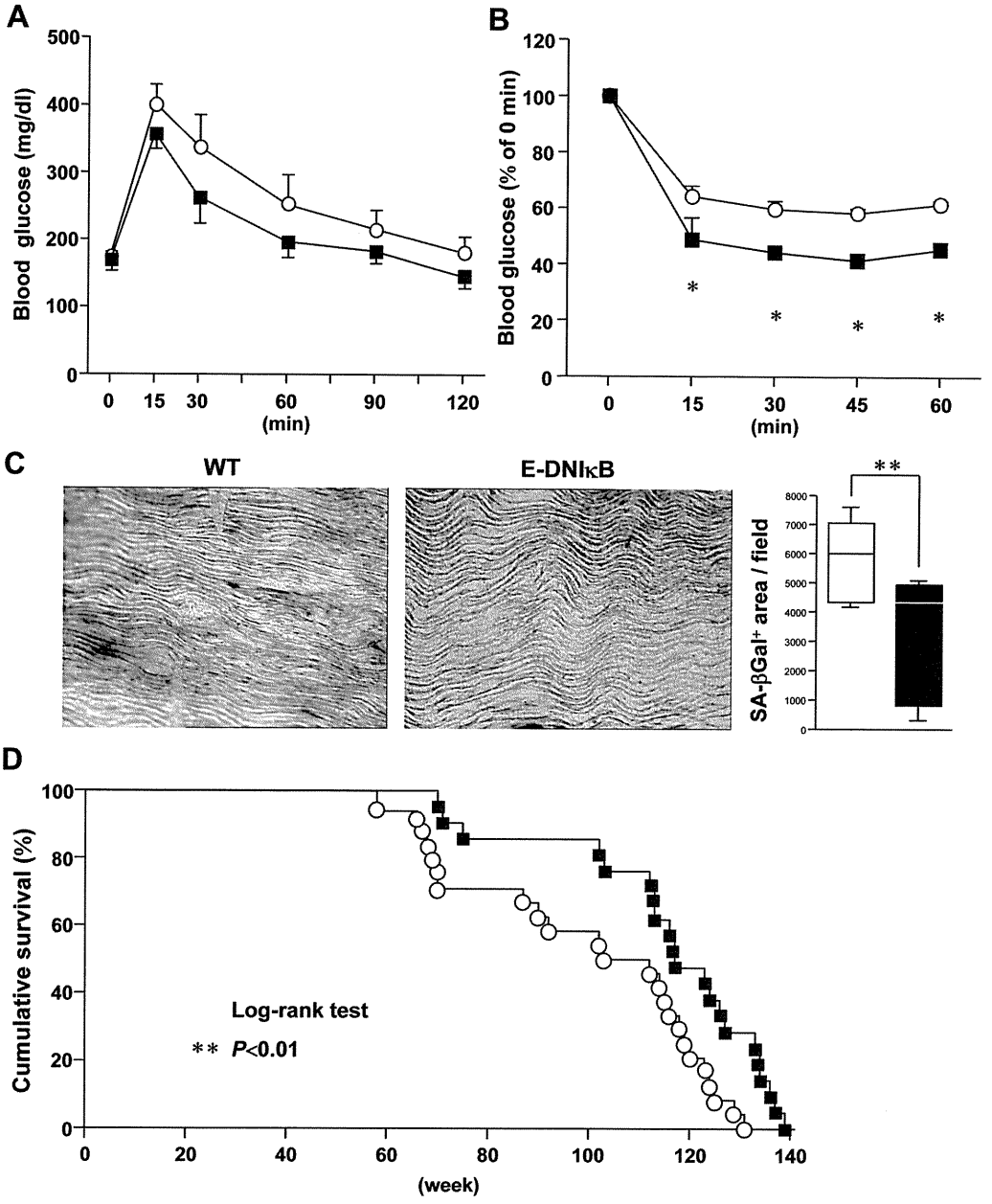

Figure 6. Blockade of nuclear factor-κB (NF-κB) signaling prevented vascular senescence and prolonged life span. **A**, Control (○; n=6) and endothelial dominant-negative IκBα transgenic (E-DNIκB; ■; n=8) mice received glucose tolerance tests with a peritoneal glucose load (1.5 g/kg body weight) after a 10-hour fast at 50 weeks of age. **B**, Insulin tolerance tests were performed in an ad libitum–fed state. Data are expressed as percentages of blood glucose levels immediately before intraperitoneal insulin loading (0.75 U/kg body weight) at 50 weeks of age. **C**, Aortas from aged control and E-DNIκB mice at 90 weeks of age were stained for senescence-associated β-galactosidase (SAβ-gal). SAβ-gal–positive areas of aortas from aged control (white bars, n=5) and E-DNIκB (black bars, n=5) mice were measured (right). Data are presented as mean±SEM. *$P<0.05$, **$P<0.01$ vs control littermate group by 1-way and repeated-measures ANOVA. WT indicates wild type. **D**, Cumulative survival of control (○; n=24) and E-DNIκB (■; n=21) mice on a normal chow diet was analyzed by the Kaplan-Meier method. **$P<0.01$ by the log-rank test.

be lower in E-DNIκB mice, although the differences were not statistically significant (Figure 6A). Insulin tolerance tests revealed better insulin sensitivity in E-DNIκB mice (Figure 6B). Thus, blockade of endothelial NF-κB signaling inhibited the development of age-related insulin resistance.

