Cardiomyocyte apoptosis induced by short-term diabetes requires mitochondrial GSH depletion

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Cardiomyocyte apoptosis induced by short-term diabetes requires mitochondrial GSH depletion. Am J Physiol Heart Circ Physiol 289: H768–H776, 2005. First published April 1, 2005; doi:10.1152/ajpheart.00038.2005.—Oxidative stress due to excessive reactive oxygen species (ROS) and depleted antioxidants such as glutathione (GSH) can give rise to apoptotic cell death in acutely diabetic hearts and lead to heart disease. At present, the source of these cardiac ROS or the subcellular site of cardiac GSH loss [i.e., cytosolic (cGSH) or mitochondrial (mGSH) GSH] has not been completely elucidated. With the use of rotenone (an inhibitor of the electron transport chain) to decrease the excessive ROS in acute streptozotocin (STZ)-induced diabetic rat heart, the mitochondrial origin of ROS was established. Furthermore, mitochondrial damage, as evidenced by loss of membrane potential, increases in oxidative stress, and reduction in mGSH was associated with increased apoptosis via increases in caspase-9 and -3 activities in acutely diabetic hearts. To validate the role of mGSH in regulating cardiac apoptosis, mitochondrial GSH depletion, increased ROS levels, and cardiac apoptosis was further aggravated compared with the untreated diabetic group. In a separate group, GSH supplementation induced a robust amplification of mGSH in diabetic rat hearts and prevented apoptosis. Our data suggest for the first time that mGSH is crucial for modulating the cell suicide program in short-term diabetic rat hearts.

mitochondria; caspase; oxidative stress; heart; glutathione

DIABETES IS A SIGNIFICANT risk factor for cardiovascular diseases, with the majority of these complications being attributed to coronary vascular pathology. However, in both human and animal models of diabetes, an additional heart muscle-specific disease in the absence of any vascular pathology has also been described (45). Probable candidates to explain this heart disease include autonomic abnormalities, metabolic disorders, abnormal contractile protein and enzyme function, interstitial fibrosis (45), and more recently, apoptosis, which is a regulated, energy-dependent cell suicide mechanism (18, 20, 33).

In human and animal models of diabetes, heart disease is associated with excessive apoptotic cell death (18, 20). It is possible that this loss of cells progressively leads to interstitial fibrosis, myocardial hypertrophy, contractile impairment, and eventual heart disease (23, 24). Various factors including protein kinase C activation (48), upregulation of the renin-angiotensin system (18), p53-mediated gene transcription (17), and proapoptotic free fatty acids (23) have been implicated in inducing myocardial apoptosis during diabetes. Interestingly, all of these different elements appear to trigger cell death by a common oxidative stress-mediated pathway (3).

Oxidative stress occurs as a result of an imbalance between the production of reactive oxygen species (ROS) and their neutralization by antioxidants. In the diabetic heart, augmented ROS production can arise from defective mitochondria, glucose autoxidation, protein glycation, and increased activity of cytosolic xanthine oxidase (2). Irrespective of their origin, ROS such as hydrogen peroxide ($H_2O_2$) can induce cell death via mechanisms including lipid peroxidation, alteration of cellular proteins, and initiation of diverse stress-signaling pathways (37). Among antioxidants, glutathione [L-$\gamma$-glutamyl-L-cysteinyl glycine (GSH)] is a nonprotein thiol that is crucial for protecting the heart against $H_2O_2$-induced damage (37). In both human and experimental models of diabetes, loss of GSH has been demonstrated and correlated to increased cardiac oxidative stress (34, 46, 47). Reduced GSH, which is present in the cytosol (cGSH; 80% of total cellular GSH) and the mitochondria (mGSH; 10–15% of total GSH), acts as a hydrogen donor in the neutralization of $H_2O_2$ and is itself converted to the inactive glutathione disulfide (oxidized glutathione; GSSG; Ref. 14). Despite the importance of mGSH in preventing mitochondrial oxidative stress, mitochondria are incapable of de novo synthesis of GSH and rely on the import of GSH from the cytosol, where it is manufactured from constituent amino acids (30).

