Nuclear-factor κB p65 is a mediator of insulin and tumour necrosis factor-alpha stimulated vascular cell adhesion molecule-1 expression in vascular endothelial cells

DZ Mackesy¹, ML Goalstone¹,²*

Abstract

Introduction
Type-2 Diabetes Mellitus is a combination of disorders that includes, but is not limited to, hyperinsulinaemia, dyslipidaemia and insulin resistance. Among the sequelae of these pathologies are inflammation and atherosclerosis. Whereas the canonical intracellular mediator of inflammation is Nuclear-Factor kappa-B (NFκB), its role in atherosclerosis has not been fully characterised. The aim of this study was to determine that nuclear-factor κB p65 is a mediator of insulin and tumour necrosis factor-alpha stimulated vascular cell adhesion molecule-1 expression in vascular endothelial cells.

Materials and methods
Vascular cells were treated with either insulin (1 nM) or Tumour Necrosis Factor-alpha (TNFα) (1 ng/mL) alone or in combination in the absence or presence of intracellular kinase biochemical inhibitors or different isoforms of a genetic inhibitor of NFκB expression in order to determine changes in Vascular Cell Adhesion Molecule-1 (VCAM-1) expression.

Results
We observed a considerable amount of cross talk between kinases led to either significant decreases or increases in VCAM-1 expression, depending on the kinases inhibited. Additionally, we determined that both insulin and TNFα-stimulated increases in VCAM-1 expression. Yet, inhibition of two different intracellular kinase pathways had significantly different effects on insulin and TNFα-stimulated VCAM-1 expression. Interestingly, only the p65 genetic inhibitor isoform of NFκB significantly decreased TNFα-stimulated VCAM-1 expression. In contrast, the p65 isoform genetic inhibitor moderately, but not significantly, effected insulin-stimulated VCAM-1 expression.

Conclusion
Intracellular kinases are not single pathway mediators of extracellular signals to intracellular events, but are inter-related highways that communicate to each other to modulated cellular responses to their environment. Interestingly, the expression of the cell surface adhesion molecule, VCAM-1, is in part mediated by the canonical mediator of inflammation, NFκB. Although NFκB regulates TNFα-stimulated VCAM-1 expression, it does not mediate insulin-stimulated VCAM-1 expression. This suggests that insulin-stimulated VCAM-1 expression is regulated in part by an NFκB-independent mediator, yet to be determined.

Introduction
Type-2 Diabetes Mellitus (T2DM) is a constellation of disorders that includes, but is not limited to, hyperinsulinaemia, dyslipidaemia and insulin resistance. These pathologies are risk factors for retinopathy, neuropathy and cardiovascular events. Vascular complications are the leading cause of morbidity and mortality in people with diabetes.

Atherosclerosis is a major consequence of vascular dysfunction and in part comes from a collection of players that leads to, vascular smooth cell proliferation, lack of vascular compliance, endothelial cell (EC) remodelling, and increased expression of EC cell surface molecules. One particular characteristic of atherogenesis is the increased expression of cell surface cellular adhesion molecules (CAMs) at the surface of vascular endothelial cells.

Although insulin is considered to be an anti-atherogenic hormone, other studies have suggested that long-term (i.e., chronic) insulin resistance accompanied by hyperinsulinaemia contributes to the pathogenesis of atherosclerosis. Hyperinsulinaemia appears to augment the effects of inflammatory cytokines, thereby significantly increasing the expression of CAMs such as vascular cell adhesion molecule-1 (VCAM-1)²⁴.

One such inflammatory cytokine is tumour necrosis factor-alpha (TNFα). TNFα is secreted by mature macrophages and endothelial cells during the progression of atherosclerosis. Interestingly, TNFα activity is linked to insulin resistance¹³, which is mediated in part by the pathways associated with extracellular signal-regulated kinases (ERK) such as ERK1/2, phosphatidylinositol-3 kinase (PI3K), p38 kinase, c-jun N-terminal kinases (JNK) and other downstream effectors such as nuclear-factor kappa-B (NFκB)¹⁴.

