Apoptosis has been observed in vascular cells, nerve, and myocardium of diabetic humans and experimental animals, although whether it contributes to or is a marker of complications in these tissues is unclear. Previous studies have shown that incubation of human umbilical vein endothelial cells (HUVECs) with 30 vs. 5 mmol/l glucose for 72 h causes a significant increase in apoptosis, possibly related to an increase in oxidative stress. We report here that this increase in apoptosis (assessed morphologically by TdT-mediated dUTP nick-end labeling staining) is preceded (24 h of incubation) by inhibition of fatty acid oxidation, by increases in diacylglycerol synthesis, the concentration of malonyl CoA, and caspase-3 activity, and by decreases in mitochondrial membrane potential and cellular ATP content. In addition, the phosphorylation of Akt in the presence of 150 μU/ml insulin was impaired. No increases in ceramide content or its de novo synthesis were observed. AMP-activated protein kinase (AMPK) activity was not diminished; however, incubation with the AMPK activator 5-aminoimidazole-4-carboxamide-riboside increased AMPK activity twofold and completely prevented all of these changes. Likewise, expression of a constitutively active AMPK in HUVEC prevented the increase in caspase-3 activity. The results indicate that alterations in fatty-acid metabolism, impaired Akt activation by insulin, and increased caspase-3 activity precede visible evidence of apoptosis in HUVEC incubated in a hyperglycemic medium. They also suggest that AMPK could play an important role in protecting the endothelial cell against the adverse effects of sustained hyperglycemia. *Diabetes* 51:159–167, 2002

Vascular disease that affects both the micro- and macrovasculature is the principal cause of morbidity and mortality in patients with diabetes (1). A wide variety of studies suggest that an early site at which these vascular complications develop is the endothelium. In particular, early abnormalities such as increases in renal and retinal blood flow, impaired vaso-dilation in response to physiological and pharmacological stimuli, and increased vascular permeability have been attributed to endothelial cell damage (1). Morphological correlates of these functional abnormalities were not initially identified; however, recent studies have shown an increase in apoptosis in various organs affected by diabetes, including the eye (2,3), heart (4,5), and vascular endothelium (5).

The Diabetes Control and Complications Trial (6) and the U.K. Prospective Diabetes Study (7,8) have linked hyperglycemia to the occurrence of microvascular disease in patients with diabetes. Although less clear-cut, a relationship between hyperglycemia and macrovascular disease (9) has also been reported. That hyperglycemia increases apoptosis in cultured endothelium was first demonstrated by Barmgartner-Parzer et al. (10) in HUVECs and since then by many others (11–13). It is well-established that apoptosis can be induced by internal signals, such as cytochrome C and apoptosis-inducing factors released from mitochondria, and that it is commonly associated with decreases in mitochondrial membrane potential and ATP production (14–16). In addition, such factors as caspases, ceramide, and oxidative stress have been implicated in its causation, whereas other factors, including shear stress, IGF-I, insulin, and Akt activation, have been shown to be antiapoptotic (17–21). Despite this, the metabolic mechanism(s) by which hyperglycemia initiates apoptosis in endothelium is incompletely understood.

This article addresses the notion that the apoptosis caused by sustained hyperglycemia in cultured HUVECs is associated with alterations in intracellular fatty-acid metabolism. Early studies showed that incubation with 5-aminoimidazole-4-carboxamide-riboside (AICAR), which activates AMPK by formation of ZMP, enhances fatty-acid oxidation in many cells (22) and inhibits apoptosis in thymocytes treated with glucocorticoids (23) and cultured fibroblasts deprived of growth factors (24). The mechanism responsible for this antiapoptotic action was not

**Hyperglycemia-Induced Apoptosis in Human Umbilical Vein Endothelial Cells**

**Inhibition by the AMP-Activated Protein Kinase Activation**

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AICAR, 5-aminoimidazole-4-carboxamide-riboside; AMPK, AMP-activated protein kinase; DAG, diacylglycerol; eNOS, endothelial nitric oxide synthase; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cell; PKC, protein kinase C; TUNEL, TdT-mediated dUTP nick-end labeling.
examined. For this reason, in the present study, the effects of AICAR on fatty-acid metabolism and on indexes of apoptosis, such as TdT-mediated dUTP nick-end labeling (TUNEL) staining and caspase-3 activity, were evaluated in HUVEC exposed to media enriched in glucose. In addition, the effect of expressing a constitutively active AMPK on caspase-3 activity in these cells was examined. A preliminary report describing parts of this work has appeared elsewhere (25).

