Forkhead Transcription Factors (FoxOs) Promote Apoptosis of Insulin-Resistant Macrophages During Cholesterol-Induced Endoplasmic Reticulum Stress

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OBJECTIVE—Endoplasmic reticulum stress increases macrophage apoptosis, contributing to the complications of atherosclerosis. Insulin-resistant macrophages are more susceptible to endoplasmic reticulum stress–associated apoptosis probably contributing to macrophage death and necrotic core formation in atherosclerotic plaques in type 2 diabetes. However, the molecular mechanisms of increased apoptosis in insulin-resistant macrophages remain unclear.

RESEARCH DESIGN AND METHODS—The studies were performed in insulin-resistant macrophages isolated from insulin receptor knockout or *ob/ob* mice. Gain- or loss-of-function approaches were used to evaluate the roles of forkhead transcription factors (FoxOs) in endoplasmic reticulum stress–associated macrophage apoptosis.

RESULTS—Insulin-resistant macrophages showed attenuated Akt activation and increased nuclear localization of FoxO1 during endoplasmic reticulum stress induced by free cholesterol loading. Overexpression of active FoxO1 or FoxO3 failed to induce apoptosis in unchallenged macrophages but exacerbated apoptosis in macrophages with an active endoplasmic reticulum stress response. Conversely, macrophages with genetic knockouts of FoxO1, -3, and -4 were resistant to apoptosis in response to endoplasmic reticulum stress. FoxO1 was shown by chromatin immunoprecipitation and promoter expression analysis to induce inhibitor of κBε gene expression and thereby to attenuate the increase of nuclear p65 and nuclear factor-κB activity during endoplasmic reticulum stress, with proapoptotic and anti-inflammatory consequences.

CONCLUSIONS—Decreased Akt and increased FoxO transcription factor activity during the endoplasmic reticulum stress response leads to increased apoptosis of insulin-resistant macrophages. FoxOs may have a dual cellular function, resulting in either proapoptotic or anti-inflammatory effects in an endoplasmic reticulum stress–modulated manner. In the complex plaque milieu, the ultimate effect is likely to be an increase in macrophage apoptosis, plaque inflammation, and destabilization. *Diabetes* **57:2967–2976, 2008**

Patients with type 2 diabetes have a strikingly increased incidence of atherosclerotic cardiovascular disease. In part, this reflects diabetic dyslipidemia characterized by increased VLDL and reduced HDL levels secondary to peripheral insulin resistance and hyperinsulinemia (1,2). Vascular insulin resistance may also play a role in the acceleration of atherosclerosis in diabetes. Decreased insulin signaling and Akt activity in vascular endothelium impair vascular nitric oxide responses and are associated with increased expression of inflammatory genes, vascular cell adhesion molecule-1, and accelerated atherosclerosis (3,4). Moreover, diabetic individuals are susceptible to complications of atherosclerotic plaque formation, reflecting plaque instability. Apoptosis of macrophages, smooth muscle cells, and endothelial cells in advanced atherosclerotic lesions is thought to lead to plaque rupture and atherothrombosis (5). Atherosclerotic lesions from patients with type 2 diabetes showed increased areas of necrotic core and increased numbers of apoptotic macrophages even after normalization for total lesion area, suggesting that macrophage apoptosis and resulting necrotic core formation may specifically contribute to plaque instability in diabetes (5).

A variety of different processes contribute to macrophage apoptosis in advanced lesions, including growth factor withdrawal, hypoxia, and oxidative and other cellular stresses. Several of these factors mediate apoptosis by activating the cellular endoplasmic reticulum stress response. Increased expression of C/EBP homologous protein (CHOP, also known as Ddit3/Gadd153) is a hallmark of endoplasmic reticulum stress, and CHOP is expressed in a subset of macrophages in advanced atherosclerotic lesions in both humans and mice (6–8). The endoplasmic reticulum stressors in atherosclerotic plaques include free cholesterol (FC), 7-oxysterols, oxidized phospholipids, and homocysteine (6,9–11). The unfolded protein response (UPR) is an adaptive response to endoplasmic reticulum stress that involves decreased translation of most proteins but increased expression of a subset of molecules involved in protein refolding or other endoplasmic reticulum functions (12). The UPR involves a balance or pro- and antiapoptotic factors and only leads to apoptosis when endoplasmic reticulum stress is overwhelming or accompanied by additional apoptotic stimuli. Endoplasmic reticulum loading of free cholesterol induces CHOP but is insufficient to cause apoptosis unless accompanied by an additional stimulus, such as ligation of the

FIG. 1. Myr-Akt rescues insulin-resistant macrophages from apoptosis. *A* and *B*: ConA-elicited peritoneal macrophages from $Insr^{+/+}$, $Insr^{-/-}$ (*A*), ob control, or *ob/ob* (*B*) mice were transduced with adenovirus containing constitutively active myristoylated Akt (Myr-Akt) or control LacZ at 500 MOI for 18 h. After the transduction, macrophages were loaded with FC for 16 h. *C*: Macrophages were treated with 5 μmol/l LY-294002 for 1 h prior and together with FC loading for 16 h and then stained with annexin V and propidium iodide for apoptosis assay. (Please see http://dx.doi.org/10.2337/db08-0520 for a high-quality digital representation of this image.)

scavenger receptor A (SRA) by modified LDL (13). The UPR is also associated with an increase in Akt phosphorylation and activity, which is thought to represent a compensatory prosurvival mechanism (14).

Macrophages from *ob/ob* mice have decreased levels of insulin receptors and show decreased phosphorylation of insulin receptor substrate 2 in response to insulin, indicating macrophage insulin resistance (15,16). To model the vascular effects of macrophage insulin resistance, we transplanted bone marrow from $Insr^{-/-}$ mice into $Ldlr^{-/-}$ recipients. This was associated with a dramatic increase in necrotic core formation, reflecting increased numbers of apoptotic macrophages (14). $Insr^{-/-}$ macrophages showed increased susceptibility to apoptosis induced by loading with free cholesterol or oxidized LDL. In $Insr^{-/-}$ macrophages, there was a marked blunting of Akt phos-

phorylation in response to endoplasmic reticulum stress (14). Insulin signaling leads to a variety of progrowth, antiapoptotic responses, notably phosphorylation and nuclear exclusion of the closely related transcription factors forkhead transcription factor 01(FoxO1), -3, and -4. The goal of the present study was to evaluate the hypothesis that decreased Akt signaling and increased activity of FoxOs during the endoplasmic reticulum stress response are responsible for the increased apoptotic response of insulin-resistant macrophages.

