Diabetes Activates Cell Death Pathway After Transient Focal Cerebral Ischemia

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It is well known that diabetes aggravates brain damage in experimental and clinical stroke subjects. Diabetes accelerates maturation of neuronal damage, increases infarct volume, and induces postischemic seizures. The mechanism by which diabetes increases ischemic brain damage is still elusive. Our previous experiments indicate that mitochondria dysfunction may play a role in neuronal death. The objective of this study is to determine whether streptozotocin-induced diabetes activates cell death pathway after a brief period of focal cerebral ischemia. Both diabetic and nondiabetic rats were subjected to 30 min of transient middle cerebral artery occlusion, followed by 0, 0.5, 3, and 6 h of reperfusion. We first determined the pathological outcomes after 7 days of recovery by histopathology, and then detected key components of programmed cell death pathway using immunocytochemistry coupled with confocal laser-scanning microscopy and Western blot analysis. The results show that the cytosolic cytochrome c increased mildly after reperfusion in nondiabetic samples. This increase was markedly enhanced in diabetic rats in both ischemic focus and penumbra. Subsequently, caspase-3 was activated and poly-ADP ribose polymerase (PARP) was cleaved. Our results suggest that activation of apoptotic cell death pathway may play a pivotal role in exaggerating brain damage in diabetic subjects. *Diabetes* 52:481–486, 2003

Both acute hyperglycemia and chronic diabetes exaggerate ischemic brain damage (1–5). The mechanisms by which diabetes aggravates ischemic brain damage are still elusive. Vascular physiological and morphological alterations are prominent in chronic diabetes and contribute to the marked damage. However, since acute hyperglycemia induced by glucose solution infusion or acute diabetes (2–3 days) induced by streptozotocin (STZ) injection exacerbates brain damage exactly in the same way as chronic diabetes enhances cerebral stroke, other mechanisms may be involved.

Previous studies have demonstrated that mitochondrial dysfunction and mitochondria-initiated apoptosis are involved in pathophysiology of cerebral ischemia (6–9). Under adverse conditions, such as calcium overload and reactive free radical generation, a mitochondrial permeability transition (MPT) pore is formed at the binding site of inner and outer membranes. The MPT pore is a nonspecific mega pore that allows ions and intramitochondrial substances with molecular masses <1,500 Da to freely equilibrate across the membrane. Release of mitochondrial substrate proteins such as cytochrome c and apoptosis inducing factor (AIF) into the cytosol activates proteolytic proteases known as caspases and the intracellular apoptotic machinery (9–12). Downstream in the caspase cascade is the activation of DNases, causing DNA fragmentation and apoptosis (13,14).

We have previously shown that acute hyperglycemia induces early release of cytochrome c into the cytosol, activates caspase-3 (a cell death executioner), and causes DNA fragmentation after a transient global cerebral ischemia (15,16). The objective of this study is to determine whether STZ-induced 1-month diabetes activates apoptotic cell death pathway after a brief period of focal cerebral ischemia.

RESEARCH DESIGN AND METHODS

Animals. A total of 71 male Wistar rats (Charles River Laboratories, Wilmington, MA), weighing 310–340 g, were used in the presented experiments. Experimental groups and number of rats in each group are given in Table 1. All animal use procedures were in strict accordance with the National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee at the University of Hawaii.

Animal surgical procedures. The animals were fasted overnight but had free access to drinking water before induction of diabetes. Diabetes was induced by a single subcutaneous injection of 40 mg/kg body wt of STZ (Sigma) freshly dissolved in 0.1 mol/l citrate buffer at pH 4.5. The presence of hyperglycemia was confirmed 48 h after STZ administration via an Ames Glucometer II analysis using a tail stick (Miles Laboratory Inc., Elkhart, IN). Animals with blood glucose concentration >16 mmol/l were selected (n = 4). This blood glucose level was chosen based on previous observation of extensive neuronal necrosis in all animals with plasma glucose levels exceeding 16 mmol/l (17). Age-matched rats were injected with an equal volume of citrate buffer to serve as nondiabetic normoglycemic controls. Before the induction of ischemia, the animals were fasted again overnight with free access to drinking water.

