Nrf2 Play an Protective Role in Oxldl Induced Fibrosis in Renal Tubular Cells

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Research Article

Keywords: CD36, Nrf2, OxLDL

Posted Date: May 17th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-482347/v1

Abstract

CD36 is a receptor of OxLDL in kidney tubular epithelial cells(NRK-52E cells),nuclear factor erythroid 2-related factor 2 (Nrf2) is a crucial factor initiate Nrf2-signaling pathway regulating oxidative stress,kelch-like ECH-associated protein 1(Keap1) is called Nrf2 inhibitor.We treated NRK-52E cells by different concentration and time of OxLDL, and Nrf2 inhibitor KeapI ,and then observed CD36 ,cytoplasmic and nuleus of Nrf2 ,E-cadherinin in NRK-52E cells by western blotting and RT-PCR methods etc.,we found that CD36 protein in OxLDL stimulated NRK-52E cells increased after enough concentration and time;Although total Nrf2 protein level did not chang significantly at 5h,10h,but decreased at 24h,48h;Nuleus Nrf2 protein level increased relatively.Meanwhile, cytoplasmic Nrf2 protein level did not change significantly; CD36 mRNA and protein expression decreased in the NRK-52E renal tubular cells treated with Nrf2 inhibitor keap1; After OxLDL stimulation and then keap1 overexpression, CD36 mRNA and protein expression also decreased; E-cadherin expression decreased in NRK-52E after KeapI over-expression treatment .These results may indicateed that CD36 could accept OxLDL and then upregulated in NRK-52E cells ; Nrf2 could be activated by OxLDL, But Nrf2 could resist oxidative stress induced by OxLDL only if it transfer to the nucleus from cytoplasm.Nrf2 may play an protective role through upregulating CD36 .

1. Introduction

As the research before, CD36 is one of the main receptor of OxLDL,which is one of injury factor of lipid associated injury to tubular interstitium,some mechanism has been proved in preadipocytes cells that oxLDL induce a peroxisome proliferator activated receptor c-independent CD36 overexpression by up-regulating nuclear factor erythroid 2 (NF-E2)-related factor 2 (Nrf2).However, is there a same mechanism in tubular cells?This study tend to explore the mechanism of CD36 in renal tubular cells .

Novel evidence is provided that OxLDL induce a peroxisome proliferator activated receptor c-independent CD36 overexpression by up-regulating nuclear factor erythroid 2 (NF-E2)-related factor 2 (Nrf2). The nuclear translocation of Nrf2 appeared to depend on PKC pathway activation. In adipocytes, the CD36 up-regulation may indicate a compensation mechanism to meet the demand of excess OxLDL and oxidised lipids in blood, reducing the risk of atherogenesis.Besides strengthening the hypothesis that OxLDL can contribute to the onset of insulin-resistance,data herein presented highlight the significance of OxLDL-induced CD36 overexpression within the cellular defence response.

2. Materials And Methods

2.1. Cell culture

Rattus norvegicus kidney tubular epithelial cell line NRK-52E (American Type Culture Collection) were plated and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum. 293T cells were obtained from American Type Culture Collection and was used for lentivitus transduction.

2.2 OxLDL preparation and treatment

OxLDL was obtained from Yiyuan Biotechnology Company (Guangzhou, China). It was sterilized by a 0.22μm membrane (Millipore Corporation,Bedford, MA) and incubated with the NRK-52E cells. Various concentrations of oxLDL were tested. 50mg/L oxLDL was incubated with the NRK-52E cells for 0h to 48hrs 0mg/L, 100mg/L, 150mg/L OxLDL were incubated with the NRK-52E cells for 0 day to 4 days. In some experiments, NRK-52E cells infected with empty p305 and p305-keapI were treated with oxLDL(150mg/L) for 2 or 3 days. Cells were processed for expression of CD36,total Nrf2,nuclear Nrf2,NFκB,E-cadherin at the end of incubation period as described below.

2.3. Immunoblotting analysis

At the end of incubation, cells were washed with PBS and lysed in lysis buffer (Bio-Rad Laboratories, Hercules, CA). Cell lysates were ultrasonic crushed and centrifuged at 13000 rpm at 4℃ for 15min to pellet cell debris. Before electrophoresis, a marker dye and 2-mercaptoethanol were added to lysates, which were then fractionated by SDS-polyacrylamide gel electrophoresis and electrotransferred onto a nitrocellulose membrane (Pall Company, USA). After blockade with 5% nonfat milk, membranes were incubated at 4℃ overnight with Rabbit polyclonal anti-CD36 antibody (Abcam, Cambridge, UK), Rabbit polyclonal anti-Nrf2 antibody (Abcam, Cambridge, UK), mouse mAb to E-cadherin (BD Biosciences, San Jose, CA, USA) or Rabbit polyclonal to NFκB (Santa Cruz Biotechnology, Inc. Dallas, TX, USA). After extensive washing in TBS-Tween 20, the membranes then were incubated with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG (Zhong shan golden bridge, Beijing, China) for 1 h at room temperature. After washing, the membranes were incubated with enhanced chemiluminescence system (ECL) detection kit (Millipore Corporation, Bedford, MA).

2.4. Nuclear protein extracts

NRK-52E cells infected with empty p305 and p305-keapI were washed with ice-cold PBS and lysed in an appropriate volume(10×cell volume) of ice-cold buffer A containing 0.5% NP-40, 1 mM DTT and 1×protease inhibitor (PI). Cell lysates were pipeted up and down several times to disrupt cell clumps, rotated for 10min at 4℃ and then centrifuged at top speed for 3min at 4℃.The supernatants (cytosolic fraction) were saved at 4℃.The pellets were washed with ice-cold buffer A and mixed with 3×volumes of ice-cold buffer B containing 1 DTT and 1× protease inhibitor (PI). The mixtures were incubated on ice for 30min with inermittent strong vortexing and spined for 15min at top speed at 4℃. The supernatants were collected and the concerntration of proteins were determined with BCA assay.