RESEARCH DESIGN AND METHODS

LDL receptor knockout, *ob/ob*, and control mice were obtained from The Jackson Laboratories (Bar Harbor, ME). Double-knockout mice were generated by crossing C57BL6J $Insr^{-/-}$ (14) and $Ldlr^{-/-}$ mice. The $Ldlr^{-/-} Insr^{+/+}$
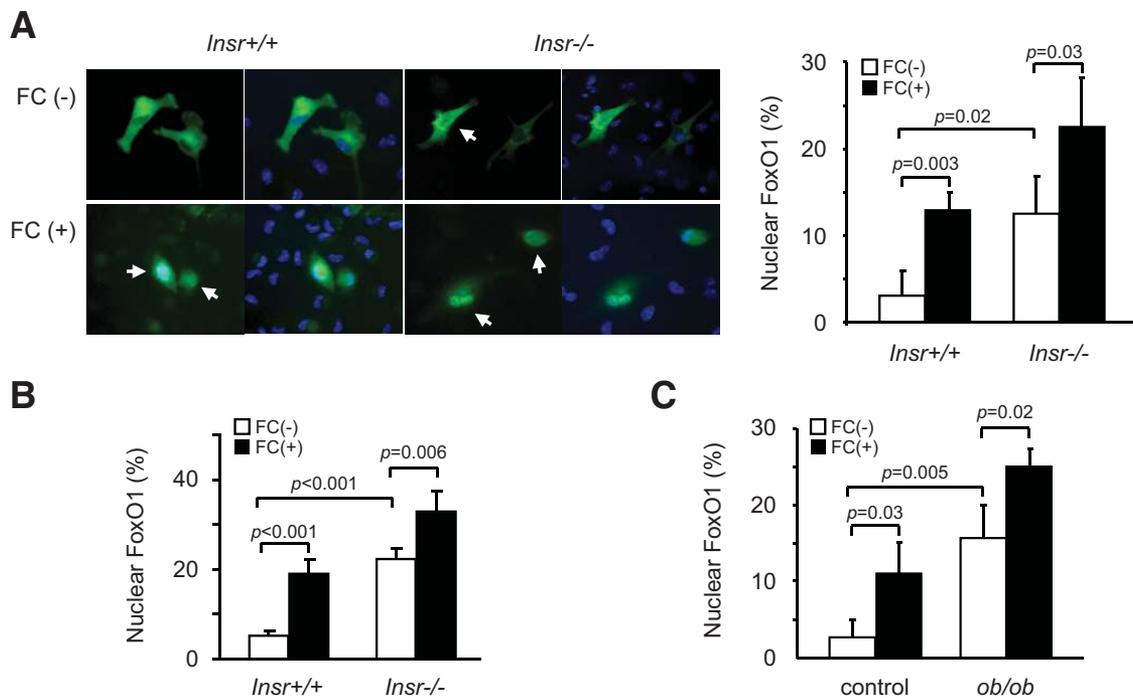
FIG. 2. FoxO is preferentially localized to the nucleus in FC-loaded or insulin-resistant macrophages. *A* and *C*: ConA-elicited peritoneal macrophages from $Insr^{+/+}$, $Insr^{-/-}$, ob control, or *ob/ob* mice were transduced with adenovirus encoding FoxO1-GFP and treated with FC for 6 h, and then the GFP signal localization was analyzed. *A*: The representative images of localization in FoxO1-GFP transduced macrophages are shown. Arrows indicate FoxO localization in nucleus. *B*: For endogenous FoxO staining, cells were fixed after 6 h of FC loading and stained with antibodies against FoxO1 and Hoechst dye. Percentage of nuclear FoxO-positive cells to total cell number (*B*) or to GFP-positive cells (randomly counted at least 100 GFP-positive cells) (*A* and *C*) was shown in figures. (Please see http://dx.doi.org/10.2337/db08-0520 for a high-quality digital representation of this image.)

littermates were used as controls. FoxO1/3/4 triple-knockout mice have been described previously (17).

Primary macrophage culture and free cholesterol loading. Peritoneal macrophages were harvested by peritoneal lavage 3 days after intraperitoneal injection of concanavalin A (ConA) (6). Macrophages were maintained in culture medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 20% L-cell conditioned medium) until 90% confluent. Bone marrow–derived macrophages were differentiated from marrow cells by culture in the aforementioned medium for 10–12 days. FC was loaded by incubating cells in culture medium containing 10 μg/ml ACAT inhibitor 58035 (Sigma) plus 100 μg/ml acetylated LDL for the indicated time periods.

Plasmid construction. Plasmids encoding FoxO1-ADA and DNA binding–deficient (DBD) FoxO1-ADA were previously described (18). The upstream sequence of mouse inhibitor of κB (IκB) ε (from −295 to 200) was amplified from mouse genomic DNA by PCR using the following primers: 5′-ACTCTC GAGTGAATGAAGACACCACGGAAC-3′ and 5′-AGTAAGCTTAGGGTCCAGC GGGATCTAACT-3′ and cloned into pGL3-Basic luciferase reporter plasmid (IκBε-295-Luc). Deletion mutants were generated by standard procedures. IκBε-295TM (triple mutant)-Luc, which contains mutations in three consensus forkhead binding motifs [AAA(C/T)A] between −295 and −159 region was generated using the QuikChange II Site-Directed Mutagenesis kit (Stratagene). Primer sequences are available on request.

Adenoviral vectors. Adenovirus encoding Myr-Akt (19), FoxO1-ADA (20), control short hairpin RNA (shRNA), FoxO1 shRNA (20), and nuclear factor-κB (NF-κB)–responsive reporter construct (21) were previously described. FoxO3a-AAA was obtained from Vector BioLabs. Amplified adenoviruses were purified with AdenoPACK100 (Sartorius) and titrated with Adeno-X Rapid Titer Kit (BD Biosciences).

Macrophage apoptosis assay. Apoptosis assays were performed by staining macrophages with Alexa 488- or 594-labeled annexin V and propidium iodide using Vybrant Apoptosis Assay kit (Invitrogen). At least three separate fields for each treatment condition were randomly selected and counted for cells (>200) positively stained for annexin V and propidium iodide and are shown as a percentage of total cell number.

p65 NF-κB localization. Nuclear and cytoplasmic proteins were extracted using Nuclear Extraction kit (Panomics). Western blot was carried out using p65 NF-κB (F-6) from Santa Cruz Biotechnology for primary antibody. LaminB1 (Santa Cruz Biotechnology) or nucleophosmin (Zymed Laboratories) were used as loading controls.

FoxO1 localization. Peritoneal macrophages were cultured in Lab-Tek II Chamber Slide System (Nalge Nunc International). For endogenous FoxO staining, cells were fixed with 2% paraformaldehyde and immunostained for FoxO1 using a mixture of rabbit polyclonal antibodies, FoxO1 (9462; Cell Signaling), FKHR (H-128; Santa Cruz Biotechnologies), and 3587 (22) overnight at 4°C, each used at a dilution of 1:100. Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen) were used as secondary antibodies. Adenovirus encoding FoxO1–green fluorescent protein (GFP) was transduced in primary macrophages at 500 multiplicity of infection (MOI) for 16 h and incubated for another 32 h before treatment. Nuclei were stained with Hoechst (Invitrogen) for 10 min.

Chromatin immunoprecipitation assays. Chromatin immunoprecipitation (ChIP) assay was performed with ChIP Assay kit (Millipore). After plasmid transfection, 6×10^6 RAW 264.7 cells were fixed with 1% formaldehyde for 30 min at room temperature. Cell lysate was sonicated for 3 min at 4°C. After preclearing with 100 μl salmon sperm DNA/protein A-Sepharose, protein lysate was immunoprecipitated with 2 μg anti-FKHR antibody (Santa Cruz Biotechnology) or an equal amount of normal rabbit IgG for 16 h at 4°C. The antibody-protein-DNA complexes were collected with 40 μl salmon sperm DNA/protein A-Sepharose. After releasing crosslink, purified DNA was subjected to PCR using the following primers: 5′-TGAATGAAGACACCACGGAA C-3′ and 5′-GCTTGCGTAGGCAGGGAGTC-3′ to amplify the −295 to −130 region of the mouse IκBε promoter.

Statistical analysis. Results are expressed as means ± SD, and statistical significance of differences was evaluated with Student's *t* test.

