Deletion of the Activated Protein-1 Transcription Factor JunD Induces Oxidative Stress and Accelerates Age-Related Endothelial Dysfunction

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**Background**—Reactive oxygen species are major determinants of vascular aging. JunD, a member of the activated protein-1 family of transcription factors, is emerging as a major gatekeeper against oxidative stress. However, its contribution to reactive oxygen species homeostasis in the vasculature remains unknown.

**Methods and Results**—Endothelium-dependent vasorelaxation was impaired in young and old JunD**−/−** mice (6 and 22 months old) compared with age-matched wild-type mice. JunD**−/−** mice displayed an age-independent decline in endothelial nitric oxide release and endothelial nitric oxide synthase activity and increased mitochondrial superoxide formation and peroxynitrite levels. Furthermore, vascular expression and activity of the free radical scavengers manganese and extracellular superoxide dismutase and aldehyde dehydrogenase 2 were reduced, whereas the NADPH oxidase subunits p47phox, Nox2, and Nox4 were upregulated. These redox changes were associated with premature vascular aging, as shown by reduced telomerase activity, increased β-galactosidase–positive cells, upregulation of the senescence markers p16**INK4a** and p53, and mitochondrial disruption. Interestingly, old wild-type mice showed a reduction in JunD expression and transcriptional activity resulting from promoter hypermethylation and binding with tumor suppressor menin, respectively. In contrast, JunD overexpression blunted age-induced endothelial dysfunction. In human endothelial cells, JunD knockdown exerted a similar impairment of the O₂−/nitric oxide balance that was prevented by concomitant NADPH inhibition. In parallel, JunD expression was reduced in monocytes from old versus young healthy subjects and correlated with mRNA levels of scavenging and oxidant enzymes.

**Conclusions**—JunD provides protection in aging-induced endothelial dysfunction and may represent a novel target to prevent reactive oxygen species–driven vascular aging. (**Circulation.** 2013;127:1229-1240.)

**Key Words:** aging ■ endothelial dysfunction ■ oxidative stress ■ vascular biology

The prevalence of cardiovascular disease is markedly age dependent and a major cause of myocardial infarction and death among elderly patients. A key alteration of aging arteries is the development of endothelial dysfunction, which is associated with a reduction in the bioavailability of nitric oxide (NO).1–3 The primary mechanism of aging-associated endothelial dysfunction is oxidative stress, a state in which the generation of reactive oxygen species (ROS) exceeds the capacity of cellular antioxidant defense systems.4 Of note, superoxide anion (O₂−) inactivates endothelium-derived NO.4–7 From the interaction of O₂− with NO, the highly reactive peroxynitrite (ONOO−) is formed.5 As a consequence, vasodilation and the antithrombotic and antiinflammatory effects mediated by the endothelium are impaired, resulting in vascular damage.5,9 Although the accumulation of ROS generation is the most widely accepted cause of vascular aging,4,10 a thorough understanding of the molecular mechanisms of altered redox signaling in endothelial cells remains elusive.
Clinical Perspective on p 1240

Activator protein-1 (AP-1) is a collection of dimeric complexes made up of different members of 3 families of DNA-binding proteins: Jun, Fos, and ATF/CREB.11–13 These members assemble to form AP-1 transcription factors with activities that are strongly influenced by their specific components and their cellular environment.12 JunD, the most recent gene of the Jun family, regulates cell growth and survival and protects against oxidative stress by modulating genes involved in antioxidant defense and ROS production. Along these lines, JunD−/− mice exhibit shortened life span and increased incidence of aggressive cancers.14–16 Recent evidence demonstrated an accumulation of ROS in JunD−/− murine embryonic fibroblasts that was reduced by treatment with ascorbate.17 Gene expression profiling of JunD−/− cells showed downregulation of several free radical scavenging enzymes associated with an increase in the expression of ROS-producing NADPH oxidase.17 In contrast, overexpression of JunD blunting redox signaling abolished ROS production and apoptosis.17,18

In this study, we tested the hypothesis that JunD may be a critical regulator of vascular homeostasis by investigating its role in ROS-driven vascular aging.

Methods

A detailed description of the methods used in this study is provided in the online-only Data Supplement.

Animals

Young (6-month-old) and old (22-month-old) male JunD−/− mice and male wild-type (WT) littermates were obtained from Institut Curie (Paris, France). Control and knockout mice shared an identical genetic background because both mice lines are of the same C57BL6/129Sv (Paris, France). Control and knockout mice shared an identical genetic background because both mice lines are of the same C57BL6/129Sv (Paris, France). Control and knockout mice shared an identical genetic background because both mice lines are of the same C57BL6/129Sv (Paris, France). Control and knockout mice shared an identical genetic background because both mice lines are of the same C57BL6/129Sv (Paris, France). Control and knockout mice shared an identical genetic background because both mice lines are of the same C57BL6/129Sv (Paris, France). Control and knockout mice shared an identical genetic background because both mice lines are of the same C57BL6/129Sv (Paris, France).

Tissue Harvesting and Organ Chamber Experiments

On the day of the experiment, mice were anesthetized by the intraperitoneal administration of 50 mg/kg sodium pentobarbital and then euthanized. The chest and abdomen were opened with a medial sternotomy. The aorta was cleaned from adhering connective tissue under a dissection microscope and immediately used for organ chamber experiments.

Measurements of Mitochondrial O2−, NO, and ONOO− From Mouse Aorta

Superoxide anion was measured in mitochondria isolated from mouse aorta by the use of electron spin resonance (ESR) spectroscopy analysis as previously reported.19 In situ measurements of NO and ONOO− were carried out with electrochemical nanosensors.20,21

Isolation of Mitochondrial and Cytosolic Fraction

Mitochondria were isolated by centrifugation as previously reported.22

Mitochondrial Swelling Assay

Isolated mitochondria (40 µg) from mouse aortas in swelling buffer [250 mmol/L sucrose, 10 mmol/L 3-(n-morpholino)propanesulfonic acid, 5 µmol/L EGTA, 2 mmol/L MgCl2, 5 mmol/L KH2PO4, 5 mmol/L pyruvate, 5 mmol/L malate] were incubated with 150 µmol/L calcium chloride (CaCl2) in a final volume of 200 µL in a 96-well plate for 20 minutes. Absorbance was read every 30 seconds at 520 nm.23

Mitochondrial DNA Damage Detection

Assessment of mitochondrial DNA damage was performed by the use of quantitative PCR–based amplification of a large fragment of mitochondrial DNA as previously described.24

Western Blotting

Frozen samples of aortas were pulverized and dissolved in lysis buffer (120 mmol/L sodium chloride, 50 mmol/L Tris, 20 mmol/L sodium fluoride, 1 mmol/L benzamidine, 1 mmol/L DTT, 1 mmol/L EDTA, 6 mmol/L EGTA, 15 mmol/L sodium pyrophosphate, 0.8 µg/mL leupeptin, 30 mmol/L p-nitrophenyl phosphate, 0.1 mmol/L phenylmethylsulfonyl fluoride, and 1% NP-40) for immunoblottting.

