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Original scientific paper

Cross talk of c-Jun N-terminal kinase and p38 map kinase modulate insulin and TNFα-stimulated VCAM-1 expression in rat aorta endothelial cells

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Abstract

VCAM-1 cell surface expression was determined by flow cytometry in rat aorta endothelial cells stimulated with insulin and/or Tumor Necrosis Factor-a in the absence or presence of short-hairpin RNA inhibitors of c-Jun N-terminal Kinase (JNK) and p38 MAP Kinase. Cells transfected with insulin alone exhibited moderate increases in cell surface VCAM-1 expression, whereas cells stimulated with TNFα alone or in combination with insulin, exhibited a significant (P, 0.05) increase in VCAM-1 surface expression. Cells transfected with shJNK alone showed increased VCAM-1 expression at the cell surface as compared to mock-transfected positive controls. In contrast, cells transfected with shp38 exhibited significant decreased insulin-stimulated VCAM-1 expression at the cell surface, but only moderate decreased VCAM-1 expression in cells stimulated by TNFα alone in combination with insulin. Interestingly, in cells first tranfected with shJNK and then shp38, VCAM-1 expression appeared to increase in an additive fashion. In contrast, cells transfected with shp38 first then shJNK, VCAM-1 expression exhibited increased VCAM-1 expression even in the presence of shp38. One can conclude that JNK is a potent negative regulator of insulin- and TNFα-stimulated cell surface expression of VCAM-1, whereas p38 is mild positive regulator of insulin-, but not TNFα-stimulated VCAM-1 expression. JNK appears to be a more potent mediator of insulin- and TNFα-stimulated cells surface VCAM-1 expression than p38 MAP Kinase. Thus, it could be a therapeutic target for amelioration of inflammation-associated VCAM-1 expression and its sequelae of events that lead to atherosclerosis.

Keywords

JNK; p38, TNFα; VCAM-1; Hyperinsulinemia; Atherosclerosis

Introduction

The transduction of external signals to internal events in cells is mediated in part by a family of enzymes called protein kinases. Kinases transfer the gamma phosphate of ATP to their respective substrates, thereby activating these substrates and their downstream effectors. The super-family of mitogen-activated protein kinases (MAPK) is one such group. Interestingly, some kinases inhibit their downstream effectors by phosphorylation of their respective substrates. For example, the phosphorylation of glycogen synthase (GS) by glycogen synthase kinase deactivates GS [1]. The pathophysiology of many diseases involves abnormal kinase activity. Thus, the characterization of kinase activity in cellular pathways and their pathophysiological sequelae have led to the better understanding of normal and pathological events,

respectively.

Atherosclerosis is a major consequence of vascular dysfunction and leads to, but is not limited, to vascular smooth cell proliferation, lack of vascular compliance, endothelial cell proliferation, neointima formation and vascular inflammatory events. One particular characteristic of endothelial pathology is the increased expression of cellular adhesion molecules (CAMs) at the surface of endothelial cells, which are located at the margins of the intima in vessels [2-4]. The expression of the CAMs is mediated in part by the activity of intracellular endothelial cell kinases, which convey receptor activation at the cell surface to downstream effectors, which in turn upregulate the production and expression of cell surface molecules.

Recently, we reported that ERK2 and Akt are negative regulators of insulin- and TNFα-stimulated VCAM-1 [5] expression. It is not uncommon for kinases to "cross-talk" and modulate the activities of downstream effectors. We report here that c-Jun N-terminal kinase (JNK) and p38 map kinase (p38) also modulate insulin and TNFα-stimulated VCAM-1 expression, but in a different aspect.