Recently, we reported (25) that short-term diabetes induces the following: cardiac GSH depletion, increased ROS levels, and cardiac apoptosis as early as 4 days after streptozotocin (STZ) administration. Interestingly, similar observations were reported when cardiomyocytes were exposed to 24 h of high glucose levels in vitro (16). In both of these studies, neither the source of ROS nor the site of GSH loss was explored. This is important because cytosolic and mitochondrial sources of ROS may affect specific pools of GSH (i.e., cGSH or mGSH) and differentially regulate apoptosis (14, 26, 38). With the use of acutely diabetic rats, the objectives of the present study were to 1) determine the source of cardiac ROS and 2) investigate the

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contribution of the respective intracellular GSH pools to cardiac apoptosis. We report that in the diabetic heart, mitochondria may be the chief source of ROS, with mGSH depletion being crucial in the activation of the cell suicide program in vivo.

MATERIALS AND METHODS

Experimental animals. This investigation conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985) and the Animal Care and Use Committee of the University of British Columbia. Male Wistar rats (220–240 g body wt) were obtained from the University of British Columbia Animal Care Unit and were maintained under a 12:12-h light-dark cycle (light from 0700 to 1900). All animals were fed laboratory-standard dry diet (PMI Feeds; Richmond, VA). Water was provided ad libitum. In some rats, diabetes was induced with a moderate dose of STZ (55 mg/kg iv), which is a selective β-cell toxin, and hyperglycemia was verified after 24 h with a glucometer (Advantage Comfort; Roche Diagnostics). All diabetic rats were kept for 4 days after STZ administration and were anesthetized with pentobarbital sodium (65 mg/kg ip); the thoracic cavity was then opened, and hearts were removed (23–25). After rats were killed, plasma insulin was measured by radioimmunoassay (Novo; Copenhagen, Denmark) using rat insulin standards.

Treatments. To examine the influence of acute diabetes on specific pools of GSH in addition to determining how manipulation of these GSH pools could affect myocardial apoptosis, diabetic rats were left untreated or were injected with l-buthionine-sulfoximine (BSO; 10 mmol/kg ip dissolved in saline) and/or diethyl maleate (DEM; 4 mmol/kg ip dissolved in corn oil) for 4 days after STZ administration. A nondiabetic group given both BSO and DEM was used to negate the possibility of nonspecific toxic effects of these agents. BSO inhibits the activity of γ-glutamyl cysteine synthetase (γ-GCS) and blocks the de novo synthesis of GSH (37), whereas DEM conjugates and inactivates all GSH within the cell (21, 22, 26). In contrast, to augment cardiac GSH, diabetic rats were treated with daily injections of GSH (400 mg/kg ip, pH 7.4) for 10 days before induction of diabetes. Administration of GSH was continued for 4 days after STZ administration.

Preparation of cardiac myocytes. Hearts were digested by retrograde perfusion of modified Joklik’s minimal essential medium containing collagenase (228 U/ml), 0.5% BSA, and 50 mM CaCl2 through the heart. Myocytes were made calcium tolerant by successive exposure to increasing concentrations of calcium (≤1 mM). Yield of cardiomyocytes (90–95% of total cell number) was determined microscopically using a Neubauer hemocytometer. Myocyte viability (generally 75–85%) was assessed as the percentage of elongated cells with clear cross-striations that excluded 0.2% Trypan blue. There was no difference in myocyte viability (percentage of live cells) or yield (total number of cells × 10⁹) between control and diabetic rats at 4 days after the induction of diabetes. Immediately after isolation, fractions of cardiomyocytes were snap frozen in liquid nitrogen for estimation of various caspases. The remaining cardiomyocytes were either fractionated for determination of subcellular pools of GSH or plated on laminin-coated culture plates as described previously (25).

