase observed from 6 hr. HUVECs exposed to H_2O_2 exhibit increased O_2^-, suggesting that H_2O_2 induced oxidative stress may be a reasonable for atherosclerosis. This increase can be reduced by the flavonoid, N-acetyl cysteine (NAC). The modulation of endothelial cell function through this mechanism may underlie the contribution of H_2O_2 to the development of vascular disease.

Highlights

- Exposure of HUVECs to H_2O_2 resulted in an increase of ICAM-1 and VCAM-1 mRNA and protein levels.
- Exposure of the HUVECs to H_2O_2 produced significant increases in superoxide anions.
- The antioxidant NAC attenuated H_2O_2-induced mRNA ICAM-1 and VCAM-1 level and superoxide anions.
- NAC may protect against atherosclerosis via preventing H_2O_2 induced injury to endothelial cells.

Keywords: Human Umbilical Vein Endothelial Cells (HUVECs); Intercellular adhesion molecule 1(ICAM-1); vascular Cell adhesion molecule 1 (VCAM-1); Hydrogen peroxide (H_2O_2).

1. Introduction

Atherosclerosis is a complex process of the formation of atherosclerotic plaques that involves various mechanisms such as inflammation and endothelial dysfunction (Yang et al., 2017). Dysfunction of the endothelial cells (ECs) plays a critical role in different pathogenesis of many serious human diseases, such as atherosclerosis and hypertension (Gimbrone and Garcia-Cardena, 2016; Steyers and Miller, 2014). Endothelial dysfunction has been postulated a main step in the initiation in the pathogenesis of atherosclerosis (Bonetti et al., 2003b; Steyers and Miller, 2014). It has also been reported that endothelial dysfunction serve as a maintenance of atherosclerosis and represents as indicator for future risk of cardiovascular events (Steyers and Miller, 2014). The phenotypic features of endothelial dysfunction include upregulations of cellular adhesion molecules, including intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (Zonneveld et al., 2014). These phenotypic alterations of the functional endothelium have been found to be associated with barrier function leading to leukocyte extravasation and increased vascular smooth muscle tone secondary to reduced processing of vasodilator substances such as nitric oxide (NO) (Bonetti et al., 2003a). During atherogenesis endothelial cells are induced via expressing adhesion molecules which recruits inflammatory monocytes into the vascular wall (Mestas and Ley, 2008). Increased intercellular monocyte adhesion expression to the endothelium is among the mechanisms leading to indication of atherosclerosis (Di Fulvio et al., 2014). Due to this association among endothelial dysfunction and inflammation in atherosclerosis, the blockage of the inflammatory procedure which occurs on the endothelial cells could be a benefit path of preventing atherosclerosis (Rubio-Guerra et al., 2010).

Endothelial dysfunction caused by increased reactive oxygen species (ROS) production has been found to be associated with the development of atherosclerosis (Aldosari et al., 2018).

The ROS family are involved in several molecules that have different effects on cellular functions, including regulation of cell growth and intercellular adhesion molecules (ICAMs) (Harrison et al., 2006; Hubbard and Rothlein, 2000). Hydrogen peroxide (H_2O_2) is a main component of ROSs generated by inflammatory and vascular cells and induces oxidative stress, which could be related to endothelial cell dysfunction (Coyle and Kader, 2007). ROS can also modify or damage DNA, RNA and proteins in cells via oxidation and peroxidation (Frank et al., 2000). It's well-known that oxidative stress (OS) occurs when there is an imbalance among oxidants and antioxidants. There are many antioxidant defence mechanisms and antioxidants have a profound impact on the expression of genes. Antioxidants include endogenous thiols or sulfhydryl containing compounds such as glutathione (GSH) and thioredoxin (Jyrkkanen et al., 2008). NAC is a thiol that is precursor to the amino acid cysteine and reduces GSH. NAC is a source of sulfhydryl groups in cells and can interact directly with ROS such as hydroxyl radical (•OH) and H_2O_2 because it is a scavenger of oxygen free radicals (Zafarullah et al., 2003).

The aim of this study was to investigate the effect of H_2O_2 on the activation of HUVECs by assessing the gene and protein expression of ICAM-1 and VCAM-1 using a quantitative PCR (qRT-PCR) and Western blot techniques. We also investigated the effects of NAC (a well-known antioxidant) on the expression of ICAM-1 and VCAM-1 on H_2O_2 treated HUVECs, and their relation to superoxide anion radical levels. This would help to understand the underlying mechanism through which the modulation of endothelial cell function may underline the contribution of H_2O_2 to the development of vascular disease.

