Published in final edited form as:

Curr Neurovasc Res. 2009 November ; 6(4): 267–278.

Preservation of Cellular Glutathione Status and Mitochondrial Membrane Potential by N-Acetylcysteine and Insulin Sensitizers Prevent Carbonyl Stress-Induced Human Brain Endothelial Cell Apoptosis

Masahiro Okouchi¹, Naotsuka Okayama², and Tak Yee Aw^{*,1}

¹Department of Molecular and Cellular Physiology, Louisiana State University Health Sciences Center, Shreveport, LA, USA

²Department of Internal Medicine and Bioregulation, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

Abstract

Oxidative stress-induced cerebral endothelial cell dysfunction is associated with cerebral microvascular complication of primary diabetic encephalopathy, a neurodegenerative disorder of long-standing diabetes, but the injury mechanisms are poorly understood. This study sought to determine the contribution of carbonyl (methylglyoxal, MG) stress to human brain endothelial cell (IHEC) apoptosis, the relationship to cellular redox status and mitochondrial membrane potential, and the protection by thiol antioxidant and insulin sensitizers. MG exposure induced IHEC apoptosis in association with perturbed cellular glutathione (GSH) redox status, decreased mitochondrial membrane potential ($\Delta\psi_m$), activation of caspase-9 and -3, and cleavage of polyADP-ribose polymerase. Insulin sensitizers such as biguanides or AMP-activated protein kinase activator, but not glitazones, afforded cytoprotection through preventing $\Delta\psi_m$ collapse and activation of caspase-9 that was independent of cellular GSH. Similarly, cyclosporine A prevented $\Delta\psi_m$ collapse, while N-acetylcysteine (NAC) mediated the recovery of cellular GSH redox balance that secondarily preserved $\Delta\psi_m$. Collectively, these results provide mechanistic insights into the role of GSH redox status and mitochondrial potential in carbonyl stress-induced apoptosis of brain endothelial cells, with implications for cerebral microvascular complications associated with primary diabetic encephalopathy. The findings that thiol antioxidant and insulin sensitizers afforded cytoprotection suggest potential therapeutic approaches.

Keywords

Human brain endothelial cells; GSH/GSSG redox status; mitochondrial apoptotic signaling; mitochondrial permeability transition pore; methylglyoxal-induced apoptosis; biguanides; AMP-activated protein kinase activator; glitazones; insulin sensitizers and endothelial cell apoptosis

INTRODUCTION

Memory and learning deficits as well as cognitive impairment and dementia accompany amygdala and hippocampal lesions and atrophy in patients with longstanding diabetes (den Heijer *et al.* 2003; Okouchi *et al.* 2007; Ryan *et al.* 2003). These collective disorders,

*Address correspondence to this author at the Department of Molecular and Cellular Physiology, LSU Health Sciences Center, 1501 Kings Highway, Shreveport, LA 71130-3932, USA; Tel: 318-675-6032; Fax: 318-675-4217; taw@lsuhsc.edu.

defined as primary diabetic encephalopathy, is a late complication of type 1 and 2 diabetes that is independent of the normal aging process, recurrent hypoglycemic episodes, or hypoxic-ischemic insults (Li *et al.* 2005; Sima *et al.* 2004; Sima and Li. 2005). Oxidative stress induced by glucose toxicity and the susceptibility of brain neurons and cerebral microvasculature to oxidative challenge are reportedly major contributing factors to primary diabetic encephalopathy (Li *et al.* 2005; Okouchi *et al.* 2007). Sustained hyperglycemia and elevated levels of reactive carbonyl compound such as methylglyoxal (MG) can induce (a) intracellular oxidative or carbonyl stress through enhanced production of reactive oxygen species (ROS) (Yim *et al.* 1995) or formation of protein aggregates with cell surface or intracellular molecular targets that initiate tyrosine kinase signaling (Akhand *et al.* 1999), and (b) mitochondrial dysfunction (Rosca *et al.* 2002) and activation of caspase cascade (Akhand *et al.* 2001). Reportedly, MG itself is an independent factor which contributed to the progression of diabetic complications (Beisswenger *et al.* 2005). Thus, carbonyl (MG) stress could play an important role in the pathogenesis of diabetic cerebral microvascular complications and primary diabetic encephalopathy. Indeed, the brain microvasculature is involved in oxidative stress-mediated brain damage and dementia progression in neurodegenerative diseases (Okouchi *et al.* 2007), and the endothelium could be an early site at which vascular complications develop (Langston *et al.* 2008; Okouchi *et al.* 2006). Diabetic brain endothelial cell function can further be compromised by elevated glucose toxicity due to hyperglycemic stress-induced impairment of the blood-brain barrier (Huber *et al.* 2006; Ulus *et al.* 2003).

Alongside elevated carbonyl stress, decreased mitochondrial glutathione (GSH) and compromised functions of GSH redox cycle enzymes, GSH peroxidase and GSSG reductase (Ulus *et al.* 2003), have been detected in diabetic brain, suggesting an association between the loss of GSH redox homeostasis and carbonyl challenge. Our recent studies demonstrated that cellular glutathione-to-glutathione disulfide (GSH/GSSG) redox shifts in favor of GSSG is, in fact, a critical event in the initiation of apoptosis in neuron-like pheochromocytoma (PC12) cells induced by carbonyl (MG, [Okouchi *et al.* 2005a]), oxidative (hydroperoxide, [Ekshyyan and Aw. 2005; Pias and Aw. 2002b; Pias *et al.* 2003]), redox (Pias and Aw. 2002a) and hyperglycemic (Okouchi *et al.* 2005b) stress. The mitochondrion is central to apoptotic regulation (Green and Reed. 1998), and current evidence implicates mitochondrial damage in diabetic neurodegeneration (Okouchi *et al.* 2007; Russell *et al.* 2002). Apoptotic signaling is mediated by the collapse of the mitochondrial membrane potential ($\Delta\psi_m$) induced by the opening of the mitochondrial permeability transition pore (mPTP) (Green and Reed. 1998; Russell *et al.* 2002). Loss of $\Delta\psi_m$ leads to depolarization of the inner mitochondrial membrane and is accompanied by the release of mitochondrial cytochrome *c* and downstream activation of effector caspases (Green and Reed. 1998; Russell *et al.* 2002). The precise role that carbonyl stress such as induced by MG, plays in cellular GSH redox imbalance, loss of $\Delta\psi_m$, and initiation of apoptosis in cerebral endothelial cells is unclear.

Insulin sensitizers such as biguanides and glitazones have been widely used for the treatment of type 2 diabetes in which they ameliorate hyperglycemia through distinct metabolic pathways (Inzucchi *et al.* 1998), acting as AMP-activated protein kinase (AMPK) activator (Huypens *et al.* 2005) or peroxisome proliferator-activated receptor (PPAR)- γ (Lovekamp-Swan and Chaffin. 2005), respectively. Lower incidences of Type 2 diabetic-associated coronary artery disease and vascular complications were associated with treatment with metformin, a member of biguanides (1998), which was shown to improve endothelial function irrespective of glycemic control (Katakam *et al.* 2000; Mather *et al.* 2001). Other studies demonstrated that metformin afforded protection of endothelial cells against hyperglycemic stress through inhibition of mPTP function irrespective of glycemic control (Detaille *et al.* 2005), suggesting that preservation of mitochondrial integrity is central to the cytoprotection by insulin sensitizers against glycemic stress. Whether biguanides preserve

mitochondrial integrity against carbonyl stress through similar mechanisms is unknown. The current study was designed to investigate the significance of carbonyl stress (MG) in cerebral microvascular endothelial apoptosis and its relationship to cellular GSH redox status and $\Delta\psi_m$, and mitochondrial apoptotic signaling. Given the effectiveness of insulin sensitizers in endothelial protection, we further investigated whether endothelial cytoprotection by biguanides and/or glitazones against MG stress was linked to the preservation of GSH redox homeostasis and mitochondrial integrity.