Estimation of ROS. The redox-sensitive dye 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H₂DCFDA) was used to assess the presence of ROS (25, 33). CM-H₂DCFDA is a cell-permeant indicator that is oxidized in the presence of ROS such as H₂O₂ whereby it emits green fluorescence. Myocytes were isolated and plated for 16 h. Thereafter, the medium was discarded and wells were loaded with CM-H₂DCFDA (5 µM for 30 min at 37°C). Subsequently, cells were washed and incubated for an additional 45 min after which green fluorescence was monitored in a fluorometer at 485/530-nm wavelengths. To identify the sources of ROS production, some cardiomyocytes were incubated with rotenone (5 µg/ml) or antimycin A (100 ng/ml) for 1 h (35). Rotenone blocks complex I of the mitochondrial electron transport chain (ETC) and decreases mitochondrial ROS generation (35). Conversely, incubation with antimycin A blocks complex III and increases mitochondrial ROS (51). Finally, N-acetylcysteine (NAC; 5 mM), which can decrease ROS production by increasing the intracellular GSH concentration (31), was used to monitor the effect of GSH supplementation in vitro. The emission was calculated per 75,000 cells in fluorescence units (FU). For confocal microscopic images, chambered coverslips were used as described before (25). Myocytes were visualized using a confocal scanning microscope at 485/530-nm wavelengths and ×300 magnification for visualization of green fluorescence (25).

Determination of mitochondrial membrane potential. The carbocyanine dye JC-1 was used to assess changes in mitochondrial membrane potential (ΔΨm; Ref. 44). At low concentrations, this dye exists as a monomer in the cytosol and yields green fluorescence at 530 nm (50). Being cationic in nature, it accumulates in mitochondria and forms dimers that exhibit red fluorescence at 590 nm. Formation of these dimers is directly proportional to the ΔΨm (44). Cells were isolated, plated, and cultured for 16 h. Thereafter, the medium was discarded, and the wells were loaded with 10 µg/ml JC-1 for 10 min, washed in ice-cold phosphate-buffered saline, and scanned immediately using a CytoFluor fluorimeter at 530/580-nm wavelengths for dimers and 485/530-nm wavelengths for the monomeric JC-1. The emission was estimated per 75,000 cells (in FU), and the red-to-green fluorescence ratio was calculated. For confocal microscopic images, chambered coverslips were used instead of culture plates. After these procedures, myocytes were visualized using a confocal scanning microscope (MRC 600; Bio-Rad) at 530/580-nm wavelengths with ×600 magnification. A single scan was performed to prevent mitochondrial damage during the experiment. The mitochondrial uncoupler carbonyl cyanide m-chlorophenylhydrazone (mCCCP; 100 µM) was used as a negative control (50).

Separation of cytosolic and mitochondrial fractions. Separation of cytosol and mitochondria was achieved by digitonin fractionation (15). In brief, digitonin (0.24 mg/ml) was added to the cardiomyocyte pellet (2 × 10⁶ cells) to disrupt the plasma membrane. After 2 min, the mixture was transferred to Eppendorf tubes that contained a 40% glycerol-silicone-oil mixture and buffer that contained (in mM) 19.8 NaCl, 50 KCl, 10 glucose, 10 HEPES, pH 7.4. The mixture was sonicated for 15–20 s. To determine fractionation efficiency, the glycerol and buffer samples were each assayed spectrophotometrically for succinate dehydrogenase (a mitochondrial marker) and lactate dehydrogenase (LDH; a cytosolic marker) activities. Because only ∼5% of the total succinate dehydrogenase was released into the buffer layer, mitochondria were recovered intact in the glycerol layer (15) before sonication. Additionally, LDH activity within the glycerol layer was also minimal (∼5%)

Estimation of GSH. Intracellular fractions were immediately subjected to deproteinization with 5% metaphosphoric acid to remove protein thiol groups, snap frozen in liquid nitrogen, and stored at −80°C. GSH determination was performed within the next 48 h. Cardiac GSH content was measured using a commercially available kit (Toyo Soda). GSH was reacted with 5′,5″-dithiobis-2-nitrobenzoic acid DTNB to produce a yellow-colored 5-thio-2-nitrobenzoic acid (TNB). The mixed disulfide GS-TNB produced in parallel, is recycled back to GSH by glutathione reductase to produce more TNB. The rate of TNB production is directly proportional to this recycling reaction, which is in turn directly proportional to the total concentration of GSH in the sample. Oxidized glutathione (GSSG) was estimated using 2-vinylpyridine (29). The difference between total GSH and GSSG amounts gave the value of the reduced GSH. Protein assays were performed according to the Bradford method using a non-deprotein-
ate portion of the samples. Values are expressed as nanomoles of GSH per milligram of protein.