RESULTS

Myr-Akt rescues the apoptotic defect in $Insr^{-/-}$ and *ob/ob* macrophages. We previously showed reduced induction of Akt phosphorylation during the UPR in $Insr^{-/-}$ macrophages compared with $Insr^{+/+}$ macrophages. To ascertain whether this might contribute to enhanced apoptosis, we used adenovirus to overexpress a constitutively active form of Akt (Myr-Akt). FC loading using AcLDL+ an ACAT inhibitor (58035) led to increased apoptosis in $Insr^{-/-}$ cells compared with $Insr^{+/+}$ cells, as reported
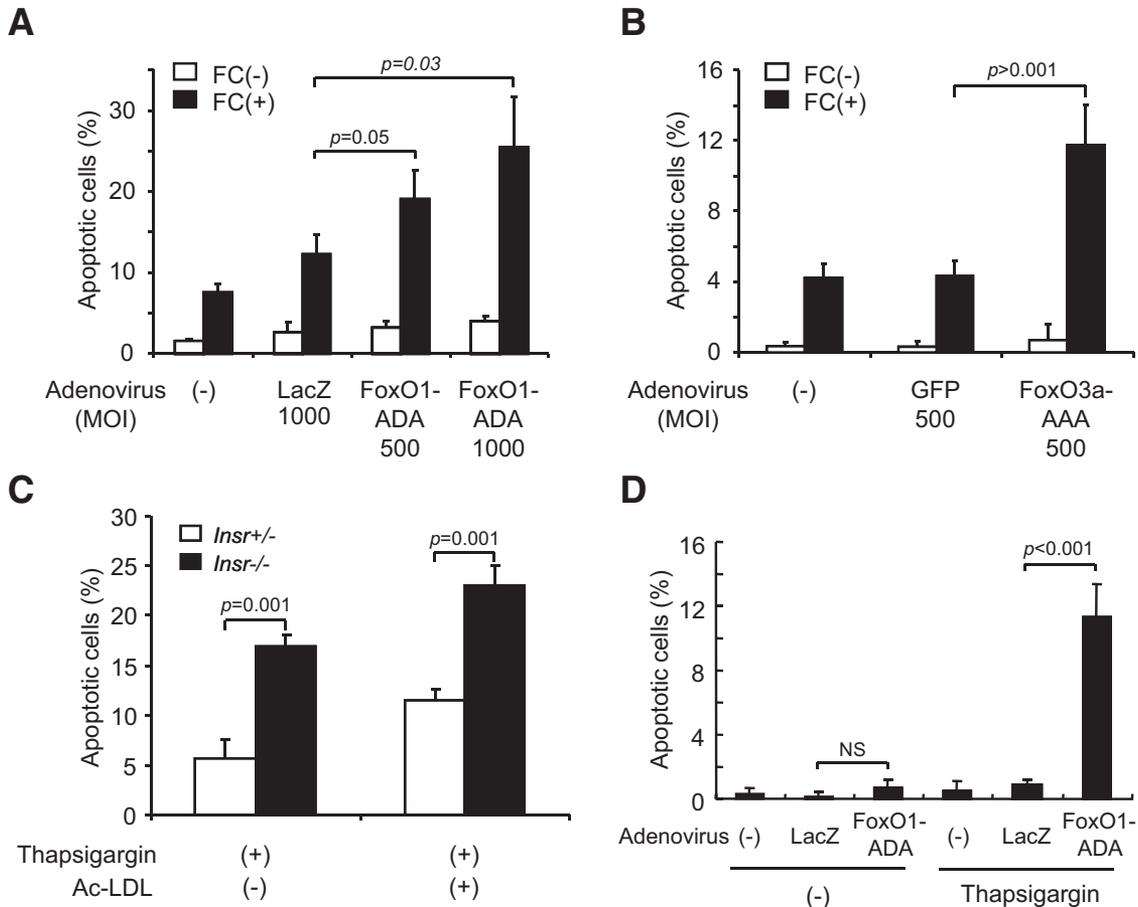
FIG. 3. FoxOs enhance endoplasmic reticulum–stressed macrophage apoptosis. ConA-elicited peritoneal macrophages were transducted with adenovirus containing constitutively active FoxO1 (FoxO1-ADA), FoxO3 (FoxO3-AAA), or controls at 500 MOI for 18 h. Macrophages were loaded with FC (*A* and *B*) or treated with 0.5 μmol/l thapsigargin (*D*) for 16 h. ConA-elicited macrophages from $Insr^{+/-}$ control or $Insr^{-/-}$ mice were treated with 0.25 μmol/l thapsigargin and/or 50 μg/ml Ac-LDL for 30 h (*C*) and stained with annexin V and propidium iodide for apoptosis assay.

previously (14). Myr-Akt expression resulted in a major reduction in apoptosis in response to FC loading in both sets of cells and virtually eliminated the difference between $Insr^{+/+}$ and $Insr^{-/-}$ cells (Fig. 1*A*). Given that transduction efficiency of adenovirus was ~60%, this indicates a substantial reversal of the effect of FC loading and insulin receptor deficiency on apoptosis. Myr-Akt expression did not affect the uptake of AcLDL or FC accumulation in macrophages (not shown). To assess whether similar responses could be observed in cells with less severe defects in insulin signaling, we also audited the antiapoptotic impact of activated Akt in insulin-resistant *ob/ob* macrophages. Enhanced apoptosis in response to FC loading was also seen in *ob/ob* macrophages, and this response was partly rescued by Myr-Akt overexpression (Fig. 1*B*). To reinforce the relevance of the phosphatidylinositol 3-kinase-Akt signaling axis in this physiological process, we assessed the impact of inhibition of endogenous Akt activity in FC-loaded macrophages, using the phosphatidylinositol 3-kinase inhibitor LY-294002. This treatment resulted in a pronounced increase in apoptosis in FC-loaded wild-type macrophages (Fig. 1*C*). These data suggest that induction of Akt activity during FC-induced endoplasmic reticulum stress provides an antiapoptotic function that is otherwise impaired in insulin-resistant macrophages.

FoxO1 and FoxO3 promote macrophage apoptosis during the UPR. Insulin signaling via phosphatidylinositol 3-kinase leads to Akt-mediated phosphorylation of FoxO1 and FoxO3 on serine residues, causing nuclear exclusion and loss of transcriptional activity (22). Additionally, FC loading of macrophages leads to activation of Jun NH_2-terminal kinase (JNK), and in other settings, JNK can phosphorylate related FoxOs (FoxO4) on different amino acids, resulting in nuclear localization and activation (23,24). We observed increased nuclear FoxO1 as a result of FC loading or insulin receptor deficiency and an additive effect of FC loading and insulin receptor deficiency in $Insr^{-/-}$ macrophages (Fig. 2*A* shows FoxO1-GFP; Fig. 2*B* shows endogenous nuclear FoxO1). *ob/ob* macrophages also showed increased nuclear FoxO1 compared with wild-type macrophages, and again, FC loading further increased levels of nuclear FoxO1 (Fig. 2*C*). Thus, both decreased insulin signaling and FC-induced endoplasmic reticulum stress are associated with increased nuclear FoxO1.

To evaluate the effect of constitutively nuclear forms of FoxOs on apoptosis, we transduced macrophages with FoxO1 or FoxO3 in which Akt target serine residues had been mutated to alanine or aspartic acid (FoxO1-ADA and FoxO3-AAA). Whereas in control macrophages, there was only a slight increase in apoptosis, the constitutively active FoxOs resulted in a robust

A
Primary Peritoneal Macrophages
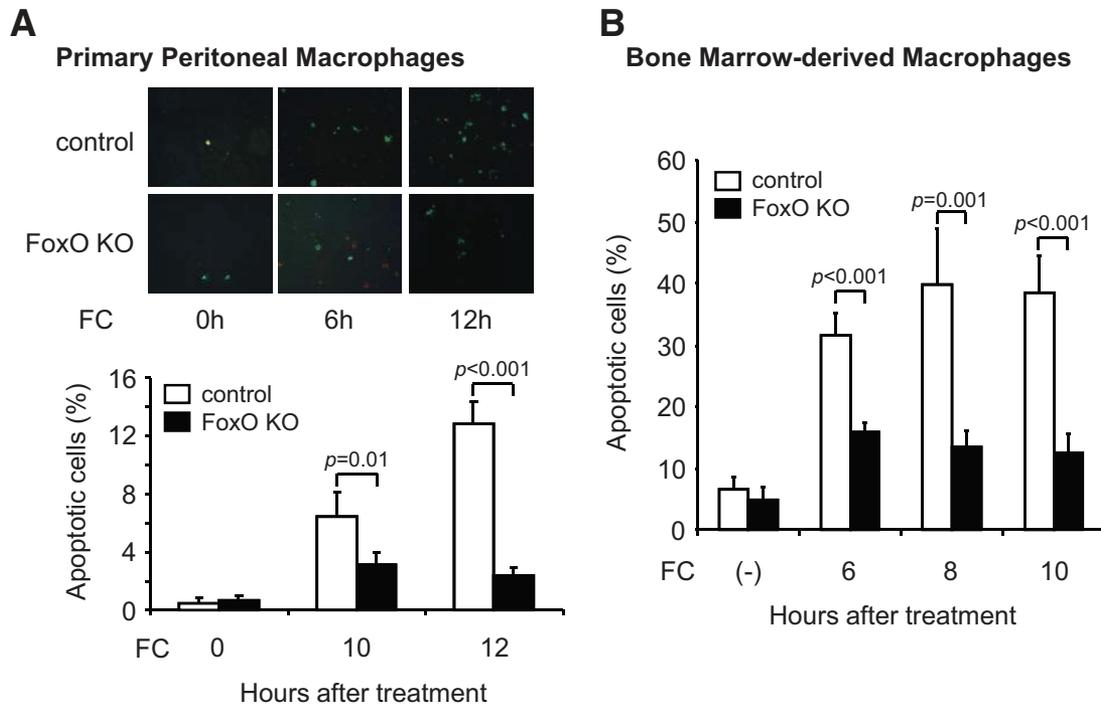
FIG. 4. Proapoptotic roles of FoxO in endoplasmic reticulum–stressed macrophages. *A*: ConA-elicited primary peritoneal macrophages from FoxO 1/3/4 knockout (FoxO KO) or control mice were left untreated or loaded with FC for 10 or 12 h and stained with annexin V and propidium iodide for apoptosis assay. *B*: Bone marrow–derived macrophages from FoxO 1/3/4 knockout or control mice were loaded with FC for the indicated hours and then subjected to the apoptosis assay. (Please see http://dx.doi.org/10.2337/db08-0520 for a high-quality digital representation of this image.)

