Blockade of the Nuclear Factor-κB Pathway in the Endothelium Prevents Insulin Resistance and Prolongs Life Spans

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Background—Nuclear factor-κB (NF-κB) signaling plays critical roles in physiological and pathological processes such as responses to inflammation and oxidative stress.

Methods and Results—To examine the role of endothelial NF-κB signaling in vivo, we generated transgenic mice expressing dominant-negative IκB under the Tie2 promoter/enhancer (E-DNIκB mice). These mice exhibited functional inhibition of NF-κB signaling specifically in endothelial cells. Although E-DNIκB mice displayed no overt phenotypic changes when young and lean, they were protected from the development of insulin resistance associated with obesity, whether diet- or genetics-induced. Obesity-induced macrophage infiltration into adipose tissue and plasma oxidative stress markers were decreased and blood flow and mitochondrial content in muscle and active-phase locomotor activity were increased in E-DNIκB mice. In addition to inhibition of obesity-related metabolic deteriorations, blockade of endothelial NF-κB signaling prevented age-related insulin resistance and vascular senescence and, notably, prolonged life span. These antiaging phenotypes were also associated with decreased oxidative stress markers, increased muscle blood flow, enhanced active-phase locomotor activity, and aortic upregulation of mitochondrial sirtuin-related proteins.

Conclusions—The endothelium plays important roles in obesity- and age-related disorders through intracellular NF-κB signaling, thereby ultimately affecting life span. Endothelial NF-κB signaling is a potential target for treating the metabolic syndrome and for antiaging strategies.

Key Words: inflammation ■ insulin resistance ■ oxidative stress ■ NF-κB

Nuclear factor-kappa B (NF-κB) is a transcription factor regulating the gene expression of numerous cytokines, growth factors, adhesion molecules, and enzymes involved in a variety of pivotal cellular processes, including responses to inflammation and oxidative stress. Without inflammatory stimuli, NF-κB is maintained in the cytoplasm in a nonactivated form by association with an inhibitor subunit, IκB. In response to activating stimuli, including tumor necrosis factor-α (TNF-α), lipopolysaccharide, and other inflammatory cytokines, IκB is phosphorylated by IκB kinase β, resulting in proteolysis of IκB. Consequently, a nuclear recognition site of NF-κB is exposed, and NF-κB is stimulated to move into the nucleus, resulting in mRNA expression of target genes, including inflammatory cytokines and adhesion molecules.

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Obesity is characterized by a state of chronic low-grade inflammation. Oxidative stress is also widely recognized as being associated with various obesity-related disorders. In insulin resistance is an important mechanism underlying obesity-related disorders, eg, diabetes mellitus, hyperlipidemia, and hypertension, collectively called the metabolic syndrome. In these metabolic states, NF-κB has been implicated in the processes of both inflammatory responses.
and oxidative stress. Indeed, blockade of the NF-κB signaling pathway by systemic administration of high-dose salicylate or global disruption of IkB kinase β reportedly suppresses inflammatory processes associated with insulin resistance in obesity and type 2 diabetes mellitus. However, the sites at which the NF-κB signaling pathway plays critical roles in these pathological processes remain to be elucidated.

The endothelium lines the entire vascular system in a single cell layer, forming an interface between vascular structures and blood. In human adults, ≈10 trillion (10^{13}) cells form an almost 1-kg organ. Endothelial cells produce and react to a wide variety of inflammation-related mediators such as cytokines, growth factors, and adhesion molecules. Endothelial injury and dysfunction are involved in the development of many diseases, including vascular diseases and inflammatory disorders. In this context, we hypothesized that NF-κB signaling in endothelial cells contributes to obesity-related disorders. Furthermore, insulin resistance and increased oxidative stress also are commonly observed in aged states. Therefore, we also hypothesized that endothelial proinflammatory responses play important roles in age-related disorders, ultimately affecting life span. Here, using the transgenic technique, we show that blockade of the intracellular NF-κB pathway in the endothelium prevents obesity- and age-related insulin resistance and enhances longevity.

**Methods**

**Animals**

Animal studies were conducted in accordance with the institutional guidelines for animal experiments at Tohoku University. The mutant cDNA for human IkBa, with alanine substitutions of 2 serine residues (32 and 36), was cloned into a transgenic vector, pSPTg.T2FpAXK, provided by Thomas N. Sato. This vector contains the Tie2 promoter, SV40 polyA signal, and Tie2 minimum enhancer fragment (Figure 1A). E-DNIkB:A^{+/+} mice were obtained by mating male KK A^{+}(A^{+/+}) mice (NipponCLEA, Shizuoka, Japan), a genetic model for obesity-diabetes syndrome, and female DNIkB mice. E-DNIkB mice were also crossed with endothelial nitric oxide (NO) synthase (eNOS)-deficient (Nos3^{-/-}) mice with the C57BL/6J background to generate E-DNIkB:Nos3^{-/-} mice.

**Isolation and Culture of Endothelial Cells**

Endothelial cells were isolated from murine lung with a MACS separation unit (Miltenyi Biotec, Surry, UK) as previously described. To quantify vascular cell adhesion molecule-1 (VCAM-1) expression, purified endothelial cells were preincubated for 1 hour and then stimulated with or without TNF-α (10 ng/mL) for 4 hours, followed by quantitative reverse-transcriptase polymerase chain reaction analysis.

**Hyperinsulinemic-Euglycemic Clamp**

Hyperinsulinemic-euglycemic clamp studies were performed as described previously. Details of the method are given in the online-only Data Supplement.

**Blood Pressure Measurement**

Systolic blood pressure in the conscious state was measured by the indirect tail cuff method with a model MK-2000 BP monitor (Muromachi Kikai, Tokyo, Japan) according to the manufacturer’s instructions. At least 6 readings were obtained for each experiment, and a mean value was assigned to each individual mouse.

