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

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters

Citation

Published Version
doi:10.2337/db08-0520

Citable link
http://nrs.harvard.edu/urn-3:HUL.InstRepos:4882674

Terms of Use
This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA
Forkhead Transcription Factors (FoxOs) Promote Apoptosis of Insulin-Resistant Macrophages During Cholesterol-Induced Endoplasmic Reticulum Stress

Takafumi Senokuchi,1 Chien-Ping Liang,1 Tracie A. Seimon,1 Seongah Han,1 Michihiro Matsumoto,1 Alexander S. Banks,1 Ji-Hye Paik,2 Ronald A. DePinho,2 Domenico Accili,1 Ira Tabas,1,3,4 and Alan R. Tall1,3,4

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 κB (IKB) gene expression and thereby to attenuate the increase of nuclear p65 and nuclear factor-kB 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

From the 1Department of Medicine, Columbia University, New York, New York; the 2Department of Medical Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts; the 3Department of Anatomy and Cell Biology, Columbia University, New York, New York; and the 4Department of Physiology and Cellular Biophysics, Columbia University, New York, New York.

Corresponding author: Takafumi Senokuchi, senokuchi@fc.kuh.kumamoto-u.ac.jp.

Received 18 April 2008 and accepted 7 August 2008.

Published online ahead of print at http://diabetes.diabetesjournals.org on 26 August 2008. DOI: 10.2337/db08-0520.

© 2008 by the American Diabetes Association. Readers may use this article as long as the work is properly cited, the use is educational and not for profit, and the work is not altered. See http://creativecommons.org/licenses/by-nc-nd/3.0/ for details.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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...
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 phosphorylation 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+/−...
control short hairpin RNA (shRNA), FoxO1 shRNA (20), and nuclear factor-
Primer sequences are available on request.

generated using the QuikChange II Site-Directed Mutagenesis kit (Stratagene).

sequence of mouse inhibitor of DBD FoxO1-ADA were previously described (18). The upstream
/H11002

I

GGGATCTAACT-3

(100 g/ml acetylated LDL for the indicated time periods.

Bone marrow–derived macrophages were differentiated from marrow cells by

fetal bovine serum and 20% L-cell conditioned medium) until 90% confluent.

culture medium (Dulbecco’s modified Eagle’s medium supplemented with 10%

in 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 dBd FoxO1-ADA (20) was amplified from mouse genomic DNA by PCR using the following primers: 5’-AAGTAGGAAGACCCACGGACC-3’ and 5’-AGTGAGCTTAGGTCCACGGGGATCTAACT-3’ and cloned into pGL3-Basic luciferase reporter plasmid (Ikebe-295-Luc). Deletion mutants were generated by standard procedures. Ikebe-295STM (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 QuickChange 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-xB
(NF-xB)-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-xB localization. Nuclear and cytoplasmic proteins were extracted
using Nuclear Extraction kit (Panomics). Western blot was carried out using
p65 NF-xB (P-6) from Santa Cruz Biotechnology for primary antibody. LaminB1 (Santa Cruz Biotechnology) or nucleophosmin (Zymed Laboratories)
were used as loading controls.

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. 2. FoxO is preferentially localized to the nucleus in FC-loaded or insulin-resistant macrophages. 

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 dBd FoxO1-ADA (20) was amplified from mouse genomic DNA by PCR using the following primers: 5’-AAGTAGGAAGACCCACGGACC-3’ and 5’-AGTGAGCTTAGGTCCACGGGGATCTAACT-3’ and cloned into pGL3-Basic luciferase reporter plasmid (Ikebe-295-Luc). Deletion mutants were generated by standard procedures. Ikebe-295STM (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 QuickChange 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-xB
(NF-xB)-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-xB localization. Nuclear and cytoplasmic proteins were extracted
using Nuclear Extraction kit (Panomics). Western blot was carried out using
p65 NF-xB (P-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) over-

expression construct (21) were previously described. Expression
of FoxO1–green fluorescent protein (GFP) was transduced in primary macro-

pheres 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 × 10⁶ 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
cell nuclei were stained with 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.)
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+/H11001/H11001 cells and Insr+/H11002/H11002 cells (Fig. 1A). 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. 1B). 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. 1C). 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.

**FoxOs in Insulin-Resistant Macrophage Apoptosis**

**FIG. 3.** FoxOs enhance endoplasmic reticulum–stressed macrophage apoptosis. ConA-elicited peritoneal macrophages were transduced 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.

**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 NH2-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. 2A shows FoxO1-GFP; Fig. 2B 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. 2C). 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
increase in apoptosis in the context of FC loading (Fig. 3A 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 \(\text{Insr}^{-/-}\) relative to \(\text{Insr}^{+/+}\) cells, consistent with our previous demonstration that \(\text{Insr}^{-/-}\) cells are more susceptible to apoptosis induced by a variety of endoplasmic reticulum stress responses (Fig. 3C). 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. 3D).

