Diabetic hearts have lower basal urocortin levels that fail to increase after cardioplegic arrest: Association with increased apoptosis and postsurgical cardiac dysfunction

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Objectives: The present study investigated the cardioprotective role of urocortin (Ucn) and its relationship with protein kinase C (PKC) ϵ and PKC δ in patients with (DMPs) and without (NDMPs) diabetes mellitus after on-pump cardiac surgery (OPCS). The molecular mechanisms responsible for the reported worse outcomes of DMP after OPCS remain unknown.

Methods: Two sequential biopsy specimens were obtained from the right atrium of 27 DMPs and 22 NDMPs before and after cardiopulmonary bypass.

Results: Postcardioplegic induction of Ucn in NDMPs ($P < .01$) was not observed in the DMPs, whose precardioplegic Ucn levels were 50% lower than those in the NDMPs ($P < .05$). In the NDMPs, cardioplegic arrest increased PKC ϵ mRNA and protein ($P < .05$); overexpression of PKC δ was not seen. In contrast, DMPs showed increased PKC δ expression ($P < .01$), with no change in PKC ϵ . Apoptosis was more than twofold greater in the postcardioplegic samples from the DMPs than in those from the NDMPs. The apoptotic myocytes were Ucn negative and exhibited nuclear relocation of PKC δ . Enhanced PKC ϵ /mitochondrial co-localization was observed in viable, Ucn-positive, myocytes. The leakage of troponin I documented in the DMPs was greater than that in the NDMPs, although the difference was not statistically significant ($P = .06$). Furthermore, despite a similar incidence of perioperative acute myocardial infarction, the DMPs did not show postoperative improvement of systolic or diastolic function, although that was seen in the NDMPs ($P < .05$).

Conclusions: Cardioplegic arrest failed to induce in DMPs myocyte overexpression of Ucn or PKC ϵ but was associated with induction and mitochondrial relocation of PKC δ , resulting in apoptosis. Failure to overexpress Ucn in the DMPs was associated with apoptosis and cardiac dysfunction and, thus, might contribute to worse postoperative outcomes. (J Thorac Cardiovasc Surg 2014;148:2296-308)

See related commentary on page 2309.

Diabetes mellitus (DM) is a well-established risk factor for the morbidity and mortality associated with on-pump cardiac surgery (OPCS).¹ Although patients with DM

(DMPs) account for 28% of all those undergoing coronary artery bypass grafting (CABG),¹ such patients constitute a larger percentage of the postoperative morbidity and mortality population, with a reported 50% to 90% increase in mortality among DMPs.¹⁻⁵ Cardiac pathologic entities such as myocardial infarction, followed by stroke, were the most common cause of late mortality after CABG in DMPs.² Compared with non-DMPs (NDMPs), the postoperative morbidity has also been greater among DMPs,^{1,6-8} with greater rates of reoperation infection,¹⁻⁷ a greater incidence of peri- and postoperative neurologic complications,⁶⁻⁸ and longer hospitalization periods.^{1,7} It has also been reported that cardiopulmonary bypass (CPB) induces greater oxidative stress in DMPs than in NDMPs, with quantitatively and qualitatively different CPB-induced and cardioplegia-induced gene expression that is potentially responsible for the different inflammatory reactions in these 2 patient groups.⁹ Additionally, both early^{1,6-8} and late^{1,3-8,10,11} mortality were greater in DMPs.

Apoptotic cell death has been implicated in the pathogenesis of the iatrogenic ischemia-reperfusion (I/R) injury

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Abbreviations and Acronyms

ATP	= adenosine triphosphate
CABG	= coronary artery bypass grafting
CPB	= cardiopulmonary bypass
DM	= diabetes mellitus
DMPs	= patients with DM
I/R	= ischemia–reperfusion
LV	= left ventricular
NDMPs	= patients without DM
OPCS	= on-pump cardiac surgery
PKC	= protein kinase C
TUNEL	= terminal deoxynucleotidyl transferase mediated nick end labeling
Ucn	= urocortin
WMSI	= wall motion score index

associated with OPCS. DNA fragmentation has been detected using terminal deoxynucleotidyl transferase mediated nick end labeling (TUNEL) staining in myocardial biopsy specimens from patients undergoing OPCS, with apoptotic rates of 0.03% and 3% to 6% in ventricular and atrial samples, respectively.^{12,13} DM was also associated with an increased occurrence of apoptotic cell death, although the underlying molecular mechanisms are poorly understood.¹⁴

Urocortin (Ucn) is a 40-amino acid member of the corticotrophin-releasing factor family¹⁵ endogenously expressed in the human heart.¹⁶ The coexpression of Ucn and its cognate receptor in cardiac myocytes suggests that endogenous Ucn might exert autocrine and/or paracrine effects on the normal and diseased human heart. Biologically active Ucn, for instance, is released from isolated myocytes exposed to simulated ischemia and confers cardioprotective properties on the culture medium that are abrogated by corticotropin-releasing factor receptor antagonists.¹⁷ In addition, plasma Ucn is increased in the circulation of patients experiencing acute myocardial infarction.¹⁸

We have previously reported that warm blood cardioplegia in NDMPs is associated with increased endogenous myocyte expression of Ucn and that Ucn-positive myocytes are viable but that Ucn-negative cells show evidence of apoptosis.¹³ Furthermore, enhanced mitochondrial translocation of protein kinase C (PKC) ϵ (an established mechanism of Ucn-mediated cardioprotection¹⁹), has been previously observed in viable Ucn-positive myocytes after cardioplegic arrest.²⁰ In contrast to the cardioprotective properties of PKC ϵ , however, PKC δ expression has been implicated in apoptosis in a variety of cellular systems, including the heart.²¹ Also, hyperglycemia has been shown to increase both PKC δ activation and apoptosis in rat ventricular myocytes in vitro.²²

Accordingly, in the present study, we have compared the induction of Ucn and the activation and translocation of PKC ϵ and PKC δ in DMPs and NDMPs undergoing OPCS.