Anesthesia was induced by inhalation of 3.5% halothane. The animals were then ventilated artificially on 1–1.5% halothane in N_2O/O_2 (70/30) during surgery. Ventilation and oxygen supply were adjusted to give a $PaCO_2$ of 35–40 mmHg, a PaO_2 over 100 mmHg, and a pH close to 7.40.

Ischemic model. A 30-min duration of transient middle cerebral artery (MCA) occlusion was induced by the intraluminal filament as described before (4). Core temperature was regularly maintained at 37°C by a heating pad. Neurological behaviors were examined after ischemia/reperfusion, and rats without neurological abnormalities were excluded (n = 7). Neurological signs included diminished resistance to lateral push, walking to the left after being pulled backwards by the tail, and spontaneous contralateral circling. Brain

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AIF, apoptosis inducing factor; ECL, enhanced chemiluminescence; GFAP, glial fibrillary acidic protein; MCA, middle cerebral artery; MPT, mitochondrial permeability transition; NIH, National Institutes of Health; PARP, poly-ADP ribose polymerase; PVDF, polyvinylidine fluoride; STZ, streptozotocin.

TABLE 1
Experimental groups and number of rats in each group

	Nondiabetic					Diabetic				
	Control	0.5 h	3 h	6 h	7 days	Control	0.5 h	3 h	6 h	7 days
Histopathology		3	3	3	6		3	3	3	6
Immuncytochemistry*	2	3	3	3		2	3	3	3	
Western blot analysis	2	4	4	4		2	4	4	4	

*Brain sections used for immunocytochemistry are derived from the same rats used for histopathology at 0.5 and 6 h of recovery.

samples were collected at the end of 30-min ischemia and after 0.5, 3, and 6 h of recirculation for histological, immunocytochemical, and Western blot analyses. For histological study, brains were sampled in additional groups of animals after 7 days of recovery. Sham-operated diabetic and nondiabetic rats were used as controls.

Evaluation of cerebral ischemic damage. At 0.5, 3, and 6 h of recovery, animals were perfusion-fixed with 4% paraformaldehyde (pH 7.4). Vibratome brain sections of 20 μm thick were stained with hematoxylin and eosin. The sections were examined by light microscopy. The same rat brains were also used for immunocytochemical staining. Brains of two additional groups, diabetic animals and nondiabetic animals, were perfusion-fixed after 7 days of recovery and embedded in paraffin. Infarct areas were measured on coronal sections at bregma −0.30 mm using the public domain NIH image program.

Immunocytochemistry. Immunocytochemical staining methods have been described before (15). In brief, vibratome sections of 50 μm were washed, and nonspecific binding sites were blocked with 3% BSA. The sections were incubated overnight with primary antibodies of cytochrome c at 1:400 (purified mouse monoclonal antibody; PharMingen), cleaved caspase-3 at 1:100 (polyclonal antibody; Cell Signaling), or poly-ADP ribose polymerase (PARP) at 1:100 (rabbit polyclonal antibody; Santa Cruz). The sections were washed and then incubated with fluorescein-conjugated affinipure secondary antibody (Jackson ImmunoResearch Laboratories). Double labeling of the above-mentioned primary antibodies with antineuronal nuclei (NeuN, 1:400) or anti-glial fibrillary acidic protein (GFAP, 1:400) antibodies conjugated with either fluorescein isothiocyanate or rhodamine red were performed. Sections were mounted on glass slides and coverslipped using Gelovatol. The slides were analyzed on a BioRad MRC1024 laser-scanning confocal microscope.

Western blot analysis. The method used for Western blot has been described before (15). Both the striatum and cortex were dissected and homogenized. The cytosolic and nuclear fractions were separated by a series of centrifugations as described before (15). Proteins were separated by 10–15% SDS-PAGE. The same amount of protein (20 μg) was applied to each lane in a slab gel of SDS-PAGE. Following electrophoresis, proteins were transferred to an immobilon-polyvinylidine fluoride (PVDF) membrane. The membrane was incubated with primary antibodies against cytochrome c at a dilution of 1:1,000, or PARP 1:500, overnight at 4°C. Membranes were incubated with horseradish peroxidase–conjugated secondary antibodies for 1 h at room temperature. Blots were then developed using the enhanced chemiluminescence (ECL) method (Amersham).