2.5. Total RNA extraction and RT-PCR analysis

Total RNAs were isolated, with the TRIZOL reagent (Invitrogen-Life technologies, USA) as described elsewhere from NRK-52E cells,NRK-52E cells infected with empty p305 and p305-keapI and cells treated with oxLDL.Quantitative real-time PCR was performed with primers in a sequence detector(Applied Biosystems). Oligonucleotide primers used for RT-PCR were as follows: 5’-GGTGTGCTCAACAGCCTTATC-

3’ and 5’-TTATGGCAACCTTGCTTATG-3’ for detecting rat CD36 mRNA,5’-GACTGGATCTGGCATAAAGA-3’,and 5’-tcaacggcacagtcaagg-3’ and 5’-actccacgacatactcagc-3’ for rat GAPDH mRNA.Expression of CD36 and Lox-1 genes were determined as the amount of CD36 relative to mRNA for GAPDH by using the comparative C_T method described in ABI sequence detection system.

2.6. Lentivirus Transfection

Plasmid p305 and p305-keapI were used to produce lentivirus in 293T cells with the packaging plasmids pMD2.BSBG, pMDLg/pRRE and pRSV-REV by $Ca_3(PO_4)_2$ transfection kit (Millipore Corporation, Bedford, MA). Infection of NRK-52E was performed with lentivirus vectors until the eGFP marker as successfully infected cells appeared completely.

2.7. Immunohistochemical staining

Histological sections (4μm thick) were mounted on poly-L-lysine-coated slides. Slides were deparaffinized in xylene and rehydrated in graded alcohols. Sections were pretreated with citrate buffer (0.01 mol/L citric acid, pH 6.0) for 20 min at 95℃. Then, at room temperature they were immersed in PBS containing 3% H_2O_2 for 10 min. After treatment with exposing them to 10% normal goat serum in PBS for 30 min at room temperature, the tissue sections were incubated at 4℃ overnight with rabbit polyclonal ant-Nrf2 (dilution 1:100). Then sections were rinsed with PBS, incubated with biotinylated goat anti-rabbit IgG for 30 min at room temperature and treated with 3,30-diaminobenzidine chromogen for 5 min at room temperature. Finally, sections were counterstained with hematoxylin for 2 min.

3. Results

3.1The expression of CD36 in NRK-52E cells after incubated with 50mg/L OxLDL.The NRK-52E cells were incubated with 50 mg/L OxLDL for 0h, 5h、10h、24h、48hrs respectively,collected cells,tested the expression of CD36 using western-blotting method. (Fig3.1a-c)

3.2 Expression of CD36 in NRK-52E cells stimulated by different OxLDL concentrations

Cell culture medium was used to prepare different concentrations of OxLDL at 0mg/L、 100mg/L、 150mg/L ,then incubated with NRK-52E cells at different concentrations of OxLDL for 2 and 4 days; Collected cells from each group, The expression of CD36 in cells was detected by Western-blotting methods, The results showed that the expression of CD36 protein in OxLDL stimulated NRK-52E cells with 150 mg/L-OxLDL was higher than that in 0 mg/L OxLDL group after 2 days and 4 days ($P<0.05$); The 4-day group was more obvious than 2-day group; Stimulating NRK-52E cells with 100 mg/L of OxLDL for 2 days, The expression of CD36 protein in the 100 mg/L group also increased ($P <0.05$), but the increase was relatively small compared with 150 mg/L ($P <0.05$); The CD36 protein expression of NRK-52E cells stimulated with 100 mg/L after 4 days was not statistically significant ($P >0.05$) . (Figure 3.2 a-b)

3.3.1 Total Nrf2 protein level after OxLDL treatment in NRK-52E cells

Using cell culture medium, the OxLDL was prepared into 50 mg/L concentration and then stimulated NRK-52E cells for 0h、5h、10h、24h、48h respectively ,Then the cells were collected and detected total Nrf2 protein levels in cells, The results showed that there was no significant change from 5 h、10h NRK-52E cells group in total Nrf2 protein level (P >0.05), After 24 hours of treatment, the results showed a decrease change compared with the control group (P <0.05), After 48 hours of treatment, the results showed a decrease change compared with the control group,but not statistically significant (P>0.05). (Figure 3.3.1 a-b)

3.3.2 Nrf2 protein levels in the nucleus and cytoplasm of NRK-52E cells after OxLDL treatment

NRK-52E cells were treated with 150mg/L OxLDL and starvated for 2 days respectively and then extracted nucleoprotein and cytoplasmic protein to detect the Nrf2 protein level respectively. The results show that Nrf2 protein in the NRK-52E nucleus of serum starvation group and OxLDL treatment group was higher than that of control group (P<0.05), and Nrf2 protein in cytoplasm of serum starvation group and OxLDL treatment group was not higher than that of control group (P >0.05). (Fig.3.3.2a-b)

3.4. KeapI effects on CD36、E-cadherin protein expression

3.4.1 Effect of Nrf2-silencing on CD36-protein expression

CD36 protein expression in NRK-52E cells after KeapI over expressed and untreated group then OXLDL stimulation (Fig.3.4.1a-b)

The NRK-52E cells were treated by KeapI overexpression(305-keap1 group) and untreated group(305-empty group) respectively ,then stimulated NRK-52E cells using 0mg/L, 150mg/L OxLDL for 2 and 3 days respectively ;The results show that CD36 expression in NRK-52E cells treated with 0mg/L OxLDL did not change in NRK-52E cells of Nrf2 inhibitor Keap1 overexpression treatment(305-keap1 group) compared with untreated control group(305-empty group); CD36 expression reduced after 150mg/L OxLDL stimulation for 2 and 3 days in KeapI-over-expressed NRK-52E cells groups compared with untreated control NRK-52E cells groups (P<0.05); (Fig. 3.4.1a-b)

3.4.2 Effect of Nrf2-silencing on CD36-mRNA

The CD36-mRNA in renal tubular cells treated with Keap1-overexpression (305-keap1 group) and OxLDL stimulation (305-Keap1-OxLDL group) compared with empty control groups (305-empty group, 305-empty-OxLDL group) (Fig.3.4.2a-c)

To determine whether the activation of Nrf2 was responsible for the renal tubular cells,mediated by OxLDL ,we treated renal tubular cells by Nrf2 inhibitor Keap1 (305-keap1 group), and the control group (305-empty group)was not treated, then both group cells were stimulated by OxLDL (305-Keap1-OxLDL

group), the results show that the CD36-mRNA of keap1-group was lower than that of empty group; and Keap1-OxLDL group showed a remarkble reduction as well comparing empty control group.