As determinations of ROS and ΔΨm were performed in myocytes plated for 16 h, cellular GSH values were also estimated after 16 h of incubation in medium 199. Briefly, after isolation, myocytes were either snap frozen immediately and deproteinized with 5% metaphosphoric acid for estimation of GSH (0 h) or cultured for an additional 16 h, after which the cells were detached and processed as described above. Although diabetic myocytes show decreased GSH content compared with nondiabetic control cells at 0 h (control, 23.8 ± 2.4; diabetic, 16.1 ± 2.0 pmol GSH/mg of protein; P < 0.05), comparison of GSH in nondiabetic and diabetic myocytes after 16 h of plating showed no additional loss of GSH (control, 21.0 ± 1.1; diabetic, 13.9 ± 2.2 pmol GSH/mg of protein).

Cardiomyocyte apoptosis. Where indicated, part of the left ventricle was fixed in 10% formalin and embedded in paraffin, and 5-μm sections were prepared. To estimate cardiomyocyte apoptosis, terminal deoxynucleotidyl (TdT)-mediated, dUTP nick-end labeling (TUNEL) assay with cardiomyocyte-specific labeling was carried out as described previously (24, 25). At least six random sections of each heart were quantified using Northern Eclipse software. Values are expressed as the number of TdT-labeled nuclei per 10^6 nuclei.

Cardiac caspase activity. Activities of cardiac caspase-3, -8, and -9 were determined using the corresponding fluorescent caspase-specific substrates Asp-Val-Glu-Asp (DEVD)-aminomethylcoumarin (AMC; Molecular Probes), Ile-Glu-Thr-Asp (IETD)-AMC (BioVision), and Leu-Glu-His-Asp (LEHD)-AMC (Biosource), respectively. Briefly, 50–75 μg of total cell protein was added to reaction buffer, which contained 50 μM of the respective substrate, and incubated at 37°C for 1 h. The enzyme-catalyzed release of AMC was quantified in a fluorimeter at 380/450-nm wavelengths.

Lipid peroxidation. As previously described (23), oxidative damage to lipids was determined by the appearance of thiobarbituric acid-reactive substances (TBARS) like malondialdehyde in each of the subcellular fractions.

Protein carbonyl content. Protein carbonyls (PCs) were assayed as an index of oxidative modification of proteins (7). Briefly, 50 μg of the fractions were added to an equal volume of 10% TCA and centrifuged at 6,000 g at 4°C for 5 min; the supernatant was discarded. The precipitated proteins were resuspended in 0.2% 2,4-dinitrophenyl hydrazine and incubated for 1 h at 37°C. Subsequently, proteins were precipitated again with TCA, centrifuged, washed with ethanol-ethyl acetate, and dissolved in 6 mM guanidine hydrochloride, and the absorbance was measured spectrophotometrically at 370 nm.

Serum LDH. As described previously (24), serum samples were isolated, kept at 4°C, and processed for LDH within 24 h using a commercially available kit (Sigma).

Statistical analysis. Values are means ± SE. One-way ANOVA and subsequent Tukey or Holm-Sidak test were used to determine differences between group mean values. The level of statistical significance was set at P < 0.05.

RESULTS

Mitochondrial dysfunction after acute diabetes. STZ administration increased blood glucose levels within 24 h, which were sustained for the next 3 days (control, 7.1 ± 0.3; diabetes, 23.4 ± 0.7 mM; P < 0.05) with a reduction in plasma insulin levels.
levels (control, 3.3 ± 0.4; diabetes, 0.7 ± 0.1 ng/ml; \( P < 0.05 \)). One of the earliest diabetes-induced alterations is generation of ROS (25, 33). To evaluate ROS levels, we utilized CM-H2DCFDA, a dye that fluoresces green when oxidized by ROS (Fig. 1, top). With the use of confocal microscopy and fluorimetry (Fig. 1, top and bottom, respectively), diabetic myocytes revealed an increase in green fluorescence, which suggests an increased prevalence of ROS within these cells. Treatment with rotenone reduced ROS production in diabetic myocytes to control levels. Diabetic cells were more sensitive toward antimycin A and demonstrated greater fold increases in ROS compared with control myocytes. Additionally, incubation with NAC decreased the fluorescence to less than control levels.