METHODS**Patient Population**

The present study strictly conformed to the principles outlined in the Declaration of Helsinki. The institutional review board of the University of Verona approved the study, and all patients selected gave informed consent before enrollment. A total of 49 patients (27 DMPs and 22 NDMPs), who were admitted for elective on-pump CABG, were recruited (Table 1). All the patients had multiple-vessel coronary disease with class II–III symptoms, according to the Canadian Cardiovascular Society Angina Classification. To avoid potential preoperative bias affecting the circulating Ucn level and the corresponding Ucn-induced intracellular pathways, only patients with stable angina, a left ventricular ejection (LV) fraction > 40%, and no significant organ comorbidities, who had been scheduled for isolated primary CABG, were enrolled in the present study (Table 2).

Surgical Procedure

The anesthesia regimen was standardized and consisted of intravenous propofol (2 mg/kg) and fentanyl (0.010 mg/kg) administration, followed by propofol infusion (150–200 μ g/kg/min) and isoflurane (0.8% inspired concentration). Neuromuscular blockade was achieved using 4 mg/h vecuronium bromide. After median sternotomy and heparin (3 mg/kg) administration, the aorta, right atrium, and inferior vena cava were cannulated. The left internal mammary artery was harvested as a pedicle and was always anastomosed to the left anterior descending artery. The internal saphenous vein was always harvested from the best side, which was detected using preoperative clinical evaluation and Doppler scanning. The management of CPB was standardized and achieved, as previously described.²³ Likewise, cardiac arrest and myocardial protection were standardized and achieved, as previously described.²³ In brief, after aortic crossclamping, cardiac arrest was induced by cold blood cardioplegia at 4°C, injected antegrade for about 3 minutes into the aortic root and then retrograde for an additional 2 minutes into the coronary sinus. A second maintenance solution (21 mEq K⁺/800 mL) at 4°C was administered retrograde every 20 minutes. After completion of the distal anastomosis and before aortic crossclamp removal, a third reperfusion dose of warm (37°C) blood cardioplegia was administered. The aortic crossclamp was then removed and construction of the proximal anastomoses begun. Inotropic drugs were not used until specimen retrieval had been completed.

Tissue Sampling and Preparation

Two sequential biopsy specimens were obtained from a virgin site in the right atrium by 2 highly experienced cardiac surgeons (G.F., A.M.). The first biopsy was taken as a control sample before starting CPB but before cardioplegia, using a small purse string suture of polypropylene 4-0, stitched on the middle lateral wall of the right atrium, far from the right appendage and quite below the superior vena cava atrial junction. The second was obtained as near as possible to the site of the previous sample, about 10 minutes after release of the aortic crossclamp, using a new purse string suture of polypropylene 4-0, also stitched on the middle lateral right atrial wall.

No clinical complications related to the sampling procedure occurred. Immediately after collection, each atrial biopsy specimen was cut into 2 parts, as previously described,²⁰ and used for immunofluorescent staining, RNA isolation, and protein isolation, respectively.

RNA Isolation and Quantitative Polymerase Chain Reaction

Total RNA was isolated from cardiac samples using an RNA isolator solution, TRIZol (Gibco-BRL, Sussex, United Kingdom), treated with

TABLE 1. Operative details, clinical characteristics, and outcome data

Variable	DMPs (n = 27)	NDMPs (n = 22)	P value
Age (y)	63.6 ± 5.0	61.5 ± 3.0	.74
Gender			.540
Male	14	12	
Female	13	10	
HbA1c (%)			.001
Mean	8.4	5.9	
Range	8.4-8.8	5.8-6.0	
Glycemia at admission (mg/dL)			.001
Median	163.6	105	
Range	163.0-170.0	98.0-105.0	
Diabetes duration			—
>3 y	6	NA	
>10 y	21	NA	
Medical management of DM			
Oral agents	5	NA	
Oral agents plus insulin	14	NA	
Insulin	8	NA	
Preoperative E	78.2 ± 8.7	74.1 ± 18.7	.3
Preoperative A	81.0 ± 15.2	79.1 ± 17.2	.683
Preoperative E/A	0.9 ± 0.2	0.9 ± 0.2	.399
Preoperative Ea	8.6 ± 1.7	8.9 ± 1.9	.504
Preoperative E/Ea	9.5 ± 2.3	8.8 ± 3.5	.424
Preoperative WMSI	1.36 ± 0.36	1.35 ± 0.43	.914
Preoperative LVEF	52.8 ± 4.2	51.2 ± 2.1	.106
CABG (n)	3.6 ± 0.6	3.8 ± 0.4	.97
Aortic crossclamp time (min)			
Median	48.2	49.2	.755
Range	42.0-56.0	49.0-51.0	
Preoperative troponin I (μg/L)	0.06 ± 0.10	0.07 ± 0.09	.681
Perioperative AMI	1/27 (3.7)	0/22 (0)	.551
Perioperative pneumonia	1/27 (3.7)	1/22 (4.5)	.702
Prolonged intubation	2/27 (7.4)	1/22 (4.5)	.578
Postoperative AKI	1/27 (3.7)	0/22 (0)	.551
Stroke	0/27 (0)	0/22 (0)	NA

