Altered Apoptosis-Related Signaling After Cardioplegic Arrest in Patients With Uncontrolled Type 2 Diabetes Mellitus

Jun Feng, MD, PhD; Yuhong Liu, MD; Nikola Dobrilovic, MD; Louis M. Chu, MD; Cesario Bianchi, MD, PhD; Arun K. Singh, MD; Frank W. Sellke, MD

Background—We investigated the effects of cardioplegic arrest and reperfusion (CP/Rep) on myocardial apoptosis and key apoptotic mediators, such as apoptosis-inducing factor, caspase 3, caspase 8, caspase 9, poly(adenosine diphosphate-ribose) polymerase, B-cell lymphoma 2 (Bcl-2) family proteins, and protein kinase C (PKC), in uncontrolled type 2 diabetic, controlled type 2 diabetic, and nondiabetic patients.

Methods and Results—Right atrial tissue was harvested pre- and post-CP/Rep from uncontrolled type 2 diabetic patients (hemoglobin A1c=9.6±0.25), controlled type 2 diabetic patients (hemoglobin A1c=6.5±0.15), and nondiabetic patients (hemoglobin A1c=5.4±0.12) undergoing coronary artery bypass grafting (n=8/group). Terminal deoxynucleotidyl transferase dUTP nick-end labeling staining was used for the identification of apoptotic cells. Total and modified apoptosis-inducing factor, Bcl-2 family proteins, phospho-PKC-α, phospho-PKC-β1, and poly(adenosine diphosphate-ribose) polymerase were quantified by immunoblotting or immunohistochemistry. At baseline, the number of apoptotic cells and expression of total apoptosis-inducing factor, Bcl-2, Bak, and Bax in the pre-CP/Rep atrial tissue from uncontrolled type 2 diabetic patients were significantly increased compared with those of nondiabetic or controlled type 2 diabetic patients (P<0.05). After CP/Rep, the amount of apoptotic cells, apoptosis-inducing factor, phospho-Bad, phospho-PKC-α, phospho-PKC-β1, and cleaved poly(adenosine diphosphate-ribose) polymerase in post-CP/Rep atrial tissue were increased in all 3 groups compared with pre-CP/Rep. These increases after CP/Rep were more pronounced in the uncontrolled type 2 diabetic group. In addition, there were significant increases in the expression of cleaved caspase 8 and caspase 9 in the basal and post-CP/Rep atrium of uncontrolled type 2 diabetic group compared with nondiabetic or controlled type 2 diabetic group.

Conclusions—Uncontrolled diabetes mellitus is associated with increases in myocardial apoptosis and expression of key apoptosis mediators at baseline and in the setting of CP/Rep. (Circulation. 2013;128[suppl 1]:S144-S151.)

Key Words: apoptosis ■ coronary artery bypass surgery ■ cardioplegia ■ cardiopulmonary bypass ■ diabetes mellitus

Diabetes mellitus is associated with increased risk of ischemic heart disease and increased morbidity and mortality after open heart operations involving cardioplegia (CP) and cardiopulmonary bypass (CPB).1,2 Previous studies have demonstrated that diabetes mellitus is associated with increased myocardial oxidative stress, attenuation of cell survival pathways, and induction of apoptosis in animal models.3–6 Recent clinical trials have also shown that glycemic control significantly reduces microvascular complications, such as retinopathy, nephropathy, and peripheral arterial disease.7,8 In addition, perioperative glucose control in some studies is associated with improved outcomes after coronary artery bypass graft surgery.9,10 Recently, we found that poorly controlled diabetes mellitus is associated with microvascular dysfunction and worsens the recovery of arteriolar endothelial and smooth muscle function after CP/CPB.11,12 However, whether diabetes mellitus, especially uncontrolled type 2 diabetes mellitus (UDM), affects apoptosis and apoptosis-related signaling after CP/CPB has not been investigated.

The goal of this research is to investigate the effect of diabetes mellitus on cardioplegic arrest and reperfusion (CP/Rep)-induced changes in myocardial apoptosis and apoptosis-related signaling. Specifically, this study was designed to directly test the effect of diabetes mellitus and CP/Rep on the development of apoptosis and apoptosis-related protein expression/localization in human atrial tissues harvested from patients with coronary artery bypass graft surgery.

