C/EBP Homologous Protein-10 (CHOP-10) Limits Postnatal Neovascularization Through Control of Endothelial Nitric Oxide Synthase Gene Expression

Céline Loinard, PhD; Yasmine Zouggari, PhD; Patricia Rueda, PhD; Bhamra Ramkhelawon, PhD; Clément Cochain, PhD; José Vilar, PhD; Alice Récalde, PhD; Adèle Richart, PhD; Dominique Charue, BSc; Micheline Duriez, BSc; Masataka Mori, PhD; Fernando Arenzana-Seisdedos, PhD; Bernard I. Lévy, MD, PhD; Christophe Heymes, PhD; Jean-Sébastien Silvestre, PhD

Background—C/EBP homologous protein-10 (CHOP-10) is a novel developmentally regulated nuclear protein that emerges as a critical transcriptional integrator among pathways regulating differentiation, proliferation, and survival. In the present study, we analyzed the role of CHOP-10 in postnatal neovascularization.

Methods and Results—Ischemia was induced by right femoral artery ligation in wild-type and CHOP-10−/− mice. In capillary structure of skeletal muscle, CHOP-10 mRNA and protein levels were upregulated by ischemia and diabetes mellitus. Angiographic score, capillary density, and foot perfusion were increased in CHOP-10−/− mice compared with wild-type mice. This effect was associated with a reduction in apoptosis and an upregulation of endothelial nitric oxide synthase (eNOS) levels in ischemic legs of CHOP-10−/− mice compared with wild-type mice. In agreement with these results, eNOS mRNA and protein levels were significantly upregulated in CHOP-10 short interfering RNA–transfected human endothelial cells, whereas overexpression of CHOP-10 inhibited basal transcriptional activation of the eNOS promoter. Using a chromatin immunoprecipitation assay, we also showed that CHOP-10 was bound to the eNOS promoter. Interestingly, enhanced postischemic neovascularization in CHOP-10−/− mice was fully blunted in CHOP-10/eNOS double-knockout animals. Finally, we showed that induction of diabetes mellitus is associated with a marked upregulation of CHOP-10 that substantially inhibited postischemic neovascularization.

Conclusions—This study identifies CHOP-10 as an important transcription factor modulating vessel formation and maturation. (Circulation. 2012;125:1014-1026.)

Key Words: angiogenesis • CHOP-10 • diabetes • eNOS • ischemia • vasculogenesis

C/EBP homologous protein-10 (CHOP-10), also known as growth arrest and DNA damage-inducible gene 153 (GADD153), is a member of the CCAAT/enhancer binding proteins (C/EBPs) family of transcriptional factors that regulate cell cycle and apoptosis. CHOP-10 plays important roles in diverse cellular responses, including the regulation of cellular differentiation and proliferation and immune and inflammatory processes and is a significant mediator of apoptosis in response to endoplasmic reticulum (ER) stress.1-3 Indeed, CHOP-10 is expressed at low levels under physiological conditions but is strongly induced at the transcription level in response to major sensor and transducer proteins on the ER membrane such as IRE1, ATF6, and PERK.4 In particular, CHOP-10 has been identified as an ER stress–induced transcription factor that is a significant mediator of β-cell dysfunction. Hence, although CHOP-10−/− mice do not have a readily detectable phenotype under basal conditions,1 β-cells from CHOP-10−/− mice are protected from apoptosis caused by accumulation of a folding-defective mutant of proinsulin or by diabetes mellitus.5,6

Clinical Perspective on p 1026

Interestingly, ischemia or hypoxia has been reported to upregulate CHOP-10 mRNA through hypoxia-inducible factor-1α–independent mechanisms.7 CHOP-10 is markedly induced after bilateral common carotid arteries occlusion.
leading to damaged neurons. Ischemia-associated apoptotic loss of neurons is decreased in CHOP-10−/− mice.8 Furthermore, hypoxia enhances CHOP-10 levels, leading to apoptosis of rat embryonic heart–derived H9c2 cells. The stable or inducible overexpression of CHOP-10 sensitizes the H9c2 cells to apoptotic cell death. In contrast, cells transiently transfected with an antisense for CHOP-10 are more resistant to hypoxia-induced apoptosis than vector control cells.1,9

Although these studies suggest an essential role of CHOP-10 in the control of apoptosis, relatively little is known about the function and significance of CHOP-10 in endothelial cells and vessel growth. Homocysteine causes ER stress and growth arrest in cultured human umbilical vein endothelial cells (HUVECs) through upregulation of CHOP-10.10 CHOP-10 is enhanced in fibroblast growth factor-2–overexpressing murine aortic endothelial cells or by recombinant vascular endothelial growth factor treatment.11 Finally, vascular endothelial growth factor-A (VEGF-A), fibroblast growth factor-2, angiogenin, and interleukin-8 are transcriptionally upregulated in multiple cell lines by various ER stress inducers.12

In the present study, we show that CHOP-10 regulates both apoptosis and endothelial nitric oxide synthase (eNOS) gene expression. In vivo, CHOP-10 deficiency increases eNOS expression and enhances vessel formation and maturation in physiological and pathological models of postischemic neovascularization. Thus, our data suggest an important role for CHOP-10 transcription factor in the regulation of vessel formation in the adult.

