

# FOXO-dependent expression of the proapoptotic protein Bim: pivotal role for apoptosis signaling in endothelial progenitor cells

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## SPECIFIC AIMS

Endothelial progenitor cells (EPCs) contribute to post-natal neovascularization. Since EPC apoptosis might be a potential mechanism to regulate the number of EPCs, we investigated the effects of oxidative stress and HMG-CoA reductase inhibitors (statins) on EPC apoptosis and the underlying mechanism. Because forkhead transcription factors (FOXO1, FOXO3a, FOXO4) exert proapoptotic effects, we elucidated the involvement of forkhead transcription factors and demonstrated that FOXO4 plays a crucial role in apoptosis signaling in EPCs. We further examined whether FOXO-dependent regulation of the proapoptotic protein Bim is involved in the protective effects of statins on oxidative stress-induced apoptosis of EPCs.

## PRINCIPAL FINDINGS

### 1. Statins prevent H<sub>2</sub>O<sub>2</sub>-induced apoptosis of EPCs

Incubation of EPCs with H<sub>2</sub>O<sub>2</sub> for 24 h resulted in a dose-dependent increase of apoptosis as assessed by annexin-V measurement. Likewise, the number of ex vivo differentiated attached DiLDL/lectin double-positive cells was reduced to 48 ± 4% of control in the presence of 500 μM H<sub>2</sub>O<sub>2</sub>. Preincubation of EPCs with the HMG-CoA reductase inhibitor atorvastatin for 24 h significantly reduced H<sub>2</sub>O<sub>2</sub>-induced apoptosis in a dose-dependent manner, a maximal inhibitory effect achieved with 0.01 μM. Consistently, mevastatin significantly blocked H<sub>2</sub>O<sub>2</sub>-induced apoptosis, suggesting a class effect of statins. The anti-apoptotic effect of atorvastatin and mevastatin was partially reversed by the product of the HMG-CoA reductase, mevalonate. Because statins and VEGF activate the anti-apoptotic PI3K/Akt pathway in EPCs, we investigated the role of

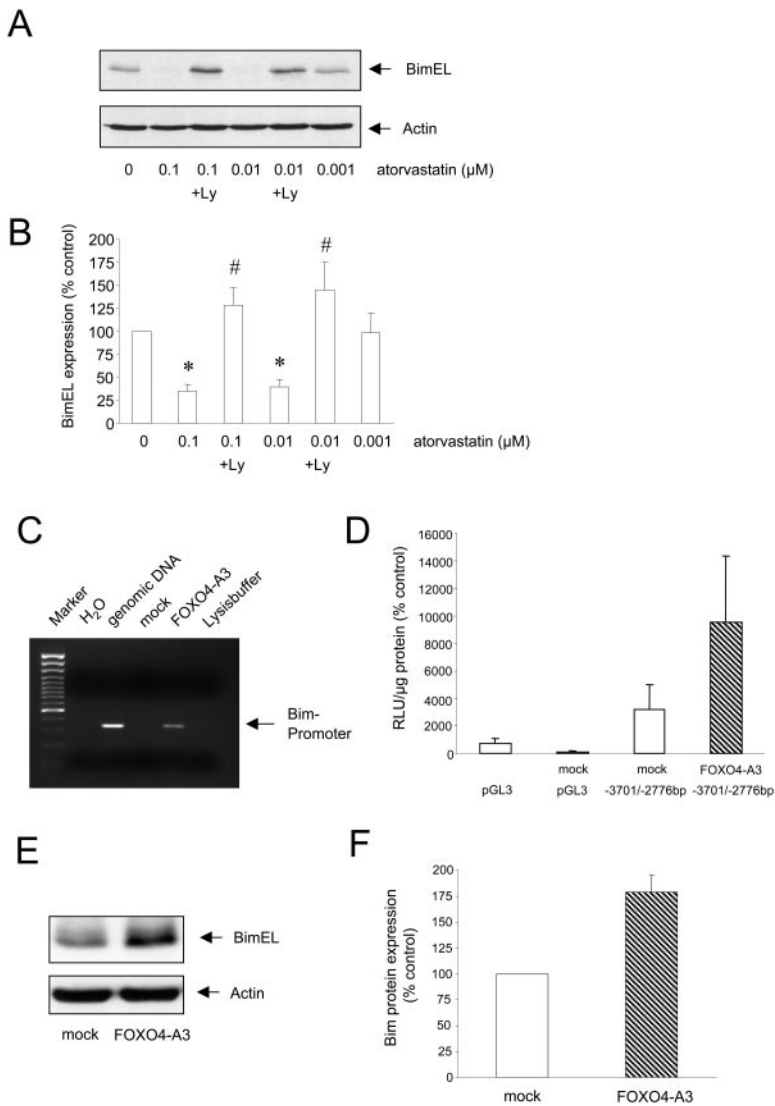
the PI3K/Akt pathway for EPC apoptosis. The PI3K inhibitor Ly294002 abolished the apoptosis inhibitory effect of atorvastatin in EPCs. VEGF, also known to activate the PI3K/Akt pathway, significantly reduced H<sub>2</sub>O<sub>2</sub>-induced EPC apoptosis in a PI3K/Akt-dependent manner. In contrast, inhibition of one anti-apoptotic downstream pathway of Akt, the NO synthase, by blockade with LNMA did not reverse the apoptosis by atorvastatin, suggesting a PI3K/Akt-dependent but NO-independent anti-apoptotic signaling pathway, which is activated by statins.

