Involvement of Endoplasmic Stress Protein C/EBP Homologous Protein in Arteriosclerosis Acceleration With Augmented Biological Stress Responses

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Background—The processes of arteriosclerosis, including atherosclerosis and vascular remodeling, are affected by interactions among numerous biological pathways such as responses to inflammation, oxidative stress, and endoplasmic reticulum stress. C/EBP homologous protein (CHOP), which is well known to induce cellular apoptosis in response to severe endoplasmic reticulum stress, is reportedly upregulated in plaque lesions.

Methods and Results—We examined the effects of CHOP deficiency on 2 types of arteriosclerosis: cuff injury–induced neointimal formation and hypercholesterolemia-induced atherosclerosis. Cuff injury–induced neointimal formation was markedly inhibited in CHOP<sup>−/−</sup> mice with suppressed aortic expression of inflammatory factors and smooth muscle cell proliferation–related proteins. A CHOP deficiency also inhibited aortic plaque formation in hypercholesterolemic apolipoprotein E<sup>−/−</sup> mice with suppressed aortic expression of inflammatory factors and oxidative stress markers. Bone marrow transplantation experiments revealed that recipient CHOP deficiency significantly suppressed both cuff injury–induced neointimal formation and hypercholesterolemia-induced atherosclerotic plaque formation to a greater extent than donor CHOP deficiency, suggesting the importance of CHOP in vascular cells for arteriosclerosis progression. Furthermore, in our in vitro experiments, in not only macrophages but also endothelial and smooth muscle cell lines, endoplasmic reticulum stress inducers upregulated inflammation-, adhesion-, or smooth muscle cell proliferation–related proteins, whereas decreased CHOP expression remarkably suppressed endoplasmic reticulum stress–induced upregulation of these proteins.

Conclusions—In addition to the well-known signaling for apoptosis induction, CHOP may play important roles in augmenting potentially pathological biological stress responses. This noncanonical role of CHOP, especially that expressed in vascular cells, may contribute to the progression of vascular remodeling and atherosclerosis. (Circulation. 2011;124:830-839.)

Key Words: atherosclerosis ▪ inflammation ▪ remodeling ▪ stress, physiological ▪ transcription factor CHOP

The mechanisms underlying the pathogenesis of arteriosclerosis such as vascular remodeling and atherosclerosis development are extremely complex and are affected by interactions among numerous biological pathways, including those of inflammation,<sup>1</sup> metabolic disorders,<sup>2</sup> and oxidative stress.<sup>3</sup> Oxidized low-density lipoprotein (LDL) is proposed to play a central role in hypercholesterolemia-induced atherosclerotic development,<sup>4</sup> not only through its incorporation into macrophages to form foam cells but also through the promotion of inflammatory cytokine release and oxidative stress in vascular walls.<sup>5</sup> In addition to inflammation and oxidative stress, several lines of evidence have suggested exacerbation of endoplasmic reticulum (ER) stress in atherosclerotic lesions.

Clinical Perspective on p 839

The ER is an organelle that has essential roles in multiple cellular processes required for cell survival and normal cellular functions. Various disturbances, including ischemia, hypoxia, oxidative injury, and viral infections, trigger protein unfolding in the ER, leading to unfolded protein responses (UPRs), also known as ER stress responses.<sup>6</sup> Recent studies have revealed that ER stress is associated with a wide range of diseases, including neurodegenerative disorders,<sup>7</sup> cancer,<sup>8</sup> and diabetes.
mellitus. In terms of atherosclerosis development, ER stress is reportedly increased in atherosclerotic lesions of apolipoprotein E (apoE)–deficient mice. Free cholesterol incorporated into macrophages induces ER stress, leading to macrophage apoptosis. In addition, ER stress is involved in oxidized LDL–induced endothelial dysfunction. Endoplasmic reticulum stress in the vascular wall may exacerbate oxidative stress and inflammation. In human subjects, ER stress in atherosclerotic plaques could be associated with acute coronary syndrome. Collectively, these findings suggest that ER stress plays important roles in atherosclerosis development.

The UPR is an adaptive response that first tends to restore ER activity and cellular homeostasis but switches toward apoptosis when ER stress is severe or prolonged. The transcription factor C/EBP homologous protein (CHOP; also known as GADD153) is a downstream component of ER stress pathways and induces cellular apoptosis. It is highly expressed in atherosclerotic plaque lesions. Thorp and colleagues reported that CHOP deficiency suppressed atherosclerotic progression in apoE–/– mice via a mechanism involving decreased atherosclerotic plaque necrosis and lesional apoptosis of macrophages. In addition to induction of apoptosis, CHOP can exacerbate ER stress by inducing the expression of genes encoding ER client proteins such as GADD34 and by rendering the ER more oxidative by inducing expression of ER oxidase 1 (ERO1α). These findings prompted us to hypothesize that CHOP is a key molecule linking several biological stress responses and thereby accelerates atherosclerosis development. Therefore, we analyzed the roles of CHOP, especially that expressed in vascular cells, in these biological responses and the resultant atherosclerotic processes such as vascular remodeling and atherosclerosis.

Methods

Animals

Animal studies were conducted in accordance with the institutional guidelines for animal experiments at Tohoku University. The CHOP–/– mice were backcrossed for at least 8 generations with C57BL/6J mice (Nippon CLEA, Shizuoka, Japan). The CHOP–/– mice were mated with female apoE–/– mice (The Jackson Laboratory, Bar Harbor, ME) to establish a line of CHOP–/–/apoE–/– mice. To accelerate the development of atherosclerosis, these mice were fed a 1.25% high-cholesterol diet from 8 weeks of age.