Detection of Membrane Translocation of the NADPH Subunit P47phox

This method is reported in the online-only Data Supplement.

Manganese and Extracellular Superoxide Dismutase Activity

Manganese superoxide dismutase and extracellular superoxide dismutase activity was measured in isolated mitochondria and aortic lysates, respectively, by use of a commercially available kit (OxiSelect Superoxide Dismutase Activity Assay, Cell Biolabs, Inc).

NADPH and Xanthine Oxidase Activity

Commercially available kits were used to assess NADPH and xanthine oxidase activity in aortic lysates of JunD−/− and WT mice (abcam).

Aldehyde Dehydrogenase 2 Activity

Aldehyde dehydrogenase 2 activity was determined in mitochondria isolated from mouse aorta of JunD−/− and WT mice according to the manufacturer’s recommendations (aldehyde dehydrogenase 2 activity assay kit, abcam).

Coimmunoprecipitation

This method is reported in the online-only Data Supplement.

Immunofluorescence

Thoracic aorta segments from all mice were embedded in optimum cutting temperature and stored at −80°C. Slices 5 µm thick were then cut, blocked with 1% BSA for 1 hour, and incubated overnight at 4°C with anti–3 nitrotyrosine (Upstate Biotechnology), CD31 (BD Biosciences), p47phox, Nox2, Nox4, p53, p16INK4a, and JunD antibodies (Santa Cruz Biotechnology, Nunningen, Switzerland). Fluorescence-labeled secondary antibodies (Alexa Fluor 546 and 488, Molecular Probes) were applied for 1 hour. Slides were successively incubated with DAPI solution (Vector Laboratories) for 10 minutes and analyzed by fluorescence microscopy (Olympus). Quantification of immunofluorescence was performed by counting stained cells on total cells.

JunD Promoter Methylation in Mouse Aorta

For each analyzed genomic DNA sample, 30 ng genomic DNA was digested with methylation-sensitive enzyme or methylation-dependent enzyme and with both methylation-sensitive and -dependent enzymes in 15 µL total volume including 5x digestion buffer overnight at 37°C.
Nuclear Extracts and JunD-Binding Activity
Nuclear protein activity and JunD-binding activity were assessed with commercially available kits (Active Motif, Rixensart, Belgium).

In Vivo Knockdown of JunD
In vivo knockdown of JunD was performed by injecting a predesigned siRNA specifically targeting JunD (Santa Cruz Biotechnology), together with a cationic transfection reagent (JetPEI, Polyplus Transfection), as previously reported.24

JunD Overexpression in Mice
Old (22 months) male mice were injected intravenously with either 40 μg cloning vector (Origene, PCMV6-Kan/Neo, PCMV6KN) or a predesigned mouse JunD cDNA clone (Origene, MC200652), together with a cationic transfection reagent (JetPEI), according to the manufacturer’s instructions.

Telomerase Activity
The catalytic activity of telomerase was assessed by quantitative PCR as described previously.24

β-Galactosidase Staining
See the online-only Data Supplement for a description of this method.

Cell Culture
Human aortic endothelial cells (passages 5–8) were cultured in EGM-2 containing 2% FCS for 12 hours and then transfected with JunD, p47phox, Nox2, Nox4, and scrambled siRNAs. After 72 hours, cells were harvested for immunoblotting, ESR measurements, and assessment of NADPH activity.

Real-time PCR
All PCR experiments were performed with the SYBR Green JumpStart kit (Sigma Aldrich). A detailed description of this method, including primer sequences, is given in the online-only Data Supplement.

Measurements of \( \text{O}_2^- \) and NO by ESR Spectroscopy
\( \text{O}_2^- \) and NO levels in intact cells were assessed by ESR spectroscopy analysis as previously described.25

siRNA Transfection
Human aortic endothelial cells were transfected at 70% to 80% confluence with the lipofectamine reagent (Invitrogen, Carlsbad, CA) for 4 hours at 37°C in EBM-2. Commercially available human JunD, p47phox, Nox2, and Nox4 siRNAs were purchased from Santa Cruz Biotechnology. A predesigned scrambled siRNA was used as a control (Microsynth, 5′ UAC ACA CUC UCG UCU C UdTdT 3′).

Subjects
Twelve young (24±3 years of age) and 14 old (65±6 years of age) male healthy volunteers were enrolled consecutively at the Blood Donation Service of the University Hospital Zürich. All of the subjects were nonsmokers, normotensive, and free of overt cardiovascular disease as determined by health history questionnaire and physical examination. All participants signed an informed consent.

Isolation of Peripheral Blood Monocytes
Peripheral blood mononuclear cells were isolated fromuffy coats by density gradient centrifugation over Ficoll-Paque. Blood monocytes were recovered by adherence to 100-mm plastic tissue culture plates (Greiner, Frinckenhausen, Germany) in the presence of 10% FCS for 1 hour at 37°C in 5% CO2/air. Nonadherent cells were removed from monocyte monolayers by washing with PBS. Isolated monocytes were cultured in RPMI medium supplemented with 10% FBS. Finally, cells were collected for real-time PCR.

Statistical Analysis
All data are presented as means±SEM. Statistical comparisons were made with the Student ttest for unpaired data and 1-way ANOVA followed by the Bonferroni post hoc test when appropriate. Spearman correlation analysis was used to assess the correlation between variables. Values of \( P<0.05 \) were considered statistically significant. All analyses were performed with GraphPad Prism Software (version 5.0).

Results

Genetic Deletion of JunD Accelerates Aging-Induced Endothelial Dysfunction
To investigate the role of JunD in vascular aging, we examined endothelium-dependent responses in 6- and 22-month-old JunD−/− and WT mice. Young JunD−/− mice showed an impairment of endothelium-dependent relaxation to acetylcholine (10−8–10−6 mol/L) that was similar to that observed in old WT mice (Figure 1A), suggesting premature endothelial aging in animals lacking the AP-1 transcription factor JunD. Moreover, old JunD−/− mice exhibited a more pronounced impairment of vasorelaxation compared with age-matched WT mice (Figure 1A). The addition of the free radical scavenger polyethylene glycol–superoxide dismutase (150 U/mL) restored acetylcholine-induced relaxation not only in old WT mice but also in JunD−/− mice regardless of age (Figure 1B). Endothelium-independent relaxation to sodium nitroprusside (10−10–10−5 mol/L) was similar in all groups (Figure 1C). These findings indicate a link among JunD deletion, ROS generation, and endothelial dysfunction.

Mitochondrial Derangement and Oxidative Stress in JunD−/+ Mice
Superoxide anion (\( \text{O}_2^- \)) was measured by ESR spectroscopy in mitochondria isolated from mouse aorta to directly assess the oxidative stress burden in the presence or absence of JunD. Mitochondrial \( \text{O}_2^- \) levels were elevated in young animals lacking JunD as in old WT littermates (Figure 2A). \( \text{O}_2^- \) production further increased in 22-month-old JunD−/+ mice (Figure 2A). To investigate the impact of JunD deletion on mitochondrial function, mitochondria isolated from mouse aortas were challenged with calcium overload (150 μmol/L CaCl2), and the rate of mitochondrial swelling was determined by light scattering. A stable absorbance at 520 nm throughout the 20-minute time course was observed in mitochondria from young WT. In contrast, mitochondria from young JunD−/+ mice displayed a significant decrease in absorbance after calcium overload (Figure 2B and Figure 1 in the online-only Data Supplement). Interestingly, young JunD−/+ mice and old WT littermates showed a comparable mitochondrial disruption (Figure 2B). A similar pattern of mitochondrial DNA fragmentation and increased caspase 3 activity was observed (Figure 2C and Figure II in the online-only Data Supplement).