NFκB is a protein complex that controls the transcription of many genes such as intercellular adhesion molecule-1 (ICAM-1), interferon-gamma (IFN-γ), interleukin-8 (IL-8), TNFα.
and vascular cellular adhesion molecule-1 (VCAM-1)\(^1\). Although it has been shown that NFκB plays a key role in regulating immune responses to infection\(^16,17\), NFκB is also linked to the pathogenesis of inflammation\(^17,18\). NFκB is a family of isoforms that shares a Rel homology domain in the N-terminus\(^15,19\). Known isoforms of NFκB are RelA (p65), RelB, c-Rel, and the precursor isoforms, p105 and p100, which eventually form the mature isoforms of p50 and p52, respectively.

This study was performed in order to determine (1) which isoforms of NFκB were activated in the presence of insulin and TNFα, (2) what effects do kinase inhibitors have on insulin and TNFα-stimulated NFκB nuclear import, and (3) the effects of a genetic inhibitor of NFκB on insulin and TNFα-stimulated VCAM-1 expression. Here we show that (1) only the p65 isoform of NFκB (p65-NFκB) was significantly activated by insulin and TNFα; (2) Wortmannin increased insulin and TNFα-stimulated nuclear import of p65-NFκB; and (3) genetic inhibition of p65-NFκB significantly decreased TNFα-stimulated, but not insulin-stimulated, total protein and surface expression of VCAM-1.

Materials and methods

The protocol of this study has been approved by the relevant ethical committee related to our institution in which it was performed.

Materials and reagents

All general lab reagents were purchased from Sigma–Aldrich (St. Louis, MO). Primary antibodies to VCAM-1 proteins and conjugated to FITC were from BD Biosciences (San Jose, CA). The primary rabbit antibody to VCAM-1 (NB1-95622) and its respective goat anti-rabbit-HRP secondary antibody (NB730-H) were from Novus Biologicals (Littleton, CO). VCAM-1/FITC-conjugated primary antibodies (553332) were from BD Biosciences (San Jose, CA). Rat aorta vascular endothelial cells were from ATCC (Manassas, VA) and culture medium was from Gibco/Invitrogen (Carlsbad, CA). Kinase inhibitors PD98059 (for MEK1/2) and Wortmannin (for phosphatidylinositide 3-kinase [PI3K]) were from Cell Signalling (Danvers, MA). SB203580 (p38 MAPK inhibitor) and SP600125 (JNK inhibitor) were from EMD-Calbiochem (Gibbstown, NJ). TNFα was from Roche Applied Science (Indianapolis, IN) and insulin was from Sigma–Aldrich (St. Louis, MO).

Cell culturing

Cells were cultured in complete growth medium (CGM) [DMEM with 4 mM L-glutamine modified by ATCC to contain 4.5 g/L glucose, 1.5 g/L sodium bicarbonate and supplemented with 10% heat-inactivated foetal bovine serum (Gibco/Invitrogen, Carlsbad, CA) and 1% Antimycotic-Antibiotic solution (Gibco)] and cultured at 37°C, 5% CO\(_2\), atmosphere. Cells were then cultured in serum-free medium (SFM) for 24 h, pre-treated in the absence or presence of indicated inhibitors for an additional hour, and then incubated in this same medium without or with insulin (1 nM) or TNFα (1 ng/mL) alone or in combination for designated times. Cells were harvested for either Western blot analysis, transfection or flow cytometry.