Plasma Metabolic Parameters

Blood glucose, plasma adiponectin, and tumor necrosis factor-α levels and serum total cholesterol, triglyceride, and free fatty acid concentrations were determined as described previously. Plasma levels of monocyte chemoattractant protein-1 (MCP-1), malondialdehyde, and 8-isoprostane were measured with an ELISA kit (R&D Systems, Inc), a TBARS Assay kit (Cayman Chemical Systems, Minneapolis, MN), an 8-isoprostane enzyme immunoassay kit (Cayman Chemical Company, Ann Arbor, MI), and an 8-isoprostane enzyme immunoassay kit (Cayman Chemical Company), respectively.

Blood Pressure Determinations

Systolic blood pressures were measured over several days in conscious mice with a tail-cuff system (Muromachikai Co, Kyoto, Japan) according to the manufacturer’s instructions. Five to 8 measurements were recorded for each mouse. Results are presented as the mean of measurements on consecutive days.

Cuff Injury of Femoral Arteries

Polyethylene PE-50 tubes (2 mm; inner diameter, 0.58 mm; outer diameter, 0.965 mm; Becton Dickinson, Franklin Lakes, NJ) were placed around the right femoral arteries of 8-week-old mice as described previously. The contralateral artery served as an uninjured control. Vessels were isolated and processed for histological analyses 3 weeks after cuff placement. The middle segment of the artery was cut at 50-μm intervals, and the thicknesses of the intima and media were measured.

Quantitative Reverse-Transcription Polymerase Chain Reaction-Based Gene Expression

Quantitative reverse-transcription polymerase chain reaction was performed as previously described. The relative amount of mRNA was calculated with a-actin mRNA for cuff injury experiments or with GAPDH mRNA for the apoE–/– mouse and in vitro experiments as the invariant control. The oligonucleotide primers are described in Table I in the online-only Data Supplement.

Immunoblotting

Immunoblot analyses were performed as previously described using antibodies to Bip (GRP78) (Santa Cruz Biotechnology, Santa Cruz, CA), mouse class A type I scavenger receptor (R&D Systems, Inc), and GAPDH (Ambion, Austin, TX).

Evaluation of Atherosclerotic Lesions

The atherosclerotic lesions were evaluated as Oil Red O–stained areas, as described previously. The aortas were removed, cleaned, cut open with the luminal surface facing up, and then immersion fixed in 10% formalin in PBS. The inner aortic surfaces were stained with Oil Red O for 30 minutes at room temperature. Quantification was performed as previously described. The relative amount of mRNA was calculated with a-actin mRNA for cuff injury experiments or with GAPDH mRNA for the apoE–/– mouse and in vitro experiments as the invariant control. The oligonucleotide primers are described in Table I in the online-only Data Supplement.

Tissue Collection and Immunohistochemical Staining

Aortas were snap-frozen in liquid nitrogen, stored at −80°C for protein and gene expression studies, and then embedded in optimum cutting temperature compound. The proximal artery was cut into subserial 6-μm cross sections and stained with antibodies to oxidized LDL (Calbiochem, San Diego, CA), MOMA-2 (Serotec, Oxford, UK), α-smooth muscle actin (Progen, Heidelberg, Germany), and proliferating cell nuclear antigen (Santa Cruz Biotechnology).

Irradiation and Bone Marrow Transplantation

The CHOP–/– and CHOP–/– recipient mice underwent lethal irradiation (10 Gy) at 6 weeks of age and then received 4×10^6 freshly prepared sterile bone marrow cells obtained from CHOP–/– and CHOP–/– mice via tail vein injection. The expression of CHOP in bone marrow cells was quantified by reverse-transcription polymerase chain reaction 2 weeks after bone marrow transplantation (BMT). The peripheral cuff was placed around the right femoral artery of each mouse 2 weeks after BMT. The mice were fed standard chow for 2 weeks after BMT and then a 1.25% high-cholesterol diet for the following 12 weeks.

Ex Vivo Treatment of Macrophages

Peritoneal macrophages were harvested from lavage of CHOP–/– and CHOP–/– mice 4 days after intraperitoneal injection of 4% thioglycollate. Macrophages were cultured in RPMI 1640 containing 10% FBS. Two hours later, nonadherent cells were flushed out and fresh medium was added. After 24 hours, the cells were incubated
with 0.25 μmol/L thapsigargin (Sigma-Aldrich Inc, St. Louis, MO) for 4 hours, 1 mmol/L peroxynitrite (Cayman Chemical Co) for 3 hours, or 100 g/mL acetyl-LDL (Biomedical Technologies Inc, Stoughton, MA) plus the acyl-CoA:cholesterol acyltransferase inhibitor CI976 (Sigma-Aldrich Inc; 10 μmol/L) for 5 hours.

Cell Culture and siRNA Transfection

Mouse endothelial MSS31 cells, provided by Dr Yasufumi Sato (Tohoku University), and human aortic smooth muscle cells (SMCs; KURABO, Osaka, Japan) were cultured in minimum essential medium containing 5% FBS and Humedia-SG2 (KURABO), respectively. Human and mouse CHOP small interfering RNAs (siRNAs) and control siRNAs were designed in conjunction with Thermo Fisher Scientific Inc (Yokohama, Japan). siRNAs and the transfection reagent DharmaFECT (Thermo Fisher scientific Inc, Yokohama, Japan) were added to the medium, followed by incubation for 24 hours at 37°C. siRNA-transfected human aortic SMCs and MSS31 were incubated with thapsigargin (0.75 μmol/L) and tunicamycin (2.5 μg/mL), respectively, for 10 hours.