### 2. Statins phosphorylate the forkhead factor FOXO4 and down-regulate the proapoptotic protein Bim

Because forkhead transcription factors are direct downstream targets of Akt and are inactivated by Akt-dependent phosphorylation of serine/threonine residues, we investigated whether statins may phosphorylate and thereby inactivate the forkhead transcription factors. Since FOXO4, but not FOXO1 or FOXO3a, was highly expressed in EPCs, we focused on FOXO4. Atorvastatin dose- and time-dependently phosphorylated FOXO4 in EPCs, with a maximum effect at 60 min. Atorvastatin-induced phosphorylation was blocked by the PI3K inhibitor Ly294002.

The proapoptotic protein Bim is a major downstream target of the forkhead transcription factor family, which has been implicated in Akt-dependent regulation of hematopoietic stem cell survival. Therefore, we analyzed the potential involvement of Bim in apoptosis of EPCs. Coincubation with atorvastatin dose-dependently down-regulated Bim expression (**Fig. 1A, B**) and abro-

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**Figure 1.** Statins down-regulate Bim expression in EPCs. *A*) EPC were incubated with atorvastatin in the presence or absence of the PI3K inhibitor Ly294002 (10  $\mu$ M) for 24 h and expression of Bim was analyzed by Western blot. Actin serves as loading control. A representative blot of 4 independent experiments is shown. *B*) Data are mean  $\pm$  SE; \* $P$  < 0.05 vs. control, # $P$  < 0.05 vs. atorvastatin. *C*) EPC were transiently transfected with GFP-tagged FOXO4-A3 and incubated in the presence of ZVAD (50  $\mu$ M) for 4 h. Cells were cross-linked and lysates were incubated with a GFP antibody for immunoprecipitation. Upon DNA isolation, amplification of Bim promoter DNA was assessed by PCR and gel electrophoresis. Genomic DNA was used as positive control. A representative micrograph of 3 independent experiments is shown. *D*) HEK 293 cells were cotransfected with the pGL3-Bim-luciferase construct (-3701/-2776 bp) and FOXO4-A3 for 18 h, and luciferase activity was measured by enzymatic reaction. Data are mean  $\pm$  SE (mock+pGL3 set as 100%; RLU=relative light units). *E*) EPCs were transiently transfected with GFP-FOXO4-A3 and incubated in the presence of ZVAD (50  $\mu$ M) for 6 h. EPCs were lysed and expression of Bim was analyzed by Western blot. Actin serves as loading control. A representative blot of 3 independent experiments is shown. *F*) Blots were scanned and expression of BimEL was quantified by densitometric analysis. Ratios for Bim/actin are shown. Data are mean  $\pm$  SE (% control).

gated the H<sub>2</sub>O<sub>2</sub>-induced increase in Bim expression in EPCs. The reduction of Bim expression by atorvastatin was dependent on the PI3K pathway, as demonstrated by the inhibitory effect of the PI3K inhibitor Ly294002 (Fig. 1A, B).

### 3. FOXO4 regulates Bim promoter activity and its expression

Having demonstrated that atorvastatin regulates phosphorylation of FOXO4 and expression of Bim, we investigated the interaction of FOXO4 with the Bim promoter in EPCs using chromatin immunoprecipitation assays. As shown in Fig. 1C, the Bim promoter interacted with FOXO4 in EPCs. Moreover, FOXO4-A3 overexpression increased luciferase activity of the -3701/-2776 bp Bim promoter construct compared with cotransfection of the empty vectors (Fig. 1D) and induced the expression of Bim in EPCs (Fig. 1E, F). Finally, we addressed the causal role of FOXO4 phosphorylation and Bim down-regulation for statin-induced promotion of EPC survival. Overexpression of

BimEL or nonphosphorylatable FOXO4-A3 construct potentially increased apoptosis of EPCs. The drastic pro-apoptotic effect was underlined by the finding that expression of GFP-BimEL or GFP-FOXO4-A3 in EPCs could only be detected in the presence of the caspase inhibitor ZVAD-fmk. Coincubation with atorvastatin did not prevent BimEL- or FOXO4-induced apoptosis, consistent with the concept that statins mediate the anti-apoptotic effect via FOXO4-dependent regulation of Bim.