Flow cytometry

Cells were grown in CGM. Growth medium was replaced with serum-free medium (SFM) for 24 h. Cells were incubated in SFM in the absence or presence of designated inhibitors for 1 h, then treated without or with insulin or TNFα alone or in combination for indicated times. Cells were carefully lifted from culture plates using NO-ZYME (Cat# 59226C) (Sigma, St. Louis, MO). Cell counts were performed using Trypan blue and a haemocytometer. Equal numbers of cells (2 × 10\(^6\) cells) from each treatment group were placed into 1.5 mL Eppendorf tubes. Cells were centrifuged at 500 × g for 6 min. The supernatants were removed and cells were resuspended in 100 μL of SFM. Four microlitres of VCAM-1/FITC-conjugated primary antibodies (BD Biosciences, San Jose, CA) were added to each 100 μL of cell suspension containing 2 × 10\(^6\) cells (otherwise 2 μL of antibody per 1 × 10\(^6\) cells) and incubated on ice for 30 min. Two millilitres of Fluorescence-Activated Cell Sorting (FACS) solution [1% BSA in PBS] were added to the cold cell antibody solution followed by centrifugation at 500 × g for 6 min. The supernatant was removed and cells were resuspended in 2 mL of Fluorescence-Activated Cell Sorting (FACS) solution in order to remove non-specific binding antibodies. This process was repeated once more and supernatant was removed and replaced with 200 μL of 1% paraformaldehyde (PFA) in FACS. The cell suspension was incubated on ice for 15 min and then read at 488 nm on a BD LSRII flow cytometer (BD Bioscience, San Jose, CA).

NFκB nuclear extract and isoform activation

Cells were grown in CGM in 6-well cell culture plates. Medium was aspirated and cells were washed twice with 2 mL of sterile PBS. Nuclear and cytoplasmic protein extracts were then isolated using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (78833) Thermo-Fisher (Waltham, MA). Protein concentrations were quantified using the Micro BSA Protein Assay Kit (23235) Thermo-Fisher for equal protein loading. NFκB family isoform activity was measured using a TransAM NFκB family kit (43296) Active Motif (Carlsbad, CA) and the protocol as
described by the manufacturer. Results were read on a BioTec ELx800 plate reader.

**Western blot analysis**

Cells were cultured in CGM until assays were performed. CGM was replaced with SFM for 24 h before treatments. Thereafter, cells were treated with or without designated inhibitors for 1 h and then treated without or with either insulin (1 nM) or TNFα (1 ng/mL) or both for indicated times. Whole cell lysates were prepared using lysis buffer (50 mM HEPES, 150 mM NaCl, 15 mM MgCl₂, 1 mM PIPES, 1 mM NaHPO₄, 1 mM DTT, 1 mM Na Vanadate, 1% TX-100, 0.05% SDS, 10 µg/mL Aprotinin and 10 µg/mL Leupeptin). Lysates were cleared of particulates by centrifugation and protein concentrations were determined in order to load lanes with equal amounts of protein. Equal protein amounts were placed in 2× Laemmli Sample Buffer, frozen overnight and then boiled for 5 min just before use. Forty microlitres of cleared lysates plus sample buffer were loaded into each well of an 8–16% Pierce Precise Protein gel (Thermo-Fisher, Waltham, MA) and were run in 1× Tris/HEPES run buffer at 100 V for 45 min. Proteins were then transferred to PVDF or nitrocellulose membranes (Millipore, Billerica, MA) using a standard wet transfer protocol. After completion of protein transfer, membranes were washed two times in 0.1% Tween in tris-buffered saline (TBS-T) solution for 10 min. Membranes were then incubated in 5% non-fat milk (milk) in TBS-T blocking solution for 2 h at room temperature, washed two times in TBS-T for 5 min each and then incubated with a designated primary antibody solution (1:1000 in 1% milk/TBS-T) overnight at 4 °C. Membranes were washed three times with TBS-T and then incubated with a designated secondary antibody (1:2000 in 1% milk/TBS-T) conjugated with horseradish peroxidase at room temperature for 2 h. Membranes were washed two times with TBS-T for 10 min at room temperature and washed once with TBS for 10 min. One millilitre of ECL (GE/Amersham) detection solution was added to each membrane and incubated for 1 min. Excess ECL was removed and membranes were exposed to HyperFilm (GE/Amersham) for visualization of proteins. Densitometry was performed using the ImageQuant TL v2005 (GE/Amersham) software program in order to represent profile bands on representative films.