In agreement with increased mitochondrial \( \text{O}_2^- \) generation, NO release from single aortic endothelial cells was markedly decreased in JunD−/+ mice compared with WT controls (Figure 2C). These findings suggest that JunD deletion promotes mitochondrial oxidative stress in endothelial cells, resulting in impaired NO release and decreased endothelial function.
impaired in both young and old JunD−/− mice compared with age-matched WT littermates (Figure 2D). Blunted NO availability was associated with an early reduction of both activating endothelial NO synthase (eNOS) Ser-1177 phosphorylation and total protein in JunD−/− mice (Figure 2E and Figure III in the online-only Data Supplement). Furthermore, ONOO− levels and 3-nitrotyrosine immunostaining were also elevated in young JunD−/− mice to an even higher level than in aged control animals (Figure 2F and 2G).

Aging Downregulates JunD Expression

In agreement with the effects of JunD deletion on endothelium-dependent relaxation, immunofluorescence analysis of the mouse aorta revealed that JunD expression is confined mostly to the vascular endothelium (Figure 3A). To strengthen the link between the AP-1 transcription factor JunD and age-dependent, ROS-mediated endothelial dysfunction, its protein expression was assessed in aortic lysates obtained from young and old WT mice. Interestingly, old mice showed a downregulation of JunD expression compared with young animals (Figure 3B). This finding was confirmed by immunofluorescence analysis showing reduced JunD-specific staining (Figure 3C). As expected, JunD−/− mice did not show any protein signal (Figure 3C).

Then, we investigated whether JunD downregulation was induced at the transcriptional level by epigenetic changes. Quantitative analysis of JunD promoter methylation, an important repressor of gene transcription, showed a significant increase in methylated CpG dinucleotides in old compared with young mice (Figure 3D).

Together with blunted expression, we found that JunD transcriptional activity is reduced in aged vessels (Figure 3E). The tumor suppressor menin, a critical modulator of JunD activity, coprecipitated with JunD only in the aorta of old mice, leading to blunted JunD phosphorylation (Figure 3F and Figure IV in the online-only Data Supplement).

To further elucidate the relevance of aging-induced JunD downregulation, silencing of JunD was performed by intravenously injecting JunD siRNA with a cationic transfection reagent. A predesigned scrambled siRNA, used as a negative control, did not affect JunD expression (Figure VA in the online-only Data Supplement). Interestingly, as observed for JunD−/− mice, siRNA-mediated knockdown of JunD exerted a significant impairment of acetylcholine-induced vasorelaxation in both young and old WT mice (Figure VB in the online-only Data Supplement).
Restoration of JunD Expression Improves Vascular Function in Old Mice

Because JunD deletion is associated with early endothelial dysfunction, we determined whether restoration of its expression exerts protective effects against age-induced, ROS-dependent endothelial dysfunction. In vivo overexpression of JunD was performed by intravenous injection of a predesigned cDNA clone. This approach resulted in a significant overexpression of JunD in the aorta of old mice, whereas injection of cloning vector did not exert any significant effect (Figure VI in the online-only Data Supplement). Interestingly, JunD overexpression improved acetylcholine-dependent relaxation compared with vector-treated mice (Figure 3G). This finding indicates that JunD is critically involved in age-dependent endothelial dysfunction.

JunD Affects the Balance Between ROS-Scavenging and ROS-Generating Enzymes

The expression and activity of manganese superoxide dismutase, extracellular superoxide dismutase, and aldehyde dehydrogenase 2 were decreased in the aorta of JunD−/− mice compared with age-matched littermates (Figure 4A–4C). Similar findings were observed for glutathione peroxidase-1 and xanthine oxidase (Figure VII in the online-only Data Supplement). In contrast, the NADPH oxidase subunits p47phox, Nox2, and Nox4 were already upregulated in young JunD−/− mice and further increased with aging (Figure 5A–5C and Figure VIII in the online-only Data Supplement). Upregulation of NADPH isoforms was confined mainly to the vascular endothelium as assessed by immunofluorescence (Figure 5A–5C). These findings were
supported by a significant increase in NADPH activity and p47phox membrane translocation in JunD−/− mice compared with age-matched littermates (Figure 5D and Figure IX in the online-only Data Supplement). Endothelial superoxide anion generation was consistently elevated in young JunD−/− mice and further increased in old mice (Figure X in the online-only Data Supplement).

To further investigate the link between JunD and NADPH in the vascular endothelium, selective downregulation of JunD was achieved with siRNA technology. Interestingly, pretreatment with the NADPH inhibitor apocynin blunted impairment of acetylcholine-induced relaxation in the aortic rings of JunD siRNA–treated young and old mice and in old WT animals (Figure 5E).

**JunD Deletion Accelerates ROS-Induced Vascular Aging**

Because JunD deletion is associated with an early burst of oxidative stress, we investigated markers of vascular senescence in age-matched WT and JunD−/− mice. Telomerase activity was blunted in young JunD−/− compared with age-matched WT mouse aorta, and no further impairment was observed on aging. This finding indicated a vascular senescence phenotype in young animals lacking JunD (Figure 6A). β-Galactosidase staining further supported the early occurrence of vascular aging in JunD−/− mice (Figure 6B). Accordingly, the expression of aging markers such as tumor suppressor p53 and the cyclin-dependent kinase inhibitor p16INK4a was increased in these mice (Figure 6C and 6D and Figure XI in the online-only Data Supplement).

**Gene Silencing of JunD in Human Endothelial Cells**

To translate the findings observed in JunD−/− mice to the human endothelium, we downregulated JunD in human aortic endothelial cells using siRNA technology. Selective JunD siRNA markedly reduced its expression, whereas scrambled siRNA did not exert any effect (Figure 7A). In line with our findings in the mouse, knockdown of JunD elicited a marked
upregulation of NADPH isoforms and increased enzyme activity (Figure 7A and 7B and Figure XII in the online-only Data Supplement). These findings were associated with $\text{O}_2^-$ overproduction and blunted NO availability as assessed by ESR spectroscopy (Figure 7C and 7D). Of interest, the NO/$\text{O}_2^-$ balance was restored by either NADPH inhibitor apocynin or concomitant knockdown of the NADPH oxidase subunits p47phox and Nox2 subunits (Figure 7C and 7D and Figure XIII in the online-only Data Supplement). In contrast, Nox4 downregulation did not exert any inhibitory effect on superoxide anion generation (Figure XIII in the online-only Data Supplement). In agreement with increased ROS generation, polyethylene glycol–superoxide dismutase blunted $\text{O}_2^-$ and preserved NO availability after silencing of JunD (Figure 7C and 7D). Moreover, we also confirmed that JunD is required for eNOS expression and activation in human endothelial cells. Indeed, eNOS protein expression and Ser1177-activating phosphorylation were significantly reduced by JunD knockdown (Figure 7E).

