Insulin-Mediated Stimulation of Protein Kinase Akt
A Potent Survival Signaling Cascade for Endothelial Cells

Corinna Hermann, Birgit Assmus, Carmen Urbich, Andreas M. Zeiher, Stefanie Dimmeler

Abstract—Insulin exerts potent antiapoptotic effects in neuronal cells and has been suggested to promote angiogenesis. Therefore, we investigated whether insulin inhibits tumor necrosis factor-α (TNF-α)–induced apoptosis in human umbilical vein endothelial cells (HUVECs). Because insulin has been shown to stimulate the protein kinase Akt, we investigated whether activation of Akt contributes to the apoptosis-suppressive effect of insulin and characterized the downstream signaling pathway. Incubation with insulin dose-dependently prevented apoptosis induced by TNF-α (50 ng/mL). The extent of apoptosis suppression by insulin was similar to the effect of vascular endothelial growth factor. Pharmacological inhibition of Akt activation or overexpression of a dominant-negative Akt mutant prevented the antiapoptotic effect of insulin. Furthermore, we investigated the effect of TNF-α on Akt phosphorylation by Western blot analysis with the use of a phosphospecific Akt antibody. Incubation of HUVECs with TNF-α induced a marked dephosphorylation of Akt. Insulin counteracted this TNF-α–induced dephosphorylation of Akt. Furthermore, we investigated the downstream signaling events. Akt has been shown to mediate its apoptosis-suppressive effects via phosphorylation of Bad or caspase-9. However, incubation with insulin did not lead to enhanced phosphorylation of Bad at Ser 136 or Ser 112. In contrast, insulin inhibited caspase-9 activity and prevented caspase-9–induced apoptosis. Mutation of the Akt site within caspase-9 significantly reduced the apoptosis-suppressive effect of insulin. The present study demonstrates an important role for insulin-mediated Akt activation in the prevention of endothelial cell apoptosis, which may importantly contribute to cell homeostasis and the integrity of the endothelium. In endothelial cells, Akt seems to mediate its antiapoptotic effect, at least in part, via phosphorylation of caspase-9 rather than Bad. (Arterioscler Thromb Vasc Biol. 2000;20:402-409.)

Key Words: endothelial cells ▪ insulin ▪ Akt kinase ▪ atherosclerosis

The survival of endothelial cells critically determines vessel growth and inflammatory processes in the vessel wall. Survival of the cells is counterbalanced by the induction of physiological cell death, apoptosis. Besides the contribution of apoptosis to endothelial injury, apoptotic cell death regulates vessel growth. Thus, recent studies have provided evidence that inhibition of endothelial cell apoptosis in combination with enhanced proliferation is implicated as the basis for angiogenesis associated with diabetic retinopathy, tumor growth, and metastasis. Interestingly, potent endothelial mitogenic stimuli, such as vascular endothelial growth factor (VEGF) as well as basic fibroblast growth factor, protect endothelial cells against apoptosis. Moreover, angiotatin, an inhibitor of neoangiogenesis, induces endothelial cell apoptosis. Thus, the balance between positive and negative regulators of endothelial cell death may determine vascular development.

Recent studies also provide evidence that activation of the serine/threonine protein kinase Akt, also known as protein kinase B or Rac kinase, is involved in embryonic vascular development and neoangiogenesis. Stimulation of the endothelium-specific receptor tyrosine kinase Tie2 triggers the activation of Akt. Moreover, hypoxia-induced expression of VEGF involves the Akt signaling pathway. Furthermore, activation of Akt kinase potently prevents apoptotic cell death. Stimulation of the protein kinase Akt was shown to be dependent on phosphoinositide 3-OH kinase (PI3K), which mediates growth factors and hypoxia-induced Akt activation.

The hormone insulin exerts various biological effects. For example, insulin is mitogenic in certain cell types and potently suppresses apoptotic cell death in fibroblasts and neuronal cells. However, the effects of insulin on endothelial cell survival are poorly defined. Therefore, the aim of the present study was to investigate the effect of insulin on endothelial cell apoptosis and to elucidate the underlying signaling events. Because the Akt kinase pathway has been shown to be involved in apoptosis suppression as well as angiogenesis, we determined the role of protein kinase Akt in the insulin-mediated inhibition of endothelial cell apoptosis.