**Biochemical inhibitors**

Time course phosphorylation assays were first conducted on cells in the presence of insulin or TNFα alone in order to determine the time points at which phosphorylation of intracellular kinase intermediates were activated. Subsequently, dose–response analyses were performed in order to determine half maximal inhibitory concentrations (IC₅₀) for inhibitors. Cells were incubated first in SFM for 24 h and then treated without or with PD98059 (10 µM), Wortmannin (15 µM), SB203580 (0.1 µM) or SP600125 (25 µM) alone for 1 h. Thereafter, cells were treated with insulin or TNFα alone, or in combination for indicated times and doses.

**Competent cell transformation, selection and glycerol stocks**

NFkB p65 shRNA plasmids (KR46841P) SABiosciences (Valencia, CA) were used to transform competent *Escherichia coli* MC1061/P3 cells (C663-03) Invitrogen (Grand Island, NY) via the provided heat shock protocol. Transformed cells were then plated on LB plates containing 1.2% agar and 100 µg/mL ampicillin (171257) Calbiochem (Darmstadt, Germany) overnight. Single colonies from each plasmid clone were then picked from the plates, and colonies were grown for 12 h in 100 µL LB broth (10855-021) Gibco/Invitrogen containing 100 µg/mL ampicillin. Cells from the LB broth were then used to prepare glycerol stocks. Each glycerol stock contained 80% cells and 20% glycerol, and were stored at (−)80°C for future use.

**E. coli growth and DNA isolation**

Glycerol stocks were grown overnight in a flask containing 100 mL LB broth (10855-021) Gibco and 100 µg/mL ampicillin (171257) Calbiochem at 37°C and shaken at 225 rpm for 24 h. Plasmid DNA was extracted using the Quantum Prep Plasmid Midiprep Kit (732-6120) Bio Rad (Hercules, CA). After isolation, the plasmid DNA was precipitated with 2 volumes of 100% ethanol.

**DNA concentrating and quantification**

Isolated DNA was centrifuged at 4°C for 15 min at 2500 RPM. The supernatant was decanted, and the DNA pellet was washed with 500 µL of 70% ethanol, vortexed, and centrifuged at 4°C for 10 min at 25000 RPM. The 70% ethanol wash was then repeated, and the DNA pellet was air dried for 10 min at 50°C using a heating block. The DNA pellet was then resuspended in 60 µL of Nuclease free H₂O and stored at −20°C. DNA was then quantified using the Nanodrop ND-1000 spectrophotometer. Each plasmid clone was verified for content via a restriction digest using psti (R0140S) New England Biolabs (Ipswich, MA). A total of 1µg of digested plasmids were then ran on a 1% agarose gel at 100 V for 45 min and compared to the plasmid map for correct band sizes.

**Transfection**

Cells were plated for 50–70% confluency in CGM in 6-well culture plates. Cell medium was aspirated and cells were washed two times with 2 mL of transfection medium (108062) Santa Cruz (Dallas, TX). Immediately after the washes, a 1 mL volume of transfection medium containing 3 µg of
plasmid DNA and 9 μL of transfection reagent (108061) Santa Cruz was added to each well. Cells were then incubated for 6 h at 37°C and 5% CO₂. One millilitre of growth medium containing two times the normal serum and antibiotics concentration (2× CGM) was added to the original transfection solution. Cells were incubated in this medium for 24 h. Subsequently, the medium was aspirated and replaced with fresh 1 × CGM and incubated for an additional 24 h.

Genetic inhibitors

After the initial transfections, each of the four provided inhibitory NFkB p65 RNA clones and the scrambled (inactive) control NFkB clone was tested for knockdown effectiveness. Total protein was extracted and quantified using the Micro BSA Protein Assay Kit (23235) Thermo Scientific. Fifty micrograms of protein were resolve on SDS-PAGE and determined by Western blot analysis. p65-NFkB was determined using a 1:1000 diluted NFkB p65 primary antibody (4764s) Cell Signalling (Boston, MA) and a 1:2000 diluted horseradish peroxidase (HRP) conjugated anti-rabbit secondary antibody (7074) Cell Signalling. After exposure to 1 mL of Enhanced Chemiluminescent (ECL) solution, blots were exposed to Blue Lite Autorad Film (Gene Mate 9024-8 × 10) and developed. Total NFkB p65 protein levels in each transfection were then quantified using densitometry analysis. Each band from active NFkB inhibitory RNA was compared to untransfected negative controls and scrambled clone transfection controls.

