ELSEVIER

Biochemical Pharmacology 63 (2002) 543–552

Biochemical Pharmacology

Mitochondrial gene expression and ventricular fibrillation in ischemic/reperfused nondiabetic and diabetic myocardium

Levente Szendrei[a], Tibor Turoczi[b], Peter Kovacs[a], Miklos Vecsernyes[a], Dipak K. Das[b], Arpad Tosaki[a,b,*]

[a]*Department of Pharmacology, First Department of Internal Medicine, School of Medicine, University of Debrecen, Debrecen, Hungary*
[b]*University of Connecticut Health Center, School of Medicine, Farmington, CT, USA*

Received 26 May 2001; accepted 5 November 2001

Abstract

We investigated the mitochondrial gene expression related to cardiac function and ventricular fibrillation (VF) in ischemic/reperfused nondiabetic and diabetic myocardium. To identify potentially more specific gene responses we performed subtractive screening, Northern blotting, and reverse transcription-polymerase chain reaction (RT-PCR) of mitochondrial genes expressed after 30 min ischemia followed by 120 min reperfusion in isolated rat hearts that showed VF or did not show VF. Cytochrome oxidase B subunit III (*COXBIII*) and ATP synthase subunit 6, studied and selected out of 40 mitochondrial genes by subtractive screening, showed an expression after 30 min ischemia (no VF was recorded) in both nondiabetic and diabetic subjects. Upon reperfusion, the down-regulation of these genes was only observed in fibrillated hearts. Such a reduction in signal intensity was not seen in nonfibrillated myocardium. In additional studies, nondiabetic and diabetic hearts, without the ischemia/reperfusion protocol, were subjected to electrical fibrillation, and a significant reduction in *COXBIII* and *ATPS6* mRNA signal intensity was observed indicating that VF contributes to the down-regulation of these genes. Cardiac function (heart rate, coronary flow, aortic flow, left ventricular developed pressure) showed no correlation between the up- and down-regulation of these mitochondrial genes in both nondiabetic and diabetic ischemic/reperfused myocardium. Our data suggest that *COXBIII* and *ATPS6* may play a critical role in arrhythmogenesis, and the stimulation of *COXBIII* and *ATPS6* mRNA expression may prevent the development of VF in both nondiabetic and diabetic ischemic/reperfused myocardium.

Keywords: Mitochondrial gene expression; Ischemia; Reperfusion; Ventricular fibrillation; Cardiac function; Isolated heart

1. Introduction

Early reperfusion of patients with acute myocardial infarction improves left ventricular dysfunction and survival, therefore, every effort must be made to increase the recovery of cardiac function and minimize sudden cardiac death caused by VF. The restoration of flow to a previously ischemic tissue is an absolute prerequisite for the survival of the myocardium, the notion has developed that it is not without hazard. It is widely believed that under certain circumstances, the act of reperfusion could induce lethal myocardial injury. One unfavorable and potentially lethal aspect of reperfusion-induced injury is the occurrence of reperfusion-induced arrhythmias [1,2]. Reperfusion arrhythmias occur within seconds of the onset of reflow and have been observed in all species studied, including humans, in whom they regularly occur during cardiac surgery and thrombolytic therapy [3–7].

Diabetes mellitus is often associated with cardiovascular complications including coronary artery lesions and diabetic cardiomyopathy. Postmyocardial infarction prognosis also appears to be worse in diabetic patients, who exhibit a higher incidence of congestive heart failure and sudden cardiac death compared to nondiabetics [8]. Diabetic myocardium exhibits a variety of abnormalities in sarcolemmal ion transport, including depression of Na^+–H^+ and Na^+–Ca^{2+} exchange processes [9,10], and inhibition of Ca^{2+} and Na^+–K^+ ATPase [11]. Sarcoplasmic reticular function also appears to be defective in diabetic myocardium with depressed ATP-dependent Ca^{2+} transport and Ca^{2+}-stimulated ATPase activity [12] via a decreased ATP synthesis [13] and electron transfer chain [14,15]. Thus, the

* Corresponding author. Tel.: +36-52-427899; fax: +36-52-427899.
E-mail address: tosaki@king.pharmacol.dote.hu (A. Tosaki).

Abbreviations: *COXBIII*, cytochrome oxidase B subunit III; *ATPS6*, ATP synthase subunit 6; HR, heart rate; CF, coronary flow; AF, aortic flow; LVDP, left ventricular developed pressure; VF, ventricular fibrillation; HIF-1, hypoxia-inducible nuclear factor-1.