The results of the present study demonstrate that insulin prevents TNF-α–induced apoptosis of endothelial cells. The
apoptosis-suppressive effects of insulin are mediated by PI3K-dependent activation of protein kinase Akt, as demonstrated by the pharmacological inhibition of PI3K and the overexpression of a dominant-negative Akt mutant. Insulin thereby stimulated the phosphorylation of Akt and, moreover, counteracted the TNF-α–induced dephosphorylation of Akt. Furthermore, the effects of Akt on endothelial cell survival are independent of p70 S6 kinase and the phosphorylation of the proapoptotic Bad protein.

**Methods**

**Cell Culture**

Human umbilical vein endothelial cells (HUVECs) were purchased from Cell Systems/Clonetics, Solingen, Germany, and were cultured in endothelial basal medium (EGM-1) supplemented with hydrocortisone (1 μg/mL), bovine brain extract (3 μg/mL), gentamicin (50 μg/mL), amphotericin B (50 μg/mL), epidermal growth factor (10 ng/mL), and 10% FCS until the third passage. After detachment with 0.05% trypsin, cells (3.5 × 10⁶ cells/mL) were washed twice with ice-cold PBS followed by incubation of the wells with 200 μL of cell lysis buffer (20 mmol/L Tris [pH 7.4], 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na₃VO₄, 1 μg/mL leupeptin, and 1 mmol/L PMSF) for 5 minutes on ice. Then the cells were scraped off the plates and sonified with a Branson Sonifier (30% duty cycle 100%) on ice. After centrifugation for 10 minutes at 20,000 g, the protein concentration was determined in the supernatant by using the Bio-Rad reagent (Bio-Rad).

Proteins (50 μg per lane) were loaded onto 8% SDS-polyacrylamide gels and blotted onto polyvinylidenedifluoride membranes. After they were blocked with 5% milk powder at room temperature for 2 hours, the antibodies were incubated as follows: for Akt or phospho-Akt (Biolabs) 1:500, 4°C overnight in Tris-buffered saline (TBS; 50 mmol/L Tris-HCl [pH 8], 150 mmol/L NaCl, and 0.6% FCS) were used. The antibodies and the control protein specific Ser 136 antibodies (1:500 in TBS, Tween-20, 3% milk powder, and 0.6% FCS) were supplied by New England Biolabs. The autoradiograms were scanned and semiquantitatively analyzed.

For detection of Bad phosphorylation, phosphospecific Ser 112 antibodies (1:500 in TBS, 0.1% Tween-20, and 0.6% FCS) were used. The antibodies and the control protein specific Ser 136 antibodies (1:500 in TBS, 0.1% Tween-20, and 0.6% FCS) were supplied by New England Biolabs. The autoradiograms were scanned and semiquantitatively analyzed.

**Caspase-9 Activity**

For detection of caspase-9 activity, 4 × 10⁶ cells were lysed in 100 μL of buffer (1% Triton X-100, 0.32 mol/L sucrose, 5 mmol/L EDTA, 1 mmol/L PMSF, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 2 mmol/L DTT, and 10 mmol/L Tris-HCl [pH 8]) for 20 minutes at 4°C, followed by centrifugation (20,000 g for 15 minutes). Caspase-9 activity was detected in the resulting supernatants by measuring the proteolytic cleavage of the fluorogenic substrate 7-amino-4-trifluoromethyl coumarin (AFC)–LEHD with AFC as the standard.
and by using an excitation wavelength of 400 nm and an emission wavelength of 505 nm. Then 70 \micro L of supernatant was incubated with 630 \micro L of assay buffer (100 mmol/L HEPES, 10% sucrose, 0.1% CHAPS [pH 7.5], 2 mmol/L DTT, 200 \micro mol/L PMSF, 10 \micro g/ml aprotinin, and 10 \micro g/ml leupeptin) for 5 minutes before starting the enzymatic reaction with 48 \micro mol/L AFC-LEHD. Protein content was analyzed with the Bio-Rad assay (Bio-Rad), and enzyme activity was calculated as moles of AFC released per milligram protein per second.