RESEARCH DESIGN AND METHODS
All chemicals were obtained from Sigma (St. Louis, MO), and radioactive materials were obtained from NEN (Boston, MA) unless otherwise stated. HUVECs and EBM-2 growth media with EGM-2 bullet kit were purchased from Clonetics (San Diego, CA). Cells in passage 5 were used.

Assessment of apoptosis by TUNEL staining. Cells were grown to confluence in EBM-2 medium with EGM-2 bullet kit in 60-mm dishes or six-well plates. The medium was then changed to Medium-199 (containing phenol red as pH indicator) supplemented with 10% fetal bovine serum (FBS; certified from Gibco), antibiotics, and additions as indicated in RESULTS. After 72 h of incubation, during which the medium was changed daily, the cells were fixed with 2% paraformaldehyde in phosphate buffer. Apoptosis was assessed without knowledge of cell source by one of us (Y.L.) by TUNEL staining with diaminobenzidine and DAB. Cells were counted using a kit obtained from Oncogene (Boston, MA). The number of stained cells was assessed using the computer software program NIH image, not by human eyes, to avoid potential subjective errors. Nuclear fragmentation was also assessed at high magnification by light microscopy to confirm apoptosis. In separate experiments, Annexin-V and propidium iodide double staining were performed to distinguish apoptosis from necrosis. Percentage apoptosis was calculated from the number of TUNEL-positive cells divided by the total number of cells counted. Three to five images obtained in a systematically random manner were analyzed in each dish, representing ~1,000–1,500 cells per dish. Because of some variation among the dishes, even in the same treatment group, eight dishes or three wells of a six-well culture plate were evaluated for each treatment group.

Assessment of apoptosis by caspase-3 activity. Although morphological assessment revealed clear changes in the number of TUNEL-positive cells, we measured caspase-3 activity to confirm that apoptosis was occurring. Cells were grown in six-well plates to reach confluence and were exposed to Medium-199 supplemented with 10% FBS with 5 or 30 mmol/l glucose ± 1 mmol/l AICAR for 24 h. In the experiments using adenovirus, cells were infected for 24 h, and medium was changed to Medium-199 supplemented with 10% FBS and 5 mmol/l glucose, and the cells were harvested 24 h later. Caspase-3 activity was assayed in cell extracts with an EnzChek Caspase-3 Assay Kit #2 (Molecular Probes, Eugene, OR) by measuring the fluorescence signal of the cleaved product by the enzyme. Values for activity were normalized for cell protein.

Constitutively active AMP-kinase adenovirus. Constitutively active AMP-kinase adenovirus was constructed from truncated rat AMP-kinase adenovirus. The virus also coexpressed GFP by a second promotor, so GFP expression could be used to assess the efficiency of infection. Preliminary studies revealed that within 24 h of infection, nearly all (~90%) the endothelial cells expressed GFP and that AMP-independent AMP-kinase activity was three- to fivefold higher than in control cells. This AMP-kinase activity is similar to that found in HUVEC treated with 1 mmol/l AICAR for 1–2 h.

