Homozygosity for an Allele Encoding Deacetylated FoxO1 Protects Macrophages From Cholesterol-Induced Inflammation Without Increasing Apoptosis

Kyoichiro Tsuchiya, Alexander S. Banks, Chien-Ping Liang, Ira Tabas, Alan R. Tall, Domenico Accili

Objective—Insulin resistance renders macrophages more prone to cholesterol-induced apoptosis by promoting nuclear localization of transcription factor FoxO1. However, FoxO1 also decreases macrophage inflammation, raising the question of how the balance between proapoptotic and antiinflammatory effects is determined. We sought to identify the mechanism whereby FoxO1 dampens inflammation without promoting apoptosis. We hypothesized that nutrient-dependent FoxO1 acetylation plays a role in this process.

Methods and Results—We generated knock-in mice bearing alleles that encode constitutively deacetylated FoxO1 and studied the ex vivo response of primary peritoneal macrophages. We show that macrophages derived from mice homozygous for constitutively deacetylated FoxO1 alleles retain antiinflammatory properties in response to free cholesterol loading, without increasing apoptosis. Deacetylated FoxO1 inhibits free cholesterol–induced Akt phosphorylation and increases levels of the nuclear factor-κB precursor p105, decreasing nuclear translocation of nuclear factor-κB p65 subunit and dampening Mek/Erk activation to prevent inflammation.

Conclusion—Deacetylated FoxO1 regulates p105 to prevent macrophage inflammation without causing apoptosis, suggesting a potential novel therapeutic approach to atherosclerosis through FoxO1 deacetylation. (Arterioscler Thromb Vase Biol. 2011;31:00-00.)

Key Words: apoptosis ■ diabetes mellitus ■ insulin resistance ■ lipids ■ macrophages

Complications from atherosclerotic cardiovascular disease are a leading cause of death.1 Macrophage inflammation in the vessel wall is a key event in the formation and rupture of atherosclerotic plaques. Various cytokines produced from lesional macrophages contribute to lesion progression.2 In early atherosclerosis, macrophages take up modified cholesterol-rich lipoproteins and store their cholesterol largely in esterified form, resulting in foam cell formation. As lesions advance, cholesteryl-ester content drops and unesterified or free cholesterol (FC) rises,3,4 likely inducing endoplasmic reticulum (ER) stress and expression of transcription factor Chop.5 The in vivo relevance of ER stress–mediated Chop induction to atherosclerosis progression is demonstrated by studies in which Chop ablation halved the number of apoptotic lesional macrophages and reduced plaque size in apolipoprotein E−/− or low-density lipoprotein receptor−/− mice.6 In addition to ER stress, FC activates the mitogen-activated protein kinase (Mapk, including Erk1/2, Jnk1/2, and p38) and Ikk /nuclear factor (Nf)-κB inflammatory pathways, possibly by inducing tumor necrosis factor-α (Tnf-α) and interleukin-6 (II-6).7

Macrophages are insulin-sensitive cells.8 Defective macrophage insulin signaling predisposes to foam cell formation in insulin-resistant states9 and impairs the ability of macrophages to relieve ER stress, resulting in greater macrophage apoptosis and plaque necrosis within advanced lesions.10 In macrophages, FoxO1, FoxO3, and FoxO4 appear to have dual functions, promoting apoptosis in the context of ER stress but decreasing inflammation in response to FC11 or lipopolysaccharide (LPS).12 FoxO1 activity is regulated by posttranslational modifications to meet the cell’s metabolic demand or stress response.13 Falling nutrient levels activate FoxO1 through dephosphorylation and deacetylation, whereas rising nutrient levels inactivate FoxO1 through Akt-dependent phosphorylation, and Cbp/p300-dependent acetylation.14

Our goal was to define approaches that leverage the antiinflammatory actions of FoxO1 for therapeutic ends without promoting apoptosis. It has been shown that hyperglycemia in type 2 diabetes leads to FoxO1 acetylation.15–17 Acetylation targets FoxO1 to nuclear PML bodies, where it undergoes deacetylation to become transcriptionally active.15 Less clear is the effect of FC signaling on macrophage FoxO1 and the...
role of FoxO1 acetylation in macrophage activation. To answer these questions, we generated knock-in mice bearing alleles that encode constitutively deacetylated FoxO1 and studied the ex vivo response of primary peritoneal macrophages to FC challenge. Deacetylated FoxO1 prevented FC-induced Akt phosphorylation and increased levels of p105, the precursor protein of NF-κB subunits. p105, in turn, promoted NF-κB p65 and p50 cytoplasmic retention and dampened Erk activation, decreasing inflammation. The data are consistent with a model in which FoxO1 deacetylation can uncouple inflammation from apoptosis in FC-loaded macrophages.