MATERIALS AND METHODS

Materials

An immortalized human brain endothelial cell line (IHEC) was kindly provided by Dr. Danica Stanimirovic (Cellular Neurobiology Group, Institute for Biological Sciences, NRC, Canada) and propagated by Dr. Steven J. Alexander at LSUHSC-Shreveport, LA. The following chemicals were obtained from Sigma Chemicals (St. Louis, MO, USA): medium-199, supplement (insulin-transferrin sodium selenite), 4'-diamidino-2-phenyl-indole (DAPI), 2,4-dinitrofluorobenzene (DNFB), 5'-dithio-bis 2-nitrobenzoic acid (DTNB), methylglyoxal (MG), *N*-acetylcysteine (NAC), cyclosporine A, 1,1-dimethylbiguanide hydrochloride (metformin), phenformin hydrochloride, 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR), carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP), valinomycin, 2,4-dinitrophenol (DNP), 2,4-dinitrofluorobenzene, protease inhibitors (aprotinin, leupeptin, phenylmethyl-sulfonyl fluoride), dithiothreitol (DTT), glutathione (GSH and GSSG), 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benza-midazolo- carbocyanin iodide (JC-1) and iodoacetic acid. Troglitazone and pioglitazone were from Cayman Chemical (Ann Arbor, Michigan, USA). Fetal bovine serum was from JRH Biosciences (Lenexa, Kansas, USA). Inhibitors of cas-pase-9 (LEHD-CHO), caspase-8 (IETD-CHO), and caspase-3 (Z-DQMD-FMK) were from Calbiochem (San Diego, CA, USA). Nitrocellulose membranes, Bio-Rad protein dye assay kit, and the SDS electrophoresis units were from BIORAD Corporation (Hercules, CA, USA). Secondary IgG anti-mouse and anti-rabbit antibodies, enhanced chemiluminescence system for Western immunoblot, and hyperfilms were from Amersham (Arlington Heights, IL, USA). Polyclonal antibody against polyADP ribose polymerase (PARP) was from Santa Cruz Biologicals (Santa Cruz, CA, USA), and those against caspase 9 and caspase 8 were from Cell Signaling Technology (Beverly, MA, USA), and Pharmingen (San Diego, CA, USA), respectively. Procaspase 3 (CPP32) antibody was from Upstate Cell Signaling Solutions (Chicago, IL, USA). Monoclonal antibody against β -actin (mouse) was from Oncogene (Cambridge, MA, USA). Fluorescent mounting media was from DAKO Corporation (Carpinteria, CA, USA). Twelve mm circle number 1 glass cover slips, collagen I-coated 60 mm culture plates, T-25 and T-75 cm² flasks were from Discovery Labware (Bedford, MA, USA). All other chemicals were of reagent grade and were purchased from local sources.

Cell Culture and Treatment with Chemical Agents

IHECs were cultured in M199 medium containing 10% heat-inactivated FBS, 1% insulin-transferrin sodiumselenite on T-75 flasks or 60 mm culture plates at 37°C in a 95 % air, 5% CO₂ humidified environment. The culture medium was changed every two days. For all experiments, cells were seeded at specified densities the day before the experiment, and on the day of the experiment, culture media were replaced with fresh serum-free M199 media. MG was added to cell cultures at a final concentration of 300 μ M. In designated experiments, cells were pretreated for 30min with NAC, 2mM; cyclosporine A, 1 μ M; metformin, 100 μ M; phenformin, 200 μ M; AICAR, 2mM; troglitazone, 10 μ M; or pioglitazone, 10 μ M; concentrations that are pharmacologically relevant and are in line with previously published values. Inhibitors of caspase-9, -8, and -3 were each prepared as 20mM stock solutions in

DMSO. Whenever present, the inhibitors were added to cell incubations at final concentrations of 10 μ M each for caspases -9 and -8 and 1 μ M for caspase-3.

Detection of Apoptosis by DAPI Staining

DAPI staining was performed according to Wang *et al.* (Wang *et al.* 1998). IHECs (1×10^5) were grown on 12 mm circular glass coverslips in 24-well plates and incubated with MG without or with the various chemical agents or caspase inhibitors for 24h. Cells were then washed with PBS and fixed with cold 2 % paraformaldehyde for 15 min, followed by fixation with cold 70 % ethanol at -20°C for 1h and stained with 1 $\mu\text{g/ml}$ DAPI for 30 min in the dark. After two additional PBS washes, slides were mounted using DAKO fluorescent mounting fluid and counted using a fluorescent Olympus B $\times$ 50 microscope with a 20 $\times$ objective. At least six fields of total and apoptotic cells were counted on each slide for a total of 500 cells.

Determination of GSH and GSSG, and Protein-SSG

IHECs (2×10^6) in 60 mm culture plates were exposed to 300 μM MG. In some experiments, cells were pretreated with NAC or insulin sensitizers. At times from 0 to 1h, cells were harvested by scraping into ice-cold 5% trichloroacetic acid (TCA). Cell suspensions were centrifuged at 10,000 $\times$ rpm (5min) to remove TCA-insoluble proteins. Soluble GSH and GSSG were determined by high-performance liquid chromatography (HPLC) (Reed *et al.* 1980). Acid supernatants were derivatized with 6mM iodoacetic acid and 1% 2,4-dinitrophenyl fluorobenzene (DNFB) to yield the S-carboxymethyl and 2,4-dinitrophenyl derivatives of GSH and GSSG. GSH and GSSG derivatives were separated on a 250mm $\times$ 4.6mm Alltech Lichrosorb NH_2 10 μm column, and cellular contents quantified by comparison to standards derivatized in the same manner. Protein-bound disulfides (protein-SSG) were measured in TCA-insoluble proteins by a modified Ellman's assay (Ellman and Lysko. 1967). Proteins were solubilized in 0.1M NaOH and neutralized to pH 8.0. Samples (0.4ml) were mixed with 0.7ml phosphate buffer (1.5mM KH_2PO_4 , 6.4mM K_2HPO_4 , 1.6mM EDTA) containing 2mM of 5, 5'-dithio-bis-2-nitrobenzoic acid, and the absorbance read at 410 nm. Total protein thiol/disulfide (protein-SH plus -SSG) and protein-SSG were determined without or with addition of N-ethylmaleimide, respectively.

Protein Expression of Procaspases -8 and 9 and polyADP Ribose Polymerase

Preparation of Total Cell Lysates—Following treatment with MG and various chemical agents, IHECs (2×10^6) on 60 mm culture plates were lysed with 500 μl lysis buffer containing 300mM NaCl, 50mM Tris-HCl, 0.5% Triton X-100, 10 $\mu\text{g/ml}$ leupeptin, 10 $\mu\text{g/ml}$ aprotinin, 1mM PMSF, and 1mM DTT for 30min at 4°C . Cells were homogenized, and whole cell extracts were stored at -20°C until Western analyses of CPP32, procaspases -8 and -9, and polyADP ribose polymerase (PARP).