### Age-Dependent Downregulation of JunD in Old Healthy Subjects

To exploit the role of the JunD gene in human vascular aging, we assessed JunD mRNA expression in peripheral monocytes obtained from young and old human subjects free of overt cardiovascular disease and risk factors. Interestingly, JunD protein and gene expression were significantly reduced in old compared with young individuals (Figure 8A and 8B). Moreover, mRNA levels significantly correlated with gene expression of scavenger manganese superoxide dismutase, extracellular superoxide dismutase (Figure 8C and 8D), and the NADPH p47phox and Nox2 subunits (Figure 8E and 8F). These results suggest that downregulation of JunD may represent an important mechanism of increased oxidative stress burden in human aging.

### Discussion

JunD is emerging as a major gatekeeper against oxidative stress, but its contribution to ROS homeostasis in the vasculature remains unknown. The present study demonstrates for the first time that genetic deletion of JunD is associated with premature endothelial dysfunction and vascular aging via ROS generation. Several lines of evidence support our conclusions. In contrast to young WT mice, age-matched mice lacking JunD showed an early impairment of endothelium-dependent relaxation that was restored by the ROS-scavenging enzyme polyethylene glycol–superoxide dismutase. A marked increase in mitochondrial $\text{O}_2^-$, paralleled by reduced endothelial NO bioavailability and elevated ONOO$^-$ concentrations, was already found in the aorta of young $\text{JunD}^{-/-}$ mice. JunD deletion was associated with downregulation of free radical scavengers and increased expression and activity of ROS-generating NADPH oxidase. JunD expression was reduced in old compared with young WT mice, implying that the protective role of this transcription factor is diminished with aging. Interestingly, overexpression of JunD rescued age-induced endothelial dysfunction. Oxidative stress burden in $\text{JunD}^{-/-}$ mice led to premature vascular senescence. Last but not least, JunD was downregulated in peripheral monocytes of old healthy subjects and correlated with scavenging and oxidant enzymes, suggesting a potential translation of our findings to the clinical context.

The AP-1 transcription factor JunD modulates different target genes involved in proliferation, growth, and survival. Gerald and colleagues previously reported increased ROS generation in immortalized $\text{JunD}^{-/-}$ cells. In contrast, redox signaling involved in tumor angiogenesis was blunted by JunD overexpression. Notably, $\text{JunD}^{-/-}$ mice display reduced life span. In the present study, double immunostaining with the endothelial marker CD31 showed that JunD is expressed in the vascular endothelium. Hence, we investigated whether its genetic deletion was associated with a premature oxidative phenotype in the vasculature.
JunD−/− mice displayed a ROS-dependent impairment of endothelium-dependent relaxation already at a young age. Indeed, the free radical scavenger superoxide dismutase restored endothelial function. In line with these findings, O2− generation, peroxynitrite levels, and 3-nitrotyrosine immunostaining were also prematurely elevated and NO

Figure 5. Western blot and immunofluorescence showing the expression of NADPH subunits (A) p47phox, (B) Nox2, and (C) Nox4 in the 4 experimental groups. Nuclei stained with DAPI (blue; magnification ×10). For each panel, bar graphs show densitometric analysis of Western blot. D, NADPH activity in aortic lysates from young and old wild-type (WT) or JunD−/− mice. E, Maximal relaxation to acetylcholine (Ach; 10−6 mol/L) before and after incubation with the NADPH inhibitor apocynin (100 µmol/L) assessed in aortic rings from young and old WT mice treated with scrambled or JunD siRNA. Results are presented as mean±SEM; n=4 to 6 per group. *P<0.05.

Figure 6. A, Bar graphs show telomerase activity in young and old wild-type (WT) or JunD−/− mice. B, Aortic cross sections showing β-galactosidase–positive cells in the 4 experimental groups (magnification ×10). C and D, Representative Western blots and immunofluorescence showing expression of the senescence markers p53 and p16INK4a in age-matched WT and JunD−/− mice. Nuclei stained with DAPI (blue; magnification ×10). For each panel, bar graphs show densitometric analysis of the Western blot. Results are presented as mean±SEM; n=4 to 5 per group. *P<0.05.
availability was blunted in young mice carrying the genetic disruption of JunD. These mice also showed increased DNA fragmentation, swelling of the mitochondria, and caspase 3 activation, markers of apoptosis. The increase in oxidative stress was associated with impaired balance between ROS-scavenging and -producing enzymes. Expression and activity of extracellular superoxide dismutase, manganese superoxide dismutase, glutathione peroxidase-1, and xanthine oxidase were significantly reduced in knockout mice, suggesting that JunD is essential for their transcription. In addition, the mitochondrial reductase aldehyde dehydrogenase 2 was almost abolished in young JunD−/− mice compared with age-matched littermates. This scavenging enzyme has recently been reported to reduce ischemia/reperfusion injury and to protect against cardiac arrhythmias.28–30 We show that JunD deletion affects aldehyde dehydrogenase 2 expression and activity in mouse aorta and that its downregulation may contribute to the abnormal vascular redox state. Our findings are in line with the established role of AP-1 in the activation of several genes encoding a set of detoxifying defensive proteins.31 In contrast, we found that p47phox, Nox2, and Nox4, key subunits of ROS-generating NADPH oxidase, were upregulated in the endothelium of young JunD−/− mice. Notably, the NADPH inhibitor apocynin was able to improve acetylcholine-induced vasorelaxation in the aorta of WT mice with downregulation of JunD induced by siRNA technology. In this regard, we have recently reported that in vivo delivery of siRNA with a cationic reagent targets the vascular endothelium.19 As observed for JunD−/− mice, silencing of JunD elicited a similar impairment of vasorelaxation. Transient knockdown of the transcription factor allowed us to investigate the effects of gene downregulation more specifically than in the setting of genetic deletion, increasing the physiological relevance of our findings. On the other hand, we also showed that overexpression of JunD blunted endothelial dysfunction in old mice. This latter experiment strongly suggests that modulation of JunD expression plays a role in ROS-mediated vascular dysfunction.