Materials and methods

Materials

All general lab reagents were purchased from Sigma-Aldrich (St. Louis, MO). Primary rabbit antibodies to JNK (9258s), p38 (9212s), and alpha-tubulin (2144S) were from Cell Signaling (Boston, MA). The primary rabbit antibody to VCAM-1 (NBP1-95622) was from Novus Biologicals (Littleton, CO) and goat anti-rabbit-secondary antibody IRDye680RD (926¬68171) was from LI-COR (Lincoln, NE). DyLight 488-conjugated anti-VCAM-1 antibody was from Thermo Fisher Scientific (Pittsburgh, PA). Four-well chamber slides were from Thermo Fisher and DAPI Mounting Medium was from Vector Labs (Burlingame, CA). Rat aorta vascular endothelial cells (RAEC) (CRL-1444) were from ATCC (Manassas, VA) and culture medium was from Life Technologies (Grand Island, NY). JNK short-hairpin RNA (shRNA) (KR43333P) and p38 shRNA (KR52703P) were obtained from SA Biosciences/Qiagen ThermoFisher (Valencia, CA). Transfection medium (sc108062) and transfection reagent (sc108061) were from Santa Cruz (Dallas, TX).

Cell culturing

RAEC were cultured in complete growth medium (CGM) [DMEM with 4 mM L-glutamine, 4.5 g/L-glucose and 1.5 g/L sodium bicarbonate) and supplemented with 10 % heat-inactivated fetal bovine serum (HI-FBS) (10438-026) (Life Technologies, Grand Island, NY) and 1 % antimycotic-antibiotic solution (15240-062) (Life Technologies) and cultured at 37 °C, 5 % CO_2 atmosphere.

Western blot analysis

Sodium dodecyl sulfate polyacrylamide electrophoresis was performed on cleared lysates. Western blot analysis was subsequently performed as previously described [6], with the following differences. After completion of protein transfer, membranes were washed in ultra-pure water for 5 min. Membranes were then incubated in 3 % non-fat milk (milk) in Tris-buffer saline (TBS) blocking solution for 1 h at room temperature and then incubated with a designated primary antibody solution (1:1000 in 3 % milk/TBS-T) overnight at 4 °C. Membranes were washed 4 times with TBS plus Tween (TBS-T) for 5 min at room temperature and then incubated with a goat anti-rabbit secondary antibody (1:5000 in 3 % milk/TBS-T) conjugated to fluorochrome IR680RD for 1 hour at room temperature. Membranes were washed 4 times with TBS-T for 5 min each time at room temperature and then incubated with a rabbit anti-tubulin primary antibody solution (1:1000 in 3 % milk/TBS-T) for 3 h at room temperature. After washing the membranes 4

times with TBS-T, the membranes were again incubated with a goat anti-rabbit secondary antibody (1:5000 in 3 % milk/TBS-T) conjugated to fluorochrome IR680RD for 1 hour at room temperature. The membranes were washed 4 times with TBS-T and allowed to dry before performing densitometry. Densitometry was performed using an Odyssey Licor system (Lincoln, NE). Alpha-tubulin protein was used to normalize VCAM-1 signals.

Preparation of shRNA stable cell lines

RAEC were grown to 50-70 % confluence in CGM in 6-well culture plates. Cells were transfected with shJNK or shp38 inhibitory plasmids alone or in combination as previously described [6]. Cells were incubated in CGM containing 2 ug/mL of Puromycin (Sigma-Aldrich) for 2-3 weeks for selection of Puromycin resistant transformants.

Dual transfection of stable cell lines

To examine the effect of simultaneous JNK and p38 knockdown on VCAM-1 expression, the JNK shRNA stable cell line (JNK KD) was transiently transfected with shp38 plasmid and the p38 shRNA stable cell line (p38 KD) was transiently transfected with the shJNK plasmid. These two protocols were carried out in order to see if any differences occurred with respect to transfection sequence. Stable cell lines were transiently transfected with vehicle or shRNA as described above and incubated for 5 hr with the DNA transfection mix. Subsequently the transfection mix was aspirated and replaced with 2.0 mL CGM. Stimulation of cells by insulin and/or TNFα occurred 48 hours after transient transfection was accomplished.

Stimulation of VCAM-1 expression

RAEC were cultured in CGM, whereas shRNA stable cell lines (e.g., JNK KD and p38 KD) were cultured in CGM containing 2 ug/mL Puromycin until assays were performed. After incubating the transfected cells for an additional 48 h, the cells were stimulated with or without insulin (10 nM) 1hour or TNFα (10 ng/mL) alone or in combination for six hours. Thereafter we evaluated for VCAM-1 expression by flow cytometry.