Materials and Methods

Experimental Animals
Acetylation-defective FoxO1 knock-in mice (FoxO1KR/KR) have been described.18

Materials
A detailed list of manufacturers is provided in the supplemental material, available online at http://atvb.ahajournals.org.

Macrophage Culture, FC Loading, Immunoprecipitation, and Immunoblotting
We harvested peritoneal macrophages from FoxO1+/+ or FoxO1KR/KR mice by peritoneal lavage 3 days after intraperitoneal injection of 4% thioglycolate and cultured them in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 0.05% antibiotic-antimycotic (Invitrogen). We loaded cells with FC by incubation in medium supplemented with compound 58035 (10 mg/L) and acetylated low-density lipoprotein (100 mg/L) for the indicated time periods.19 Protein analysis was carried out as described.15,16

Immunocytochemistry
We performed FoxO1 immunocytochemistry as described.16 We measured apoptosis by Alexa 488–labeled annexin V and propidium iodide staining (Vybrant Apoptosis Assay kit, Invitrogen).

Quantitative Reverse Transcription–Polymerase Chain Reaction and ELISA
We performed quantitative reverse transcription–polymerase chain reaction using DyNAmo HS SYBR Green qPCR Kit (Finnzymes) as described.15

Adenoviruses
We have described adenoviruses encoding LacZ, green fluorescent protein–tagged FoxO1 wild-type (WT) and FoxO1-KR, hemagglutinin-tagged FoxO1-ADA, and NF-κB-luciferase.15,19,20,21

Luciferase Assays
We measured luciferase activity in peritoneal macrophages transfected with NF-κB reporter construct using the Dual Luciferase Reporter assay system (Promega).

Statistical Analysis
Data are shown as mean±SE. We determined statistically significant differences (P<0.05) using the unpaired t test or ANOVA with the Dunn post hoc test.

Results

FC Loading Induces FoxO1 Acetylation in Peritoneal Macrophages
We examined the effect of FC loading on FoxO1 acetylation and subcellular localization. FC induced FoxO1 acetylation in FoxO1+/+ but not FoxO1KR/KR macrophages (Figure 1A). The latter also showed increased nuclear FoxO1 in the basal state that was unaffected by FC loading, whereas the number of WT macrophages with predominantly nuclear FoxO1 doubled in response to FC loading (Figure 1B and 1C). FoxO1 regulation by FC loading in macrophages is reminiscent of its regulation by high glucose/oxidative stress in pancreatic β-cells.15 The findings indicate that FC challenge phenocopies nutrient excess (high glucose) to promote FoxO1 acetylation, whereas deacetylation is required to keep FoxO1 in the nucleus.

Blunted Induction of Inflammatory Cytokines in FC-Loaded FoxO1KR/KR Macrophages
FC loading increased levels of mRNAs encoding monocyte chemotactant protein-1 (Mcp-1), Tnf-α, Il-6, Il-1β, Rantes, macrophage inflammatory protein–1α (Mip-1α), Mip-1β, Mip-2, Tgf-β1, and osteopontin (encoded by Spp1) between 2- and 8-fold in WT macrophages, but these effects were absent (Mcp-1, Tnf-α, Il-6, Mip-1β, Mip-2) or blunted (Rantes, Tnf-α, Il-1β, Mip-2) in FoxO1KR/KR macrophages (Figure 1D). Levels of Mip-1α, Tgf-β1, and Spp1 were decreased also in the basal state in FoxO1KR/KR macrophages (Figure 1D). In contrast, levels of the antiinflammatory cytokine Il-10 were comparable between the 2 genotypes. The changes in mRNA were paralleled by a 5-fold increase in Mcp-1 release in Mip-1 in the culture medium in WT macrophages that was totally absent in FoxO1KR/KR macrophages (Figure 1E). Release of Tnf-α and Il-6 increased >100-fold in FoxO1+/+ cells and was blunted by 40% and 60%, respectively, in FoxO1KR/KR cells (Figure 1F and 1G).