Detection of Apoptosis

For morphological staining of nuclei, cells were centrifuged (10 minutes, 700g), fixed in 4% formaldehyde, and stained with 4',6-diamidino-2-phenylindole (DAPI; 0.2 \micro g/ml in 10 mmol/L Tris-HCl [pH 7], 10 mmol/L EDTA, and 100 mmol/L NaCl) for 20 minutes. Five hundred cells were counted by 2 independent investigators blinded to the nature of the experiment, and the percentage of apoptotic cells per total number of cells was determined.

For internucleosomal DNA laddering, 10^6 cells were removed from the culture flask, washed with PBS, and incubated in lysis buffer (5 mmol/L Tris-HCl [pH 8], 20 mmol/L EDTA, and 0.5% Triton X-100) for 15 minutes at 4°C. Then the samples were incubated with RNAse A for 1 hour at 37°C, followed by addition of a final concentration of 0.5 mg/ml proteinase K and 1% SDS; afterward, the samples were incubated overnight at 65°C. After isolation of DNA by phenol-chloroform extraction, the DNA was precipitated with 70% isopropanol and 0.1 mol/L NaCl. The resulting pellet was resolved in Tris-EDTA buffer (10 mmol/L Tris-HCl [pH 8] and 1 mmol/L EDTA), and the DNA samples were incubated with 5 U of Klenow polymerase and 0.5 \micro Ci of [32P]dCTP in the presence of 10 mmol/L Tris-HCl [pH 7.5] and 5 mmol/L MgCl2, for 10 minutes at room temperature according to Rösl.16 The reaction was terminated by addition of 10 mmol/L EDTA, and the unincorporated nucleotides were removed with Sephadex G-50 spin columns. Labeled DNA fragments were separated on a 1.0% agarose gel, transferred to nitrocellulose membranes, and exposed to x-ray film.

Transfection

The plasmid encoding the dominant-negative Akt mutant (Aktmt; truncated form) was kindly donated by Dr Julian Downward (Imperial Cancer Research Fund, London, UK).19 After digestion with HindIII/EcoRI, Aktmt was subcloned into the respective sites (EcoRV/EcoRI) of pcDNA3.1 (Invitrogen). Caspase-9 was cloned by polymerase chain reaction (PCR) into the EcoRV/BamHI sites of the pcDNA3.1 vector. Ser 196 was mutated by PCR-directed mutagenesis into alanine. HUVECs were cotransfected with pcDNA3.1-LacZ and either pcDNA3.1-Aktmt or pcDNA3.1 control vector lacking an insert as described.2 For this purpose, 150 \micro L of EGM-1 without FCS was mixed with 3 \micro g of plasmids (1 \micro g of pcDNA3.1-LacZ and 2 \micro g of pcDNA3.1-Aktmt or pcDNA3.1) and 30 \micro L of Superfect and incubated for 10 minutes at room temperature. During the incubation time, EGM-1 with 10% FCS was removed from the cell-culture plates, and HUVECs were washed once in EGM-1 without FCS. Complete medium (1 mL) with 10% FCS was added to the plasmid-Superfect mixture, and HUVECs were incubated with this mixture for 3 hours at 37°C. After incubation, the culture medium was removed, 3 mL of fresh EGM-1 with 10% FCS was added, and HUVECs were incubated for 24 hours to allow for protein expression. The transfected cells were identified by β-galactosidase staining. Then the plates were centrifuged to pellet the detached cells. The cells were subsequently fixed in 2% formalin–0.2% glutaraldehyde, and β-galactosidase activity was determined by incubation with 40 \micro g/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside for 6 hours at 37°C. The transfection efficiency in this study was 25±0.2% in pcDNA3.1- and pcDNA3.1-LacZ–cotransfected cells. Transfection with 3 \micro g of pcDNA3.1–LacZ results in an ~53±16% transfection efficiency. Viable versus dead (stained) cells were counted by 2 blinded investigators, and results were expressed as dead/total cells×100. In addition, necrotic cell death was excluded by measuring lactate dehydrogenase release, thus indicating that death of the transfected cells was caused by apoptosis.

Statistical Analysis

Data are expressed as mean±SEM from at least 3 independent experiments. Statistical analysis was performed with ANOVA followed by a modified least significant difference test (SPSS Software).