0006-2952/02/$ – see front matter
PII: S0006-2952(01)00913-3

expression of some mitochondrial genes (e.g. *COXBIII* and *ATPS6*) may relate to the function of various ion channels and ion exchange processes, and could play a role in arrhythmogenesis in ischemic/reperfused myocardium because arrhythmias are known to be related to disturbances in ion metabolism. In order to determine whether diabetes affects cardiac susceptibility to reperfusion injury we compared (i) the expression of mitochondrial genes (*COXBIII* and *ATPS6*, selected out of 40 mitochondrial genes by a preliminary screening) using molecular biology techniques, (ii) their relationship to VF, and (iii) myocardial function in ischemic/reperfused, diabetic and nondiabetic hearts. In additional investigations, (iv) in isolated hearts obtained from nondiabetic and 8-week diabetic rats were subjected to electrically-induced VF in order to study the VF-induced *COXBIII* and *ATPS6* expression. Although many mechanisms have been proposed to explain the causes of arrhythmias, no work has been done, to our knowledge, concerning the development of arrhythmias related to mitochondrial gene expression in ischemic/reperfused myocardium. Long QT syndrome and idiopathic VF, as known up to now, are the cardiac disorders based on genetic mutation and cause sudden cardiac death from arrhythmias [16–18]. Thus, our study may offer a further understanding of the arrhythmogenic mechanism(s) at a molecular level and identify some mitochondrial genes responsible for arrhythmogenesis in ischemic/reperfused hearts.

2. Materials and methods

2.1. Animals, induction of diabetes, and isolated heart preparation

Male Sprague–Dawley rats (320–350 g) were used for all studies. Animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research prepared by the National Academy of Sciences (Publication No. 86-23, revised 1985). Diabetes mellitus was induced by i.v. injection of streptozotocin (55 mg/kg). Nondiabetic age-matched controls were injected with an equivalent volume of vehicle. Diabetes was confirmed by the presence of hyperglycemia. Rats were anesthetized with i.p. pentobarbital (60 mg/kg) and then given intravenous heparin (500 IU/kg). After thoracotomy, the heart was excised, and the aorta and left atrium were cannulated. Hearts were initially perfused according to Langendorff then preparations were switched to the working mode as previously described [19].

2.2. Indices measured

Serum glucose was measured by a spectrophotometer using standard assay kits. ECG was registered by a recorder (Haemosys, Experimetria) throughout the experimental period by two silver electrodes attached directly to the heart. The incidence of reperfusion-induced VF, in our model, is very high (about 80–90%) in both nondiabetic and diabetic subjects.

Therefore, in order to get 6 nonfibrillated nondiabetic and 6 nonfibrillated diabetic hearts, 31 and 41 hearts were needed to be studied, respectively, and subjected to 30 min ischemia followed by 2 hr reperfusion. In hearts developed VF (25 out of 31 nondiabetics and 35 out of 41 diabetics) upon reperfusion were further divided into two subgroups (six hearts in each subgroup): (i) the duration of reperfusion-induced VF was less than 3 min, and (ii) longer than 10 min. If the duration of VF was less than 3 min (spontaneous defibrillation occurred), the VF was defined as reversible VF. If the duration of VF was sustained until the end of the first 10 min of reperfusion, the VF was considered to be irreversible. In this latter group, after 10 min of VF, hearts were electrically defibrillated and reperfused for an additional 110 min. Heart rate (HR), coronary flow (CF), aortic flow (AF), and left ventricular developed pressure (LVDP) were also recorded.

2.3. Northern blot

Hybridizations were performed as previously described [20]. RNA was prepared from the left ventricle (about 100 mg), and 5 μg total RNA was subjected to electrophoresis in formaldehyde-containing 1% agarose gels and transferred to nylon membranes by standard capillary transfer. Hybridization was carried out with ^{32}P-labeled probes in a hybridization oven using an aqueous exclusion rate-enhancing solution (QuickHyb, Stratagene). After autoradiography and automated radiometric scanning, stripping and re-probing for housekeeping gene mRNA levels (*GAPDH*), the membranes' RNA was routinely employed for standardization of quantitative measurements.

2.4. RT-PCR

Aliquots containing 50 ng total RNA or 20 ng mRNA were digested with amplification grade DNAse I (Gibco-BRL) to remove any DNA contamination and reverse transcribed into cDNA using 0.1 μg random 9-mer primers (Stratagene). Quantitation was done as described by Feldman *et al.* [21]. Aliquots corresponding to 0.1 μg initial total RNA was amplified for 12–26 cycles using specific primers (5′-CGAACCTGAGCCCTAATA and 5′-GTAGCTCCTCCGATTAGA, 312 bp for ATPase 6; 5′-CTTAGCATCAGGAGTCTC and 5′-TATCATGCTGCGGCTTCA, 338 bp for cytochrome oxidase III) in an optimized reaction using the AmpliTaq PCR kit. Fragments were radiolabeled by the use of 10 μCi [^{32}P]dCTP per reaction and five 1/10 volume aliquots were taken every 2 cycles. Unincorporated nucleotides were separated on 3% agarose gel electrophoresis and radioactivity incorporated in amplified fragments measured by liquid scintillation.

