ROLE OF INFLAMMATION AND ENDOTHELIAL DYSFUNCTION OF CORONARY ARTERIOLES IN TYPE 2 DIABETES

A Thesis

by

JI YEON YANG

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2008

Major Subject: Biomedical Sciences

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ABSTRACT

Role of Inflammation and Endothelial Dysfunction

of Coronary Arterioles in Type 2 Diabetes. (August 2008)

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Co-Chairs of Advisory Committee: Dr. Cuihua Zhang
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We hypothesized that the interaction between tumor necrosis factor alpha (TNFα)/nuclear factor-kappaB (NFκB) via activation of IKKβ may amplify one another resulting in the evolution of vascular disease and insulin resistance associated with diabetes. The interaction between TNFα and monocyte chemoattractant protein-1 (MCP-1) may contribute to the evolution of vascular inflammation and endothelial dysfunction in coronary arterioles in type 2 diabetes. To test this hypothesis, endothelium-dependent (ACh) and –independent (SNP) vasodilation of isolated, pressurized coronary arterioles (40-100 μm) from mLepr^{db} (heterozygote, normal), Lepr^{db} (homozygote, diabetic) and Lepr^{db} mice null for TNFα (db^{TNF-}/db^{TNF-}) were examined. Although dilation of vessels to SNP was not different between Lepr^{db} and mLepr^{db} mice, dilation to ACh was reduced in Lepr^{db} mice. The NFκB antagonist, MG-132, IKKβ inhibitor, sodium salicylate (NaSal), or Anti-MCP-1 partially restored endothelium-dependent coronary arteriolar dilation in Lepr^{db} mice. Protein expression of IKKα and IKKβ were higher in Lepr^{db} than in mLepr^{db} mice. The expression of IKKβ, but not the expression of IKKα was increased in db^{TNF-}/db^{TNF-} mice. Lepr^{db} mice showed increased insulin resistance, but NaSal

improved insulin sensitivity. Protein expression of TNFα, NFκB, phosphorylation of IKKβ and JNK were greater in $Lepr^{db}$ mice, but NaSal attenuated protein expression of them in $Lepr^{db}$ mice. The ratio of phosphorylated IRS-1 at Ser307 (pIRS-1)/IRS-1 protein expression was elevated in $Lepr^{db}$ mice; both NaSal and JNK inhibitor SP600125 reduced pIRS-1/IRS-1 in $Lepr^{db}$ mice. MG-132 or neutralization of TNFα reduced superoxide production in $Lepr^{db}$ mice. Anti-MCP-1 attenuated superoxide production and protein expression of nitrotyrosine (N-Tyr), which is an indicator of peroxynitrite production, in isolated coronary arterioles of $Lepr^{db}$ mice. Immunostaining results showed that expression of MCP-1 and vascular cellular adhesion molecule-1 (VCAM) is co-localized with endothelial cells and macrophages. Anti-TNFα or anti-MCP-1 markedly reduced macrophage infiltration and the number of MCP-1 positive cells. Neutralization of TNFα or anti-MCP-1 reduced the expression of adhesion molecules. In conclusion, our results indicate that the interaction between NFκB and TNFα signaling induces activation of IKKβ. In addition, TNFα and TNFα-related signaling, including the expression of MCP-1 and adhesion molecules, further exacerbates oxidative stress leading to endothelial dysfunction in type 2 diabetes.

DEDICATION

To mom, dad and my husband, Min Jae Song. This project is dedicated to you.

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I would like to acknowledge those individuals who have given me motivation to succeed, financial assistance, and most of all their friendship. First, my advisor, Professor Cuihua Zhang, for the guidance and help I received during my master's thesis research. I wish to express my warm and sincere thanks to Professor Louise Abbott for her detailed and constructive comments. Also I would like to appreciate Professor Glen A. Laine, Cristine L. Heaps and Cindy Meininger for agreeing to serve as my committee co-chair and members. Without doubt, I could not have completed this step of my life without my many friends, family, and mentors who have assisted me in many forms and manners. Mom and Dad, you are truly the best. I am indebted to you for all your love and encouragement. Thanks to my wonderful and beautiful sister and brother. My lab members: Yoonjung Park, Hanarui Zhang, Sewon Kim, Darcey Klaahsen, XiuPing Chen, Xue Gao, Stefano Capobianco, Yuan Gao. I thank you for the positive things, large and small, that you have added to my life. This study was supported by an Atorvastatin Research Award from Pfizer (2004-37), an American Heart Association SDG (110350047A), and NIH grants (RO1-HL077566 and RO1-HL085119).

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CHAPTER I

INTRODUCTION

A. Feed-forward Signaling of TNFα and NFκB via IKKβ Pathway Induces Insulin Resistance and Coronary Arteriolar Dysfunction in Type 2 Diabetic Mice

The prevalence of obesity and diabetes has led to a significant increase in the complications associated with cardiovascular disease. Many of these complications can be linked to a dysregulation of the body's immune system.[1,2] Tumor necrosis factor alpha (TNFα) is a pro-inflammatory cytokine that has been implicated in cardiovascular diseases.[3,4] We previously found that increases in TNFα expression induces activation of NAD(P)H oxidase and the production of reactive oxidative species (ROS), leading to endothelial dysfunction in type 2 diabetes.[5] Importantly, TNFα activates the transcription nuclear factor-kappaB (NFκB), which regulates the expression of genes involved in inflammation, oxidative stress and endothelial dysfunction.[5] TNFα also initiates the signaling cascades via the inhibitor of NFκB (IκB) kinase (IKK) complex, which contains IKKα and IKKβ. The inhibitory protein IκBα is phosphorylated, ubiquitinated and degraded by proteasomes, releasing NFκB to translocate into the

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This thesis follows the style of *Circulation Research*.

nucleus. Under normal physiological conditions, the inflammatory response is terminated by binding NFκB with the inhibitory protein IκB.[6,7] Investigation of TNFα-mediated NFκB pathway is needed to identify specific pro-inflammatory agents in type 2 diabetes.

Salicylates, including aspirin and sodium salicylate (NaSal) are common nonsteroidal anti-inflammatory drugs (NSAIDs), which have anti-platelet and anti-inflammatory effects. Aspirin inhibits prostaglandin production, but NaSal has a prostaglandin-independent effect. NaSal inhibits the activity of IKKβ, which is required for the activation of NFκB. IKKβ, the molecular target of NaSal, is associated with the rise of insulin resistance.[8,9] Recent work suggests that specific antagonism of the NFκB inflammatory pathway through the inhibition of IKKβ reduces acute myocardial damage following ischemia-reperfusion injury[10] and insulin action plays a critical role in reducing myocardial infarct size by increasing cardiac myocyte metabolism.[11-13] We previously found that TNFα-activated Jun N-terminal kinase (JNK), which mediates $O_2^{\cdot -}$ production and impairs endothelium-dependent vasodilation in coronary arterioles.[14] NaSal prevents TNFα-induced JNK activation from blocking insulin signaling via a serine phosphorylation.[15] Therefore, we evaluated 1) whether reciprocal interactions between TNFα and NFκB via activation of IKKβ accentuate the evolution of vascular disease in type 2 diabetes; and 2) whether NaSal restores endothelial dysfunction and insulin resistance by inhibiting IKKβ/NFκB activity thereby preventing JNK activation in coronary arterioles from type 2 diabetes ($Lepr^{db}$), type 2 diabetic mice null for TNFα (db^{TNF-}/db^{TNF-}) and lean control ($mLepr^{db}$) mice.

B. Role of MCP-1 in TNFα-induced Endothelial Dysfunction in Type 2 Diabetic Mice

Diabetes is accompanied by a maturing inflammatory response that consists of increased TNFα and monocyte chemoattractant protein-1 (MCP-1) expression in adipocytes and the lumen of vessels.[16] At the tissue level, diabetic inflammation enhances expression of adhesion molecules, which facilitates macrophage infiltration.[17~19] The progression of vascular disease in type 2 diabetes is caused by the complex interaction of many factors. We previously found that increases in TNFα expression induces activation of NAD(P)H oxidase and production of reactive oxidative species (ROS), leading to endothelial dysfunction in type 2 diabetes.[5] Additionally, TNFα is reported to enhance MCP-1 expression;[20] thus the interaction between these two systems may contribute significantly to the evolution of vascular disease in diabetes. MCP-1 is a major component of adipose tissue that contributes to abnormal lipid metabolism[21] and is expressed by a variety of activated cells (e.g., endothelial cells, monocytes, and smooth muscle cells). MCP-1 has been thought to be essential in defending the host against foreign organisms and is an important mediator in chronic inflammation.[22] Many studies suggest that MCP-1 plays a detrimental role in the development of vascular disease through recruitment of monocytes that accumulate in/on vascular endothelium and may augment vascular disease at the inflammatory site.[23-25] Monocytes that migrate from the peripheral vasculature into the myocardium undergo maturation to macrophages, which then release proteolytic enzymes and damage the myocardium.[26] The action of MCP-1 on the stages of monocyte recruitment (rolling and/or adhesion) is associated with the activation of specific cell surface proteins.

In this regard, adhesion molecules (e.g.,vascular cell adhesion molecule-1 [VCAM], intracellular adhesion molecule-1 [ICAM], and E-selectin as well as MCP-1) are responsible for myocardial injury and cardiac contractile dysfunction.[27]

Thus, the goal of this study was to document the cellular and molecular mechanisms involved in MCP-1 in the coronary microcirculation in TNFα-induced endothelial dysfunction in type 2 diabetes. We examined the role of MCP-1 in type 2 diabetes, and tested whether the interaction of TNFα and MCP-1 amplifies the signaling of adhesion molecules and macrophage infiltration. Endothelial function and the production of ROS were determined in isolated coronary arterioles (40-100 μm) from genetically modified mice with type 2 diabetes ($Lepr^{db}$) with and without treatment with MCP-1 neutralizing antibody. We also tested the co-localization of MCP-1, CD68 and adhesion molecules in type 2 diabetes using immunohistochemical analysis.

CHAPTER II

METHODS

A. Animal Treatment

1. Animal Model

The procedures followed were in accordance with approved guidelines set by the Laboratory Animal Care and Use Committee at Texas A&M University. Heterozygote controls (C57BLKS/J, mLepr^{db}), homozygote type 2 diabetes (C57BLKS/J, Lepr^{db}) and Lepr^{db} null for TNFα (db^{TNF-}/db^{TNF-}) mice were purchased from Jackson Laboratory and maintained on a normal rodent chow diet. Our studies utilized 12-16 week-old, 20-30 g mLepr^{db}, 40-60 g Lepr^{db} and db^{TNF-}/db^{TNF-} mice of either sex. The cross (db^{TNF-}/db^{TNF-}) of Lepr^{db} with TNFKO is heterozygous for Lepr^{db} and homozygous for TNF KO (TNF^{-}/$^{-}$).The db^{TNF-}/db^{TNF-} mice show the phenotypes of hyperglycemia and obesity that is consistent with diabetes and the penetrance of the leptin receptor mutation. The obese mice from the second round of breeding of Lepr^{db} and TNF^{-}/$^{-}$ mice were used in experimentation.

2. Neutralization and Treatment

The neutralizing antibody to TNFα[28] is 2E2 monoclonal antibody (2E2 MAb. 94021402, NCI Biological Resources Branch). At 12-16 weeks of age, all mice received anti-TNF (2E2 MAb. 0.625 mg/ml/kg/day, 3 days, i.p.); dosage was based on our estimates of TNFα expression, which was estimated to be in the low ng or pg range. This amount was able to neutralize 10-100 fold more than the estimated levels of TNFα.

MG132 tripeptide (Z-Leu-Leu-Leu- aldehyde, Sigma, dissolved in dimethyl sulfoxide [DMSO]) is a peptide aldehyde proteasome inhibitor. Ultimately preventing IκBα degradation by the proteasome restricts NFκB activity in vitro.[7] $Lepr^{db}$ mice were injected (10 mg/kg/day for 3 days, i.p.).[29] The contribution of NAD(P)H oxidase in generating $O_2^{\cdot -}$ was assessed by treating mice with the NAD(P)H oxidase inhibitor apocynin (100 mg/kg/day, i.p. for 3 days). We administered NaSal (Calbiochem; 6 mg/ml in drinking water for 7-10 days, pH 7) to $Lepr^{db}$ and $mLepr^{db}$ mice to determine whether blocking IKKβ affects their blood glucose level. To determine whether blockage of JNK affects insulin receptor substrate-1 (IRS-1) activity, we also administered the JNK inhibitor, SP600125 (CalBiochem, 10 mg/kg/day in 0.05 ml DMSO, i.p. for 7 days) to $Lepr^{db}$ mice.[30] Mice were treated with the neutralizing antibody to MCP-1 (anti-MCP-1; 200 mg/kg/day, i.p. for 3 days, MURINE; Biovision) to block MCP-1 signaling.

B. Measurement of Blood Parameters

Blood was obtained from the vena cava after anesthesia with sodium pentobarbital (50 mg/kg; i.p.) and exposure of the vein. Whole blood samples were spun at 3,000 rpm for 10 minutes, and serum was stored at -80°C until analysis.

1. Measurement of Glucose

We used a OneTouch Ultramini glucometer (LifeScan) for measuring blood glucose in $mLepr^{db}$, $Lepr^{db}$ and $Lepr^{db}$ mice treated with NaSal at the same time (8:00-10:00AM) throughout the protocol.

2. Serum Cholesterol Level

Serum cholesterol level was measured with the Cholesterol/Cholesteryl Ester Quantitation Kit (Biovision) using spectrophotometry (Multiskan MCC, Fisher Scientific). A large portion of cholesterol in blood is in the form of cholesteryl esters. Cholesterol esterase hydrolized cholesteryl ester into cholesterol. Cholesterol is then oxidized by cholesterol oxidase to yield H_2O_2. The produced H_2O_2 interacts with a sensitive cholesterol probe.