Results

Effects of Insulin on TNF-α-Induced Apoptosis in HUVECs

To investigate the effect of insulin on endothelial cell survival, apoptosis of HUVECs was induced by TNF-α as previously described.20 HUVECs were cultivated in FCS-containing medium and incubated with TNF-α (50 ng/ml) for 18 hours. Apoptosis was detected by morphological analysis of DAPI-stained cells as shown in Figure 1A. TNF-α-stimulated apoptosis in 7.5±2.3% of the cells compared with 2.9±1% in unstimulated control cells. Incubation with insulin dose-dependently inhibited TNF-α–induced apoptosis (Figure 1B). Inhibition of TNF-α–induced apoptosis by 100 nmol/L insulin was further confirmed by prevention of the

![Figure 2](http://atvb.ahajournals.org/Downloaded from http://atvb.ahajournals.org/Downloaded from http://atvb.ahajournals.org/)
expression. Then the transfected cells were incubated in FCS-containing medium with either TNF-α (50 ng/mL) alone or insulin (100 nmol/L) for 18 hours. After β-galactosidase staining, dead versus viable stained cells were counted, and results were expressed as mean ± SEM, n = 3. *P < 0.005, vs TNF-α+Ins+Aktmt.

typical DNA laddering (Figure 1C). These findings indicate that insulin protects endothelial cells against apoptosis induced by TNF-α. In addition, incubation of endothelial cells with insulin reduced the basal levels of apoptosis by ~21%. Moreover, apoptosis induced by angiotensin II (Ang II) was completely prevented by insulin (Ang II 5.2 ± 0.25% compared with Ang II plus insulin 2.6 ± 0.1%, P < 0.05), indicating a general antiapoptotic action of insulin.

Furthermore, we compared the apoptosis-suppressive effect of insulin with that of VEGF.1,7 VEGF dose-dependently prevented TNF-α-induced apoptosis with maximal effects at 100 ng/mL (with TNF-α, 7.5 ± 2.3% apoptotic cells compared with TNF-α plus VEGF 2.2 ± 0.7%, P < 0.05). Moreover, similar results were obtained when apoptosis was induced by growth factor withdrawal. Incubation of HUVECs for 24 hours in EGM-1 without supplements induced apoptosis in 57 ±7% of the cells. Coincubation with VEGF (100 ng/mL) or insulin (100 nmol/L) significantly reduced apoptosis to 35 ±7% and 27 ±9%, respectively (P < 0.001). Thus, the apoptosis-suppressive effect of insulin is essentially equipotent to the effect of VEGF.

Effects of Insulin on the PI3K/Akt Pathway in HUVECs

Insulin has been shown to stimulate the protein kinase Akt via activation of PI3K.21 To elucidate the effects of insulin on Akt in endothelial cells, Akt phosphorylation was determined by Western blotting with a phosphospecific Akt antibody, which has been shown to correlate with enzyme activity.7 Therefore, HUVECs were serum-starved for 12 hours and then incubated with insulin. Insulin dose- and time-dependently increased Akt phosphorylation (Figures 2A and 2B), with a maximal increase of ~3-fold after incubation with 100 nmol/L insulin for 1 hour. In the presence of serum, insulin also induced a 2-fold increase in Akt phosphorylation. Western blot analysis with antibodies against total Akt revealed equal protein levels in all samples, thus demonstrating that the increased amount of phosphorylated Akt after insulin stimulation was not due to increased expression of Akt (data not shown). In addition, reprobing of the Western blot with an anti-actin antibody demonstrated equal loading (Figures 2A and 2B).

To verify the contribution of PI3K in mediating Akt phosphorylation, the effect of PI3K inhibitors was investigated. Preincubation of HUVECs with the PI3K inhibitors wortmannin (20 nmol/L) or Ly-294002 (10 μmol/L) completely prevented the insulin-induced Akt phosphorylation, as illustrated in Figure 2C.