Data presented as mean ± standard deviation or n (%), unless otherwise noted. *DMPs*, Patients with diabetes mellitus; *NDMPs*, patients without DM; *HbA1c*, hemoglobin A1c; *DM*, diabetes mellitus; *WMSI*, wall motion score index; *LVEF*, left ventricular ejection fraction; *CABG*, coronary artery bypass grafting; *AMI*, acute myocardial infarction; *AKI*, acute kidney injury; *NA*, not applicable.

DNase (Promega, Madison, Wis), and subsequently reversed transcribed with Superscript II reverse transcriptase (Gibco BRL, Life Technologies, Carlsbad, Calif) and oligodT (18-mer) primer.²⁰ Real-time, 1-step polymerase chain reaction for specific mRNA was performed using the QuantiTect SYBR Green polymerase chain reaction kit (Qiagen, Hilden, Germany) and LightCycler 1.5 instrument (Roche Diagnostics, Indianapolis, Ind), according to the manufacturer's instructions. The following specific primers were used to quantitate, by polymerase chain reaction, the transcript levels of the genes of interest. The Ucn primer sequences were 5'-GCTTGCTGGTGAAAGGACC-3' (sense) and 5'-CTTGCCACCGAGTCGAAT-3' (antisense). The PKCε primer sequences were 5'-ACCAAGCAGAAGACCAACAG-3' (sense) and 5'-TTCCTATGACACCCAGATG-3' (antisense). The PKCδ sequences were 5'-GGAAGAAGCAATGGTCCAAG-3' (sense) and antisense 5'-GTATTATGTGGGAGAAAATG-3' (antisense).

TABLE 2. Echocardiographic and biochemical results stratified by group

Variable	Preoperative	Postoperative	P value
DMPs			
E	78.2 ± 8.7	78.7 ± 11.5	.881
A	81.0 ± 15.2	71.1 ± 12.6	.008
E/A	0.9 ± 0.2	1.1 ± 0.2	.017
Ea	8.6 ± 1.7	9.0 ± 1.3	.73
E/Ea	9.5 ± 2.3	8.8 ± 1.8	.209
WMSI	1.36 ± 0.36	1.38 ± 0.42	.756
LVEF	52.8 ± 4.2	55.4 ± 7.5	.136
NDMPs			
E	74.1 ± 18.7	91.9 ± 9.1	.002
A	79.1 ± 17.2	68.3 ± 12.6	.008
E/A	0.9 ± 0.2	1.4 ± 0.3	.001
Ea	8.9 ± 1.9	10.5 ± 1.7	.002
E/Ea	8.8 ± 3.5	8.8 ± 1.6	.972
WMSI	1.35 ± 0.43	1.17 ± 0.21	.49
LVEF	51.2 ± 2.1	58.6 ± 7.2	.0001

Troponin I levels preoperatively from peripheral blood, intraoperatively at reperfusion 10 minutes after aortic declamping and at 6, 12, and 48 hours postoperatively: for DMPs, 0.06 ± 0.10 , 1.52 ± 0.83 , 5.38 ± 3.94 , 11.09 ± 13.59 , and 14.57 ± 20.76 μg/L ($P^a = .0001$); for NDMPs, 0.07 ± 0.09 , 1.05 ± 0.72 , 3.09 ± 1.28 , 4.04 ± 4.82 , and 5.13 ± 13.53 μg/L ($P^a = .001$), respectively. $P^b = .037$; $P^c = .050$. *DMPs*, Patients with diabetes mellitus; *WMSI*, wall motion score index; *LVEF*, left ventricular ejection fraction; *NDMPs*, patients without diabetes mellitus; P^a , time P value; P^b , group P value; P^c , group × time P value.

Immunoblotting Material and Approach

The types and sources of the antibody used for the immunoblotting procedure were as follows. The antibody against actin (matrix protein p84 [heat shock protein 60]) and total PKCε and PKCδ were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif). The anti-phospho-PKCε (Ser729) and anti-phospho-PKCδ (Ser665) rabbit polyclonal antibody were purchased from Upstate Biotechnology (Millipore Corp, Lake Placid, NY). All secondary antibodies were conjugated to horseradish peroxidase, and the ensuing immunoreactive bands were developed using a Western lightning chemiluminescence kit (PerkinElmer Life Science, Waltham, Mass).