Western Analyses—Equal volumes of 2 $\times$ sample buffer were added to total cell lysates (20 or 40 μg protein). Samples were boiled (5min), resolved on 8, 10 or 12 % acrylamide gels (100V, 90min), and transferred to nitrocellulose membranes. The membranes were probed individually with CPP32 (1:1000), procaspase 9 (1:1000), procaspase 8 (1:1000), or PARP (1:250) antibodies followed by horseradish peroxidase-conjugated secondary antibodies (1:1000) that corresponded to the respective primary antibodies (mouse, rabbit or goat). Chemiluminescence was detected with an ECL Western blotting detection reagent according to manufacturer's recommendation. Each membrane was stripped (6.25mM Tris pH 6.7, 2% SDS, 100mM mercaptoethanol) and probed for β -actin to verify equal protein loading.

Flow Cytometry

Mitochondrial membrane potential, $\Delta\psi_m$, was detected using the fluorescent cationic dye, JC-1, and flow cytometry (Ekshyyan and Aw. 2005). In control cells with intact $\Delta\psi_m$, JC-1 bears a delocalized positive charge, accumulating and aggregating in the mitochondrial matrix where it fluoresces red. $\Delta\psi_m$ collapse in apoptotic cells causes JC-1 to remain in the cytosol as a green fluorescent monomeric form. Mitochondrial depolarization is determined as decreased red-to-green fluorescence intensity ratio, i.e., fluorescence emission shift from red (590nm) to green (525nm). Thus, apoptotic cells, characterized by decreased $\Delta\psi_m$, will exhibit low red-to-green fluorescence ratio. A combination of FCCP (10 μ M), valinomycin (10 μ M), and DNP (1mM) induces essentially complete mitochondrial depolarization and was used as a positive control. IHECs (1 $\times$ 10⁶) were cultured in 6-well plates and exposed to 300 μ M MG for 4 and 6h. In some experiments, cells were pretreated with cyclosporine A, NAC, metformin, phenformin, AICAR, troglitazone, or pioglitazine at the designated concentrations. Following treatment with these agents, IHECs were collected and incubated with 5 μ g/ml of JC-1 at 37°C for 15min in a 5% CO₂ incubator. After washing, cells were analyzed on a FACS Caliber flow cytometry (Becton Dickinson, San Jose, CA, USA).

Protein Assay

Protein was measured using the Bio-Rad Protein Assay kit according to manufacturer's protocol.

Statistical Analysis

Results are expressed as mean $\pm$ SEM. Data were analyzed using a one-way ANOVA with Fisher corrections for multiple comparisons. $P < 0.05$ were considered statistically significant.

RESULTS

MG-Induced IHEC Apoptosis was Associated with Cellular GSH Redox Imbalance and Prevented by NAC

Initial studies showed that MG dose-dependently induced IHEC apoptosis at 24h over the range of 100–1000 μ M (8–30%). A concentration of 300 μ M MG elicited between 16–18% cell apoptosis and was used in subsequent experiments to examine the relationships between apoptosis and cellular GSH redox, mitochondrial apoptotic signaling and cytoprotection by NAC and insulin sensitizers. Pretreatment with NAC significantly attenuated MG-induced IHEC apoptosis; however, the addition of NAC at 1h post-MG treatment did not confer cytoprotection (Fig. 1A), suggesting a role for early redox signaling in MG-induced IHEC apoptosis. To further explore the relationship between MG stress and redox status, we determined cellular GSH/GSSG contents. Time course experiments (0–1h) showed that 300 μ M MG induced GSH oxidation maximally at 15min (data not shown), indicating an early and rapid induction of GSH redox imbalance. At 15min, MG caused significant decreases in GSH and GSSG levels (Fig. 1B). The decreases in GSSG corresponded to significant increases in protein-bound disulfides (protein-SSG, Fig. 1C), suggesting GSSG-mediated oxidation of protein thiols and involvement of soluble and protein bound thiol/disulfide pools in redox homeostasis. Total GSH pool (soluble GSH/GSSG plus protein-bound thiol/disulfide) was unaltered, indicating that MG elicited redistribution rather than loss of the cellular thiol-disulfide pools. The combined decrease in GSH and increase in protein-SSG yielded an attenuated GSH-to-disulfide ratio (Fig. 1D), an index of elevated oxidative stress. Pretreatment with NAC restored cell GSH, attenuated GSSG (Fig. 1B) and protein-SSG formation (Fig. 1C), and resulted in highly reduced GSH-to-disulfide ratios (Fig. 1D).

MG Induced the Mitochondrial Pathway of Apoptosis: Activation of Caspases -9 and -3

To evaluate mitochondrial involvement in MG-mediated endothelial cell apoptosis, IHECs were treated with caspase inhibitors of mitochondrial (caspase-9) or death receptor (caspase-8) signaling. IHEC apoptosis was significantly attenuated by inhibition of caspases-9 and -3, but not of caspase-8 (Fig. 2A), indicating that MG mediated IHEC apoptosis occurred through direct mitochondrial signals. The activation of caspases-9 and -3 was examined as further evidence of mitochondrial apoptotic signaling. Basal procaspase-9 level was high and time-dependently decreased from 2–10h post-MG treatment (Fig. 2B), consistent with cleavage of the pro-enzyme to active caspase-9. Caspase-3 activation was evaluated by cleavage of procaspase-3 (CPP-32) and caspase-3-mediated cleavage of its target substrate, polyADP ribose polymerase (PARP). MG elicited significant decreases in the expression of CPP32 at 4–10h (Fig. 2A), consistent with caspase-3 cleavage/ activation. A prominent protein band that corresponded to naive PARP (116 kDa) was present in unchallenged control cells and was time-dependently cleaved to its 85 kDa product from 6–10h (Fig. 2B) following MG exposure, a kinetic time frame that was preceded by caspase-3 activation at 4h. Procaspase-8 expression levels and activation status were unchanged at all time points (Fig. 2B), thus validating non-involvement of the death receptor pathway.

Role of Mitochondrial Membrane Potential ($\Delta\psi_m$) and Permeability Transition in MG-Induced IHEC Apoptosis: Protection by NAC and Cyclosporine A

Changes in $\Delta\psi_m$ (Figs. 3A, B) were assessed using fluorescent JC-1 dye and flow cytometry. Unchallenged control cells exhibited intact $\Delta\psi_m$, represented by high red-to-green fluorescence ratios. A heterogeneous population of cells exhibiting different proportions of red-to-green fluorescence ratios was observed (Fig. 3A). Combination treatment with FCCP, valinomycin, and dinitrophenol induced >90% cells that exhibited low red-to-green fluorescence ratios (Figs. 3A, B), indicative of membrane depolarization and collapse of $\Delta\psi_m$. Exposure to MG for 4h or 6h resulted in time-dependent increases in the fraction of cells with low red-to-green fluorescence ratios, consistent with significant loss of $\Delta\psi_m$ (Figs. 3A, B). MG-induced collapse of $\Delta\psi_m$ at both time points was abrogated by NAC or cyclosporine A, indicating the preservation of $\Delta\psi_m$ by restored GSH status (NAC) and inhibition of mPTP function (cyclosporine A) (Figs. 3A, B). Interestingly, NAC and cyclosporine A treatment resulted in cell distributions that seem to reflect two subpopulations with subpopulation delineation being more prominent in cyclosporine A-treated cells (Fig. 3A). The reason for these differences in cell distribution is unclear and could be related to their respective mechanisms of protection, namely, redox restoration (NAC) versus mPTP preservation (cyclosporine A). Accordingly, pretreatment of IHECs with cyclosporine A, significantly attenuated MG-induced apoptosis (Fig. 3C) validating a role for mitochondrial permeability transition (MPT) in mediating apoptotic death.