Methods

Hindlimb Ischemia

Experiments were conducted according to the French veterinary guidelines and those formulated by the European Community for experimental animal use (L358–86/609/EEC). CHOP-10−/− deficient mice (8 weeks old; weight, 28±3 g) were obtained from Dr Mori.9 Diabetic and nondiabetic 8-week-old wild-type (WT) C57BL/6 mice, CHOP-10−/− deficient mice, eNOS-deficient mice, and CHOP-10/eNOS double-deficient animals underwent surgical ligation of the proximal part of the right femoral artery, as described previously.13 To induce diabetes mellitus, C57BL/6 WT and CHOP-10−/− mice were injected intraperitoneally with 60 mg/kg streptozotocin in saline or sodium citrate buffer (0.05 mol/L, pH 4.5), daily for 5 days, as described previously.13 Mice were also treated with or without the antioxidant N-acetyl-l-cysteine (NAC; 200 mg/kg per day IP). In additional experiments, the superoxide dismutase mimetic 4-hydroxy-TEMPO (TEMPOL, 20 mg/kg per day; Sigma Aldrich) and/or antioxidants directed against CHOP-10 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) and/or antibodies directed against CHOP-10 (1:100; Santa Cruz Biotechnology) and/or fluorescein isothiocyanate/Griffonia Simplicifolia Agglutinin isolectin B4 (1:100; Sigma) for 30 minutes at room temperature. Finally, sections were incubated with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (1:10 000; Sigma) for 10 minutes at room temperature. Cy5 and TRITC-labeled secondary antibodies (1:200; Jackson Immunoresearch) were then used to reveal CHOP-10−/− and eNOS-positive stainings, respectively.

Flow Cytometry Analysis

For analysis of eNOS expression in infiltrating cells, bone marrow mononuclear cells (BM-MNCs) were isolated from femurs and tibias of C57BL/6 WT and CHOP-10−/− deficient mice, stained with carboxyfluorescein succinimidyl ester (CFSE) (10 μmol/L; Sigma Aldrich) for 10 minutes, and intravenously injected into C57BL/6 WT mice with hindlimb ischemia. After 2 days, ischemic quadriceps, gastrocnemius, and tibialis anterior muscles were weighed, minced, and digested in 450 U/mL collagenase I, 125 U/mL collagenase XI, 60 U/mL DNase1, and 60 U/mL hyaluronidase (Sigma Aldrich) for 1 hour at 37°C. All cell suspensions were layered on Histopaque 1083 (Sigma Aldrich) for gradient density centrifugation, and CFSE-positive cells were selected with the use of a FACSaria cell sorter (BD Biosciences).

For analysis of postischemic inflammatory response, 10-week-old C57BL/6 mice underwent medialullar aplasia by total body irradiation (9.5 Gy). Bone marrow cells were then isolated from femurs and tibias of CD45.2 C57BL/6 WT and CD45.2 CHOP-10−/− mice and intravenously injected into irradiated animals. After 8 weeks, mice underwent surgical ligation of the right femoral artery, as described above. Mice were then euthanized 2 days after femoral artery ligation. Ischemic gastrocnemius and tibialis anterior muscles were weighed, minced, and digested as described previously.14 All cell suspensions were layered on Histopaque 1083 (Sigma Aldrich) for gradient density centrifugation. Blood cells and ischemic muscle cells were labeled with fluorescein isothiocyanate–conjugated anti-CD45.1 (BD Biosciences), allophycocyanin-conjugated anti-CD45.2 (BD Biosciences), Pacific Blue–conjugated anti-CD11b (clone: M1/70; eBioscience), R-phycocerythrin–conjugated anti-Ly6G (clone: 1A8; BD Pharmingen), R-phycocerythrin–conjugated anti-NK-1.1 (clone: PK136), biotin anti-Ly6C (clone: AL-21; BD Pharmingen), PerCP streptavidin (BD Pharmingen), R-phycocerythrin-Cy7–conjugated anti-CD3e (BD Pharmingen), and R-phycocerythrin–conjugated anti-CD45R (clone: RA3–6B2; eBioscience). Cells were analyzed by flow cytometry with the use of an LSRII device (Becton Dickinson).

Cell Culture

HUVECs were plated in 6-well plates and incubated at 37°C in a 5% CO2 atmosphere. HUVECs were obtained from Promocell (Heidelberg, Germany) and cultured in endothelial cell basal medium with supplement pack. All experiments were performed between passages 3 and 6.

RNA Interference

CHOP-10–specific short interfering RNA (siRNA) duplexes (siGENOME SMARTpool) were purchased from Dharmacon. Transfection was performed according to the manufacturer’s instructions.
Luciferase Assay

Reporter gene constructs were generated with the use of the vector plasmid pGL3-Basic (Promega), which contains the luciferase gene. pGL3-eNOS-3500 contained a 3.5-kb human eNOS promoter fragment. Transfection of the GADD153 gene into HUVECs was performed with the use of an expression plasmid vector encoding human GADD153 cDNA or the pcDNA3.9 HUVECs were transiently transfected with 2 μg of reporter gene and 2 μg of indicated plasmids with the use of lipofectamine transfection reagent (Invitrogen, Fischer) for 3 hours at 37°C. After 24 hours of incubation, cells were lysed in passive lysis buffer (Promega), and luciferase activity was measured with the Luciferase Assay System (Promega) with a luminometer (Fluoroskan Ascent FL, Labsystems).