2. Materials and Methods

2.2. Cell culture and treatment

The HUVECs were grown in culture flasks in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) containing 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 Units/ml penicillin and 100 µg/ml streptomycin at pH 7.4. The cells were incubated in T 75 cm^2 tissue culture flasks at 37°C under a humidified 5% CO_2 and 95% air. Cells used in this study were passaged every three days. Briefly, HUVECs were grown on six-well culture plates at $2x10^4$ cell/well in DMEM at 37°C and 5% CO_2, medium was changed every 48hr until cells were 80-90% confluent. Cell viability was evaluated by Trypan blue exclusion. The cells were washed twice with PBS and then treated with 100 µM H_2O_2 for 2, 6, 12 and 24 hrs. After treatment, cells were harvested, washed twice with PBS, and then used in experiments.

2.2. Reverse transcription and real-time PCR

Total RNA was isolated from the HUVECs using the TRIzol® following the manufacturer's (Invitrogen) instructions. The first strand of the cDNA was synthesized using an ImProm-II™ Reverse Transcription System reaction following the manufacturer's instructions (Promega) according to the procedure as described previously (Habas et al., 2017). Quantitative real-time PCR was performed using SYBR® Green PCR Master Mix (Applied Biosystems) with StepOnePlus™ real-time PCR instrument (Applied Biosystems) under the following conditions: 95°C for 2 min, followed by 40 cycles of 95°C for 30 s and 72°C for 5 s. Quantitative real-time PCR was performed using SYBR® Green PCR Master Mix (Applied Biosystems) with StepOnePlus™ real-time PCR instrument (Applied Biosystems) under the following conditions: 95°C for 2 min, followed by 40 cycles of 95°C for 30 s.

2.3. Western blot analysis

Treated cells were lysed in lysis buffer (60 mM Tris, 2 % SDS, 100 mM DTT) and quantified using a commercial kit (Bradford Protein Assay, Biorad, UK) according to the manufacturer's specifications. 40 μg of total protein electrophoresed in a sodium dodecyl sulfate-polyacrylamide gel (10%) and transferred to nitrocellulose membranes. The membranes were then blocked for 1 hour with blocking buffer (PBS, 0.5 % skim milk powder, and 0.1 % Tween-20), and incubated at 4 °C overnight with anti-ICAM-1 (1:2000, Cell Signaling Technology, UK), anti-VCAM-1 (1:2000, Cell Signaling Technology, UK), and mouse monoclonal anti-GAPDH (1:1,000; Abcam, Cambridge, UK). The membranes were then incubated with anti-rabbit secondary antibody (1:1,000; Abcam, Cambridge, UK). The proteins were visualized using an enhanced chemiluminescence assay kit (GE Healthcare, UK). Digital images were captured and densitometry was performed utilizing Image J software (Quantity One, Biorad).

2.4. Evaluation of superoxide anion production

The production of superoxide anions by H_2O_2 was measured by nitroblue tetrazolium (NBT) assay. In briefly, following the treatment, the plate was aspirated and medium was replaced with phenol red-free media (M199) (Sigma-Aldrich, UK), containing 1 mg/ml NBT with and without 100μM H_2O_2. After incubation at 37°C and 5% CO_2 for 90 mins, the HUVECs were directly lysed with lysis solution (90% DMSO, 0.1% SDS and 0.01 M of NaOH), allowing the release of the NBT-diformazan product into the lysate. The release of superoxide anions was measured spectrophotometrically at a wavelength of 750 nm using microplate reader MRX II (Dynex Technologies, Chantilly, USA).

2.5. Statistical analysis

Data are expressed as mean ± SEM of at least three independent experiments with three replicates per experimental group. Comparisons were made by one-way ANOVA; p values < 0.05 were considered significant.

3. Results

3.1. Detection of ICAM-1 and VCAM-1 mRNA after treated with H_2O_2

The expression of ICAM-1 and VCAM-1 mRNA in HUVEC cells after 2, 6, 12 and 24 hrs incubation with H_2O_2 was examined to determine whether HUVEC cells have the capacity to be a source of inducible ICAM-1 and VCAM-1 by monitoring the upregulation of ICAM-1 and VCAM-1expression, well-known endothelial cell markers.

