Akt pathway is hypoactivated by synergistic actions of diabetes mellitus and hypercholesterolemia resulting in advanced coronary artery disease

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1Cardiovascular Division, Hospital of the University of Pennsylvania and Cardiovascular Institute, University of Pennsylvania, 2Penn Epilepsy Center and Department of Neurology, University of Pennsylvania, 3Department of Radiology, Hospital of the University of Pennsylvania, and 4Department of Medicine, Institute for Diabetes, Obesity, and Metabolism, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania

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Akt pathway is hypoactivated by synergistic actions of diabetes mellitus and hypercholesterolemia resulting in advanced coronary artery disease. Am J Physiol Heart Circ Physiol 299: H699–H706, 2010. First published July 2, 2010; doi:10.1152/ajpheart.00071.2010.—Atherosclerosis is an inflammatory process leading to enhanced cellular proliferation, apoptosis, and vasa vasorum (VV) neovascularization. While both diabetes mellitus (DM) and hypercholesterolemia (HC) predispose to atherosclerosis, the precise interaction of these risk factors is unclear. Akt is a central node in signaling pathways important for inflammation, and we hypothesized that DM/HC would lead to aberrant Akt signaling and advanced, complex atherosclerosis. DM was induced in pigs by streptozotocin and HC by a high-fat diet. Animals were randomized to control (non-DM, non-HC), DM only, HC only, and DM/HC groups. Coronary artery homogenates were analyzed by immunoblotting for proteins involved in the Akt pathway, including phosphorylated (p)-Akt (Ser473), p-GSK-3β (Ser9), activated NF-κB p65, and VEGF. Immunohistochemical staining for Ki67 (cell proliferation), terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) (apoptosis), and von Willebrand factor (vWF) (neovascularization) was performed. Neovascularization was visualized with micro-computed tomography (CT). Only DM/HC animals developed advanced atherosclerosis and showed decreased p-Akt (Ser473) and p-GSK-3β (Ser9) levels ($P < 0.01$ and $P < 0.05$, respectively). DM/HC arteries demonstrated increased cellular proliferation ($P < 0.001$), apoptosis ($P < 0.01$), and activation of NF-κB p65 ($P < 0.05$). Induction of DM/HC also resulted in significant VV neovascularization by enhanced VEGF expression ($P < 0.05$), increased vWF staining ($P < 0.01$), and increased density by micro-CT. In conclusion, DM and HC synergistically resulted in complex atherosclerosis associated with attenuated p-Akt (Ser473) levels. Aberrant Akt signaling correlated with increased inflammation, cellular proliferation, apoptosis, and VV neovascularization. Our results revealed a synergistic effect of DM and HC in triggering abnormal Akt signaling, resulting in advanced atherosclerosis.

The combination of diabetes mellitus (DM) and hypercholesterolemia (HC) greatly increases the risk for the development and complications of coronary artery disease (CAD) (23, 48). At any given serum cholesterol level the mortality rates from CAD are three- to fivefold greater in diabetics than nondiabetics (51), and over 80% of diabetics will die of vascular related causes. However, the molecular mechanisms driving the pathogenic vascular processes are not fully elucidated, and there are limited data illustrating how DM and HC act in concert to affect disease development and progression.

The serine/threonine kinase Akt [or protein kinase B (PKB)] is a central signaling node involved in cell growth, proliferation, differentiation, apoptosis, angiogenesis, and inflammation (36, 49). A growing number of disease processes have been linked to aberrant loss or gain of Akt activation, including numerous cancers and type 2 DM (29, 35). Many of the cellular processes regulated by Akt also play a central role in atherosclerosis, and so Akt signaling dysfunction may affect atherosclerosis development and progression.