Flow cytometry

Mock transfected RAEC (control), stably transfected JNK knockdown (JNK KD) and p38 knockdown (p38 KD) cell lines were inoculated into 6-well tissue culture dishes, transiently transfected with vehicle, shp38 or shJNK plasmids, respectively, and allowed to settle for 48 hours. Cells were then stimulated without or with insulin or TNFα alone, or combination as described above. The cells were washed twice with 2 mL of 1X PBS (Gibco). The PBS was aspirated and 0.5 mL of Cell Dissociation Solution Non-Enzymatic (Sigma-Aldrich) was added to each well. After incubating the cells at 37 °C and 5 % CO_2 for 30 min, 1 mL of 1 % bovine serum albumin (BSA, Sigma-Aldrich) in PBS was added to the cells and then were gently triturated into a single cell suspension. The cells were transferred to 5 mL Falcon polystyrene round bottom tubes (Thermo Scientific) and centrifuged at 500 x g for 5 min. After aspirating the supernatants, the cells were resuspended in 3 mL 1 % BSA, pelleted at 500 x g by centrifugation, and the supernatants removed by aspiration. The cells were resuspended in 200 uL of 1 % BSA. Two microliters of DyLight 488-conjugated anti-VCAM-1 antibody (Life Technologies, Grand Island New York) were added to each tube and the cells were resuspended by vortexing. The cells were incubated in the dark for 30 min at room temperature. The cells were centrifuged, washed twice with 3 mL 1% BSA and resuspended in 200 uL of 1 % paraformaldehyde (PFA, Electron Microscopy Sciences, Hatfield, PA). After incubating the cells for 5 min at room temperature, the cells were diluted with an additional 300 uL of PBS and analyzed using flow cytometry. The experiments were run on a BD LSRII (BD Biosciences, San Jose, CA). MFI and gating

percentages as part of data analysis was done using BD FACSDiva v6 software.

Chamber slide cell preparation

2 x 10^5 of RAEC control, JNK KD or p38 KD stable cell lines were plated separately in 1 mL of CGM in each well of a 4-well chamber slide and allowed to grow for 24 h, 37 °C and 5 % CO_2. The medium was aspirated and 1.0 mL of fresh CGM was applied to the cells. JNK KD and p38 KD were mock transfected, or transiently transfected with shp38 or shJNK, respectively for 48 hours. Cells were then treated with either vehicle, TNFα (10 ng/mL) or insulin (10 nM) alone, or TNFα (10 ng/mL) plus insulin (10 nM). The medium was aspirated and the cells were washed three times with PBS and then incubated in 400 µL of 4 % paraformaldehyde in PBS for 30 minutes. The medium was aspirated and washed three times with 1 mL of PBS. The final PBS wash was aspirated and 400 µL of a 1:1000 DyLight anti-VCAM-1 antibody solution in 1% BSA was added to each chamber and incubated for 30 minutes at room temperature. The cells were then washed three times with 500 µL of 1 % BSA. The chamber walls were removed and one drop of DAPI Mounting Medium was added to each group of cells on the slide. Cells were then sealed with a glass cover slip using clear nail polish. Slides were kept in a dark refrigerator until microscopic visualization.

Confocal microscopy

A single, non-confluent monolayer of cells was imaged with a Leica TSC SP8X white light laser scanning confocal microscope (Leica Microsystems GmbH. Ernst-Leitz-Straße 17-37 Wetzlar, 35578 Germany). All image acquisitions were carried out using the Leica Application Suite X (version 1.1.0.12420, LASX AF). Excitation of the DAPI channel was accomplished using a 405nm diode laser with an excitation intensity level of 2.67 %. Emission signal was captured with standard PMT Channel 1 and an emission gap of 430 – 480 nm. The Leica Supercontinuum white light excitation laser line (488nm) with 3 % intensity level was used to for Alexa fluor 488. Emission signals were captured with the Leica HyD 2 detector (Hybird 2 PMT) with an emission gap of 505 – 555 nm.