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation assay was performed according to the manufacturer’s instructions (Ademtech, Pessac, France). Briefly, cell lysates were incubated with an antibody against CHOP-10 (Santa Cruz Biotecnology) or polyclonal IgG (Jackson ImmunoResearch Laboratories, Inc). The isolated DNA was amplified by polymerase chain reaction (PCR) with primers corresponding to a 465-bp fragment forward: 5’-GGGGCTGGGAGGGGAAGG -3’ and reverse: 5’-CCTGCCCATGTATCCCTGGTG-3’ and to a 243-bp fragment (forward: 5’-GGGGCTGGGAGGGGAAGG GAT-3’ and reverse: 5’GGAGGTTGGCGAAGGTGGACG3’) of the human ENOS promoter.

Analysis of Protein Expression

Tibial anterior muscles from ischemic and nonischemic hindlimbs, aortas, and HUVECs were homogenized in RIPA buffer (Tris-HCl 50 mmol/L, pH 7.4, NaCl 150 mmol/L, EDTA 1 mmol/L, Triton X-100 1%, deoxycholate 1%, sodium dodecyl sulfate 0.1%, with protease and phosphatase inhibitors). BM-MNCs were homogenized in lysis buffer (10 mmol/L Tris-HCl, pH 7.4, 1 mmol/L sodium orthovanadate, sodium dodecyl sulfate 1% with protease and phosphatase inhibitors). Proteins were resolved by 9% or 12% denaturing gel electrophoresis and blotted onto nitrocellulose sheets (Biorad, 0.2 μm). Antibodies against VEGF-A (1:1000; Santa Cruz Biotecnology), eNOS (1:1000; Becton Dickinson), and CHOP-10 (1:1000; Alexis, Cogger) were used for immunoblotting. As a protein loading control, membranes were stripped and incubated with a monoclonal antibody directed against GAPDH (1:10 000; Abscyss) or β-actin (1:10 000; Sigma), and specific chemiluminescent signal was detected as described previously.14

Quantitative Reverse Transcription PCR

cDNA synthesis was performed with QuantiTect Reverse Transcription Kit (Qiagen). PCR was performed on an ABI prism 7700 with the Power SYBR Green PCR Master Mix (Applied Biosystems). Mouse GAPDH was used to normalize sample amplification. The following oligonucleotides (Applied Biosystems) served as primers: GAPDH forward: 5’-CGTCCCGTACAAATAAGTGTGAA-3’, and reverse: 5’-GCCGGTAGTTGGCATATCGGAAACA-3’; eNOS forward: 5’-GGCAGCCTGGAGGATCCAC-3’; and reverse: 5’-GATAGTAGAAAATTGGGCGACCCTA-3’. and reverse: 5’-TTTCGCGTGCTGTCCTTCCCTC-3’.

Isolation and Treatment of BM-MNCs

BM-MNCs were obtained by flushing tibia and femur of diabetic and nondiabetic WT, CHOP-10–null, eNOS-null, and CHOP-10/eNOS–deficient mice. Low-density mononuclear cells were then isolated by centrifugation on a Ficoll gradient, as described previously.15 Five hours after hindlimb ischemia, control animals received intravenous injections of 1×10^6 BM-MNCs. Neovascularization reaction was assessed at day 14 after ischemia, as described above. CHOP-10–deficient BM-MNCs were also treated with or without N^6-nitro-L-arginine methyl ester (L-NAME) (NOS inhibitor, 10−7 mol/L; Sigma, St Quentin Fallavier, France) for 1 hour before injection. The ability of cultured BM-MNCs to differentiate into cells with endothelial phenotype was revealed by dual-positive staining for both AcLDL-Dil and GSA IB-4 (Griffonia Simplicifolia Agglutinin isolectin B4), as described previously.13

Apoptosis

Frozen tissue sections (7 μm) from calf muscle were fixed with 4% paraformaldehyde and incubated with phosphate-buffered saline/bovine serum albumin and Triton 0.1% for 20 minutes at room temperature. Sections were then incubated with the In Situ Cell Death Detection Kit, Fluorescein (Roche Applied Science) for 1 hour at 37°C and with DAPI (Sigma) for 30 minutes at room temperature. Cultured cells were incubated in serum-free medium or in serum-free medium with H2O2 (0.075 mmol/L+FeSO4 0.1 mmol/L) for 30 minutes, washed, and incubated in serum-free medium. Then they were treated with 10 μmol/L homocysteine or serum-free medium for 16 hours. The percentage of positively stained cells nuclei was determined by random counting of 10 fields per dish. The index of apoptosis was determined by counting the number of Hoechst 33342 (1 μg/mL; Sigma)–stained nuclei of cells. Positive controls were obtained after DNase treatment of the cultured cells. Immunofluorescent images were obtained with the use of a DMRP Leica microscope with a JVC color video camera KY-F50 and were counted by coupling to an imaging analysis system (Histolab software, Microvision, France).