In contrast, the response of FoxO1KR/KR macrophages to the Toll-like receptor-2 agonist zymosan, and to the Toll-like receptor-4 agonist LPS did not show a clear-cut antiinflammatory pattern and, in some instances (eg, LPS-stimulated Il-6; zymosan- and LPS-stimulated Il-10, and LPS-stimulated Tnf-α induction) were more pronounced in FoxO1KR/KR than in FoxO1+/+ macrophages (Supplemental Figure IA and IB).

FoxO1-KR Does Not Increase FC-Induced Apoptosis
FC increases macrophage apoptosis by activating the Chop branch of the unfolded protein response.5,6 The phosphorylation-defective mutant FoxO1, FoxO1-ADA (in which the 3 main sites of insulin-induced phosphorylation have been mutated),22 is constitutively nuclear and exacerbates this effect.11 As the FoxO1-KR mutant was also predominantly nuclear, we expected it to induce apoptosis. However, we failed to detect differences in Bip and Chop mRNA (Supplemental Figure IIA), or in the number of apoptotic macrophages in response to FC between FoxO1+/+ and FoxO1KR/KR cells (Supplemental Figure IIB and IIC). Apoptosis induced by thapsigargin, an inhibitor of the ER Ca2+ pump sarcoplasmic/ER calcium ATPase (Serca) that promotes ER stress,23 was also comparable between the 2 cell types, as was expression of type A scavenger receptor (a mediator of acetylated low-density lipoprotein uptake24), Toll-like receptor-4, and Tnf receptor 1, all of which are required for ER stress–induced Mapk activation25,26 (Supplemental Figure IIID).
Figure 1. Free cholesterol (FC)-induced inflammatory cytokine expression and protein secretion in macrophages. A, Western blots of FC-loaded FoxO1+/+ (+/+ ) and FoxO1KR/KR macrophages (KR/KR) macrophages, immunoprecipitated (IP) with anti-FoxO1 antibody followed by immunoblotting with anti-acetyl-lysine (ac-Lysine) or total FoxO1 antibodies. B, Representative immunohistochemical
Impaired Mek/Erk Activation in FoxO1KR/KR Macrophages

Macrophage FC loading increases Mcp-1, Tnf-α, and Il-6 via the Nf-κB or Mapk pathway or both7 (Supplemental Figure III). We interrogated the contribution of these 2 mechanisms to the blunted inflammatory response of FoxO1KR/KR macrophages. FC rapidly induced Mek phosphorylation by ∼2-fold and promoted biphasic Erk phosphorylation in FoxO1+/− macrophages; these responses were significantly attenuated in FoxO1KR/KR macrophages (Figure 3A–3C). In contrast, Jnk and p38 phosphorylation increased up to 8-fold and were comparable in both cell types (Figure 3A and 3D–3E).

Distinct Signaling Pathways Are Elicited by Phosphorylation-Defective Versus Acetylation-Defective FoxO1 Mutants

We have previously shown that the phosphorylation-defective FoxO1-ADA has antiinflammatory effects in macrophages.11 As the FoxO1-KR mutant is also predominantly nuclear (although not to the same extent as the phosphorylation-defective mutant), we considered the possibility that the observed effects might simply reflect a gain of FoxO1 function, and be unrelated to its acetylation state. To investigate this point, we performed ex vivo experiments by transducing primary peritoneal macrophages with FoxO1-WT, FoxO1-ADA (phosphorylation-defective), and FoxO1-KR (acetylation-defective) and measured their response to FC loading. FoxO1-KR and WT-FoxO1 inhibited FC-induced Akt phosphorylation on Ser473 and Thr308, whereas FoxO1-ADA increased it (Supplemental Figure IVA, IVC). Likewise, FC induced Mek and Erk phosphorylation (Supplemental Figure IVA, IVB, and IVF). FoxO1-WT or FoxO1-ADA had no effect on this response, whereas FoxO1-KR blunted it (Supplemental Figure IVA, IVB, and IVE). These data indicate that deacetylated FoxO1 has a unique effect that modulates inflammatory pathways in FC-loaded macrophages.

Figure 1 (Continued). Images of FoxO1 subcellular localization. C, Percentage of cells with nuclear FoxO1. All images are representative of at least 3 independent experiments. Scale bar=10 μm. **P<0.01 and ***P<0.001 between the indicated groups. D, Time course analyses of mRNAs expression in FoxO1+/− (+/−, open circles) and FoxO1KR/KR macrophages (KR/KR, closed circles) loaded with FC (n=5). *P<0.05, **P<0.01, and ***P<0.001 vs +/+. E to G, Peptide levels of monocyte chemoattractant protein-1 (Mcp-1) (E), tumor necrosis factor-α (Tnf-α) (F), and Il-6 (G) in conditioned medium from macrophages loaded with FC for 24 hours (n=4). *P<0.05, **P<0.01, and ***P<0.001 between the indicated groups.