Statistics

Data were analysed by either unpaired Student’s t test (two groups) or ANOVA with subsequent Tukey post-test (several groups) as indicated. A "P" value of <0.05 was considered significant. Results were expressed as the mean ± SEM of three or more independent experiments.

Results

We first wanted to determine whether insulin and TNFα had the same effect on activation of all isoforms of NFkB. Using ELISA analysis of activated NFkB isofrom array, we observed that insulin and TNFα significantly (P < 0.05) activated the p65 isoform of NFkB (Figure 1). Interestingly, insulin did not decrease the effect of TNFα-stimulated p65-NFkB activation, but augmented it.

Effects of biochemical inhibitors on insulin and TNFα-stimulated nuclear import of NFkB

Since NFkB is downstream of many kinase-mediated pathways, we asked, “what effects would be seen on insulin and TNFα-stimulated p65-NFkB nuclear import in cells pre-treated with biochemical kinase inhibitors”? The MEK1/2 inhibitor (PD98059, [10 μM]), the p38 inhibitor (SB203580, [100 nM]) and the c-jun N-terminal kinase (JNK) inhibitor (SP600125, [25 μM]), moderately, but significantly (P < 0.05) inhibited insulin-stimulated p65-NFkB nuclear import (Figure 2). In contrast, none of these inhibitors affected TNFα-stimulated p65-NFkB nuclear import. What piqued our interest was the increase in percent nuclear amounts of p65-NFkB in the presence of the PI3K inhibitor, Wortmannin (15 μM), and cells treated with either TNFα alone or TNFα plus insulin. Here, we observed a glimpse of insulin resistance affecting an increase in the nuclear import of the inflammation-related mediator, NFkB.

Genetic inhibition of NFkB expression

It is well known that biochemical inhibitors do not always have single targets. Thus, we wanted to determine the effects of genetic inhibition of p65-NFkB on insulin and TNFα-stimulated VCAM-1 expression. Since it appeared that only the p65-NFkB isoform of NFkB was affected by insulin and TNFα, we decided to focus on p65-NFkB genetic RNA inhibition. We transfected different cell samples with either a scrambled (inactive) NFkB RNA (scNFkB) construct or one of four different active inhibitor p65-NFkB RNA inhibitor (siNFkB 1–4) constructs and blotted for total p65-NFkB.


Figure 1: Effects of insulin and TNFα on activation of different isoforms of NFkB isoforms. Cells were serum-starved for 24 h and then treated without or with insulin (1 nM) or TNFα (1 ng/ml) alone or in combination for 1 h. Activation of NFkB was determined by ELISA, using the TransAM™ methodology as described in the “Materials and methods” section. Activated isoforms of NFkB are expressed as percent of negative controls and mean ± SEM of four experiments. *P < 0.05 compared to negative controls. #P < 0.05 vs TNFα alone.

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FOR CITATION PURPOSES: Mackesy DZ, Goalstone ML. Nuclear-factor kB p65 is a mediator of insulin and tumour necrosis factor-alpha stimulated vascular cell adhesion molecule-1 expression in vascular endothelial cells. OA Inflammation 2013 Jun 01;1(1):7.
Using clone #3, we then wanted to determine whether decreased surface VCAM-1 expression was significantly greater than that seen for TNFα alone. Interestingly, in the presence of siNFkB, TNFα-stimulated, but not insulin-stimulated, surface VCAM-1 expression was increased by 25% as compared to positive controls. Additionally, in the presence of siNFkB and the combination of insulin plus TNFα surface expression of VCAM-1 decreased 38%, as compared to positive controls, but did not reach the same levels as insulin alone.