increase in apoptosis in the context of FC loading (Fig. 3*A* and *B*). These findings suggest that FoxO1 and FoxO3 have a major role in promoting apoptosis in cells undergoing the UPR in response to FC loading but not in unstressed cells. To determine whether this was a general feature of the endoplasmic reticulum stress response, we subjected macrophages to a different form of endoplasmic reticulum stress by treating them with thapsigargin, an agent that inhibits the endoplasmic reticulum Ca^{2+} pump sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA). First, we showed that thapsigargin caused an increased amount of apoptosis in $Insr^{-/-}$ relative to $Insr^{+/-}$ cells, consistent with our previous demonstration that $Insr^{-/-}$ cells are more susceptible to apoptosis induced by a variety of endoplasmic reticulum stress responses (Fig. 3*C*). Second, we showed a dramatic increase in apoptosis in cells treated with both FoxO1-ADA and thapsigargin but not in cells treated with either alone (Fig. 3*D*).

To evaluate further the role of FoxOs in apoptotic responses, we assessed the impact of FoxO1 or FoxO3 knockdown via shRNA or siRNA (small interfering RNA) approaches, respectively. The knockdown was ~50% effective on an mRNA level for each transcription factor (supplementary Fig. S1*A*, which is available in an online appendix at http://dx.doi.org/10.2337/db08-0520). There was a significant reduction of apoptosis with knockdown of either transcription factor in FC-loaded $Insr^{+/+}$ and $Insr^{-/-}$ cells. The combined knockdown of FoxO1 and FoxO3 caused a further reduction in apoptosis to a similar final level in $Insr^{+/+}$ and $Insr^{-/-}$ cells (supplementary Fig. S1*B*). Comparable results were obtained in *ob/ob* macrophages (supplementary Fig. S1*C*). These findings indicate that FoxO1 and FoxO3 are involved in the apoptosis brought about by FC loading and mediate the

enhanced apoptotic response observed in $Insr^{-/-}$ and *ob/ob* macrophages.

Macrophages express all three of the related FoxO isoforms, FoxO1, -3, and -4. To confirm the role of endogenous FoxOs in enhancing an apoptotic response during endoplasmic reticulum stress, we evaluated the response of primary peritoneal macrophages and bone marrow–derived macrophages with combined knockouts of FoxO1/3/4, obtained by injecting polyinosine-polycytidylic acid into the *Mx-Cre* transgenic mice containing floxed transgenes for each of the three FoxOs (17). Efficient knockout of all three FoxOs in bone marrow and peritoneal macrophages was confirmed by PCR (supplementary Fig. S2). This genetic system showed a markedly decreased apoptotic response of the triple-knockout macrophages in response to FC loading in both types of macrophages (Fig. 4*A* and *B*).

Mechanisms of FoxO-induced apoptosis during endoplasmic reticulum stress. We next considered three potential mechanisms to explain the effect of active FoxOs: *1*) induction of UPR genes, *2*) enhancement of the effects of SRA ligation in the context of the UPR (25), and *3*) modification of the apoptotic response to the UPR. Previous studies have shown that FoxO1 induces CHOP expression during differentiation of preadipocytes into adipocytes (26), suggesting that FoxO1-ADA could be enhancing apoptosis by increasing the UPR. However, CHOP mRNA and protein were not further induced by FoxO1-ADA or FoxO3-AAA in control, FC-loaded, or thapsigargin-treated macrophages (supplementary Fig. S3*A–C*; data not shown). A combination of UPR induction and ligation of the SRA (e.g., by the SRA ligand fucoidan) is required to see an apoptotic response in FC-loaded macrophages, and this response is much more prominent in $Insr^{-/-}$ than in $Insr^{+/+}$ macrophages (25). If FoxO1-ADA
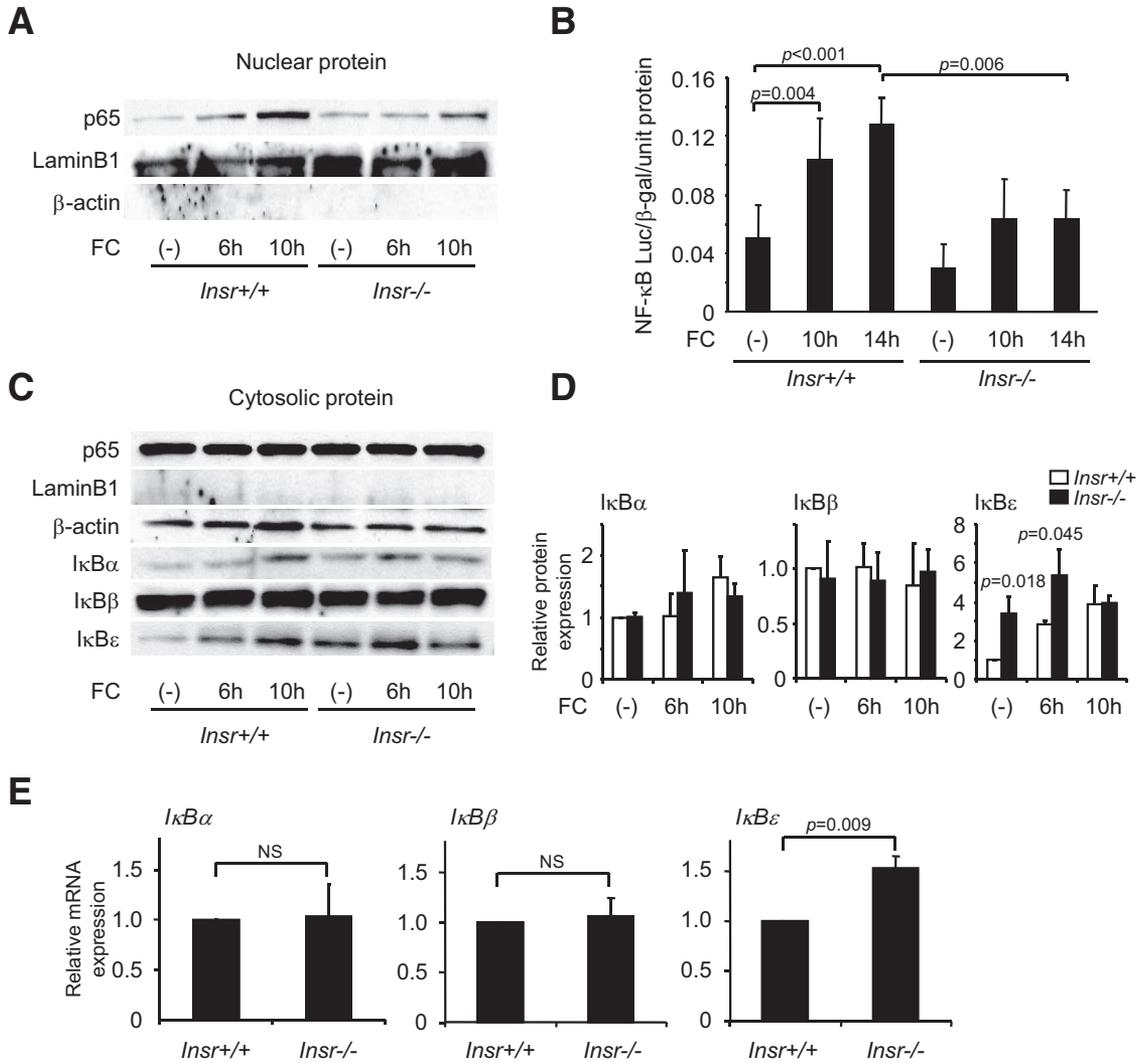
FIG. 5. Attenuated NF-κB activity and increased IκBε in *Insr*⁻/⁻ macrophages. ConA-elicited peritoneal macrophages from *Insr*⁺/⁺ and *Insr*⁻/⁻ mice were loaded with FC for the indicated hours. Cells were lysed and subjected to nuclear fractionation. *A* and *C*: Nuclear (15 µg) (*A*) and cytoplasmic (50 µg) (*B*) protein were analyzed by SDS-PAGE and immunoblotting for p65 NF-κB, the nuclear marker LaminB1, IκBs, and the cytoplasmic marker β-actin. Total RNA was extracted from ConA-elicited peritoneal macrophages from *Insr*⁺/⁺ and *Insr*⁻/⁻ mice for quantitative real-time PCR. *E*: Gene expression was normalized to β-actin expression. *D*: Data were quantified from three independent Western blot experiments. ConA-elicited peritoneal macrophages were transduced with adenovirus containing NF-κB–responsive luciferase reporter construct and LacZ at 500 and 100 MOI, respectively, for 18 h. After FC loading for the indicated hours, cells were lysed and subjected to luciferase assay. *B*: Reporter luciferase activity was normalized to transduction efficiency evaluated by β-galactosidase activity per microgram protein.