The total RNA was prepared and the results of RT-PCR for the expression of ICAM-1 and VCAM-1 mRNA and β-actin (control housekeeping gene) mRNA were shown in Figure 1. Exposure to H_2O_2 readily induced up-regulation of ICAM-1 and VCAM-1 expression on HUVEC cells, which normally express low levels of VCAM-1. H_2O_2 induced ICAM-1 and VCAM-1 expression in a time-dependent manner 6, 12 and 24 hrs with detectable levels of ICAM-1 and VCAM-1up-regulation from 100 μM of H_2O_2 for 2 hrs (Figure 1). The up-regulation of ICAM-1 and VCAM-1up was detectable by both RT-PCR and qPCR analysis (Figures 1 and 2). Thus, HUVEC cells treated with 100μM H_2O_2 in the presence of 2 mM NCA for 24 hrs showed significant decreases in the mRNA level of ICAM-1 and VCAM-1compared with control ($^{\#\#}p \leq 0.01$; Figures 2 and 3). These results suggested that H_2O_2 could be a critical molecule in activation ICAM-1 and VCAM-1 up-regulation.

The ICAM-1 and VCAM-1 expression at the protein level was also confirmed by quantitative Western blot analysis (Figures 4 and 5). Exposure to H_2O_2 readily induced up-regulation of ICAM-1 and VCAM-1 expression on HUVEC cells, which normally express low levels of VCAM-1. A statistically significant increase was shown in the levels of catalase protein at 2 hrs (***p<0.001). A statistically significant increase was shown in the levels of catalase protein at 6, 12 and 24 hrs (*p<0.05), (**p<0.01) and (***p<0.001) respectively as shown in Figures 4 A and B and Figure 5 A and B.

NAC mediated expression of both ICAM-1 and VCAM-1 in HUVEC cells was also investigated. HUVECs treated with 100µM H_2O_2 and 2 mM NAC at 24hrs showed significant reductions in mRNA expression level of ICAM-1 and VCAM-1 compared with the same cells treated with 100µM H_2O_2 alone at 24 hrs ($^{\#\#}p<0.01$) (Figures 2 and 3).

3.2. Detection of superoxide anion in HUVEC by NBT assay

The generation of superoxide anion was detected by NBT assay. Cells treated with H_2O_2 at 2, 6, 12 and 24 hrs showed clear increased production of superoxide anion, which were significantly increased compared to the control ($^{*}p<0.05$), ($^{**}p<0.01$) and ($^{***}p<0.001$) (Figure 6). NCA is a potent suppressor for H_2O_2 induced cytotoxic effects, involving caspase activation and cell detachment. Thus, co-treatment with NAC and H_2O_2 was studied in HUVECs in order to investigate whether NAC might facilitate a reduction in superoxide anion overproduction, and thus acting as a cellular detoxifier of superoxide anions. HUVECs treated with 100µM H_2O_2 and 2 mM NAC at 24hrs showed significant reductions in superoxide anion production levels compared with the same cells treated with 100µM H_2O_2 alone at 24hrs ($^{\#\#}p<0.01$) (Figure 6).

4. Discussion

Atherosclerosis has been documented as a chronic inflammatory disease and oxidative stress plays an essential role in its initiation and development (Libby, 2006). It has been reported that an increased generation of ROS including O_2^-, H_2O_2 and vascular inflammation play critical keys in endothelial dysfunction (Ugusman et al., 2011a). ECs in human atherosclerotic injury have upregulated cell adhesion molecules expression such as ICAM-1and VCAM-1 (Ugusman et al., 2011b).

In this study, endothelial cells exposed to H_2O_2 showed increased O_2^-, which leads to upregulation of both intercellular and vascular adhesion molecule 1 (ICAM-1 and VCAM-1).

Consistent with previous reports (Bradley et al., 1993; Cai, 2005), our results showed that H_2O_2 was a potent ICAM-1 and VCAM-1 inducer in endothelial cells at both mRNA expression and protein levels. RT-PCR and Western blot results suggested that H_2O_2 induced the membrane expression of ICAM-1 and VCAM-1 after treating HUVECs cells with H_2O_2. This result implied that H_2O_2-induced oxidative stress may be a sensible model for atherosclerosis. Endothelial dysfunction is considered an early indicator of atherosclerosis and therefore our result suggested a possible role for H_2O_2 in the development of atherosclerosis. However, further studies are necessary to clarify the pathogenic effect of H_2O_2 and its underlying molecular mechanism in the atherosclerosis.

Activation of endothelial cells is early stage of atherosclerosis and induction of several intracellular signalling pathways, resulting to upregulation of some proteins such as CAMs (Galkina and Ley, 2009). It has been showed that during early stages of atherosclerosis, circulating mononuclear cells (MNCs) bind to ECs and migrate into the sub-endothelial space promoting the activation of ECs. ICAM-1 expression is upregulated during this time, leading to EC activation and the progression of atherosclerosis (Badimon et al., 2012). The expression of leukocyte and endothelial cell adhesion molecules is extremely important for the emigration of leukocytes through an inflammatory response. The significance of the inflammatory response during early development of atherosclerosis is indicated via the unregulated expression of adhesion molecules (Lee et al., 2010).