3. Serum Insulin Level

Insulin was measured with the use of a commercial kit, insulin (Mouse) Ultrasensitive EIA (ALPCO Diagnostics). Insulin concentration was measured by spectrophotometry (Multiskan MCC, Fisher Scientific).

4. HOMA-IR

Insulin resistance was determined by utilizing the homeostasis model assessment; HOMA-IR using the following formula:

HOMA-IR= ((non-fasting glucose [mmol/L]) × (non-fasting insulin [mU/L]))/22.5.

C. Functional Study

1. Preparation of Coronary Microvessels

The techniques for identification and isolation of coronary microvessels were described in detail previously.[31,32] Coronary arterial microvessels (<100 μm) were carefully dissected out for in vitro study. After isolation, the microvessel was cannulated with two glass micropipettes filled with filtered albumin-PSS, and the outside of the microvessel

was securely tied to the pipettes with 11-O ophthalmic suture. The preparation was transferred to the stage of an inverted microscope coupled to a CCD camera and video micrometer (Figure 1). To determine whether NFκB, IKKβ, or MCP-1 was playing a role in endothelial injury in type 2 diabetes, the endothelium-dependent vasodilator acetylcholine (ACh, 0.1 nmol/L to 10 μmol/L), the endothelial independent vasodilator sodium nitroprusside (SNP, 0.1 nmol/L to 10 μmol/L)-induced, and flow-induced vasodilation (NO-mediated, endothelial dependent but agonist independent; 4 to 60 cm H_2O) were assessed in coronary arterioles in mLepr^{db}, Lepr^{db}, and Lepr^{db} mice treated with MG132, NaSal, or anti-MCP-1. Flow was established by the production of a pressure drop across the vessel, and linearly related to the pressure drop (ΔP).

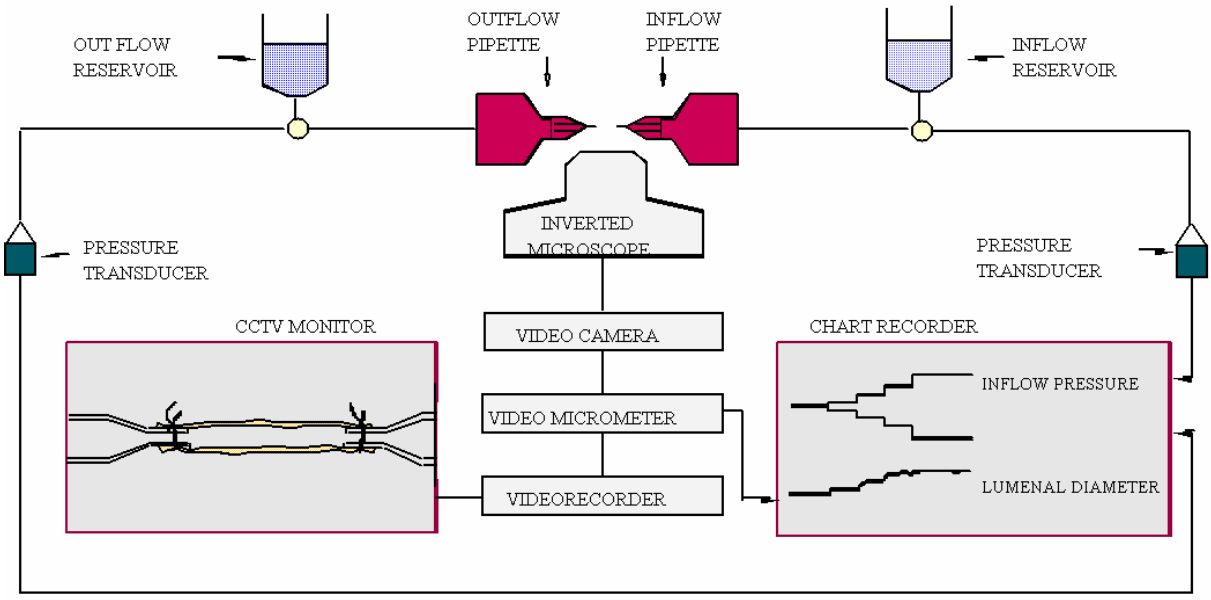

Figure 1. Isolated vessel set-up for coronary arteries.

2. Data Analysis

At the end of each experiment, the vessel was relaxed with 100 μmol/L SNP to obtain its maximal diameter at 60 cm H_2O intraluminal pressure.[32] All diameter changes to pharmacological agonists were normalized to the control diameter and expressed as a percentage of control diameter. Functional data are presented as mean±SEM, except as specifically stated (e.g. as mean±SD for molecular study). Statistical comparisons of vasomotor responses under various treatments were performed with two-way ANOVA and intergroup differences were tested with Bonferonni Inequality. Significance was accepted at $P<0.05$.

3. Insulin Tolerance Test

The tail was nicked with a fresh razor blade by horizontal cut of the tip and the OneTouch Ultramini glucometer was used to measure baseline blood glucose. A total of 0.75 units per kg body weight of diluted insulin from porcine pancreas (Sigma) was injected into the intraperitoneal cavity. Blood samples were taken for glucose determinations 0, 30, 60, and 120 minutes later. Blood was sampled from the tail of each mouse by gently massaging a small drop of blood onto the glucometer strip.

D. Molecular Study

1. Electron Paramagnetic Resonance (EPR) Spectroscopy

Homogenates of (4-6 isolated coronary arterioles) were prepared in a 50 mmol/L phosphate buffer containing 0.01 mmol/L EDTA. The homogenate was then subjected to low-speed centrifugation (1000 g) for 10 minutes to remove unbroken cells and tissue debris. The supernatants containing 2 mmol/L CPH (1-hydrox-3 carboxypyrrolidine)

were incubated for 30 minutes at 37 °C and frozen quickly in liquid nitrogen. EPR spectroscopy was performed at room temperature using a Bruker EMX spectrometer and 1 mm diameter capillaries. Superoxide quantitation from the EPR spectra was determined by double integration of the peaks, with reference to a standard curve from horseradish peroxidase generation of the anion from standard solutions of hydrogen peroxide, using p-acetamidophenol as the co-substrate,[33,31] then normalized by protein concentration.

2. Immunofluorescence

Mouse heart was perfused with PBS and then perfused again with 4 % paraformaldehyde. Isolated heart tissue was fixed in 4 % paraformaldehyde diluted in PBS 0.1 M (overnight at 4 °C). Fixed heart tissue was perfused in 20 % sucrose diluted in PBS 0.1 M (overnight at 4 °C). Once the heart was completely perfused, the tissue was embedded in OCT and snap-frozen at –60 °C and stored at –80 °C. Frozen tissues were sectioned at 5 µm thick, using cryostat (Leica) and slides allowed to dry at room temperature; then the tissues were incubated in 0.3 % Triton-100 for 30 minutes and washed in PBS 3 times. 5 % goat serum was used for 30 minutes to block nonspecific binding. Anti-rat primary MCP-1, VCAM-1 antibodies (Abcam) and anti-rabbit primary α-actin, von Wilibrand factor (VWF), anti-mouse primary CD68 (Abcam) as monocyte/macrophage marker were incubated sequentially overnight at 4 °C. Sections were washed 3 times in PBS. Appropriate secondary FITC-(green color) and Texas Red-(red color) conjugated antibodies were incubated with sections for 4 hours and washed off. Sections were finally mounted in an anti fading agent (Slowfade gold with

DAPI, Molecular Probes), and then the slides were observed and analyzed with the use of a fluorescence microscope (Zeiss Axioplan microscope with a 40X objective or a Nikon microscope with a 63X objective).

3. Immunoblotting

Coronary arterioles (4-6 vessels per sample) or heart tissues were separately homogenized and sonicated in lysis buffer (Cellytic™ MT Mammalian Tissue Lysis/Extraction Reagent, Sigma). Protein concentrations were assessed using a BCA™ Protein Assay Kit (Pierce), and equal amounts of protein were separated by SDS-PAGE and transferred to nitrocellulose or PVDF membranes (Pierce Biotechnology). IKKα (Santa Cruz), IKKβ (Abcam), p-IKKβ (Cell Signal), IκBα (Santa Cruz), p-IκBα (Santa Cruz), NFκB (Santa Cruz), TNFα (Santa Cruz), N-Tyr (Abcam), JNK, p-JNK (Abcam), IRS-1, and pIRS-1 (Millipore) antibodies were utilized to assess protein expression in $mLepr^{db}$, $Lepr^{db}$, and $Lepr^{db}$ mice treated with anti-TNF (0.625 mg/ml/kg/day, i.p. for 3 days), NAD(P)H oxidase inhibitor apocynin (100 mg/kg/day, i.p. for 3 days),[34] NFκB antagonist MG132 (10 mg/kg/day, i.p. for 3 days), NaSal (6 mg/ml in drinking water), or SP600125 (10 mg/kg/day in 0.05 ml DMSO, i.p. for 7 days). MCP-1 (Abcam), N-Tyr (Abcam, an indicator for peroxynitrite-mediated tissue injury), VCAM, ICAM, and E-selectin (Santa Cruz) protein expression were determined with $mLepr^{db}$, $Lepr^{db}$, and $Lepr^{db}$ mice treated with anti-TNFα (0.625 mg/ml/kg/day, i.p. for 3 days). Signals were visualized by enhanced chemiluminescence (ECL, Amersham), and quantified by Fuji film imaging software.

4. Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RNA is first reverse transcribed into cDNA using a reverse transcriptase as described. The resulting cDNA is used as templates for subsequent PCR amplification using primers specific for one or more genes. RT-PCR can also be carried out as one-step RT-PCR in which all reaction components are mixed in one tube prior to starting the reactions.

Total RNA was extracted from heart tissue using Trizol reagent (Life Technologies Inc), and was processed directly to cDNA using the SuperScript™ III Reverse Transcriptase (Life Technologies Inc). The primers of IKKα and IKKβ were designed (Beacon software) and synthesized (Invitrogen). cDNA was amplified using a qRT-PCR Kit with SYBR® Green (Life Technologies Inc). Data were calculated by 2-ΔΔCT method and presented as fold change of transcripts for IKKα and IKKβ gene in $Lepr^{db}$ mice normalized to β-actin, compared with $mLepr^{db}$ mice (defined as 1.0 fold). ΔΔCT=(CT-target-CT- β-actin) $Lepr^{db}$ - (CT-target-CT- β-actin) $mLepr^{db}$

CHAPTER III

DATA ANALYSIS I

Table 1. Serum parameters in mice with NaSal.

Groups	m Lepr^{db}	Lepr^{db}	m Lepr^{db} +NaSal	Lepr^{db} +NaSal
Body weight, g	25.82±1.5	50.53±3.17*	24.1±0.6	47.81±4.7*
Abdominal girth, cm	8.05±0.5	11.9±0.4*	8.3±0.4	11.75±0.6*
Glucose, mg/dl (non-fasting)	139±17.26	442±52.38*	122.25±16	234.5±57.08$^{\#}$
Cholesterol level, µg/µl	1.12±0.23	1.8±0.4*	1.24±0.32	1.8±0.25*
Insulin, ng/ml (non-fasting)	2.234±0.73	2.58±0.52	2.8±0.45	2.8±0.24
HOMA-IR	18.3±6.1	67.5±12.3*	20.3±4.4	38.9±9.2$^{\#}$

Body weight and serum parameters were measured at 12-16 weeks for the different strains of mice (Table 1). Lepr^{db} mice were treated with NaSal for 7-10 days and serum was collected from non-fasting mice in the morning. Non-fasting measurements were required since the Lepr^{db} murine model was intolerant to a fasting protocol. Data represent mean ± SD, n=8. *$p<0.05$ vs mLepr^{db}, $^{\#}p<0.05$ vs Lepr^{db}.

A. Roles of IKKα and IKKβ in Type 2 Diabetes

To evaluate the roles of IKKα and IKKβ in diabetes, Lepr^{db} mice were treated with anti-TNF, NAD(P)H oxidase inhibitor apocynin, and NFκB antagonist MG132. Protein expression of IKKα and IKKβ were increased in Lepr^{db} mice (Figure 2). IKKβ expression was significantly attenuated in Lepr^{db} mice treated with MG-132 or apocynin,

or in db^{TNF-}/db^{TNF-} mice vs. $Lepr^{db}$ mice. However, the alteration of IKKα expression was not observed in $Lepr^{db}$ mice treated with neutralizing antibody to TNFα, or in db^{TNF-}/db^{TNF-} mice. These findings suggest a link among IKKβ, NFκB, NAD(P)H oxidase and TNFα in coronary arterioles from mice with type 2 diabetes.