NO Production

NO production in BM-MNCs was assessed by measuring intracellular nitration of NO-sensitive fluorochrome 4,5-diaminofluorescein diacetate (DAF-2/DA; Alexis Biochemicals). Briefly, BM-MNCs were incubated with 10 μmol/L DAF-2/DA for 3 hours (37°C). Supernatants were then removed, and cells were washed in DAF-2/DA–free buffer followed by immediate fluorescence-activated cell sorting analysis. A Becton Dickinson FACSCalibur analyzer was used to quantify fluorescence (excitation wavelength, 488 nm; emission wavelength, 530 nm) at the single-cell level, and data were analyzed with the use of Cellquest version 3.3 (Becton Dickinson) software.

Statistical Analysis

Results were expressed as mean±SEM. To accommodate unequal variance, Welch’s ANOVA was used, and comparisons between groups were then performed with Games-Howell’s test when the ANOVA test was statistically significant. When variances were homogeneous, Student t test was used for comparing 2 groups, and 1-way ANOVA was used to compare each parameter when there were ≥3 independent groups. Comparisons between groups were performed with the post hoc Bonferroni t test when the ANOVA test was statistically significant.

Results

Ischemia Upregulates CHOP-10 Levels

We first analyzed the expression of CHOP-10 in the ischemic skeletal muscle. Two days after ischemia, CHOP-10 mRNA and protein levels were significantly increased in ischemic compared with nonischemic calf muscle (Figure 1A through 1C) and returned to basal levels at day 21 (Figure 1A and 1B), suggesting that CHOP-10 may be involved in the acute phase of postischemic vessel growth. Of note, CHOP-10 contents were also increased in the adductor muscle (Figure 1 in the online-only Data Supplement). Moreover, treatment with the oxidative stress inhibitors NAC or TEMPOL abrogated the ischemia-induced CHOP-10 upregulation, highlighting a role for reactive oxygen species in the regulation of CHOP-10 (Figure 1A through 1C). Finally, we showed that CHOP-10 is expressed in ischemic capillaries structure and is colocalized
with eNOS-positive staining and isolectin B4 staining, a specific marker of endothelial cells (Figure 1D).

**CHOP-10–Dependent Regulation of eNOS Expression**
To investigate the role of CHOP-10 in the regulation of angiogenesis, we assessed the effect of CHOP-10 on 2 key proangiogenic factors involved in postischemic neovascularization: VEGF-A and eNOS. Unexpectedly, whereas VEGF-A protein levels were unchanged (Figure II in the online-only Data Supplement), eNOS mRNA and protein levels were increased in ischemic muscle of CHOP-10 knockout mice compared with WT mice as early as day 2 after the onset of ischemia and throughout the experiment (Figure 2A). eNOS mRNA and protein levels were also significantly increased in the aorta of CHOP-10–null animals compared with WT animals (Figure 2B), suggesting that eNOS is a CHOP-10–regulated gene in vivo. Consistent with these results, treatment of cultured HUVECs with homocysteine (5 nmol/L, 4 hours) increased CHOP-10 mRNA levels (P<0.01; Figure 2C and Figure III in the online-only Data Supplement) and downregulated eNOS mRNA and protein contents (Figure 2D and 2E). Interestingly, CHOP-10 siRNA prevented the downregulation of eNOS mRNA and protein levels induced by homocysteine (Figure 2D and 2E). Finally, overexpression of CHOP-10 significantly inhibited the basal transcriptional activation of the eNOS promoter, as assessed by a reporter gene assay with the use of a fragment of the human eNOS gene (Figure 2F). Indeed, promoter analysis revealed that the eNOS promoter contains a conserved optimal CHOP-10–responsive element (CCAAT; http://www.cbrc.jp/research/db/TFSEARCH.html). Chromatin immunoprecipitation assays demonstrated that CHOP-10 bound to the eNOS promoter encompassing this DNA-binding element in position 757 to 1222 bp and 2843 to 3086 bp. However, no binding was detected when immunoprecipitation was performed with the use of IgG, which confirmed the specificity of the CHOP-10/eNOS promoter interaction (Figure 2G).

**CHOP-10 Is a Modulator of Postnatal Neovascularization**
To determine the in vivo significance of these findings, we assessed the role of CHOP-10 in postischemic vessel growth.
Angiographic score was increased by 2-fold in CHOP-10 knockout animals compared with WT mice (Figure 3A). This was confirmed by capillary density evaluation and measurements of foot perfusion and arteriolar number (Figure 3B through 3D). To further support the role of eNOS in these effects, we generated CHOP-10/eNOS double-deficient animals. Interestingly, the proangiogenic effect of CHOP-10 deficiency was fully blunted...
in these animals. In this regard, postischemic vessel growth was similar in CHOP-10/eNOS−deficient animals and eNOS knockout mice (Figure 3). Altogether, these results support the concept that transcriptional repression of eNOS by CHOP-10 might contribute to the antiangiogenic effects of CHOP-10.