Statistical Analysis

All statistical analyses were performed with the SPSS version 15.0 (SPSS Japan Inc, Tokyo, Japan). All data were tested for normality by the Kolmogorov-Smirnov test. When data were normally distributed, the statistical significance of differences was assessed with the unpaired t test and 1-way ANOVA, followed by Tukey post hoc analyses. When data were not normally distributed, the statistical significance of differences was judged on the basis of P values with the Mann–Whitney U test.

Results

C/EBP Homologous Protein Deficiency Suppresses Neointimal Formation Induced by Cuff Injury

The CHOP+/+ and CHOP–/– mice were fed normal chow and comparatively analyzed at 11 weeks of age. As reported previously,29 CHOP deletion did not significantly affect overall body weight, blood glucose, blood pressure, or plasma lipid parameters (Figure IA through ID in the online-only Data Supplement). Although plasma adiponectin levels were slightly higher in CHOP–/– mice, no significant differences were observed in serum MCP-1 or tumor necrosis factor-α levels between CHOP+/+ and CHOP–/– mice (Figure IE in the online-only Data Supplement).

First, to assess the role of CHOP in vascular remodeling, we evaluated the effects of CHOP deficiency on neointimal formation induced by cuff injury. There were no apparent histological differences between uninjured femoral arteries in CHOP+/+ and CHOP–/– mice (Figure 1A). In contrast, in
cuff-injured femoral arteries, CHOP−/− mice exhibited significant suppression of intimal hyperplasia (Figure 1A). The intimal hyperplasia was also quantified as intimal-to-luminal and intimal-to-medi-al area ratios, which were decreased by 56.4% and 56.0%, respectively, in CHOP−/− mice compared with CHOP+/+ mice (Figure 1B).

Arterial wall intimal hyperplasia is reportedly attributable to heightened inflammatory reactions induced by interactions between recruited leukocytes and migrating SMCs. Therefore, we next examined the local expression of genes related to vascular remodeling in cuff-injured femoral arteries 7 days after cuff placement. First, CHOP was markedly upregulated by cuff injury in CHOP+/+ mice (Figure II in the online-only Data Supplement). In uninjured femoral arteries, there were no significant differences in the expression of inflammatory factors or vascular SMC proliferation–related factors between CHOP+/+ and CHOP−/− mice (Figure 1C and 1D). In contrast, in cuff-injured femoral arteries, expression of inflammatory factors such as interleukin (IL)-1β, IL-6, and MCP-1 was significantly suppressed in CHOP−/− mice compared with CHOP+/+ mice (Figure 1C). In addition, CHOP deficiency inhibited increased expression of vascular SMC proliferation–related factors such as stromal cell-derived factor-1α (also known as CXCL12), early growth response protein-1, and platelet-derived growth factor-B (Figure 1D). As a result, cuff injury–induced expression of α-smooth muscle actin was markedly suppressed in the arteries of CHOP−/− mice (Figure IIIA in the online-only Data Supplement). Immunostaining with antibodies against α-smooth muscle actin revealed that CHOP deficiency decreased α-smooth muscle actin staining in the neointima of cuff-injured arteries (Figure IIIB in the online-only Data Supplement). Furthermore, proliferating cell nuclear antigen–positive cells in the neointima were significantly decreased in CHOP−/− mice (Figure IIIC in the online-only Data Supplement). Taken together, these findings suggest that CHOP exacerbates cuff injury–induced inflammation and SMC proliferation, leading to vascular remodeling.

C/EBP Homologous Protein Deficiency Suppressed Hypercholesterolemia-Induced Atherosclerosis in ApoE−/− Mice

Next, to examine whether CHOP is involved in hypercholesterolemia-induced atherosclerosis, CHOP−/− mice were crossed with apoE-deficient mice, resulting in the generation of CHOP+/−;apoE−/− double-knockout mice. The CHOP+/+;apoE−/− and CHOP−/−;apoE−/− mice were fed a 1.25% high-cholesterol diet starting at 8 weeks of age. In this hypercholesterolemia model, body weight, blood glucose, blood pressure, and lipid parameters were similar in CHOP+/+;apoE−/− and CHOP−/−;apoE−/− mice (Figure IV in the online-only Data Supplement). Interestingly, atherosclerosis development was significantly inhibited by CHOP deficiency. The atherosclerotic areas were decreased by 31% in the whole aortas of CHOP−/−;apoE−/− compared with those of CHOP+/+;apoE−/− mice at 32 weeks of age, as defined by Oil Red O staining (Figure 2A). Histological analyses of aortic root cross sections of CHOP+/+;apoE−/− and CHOP−/−;apoE−/− (n=5, each) mice revealed that CHOP deficiency modestly inhibited atherosclerosis development even at 20 weeks of age (Figure V in the online-only Data Supplement).