3.5 E-cadherin protein expression in tubular cells treated by Keap1

To determine the relation between the Nrf2 and renal tubbular fibrosis,we treated the tubular cells by Nrf2 inhibitor 305-keap1(305-keapI group),and then detected the fibrosis factor E-cadherin protein expression in the treated cells group and control group,the results show that E-cadherin protein expression in 305-keapI group reduced compared with that in control group.

Discussion

Hyperglycemia and hyperlipidemia often occur and persist in diabetic nephropathy. There is evidence that abnormal lipid metabolism is an important factor affecting oxidative stress in diabetes mellitus and plays an important role in the occurrence and development of renal interstitial damage[1] [2]Oxidized low density lipoprotein (OxLDL) is the main damage factor of abnormal lipid metabolism. OxLDL is recognized as a serum indicator to observe oxidative stress and used in cytological studies of oxidative stress as well.However, it is not clear how abnormal lipid metabolism leads to oxidative stress and ultimately renal interstitial injury and the signaling pathway was not clear so far.

The past investigations demonstrated that OxLDL play a crucial role in the pathogenesis of atherosclerosis,[3][4][5] regulate the expression of growth, transcription factors and cytokines and affect cell activity.[6] [7]Past cytological studies have found that OxLDL in preadipocytes is a specific, saturable process mediated by CD36 that affects adipose tissue homeostasis by inhibiting the differentiation of preadipocytes[8]. Massimo's and others studies confirm that OxLDL regulate the CD36 ,OxLDL receptors through Nrf2 pathway in preadipocytes[8]. Our study aimed to investigate the OxLDL mechanism and signaling pathway in renal tubular cells in the same high glucose and high fat state.

Our study found that CD36 protein ,being OxLDL membrane receptor,increased in the tubular NRK-52E cells treated by OxLDL.This result demonstrated that CD36 was upregulated by OxLDL ,and correlated with OxLDL concentration and time, 150mg/L OxLDL influenced and upregulated CD36 was more obvious than 50mg/L□100mg/L OxLDL; CD36 protein expression was higher upregulated according to longer time and higher concentration of OxLDL;CD36 protein chanded at least 50 mg/L 24 hours OxLDL intervention , demonstrating that OxLDL mediated damage to renal tubules progress slowly; and our study firstly confirmed that OxLDL receptor, CD36 in tubular cells play a role being an oxidized LDL receptor during hyperlipemia and oxidative stress, CD36 upregulation suggests a coping mechanism after excessive OxLDL in hyperlipidemia, and thus reduce the risk of renal tubular injury caused by OxLDL.

We also found that not only CD36 was up-regulated , but also Nrf2 changed in high OxLDL environment, There was no significant change in the level of total Nrf2 protein after 50mg/L 5 h□10h OxLDL

treatment,but decreased at 24 and 48 hours of treatment. This result mean that OxLDL mediated Nrf2 pathway need enough long time ,This is consistent with previous studies in preadipocytes[9][10][11][12].

Our studies also show that Nrf2 protein level in nuclear of NRK-52E cells treated with OxLDL and starvation for 2 days is relatively elevated, while Nrf2 protein level in the cytoplasm is relatively decreased, indicating that OxLDL stimulation initiates oxidative stress and activates Nrf2 signaling pathway by causing Nrf2 transfer from cytoplasm to nucleus, continuing to regulate downstream gene HO-1 then .[8] Our study may confirm Nrf2 regulation mechanism in renal tubular cell in oxidative stress and abnormal lipid metabolism condition, The linkage of CD36Nrf2 protein levels after OxLDL treatment in renal tubular cells probably suggests that OxLDL initiate the Nrf2 protective mechanism by up-regulating CD36 .

Cytoplasmic inhibitory factor, kelch-like ECH-associated protein 1(Keap1), is a chemoreceptor inducing Nrf2 activation by oxides and electrophiles, and also a regulator of Nrf2 degradation mediated by ubiquitin-protease system , which plays a central role in regulation of Nrf2 signaling pathway[11][12]. Keapl has been shown to be a shuttle protein capable of reciprocating between the cytosol and the nucleus.once the intracellular redox homeostasis is restored, Keapl is able to shuttle from the cytosol into the nucleus,move up Nrf2 from ARE,and then the complex of Nrf2-Keapl shift out of the nucleus again.the Nrf2-Keapl complex is again degraded in the cytoplasm by Cul3-Rbxl-E3 dependent ubiquitin enzyme mechanisms. Nrf2 maintain low level expression again, Nrf2 signal pathway is closed.[13][14] Therefore, Keap1 can be considered as a molecule that inhibits Nrf2 transfer to the nucleus and work.. therefore Keap1called a Nrf2 inhibitor.[15-17]

Our study results showed that CD36 mRNA and protein expression decreased in the NRK-52E renal tubular cells treated with Nrf2 inhibitor keap1; After OxLDL stimulation and then keap1 overexpression treatment, CD36 mRNA and protein expression of renal tubular cells also decreased.These results suggest that Nrf2 play a role in regulating OxLDL receptor--CD36 ,activate the protective mechanism aiming to oxidative stress by up-regulating CD36 .

Strutz etc[18] firstly proposed the concept of tubular phenotypic transformation (Epithelial-mysenchymal transition,EMT) in model of renal tubular basement membrane cells, which can be transformed into MFB cells and express vimentin, a interstitial marker[19]The main feature of renal tubular cells after EMT is reduction or lost of E- calcium adhesion protein (E-cadherin) expression[20],renal tubular epithelial cells maintain the integrity of cell morphology, structure and function through various cell adhesion mechanisms such as E-cadherin, and the loss of E-cadherin leads to the transformation of primary epithelial cell characteristics to non-epithelial cell functions[21]Therefore, E-cadherin is the main molecule marker of epithelial adhesion and phenotype and can be used as an important marker of EMT.Reduction of E-cadherin expression can be used as a marker of renal tubular EMT and fibrosis. Therefore, we chose E-cadherin as the molecule marker of renal tubular cell fibrosis caused by OxLDL in this study[21-22].