**Effects of inhibitory NFκB RNA on total VCAM-1 expression**

We followed these experiments with the same siNFkB and determined changes in total VCAM-1 protein in cells treated with insulin or TNFα alone or in combination (Figure 5). Again, we observed significant (P < 0.05) decreases in TNFα-stimulated and insulin plus TNFα-stimulated total VCAM-1 expression in the presence of siNFkB. However, this effect was not seen in insulin-stimulated total VCAM-1 expression. Inhibitor NFκB RNA (siNFkB) decreased TNFα-stimulated and insulin plus TNFα-stimulated total VCAM-1 expression 55% and 39%, respectively.

**Discussion**

In the United States alone, there are over 24 million people reported to have diabetes and countless others undiagnosed. T2DM is a syndrome of multiple disorders that starts with insulin resistance and eventually leads to a multitude of pathologies. Cardiovascular complications are the most prevalent pathology found associated with T2DM. Insulin resistance and hyperinsulinaemia are risk factors for cardiovascular diseases (CVD). 1,18 and appear to be effectors of inflammation as well. Inflammation of the vasculature is associated with the expression of cellular adhesion molecules.

Statistically, neither the presence of the scrambled NFκB (scNFkB) alone nor the active, inhibitory RNA form of NFκB (siNFkB) alone increased surface expression of VCAM-1 above that observed for negative controls. Yet in the presence of siNFkB, TNFα-stimulated, but not insulin-stimulated, surface VCAM-1 expression was significantly (P < 0.05) decreased 25% as compared to positive controls. Additionally, in the presence of siNFkB and the combination of insulin plus TNFα surface expression of VCAM-1 decreased 38%, as compared to positive controls, but did not reach the same levels as insulin alone.

**Competing interests:** none declared. Conflict of interests: none declared. All authors abide by the Association for Medical Ethics (AME) ethical rules of disclosure.

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molecules on the surface of vascular endothelial cells. VCAM-1 is one such adhesion molecule and appears to be upregulated in the state of insulin resistance and hyperinsulinaemia. TNFα is a well-characterised inflammatory cytokine and its presence is robustly correlated with atherogenesis. In contrast, insulin’s role in atherogenesis is hotly debated. Some argue that insulin stimulates an increase in nitric oxide (NO) and thus either maintains arterial plasticity, or decreases systemic inflammation. Both of which appear to inhibit atherogenesis. Others argue that hyperinsulinaemia, which accompanies insulin resistance, increases the production of endothelial and vascular smooth muscle cells and adds to the inflammatory response. On first observation, the two sides seem incompatible. Yet, if one looks closer, different concentrations of insulin appear to have different effects. At physiologic concentrations, insulin has a protective effect. Yet at higher than physiologic concentrations, chronic hyperinsulinaemia appears to have a detrimental effect by, but not limited to, increasing the expression of CAMs and deleterious cytokines.

One possible mechanism for increased expression of CAMs during insulin resistance and the concomitant state of hyperinsulinaemia is insulin’s ability to prepare cells to be more responsive to potent cytokines and thereby increase CAM expression. Previous studies have demonstrated that hyperinsulinaemia is positively associated with (1) insulin resistance, (2) increased expression of TNFα, and (3) CAM expression; all of which are linked to inflammatory conditions and atherosclerosis. Since hyperinsulinaemia and TNFα appear to be associated with inflammation of the arterial endothelium, we were interested in determining whether insulin and TNFα-stimulated the canonical mediator of inflammation, NFκB. Since NFκB is a family of transcription factors, we determined which member of this family was involved in insulin and TNFα stimulation and whether this family member was important to insulin and TNFα-stimulated VCAM-1 expression. Our ELISA experiments demonstrated that the p65-NFκB isoform is the mediator of insulin and TNFα signalling to total protein and surface expression of VCAM-1. Moreover, the current study’s ELISA assays matched our previous results for Western blot analysis of nuclear import of p65-NFκB.