Because BM-MNCs have been shown to contribute to postnatal vessel growth, we also assessed the ability of CHOP-10 to control BM-MNC–related effects. WT BM-MNCs expressed CHOP-10 mRNA levels (data not shown). eNOS mRNA and protein levels were increased by 7.4- and 3-fold, respectively, in CHOP-10 knockout BM-MNCs compared with WT BM-MNCs (Figure 4A). This increase was associated with enhanced NO production (Figure 4B). Of interest, eNOS mRNA and protein levels were also upregulated in CFSE-labeled BM-MNCs infiltrating the ischemic leg (Figure 4C). Intravenous administration of CHOP-10 knockout BM-MNCs markedly increased postischemic neovascularization compared with that of WT BM-MNCs. Inhibition of eNOS activity by pretreatment with L-NAME or by using CHOP-10/eNOS double-deficient cells fully abrogated the proangiogenic effect of CHOP-10 knockout BM-MNCs (Figure 4D through 4F).

To support the role of CHOP-10 in endogenous BM-MNC–related effects, we also analyzed postischemic revascularization in WT CD45.1 mice reconstituted with bone marrow isolated from CD45.2 C57BL/6 WT or CD45.2 CHOP-10−deficient mice. After hindlimb ischemia, neovascularization was increased in WT mice reconstituted with CHOP-10 knockout bone marrow compared with WT mice reconstituted with WT bone marrow,
demonstrating that endogenous BM-MNC–derived CHOP-10 is important for basal postischemic neovascularization (Figure 5A). This effect was associated with activation of postischemic inflammatory response. In ischemic muscles, we showed that the numbers of CD45.2-positive cells, monocytes (CD45.2\(^{+}\)/H\(11001^{+}/\)CD11b\(^{+}/\)Ly6G\(^{+}\) cells), and T lymphocytes (CD45.2/CD3\(^{+}\) cells) were higher in WT mice reconstituted with CHOP-10 knockout bone marrow compared with WT mice reconstituted with WT bone marrow (Figure 5B), indicating that the increase in postischemic immunoinflammatory response may also participate in the beneficial effects observed in this context. Similarly, the number of CD45.2-positive cells was higher in the blood of WT mice reconstituted with CHOP-10 knockout bone marrow compared with WT mice reconstituted with WT bone marrow (Figure 5C). In addition, CHOP-10–null BM-MNCs were more resistant to H\(_2\)O\(_2\)- and homocysteine-induced apoptosis through an NO-independent pathway (Figure IV in the online-only Data Supplement). In contrast, the ability of BM-MNCs to differentiate into cells with an endothelial phenotype was unaffected by CHOP-10 deficiency (Figure IV in the online-only Data Supplement).

To determine the critical source of CHOP-10 (ie, bone marrow–derived cells or endothelial cells) in terms of the neovascularization reaction, we also analyzed vessel growth in CHOP-10 knockout mice reconstituted with bone marrow isolated from C57BL/6 WT or CHOP-10–deficient animals. We showed that postischemic revascularization was higher in chimeric WT mice reconstituted with CHOP-10 knockout bone marrow compared with chimeric WT mice reconstituted with WT bone marrow (Figure 5A). Altogether, our results suggest that CHOP-10 may regulate postischemic vessel growth by modulating the angiogenic contributions of both endothelial cells and BM-MNCs to neovascularization.
Figure 5. A, Quantitative evaluation of microangiography and capillary density in ischemic wild-type (WT) or C/EBP homologous protein-10 (CHOP-10)–deficient (CHOP-10 KO) mice lethally irradiated and transplanted with bone marrow mononuclear cells (BM-MNC) isolated from WT or CHOP-10–deficient animals. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01 vs chimeric WT animals transplanted with WT BM-MNC; †P < 0.05 vs chimeric WT mice transplanted with CHOP-10 KO BM-MNC; n = 10.

B, Top, Representative flow cytometry scattergraph of Ly6C-positive cells. Plots are gated on CD11b-positive cells. Ly6C expression was divided into low- and high-positive cells. Number in each quadrant indicates percentages of cells. Bottom, Quantitative and phenotypic analysis of infiltrating CD45.2 BM-MNC isolated from WT or CHOP-10 KO mice transplanted into lethally irradiated CD45.1 WT animals. The number of CD45.2+ (total number of cells); CD45.2+/CD11b+/Ly6G– (monocytes; the granulocyte marker Ly6G discriminates neutrophils from cells expressing monocytic markers); CD45.2+/CD3+ (T lymphocytes); and CD45.2+/B220hi (B lymphocytes) was measured in the ischemic muscle of chimeric CD45.1 WT mice 2 days after the onset of ischemia. Data are presented as mean ± SEM. *P < 0.05 vs...
CHOP-10 Upregulation Participates in Diabetes Mellitus–Induced Impairment of Vessel Formation

To further validate the in vivo relevance of CHOP-10 for postnatal neovascularization, we investigated the effect of CHOP-10 deficiency in diabetes mellitus. Diabetes mellitus–induced reactive oxygen species overproduction impairs postischemic neovascularization. Of interest, CHOP-10 has been shown to be increased by reactive oxygen species and to trigger oxidative stress–induced β-cell apoptosis in diabetes mellitus.

Blood glucose levels were similar in diabetic WT and diabetic CHOP-10 knockout animals (591 versus 595 mg/dL, respectively; n=13).