were activating the UPR in cells undergoing FC loading by incubation with Ac-LDL + 58035, it would be anticipated that fucoidan would increase apoptosis in cells expressing FoxO1-ADA. However, the combination of FoxO1-ADA and fucoidan did not increase apoptosis (supplementary Fig. S3*D*). Moreover, in thapsigargin-treated *Insr*⁻/⁻ cells, addition of the SRA ligand Ac-LDL did not lead to a further increase in the level of apoptosis beyond that observed in *Insr*⁺/⁻ cells (Fig. 3*C*). Together, the findings tend to exclude mechanisms 1 and 2.

***Insr*⁻/⁻ and *ob/ob* macrophages display increased IκBε and decreased nuclear p65 during the UPR.** We next considered the possibility that FoxOs were modifying the response to the UPR in a way that led to increased apoptosis (mechanism 3). The UPR leads to an increase in nuclear p65 and NF-κB activity and induction of antiapoptotic genes (27,28). FC loading also leads to increased p65 and NF-κB activity (29), and blocking this response by overexpressing the IκBα super-repressor resulted in increased apoptosis (supplementary Fig. S4), similar to

previous results (30,31). The induction of nuclear p65 (Fig. 5*A*) and NF-κB activity (Fig. 5*B*) during FC loading was attenuated in *Insr*⁻/⁻ cells, and NF-κB activity was also reduced in FC-loaded *ob/ob* macrophages (supplementary Fig. S5).

FoxO3 knockout mice show lymphoid hyperplasia and autoimmune phenomena reflecting expansion of T-cell populations (32), reflecting prolonged lymphocyte survival related to increased nuclear p65 and NF-κB activity. The increase in nuclear p65 was explained by a decrease in the mRNA and protein of IκBβ and IκBε (32). We found an increased level of IκBε but not IκBα or IκBβ in *Insr*⁻/⁻ cells in the basal state and during the early response (6 h) to FC loading (Fig. 5*C* and *D*). IκBε mRNA was similarly increased in *Insr*⁻/⁻ cells (Fig. 5*E*). After 10 h of FC loading, the differential response of IκBε was eliminated because of a more prominent increase of IκBε in *Insr*⁺/⁺ cells than in *Insr*⁻/⁻ cells, likely reflecting the more robust increase of nuclear p65 in *Insr*⁺/⁺ cells (Fig. 5*A*) and the subsequent feedback induction of IκBε (33,34). *ob/ob*
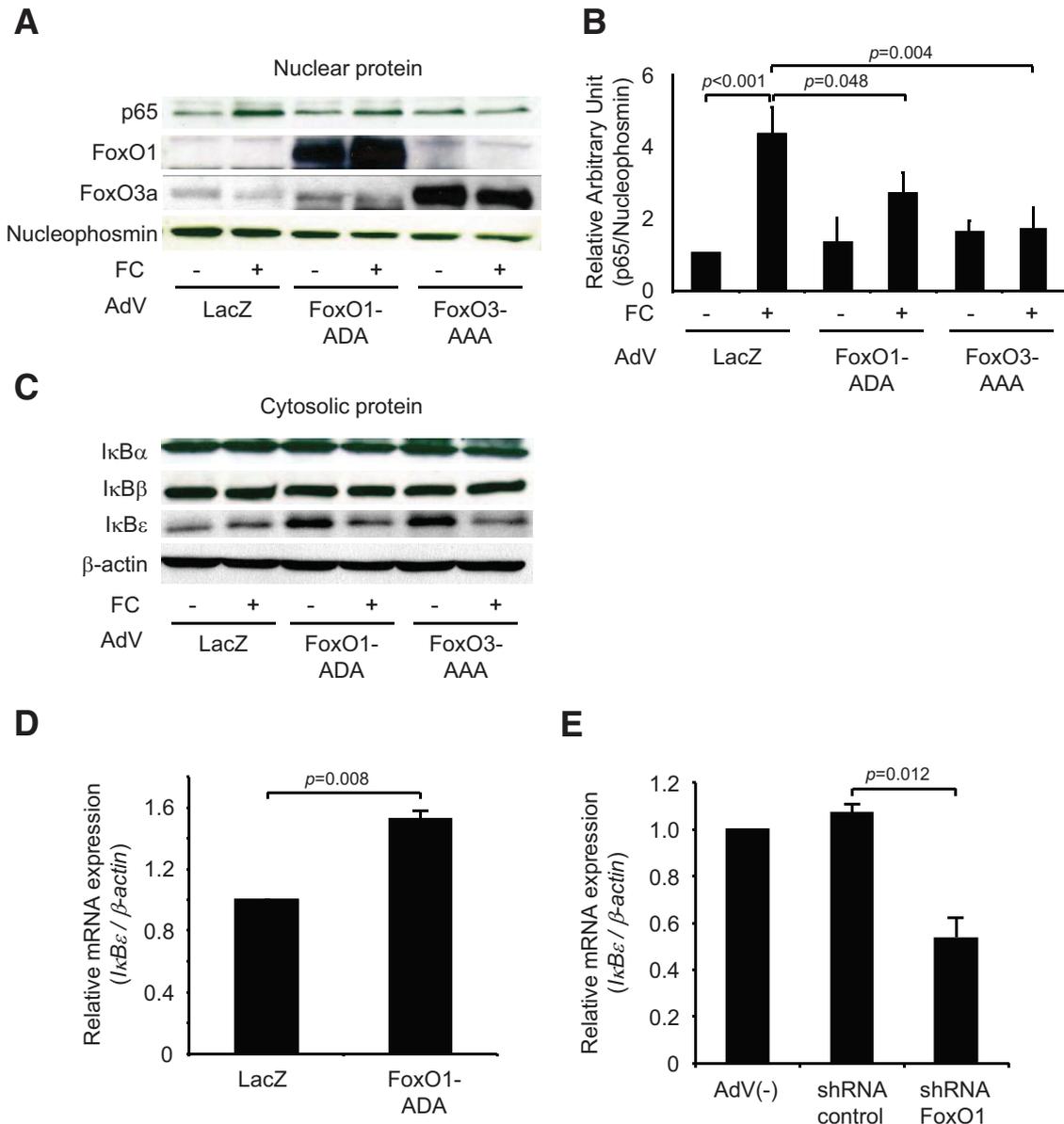
FIG. 6. FoxOs decrease FC-induced NF-κB nuclear translocation and increase IκBε. ConA-elicited peritoneal macrophages were transduced with adenovirus encoding FoxO1-ADA or FoxO3-AAA for 18 h followed by loading with FC for 10 h. Cells were lysed and subjected to nuclear fractionation. *A* and *C*: Nuclear (15 μg) and cytoplasmic (50 μg) proteins were analyzed by SDS-PAGE and immunoblotting for p65 NF-κB, the nuclear marker nucleophosmin, IκBs, and the cytoplasmic marker β-actin. *B*: Data were quantified from three independent Western blot experiments. Total RNA was extracted for quantitative PCR from the macrophages transducted with adenovirus encoding FoxO-ADA or specific shRNA for FoxO1 (shFoxO) after 18 or 36 h of transduction, respectively. *D* and *E*: Gene expression was normalized to β-actin expression. (Please see http://dx.doi.org/10.2337/db08-0520 for a high-quality digital representation of this image.)

macrophages also showed increased levels of IκBε (supplementary Fig. S5*B* and *C*). Because IκBs bind p65 in the cytosol (33–36), these findings offer a plausible explanation for the decreased nuclear p65 and NF-κB activity seen in *Insr*$^{-/-}$ and *ob/ob* macrophages.