**Muscle Blood Flow Measurement**

Muscle blood flow was measured with the fluorescent microsphere method as previously described. Details of the method are given in the online-only Data Supplement.

**Histological Analysis**

Tissues sections were prepared and analyzed as described in the online-only Data Supplement. Total adipocyte areas were traced manually and analyzed. White adipocyte areas were measured in ≧100 cells per mouse in each group as described previously.

**Detection of Cellular Senescence**

Cellular senescence was evaluated by senescence-associated β-galactosidase staining. Senescence-associated β-galactosidase was detected with a senescence detection kit (BioVision, Milpitas, CA) as previously described. Senescence-associated β-galactosidase-positive areas were quantified by Easy Access (AD Science Co, Chiba, Japan).

**Statistical Analysis**

All data are expressed as mean±SEM. Images shown are representative of data from 3 independent experiments. All statistical analyses were performed with Ekuseru-Tokei 2010 statistical software (Social Survey Research Information Co, Ltd, Tokyo, Japan). Normality was tested with the Kolmogorov-Smirnov test. When data were normally distributed, the statistical significance of differences was assessed by 1-way ANOVA. Multiple experimental groups were compared by use of a Bonferroni test. Data were analyzed with nonparametric ANOVA (Kruskal-Wallis) when conformity to a normal distribution was not confirmed. Repeated-measures ANOVA was used to compare data obtained by serial measurements over time between the 2 experimental groups. Survival rates were compared between E-DNIkB mice and control littermates by the Kaplan-Meier method with log-rank tests. In all analyses, values of P<0.05 were accepted as statistically significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

**Construction of the Transgene and Generation of the E-DNIkB Mice**

To block the NF-κB signaling in endothelial cells, we generated transgenic mice (E-DNIkB mice) in which the dominant-negative form of human IkBa (DNIkBa), with alanine substitutions of 2 serine residues (32 and 36), was expressed under the control of the Tie2 enhancer/promoter (Figure 1A). Immunoblotting of lung lysates confirmed the expression of transgene-derived human IkBa, ie, DNIkB, with a slightly higher molecular weight than the murine, ie, endogenous, IkBa protein (Figure 1B). Transgene expression was apparent in the lung, in which the endothelium is abundant, but was only faintly detectable in other tissues such as the liver, pancreas, muscle, and adipose tissue (Figure 1B). In addition, endogenous IkBa proteins were degraded whereas DNIkB remained essentially intact without degradation after TNF-α stimulation (Figure 1C). Immunostaining of lung tissue revealed that movement of NF-κB to the nucleus in response to TNF-α stimulation was markedly inhibited by DNIkB expression (Figure 1D).

To further confirm the functional inhibition of endothelial NF-κB signaling, we isolated endothelial cells from murine...
Figure 1. Generation of endothelial dominant-negative IκBα transgenic (E-DNIκB) mice. A. The construct of the transgene for generating E-DNIκB mice. B. Extracts of various tissues, as indicated, obtained from control and E-DNIκB mice were immunoblotted with anti-IκBα antibody. Exogenous (human) IκBα has a slightly higher molecular weight than endogenous (murine) IκBα. WT indicates wild type. C. Lung extracts of nontransgenic (control) and E-DNIκB mice were obtained 30 minutes after injection of tumor necrosis factor-α (TNF-α; 25 μg/kg), followed by immunoblotting with anti-IκBα antibody. D. Sections of thoracic aortas obtained from control and E-DNIκB mice were immunostained with anti-p65 (nuclear factor-κB [NF-κB] subunit) antibody. L indicates lumen; A, adventitia. Arrows indicate endothelial nuclei. E, Endothelial cells were isolated from lungs of control (white bars) and E-DNIκB (black bars) mice (n=6 per group) by use of a MACS separation unit. Purified endothelial cells were stimulated with or without TNF-α (10 ng/mL) for 4 hours, followed by analysis of vascular cell adhesion molecule-1 (VCAM-1) expression by quantitative reverse-transcriptase polymerase chain reaction (RT-PCR). F, Circulating blood cells from control (white bars) and E-DNIκB (black bars) mice (n=5 per group) were stimulated with or without lipopolysaccharide (100 ng/mL) for 3 hours, followed by analysis of TNF-α expression by quantitative RT-PCR analysis. In E and F, the relative amounts of mRNA were calculated with β-actin mRNA as the invariant control. Data are presented as mean±SEM. *P<0.05, **P<0.01 by 1-way ANOVA and Kruskal-Wallis tests.
lung and analyzed the expression of VCAM-1, a target gene of NF-κB. In isolated endothelial cells from wild-type mice, TNF-α upregulated VCAM-1 expression, whereas basal and TNF-α–induced VCAM-1 expression was markedly inhibited in isolated endothelial cells from E-DNIkB mice (Figure 1E). In contrast, in circulating cells from E-DNIkB mice, neither basal nor lipopolysaccharide-induced TNF-α expression was suppressed (Figure 1F). These findings demonstrate that NF-κB signaling is functionally blocked specifically in endothelial cells of the E-DNIkB mice used in this study.

**Young E-DNIkB Mice Showed No Apparent Phenotypic Differences Compared With Control Mice**

First, we analyzed glucose metabolism in these mice on a normal chow diet at 8 weeks of age. Food intakes (Figure 2A) and body weights (Figure 2B) were similar in E-DNIkB mice and their wild-type littermate controls. Glucose tolerance tests revealed no significant differences in blood glucose levels before and after glucose loading between these 2 groups (Figure 2C). In addition, blood glucose levels on insulin tolerance tests did not differ significantly between these 2 groups (Figure 2D). In contrast, a hyperinsulinemic-euglycemic clamp study, a more sensitive procedure for estimating insulin sensitivity, showed increased glucose infusion rates in E-DNIkB mice (Figure 2E). Because hyperinsulinemia during the clamp procedure suppresses hepatic glucose production, these findings indicate slight improvement of insulin sensitivity in insulin-responsive tissues, mainly muscle. Furthermore, systolic blood pressure was also similar in these 2 groups (Figure 2F). Thus, although young and on a normal chow diet, E-DNIkB mice showed no apparent phenotypic differences from control mice, except for slightly improved insulin sensitivity.