FoxO1-KR Inhibits FC-Induced Akt Phosphorylation

In view of the role of Akt in modulating the macrophage antiapoptotic and antiinflammatory responses,27 we examined the effects of FC loading on Akt activity in macrophages. In the early stages of FC loading, Akt phosphorylation on Ser473 and Thr308 was increased in FoxO1+/− macrophages, but this effect was decreased by ∼80% in FoxO1KR/KR macrophages (Figure 2A–2C). Phosphorylation of the Akt substrate glycogen synthase kinase 3β was also decreased in FoxO1KR/KR macrophages (Figure 2A and 2D). In contrast, there was no difference in insulin-induced Akt phosphorylation between the 2 cell types (Figure 2E–2H). Thus, Akt activation by FC is selectively impaired in FoxO1KR/KR macrophages.

Figure 2. Free cholesterol (FC)– and insulin-induced Akt and glycogen synthase kinase 3β (GSK3β) phosphorylation in macrophages. A to D, Representative immunoblots (A) and quantification of phospho-(p) Akt Ser473 (B), phospho-Akt Thr308 (C), and phospho-GSK3β (D) at the indicated times. E to H, Representative immunoblots (E) and quantification of phospho-Akt Ser473 (F), phospho-Akt Thr308 (G), and phospho-GSK3β (H) in FoxO1+/− (+/−) and FoxO1KR/KR (KR/KR) macrophages stimulated with insulin (10 μmol/L) for the indicated times. Band intensity was quantified from 3 independent experiments. *P<0.05, **P<0.01, and ***P<0.001 vs +/−.

Figure 3. Impaired Mek/Erk activation in FoxO1KR/KR macrophages. A, FC-induced Mcp-1 (n=9), Tnf-α (n=9), and Il-6 (n=9) in macrophages stimulated with FC (B), Mapk pathway or both7 (Supplemental Figure III). We interrogated the contribution of these 2 mechanisms to the blunted inflammatory response of FoxO1KR/KR macrophages. FC rapidly induced Mek phosphorylation by ∼2-fold and promoted biphasic Erk phosphorylation in FoxO1+/− macrophages; these responses were significantly attenuated in FoxO1KR/KR macrophages (Figure 3A–3C). In contrast, Jnk and p38 phosphorylation increased up to 8-fold and were comparable in both cell types (Figure 3A and 3D–3E).

Figure 1 (Continued). Images of FoxO1 subcellular localization. C, Percentage of cells with nuclear FoxO1. All images are representative of at least 3 independent experiments. Scale bar=10 μm. **P<0.01 and ***P<0.001 between the indicated groups. D, Time course analyses of mRNAs expression in FoxO1+/− (+/−, open circles) and FoxO1KR/KR macrophages (KR/KR, closed circles) loaded with FC (n=5). *P<0.05, **P<0.01, and ***P<0.001 vs +/+. E to G, Peptide levels of monocyte chemoattractant protein-1 (Mcp-1) (E), tumor necrosis factor-α (Tnf-α) (F), and Il-6 (G) in conditioned medium from macrophages loaded with FC for 24 hours (n=4). *P<0.05, **P<0.01, and ***P<0.001 between the indicated groups.
Accordingly, we observed a 2-fold increase in nuclear content of the Nf-κB p65 subunit following FC loading of FoxO1/HR/HR macrophages (Figure 4B–4D). In contrast, levels of nuclear p65 were unchanged in FC-treated FoxO1KR/KR macrophages (Figure 4B–4D). To correlate these changes with NF-κB function, we assessed the activity of an NF-κB reporter gene transduced into FoxO1/HR/HR or FoxO1KR/KR cells. In FoxO1/HR/HR cells, FC induced an 8-fold increase of NF-κB-luciferase activity, whereas the effect of FC was decreased by 40% in FoxO1KR/KR cells (Figure 4E).