Furthermore, systolic blood pressure was significantly lower in E-DNIκB mice than in wild-type controls of the same age (Figure VIIIA in the online-only Data Supplement). To determine whether eNOS mediates age-related blood pressure elevation via endothelial NF-κB signaling, we next crossed E-DNIκB mice with eNOS-deficient ($Nos3^{-/-}$) mice. Whereas endothelial DNIκB expression suppressed blood pressure elevation in $Nos3^{+/+}$ mice, eNOS deficiency blunted the inhibitory effects of DNIκB on blood pressure elevation (Figure VIIIA in the online-only Data Supplement). These findings indicate involvement of eNOS in protection from age-related hypertension in E-DNIκB mice.

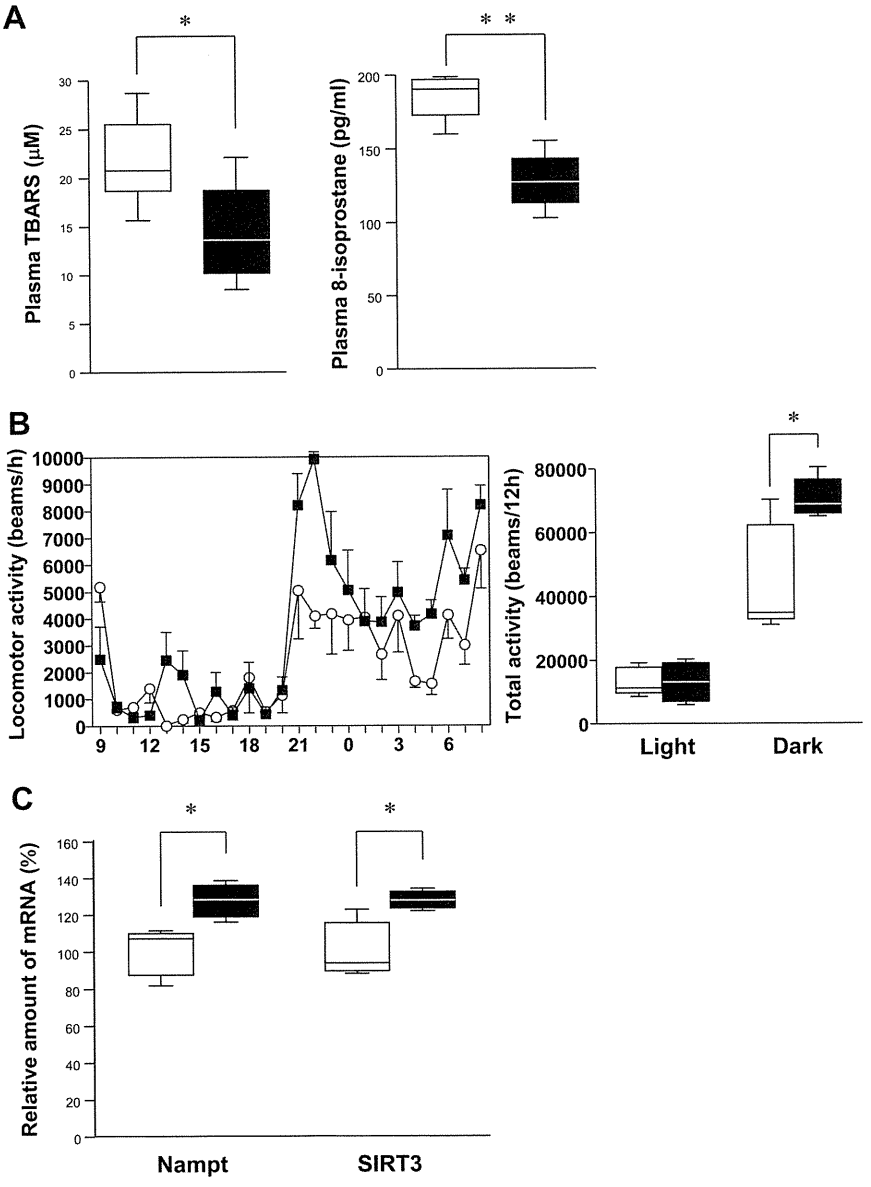

Figure 7. Increments in locomotor activity and upregulation of mitochondrial prosurvival genes in endothelial dominant-negative IκBα transgenic (E-DNIκB)mice. **A**, Plasma thiobarbituric acid–reactive substance (TBARS) and 8-isoprostane concentrations were measured in aged control (white bars, n=6) and E-DNIκB (black bars, n=8) mice at 50 weeks of age. **B**, Locomotor activities of aged control (○; n=5) and E-DNIκB (■; n=5) mice at 50 weeks of age (left). Estimated activities of aged control and E-DNIκB mice in 12-hour light and dark periods (right) were calculated. **C**, Aortic gene expression of mitochondrial prosurvival genes in 50-week-old aged control and E-DNIκB mice was analyzed by reverse-transcriptase polymerase chain reaction. The relative amounts of mRNA were calculated with β-actin mRNA as the invariant control. Data are presented as means±SEM. Nampt indicates nicotinamide phosphoribosyltransferase n=5 in each group. *$P<0.05$, **$P<0.01$ by 1-way ANOVA.