Diabetes mellitus markedly upregulated CHOP-10 mRNA and protein levels in ischemic calf muscle (Figure 6A and 6B). Interestingly, treatment with the oxidative stress inhibitor NAC abolished the ischemia-induced increase of CHOP-10 in diabetic tissue, suggesting that diabetes mellitus–induced reactive oxygen species overproduction controls CHOP-10 levels (Figure 6A and 6B).
In the ischemic calf, CHOP-10 deletion led to an increase in eNOS mRNA and protein levels in diabetic CHOP-10–deficient mice compared with the diabetic WT group (Figure 6C and 6D). eNOS mRNA and protein levels were also increased in the aorta of diabetic CHOP-10–deficient mice compared with those of WT diabetic animals (Figure 6E and 6F). There was no significant difference in the VEGF contents of WT and CHOP-10–deficient diabetic animals (Figure V in the online-only Data Supplement). CHOP-10 deletion was also associated with a decrease in the number of apoptotic cells at 2 (7.5-fold; P<0.001) and 7 (45-fold; P<0.001) days after the onset of ischemia in diabetic CHOP-10–deficient mice compared with diabetic WT animals (Figure V in the online-only Data Supplement). Interestingly, CHOP-10 deletion–induced changes in eNOS and apoptosis were associated with activation of vessel growth despite the diabetic environment. Hence, angiographic score (3.3-fold; P<0.01; Figure 7A), capillary density (1.8-fold; P<0.001; Figure 7B), and foot perfusion (2.2-fold; P<0.001; Figure 7C) were increased in the diabetic CHOP-10 knockout group compared with the diabetic WT group.

Similarly, the diabetic BM-MNC proangiogenic effect was enhanced by CHOP-10 deficiency. CHOP-10 mRNA and protein levels were increased in diabetic BM-MNCs compared with WT and NAC-treated diabetic BM-MNCs (Figure VI in the online-only Data Supplement). eNOS mRNA (2-fold; P<0.001; Figure VI in the online-only Data Supplement) and protein (2.4-fold; P<0.001; Figure VI in the online-only Data Supplement) levels were increased in BM-MNCs isolated from diabetic CHOP-10 knockout compared with those isolated from diabetic WT mice. This increase in eNOS content was associated with an upregulation of NO production (Figure VI in the online-only Data Supplement). Interestingly, angiographic score (1.8-fold; P<0.05; Figure 8A), capillary density (1.8-fold; P<0.001; Figure 8B), and foot perfusion (1.7-fold; P<0.01; Figure 8C) were increased in ischemic WT mice treated with diabetic CHOP-10 knockout BM-MNCs compared with those receiving diabetic WT BM-MNCs. Furthermore, the number of cells double positive for Dil-LDL and GSA-IB4 lectin was increased by CHOP-10 deficiency (Figure 8D), whereas the number of apoptotic cells was reduced, suggesting that CHOP-10 participates in diabetes mellitus–induced apoptosis and inhibition of BM-MNC differentiation and that a loss of CHOP-10 function activates postnatal vasculogenesis despite the diabetic microenvironment (Figure 8E).

Discussion

Although CHOP-10 transcription factor of the CEBP family may be involved in cell differentiation, growth, and survival, its role in the regulation of postnatal neovascularization has not been defined. This study identifies CHOP-10 as a major modulator of vessel formation and maturation.

The physiological significance of CHOP-10 in the vasculature is validated by the observation that ischemia and diabetes mellitus–induced reactive oxygen species overproduction upregulated CHOP-10 levels and that CHOP-10 deficiency markedly improved posts ischemic vessel growth in control animals but also in a pathological setting, such as diabetes mellitus. Of note, CHOP-10 is only 1 of multiple mechanisms that contribute to diabetes mellitus–induced impairment of posts ischemic vessel growth.13,16

Our data demonstrated that the transcriptional repression of eNOS by CHOP-10 greatly contributes to the antiangiogenic effects of CHOP-10. Indeed, CHOP-10 is a negative modulator of the activity of C/EBP-like proteins, and C/EBP response elements have been shown in eNOS gene promoter.17 As such, knockdown of CHOP-10 gene expression with the use of specific siRNA induced a marked increase in eNOS mRNA and protein levels in homocysteine-treated endothelial cells. Moreover, eNOS expression was enhanced in aorta and ischemic muscle of CHOP-10–deficient mice, indicating that eNOS is a CHOP-10–regulated gene in vivo. Evidence for a direct effect of CHOP-10 on eNOS transcription was obtained by chromatin immunoprecipitation and the
observation that overexpression of a gain of function of CHOP-10 significantly inhibited basal promoter activity of a reporter gene driven by the human eNOS promoter. Finally, activation of vessel growth associated with CHOP-10 deficiency was fully abrogated in mice lacking eNOS.

Endothelial-derived NO is essential for blood vessel growth after arterial occlusion mediating endothelial cell growth,18 migration,19 vascular remodeling,20 angiogenesis,18,21 vasodilation,22 and bone marrow–derived cell-related functions.13,23 Hence, activation of eNOS-related signaling induced by CHOP-10 deficiency may trigger numerous pathways required for postnatal angiogenesis and vasculogenesis. Although there is some in vitro evidence indicating that NO may upregulate VEGF gene expression in various cell types,24–26 CHOP-10 knockout mice, despite eNOS upregulation, have normal levels of VEGF. Similarly, basal and time-dependent VEGF expressions in ischemic muscles do not differ between eNOS-overexpressing and WT mice.27 The reason for this discrepancy remains enigmatic but may underscore the multiple cellular and molecular mechanisms involved in the regulation of VEGF in the ischemic tissues.