Akt activity is induced after activation of phosphatidylinositol 3-kinase (PI3K) by a variety of growth factors. For example, insulin binds to growth factor receptor tyrosine kinases (RTKs), which results in the insulin receptor substrate (IRS) family of adaptor molecules engaging and activating PI3K at the plasma membrane. PI3K phosphorylates phosphoinositides to produce the lipid second messenger phosphatidylinositol 3,4,5-trisphosphate (P(1,4,5)P3), which binds to Akt, recruiting the molecule to the plasma membrane. Akt is subsequently phosphorylated at the Thr308 position by 3-phosphoinositide-dependent kinase (PDK1) and at the Ser473 position by the mammalian target of rapamycin–rictor kinase complex (mTORc), resulting in the activated, phosphorylated form of Akt, p-Akt (10, 14, 19, 49, 50, 54).

When activated, Akt phosphorylates GSK-3β (p-GSK-3β), which in turn inactivates this kinase, leading to increased cell survival by blocking the function of proapoptotic proteins such as Bad and decreasing the transcription of death genes by phosphorylation of the forkhead transcription factors (10). By inactivating GSK-3β, p-Akt also prevents the degradation of the antiapoptotic substrate myeloid cell leukemia-1 (Mcl-1) (61). The effects of Akt-dependent phosphorylation of GSK-3β on cellular proliferation are not completely understood. Activated Akt has been shown to enhance the stability of proteins responsible for the G1-to-S phase cell cycle transition, such as cyclin D, cyclin E, c-jun, and c-myc (36). However, p-Akt has also been shown to block cell cycle progression by phosphorylating and inhibiting p21 (24, 38, 62). Akt plays a direct role in NF-κB activation and subsequent inflammation by enhancing the deg-
radation of the NF-κB inhibitor IκB (28) and is involved in modulating the chemotactic response of neutrophils and macrophages to inflammatory foci (30). Finally, Akt plays an important role in angiogenesis by causing increased production of hypoxia-inducible factor (HIF-1α and HIF-2α) transcription factors, leading to increased expression and secretion of VEGF (36). In summary, while activated Akt appears to play a major role in maintaining cellular homeostasis and is considered antiatherosclerotic, hypoaevation of Akt may help drive the development of atherosclerosis.

The role of the Akt signaling pathway and CAD has not been defined. Since patients with DM and HC have more complex CAD (41), we hypothesized that DM and HC synergistically effect Akt signaling and are associated with the development of complex atherosclerosis. We evaluated this association by comparing the Akt signaling pathway in DM/HC animals, which develop complex disease (20, 40), to Akt signaling in control, DM-only, and HC-only animals, which do not.

MATERIALS AND METHODS

Animals and experimental protocol. All animal procedures conformed to U.S. Department of Agriculture regulations and requirements and were approved by the University of Pennsylvania Animal Care and Use Committee. Yorkshire domestic male swine weighing 20–25 kg (Archer Farms, Darlington, MD) were randomized into one of four groups: control (non-DM, non-HC, n = 9), DM only (n = 5), HC only (n = 5), and DM/HC (n = 10). An additional four DM/HC animals were used to evaluate the temporal effects of DM/HC on Akt signaling. DM was induced by the intravenous administration of 125 mg/kg of streptozotocin (Sicor Pharmaceuticals, Irvine, CA), while HC was induced by an atherogenic diet, which was continued until death (0.5% cholesterol, 10% lard, and 1.5% sodium cholate; Animal Specialties, Quakertown, PA) (20, 40, 57). Exogenous insulin was administered to U.S. Department of Agriculture regulations and require-