ROS generation in diabetic heart may be regulated by changes in \( \Delta \psi_m \) (5, 27). With the use of confocal microscopy with JC-1, a dye that fluoresces green when oxidized by ROS (Fig. 1, top), with the use of confocal microscopy and fluorimetry (Fig. 1, top and bottom, respectively), diabetic myocytes revealed an increase in green fluorescence, which suggests an increased prevalence of ROS within these cells. Treatment with rotenone reduced ROS production in diabetic myocytes to control levels. Diabetic cells were more sensitive toward antimycin A and demonstrated greater fold increases in ROS compared with control myocytes. Additionally, incubation with NAC decreased the fluorescence to less than control levels.

Cardiac GSH and apoptosis after acute diabetes. As reported previously, estimation of cardiac apoptosis revealed a nearly fourfold increase in TUNEL-positive nuclei in diabetic hearts (Fig. 3, top). As the most important cardiac antioxidant, in addition to being a key regulator of apoptosis (16, 25), reduced GSH was estimated in both the cytosolic and mitochondrial fractions. Interestingly, unlike the cytoplasmic pool, a depletion of mGSH with an increase in mitochondrial GSSG (Fig. 3, bottom) was observed in 3-day diabetic rat hearts, which implicates the importance of this specific pool of GSH in the cell suicide process.

GSH modulation and effects on cardiac apoptosis. To further investigate the relationship between GSH and cardiac apoptosis, we used different GSH-depleting agents and quantified TUNEL-positive nuclei in diabetic hearts. BSO, which is known to inhibit de novo synthesis of GSH, decreased cGSH but not mGSH (Fig. 4, top left and top right, respectively) in diabetic hearts; this is likely due to the fact that mitochondria lack \( \gamma \)-GCS, the target enzyme for BSO (30). In an attempt to also influence mGSH, DEM, which is an agent that conjugates GSH and depletes both mGSH and cGSH in vitro, was used (21, 26). Like BSO, DEM induced a substantial decrease in cGSH but was ineffective in reducing mGSH in vivo (Fig. 4, top left and top right, respectively). Interestingly, although both BSO and DEM treatments brought about substantial reductions in cGSH, they were ineffective in augmenting...
cardiomyocyte apoptosis in diabetic hearts (Fig. 4, bottom). Although coadministration of BSO and DEM did not further decrease cGSH levels compared with DEM only, mGSH was almost undetectable with this treatment (Fig. 4, top right). In this setting, cardiac apoptosis was further aggravated compared with the untreated diabetic group (Fig. 4, bottom). In control animals, similar treatment resulted in depletion of only cGSH (untreated controls, 18.7 ± 3.9; control rats treated with BSO and DEM, 9.7 ± 3.1 pmol/mg protein; P < 0.05) and not mGSH (untreated controls, 7.2 ± 1.9; control rats treated with BSO and DEM, 6.3 ± 0.9 pmol/mg protein). Apoptotic rates also remained unchanged compared with untreated control animals (data not shown).

In an attempt to prevent cardiac GSH loss, animals were pretreated with exogenous GSH before the induction of diabetes, and the treatment continued until termination. Unexpectedly, GSH supplementation decreased cGSH levels (Fig. 4, top). However, there was a robust amplification in mGSH content in diabetic hearts (Fig. 4, top right) together with a substantial decrease in the apoptotic index to control levels (Fig. 4, bottom).

Cardiac oxidative stress and caspase activity. Having implicated mGSH loss as a key participant in cardiac apoptosis, we investigated the outcome of GSH depletion on the initiation pathways of apoptosis. Within 3 days of diabetes, mitochondrial lipid (mTBARS) and protein (mPC) oxidation increased (Fig. 5, top) with no change in these parameters in the cytosol (data not shown). Among the caspase family, caspase-9 is the initiator caspase of the mitochondrial apoptotic pathway, and caspase-8 is the initiator caspase of the "death receptor"-mediated pathway. Both of these caspases ultimately activate caspase-3, which is the common effector caspase of both of the above pathways. On induction of diabetes, we observed an increase in caspase-9 and -3 activities only (Fig. 5, bottom) and no change in caspase-8 activity (untreated control rats, 20.2 ± 2.5; untreated diabetic rats, 19.7 ± 2.5 FU/mg protein). Taken together, these data suggest that after induction of acute diabetes, the mitochondrial death pathway is important in precipitating cardiac apoptosis (1).