In addition, it is likely that CHOP-10 controls several other target genes implicated in vessel formation and stabilization, such as those modulating the apoptotic pathway. Indeed, CHOP-10 has been shown to mediate ER stress–induced cell death through downregulation of Bcl2 and enhanced oxidant injury.3 In this regard, homocysteine- and H2O2-induced BM-MNC death, as well as the number of apoptotic cells in ischemic tissue, was reduced in CHOP-10 knockout mice. It is noteworthy that eNOS deficiency did not affect the number

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**Figure 8.** Quantitative evaluation of microangiography (A), capillary density (B), and foot perfusion (C) in ischemic wild-type (WT) mice receiving bone marrow mononuclear cells (BM-MNC) isolated from WT, diabetic WT (Diab WT), and diabetic C/EBP homologous protein-10 (CHOP-10) knockout (KO) (Diab CHOP-10 KO) mice 14 days after ischemia. Results are expressed as a ratio of ischemic (Isch) to nonischemic (N.Isch) legs. Data are presented as mean ± SEM. With 3 possible comparisons for Bonferroni correction, *P < 0.016 was considered significant. **P < 0.0033 vs mice treated with WT BM-MNC; †P < 0.016, ‡P < 0.0033 vs mice treated with diabetic WT BM-MNC; n = 9. D, Quantification of AcLDL-Dil- and BS-1 lectin–positive cells of cultured BM-MNC isolated from WT, diabetic WT, and diabetic CHOP-10 KO animals. With 3 possible comparisons for Bonferroni correction, *P < 0.016 was considered significant. **P < 0.0033 vs mice treated with WT BM-MNC; †P < 0.016 vs mice treated with diabetic WT BM-MNC. E, Number of cells positive for both Hoechst and terminal deoxyribonucleotidyl transferase dUTP nick end labeling (TUNEL) staining after treatment of BM-MNC with H2O2 (0.075 mmol/L) or homocysteine (HOMO) (10 nmol/L). CONT indicates control. Data are presented as mean ± SEM. With 3 possible comparisons for Bonferroni correction, *P < 0.016 vs WT BM-MNC; †P < 0.016 vs diabetic WT BM-MNC. n = 9.
of apoptotic cells, suggesting that CHOP-10 controls cell death through an eNOS-independent pathway.

This study also highlights the role of ER stress in adult neovascularization. The ER has emerged as a major site of cellular homeostasis, particularly for the unfolded protein response, which has been shown to play a major role in cancer and many other diseases. Under various conditions such as disturbance of Ca\(^{2+}\) homeostasis, hypoxia, hypoglycemia, and treatment with various agents, ER functions are disturbed and ER stress is induced. For example, the inducible ER chaperone GRP78/BiP, which is expressed at high levels in a variety of tumors and confers drug resistance in both proliferating and dormant cancer cells.\(^{29}\) Hence, activation of CHOP-10 in the setting of ischemia and its angiostatic effect may be part of the evolutionarily conserved mechanism that activates both proapoptotic and survival pathways to allow eukaryotic cells to adapt to ER stress.

In both genetic and diet-induced models of insulin resistance, CHOP deficiency improves glycemic control, expands β-cell mass, and promotes cell survival.\(^6\) Interestingly, mice deficient for VEGF-A in pancreatic islets have reduced insulin gene expression levels and impaired glucose tolerance.\(^{30,31}\) In this regard, the capillary network is essential for fine-tuning blood glucose regulation,\(^{32}\) suggesting that the angiostatic potential of CHOP-10 may participate in β-cell dysfunction under conditions of increased insulin demand. It is noteworthy that in our experimental conditions, the effect of CHOP-10 deficiency on postnatal vessel growth is not associated with changes in blood glucose levels.

In conclusion, this work suggests that CHOP-10 functions as a major regulator of postnatal neovascularization through regulation of eNOS-related signaling.

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Disclosures

None.

References


**CLINICAL PERSPECTIVE**

Understanding the molecular and cellular mechanisms involved in the regulation of vessel growth and maturation in the setting of tissue ischemia is of major importance and may trigger the development of innovative strategies of therapeutic angiogenesis in the treatment of ischemic diseases. In the present study, we analyzed the role of C/EBP homologous protein-10 (CHOP-10) in postischemic revascularization. CHOP-10 is a novel developmentally regulated nuclear protein that emerges as a critical transcriptional integrator among pathways regulating differentiation, proliferation, and survival. This study identifies CHOP-10 as a major modulator of vessel formation and maturation. The physiological significance of CHOP-10 in the vasculature is validated by the observation that ischemia and diabetes mellitus–induced oxidative stress upregulated CHOP-10 levels and that CHOP-10 deficiency markedly improved postischemic vessel growth in control animals but also in a pathological setting, such as diabetes mellitus. Finally, our data demonstrated that the transcriptional repression of endothelial nitric oxide synthase by CHOP-10 greatly contributes to the antiangiogenic effects of CHOP-10.