Our study results showed that E-cadherin protein expression decreased in renal tubular cells treated with Keap1.This result may demonstrate that Nrf2 probably plays an inhibitory role in the EMT process in renal tubular cells,and confirm that Nrf2 has a certain inhibitory effect on renal interstitial tubules fibrosis process, and play an protective effect on renal tubulointerstitium.The protective effect of Nrf2 is finally reflected in the inhibition of EMT response by reducing the loss of E-cadherin and inhibiting the fibrosis process in renal tubular cells .

Overall, the above results suggest that Nrf2 plays a crucial role as a transcriptional regulator of oxidative stress in OxLDL induced renal fibrosis; excessive OxLDL in renal tubular cells not only up-regulate CD36, but also activate the Nrf2 signaling pathway, leading to Nrf2 transferring from cytoplasm to nucleus, and this transferring may regulate CD36 later, mainly cause CD36 up-regulation,thus strengthening the ability of CD36 defensive clearance for OxLDL , and then play a certain protective role; E-cadherin should be most worthy of further study and discussion on how to minimize tubular cell injury and how to delay nephropathy finally. More research should focus on the future therapeutic targets.

Declarations

Acknowledgement

I really appreciate Professor Shan Lin for her support during my study.Without her help ,I could not complete my experiment and clinical work at the same period.

Data statement

All data included in this study are available upon request by contact with the corresponding author.

Statement of Ethics

The research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki.

Conflict of Interest Statement

All the authors declared no competing interests.

Funding Sources

This work was supported by funding from Tianjin Municipal Health Bureau (2011 KZ105)

Author Contributions

Xiang-Long was responsible for the study concept and design and drafted the manuscript.Zhe-Liu support the concept and supervised the experiments .Xiang-Long and Yanan-Sun performed the experiments and collected data. Hong-Zhang gave advices.

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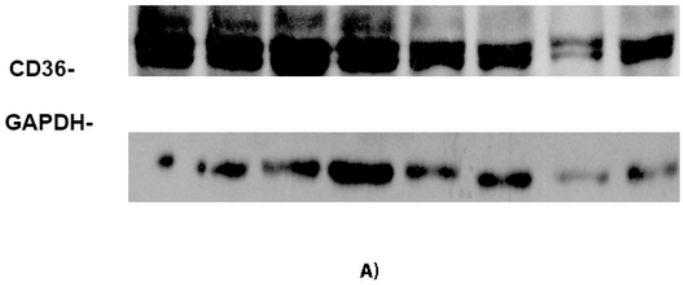
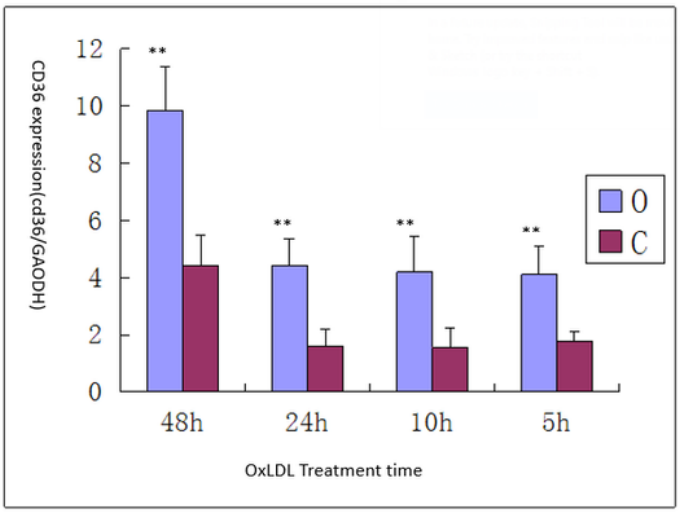
Figures



A)



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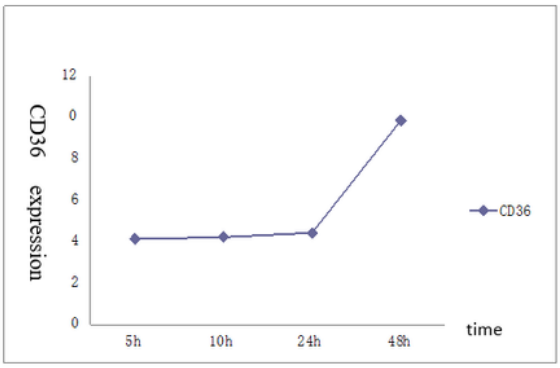


C)

Figure 1

a CD36 expression in NRK-52E cells incubated by 50mg/L OxLDL(C:control) The expression of CD36 in NRK-52E cells incubated by 50mg/L OxLDL ,OxLDLD was made to different concentration ,and incubated NRK-52E cells for different time 0h、5h、10h、24h、48hrs.Collected all groups of cells and test CD36 level by Western-blotting、the results show the CD36 level of 5h、10h、24h、48hours groups increased (P＜0.01);and CD36 level of 24h、48hrs groups more apparently. b CD36 expression in NRK-52E 50mg/L

OxLDLO:OxLDL(C:control ; *:P0.05;**: P0.01 C CD36 expression in NRK-52E cells incubated with different time of OxLDL