**E-DNIkB Mice Were Protected From Obesity-Induced Insulin Resistance**

We next analyzed the effects of endothelial NF-κB signaling blockade on glucose metabolism and insulin sensitivity in...
states of obesity. First, E-DNIκB mice with the C57BL/6J background were placed on a high-fat diet starting from 8 weeks of age. Body weights were similarly increased in E-DNIκB mice and their wild-type littermate controls (Figure 3A). However, after 20 weeks of high-fat loading, differences in glucose tolerance and insulin sensitivity became evident. Glucose and insulin tolerance tests revealed that blockade of endothelial NF-κB signaling significantly protected these mice from the development of glucose intolerance (Figure 3B) and insulin resistance (Figure 3C). Furthermore, systolic blood pressure was significantly lower in E-DNIκB mice (Figure 3D). These findings indicate that inhibition of endothelial NF-κB signaling prevents obesity-induced disorders such as insulin resistance, glucose intolerance, and hypertension.

The prevention of insulin resistance and hypertension achieved by inhibiting endothelial NF-κB signaling was observed in genetically obese (A²/+) mice in earlier periods. Body weights were slightly lower in E-DNIκB;A²/+ than in control littermate A²/+ mice at 16 weeks of age (Figure I in the online-only Data Supplement). Glucose (Figure 4A) and insulin (Figure 4B) tolerance tests revealed markedly better glucose tolerance and insulin sensitivity with blockade of endothelial NF-κB signaling. In contrast, hepatic expression of gluconeogenic genes, phosphoenolpyruvate carboxykinase, and glucose-6-phosphatase did not differ significantly between the 2 groups (Figure II in the online-only Data Supplement), suggesting that muscle is the major tissue responsible for improvement of insulin sensitivity achieved by blockade of endothelial NF-κB signaling. Systolic blood pressure was also significantly lower in E-DNIκB;A²/+ mice than in A²/+ littermates (Figure 4C). In addition, aortic expression of adhesion molecules such as VCAM-1 and E-selectin was decreased in E-DNIκB;A²/+ mice (Figure 4D). Simultaneously, eNOS expression was significantly increased (Figure 4D), which may have contributed to the lower blood pressures.

**Obesity-Associated Macrophage Infiltration Into Adipose Tissue Was Markedly Inhibited in E-DNIκB Mice**

Obesity is a chronic state of low-grade inflammation leading to insulin resistance and oxidative stress. Macrophage infiltration into white adipose tissue reportedly contributes to the underlying mechanism. Therefore, we histologically analyzed white adipose tissue, an important site of obesity-related inflammation. The adipocyte sizes and weights of epididymal fat tissue were smaller in E-DNIκB;A²/+ mice than in control A²/+ littermates, whereas liver weights did not differ significantly (Figure 4E and Figure I in the online-only Data Supplement). These findings suggest that less adipose contributes to the better glucose tolerance in E-DNIκB;A²/+ mice. Although immunohistochemical staining with antibodies against MOMA-2, a macrophage marker, revealed massive infiltration of macrophages into adipose tissue in A²/+ littermates, macrophage infiltration was markedly inhibited by endothelial blockade of NF-κB signaling (Figure 4E). The inhibition of macrophage infiltration was quantitatively confirmed by reverse-transcriptase polymerase chain reaction. Expression of F4/80 and inducible NO synthase was significantly lower in adipose tissues of E-DNIκB;A²/+ mice than in those of control littermate A²/+ mice (Figure 4F). These findings indicate that endothelial NF-κB signaling is involved in obesity-induced macrophage infiltration into adipose tissue. In addition, E-selectin expression was significantly decreased and VCAM-1 and intercel-
lular adhesion molecule-1 tended to be downregulated in adipose tissues of E-DNIκB;A\(^{+/+}\) mice, whereas angiogenesis markers such as Tie2 and vascular endothelial growth factor did not differ (Figure III in the online-only Data Supplement). Thus, suppression of endothelial expression of adhesion molecules might be involved in the decrease in macrophage infiltration into adipose tissue observed in E-DNIκB;A\(^{+/+}\) mice.

Plasma leptin, TNF-\(\alpha\), and monocyte chemoattractant protein-1 tended to be lower whereas adiponectin was significantly higher in E-DNIκB;A\(^{+/+}\) mice than in A\(^{+/+}\) littermates (Figure IV in the online-only Data Supplement). Furthermore, plasma levels of the oxidative stress markers thiobarbituric acid–reactive substance and 8-isoprostane were significantly lower in E-DNIκB;A\(^{+/+}\) mice than in A\(^{+/+}\) littermates (Figure 4G). Aortic expression of the antioxidant enzymes manganese superoxide dismutase and glutathione peroxidase was significantly suppressed in E-DNIκB;A\(^{+/+}\) mice (Figure V in the online-only Data Supplement), suggesting amelioration of oxidative stress in vascular cells. Taken together, these observations indicate that endothelial NF-κB signaling is involved in obesity-associated inflammation and oxidative stress.