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 littermates 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 littermates ($P=0.0095$ 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;Nos3$^{-/-}$ 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,[35] and constitutive NO production by endothelial cells reportedly inhibits its 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 obese[29] and aged[38] 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-DNIκB 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.[43] In these knockout mice as well, Nampt 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.[44] 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.[45] 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.[46] Indeed, blockade of endothelial NF-κB signaling suppresses hypercholesterolemia-induced atherosclerosis caused by apolipoprotein E deficiency.[47] 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

1. Hayden MS, Ghosh S. Shared principles in NF-kappaB signaling. *Cell*. 2008;132:344–362.
2. Karin M, Yamamoto Y, Wang QM. The IKK NF-kappa B system: a treasure trove for drug development. *Nat Rev Drug Discov*. 2004;3:17–26.
3. Hotamisligil GS. Inflammation and metabolic disorders. *Nature*. 2006;444:860–867.
4. Semenkovich CF. Insulin resistance and atherosclerosis. *J Clin Invest*. 2006;116:1813–1822.
5. Alberti KG, Zimmet P, Shaw J. The metabolic syndrome: a new worldwide definition. *Lancet*. 2005;366:1059–1062.
6. Katagiri H, Yamada T, Oka Y. Adiposity and cardiovascular disorders: disturbance of the regulatory system consisting of humoral and neuronal signals. *Circ Res*. 2007;101:27–39.
7. Arkan MC, Hevener AL, Greten FR, Maeda S, Li ZW, Long JM, Wynshaw-Boris A, Poli G, Olefsky J, Karin M. IKK-beta links inflammation to obesity-induced insulin resistance. *Nat Med*. 2005;11:191–198.
8. Yuan M, Konstantopoulos N, Lee J, Hansen L, Li ZW, Karin M, Shoelson SE. Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of IKKbeta. *Science*. 2001;293:1673–1677.
9. Kim JK, Kim YJ, Fillmore JJ, Chen Y, Moore I, Lee J, Yuan M, Li ZW, Karin M, Perret P, Shoelson SE, Shulman GI. Prevention of fat-induced insulin resistance by salicylate. *J Clin Invest*. 2001;108:437–446.
10. Aird WC. Phenotypic heterogeneity of the endothelium, I: structure, function, and mechanisms. *Circ Res*. 2007;100:158–173.
11. Rocha VZ, Libby P. Obesity, inflammation, and atherosclerosis. *Nat Rev Cardiol*. 2009;6:399–409.
12. Kim JA, Montagnani M, Koh KK, Quon MJ. Reciprocal relationships between insulin resistance and endothelial dysfunction: molecular and pathophysiological mechanisms. *Circulation*. 2006;113:1888–1904.
13. Hasegawa Y, Ogihara T, Yamada T, Ishigaki Y, Imai J, Uno K, Gao J, Kaneko K, Ishihara H, Sasano H, Nakauchi H, Oka Y, Katagiri H. Bone marrow (BM) transplantation promotes beta-cell regeneration after acute injury through BM cell mobilization. *Endocrinology*. 2007;148:2006–2015.
14. Imai J, Katagiri H, Yamada T, Ishigaki Y, Suzuki T, Kudo H, Uno K, Hasegawa Y, Gao J, Kaneko K, Ishihara H, Niijima A, Nakazato M, Asano T, Minokoshi Y, Oka Y. Regulation of pancreatic beta cell mass by neuronal signals from the liver. *Science*. 2008;322:1250–1254.
15. Marelli-Berg FM, Peek E, Lidington EA, Stauss HJ, Lechler RI. Isolation of endothelial cells from murine tissue. *J Immunol Methods*. 2000;244:205–215.
16. Uno K, Katagiri H, Yamada T, Ishigaki Y, Ogihara T, Imai J, Hasegawa Y, Gao J, Kaneko K, Iwasaki H, Ishihara H, Sasano H, Inukai K, Mizuguchi H, Asano T, Shiota M, Nakazato M, Oka Y. Neuronal pathway from the liver modulates energy expenditure and systemic insulin sensitivity. *Science*. 2006;312:1656–1659.
17. Iwamoto T, Kita S, Zhang J, Blaustein MP, Arai Y, Yoshida S, Wakimoto K, Komuro I, Katsuragi T. Salt-sensitive hypertension is triggered by Ca^{2+} entry via Na^{+}/Ca^{2+} exchanger type-1 in vascular smooth muscle. *Nat Med*. 2004;10:1193–1199.
18. Kubis N, Richer C, Domergue V, Giudicelli JF, Levy BI. Role of microvascular rarefaction in the increased arterial pressure in mice lacking for the endothelial nitric oxide synthase gene (eNOS3pt−/−). *J Hypertens*. 2002;20:1581–1587.
19. Gao J, Katagiri H, Ishigaki Y, Yamada T, Ogihara T, Imai J, Uno K, Hasegawa Y, Kanzaki M, Yamamoto TT, Ishibashi S, Oka Y. Involvement of apolipoprotein E in excess fat accumulation and insulin resistance. *Diabetes*. 2007;56:24–33.
20. Kunieda T, Minamino T, Nishi J, Tateno K, Oyama T, Katsuno T, Miyauchi H, Orimo M, Okada S, Takamura M, Nagai T, Kaneko S, Komuro I. Angiotensin II induces premature senescence of vascular smooth muscle cells and accelerates the development of atherosclerosis via a p21-dependent pathway. *Circulation*. 2006;114:953–960.
21. Kim I, Moon SO, Kim SH, Kim HJ, Koh YS, Koh GY. Vascular endothelial growth factor expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin through nuclear factor-kappa B activation in endothelial cells. *J Biol Chem*. 2001;276:7614–7620.
22. Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA, Chen H. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest*. 2003;112:1821–1830.
23. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW Jr. Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest*. 2003;112:1796–1808.
24. Nishimura S, Manabe I, Nagasaki M, Seo K, Yamashita H, Hosoya Y, Ohsugi M, Tobe K, Kadowaki T, Nagai R, Sugiura S. In vivo imaging in