Identification of p105 (Nfkβ1) as Mediator of the Antiinflammatory Effects of FoxO1-KR

The combined impairment of NF-κB and Mek/Erk activities in FoxO1KR/KR macrophages led us to formulate the testable hypothesis that a shared regulator of both pathways was responsible for this reduction. The product of the Nfkβ1 gene, p105, is the precursor of NF-κB p50 and functions as an IκB-like molecule by sequestering p65, p50, and c-Rel in the cytoplasm and preventing their binding to DNA. p105 also inhibits the Mek/Erk pathways by forming stoichiometric complexes with the Mek inhibitor TPL-2/Cot. We detected a 4-fold increase in p105 protein levels (Figure 5A and 5B), associated with a 2-fold rise of cytosolic p50 levels in FoxO1KR/KR macrophages (Figure 5C), consistent with increased extranuclear retention of this subunit. In contrast, nuclear levels of p50 increased only by 30% in FoxO1KR/KR cells (Figure 5D). The increase of p105 protein was associated with a 30% rise of Nfkβ1 mRNA in FoxO1KR/KR macrophages (Figure 5E), suggesting that the main mechanism of increased p105 levels is not increased Nfkβ1 transcription. Transduction of FoxO1-KR adenovirus in primary peritoneal macrophages also increased p105 protein levels (Figure 5F), albeit to a lower extent, probably reflecting the limited efficiency of adenoviral transduction compared with
studying a homogeneous population of FoxO1KR/KR macrophages. The increase was independent of changes in Nfkb1 mRNA (data not shown), consistent with a transcription-independent mechanism. In contrast, FoxO1-WT and FoxO1-ADA had no effect on p105 (Figure 5F and 5G). These data indicate that p105 is a specific, albeit indirect (nontranscriptional) target of deacetylated FoxO1.

Figure 5. Nfkb1 and p105 levels in macrophages. A to D, Representative immunoblots (A) and quantification of cytosolic p105 (B) and p50 (C) and nuclear p105 (D) in FoxO1+/+ (+/+) and FoxO1KR/KR (KR/KR) macrophages loaded with free cholesterol (FC) for the indicated times. Loading control (α-tubulin and nucleophosmin [NPM]) is shown in Figure 6B. E, Nfkb1 levels in FoxO1+/+ (+/+) and FoxO1KR/KR (KR/KR) macrophages (n=6). F and G, Representative immunoblots (F) and quantification of p105 and actin levels (G) in cytosolic fractions from FoxO1+/+ macrophages transduced with adenovirus (AdV) encoding LacZ, FoxO1-WT, FoxO1-KR, or FoxO1-ADA. Band intensity was quantified from 3 independent experiments. WT indicates wild-type. *P<0.05 and **P<0.01 vs +/+. The effect of FoxO1-KR on p105

Phosphatidylinositol 3-Kinase Inhibition Mimics the Effect of FoxO1-KR on p105

Akt phosphorylates Ikk,33,34 triggering p105 proteolysis and Nf-κB/Mek/Erk activation. In addition, the Akt substrate glycogen synthase kinase-3 (GSK3),35 stabilizes p105 through phosphorylation, preventing its degradation. We reasoned that if FoxO1-KR increased p105 by inhibiting Akt, its effects should be phenocopied by pharmacological inhibition of phosphatidylinositol 3-kinase. Indeed, the phosphatidylinositol 3-kinase inhibitor LY294002 inhibited Akt phosphorylation and glycogen synthase kinase 3β phosphorylation and increased p105 protein levels (Figure 6A and 6B) independently of mRNA (Figure 6C). LY294002 also inhibited FC-induced Mcp-1, Tnf-α, IL-6, IL-1β, Mip-1β, and Mip-2 expression in macrophages (Figure 6D). In contrast, Mip-1α, Tgf-β1, and Spp1, whose induction by FC was blunted in FoxO1KR/KR macrophages, were unaffected by LY294002 (Figure 6D). These data provide indirect support for the
hypothesis that the antiinflammatory effects of FoxO1-KR are mediated by its ability to decrease Akt phosphorylation. 

**FoxO1-KR Does Not Act Through IκBζ, the Main Target Gene of FoxO1-ADA**

We have previously shown that phosphorylation-deficient FoxO1-ADA blunts Nf-κB activation by FC and increases IκBζ expression,11 leading to p65 retention in the cytoplasm. In the light of this precedent, we considered the possibility that FoxO1-KR regulated expression of IκBζ, leading to increased levels of adhesion molecules and tissue factor and migration of vascular smooth muscle cells. We now show that FC increases FoxO1 acetylation and that constitutively deacetylated FoxO1 prevents FC-induced Nf-κB activity independent of IκBζ and provide more evidence for a deacetylation-specific mechanism of reduced inflammation.