Blood Flow and Mitochondrial Contents Were Increased in Muscles of E-DNIκB;A\(^{+/+}\) Mice

Endothelial adhesion molecule expression is involved in the leukocyte-endothelium interaction, which reportedly affects the microcirculation.\(^{24}\) In addition, endothelium-derived NO is widely recognized as a major vasodilator\(^{25}\) that modulates mitochondrial biogenesis.\(^{26}\) Because endothelial blockade of NF-κB signaling decreased and increased aortic expression of vascular adhesion molecules and eNOS, respectively (Figure 4D), we first measured blood flow in muscle, a major

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**Figure 4.** Blockade of endothelial nuclear factor-κB (NF-κB) signaling prevented adipose macrophage infiltration in genetically obese mice. **A.** Male KK A\(^{+/+}\) (A\(^{+/+}\)) control mice (C) and endothelial dominant-negative IkB\(\alpha\) transgenic mice mated with male KK A\(^{+/+}\) (E-DNIκB;A\(^{+/+}\)) received glucose tolerance tests with a peritoneal glucose load (0.5 g/kg body weight) after a 10-hour fast at 16 weeks of age. B. Insulin tolerance tests were performed in an ad libitum–fed state at 16 weeks of age. Data are expressed as percentages of blood glucose levels immediately after intraperitoneal insulin loading (2.5 U/kg body weight). C. Systolic blood pressures were measured in A\(^{+/+}\) control (white bars) and E-DNIκB;A\(^{+/+}\) (black bars) mice at 16 weeks of age. D. Aortic gene expression of vascular cell adhesion molecule-1 (VCAM-1), E-selectin, and endothelial nitric oxide synthase (eNOS) was analyzed in A\(^{+/+}\) control and E-DNIκB;A\(^{+/+}\) mice at 16 weeks of age by reverse-transcriptase polymerase chain reaction (RT-PCR). E. Epididymal fat from A\(^{+/+}\) control and E-DNIκB;A\(^{+/+}\) mice were immunostained with anti-MOMA2 antibody (left). Cell diameters were measured (right). F. Gene expression of macrophage markers in epididymal fat was analyzed in A\(^{+/+}\) control and E-DNIκB;A\(^{+/+}\) mice by RT-PCR. In D and F, the relative amounts of mRNA were calculated with β-actin mRNA as the invariant control. G. Plasma concentrations of the oxidative stress markers thiobarbituric acid–reactive substance (TBARS) and 8-isoprostane were measured in A\(^{+/+}\) control and E-DNIκB;A\(^{+/+}\) mice. Data are presented as mean±SEM. n=5 in A\(^{+/+}\) control and n=7 in E-DNIκB;A\(^{+/+}\) mice. *P<0.05 by 1-way repeated-measures ANOVAs and Kruskal-Wallis tests.
insulin-sensitive tissue, using the fluorescent microsphere method. Blockade of endothelial NF-κB signaling significantly increased blood flow in the muscles of Ay/−/− mice (Figure 5A), suggesting involvement in the prevention of obesity-induced insulin resistance. Next, we examined mitochondrial protein expression and mitochondrial DNA contents. Mitochondrial proteins such as cytochrome c, uncoupling protein-3, and medium-chain acyl-CoA dehydrogenase were upregulated in muscles of E-DNIκB;Ay/−/− mice compared with Ay/−/− littermate controls (Figure 5B). Mitochondrial DNA contents were significantly higher in muscles of E-DNIκB;Ay/−/− mice (Figure 5C). These findings indicate that blockade of endothelial NF-κB signaling enhances mitochondrial biogenesis in muscle.

Then, we assessed whether locomotor activity was affected by blockade of endothelial NF-κB signaling. Interestingly, E-DNIκB mice exhibited significant increments in locomotor activity during the 12-hour dark phase, whereas locomotor activities did not differ during the 12-hour light phase (Figure 5D). Compatible with this, oxygen consumption was also increased in E-DNIκB mice during the dark phase but unchanged in the light phase (Figure VI in the online-only Data Supplement). Because muscle blood flow and mitochondrial function are reportedly involved in insulin sensitivity, these enhancements associated with increased locomotor activity may underlie the protection from insulin resistance in response to blockade of endothelial NF-κB signaling.

E-DNIκB Mice Were Protected From Age-Related Insulin Resistance and Blood Pressure Elevation

Insulin resistance, elevated blood pressure, and increased oxidative stress, which were prevented in obese E-DNIκB mice, are also commonly observed in aged states. Therefore, we next examined the effects of endothelial NF-κB blockade on age-related metabolic deteriorations and vascular senescence using 50-week-old E-DNIκB mice on a standard chow diet. Food intakes were similar (Figure VIIA in the online-only Data Supplement), but body weights were slightly lower in aged E-DNIκB mice than in wild-type littermates (Figure VIIIB in the online-only Data Supplement). Blood glucose levels after glucose loading tended to
To determine whether eNOS mediates age-related blood pressure elevation via endothelial NF-κB signaling, we next crossed E-DNικB mice with eNOS-deficient (Nos3−/−) mice. Whereas endothelial DNικB expression suppressed blood pressure elevation in Nos3+/− mice, eNOS deficiency blunted the inhibitory effects of DNικB on blood pressure elevation (Figure VIII in the online-only Data Supplement). These findings indicate involvement of eNOS in protection from age-related hypertension in E-DNικB mice.
Blockade of NF-κB Signaling Prevented Vascular Senescence and Prolonged Life Span in Mice

Next, we analyzed vascular senescence by β-galactosidase staining of the aortas of aged E-DNIκB mice at 90 weeks of age because endogenous β-galactosidase activity is reportedly increased in senescent states.20 As shown in Figure 6C, β-galactosidase staining was much weaker in the aortas of E-DNIκB mice, suggesting prevention of vascular senescence by endothelial blockade of NF-κB signaling. These findings prompted us to hypothesize that endothelial NF-κB signaling affects longevity. Therefore, we analyzed the life spans of these mice. E-DNIκB mice and control littersmates were fed a standard chow diet ad libitum and maintained in regular housing until death. Intriguingly, E-DNIκB mice exhibited significantly prolonged life spans compared with control littersmates ($P<0.005$ by log-rank test; Figure 6D). Thus, blockade of endothelial NF-κB signaling may prevent age-related metabolic deterioration and vascular senescence and thereby increase longevity.