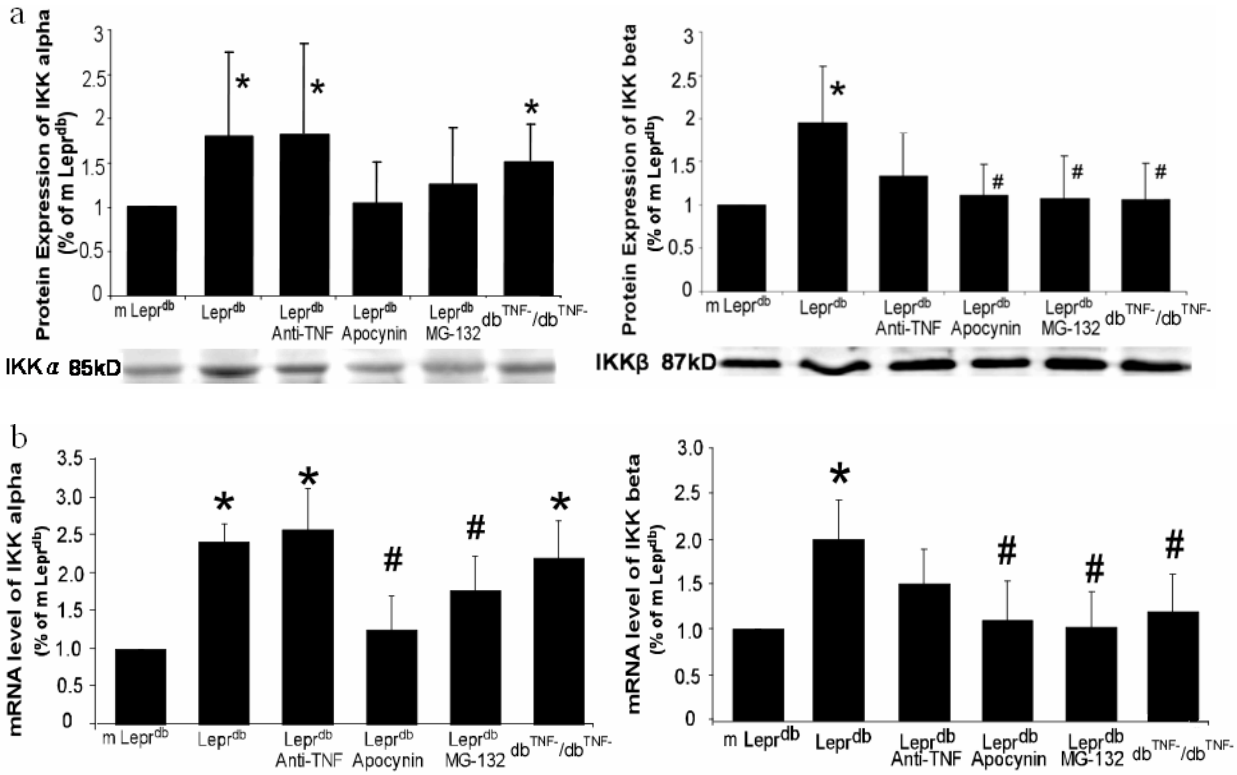

Figure 2**.** Analysis of IKK subunit expression. (a) Anti-TNF attenuated IKKβ, not IKKα protein expression in $Lepr^{db}$ mice. (b) mRNA expression of IKKα and IKKβ are presented as relative (i.e., -fold) changes in $Lepr^{db}$ compared with control mice. Data are normalized to β-actin expression levels. Data represent mean±SD, n=4. * $p<0.05$ vs. $mLepr^{db}$; # $p<0.05$ vs. $Lepr^{db}$.

B. Activation of IKKβ, NFκB and Degradation of IκBα-induced by TNFα

Phosphorylation of IKKβ activates IκBα to decrease NFκB inhibitory action of IκBα. In $Lepr^{db}$ mice heart, protein expression of p-IKKβ was higher, but expression of p-IKKβ was attenuated by anti-TNF, MG132, apocynin or in db^{TNF-}/db^{TNF-} mice (Figure 3). In $Lepr^{db}$ mice heart, protein expression of IκBα was lower (Figure 4a) vs. $mLepr^{db}$ mice. However, protein expression of p-IκBα was higher in $Lepr^{db}$ vs. $mLepr^{db}$ mice. Protein expression of p-IκBα was attenuated in db^{TNF-}/db^{TNF-} and $Lepr^{db}$ mice treated anti-TNF, apocynin or MG-132 vs. $mLepr^{db}$ mice (Figure 4b). In $Lepr^{db}$ mice heart, protein expression of NFκB was higher, but apocynin, MG-132 or db^{TNF-}/db^{TNF-} mice presented a reduced protein expression of NFκB (Figure 4c).

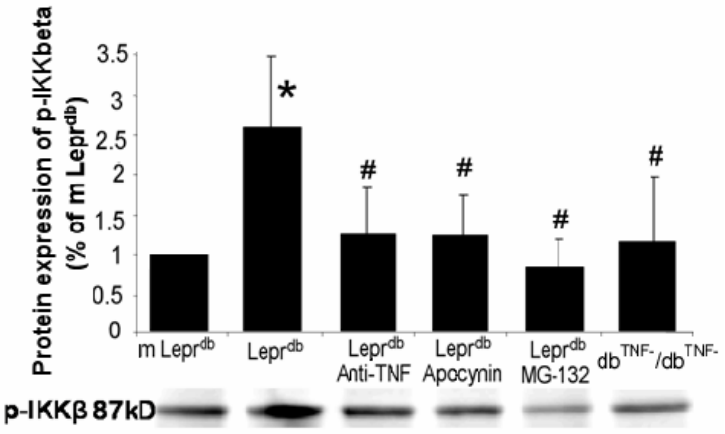

Figure 3. IKKβ activity. Protein expression of p-IKKβ was higher in $Lepr^{db}$ vs. $mLepr^{db}$. Data represent mean±SD, n=4. *$p<0.05$ vs. $mLepr^{db}$; #$p<0.05$ vs. $Lepr^{db}$.

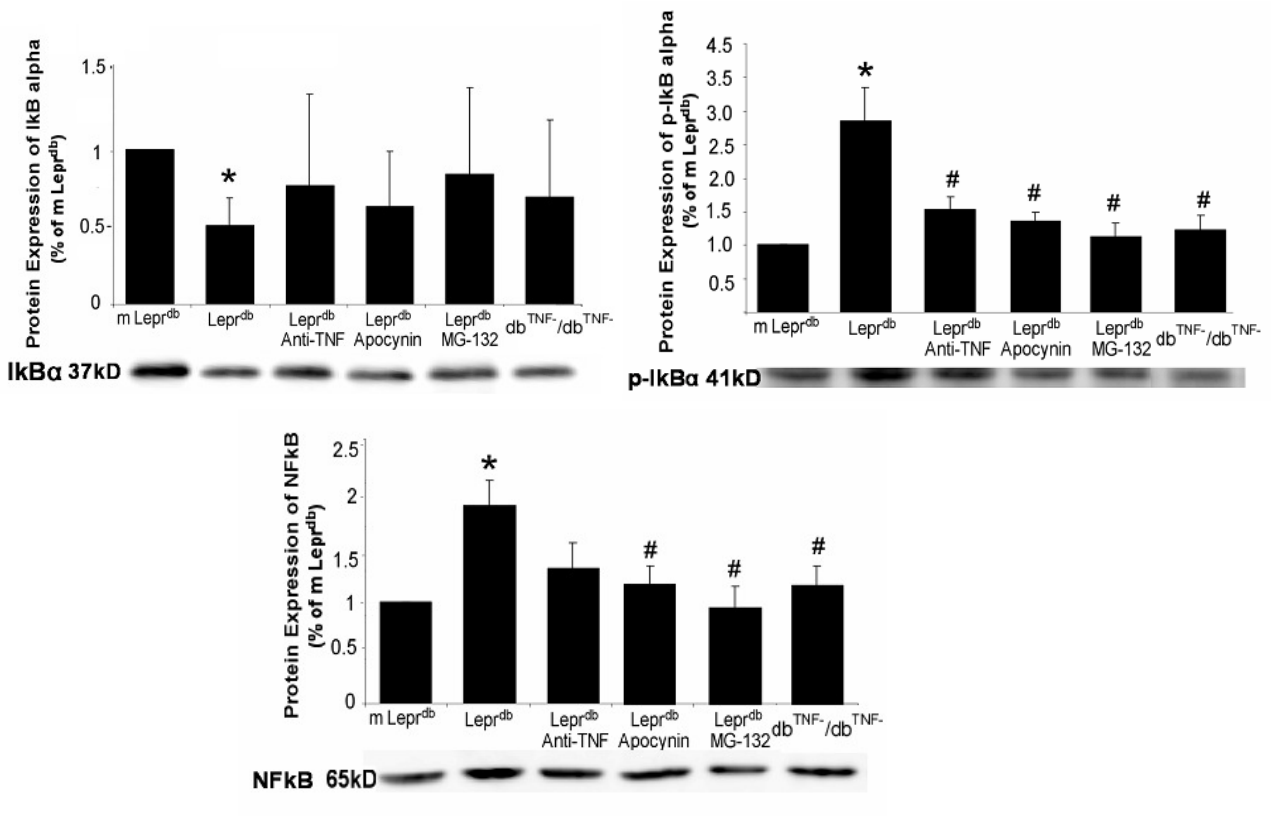

Figure 4. Analysis of IκBa and NFκB expression. Data represent mean±SD, n=4. *p<0.05 vs. mLepr^{db}; #p<0.05 vs. Lepr^{db}.

C. Role of Oxidative Stress in Type 2 Diabetes-induced Vascular Dysfunction

Vasodilation to the endothelium-dependent vasodilator ACh was significantly impaired in Lepr^{db} vs. mLepr^{db} mice (Figure 5a), but NFκB antagonist MG-132 partially restored endothelium-dependent coronary arteriolar dilation in Lepr^{db} mice. The EPR result showed that $O_2^{\cdot -}$ production was elevated and MG-132 decreased $O_2^{\cdot -}$ production in Lepr^{db} vs. mLepr^{db} mice (Figure 5b).

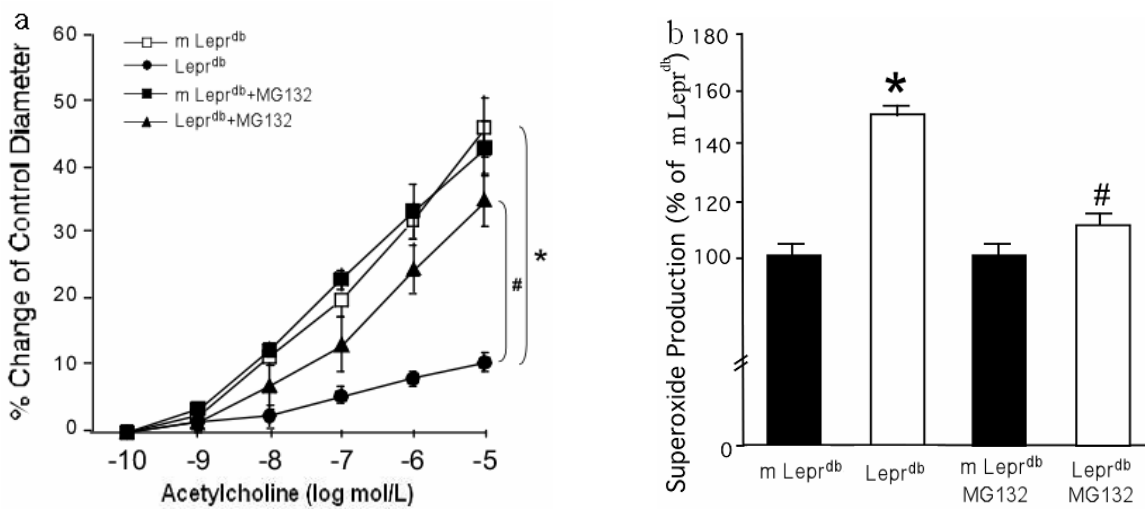

Figure 5. NFκB induces $O_2^{\cdot -}$ production. (a) Lepr^{db} mice impaired but MG132 treatment significantly restored ACh-induced endothelial-dependent vasodilation. (b) $O_2^{\cdot -}$ production from isolated coronary arterioles was higher in Lepr^{db} mice vs. mLepr^{db} mice, and MG132 attenuated $O_2^{\cdot -}$ production in Lepr^{db} mice. Values were normalized to the mean value of mLepr^{db}, n=5. *p<0.05 vs. mLepr^{db}; #p<0.05 vs. Lepr^{db}.

D. Effect of NaSal on Improvement of Insulin Sensitivity

Blood glucose, body weight, abdominal girth, lipid levels and insulin levels were higher in Lepr^{db} vs. mLepr^{db} mice. There were no significant differences in body weight, abdominal girth, lipid levels and insulin levels before and after treatment with NaSal in Lepr^{db} mice (See Table 1). Lower values of glucose concentration and HOMA-insulin resistance were found in Lepr^{db} mice treated with NaSal. The average body weight loss of Lepr^{db} mice was 2.3 ± 2.5 mg after 7 days administration with 6 mg/ml of NaSal in drinking water. Insulin tolerance test (Figure 6) showed a significant difference in the

glucose clearance rate at 120 minutes after insulin administration in Lepr^{db} mice treated with NaSal compared to Lepr^{db} mice.

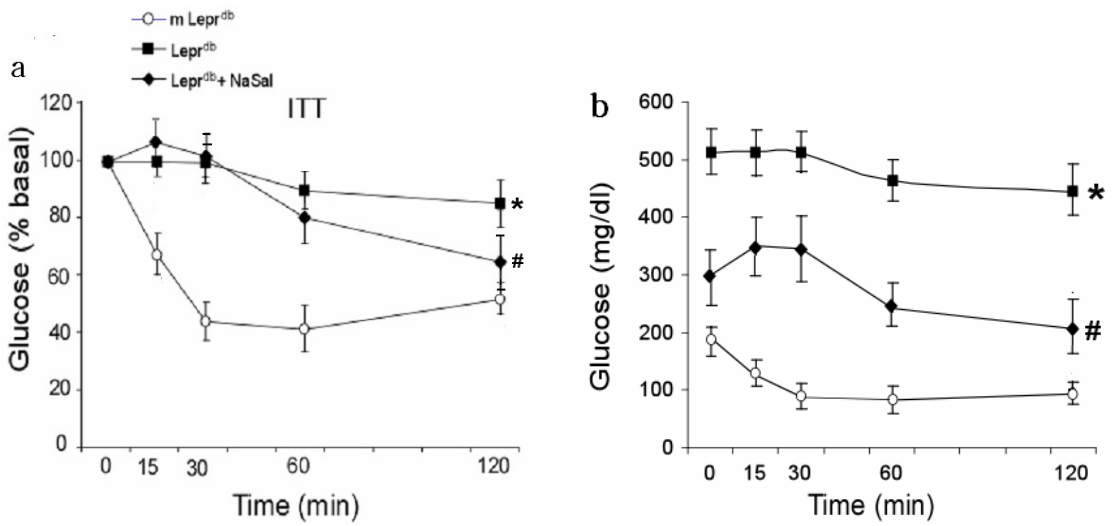

Figure 6. Insulin tolerance test. Insulin was injected (0.75 U/kg, i.p.) and blood samples were taken for glucose determinations 0, 30, 60, and 120 minutes later. (a) Insulin tolerance test showed that the glucose clearance rate at 120 minutes after insulin administration in Lepr^{db} mice treated with NaSal was significantly higher compared to Lepr^{db} mice. The value was normalized to the basal glucose level. (b) Actual glucose level. Data represent mean±SEM, n=6-8. *$p<0.05$ vs. mLepr^{db}; #$p<0.05$ vs. Lepr^{db}.