mice reveals local cell dynamics and inflammation in obese adipose tissue. *J Clin Invest*. 2008;118:710–721.
25. Huang PL, Huang Z, Mashimo H, Bloch KD, Moskowitz MA, Bevan JA, Fishman MC. Hypertension in mice lacking the gene for endothelial nitric oxide synthase. *Nature*. 1995;377:239–242.
26. Nisoli E, Clementi E, Paolucci C, Cozzi V, Tonello C, Sciorati C, Bracale R, Valerio A, Francolini M, Moncada S, Carruba MO. Mitochondrial biogenesis in mammals: the role of endogenous nitric oxide. *Science*. 2003;299:896–899.
27. Gervais M, Demolis P, Domergue V, Lesage M, Richer C, Giudicelli JF. Systemic and regional hemodynamics assessment in rats with fluorescent microspheres. *J Cardiovasc Pharmacol*. 1999;33:425–432.
28. Duplain H, Burcelin R, Sartori C, Cook S, Egli M, Lepori M, Vollenweider P, Pedrazzini T, Nicod P, Thorens B, Scherrer U. Insulin resistance, hyperlipidemia, and hypertension in mice lacking endothelial nitric oxide synthase. *Circulation*. 2001;104:342–345.
29. Lowell BB, Shulman GI. Mitochondrial dysfunction and type 2 diabetes. *Science*. 2005;307:384–387.
30. Chan DC. Mitochondria: dynamic organelles in disease, aging, and development. *Cell*. 2006;125:1241–1252.
31. Lanza IR, Short DK, Short KR, Raghavakaimal S, Basu R, Joyner MJ, McConnell JP, Nair KS. Endurance exercise as a countermeasure for aging. *Diabetes*. 2008;57:2933–2942.
32. Lin SJ, Kaeberlein M, Andalis AA, Sturtz LA, Defossez PA, Culotta VC, Fink GR, Guarente L. Calorie restriction extends *Saccharomyces cerevisiae* lifespan by increasing respiration. *Nature*. 2002;418:344–348.
33. De Martin R, Hoeth M, Hofer-Warbinek R, Schmid JA. The transcription factor NF-kappa B and the regulation of vascular cell function. *Arterioscler Thromb Vasc Biol*. 2000;20:E83–E88.
34. Cao Y. Angiogenesis modulates adipogenesis and obesity. *J Clin Invest*. 2007;117:2362–2368.
35. Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*. 1987; 327:524–526.
36. Peng HB, Libby P, Liao JK. Induction and stabilization of I kappa B alpha by nitric oxide mediates inhibition of NF-kappa B. *J Biol Chem*. 1995; 270:14214–14219.
37. Adler AS, Kawahara TL, Segal E, Chang HY. Reversal of aging by NFkappaB blockade. *Cell Cycle*. 2008;7:556–559.
38. Petersen KF, Befroy D, Dufour S, Dziura J, Ariyan C, Rothman DL, DiPietro L, Cline GW, Shulman GI. Mitochondrial dysfunction in the elderly: possible role in insulin resistance. *Science*. 2003;300:1140–1142.
39. Terjung RL, Zarzeczny R, Yang HT. Muscle blood flow and mitochondrial function: influence of aging. *Int J Sport Nutr Exerc Metab*. 2002;12:368–378.
40. Sinclair DA, Lin SJ, Guarente L. Life-span extension in yeast. *Science*. 2006;312:195–197.
41. Lin SJ, Defossez PA, Guarente L. Requirement of NAD and SIR2 for life-span extension by calorie restriction in *Saccharomyces cerevisiae*. *Science*. 2000;289:2126–2128.
42. Yang H, Yang T, Baur JA, Perez E, Matsui T, Carmona JJ, Lamming DW, Souza-Pinto NC, Bohr VA, Rosenzweig A, de Cabo R, Sauve AA, Sinclair DA. Nutrient-sensitive mitochondrial NAD+ levels dictate cell survival. *Cell*. 2007;130:1095–1107.
43. Benigni A, Corna D, Zoja C, Sonzogni A, Latini R, Salio M, Conti S, Rottoli D, Longaretti L, Cassis P, Morigi M, Coffman TM, Remuzzi G. Disruption of the Ang II type 1 receptor promotes longevity in mice. *J Clin Invest*. 2009;119:524–530.
44. Li XC, Zhuo JL. Nuclear factor-kappaB as a hormonal intracellular signaling molecule: focus on angiotensin II-induced cardiovascular and renal injury. *Curr Opin Nephrol Hypertens*. 2008;17:37–43.
45. Higuchi S, Ohtsu H, Suzuki H, Shirai H, Frank GD, Eguchi S. Angiotensin II signal transduction through the AT1 receptor: novel insights into mechanisms and pathophysiology. *Clin Sci*. 2007;112:417–428.
46. Ross R. Atherosclerosis: an inflammatory disease. *N Engl J Med*. 1999; 340:115–126.
47. Gareus R, Kotsaki E, Xanthoulea S, van der Made I, Gijbels MJ, Kardakaris R, Polykratis A, Kollias G, de Winther MP, Pasparakis M. Endothelial cell-specific NF-kappaB inhibition protects mice from atherosclerosis. *Cell Metab*. 2008;8:372–383.