Similarly in the obesity model, plasma levels of oxidative stress markers were significantly lower in aged E-DNIκB mice at 50 weeks of age than in wild-type controls of the same age (Figure 7A). Blood flow in muscles was increased in aged E-DNIκB mice compared with wild-type littermates, whereas eNOS deficiency blunted the effects of increased blood flow in aged E-DNIκB:Bos3−/− mice (Figure VIIIB in the online-only Data Supplement). Furthermore, blockade of endothelial NF-κB signaling enhanced locomotor activity during the 12-hour dark phase with no significant alterations during the 12-hour light phase (Figure 7B).

Oxidative damage to mitochondria reportedly contributes to aging and various age-related disorders.30 In human subjects, endurance exercise reportedly enhances mitochondrial SIRT3 expression.31 Upregulation of nicotinamide phosphoribosyltransferase (Nampt) and SIRT3, which are highly expressed in mitochondria, is linked to life-span extension in the context of caloric restriction.32 Therefore, we next examined the expression of Nampt and a mitochondrial sirtuin,
SIRT3, in the aortas of 50-week-old E-DNIκB and control mice. In E-DNIκB mice, both Nampt and SIRT3 were actually upregulated (Figure 7C) despite no food intake differences (Figure VIIA in the online-only Data Supplement). Thus, decreased oxidative stress, enhanced active-phase locomotor activity, and upregulation of mitochondrial prosurvival genes might contribute to the life-span prolongation resulting from NF-κB signaling blockade in the endothelium.

Discussion

The endothelium forms an interface between vascular structures and blood. Endothelial cells produce and react to a wide variety of mediators, including cytokines, growth factors, vasoactive substances, and chemokines, as well as adhesion molecules. Therefore, in this study, we focused on proinflammatory responses in the endothelium in an effort to elucidate the role of endothelial NF-κB signaling. We blocked this signaling in vivo by expressing the dominant-negative form of IκBα in endothelial cells using the transgenic procedure. Although E-DNIκB mice displayed no overt phenotypic changes when young and lean, they were protected from the development of both obesity-induced and age-related insulin resistance. Furthermore, intriguingly, E-DNIκB mice were also protected from vascular senescence and showed extended longevity.

The mechanisms underlying protection from obesity-induced insulin resistance in E-DNIκB mice are likely to involve suppression of macrophage infiltration into adipose tissue. Recent studies have demonstrated that macrophage infiltration into white adipose tissues is increased in obesity, raising levels of proinflammatory cytokines such as TNF-α.22,23 Such macrophage infiltration into adipose tissue may be triggered by the interaction between endothelial cells and macrophages via adhesion molecules. The expression of adhesion molecules is reportedly regulated by NF-κB in the endothelium.33 In the present study, the expression of vascular adhesion molecules was actually decreased in E-DNIκB mice. A series of recent reports have indicated angiogenic factors to be involved in the development of obesity in adipose tissue.34 However, adipose expression of Tie2 and vascular endothelial growth factor did not differ between E-DNIκB; Ay/+ mice and control Ay/+ littermates, suggesting minimal effects of endothelial NF-κB signaling on angiogenesis in adipose tissue. In addition, proinflammatory cytokines secreted by macrophages in adipose tissue may further activate the endothelial NF-κB pathway, producing a deleterious cycle. Indeed, epididymal fat weight was significantly lower and plasma adiponectin was higher in E-DNIκB; Ay/+ mice than in Ay/+ littermates. Therefore, interruption of this deleterious cycle by blockade of endothelial NF-κB signaling is speculated to contribute to protection from obesity-related chronic inflammation and increased oxidative stress in E-DNIκB mice.

Decreased eNOS production may lead to a reduction in microcirculatory blood flow and elevated blood pressure. Endothelium-derived NO is a major vasodilator,15 and constitutive NO production by endothelial cells reportedly inhibits adhesion molecule expression through stabilization of IκB.36 On the other hand, in this study, eNOS expression was enhanced in E-DNIκB mice, suggesting that endothelial NF-κB signaling per se negatively regulates eNOS expression. Furthermore, in NOS3−/− mice, endothelial DNIκB expression did not significantly suppress age-related hypertension or increase blood flow in muscle, indicating the involvement of eNOS in the antihypertensive effects of endothelial NF-κB blocking. NO production and NF-κB activation affect each other in endothelial cells. NF-κB activation decreases eNOS expression, resulting in decreased NO production and thus further NF-κB activation, producing a vicious cycle that further decreases microcirculation and increases proinflammatory responses and oxidative stress. The attenuation of microcirculatory blood flow observed in eNOS-deficient mice decreases transcapillary passage of insulin to metabolically active tissues such as muscle, thereby contributing to impairment of insulin action.18 Negative regulation of eNOS expression by NF-κB in endothelial cells is thus another important mechanism underlying insulin resistance and hypertension.

Insulin resistance, elevated blood pressure, and increased oxidative stress are commonly observed not only in obese but also in aged states. These age-related metabolic deteriorations were also prevented in E-DNIκB mice, in association with increased muscle blood flow and decreased oxidative stress markers. Furthermore, it is noteworthy that E-DNIκB mice were protected from vascular senescence and lived longer even under normal chow-fed conditions. At the cellular level, NF-κB has been implicated in age-dependent induction of cellular senescence.37 However, in this study, blockade of NF-κB signaling selectively in endothelial cells affected vascular senescence of the whole aorta and the life spans of the model animals. Therefore, intracellular events in the endothelium alone cannot explain these antiaging phenotypes manifesting in the whole body. Relatively early deaths of E-DNIκB mice (60–100 weeks of age) were decreased and maximum life span was longer in E-DNIκB mice, suggesting that endothelial NF-κB blockade prevents both fatal morbidities and senescence. Amelioration of insulin resistance and decreased oxidative stress likely contribute to these systemic antiaging phenotypes.