Histology/immunohistochemistry. Movat pentachrome staining was performed on paraffin-embedded 5-μm sections. For immunohistochemistry, after deparaffinizing and two 5-min washes in PBS, the sections were blocked for 20 min with a solution of 2% unconjugated goat anti-mouse IgG in PBS. Indicated dilutions of the primary antibodies were added to the slides and incubated for 2 h at room temperature, followed by two 5-min washes with PBS. Biotinylated secondary IgG diluted at 1:100 was added to the slides and incubated for 30 min. After two additional 5-min PBS washes, the tissue sections were treated with a 3% solution of H2O2 to quench endogenous peroxidase activity. After two additional 5-min washes, the slides were incubated for 30 min in solution containing the avidin-biotin-peroxidase complex. Diaminobenzidine/H2O2 substrate was allowed to react with the peroxidase-labeled tissue sections for 20 min. After two 1-min rinses in distilled water, the tissue sections were counterstained with hematoxylin and eosin, cleared, and dehydrated by successive gradations through 70%, 95%, and 100% ethanol, followed by a final passage in xylene. The slides were mounted and analyzed by bright field microscopy with a Leica DM4000 B microscope (Bannockburn, IL) and a Leica DC 480 camera.

Proliferating cells were detected by staining with an antibody to Ki67. Quantification of Ki67-positive staining was performed by morphometric analysis of slides at ×400 magnification by two techni-
cians in a blinded manner. The area of the tissue that exhibited immunostaining was quantified within the arterial medial and intimal layers, and the percentage of Ki67-positive cells was determined with Image-Pro Plus (Media Cybernetics) software.

Apoptosis was evaluated with the terminal deoxynucleotidyltrans-
ferase-mediated dUPT nick end labeling (TUNEL) TACS Blue Label kit (R&D Systems, Minneapolis, MN). Briefly, 5-μm sequential sections were deparaffinized as described for the immunohistochemical analysis and treated with protease K. After quenching of endogenous peroxidase activity with H2O2, the slides were washed with PBS. TUNEL reaction mixture was then added to tissue sections, followed by endogenous peroxidase quenching in a humidified chamber for 60 min at 37°C. The slides were then rinsed in PBS, color developed, and counterstained with nuclear fast red. Analysis of the slides was performed under a bright field microscope with Image-Pro Plus for quantifica-
tion.

Quantification of vasa vasorum (VV) area and density was made on arterial segments (n = 6/artery) stained with vWF antibody. Comput-
erized analysis with Image-Pro Plus under ×50 magnification was performed by an investigator in a blinded manner, and results were independently verified by a second investigator. The area bordered by the external elastic lamina and the outer adventitia, excluding muscle, adipose tissue, and fibrous tissue, was analyzed with a technique described by Edelman et al. (13). The concentration (total number of vasa/mm2 of vessel wall) and area density (area of vasa/mm2 of vessel wall) of VV for each section were then calculated.
Micro-computerized tomography evaluation of VV neovascularization. To analyze the development of the VV, three arteries from each group were imaged with micro-computerized tomography (CT). After removal of the heart, glass cannulas were tied at the coronary orifices and injected with 500 ml of heparinized saline (0.9% sodium chloride + 5,000 IU unfractionated heparin) for 30 min at physiological pressure to clear the vascular system. A low-viscosity, radiopaque liquid polymer compound (MV-122; Canton Biomedical Products, Boulder, CO) was injected through the cannulas until the injected mass flowed freely from the arterial vent. The heart was then immersed in 10% buffered formalin and refrigerated at 4°C overnight to allow compound polymerization. On the following day, arterial segments 60–80 mm in length were removed and placed in a 95% alcohol solution for 48 h. At successive 24-h intervals, the glycerin concentration was raised from 30%, 50%, 75%, and finally 100% to completely dehydrate the segments. The specimen was rinsed with acetone, left in the open air for 24 h, and embedded in paraffin molds for three-dimensional micro-CT imaging. All samples underwent micro-CT imaging at 24-μm isotropic resolution with an Explore Locus SP specimen scanner (GE Healthcare).

Ex vivo treatment of coronary arteries with insulin. Coronary arteries from each of the four groups were incubated immediately after collection in DMEM containing 1 mM insulin for 5 min. The arteries were then processed for Western blot analysis with p-Akt (Ser473) and Akt antibodies.