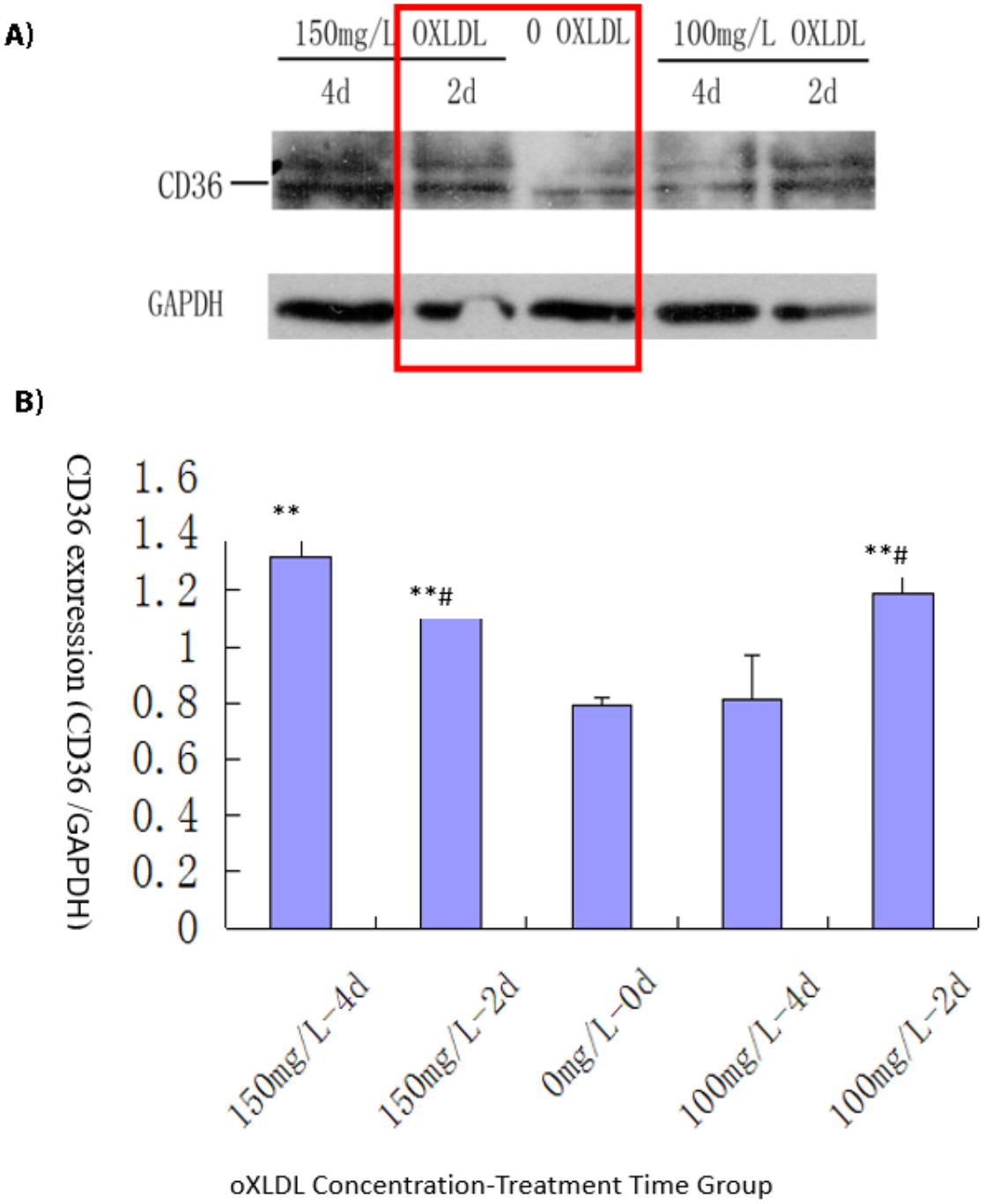


Figure 2

a expression of CD36 after treatment of NRK-52E cells with different concentrations. b expression of CD36 after treatment of NRK-52E cells with different concentrations *: P <0.05; **: P<0.01; #:2 d compared with the 4-day group).