Data analysis

Data were analyzed by either unpaired Student's *t* test (two groups) or ANOVA with subsequent Tukey posttest (several groups) as indicated. A "P" value of less than 0.05 was considered significant. Results were expressed as the mean ± Standard Error of the Mean (SEM) of three or more independent experiments.

Results

In our first set of experiments, we determined which shRNA plasmid clone of each kinase would affect the greatest decrease in total cell content of that kinase (Figure 1). Clone #2 for JNK and clone #1 for p38 were the most effective in downregulating their respective kinases.

Using these two clones we then established stable RAEC cell lines that would constitutively express decreased levels of their respective kinases. RAEC cell lines stably transfected with shJNK and shp38 were denoted as JNK KD and p38 KD, respectively. In contrast, transiently transfected cells were named shJNK and shp38, correspondingly. Additionally, we determined that not only single transfections effected down regulation of the respective proteins, but double transfections down regulated both proteins (Figure 2).

In previous studies, we noted that expression of total VCAM-1 was not necessarily indicative of expression of VCAM-1 at the cell surface [7,8]. Additionally, since the thrust of these experiments was to define changes in amounts of VCAM-1 protein at the cell surface (i.e., where adhesion to monocytes occurs), we performed only flow cytometry and confocal microscopy experiments and not Western blot

analysis.

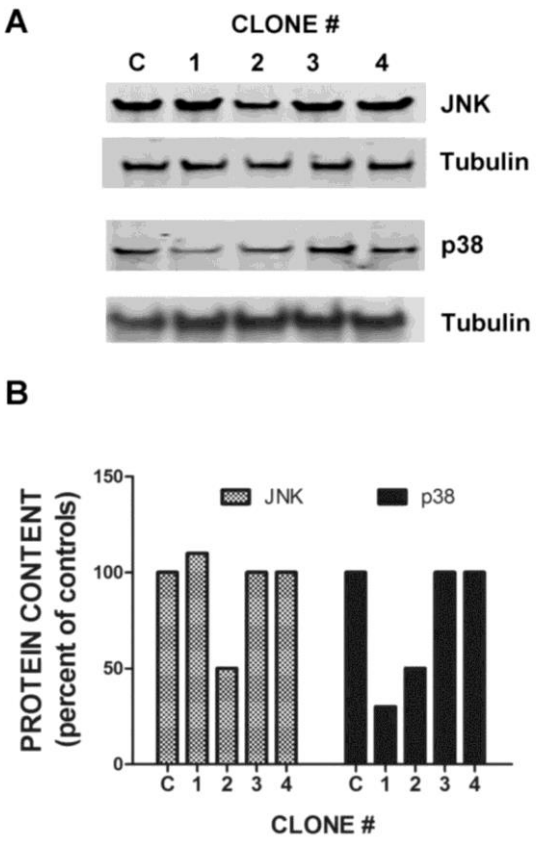

Figure 1. Transfection efficiency and knockdown by shJNK and shp38 clones in RAEC. Cells were transiently transfected with individual shJNK or shp38 clones as described in Methods. Tubulin was used as loading controls. (A) Western blot expression profiles of total JNK and p38 protein indicated changes in total JNK and p38 protein in the presence of designated shRNA clones. Total JNK and p38 kinase proteins were resolved by SDS-PAGE and determined by Western blot analysis. (B) Figure represents the expression of JNK and p38 in the presence of scrambled control (C) clones or designated numbered clones provided by the supplier. The numbers on the "x" axis represent the clone number. The expression of VCAM-1 in mock transfected control (C) cells was set to 100%. Expression of clones 1 - 4 are normalized to the expression of the scrambled shRNA controls (C).