Biguanides and AICAR, but Not Glitazones Prevented MG-induced IHEC Apoptosis through Protection Against Mitochondrial Depolarization and Caspase-9 Activation

Biguanides and AICAR (a direct AMPK activator) were shown to prevent MPT and hyperglycemia-induced endothelial cell apoptosis (Detaille *et al.* 2005; Ido *et al.* 2002). To test whether insulin sensitizers preserve mitochondrial potential and prevent IHEC apoptosis during carbonyl stress, we determined $\Delta\psi_m$ and caspase-9 activation. Among the various agents, biguanides, e.g., metformin and phenformin or AICAR, but not glitazones, e.g., troglitazone or pioglitazone, significantly attenuated MG-induced loss of $\Delta\psi_m$ (Fig. 4A); less than 5% cells treated with biguanides or AICAR exhibited low red-to-green fluorescence (Fig. 4B). Metformin caused a delay in activation of caspase-9 (12h post MG as compared to 4h in cells treated with MG alone), while there was no MG-induced activation of caspase-9 activation in cells pretreated with AICAR over 16h (Fig. 5A). MG-induced caspase-9 activation in troglitazone-treated cells occurred between 4–16h which

kinetically resemble that in cells treated with MG alone (Fig. 5A). Accordingly, AICAR and biguanides (metformin, phenformin) significantly prevented MG-induced IHEC apoptosis, while both glitazones (troglitazone, pioglitazone) were without effect (Fig. 5B).

Biguanides and Glitazones did not Restore GSH Redox Imbalance Caused by MG

Given that metformin can improve antioxidant systems (Pavlovic *et al.* 2000), we determined whether the preservation of $\Delta\psi_m$ and IHEC viability by biguanides was related to the restoration of cellular GSH redox balance. The results show that neither AICAR, biguanides or glitazones, prevented MG-induced decreases in GSH and GSSG (Figs. 6A, B). MG-induced increases in protein-SSG formation (Fig. 6C) and attenuated GSH-to-disulfide ratios (Fig. 6D) were similarly unchanged by these insulin sensitizers. The presence of glitazones, in fact, exaggerated cellular GSH/GSSG decreases and potentiated protein-SSG formation (Fig. 6). This finding that insulin sensitizers failed to restore GSH redox status confirms our suggestion that the induction of redox imbalance by MG was upstream of MG-induced loss of $\Delta\psi_m$, the target site of protection by AICAR and biguanides.

DISCUSSION

Our current study has provided the first evidence of a redox-dependent susceptibility of human brain endothelial cells to carbonyl stress. This conclusion is supported the finding that MG-induced cellular GSH redox imbalance kinetically preceded endothelial cell apoptosis, both of which were prevented by NAC in accordance with restored GSH redox status. Furthermore, the result that NAC failed to protect against apoptosis when given at a time after peak MG-induced GSH oxidation suggests that the initial disruption of GSH redox balance was a crucial upstream event in endothelial apoptotic signaling. NAC could function as a reductant in MG elimination and spare the cellular GSH and protein-SH pools or serve as GSH precursor. Interestingly, inhibition of *de novo* GSH synthesis did not attenuate GSH levels in NAC-treated cells (data not shown), consistent with an antioxidant effect of NAC under these conditions. Accumulated evidence from our laboratory has established that GSH oxidation is a major contributor to cell apoptosis mediated by oxidative, carbonyl or hyperglycemic stress in brain endothelial cells (Okouchi *et al.* 2006) and PC12 cells (Ekshyyan and Aw. 2005; Okouchi *et al.* 2005a, b; Pias and Aw. 2002a, b; Pias *et al.* 2003). Previous findings that elevated tissue GSH attenuated levels of ROS and apoptosis in the brain (Drake *et al.* 2002) support our suggestion that the cellular redox status is an important determinant of cerebral apoptotic vulnerability, including endothelial cells. The mechanism by which MG-induced GSH redox shifts mediates human brain endothelial cell apoptosis is unresolved. One mechanism may involve target-specific thiol oxidation of redox-sensitive mPTP to regulate pore opening and closure. GSH has been shown to interact with mPTP to control the threshold potential for pore opening, and this event is closely associated with the function of Bax and Bcl-2 in regulating cytochrome *c* translocation from the mitochondria to the cytosol (Costantini *et al.* 1996; Halestrap *et al.* 1997). Bcl-2 function was reportedly blocked by thiol oxidation of mPTP through formation of disulfide crosslinks (Zamzami *et al.* 1998). Our current findings that MPT was involved in MG-mediated apoptosis and that NAC effectively prevented mitochondrial depolarization are consistent with a redox regulatory mechanism of mPTP function.

The sequelae of events in MG-mediated human brain endothelial cell apoptosis are consistent with mitochondrial initiated apoptotic signaling (Okouchi *et al.* 2005a). The time courses of loss of GSH redox balance, activation of caspase-9 and -3, and cleavage of PARP is the first reported kinetic sequence of mitochondrial signaling events for MG-induced apoptosis in human brain endothelial cells (Fig. 7). Moreover, the kinetics of caspase-9 and -3 activation (1–4h) are consistent with these events being downstream of the induction of GSH redox imbalance (15 min) and upstream of apoptotic death (24h). These findings

support the contention that redox activation of mitochondrial signaling determines apoptosis progression during MG challenge, a mechanism that is shared by hydroperoxides (Ekshyyan and Aw. 2005; Pias and Aw. 2002b; Pias *et al.* 2003). Dysfunctional mitochondria (Santos *et al.* 2001) and mitochondria-dependent cytochrome *c* translocation and caspases activation and PARP cleavage (Skaper. 2003) have been implicated in the vulnerability of the diabetic brain to oxidative stress.

A novel observation is that pharmacologically relevant concentrations of biguanides and AICAR prevented carbonyl stress-induced endothelial mitochondrial depolarization, the same extent of protection as the thiol antioxidant NAC, and cyclosporine A, a well-known agent in the prevention of oxidative stress-induced mitochondrial depolarization and apoptotic signaling (Soriano *et al.* 2004). In primary neurons, metformin was neuroprotective against etoposide-induced apoptosis through mitigating the apoptotic cascade, namely permeability transition, dissipated mitochondrial membrane potential and cytochrome *c* release (El-Mir *et al.* 2008). Since cyclosporine A cannot be widely used clinically due to severe side effects, the potency of biguanides and AICAR in preventing $\Delta\psi_m$ loss could offer a new and alternate therapeutic approach for cytoprotection of human brain endothelial cells against carbonyl stress. The precise mechanism by which biguanide insulin sensitizers protect against MG-induced mitochondrial depolarization is not completely understood. AICAR has been shown to prevent hyperglycemia-induced loss of $\Delta\psi_m$ through enhancing Akt signaling and endothelial nitric oxide synthetase (eNOS)-dependent nitric oxide production (Ido *et al.* 2002). A similar mechanism in cardioprotection against ischemia- reperfusion injury was recently documented for metformin (Gundewar *et al.* 2009); significantly, cardioprotection was abrogated in mice deficient in functional AMP kinase (AMPK) or eNOS ((Gundewar *et al.* 2009). Other studies by Bhamra *et al.* (2008) further suggested that the metformin-mediated cardioprotection involved inhibition of mPTP opening through PI3K-dependent Akt phosphorylation. It is conceivable that AICAR can preserve endothelial hexokinase-mPTP interaction, given the role of AMPK in controlling mitochondrial hexokinase binding to the voltage-dependent anion channel of mPTP (Detaille *et al.* 2005), the disruption of which induced mitochondrial cytochrome *c* release and apoptosis (Majewski *et al.* 2004). Similarly, metformin can accumulate in the mitochondria and directly influence mitochondrial hexokinase binding (Marchetti *et al.* 2004). Interestingly, metformin was shown to inhibit mitochondrial respiratory chain complex I which has been suggested to prevent mPTP opening as an adaptive response to decreased mitochondrial ROS generation and attenuated oxidative stress (Detaille *et al.* 2005; Marchetti *et al.* 2004). Consistent with an antioxidant role, metformin reportedly attenuate ROS generation (Gallo *et al.* 2005, Bellin *et al.* 2006). Since metformin did not restore endothelial GSH redox balance, our data suggests that $\Delta\psi_m$ preservation was unlikely to be the result of a direct antioxidant effect of metformin.