Metabolic studies. As described by others (10) and as shown here (Fig. 1), incubation in media containing 30 mmol/l glucose does not increase apoptosis in HUVEC after 24 h, but it does by 72 h. For this reason, metabolic and signaling changes antedating the increase in TUNEL staining were evaluated in cells incubated for 24 h in Medium-199 supplemented with 10% FBS and 50 mmol/l glucose. Glucose oxidation was assessed by determining 14CO2 released into the medium during a 2-h incubation with 14C-glucose. In separate experiments, free fatty oxidation was determined from H2O production as previously described using [3H]palmitic acid as a substrate (28). For this measurement, the cells were first preincubated for 24 h with radioactive palmitate, because unless intracellular lipid pools are prelabeled, the rate of fatty-acid oxidation was greatly underestimated (29). Incorporation of label into cell diacylglycerol was assessed after its separation by thin-layer chromatography. Cell protein was measured by the bicinchoninic acid (BCA) assay.

Mitochondrial membrane potential and ATP measurement. Mitochondrial membrane potential was estimated by incubating the cells for 15 min with a depolarizing buffer containing HEPES pH 7.4, 20 mmol/l NaCl, 110 mmol/l KCl, 2 mmol/l MgCl2, 1.5 mmol/l CaCl2, 1 mmol/l NaPO4, and 0.01 μCi/ml [3H]tetraphenylphosphonium (TPP; from Amersham, Piscataway, NJ). The final concentration of TPP was 5 mmol/l, well below the concentration reported to inhibit mitochondrial respiration (30). After incubation, the cells were washed with ice-cold phosphate-buffered saline three times and solubilized with NaOH, and the radioactivity and protein content were measured. Cellular ATP levels were determined with a luminometric assay kit from Sigma as described previously (28).

Akt Western blot. Confluent cells in 100-mm dishes were incubated in Medium-199 supplemented with 1% FBS for 24 h and then were incubated with 150 μU/ml insulin for 20 min. Cells were harvested with a lysis buffer containing 20 mmol/l Tris-HCl (pH 8.0), 100 mmol/l NaCl, 25 mmol/l b-glycerophosphate, 1 mmol/l EDTA, 1 mmol/l EGTA, 1 mmol/l NaVO3, 1% NP-40, 1 mmol/l DTT, 1 mmol/l phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml pepstatin-A. Proteins were separated by SDS-PAGE and transferred onto a polyvinylidene fluoride membrane. Phosphorylation of Akt was assessed with anti–phospho-S473 Akt antibody. After stripping, total Akt with an antibody obtained from Cell Signaling Technologies (Beverly, MA) was measured.

Ceramide and serine incorporation into ceramide. Ceramide levels were assessed in lipid extracts of cells grown in 100-mm dish by converting ceramide to ceramide-1-phosphate using diacylglycerol (DAG)-kinase and 32P-ATP followed by thin-layer chromatography (31). Serine incorporation into ceramide was assessed in cells incubated with 3H-serine (final specific activity, ~1,000 dpm/μmol) for 16 h.

Malonyl-CoA. Malonyl-CoA was measured in a neutralized 0.6-N perchloric acid cell extract by the method described by Poser and Lynen (32). Fatty acid synthase was purified from Baker’s yeast (33). AMP-kinase activity was assayed in ammonium sulfate precipitates of the cell homogenate by measuring the phosphorylation of SAMS peptide (28).

Statistics. Statistical analysis was done by the general linear models procedure in SAS program on a Macintosh computer.

RESULTS
Hyperglycemia-induced apoptosis is inhibited by AICAR and by expression of constitutively active AMP-kinase. As shown in Fig. 1, the percentage of TUNEL-positive cells was similar (3–4%) in HUVEC incubated for 24 h in the EBM-2 media containing 5 or 30 mmol/l glucose. In addition, no differences in cell number, morphology, or protein were observed at this time (data not shown). By 72 h, however, a significantly greater percentage of cells (18 vs. 13%; P < 0.05) incubated at the higher glucose concentration were apoptotic. As also shown in Fig. 1, AICAR had no affect on apoptotic rate in cells incubated in 30 mmol/l glucose after 24 h, but it completely prevented the increase in apoptosis caused by hyperglycemia at 72 h. No increase in apoptosis was observed at 72 h in an osmotic control in which cells were incubated with 5 mmol/l glucose and 25 mmol/l mannitol, in agreement with the findings of others (11) (data not shown).