Statistical analysis. Numerical data are expressed as means ± SD, unless otherwise noted. Comparisons of multiple groups were made by analysis of variance (ANOVA), and if significant the Scheffé method was performed to evaluate intergroup differences. SPSS version 12 was used for statistical analysis. A P value < 0.05 was considered significant.

RESULTS

In total 33 animals were used in this study, of which 29 were killed at ~24 wk after induction, 2 DM/HC animals at 1 mo after induction, and 2 DM/HC animals at 3 mo. At baseline, serum glucose levels averaged 65.0 ± 5.4 mg/dl and cholesterol levels 91.0 ± 5.2 mg/dl. At death, blood glucose levels were significantly higher in the DM (441.3 ± 33.3 mg/dl) and DM/HC (415.0 ± 50.0 mg/dl) groups compared with control (64.7 ± 5.4 mg/dl) and HC (85.2 ± 30.2 mg/dl) groups (P < 0.001). Both control and HC animals rapidly gained weight during the course of the experiment (77.8 ± 4.5 kg and 85.2 ± 30.2 kg, respectively, at death), while DM and DM/HC animals had significantly reduced body weight gain (31.5 ± 1.6 kg and 44.1 ± 1.7 kg, respectively; P < 0.01). Despite identical diets and feeding protocols, serum cholesterol levels were increased in the DM/HC group (619 ± 100 mg/dl) compared with the HC group (326 ± 54 mg/dl) (P < 0.01), indicating an effect of DM on subsequent serum cholesterol levels.

Histology of coronary lesions. Control and DM animals did not develop atherosclerotic lesions (Fig. 1, A and B). HC pigs (Fig. 1C) exhibited moderate coronary atherosclerosis, which was relatively homogeneous in appearance and characterized as pathological intimal thickening (55). Sections collected from DM/HC pigs exhibited extensive complex atherosclerosis demonstrating a range of high-risk lesions including fibroatheromas, thin-fibrous cap atheromas, and fibrocalcific lesions (Fig. 1D). This observation is consistent with previously pub-
lished data by our group (40, 57) and demonstrates a synergistic effect of DM and HC in the development of complex coronary atherosclerosis. By morphometry the percent stenosis for HC animals averaged 27.5 ± 23.9%, intimal area 0.79 ± 1.41 mm², and intimal-to-medial ratio 0.67 ± 1.54. For DM/HC animals the percent stenosis was similar at 27.7 ± 27.8%, intimal area 0.74 ± 1.45 mm², and intimal-to-medial ratio 0.78 ± 1.20. The extent of disease is similar in both HC and DM/HC animals.

Effect of DM/HC on Akt pathway. Previous studies using mouse models have shown that Akt exerts vascular protection against atherosclerosis (16), so we hypothesized that the complex atherosclerosis observed in the DM/HC group would be associated with aberrant Akt signaling. Indeed, induction of DM and HC led to significantly decreased p-Akt (Ser473) levels (P < 0.01), whereas DM or HC alone did not (Fig. 2). This suggests that DM and HC may act synergistically to modulate the Akt pathway.

Phosphorylation of Akt results in subsequent phosphorylation of GSK-3β at the Ser9 site, thereby inactivating the enzyme. GSK-3β regulates important cellular processes involved in atherosclerosis such as cellular survival and cellular proliferation (36), so we investigated the effect of DM/HC on GSK-3β kinase phosphorylation. Induction of DM and HC acted synergistically to decrease the phosphorylation of GSK-3β (Ser9) (P < 0.05, Fig. 2), thus leading to activation of the enzyme. Control, DM, and HC groups did not display this aberrant signaling (Fig. 2). Hence, the combination of DM and HC was associated with enhanced activation of GSK-3β, thus implicating a potential role of aberrant downstream Akt signaling in the development of advanced atherosclerosis.