In contrast to biguanides, it is notable that glitazones failed to prevent MG-induced mitochondrial depolarization and endothelial cell apoptosis. Glitazones have been shown to increase apoptosis in vascular smooth muscle cells (Redondo *et al.* 2005), and lymphoma cells (Ray *et al.* 2005). In these instances, the promotion of cell apoptosis occurred in conjunction with mitochondrial apoptotic signaling involving proapoptotic shifts in the expression of Bax/Bcl-2, activation of caspases-9 and -3, and cleavage of PARP. Moreover, glitazones reportedly decreased $\Delta\psi_m$ in lymphoma cells (Ray *et al.* 2005), in agreement with our current findings in endothelial cells. Our results further show that glitazone exacerbated endothelial GSH redox imbalance, a major contributor to cellular apoptosis (Ekshyyan and Aw. 2005; Okouchi *et al.* 2005a, b; Pias and Aw. 2002a, b; Pias *et al.* 2003). Enhanced mitochondrial ROS production has been implicated in the mitochondrial toxicity of glitazones (ciciglitazone, rosiglitazone) and lethality of glioma cells independent of glycemic control (Perez-Ortiz *et al.* 2004, 2007). Interestingly, recent studies by Rachek *et*

al. (2009) implicate mitochondrial DNA damage as an initiating event in troglitazone (not rosiglitazone)-induced mitochondrial dysfunction and hepatotoxicity. Whether these mitochondrial mechanisms have clinical importance and contribute to glitazone-induced toxicity in diabetic patients remains to be fully investigated. To date, troglitazone has been withdrawn from the market owing to serious hepatotoxicity; the market status of other glitazones (pioglitazone, ciglitazone, rosiglitazone), also known to elicit mitochondrial toxicity experimentally, is uncertain.

The maintenance of mitochondrial integrity is critical to normal insulin function. Support for the hypothesis that mitochondrial dysfunction compromised insulin secretion and insulin action, the two prominent features of type II diabetes, comes from early studies of mitochondrial diabetes, a hereditary disorder characterized by mitochondrial DNA (mtDNA) damage and/or mutation (Silva *et al.* 2000, Lowell and Shulman, 2005, Wiederkehr and Wollheim, 2006). Associated with mitochondrial failure is attenuated ATP production and pyruvate cycling, reportedly two key factors that couple mitochondrial metabolism to β -cell insulin secretion (Wiederkehr and Wollheim, 2006). Recent evidence suggests a role for mutated or suppressed HNF (hepatic nuclear factor)-1 α gene in mitochondrial dysfunction- impaired glucose-stimulated insulin secretion in INS-1 cells and islets from Hnf-1 α null mice; notably, mitochondrial uncoupling was significantly higher in dominant negative-HNF-1 α cells with consequent decrease in rates of ATP synthesis (Pondgratz *et al.* 2009). Oxidative stress through ROS could similarly decrease β -cell secretory function owing to persistent damage to mitochondrial respiratory proteins (Li *et al.* 2009). While decreased mitochondrial activity has been associated with impairment of insulin action (Lowell and Shulman 2005, Wiederkehr and Wollheim 2006), its mechanistic contribution to the development of insulin resistance is incompletely understood. One interesting aspect of the relationship between mitochondrial dysfunction and insulin action is that insulin signaling appears to preserve mitochondrial function. This notion is supported by recent evidence that insulin-like growth factor-1 signaling prevent high glucose-induced apoptosis in human endothelial cells through attenuating mitochondrial dysfunction and cytochrome *c* release (Li *et al.* 2009). The signal transduction mechanism by which insulin protects mitochondrial integrity is unclear, and should provide a fruitful avenue for future investigation.

In summary, we have demonstrated that carbonyl stress-induced human brain endothelial cell apoptosis is correlated with an early disruption of cellular GSH redox balance and activation of mitochondrial apoptotic signaling (Fig. 7). Cytoprotection by thiol antioxidant agents such as NAC occurred primarily via restored GSH redox homeostasis and secondary $\Delta\psi_m$ preservation, while cyclosporine A directly prevented $\Delta\psi_m$ loss. Pharmacologic levels of biguanides or AICAR, but not glitazones mediated endothelial cell viability by preventing carbonyl stress-induced $\Delta\psi_m$ collapse that was unrelated to preservation of endothelial GSH redox balance. Taken together, the current study provides key insights into carbonyl stress-mediated damage to brain endothelial cells and the therapeutic potential of thiol antioxidant and/or AMPK-dependent insulin sensitizers in preserving endothelial integrity. Furthermore, the current results will have broader implications for cerebral microvascular complications and primary diabetic encephalopathy and their management.

Acknowledgments

We acknowledge Deborah Chervenak and Lijia Yin for excellent technical assistance with flow cytometry. This study was supported by a grant from the National Institutes of Health, DK44510.

ABBREVIATIONS

AICAR	5-Aminoimidazole-4-carboxamide-1- β -D-ribofuranoside
AMPK	AMP-activated protein kinase
BBB	Blood-brain barrier
BSO	Buthionine sulfoximine
CNS	Central nervous system
CPP-32	Procaspase-3
DAPI	4',6-Diamidino-2-phenylindole
DNFB	2,4-Dinitrofluorobenzene
$\Delta\Psi_m$	Mitochondrial membrane potential
mPTP	Mitochondrial permeability transition pore
DTNB	5'-Dithio-bis 2-nitrobenzoic acid
DTT	Dithiothreitol
DNP	2,4-Dinitrophenol
FCCP	Carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone
GLUT	Glucose transporter
GSH	Glutathione
GSSG	Glutathione disulfide
HPLC	High-performance liquid chromatography
IHEC	Human brain endothelial cells
MG	Methylglyoxal
MPT	Mitochondrial permeability transition
NAC	N-acetylcysteine
PC12 cells	Naive pheochromocytoma cells
PARP	polyADP ribose polymerase
PPAR	Peroxisome proliferator-activated receptor
Protein-SSG	Protein-glutathione mixed disulfide
ROS	Reactive oxygen species
TCA	Trichloroacetic acid

REFERENCES

- UK Prospective Diabetes Study (UKPDS) Group. Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). *Lancet*. 1998; 352:854–865. [PubMed: 9742977]
- Akhand AA, Hossain K, Mitsui H, Kato M, Miyata T, Inagi R, Du J, Takeda K, Kawamoto Y, Suzuki H, Kurokawa K, Nakashima I. Glyoxal and methylglyoxal trigger distinct signals for map family kinases and caspase activation in human endothelial cells. *Free Radic Biol Med*. 2001; 31:20–30. [PubMed: 11425486]