E. Role of NaSal in Vascular Dysfunction in Type 2 Diabetes

To establish the role of IKKβ in the signaling pathway, we studied whether the blockade of IKKβ improves endothelium-dependent vasodilation in Lepr^{db} mice.

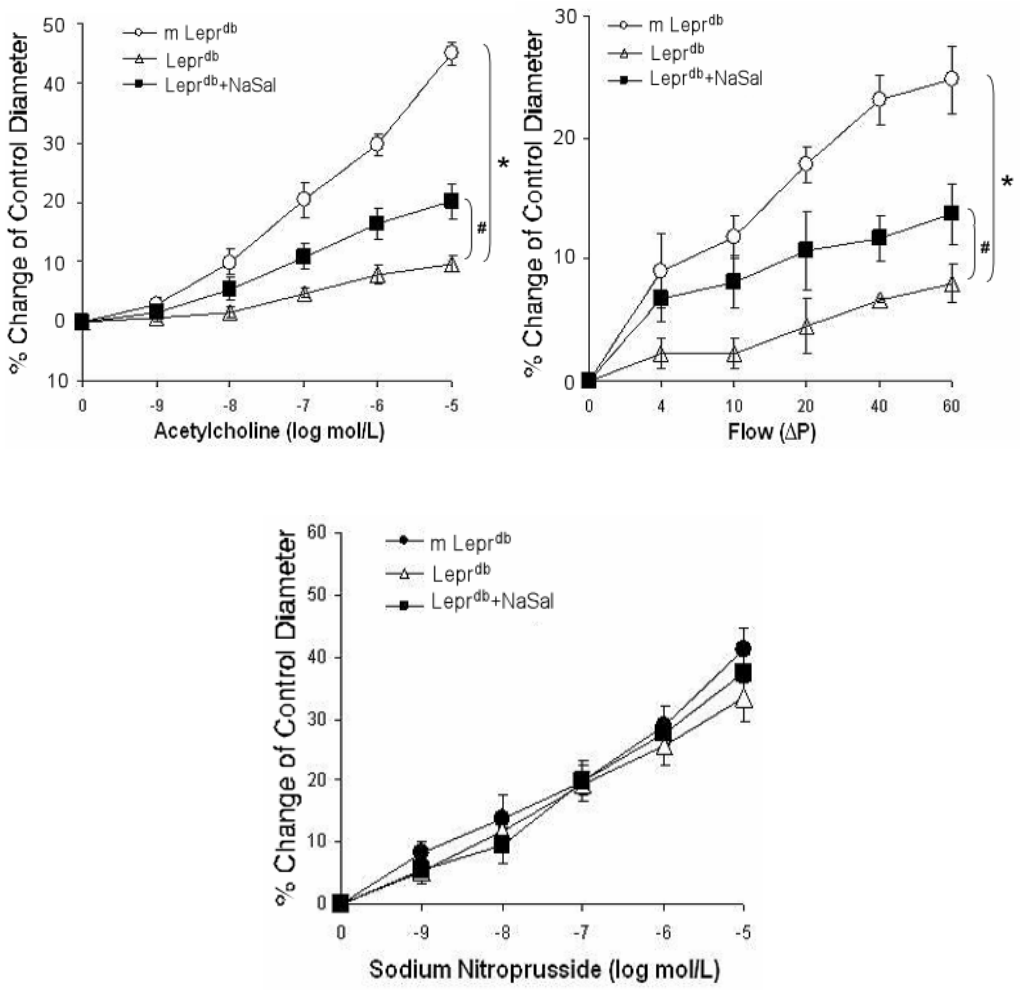

Figure 7. NaSal improves arteriolar function. (a) ACh-induced vasodilation in isolated mice coronary arterioles was blunted in Lepr^{db} vs. mLepr^{db} mice, and NaSal partially restored this dilation in Lepr^{db} mice. (b) NaSal partially restored flow-induced vasodilation in Lepr^{db} mice. (c) SNP-induced dilation in coronary arterioles was identical between mLepr^{db} and Lepr^{db} mice. NaSal did not affect SNP-induced vasodilation in Lepr^{db} mice. n=5; *p<0.05 vs. mLepr^{db}; #p<0.05 vs. Lepr^{db}.

Functional results (Figure 7) showed that NaSal partially restored the ACh- and flow-induced endothelium-dependent vasodilation in $Lepr^{db}$ mice. SNP-induced vasodilation was equivalent in $mLepr^{db}$ and $Lepr^{db}$ mice, indicating that function of smooth muscle was preserved.

F. Insulin Resistance Enhanced Activity of JNK and IKKβ in Type 2 Diabetes

Western blotting (Figure 8) showed that protein expression of TNFα, NFκB, phosphorylation of IKKβ and phosphorylation of JNK were greater in $Lepr^{db}$ mice heart, but NaSal attenuated the protein expression of TNFα, NFκB, phosphorylation of IKKβ and phosphorylation of JNK in $Lepr^{db}$ mice heart. Phosphorylation of insulin-receptor substrate-1 on serine site blocks insulin signaling transduction in diabetes. Protein expression of IRS-1 (Figure 9a) was significantly reduced in $Lepr^{db}$, $Lepr^{db}$ treated with NaSal and $Lepr^{db}$ treated with JNK inhibitor SP600125 vs. $mLepr^{db}$ mice. The ratio of pIRS-1 and IRS-1 was significantly higher in $Lepr^{db}$, $Lepr^{db}$ treated with NaSal and $Lepr^{db}$ treated with JNK inhibitor SP600125 vs. $mLepr^{db}$ mice. However, protein expression of phosphorylation of IRS-1 (pIRS-1, Ser307) was unaffected (Figure 9b) in $Lepr^{db}$, $Lepr^{db}$ treated with NaSal and $Lepr^{db}$ treated with JNK inhibitor SP600125 vs $mLepr^{db}$.

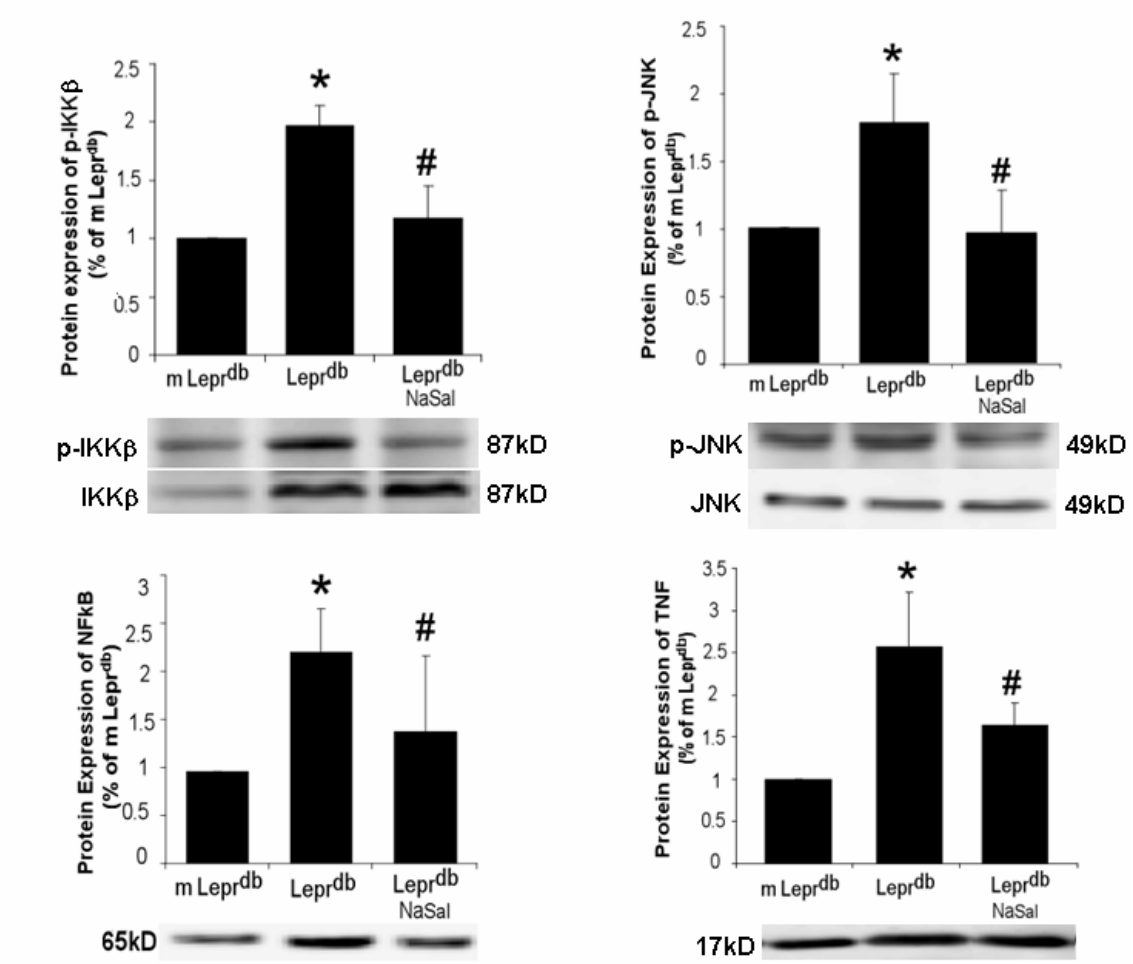

Figure 8. Effect of NaSal on IKKβ and JNK activity. Protein expression of p-IKKβ, p-JNK, NFκB and JNK were greater in Lepr^{db} mice vs. mLepr^{db} mice, but NaSal decreased protein expression of p-IKKβ, p-JNK, NFκB and JNK, without affecting protein expression of IKKβ and JNK. Data represent mean±SD, n=5. *$p<0.05$ vs. mLepr^{db} ; #$p<0.05$ vs. Lepr^{db}.

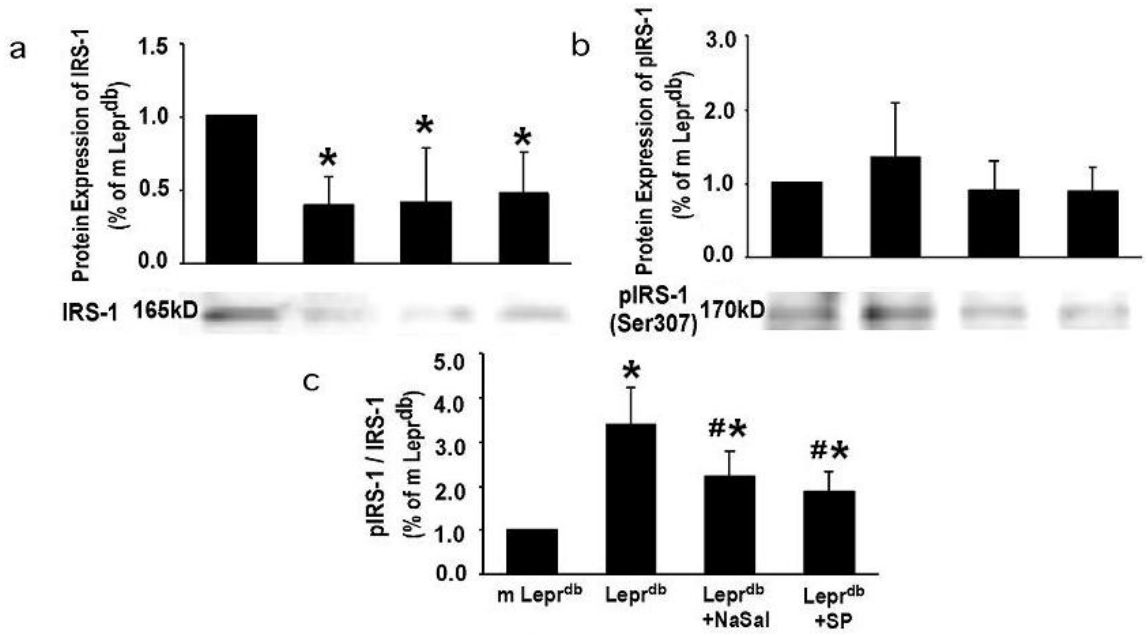

Figure 9. Protein expression of IRS-1 ser307. (a) Total insulin receptor substrate-1 (IRS-1) protein expression was decreased in Lepr^{db} vs. mLepr^{db} mice. NaSal and SP600125 treatment did not increase IRS-1 expression. (b) p-IRS-1 (Ser307) protein expression was elevated in Lepr^{db} compared with mLepr^{db} control mice and both NaSal and SP600125 treatment appeared to reduce p-IRS-1 protein expression although there was no statistically significant difference between groups. (c) Ratio of p-IRS-1 to total IRS-1 was significantly higher in Lepr^{db} vs. mLepr^{db} mice. NaSal and SP600125 treatment reduced p-IRS-1/IRS1. Data represent mean±SD, n=8. *P<0.05 vs. mLepr^{db}; # P<0.05 vs. Lepr^{db}.