Aortic sections from CHOP−/−;apoE−/− and CHOP+/+;apoE−/− mice were immunostained with antibodies against the macrophage marker MOMA-2, α-smooth muscle actin, and oxidized LDL (Figure VI in the online-only Data Supplement). A CHOP deficiency apparently decreased depositions of macrophages, SMCs, and oxidized LDL in the plaques of apoE−/− mice. These findings were compatible with aortic gene expression. The aortic expression of F4/80, another macrophage marker, was significantly decreased in CHOP−/−;apoE−/− mice (Figure 2B). In addition, aortic expression of inflammatory factors such as MCP-1 and IL-6 (Figure 2C); the ER chaperone Bip (Figure 2D); SMC proliferation–related genes such as transforming growth factor-β1, platelet-derived growth factor-B, and platelet-derived growth factor-RB (Figure 2E); and scavenger receptors such as mouse class A type I scavenger receptor and CD36 (Figure 2F) was significantly decreased in CHOP−/−;apoE−/− mice. Decrements in protein expression of Bip and mouse class A type I scavenger receptor were confirmed by immunoblotting (Figure VII in the online-only Data Supplement). Furthermore, the plasma adiponectin level was increased whereas MCP-1 and tumor necrosis factor-α were significantly decreased in CHOP−/−;apoE−/− mice (Figure 2G). Oxidative stress reportedly decreases plasma adiponectin. In addition, CHOP deficiency markedly decreased plasma levels of oxidative stress markers such as malondialdehyde and 8-isoprostan (Figure 2H). Thus, CHOP appears to be involved in hypercholesterolemia-induced vascular inflammation and systemic oxidative stress, leading to atherosclerosis development.

C/EBP Homologous Protein Deficiency Suppressed Gene Expressions of Inflammation-Related Factors and Vascular Adhesion Molecules in Early-Stage Atherosclerosis

To examine the effects of CHOP deficiency on early-stage atherosclerosis, mRNA expression of inflammatory factors and vascular adhesion molecules was analyzed in the aortas of CHOP−/−;apoE−/− and CHOP+/+;apoE−/− mice at 14 weeks of age, when there were no apparent differences in Oil Red O–stained plaque areas between these 2 groups (Figure VIII in the online-only Data Supplement). Aortic expression of F4/80 and inflammatory factors such as MCP-1, IL-1β, and IL-6 was significantly decreased in CHOP−/−;apoE−/− mice compared with CHOP+/+;apoE−/− mice (Figure 3A), suggesting decreased aortic inflammation. In addition, expression of the vascular cell adhesion molecule-1 was decreased in CHOP−/−;apoE−/− mice (Figure 3B). A CHOP deficiency also suppressed the aortic expression of GADD34 and ERO1α (Figure 3C), which are reportedly upregulated downstream from CHOP by ER stress. Furthermore, antioxidant enzymes such as manganese superoxide dismutase, glutathione-S-transferase, and catalase, which are upregulated in response to oxidative stress, were attenuated by CHOP deficiency (Figure 3D). These findings suggest that CHOP deficiency protects the arterial wall from both hypercholesterolemia-induced ER stress and oxidative stress. ERO1α downregulation may explain the decreased oxidative stress observed in CHOP−/−;apoE−/− mice. Thus, CHOP is
likely to play important roles in promoting inflammation and adhesion molecule expression through oxidative stress and ER stress, thereby leading to atherosclerosis development.

C/EBP Homologous Protein, Especially in Vascular Cells, Is Involved in Cuff Injury–Induced Vascular Remodeling and Hypercholesterolemia-Induced Atherosclerosis

Thus, whole-body CHOP deficiency suppressed 2 types of arteriosclerosis, ie, cuff injury-induced neointimal formation and hypercholesterolemia-induced atherosclerosis. Both types of arteriosclerosis are well known to be caused by interactions between vascular cells such as endothelial cells and SMCs and hematopoietic cells such as macrophages. To determine which CHOP, that expressed in vascular cells or that expressed in hematopoietic cells, is important for these types of arteriosclerosis, we performed BMT experiments.

First, a series of BMT experiments, CHOP+/+ to CHOP+/−, CHOP−/− to CHOP+/+, CHOP+/+ to CHOP+/−, and CHOP+/− to CHOP−/−, was carried out in 6-week-old mice followed by external vascular cuff placement on the femoral arteries 2 weeks after BMT. At that time point, ie, 2 weeks after BMT, CHOP expression of BM cells in CHOP−/− to CHOP+/+ BMT mice was markedly low (Figure IXA in the online-only Data Supplement), suggesting successful reconstitution of BM after transplantation. In addition, peripheral white blood cells decreased rapidly until day 3 after lethal irradiation and then recovered to pre-BMT levels within 13 days (Figure IXB in the online-only Data Supplement), suggesting successful reconstitution of BM after transplantation. Both CHOP+/+ to CHOP+/− BMT mice, arterial expression of both F4/80 and CD45 was dramatically increased, by 22- and 53-fold, respectively, during cuff injury, suggesting that almost all hematopoietic cells, including macrophages, in cuff-injured arteries had infiltrated the vascular wall after BMT and cuff placement (Figure X in the online-only Data Supplement).

In coordination to promote cuff injury–induced vascular remodeling, macrophages, in cuff-injured arteries had infiltrated the vascular wall after BMT and cuff placement (Figure X in the online-only Data Supplement). Under these conditions, neointimal formation was markedly suppressed in the CHOP−/− to CHOP−/− BMT mice compared with the CHOP+/+ to CHOP−/− BMT mice. Both CHOP−/− to CHOP+/− and CHOP+/+ to CHOP−/− mice exhibited intermediate degrees of neointimal formation, but recipient CHOP deficiency alone more strongly suppressed neointimal formation than donor deficiency alone (Figure 4). These findings suggest that CHOP expressed in hematopoietic and vascular cells may act in coordination to promote cuff injury–induced vascular remodeling.
remodeling, although vascular cell CHOP plays the more important role.