Our results also showed that ICAM-1 and VCAM-1 was measurable in non-treated cells in similar way to available data. It is well known that HUVECs express low level of ICAM-1 and VCAM-1 under basal conditions (Kinashi and Springer, 1994; van Buul et al., 2004). Our results were in line with a number of studies which investigated the role of H_2O_2 on adhesion molecule expression in other types of vascular cells.

We further examined the effect of NAC on the H_2O_2-induced ICAM-1 and VCAM-1 gene expression. The pre-treatment of HUVECs with NAC inhibited H_2O_2-induced ICAM-1 and VCAM-1 mRNA expression. Our results showed that ICAM-1 and VCAM-1 mRNA expression were significantly reduced by the antioxidant NAC.

Previous clinical and experimental studies showed the protective effect of NAC on reducing ICAM-1 adhesion molecule expression in ECs associated with a higher incidence of cardiovascular patients (Rains and Jain, 2015). Our results showed that NAC significantly reduced expression of adhesion molecules ICAM-1 and VCAM-1 at mRNA levels in HUVECs treated with H_2O_2 at 24hr ($^{\#\#}p<0.01$). These result suggested that antioxidants such as NAC may potentially protect against H_2O_2 endothelial dysfunction. NAC is a reduced thiol, a molecule with a sulfhydryl group which has several biological functions. It's a pharmacological precursor of L-cysteine, which functions as an anti-oxidant, and is a precursor of GSH, a tripeptide present in high concentrations in most cells. NAC, which is necessary for GSH regeneration, is a direct scavenger of free radicals and also inhibits inducible nitric oxide (NO) synthase expression along with the expression of intercellular adhesion molecule-1 (ICAM-1) (Jiang et al., 2005). Our results of NAC and H_2O_2 co-incubation indicate that H_2O_2 can induce ROS generation, which in turn might promote endothelial adhesion molecule expression through the activation of NF-kB and subsequently promote early atherogenesis in cardiovascular patients. Many studies on NAC therapy in disapprovingly ill patients have yielded conflicting results. Some results have implied that the effects of NAC could be positive for certain mechanisms of disease such as endothelial cell activation (Heller et al., 2001).

NAC has a comprehensive range of actions and potential applications across many conditions and systems. As a drug, NAC represents the perfect xenobiotic, efficient at directly entering endogenous biochemical developments as a result of its own metabolism. Furthermore, NAC

may cross the blood barrier. This provides a possibility to discover doses and period of treatment with NAC to achieve cytoprotection in cardiovascular diseases.

5. Conclusion

In this study, we showed that H_2O_2 can significantly induced ECs activation through both increased mRNA expression and protein up-regulation of ICAM-1 and VCAM-1 adhesion molecules in cultured HUVECs. In addition, our study showed that co-treatment with NAC significantly attenuated the effect of H_2O_2 administration on the mRNA expressions of adhesion molecules in cultured HUVECs. This implied and emphasised the role of ROS in the endothelial cell damage sustained, and implied a potential use of NAC in the treatment of cardiovascular diseases.

Conflict of interest

The authors declared that there are no conflicts of interest.

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Figure legends

Figure 1 RT-PCR investigations of ICAM-1 (A) and β-actin (B) mRNA gene in HUVECs. Lane 1: DNA ladder, Lane 2: control, Lanes 3, 4, 5 and 6: HUVEC treated with H_2O_2 at 2, 6, 12 and 24 hrs respectively.

Figure 2 Effect of H_2O_2 on the ICAM-1 mRNA expression in HUVECs by qPCR. The cells were harvested at 0, 2, 6, 12 and 24 hrs for total RNA isolation and expression of the ICAM-1 mRNA were determined by qPCR. Data in figure shows change of ICAM-1 mRNA level at each time point. *p<0.05, **p<0.01 and ***p<0.001 compared with non-treated cells. $^{\#\#}p$<0.01 H_2O_2 treatment at 24hr compared with H_2O_2 and NAC co-treatment at 24hr.