Statistics. Histological outcomes were analyzed by unpaired Student's t test. Relative density of cytochrome c and PARP were measured with a Photoshop image program. Comparison of recirculation groups with sham-operated controls in diabetic and nondiabetic animals were made by ANOVA followed by post hoc Scheffe's test. Comparison between diabetic and nondiabetic animals at the identical recirculation time point was made by unpaired Student's t test. $P < 0.05$ was regarded as statistically significant. All tests were two-sided.

RESULTS

Physiological variables. Physiological variables were measured at 5 min prior and 20 min after the induction of ischemia. Mean $PaCO_2$ was maintained at 34–43 mmHg, PaO_2 106–123 mmHg, arterial pH 7.40–7.46, blood pressure 103–117 mmHg, and body temperature 36.7–37.4°C. There were no statistical differences for the above parameters between diabetic and nondiabetic groups. As expected, plasma glucose concentrations were significantly higher in diabetic animals (16.4–22.6 mmol/l) than in nondiabetic animals (5.3–5.6 mmol/l) ($P < 0.001$).

Ischemic brain damage in diabetic and nondiabetic rats. The brain damage in both nondiabetic and diabetic rats was evaluated after 0.5, 3, 6 h, and 7 days of recovery following a 30-min MCA occlusion. In nondiabetic animals subjected to stroke, no damage was observed after 30 min of recovery while mild selective neuronal necrosis was observed after 3 and 6 h of recovery. The damage was restricted to the striatum without involvement of cortical structures. In diabetic animals, moderate selective neuronal necrosis was present in the striatum after 30 min of reperfusion, increased after 3 h of recovery, and massive tissue destruction and infarction were seen after 6 h of recirculation (Fig. 1*A–D*). In the cortex of the diabetic rats, a few scattered dark neurons were noticed after 30 min, moderate selective neuronal necrosis after 3 h, and micro-infarction after 6 h of recovery (Fig. 1*E–H*). After 7 days of recovery, the damage was further advanced in nondiabetic but not in diabetic rats, implying the full damage was already reached after 6 h of recirculation in diabetic rats. In nondiabetic rats, micro-infarction foci emerged in the striatum and selective neuronal necrosis was extended to the cortex. In diabetic rats, infarction was observed in both the striatum and cortex. At 7 days after recovery, the mean damage area (infarct and selective neuronal necrosis) was significantly larger in diabetic rats than in nondiabetic rats (38.7 ± 14.7 vs. 15.7 ± 6.3%, $P = 0.005$).

Release of cytochrome c. Cytochrome c release was

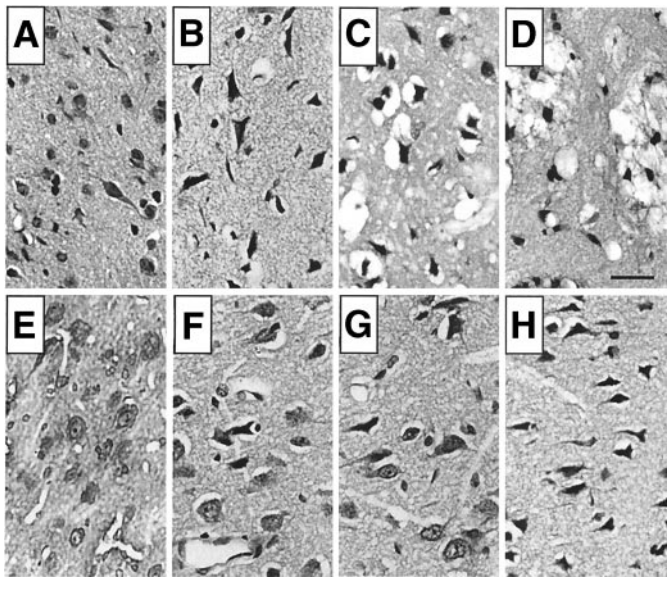

FIG. 1. Representative photomicrographs showing the brain damage in the striatum (*A–D*) and cortex (*E–H*) in diabetic animals subjected to 30-min MCA occlusion. In the striatum, compared with the control (*A*), neuronal necrosis is marked at 30 min (*B*) while infarction is notable at 3 h (*C*) and 6 h (*D*) of reperfusion. In the cortex, the control (*E*) does not have any damaged neurons, while selective neuronal death is evident after 30 min (*F*) and 3 h (*G*), and infarction is evident after 6 h (*H*) of recovery. Hematoxylin and eosin staining. Bar = 50 μm.