The question remains, though, does insulin in the context of insulin resistance/hyperinsulinaemia exacerbate or mitigate the existing conditions of TNFα-stimulated VCAM-1 expression? Moreover, what are the other molecular mechanism(s) that play a role in this process? Insulin resistance is frequently defined in molecular terminology as a post-insulin receptor dysfunction. It is commonly believed that perturbation of the phosphatidylinositol-3 kinase (PI3K) and Akt signal pathway leads to dysfunction in intracellular insulin signalling: a down regulation of translocation of glucose transporters to the membrane and decreased uptake of glucose. Yet, there may be other effects of this perturbation. For example, upregulation or “disinhibition” of other kinase pathways or mediators may be affected by PI3K dysregulation. One such example is the activation of the canonical inflammation mediator, NFκB. We report here that inhibition of PI3K increases TNFα-stimulated and insulin plus TNFα-stimulated NFκB nuclear import (Figure 2).

We have shown in this study that high-physiological concentrations of insulin increased the expression of surface and total VCAM-1 above that seen for quiescent cells. Yet, contrary to some investigators, the presence...
of hyperinsulinaemia did not have a diminutive effect on TNFα-stimulated expression of surface or total VCAM-1 protein.

We have demonstrated that a strong relationship among insulin, TNFα, NFκB activation and VCAM-1 expression exists. Our observations appear to be in concert with Wissink et al. who demonstrated that TNFα-stimulated the cellular adhesion molecule, ICAM-1, via activation of NFκB as well. Thus, there appears to be a common relationship among inflammatory cytokines, cells surface receptors and the canonical inflammatory mediator, NFκB.

It appears that in response to the presence of insulin and TNFα the p65 (RelA) isoform of NFκB was the most activated member of the NFκB family to be stimulated by these analogues (Figure 1). We have previously demonstrated that in the presence of both insulin and TNFα nuclear import of NFκB was accelerated, suggesting at least an additive effect of these two cytokines and no amelioration of the effects of insulin on TNFα action. Yet, interestingly what also increased nuclear import of NFκB was the presence of the PI3K inhibitor, Wortmannin, in cells treated with either TNFα alone or the combination of TNFα plus insulin (Figure 2). This indicated that dysregulation of the PI3K pathway, as seen in insulin resistance, may contribute to vascular inflammation. Yet, what was perplexing was that genetic inhibition of p65-NFκB only inhibited TNFα-stimulated, but not insulin-stimulated, expression of surface (Figure 4) and total protein (Figure 5) of VCAM-1. Thus, we had to come to a reconciliation that would resolve this issue. Another pathway mediator, and unknown or yet to be fully characterised kinase, may be involved in intracellular insulin signalling and VCAM-1 expression (Figure 6); that is an NFκB-independent pathway. This will be the basis of our future research.

Conclusion

This study demonstrates that (1) insulin and TNFα increase VCAM-1 expression, (2) hyperinsulinaemia does not mitigate TNFα-stimulated increases in VCAM-1 expression, (3) the p65 (RelA) isoform of NFκB is a mediator of insulin and TNFα VCAM-1 expression, (4) WT increases TNFα-stimulated and insulin plus TNFα-stimulated p65-NFκB activation and nuclear import and (5) the genetic inhibition of p65 (RelA) NFκB significantly (P < 0.05) inhibits TNFα-stimulated, but not insulin-stimulated, VCAM-1 expression. It is the hope of this study and those to come to provide information that will lead to therapeutic strategies in the pathogenesis of atherosclerosis.
Acknowledgements
This work was supported by the Research Service of the Department of Veterans Affairs (to M.L.G.), in which Dr. Goalstone is a recipient of a VA Career Development Award. We would also like to acknowledge the assistance of Harsh Pratap (Flow Cytometry technician for the Mucosal and Vaccine Research Colorado) of the Eastern Colorado Health Care Service (Denver VAMC).

References
19. Granic I, Dolga AM, Nijholt IM, van Dijk G, Eisel UL. Inflammation and NF-κB.
Figure 6: Hypothetical models of intracellular signalling of insulin- and TNFα-stimulated NFκB activation and VCAM-1 expression. Current proposed model of insulin and TNFα intracellular signalling to VCAM-1 expression with respect to this study’s results. Here we hypothesize an unknown mediator (?) that may play a significant role in insulin and TNFα-mediated VCAM-1 expression.