Figure 3 Effect of H_2O_2 on the VCAM-1 mRNA expression in HUVECs by qPCR. The cells were harvested at 0, 2, 6, 12 and 24 hrs for total RNA isolation and expression of the VCAM-1 mRNA were determined by qPCR. Data in figure shows change of VCAM-1 mRNA level at each time point. *p<0.05, **p<0.01 and ***p<0.001 compared with non-treated cells. $^{\#\#}p$<0.01 H_2O_2 treatment at 24hr compared with H_2O_2 and NAC co-treatment at 24hr.

Figure 4 Effect of H_2O_2 on ICAM-1 expression in HUVECs by Western blot. HUVECs were incubated with or without H_2O_2 for 2, 6, 12 and 24 hrs. **(A):** shows a representative blot indicating a protein band of 90 kDa representing ICAM-1. **(B):** data (mean ± SE) are shown as a bar graph of densitometry data from 3 different donors (n=3). *p<0.05, **p<0.01 and ***p<0.01 compared with non-treated HUVECs.

Figure 5 Effect of H_2O_2 on VCAM-1 expression in HUVECs by Western blot. HUVECs were incubated with or without H_2O_2 for 2, 6, 12 and 24 hrs. **(A):** shows a representative blot indicating a protein band of 95 kDa representing VCAM-1. **(B):** data (mean ± SE) are shown as a bar graph of densitometry data from 3 different donors (n=3). *p<0.05, **p<0.01 and ***p<0.01 compared with non-treated HUVECs.

Figure 6 Effect of H_2O_2 and NAC co-incubation on superoxide anion production in HUVECs using NBT assay. HUVECs were grown in complete medium and either treated with 100 µM H_2O_2 alone or with 100µM H_2O_2 and 2 mM NAC for different time course 2, 6, 12 and 24 hrs. Non-treated cells were considered as negative control. The results are expressed as mean (± SE) from 3 different donors (n=3). *p<0.05, **p<0.01 and ***p<0.01 versus non-treated cells. $^{\#\#}p$<0.01 H_2O_2 treatment at 24hr alone compared with H_2O_2 and NAC co-treatment cells at 24hr.

Figure 1

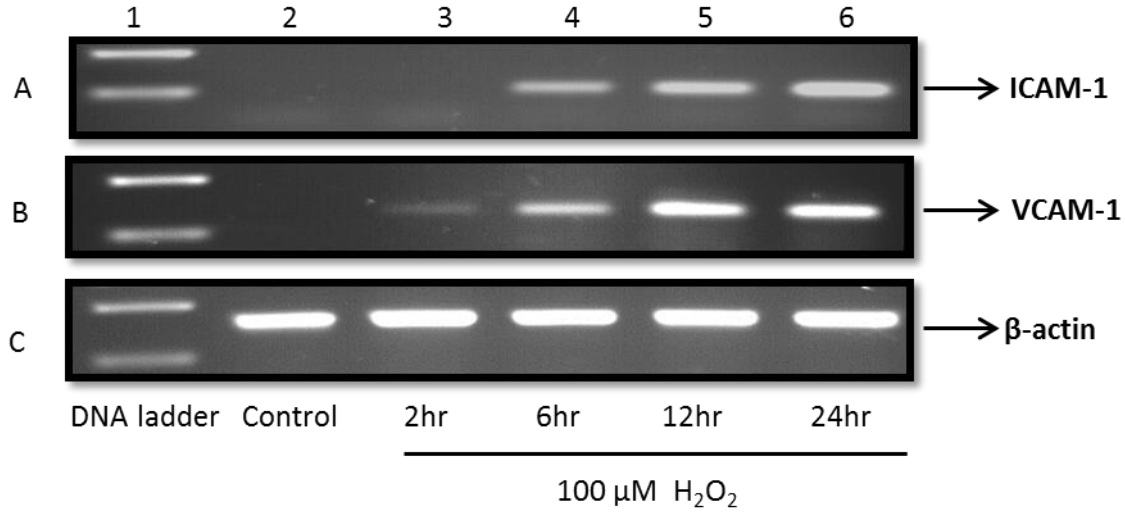
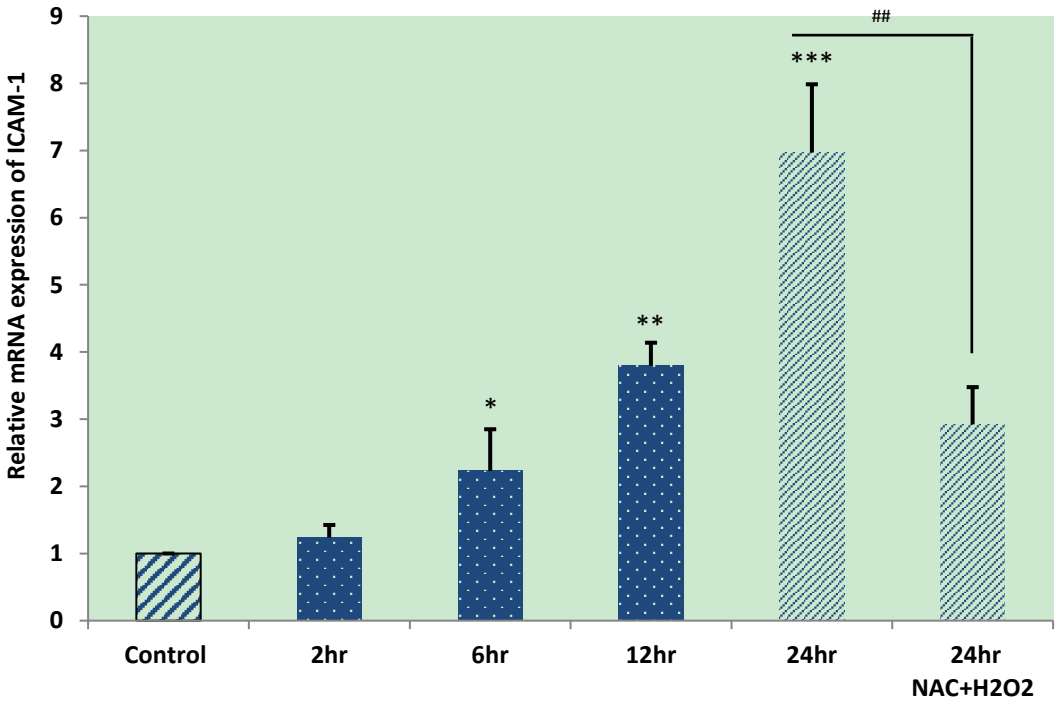