In addition, mitochondrial function may be involved in the underlying mechanism. Mitochondrial dysfunction in muscle promotes the development of insulin resistance in obese29 and aged38 human subjects and contributes to aging and various age-related disorders.30 Mitochondrial dysfunction is also linked to decreased muscle blood flow with aging.39 Because eNOS reportedly modulates mitochondrial biogenesis,26 eNOS upregulation may contribute to enhanced mitochondrial contents in muscles of E-DNIκB mice. Furthermore, endurance exercise, which is considered to confer life-span-extending effects, enhances the expression of a mitochondrial sirtuin, SIRT3, in human subjects.31 SIRT3 was initially reported to be linked to caloric restriction–induced cell survival.40,41 Nampt provides mitochondrial NAD+ as the cosubstrate for SIRT3, and enhanced Nampt and SIRT3 expression maintains mitochondrial viability and promote cell survival.42 In the present study, aortic expression of Nampt and SIRT3 was significantly upregulated in aged
E-DNIXB mice, suggesting contributions of the sirtuin pathway to the antisenescence and prolonged longevity phenotypes. Thus, various mechanisms derived from endothelial NF-κB signaling, including systemic locomotion, systemic oxidative stress, peripheral blood flow, and mitochondrial sirtuins, may influence longevity in a complex manner (Figure IX in the online-only Data Supplement).

It was recently reported that global disruption of the angiotensin II type 1 receptor promotes longevity. In these knockout mice as well, Nampy and SIRT3 were upregulated in the kidney. The angiotensin II type 1 receptor pathway activates a variety of intracellular signaling pathways, including NF-κB signaling. However, although a growing body of evidence for angiotensin II signaling in smooth muscle cells has accumulated, much less is known about endothelial angiotensin II signal transduction and function. The present study suggests the specific importance of NF-κB signaling in endothelial cells for the mechanism underlying the life-span extension observed in globally angiotensin II type 1 receptor–deficient mice. Although further intensive studies are necessary to elucidate the precise mechanisms, the endothelium apparently plays important roles in determining life span.

Adhesion molecule expression in the endothelium is well known to promote atherosclerotic plaque formation. Indeed, blockade of endothelial NF-κB signaling suppresses hypercholesterolemia-induced atherosclerosis caused by apolipoprotein E deficiency. Therefore, endothelial NF-κB signaling is apparently involved in the development of obesity-related disorders, including insulin resistance, hypertension, and atherosclerosis, via a variety of processes in different tissues. Furthermore, in this study, selective blockade of endothelial NF-κB signaling not only prevented age-related insulin resistance but also inhibited senescence and increased longevity with normal chow feeding. Thus, endothelial NF-κB signaling is a potential target for treatment of the metabolic syndrome and for antiaging strategies.

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Disclosures

None.

References

Insulin resistance is an important mechanism underlying obesity-related disorders, eg, diabetes, hyperlipidemia, and hypertension, collectively called the metabolic syndrome. In particular, inflammation and oxidative stress are well known to play important roles in the pathogenesis of this systemic syndrome and the resultant development of atherosclerosis. A transcription factor, nuclear factor-κB (NF-κB), has been considered to mediate the responses to both inflammation and oxidative stress intracellularly. However, the site(s) at which the NF-κB signaling pathway plays critical roles in these pathological processes remains to be elucidated. This study focused on the roles of endothelial NF-κB signaling, ultimately impacting lifespan. Blockade of endothelial NF-κB signaling is a potential target not only for treating the metabolic syndrome, but also for anti-aging strategies.
Blockade of the Nuclear Factor-κB Pathway in the Endothelium Prevents Insulin Resistance and Prolongs Life Spans
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SUPPLEMENTAL MATERIAL

Supplemental Methods

Animals

Animal studies were conducted in accordance with the institutional guidelines for animal experiments at Tohoku University. The animals were housed in an air-conditioned environment, with a 12-h light-dark cycle, and fed a regular unrestricted diet or a high-fat diet consisting of 15.3% (wt/wt) fat (Quick Fat; Nippon CLEA, Shizuoka, Japan) starting at 8 weeks of age. The mutant cDNA for human IκBα, with alanine substitutions of two serine residues (32 and 36), was cloned into a transgenic vector, pSPTg.T2FpAXK, provided by Thomas N. Sato. This vector contains the Tie2 promoter, SV40 polyA signal and Tie2 minimum enhancer fragment. To generate transgenic mice, the construct cDNA was linearized with SalI digestion and microinjected into fertilized oocytes by Oriental Yeast Co. Genotyping was performed by PCR of tail DNA using the primers 5’-CCATGCGACGCGGGAAGTC-3’ and 3’-CGGAGCTCAGGATCACA-5’. The two lines of transgenic mice used in the experiment had similar phenotypes. Founder mice were backcrossed for at least 6 generations with C57BL/6J mice (The Jackson Laboratory, ME, USA). E-DNIκB;A/Y/+ mice were obtained by mating male KK A/Y (A/Y+) mice (Nippon CLEA, Shizuoka, Japan), a genetic model for obesity-diabetes syndrome, and female E-DNIκB mice. Male E-DNIκB Tg/+; A/Y/+ and littermate control male A/Y/+ mice were used in the experiment. E-DNIκB mice were crossed with endothelial nitric oxide synthase (eNOS)-deficient (Nos3−/−) mice with the C57BL/6J background1 (The Jackson Laboratory, ME, USA) to generate E-DNIκB;Nos3−/− mice. Littermate Nos3−/− mice were used as controls in these experiments.
**Blood analysis**

Blood glucose was determined as described previously.² Plasma TBARS and 8-isoprostane levels were measured with Assay Kits (Cayman Chemical Co, MN, USA).