2.5. Imaging of mitochondrial genes

Radioactivity within bands of interest was estimated by phosphorimage analysis (Molecular Dynamics Inc.) corrected for background, and normalized to hybridization with *GAPDH* cDNA as described below. Storage phosphor screens were placed over the sample in a sample cassette and stored at room temperature for 48 hr. During incubation, radiation from the labeled RNA in the blot excites electrons in the phosphor to a metastable excited state. The screen was then placed in a Molecular Dynamics PhosphorImager, in which it was scanned with a laser beam inducing further excitation of the previously excited electrons to an unstable state, the decay of which is accompanied by the emission of visible light. The intensity of emitted light was measured and stored in a data file as a function of the position of the scanning laser, which corresponds to the location of exciting radiation on the blot. Each band in an image displayed on the screen was marked by a rectangle and the band or spot intensity within the marked area was then integrated.

2.6. cDNA library construction and analysis of mitochondrial genes

Ten microgram poly(A)$^+$ RNA from control and ischemic hearts (pooled samples) were used for the construction of a cDNA library in a pUC-derived plasmid vector [22,23]. All necessary reagents, including the complete *E. coli* cells, were from Gibco-BRL (Superscript^{TM} System for Plasmid Cloning). First strand synthesis of cDNA was carried out using a special oligo(dT)-blunt end *Not*I primer-adapter instead of the oligo(dT) primer. After RNA-primed synthesis of second strand cDNA, *Sal*I adapters were added followed by digestion with *Not*I to create the compatible terminus at the poly(dT) end of the ds-cDNA, and 5 ng were used for oriented ligation into a *Not*I/*Sal*I-precut pSPORT 1 plasmid and 1/4 of the ligation reaction used to transform 100 μL competent MAX efficiency DH5α *E. coli* cells. After determination of transformation efficiency, cells were plated at 1×10^3 to 2×10^3 cfu/85 mm plate for 10–12 hr. After 4 hr at 37° shaking with 4 mL per plate TB medium overlay followed by pooling of the medium, overnight liquid culture originated from 5.2×10^5 cfu was kept frozen for repeated library platings for screening.

2.7. Subtractive screening of mitochondrial genes

Subtracted cDNA probes were prepared by modification of the technique described by Rhyner *et al.* [24]. Briefly, 5 μg of poly(A)$^+$ RNA extracted from control, ischemic and reperfused diabetic, and nondiabetic myocardium was subjected to reverse transcription for 1 hr at 42° using 1000 U of a RNAse H M-MLV reverse transcriptase (Superscript^{TM} II, Gibco-BRL), 1 mg random 9-mer primers (Stratagene), dNTP mixture (0.5 mol/L final concentration with 1 μCi[α-^{32}P]dCTP tracer), 100 U placental RNAse inhibitor (Promega) and appropriate buffer in a 125 μL reaction. The cDNA was subjected to two rounds of hybridization at 68° in 30 μL of 2 mol/L phosphate buffer, pH 6.8 with a 10-fold excess (w/w) of poly(A)$^+$ RNA from control tissue, and the non-hybridized cDNA separated after each round of hybridization by hydroxyapatite chromatography on thermo-jacketed (60°) columns using 0.05 mol/L phosphate buffer. The cDNA enriched in specifically expressed sequences was desalted, purified, and subjected to random-primed second strand synthesis for radiolabeling to high specific activity ($<0.5 \times 10^9$ cpm/μg). Replicas from cDNA library platings at 1×10^3 to 2×10^3 cfu per plate (85 mm plates) were made by colony lifts on nylon filters (CL/P, Bio-Rad) and screened by hybridization with this probe.

2.8. DNA sequencing and gene identification

The Sanger *et al.* dideoxy-mediated chain-extension/chain-termination method was used [25] with denatured plasmid DNA as substrate (pUC 19-derived pSPORT 1 vector; Gibco-BRL). The M13/pUC 'universal' and T7/T3α sequencing primers and the Sequenase ver 2.0 kit (USB) were routinely used in ^{35}S-labeled reactions and bands separated on 47 cm 6% polyacrylamide/urea gels with double loading (2.5 and 5 hr running at 2000 V). Pearson–Lipman algorithms [26] were used for similarity searches to DNA sequences in the GenBank and EMBL databases.

2.9. Experimental time course

Hearts were obtained from two populations of rats: (i) age-matched nondiabetics which were the same age as diabetic rats and were injected with citrate buffer 8 weeks prior to the study; (ii) diabetic rats which were injected with streptozotocin (55 mg/kg, i.v.) 8 weeks prior to the isolation of the hearts and induction of 30 min normothermic global ischemia followed by 2 hr reperfusion. Myocardial function (HR, CF, AF, and LVDP) was measured before ischemia and after 2 hr reperfusion. In additional studies, in order to simulate the period of 10 min of irreversible VF, without the ischemic/reperfused protocol, isolated hearts were electrically fibrillated (20 Hz, 1200 beats/min) using a 5 V square-wave pulses of 1 ms duration for 10 min in Langendorff mode. Then hearts were defibrillated (if it was necessary), switched to working mode, and perfused for additional 110 min. mRNA expression was determined at the end of each experiment.

2.10. Statistics

HR, CF, AF, and LVDP were expressed as the mean ± SEM. Two-way analysis of variance was first carried out

to test any difference between the mean values of all groups. If differences were established, the values of the diabetic groups were compared with those of the nondiabetic age-matched controls using the Bonferroni correction. A change of $P < 0.05$ was considered to be significant.