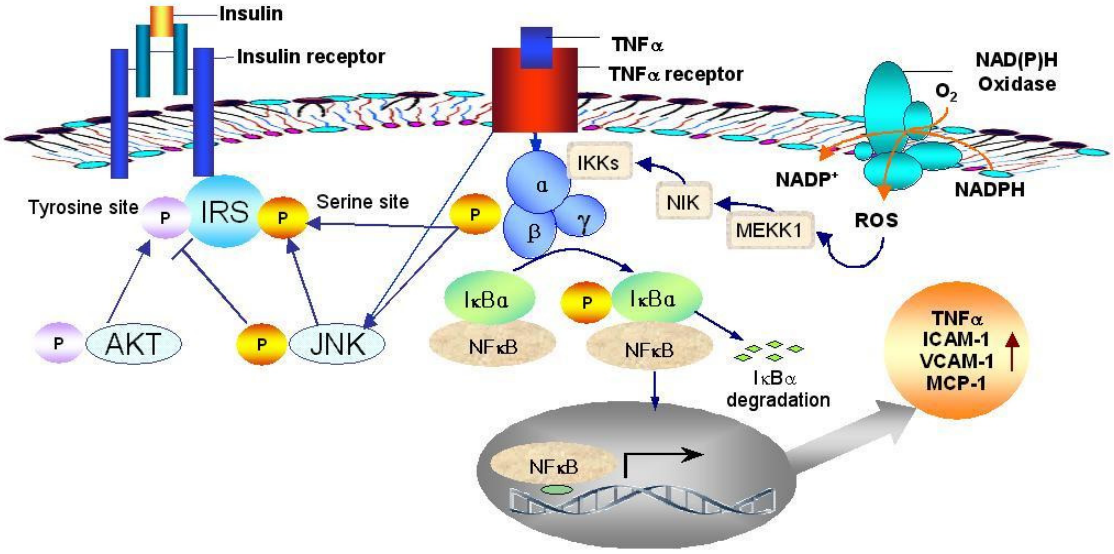

Figure 10. Putative TNFα/ NFκB and insulin signaling pathways. This schematic shows the correlative and causative relationship between inflammation and insulin resistance and depicts our proposed mechanism(s) by which NaSal sensitizes insulin signaling. TNFα triggers the activation of IKKβ (rather than IKKα) to initiate processes leading to inflammation. NFκB is free to translocate and bind to DNA on the target genes when IκBα is phosphorylated by IKKβ, ubiquitinated and degraded by S26 proteasome. NFκB is involved in many aspects of the inflammatory response, e.g. induction of TNFα, MCP-1, and adhesion molecules. NFκB itself is induced by stimuli such as pro-inflammatory cytokines. On the other hand, JNK and IKKβ induce IRS-1 serine phosphorylation to block insulin signaling in type 2 diabetes.

CHAPTER IV

DATA ANALYSIS II

Table 2. Serum parameters in mice with anti-MCP-1.

Groups	m Lepr^{db}	Lepr^{db}	Lepr^{db} +anti-MCP-1
Body weight, g	25.82±1.5	48.45±2.3*	49.33±2.7*
Glucose, mg/dl(non-fasting)	139±17.26	437.5±44.5*	446.12±35.2*
Abdominal girth, cm	8.05±0.5	11.9±0.4*	11.8±0.39*
Cholesterol level, μg/ μl	1.12±0.23	1.8±0.4*	1.57±0.4*
Insulin, ng/ml(non-fasting)	2.234±0.73	2.58±0.52	2.59±0.488

0.2g/kg/day of anti-MCP-1 i.p. for 3day, n=8

Glucose concentration and cholesterol level are higher in Lepr^{db} and Lepr^{db} mice treated with anti-MCP-1 (0.2 g/kg/day, 3 days) than in mLepr^{db} control mice ($P<0.05$, n=8). Abdominal girth was higher in Lepr^{db} and Lepr^{db} mice treated with anti-MCP-1 vs. mLepr^{db} on the day of surgery (n=8). * $p<0.05$ vs. mLepr^{db} ; # $p<0.05$ vs. Lepr^{db}.

A. TNFα and MCP-1 Amplification of Signaling in Coronary Arterioles in Type 2 Diabetes

We determined if TNFα and MCP-1 interact in their protein expressions. Protein expression of TNFα and MCP-1 from isolated coronary arterioles was analyzed in mLepr^{db}, Lepr^{db} and Lepr^{db} mice treated with anti-TNFα or anti-MCP-1. Western blot analysis (Figure 11) revealed that anti-TNFα markedly decreased MCP-1 expression and

similarly anti-MCP-1 decreased TNFα expression, indicating there is an association between MCP-1 and TNFα signaling.

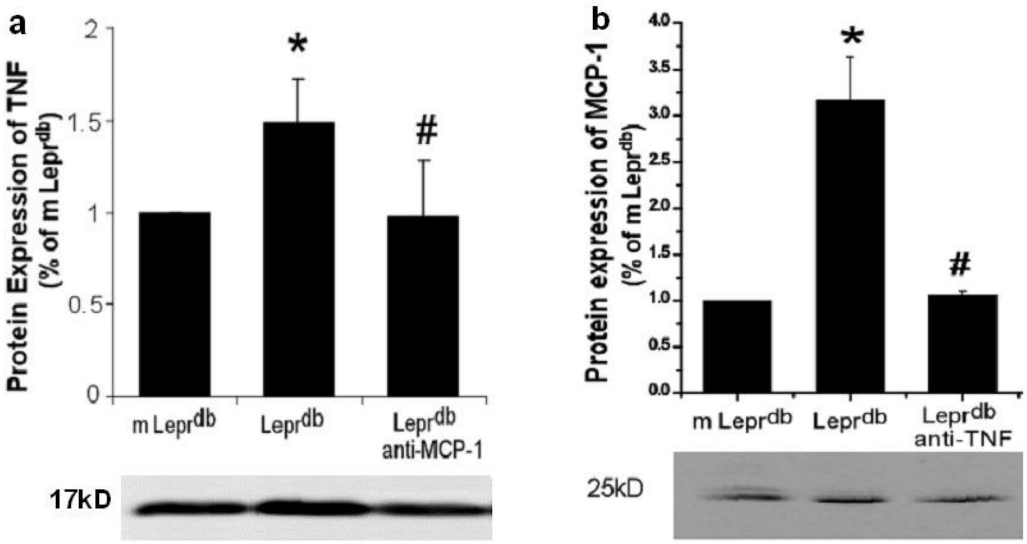

Figure 11. Interaction between TNF and MCP-1. (a) Protein expression of TNFα from isolated coronary arterioles was higher in Lepr^{db} vs. mLepr^{db} mice, but anti-MCP-1 attenuated TNFα expression in Lepr^{db} mice. (b) MCP-1 expression was elevated in Lepr^{db} vs. mLepr^{db} mice, but anti-TNFα attenuated MCP-1 expression in Lepr^{db} mice. Data represent mean±SD, n=4. * $p<0.05$ vs. mLepr^{db} ; # $p<0.05$ vs. Lepr^{db}.

B. Cellular Source of MCP-1 Expression in Type 2 Diabetes

Immunostaining (Figure 12) showed that MCP-1 protein expression (red) was present in endothelial cells, but not in vascular smooth muscle cells. Lean control (mLepr^{db}) heart (A,B,C), Lepr^{db} mice (D,E,F), Lepr^{db} mice with anti-TNFα treatment

(G,H,I) and Lepr^{db} mice with anti-MCP-1 treatment (J,K,L). The expression of MCP-1 was decreased in Lepr^{db} mice with anti-TNFα and anti-MCP-1 treatment. Negative control (M,N,O) shows an absence of staining in vessels with the secondary antibodies. Additionally, MCP-1 expression was higher in Lepr^{db} than in mLepr^{db} mice, but anti-TNFα or anti-MCP-1 decreased MCP-1 expression in Lepr^{db} mice. Lepr^{db} heart yielded a high percentage (70 %) of MCP-1 positive staining in the vessels, but a lower percentage of MCP-1 positive staining was presented in Lepr^{db} mice treated with anti-MCP-1 (30 %) or anti-TNFα (40 %) comparable to that in mLepr^{db} mice (15%). We quantified MCP-1 expression in vessels by counting the specific stained MCP-1 per section and normalized to mean value of mLepr^{db} mice (data not shown). Experiments were performed without the primary antibodies to test whether or not staining was related to the non specific binding of the secondary antibodies, which showed no staining in heart sections, indicating that the signals were due to specific binding of the primary antibody.

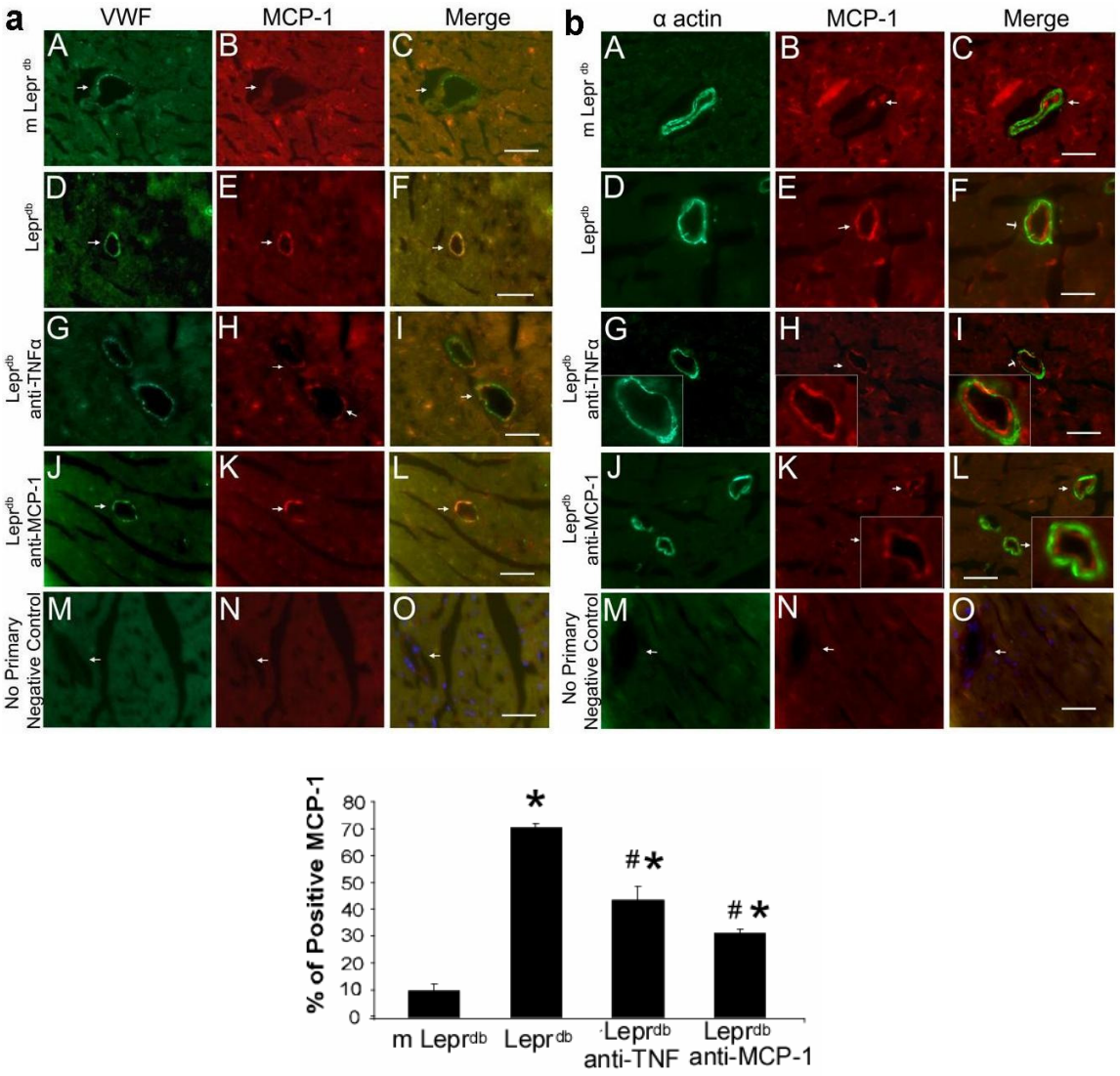

Figure 12. Co-localization of MCP-1 in microvessels. (A) Dual fluorescence combining MCP-1 and endothelial cells (von Willebrand factor [VWF] or vascular smooth muscle cells (α-actin, Figure 12b) or using specific antibodies followed by a fluorescent-labeled secondary antibody. (B) Dual fluorescence combining MCP-1 with markers for smooth muscle [α-actin]. Bar size is 50 μm. Original Magnification x 40. Data shown are representative of 4 separate experiments. * $p<0.05$ vs. $mLepr^{db}$; # $p<0.05$ vs. $Lepr^{db}$.

C. Role of MCP-1 in Type 2 Diabetes-Induced Vascular Dysfunction

Vasodilation to the endothelium-dependent vasodilator Ach was impaired in $Lepr^{db}$ mice compared to $mLepr^{db}$ mice (Figure 13). Conversely, neutralizing antibody to MCP-1 partially restored coronary arteriolar dilation-induced by Ach in $Lepr^{db}$ mice. To address whether over-expression of MCP-1 influences enhanced oxidative stress in $Lepr^{db}$ mice, we analyzed protein expression of N-Tyr (Figure 14b) to infer the concentration of peroxinitrite ($ONOO^-$), and measured $O_2^{\cdot -}$ production (Figure 14a). Multiple bands revealed that protein expression of N-Tyr was higher in $Lepr^{db}$ mice vs $mLepr^{db}$ mice, but neutralization of MCP-1 attenuated protein expression of N-Tyr in $Lepr^{db}$ mice. The EPR result showed that $O_2^{\cdot -}$ production was elevated and neutralization of MCP-1 decreased $O_2^{\cdot -}$ production in $Lepr^{db}$ mice.