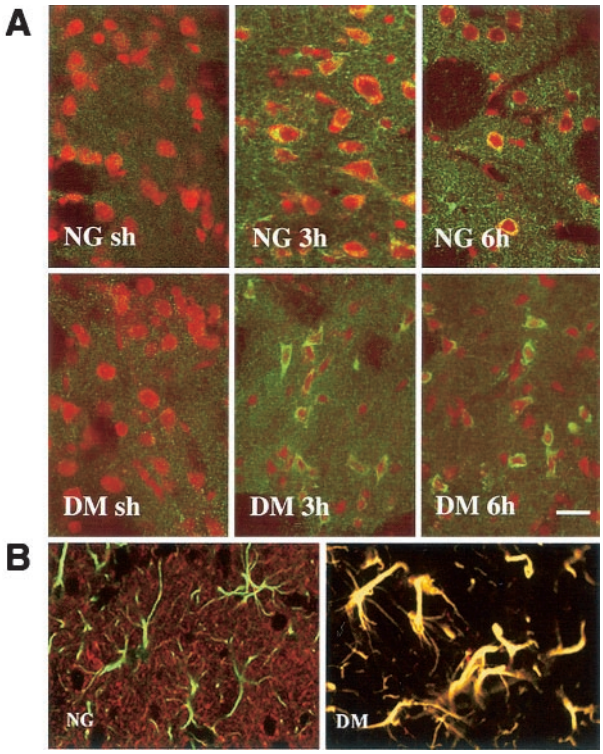

FIG. 2. Double-immunostained confocal images for cytochrome c in nondiabetic normoglycemic (NG) and diabetic (DM) rats in the striatum subjected to sham-operation (sh) or to 30 min transient focal ischemia followed by 3 and 6 h of recovery. *A*: Cytochrome c release in neurons. NeuN, a marker for neuron cells, is stained with red fluorescence and cytochrome c is stained with green fluorescence. Overlay of red and green generates yellow color. Cytosolic cytochrome c release is evident in diabetic sections after 3 and 6 h of reperfusion while it is faint in nondiabetic sections. *B*: Cytochrome c localization in glia cells after 3 h of recovery. Glial cells are marked with green fluorescence and cytochrome c with red. Cytochrome c colocalizes with GFAP-positive glial cells, as reflected in yellow color, in diabetic but not in nondiabetic sections. Bar = 50 μm.

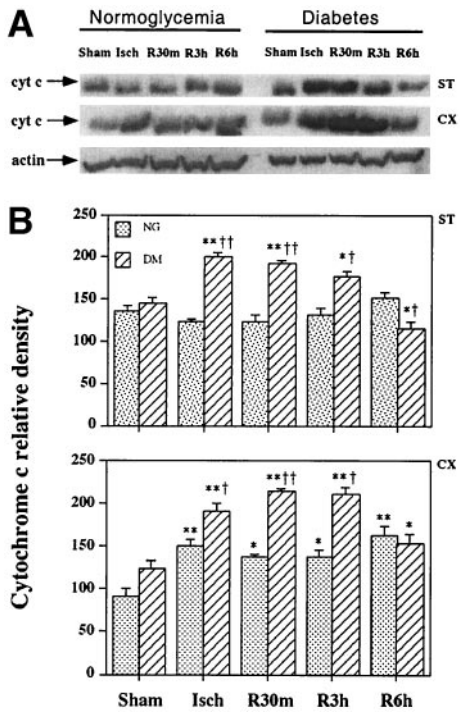

FIG. 3. *A*: Western blot analysis of cytochrome c in the cytosolic fraction from the striatum and cortex after 30-min transient focal ischemia. Cytochrome c level is elevated mildly at 6 h in the striatum and at 0–6 h in the cortex. The release was markedly enhanced by diabetes. *B*: Semiquantitative changes of cytochrome c in the striatum (ST) and cortex (CX). Data are mean ± SD (n = 4 per time point). *P < 0.05, **P < 0.01 vs. control (ANOVA followed by Scheffe's test); † P < 0.05; ††P < 0.01 vs. normoglycemic samples at an identical time point (unpaired Student's t test).