1
2
3
4
5
6
A
ICAM-1
B
VCAM-1
C
β-actin
DNA ladder
Control
2hr
6hr
12hr
24hr
100 µM H2O2

Figure 2


Relative mRNA expression of ICAM-1
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Control
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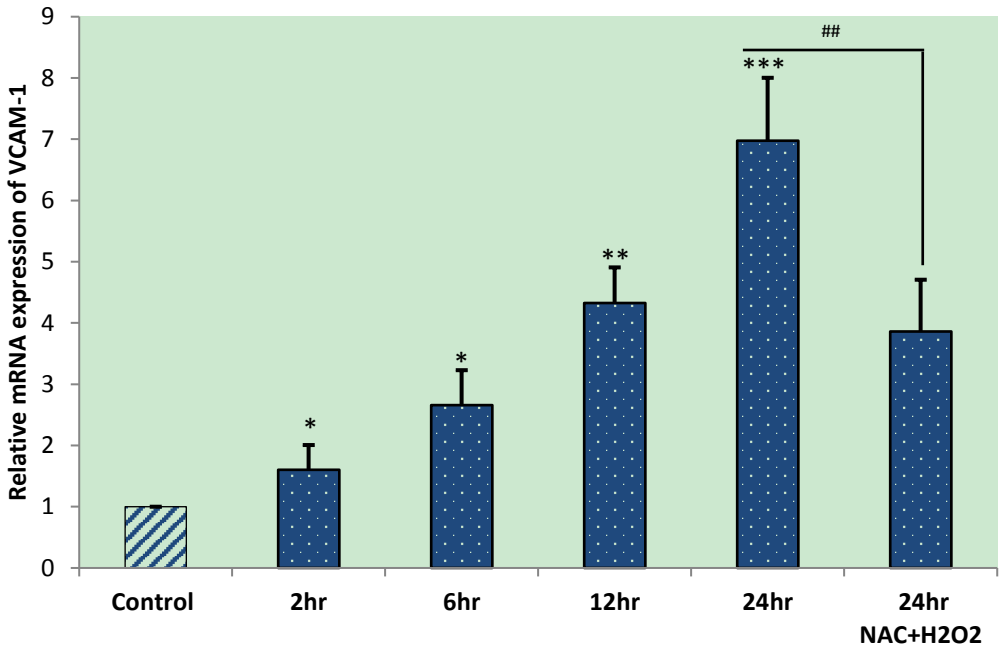
NAC+H2O2