Insulin Inhibits TNF-α-Induced Apoptosis via the PI3K/Akt Pathway

Having demonstrated that insulin stimulates Akt phosphorylation via PI3K in HUVECs, we tested the contribution of Akt in the apoptosis-suppressive effect of insulin. Inhibition of PI3K by wortmannin (20 nmol/L) or Ly-294002 (10 μmol/L) completely abrogated the apoptosis-suppressive effect of insulin (100 nmol/L) on TNF-α-induced apoptosis (Figure 3A and data not shown). The compounds did not significantly enhance TNF-α-induced apoptosis (Figure 3A) and further elicited only a minor effect on basal apoptosis (Ly-294002 171 ±31% and wortmannin 106 ±15%). To test whether the activation of Akt is involved in the antiapoptotic effect of insulin, we transfected HUVECs with Aktmt, a dominant-negative Akt mutant. Overexpression of the dominant-negative Aktmt inhibited insulin-stimulated Akt activation (data not shown). Moreover, the antiapoptotic effect of insulin on TNF-α-induced apoptosis was completely abolished in cells transfected with dominant-negative Aktmt, whereas insulin still exerted protective effects in cells transfected with a control vector (Figure 3B). Transfection of the cells with dominant-negative Aktmt did not enhance basal or TNF-α-induced apoptosis (Figure 3B).

TNF-α Induces Dephosphorylation of Akt

To further test whether TNF-α affects basal Akt activity, HUVECs were cultured in FCS-containing medium to allow for basal Akt activation (see the first lane in Figure 4A) and then incubated with TNF-α for various times. TNF-α (50 ng/mL) induced a time-dependent decrease in basal Akt phosphorylation (Figure 4A). Insulin prevented the dephosphorylation of Akt induced by TNF-α (Figure 4A), thus supporting the important role of Akt in preventing apoptosis. Recently, it has been shown that the protease family of caspas, which are activated by TNF-α, specifically cleave the protein kinase Akt, thereby lowering the amount of Akt.22
However, Western blotting with antibodies against phosphorylated and unphosphorylated forms of Akt did not reveal a decrease of Akt protein levels after TNF-α stimulation (Figure 4B), thus suggesting that TNF-α induces the dephosphorylation of Akt but not the cleavage of Akt. A recent study showed that ceramides trigger the dephosphorylation of Akt.23 Because TNF-α is known to stimulate ceramide release,24 we investigated whether SPP, a metabolite of ceramide that is known to inhibit ceramide-induced apoptosis25 can prevent TNF-α–induced Akt dephosphorylation. As shown in Figure 4C, coinubation of HUVECs with TNF-α (50 ng/mL) and SPP (1 μmol/L) completely blocked the TNF-α–induced dephosphorylation of Akt and moreover enhanced the phosphorylation of Akt compared with unstimulated controls. In addition, SPP inhibited TNF-α–induced apoptosis (Figure 4D), suggesting an important role for ceramide in TNF-α–induced Akt dephosphorylation.

**Downstream Effects of Akt**

Having demonstrated that insulin counteracts TNF-α–induced dephosphorylation of Akt, thereby preventing TNF-α–induced apoptosis, we investigated the role of potential downstream targets of Akt, p70 S6 kinase26 and the Bcl-2–like proapoptotic protein Bad. However, the antiapoptotic effect of insulin was not prevented by the p70 S6 kinase inhibitor rapamycin (TNF-α 257±40%, TNF-α plus insulin 157±26%, TNF-α plus insulin plus rapamycin 159±37%, and rapamycin alone 121±30%). Furthermore, insulin did not stimulate the phosphorylation of Bad as detected by Western blotting with phosphospecific antibodies against the Akt sites Ser 112 and Ser 136, although the control proteins were detected (Figure 5A). Moreover, incubation of HUVECs with insulin did not induce a mobility shift of Bad protein on the SDS–polyacrylamide gel electrophoresis gels (data not shown).

Insulin has been shown to stimulate the production of NO in endothelial cells in a PI3K-dependent manner.27 Moreover, we have recently shown that the phosphorylation of endothelial NO synthase by Akt enhances the enzymatic activity of NO synthase.28 However, inhibition of NO synthase by treatment of the cells with L-NAME, 1 mmol/L) did not reduce the antiapoptotic effect of insulin (Figure 5B), suggesting that the Akt-mediated antiapoptotic effect of insulin is independent of the generation of NO.