The oxidant-rich milieu observed in young JunD−/− mice was associated with increased NO breakdown, as indicated by elevated ONOO− and protein nitrosylation. However, we also found a decreased expression of eNOS in the aorta of

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**Figure 7.** A, Representative Western blots and densitometric quantification of NADPH subunits after JunD knockdown in human aortic endothelial cells (HAECs). Scrambled siRNA (scr.siRNA) was used as control. B, Bar graphs show NADPH oxidase activity in the presence or absence of JunD silencing. C and D, Electron spin resonance spectroscopy analysis of superoxide anion (O₂⁻) and nitric oxide (NO) production in HAECs in the presence or absence of JunD knockdown, the NADPH inhibitor apocynin, and polyethylene glycol-superoxide dismutase (PEG-SOD). E, Representative Western blots and densitometric quantification of endothelial nitric oxide synthase (eNOS) and activating eNOS Ser1177 phosphorylation (p) after JunD knockdown. Results are presented as mean±SEM; n=6 to 9 per group.
these mice that may contribute to the reduced NO bioavailability. Previous evidence reported that AP-1 is required for eNOS transcription.\textsuperscript{32} Interestingly, our findings suggest that AP-1 complexes lacking JunD may not be able to warrant eNOS gene expression. JunD inactivation also blunted eNOS Ser1177 phosphorylation, in line with the notion that ROS suppress enzyme activity.\textsuperscript{33} In addition, we showed that oxidative stress in \textit{JunD}−/− mice was associated with early features of vascular aging, including reduced telomerase activity, increased \(\beta\)-galactosidase staining, and upregulation of the senescence markers p53 and p16\textsuperscript{INK4a}. Importantly, the extent of vascular senescence observed in young \textit{JunD}−/− mice was similar to that seen in old WT mice, suggesting that JunD deletion accelerates ROS-driven vascular aging. The importance of premature senescence in this setting is strengthened by the notion that cardiovascular disease, in particular myocardial infarction and stroke, exhibits a strong age dependency.\textsuperscript{34}

To test JunD role in the human endothelium, JunD siRNA–mediated knockdown was performed in human aortic endothelial cells. Silencing of JunD increased NADPH activity and ROS generation, whereas concomitant treatment with apocynin or knockdown of p47phox and Nox2 subunits prevented oxidative stress, confirming that JunD also is a critical modulator of NADPH activity in human endothelial cells. In contrast, gene silencing of Nox4 did not abolish superoxide production, in line with the observation that this subunit is involved in \(\text{H}_2\text{O}_2\) generation.\textsuperscript{35} Although Nox4 is upregulated in aging\textsuperscript{36} and Nox4-derived \(\text{H}_2\text{O}_2\) has recently been linked to DNA damage, mitochondrial dysfunction, and senescence phenotypes,\textsuperscript{36–38} other evidence suggests a protective role against vascular ischemic or inflammatory stress.\textsuperscript{39,40} Whether the observed Nox4 upregulation represents a futile compensatory mechanism or contributes to vascular aging in \textit{JunD}−/− mice remains unclear.

Another important finding of the present study is that JunD expression was downregulated in old compared with young WT animals. Because oxidative stress contributes to aging, we speculate that JunD is crucial in maintaining a redox balance in the vasculature, whereas its deletion is associated with premature ROS-mediated endothelial dysfunction. This conclusion is supported by our previous work showing that normalization of ROS prevents age-related vascular dysfunction.\textsuperscript{41} In our effort to elucidate the molecular basis of age-dependent JunD downregulation, we investigated the epigenetic modulation of JunD expression at the transcriptional level. Quantitative analysis of JunD promoter methylation showed a significant hypermethylation of CpG dinucleotides in old compared with young mice. Indeed, DNA methylation is an important repressor of gene transcription in mammals.\textsuperscript{42} Besides epigenetic changes, we found that JunD transcriptional activity was reduced in aortas of aged compared with young mice. In this regard, it was recently shown that the tumor suppressor menin binds JunD, leading to inhibition of its activity.\textsuperscript{43} Accordingly, in our experimental setting, menin coprecipitates with JunD only in aged, not in young, vessels, and this interaction inhibits JunD-activating phosphorylation. Hence, transcriptional and posttranslational modification may explain the decrease in JunD expression and activity in the vasculature of old mice.

Figure 8. A, Box plots show JunD mRNA and (B) representative Western blot with quantification of JunD protein expression in peripheral blood monocytes from young and old healthy subjects. C through F, Gene expression of manganese superoxide dismutase (MnSOD), extracellular superoxide dismutase (ecSOD), p47phox, and Nox2 and relative correlations with JunD transcript. Results are presented as mean±SEM; \(n=12\) to \(14\) per group. TBP indicates TATA-binding protein.
Of note, JunD gene expression was downregulated in peripheral monocytes of old healthy subjects and significantly correlated with ROS-scavenging and -generating enzymes. Although we did not confirm the age-dependent decline of endothelial function in our cohort, several studies have reported a strong correlation between age and endothelial dysfunction.44–46

Conclusions
The present work demonstrates for the first time that JunD is critically involved in age-induced oxidative stress in the endothelium and controls vascular senescence. The strength of our study is the consistent observation of JunD as a protector of endothelial homeostasis in different experimental settings, including knockout mice, human endothelial cells, and healthy individuals. These findings may provide the rationale to pharmacologically modulate JunD expression for the prevention of cardiovascular disease.

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Disclosures
None.

References


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**CLINICAL PERSPECTIVE**

The prevalence of cardiovascular disease is markedly age dependent and a major cause of myocardial infarction and death. A key alteration of aging arteries is the development of endothelial dysfunction, which is associated with a reduction in nitric oxide bioavailability. The primary mechanism of aging-associated endothelial dysfunction is oxidative stress, a state in which generation of reactive oxygen species exceeds the capacity of cellular antioxidant defense systems. Although the accumulation of reactive oxygen species is the most widely accepted cause of vascular aging, a thorough understanding of the molecular mechanisms underlying altered redox signaling in endothelial cells is required. JunD, a member of the activated protein-1 family of transcription factors, is emerging as a major gatekeeper against oxidative stress. However, its contribution to reactive oxygen species homeostasis in the vasculature remains unknown. In the present study, we have shown that JunD is critically involved in age-induced vascular dysfunction. Genetic deletion of JunD was associated with increased mitochondrial oxidative stress, deregulation of scavenger and oxidant enzymes, blunted endothelial nitric oxide synthase activity and vasorelaxation, and early vascular senescence. Interestingly, JunD expression and activity declined with age by means of promoter-related epigenetic changes and interaction with the tumor suppressor menin. Overexpression of JunD rescued age-related endothelial dysfunction. These findings may provide the rationale to pharmacologically target JunD in reactive oxygen species–driven, aging-associated cardiovascular disease.
Deletion of the Activated Protein-1 Transcription Factor JunD Induces Oxidative Stress and Accelerates Age-Related Endothelial Dysfunction


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SUPPLEMENTAL MATERIAL for

Deletion of the AP-1 Transcription Factor JunD
Induces Oxidative Stress and Accelerates Age-Related
Endothelial Dysfunction

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Supplemental Methods

Tissue Harvesting
On the day of the experiment, mice were anesthetized through the intraperitoneal administration of 50 mg/kg sodium pentobarbital and then euthanized. The chest and abdomen were opened with a medial sternotomy. The entire aorta from the heart to the iliac bifurcation was excised and placed immediately in cold modified Krebs-Ringer bicarbonate solution (pH 7.4, 37°C, 95% O2; 5% CO2) of the following composition (mmol/L): NaCl (118.6), KCl (4.7), CaCl2 (2.5), KH2PO4 (1.2), MgSO4 (1.2), NaHCO3 (25.1), glucose (11.1), and calcium EDTA (0.026). The aorta was cleaned from adhering connective tissue under a dissection microscope and immediately used for organ chamber experiments or snap-frozen in liquid nitrogen and stored at -80°C.