CLINICAL PERSPECTIVE

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. By expressing the dominant-negative IκB mutant in the endothelium using the transgenic procedure, the NF-κB signaling pathway was blocked selectively in endothelial cells. These mice were protected from the development of both obesity- and age-related insulin resistance. Furthermore, importantly, these mice exhibited prolonged lifespans. In addition to the decrease in relatively early deaths, maximum lifespan was shown to be longer in these transgenic mice. Vascular senescence was markedly inhibited by blockade of endothelial NF-κB. Thus, the endothelium plays important roles in obesity- and age-related disorders through intracellular NF-κB signaling, ultimately impacting lifespan. Blockade of endothelial NF-κB signaling apparently protects the whole body from both fatal morbidities at earlier ages and the development of senescence. Amelioration of insulin resistance and decreased oxidative stress are likely to contribute to these beneficial phenotypes. Therefore, endothelial NF-κB signaling is a potential target not only for treating the metabolic syndrome, but also for anti-aging strategies.

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 Sal1 digestion and microinjected into fertilized oocytes by Oriental Yeast Co. Genotyping was performed by PCR of tail DNA using the primers 5'-CCATGCGAGCGGGAAGTC-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 background[1] (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 $\cdot$ kg^{-1} $\cdot$ 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.[5] Antibodies to IκB-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

1. Hasegawa Y, Ogihara T, Yamada T, Ishigaki Y, Imai J, Uno K, Gao J, Kaneko K, Ishihara H, Sasano H, Nakauchi H, Oka Y, Katagiri H. Bone marrow (BM) transplantation promotes beta-cell regeneration after acute injury through BM cell mobilization. *Endocrinology.* 2007;148:2006-2015.
2. Ishigaki Y, Katagiri H, Yamada T, Ogihara T, Imai J, Uno K, Hasegawa Y, Gao J, Ishihara H, Shimosegawa T, Sakoda H, Asano T, Oka Y. Dissipating excess energy stored in the liver is a potential treatment strategy for diabetes associated with obesity. *Diabetes.* 2005;54:322-332.
3. Yamada T, Katagiri H, Ishigaki Y, Ogihara T, Imai J, Uno K, Hasegawa Y, Gao J, Ishihara H, Niijima A, Mano H, Aburatani H, Asano T, Oka Y. Signals from intra-abdominal fat modulate insulin and leptin sensitivity through different mechanisms: neuronal involvement in food-intake regulation. *Cell Metab.* 2006;3:223-229.
4. Uno K, Katagiri H, Yamada T, Ishigaki Y, Ogihara T, Imai J, Hasegawa Y, Gao J,

Kaneko K, Iwasaki H, Ishihara H, Sasano H, Inukai K, Mizuguchi H, Asano T, Shiota M, Nakazato M, Oka Y. Neuronal pathway from the liver modulates energy expenditure and systemic insulin sensitivity. *Science.* 2006;312:1656-1659.