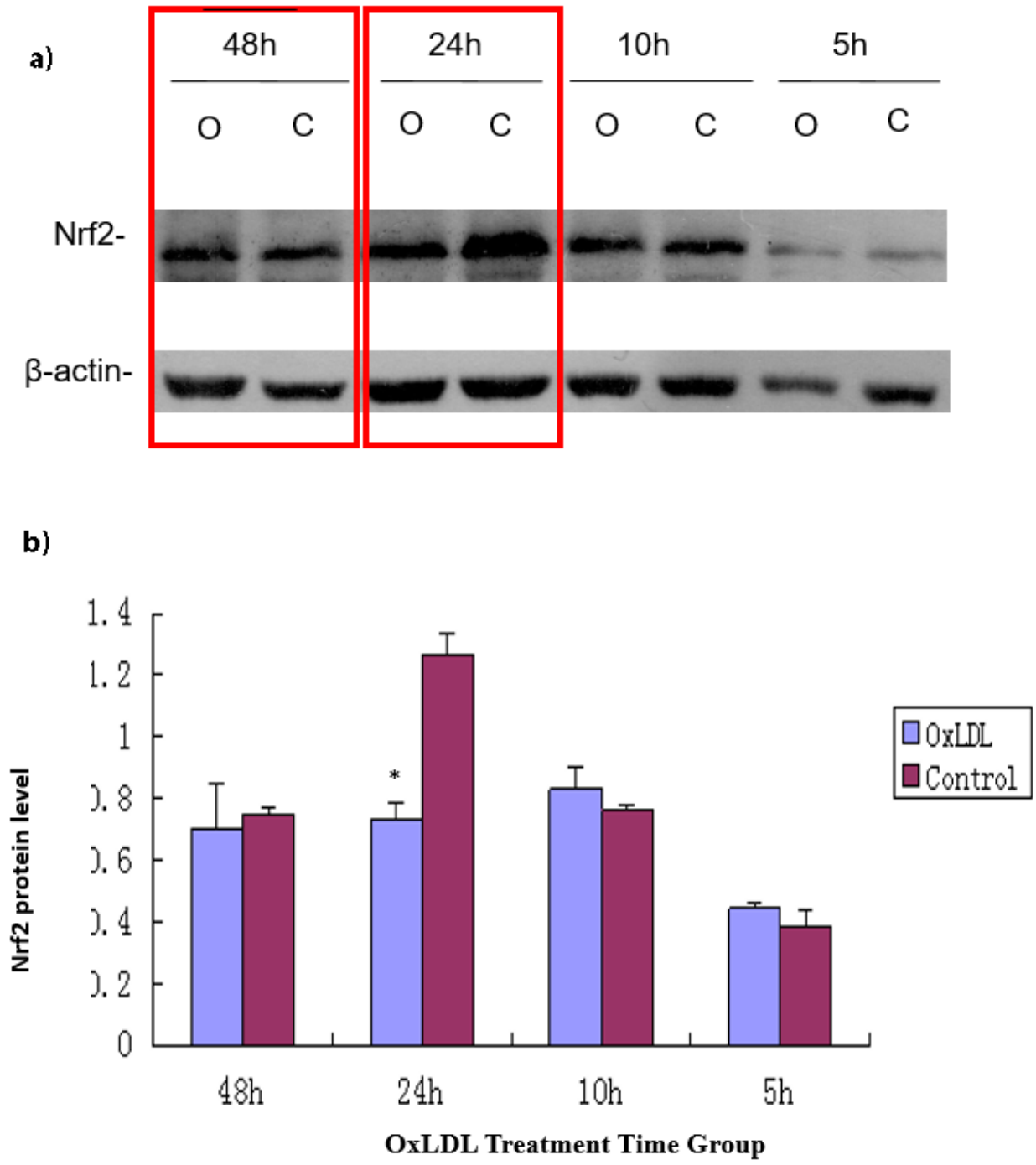


Figure 3

a Total protein levels detected a 50mg/L O x LDL treated NRK-52E cells (note: C□control;) Figure 3.3.1 O□O x LDL processing). b Total protein levels detected b 50mg/L OXLDL treated NRK-52E cells (note: C□ control;O□OXLDL treatment; * Compared with the control group P <0.05))

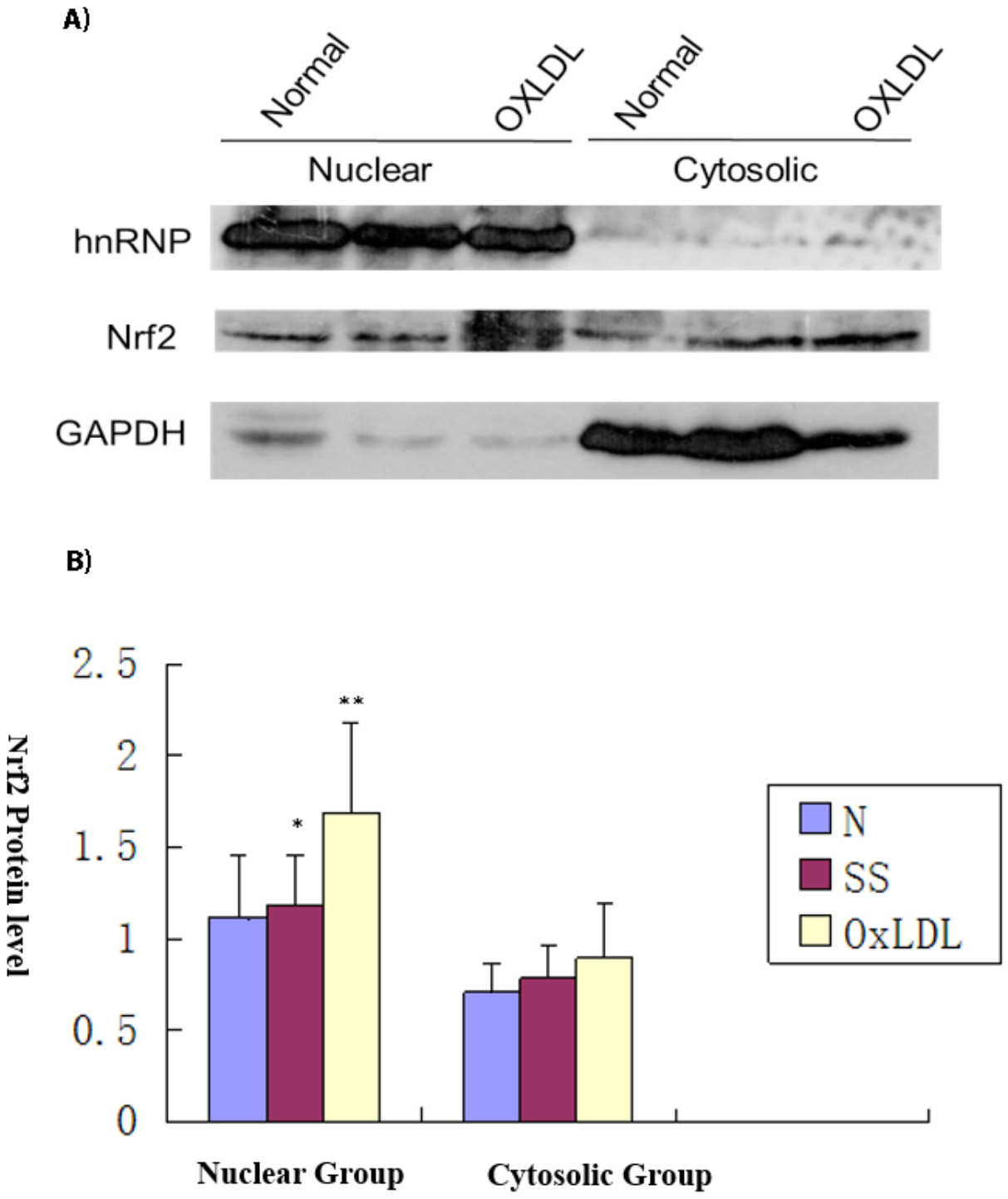


Figure 4

a Nrf2 protein expression in nuleus and cytoplasm of NRK-52E cells treated with 150mg/L OxLDL and serum starvation (serum starvation,SS) b levels of nuclear protein and cell protein were detected after 150mg/L OxLDL and serum starvation Nrf2 treated cells. (Note: N： normal control group; SS： serum starvation group; OxLDL：OxLDL processing group; * compared with the control groupP＜0.05, ** Compared with the control group P<0.01).