Recent studies provide evidence that Akt phosphorylates caspase-9 at Ser 196 and thereby inhibits its activity.29 Indeed, insulin inhibited the TNF-α–stimulated caspase-9 activity in a wortmannin-sensitive manner (TNF-α

---

**Figure 4.** TNF-α induces Akt dephosphorylation. A, Effect of insulin on TNF-α–induced dephosphorylation of Akt. HUVECs were incubated in EGM-1 with FCS and stimulated with TNF-α (50 ng/mL) in the presence or absence of insulin (100 nmol/L) for the time indicated, and Western blotting with a phosphospecific Akt antibody was performed. Phosphorylated Akt and unphosphorylated Akt were used as positive and negative controls, respectively. The blots were reprobed with an anti-actin antibody to demonstrate equal loading. B, Effect of TNF-α on Akt protein levels. HUVECs were cultured in EGM-1 with FCS and stimulated for the time indicated with TNF-α (50 ng/mL), and the phosphorylation of Akt was detected by Western blotting with a phosphospecific antibody. Then the blot was reprobed with an anti-Akt antibody that recognizes the phosphorylated and unphosphorylated Akt protein (lower panel). C, Effect of SPP on TNF-α–induced Akt dephosphorylation. HUVECs were incubated with TNF-α (50 ng/mL) and SPP (1 μmol/L) for the indicated times, and phosphorylation of Akt was detected by Western blot analysis with a phosphospecific Akt antibody. D, HUVECs were incubated with TNF-α (50 ng/mL) for 18 hours in the presence or absence of SPP (1 μmol/L), and apoptosis was determined by fluorescence staining of the nuclei with DAPI. Data are mean±SEM, n=3. *P<0.05, vs TNF-α.
TNF-α-induced apoptosis combined, processes mediated by growth factors and determined by cell proliferation and the inhibition of cell death. 

Here, we demonstrate that insulin protects endothelial cells from induction of apoptosis induced by TNF-α. 

The present study shows, for the first time, the inhibition of endothelial cell apoptosis by insulin and links this cell-protective effect of insulin to the activation of Akt. In addition, our findings demonstrate that insulin not only protects endothelial cells from induction of apoptosis induced by TNF-α or Ang II. 

The antiapoptotic effect of insulin is thereby equipotent to the potent angiogenic factor VEGF. Furthermore, the present study demonstrates that the apoptosis-suppressive effects of insulin are mediated by activation of the protein kinase Akt.

The effects of insulin on protein synthesis, glucose uptake, and cellular growth are known to be mediated via the insulin receptor and the subsequent stimulation of insulin receptor substrate-1–mediated activation of PI3K. In line with these general considerations, the insulin-mediated activation of Akt in HUVECs was PI3K dependent, as confirmed by our use of the pharmacological PI3K inhibitors wortmannin and Ly-294002. In nonendothelial cells, the apoptosis-suppressive effect of insulin has been shown to be mediated by PI3K-dependent activation of Akt. Indeed, similar results were obtained in the present study, wherein TNF-α–induced apoptosis of endothelial cells was inhibited by insulin in a PI3K-dependent fashion. Moreover, overexpression of the dominant-negative Akt mutant, Aktmt, completely abolished the apoptosis-suppressive effect of insulin, indicating that the insulin-induced, PI3K-dependent activation of Akt is indeed necessary for the antiapoptotic function of insulin in HUVECs. 

In addition, we excluded a potential role for p70 S6 kinase, which is known to be activated by PI3K/Akt, in the antiapoptotic effect of insulin. Inhibition of p70 S6 kinase by rapamycin did not affect the inhibition of apoptosis by insulin. This result is consistent with recent data showing that PI3K-dependent activation of Akt, but not of p70 S6 kinase, is necessary for the prevention of apoptosis in rat-1 cells and neuronal cells.

Taken together, these findings indicate that insulin exerts antiapoptotic effects via the protein kinase Akt in a variety of cellular models, suggesting a general antiapoptotic principle. 

**Discussion**

Angiogenesis, the development of new blood vessels, is a highly regulated process that is important for the neovascularization of new tissue and revascularization after ischemic infarction and counteracts the vascular regression after injury of the blood vessel. Angiogenesis is also involved in several pathological processes, like vascularization of tumor tissue or proliferative diabetic retinopathy. Angiogenesis is mainly determined by cell proliferation and the inhibition of cell death combined, processes mediated by growth factors and hormones. Here, we demonstrate that insulin protects endothelial cells from induction of apoptosis induced by TNF-α or Ang II. The antiapoptotic effect of insulin is thereby equipotent to the potent angiogenic factor VEGF. Furthermore, the present study demonstrates that the apoptosis-suppressive effects of insulin are mediated by activation of the protein kinase Akt.