5. Imai J, Katagiri H, Yamada T, Ishigaki Y, Suzuki T, Kudo H, Uno K, Hasegawa Y, Gao J, Kaneko K, Ishihara H, Niijima A, Nakazato M, Asano T, Minokoshi Y, Oka Y. Regulation of pancreatic beta cell mass by neuronal signals from the liver. *Science.* 2008;322:1250-1254.

6. Mashimo T, Voigt B, Kuramoto T, Serikawa T. Rat Phenome Project: the untapped potential of existing rat strains. *J Appl Physiol.* 2005;98:371-379.

7. Kubis N, Richer C, Domergue V, Giudicelli JF, Levy BI. Role of microvascular rarefaction in the increased arterial pressure in mice lacking for the endothelial nitric oxide synthase gene (eNOS3pt-/-). *J Hypertens.* 2002;20:1581-1587.

Supplemental Figure Legends

Supplemental Figure 1. Body compositions of $A^y/+$ control and E-DNIκB;$A^y/+$ mice

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

Supplemental Figure 2. Hepatic expressions of gluconeogenic genes from $A^y/+$ control and E-DNIκB;$A^y/+$ mice

Hepatic expressions of gluconeogenic genes from $A^y/+$ control (white bars, n=4) and E-DNIκB;$A^y/+$ (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.

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

Expressions of adhesion molecules and angiogenesis markers in epididymal fat tissues from $A^y/+$ control (white bars, n=5) and E-DNIκB;$A^y/+$ (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^y/+) mice

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

Supplemental Figure 5. Aortic expressions of anti-oxidant enzymes were suppressed in E-DNIκB;A^y/+ mice

Aortic gene expressions of anti-oxidant enzymes from A^y/+ control (white bars, n=5) and E-DNIκB;A^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^y/+ control littermate group by one-way ANOVA.

Supplemental Figure 6. Oxygen consumption during the dark phase was increased in E-DNIκB;A^y/+ mice

Oxygen consumption of A^y/+ control littermates (white bars, n=4) and E-DNIκB;A^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^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-DNIκB mice at 50 weeks of age. Data are presented as means ± SEM. *P<0.05 compared with control littermate group by one-way ANOVA.

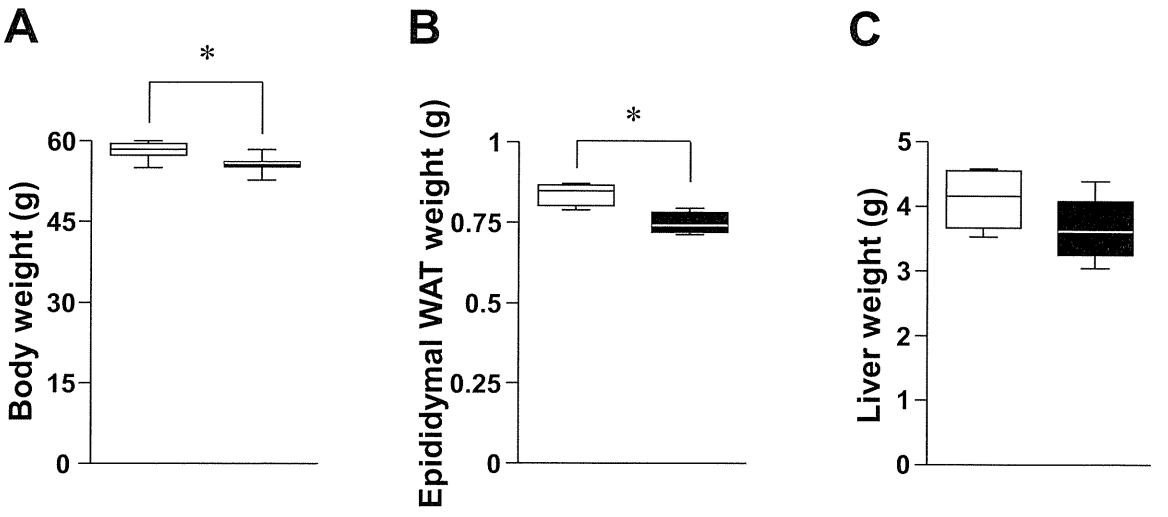
Supplemental Figure 8. eNOS deficiency suppresses the effects of endothelial DNIκB expression on blood pressure and muscle blood flow

(A) Blood pressures and (B) gastrocnemius muscle blood flows of wild-type, E-DNIκB, control $Nos3^{-/-}$ and E-DNIκB;$Nos3^{-/-}$ 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.