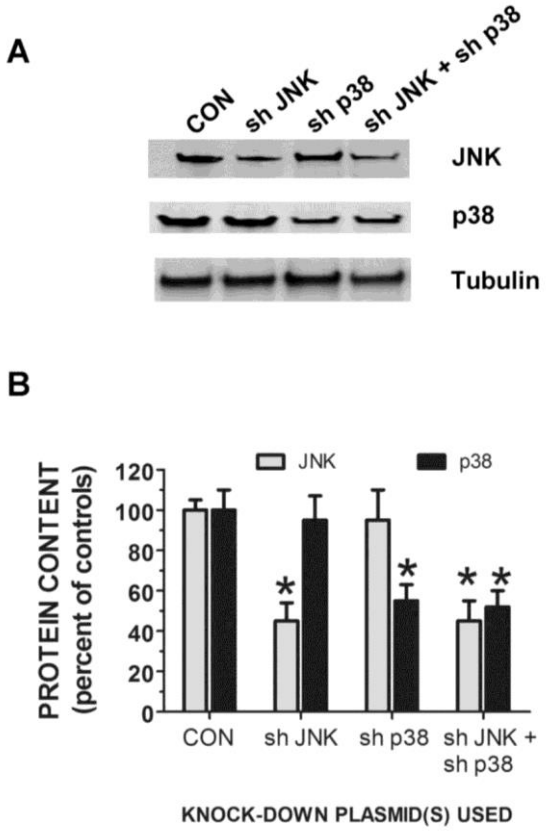

Figure 2. Expression of JNK and p38 total protein in RAEC transfected with shJNK and shp38 plasmids. Cells were transfected with clone-2 shJNK or clone-1 shp38 alone or both and cultured for 24 h. (A) Total JNK and p38 proteins were resolved by SDS-PAGE and determined by Western blot analysis. (B) Graph represents the protein content of JNK (light gray bars) and p38 protein (solid black bars) as percent of controls (CON) and results are expressed as the mean ± SEM for four independent experiments. *, $P < 0.05$.

We challenged cell groups with insulin (10 nM) or TNFα (10 mg/mL) alone or in combination and determined changes in surface VCAM-1 expression via flow cytometry. Interestingly, JNK KD cells transiently transfected with shp38 (Figure 3A) exhibited a different profile from that seen with p38 KD transiently transfected with shJNK (Figure 3B). In all instances, except in insulin only treated cells, VCAM-1 significantly ($P < 0.05$) increased above positive controls. In comparison, VCAM-1 was significantly ($P < 0.05$) decreased in the presence of insulin alone in p38 KD cells as compared to p38 KD positive controls. In contrast, VCAM-1 surface expression neither increased nor decreased significantly in the presence of TNFα alone or in combination with insulin as compared to positive controls in p38KD cells. Yet, in p38 KD + shJNK

cells, TNFα alone and in combination with insulin exhibited significant ($P < 0.05$) increases in VCAM-1 expression as compared to positive controls.

In order to corroborate the flow cytometry results, we performed confocal microscopy of control, JNK KD ± shp38 and p38 KD ± shJNK cells when stimulated with insulin or TNFα alone or in combination (Figure 4). The confocal micrographs appeared to agree with our flow cytometry measurements with regard to expression of VCAM-1 at the cell surface.