- Akhand AA, Kato M, Suzuki H, Liu W, Du J, Hamaguchi M, Miyata T, Kurokawa K, Nakashima I. Carbonyl compounds crosslink cellular proteins and activate protein-tyrosine kinase p60c-Src. *J Cell Biochem.* 1999; 72:1–7. [PubMed: 10025661]
- Beisswenger PJ, Drummond KS, Nelson RG, Howell SK, Szewergold BS, Mauer M. Susceptibility to diabetic nephropathy is related to dicarbonyl and oxidative stress. *Diabetes.* 2005; 54:3274–3281. [PubMed: 16249455]
- Bellin C, de Wiza DH, Wiernsperger NF, Rosen P. Generation of reactive oxygen species by endothelial and smooth muscle cells: influence of hyperglycemia and metformin. *Horm Metab Res.* 2006; 38:732–739. [PubMed: 17111300]
- Bharma GS, Hausenloy DJ, Davidson SM, Carr RD, Wynne AM, Mocanu MM, Yellon DM. Metformin protects the ischemic heart by the Akt-mediated inhibition of mitochondrial permeability transition pore opening. *Basic Res Cardiol.* 2008; 103:274–284. [PubMed: 18080084]
- Costantini P, Chernyak BV, Petronilli V, Bernardi P. Modulation of the mitochondrial permeability transition pore by pyridine nucleotides and dithiol oxidation at two separate sites. *J Biol Chem.* 1996; 271:6746–6751. [PubMed: 8636095]
- den Heijer T, Vermeer SE, van Dijk EJ, Prins ND, Koudstaal PJ, Hofman A, Breteler MM. Type 2 diabetes and atrophy of medial temporal lobe structures on brain MRI. *Diabetologia.* 2003; 46:1604–1610. [PubMed: 14595538]
- Detaille D, Guigas B, Chauvin C, Batandier C, Fontaine E, Wiernsperger N, Leverve X. Metformin prevents high-glucose-induced endothelial cell death through a mitochondrial permeability transition-dependent process. *Diabetes.* 2005; 54:2179–2187. [PubMed: 15983220]
- Drake J, Kanski J, Varadarajan S, Tsoras M, Butterfield DA. Elevation of brain glutathione by gamma-glutamylcysteine ethyl ester protects against peroxynitrite-induced oxidative stress. *J Neurosci Res.* 2002; 68:776–784. [PubMed: 12111838]
- Ekshyyan O, Aw TY. Decreased susceptibility of differentiated PC12 cells to oxidative challenge: relationship to cellular redox and expression of apoptotic protease activator factor-1. *Cell Death Differ.* 2005; 12:1066–1077. [PubMed: 15877105]
- Ellman GL, Lysko H. Disulfide and sulfhydryl compounds in TCA extracts of human blood and plasma. *J Lab Clin Med.* 1967; 70:518–527. [PubMed: 6038554]
- El-Mir MY, Detaille D, R-Villanueva G, Delgado-Esteban M, Guigas B, Attia S, Fontaine E, Almeida A, Leverve X. Neuroprotective role of antidiabetic drug metformin against apoptotic cell death in primary cortical neurons. *J Mol Neurosci.* 2008; 34:77–87. [PubMed: 18040888]
- Gallo A, Ceolotto G, Pinton P, Iori E, Murphy E, Rutter GA, Rizzuto R, Semplicini A, Avogaro A. Metformin prevents glucose-induced protein kinase C-beta2 activation in human umbilical vein endothelial cells through an antioxidant mechanism. *Diabetes.* 2005; 54:1123–1131. [PubMed: 15793252]
- Green DR, Reed JC. Mitochondria and apoptosis. *Science.* 1998; 281:1309–1312. [PubMed: 9721092]
- Halestrap AP, Woodfield KY, Connern CP. Oxidative stress, thiol reagents, and membrane potential modulate the mitochondrial permeability transition by affecting nucleotide binding to the adenine nucleotide translocase. *J Biol Chem.* 1997; 272:3346–3354. [PubMed: 9013575]
- Gundewar A, Calvert JW, Jha S, Toedt-Pingel I, Ji SY, Numez D, Ramachandran A, Anaya-Cisneros M, Tian R, Lefer DJ. Activation of AMP-activated protein kinase by metformin improves left ventricular function and survival in heart failure. *Circulation.* 2009; 104:403–411.
- Huber JD, VanGilder RL, Houser KA. Streptozotocin-induced diabetes progressively increases blood-brain barrier permeability in specific brain regions in rats. *Am J Physiol Heart Circ Physiol.* 2006; 291:H2660–H2668. [PubMed: 16951046]
- Huypens P, Quartier E, Pipeleers D, Van de Casteele M. Metformin reduces adiponectin protein expression and release in 3T3-L1 adipocytes involving activation of AMP activated protein kinase. *Eur J Pharmacol.* 2005; 518:90–95. [PubMed: 16039647]
- Ido Y, Carling D, Ruderman N. Hyperglycemia-induced apoptosis in human umbilical vein endothelial cells: inhibition by the AMP-activated protein kinase activation. *Diabetes.* 2002; 51:159–167. [PubMed: 11756336]

- Inzucchi SE, Maggs DG, Spollett GR, Page SL, Rife FS, Walton V, Shulman GI. Efficacy and metabolic effects of metformin and troglitazone in type II diabetes mellitus. *N Engl J Med.* 1998; 338:867–872. [PubMed: 9516221]
- Katakam PV, Ujhelyi MR, Hoenig M, Miller AW. Metformin improves vascular function in insulin-resistant rats. *Hypertension.* 2000; 35:108–112. [PubMed: 10642283]
- Langston W, Circu ML, Aw TY. Insulin stimulation of gamma-glutamylcysteine ligase catalytic subunit expression increases endothelial GSH during oxidative stress: influence of low glucose. *Free Radic Biol Med.* 2008; 45:1591–1599. [PubMed: 18926903]
- Li N, Brun T, Cnop M, Cunha DA, Eizirik DL, Maechler P. Transient oxidative stress damages mitochondrial machinery inducing persistent b-cell dysfunction. *J Biol Chem.* 2009 in press M109.024323.
- Li Y, Wu H, Khardori R, Song YH, Lu YW, Geng YJ. Insulin-like growth factor-1 receptor activation prevents high glucose-induced mitochondrial dysfunction, cytochrome c release and apoptosis. *Biochem Biophys Res Commun.* 2009; 384:259–264.
- Li ZG, Zhang W, Sima AA. The role of impaired insulin/IGF action in primary diabetic encephalopathy. *Brain Res.* 2005; 1037:12–24. [PubMed: 15777748]
- Lovekamp-Swan T, Chaffin CL. The peroxisome proliferator-activated receptor gamma ligand troglitazone induces apoptosis and p53 in rat granulosa cells. *Mol Cell Endocrinol.* 2005; 233:15–24. [PubMed: 15767042]
- Lowell BB, Shulman GI. Mitochondrial dysfunction and type 2 diabetes. *Science.* 2005; 307:384–387. [PubMed: 15662004]
- Majewski N, Nogueira V, Bhaskar P, Coy PE, Skeen JE, Gottlob K, Chandel NS, Thompson CB, Robey RB, Hay N. Hexokinase-mitochondria interaction mediated by Akt is required to inhibit apoptosis in the presence or absence of Bax and Bak. *Mol Cell.* 2004; 16:819–830. [PubMed: 15574336]
- Marchetti P, Del Guerra S, Marselli L, Lupi R, Masini M, Pollera M, Bugliani M, Boggi U, Vistoli F, Mosca F, Del Prato S. Pancreatic islets from type 2 diabetic patients have functional defects and increased apoptosis that are ameliorated by metformin. *J Clin Endocrinol Metab.* 2004; 89:5535–5541. [PubMed: 15531508]
- Mather KJ, Verma S, Anderson TJ. Improved endothelial function with metformin in type 2 diabetes mellitus. *J Am Coll Cardiol.* 2001; 37:1344–1350. [PubMed: 11300445]
- Okouchi M, Ekshyyan O, Maracine M, Aw TY. Neuronal apoptosis in neurodegeneration. *Antioxid Redox Signal.* 2007; 9:1059–1096. [PubMed: 17571960]
- Okouchi M, Okayama N, Alexander JS, Aw TY. NRF2-dependent glutamate-L-cysteine ligase catalytic subunit expression mediates insulin protection against hyperglycemia-induced brain endothelial cell apoptosis. *Curr Neurovasc Res.* 2006; 3:249–261. [PubMed: 17109620]
- Okouchi M, Okayama N, Aw TY. Differential susceptibility of naive and differentiated PC-12 cells to methylglyoxal-induced apoptosis: influence of cellular redox. *Curr Neurovasc Res.* 2005a; 2:13–22. [PubMed: 16181096]
- Okouchi M, Okayama N, Aw TY. Hyperglycemia potentiates carbonyl stress-induced apoptosis in naive PC-12 cells: relationship to cellular redox and activator protease factor-1 expression. *Curr Neurovasc Res.* 2005b; 2:375–386. [PubMed: 16375719]
- Pavlovic D, Kocic R, Kocic G, Jevtovic T, Radenkovic S, Mikic D, Stojanovic M, Djordjevic PB. Effect of four-week metformin treatment on plasma and erythrocyte antioxidative defense enzymes in newly diagnosed obese patients with type 2 diabetes. *Diabetes Obes Metab.* 2000; 2:251–256. [PubMed: 11225659]
- Perez-Ortiz JM, Tranque P, Vaquero CF, Domingo B, Molina F, Calvo S, Jordan J, Cena V, Llopis J. Glitazones differentially regulation primary astrocytes and glioma cell survival. Involvement of reactive oxygen species and peroxisome proliferator-activated receptor-gamma. *J Biol Chem.* 2004; 279:8976–8985. [PubMed: 14699130]
- Perez-Ortiz JM, Tranque P, Burgos M, Vaquero CF, Llopis J. Glitazones induce astrogloma cell death by releasing reactive species from mitochondria: modulation of cytotoxicity by nitric oxide. *Mol Pharm.* 2007; 72:407–417.