### 4. Role of FOXO4 and Bim in HUVEC

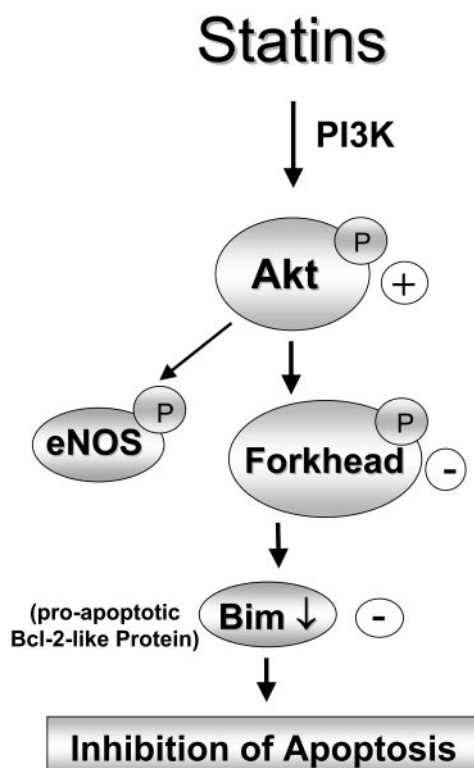
To further substantiate a pivotal role for FOXO4-dependent regulation of Bim to mediate the anti-apoptotic effects of statins in EPCs, we made use of the differential expression profile of the forkhead factor family members in HUVEC as compared with EPCs. Statins had no effect on Bim expression in HUVEC, although statins activate Akt in HUVEC. Surprisingly, overexpression of a constitutively active GFP-FOXO4-A3 did not increase apoptosis in HUVEC.

Likewise, overexpression of GFP-BimEL induced only a minor nonsignificant increase in apoptosis of HUVEC, although transfection efficiency was higher in HUVEC compared with EPCs ( $33 \pm 6\%$  vs.  $21 \pm 5\%$ , respectively). Thus, Akt-dependent signaling pathways regulating apoptosis differ between mature endothelial cells and EPCs. The FOXO4-dependent regulation of Bim appears to be a rather specific signaling pathway in EPCs.

## CONCLUSIONS

The data of the present study demonstrate that reactive oxygen species, which are known to be increased in patients with CAD or diabetes, profoundly reduce the number of viable EPCs by inducing apoptosis. Although recent data demonstrated that EPCs are more protected against oxidative stress-induced apoptosis than HUVEC, high levels of  $H_2O_2$  ( $\geq 500 \mu M$ ) increased apoptosis  $\sim 2.5$ - to 4-fold in EPCs. The proatherosclerotic factor oxidized LDL reduced EPC numbers to a similar extent, suggesting that apoptosis is one regulatory mechanism for EPC survival. Thereby, up-regulation of the forkhead factor-dependent proapoptotic protein Bim appears to play a pivotal role for apoptosis signaling in EPCs. Statin-mediated activation of the PI3K/Akt signaling pathway abrogated increased Bim expression and apoptosis induction in EPCs by phosphorylation-mediated inactivation of FOXO4. The causal role of FOXO4 phosphorylation was demonstrated in the present study by using a FOXO4 construct that lacks the known Akt phosphorylation motifs and thereby prevents Akt-dependent inactivation.

Little is known about the individual biological function of the different forkhead transcription factors. FOXO1, FOXO3a, and FOXO4 contain Akt phosphorylation sites at serine and threonine residues. Moreover, overexpressed FOXO1, FOXO3a, and FOXO4 were shown to regulate Bim expression in hematopoietic cells. These data would suggest a similar effect of the different forkhead transcription factors on the target genes. However, first studies indicate that the biological activity of FOXO1 and FOXO3a might differ from FOXO4; moreover, the expression profile is different. FOXO4 was shown to be highly expressed in hematopoietic cells; FOXO1 and FOXO3a are expressed more ubiquitously. This is consistent with the findings of the present study, which demonstrates a higher expression of FOXO4 in EPCs (which more closely resemble the hematopoietic cell type) compared with mature endothelial cells. However, the differences in apoptosis signaling between EPCs and mature HUVEC may involve additional downstream signals beyond the differential expression of forkhead



**Figure 2.** Schematic illustrations of our findings.

transcription factors. Whereas our data rule out a differential alternative splicing of Bim, overexpression of BimEL only induced a minor apoptotic response in HUVEC compared with EPC. Since the expression levels and transfection efficiency of BimEL favored HUVEC, this would imply a possible post-transcriptional inactivation of Bim in HUVEC. Indeed, the proapoptotic activity of Bim is known to be post-transcriptionally regulated by association with the dynein motor complex. Another possibility could be the phosphorylation of BH3-only proteins, as has been shown for BAD or Bid.

Taken together, the data of the present study demonstrate that statins inhibit apoptosis of EPCs by down-regulation of the proapoptotic protein Bim (**Fig. 2**). The down-regulation of Bim involves statin-induced phosphorylation of the forkhead factor FOXO4, which inactivates the transcription factor and thereby prevents Bim expression. The potential importance of FOXO4 for regulation of EPCs is underlined by the finding that EPCs derived from patients with CAD reveal significantly higher expression levels than healthy controls and showed increased basal apoptosis. These data suggest that selective targeting of the FOXO4-Bim pathway may provide an important therapeutic strategy to improve EPC function in patients with coronary artery disease. FJ