**Discussion**

The pathogenesis of inflammation in cardiovascular atherosclerotic disease is multifactorial and involves generation of cytokines, free radical formation, hemodynamic stress, hypertension, infections, and accumulation of oxidized phospholipids and 7-oxysterols. FC accumulation in macrophages promotes inflammation through cytokine production, leading to increased levels of adhesion molecules and tissue factor and migration of vascular smooth muscle cells. We now show that FC increases FoxO1 acetylation and that constitutively deacetylated FoxO1 prevents FC-induced inflammation without promoting apoptosis. The latter finding sets this FoxO1 mutant apart from a phosphorylation-defective mutant that we have previously shown to inhibit inflammation and promote FC-dependent apoptosis. Interestingly, constitutively active Akt prevents FC-induced macrophage apoptosis in insulin-resistant macrophages, whereas phosphatidylinositol 3-kinase-Akt inhibition by LY294002 increases it. These findings suggest that FoxO1-KR activates additional antiapoptotic pathways to offset its proapoptotic properties macrophages. As FoxO1 phosphorylation and acetylation are regulated in response to different physiological cues and disease states, the present findings have implications that go beyond the FC loading model.

We propose a novel mechanism to explain the findings (Supplemental Figure VI). FoxO1 deacetylation blocks FC-dependent Akt phosphorylation, leading to decreased IκBζ expression, reduced cytokine expression, and reduced inflammation. We identify the product of the Nfkbi gene p105 as a nontranscriptional target of deacetylated FoxO1 that potently mediates its antiinflammatory effects. The data provide further evidence that deacetylation fine-tunes FoxO1 function through target gene selection; thus, the phosphorylation-defective and acetylation-defective mutants have opposite effects on Akt phosphorylation and selective effects on Nfkbi and IκBζ expression.11,21 Identifying the mechanism of the differential effects on Akt phosphorylation poses a daunting challenge for future work, as does the identification of coregulatory mechanisms whereby FoxO proteins can regulate gene expression without direct DNA binding.20

Akt regulates inflammation by phosphorylating Ikkα on Thr23 and activating Nf-κB. Pharmacological inhibition of Ikk increases p105 levels, and inhibits LPS-stimulated Nf-κB and Mek/Erk activation in macrophages. On the other hand, p105 gain-of-function fails to affect LPS-induced Nf-κB and Mek/Erk signaling, suggesting that this mechanism is specific for certain inflammatory stimuli, but not all inflammatory stimuli. Consistent with this observation, we have observed that FoxO1KR/KR macrophages are resistant to FC-induced but not to zymosan- or LPS-induced activation of inflammatory cytokines. It is also of interest to note that pharmacological inhibition of phosphatidylinositol 3-kinase-Akt mimics the FoxO1-KR effect to raise p105 levels but has narrower effects on cytokine induction by FC, suggesting that not all effects of FoxO1-KR can be explained by its inhibition of Akt. We propose that decreased Akt activity by FoxO1-KR attenuates p105 proteolysis by 2 separate mechanisms: Ikk inhibition, leading to Nf-κB and Mek inhibition, and decreased glycogen synthase kinase 3β phosphorylation, which has been shown to prevent constitutive processing/degradation of p105 by increasing its stability.

The present study confirms the biphasic activation pattern of Mek and Erk. This could be due to delayed activation of certain subsets of Mapks or to autocrine signaling by cytokines produced from FC-stimulated macrophages, such as CXC-ligand 4 and IL-10.

The role of FoxO1 in the development of atherosclerosis varies in different cells and tissue types. Insulin receptor-deficient macrophages, in which FoxO1 is constitutively active, display antiinflammatory responses to FC but are prone to apoptosis. Conversely, in vascular endothelial cells, FoxO1 gain-of-function promotes peroxynitrite and reactive oxygen species generation, predisposing to inflammation. This finding is consistent with the observation that endothelial-cell specific ablation of insulin receptors (a condition associated with increased nuclear FoxO1) in apolipoprotein E−/− mice worsens atherosclerosis, as does Akt1 ablation, pinpointing vascular endothelial cells as a key site of atherosclerotic lesion development in insulin resistance.

In conclusion, our study demonstrates that FoxO1 deacetylation is an important regulatory mechanism in cholesterol-laden macrophages, with the potential to uncouple inflammation from apoptosis. As these mechanisms underlie the progression of atherosclerotic plaques from benign to unstable lesions, it can be envisioned that treatments promoting FoxO1 deacetylation will favorably affect cardiovascular outcomes in type 2 diabetes.
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Disclosures

None.