The effects of insulin on protein synthesis, glucose uptake, and cellular growth are known to be mediated via the insulin receptor and the subsequent stimulation of insulin receptor substrate-1–mediated activation of PI3K. In line with these general considerations, the insulin-mediated activation of Akt in HUVECs was PI3K dependent, as confirmed by our use of the pharmacological PI3K inhibitors wortmannin and Ly-294002. In nonendothelial cells, the apoptosis-suppressive effect of insulin has been shown to be mediated by PI3K-dependent activation of Akt. Indeed, similar results were obtained in the present study, wherein TNF-α–induced apoptosis of endothelial cells was inhibited by insulin in a PI3K-dependent fashion. Moreover, overexpression of the dominant-negative Akt mutant, Aktmt, completely abolished the apoptosis-suppressive effect of insulin, indicating that the insulin-induced, PI3K-dependent activation of Akt is indeed necessary for the antiapoptotic function of insulin in HUVECs. 

In addition, we excluded a potential role for p70 S6 kinase, which is known to be activated by PI3K/Akt, in the antiapoptotic effect of insulin. Inhibition of p70 S6 kinase by rapamycin did not affect the inhibition of apoptosis by insulin. This result is consistent with recent data showing that PI3K-dependent activation of Akt, but not of p70 S6 kinase, is necessary for the prevention of apoptosis in rat-1 cells and neuronal cells.

Taken together, these findings indicate that insulin exerts antiapoptotic effects via the protein kinase Akt in a variety of cellular models, suggesting a general antiapoptotic principle.

The present study shows, for the first time, the inhibition of endothelial cell apoptosis by insulin and links this cell-protective effect of insulin to the activation of Akt. In addition, our findings demonstrate that insulin not only counteracts apoptosis due to growth factor withdrawal but also prevents TNF-α receptor–mediated cell death, which occurs in the presence of growth factors. TNF-α has been shown to mediate its proapoptotic effect via specific signaling pathways, leading to activation of the caspase cascade. Our
data now extend these findings, giving evidence that TNF-α dephosphorylates and deactivates Akt kinase. The dephosphorylation of Akt kinase therefore precedes the activation of caspases and cell death. Two to 3 hours after TNF-α stimulation, Akt was maximally dephosphorylated, whereas apoptosis induction and caspase activation were observed between 12 and 18 hours afterward under our experimental setting.20 A recent study suggests that caspase-induced cleavage of Akt may contribute to cell death.22 However, our data demonstrate that TNF-α treatment does not affect the protein level of Akt but significantly reduces its phosphorylation. These data are in accordance with those of Kennedy et al34 or Cardone et al,29 who demonstrated that the protein kinase Akt is not a substrate for caspases but, in contrast, directly inhibits caspase activation. Thus, inhibition of Akt by TNF-α might have a major impact on the TNF-α-induced activation of caspases and the induction of apoptosis. The TNF-α-induced dephosphorylation of Akt is therefore likely mediated via the ceramide pathway. In fact, the ceramide metabolite SPP, which antagonizes ceramide actions,25 prevented dephosphorylation of Akt, thus correlating with the inhibition of TNF-α-induced apoptosis.

The TNF-α-induced dephosphorylation of Akt was abrogated by coincubation with insulin. Thus, insulin not only stimulates basal Akt activity but also reverses the dephosphorylation of Akt induced by TNF-α. The interference of insulin in TNF-α-activated Akt dephosphorylation could be explained by enhanced activation of Akt, thus compensating for the induced dephosphorylation, or by inhibition of the signaling cascade leading to Akt dephosphorylation. Whereas preincubation of TNF-α and C2-ceramide has been shown to inhibit insulin signaling,35,36 the present study now demonstrates that insulin, when added before or simultaneously (data not shown), can prevent TNF-α–induced signaling in endothelial cells.