Western Blotting

The pre- and postcardioplegic cardiac specimens were homogenized in lysis buffer (100 mmol/L NaCl, 0.1% Triton X-100, 50 mmol/L Tris; pH 8.3), loaded on 8% sodium dodecyl sulfate-polyacrylamide gel, separated by gel electrophoresis, transferred onto a nitrocellulose membrane, and probed with relevant primary antibody. The assessment of Ucn protein (molecular weight, 4693.52 Daltons) was performed as previously described using a Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis system.^{20,24}

Mitochondrial, Nuclear, and Cytoplasmic Protein Fractionation, Characterization, and Assessment

The mitochondrial, nuclear, and cytosolic fractions were prepared using commercially available kits (mitochondria isolation kit for tissue, Pierce Protein Research Products, Thermo Fisher Scientific, Rockford, Ill; and nuclear extraction kit, Millipore, Billerica, Mass), according to the manufacturer's instructions. Equal amounts (20 μg) of nuclear, mitochondrial, and cytoplasmic proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as previously described,²⁰ and were subsequently probed with antibody against total and phosphorylated PKCε and PKCδ. Anti-cytochrome oxidase subunit IV, anti-nuclear matrix protein p84, and anti-actin antibody were used to probe for mitochondrial, nuclear, and cytoplasmic fractions, respectively.²⁵

FIGURE 1. A, Quantitative polymerase chain reaction of urocortin messenger RNA (mRNA) levels in cardiac samples collected before and after cardioplegic (CP) arrest from patients without diabetes mellitus (NDMPs) and patients with diabetes mellitus (DMPs). **B,** Expression of the urocortin protein, as determined by Western blotting of extracts from pre- and post-CP atrial tissues. A total of 54 samples from DMPs (27 taken before cardioplegia and 27 after release of aortic crossclamping) and 44 samples from NDMPs (22 obtained before cardioplegia and 22 after release of aortic crossclamping) were processed. To obtain adequate amounts of protein and mRNA, the pre- and post-CP samples collected from 3 patients were combined and processed together, with the exception of 1 pre- and post-CP batch from NDMPs composed of 4 samples. The blot shown is representative of 3 different blots. Marker proteins with the indicated molecular masses were also used.

Immunocytochemistry Assessment

Adjacent 5-μm myocardial sections were stained with TUNEL reagents (using a rhodamine fluorochrome) and/or antibody against Ucn, phosphorylated PKCε, and phosphorylated PKCδ. Anti-cytochrome oxidase subunit IV antibody was used to specifically label mitochondria. When indicated, the myocardial sections were counterstained with propidium iodide or TO-PRO-3 (Life Technologies), as previously described.^{13,20,25,26} After washing and incubation with suitable secondary antibodies (DakoCytomation, Glostrup, Denmark), the sections were analyzed by a confocal microscopist (T.M.S.), who was unaware of the origin and sequence of the specimens. The data

are expressed as the mean ± standard deviation of 12 to 15 high power fields.

Clinical Data

High-sensitivity troponin I was assayed preoperatively from peripheral blood, intraoperatively at reperfusion, 10 minutes after aortic de-clamping, and, from the peripheral blood, at 6, 12, and 48 hours postoperatively.

The LV ejection fraction was calculated using routine transthoracic echocardiography according to the Simpson method. The wall motion score index (WMSI) was calculated using the 16-segment model for

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FIGURE 2. A, Quantitative polymerase chain reaction of protein kinase C (*PKC*) ϵ urocortin messenger RNA (mRNA) levels from pre- and postcardioplegic (CP) myocardial samples from patients without (NDMPs) and with (DMPs) diabetes mellitus. ** $P < .01$ and * $P < .05$. B, Expression of total and phosphorylated PKC ϵ protein, as determined by Western blotting of extracts from nondiabetic and diabetic human hearts before and after CP arrest. A total of 54 samples from DMPs (27 taken before cardioplegia and 27 after release of aortic crossclamping) and 44 samples from NDMPs (22 obtained before cardioplegia and 22 after release of aortic crossclamping) were processed. To obtain adequate amounts of proteins and mRNA, pre- and post-CP samples collected from 3 different patients were combined and processed together, with the exception of 1 pre- and post-CP batch from NDMPs composed of 4 samples. The data shown are representative of 3 different experiments.

LV segmentation, as recommended by the American Society for Echocardiography,²⁷ before admission to the operating room and at 96 hours postoperatively. The LV myocardial velocities were also recorded at the mitral annulus using pulsed-wave tissue Doppler imaging to investigate perioperative diastolic function, at the same measurement points

(before admission to the operating room and 96 hours postoperatively) used for WMSI.²⁸

The other data collected included age, gender, hemoglobin A1c, and serum glycemia at admission; number of CABG procedures; aortic cross-clamp time; percentage of patients with perioperative acute myocardial

infarction; prolonged (>48 hours) ventilation; and incidence of pneumonia, acute renal insufficiency, and stroke.

Statistical Analysis

For the molecular data, significance was evaluated using the 2-tailed *t* test. For the baseline data and clinical characteristics, the continuous variables were first tested for normality using the Shapiro-Wilk test and then compared using the Student *t* test or Mann Whitney *U* test, accordingly. The categorical variables were compared using the Fisher exact test and ordinal variables using the Mann Whitney *U* test.

Repeated measures analysis of variance with Bonferroni's correction for multiple measurements was used to compare serial data related to perioperative troponin I leakage. The reported *P* values include “*P*^a” (time-*P*), assessing change over time of the measured variable; “*P*^b” (group-*P*), assessing the level of difference between groups; and “*P*^c” (group × time *P*) assessing the group–time interaction. The paired samples *t* test was used to investigate the perioperative changes of the echocardiographic data. Statistical analysis was performed using the Statistical Package for Social Sciences program for Windows, version 15.0 (SPSS, Inc, Chicago, Ill).