Figure 3


Relative mRNA expression of VCAM-1
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Control
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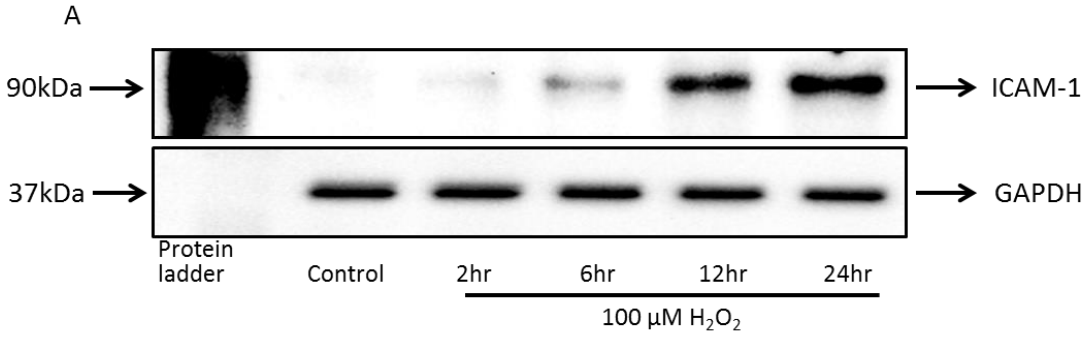
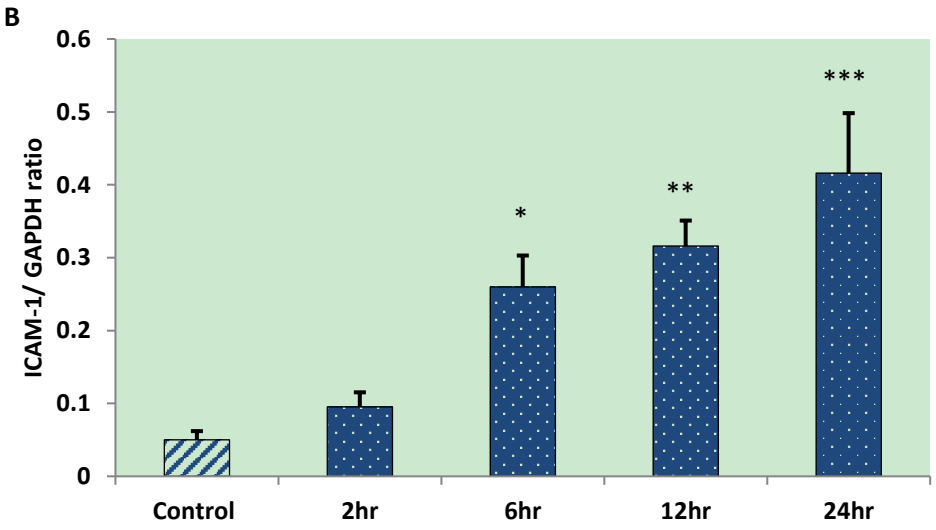
24hr
24hr
NAC+H2O2

Figure 4


A
90kDa
ICAM-1
37kDa
GAPDH
Protein ladder
Control
2hr
6hr
12hr
24hr
100 μM H_2O_2


B
ICAM-1/ GAPDH ratio
0.6
0.5
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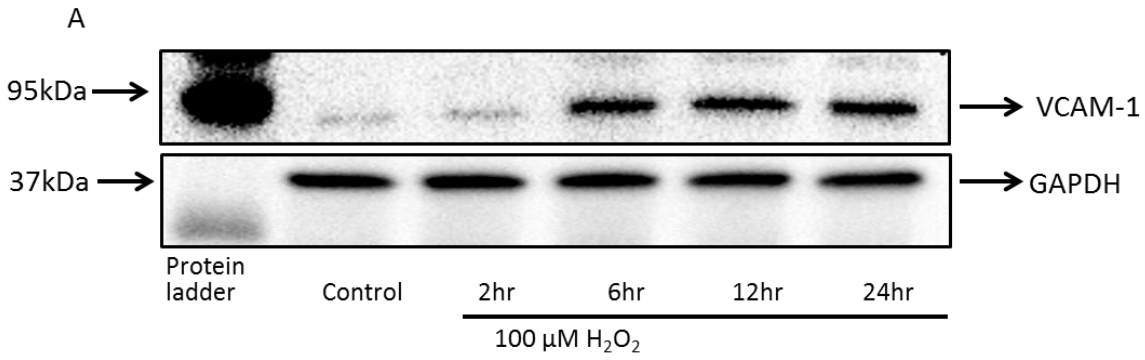
0.1
0
*
**
***
Control
2hr
6hr
12hr
24hr

Figure 5


A
95kDa
37kDa
VCAM-1
GAPDH
Protein ladder
Control
2hr
6hr
12hr
24hr
100 µM H_2O_2