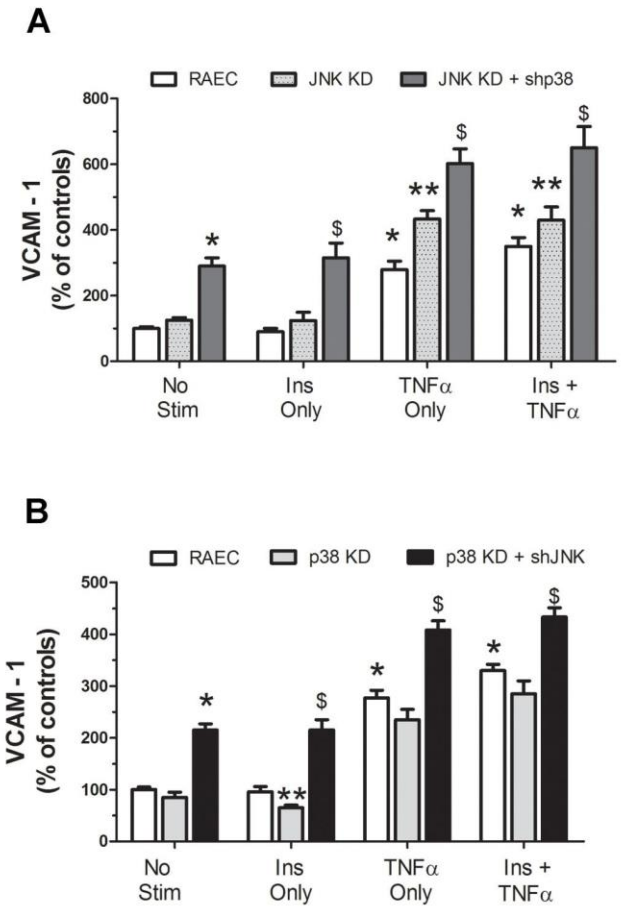

Figure 3. Changes in expression of VCAM-1 on the cell surface of RAEC were measured by flow cytometry. RAEC (controls), JNK KD and p38KD cells were transfected with either scramble control shRNA, shJNK, shp38 or both, depending on the desired combinations. Thereafter, the cells were stimulated with vehicle (PBS) or insulin (10 nM) for one hour or TNFα (10 ng/mL) for 6 hours or both. VCAM-1 expression at the cell surface was analyzed by flow cytometry as described in Methods. (A) Cells were stably transfected with scrambled control shRNA (open bars) or shJNK (JNK KD) (stippled bars) alone or in combination with transiently transfected shp38 (solid gray bars). (B) Cells were stably transfected with scrambled control shRNA (open bars) or shp38 (p38 KD) (light gray bars) alone or in combination with transiently transfected shJNK (solid black bars). Graphs represent the percent of surface VCAM-1 expression compared to unstimulated, mock-transfected cells and results are expressed as mean ± SEM for five independent experiments.*, $P < 0.05$ vs. non-stimulated, mock-transfected controls.**, $P < 0.05$ vs. positive controls. $, $P < 0.05$ versus single-transfected cells. n = 5.

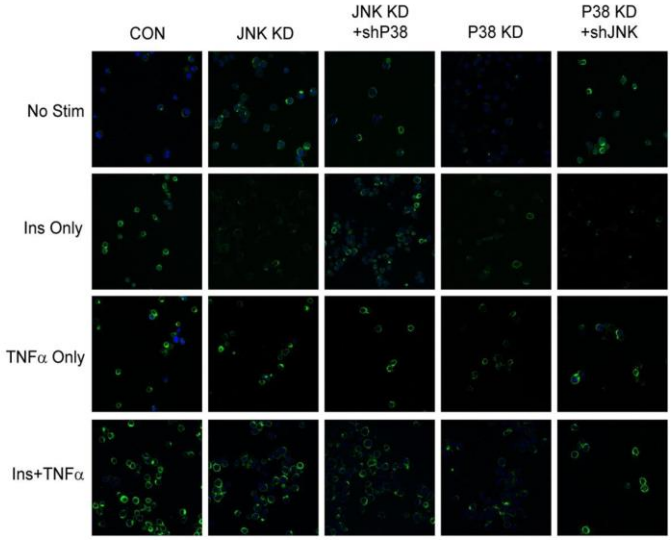

Figure 4. RAEC transfected with either shJNK (JNK KD) or shp38 (p38 KD) alone or in combinations exhibited differential surface expression of VCAM-1 in cells stimulated with insulin and TNFα as determined by confocal microscopy. Cells were plated and stimulated as described in Methods. Surface VCAM-1 was detected by immunocytochemistry as described in Methods and visualized by confocal microscopy. (CON), controls, scrambled shRNA. (JNK KD) cells stably transfected with shJNK. (p38 KD) cells stably transfected with shp38. (JNK KD + shp38) cells stably transfected with shJNK and transiently transfected with shp38. (p38KD + shJNK), cells stably transfected with shp38 and transiently transfected with shJNK. (No Stim), no stimulation by insulin or TNFα. (Ins Only) cells stimulated with 10 nM insulin for 1 hour. (TNFα) cells stimulated with 10 ng/mL of TNFα for 6 hours. (Ins + TNFα) cells stimulated with insulin for one hour and TNFα for six hours.

Discussion

Here we report that JNK is a negative mediator of insulin and TNFα-stimulated VCAM-1 expression. In contrast, p38 kinase appears to be a positive regulator in the insulin signaling system, but plays a less significant role in the TNFα signaling pathway. Additionally, JNK appears to be a more important mediator of VCAM-1 expression than p38 kinase in the insulin and JNK signaling pathways.