Organ chamber experiments
For endothelial function experiments, aortas were cut into 2-3 mm rings which were connected to an isometric force transducer (Multi-Myograph 610M, Danish Myo Technology, Denmark), suspended in an organ chamber filled with 5 mL Krebs-Ringer bicarbonate solution (37°C, pH 7.4), and bubbled with 95% O2, 5% CO2. Isometric tension was recorded continuously. After a 30 minute equilibration period, rings were gradually stretched to the optimal point of their length–tension curve as determined by the contraction in response to potassium chloride (100 mmol/L). Concentration–response curves were obtained in a cumulative fashion.1 Several rings cut from the same artery were studied in parallel. Responses to acetylcholine (Ach, 10⁻⁹ to 10⁻⁶ mol/L, Sigma Aldrich) in the presence or the absence of polyethylene glycol-superoxide dismutase (PEG-SOD, 150 U/mL, Sigma Aldrich) and apocynin (100 µM, Sigma Aldrich) were recorded during submaximal contraction to norepinephrine (NE, 10⁻⁶ mol/L). PEG-SOD
and apocynin were added to the organ chamber 30 and 60 minutes before the assessment of endothelium-dependent relaxation to Ach, respectively. The nitric oxide (NO) donor sodium nitroprusside (SNP, $10^{-10}$ to $10^{-5}$ mol/L, Sigma Aldrich) was added to test endothelium-independent relaxation. Relaxations were expressed as percentages of pre-contraction to norepinephrine.

**Measurements of mitochondrial $O_2^-$ by ESR spectroscopy**

$O_2^-$ generation in mitochondria isolated from mouse aorta was assessed by electron spin resonance (ESR) spectroscopy analysis using the spin trap 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine (CMH). Twenty five µg of isolated mitochondria were resuspended in Krebs Hepes Buffer (KHB, PH 7.35) containing desferoxamine (25µM) and DETC (5µM). $O_2^-$ production was determined by following the oxidation of CP-H to paramagnetic 3-carboxy-proxyl (CP). Signals were quantified by measuring the total amplitude after correction of baseline and subtraction of background signals.

**Measurements of endothelial NO and ONOO$^-$ from mouse aorta**

In situ measurements of NO and ONOO$^-$ were carried out with three electrochemical nanosensors combined into one working unit with a total diameter of 2.0 - 2.5 µm. Their design was based on previously developed and well-characterized chemically modified carbon-fiber technology. Amperometry was performed with a computer-based Gamry VFP600 multichannel potentiostat. A current at the peak potential characteristic for NO (0.65 V) oxidation and ONOO$^-$ (-0.40 V) reduction was directly proportional to the local concentrations of these compounds in the immediate vicinity of the sensor. Linear calibration curves (current vs. concentration) were constructed for each sensor from 10 nmol/L to 2 µmol/L before and after measurements with aliquots of NO and ONOO$^-$ standard solutions, respectively. At a constant distance of the sensors from the surface of the endothelial cell, the reproducibility of measurements is high (5-12%). The consumption
of redox species by nanosensors depends on the area of the electrode (< 0.12 µm²) and the duration time of electrolysis (~5-10 s). For the amperometric measurements used, it varied between 0.04-0.1% of the NO and ONOO⁻ peak concentration. This value is negligible compared with the experimental error. The position of nanosensors (x,y, z coordinates) versus the endothelial cell was established with the help of a computer controlled micromanipulator. In order to establish a constant distance from cells, the module of sensors was lowered until it reached the surface of the cell membrane. After that, the sensors were slowly raised 4 ± 1 µm (z coordinates) from the surface of cells. The sensors were then moved horizontally (x, y coordinates) and positioned above a surface of randomly chosen single endothelial cells in an aortic ring. Acetylcholine (Ach), were then injected with a nanoinjector that was also positioned by a computer controlled-micromanipulator.

**Isolation of mitochondrial and cytosolic fraction**

Thoracic aortas were suspended in the mitochondrial buffer containing 10mM MOPS (pH 7.2), 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 10 µg/mL leupeptin, 10 µg/mL aprotinin, and 0.25 M sucrose, and gently homogenized with a Dounce homogenizer (30 strokes). The homogenate was centrifuged at 750g for 10 minutes at 4°C to remove nuclei and unbroken cells, and the supernatant was centrifuged at 10,000g for 15 minutes. The resultant mitochondrial pellet was resuspended in 40 µL of standard lysis buffer (see Western blot for details) while the supernatant was collected as the cytosolic fraction.

**Mitochondrial DNA damage detection**

Briefly, each 30 ng of total DNA was used in two PCR reactions, one amplifying a short 117-bp product of mtDNA and the other amplifying a long fragment of 10-kb product of mtDNA. The amount of PCR products from the 10-kb amplification reflects the quality of the template mtDNA, whereas the 117-bp amplification serves as an internal control for
template concentration. Primer sequences are shown in Supplemental Table 1. PCR reaction was run with the following conditions: 10-kb PCR, denaturation at 94°C for 15 seconds, annealing, and extension at 68°C for 12 minutes for 20 amplifying cycles, followed by a final 10 minutes extension; 117-bp PCR, 94°C for 30 seconds, 60°C for 45 seconds, 72°C for 45 seconds for 20 cycles, and followed by final extension at 72°C for 10 minutes. mtDNA integrity was determined by calculating the ratios of amount of 10 kb products to that of 117-bp products.

**Western blotting**

Frozen samples of aorta and HAECs were lysed for immunoblotting (120 mmol/L sodium chloride, 50 mmol/L Tris, 20 mmol/L sodium fluoride, 1 mmol/L benzamidine, 1 mmol/L DTT, 1 mmol/L EDTA, 6 mmol/L EGTA, 15 mmol/L sodium pyrophosphate, 0.8 µg/mL leupeptin, 30 mmol/L p-nitrophenyl phosphate, 0.1 mmol/L PMSF, and 1% NP-40) for immunoblotting. Cell debris were removed by centrifugation (12 000 g) for 10 minutes at 4°C. The samples (20 µg) were added with 5X Laemmli’s SDS-PAGE sample buffer (0.35 mol/L Tris-Cl, pH 6.8, 15% SDS, 56.5% glycerol, 0.0075% bromophenol blue), followed by heating at 99°C for 5 minutes, and then subjected to 10% SDS-PAGE gel for electrophoresis. Proteins were then transferred onto Immobilon-P filter papers (Millipore AG, Bedford, MA) with a semidry transfer unit (Hoefer Scientific, San Francisco, CA). The membranes were then blocked with 5% milk in TBS-Tween buffer (0.1% Tween 20; pH 7.5) for 1 hour at room temperature and incubated with JunD, eNOS, GpX1, p47phox, Nox2, Nox4, ALDH2, p16INK4a and p53 antibodies (Santa Cruz Biotechnology, Nunningen, Switzerland); phospho eNOS Ser-1177 (Transduction Laboratories, Lexington, USA); MnSOD and ecSOD (Upstate Biotechnology, USA). Anti-rabbit and anti-mouse secondary antibodies were bought from GE Healthcare (Buckinghamshire, United Kingdom). The immunoreactive bands were detected by an enhanced chemiluminescence.
system (Millipore, Billerica, USA). Related signals were quantified using a Scion image software (Scion Corp, Frederick, USA).