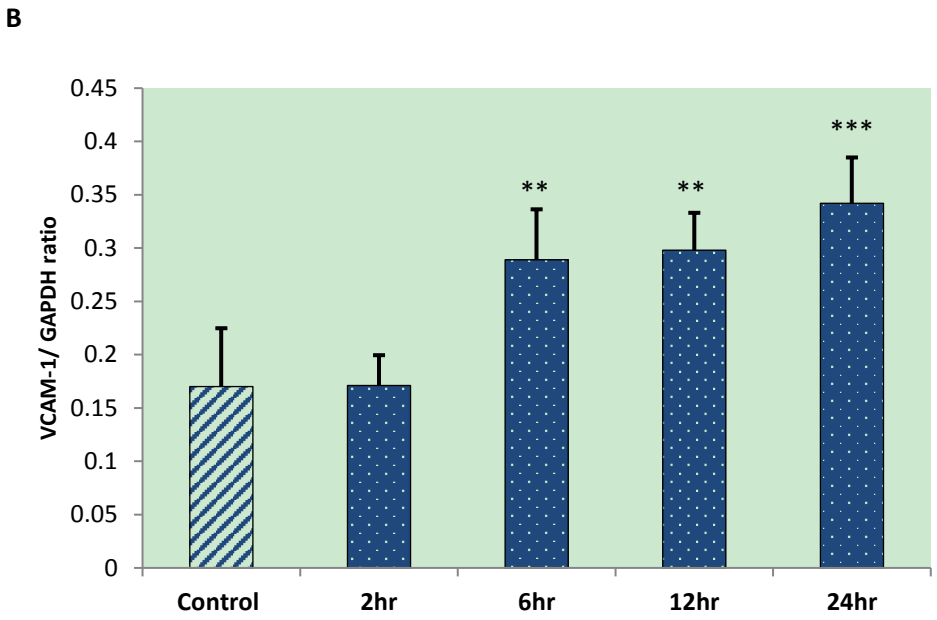
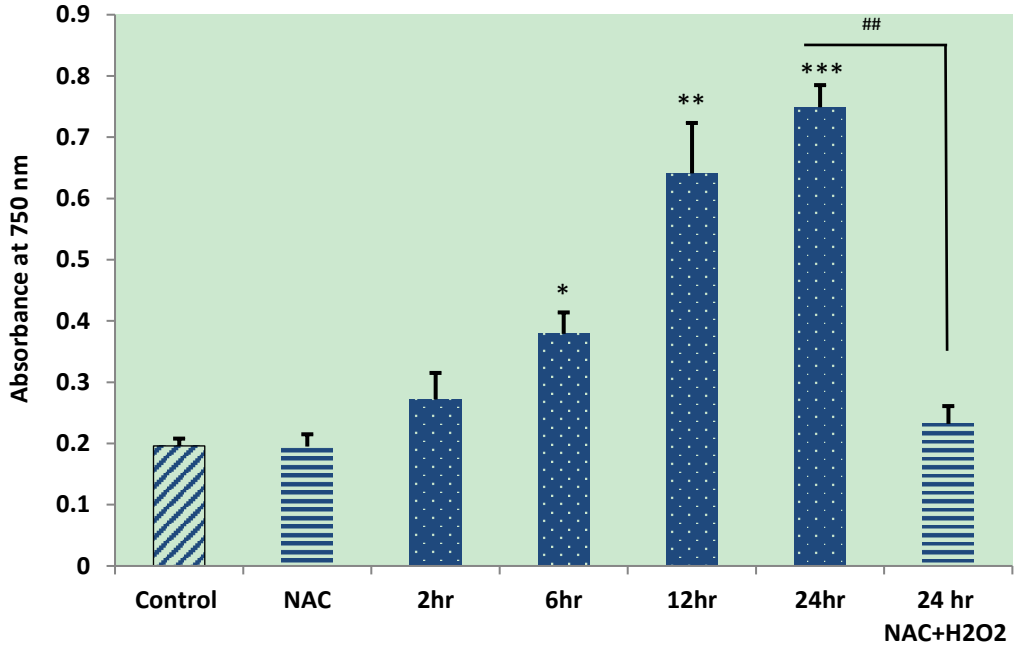

B
VCAM-1/ GAPDH ratio
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
**
**
***
Control
2hr
6hr
12hr
24hr

Figure 6


Absorbance at 750 nm
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
**
***
##
Control
NAC
2hr
6hr
12hr
24hr
24 hr NAC+H2O2