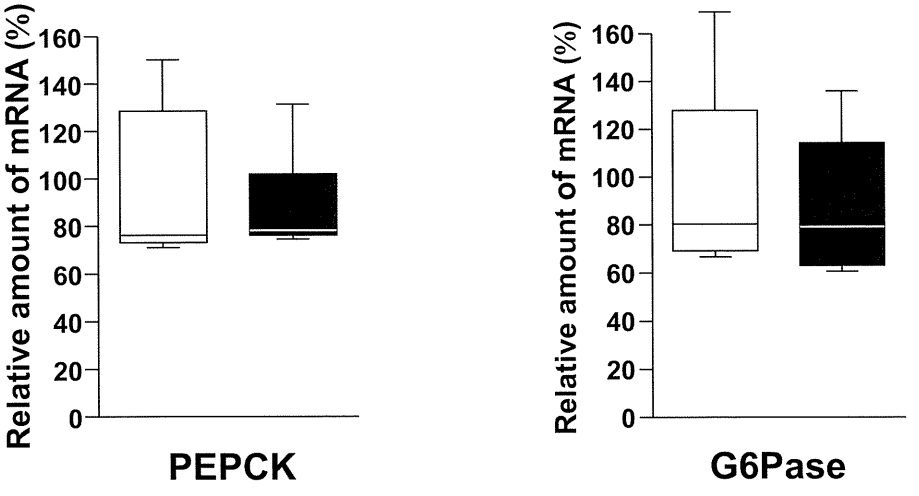
Supplemental Figure 9. Schematic diagram illustrating the proposed functions of endothelial NF-κB signaling

Supplemental Table 1. Sequences of Quantitative RT-PCR primers

Supplemental Figure 1



Supplemental Figure 2



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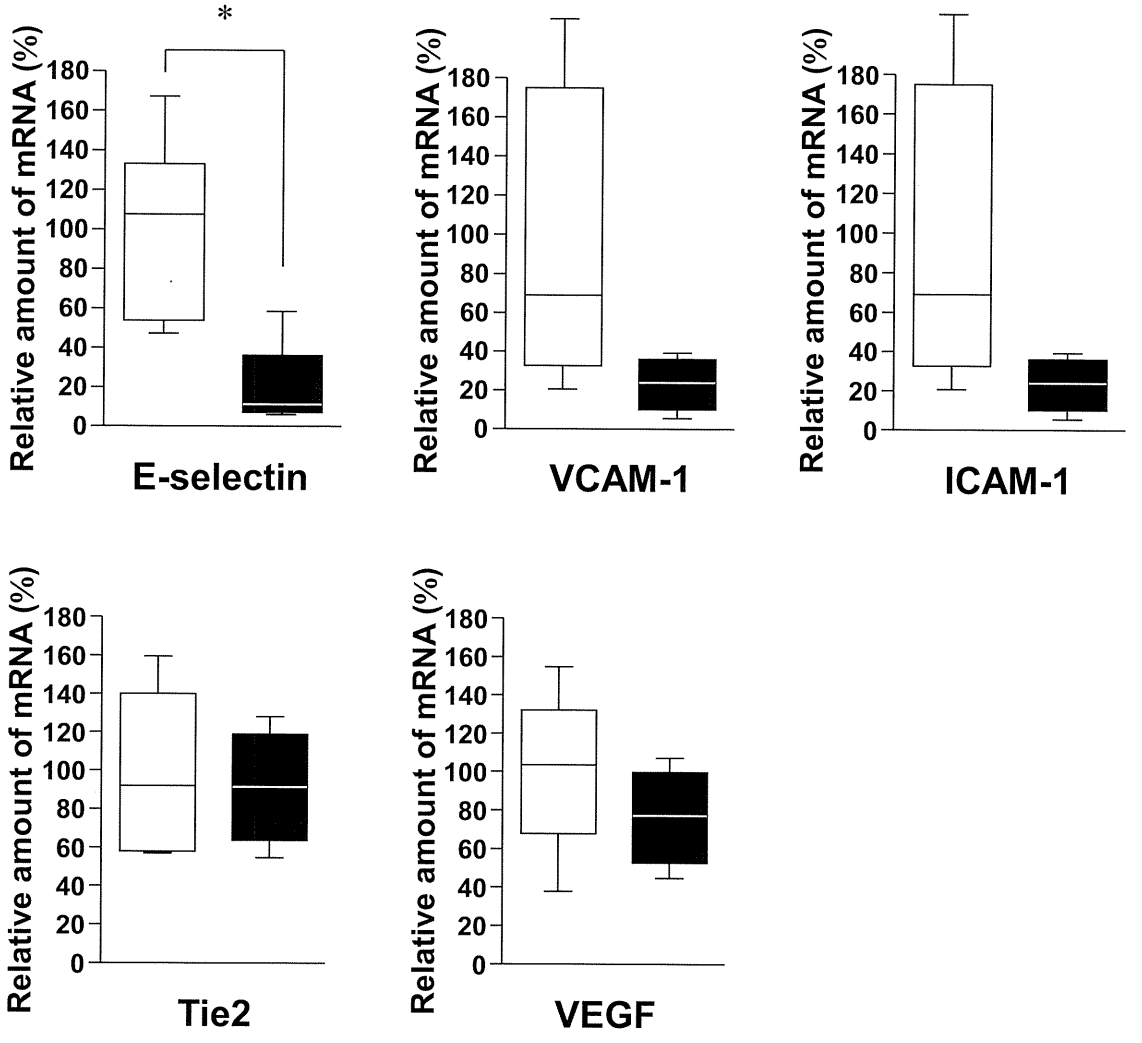