**Glucose and insulin tolerance tests**

Glucose and insulin tolerance tests were performed as described previously.³ Glucose tolerance tests were performed on fasted (10 h) mice. Mice were injected with glucose into the intraperitoneal space, and blood glucose was assayed immediately before and at 15, 30, 60, 90 and 120 min postadministration. Insulin tolerance tests were performed on fed mice. Mice were injected with human regular insulin (Eli Lilly, Kobe, Japan), and blood glucose was assayed immediately before and at 15, 30, 45 and 60 min postinjection.

**Hyperinsulinemic-euglycemic clamp**

Hyperinsulinemic-euglycemic clamp studies were performed as described previously.⁴ Chronically cannulated, conscious and unrestrained mice were fasted for 6 h before the study. Insulin (500 mU · kg⁻¹ · min⁻¹) was infused throughout the clamp study. Blood glucose was monitored every 5 min via carotid arterial catheter samples. Glucose was infused at a variable rate to maintain blood glucose at 120 mg/dl. The glucose infusion rate was calculated as described previously.⁴

**Histological analysis**

Tissues sections were removed, fixed with 10% formalin, and embedded in paraffin. The streptavidin–biotin (SAB) method was performed using a Histofine SAB-PO kit
(Nichirei, Tokyo, Japan) for immunostaining with antibodies against NFkB p65 (C20) (Santa Cruz Biotechnology, CA, USA) and MOMA2 (Serotec Immunological Excellence, Oxford, UK). Slides were next incubated with the biotinylated IgG for 1h and then with peroxidase-conjugated streptavidin for 30 min at room temperature. Finally, immunoreactivity was visualized by incubation with a substrate solution containing 3,3′-diaminobenzidine tetrahydrochloride (DAB). For experiments involving TNF-α induced p65 translocation, mice were injected with recombinant murine TNF-α (R&D Systems Inc, MN, USA), and killed 30 minutes later.

**Immunoblotting**

Lung samples obtained from mice were homogenized and subjected to immunoblotting as described previously. Antibodies to IkB-α (C-15, 21) (Santa Cruz Biotechnology, CA, USA) were commercially obtained.

**Quantitative RT-PCR–based gene expression**

Total RNA was isolated from mouse tissues with Isogen (Wako Pure Chemical, Osaka, Japan), and cDNA was synthesized with a Cloned AMV First Strand Synthesis Kit (Invitrogen, MD, USA) using 5 µg of total RNA. cDNA synthesized from total RNA was evaluated using real-time quantitative PCR (Light Cycler Quick System 350S; Roche Diagnostics). The relative amount of mRNA was calculated with β-actin as the invariant control. Samples from skeletal muscle were calculated with α-actin as the invariant control. The oligonucleotide primers are described in Table S1. The mitochondrial DNA content was quantified using a sequence detection system (QIAGEN Inc., CA, USA). Total DNA was extracted from gastrocnemius muscles. The reactions were performed as follows: initial denaturing step at 95°C for 10 minutes and 40 cycles of 95°C for 15
seconds and 60°C for 1 minute. A melting curve was analyzed to check the specificity of the PCR product. The primer sequences were: 5’-GCC TTT CAG GAA TAC CAC GA-3’ and 5’-CCA ATT TTA GGG GGT TCG AT-3’ (GenBank NC 005089). The relative amounts of mitochondrial DNA were calculated with α-actin mRNA as the invariant control.

**Oxygen consumption**

Oxygen consumption was measured with an O2/CO2 metabolism measuring system (model MK-5000RQ; Muromachi Kikai, Tokyo, Japan) as described previously.²

**Locomotor activity**

Spontaneous locomotor activities of mice were analyzed with an infrared activity monitor (Supermex; Muromachi Kikai, Tokyo, Japan), as described previously.⁶ In this system a sensor monitors motion in multiple zones of the cage and movement of animal in the X, Y and Z axis can be determined. Mice were first acclimatized to the cages and housed individually for 4 days before measurement were taken. Food and water were provided *ad libitum*. All counts were automatically recorded at 60-min intervals and totalled for both the 12- hour-light and 12-hour-dark period. Data were averaged over the 3-day period of measurement.

**Muscle blood flow measurement**

A catheter was inserted in the carotid artery for injection of fluorescent microspheres, a second one was inserted in the femoral artery for reference blood sample withdrawal. After stabilization of hemodynamic parameters, 200 µl of yellow-green fluorescent
microspheres (Triton, CA, USA) were injected into the carotid artery with an injection syringe over 10 s followed by 0.1 ml of saline. A reference blood sample was withdrawn from the femoral artery at a rate of 0.25 ml/min, into a pre-weighed heparinated syringe, starting 10 s before microsphere injection and lasting for a total of 60 s. The syringe containing the blood sample was weighed and the blood was digested with 250 µl of 16N KOH. Mice were sacrificed and skeletal muscles were weighed and digested in 4 ml of 4N KOH with 20% Tween 80. After 24 h, the digested tissues were filtered individually and processed for fluorescence quantification.7

Supplemental References


Supplemental Figure Legends

Supplemental Figure 1. Body compositions of Ay/+ control and E-DNIκB;Ay/+ mice

(A) Body weights of Ay/+ control (white bars) and E-DNIκB;Ay/+ (black bars) mice at 20 weeks of age. (B) Epididymal fat weights of Ay/+ control and E-DNIκB;Ay/+ mice at 20 weeks of age. (C) Liver weights of Ay/+ control and E-DNIκB;Ay/+ mice at 20 weeks of age. Data are presented as means ± SEM. *P<0.05 compared with Ay/+ control littermate group by one-way ANOVA. n=5 in Ay/+ control and n=6 in E-DNIκB;Ay/+ mice.