Methods

Human Subjects and Tissue Harvesting

Samples of right atrial appendage were harvested from patients undergoing coronary artery bypass graft surgery before and after exposure of the heart to blood cardioplegia and short-term reperfusion under...
conditions of CPB. Samples were handled in a nontraumatic fashion. Double 3-0 polypropylene purse-string sutures (Ethicon, Somerville, NJ) were placed in the atrial appendage. The first sample of atrial appendage was harvested pre-CP/Rep. During placement of the venous cannula, the superior suture was tightened to secure the venous cannula. The inferior suture remained loose to allow this portion of the atrium to be exposed to blood cardioplegia and reperfused (post-CP/ Rep) after removal of the aortic cross-clamp. An initial 600 to 800 mL of cold-blood (0°C–4°C) hyperkalemic (15 mmol/L K+) cardioplegic solution was delivered antegrade into the aortic root. This was followed at 8- to 20-minute intervals with 200 to 300 mL of cold cardioplegic solution (15 mmol/L K+). The composition of cardioplegic solutions has been previously described in detail.10,12

The second sample of atrial tissue (post-CP/Rep) was harvested between the purse-strings during removal of the venous cannula. Sections of right atrium were immediately frozen in liquid nitrogen (immunoblotting) or fixed in 10% formalin for 24 hours followed by paraffinization and sectioning into 5-μm slices (immunofluorescent staining).

Hemoglobin A1C was measured in all patients. The patients were then divided into the following 3 groups: (1) patients with a normal HgbA1c and no history or treatment for diabetes mellitus were considered nondiabetic (ND); (2) patients with a history of diabetes mellitus with HgbA1c >5.5 and <7 were considered controlled diabetic (CDM); and (3) patients with HgbA1c ≥8.5 were considered UDM. Patients who also had valve surgery, cross-clamp time >160 minutes, or CPB time >180 minutes were excluded from the study. Of the total 110 patients enrolled, 8 randomly chosen patients from 110 cases in each group were included for analysis in this study. All procedures were approved by the Institutional Review Board of Rhode Island Hospital, Alpert Medical School of Brown University, and informed consent was obtained from all enrolled patients as required by the institutional review board.

Immunoblot

Atrial tissue from 8 patients per group was dissected and cleaned of fat and connective tissues and then solubilized in SDS-PAGE buffer. Total protein (40 μg) was fractionated on an 8% to 16% SDS-PAGE gel and then transferred to a polyvinylidene difluoride membrane (Millipore Corporation). Ten photographs (magnification, ×200) of each tissue section were taken. The nuclei were viewed and manually counted by 2 observers who were blinded to the experimental conditions. The number of apoptotic cardiomyocytes was calculated in an average of 10 microscopic fields in each sample and expressed as the percentage of the total number of cardiomyocyte nuclei.13–16

Immunofluorescence Microscopy

The detailed methods have been previously described.10–12 After PBS wash, atrial tissue sections were incubated overnight with anti–phospho-protein kinase C (PKC)-α, anti–phospho-PKC-β1 (ABCAM), and cleaved PARP (Cell Signaling Tech) at 4°C.

Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling Staining

Right atrial myocardial tissue was fixed in formalin for 24 hours, embedded in paraffin, and cut into 4-μm sections. The apoptotic cells were identified by terminal deoxynucleotidyl transferase dUTP nick-end labeling using an apoptosis detection kit according to the manufacturer’s protocol (Millipore Corporation). Ten photographs (magnification, ×200) of each tissue section were taken. The nuclei were viewed and manually counted by 2 observers who were blinded to the experimental conditions. The number of apoptotic cardiomyocytes was calculated in an average of 10 microscopic fields in each sample and expressed as the percentage of the total number of cardiomyocyte nuclei.13–16

Data Analysis

Data in the table are presented as mean and SEM. One-way ANOVA tests followed by Kruskal–Wallis multiple comparison tests were used to analyze clinical, Western blot, and terminal deoxynucleotidyl transferase dUTP nick-end labeling–positive cell data among the 3 groups (GraphPad Software, Inc, San Diego, CA). The Fisher exact test was used to analyze categorical data of patient characteristics. P<0.05 was considered significant.

Results

Patient Characteristics

Patient characteristics are listed in the Table. All patients (n=24, 8/group) with preoperative hypertension were on antihypertensive medication (β-blocker, aspirin, calcium channel blocker, or angiotensin-converting enzyme inhibitor). The preoperative