FoxO1 binds and activates the *IκBε* promoter. We next determined whether the increase in *IκBε* gene expression in *Insr*$^{-/-}$ cells could be linked to increased nuclear FoxOs. Consistent with this possibility, overexpression of constitutively active FoxO1 or FoxO3 resulted in increased *IκBε* mRNA and protein levels without changes in IκBα or IκBβ isoforms and a decrease in the nuclear localization of p65 in response to FC loading (Fig. 6*A–D*). The increase in IκBε was seen in non–FC-loaded macrophages but not after 10 h of FC loading, similar to the loss of the differential response of *Insr*$^{+/+}$ and *Insr*$^{-/-}$

macrophages after 10 h of FC loading (Figs. 6*C* and 5*C*). Knockdown of endogenous FoxO1 by shRNA resulted in decreased expression of *IκBε* mRNA (Fig. 6*E*). These experiments suggest that increased IκBε levels reflect increased activity of FoxOs and lead to decreased nuclear p65 in *Insr*$^{-/-}$ macrophages.

To see whether the *IκBε* gene might be a direct target of FoxOs, we overexpressed FoxO1-ADA and FoxO3-AAA in cells transfected with an *IκBε* promoter-luciferase reporter gene. A construct containing 295 bp of upstream sequence showed transactivation by FoxOs, whereas a construct containing only 159 bp of upstream sequence did not (Fig. 7*A* and *B*). ChIP analysis revealed occupancy of the −159 to −295 bp region of the *IκBε* promoter by FoxO1 (Fig. 7*C*, *top panel*). We identified three putative FoxO binding core sequences [AAA(C/T)A] in this region,
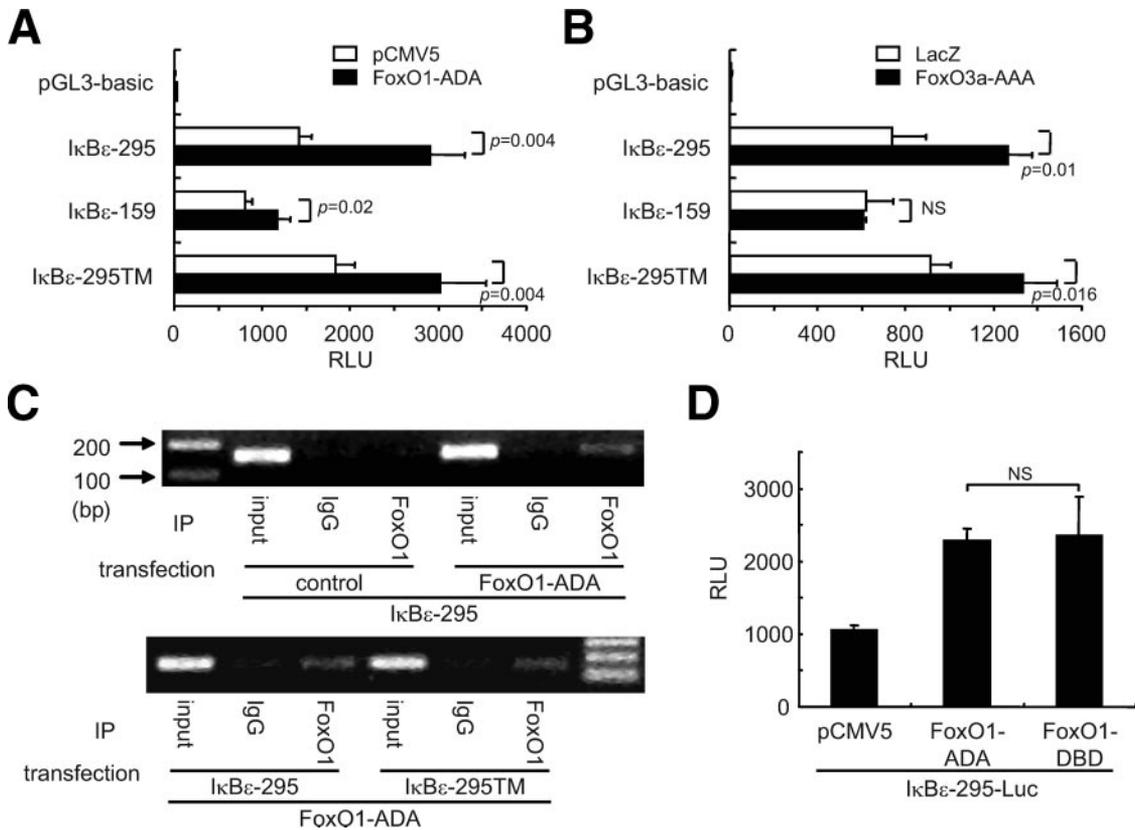
FIG. 7. FoxO upregulates IκBε transactivity by indirect interaction. RAW 264.7 cells were cultured in a 24-well plate and cotransfected with luciferase reporter plasmids (control pGL3-basic, IκBε-295, IκBε-159, or IκBε-295TM [triple mutant] [0.8 μg/well] with pRL-CMV *Renilla* luciferase vector [12.5 ng/well]) and FoxO1-ADA, DBD-FoxO1-ADA plasmid (0.8 μg/well), or FoxO3-AAA adenovirus (500 MOI) with Lipofectamine transfection reagent and cultured for 18 h. *A*, *B*, and *D*: Cells were lysed for luciferase activity measurement. The reporter luciferase activity was normalized to cotransfected *Renilla* luciferase activity. For ChIP assay, 6×10^6 of RAW 264.7 cells were cultured in a 10-cm dish. Cells were cotransfected with 6.4 μg IκBε-295 or IκBε-295TM and 6.4 μg control or FoxO1-ADA plasmid with Lipofectamine transfection reagent and cultured for 18 h. *C*: Cells were then fixed and subjected to ChIP assay.

one of which at −260 is conserved among rodents, humans, and nonhuman primates. However, using a DBD FoxO1 mutant, we still obtained transactivation of the *IκBε* promoter, consistent with the conclusion that FoxO1 activated *IκBε* transcription without direct DNA binding (Fig. 7*D*) (18,37,38). Moreover, the ChIP analysis showed binding of FoxO1 to the *IκBε* promoter containing mutations in all three potential FoxO binding sites (Fig. 7*C*, *bottom panel*). Thus, FoxO1 transactivates the proximal *IκBε* promoter likely by interacting with other molecules that directly bind the DNA in the −159 to −295 region. These findings are consistent with previous studies showing that a subset of FoxO1/3 target genes is induced without direct binding of FoxOs to target promoters (18,37,38).

Decreased expression of inflammatory and antiapoptotic genes in FC-loaded *Insr*⁻/⁻ macrophages. Previous reports have suggested either increased apoptosis or decreased inflammatory gene expression in *Insr*⁻/⁻ macrophages (14,39). The finding of reduced NF-κB activity in these cells suggested a common mechanism to explain both increased apoptosis and reduced inflammatory responses. Consistent with this idea, we found decreased expression of several antiapoptotic genes (known targets of NF-κB) and some but not all proinflammatory genes in *Insr*⁻/⁻ cells treated with FC loading (Fig. 8).