Supplemental Figure 2. Hepatic expressions of gluconeogenic genes from Ay/+ control and E-DNIκB;Ay/+ mice

Hepatic expressions of gluconeogenic genes from Ay/+ control (white bars, n=4) and E-DNIκB;Ay/+ (black bars, n=6) mice at 20 weeks of age were analyzed by RT-PCR. The relative amounts of mRNA were calculated with β-actin mRNA as the invariant control. Data are presented as means ± SEM.

Supplemental Figure 3. Adipose expressions of adhesion molecules and angiogenesis markers from Ay/+ control and E-DNIκB;Ay/+ mice

Expressions of adhesion molecules and angiogenesis markers in epididymal fat tissues from Ay/+ control (white bars, n=5) and E-DNIκB;Ay/+ (black bars, n=4) mice at 20 weeks of age were analyzed by RT-PCR. The relative amounts of mRNA were calculated with β-actin mRNA as the invariant control. Data are presented as means ± SEM. *P<0.05 compared with control littermate group by one-way ANOVA.
Supplemental Figure 4. Blockade of endothelial NF-κB signaling prevented insulin resistance in genetically obese (A\textsuperscript{Y/+}) mice

Plasma levels of adipokines, inflammatory-related cytokines and insulin in A\textsuperscript{Y/+} control (white bars, n=5) and E-DNIκB;A\textsuperscript{Y/+} (black bars, n=6) mice were measured. Data are presented as means ± SEM. *P<0.05 compared with A\textsuperscript{Y/+} control littermate group by one-way ANOVA.

Supplemental Figure 5. Aortic expressions of anti-oxidant enzymes were suppressed in E-DNIκB;A\textsuperscript{Y/+} mice

Aortic gene expressions of anti-oxidant enzymes from A\textsuperscript{Y/+} control (white bars, n=5) and E-DNIκB;A\textsuperscript{Y/+} (black bars, n=6) mice at 20 weeks of age were analyzed by RT-PCR. The relative amounts of mRNA were calculated with β-actin mRNA as the invariant control. Data are presented as means ± SEM. *P<0.05, **P<0.01 compared with A\textsuperscript{Y/+} control littermate group by one-way ANOVA.

Supplemental Figure 6. Oxygen consumption during the dark phase was increased in E-DNIκB;A\textsuperscript{Y/+} mice

Oxygen consumption of A\textsuperscript{Y/+} control littermates (white bars, n=4) and E-DNIκB;A\textsuperscript{Y/+} mice (black bars, n=4) were measured at 16 weeks of age. Data are presented as means ± SEM. *P<0.05 compared with A\textsuperscript{Y/+} control littermate group by one-way ANOVA.

Supplemental Figure 7. Protection from age-related body weight gain in E-DNIκB mice

(A) Food intakes of control (white bars, n=5) and E-DNIκB (black bars, n=6) of
50-week-old mice maintained on a normal chow diet. (B) Body weights of aged control and E-DNIkB mice at 50 weeks of age. Data are presented as means ± SEM. *P<0.05 compared with control littermate group by one-way ANOVA.

**Supplemental Figure 8.** eNOS deficiency suppresses the effects of endothelial DNIkB expression on blood pressure and muscle blood flow

(A) Blood pressures and (B) gastrocnemius muscle blood flows of wild-type, E-DNIkB, control Nos3−/− and E-DNIkB;Nos3−/− mice at 50-60 weeks of age. Data are presented as means ± SEM. *P<0.05 compared with control littermate group by one-way ANOVA. n=4-5 in each group.

**Supplemental Figure 9.** Schematic diagram illustrating the proposed functions of endothelial NF-κB signaling

**Supplemental Table 1.** Sequences of Quantitative RT-PCR primers
Supplemental Figure 2

Relative amount of mRNA (%)

PEPCK

G6Pase

Relative amount of mRNA (%)

0 20 40 60 80 100 120 140 160

0 20 40 60 80 100 120 140 160
Supplemental Figure 3

**VEGF Tie2**

Relative amount of mRNA (%)

**E-selectin**

Relative amount of mRNA (%)

**VCAM-1**

Relative amount of mRNA (%)

**ICAM-1**

Relative amount of mRNA (%)

**Tie2**

Relative amount of mRNA (%)

**VEGF**

Relative amount of mRNA (%)
Supplemental Figure 4

A. Plasma adiponectin (µg/ml) compared to B. Plasma leptin (ng/ml).

C. Plasma TNF-α (pg/ml) compared to D. Plasma MCP-1 (pg/ml).

* indicates a statistically significant difference.
Supplemental Figure 5

MnSOD

Relative amount of mRNA (%)

0 20 40 60 80 100 120 140

glutathion peroxidase

Relative amount of mRNA (%)

0 20 40 60 80 100 120 140

* 

**
Supplemental Figure 6
Supplemental Figure 7

A

Food intake (g/day)

B

Body weight (g)

*
Supplemental Figure 8

A

![Graph A]

B

![Graph B]
Supplemental Figure 9

- NF-κB signalling
- Endothelium
  - Inflammatory response
  - Adhesion molecule expression
  - Mφ infiltration into adipose tissue
  - Blood flow in muscle
  - Blood pressure
  - Locomotor activity
  - Mitochondrial biogenesis
  - Mitochondrial sirtuins
  - Insulin resistance
  - Longevity

- Inflammation
- Oxidative stress
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