**Detection of membrane translocation of NADPH subunit p47phox**

Segments of thoracic aortas were homogenized in a lysis buffer [50 mmol/L HEPES (pH 7.4), 50 mmol/L NaCl, 1 mmol/L MgCl₂, 2 mmol/L EDTA, 1 mmol/L PMSF, 10 mg/ml leupeptin, 1 mmol/L NaVO₄, and 0.1% Triton X-100] for 5 minutes on ice. Aortic lysates were centrifuged at 15 000 rpm at 4°C for 15 minutes. After the supernatant had been collected as the cytosol fraction, the pellet was resuspended in 1% Triton X-100 in the lysis buffer and centrifuged at 15 000 rpm and 4°C for 15 minutes. The supernatant was then collected as the membrane fraction. Equal amounts (25 μg) of protein from each fraction were subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblotting with anti-p47phox antibody (Santa Cruz Biotechnology, Nunningen, Switzerland).

**Co-immunoprecipitation**

Segments of mouse aorta were lysed in ice-cold lysis buffer containing 50 mmol/L Tris-HCl, pH 7.4, 100 mmol/L NaF, 15 mmol/L Na₄P₂O₇, 1 mmol/L Na₃VO₄, 1% Triton X-100, and 1 mmol/L phenylmethylsulfonyl fluoride. Lysates were centrifuged at 10,000 g to remove insoluble material. For immunoprecipitation, precleared lysates were incubated with anti-menin1 antibody overnight at 4°C. Lysates were precleared by incubation with 50 μL of protein A/G-agarose for 2 h at 4°C with rocking. Agarose beads were pelleted by centrifugation at 1000 g. Immunoprecipitated proteins were eluted from the beads by boiling for 5 min in SDS sample buffer and immunoblotted with anti-JunD antibody (1:1000 dilution). Bound antibody was visualized using an enhanced chemiluminescence system (Millipore, Billerica, USA) after incubation of the blot with peroxidase-conjugated secondary antibody for 1 h.
JunD promoter methylation in mouse aorta

For each analyzed genomic DNA sample, 30 ng of genomic DNA was digested with methylation sensitive enzyme (Ms) or with methylation dependent enzyme (Md) and also with both methylation sensitive and dependent enzymes (Msd) in 15µl total volume including 5X digestion buffer overnight at 37°C. In parallel, 30 ng of each genomic DNA sample was mock-treated (Mo) under identical conditions with the exception that water was substituted for digestion enzyme. Following digestion, enzyme activity was heat inactivated at 65°C for 20 min. A total of 6 ng digested DNA was analyzed by quantitative real-time PCR. A predesigned primer for mouse JunD promoter was obtained from SA Biosciences (Frederick, MD, USA). Standard quantitative PCR cycling conditions were used with a ‘hot’ plate read of 72°C for 1 min. The melt curve of each amplicon was calculated within a temperature gradient from 60 to 95°C at 1°C increments with a 15 s hold time for each read. The cycle number at which the Ms and Md digested sample crossed the threshold was subtracted from the cycle number at which the Mo-treated sample crossed the threshold to determine the ΔCt of the locus. Since Ms/Md digests only DNA including purine-5mC, thereby decreasing the amplifiable copies of loci containing DNA methylation and increasing the Ct relative to the mock-treated sample, increasing ΔCt values reflect increasing levels of local DNA methylation.

Nuclear extracts and JunD binding activity

Nuclear protein was obtained by using a nuclear extraction kit (Active Motif, Rixensart, Belgium). Aorta segments were lysed in hypotonic buffer for 15 min before centrifugation, isolated nuclei were resuspended in a hypertonic buffer, and nuclear protein was extracted by incubation on a rotator for 30 min. Nuclear and cytosolic fractions were collected after centrifugation. The DNA binding reaction was carried out with 5 µg of nuclear protein in a
96-well plate coated with consensus sequences for JunD for 1 h at room temperature. After washing, JunD antibody (Active Motif) was added and incubated for 1 h, followed by incubation with a horseradish peroxidase-conjugated secondary antibody. Finally, JunD DNA binding was assessed spectrophotometrically at 450 nm.

**In vivo knockdown of JunD**

In vivo knockdown of JunD was performed by injecting a predesigned siRNA specifically targeting JunD (Santa Cruz, USA). A scrambled-siRNA was used as a negative control (Microsynth, 5'-UAC ACA CUC UCG UCU CU dTdT-3'). Amount of JunD siRNA was selected based on dose optimization studies (data not shown). The siRNA mix at the final dose of 1.6 mg/kg was incubated with the in vivo-jetPEI delivery reagent (Polyplus Transfection) for 15 min at room temperature and injected i.v in a final volume of 100 μL, as previously reported. Successful knockdown of JunD was assessed by Western blot in aortic lysates. We have previously characterized the distribution of siRNA injected together with the vivo-jetPEI delivery reagent. Labelling of siRNA sequences with a fluorescent tag showed its exclusive distribution to the endothelium within the vessel wall.

**JunD overexpression in mice**

Old male mice (22 months) were injected i.v either with 40μg of cloning vector (Origene, PCMV6-Kan/Neo, PCMV6KN) or a predesigned mouse JunD cDNA clone (Origene, MC200652) together with a cationic transfection reagent (JetPEI, Polyplus Transfection), according to the manufacturer's instructions (http://www.polyplus-transfection.com). I.v. injections were repeated every other day for 1 week and mice harvested for organ chamber experiments, as described in the methods section. Time course studies showed that JunD overexpression was already evident after 24 hours and injections were repeated to warrant a stable overexpression before ex-vivo assessment of endothelial function.
Telomerase activity

The catalytic activity of telomerase was assessed by quantitative PCR, as described previously. Segments of mouse aorta were homogenized in CHAPS buffer and centrifuged at 4°C. Two different protein concentrations, 0.5 μg and 1 μg, were employed to document the specificity of the assay. Lysates were incubated in a solution containing reverse transcriptase reaction mix and Taq polymerase (TRAPEZE RT Telomerase Detection Kit, Chemicon) at 30°C for 30 minutes. Telomerase positive cells were used as positive control while CHAPS buffer in the absence of protein lysates was used as negative control. PCR cycling conditions were as follows: 95°C for 2.0 minutes; 40 cycles of 94°C for 15 seconds; and 59°C for 60 seconds. Data were collected at the 59°C stage of each cycle.