Next, BMT experiments were performed with 8-week-old CHOP^+/+;apoE^−/− and CHOP^−/−;apoE^−/− mice. Two weeks after BMT, high-cholesterol chow feeding was started and continued until 22 weeks of age. Both CHOP^−/−;apoE^−/− to CHOP^+/+;apoE^−/− and CHOP^+/+;apoE^−/− to CHOP^−/−;apoE^−/− mice apparently exhibited intermediate degrees of plaque formation. However, recipient CHOP deficiency significantly decreased atherosclerotic areas with donor CHOP deficiency were not statistically significant. In addition, recipient CHOP deficiency more strongly decreased atherosclerotic areas than donor CHOP deficiency were not statistically significant.

Endoplasmic Reticulum Stress–Induced Biological Stress Responses That Were Suppressed by Decreased C/EBP Homologous Protein Expression in Macrophages and Vascular Cells

To determine the molecular mechanisms underlying the atherogenic actions of CHOP, we examined the effects of decreased CHOP expression on ER stress responses in macrophages and in SMC and endothelial cell lines in vitro. First, in peritoneal macrophages obtained from wild-type mice, treatment with thapsigargin, an ER stress inducer, markedly increased CHOP expression. In addition, inflammatory cytokines such as IL-6, MCP-1, and tumor necrosis factor-α were markedly upregulated in wild-type macrophages. These inflammatory responses to thapsigargin were significantly suppressed in macrophages obtained from CHOP^−/− mice (Figure 6A). Furthermore, peroxynitrite and free cholesterol also increased the expression of both CHOP and inflammatory cytokines, but CHOP deficiency suppressed the inflammatory cytokine upregulation in macrophages (Figure XI in the online-only Data Supplement). Thus, CHOP upregulation appears to be involved in ER stress–induced inflammatory responses in macrophages.

Next, we examined alterations in gene expression with CHOP knockdown in a human aortic SMC line in response to thapsigargin treatment. In human aortic SMCs, thapsigargin upregulated CHOP and proliferation-related genes such as platelet-derived growth factor-B and stromal cell–derived factor-1α, as well as the inflammatory factor MCP-1 (Figure 6B). Knockdown of CHOP significantly suppressed the upregulation of proliferation- and inflammation-related factors. In addition, in a murine endothelial cell line, MSS31, tunicamycin, another ER stress inducer, increased the expression of ER stress–related proteins such as CHOP, GADD34, and ERO1α, but these increments were significantly inhibited by CHOP knockdown (Figure 6C), suggesting a role of CHOP in accelerating UPRs. Furthermore, CHOP knockdown blocked tunicamycin-induced upregulation of vascular cell adhesion molecule-1 in endothelial cells. These in vitro experiments revealed that ER stress promotes the proliferation of SMCs and adhesion molecule upregulation in endothelial cells. Thus, CHOP augments these stress responses in vascular cells. Collectively, CHOP, in both macrophages and vascular cells, is likely to exert atherogenic effects via heightened biological stress responses, including inflammation-, adhesion-, or SMC proliferation–related protein upregulation.

Discussion

The first important result obtained in this study is that CHOP deficiency inhibited cuff injury–induced neointimal formation. Neointimal formation, which is an important feature of restenosis after angioplasty of human coronary arteries, critically involves the proliferation and migration of vascular SMCs. In the cuff injury model, adventitial inflammation is considered to be a major cause of medial SMC migration to the subendothelial space. In the present study, CHOP deficiency suppressed aortic expression of inflammatory factors such as MCP-1 (Figure 3), but these increments were significantly inhibited by CHOP knockdown (Figure 6B). Knockdown of CHOP significantly suppressed the upregulation of proliferation- and inflammation-related factors. In addition, in a murine endothelial cell line, MSS31, tunicamycin, another ER stress inducer, increased the expression of ER stress–related proteins such as CHOP, GADD34, and ERO1α, but these increments were significantly inhibited by CHOP knockdown (Figure 6C), suggesting a role of CHOP in accelerating UPRs. Furthermore, CHOP knockdown blocked tunicamycin-induced upregulation of vascular cell adhesion molecule-1 in endothelial cells. These in vitro experiments revealed that ER stress promotes the proliferation of SMCs and adhesion molecule upregulation in endothelial cells. Thus, CHOP augments these stress responses in vascular cells. Collectively, CHOP, in both macrophages and vascular cells, is likely to exert atherogenic effects via heightened biological stress responses, including inflammation-, adhesion-, or SMC proliferation–related protein upregulation.
factors and growth factors that induce vascular SMC proliferation. In addition, BMT experiments revealed a small but significant effect of CHOP donor deficiency. Because almost all hematopoietic cells, which were detected in the aorta 3 weeks after cuff placement, had infiltrated the aorta after cuff placement following bone marrow replacement, the small but significant effect of CHOP donor deficiency actually reflects the positive contribution of CHOP expressed in hematopoietic cells. On the other hand, recipient CHOP deficiency more strongly suppressed cuff injury–induced neointimal formation than donor CHOP deficiency, indicating the importance of recipient CHOP in vascular remodeling. We cannot rule out the possibility that CHOP expressed in recipient cells other than vascular cells is involved in vascular remodeling. However, taken together with the findings that CHOP deficiency suppressed vascular expressions of inflammatory factors and stress-related proteins and that CHOP in endothelial cells and vascular SMCs contributes to upregulation of these factors in vitro, CHOP in vascular cells is likely to play important roles. Thus, CHOP expressed in both hematopoietic cells and vascular cells, especially that expressed in vascular cells, contributes to vascular remodeling. Our in vitro results further support the notion that CHOP expressed in hematopoietic and vascular cells acts in a coordinated fashion to exacerbate arterial inflammation, which leads to vascular remodeling.