DISCUSSION

Our findings are consistent with the hypothesis that a blunted Akt response in insulin-resistant macrophages promotes apoptosis during the endoplasmic reticulum stress response and show that a major underlying mechanism involves increased activity of FoxO transcription factors. FoxO1 binds the promoter and induces expression of IκBε, blunting the p65/NF-κB response to endoplasmic reticulum stress, with proapoptotic and anti-inflammatory consequences. These studies are likely relevant to the mechanisms of increased macrophage apoptosis, necrotic core formation, and plaque destabilization in type 2 diabetic individuals (5) and suggest that insulin-sensitizing agents, such as peroxisome proliferator–activated receptor-γ agonists that reduce macrophage insulin resistance in vivo (15), could have beneficial effects in diabetic atherosclerosis by promoting plaque stabilization.

The closely related FoxO transcription factors FoxO1, -3, and -4 have a variety of different roles in modulating cell cycle control, apoptosis, and antioxidant responses with substantial redundancy and variable effects dependent on cell type and context (40). A recent study has revealed fundamental and overlapping roles of FoxO1, -3, and -4 in cell cycle control, induction of antioxidant molecules, and protection from apoptosis in hematopoietic stem cells (41). In contrast, in the present study, macrophages with knockout of all three FoxOs showed increased survival during endoplasmic reticulum stress induced by FC loading or growth factor withdrawal. Overexpression of FoxOs in various immortalized cell lines has been shown to induce apoptosis, and FoxO1
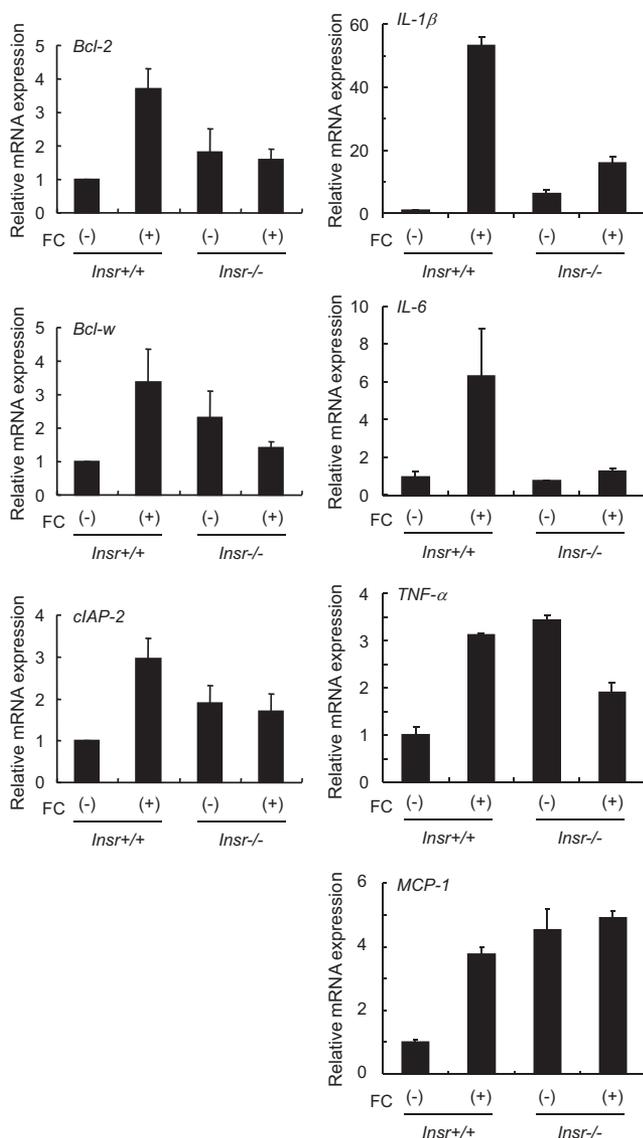
FIG. 8. Antiapoptotic and inflammatory gene expression was attenuated in FC-loaded $Insr^{-/-}$ macrophages. ConA-elicited peritoneal macrophages from $Insr^{+/+}$ control and $Insr^{-/-}$ mice were left untreated or loaded with FC for 12 h, and total RNA was extracted for quantitative real-time PCR. Gene expression was normalized to β-actin expression.

and/or FoxO3 have been found to directly target a variety of proapoptotic factors, such as Bim, Fas-ligand, and TRAIL (tumor necrosis factor–related apoptosis-inducing ligand). However, increased expression of these molecules did not appear to be involved in the increased apoptosis shown by primary cultures of $Insr^{-/-}$ macrophages (C.P.L., unpublished data). CHOP induction of Bim increases apoptosis in response to the UPR in a variety of cell types, including peritoneal macrophages (42). Measurements of Bim mRNA and protein showed increased expression in response to FC loading, but this response was not increased further in $Insr^{-/-}$ macrophages (supplementary Fig. S6). Importantly, although FoxO1/3 overexpression or knockdown had little effect on macrophage apoptosis in the basal condition, there were major effects during the endoplasmic reticulum stress response, defining a physiological role of these transcription factors in modulating macrophage apoptosis as part of the UPR. Thus, our findings

suggest that the outcome of FoxO-dependent transcription is critically dependent on the local context and that the definition of FoxO proteins as pro- or antiapoptotic is an oversimplification.

At least a part of the role of FoxOs in promoting apoptosis during endoplasmic reticulum stress involved an attenuation of the increased NF-κB response that is characteristic of the UPR and has an antiapoptotic function in this context (43). The mechanism of increased NF-κB activity during the UPR is not well understood (12). However, the blunting of this response in $Insr^{-/-}$ cells appears to involve a specific increase in levels of IκBε that likely retains p65 in the cytoplasm. Our studies establish the underlying molecular mechanism to be transactivation of the proximal $I\kappa B\varepsilon$ promoter via an indirect binding of FoxO1/3. However, this is not necessarily the only mechanism by which FoxOs influence apoptosis during the endoplasmic reticulum stress response, and this area warrants additional investigation.

Our findings are likely relevant to the increased complications of atherosclerosis suffered by diabetic individuals. Increased numbers of apoptotic macrophages associated with large necrotic cores and thin caps are characteristic of unstable plaques (44,45), and unstable plaques show evidence of increased endoplasmic reticulum stress responses (7). A number of different potential endoplasmic reticulum stressors, including an increased content of free cholesterol, are likely relevant in advanced plaques (45,46). Although most of our studies were performed using the FC loading method to induce apoptosis, the increase in apoptosis of insulin-resistant macrophages during the endoplasmic reticulum stress response appears to be generic, whether induced by FC loading, oxidized LDL, thapsigargin, or growth factor withdrawal (14), and thus is likely relevant to increased atherosclerotic complications in diabetic individuals. Interestingly, administration of an ACAT (acyl-coenzyme A: cholesterol acyltransferase) inhibitor (pactimibe) to humans led to a worsening of coronary atherosclerosis, especially in diabetic patients and subjects with BMI >30 (47), suggesting direct relevance of mechanisms defined here to diabetic atherosclerosis.

Diminished levels of nuclear p65 and NF-κB activity observed in $Insr^{-/-}$ cells during the endoplasmic reticulum stress response were associated with both increased apoptosis and decreased expression of some inflammatory genes, reflecting the well-known role of NF-κB in inducing these genes. This could perhaps explain previous findings of both increased apoptosis and decreased inflammation in different studies involving insulin receptor–deficient bone marrow cells in murine atherosclerosis models (14,39). However, in the complex milieu of advanced atherosclerotic plaques, it is likely that increased apoptosis of macrophages is associated with a clearance defect of apoptotic cells, leading to postapoptotic necrosis and inflammatory changes (45). Also, it is notable that in the basal state, $Insr^{-/-}$ macrophages showed increased expression of a number of different inflammatory genes (Fig. 8). Overall, our studies suggest that insulin-resistant macrophages are more likely to undergo apoptosis and to promote plaque inflammation.