Beta-galactosidase staining

Senescence-associated β-galactosidase (SA-β-gal) activity is detectable using the artificial substrate 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) and allows the identification of senescent cells in vivo. The ability to induce SA-β-gal activity is a manifestation of residual lysosomal activity at suboptimal pH (pH 6). It becomes detectable in the course of senescence because of the increased lysosomal content in senescent cells. Thoracic aorta segments from all the experimental groups were embedded in optimum cutting temperature and stained for β-galactosidase activity. Briefly, aortas were fixed in Fixative Solution (25% glutaraldehyde in PBS/MgCl₂) for 1h and incubated at 37°C for 6h with fresh Staining Solution (0.6 M potassium ferricyanide, 0.6M potassium ferrocyanide, 1 M MgCl₂, 10% Igepal, 10% sodium deoxycholate, 40 mg/ml X-gal solution, PBS). Staining was evident in 6 hours. SA-β-gal positive cells were visualized under an optical microscope (Olympus, 10x magnification).
**Cell culture**

Human aortic endothelial cells (HAECs, passages 5 to 8) were purchased from Clonetics (Allschwil, Switzerland) and grown in fibronectin-coated 75cm² flasks in optimized endothelial growth medium-2 (EGM-2, Clonetics, Walkersville, USA) supplemented with 10% FCS. Cells were detached by using Tripsin/EDTA for 90 seconds and reseeded in fibronectin-coated 3 cm²-cell culture dishes or 12 multiwell plates. For experiments, HAECs were cultured in EGM-2 containing 2% FCS for 12 h and then transfected with JunD, p47phox and scrambled siRNAs. After 72 hours cells were harvested for immunoblotting, ESR measurements and assessment of NADPH activity.

**Real-time PCR**

Total RNA was extracted from human peripheral blood monocytes using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s recommendations. Conversion of total cellular RNA to cDNA was carried out with Moloney murine leukemia virus reverse transcriptase and random hexamer primers (Amersham Biosciences, Piscataway, NJ) in a final volume of 33 μl using 1 μg of RNA. Real time PCR was performed in an MX3000P PCR cycler (Stratagene, Amsterdam, The Netherlands) by using the SYBR Green JumpStart kit (Sigma). Each reaction (25 μl) contained 2 μl cDNA, 10 pmol of each primer, 0.25 μl of internal reference dye and 12.5 μl of JumpStart Taq ReadyMix (containing buffer, dNTPs, stabilizers, SYBR Green, Taq polymerase and JumpStart Taq antibody). Primers for JunD, MnSOD, ecSOD, p47phox, Nox2, Nox4, 117-pb and 10-kb were synthesized by Microsynth and relative sequences are shown in Supplemental Table1. The amplification program consisted of 1 cycle at 95 °C for 10 min, followed by 40 cycles with a denaturing phase at 95 °C for 30 s, an annealing phase at 56 °C for 30 s, and an elongation phase at 72 °C for 30 s. A melting curve analysis was performed after
amplification to verify the accuracy of the amplicon, and PCR products were analyzed on an ethidium bromide stained 1% agarose gel. Data were normalised to results obtained with primers specific for human TBP and mouse GAPDH.

**Measurement of O\textsubscript{2}\textsuperscript{-} and NO in HAECs**

$O_2^-$ generation in intact cells was assessed by electron spin resonance (ESR) spectroscopy analysis using the spin trap 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine (CMH). $O_2^-$ production was determined by following the oxidation of CP-H to paramagnetic 3-carboxy-proxyl (CP).\textsuperscript{7} Endothelial NO release was examined by ESR spectroscopy analysis with the use of the spin-trap colloid Fe(DETC)\textsubscript{2}. Briefly, cells were washed and resuspended in 900 µL of Krebs-HEPES buffer (37°C), 300 µL of colloid Fe(DETC)\textsubscript{2} (final concentration 285 µmol/L) was added to each sample and incubated at 37°C for 60 minutes. Samples were scraped on ice, cells were collected from each sample and tubes were centrifuged at 4°C at 5000 rpm for 10 minutes. Cells were resuspended and aspirated into 1 mL syringes which were frozen immediately in liquid nitrogen.\textsuperscript{5} All ESR spectra relative to $O_2^-$, NO and ONOO\textsuperscript{-} measurements were recorded with the use of NOX-E.5-ESR spectrometer (Bruker, Bremen, Germany). Signals were quantified by measuring the total amplitude after correction of baseline and subtraction of background signals.
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**Supplemental Table 1.** Primers used for real-time PCR
Supplemental Figures

**Supplemental Figure 1.** Bar graphs showing percentage decrease of 520-nm absorbance at 20 minutes in calcium-treated mitochondria isolated from aorta of young and old WT or JunD−/− mice is shown. Results are presented as mean ± SEM; n=4 per group.

**Supplemental Figure 2.** Caspase 3 activity in aortic lysates of young and old WT or JunD−/− mice. Results are presented as mean ± SEM; n=4 per group.
Supplemental Figure 3. Densitometric quantification of activating eNOS Ser1177 phosphorylation and eNOS expression in the 4 experimental groups. Results are presented as mean ± SEM; n=4 per group.

Supplemental Figure 4. Densitometric quantification of activating JunD phosphorylation in young and old WT mice. Results are presented as mean ± SEM; n=4 per group.
Supplemental Figure 5. (A) Representative Western blot showing JunD knockdown in aortic lysates of WT mice receiving scrambled or JunD siRNA i.v. (B) Maximal endothelium-dependent relaxations to Ach in young and old WT mice receiving scrambled or JunD siRNA. Results are presented as mean ± SEM; n=6-7 per group. *p<0.05

Supplemental Figure 6. Representative Western blot and densitometric quantification showing JunD expression in aortic lysates of old WT mice (22 months) treated with vector or predesigned JunD cDNA clone. Results are presented as mean ± SEM; n=7 per group.
Supplemental Figure 7. (A) Representative Western blot and densitometric quantification of GPX1 in age-matched WT and JunD⁻/⁻ mice. (B) Xanthine oxidase activity in aortic lysates from the 4 experimental groups. Results are presented as mean ± SEM; n=4 per group. GPX1, glutathione peroxidase 1.

Supplemental Figure 8. Quantification of immunofluorescence for the NADPH subunits p47phox, Nox2 and Nox4. Analysis was performed by counting positive cells on total cells. Results are presented as mean ± SEM; n=3-4 per group.
**Supplemental Figure 9.** Representative Western blot and densitometric quantification showing membrane translocation of the NADPH subunit p47phox in aortic lysates of age matched WT and JunD\(^{-/-}\) mice. Results are presented as mean ± SEM; n=4 per group.

**Supplemental Figure 10.** Electrochemical analysis of superoxide anion (O\(_2^\cdot\)) in single endothelial cells from aortic rings of young and old WT or JunD\(^{-/-}\) mice. Results are presented as mean ± SEM; n=4 per group.
Supplemental Figure 11. Quantification of immunofluorescence for the aging markers p53 and p16\(^{INK4a}\). Analysis was performed by counting positive cells on total cells. Results are presented as mean ± SEM; n=3-4 per group.

Supplemental Figure 12. Densitometric quantification of p47phox, Nox2 and Nox4 expression following JunD silencing in HAECs. Results are presented as mean ± SEM; n=4-5 per group.
Supplemental Figure 13. (A-C) siRNA-mediated knockdown of p47phox, Nox2 and Nox4 in HAECs. (D) ESR analysis showing superoxide anion generation following JunD knockdown with or without concomitant silencing of NADPH isoforms. Results are presented as mean ± SEM; n=5 per group.
Supplemental References