Another important aspect of the present study is that activation of CHOP was shown to play an important role in hypercholesterolemia-induced progression of atherosclerosis; CHOP is well known to be a strong mediator of apoptosis induction in cells exposed to substantial ER stress. Aortic expression of CHOP is enhanced as atherosclerosis pro-
In atherosclerotic lesions, apoptotic cells, which are derived mainly from macrophages but to a lesser extent endothelial and SMCs, are increased. Apoptotic macrophages release proteases and inflammatory factors and induce lipid core formation. Free cholesterol accumulation in the ER membranes of macrophages reportedly induces the UPR, leading to CHOP-mediated apoptotic cell death. Thorp and colleagues reported that CHOP deficiency suppressed atherosclerotic progression in apoE−/− mice, which is consistent with our results. They concluded that the mechanism underlying atherosclerotic inhibition with CHOP deficiency involves decreased atherosclerotic plaque necrosis and lesional apoptosis of macrophages. The CHOP-mediated apoptosis in macrophages also reportedly contributes to the instability of atherosclerotic plaques, suggesting the importance of macrophage CHOP. In addition to the roles of macrophage CHOP, our study shows vascular CHOP to have a significant role. First, BMT experiments revealed that CHOP deficiency in recipient mice more strongly suppressed atherosclerosis development than that in donor mice. We cannot rule out the possibility that residual macrophages that had infiltrated the aorta before BMT weakened the suppressive effects of donor CHOP deficiency. Even if this were the case, however, the significant effects of recipient CHOP deficiency indicate that CHOP expressed in vascular cells plays an important role in atherosclerosis development. In addition, global CHOP deficiency decreased aortic macrophage deposition per se in the early stage, eg, at 14 weeks of age, leading to suppression of atherosclerosis development at 24 weeks of age. Therefore, decreased macrophage infiltration in the early stage is likely to be an important mechanism by which CHOP deficiency suppresses atherosclerosis development. Endothelial adhesion molecule expression was decreased by CHOP deficiency, and in vitro experiments revealed that CHOP knockdown in endothelial cells inhibited upregulation of the endothelial adhesion molecule. Together, these findings suggest that suppression of endothelial adhesion molecule expression by CHOP deficiency is involved in the infiltration of macrophages into the vascular wall. Therefore, suppressed macrophage-endothelium interaction resulting from CHOP deficiency in both cells is likely to contribute to inhibition of atherosclerosis development. In vitro experiments also revealed that CHOP knockdown in SMCs suppressed upregulation of inflammatory factors. Supporting this notion, CHOP...
deficiency reduced aortic expression and plasma concentrations of inflammatory factors and oxidative stress markers. Thus, in addition to the reported mechanism involving macrophage apoptosis, vascular CHOP in endothelial cells and SMCs plays important roles in atherosclerosis development via augmentation of macrophage recruitment and inflammatory and stress responses.

Interestingly, CHOP deficiency suppressed aortic expressions of Bip and GADD34 in apoE⁻/⁻ mice, suggesting that CHOP also plays a role in accelerating UPRs other than those involved in the induction of apoptosis. This finding is consistent with a previous report indicating that, in CHOP⁻/⁻ cells, GADD34 protein is downregulated in association with sustained elevation of eukaryotic initiation factor-2 α phosphorylation, followed by translation repression, resulting in reduced ER stress. In addition, several reports have indicated that the UPR is closely related to inflammatory responses. Inositol-requiring enzyme-1α activates the c-jun N-terminal kinase pathway, increasing the levels of tumor necrosis factor-α, IL-6, and MCP-1 through activation of the activator protein-1 transcription factor complex. The nuclear factor-κB pathway can also be activated by PKR-like ER kinase signaling in both endothelial cells and macrophages. Furthermore, CHOP directly induces expression of ERO1α, which is required for disulphide formation in protein folding and contributes to elevation of reactive oxygen species in ER-stressed cells. Induction of ERO1α by CHOP might be involved in CHOP-mediated accumulation of reactive oxygen species. Consistent with these previous studies, here, CHOP deficiency suppressed ERO1α expression in the aortas of apoE⁻/⁻ mice. In addition, aortic expression of antioxidant enzymes was downregulated in CHOP⁻/⁻;apoE⁻/⁻ mice, suggesting a decrement in hypercholesterolemia-induced oxidative stress. Consistent with this notion, oxidative markers such as malondialdehyde and 8-isoprostane and adiponectin levels in apoE⁻/⁻ mice were markedly decreased and increased, respectively, by CHOP deficiency. Thus, in addition to the well-known “canonical” signaling that triggers apoptosis induction, CHOP may play important roles in augmenting potentially pathological biological responses. This “noncanonical” role of CHOP may contribute to hypercholesterolemia-induced exacerbation of oxidative stress not only in the aorta but also systemically, leading to atherosclerosis progression.

Conclusions

This study provides strong evidence of the impact of CHOP, especially that expressed in vascular cells, on arteriosclerosis formation. The underlying mechanisms involve augmentation of unfavorable, ie, pathological, stress responses. This noncanonical role of CHOP is important in vascular remodeling and atherosclerosis. Therefore, blockade of the CHOP signaling pathway is a promising therapeutic strategy for atherosclerosis induced by hypercholesterolemia and restenosis after angioplasty.

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Disclosures

None.