To evaluate further the role of FoxOs in apoptotic responses, we assessed the impact of FoxO1 or FoxO3 knockout via shRNA or siRNA (small interfering RNA) approaches, respectively. The knockdown was assessed by qPCR for apoptosis assay.

To evaluate further the role of FoxOs in apoptotic responses, we assessed the impact of FoxO1 or FoxO3 knockout via shRNA or siRNA (small interfering RNA) approaches, respectively. The knockdown was assessed by qPCR for apoptosis assay.

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 polynosine-polyctydilic acid into the \(\text{Mx-Cre}\) 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. 4A 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. S3A–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 \(\text{Insr}^{-/-}\) than in \(\text{Insr}^{+/+}\) macrophages (25). If FoxO1-ADA...
NF-κB activity and increased IkBε in Insr<sup>−/−</sup> macrophages. ConA-elicited peritoneal macrophages from Insr<sup>+/+</sup> and Insr<sup>−/−</sup> mice were loaded with FC for the indicated hours. Cells were lysed and subjected to luciferase assay. 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, IkBε, and the cytoplasmic marker β-actin. Total RNA was extracted from ConA-elicited peritoneal macrophages from Insr<sup>+/+</sup> and Insr<sup>−/−</sup> mice for quantitative real-time PCR. B: 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-CLDL + 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. S4). FOXOs IN INSULIN-RESISTANT MACROPHAGE APOPTOSIS

FIG. 5. Attenuated NF-κB activity and increased IkBε in Insr<sup>−/−</sup> macrophages. ConA-elicited peritoneal macrophages from Insr<sup>+/+</sup> and Insr<sup>−/−</sup> 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, IkBε, and the cytoplasmic marker β-actin. Total RNA was extracted from ConA-elicited peritoneal macrophages from Insr<sup>+/+</sup> and Insr<sup>−/−</sup> 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.

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 IkBβ and IkBε (32). We found an increased level of IkBε but not IkBα or IkBβ in Insr<sup>−/−</sup> cells in the basal state and during the early response (6 h) to FC loading (Fig. 5C and D). IkBε mRNA was similarly increased in Insr<sup>−/−</sup> cells (Fig. 5E). After 10 h of FC loading, the differential response of IkBε was eliminated because of a more prominent increase of IkBε in Insr<sup>+/+</sup> cells than in Insr<sup>−/−</sup> cells, likely reflecting the more robust increase of nuclear p65 in Insr<sup>−/−</sup> cells (Fig. 5A) and the subsequent feedback induction of IkBε (33,34). ob/ob
macrophages also showed increased levels of IkBα (supplementary Fig. S5B and C). Because IkBα 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 IkBα promoter.** We next determined whether the increase in IkBα 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 IkBα mRNA and protein levels without changes in IkBα or IkBβ isoforms and a decrease in the nuclear localization of p65 in response to FC loading (Fig. 6A–D). The increase in IkBα 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. 6C and 5C). Knockdown of endogenous FoxO1 by shRNA resulted in decreased expression of IkBα mRNA (Fig. 6E). These experiments suggest that increased IkBα levels reflect increased activity of FoxOs and lead to decreased nuclear p65 in Insr−/− macrophages.

To see whether the IkBα gene might be a direct target of FoxOs, we overexpressed FoxO1-ADA and FoxO3-AAA in cells transfected with an IkBα 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. 7A and B). ChIP analysis revealed occupancy of the −159 to −295 bp region of the IkBα promoter by FoxO1 (Fig. 7C, top panel). We identified three putative FoxO binding core sequences [AAA(C/T)A] in this region,
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. 7D) (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. 7C, 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
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 \( \text{Insr}^{-/-} \) 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 \( \text{Insr}^{-/-} \) 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-\( \kappa \)B response that is characteristic of the UPR and has an antiapoptotic function in this context (43). The mechanism of increased NF-\( \kappa \)B activity during the UPR is not well understood (12). However, the blunting of this response in \( \text{Insr}^{-/-} \) cells appears to involve a specific increase in levels of IkB\( \alpha \) that likely retains \( \text{p65} \) in the cytoplasm. Our studies establish the underlying molecular mechanism to be transactivation of the proximal IkB\( \alpha \) 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 \( \text{p65} \) and NF-\( \kappa \)B activity observed in \( \text{Insr}^{-/-} \) 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-\( \kappa \)B 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, \( \text{Insr}^{-/-} \) 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.

**ACKNOWLEDGMENTS**

T.S. is the recipient of a research fellowship from the Manpei Suzuki Diabetes Foundation of Tokyo. This work
was supported by National Institutes of Health Grants HL-75662 and HL-87123.
We thank Dr. Alexander Banks for the adenovirus encoding FoxO1-GFP.

REFERENCES

34. Whiteside ST et al.: FoxOs IN INSULIN-RESISTANT MACROPHAGE APOPTOSIS
35. Tothova Z et al.: FoxOs are critical mediators of hematopoietic stem cell survival and development. Dev Cell 9:261–270, 2005