RESULTS

In line with our previous findings, the hearts from NDMPs exposed to cardioplegic arrest and subsequent reperfusion exhibited a significant induction of Ucn at both the mRNA (268% of basic levels; *P* < .01) and the protein (threefold increase; *P* < .0026) level. In contrast, the hearts from the DMPs showed a significantly lower basal expression of Ucn at RNA (50% lower) and protein (85% lower) levels (*P* < .05 vs NDMP basal levels) that was not significantly increased by OPCS (*P* = NS; Figure 1). In the hearts from DMPs, cardioplegic arrest was associated with increased transcription of PKCε mRNA (230% of internal control; *P* < .05) and a threefold increased expression of total PKCε protein (*P* < .05), with a 4.2-fold increase in phosphorylated PKCε (*P* < .01). In contrast, in the hearts of the DMPs, the precardioplegic level of PKCε mRNA, which was approximately 30% lower than that of the hearts of the NDMPs, did not increase after cardioplegic arrest. Similarly, total PKCε protein was not augmented after cardioplegic arrest in the DMPs nor was any increase in PKCε phosphorylation detected (Figure 2).

In the hearts of the NDMPs, PKCδ was detectable at both the mRNA and the protein level; however, OPCS did not result in any increase in the levels of the transcript, total protein, or its phosphorylation. In contrast, as depicted in Figure 3, the DPMs' hearts showed a baseline expression of PKCδ that was significantly greater at the mRNA and protein levels than that in the NDMPs' control hearts and was further increased after cardioplegia (mRNA, 269% of basic level; protein, 2.4-fold increase; *P* < .01), along with enhanced PKCδ phosphorylation (Figure 3).

Apoptotic cell death, assessed by TUNEL staining, was more than twofold greater in the postcardioplegic samples from the DMPs (6.5% ± 1.8%) than that in the NDMPs (2.9% ± 0.7%; Figure 4, A). Nuclear translocation of PKCδ (2.4-fold increase) was only seen in the

postcardioplegic samples from DMPs (Figure 4, B, and Table 3) but not in the samples from the NDMPs (data not shown).

Cardiac cells showing cytosolic staining for Ucn never exhibited nuclear relocation of PKCδ or TUNEL-positive staining (Figure 5, A). Furthermore, co-localization of nuclear PKCδ and TUNEL positivity was consistently seen in Ucn-negative myocytes (Figure 5, B, and Table 4).

In contrast, mitochondrial relocation of PKCε was only detected in postcardioplegic samples from the NDMPs. In line with our previous findings,¹⁸ PKCε/mitochondria co-localization was observed in viable myocytes showing positive staining for Ucn (Figure 5, A-D, and Table 4).

Clinical Data

The clinical baseline and operative data proved comparable between the 2 groups, with the exception of the baseline serum glycemic value and percentage of hemoglobin 1c, which were greater in the DMPs (Table 1). No differences were recorded in the baseline serum troponin I or in any of the preoperative echocardiographic systolic and diastolic indexes (Table 1). A similar clinical outcome was also evident in terms of the incidence of perioperative acute myocardial infarction, pneumonia, prolonged ventilation, and acute renal insufficiency (Table 1). No stroke events were seen during the study period.

However, when perioperative leakage of troponin I was considered, both groups demonstrated a time-dependent increase in postoperative troponin I, although the DMPs had, overall, significantly greater leakage than did the NDMPs (Table 2).

Furthermore, despite a similar incidence of perioperative acute myocardial infarction, the DMPs did not show any improvement in their systolic function (using both the WMSI and LV ejection fraction), which was significantly improved in the NDMPs (Table 2). Similarly, the echocardiographic indexes of diastolic function in the DMPs remained comparable (with the exception of the E/A ratio) between the preoperative and postoperative periods, but almost all had improved significantly in the NDMPs after surgery (Table 2).

DISCUSSION

The mechanisms by which DM heightens the morbidity and mortality associated with OPCS are poorly understood. However, blood glucose levels seem to play a role, because hyperglycemia was found to influence long-term survival after CABG^{2,29} and was an independent risk factor for mortality in patients undergoing cardiac surgery.³⁰ Likewise, stricter perioperative glucose control was shown to reduce mortality in DMPs undergoing CABG.³¹⁻³³

FIGURE 3. A, Quantitative polymerase chain reaction of protein kinase C (PKC) δ urocortin messenger RNA (mRNA) levels from pre- and post-cardioplegic (CP) myocardial samples of patients without (NDMPs) and with (DMPs) diabetes mellitus. $**P < .01$ and $*P < .05$. B, Western blotting of extracts from nondiabetic and diabetic human hearts before and after cardioplegic arrest showing expression of total and phosphorylated PKC δ protein. A total of 54 samples from DMPs (27 taken before cardioplegia and 27 after release of aortic crossclamping) and 44 samples from NDMPs (22 obtained

Moreover, both preoperative^{2,34} and postoperative³⁵ hyperglycemia have been reported as important predictors of late mortality after CABG. A recent report by Voisine and colleagues⁹ clearly showed qualitative and quantitative differences in gene expression profiles from cardiac myocytes of DMPs compared with NDMPs, in particular, those related to the inflammatory response and oxidative stress after CPB with cardioplegic arrest. However, the molecular mechanisms activated in DM after cardioplegic arrest that contribute to the worse perioperative outcomes for this high-risk cohort of patients remain poorly understood. The molecular mechanisms reported in the present study could contribute to a better understanding and improved management of the response of the DM heart to cardioplegic arrest.