Treatment of diabetic rats with BSO or DEM increased cytosolic oxidative stress [TBARS: untreated diabetic rats, 23.8 ± 2.5; diabetic rats treated with BSO, 36.9 ± 4.9; diabetic rats treated with DEM, 36.2 ± 3.6 nM malondialdehyde/mg protein; P < 0.05; PC content: untreated diabetic rats, 98.5 ± 21.1; diabetic rats treated with BSO, 212.6 ± 16.3; diabetic rats treated with DEM, 208 ± 28.8 arbitrary units (AU)/mg protein; P < 0.05] and caspase-8 activity (untreated diabetic rats, 19.7 ± 2.5; diabetic rats treated with BSO, 33.1 ± 2.7; diabetic rats treated with DEM, 36 ± 3.2 FU/mg protein; P < 0.05) compared with the untreated diabetic group. These treatments were ineffective in further aggravating either mitochondrial oxidative stress (Fig. 5, top) or caspase-9 and -3 activities (Fig. 5, bottom). Coadministration of both BSO and DEM led to a depletion of mGSH, specific increases in mitochondrial oxidative stress (Fig. 5, top) and caspase-9 and -3 activities (Fig. 5, bottom), and no change in caspase-8 (data not shown) compared with untreated diabetic rats. Coadministration of BSO and DEM in control animals did not influence either oxidative stress or cardiac caspase activities (Fig. 5), which suggests that it is not simply depletion of GSH but the added...
capable of modifying various cellular structures. Should the acute diabetes (1).

DISCUSSION

Changes in ROS exceed their neutralization by antioxidants, oxidative “stress” and premature cellular demise may occur (16, 23, 25). In both clinical and experimental models of diabetes, oxidative stress is believed to be a key participant in causing cardiac injury (3). Using CM-H2DCFDA, we confirmed the presence of ROS such as H$_2$O$_2$ and OH$^-$ in cardiomyocytes isolated from acutely diabetic rats (41). Because this increase in ROS was independent of any change in antioxidants such as GSH over the incubation period of 16 h, these data implied that ROS generation occurs due to an intrinsic defect in diabetic cardiomyocytes rather than as a consequence of the experimental protocol. Given that under normal conditions 1–2% of all oxygen consumed is converted to ROS by electron transfer in the mitochondrial ETC (28), we assessed the contribution of this pathway toward ROS generation in acutely diabetic rat hearts. In this regard, ubisemiquinone at complex III appears to be a major site for cardiac mitochondrial ROS generation (35). Using rotenone (an inhibitor of the upstream complex I), we were able to decrease the excess ROS production in diabetic cardiomyocytes to control levels, likely as a consequence of inadequate electron transfer to the downstream complex III (35). These results with rotenone precluded examination of the role of extramitochondrial ROS-generating pathways in the acutely diabetic heart. As an alternate strategy, antimycin A, which inhibits electron transfer from complex III and thereby prolongs the half-life of ubisemiquinone and increases ROS, was used (35). Antimycin A augmented ROS generation in both control and diabetic cardiomyocytes. However, the fold increase in ROS was greater in diabetic compared with control myocytes, which supports our conclusion that in diabetes, cardiac ROS are predominantly mitochondrial in origin.