The presumed mechanisms for this phenomenon may lie in three regions of the cell: (1) the receptors for insulin and TNFα, (2) the respective proximal/downstream kinase mediators of these receptors and (3) common crosstalk of downstream mediators (i.e. the signal pathway kinases) of these upstream pathways. Since there is no promiscuity between the receptors of insulin and TNFα the only additive effect that could exist would be via common downstream pathways and effectors.

One of the most important mediators of the insulin receptor pathway is the adaptor protein Src homology 2 (Shc) protein [9] and the associated rodent associated sarcoma (Ras) protein [10,11]. In turn these two proteins transduce external hormone information (insulin) to the internal signal pathway mediator JNK [12]. Important mediators of the TNFα receptor are the tumor necrosis factor receptor type 1-associated DEATH domain (TRADD) protein, Inhibitor of KB Kinase (IKK) and JNK [13]. Further downstream of these adaptor proteins are the proximal kinases p38 MAP kinase and Apoptosis Signal-regulating Kinase-1 (ASK1). Both JNK and p38 MAP kinase regulate their respective signal transducer and activator of transcription (STAT) proteins [14].

There are two interesting results noted in this study: (1) in the JNK KD + shp38 or p38 KD + shJNK transfected cells, insulin plus TNFα elicited additive effects of VCAM-1 expression as compared to VCAM-1 expression in insulin or TNFα-stimulated control (mock-transfected) cells; and (2) there appears to be a lack of potentiation of insulin on TNFα-stimulated VCAM-1 expression (Figure 3).

Insulin alone in control stimulated cells does not significantly increase surface VCAM-1 greater than that seen for non-stimulated controls (Figure 3). In JNK KD cells insulin alone did not significantly ($P = 0.08$) increase VCAM-1 expression greater than stimulated controls. In p38

KD cells, insulin significantly ($P = 0.01$) decreased VCAM-1 expression as compared to insulin-stimulated controls, suggesting that p38 is a positive regulator of insulin-stimulated VCAM-1 expression. However, as seen in JNK KD + shp38 cells and p38 KD + shJNK cells, insulin stimulated VCAM-1 expression is significantly ($P < 0.05$) greater than that seen for stimulated controls. These data suggest that p38 regulation of insulin-stimulated VCAM-1expression is very different than that seen for TNFα-stimulated VCAM-1 expression.

TNFα-stimulated VCAM-1 expression shows a different profile than insulin in the two cell lines. First, in control cells TNFα significantly stimulated an increase in VCAM-1 compared to non-stimulated controls (Figure 3). This suggests that TNFα is a positive regulator of VCAM-1 expression. In JNK KD cells, TNFα stimulated and increase of VCAM-1 significantly ($P < 0.05$) greater than that seen in stimulated control cells. Interestingly this effect was not observed in p38 KD cells. However, in JNK KD + shp38 cells and p38 KD cells + shJNK, TNFα-stimulated VCAM-1 expression was greater than that seen for JNK KD and p38 KD cells alone. This suggests again that JNK is a major regulator of TNFα-stimulated VCAM-1 expression.

Another interesting phenomenon was seen in cells stimulated with insulin plus TNFα. Neither in JNK KD cells nor in p38 KD cells did the combination of insulin and TNFα increase VCAM-1 expression greater than TNFα alone. This phenomenon may have occurred because cells were not incubated in insulin for more than one hour. Longer periods (e.g., 12 or 24 h) of insulin stimulation may show different VCAM-1

expression effects and graph profiles. Thus, the detrimental aspects of insulin at short incubation times do not augment the effects of TNFα on VCAM-1 expression. We have begun to address this issue and will report on these results in future communications.

A third issue must be addressed. In our model (Figure 5), p38 acts as a positive regulator of insulin towards the expression of VCAM, but a negative regulator for TNFα. Additionally, both p38 and JNK act negatively on the unknown signal pathway intermediate "A" in the insulin signaling model, but do not appear to have any influence on the intermediate "B". These possible scenarios would account for the differences seen in insulin-stimulated VCAM-1 expression in JNK KD cells versus p38 KD cells.