References

CLINICAL PERSPECTIVE

Complex interactions among numerous biological pathways are implicated in the pathogenesis of arteriosclerosis such as atherosclerosis and vascular remodeling. In particular, responses to inflammation and oxidative stress have been considered to play central roles in arteriosclerosis development. In addition, recent studies revealed endoplasmic reticulum stress to be associated with atherosclerosis involving free cholesterol–induced macrophage apoptosis. However, details of the molecular mechanisms of interactions among classic atherogenic actions and endoplasmic reticulum stress responses remained to be elucidated. This study focused on the transcription factor C/EBP homologous protein (CHOP), which is well known to be induced by endoplasmic reticulum stress, mediating apoptotic cell death. Here, using CHOP-deficient mice, we show that CHOP plays important roles in accelerating 2 types of arteriosclerosis: cuff injury–induced neointimal formation and hypercholesterolemia–induced atherosclerosis. Augmented inflammatory and oxidative stress responses mediated by CHOP are important underlying mechanisms. Furthermore, CHOP, especially that expressed in hematopoietic and vascular cells, is involved in inflammatory interactions among macrophages, endothelial cells, and vascular smooth muscle cells, acting in a coordinated fashion to promote arteriosclerosis development. Thus, these observations of this noncanonical role of CHOP may lead to a better understanding of the molecular pathogenesis of vascular remodeling and arteriosclerosis. Furthermore, given that neointimal formation is an important feature of postangioplasty restenosis of human coronary arteries, this study provides potential strategies for the prevention of cardiovascular diseases and the advancement of coronary intervention therapies.
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Involvement of Endoplasmic Stress Protein CHOP in Arteriosclerosis Acceleration with Augmented Biological Stress Responses

First Author: Junhong Gao
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Figure I

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Panel A: Body weight (g) vs. CHOP (+/+ or −/−).

Panel B: Blood glucose (mg/dl) vs. CHOP (+/+ or −/−).

Panel C: Blood pressure (mmHg) vs. CHOP (+/+ or −/−).

Panel D: Total cholesterol (mg/dl) vs. CHOP (+/+ or −/−).

Panel E: Triglyceride (mg/dl) vs. CHOP (+/+ or −/−).

Panel F: NEFA (mEq/l) vs. CHOP (+/+ or −/−).

Panel G: Adiponectin (µg/ml) vs. CHOP (+/+ or −/−).

Panel H: MCP-1 (pg/ml) vs. CHOP (+/+ or −/−).

Panel I: TNF-α (pg/ml) vs. CHOP (+/+ or −/−).
Figure II

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- **CHOP** +/+ -/-
- **Cuff** (-) (+)

**Statistical significance:**
- **(++)**
- **(*)**
Figure III

A

\[
\begin{array}{ccc}
\text{CHOP} & +/+ & -/- \\
\text{Cuff} & (-) & (+) \\
\hline
0 & 1.0 & 2.0 & 3.0 & 4.0
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\]

** *

B

CHOP+/+

SMA

CHOP−/−

C

CHOP+/+

PCNA

CHOP−/−

PCNA positive cells (% vs CHOP+/+ mice)

**

0 50 100 150

CHOP +/+ −/−
Figure IV

A. Body weight (g) vs. CHOP condition (+/+ or −/−).

B. Blood glucose (mg/dl) vs. CHOP condition (+/+ or −/−).

C. Blood pressure (mmHg) vs. CHOP condition (+/+ or −/−).

D. Total cholesterol (mg/dl), triglyceride (mg/dl), and NEFA (mEq/l) vs. CHOP condition (+/+ or −/−).
Figure V

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CHOP+/+; apoE−/−

CHOP−/−; apoE−/−

plaque area ratio (%)
Figure VI-A

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Figure VI-B

CHOP+/+; apoE−/−

X40

X200

CHOP−/−; apoE−/−

X40

X200

MOMA-2

SMA

oxLDL
Figure VII

Gao J et al.

**Bip**

**SR-AI**

**GAPDH**

<table>
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<th>CHOP−/−; apoE−/−</th>
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Figure VIII

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CHOP^+/+; apoE^−/−

CHOP^−/−; apoE^−/−

Oil red O-positive area ratio (%)
Figure X

Gao J et al.
Figure XI

A

CHOP  

IL-6  

MCP-1  

TNF-α  

B

CHOP  

IL-6  

MCP-1  

TNF-α  

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Supplemental Material

Supplemental Figure Legends

**Figure I** CHOP deficiency caused no gross phenotypic differences

CHOP+/+ and CHOP-/- (n=5 per group) mice were fed normal chow and subjected to measurement of body weight (A), blood glucose (B), blood pressure (C), plasma levels of lipid parameters (D) such as total cholesterol (left), triglyceride (middle) and non-esterified fatty acids (NEFA) (right), and plasma levels of adipokines (E), such as adiponectin (left), MCP-1 (middle) and TNF-α (right), at 11 weeks of age. Data are presented as means ± SE.*P < 0.05 by the unpaired t test.

**Figure II** Arterial CHOP expression was up-regulated by cuff injury

Arterial expression of CHOP in cuff-injured and uninjured arteries of CHOP+/+ and CHOP-/- (n=7 per group) mice 7 days after cuff placement were quantified by RT-PCR. Data are presented as means ± SE. **P < 0.01 by one-way ANOVA.