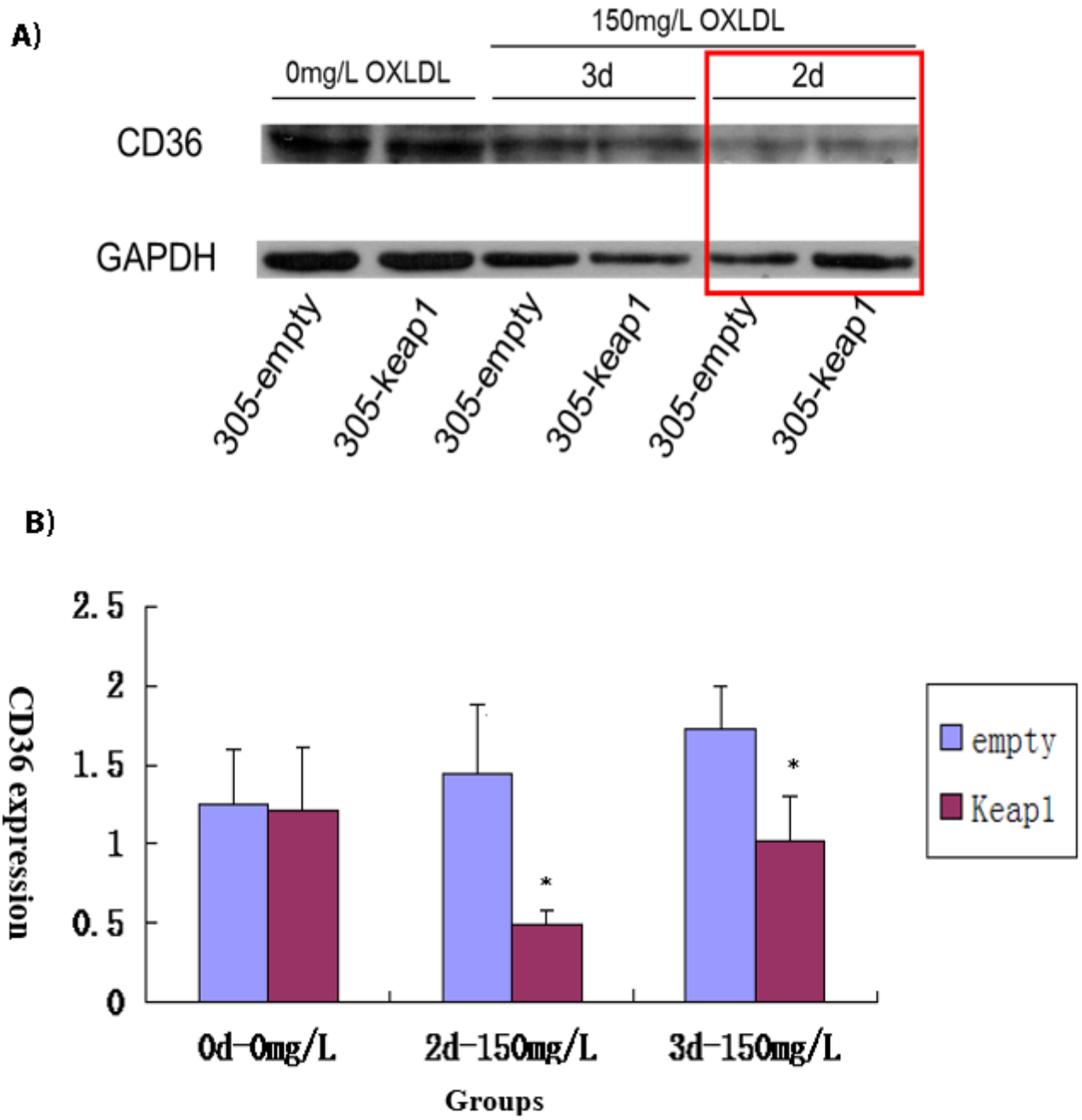
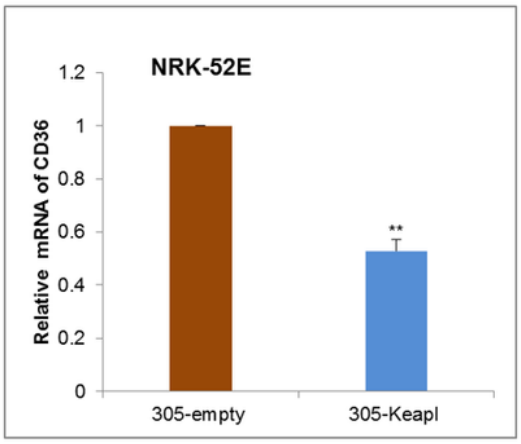
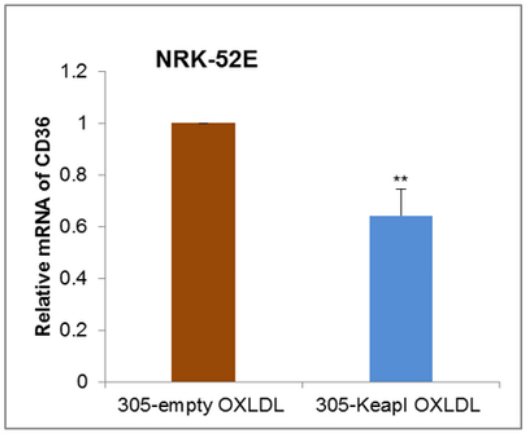


Figure 5

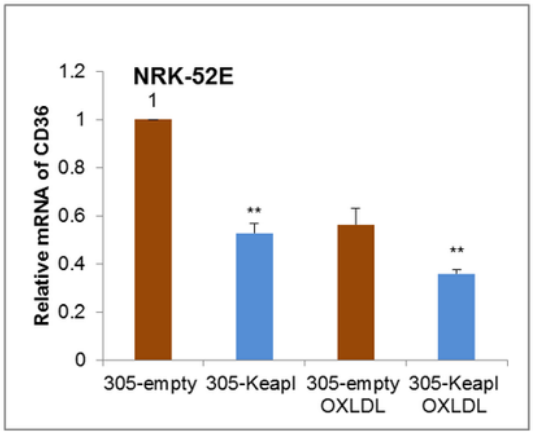
a CD36 expression in NRK-52E cells after Keapl over expressed and untreated and OXLDL stimulation b CD36 expression in NRK-52E cells after Keapl over expressed and and untreated control NRK-52E cells groups then OXLDL stimulation(Note :*: P <0.05 compared with control group ;** compared with control group, P <0.01).