3. Results

3.1. Subtractive screening

We generated a specific cDNA library in plasmid pSPORT 1, using as starting material left ventricular tissue from rat hearts subjected to 30 min ischemia. To underrepresent genes having allogenic variability, pooled poly(A)$^+$ RNA is used for cloning. The size of the obtained library was estimated to be 340,000 primary recombinants, and the median insert size is between 1.5 and 2.0 kb. Single stranded tester cDNA, reverse transcribed from aliquots of the same pooled ischemic heart mRNA, was repeatedly subtracted with driver mRNA from nonischemic hearts to produce a subtracted probe. Out of 40 putative positive clones that we selected, rapid screening by hybridization of control and ischemic RNA with probes made from the corresponding cloned inserts revealed 28 clones of apparently overexpressed genes. When we processed DNA sequencing and database searching, these clones of ischemia-expressed genes were found to contain copies of eight different genes (Table 1). Partial sequencing from the 3′- and 5′-ends (200–300 bp) followed by similarity searches against sequences stored in the GenBank and identified six of these genes (Table 1). They are genes that coded for cytochrome oxidase subunits I–III, for *ATPS6* and *8*, and for cytochrome *b*.

3.2. mRNA expression of the identified genes

Fig. 1 shows the results of Northern hybridization performed by the probe for *COXBIII*. The up-regulation of *COXBIII* was observed as early as 30 min following the onset of ischemia ($^{\#}P < 0.05$ vs. controls) (Fig. 1, lane 2) and after 120 min of reperfusion (Fig. 1, lane 3) in nondiabetic and nonfibrillated hearts ($^{\#}P < 0.05$ vs. controls). In the irreversible fibrillated (Fig. 1, lane 4) and reversible fibrillated (Fig. 1, lane 5) nondiabetic myocardium, upon reperfusion, a significant reduction in the expression of *COXBIII* mRNA was observed ($^{*}P < 0.05$), respectively. In diabetic ischemic (Fig. 1, lane 7) and reperfused (Fig. 1, lane 8) hearts, the up-regulation of *COXBIII* was significantly increased ($^{+}P < 0.05$), respectively, in comparison with the diabetic nonischemic (Fig. 1, lane 6) myocardium. In diabetic fibrillated hearts (data not shown), the signal of *COXBIII* was not detected, therefore, any quantitative or statistical analysis was not possible to be done in these groups.

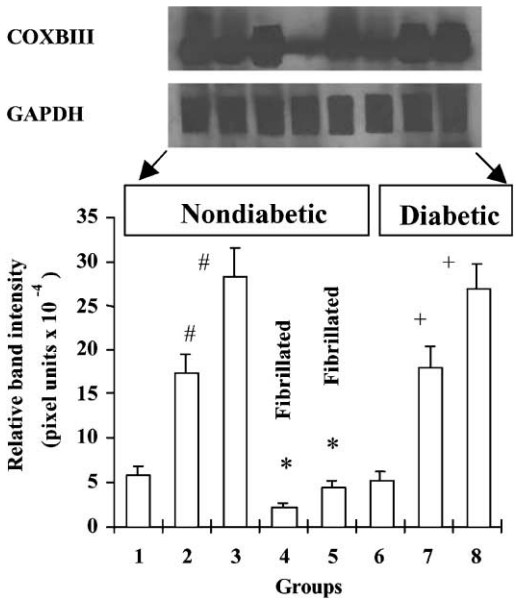

Fig. 1. The expression of *COXBIII*. In nonischemic hearts, the signal of *COXBIII* (blots, upper part) was detected (lane 1, nondiabetic). Hearts were subjected to 30 min ischemia (lane 2, nondiabetic), and 30 min ischemia followed by 2 hr reperfusion (lane 3, nondiabetic), and no VF was registered. In hearts developed irreversible (lane 4, nondiabetic) or reversible VF (lane 5, nondiabetic), a marked down-regulation in *COXBIII* mRNA was detected. In diabetics, the same expression was observed: lane 6, nonischemic diabetic; lane 7, ischemic diabetic; lane 8, ischemic/reperfused nonfibrillated diabetic. Signal intensity of *COXBIII* was not detected in fibrillated diabetics, therefore, these lanes are not depicted. Lower part shows the quantitative values (ratio between *COXBIII* and *GAPDH*) of six hearts in each group. $^{\#}P < 0.05$ compared to the nonischemic nondiabetic hearts (group 1). $^{*}P < 0.05$ compared to the ischemic/reperfused nonfibrillated group (group 3). $^{+}P < 0.05$ compared to the nonischemic diabetic myocardium (group 6).