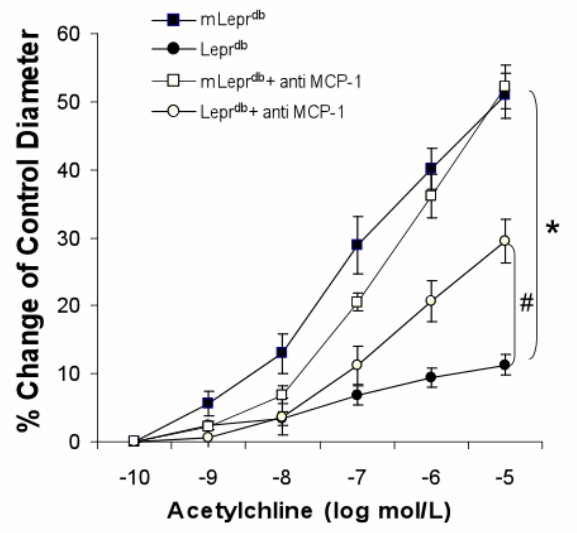

Figure 13. Role of MCP-1 on endothelial function. Neutralizing antibodies to MCP-1 restored coronary arteriolar dilation to Ach in $Lepr^{db}$ mice, but did not affect the vasodilation to Ach in $mLepr^{db}$ mice. * $p<0.05$ vs. $mLepr^{db}$; # $p<0.05$ vs. $Lepr^{db}$.

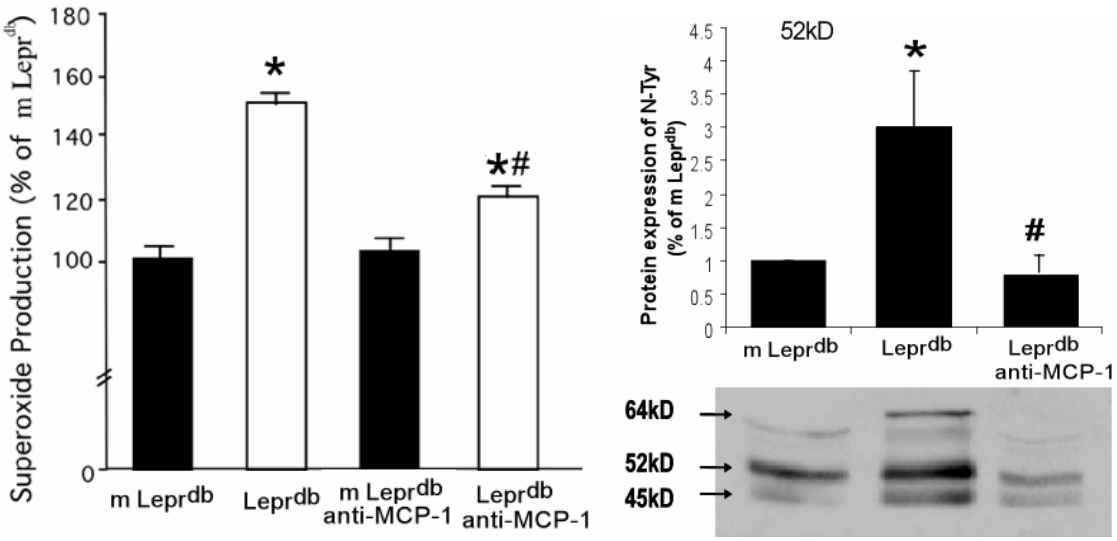

Figure 14. Effect of neutralizing MCP-1 in Lepr^{db} on oxidative stress. (a) Superoxide anion ($O_2^{\cdot -}$) production (n=6) is higher in Lepr^{db} mice than in lean controls (mLepr^{db}). Anti-MCP-1 reduced the production of superoxide in Lepr^{db} mice. (b) Protein expression of nitrotyrosine from coronary arterioles was determined. The results are shown for nitrotyrosine bands migrating at 52- and 45-kDa. Both 52- and 45-kDa bands of nitrotyrosine are higher in Lepr^{db} mice than in mLepr^{db} mice and Lepr^{db} mice treated with anti-MCP-1. Data represent mean±SD, n=4. * $p<0.05$ vs. mLepr^{db} ; # $p<0.05$ vs. Lepr^{db}.

D. TNFα and MCP-1-induced Macrophage Recruitment and Activation

We further investigated the biological function of MCP-1; responsible for chemoattraction of circulating monocyte-derived macrophages. Immunostaining analysis (Figure 13), revealed co-expression of the monocyte/macrophage marker CD68 and MCP-1 expression was increased about 3 fold in Lepr^{db} vs. mLepr^{db} mice. Anti-TNFα or

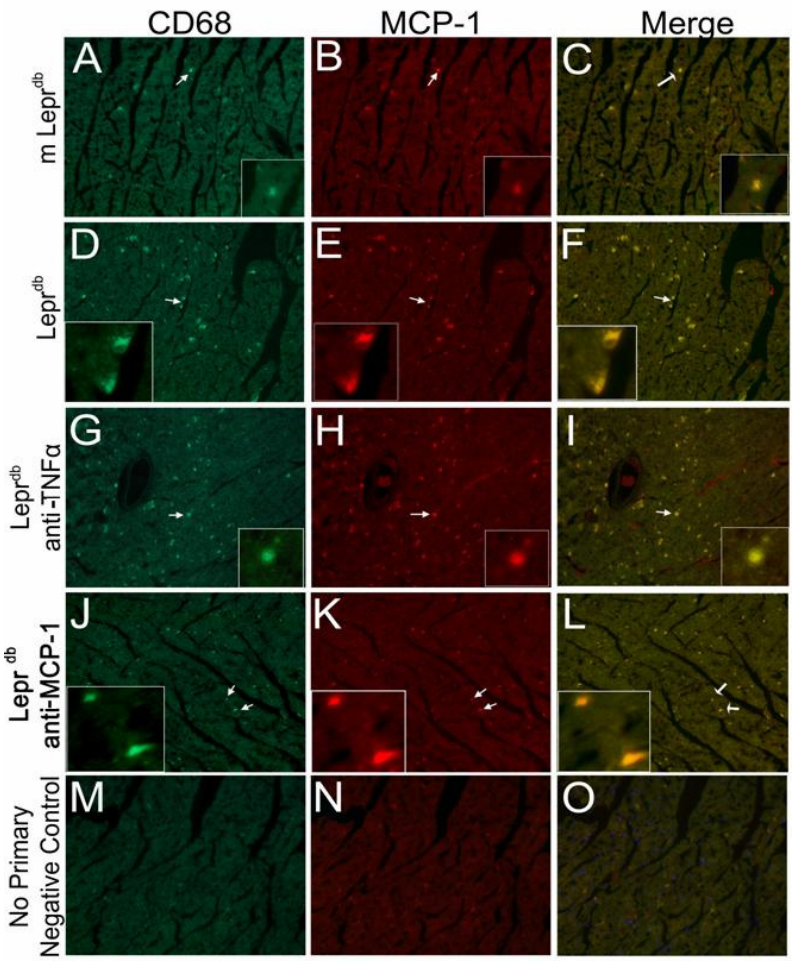

Figure 15. Co-localization of MCP-1 in macrophages. Heart sections from lean control (mLepr^{db}) (A,B,C), Lepr^{db} (D,E,F), and Lepr^{db} mice with anti-TNFα treatment (G,H,I) and Lepr^{db} mice with anti-MCP-1 treatment (J,K,L). Macrophage infiltration is significantly increased in Lepr^{db} mice compared to mLepr^{db} mice and decreased after anti-TNFα and anti-MCP-1 treatment to Lepr^{db} mice. Negative control (M,N,O) shows an absence of staining in vessels with only the secondary antibodies. Original magnification 16X. Data shown are representative of 4 separate experiments. * $p<0.05$ vs. mLepr^{db} ; # $p<0.05$ vs. Lepr^{db}.

anti-MCP-1 treatment reduced infiltration of macrophages by 2 and 1.5 fold, respectively, in Lepr^{db} mice, which suggests that coronary arteriolar dysfunction might be associated with macrophage influx. We quantified macrophage infiltration by counting the specifically stained macrophages per equal unit area and normalized to the mean value of mLepr^{db} mice.

E. TNFα and MCP-1-induced Adhesion Molecules and Macrophage Infiltration

Dual fluorescence combining VCAM with markers for endothelial cells (von Willebrand factor [VWF]) (A~F), vascular smooth muscle cells [α-actin] (G~O) or macrophages [CD68] (P~R) using specific antibodies followed by a fluorescent labeled secondary antibody. Arrows show co-localization of VCAM in endothelial cells and specific stain of VCAM inside vascular smooth muscle cells or co-localization of VCAM in infiltrated macrophage in diabetic mice (Lepr^{db}). No primary antibody control (S~U) shows an absence of staining in vessels with secondary antibodies. Figure 16a shows increased protein expression of VCAM-1 (red) in the heart of Lepr^{db} vs mLepr^{db} mice, which is consistent with the elevated protein expression detected by western blot analysis (Figure 16 b-d). We analyzed protein expression of adhesion molecules; VCAM, ICAM, and E-selectin to test whether they are correlated with increased expression of cytokines (e.g., TNFα and MCP-1) in Lepr^{db} mice. The concentration of adhesion molecules was elevated in Lepr^{db} mice vs. mLepr^{db} mice but attenuated after administration with anti-TNFα.

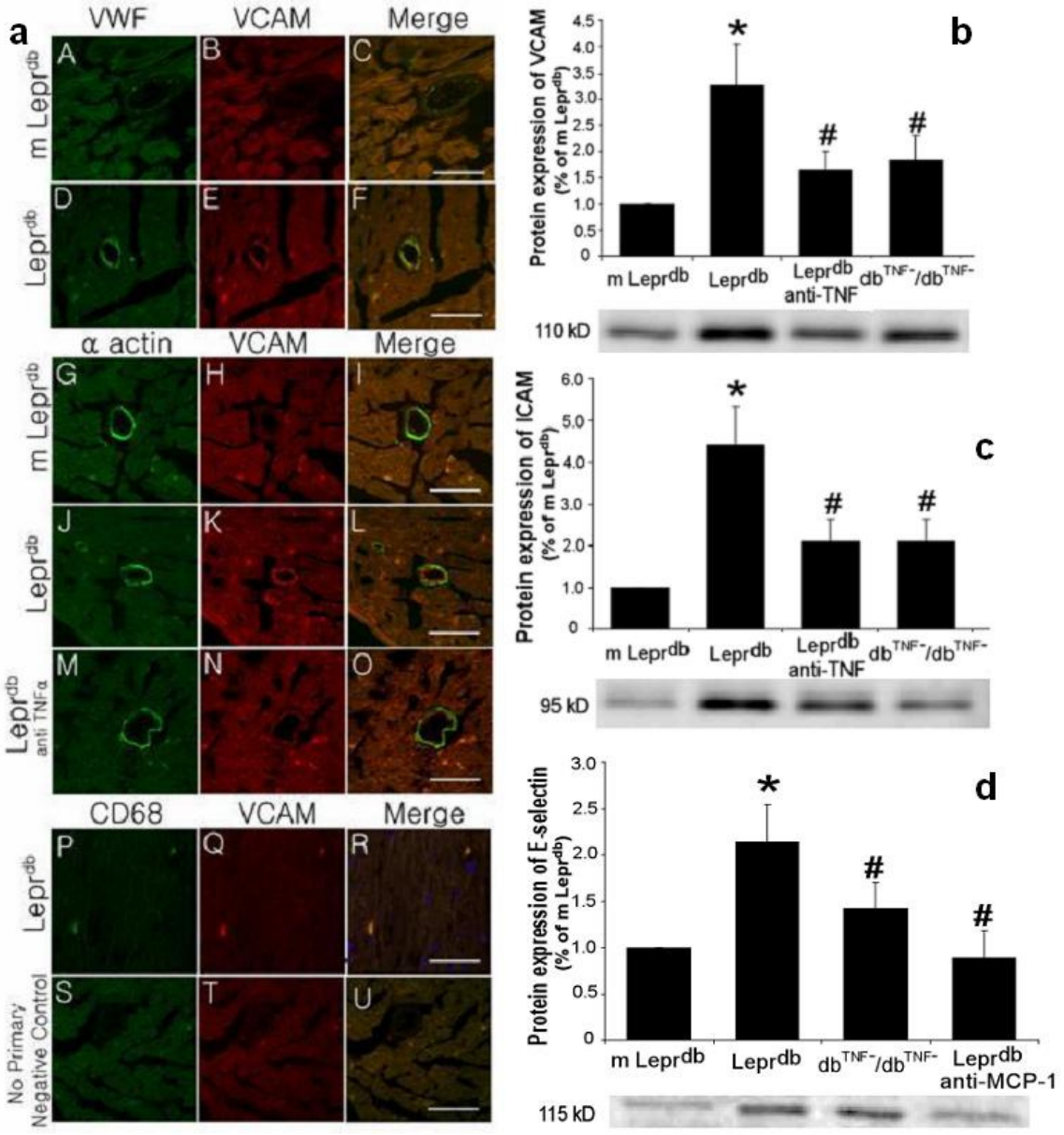

Figure 16. Analysis of adhesion molecule expression. (a) Bar size is 50μm. Original magnification: 63X. Data shown are representative of 4 separate experiments. (b) Protein expression of VCAM, (c) ICAM, and (d) E-selectin from coronary arterioles was determined. Data represent mean±SD, n=4. * $p<0.05$ vs. $mLepr^{db}$; # $p<0.05$ vs. $Lepr^{db}$.