In addition to inducing oxidative stress and negative inotropism in the heart,²² hyperglycemia has been found to induce mitochondrial dysfunction, cytochrome-c release, and apoptosis.³⁶ Hyperglycemia is a powerful activating signal for cardiac PKC isozymes, which in turn have been associated with modulation of apoptosis.²² In primary cultures of serum-starved adult rat ventricular myocytes, hyperglycemia induced apoptotic cell death, which was attenuated by administration of peptide inhibitors of PKC beta-1/beta-2, and zeta, although not PKCε (epsilonV1-2).²² In contrast, the PKCε translocation activator (psi epsilon RACK) abolished hyperglycemia-induced apoptosis, strongly suggesting a cardioprotective role for PKCε in hyperglycemia-induced apoptosis.³⁶

Activation of PKCδ, another PKC isoform, has been reported in ventricular myocytes under hyperglycemic conditions.²² In adult rat ventricular myocytes, treatment with hyperglycemia (16.5 mM) for 24 hours resulted in a greater than threefold increase in apoptosis compared with baseline and in membrane translocation and activation of PKCδ.²² Hyperglycemia-induced apoptosis was attenuated by the PKCδ-specific translocation inhibitor peptide (deltaV1-1).^{22,32} In an isolated perfused rat heart model of I/R injury, the inhibition of PKCδ prevented reperfusion injury, and activation of PKCε mimicked ischemic preconditioning, confirming the opposite effects exerted in vitro and ex vivo by PKCδ and PKCε in the context of I/R injury.³⁶

In our study, we have shown for the first time that, in the hearts of DMPs, the baseline cardiomyocyte expression of Ucn, a well-known cardioprotective agent, is roughly one half that in the hearts of NDMPs. Also, in contrast to the NDMP hearts, it was not increased after cardioplegia. The

reduced Ucn in the hearts of the DMPs was associated, not only with a reduced baseline expression of PKCε, which failed to relocate after cardioplegic arrest, but also by overexpression of PKCδ, whose postcardioplegic translocation to the nuclei was seen in apoptotic myocytes. We previously reported that human hearts exposed to mild forms of surgical ischemia exhibited PKCε phosphorylation and mitochondrial translocation, which promoted cell survival, but only in myocytes expressing Ucn. Thus, a low baseline Ucn level, together with an inability to increase its expression after cardioplegic arrest, can contribute to making diabetic hearts more susceptible to apoptosis, which would, in turn, contribute to adverse postoperative outcomes.

Different volatile anesthetics, including isoflurane, have been shown to provide cardioprotection in patients undergoing CABG with CPB.³⁷ The mechanism of anesthetic-induced cardioprotection seems to be related to PKCε-induced preconditioning by delayed opening of the mitochondrial permeability transition pore.^{38,39} Because all the patients enrolled in our study received the same anesthesia regimen, it is possible that DMPs might benefit to a lesser extent from anesthetic-induced cardioprotection owing to the low preoperative levels and the lack of postoperative induction of PKCε.

We have previously shown that in the rat heart exposed to I/R injury, Ucn exerts a cytoprotective action that is independent of an energy-sparing mechanism secondary to a negative inotropic effect and is associated with both recovery of cardiac performance and reduced depletion of endogenous high-energy phosphates.⁴⁰ The degree of cardiac dysfunction after I/R injury reflects the level of myocyte injury and death. Cell death after injury, and the form that it takes, is largely dependent on the intracellular adenosine triphosphate (ATP) levels and other high energy phosphates.⁴¹ For example, viable cells will have an ADP/ATP ratio of <0.11, apoptotic cells a ratio of 0.11 to 1.0, and necrotic cells, a ratio of >1.5.⁴² The Ucn-induced recovery of ATP stores, with a reduction of the intracellular ADP/ATP ratio, might allow damaged myocytes that would otherwise die by necrosis to die by the alternative apoptotic pathway. Because necrosis, unlike apoptosis, is associated with the release of intracellular contents and a subsequent inflammatory reaction, a reduction in the proportion of necrotic death will result in a smaller final lesion, with functional benefit. The greater troponin release and the doubled percentage of apoptotic cell death documented in the DMPs would be consistent with this interpretation. Accordingly, supplementation of cardioplegic solutions with exogenous

before cardioplegia and 22 after release of aortic crossclamping) were processed. To obtain adequate amounts of proteins and mRNA, pre- and postcardioplegic samples collected from 3 different patients were combined and processed together, with exception of 1 pre- and postcardioplegic batch from NDMPs composed of 4 samples. The data shown are representative of 3 different experiments.