Fig. 2 shows the results of Northern hybridization experiments performed using probes for *ATPS6* (probes prepared from the corresponding clones, and the probe is the 2.3 kb *Pst*I/*Xba*I fragment of the rat clone). The up-regulation of *ATPS6* (this was not so pronounced as in *COXBIII*) was observed, although the data were not statistically significant, after 30 min ischemia (Fig. 2, lane 2) and after 2 hr reperfusion (Fig. 2, lane 3) in the nondiabetic/nonfibrillated myocardium. In irreversibly fibrillated (Fig. 2, lane 4) and reversibly fibrillated (Fig. 2, lane 5) hearts, upon reperfusion, a marked down-regulation in *ATPS6* was observed. In diabetic ischemic (Fig. 2, lane

Table 1
Screening results

Gene	Clones	Message length (kb)	Location
ATP synthase, subunit 6	3	0.7	mtDNA, heavy strand
ATP synthase, subunit 8	2	0.5	mtDNA, heavy strand
Cytochrome *b*	3	1.2	mtDNA, heavy strand
Unknown	1	1.1	Nuclear
Unknown	1	1.9	Nuclear

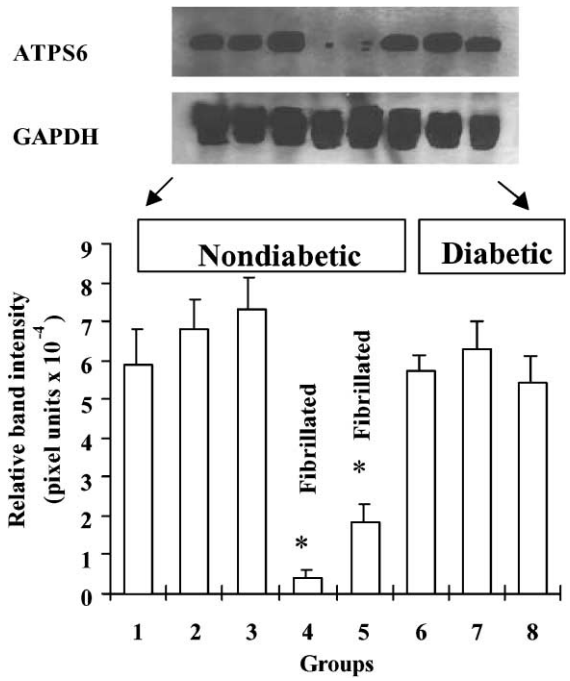

Fig. 2. The expression of *ATPS6*. In nonischemic myocardium (blots, upper part), the signal of *ATPS6* can be detected (lane 1, nondiabetic). Hearts subjected to 30 min ischemia, the expression of *ATPS6* was detected (lane 2, nondiabetic). After 2 hr reperfusion, a further increase in *ATPS6* signal intensity was observed in nonfibrillated myocardium (lane 3, nondiabetic). In hearts showed irreversible VF (lane 4, nondiabetic) or reversible VF (lane 5, nondiabetic), a marked reduction in *ATPS6* was observed. In diabetics, the trend of *ATPS6* expression was similar to the nondiabetic subjects: lane 6, nonischemic diabetic; lane 7, ischemic diabetic; lane 8, ischemic/reperfused nonfibrillated diabetic. Signal intensity of *ATPS6* was undetectable in fibrillated diabetics, therefore, these lanes are not depicted. The lower part shows quantitative values (ratio between *ATPS6* and *GAPDH*) of six hearts in each group. $^{*}P < 0.05$ compared to the ischemic/reperfused nonfibrillated group (group 3).