- Pias EK, Aw TY. Apoptosis in mitotic competent undifferentiated cells is induced by cellular redox imbalance independent of reactive oxygen species production. *FASEB J*. 2002a; 16:781–790. [PubMed: 12039859]
- Pias EK, Aw TY. Early redox imbalance mediates hydroperoxide-induced apoptosis in mitotic competent undifferentiated PC-12 cells. *Cell Death Differ*. 2002b; 9:1007–1016. [PubMed: 12181751]
- Pias EK, Ekshyyan OY, Rhoads CA, Fuseler J, Harrison L, Aw TY. Differential effects of superoxide dismutase isoform expression on hydroperoxide-induced apoptosis in PC-12 cells. *J Biol Chem*. 2003; 278:13294–13301. [PubMed: 12551919]
- Pongratz RL, Kibbey RG, Kirkpatrick CL, Zhao X, Pontoglio M, Yaniv M, Wolheim CB, Shulman GI, Cline GW. Mitochondrial dysfunction contributes to impaired insulin secretion in INS-1 cells with dominant-negative mutations of HNF-1(alpha) and in HNF-1(alpha)-deficient islets. *J Biol Chem*. 2009; 284:16808–16821. [PubMed: 19376774]
- Rachek LI, Yuzefovych LV, LeDoux SP, Julie NL, Wilson GL. Troglitazone, but not rosiglitazone, damages mitochondrial DNA and induces mitochondrial dysfunction and cell death in human hepatocytes. *Toxicol Appl Pharmacol*. 2009 [Epub ahead of print].
- Ray DM, Akbiyik F, Bernstein SH, Phipps RP. CD40 engagement prevents peroxisome proliferator-activated receptor gamma agonist-induced apoptosis of B lymphocytes and B lymphoma cells by an NF-kappaB-dependent mechanism. *J Immunol*. 2005; 174:4060–4069. [PubMed: 15778364]
- Redondo S, Ruiz E, Santos-Gallego CG, Padilla E, Tejerina T. Pioglitazone induces vascular smooth muscle cell apoptosis through a peroxisome proliferator-activated receptor-gamma, transforming growth factor-beta1, and a Smad2-dependent mechanism. *Diabetes*. 2005; 54:811–817. [PubMed: 15734860]
- Reed DJ, Babson JR, Beatty PW, Brodie AE, Ellis WW, Potter DW. High-performance liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide, and related thiols and disulfides. *Anal Biochem*. 1980; 106:55–62. [PubMed: 7416469]
- Rosca MG, Monnier VM, Szveda LI, Weiss MF. Alterations in renal mitochondrial respiration in response to the reactive oxoaldehyde methylglyoxal. *Am J Physiol Renal Physiol*. 2002; 283:F52–F59. [PubMed: 12060586]
- Russell JW, Golovoy D, Vincent AM, Mahendru P, Olzmann JA, Mentzer A, Feldman EL. High glucose-induced oxidative stress and mitochondrial dysfunction in neurons. *FASEB J*. 2002; 16:1738–1748. [PubMed: 12409316]
- Ryan CM, Geckle MO, Orchard TJ. Cognitive efficiency declines over time in adults with Type 1 diabetes: effects of micro- and macrovascular complications. *Diabetologia*. 2003; 46:940–948. [PubMed: 12819900]
- Santos MS, Santos DL, Palmeira CM, Seica R, Moreno AJ, Oliveira CR. Brain and liver mitochondria isolated from diabetic Goto-Kakizaki rats show different susceptibility to induced oxidative stress. *Diabetes Metab Res Rev*. 2001; 17:223–230. [PubMed: 11424235]
- Silva JP, Kohler M, Graff C, Oldfors A, Magnuson MA, Berggren P-O, Larsson N-G. Impaired insulin secretion and b-cell loss in tissue-specific knockout mice with mitochondrial diabetes. *Nature Gen*. 2000; 26:336–340.
- Sima AA, Kamiya H, Li ZG. Insulin, C-peptide, hyperglycemia, and central nervous system complications in diabetes. *Eur J Pharmacol*. 2004; 490:187–197. [PubMed: 15094085]
- Sima AA, Li ZG. The effect of C-peptide on cognitive dysfunction and hippocampal apoptosis in type 1 diabetic rats. *Diabetes*. 2005; 54:1497–1505. [PubMed: 15855338]
- Skaper SD. Poly(ADP-Ribose) polymerase-1 in acute neuronal death and inflammation: a strategy for neuroprotection. *Ann NY Acad Sci*. 2003; 993:217–228. discussion 287–8. [PubMed: 12853316]
- Soriano ME, Nicolosi L, Bernardi P. Desensitization of the permeability transition pore by cyclosporin A prevents activation of the mitochondrial apoptotic pathway and liver damage by tumor necrosis factor-alpha. *J Biol Chem*. 2004; 279:36803–36808. [PubMed: 15201276]
- Ulus NN, Sahilli M, Avci A, Canbolat O, Ozansoy G, Ari N, Bali M, Stefek M, Stolc S, Gajdosik A, Karasu C. Pentose phosphate pathway, glutathione-dependent enzymes and antioxidant defense during oxidative stress in diabetic rodent brain and peripheral organs: effects of stobadine and vitamin E. *Neurochem Res*. 2003; 28:815–823. [PubMed: 12718433]