<table>
<thead>
<tr>
<th>Table. Patient Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient Characteristics</td>
</tr>
<tr>
<td>Age, y*</td>
</tr>
<tr>
<td>Male/female (n)</td>
</tr>
<tr>
<td>HgbA1c, %*</td>
</tr>
<tr>
<td>Patient blood glucose, mg/dL</td>
</tr>
<tr>
<td>Cardioplegia</td>
</tr>
<tr>
<td>Preoperative insulin (n)</td>
</tr>
<tr>
<td>Intraoperative insulin (n)</td>
</tr>
<tr>
<td>Obesity (BMI &gt;30 kg/m²)</td>
</tr>
<tr>
<td>Atrial fibrillation (n)</td>
</tr>
<tr>
<td>Hypercholesterolemia (n)</td>
</tr>
<tr>
<td>Hypertension (n)</td>
</tr>
<tr>
<td>Duration of CPB, min*</td>
</tr>
<tr>
<td>Cross-clamp time, min*</td>
</tr>
<tr>
<td>CABG only (n)</td>
</tr>
<tr>
<td>No. of grafts</td>
</tr>
</tbody>
</table>

BMI indicates body mass index; CABG, coronary artery bypass graft; CPB, cardiopulmonary bypass; and HgbA1c, hemoglobin A1C.

*Data expressed as mean ± standard deviation.
†vs nondiabetes mellitus.
hemoglobin A1C levels were 9.6±0.25 in UDM patients, 6.5±0.15 in CDM patients, and 5.4±0.12 in ND patients.

Effect of Diabetes Mellitus and CP/Rep on Levels of AIF, Caspases, and PARP

There were no significant differences in the expression of total AIF, total PARP, and total/cleaved caspase 3 proteins in the pre-CP/Rep atrial tissue between ND and CDM groups (Figure 1A–1D and Figure 2A–2C). In contrast, the expression of total AIF in the pre-CP/Rep atrial tissue from UDM patients was significantly increased compared with that of ND patients (P<0.05; Figure 1A and 1B). The levels of AIF in post-CP/Rep atrial tissue were increased in all 3 groups compared with that of pre-CP/Rep (ND: P=0.04; CDM:

![Figure 1. A. Representative immunoblots of human atrial tissues for apoptosis-inducing factor (AIF). B. Immunoblot quantification shows significant enhanced expression of AIF in the uncontrolled type 2 diabetic group (UDM); *P<0.05 vs pre–cardioplegic arrest and reperfusion (CP/Rep); @P<0.05 vs pre-CP/cardiopulmonary bypass (CPB) nondiabetic (ND) group; #P<0.05 vs post-CP/Rep-ND or controlled type 2 diabetes mellitus (CDM). C. Representative immunoblots of human atrial tissues for total poly(adenosine diphosphate-ribose) polymerase (PARP). D. Immunoblot quantification shows no significant changes in total PARP protein expression among the 3 groups; n=8/group.](image)

![Figure 2. A. Representative immunoblots of human atrial tissues for total/cleaved caspase 3, cleaved caspase 8, and cleaved caspase 9; immunoblot quantification shows no significant changes in the expression of total caspase 3 (B) and cleaved caspase 3 (C) among the 3 groups; D, immunoblot quantification of cleaved caspase 8; E, immunoblot quantification of cleaved caspase 9; *P<0.05 vs pre–cardioplegic arrest and reperfusion (CP/Rep); @P<0.05 vs pre-CP/Rep-nondiabetes mellitus (ND); #P<0.05 vs post-CP/Rep-ND or controlled type 2 diabetes mellitus (CDM); n=8/group. UDM indicates uncontrolled type 2 diabetes mellitus.](image)
These increases after CP/Rep were more pronounced in the UDM group (AIF: $P=0.009$ versus ND; $P=0.01$ versus CDM). There were no significant changes in the expression of total AIF in the post-CP/Rep atrial tissue between ND and CDM groups. The pre-CP/Rep levels of total PARP and caspase 3 were slightly, but insignificantly increased in the UDM group compared with those of ND or CDM group (Figure 1C and 1D and Figure 2A and 2B). There was no difference in the expression of total/cleaved caspase 3 and total PARP between pre- and post-CP/Rep tissues in the 3 groups (Figure 1C and 1D and Figure 2A–2C). However,

$P=0.02$; UDM: $P=0.01$, respectively; Figure 1A and 1B).
pre-CP/Rep levels of cleaved caspase 8 and cleaved caspase 9 were significantly higher in the UDM group than that of ND ($P=0.001$) or CDM ($P=0.01$; Figure 2A, 2D, and 2E) group. CP/Rep further induced more caspase 8 and caspase 9 cleavage in diabetic myocardium, and these changes were more pronounced in the UDM group.