Similar results were obtained with Annexin-V staining. Of note, <5% of the Annexin-V–positive cells demonstrated nuclear staining with propidium iodide, indicating that they were predominantly apoptotic and not necrotic (data not shown). As shown in Fig. 1, an increase in caspase-3 activity, which is thought to be an early signal of apoptosis, was evident in cells incubated with 30 mmol/l glucose for 24 h (1,225 ± 42 arbitrary units vs. 1,002 ± 61 for 5 mmol/l glucose; P < 0.01; n = 5), and it was also completely prevented by addition of AICAR (833 ± 14 arbitrary units; P < 0.01 vs. 30 mmol/l glucose). A similar decrease in caspase-3 activity was observed when AMPK activity was increased by infecting HUVEC with constitutively active AMPK using an adenoviral vector (Fig. 1).

Changes in mitochondrial function and cellular ATP are prevented by AICAR. As a first step in delineating
FIG. 1. Effects of AICAR on hyperglycemia-induced apoptosis. A: HUVECs were incubated for 72 h in Medium-199 + 10% FBS ± 1 mmol/1 AICAR at the indicated glucose concentration. Typical TUNEL staining (dark brown) with methylene blue counterstaining. TUNEL-positive cells showed a typical apoptotic configuration with shrinkage and nuclear fragmentation. Representative pictures are shown. (See RESEARCH DESIGN AND METHODS for details). B: The percentage of TUNEL-positive cells was determined in three wells of six-well plates (24 h) or in eight of 60-mm dishes. Data are means ± SD; *P < 0.05, vs. 5 mmol/l glucose; †P < 0.05, vs. 30 mmol/l glucose alone. C: Caspase-3 activity in HUVEC incubated for 24 h in 5 or 30 mmol/l glucose in the presence or absence of AICAR (left; results are means ± SE; n = 5); *P < 0.05, vs. 5 mmol/l glucose; †P < 0.05, vs. 30 mmol/l glucose alone and HUVEC infected with a GFP-tagged control adenovirus or the adenovirus linked to constitutively active AMPK (right; results are means ± SE; n = 6); *P < 0.05, 5 mmol/l glucose with GFP alone; †P < 0.05, vs. 30 mmol/l glucose with GFP alone. Approximately 95% of the cells showed positively for GFP. AMPK activity was three- to fourfold higher in the cells infected with the constitutively active AMPK virus.
events that precede the increase in TUNEL staining, mitochondrial membrane potential and ATP content were evaluated in HUVEC incubated for 24 h at the two glucose concentrations. Although we did not estimate absolute membrane potential, accumulated TPP^{+}/H^{+} was reduced significantly after incubation for 24 h at 30 mmol/l glucose, suggesting that it was diminished. This effect was totally prevented by AICAR (Fig. 2). Very similar changes were observed in cellular ATP levels, which were diminished after incubation with 30 mmol/l glucose but were, if anything, higher than control values when AICAR was added to the medium.

**Sustained hyperglycemia alters intracellular fatty-acid and glucose metabolism and Akt activation but does not alter ceramide content or de novo synthesis.** We showed previously that incubation of HUVEC for 2 h in media containing 30 mmol/l vs. 5 mmol/l glucose causes no changes in AMPK activity, malonyl-CoA concentration, fatty-acid oxidation, or lactate release. In contrast, incubation at this glucose concentration for 24 h resulted in increases in the concentration of malonyl-CoA, diminished fatty-acid oxidation, and an increased incorporation of both radioactive glucose and radioactive palmitate into diacylglycerol (Fig. 3). In addition, it impaired Akt phosphorylation in the presence of insulin (Fig. 4). Like the decrease in mitochondrial membrane potential and increases in caspase-3 activity caused by hyperglycemia, these changes were completely prevented by incubation with AICAR; indeed, the concentration of malonyl-CoA was even lower than that in cells incubated with 5 mmol/l glucose. AMPK activity tended to be higher after 24 h of incubation with 30 mmol/l glucose; however, whether it was inappropriately low, in light of the decrease in ATP content (and presumably an increased AMP/ATP ratio), was not ascertained.