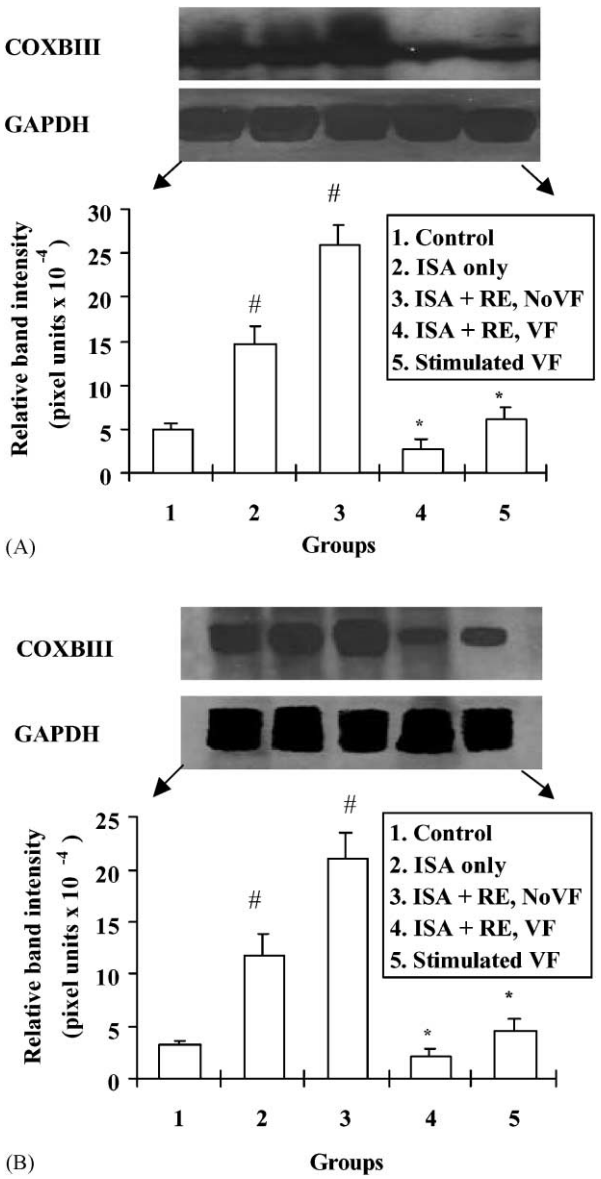

Fig. 3. Effects of reperfusion- and electrically-induced VF on *COXBIII* mRNA expression in nondiabetic (A) and diabetic (B) myocardium. Upper parts of A and B show representative pictures. Group 1: nonischemic controls. Group 2: hearts were subjected to 30 min ischemia (ISA). Group 3: hearts were subjected to 30 min ISA followed by 2 hr reperfusion (RE), and VF was not developed (NoVF). Group 4: hearts were subjected to 30 min ISA followed by 2 hr RE, and irreversible VF was developed. Group 5: hearts were electrically fibrillated for 10 min (simulation of irreversible VF) followed by 110 min perfusion. Mean ± SEM. $^{\#}P < 0.05$ compared to the nonischemic hearts (group 1). $^{*}P < 0.05$ compared to the ischemic/reperfused nonfibrillated group (group 3).

7) and reperfused (Fig. 2, lane 8) myocardium, the regulation of *ATPS6* was almost the same (nonsignificant change) as it was observed in nondiabetic/nonfibrillated hearts. In diabetic fibrillated hearts, the signals of *ATPS6* were completely undetectable (data not shown), thus, any statistical analysis was not possible to be done.

Fig. 3 shows the expression of *COXBIII* in nondiabetic (A) and diabetic (B) myocardium. Thus, after 30 min ischemia, the expression of *COXBIII* mRNA was observed (Fig. 3A, group 2) ($^{\#}P < 0.05$) compared to the nonischemic control group (Fig. 3A, group 1). This expression was further increased ($^{\#}P < 0.05$) after 30 min ischemia followed by 2 hr reperfusion in the nonfibrillated myocardium (Fig. 3A, group 3). However, in the ischemic/reperfused fibrillated group (Fig. 3A, group 4) a significant reduction in the expression of *COXBIII* mRNA was detected. The same significant reduction in *COXBIII* expression was also observed in the electrically fibrillated myocardium (Fig. 3A, group 5). The trend in the changes of *COXBIII* mRNA expression was also the same in diabetic subjects (Fig. 3B).

Fig. 4 depicts the results of *ATPS6* mRNA expression in nondiabetic (A) and diabetic (B) hearts. Thus, ischemia/reperfusion-induced and electrically-induced VF (groups 4 and 5) resulted in a significant reduction in signal intensity in both nondiabetic and diabetic hearts.

Table 2 shows RT-PCR analysis for *ATPS6* and *COXBIII* in nonfibrillated and fibrillated nondiabetic and diabetic ischemic and reperfused myocardium. Results were

