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, macropahges 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 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.

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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*<sup>−/−</sup>/*Insr*<sup>+/+</sup> mice into *Ldlr*<sup>−/−</sup> recipients. This was associated with a dramatic increase in necrotic core formation, reflecting increased numbers of apoptotic macrophages (14). *Insr*<sup>−/−</sup> macrophages showed increased susceptibility to apoptosis induced by loading with free cholesterol or oxidized LDL. In *Insr*<sup>−/−</sup> 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 C57BL/6J *Insr*<sup>−/−</sup> (14) and *Ldlr*<sup>−/−</sup> mice. The *Ldlr*<sup>−/−</sup> *Insr*<sup>−/−</sup>
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 incubating cells in culture medium containing 10 μg/ml acetylated LDL for the indicated time periods.

**Chromatin immunoprecipitation assays.** Chromatin immunoprecipitation (ChIP) assay was performed with ChIP Assay kit (Millipore). After plasmid transfection, six × 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 precleaving 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 l salmon sperm DNA/protein A-Sepharose. After releasing crosslink, purified DNA was subjected to PCR using the following primers: 5′-TGAATGAGACACCGGAGTG-3′ and 5′-GCTTGCGTAGGCAGGGAGTC-3′ and cloned into pGL3-Basic luciferase reporter plasmid (Stratagene). Primer sequences are available on request.

**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.

**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.
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 $\text{Insr}^{+/+}$ and $\text{Insr}^{-/-}$ 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 $\text{ob}/\text{ob}$ macrophages. Enhanced apoptosis in response to FC loading was also seen in $\text{ob}/\text{ob}$ 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 $\text{Insr}^{-/-}$ control or $\text{Insr}^{-/-}$ 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 $\text{Insr}^{-/-}$ macrophages (Fig. 2A shows FoxO1-GFP; Fig. 2B shows endogenous nuclear FoxO1). $\text{ob}/\text{ob}$ 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 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. 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 knockdown via siRNA or siRNA (small interfering RNA) approaches, respectively. The knockdown was ~50\% effective on an mRNA level for each transcription factor (supplementary Fig. S1A, 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 knockout of FoxO1 and FoxO3 caused a further reduction in apoptosis to a similar final level in Insr\(^{+/+}\) and Insr\(^{-/-}\) cells (supplementary Fig. S1B). Comparable results were obtained in ob/ob macrophages (supplementary Fig. S1C). 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 polynosine-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. 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 fusidian) 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
were activating the UPR in cells undergoing FC loading by incubation with Ac-βCLDL + 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. S3D). Moreover, in thapsigargin-treated insr−/− cells, addition of the SRA ligand Ac-βCLDL did not lead to a further increase in the level of apoptosis beyond that observed in insr−/− cells (Fig. 3C). Together, the findings tend to exclude mechanisms 1 and 2.

**insr−/− and ob/ob macrophages display increased IkBε 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 IkBα super-repressor resulted in increased apoptosis (supplementary Fig. S4), similar to previous results (30,31). The induction of nuclear p65 (Fig. 5A) and NF-κB activity (Fig. 5B) 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 IkBε and IkBe (32). We found an increased level of IkBε but not IkBα or IkBβ in insr−/− cells in the basal state and during the early response (6 h) to FC loading (Fig. 5C and D). IkBe mRNA was similarly increased in insr−/− cells (Fig. 5E). After 10 h of FC loading, the differential response of IkBε was eliminated because of a more prominent increase of IkBe in insr−/− cells than in insr−/− cells, likely reflecting the more robust increase of nuclear p65 in insr−/− cells (Fig. 5A) and the subsequent feedback induction of IkBe (33,34). ob/ob
macrophages also showed increased levels of IkBα (supplementary Fig. S5B and C). Because IkBs 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<sup>−/−</sup> and ob/ob macrophages.

**FoxO1 binds and activates the IkBε promoter.** We next determined whether the increase in IkBε gene expression in Insr<sup>−/−</sup> cells could be linked to increased nuclear FoxOs. Consistent with this possibility, overexpression of constitutively active FoxO1 or FoxO3 resulted in increased IkBε 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 IkBε 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<sup>−/−</sup> and Insr<sup>−/−</sup> macrophages after 10 h of FC loading (Figs. 6C and 5C). Knockdown of endogenous FoxO1 by shRNA resulted in decreased expression of IkBε mRNA (Fig. 6E). These experiments suggest that increased IkBε levels reflect increased activity of FoxOs and lead to decreased nuclear p65 in Insr<sup>−/−</sup> macrophages.

To see whether the IkBε gene might be a direct target of FoxOs, we overexpressed FoxO1-ADA and FoxO3-AAA in cells transfected with an IkBε 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 IkBε 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 IkBε promoter, consistent with the conclusion that FoxO1 activated IkBε transcription without direct DNA binding (Fig. 7D) (18,37,38). Moreover, the ChiP analysis showed binding of FoxO1 to the IkBε promoter containing mutations in all three potential FoxO binding sites (Fig. 7C, bottom panel). Thus, FoxO1 transactivates the proximal IkBε 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<sup>−/−</sup> macrophages. Previous reports have suggested either increased apoptosis or decreased inflammatory gene expression in Insr<sup>−/−</sup> 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<sup>−/−</sup> 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 IkBε, 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 insulin receptor–deficient macrophages (42). Measurements of Bim mRNA and protein showed increased expression in response to FC loading, but this response was not increased further in insulin receptor–deficient 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 IκBα that likely retains p65 in the cytoplasm. Our studies establish the underlying molecular mechanism to be transactivation of the proximal IκBα 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.

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