- Wang X, Martindale JL, Liu Y, Holbrook NJ. The cellular response to oxidative stress: influences of mitogen-activated protein kinase signalling pathways on cell survival. *Biochem J.* 1998; 333(Pt 2): 291–300. [PubMed: 9657968]
- Wiederkehr A, Wollheim CB. Implication of mitochondria in insulin secretion and action. *Endocrinol.* 2006; 147:2643–2649.
- Yim HS, Kang SO, Hah YC, Chock PB, Yim MB. Free radicals generated during the glycation reaction of amino acids by methylglyoxal. A model study of protein-cross-linked free radicals. *J Biol Chem.* 1995; 270:28228–28233. [PubMed: 7499318]
- Zamzami N, Marzo I, Susin SA, Brenner C, Larochette N, Marchetti P, Reed J, Kofler R, Kroemer G. The thiol crosslinking agent diamide overcomes the apoptosis-inhibitory effect of Bcl-2 by enforcing mitochondrial permeability transition. *Oncogene.* 1998; 16:1055–1063. [PubMed: 9519879]

Fig. 1. MG induces IHEC apoptosis and GSH redox imbalance: Protection by NAC

IHECs were challenged with 300 μ M MG in the absence or presence of 2mM NAC that was added at 30min before (NAC) or at 60 min after (postNAC) exposure to MG. **A**, Apoptosis was determined at 24h and results are means $\pm$ SEM for n = 5 preparations. # p <0.05 vs. control (without MG); * p <0.05 vs. MG-treatment. Cellular GSH/GSSG and protein-SSG were determined at 15 min. **B**, GSH (left panel) and GSSG (right panel); **C**, protein-SSG; **D**, GSH-to-Disulfide ratio. The disulfide pool comprises acid-soluble GSSG plus protein-SSG. Values are means $\pm$ SEM for n = 9 preparations. In each instance, p <0.05 vs. controls (without MG); * p <0.05 vs. MG treatment.

Fig. 2. Attenuation of MG-induced IHEC apoptosis by caspase inhibitors and kinetics caspase activation

A, IHECs were preincubated for 30min without or with inhibitors of caspases-3, -9, or -8 prior to 300 μ M MG treatment as described in Methods. Apoptosis was determined at 24h, and results are means $\pm$ SEM for n = 5 preparations. [#]*p* < 0.05 vs control (without MG); **p* < 0.05 vs MG treatment. **B**, IHECs were treated with 300 μ M MG for designated times of 0 - 10h and the kinetics of caspase activation and PARP cleavage were determined by Western blot analyses in total cell lysates. Results shown are one representative of three immunoblots for each protein. β -actin verified equal protein loading.

Fig. 3. Inhibition of MG-induced mitochondrial depolarization by NAC and cyclosporine A
A, IHECs were treated with 300 μ M MG for 4h or 6h, incubated with 5 μ g/ml JC-1 for 15min, and processed for flow cytometry. In some experiments, cells were pretreated with 2mM NAC or 1 μ M cyclosporine A prior to MG exposure. Changes in $\Delta\psi_m$ were detected using the fluorescent cationic dye JC-1 and flow cytometry as described in Materials and Methods. Mitochondrial depolarization was determined as a decrease in the red-to-green fluorescence intensity ratio, i.e., fluorescence emission shift from red (590nm) to green (525nm). A combination of FCCP (10 μ M), valinomycin (10 μ M), and DNP (1mM) was used as positive control. The designated R2 and R3 regions represent cell populations that exhibit high (R2) or low (R3) red-to-green fluorescence ratio, consistent with high and

low $\Delta\psi_m$, respectively. Results shown are one representative of five separate experiments.

B, The proportion of cells in R3 regions were quantified and expressed as percent cells with decreased $\Delta\psi_m$ $^{\#}p<0.05$ vs. control (without MG); $^*p<0.05$ vs. MG treatment. **C**, IHECs were pretreated with 1 μ M cyclosporine A for 30min prior to exposure to 300 μ M MG. Apoptosis was determined at 24h, and results are means $\pm$ SEM for n = 5 preparations. $^{\#}p<0.05$ vs. control (without MG); $^*p<0.05$ vs. MG treatment.

Fig. 4. Biguanides and AICAR, but not glitazones protect against MG-induced mitochondrial depolarization

A, IHECs were treated with 300 μ M MG for 4h or 6h in the absence or presence of biguanides (100 μ M metformin, 200 μ M phenformin), AICAR (2mM), or glitazones (10 μ M troglitazone, or 10 μ M pioglitazone). Changes in $\Delta\psi_m$ were detected using the fluorescent cationic dye JC-1 and flow cytometry as described in Materials and Methods. The designated R2 and R3 regions represent cell populations that exhibit high (R2) or low (R3) red-to-green fluorescence ratio, consistent with high and low $\Delta\psi_m$, respectively. Results shown are one representative of four separate experiments. **B**, The proportion of cells in R3 regions were quantified and expressed as percent cells with decreased $\Delta\psi_m$. Values are means $\pm$ SEM for n = 4 preparations. [#] p <0.05 vs. control (without MG); ^{*} p <0.05 vs. MG treatment.

Fig. 5. Biguanides and AICAR, but not glitazones prevent MG-induced caspase-9 activation and IHEC apoptosis

A, IHECs were incubated with 300 μ M MG for 0–16h in the absence or presence of metformin (100 μ M), AICAR (2mM), or troglitazone (10 μ M). Total cell lysates were prepared for Western blot analyses of pro-caspase 9. Results shown are one representative of three immunoblots for each protein. β -actin verified equal protein loading. **B**, IHECs were incubated with 300 μ MG without or with biguanides (100 μ M metformin or 200 μ M phenformin), AICAR (2mM), or glitazones (10 μ M troglitazone or 10 μ M pioglitazone). Apoptosis was determined at 24h and results are means $\pm$ SEM for n = 5 preparations. # p <0.05 vs. control (without MG); * p <0.05 vs. MG treatment.

Fig. 6. Insulin sensitizers did not prevent MG-induced GSH redox imbalance

IHECs were preincubated for 30 min without or with biguanides (100 μ M metformin or 200 μ M phenformin), 2mM AICAR, or glitazones (10 μ M troglitazone or 10 μ M pioglitazone) prior to 300 μ M MG exposure. At 15 min, cells were harvested for measurements of cellular GSH, GSSG and protein-SSG. **A**, GSH; **B**, GSSG; **C**, protein-SSG; **D**, GSH-to-Disulfide ratio. The disulfide pool comprises acid-soluble GSSG plus protein-SSG. Values are means $\pm$ SEM for $n = 6$ preparations. In each instance, $p < 0.05$ vs. controls (without MG); $*p < 0.05$ vs. MG plus AICAR or MG plus biguanides.

Fig. 7. Working model of the mitochondrial pathway of MG-induced IHEC apoptosis and sites of inhibition by NAC, cyclosporine A, and insulin sensitizers

The model proposes that methyglyoxal (MG) induces cellular GSH redox imbalance through generalized oxidative stress and/or oxidation of protein thiols, leading to loss of $\Delta\psi_m$, permeability transition, and initiation of the apoptotic cascade involving mitochondrial cytochrome *c* release and caspase activation. In some cell types, MG can directly activate caspases via protein carbonyl compound crosslinks (Akhand *et al.* 2001). NAC or cyclosporine A protects against MG-induced apoptosis through distinct mechanisms; NAC through restored cellular GSH balance and cyclosporine A through $\Delta\psi_m$ preservation. Biguanides and AICAR similarly protect against MG-induced $\Delta\psi_m$ collapse and caspase-9 activation. Glitazones tend to exacerbate MG-induced GSH redox imbalance, and have minimal effects on MG-induced loss of $\Delta\psi_m$ and apoptosis. The dotted lines indicate the location of substrate (NAC) entry and inhibitor (cyclosporine A, biguanides, and AICAR)

actions. Dashed line indicates glitazone potentiation of GSH redox imbalance. (+) and (–) refers to potentiation and inhibitory effects, respectively.