detected using immunocytochemistry and Western blot analysis. Compared with controls, 30-min focal ischemia in nondiabetic rats caused a mild release of cytochrome c in the striatum, while the same duration of ischemia in diabetic rats resulted in a massive cytochrome c release after 3 and 6 h of recovery (Fig. 2*A*). Similarly, cytochrome c was faintly detected in the cortex in nondiabetic rats and a relatively strong staining was present in diabetic rats after 3 and 6 h of recovery (data not shown). Double staining of cytochrome c and GFAP revealed that cytochrome c was colocalized with GFAP-positive astroglial cells (Fig. 2*B*) in diabetic but not in nondiabetic rats after 3 h of recovery. Western blot analysis revealed that nondiabetic ischemia induced mild cytochrome c release after 0–6 h of recirculation in the cortex but not in the striatum, while diabetic ischemia led to marked cytochrome c increase in both the cortex and striatum (Fig. 3). Thus, cytochrome c release in diabetic rats was already evident at the end of ischemia, peaked between 30 min and 3 h, and returned close to basal levels at 6 h of recovery .

Activation of caspase-3. In nondiabetic animals, there was no caspase-3 activation observed in up to 6 h of recovery in neuronal or glia cells in either striatum or cortex. In contrast, diabetic animals showed increased numbers of caspase-3–positive neurons in both cortex and striatum as detected by immunocytochemistry using cleaved caspase-3 antibody. Cleaved caspase-3 was present in the cytoplasm after 3 h and in the nuclei after 6 h of recovery (Fig. 4*A*). Activated caspase-3 was not detected in GFAP-positive astroglial cells.

Nuclear cleavage of PARP. Consistent with the results of caspase-3 activation, confocal imaging revealed that PARP was cleaved in diabetic animals after 3 and 6 h of recovery, but it was not cleaved in nondiabetic animals. The cleaved PARP was located in the nuclei of the neurons in both cortex and striatum. Double staining of PARP and GFAP revealed PARP was not colocalized with GFAP-positive astroglial cells. A set of confocal images illustrating PARP cleavage in the striatum is shown in Fig. 4*B*. Western blot analyses of nuclear fractions showed that cleaved PARP increased after 0.5–6 h of recovery in the striatum and cortex in both nondiabetic and diabetic samples. Compared with nondiabetic ones, diabetic animals had increased levels of PARP cleavage in the striatum (Fig. 5).

DISCUSSION

The present experiments demonstrated that a transient focal ischemia of 30-min duration in diabetic animals causes more damage than in nondiabetic animals. Furthermore, we observed the presence of damage very early in reperfusion phases for diabetic animals. While there was no damage observed after 30 min of recovery in nondiabetic animals, a moderate damage was already observed in diabetic ones. After 7 days of recovery, damage in nondiabetic animals was moderate, whereas damage in diabetic