**Figure 5.** A, Representative immunoblots of human atrial tissues for total Bak. B, Immunoblot quantification shows significantly increased Bak expression in the uncontrolled type 2 diabetic (UDM) group. C, Representative immunoblots of human atrial tissues for Bak. D, Immunoblot quantification shows significantly increased expression of Bak in the UDM. E, Representative immunoblots of human atrial tissues for Bax. F, Immunoblot quantification shows significantly increased expression of BNIP in UDM at baseline; $\#P<0.05$ vs pre-cardioplegic arrest and reperfusion (CP/Rep)-nondiabetes mellitus (ND) or controlled type 2 diabetes mellitus (CDM); $\Delta P<0.05$ vs post-CP/Rep-ND or CDM; $n=8/group$.

**Figure 6.** Immunolocalization of phospho-protein kinase C (PKC)-$\alpha$, phospho-PKC-$\beta$, and cleaved poly(adenosine diphosphate-ribose) polymerase (PARP) in human atrial myocardium (magnification, $\times200$). Tissue slices were stained for phospho-PKC-$\alpha$ (A, red) or phospho-PKC-$\beta$ (B, red) or cleaved PARP (E, green); matched negative controls are displayed in the last column; C, phospho-PKC-$\alpha$ density analysis; D, phospho-PKC-$\beta$ density analysis; $^P<0.05$ vs pre-cardioplegic arrest and reperfusion (CP/Rep); $\#P<0.05$ vs pre-CP/Rep-nondiabetes mellitus (ND) or controlled type 2 diabetes mellitus (CDM); $\triangle P<0.05$ vs post-CP/Rep-ND or CDM; $n=6/group$. UDM indicates uncontrolled type 2 diabetes mellitus.
Expression of Bcl-2 Family Proteins
The pre-CP/Rep expression of Bcl-2, Bak, and Bax in the UDM myocardium was significantly higher than that of ND or CDM (Figures 3–5; P<0.05). The post-CP/Rep levels of Bcl-2, Bak, and Bax protein expression in the UDM myocardium were also significantly higher than that of ND or CDM (Figures 3–5; P<0.05). In contrast, there were no significant differences in the expression of phospho-Bcl-2 (Figure 3A and Figure 3C) and total Bad (Figure 4A and Figure 4B) among 3 groups or between pre- and post-CP/Rep. The pre-CP/Rep expression of phospho-Bad in the UDM group was significantly higher than that of ND or CDM (P<0.05, Figure 4C and Figure 4D). The pre-CP/Rep levels of phospho-Bad were significantly increased in the post-CP/Rep period in all 3 groups and these increases were more pronounced in the UDM group (P<0.05, Figure 4D). The basal expression of Bcl-2/adenovirus E1B 19 kDa protein-interacting protein 3 in the UDM group was significantly higher than that in the ND group (P=0.01; Figure 5E and 5F). The post-CP/Rep level of Bcl-2 adenovirus E1B 19 kDa protein-interacting protein 3 tended to be decreased, but not to a significant degree, compared with that of pre-CP/Rep in the UDM group.

Effect of Diabetes Mellitus and CP/Rep on Myocardial Distribution of Phosphorylated PKC-α and PKC-β1 and Cleaved PARP
Immunofluorescent staining of right atrial tissues displayed a relatively weak signal (red) for phospho-PKC-α (Figure 6A) and phospho-PKC-β1 (Figure 6B) localized to cardiomyocytes in the pre-CP/Rep samples and stronger signals in the post-CP/Rep samples. Further density analysis shows that basal (pre-) and post-CP/Rep levels of phospho-PKC-α and phospho-PKC-β1 were higher in the UDM myocardium than that of ND or CDM (Figure 6C and 6D; P<0.05, respectively). Immunostaining also demonstrated a relatively weak signal (green) for cleaved PARP localized to the cardiomyocytes in the pre-CP/Rep samples and stronger signals in the post-CP/Rep samples (Figure 6E). This alteration was more pronounced in the UDM group. Negative controls documented a low level of background fluorescence.

Apoptosis
There was no significant difference in the number of apoptotic cells in the pre-CP/Rep atrial myocardium between ND and CDM groups. In contrast, the number of apoptotic cells in the pre-CP/Rep atrial tissue from UDM patients was significantly increased compared with that of ND patients (P<0.05; Figure 7A and 7B). The number of apoptotic cells in post-CP/Rep atrial tissue was increased in all 3 groups compared with pre-CP/Rep. These increases were more profound in the UDM group (P<0.05 versus ND; Figure 7A and 7B). In contrast, there were no significant differences in the development of terminal deoxynucleotidyl transferase dUTP nick-end labeling–positive cells in the post-CP/Rep atrial tissue between ND and CDM groups.