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smooth muscle cells is enhanced by 7-ketocholesterol and lysophosphatidylcholine independently of their effect on nitric oxide generation. *Atherosclerosis*. 2001;159:325–332.


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Detailed Methods

Materials. We obtained acetylated-human LDL (acLDL) from Biomedical Technologies; compound 58035, LPS, zymosan, Bay 11-7082, SB203580, SP600125 and LY294002 from Sigma; U0126 from Cayman Chemical. The concentrations of inhibitors used in the present study were chosen from previous studies. Sources of antibodies are as follows: acetyl-lysine, Mek1/2, phospho-Mek1/2, p38, phospho-p38, Erk1/2, phospho-Erk1/2, Jnk1/2, phospho-Jnk1/2, nucleophosmin, Akt, phospho-Akt (Ser-473 and Thr-308), phospho-GSK3β and GSK3β from Cell Signaling; TnfR1, FoxO1, p105/50, p65, and α-tubulin from Santa Cruz Biotechnology; SR-A from TransGenic; TLR-4 from Invitrogen; and actin from EMD4bioscience.

Immunocytochemistry. We cultured peritoneal macrophages in Lab-Tek II Chamber Slide System (Nalgene Nunc International), fixed them with 4% paraformaldehyde and performed FoxO1 immunocytochemistry using three antisera as described. FITC-conjugated anti-rabbit IgG (Jackson ImmunoResearch) were used as secondary antibodies. We measured apoptosis by Alexa 488-labeled annexin V and propidium iodide staining (Vybrant Apoptosis Assay kit, Invitrogen). For each condition, we randomly selected four separate fields, counted > 200 positive cells and plotted them as percentage of total cells.

Quantitative RT-PCR and ELISA. We extracted RNA using TRIzol (Invitrogen), synthesized cDNA with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), and performed quantitative RT-PCR using DyNAmo HS SYBR Green qPCR Kit (Finnzymes) in a Chromo4 Real-Time PCR Detection System (Bio-Rad). Primer sequences are available on request. We used ELISA to measure protein levels of Mcp-1 (Invitrogen), Tnf-α, and Il-6 (BD Bioscience).

Adenoviruses. We have previously described adenoviruses encoding LacZ, GFP-tagged FoxO1-WT and FoxO1-KR, HA-tagged FoxO1-ADA, and nuclear factor-κB (Nf-κB)-luciferase. We transduced macrophages at MOI 500, 18- to 24-h prior to experiments.

Immunoprecipitation and Immunoblotting. We lysed cells in buffer containing 2% SDS, 50mM Tris-HCl, 5mM EDTA, and protease/phosphatase inhibitors (Thermo Scientific), sonicated the extract and clarified it by centrifugation, followed by separation of nuclear
and cytoplasmic fractions using NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific). We performed immunoblotting followed by ECL detection (GE Lifescience), and quantified band intensity using NIH ImageJ 1.41 and normalization with gel loading controls. We carried out immunoprecipitation as described 7, 8.

Luciferase assays. We measured luciferase activity in peritoneal macrophages transfected with Nf-κB reporter construct using Dual Luciferase reporter Assay System (Promega). We normalized reporter activity by protein concentration or cotransfected Renilla luciferase, as appropriate.

Statistical analysis. We show data as mean ± SE. We determined statistically significant differences ($P < 0.05$) using unpaired $t$-test or ANOVA with Dunn’s post hoc test.
SUPPLEMENTAL FIGURE LEGENDS

Suppl. Figure I. Zymosan- and LPS-induced inflammatory cytokine expression in macrophages. Quantitative time course analyses of Mcp-1, II-6, and Tnf-α in Foxo1+/+ (+/+, open circles) and Foxo1KR/KR macrophages (KR/KR, closed circles) loaded with (A) zymosan (100µg/ml) and (B) LPS (10ng/ml) (n=4). * P < 0.05, ** P < 0.01, and *** P < 0.001 vs. KR/KR.

Suppl. Figure II. FC–induced ER stress and apoptosis in macrophages. (A) Bip and Chop mRNA levels in Foxo1+/+ (+/+) and Foxo1KR/KR (KR/KR) macrophages loaded with FC for the indicated times. (B) Annexin V (green) and propidium iodide (red) staining in macrophages loaded with FC or thapsigargin (Tg, 2µM) for 24 h. (C) Percentage of positive cells. (D) SR-A, TLR-4, TnfR1, and α-tubulin western blots in WT (+/+) and FoxO1-KR (KR/KR) macrophages. ns: not significant.