Having demonstrated that the activity of Akt is an important survival signal for endothelial cells, we further investigated the downstream effector pathways. A recent study gives evidence that the apoptosis-suppressive effect of Akt is mediated via the proapoptotic Bcl-2 family member Bad, which is inactivated by Akt-dependent phosphorylation.12 Bad, in its unphosphorylated form, is thought to induce cell death via heterodimerization or homodimerization. Growth factors as well as interleukin-3 and interleukin-4 have been shown to suppress apoptosis by inducing phosphorylation of Bad at Ser 112 or Ser 136, which can be confirmed by its migration on SDS-polyacrylamide gel electrophoresis (PAGE) gels.12,37 Hence, phosphorylation of Bad at Ser 136, which has been shown to be Akt dependent, seems to be the basic requirement for the antiapoptotic action of Bad. However, we could not detect a modification of Bad migration on SDS-PAGE after incubation with insulin. Furthermore, no phosphorylation of Ser 112 or Ser 136 was detectable in insulin-stimulated HUVECs on Western blots treated with phosphospecific antibodies, although the control protein was recognized. These data suggest that Akt may affect other targets besides Bad. Indeed, this suggestion is supported by a recent publication demonstrating that Akt-mediated cell survival is not causally related to phosphorylation of Bad protein.37

Recent studies provide evidence that Akt stimulates the phosphorylation of endothelial NO synthase and thereby activates the enzyme.28 However, blocking the insulin-stimulated generation of NO by LNMMA, an inhibitor of NO synthase, did not reduce the apoptosis-suppressive effect of insulin on TNF-α-induced apoptosis of HUVECs.

Finally, we analyzed the contribution of Akt-stimulated phosphorylation of Ser 196 within caspase-9, which inhibits caspase-9 activity.29 Indeed, TNF-α–induced caspase-9 activity was reduced by insulin in a P13K-dependent manner. Moreover, caspase-9–induced apoptosis was significantly prevented by insulin. Most important, apoptosis induced by overexpression of a caspase-9 mutant lacking the Akt phosphorylation site Ser 196 was only partially reversed by insulin. Taken together, the suppression of apoptosis by insulin appears to be at least in part mediated by Akt-stimulated caspase-9 phosphorylation and inactivation, whereas the other Akt substrates Bad, endothelial NO synthase, or p70 S6 kinase do not seem to be involved. A very recent study demonstrated that the inhibition of glycogen synthase kinase-3 by Akt-dependent phosphorylation prevented growth factor withdrawal–induced apoptosis.38 Further studies are required to elucidate the role of glycogen synthase kinase-3 in insulin-mediated inhibition of endothelial cell apoptosis.

To summarize, this study reveals an important role for Akt in the survival of endothelial cells. The proposed signal transduction pathways suggested by the data of the present study are illustrated in Figure 6. The antiapoptotic effect of insulin and VEGF (data not shown) is mediated via activation of Akt in a P13K-dependent manner. The cell-protective effects of insulin in endothelial cells are mediated not only by enhancing basal activity of the protein kinase Akt but also by abrogating the TNF-α–induced Akt dephosphorylation.

Beyond that, this study now indicates that insulin prevents apoptotic death of endothelial cells induced by inflammatory stimuli and the proatherosclerotic factor Ang II. This effect may importantly contribute to cell homeostasis and protect the integrity of the endothelium. This may be of general importance for angiogenesis, for which an undisturbed proliferation of cells is a prerequisite. In fact, angiogenesis occurs as a natural reaction to chronic ischemia. Because the time course of spontaneous neovascularization under pathophysiological conditions is too slow, selective stimulation of the Akt pathway may provide a novel therapeutic approach to enhance the neovascularization process.

Figure 6. Insulin counteracts TNF-α–induced Akt dephosphorylation: proposed signaling events.
Acknowledgment

This work was supported by grants from the Deutsche Forschungsgemeinschaft Di 600/2–3 and SFB-553. C.H. has a fellowship from Boehringer Ingelheim Fond.

References


Hermann et al
Insulin Inhibits Endothelial Cell Apoptosis
Insulin-Mediated Stimulation of Protein Kinase Akt: A Potent Survival Signaling Cascade for Endothelial Cells
Corinna Hermann, Birgit Assmus, Carmen Urbich, Andreas M. Zeiher and Stefanie Dimmeler

doi: 10.1161/01.ATV.20.2.402

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2000 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/20/2/402

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at: http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/