Effects of hypophosphorylated Akt on markers of inflammation, including cellular proliferation, apoptosis, and activation of NF-κB. DM/HC arteries demonstrated a highly significant increase in cellular proliferation as demonstrated by Ki67 staining (P < 0.001) compared with other experimental groups (Fig. 3A). Hypophosphorylation of Akt was also associated with increased levels of cellular apoptosis as shown by the results of TUNEL staining (P < 0.01, Fig. 3B). Interestingly, apoptotic cells were generally located either within the fibrous cap or within the necrotic core (Fig. 3B), suggesting increased plaque vulnerability. Finally, we investigated the synergistic effect of DM and HC on the activation of the proinflammatory and prosurvival transcription factor NF-κB, which is activated...
in atherogenesis (11, 58). The results showed that while DM or HC alone had no effect on NF-κB p65 activation, the combination of DM and HC resulted in a significant increase in enzyme activation (P < 0.05, Fig. 3C).

**Effect of DM/HC on vasa vasorum neovascularization.** Enhanced VV neovascularization has been implicated in the development of atherosclerosis by providing inflammatory cells access to sites of inflammation (22, 31), and insofar as Akt signaling pathway plays a key role in regulating angiogenesis, we determined the effect of DM/HC on plaque neovascularization. DM/HC animals demonstrated a significantly increased density of VV by vWF staining (P < 0.01) compared with other groups (Fig. 4A), a finding confirmed by direct visualization of the VV by micro-CT (Fig. 4B). Enhanced VV neovascularization was associated with increased expression of VEGF in the DM/HC group (P < 0.05, Fig. 4C).

**p-Akt and p-GSK-3β levels early after DM/HC induction.** To assess the temporal effect of DM/HC on Akt and GSK-3β phosphorylation we determined their levels 1 and 3 mo after DM/HC induction. The results demonstrated a mild effect on Akt hypoactivation at 1 mo and a greater effect at 3 mo (Fig. 5).

**Effect of ex vivo insulin treatment of coronary arteries on Akt signaling.** We tested the hypothesis that insulin therapy may normalize aberrant Akt signaling associated with the induction of DM and HC by treating ex vivo arteries with insulin and analyzing Akt phosphorylation. The results demonstrate that p-Akt (Ser473) levels were restored to normal levels in DM/HC arteries treated with insulin (Fig. 6).

**DISCUSSION**

Patients with both DM and HC have an increased risk of developing CAD, but more importantly, an increased risk of developing complications of CAD, i.e., death, myocardial infarction, and stroke. Increased oxidative stress, inflammation, and transvascular LDL transport appear to play critical roles in diabetic cardiovascular disease, resulting in larger necrotic cores and a greater influx of macrophages and T lymphocytes (5, 41, 46). In the DM/HC pig model, complex atherosclerosis was associated with hypoactivation of the Akt signaling pathway. This abnormal signaling was associated with reduced phosphorylation of GSK-3β (disinhibiting GSK-3β), increased activation of NF-κB p65, and increased expression of VEGF.

As a result, DM/HC animals exhibited increased cellular proliferation, apoptosis, inflammation, and VV neovascularization. The effects of DM/HC on reducing Akt activation were noted early after DM/HC induction and could be rapidly reversed by the administration of exogenous insulin. In addition to shedding light on the pathophysiology of DM/HC-induced atherosclerosis, these results suggest that the importance of maintaining physiological insulin levels extends beyond glycemic control.