A)



B)

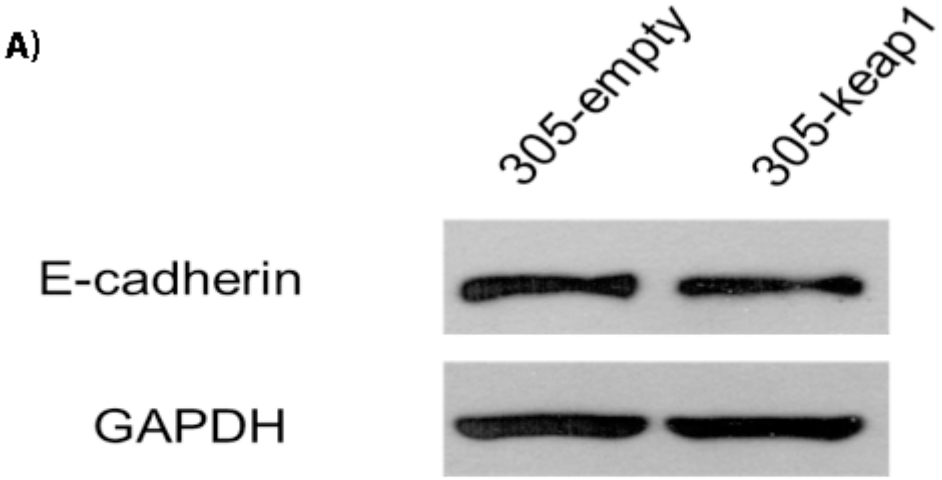


C)

Figure 6

a. Comparison of a treatment group (305- keap1 group) with untreated control group (305-empty group) (Note :*: P<0.05 compared with control group ;** compared with control group, P<0.01). b.Comparison of CD36-mRNA in Nrf2 inhibitor 305- Keap1 transfection +OxLDL treatment group (305-Keap1-OxLDL group) with 305-keap1 untreated control group (305-empty OxLDL group) (note :*: comparison with control group, P<0.05; ** Compared with the control group, P<0.01). c. Comparison of CD36-mRNA in Nrf2

inhibitor 305- Keap1 transfection and OxLDL treatment group (305-Keap1-OxLDL group) with only 305-keap1 transfected treatment group (305-keap1 group) , 305-keap1 untreated control group (305-empty group),305-Keap1 untreated but OxLDL treatment group (305- empty Keap1-OxLDL group)(note :*: comparison with control group, P<0.05; ** Compared with the control



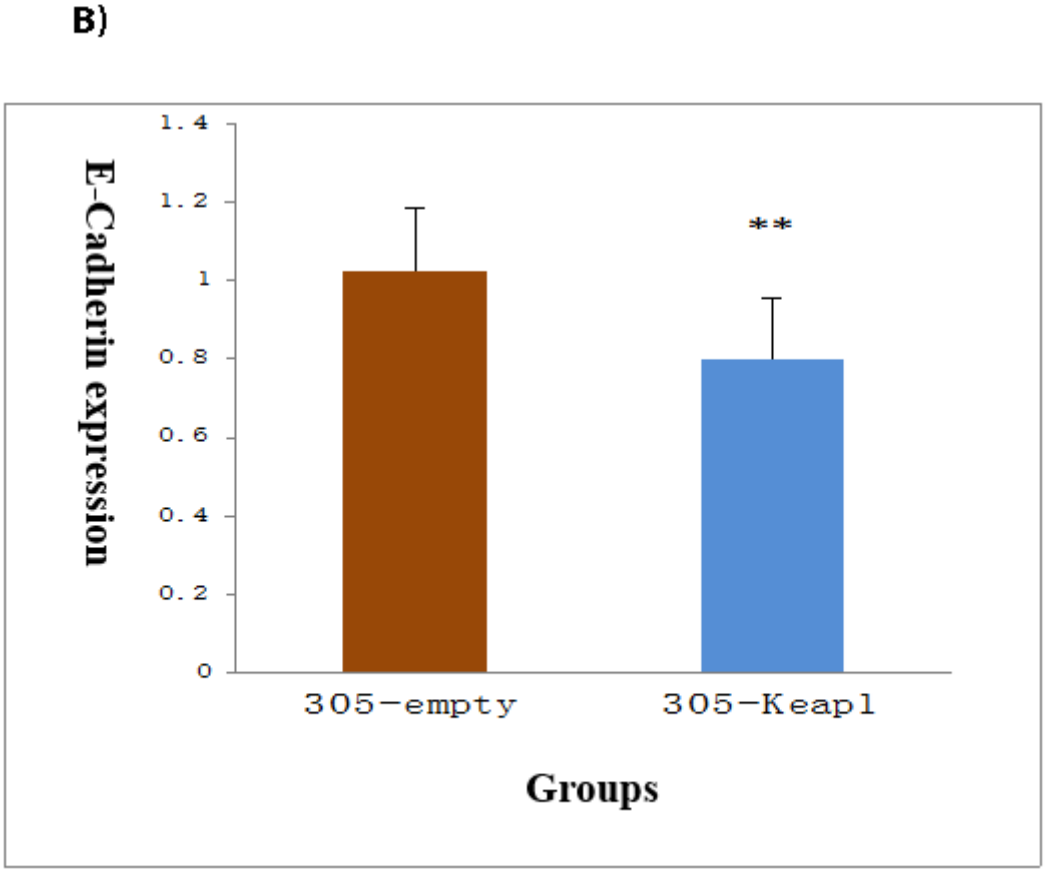


Figure 7

a E-cadherin expression in 305-keapI NRK-52E cells group compared with control group cells b E-cadherin expression in 305-keapI NRK-52E group compared with control group cells(Note: control group, $P<0.05$).