Suppl. Figure III. FC–induced inflammatory cytokine expression in response to Mapk or Nf-κB inhibitors. Mcp-1, Tnf-α, and II-6 mRNA in Foxo1+/+ macrophages pretreated with IκBα phosphorylation inhibitor Bay 11-7085 (Bay, 2µM), Mek1/2 phosphorylation inhibitor U0126 (U, 10µM), p38 phosphorylation inhibitor SB203580 (SB, 20µM), and Jnk1/2 phosphorylation inhibitor SP600125 (SP, 10µM) for 1h followed by FC loading for 8h (n=5). * P < 0.05, ** P < 0.01, and *** P < 0.001 between the indicated groups.

Suppl. Figure IV. FC activates Akt and Mapk in macrophages transduced with adenovirus encoding FoxO1 mutants. (A) Representative immunoblots and (B-E) quantification of phospho-Akt Ser-473 (B), phospho-Akt Thr-308 (C), phospho-Mek1/2 (D), and phospho-Erk1/2 (E) in Foxo1+/+ macrophages transduced with adenovirus (AdV) encoding LacZ, FoxO1-WT, FoxO1-KR, or FoxO1-ADA for 24h followed by FC loading for the indicated times. Band intensity was quantified from three independent experiments. * P < 0.05, ** P < 0.01, and *** P < 0.001 vs. LacZ-transduced macrophages.

Suppl. Figure V. IκBs mRNA levels in macrophages. (A) IκBa, IκBβ, and IκBε mRNA levels in Foxo1+/+ (+/+) and Foxo1KR/KR (KR/KR) macrophages loaded with FC for 8h (n=5), or (B) in WT macrophages transduced with adenovirus (AdV) encoding LacZ, FoxO1-WT, FoxO1-KR, or FoxO1-ADA for 24h (n=5). * P < 0.05.

Suppl. Figure VI. Model of FoxO1 function in macrophages. (A) FoxO1
deacetylation (KR mutant) inhibits FC-induced Akt phosphorylation. This leads to (i) decreased IκBα phosphorylation and Nf-κB activity, and (ii) increased p105 and reduced Mek/Erk activity. These changes decrease inflammation, without increasing apoptosis. (B) FoxO1 dephosphorylation (FoxO1-ADA) increases FC-induced Akt phosphorylation, and increases IκBε expression, leading to Nf-κB inhibition, lower inflammation and increased apoptosis. ↑: increased, ─→: not changed, ↓: decreased.
SUPPLEMENTAL REFERENCES


Suppl. Figure II

A

BiP

Chop

mRNA (fold increase)

Time (h)

0 4 8 12 24

0 4 8 12 24

B

Control FC Tg

+/+

KR/KR

C

Apoptotic cells (% cells)

Control FC Tg

+/+

KR/KR

D

+/+

KR/KR

SR-A

TLR-4

TnfR1

α-tubulin

Supplemental Material

SR-A

α-tubulin
Suppl. Figure III

**Mcp-1**

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**Tnf-α**

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**Il-6**

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Suppl. Figure IV

A

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B

C

D

E

Supplemental Material
Suppl. Figure V

A

IkBα

mRNA (fold increase)

FC (h) 0 8 0 8

+/+ KR/KR

IkBβ

mRNA (fold increase)

FC (h) 0 8 0 8

+/+ KR/KR

IkBε

mRNA (fold increase)

FC (h) 0 8 0 8

+/+ KR/KR

B

IkBα

mRNA (fold increase)

AdV LacZ FoxO1-WT FoxO1-KR FoxO1-ADA

IkBβ

mRNA (fold increase)

AdV LacZ FoxO1-WT FoxO1-KR FoxO1-ADA

IkBε

mRNA (fold increase)

AdV LacZ FoxO1-WT FoxO1-KR FoxO1-ADA

*
Suppl. Figure VI

A

FoxO1-KR

FC-loading → pAkt↓ → IKK

pIκBα↓ → Nf-κB↓ → Apoptosis↑

p105↑ → pMek/pErk↓

B

FoxO1-ADA

FC-loading → pAkt↑ → IKK

IkBε↑ → Nf-κB↓ → Apoptosis↑

p105→ → pMek/pErk↓

Stimulation → Inhibition