Our results demonstrate that decreased activation of Akt plays a role in the development of severe atherosclerosis. Hypophosphorylation of Akt inactivates the enzyme, thereby effecting...
important downstream processes regulating atherosclerosis and inflammation. In the double-knockout (ApoE−/−/Akt1−/−) mouse models, loss of Akt leads to severe atherosclerosis and occlusive CAD, as activated Akt is thought to exert vascular protection against atherosclerosis (16). In addition, macrophages from the double-knockout mice are more susceptible to oxidized LDL-induced apoptosis than control cells, indicating that the Akt pathway is important for macrophage survival and macrophage-dependent vascular inflammation (16). The finding in endotoxemic mice that reduced Akt activity resulted in enhanced inflammation (6, 33) is of particular importance given the role of oxidized LDL in inducing the initial inflammatory vascular response that progresses to atherosclerosis (56). Furthermore, the neointima of stented arteries from diabetic rats (both type 1 and type 2) exhibit attenuated p-Akt levels, which in turn correlate with increased neointimal area (27). While data from rodent models have linked attenuated p-Akt levels with the development of atherosclerosis, the present studies show that hypoactivated Akt was associated with complex, advanced CAD, the phenotype thought responsible for acute ischemic cardiac death and acute coronary syndromes. We conclude that the loss of Akt activation results in loss of vascular protection, contributing to increased levels of vascular inflammation and disease progression.

Interestingly, the combination of both risk factors was required to cause significant aberrant Akt signaling, demonstrating a synergistic effect of DM and HC on Akt phosphorylation and the development of complex atherosclerosis. The mechanism by which type 1 DM causes hypophosphorylated Akt has been well studied, as insulin is one growth factor that can activate PI3K by binding to RTKs, leading to the formation of PIP3 and the subsequent phosphorylation of Akt by PDK1 and activate PI3K by binding to RTKs, leading to the formation of

Hypophosphorylated Akt thus led to disinhibition of GSK-3β (Fig. 2). In regard to apoptosis, activated GSK-3β leads to the phosphorylation and degradation of the antiapoptotic BCL-2 family member Mcl-1, leading to increased apoptosis (37). Furthermore, previous work in mice has shown that the absence of Akt induces increased vascular smooth muscle cell apoptosis (17). In DM/HC pigs, disinhibition of GSK-3β by hypophosphorylated Akt was associated with increased cellular apoptosis consistent with these findings (Fig. 3B). GSK-3β may also have direct effects on atherosclerosis development, as previous studies have demonstrated that GSK-3β participates in activation of NF-κB, an important mediator of cell proliferation and inflammation (25, 45, 53, 59), results confirmed in the present study (Fig. 3, A and C). In hyperglycemic ApoE-deficient mice treatment with an inhibitor of GSK-3β, valproic acid, had antiatherogenic effects (4), providing further evidence that GSK-3β activation may be playing a direct role in the development of advanced atherosclerosis.

The DM/HC state also affected the major proangiogenic signaling mediator VEGF, leading to increased neovascularization within lesions. Multiple studies have demonstrated the importance of VV neovascularization in the development of atherosclerosis (2, 18, 22, 31, 32, 42, 44), and VEGF-induced neovascularization has been postulated to be a therapeutic target to reduce atherosclerosis (21, 43). An interesting finding from our experiments is that VEGF-induced neovascularization was present despite hypoactivation of Akt. Previous studies primarily performed on cell culture models have shown that Akt activation is both necessary and sufficient to enhance VEGF expression and angiogenesis (12, 26, 39) and that inhibition of Akt decreases VEGF expression (7, 15, 52). However, in our model, VEGF expression and VV neovascularization were induced in the setting of hypophosphorylated Akt. There are potential explanations for this apparent uncoupling of Akt and VEGF signaling. Akt signaling is complicated by multiple negative feedback loops and redundant pathways, and other AGC kinase family members have been shown to activate pathways previously thought to be unique Akt targets (for review see Ref. 35). For example, S6 kinase 1 (S6K1) can phosphorylate GSK-3 on its Akt site under conditions of elevated mTORc1-S6K1 signaling, triggering a negative feedback loop that blocks activation of Akt (36, 60). It is feasible that the induction of DM and HC may activate an alternative AGC kinase that drives the expression of VEGF and a negative

Fig. 6. Ex vivo insulin treatment of coronary arteries normalizes Akt signaling in the DM/HC group. A: Western blot analyses. B: densitometry results.

A

B
effect on Akt phosphorylation early may prove helpful in the primary prevention of CAD in patients with diabetes.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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