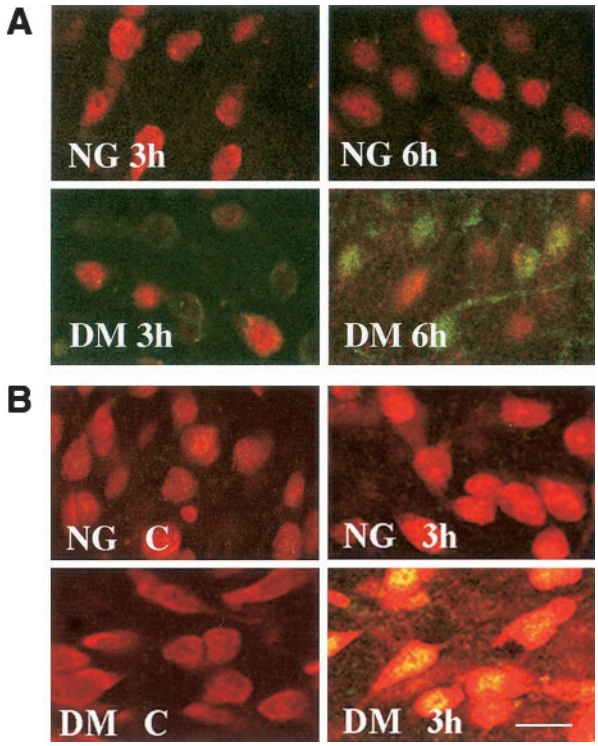

FIG. 4. *A*: Double-immunostained confocal images of caspase-3 in the striatum in normoglycemic (NG) and diabetic (DM) rats subjected to 30-min transient focal ischemia followed by 3 and 6 h of reperfusion. Neurons stained with NeuN are in red, and caspase-3 is stained with green. Colocalization of the two proteins is in yellow, indicating caspase-3 in neurons. There is no observed increase of caspase-3 up to 6 h of reperfusion in normoglycemic animals, while a great increase of caspase-3 levels is observed in diabetic rats after 3 and 6 h of reperfusion. *B*: Double-immunostained confocal images of cleaved PARP in the striatum in normoglycemic and diabetic rats at 3 h of reperfusion. Neurons stained with NeuN are in red, and cleaved PARP is stained with green. The yellow color indicates that cleaved PARP is located in the neuronal nuclei of the diabetic but not the normoglycemic animals. Bar = 50 μm.

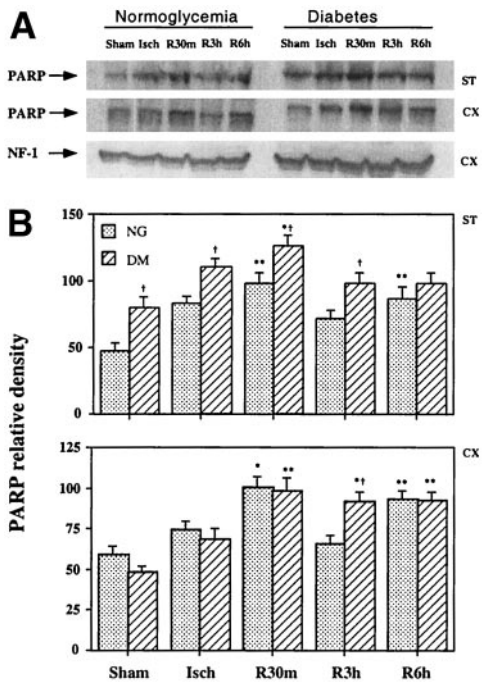

FIG. 5. *A*: Western blot analysis of cleaved PARP in nuclear fraction from the striatum and cortex after 30-min transient focal ischemia. Cleaved PARP level is elevated mildly after 3 and 6 h of reperfusion in both the striatum and cortex. The increases were further enhanced in diabetic relative to nondiabetic rats. *B*: Semiquantitative changes of cleaved PARP after 30-min transient focal ischemia in striatum (ST) and neocortex (CX). Data are mean ± SD (n = 4 per time point). *P < 0.05, **P < 0.01 vs. control (ANOVA followed by Scheffe's test); †P < 0.05; ††P < 0.01 vs. normoglycemic samples at identical time point (unpaired Student's t test).

animals was severe after 6 h of reperfusion, with little change after 7 days of recovery, implying the brain damage is matured after 6 h of recovery in diabetic animals. These histological findings are consistent with previous data published by this laboratory and others (4,18). It has been demonstrated before by Du et al. (18) that a brief period of 30-min focal ischemia in normoglycemic rats leads to brain damage in a surprisingly delayed fashion. Infarctions developed after 3 days and full maturation was reached by 2 weeks after recirculation (18). The damage is usually confined to the striatum, and a large number of neurons surrounding the infarction exhibited prominent TUNEL staining and internucleosomal DNA fragmentation (18). If preischemic hyperglycemia or diabetes exists, the damage evolves much faster. Infarctions developed after 2 h and matured after 4–6 h of recovery (4,5). In addition, cortical infarctions developed in hyperglycemic animals (3–5).