In conclusion, this work suggests that CHOP-10 functions as an important regulator of postnatal neovascularization. Because downregulation of CHOP-10 promotes neovascularization, the regulation of CHOP-10 expression and activity may pave the way for new therapeutic strategies designed to increase vessel growth in the setting of ischemia. In contrast, forced expression or activation of CHOP-10 might limit unwanted neovascularization associated with tumor development or retinopathy.
C/EBP Homologous Protein-10 (CHOP-10) Limits Postnatal Neovascularization Through Control of Endothelial Nitric Oxide Synthase Gene Expression

Céline Loinard, Yasmine Zouggari, Patricia Rueda, Bhama Ramkhelawon, Clément Cochain, José Vilar, Alice Récalde, Adèle Richart, Dominique Charue, Micheline Duriez, Masataka Mori, Fernando Arenzana-Seisdedos, Bernard I. Lévy, Christophe Heymes and Jean-Sébastien Silvestre

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Figure 1. (A) Quantification of CHOP-10 mRNA (A) and protein (B) levels in the ischemic (WT I) and non ischemic (WT NI) adductor muscle of WT mice. Results were expressed as a percentage of non ischemic muscle at day 0. *p<0.05, **p<0.01 versus non ischemic muscle at day 0. n=8 per group.
Figure 2. (A) Quantification of VEGF-A protein levels in calf muscle of WT, CHOP-10 KO and eNOS/CHOP-10 KO mice. Results were expressed as a ratio of ischemic to non-ischemic legs. *p<0.05 versus wild-type mice. n=8 per group. (B) Quantitative evaluation of the number of apoptotic cells in ischemic calf muscle of WT and CHOP-10 KO mice, 2 days after ischemia. 3 possible comparisons, for Bonferroni correction, p<0.016 was considered significant. *p<0.016 versus wild-type mice. n=8 per group.
Figure 3. Quantification of mRNA (A) and protein (B) levels of CHOP-10 in cultured HUVEC treated with homocysteine (5nM) for 4, 8 or 16 hours. 6 possible comparisons, for Bonferroni correction, a value of $p<0.008$ was considered significant. *$p<0.008$, **$p<0.0016$ versus HUVEC wild-type; †$p<0.008$, ††$p<0.0016$ versus HUVEC 4 hours. n=10 per group.
Figure 4. Wild-type mice were reconstituted with bone marrow from wild-type or CHOP-10 KO animals. 8 weeks after bone marrow transplantation, inflammatory cells number was evaluated in blood of mice without hindlimb ischemia. Upper, representative flow cytometry scattergraph, percentages were derived from cells positive for CD3 and B220. Lower, Quantitative analysis of the number of CD45 (total number of cells) ; CD45/CD11b+/Ly6G- (monocytes) ; CD45/CD3+ (T lymphocytes) and CD45/ B220High (B Lymphocytes) in the blood of chimeric WT mice. n=5 per group.
Figure 5 (A) Number of cultured BM-MNC positive for both Hoescht and TUNEL stainings after treatment with H2O2 or homocysteine (HOMO). BM-MNC were isolated from wild-type (WT), CHOP-10-deficient (CHOP-10 KO) or eNOS/CHOP-10 double deficient mice. (B) Quantification of AcLDL-Dil and BS-1 lectin-positive cells derived from WT, CHOP-10 KO and eNOS/CHOP-10 double KO BM-MNC. Data are presented as mean ± SEM. 3 possible comparisons, for Bonferroni correction, p<0.016 was considered significant. *p<0.016 versus WT BM-MNC; †p<0.016 versus CHOP-10 KO BM-MNC. n=7 per group.
Figure 6. (A) Quantification of VEGF-A protein levels in calf muscle of wild-type (WT), diabetic WT (Diab WT) and diabetic CHOP-10 KO (Diab CHOP-10 KO) mice. Data are presented as mean ± SEM. *p<0.05 versus WT, $p<0.05 versus Diab WT. (B) Quantitative evaluation of the number of apoptotic cells in ischemic calf muscle and in calf muscle of diabetic WT (Diab WT) and diabetic CHOP-10 KO (Diab CHOP-10KO) mice, 2 and 7 days after ischemia. Nd indicates not detected. Data are presented as mean ± SEM. 6 possible comparisons, for Bonferroni correction, a value of p<0.008 was considered significant . *p<0.008 versus diabetic wild-type mice. n=8 per group.
Figure 7. (A) Quantification of CHOP-10 mRNA and protein levels in BM-MNC of wild-type (WT), diabetic WT (Diab WT) and diabetic CHOP-10 KO mice (Diab CHOP-10KO). (B) Quantification of eNOS mRNA and protein levels and NO production in BM-MNC of wild-type (WT), diabetic WT (Diab WT) and diabetic CHOP-10 KO mice (Diab CHOP-10KO). Data are presented as mean ± SEM. 3 possible comparisons, for Bonferroni correction, a value of p<0.016 was considered significant *p<0.016, **p<0.002 versus WT; †p<0.016, ††p<0.002 versus diabetic WT. n= 7.