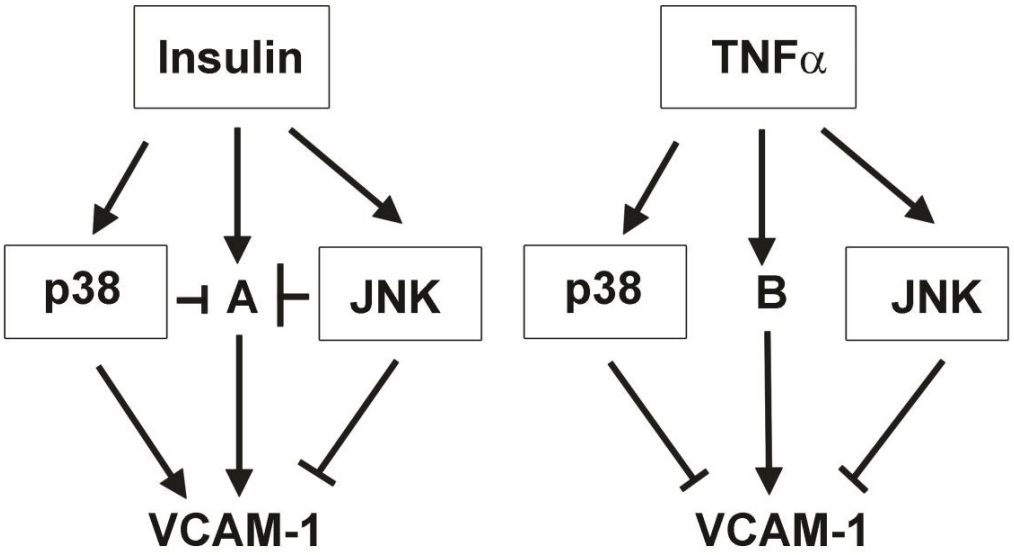

Figure 5. Model. Surface VCAM-1 expression in RAEC stimulated by insulin and TNFα is mediated in part by JNK and p38 kinase. A decrease in JNK alone has a reciprocal increase in VCAM-1 expression. A decrease in p38 alone decreases VCAM-1 expression in insulin-stimulated cells, but not TNFα-stimulated cells. A decrease in both JNK and p38 causes a significant increase in cell surface VCAM-1 expression greater than that seen in JNK KD cells alone.

Finally, it is of interest to note that VCAM-1 expression was significantly ($P < 0.05$) increased in non-stimulated JNK KD + shp38 cells and p38K KD + shJNK cells greater than that seen in non-stimulated controls (Figure 3). This suggests that the lack of regulation from both JNK and p38 can, in and of itself, cause an increase in VCAM-1 expression at the cell surface. This then would be detrimental to the vasculature. Thus, the constitutive expression of JNK and p38 is important to the quiescent properties of vascular endothelial cells.

Diabetes is a risk factor for cardiovascular disease (CVD) [15,16] and within the spectrum of CVD are the effects of atherosclerosis. Atherosclerosis is characterized by remodeling of the arterial vessel causing an increase in the endothelial layer, decrease in lumen diameter and decrease in blood flow; all of which are related to the inflammatory conditions that are regulated in part by hyperinsulinemia and TNFα [16,17]. Here we report that insulin and TNFα stimulated VCAM-1 expression of VCAM-1 in RAEC is mediated in part by the intracellular kinases, JNK and p38. Additionally, these two internal cellular pathway mediators appear to "cross-talk" with one another in regulating the expression of VCAM-1 at the surface of aorta endothelial cells (Figure 5). It is the increase in VCAM-1 that accentuates the inflammatory process seen in arteries of patients with diabetes [18]. Other cellular adhesion molecules, such as intercellular adhesion molecule (ICAM), may be involved with arterial inflammation [19-22].

Future studies are targeted for ICAM experiments. Thus, the activities of JNK and p38 are fodder for

further research and may very well be therapeutic targets for the amelioration of inflammation in the arteries that is associated with atherosclerosis.

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