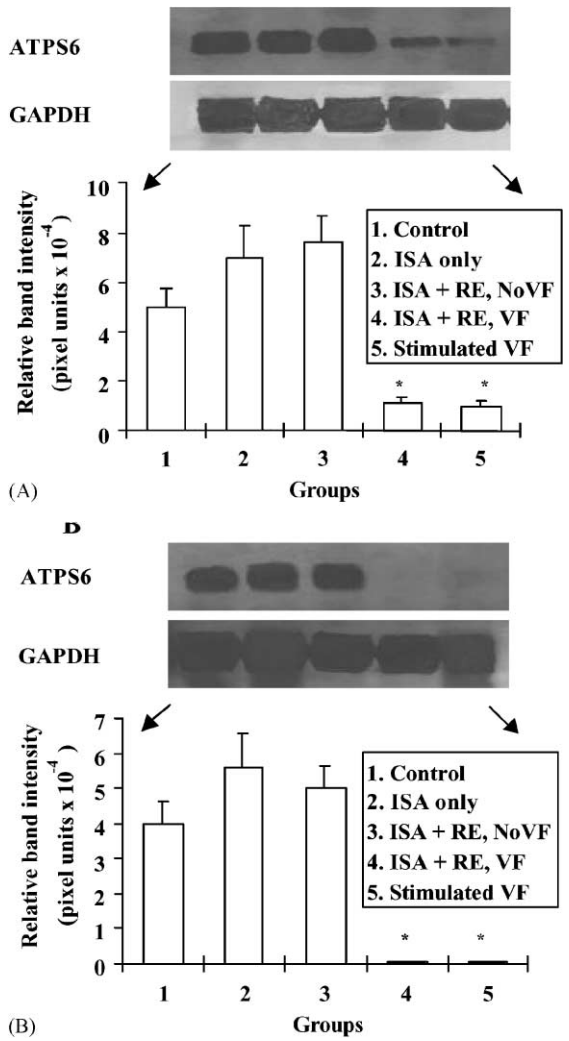

Fig. 4. Effects of reperfusion- and electrically-induced VF on *ATPS6* mRNA expression in nondiabetic (A) and diabetic (B) myocardium. Upper parts of A and B show representative pictures. Group 1: nonischemic controls. Group 2: hearts were subjected to 30 min ischemia (ISA). Group 3: hearts were subjected to 30 min ISA followed by 2 hr reperfusion (RE), and VF was not developed (NoVF). Group 4: hearts were subjected to 30 min ISA followed by 2 hr RE, and irreversible VF was developed. Group 5: hearts were electrically fibrillated for 10 min (stimulation of irreversible VF) followed by 110 min perfusion. Mean ± SEM. $^{*}P < 0.05$ compared to the ischemic/reperfused nonfibrillated group.

normalized with regard to *GAPDH* quantification in the same sample and expressed as the ratio to basal expression. Thus, the results show (Table 2) that the expression of *ATPS6* and *COXBIII* was observed after ischemia in both nondiabetic and diabetic hearts. Upon reperfusion, the same expression was observed in nonfibrillated nondiabetic, and diabetic myocardium (Table 2). In fibrillated hearts (Table 2), upon reperfusion, the down-regulation of *ATPS6* (8 ± 3 mRNA relative amount in nondiabetics) and *COXBIII* (6 ± 1 mRNA relative amount in nondiabetics) was observed in nondiabetic myocardium, but these mitochondrial genes were not detected by RT-PCR in diabetic fibrillated subjects (Table 2).

Cardiac function (AF and LVDP) was reduced (Table 4) in diabetic myocardium (serum glucose: 508 ± 31 mg/dL) in comparison with the age-matched (Table 3) nondiabetic (serum glucose: 128 ± 31 mg/dL) hearts. Reperfusion resulted in a relatively weak postischemic cardiac function in nondiabetic nonfibrillated myocardium (Table 3). In ischemic/reperfused nondiabetic hearts, where ischemia/reperfusion-induced damage superimposed by reperfusion-induced VF, a further significant reduction in postischemic cardiac function was observed (Table 3). These results are consistent with the known ability of VF to enhance reperfusion-induced injury, and in the presence of a superimposed acute ischemic event by VF, VF further increased the postischemic cardiac functional damage. In electrically fibrillated nondiabetic myocardium (Table 3), the postfibrillated cardiac function was significantly improved in comparison with the ischemic/reperfused and fibrillated group. In diabetic ischemic/reperfused, fibrillated and nonfibrillated hearts (Table 4), the trend in the recovery of postischemic cardiac function was the same as it was observed in the nondiabetic group, however, cardiac function and contractility were significantly reduced in diabetic groups compared to the age-matched nondiabetic values (Table 3). The postfibrillated recovery in diabetics (Table 4) resulted in a significantly improved cardiac function compared to those hearts subjected to ischemia/reperfusion in the presence of VF.

Table 2
Quantitative RT-PCR for *COXBIII* and *ATPS6* (mRNA relative amount, ratio to the control *GAPDH*) in nonfibrillated and fibrillated diabetic and nondiabetic hearts

	Before ISA		After ISA		After RE	
	Nondiabetic	Diabetic	Nondiabetic	Diabetic	Nondiabetic	Diabetic
ATPase subunit 6						
In nonfibrillated hearts	38 ± 8	24 ± 7	$56 \pm 6^{*}$	$49 \pm 9^{\#}$	$67 \pm 8^{*}$	$48 \pm 7^{\#}$
In fibrillated hearts	ND	ND	ND	ND	$8 \pm 3^{\blacklozenge}$	UD
Cytochrome oxidase III						
In nonfibrillated hearts	25 ± 5	13 ± 3	$48 \pm 6^{*}$	$33 \pm 6^{\#}$	$58 \pm 8^{*}$	$42 \pm 5^{\#}$
In fibrillated hearts	ND	ND	ND	ND	$6 \pm 1^{\blacklozenge}$	UD

In each group, $n = 6$, mean ± SEM. Comparisons (∗) and (#) were made to the nondiabetic and diabetic control groups before ischemia (ISA), respectively. Comparisons (◆) were made to the nondiabetic nonfibrillated group. The symbols, ∗, #, ◆, $P < 0.05$. Reperfusion, RE; not detected, ND; under detectable level, UD.