To assess whether alterations in ceramide contributed to these events and particularly the decrease in membrane potential, we attempted to measure ceramide content. Ceramide was not detectable in HUVEC incubated with either 5 or 30 mmol/l glucose. In contrast, HUVEC incubated in a medium containing 5 mmol/l glucose and 0.1 mmol/l palmitate for 24 h showed a ceramide band on autoradiography but no increase in TUNEL staining (data not shown). Consistent with these findings, we found no increase in serine incorporation into ceramide (presumably a measure of de novo synthesis) in cells incubated for 24 h in 30 vs. 5 mmol/l glucose. Observed values were 8.4 ± 0.5, 10.8 ± 0.4, and 9.0 ± 1.2 pmol/mg (n = 4) in cells incubated in 5 and 30 mmol/l glucose and 30 mmol/l glucose plus AICAR, respectively.

**Glucose metabolism.** Hyperglycemia caused a small but significant increase in lactate release by HUVEC but did not alter either pyruvate release or glucose oxidation (Fig. 5). Incubation with AICAR diminished both lactate and pyruvate release, as it has been reported to do in endothelial cells incubated for 2 h with 5 mmol/l glucose (28). In addition, and in contrast to the latter situation, it caused glucose oxidation to diminish by 60% (Fig. 5).

**DISCUSSION**

The principal findings of this study are fourfold. 1) Hyperglycemia-induced apoptosis in HUVEC, judged by TUNEL staining, is preceded by inhibition of fatty-acid oxidation, increases in fatty-acid esterification and the concentration of malonyl-CoA, and decreases in mitochondrial membrane potential and cellular ATP content. 2) These metabolic alterations are associated with an increase in caspase-3 activity and an impaired ability of insulin at a physiological concentration to activate (phosphorylate) Akt. 3) Incubation with the cell-permeable AMPK activator AICAR prevents all of these changes. 4) Overexpression of...
a constitutively active AMPK prevents the activation of caspase-3 by sustained hyperglycemia, suggesting that the antiapoptotic effect of AICAR is AMPK-mediated.

The finding that hyperglycemia of several days’ duration increases apoptosis in cultured HUVEC, as judged by cellular morphology and TUNEL staining, confirms the results of earlier investigations (10–12). Recent studies have attributed this effect of hyperglycemia to increases in oxidative stress and intracellular Ca\(^{2+}\) that, in turn, lead to sequential increases in JNK/SAPK and caspase-3 activity (12). The results of the present study suggest involvement of two other events that have been implicated in causing apoptosis in other settings (14,16). One of these is mitochondrial dysfunction, as evidenced by the loss of mitochondrial membrane potential (Ψ) and a decrease in cellular ATP content, and the other is an impaired ability of insulin to stimulate the phosphorylation (and presumably the activation) of Akt. As recently reviewed (20), Akt plays a pivotal role in preventing apoptosis in a variety of settings. In particular, its activation is crucial for the ability of such factors as shear stress, insulin, and IGF-I and other growth factors (e.g., VEGF) to inhibit apoptosis in cultured endothelium (21,34).

Increases in DAG mass attributable to de novo synthesis and increased protein kinase C (PKC) activity have been described in endothelial cells incubated for 3 days in a

FIG. 3. Fatty-acid metabolism (A and B), AMP-kinase (C), and malonyl-CoA (D). All parameters were measured in HUVECs incubated with Medium-199 + 10% FBS ± 1 mmol/l AICAR for 24 h. Fatty-acid oxidation was measured over 2 h after 24 h of prelabeling with \(^3\)H-palmitate. Data are means ± SE (n = 4–6). *P < 0.05, vs. 5 mmol/l glucose; †P < 0.05, vs. 30 mmol/l glucose.
glucose; † for this is the observation that overexpression of a constitutive active AMPK virus should resolve more definitively whether the effects of AICAR are solely due to AMPK activation.