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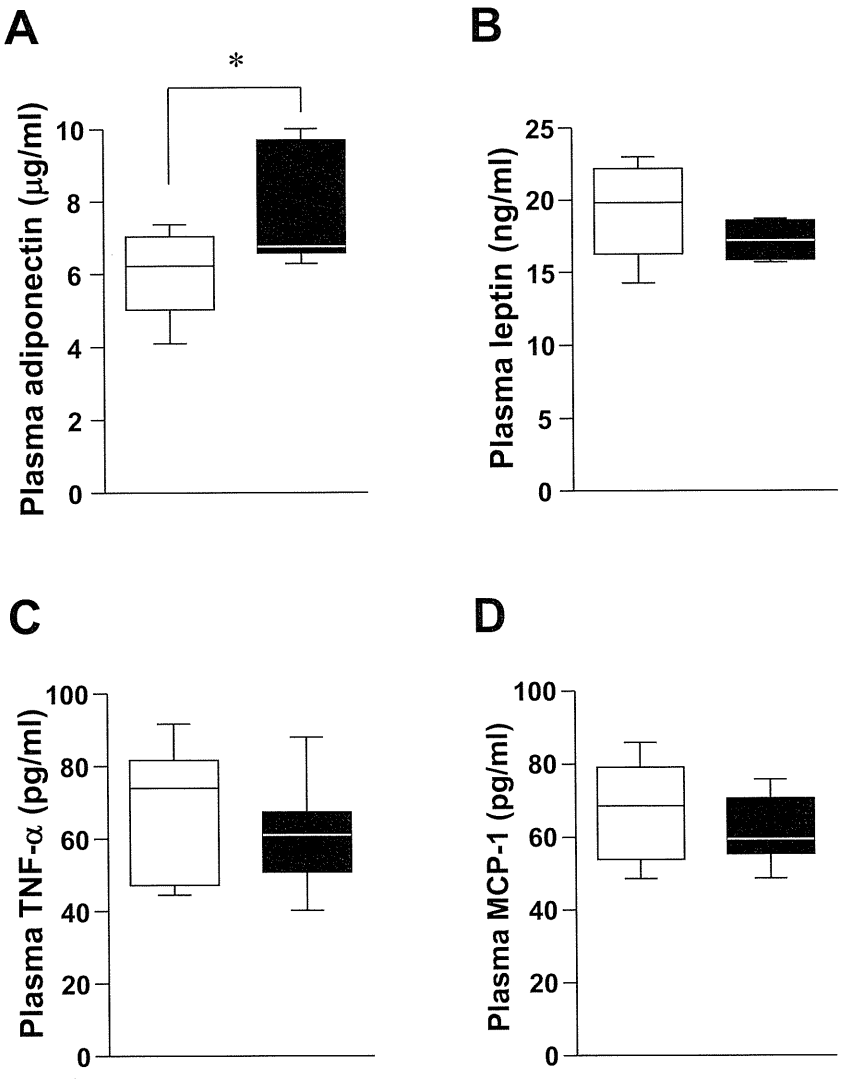
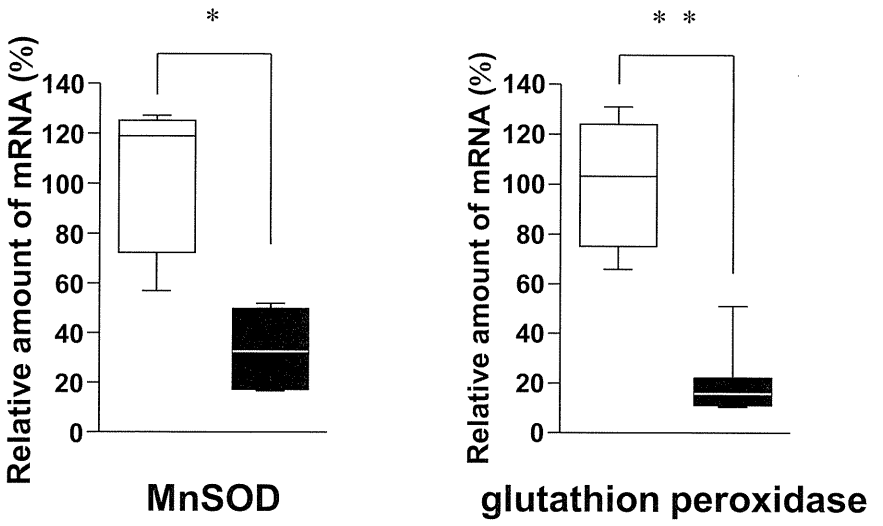


Supplemental Figure 5



Supplemental Figure 6