Amplification of mitochondrial ROS may result from changes in $\Delta\psi_m$ (crucial in maintaining electron flux in the ETC and thereby minimizing ROS generation). With the use of confocal microscopy and measurement of the red ($\Delta\psi_m < -100$ mV)-to-green ($\Delta\psi_m > -100$ mV) fluorescence ratio of the dye JC-1, a loss of negative $\Delta\psi_m$ was observed in diabetic cardiomyocytes, which suggests that ROS generation may indeed be related to changes in $\Delta\psi_m$ (50). Other than its role in ROS generation, the commitment of a cell toward apoptosis may become irreversible after collapse of $\Delta\psi_m$ (27). In this regard, changes in $\Delta\psi_m$ induce the opening of “channels” in the mitochondrial membrane (permeability transition pores) that lead to release of low-molecular weight proapoptotic factors from the mitochondria such as cytochrome c and apoptosis-inducing factor. These in turn can specifically activate initiator caspases such as caspase-9 and effector caspses such as caspase-3 and lead to apoptosis (27). Because caspase-9 and -3 were both increased along with a substantial increase in cardiac apoptotic death after only 3 days of diabetes, our data suggest that the “mitochondrial” pathway of apoptosis may predominate at this early time point.

In addition to changes in $\Delta\psi_m$, permeability transition pore formation has also been linked to insufficiency in antioxidants such as GSH and oxidative stress (5). In this study, although cGSH remained unaltered, diabetic hearts were characterized by a decrease in mGSH with a parallel increase in mitochondrial oxidative stress. A decrease in mGSH induces extensive ROS-induced damage to mitochondrial membranes that in turn causes a collapse of $\Delta\psi_m$ and activation of the mitochondrial

Fig. 4. Effects of different treatments on GSH and cardiac apoptosis. In an effort to deplete cardiac GSH, daily doses of l-buthionine-sulfoximine (BSO; 10 mmol/kg) and/or diethyl maleate (DEM; 4 mmol/kg) were administered for 3 days. For exogenous GSH supplementation, control rats were administered GSH (400 mg/kg) for 10 days before streptozotocin administration, and the treatment was continued for an additional 4 days [diabetic (DIA) + GSH rats]. After these treatments, rats were killed and hearts were isolated and used either intact or after isolation of cardiomyocytes. Myocardial GSH in subcellular fractions from diabetic hearts (top) and cardiac apoptosis as determined by TUNEL staining (bottom) are shown. DIA + DEM, DEM-treated diabetic rats; DIA + BSO + DEM, DEM- and BSO-treated diabetic rats. Results are means ± SE of 6 rats/group; *$P$ < 0.05, significantly different from untreated diabetic (UTX DIA) rats; #$P$ < 0.05, significantly different from BSO-treated diabetic (DIA + BSO) rats.

effect of the prevailing diabetic environment that promotes cell death in diabetic hearts.

Because severe GSH depletion may precipitate necrotic death (9, 24), serum LDH was measured and found to be unchanged in all groups, which suggests that cell death was predominantly apoptotic in nature (data not shown). GSH supplementation prevented cardiac apoptosis, reduced mitochondrial oxidative stress (Fig. 5, top) and activities of caspase-9 and -3 (Fig. 5, bottom); therefore, these data further confirm mitochondrial participation in cardiac apoptosis during acute diabetes (1).

ROS are unstable oxygen-containing molecules that are capable of modifying various cellular structures. Should the...
death pathway (Fig. 6; Refs. 5, 39). Because similar results have also been reported in chronically diabetic rodent hearts (46, 47), these data imply that loss of mGSH and the consequent ROS-induced damage may be an essential cause for the diabetes-induced early apoptosis of diabetic rat hearts.

To further examine the relationship between the different subcellular pools of GSH and apoptosis, we used BSO and DEM, agents that are known to deplete cellular GSH via different mechanisms. Administration of BSO, an inhibitor of γ-GCS activity, resulted in a 30% decline in cGSH with no change in mGSH or apoptosis. To overcome the resistance of mGSH to change, DEM, an agent that irreversibly inactivates all preformed subcellular pools of GSH, was used (21, 26). Although DEM decreased cGSH (by almost 70%), it was also incapable of either reducing mGSH or increasing cardiac apoptosis compared with untreated diabetic hearts. In other studies, DEM treatment before ischemia-reperfusion was also incapable of worsening damage in rat hearts (6). It has been suggested that although DEM administration decreases mGSH in vitro, conjugation of cellular GSH with DEM in vivo triggers a compensatory increase in γ-GCS activity (10, 43), and newly formed GSH is transferred preferentially to the