CHAPTER V

SUMMARY AND CONCLUSION

First, our results in feed-forward Signaling of TNFα and NFκB via IKKβ Pathway suggest that the interaction of NFκB and TNFα signaling induces activation of IKKβ and amplifies oxidative stress, leading to endothelial dysfunction in coronary arterioles of $Lepr^{db}$ mice, a model for obesity and type 2 diabetes. Importantly, our findings also support the concept that feed-forward signaling of TNFα and NFκB via the IKKβ pathway induces insulin resistance and coronary arteriolar dysfunction in type 2 diabetes based on the following observations: NFκB antagonist MG-132, or IKKβ inhibitor NaSal, restored endothelium-dependent coronary arteriolar dilation in $Lepr^{db}$ mice, but the responses in $mLepr^{db}$ mice were unaffected. Protein expression of IKKα and IKKβ were higher in $Lepr^{db}$ than in $mLepr^{db}$ mice; the expression of IKKβ, but not the expression of IKKα, were attenuated by MG-132, NAD(P)H oxidase inhibitor apocynin or in db^{TNF-}/db^{TNF-} mice. Insulin resistance was increased in $Lepr^{db}$ mice and NaSal improved insulin sensitivity. Protein expression of TNFα, NFκB, phosphorylation of IKKβ and JNK were greater in $Lepr^{db}$ mice, but NaSal attenuated protein expression of TNFα, NFκB, phosphorylation of IKKβ and JNK in $Lepr^{db}$ mice. The pIRS-1/IRS-1 ratio was elevated in $Lepr^{db}$ compared with $mLepr^{db}$ mice. Both NaSal and the JNK inhibitor SP600125 reduced the pIRS-1/IRS-1 ratio in $Lepr^{db}$ mice. MG-132 (Figure 5b) or anti-TNFα[5] reduced $O_2^{\cdot -}$ production in $Lepr^{db}$ mice. NFκB induces TNFα signaling to accentuate oxidative stress and endothelial dysfunction via an IKKβ dependent

mechanism, which may be associated with inflammatory and insulin signaling pathways in type 2 diabetes. Our findings are consistent with NFκB involvement, by interacting with TNFα, thereby inducing IKKβ and NAD(P)H oxidase, in endothelial dysfunction in type 2 diabetes. The present molecular results further support our previous physiological observations that increases in TNFα expression induce activation of NAD(P)H oxidase and production of ROS, leading to endothelial dysfunction in type 2 diabetes.[5]

A. Roles of Interaction of TNFα and NFκB Signaling on Impaired Coronary Arteriolar Responses in Type 2 Diabetes

The inducible transcription factor NFκB regulates the expression of genes encoding oxidants, cytokines, chemokines and adhesion molecules, which are associated with inflammation and activated by gene products of NFκB, e.g., a feed-forward interaction.[5,35-37] These pro-inflammatory agents play a detrimental role in vascular pathology and TNFα initiates signaling cascades that converge on the IKK complex via the NFκB signaling pathway. We previously found that TNFα signaling leads to oxidative stress via NAD(P)H oxidase activation and perhaps via activating NFκB, which in turn may lead to further increases in TNFα expression leading to endothelial dysfunction in type 2 diabetic mice.[5] TNFα is an inducer of IKKβ and IκBα in the classical NFκB pathway. Thus, TNFα initiates signaling cascades predominantly acting through IKKβ.[38,39] In this study, we examined whether feed-forward interaction between TNFα and NFκB, via the IKKβ pathway, amplify one another toward the evolution of

vascular disease and insulin resistance in type 2 diabetes. Our western blotting results showed that protein expression of IKKα and IKKβ were higher in $Lepr^{db}$ than in $mLepr^{db}$ mice. MG-132, NAD(P)H oxidase inhibitor, apocynin, or db^{TNF-}/db^{TNF-} mice attenuated the expression of IKKβ, but not the expression of IKKα. This points to a linkage among IKKβ, NFκB, NAD(P)H oxidase and TNFα in contributing to type 2 diabetes.

Biological activity of NFκB is controlled mainly by IκBα protein, which binds to NFκB in the cytoplasm and inhibits DNA binding activity. IκB blocks a nuclear translocation signal to inactivate NFκB in the cytoplasm and removes NFκB bound to promoters in the nucleus.[6] MG-132 has been shown to inhibit the degradation of IκBα. Our results showed that protein expression of IκBα was lower. However, protein expression of p-IκBα was higher in $Lepr^{db}$ mice compared to $mLepr^{db}$ mice. In db^{TNF-}/db^{TNF-} and $Lepr^{db}$ mice treated with anti-TNF, apocynin or MG-132, protein expression of p-IκBα was attenuated. In $Lepr^{db}$ mice, protein expression of NFκB was higher, but apocynin, MG-132 or db^{TNF-}/db^{TNF-} mice reduced protein expression of NFκB, indicating that TNFα, NAD(P)H oxidase and IκBα degradation may increase the activity of NFκB. Our functional results showed that ACh-induced vasodilation was impaired in $Lepr^{db}$ mice vs. $mLepr^{db}$ mice, but NFκB antagonist MG-132 partially restored ACh-induced coronary arteriolar dilation in $Lepr^{db}$ mice. The EPR results showed that $O_2^{\cdot -}$ production was elevated and MG-132 decreased $O_2^{\cdot -}$ production in $Lepr^{db}$ mice, which indicates that blocking NFκB transcriptional action markedly attenuated $O_2^{\cdot -}$ production in isolated coronary arterioles in $Lepr^{db}$ mice. This supports the concept that diabetes

increases NAD(P)H oxidase activity through the interaction of TNFα/NFκB signaling, which in turn accentuates oxidative stress and induces endothelial dysfunction via an IKKβ activation. Our results are consistent with the concept that IKKβ is a putative activator of NFκB, which is thought to alter subsequent gene expression in this pathway.[35-37]

B. The Role of IKKβ in Vascular Dysfunction in Type 2 Diabetes

NaSal is an IKKβ inhibitor that interrupts the cascade of molecular events leading to inflammation. Yuan et al. showed that reduced signaling through the IKKβ pathway inhibition by NaSal in obese mice is accompanied by improved insulin sensitivity.[9] Recent studies noted that NFκB blockade (by inhibiting IKKβ activity) decreased myocardial injury and preserved cardiac function following ischemia-reperfusion,[10] which supports the concept that the inflammatory response participates in the development of heart failure.[39] Our results showed that phosphorylation of IKKβ increases in diabetes, but blockade of NFκB and NAD(P)H oxidase and db^{TNF-}/db^{TNF-} mice attenuated phosphorylation of IKKβ, suggesting that 1) IKKβ activity increases in diabetes; and 2) phosphorylation of IKKβ increases in diabetic mice by activation of NAD(P)H oxidase and TNFα. This indicates that TNFα-induced phosphorylation of IKKβ is followed by phosphorylation of IκBα to release NFκB into the nucleus.

The pathogenesis of endothelial dysfunction in diabetes is initially related to a decrease in NO synthesis or inactivation of NO due to increased endothelial production of ROS.[41] An underlying mechanism proposed for TNFα induced endothelial

dysfunction is that TNFα signaling leads to oxidative stress via NAD(P)H activation, which in turn may lead to reduced NO bioactivity.[5,34] We identified a link between vascular dysfunction and insulin resistance in that treatment with NaSal in coronary microcirculation resulted in an improvement of endothelial dysfunction in diabetic $Lepr^{db}$ mice. The NO donor SNP induced an identical vasodilation in $Lepr^{db}$ and $mLepr^{db}$ mice, but ACh-induced vasodilation was impaired in isolated coronary arterioles in diabetic $Lepr^{db}$ mice, which indicates that endothelium-dependent vasodilation is impaired in diabetes. Most importantly, NFκB antagonist MG132 or IKKβ inhibitor NaSal partially restored endothelium-dependent coronary arteriolar dilation in type 2 diabetes. These results further support our hypothesis that TNFα and NFκB signaling via an IKKβ dependent mechanism plays a key role in endothelial dysfunction in diabetes.

Blood glucose, body weight, abdominal girth, lipid level and insulin level were higher in $Lepr^{db}$ vs. $mLepr^{db}$ mice, and there were no significant differences in body weight, abdominal girth, lipid level and insulin level before and after treatment with NaSal. However, lower values of glucose concentration and HOMA-insulin resistance found in $Lepr^{db}$ mice treated with NaSal indicate that NaSal improved insulin sensitivity in $Lepr^{db}$ mice (Table 1). Insulin tolerance test in Figure 6 shows that there was a significant difference in the glucose clearance rate at 120 minutes after insulin administration in $Lepr^{db}$ mice treated with NaSal compared to $Lepr^{db}$ mice. Our results indicate that hyperglycemia and insulin resistance in $Lepr^{db}$ mice were amended by a short-term treatment with NaSal via a downregulation of IKKβ and NFκB signaling.

Increased phosphorylation of IKKβ may also contribute to the development or progression of the vascular disease and hyperglycemia in type 2 diabetes.

C. Link between Inflammation-induced Insulin Resistance and Vascular Dysfunction

Coronary artery disease causes acute myocardial infarction, which is associated with significant morbidity and mortality in patients with diabetes. Diabetes is associated with alterations in cardiac energy metabolism, characterized by reduced glucose utilization, and increased utilization of fatty acids as a metabolic substrate.[42] The data show that insulin may also modulate cardiac myocyte metabolism through paracrine mechanisms by activating insulin receptors in the heart,[11] thereby decreasing myocardial infarct size and increasing cardiac contractility.[12,13] Human type 2 diabetes is currently characterized by defects in both insulin action and insulin secretion, which lack the focus needed to identify a single molecular abnormality underlying these features, if indeed one exists. Insulin-receptor substrates (IRS proteins) may be involved in type 2 diabetes[43] and tyrosine phosphorylation activates insulin IRS-1, which further leads to the translocation of glucose transporters (GLUT4) to the cell surface.[44] Impaired transcription of the major cardiac glucose transporter GLUT4 is seen within 4 days after the induction of diabetes.[45] IKKβ may affect glucose metabolism by altering NFκB transcriptional action to express GLUT4.[46] JNK is a main regulatory molecule that contributes to coronary arteriolar dysfunction and insulin resistance.[14,47] IRS-1 tyrosine phosphorylation by Akt induces insulin signal transduction.[15] Serine phoshphorylation by JNK can downregulate or inactivate IRS-1. The impaired activation of Akt and enhanced activation of JNK by TNFα is correlated with insulin resistance.[47] Sholeson et

al. reported that inflammation-induced insulin resistance and noted that two signaling pathways are linked to the proinflammatory effects of obesity and type 2 diabetes. Both NFκB/IKKβ and JNK pathways are activated by TNFα in liver or adipose tissue. Genetic disruption of NFκB and JNK signaling pathways has been shown to improve insulin resistance.[1] Consistent with the findings described above, inhibition of IKKβ or JNK activity by NaSal significantly reduced blood glucose level and augmented insulin signaling in cardiac tissue. Our results showed that protein expression of TNFα and NFκB were higher in diabetic mice, but NaSal attenuated protein expression of TNFα and NFκB. Specifically, NaSal attenuated phosphorylation of IKKβ and JNK without altering protein expression of IKKβ and JNK. This suggests that NaSal interrupts phosphorylation of IKKβ, which may in turn decrease phosphorylation of JNK. TNFα activates the IKKβ and JNK signaling pathway and, conversely, the expression of the TNFα gene is regulated by NFκB through activation of IKKβ. IKKβ and c-JNK phosphorylate specific serine sites (e.g., Ser307) on the insulin receptor and /or insulin receptor substrate-1 (IRS-1). Serine phosphorylation of IRS-1 blocks insulin-stimulated tyrosine phosphorylation at IRS-1 and reduces downstream insulin signaling, resulting in reduced GLUT4 translocation and insulin resistance.[48,49]

To further elucidate the signaling pathway involved in the recovery of insulin action in cardiac tissue, we tested whether insulin receptor substrate-1 serine phosphorylation is attenuated by SP600125 and NaSal. Phosphorylated IRS-1 (pIRS-1) protein expression was elevated in $Lepr^{db}$ compared with $mLepr^{db}$ mice; both NaSal and JNK inhibitor SP600125 reduced pIRS-1 protein expression in $Lepr^{db}$ mice. Our results

indicate that TNFα, phosphorylation of JNK and IKKβ, and NFκB contribute to insulin resistance and vascular dysfunction probably via activation of IKKβ in type 2 diabetes. Interestingly, NaSal and JNK inhibitor SP600125 sensitize insulin signaling by preventing serine phosphorylation on IRS-1 from IKKβ and JNK. This indicates NaSal inhibits phosphorylation of IKKβ and JNK in insulin resistant diabetic murine hearts. The pIRS-1/IRS-1 ratio was elevated in $Lepr^{db}$ compared with m$Lepr^{db}$ mice; both NaSal and JNK inhibitor SP600125 reduced the pIRS-1/IRS-1 ratio in $Lepr^{db}$ mice. The postulated mechanism is that activation of IKKβ may directly stimulate JNK activation or cause an increase in TNFα expression. Consequently, this activation could aggravate insulin resistance by further limiting insulin signaling.

In summary, our results demonstrated that TNFα was correlated with IKKβ, but not IKKα, to induce NFκB activation. Inhibition of IKKβ restored ACh-induced endothelium-dependent vasodilation in coronary arterioles in diabetes. Diabetic mice treated with NaSal showed a decrease in glucose level and a reversal of insulin resistance. Both IKKβ and JNK activation were increased in $Lepr^{db}$ mice and NaSal attenuated their activation without altering their expression levels. Overall, our molecular and functional results show that IKKβ is a crucial component of the biochemical pathway responsible for vascular dysfunction, inflammation, and insulin resistance in type 2 diabetic murine hearts. As noted above, diabetic mice treated with MG132 or NaSal showed partially restored ACh-induced vascular dysfunction. Blockade of IKKβ activity not only preserved coronary arteriolar vasodilation, but also prevented insulin resistance in type 2 diabetic mice. Although the current study

confirmed that NaSal amended whole body insulin sensitivity and demonstrated that IKKβ and NFκB signaling is associated with insulin resistance and inflammation in diabetic murine hearts, such a proposal needs to further validate how direct insulin signaling to coronary arterioles affects vessel function. Nevertheless, anti-diabetic effects of NaSal *in vivo* were observed in our study. Understanding these mechanisms regulating heart disease processes will provide new therapies to resolve these problems.