**Figure III** Cuff injury-induced neointimal SMC proliferation was suppressed in CHOP deficient mice

Expressions of α-smooth-muscle isoform of actin (SMA) in cuff-injured and uninjured arteries 3 weeks after cuff placement in CHOP+/+ and CHOP-/- (n=7 per group) mice were quantified by RT-PCR adjusted with GAPDH (A). Immunological stainings against SMA (B) and PCNA (C; upper panel) were prepared from cuff injured femoral arteries 3 weeks after placement in CHOP+/+ (left) and CHOP-/- (right) mice. The relative amounts of PCNA positive cells in neointimal lesions (n=14) were presented as the ratio to CHOP +/+ mice (n=9) (C; lower panel). *P < 0.05, **P < 0.01 by the
unpaired \( t \) test or one-way ANOVA.

**Figure IV** CHOP deficiency caused no gross phenotypic differences in apoE\(-/-\) mice

CHOP\(+/+-\);apoE\(-/-\) \( (n=18) \) and CHOP\(-/-\);apoE\(-/-\) \( (n=13) \) mice were fed 1.25% high cholesterol chow starting at 8 weeks of age and subjected to measurement of body weight (A), blood glucose (B), blood pressure (C), plasma levels of lipid parameters (D) such as total cholesterol (left), triglyceride (middle), and non-esterified fatty acids (NEFA) (right). Data are presented as means ± SE.

**Figure V** Aortic root cross-sections from 20-week-old CHOP\(+/+-\);apoE\(-/-\) and CHOP\(-/-\);apoE\(-/-\) mice

Aortic root cross-sections were prepared from 20-week-old CHOP\(+/+-\);apoE\(-/-\) \( (n=5) \) and CHOP\(-/-\);apoE\(-/-\) \( (n=5) \) mice and stained with hematoxylin and eosin. Representative histological findings are shown in the left panels. Atherosclerosis was evaluated as plaque areas, which were expressed as percentages of the total lumen areas in CHOP\(+/+-\);apoE\(-/-\) \( (n=5) \) and CHOP\(-/-\);apoE\(-/-\) \( (n=5) \) mice. Data are presented as means ± SE. **\( P < 0.01 \) by the unpaired \( t \) test.

**Figure VI** Immunohistological findings of aortic plaque lesions from 20-week-old CHOP\(+/+-\);apoE\(-/-\) and CHOP\(-/-\);apoE\(-/-\) mice

Aortic sections from 20-week-old CHOP\(+/+-\);apoE\(-/-\) (upper panels) and CHOP\(-/-\);apoE\(-/-\) (lower panels) mice fed 1.25% high cholesterol chow were immunostained with antibodies against MOMA-2 (left), SMA (middle) or oxidized LDL (right) (A). A series of immunological staining with control antibodies, i.e. rat IgG2b as
a control for MOMA-2 (left), mouse IgG2a as a control for SMA (middle), rabbit immunoglobulin as a control for oxidized LDL (right), were performed in the corresponding aortic samples (B). Magnifications of upper and lower panels are x40 and x200, respectively. Representative immunostaining findings from all samples (n=7 per group) are shown.

**Figure VII Protein expressions of Bip and SR-AI in the aorta**

Tissue protein extracts of whole aorta samples from 24-week-old CHOP+/+;apoE-/-, CHOP-/-;apoE/- mice were subjected to immunoblotting with antibodies to Bip (upper panels), SR-AI (middle panels) and GAPDH as a loading control (lower panel).

**Figure VIII Aortic root cross-sections from 14-week-old CHOP+/+;apoE-/ and CHOP-/-;apoE-- mice**

Aortic root cross-sections were prepared from 14-week-old CHOP+/+;apoE-/- and CHOP-/-;apoE/- mice and stained with oil red O. Representative histological findings are shown in the left panels. Atherosclerosis was evaluated as oil red O-stained areas, which were expressed as percentages of the total lumen areas in CHOP+/+;apoE-/ (n=3) and CHOP-/-;apoE-/ (n=3) mice. Data are presented as means ± SE.

**Figure IX Reconstitution of BM cells 2 weeks after BMT**

Bone marrow cell expressions of CHOP 2 weeks after BMT were quantified by RT-PCR in CHOP+/+ to CHOP+/+ (n=5), CHOP-/- to CHOP+/+ (n=5), CHOP +/+ to CHOP-/- (n=3) and CHOP-/- to CHOP-/- (n=3) BMT mice (A). Time course of peripheral WBC counts from the day before BMT to 13 days after BMT (B). Data are presented as means
± SE. **$P < 0.01$ by one-way ANOVA.

Figure X Macrophage and hematopoietic cell markers before and after cuff placement
Arterial expressions of F4/80 and Ptprc (CD45) before (2 weeks after BMT) ($n=6$), and 3 weeks after ($n=8$) cuff placement were quantified by RT-PCR in CHOP$^{+/+}$ to CHOP$^{+/+}$ BMT mice. Data are presented as means ± SE. **$P < 0.01$ by the unpaired $t$ test.

Figure XI
CHOP deficiency suppressed peroxynitrite- and free cholesterol-induced inflammatory responses of macrophages.

mRNA expressions of CHOP and inflammatory cytokines in peritoneal macrophages from CHOP$^{+/+}$ ($n=9$) and CHOP$^{-/-}$ ($n=9$) mice in response to peroxynitrite (A) and free cholesterol (B) treatments, were evaluated by RT-PCR. Peritoneal macrophages from CHOP$^{+/+}$ ($n=5$) and CHOP$^{-/-}$ ($n=6$) mice without these ER stress-inducing agents were also subjected to analyses of mRNA expressions. *$P < 0.05$, **$P < 0.01$ by the one-way ANOVA.