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**Fig. 5.** Mitochondrial oxidative stress and caspase activities in control and diabetic hearts. Control hearts were either left untreated (UTX CON) or were treated with BSO and DEM (CON+BSO+DEM). Diabetic rats were either left untreated or were treated with BSO (DIA+BSO), DEM (DIA+DEM), BSO and DEM (DIA+BSO+DEM), or GSH (DIA+GSH). Mitochondrial oxidative stress (top) was estimated by lipid peroxidation and the appearance of thiobarbituric acid-reactive substances (mTBARS) and protein carbonyl [mPC; measured in arbitrary units (AU)/mg protein] content. Quantitative estimation of cardiac caspase activities (bottom) was determined using the corresponding fluorescent caspase-specific substrates DEVD-AMC (for caspase-3) and LEHD-AMC (caspase-9). Results are means ± SE of 6 rats/group; *P < 0.05, significantly different from control animals; #P < 0.05, significantly different from untreated diabetic rats.

**Fig. 6.** Proposed scheme of myocardial apoptosis in acute diabetes. Diabetes induces a loss of mitochondrial GSH and an increase in ROS. In turn, ROS can propagate lipid and protein oxidation, which leads to loss of mitochondrial membrane potential (ΔΨm). As a consequence, this loss of ΔΨm may further exacerbate ROS generation, pore formation, and activation of caspase-9 and -3, which are followed by apoptosis.
mitochondria. In this regard, during extensive cGSH depletion, the carrier system responsible for the transport of GSH may act to preserve mGSH levels (22, 30). We speculated that abrogation of mGSH in vivo would only be possible if the compensatory rise in γ-GCS activity were prevented after DEM administration. To achieve this goal, BSO and DEM were coadministered over 3 days to STZ-diabetic rats. Using this novel approach, we were successful in depleting mGSH in diabetic rat hearts. Under these conditions, mitochondrial lipid peroxidation, protein oxidation, and apoptosis were exaggerated, which suggests an obligatory role for mGSH in minimizing oxidative stress and apoptosis in acutely diabetic rat hearts. Because cGSH levels were similar to those seen with DEM alone, it is unlikely that the increased cytotoxicity was due to cGSH depletion.

Exogenous GSH supplementation in pigs significantly increased myocardial GSH content and resistance to ischemia-reperfusion injury (36, 49). Additionally, apoptosis and oxidative stress in isolated hearts perfused with high levels of glucose were attenuated by addition of GSH to the perfusion buffer (4). To prevent depletion of cardiac GSH after induction of diabetes, rats were administered exogenous GSH. In acutely diabetic rat hearts, apoptosis and mitochondrial oxidative stress were averted by GSH supplementation. Measurement of GSH in the different pools revealed a selective increase in mGSH only that is likely reflective of the need to protect the diabetic mitochondria from oxidative damage. Unexpectedly, cGSH levels decreased, and the mechanism responsible for such an effect is presently unknown.

Two independent pathways can cause cardiac apoptosis, and cellular GSH levels influence both the death receptor-mediated “extrinsic” and the mitochondrial “intrinsic” pathways (19, 42). Caspase-8 and -9 are initiator caspases for the extrinsic and intrinsic pathways, respectively (1). In our study, induction of diabetes was only associated with caspase-9 activation; this effect was amplified using BSO and DEM in combination. Because similar results have been reported in diabetic kidney and neuronal cells (11, 40) and because BSO and DEM increased caspase-8 without intensifying apoptosis, our data point toward a major role for the mitochondrial apoptotic pathway in acutely diabetic heart.

In summary, at least after short-term diabetes, cardiac oxidative stress and apoptosis appear to have mitochondrial origins, and mGSH but not cGSH plays an essential role. In both Type 1 and Type 2 diabetic patients, a lack of blood GSH has been reported (12, 13, 32), and recently, Darmann et al. (8) have proposed that this depletion in erythrocytes is a consequence of accelerated GSH utilization that can be ameliorated with dietary supplementation of GSH. Thus using antioxidants such as GSH may be an effective option for scavenging mitochondrial ROS and preventing diabetic heart disease (28).

REFERENCES