Discussion
The results of the present study demonstrate increased myocardial apoptosis in poorly controlled diabetic patients. These changes may contribute to the less favorable postoperative outcomes after cardiac surgery in this patient population. Previous studies have demonstrated that apoptosis-related signaling in animal and human myocardium is significantly modified after CP/Rep and CPB. In the present study, we also found that AIF, Bcl-2 family proteins, caspase 8, caspase 9, PARP, and PKC were activated or upregulated after CP/Rep. In addition, poorly controlled diabetes mellitus enhanced the baseline myocardial expression of AIF, Bcl-2, phospho-Bad, Bak, Bax, cleaved caspase 8, cleaved caspase 9, and cleaved PARP. Interestingly, poorly controlled diabetes mellitus further enhanced the expression of AIF, Bcl-2, phospho-Bad, Bak, Bax, cleaved caspase 8, cleaved caspase 9, and cleaved PARP after CP/Rep.

Figure 7. Paraffin sections stained with terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. TUNEL-positive nuclei stain brown (arrows), and TUNEL-negative nuclei stain green (magnification, ×200). Post-cardioplegic arrest and reperfusion (CP/Rep) sections of right atrial tissue showed significantly increased amount of TUNEL-positive nuclei (arrows); *P<0.05 vs pre-CP/Rep; @P<0.05 vs pre-CP/Rep nondiabetes mellitus (ND); #P<0.05 vs post-CP/Rep-ND, n=6/group. CDM indicates controlled type 2 diabetic mellitus; and UDM, uncontrolled type 2 diabetes mellitus.
These findings suggest that both proapoptotic and antiapoptotic (Bcl-2) proteins in human myocardium were modified by diabetes mellitus and CP/Rep. The increased Bcl-2 protein expression in poorly controlled diabetic myocardium may be a compensatory effect. In addition, the increases in cleaved caspase 8 and caspase 9 observed in the present study suggest that CP/Rep and diabetes mellitus may activate both the intrinsic and extrinsic apoptosis pathways.

Previous studies have shown that treatment with a PKC-β inhibitor improved myocardial function in animal models of diabetes mellitus. In the present study, we demonstrate that diabetes mellitus significantly upregulates phospho-PKC-α and phospho-PKC-β1 in atrial cardiomyocytes at baseline. After CP/Rep, phospho-PKC-α and phospho-PKC-β1 in uncontrolled diabetes mellitus remain higher than that in ND patients, which may partially explain why CP/Rep increases the development of apoptosis in the diabetic myocardium.

We have recently found that protein oxidation was significantly increased in the human tissues harvested from the poorly controlled diabetic patients in the setting of CPB. These increases in oxidative stress may also contribute to apoptosis in the poorly controlled diabetic heart. Taken together, hyperglycemia, AIF, Bcl-2 family protein upregulation, PKC activation, and oxidative stress may cumulatively participate in the development of myocardial apoptosis of the diabetic heart.

There are several limitations of the current study that deserve mention. First, this work should be considered a pilot study considering the relatively small number of patients and samples. Another limitation is the heterogeneity of the patients; although they were reasonably well matched, there were differences in medications and the incidence of coexisting illnesses that may affect the findings. This is a limitation of all studies dealing with patients. Third, little clinical outcome data were presented. Fourth, the effects of insulin therapy might have also affected the development of myocardial apoptosis in poorly controlled diabetic patients.

In conclusion, diabetes mellitus, especially poorly controlled diabetes mellitus, is associated with increased myocardial apoptosis in the setting of CP/Rep and cardiac surgery. The enhanced expression/activation of AIF, Bcl-2, Bax, Bad, Bak, Bcl2/adenvirus E1B 19 kDa protein-interacting protein 3, caspase 8, caspase 9, and PARP may cumulatively contribute to the development of myocardial apoptosis in poorly controlled diabetic patients.

Acknowledgments

The authors thank all nurses, physician assistants, and perfusionists at the Lifespan Hospitals for collecting tissue samples and patient data.

Sources of Funding

This research project was supported, in part, by the National Heart, Lung, and Blood Institute HL-46716 and HL-69024 (Dr Sellke). Dr Chu was supported by National Institutes of Health training grants (T32-HL094300) and the Irving Bard Memorial Fellowship.

Disclosures

None.

References


Altered Apoptosis-Related Signaling After Cardioplegic Arrest in Patients With Uncontrolled Type 2 Diabetes Mellitus
Jun Feng, Yuhong Liu, Nikola Dobrilovic, Louis M. Chu, Cesario Bianchi, Arun K. Singh and Frank W. Sellke

_Circulation_. 2013;128:S144-S151
doi: 10.1161/CIRCULATIONAHA.112.000332

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/128/11_suppl_1/S144

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org//subscriptions/