In light of the alterations in fatty-acid metabolism caused by hyperglycemia, the effect of palmitate on apoptosis has been examined in preliminary studies. In keeping with findings in cardiomyocytes (40,45,46) and pancreatic islets (47), incubation with palmitate (0.5 mmol/l) markedly increased apoptosis in HUVEC (data not shown). In contrast to cells incubated with 30 mmol/l glucose, this was preceded by an increase in ceramide synthesis, which, like the apoptosis, was prevented by AICAR. Similar observations have been made in cultured retinal pericytes (48). Whether these findings reflect different metabolic mechanisms whereby hyperglycemia and palmitate cause apoptosis remains to be determined. In this context, the contribution of ceramide to palmitate-induced apoptosis in other cell types has been questioned (49). Furthermore, it has been shown that fatty acids that do not enhance de novo ceramide synthesis can also increase apoptosis in cultured endothelium (50).

The relevance of hyperglycemia and free fatty acid–induced apoptosis to the pathogenesis of diabetic complications is still unsettled. The demonstration that apoptosis increases in myocardium (4,5), vascular endothelium (5), retina (2), and neural tissues (51) of diabetic humans and experimental animals suggests that if it is not an integral event, then it is at least a marker of complications in many tissues. Of particular note is the finding of Frustaci et al. (5) that apoptosis is increased by 61- and 85-fold in endothelial cells and cardiomyocytes, respectively, in ventricular myocardial biopsies obtained from diabetic humans. It is interesting that they found that apoptosis in both of these cells was strongly associated with positive staining for nitrotyrosine, suggesting a correlation with oxidative stress and peroxynitrate generation.

A final question raised by the present study is, “What role does AMPK play in the physiology of the normal endothelial cell?” Hardie and Carling (22), who first demonstrated that AMPK is regulated by changes in the AMP/ATP ratio, suggested that it serves as a fuel gauge that responds to a decrease in the energy state of a cell by increasing ATP generation (e.g., by increasing fatty-acid oxidation) and diminishing the use of ATP for certain purposes (e.g., fatty-acid synthesis). In keeping with this hypothesis, it has been shown that AMPK activity increases acutely in response to a variety of stresses, for example, glucose; † for this is the observation that overexpression of a constitutive active AMPK virus should resolve more definitively whether the effects of AICAR are solely due to AMPK activation.

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including postischemia hypoxia and hyperosmolarity in many cells, and in skeletal muscle in response to exercise (50,51). Because AMPK seems to activate eNOS in endothelium (42) and exercising muscle (52), an interesting possibility is that it regulates the increase in endothelial cell NO generation caused by shear stress. In addition, we have observed that AMPK in HUVEC is acutely activated by incubation in a glucose-free medium (29) and by hydrogen peroxide (Y. Ido, J. Keany, N. Ruderman, unpublished data), suggesting that it could play a role in the response of the endothelial cell to a wide variety of perturbations. Finally, recent studies suggest that AMPK in muscle can be activated in vivo by treatment with metformin (53) and by leptin infusion (54). Whether similar changes occur in endothelium and, if so, what their physiological importance is remain to be determined.

In conclusion, the results indicate that hyperglycemia-induced increases in TUNEL staining in cultured HUVEC are preceded by changes in intracellular fatty-acid metabolism, mitochondrial dysfunction, caspase-3 activation, and a decreased ability of insulin to activate Akt. They also indicate that all of these events are prevented by incubation with AICAR, which seems to act by activating AMPK. Additional studies are needed to establish the mechanisms

FIG. 5. Glucose metabolism: lactate production (A), pyruvate production (B), glucose oxidation (C), and glucose incorporation into DAG (D). All parameters were measured in HUVECs incubated with Medium-199 + 10% FBS ± 1 mmol/l AICAR for 24 h. Glycolysis (lactate and pyruvate release) and glucose oxidation were measured over 2 h in media containing [U-14C]glucose. Data are means ± SE (n = 6). *P < 0.05, vs. 5 mmol/l glucose; †P < 0.05, vs. 30 mmol/l glucose.
responsible for these findings and their physiological relevance.

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