Second, our results in role of MCP-1 in TNFα-induced endothelial dysfunction suggest that MCP-1 interacts with TNFα to amplify adhesion molecules and oxidative stress, thereby contributing to vascular dysfunction in coronary microcirculation in type 2 diabetes. Our molecular evidence indicated that the expression of TNFα and MCP-1 was significantly increased in $Lepr^{db}$ mice; but, anti-MCP-1 decreased TNFα protein expression and anti-TNFα attenuated MCP-1 expression. Antibody neutralization of MCP-1 prevented coronary endothelial dysfunction and reduced $ONOO^-$ and $O_2^{\cdot -}$ generation and formation of N-Tyr in $Lepr^{db}$ mice. Administration of anti-TNFα or anti-MCP-1 to $Lepr^{db}$ mice resulted in attenuation in cardiac macrophage accumulation comparable to that in the control, suggesting that an interaction of MCP-1 and TNFα may accentuate the process of macrophage accumulation. Most importantly, blockade of TNFα or MCP-1 attenuated the expression of adhesion molecules (e.g., VCAM, ICAM, and E-selectin) in isolated coronary vessels in diabetes. We conclude that interactions of TNFα and MCP-1 signaling contribute to oxidative stress by increasing inflammatory cell accumulation and induce endothelial dysfunction in coronary arterioles in diabetic $Lepr^{db}$ mice.

D. Roles of TNFα and MCP-1 in Type 2 Diabetes

The important manifestation of diabetes-induced microvascular injury in the heart is a diminution in coronary blood flow. This is emphasized by clinical data showing ~25% of patients with acute myocardial infarction do not have adequate restoration of myocardial tissue perfusion in the infarct region, despite coronary artery recanalization.[39] We previously found that increases in TNFα expression induces activation of NAD(P)H oxidase and production of ROS, leading to endothelial dysfunction in type 2 diabetes.[5] The endothelium-derived NO is an important endogenous vasodilator that regulates microvascular tone.[50] Consistent with our previous studies, our present study shows that neutralization of MCP-1 decreased TNFα protein expression and prevented coronary endothelial dysfunction in $Lepr^{db}$ mice. Our western blot results indicated that the expression of TNFα and MCP-1 were significantly increased in $Lepr^{db}$ mice; but, anti-MCP-1 decreased TNFα protein expression and anti-TNFα attenuated MCP-1 expression. The evidence from our immunostaining in $Lepr^{db}$ mice showed that endothelial cells expressed MCP-1 (over 4.6-fold in microvessels), but $Lepr^{db}$ mice with anti-TNFα had attenuated MCP-1 expression (over 2.6-fold) in endothelial cells.

MCP-1 is an important mediator in response to acute tissue injury that plays a putative role in host defense.[27] Recent evidence suggests that MCP-1 also plays a role in the evolution of vascular disease through inducing adhesion molecule expression on/in endothelium and recruiting monocytes/macrophages at the inflammatory site.[22-24] Our results in Table 2 show that anti-MCP-1 did not decrease insulin levels, but slightly

affected lipid metabolism by decreasing circulating cholesterol levels. The results suggest that MCP-1 plays a role in upregulating cholesterol level in blood. Despite the similarities in glucose, body weight, cholesterol level, insulin level and abdominal girth in diabetic animals, endothelial function was better in $Lepr^{db}$ mice treated with anti-MCP-1. This suggests that MCP-1 signaling plays a pivotal role in attenuating TNFα protein expression, and TNFα is the key cytokine that induces endothelial dysfunction in type 2 diabetes.[5]

E. The Role of MCP-1 in ROS Production in Coronary Arterioles in Type 2 Diabetes

The link between diabetes and inflammation leading to vascular disease is associated with enhanced oxidative stress.[5] Nitration of proteins is caused by peroxynitrite, a ROS formed by the reaction of nitric oxide (NO) with $O_2^{\cdot -}$,[51] which further consumes NO and increases oxidative stress. Numerous studies have noted that nitration of tyrosine occurs at high levels of peroxynitrite ($ONOO^-$) during the pathogenesis of human atherosclerosis (Figure 17).[40,52]

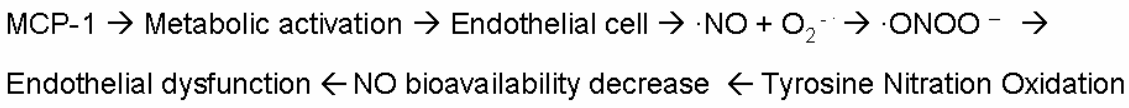

Figure 17. Potential pathway of tyrosine nitration oxidation.

In particular, decrease in expression of constitutive nitric oxide synthase (NOS) and inactivation of NO has been implicated in the endothelial dysfunction seen in type 2 diabetes.[40] Previous work detailed the role of TNFα, NFκB, and ROS in inflamed heart and vessels and suggested that they trigger NFκB activation to induce TNFα protein expression.[5,53] The interaction between activated macrophages and endothelial cells produces an oxidative stress that affects the activation state of transcription factor NFκB.[24] To test if MCP-1 enhances macrophage infiltration and induces ROS, macrophages derived from circulating monocytes were characterized by CD68 staining, which specifically labels macrophages and monocytes. Our immunostaining results showed that $Lepr^{db}$ mice expressed MCP-1 in macrophage (over 3-fold in heart sections), but anti-TNFα attenuated MCP-1 expression (2-fold in heart sections), which suggest that TNFα up-regulates MCP-1 expression in macrophages. Antibody neutralization of MCP-1 reduced $ONOO^-$ and $O_2^{\cdot -}$ generation and formation of N-Tyr in $Lepr^{db}$ mice. This result suggests that TNFα initiates the interaction of macrophages and endothelial cells via upregulation of MCP-1 signaling, which results in macrophage infiltration and production of ROS. Based on the evidence that MCP-1 contains a kB site upstream from its promoters,[24,54] NFκB may regulate both TNFα and MCP-1 gene expression in endothelial cells, thereby possibly inducing macrophage infiltration leading to vascular disease.

One of the major events of endothelial dysfunction is vascular inflammation, which is associated with impaired vascular control. It appears that MCP-1 residing in the endothelium contributes to the regulation of vasodilation under diabetic conditions

because anti-MCP-1 significantly enhanced endothelium-dependent coronary arteriolar dilations and decreased $O_2^{\cdot -}$ production in isolated blood vessels from diabetic mice. Our results support an endogenous role of vascular MCP-1 in regulating NO-mediated dilation of coronary microvessels in type 2 diabetes. Consistently, our molecular results showed that anti-MCP-1 decreased TNFα protein expression; anti-TNFα attenuated MCP-1 expression; and antibody neutralization of MCP-1 reduced $ONOO^-$ and $O_2^{\cdot -}$ generation and formation of N-Tyr in $Lepr^{db}$ mice. Of particular importance to this study is that MCP-1 and TNFα signaling plays a pivotal role in endothelial dysfunction, consistent with what might be expected in an inflammatory disease in coronary arterioles.

F. Enhancement of Adhesion Molecules by MCP-1 and TNFα in Endothelial Cells in Diabetes

Endothelial cells exert broad functions to maintain vessel homeostasis. The modification of the pattern of gene expression in the endothelial cells is a critical step during the progression of vascular disease. In endothelial cells, NFκB regulates the inducible expression of genes encoding chemokines and adhesion molecules.[55] In our studies, the inflammatory cytokine TNFα has been explored as a factor in initiation or progression of vascular disease.[5,28] Inflammatory mediators, MCP-1 and adhesion molecules, have also been implicated in the inflammatory processes related to TNFα in the development of vascular disease in type 2 diabetes.[55] Elevated adhesion molecules in the vessels have been thought to be significantly associated with an increase in the risk of coronary arterial disease.[16] E-selectin is important in monocyte arrest or the transition from slow rolling to firm adhesion. VCAM and ICAM are critical for firm attachment of

monocyte in the recruitment cascade.[17] Immunostaining data showed that macrophages and endothelial cells express adhesion molecules and western blot analysis revealed that TNFα and MCP-1 increased expression of adhesion molecules (e.g., VCAM, ICAM, and E-electin), which suggest that elevated expression of adhesion molecules is the result of the release of cytokines, TNFα and MCP-1, from endothelial cells and macrophages in $Lepr^{db}$ mice. The actions of adhesion molecules are favored in small vessels (~ 50μm) compared to that seen in the large coronary arteries under normal conditions because of the lower flow and shear stress in diabetes.

Monocyte-derived macrophages are mainly inflammatory cells. Macrophages contribute to the oxidation of LDL and oxidized LDL acts on mature macrophages to form macrophage foam cells.[21,56] Initial low expression of adhesion molecules results in low affinity to ligands, and a signal from MCP-1 is essential to mobilize adhesion molecules for firm adhesion.[22] Inflammatory mediators such as MCP-1 and adhesion molecules have been implicated in the inflammatory processes related to TNFα in the development of vascular disease in type 2 diabetes. Co-localization of MCP-1 and VCAM-1 with macrophages was observed in the myocardium, suggesting that MCP-1 and cell adhesion molecules play a role in monocyte transmigration into the heart. ICAM-1 and E-selectin also co-exist in endothelial cells and macrophages (data not shown).

Taken together, MCP-1 may play a critical role in inflammation-induced endothelial dysfunction in coronary arterioles in diabetes by increasing adhesion molecule expression in endothelial cells, thereby inducing macrophage infiltration at the

inflammatory site (Figure 18) without influencing the development of obesity or insulin resistance.

In this study, we found that beyond monocyte recruitment, MCP-1 is critical in TNFα-induced endothelial dysfunction in type 2 diabetes. The postulated mechanism is that MCP-1-induced the production of $O_2^{\cdot -}$ and $ONOO^-$ in vascular endothelial cells, which then attenuated NO bioavailability and reduced NO mediated vasodilation in the coronary microcirculation in type 2 diabetes. Furthermore, inflammatory cell infiltration was enhanced via overexpression of TNFα and MCP-1-induced upregulation of cell adhesion molecules in coronary arterioles in type 2 diabetes. Our findings demonstrate that the endothelial dysfunction occurring in type 2 diabetes is the result of the effects of the inflammatory cytokine TNFα and TNFα–related signaling, including the expression of MCP-1, which also exacerbates the oxidative stress.

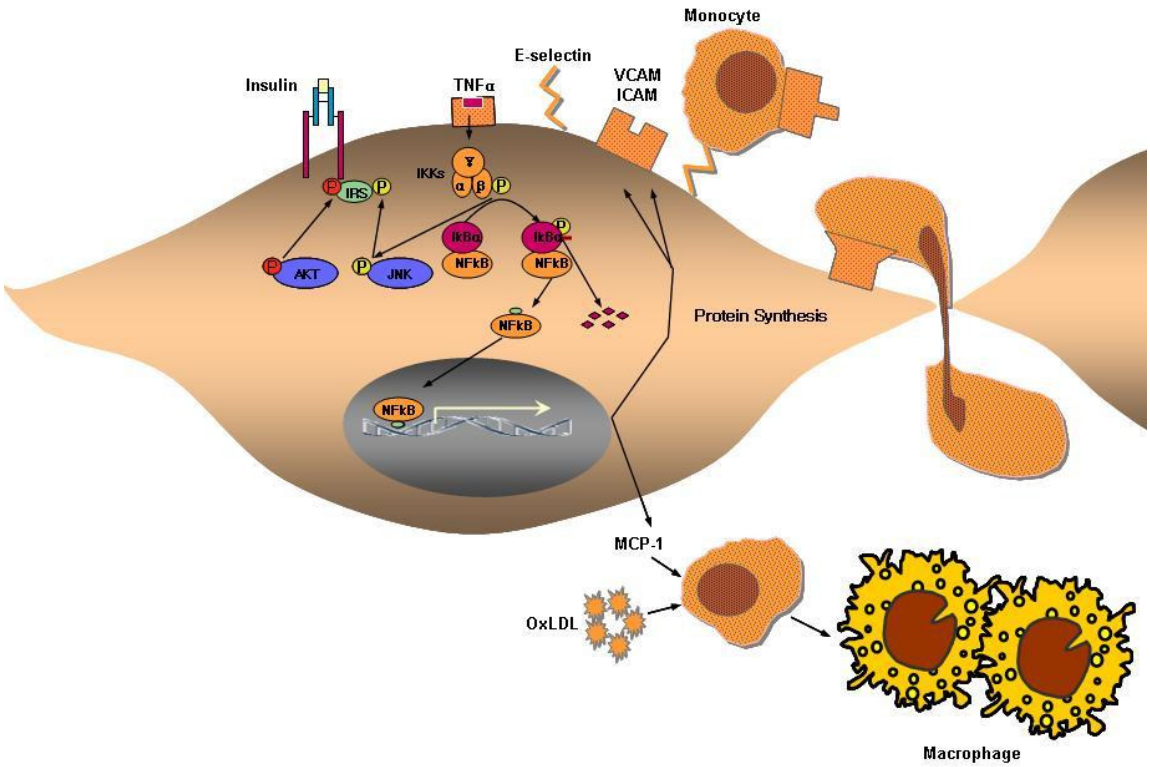

Figure 18. Interactions among TNF, MCP-1 and adhesion molecules in endothelium. We illustrate a new aspect of the regulation of TNF-dependent signal transduction. Serine site phosphorylation by Akt is blocked and JNK phosphorylate at tyrosine site of insulin receptor substrate, leading to insulin resistance in type 2 diabetes. Interaction of TNF and MCP-1 induces expression of adhesion molecules, leading to increase of macrophage infiltration and oxidative stress, which further exacerbate vascular dysfunction.

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