FIGURE 4. A, Occurrence of apoptotic cell death as assessed by terminal deoxynucleotidyl transferase mediated nick end labeling (*TUNEL*) staining at the end of cardioplegic (*CP*) arrest in myocardial sections from the hearts of patients without (*NDMPs*) and with (*DMPs*) diabetes mellitus. A total of 5 different sections for each collected sample were analyzed. Data are expressed as the mean $\pm$ standard deviation of 12 to 15 high-power fields. B, Nuclear and

TABLE 3. Percentages of cardiac cells with positive labeling for pPKCe, pPKCδ, urocortin, and TUNEL in DMPs and NDMPs before and after cardioplegic arrest

	pPKCe-positive cytosol		pPKCδ-positive cytosol		pPKCe/mitochondria co-localization		pPKCδ/nuclei co-localization		Urocortin-positive myocytes		TUNEL	
	DMPs	NDMPs	DMPs	NDMPs	DMP	NDMP	DMP	NDMP	DMP	NDMP	DMP	NDMP
Before CP	2.7 ± 0.3	7.1 ± 0.8	6.8 ± 1.1	1.6 ± 0.3	0.8 ± 0.3	2.4 ± 0.4	0.9 ± 0.2	<0.1	0.9 ± 0.3	2.7 ± 0.5	1.2 ± 0.4	<0.1
After CP	3.4 ± 0.4	26.7 ± 3.7	19.3 ± 2.8	2.2 ± 0.5	1.4 ± 0.5	22 ± 2.8	7.9 ± 1.8	3.3 ± 0.5	1.6 ± 0.5	23 ± 2.6	6.4 ± 1.3	2.9 ± 0.6
P value*	.039	.019	.023	.069	.042	.002	.009	.019	.062	.003	.006	.037

Data presented as mean percentage ± standard deviation of 12-15 high power fields. Five different sections for each collected sample were analyzed. *pPKC*, Phosphorylated protein kinase C; *TUNEL*, terminal deoxynucleotidyl transferase mediated nick end labeling; *DMPs*, patients with diabetes mellitus; *NDMPs*, patients without diabetes mellitus; *CP*, cardioplegia. *Postcardioplegic specimens versus corresponding precardioplegic samples.

Ucn warrants investigation to enhance the intrinsic cardio-protective mechanisms of the diabetic heart, thus reducing the risk of postoperative cardiac dysfunction in DMPs exposed to the inescapable I/R injury associated with OPCS.

Clinical Inferences

The main clinical finding of the present study was that despite a similar clinical outcome between the DMPs and NDMPs (documented by the negligible and comparable incidence of perioperative acute myocardial infarction, pneumonia, acute renal insufficiency, and the need for prolonged intubation), DMPs had greater perioperative troponin I leakage and suboptimal echocardiographic results. It is well known that perioperative troponin I leakage reflects the quality of myocardial protection and that its value has prognostic implications for mid- to long-term follow-up.^{43,44} Furthermore, variable degrees of myocardial damage with systolic dysfunction will occur even after successful myocardial revascularization because of iatrogenic myocardial I/R injury.^{9,43,45} Accordingly, suboptimal myocardial protection strategies, as shown by the molecular results reported in the present study and further documented by the perioperative troponin I leakage, might help to understand the worse prognosis of DMPs compared with NDMPs reported in published studies.^{1,5-8} The greater perioperative troponin leakage also helps to understand why the DMPs did not show any postoperative recovery in the WMSI and LV ejection fraction,^{43,44} the opposite of that seen in the NDMPs.

It has recently been demonstrated that DMPs have greater post-CABG diastolic dysfunction compared with NDMPs,⁴⁶ with a linear correlation between the duration of DM,⁴⁷ and that both diastolic^{46,48} and systolic⁴⁹ dysfunction after CABG significantly impair outcome. Moreover, contradictory results for LV diastolic function after CABG have been reported. Hedman and colleagues²⁸ showed early improvement of diastole after CABG. However, Diller and

colleagues⁵⁰ demonstrated early improvement followed by a late decline of LV relaxation that had reached the preoperative values within 18 months postoperatively. Other investigators have found CPB-related early impairment of LV relaxation.⁵¹ Our data could help to clarify these contradictory results, because myocardial relaxation during diastole is an energy-dependent process requiring an effective oxygen supply²⁸; therefore, less adequate techniques of myocardial protection or a less effective ability to resist ischemia and ischemia-reperfusion during the crossclamp time or at reperfusion, respectively, could induce myocardial contracture.^{52,53} Thus, it could be expected that myocardial tissues unable to adequately counteract ischemia and I/R injury, such as the myocardial cells of DMPs, would show impaired postoperative relaxation, resulting in LV diastolic dysfunction. Thus, the absence of any postoperative recovery from the preoperative diastolic dysfunction seen in our series of DMPs can be considered the net result between the beneficial effects of myocardial revascularization and the deleterious effects of preoperative ischemia and intraoperative I/R injury, together with a “less adequate” strategy of myocardial protection during the crossclamp time (ie, a less adequate capacity to counteract the unavoidable intraoperative I/R injury). Therefore, given the comparable preoperative status and the similar successful myocardial revascularization in both groups, the worse diastolic function of the DMPs can be ascribed to a worse ability of the myocardial cells to withstand I/R injury. More studies are mandatory to better understand the clinical effect of these molecular mechanisms. However, the results from the present study have confirmed the urgent need for improved myocardial protection strategies for DMPs referred for CABG, given the profound effect on outcome of high troponin leakage^{43,44} and any decline in postoperative systolic and/or diastolic function.⁴⁷⁻⁴⁹ The increasing knowledge of the molecular mechanisms

mitochondrial translocation of protein kinase C (*PKC*)δ in the human heart exposed to CP arrest as assessed by Western blotting in protein extracts from cardiac samples of NDMPs and DMPs collected before and after CP. Matrix protein p84, cytochrome oxidase subunit IV (*COX IV*), and actin were used as specific markers of the nuclear, mitochondrial, and cytosolic fractions, respectively.