A central finding in this study was that hyperglycemia caused an early release of mitochondrial matrix component cytochrome c, activation of caspase-3, and cleavage of PARP. It is hypothesized that MPT pore is activated by depolarization of the inner membrane, by increased oxidative stress, by elevated mitochondrial calcium load, and by high pH (19–21) (in vitro results [11,12,22]). Results reported from several laboratories have shown that hyperglycemia enhances free radical generation in vitro and in vivo (23–28), including animal cerebral ischemia models (29,30). Thus, hyperglycemia may trigger MPT by increasing free radical generation. Acidosis is a main feature of hyperglycemic ischemia. Acidic pH was shown to inhibit MPT pore in de-energized mitochondrial preparations (31,32). However, this is contradictory to what occurred in vivo since hyperglycemia and enhanced acidosis aggravate neuronal damage (4,5,33,34). A recent study shed light on this issue. Using energized mitochondria preparation from both brain and heart, Kristián et al. (35) demonstrated that acidosis promotes, rather than prevents, the formation of the MPT under conditions mimicking those occurring in vivo in terms of mitochondrial calcium content, P_i concentration, and pH value. We have also previously observed that hyperglycemia causes mitochondrial swelling at early reperfusion phase as detected by electron microscopy (16).

The pore allows calcium to leave the mitochondria and other ions (including H^+) to equilibrate across the inner mitochondrial membrane, leading to the dissipation of the H^+ gradient with a collapse of the transmembrane potential, cessation of ATP synthesis, and uncoupling of oxidative phosphorylation (10–12,22). The pore also allows cytochrome c and other matrix proteins with a mass <1,500 Da to be released from mitochondria. We observed cytochrome c release in diabetic animals as early as 30 min after reperfusion and persisting for at least 3 h. Thus, early mitochondrial damage is a characteristic feature of hyperglycemia-mediated ischemic brain damage.

The released cytochrome c binds Apaf-1 and procaspase-9 to form an apotosome, which activates a family of caspases. Caspases are considered the executioners of cell death since they cause the activation of calpains, caspase-activated endonuclease CAD/DFF40 (36,37), as well as cleavage of a DNA repair enzyme PARP, with an ensuing breakdown of nuclear structure and bioenergetic metabolism (38,39). Caspase-3 is one of the several critical pro-apoptotic executioner proteases. In the present study, marked cytochrome c release was observed in diabetic animals after ischemia/reperfusion, and subsequently, caspase-3 was activated and PARP was cleaved in diabetic animals. These findings suggest that mitochondria dysfunction and mitochondria-initiated cell death pathway, which involves cytochrome c release, caspase-3 activation, and PARP cleavage, may play a key role in mediating diabetes-enhanced ischemic brain damage. This notion is consistent with previous observations that mitochondria MPT pore blocker cyclosporin A reduced hyperglycemia-exaggerated brain damage after ischemia (40).

Mitochondrial dysfunction has been shown to play a critical role in mediating both apoptotic and necrotic cell death (41,42). On one hand, formation of the MPT pore causes release of cytochrome c and other pro-apoptotic molecules, which subsequently activate caspase cascades that sets apoptosis in motion (43). On the other hand, the MPT hinders ATP synthesis and increases ATP hydrolysis. If the cell cannot maintain its ATP content at a certain level, the cell will die of necrosis. In addition, caspase activates PARP. Excessive activation of PARP can deplete ATP that is consumed in regeneration of nicotin-amide-adenosine dinucleotide, leading to necrotic cell death (44). Therefore, the MPT pore is a regulator for both apoptosis and necrosis. Although the apoptotic cell death pathway is activated in diabetic ischemic rats, it is not clear whether the neurons die of apoptosis or necrosis. In our previous publication (16) we demonstrated that hyperglycemic ischemia resulted in increased number of TUNEL positive neurons and the laddered patterning of DNA fragmentation. Yet the ultrastructural alterations in these rats did not fulfill the criteria of apoptotic morphology. Thus, it is likely that activation of the cell death pathway is common at certain stages for both apoptotic and necrotic cell death, or alternatively, that ischemic cell death is a unique process that consists of components of both apoptosis and necrosis. Although it remains undefined whether the neurons in diabetic rats died of apoptosis or necrosis after ischemia and reperfusion, it is clear that early activation of the cell death pathway is a conspicuous feature of hyperglycemia/diabetes-enhanced ischemic brain damage.

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