REFERENCES

1. Ginsberg HN: Insulin resistance and cardiovascular disease. *J Clin Invest* 106:453–458, 2000
2. Reaven GM: Multiple CHD risk factors in type 2 diabetes: beyond hyperglycaemia. *Diabetes Obes Metab* 4 (Suppl. 1):S13–S18, 2002
3. Fernandez-Hernando C et al.: Loss of akt1 leads to severe atherosclerosis and occlusive coronary artery disease. *Cell Metab* 6:446–457, 2007
4. Rask-Madsen C et al.: Loss of insulin signaling in vascular endothelium accelerates atherosclerosis in apolipoprotein E knockout mice (Abstract). *AHA Abstract Circulation.* Dallas, TX, American Heart Association, 2006 (Presentation no. 260)
5. Burke AP et al.: Morphologic findings of coronary atherosclerotic plaques in diabetics: a postmortem study. *Arterioscler Thromb Vasc Biol* 24:1266–1271, 2004
6. Feng B et al.: The endoplasmic reticulum is the site of cholesterol-induced cytotoxicity in macrophages. *Nat Cell Biol* 5:781–792, 2003
7. Myoishi M et al.: Increased endoplasmic reticulum stress in atherosclerotic plaques associated with acute coronary syndrome. *Circulation* 116:1226–1233, 2007
8. Zhou J et al.: Association of multiple cellular stress pathways with accelerated atherosclerosis in hyperhomocysteinemic apolipoprotein E-deficient mice. *Circulation* 110:207–213, 2004
9. Gargalovic PS et al.: The unfolded protein response is an important regulator of inflammatory genes in endothelial cells. *Arterioscler Thromb Vasc Biol* 26:2490–2496, 2006
10. Hossain GS et al.: TDAG51 is induced by homocysteine, promotes detachment-mediated programmed cell death, and contributes to the development of atherosclerosis in hyperhomocysteinemia. *J Biol Chem* 278:30317–30327, 2003
11. Pedruzzi E et al.: NAD(P)H oxidase Nox-4 mediates 7-ketocholesterol-induced endoplasmic reticulum stress and apoptosis in human aortic smooth muscle cells. *Mol Cell Biol* 24:10703–10717, 2004
12. Marciniak SJ, Ron D: Endoplasmic reticulum stress signaling in disease. *Physiol Rev* 86:1133–1149, 2006
13. Seimon TA et al.: Combinatorial pattern recognition receptor signaling alters the balance of life and death in macrophages. *Proc Natl Acad Sci U S A* 103:19794–19799, 2006
14. Han S et al.: Macrophage insulin receptor deficiency increases ER stress-induced apoptosis and necrotic core formation in advanced atherosclerotic lesions. *Cell Metab* 3:257–266, 2006
15. Liang CP et al.: Increased CD36 protein as a response to defective insulin signaling in macrophages. *J Clin Invest* 113:764–773, 2004
16. Liang CP et al.: The macrophage at the crossroads of insulin resistance and atherosclerosis. *Circ Res* 100:1546–1555, 2007
17. Paik JH et al.: FoxOs are lineage-restricted redundant tumor suppressors and regulate endothelial cell homeostasis. *Cell* 128:309–323, 2007
18. Kitamura T et al.: A Foxo/Notch pathway controls myogenic differentiation and fiber type specification. *J Clin Invest* 117:2477–2485, 2007
19. Nakae J et al.: The forkhead transcription factor Foxo1 (Fkhr) confers insulin sensitivity onto glucose-6-phosphatase expression. *J Clin Invest* 108:1359–1367, 2001
20. Matsumoto M et al.: Dual role of transcription factor FoxO1 in controlling hepatic insulin sensitivity and lipid metabolism. *J Clin Invest* 116:2464–2472, 2006
21. Hellerbrand C et al.: Inhibition of NFkappaB in activated rat hepatic stellate cells by proteasome inhibitors and an IkappaB super-repressor. *Hepatology* 27:1285–1295, 1998
22. Nakae J et al.: Insulin stimulates phosphorylation of the forkhead transcription factor FKHR on serine 253 through a Wortmannin-sensitive pathway. *J Biol Chem* 274:15982–15985, 1999
23. Essers MA et al.: FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK. *EMBO J* 23:4802–4812, 2004
24. Kawamori D et al.: The forkhead transcription factor Foxo1 bridges the JNK pathway and the transcription factor PDX-1 through its intracellular translocation. *J Biol Chem* 281:1091–1098, 2006
25. Devries-Seimon T et al.: Cholesterol-induced macrophage apoptosis requires ER stress pathways and engagement of the type A scavenger receptor. *J Cell Biol* 171:61–73, 2005
26. Murphy CT et al.: Genes that act downstream of DAF-16 to influence the lifespan of *Caenorhabditis elegans. Nature* 424:277–283, 2003
27. Deng J et al.: Translational repression mediates activation of nuclear factor kappa B by phosphorylated translation initiation factor 2. *Mol Cell Biol* 24:10161–10168, 2004
28. Jiang HY et al.: Phosphorylation of the alpha subunit of eukaryotic initiation factor 2 is required for activation of NF-kappaB in response to diverse cellular stresses. *Mol Cell Biol* 23:5651–5663, 2003
29. Li Y et al.: Free cholesterol-loaded macrophages are an abundant source of tumor necrosis factor-alpha and interleukin-6: model of NF-kappaB- and map kinase-dependent inflammation in advanced atherosclerosis. *J Biol Chem* 280:21763–21772, 2005
30. Cui D et al.: Pivotal advance: macrophages become resistant to cholesterol-induced death after phagocytosis of apoptotic cells. *J Leukoc Biol* 82:1040–1050, 2007
31. Thorp E et al.: Pioglitazone increases macrophage apoptosis and plaque necrosis in advanced atherosclerotic lesions of nondiabetic low-density lipoprotein receptor-null mice. *Circulation* 116:2182–2190, 2007
32. Lin L et al.: Regulation of NF-kappaB, Th activation, and autoinflammation by the forkhead transcription factor Foxo3a. *Immunity* 21:203–213, 2004
33. Li Z, Nabel GJ: A new member of the I kappaB protein family, I kappaB epsilon, inhibits RelA (p65)-mediated NF-kappaB transcription. *Mol Cell Biol* 17:6184–6190, 1997
34. Whiteside ST et al.: I kappa B epsilon, a novel member of the I kappa B family, controls RelA and cRel NF-kappa B activity. *EMBO J* 16:1413–1426, 1997
35. Baeuerle PA, Baltimore D: I kappa B: a specific inhibitor of the NF-kappa B transcription factor. *Science* 242:540–546, 1988
36. Beg AA et al.: I kappa B interacts with the nuclear localization sequences of the subunits of NF-kappa B: a mechanism for cytoplasmic retention. *Genes Dev* 6:1899–1913, 1992
37. Liu ZP et al.: Phenotypic modulation of smooth muscle cells through interaction of Foxo4 and myocardin. *Dev Cell* 9:261–270, 2005
38. Ramaswamy S et al.: A novel mechanism of gene regulation and tumor suppression by the transcription factor FKHR. *Cancer Cell* 2:81–91, 2002
39. Baumgartl J et al.: Myeloid lineage cell-restricted insulin resistance protects apolipoproteinE-deficient mice against atherosclerosis. *Cell Metab* 3:247–256, 2006
40. Burgering BM et al: Cell cycle and death control: long live Forkheads. *Trends Biochem Sci* 27:352–360, 2002
41. Tothova Z et al.: FoxOs are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress. *Cell* 128:325–339, 2007
42. Puthalakath H et al.: ER stress triggers apoptosis by activating BH3-only protein Bim. *Cell* 129:1337–1349, 2007
43. Mauro C et al.: Central role of the scaffold protein tumor necrosis factor receptor-associated factor 2 in regulating endoplasmic reticulum stress-induced apoptosis. *J Biol Chem* 281:2631–2638, 2006
44. Libby P et al.: Macrophages and atherosclerotic plaque stability. *Curr Opin Lipidol* 7:330–335, 1996
45. Tabas I: Consequences and therapeutic implications of macrophage apoptosis in atherosclerosis: the importance of lesion stage and phagocytic efficiency. *Arterioscler Thromb Vasc Biol* 25:2255–2264, 2005
46. Tabas I et al.: Subendothelial lipoprotein retention as the initiating process in atherosclerosis: update and therapeutic implications. *Circulation* 116:1832–1844, 2007
47. Nissen SE et al.: Effect of ACAT inhibition on the progression of coronary atherosclerosis. *N Engl J Med* 354:1253–1263, 2006