FIGURE 5. Two adjacent 5- μ m myocardial sections serially cut from the same postcardioplegic wax block of a patient with diabetes. The sections were stained with (A) an antibody against urocortin (*white asterisk*) and rhodamine-based terminal deoxynucleotidyl transferase mediated nick end labeling (TUNEL) reagents (*white arrow*) or (B) an antibody against phosphorylated protein kinase C (PKC) δ (*yellow arrow*). Secondary antibodies conjugated with fluorescein were used to label the primary antibody against urocortin (A, bright green cytosol) and phosphorylated PKC δ (B, bright green nuclei). A, Urocortin-negative cells were TUNEL negative, but, (B) adjacent urocortin-negative cells with apoptotic nuclei stained red by TUNEL reagents also exhibited nuclear translocation of PKC δ . A total of 54 samples from diabetic patients (27 taken before cardioplegia and 27 after release of aortic crossclamping) and 44 samples from nondiabetic patients (22 obtained before cardioplegia and 22 after release of aortic crossclamping) were processed. Five different sections for each collected sample were analyzed. A and B, The images depicted were representative of 27 myocardial samples harvested from diabetic patients after cardioplegic arrest. Data are expressed as the mean $\pm$ standard deviation of 12 to 15 high-power fields. Myocardial sections from atrial tissues collected from a nondiabetic patient after cardioplegic arrest. C, The first section was stained with primary antibody against urocortin and a secondary antibody conjugated with fluorescein. Positive cells appear *bright green*. D, The second section was stained with 2 different primary antibodies (anti-cytochrome oxidase subunit IV [COX IV], a specific mitochondrial marker, and anti-phosphorylated PKC δ) and finally counterstained with the nuclear marker TOPRO-3 (*blue staining*). COX IV stained bright red by a secondary antibody conjugated with rhodamine. E, PKC δ stained *bright green* by a secondary antibody conjugated with fluorescein. F, An overlay of the 3 stains is shown. The percentage of co-localization of phosphorylated PKC δ and mitochondria was digitally quantified using image analyzer software. D, Visually, an overlap of “*green*” phosphorylated PKC δ and “*red*” mitochondria resulted in a *bright orange* fluorescent signal that could be observed in the merged images. Five different sections for each collected sample were analyzed. C-F, Representative images of 22 myocardial samples harvested from nondiabetic patients after cardioplegic arrest. Data are expressed as the mean $\pm$ standard deviation of 12 to 15 high-power fields.

underlying perioperative systolic and diastolic dysfunction in DMPs undergoing CABG, and the potential beneficial effects of Ucn, possibly incorporated into cardioplegic

solutions, on the intracellular pathways modulating post-CABG myocardial cell survival and apoptosis could positively affect the post-CABG outcome of DMPs.

TABLE 4. Association of PKCε mitochondrial relocation and PKCδ nuclear relocation with urocortin and TUNEL staining in myocardial samples after cardioplegic arrest

Myocytes	Post-CP pPKCε/mitochondria co-localization		Post-CP pPKCδ/nuclei co-localization	
	DMPs	NDMPs	DMPs	NDMPs
Overall co-localization	1.4 ± 0.5; P = .048	22 ± 2.8; P = .0001	7.9 ± 1.8; P = .016	3.3 ± 0.5; P = .042
Urocortin				
Positive	1.1 ± 0.3; P = .00033	18 ± 2.1; P = .00028	<0.1; P = <.00001	<0.1; P = <.00001
Negative	0.3 ± 0.2; P = .00018	4 ± 0.6; P = .00027	7.9 ± 1.8; P = <.00001	3.3 ± 0.5; P = <.00001
TUNEL				
Negative	1.4 ± 0.5; P = 1	22 ± 2.8; P = 1	1.5 ± 0.4; P = .048	0.4 ± 0.2; P = .0023
Positive	<0.1; P <.00001	<0.1; P <.00001	6.4 ± 1.3; P = .0023	2.9 ± 0.6; P = .0041

Data in percentages presented as mean ± standard deviation of 12-15 high-power fields. Five different sections for each collected sample were analyzed. PKC, Protein kinase C; TUNEL, terminal deoxynucleotidyl transferase mediated nick end labeling; CP, cardioplegia; DMPs, patients with diabetes mellitus; NDMPs, patients without diabetes mellitus.

Study Limitations

One potential limitation of our study was the use of atrial rather than ventricular specimens. However, the choice of atrial tissue was dictated by ethical constraints, because atrial, unlike ventricular, biopsy carries no significant morbidity and provides full-thickness samples that are readily reproducible. Although the atrial myocardium differs from the ventricular myocardium with respect to the relative percentage of myocyte, endothelial, connective, and neural elements, all these cell types are also present in ventricular tissue and demonstrate similar ultrastructural changes in human heart disease.⁹ It has also been shown that the gene profiles show similar patterns of expression in both ventricular and atrial tissue before and after cardioplegic arrest.⁹

Finally, the present study was not intended for clinical purposes; therefore, the related clinical results must be considered as descriptive only (our study was underpowered to detect differences in the major clinical outcome variables).

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