Table 3
Cardiac function in nonfibrillated ischemic/reperfused, fibrillated ischemic reperfused, and electrically fibrillated nondiabetic myocardium

Groups	Preischemic values (preischemic)				After 60 min RE				After 120 min RE			
	HR	CF	AF	LVDP	HR	CF	AF	LVDP	HR	CF	AF	LVDP
Time-matched control perfusion	311 ± 8	26.3 ± 1.0	50.9 ± 2.0	17.8 ± 0.3	299 ± 9	24.9 ± 1.8	49.2 ± 1.4	16.9 ± 0.5	300 ± 7	25.6 ± 1.3	47.9 ± 2.2	16.0 ± 0.5
ISA/RE nonfibrillated	307 ± 7	27.1 ± 1.2	52.0 ± 1.8	17.4 ± 0.4	296 ± 8	20.0 ± 1.3	22.0 ± 1.2	14.2 ± 0.3	294 ± 9	20.4 ± 1.0	21.6 ± 1.4	14.2 ± 0.4
ISA/RE fibrillated	319 ± 8	28.0 ± 0.7	51.0 ± 1.4	17.9 ± 0.5	296 ± 6	16.7 ± 1.1*	11.2 ± 0.6*	11.0 ± 0.4*	298 ± 7	17.1 ± 0.8*	10.6 ± 0.6*	11.0 ± 0.6*
Electrically fibrillated	310 ± 8	26.5 ± 1.0	52.6 ± 2.4	17.6 ± 0.4	293 ± 9	21.0 ± 1.3#	23.0 ± 1.5#	15.0 ± 0.5#	288 ± 8	20.2 ± 1.0#	22.0 ± 1.4#	15.1 ± 0.5#

In each group, $n = 6$, mean ± SEM, $^{*}P < 0.05$ compared to the ISA/RE nonfibrillated group, $^{\#}P < 0.05$ compared to the ISA/RE fibrillated group. HR, heart rate (beats/min); CF, coronary flow (mL/min); AF, aortic flow (mL/min); LVDP, left ventricular developed pressure; ISA, ischemia; RE, reperfusion.

Table 4
Cardiac function in nonfibrillated ischemic/reperfused, fibrillated ischemic reperfused, and electrically fibrillated diabetic myocardium

Groups	Preischemic values (preischemic)				After 60 min RE				After 120 min RE			
	HR	CF	AF	LVDP	HR	CF	AF	LVDP	HR	CF	AF	LVDP
Time-matched control perfusion	306 ± 8	26.1 ± 1.2	39.0 ± 1.6	16.1 ± 0.5	291 ± 9	25.3 ± 1.0	36.8 ± 1.7	15.4 ± 0.6	295 ± 8	24.2 ± 2.0	34.2 ± 2.1	14.7 ± 0.5
ISA/RE nonfibrillated	300 ± 7	25.7 ± 1.0	38.2 ± 1.1	15.7 ± 0.6	286 ± 7	19.8 ± 1.2	19.7 ± 1.0	13.0 ± 0.4	284 ± 9	18.7 ± 1.8	17.4 ± 1.7	12.1 ± 0.4
ISA/RE fibrillated	309 ± 9	26.8 ± 0.9	37.2 ± 1.8	15.8 ± 0.7	288 ± 8	16.9 ± 0.8	6.5 ± 0.8*	8.0 ± 0.6*	285 ± 9	16.0 ± 0.9	5.1 ± 0.6*	7.0 ± 0.5*
Electrically fibrillated	299 ± 8	25.2 ± 0.7	36.8 ± 2.0	15.5 ± 0.6	279 ± 9	19.7 ± 0.7#	13.8 ± 1.0#	12.4 ± 0.5#	276 ± 8	18.6 ± 0.8#	13.7 ± 0.8#	11.9 ± 0.4#

In each group, $n = 6$, mean ± SEM, $^{*}P < 0.05$ compared to the ISA/RE nonfibrillated group, $^{\#}P < 0.05$ compared to the ISA/RE fibrillated group. HR, heart rate (beats/min); CF, coronary flow (mL/min); AF, aortic flow (mL/min); LVDP, left ventricular developed pressure; ISA, ischemia; RE, reperfusion.

4. Discussion

Ischemic episodes elicit different changes in the expression of several genes, arrhythmogenesis, and cardiac function in the myocardium [27,28]. The electrophysiology of arrhythmogenesis [29] including the mechanism(s) of VF, e.g. re-entry, is very well defined [30–32], and only a few data, to our knowledge, are available to explain the mechanism of VF at genetic level [17]. The long QT syndrome and idiopathic VF are cardiac disorders based on genetic mutation causing lethal arrhythmias and sudden cardiac death [16,18].

Efforts were also made to study the mechanism of idiopathic VF indicating that mutations in cardiac ion-channel genes may contribute to the risk of fibrillation and sudden cardiac death [33,34]. This idea led us to speculate that mitochondrial genes [35,36], because mitochondria are the locus of oxidative phosphorylation, may primarily play an important role in the regulation of arrhythmogenesis in ischemic/reperfused myocardium.

In the present study, we have examined the dependence of reperfusion-induced VF on mitochondrial mRNA regulation in perfused nondiabetic and diabetic rat hearts. In addition, we have analyzed the relationship between VF and mitochondrial gene expression focusing on *COXBIII* and *ATPS6*, without the ischemic/reperfused protocol, in electrically fibrillated myocardium. Our data show an increase, studied and preselected by subtractive screening from 40 mitochondrial genes, in the expression of the two mitochondrial-encoded genes, *COXBIII* and *ATPS6*, after 30 min ischemia, and a down-regulation of these two genes after 2 hr reperfusion in fibrillated hearts. In nonfibrillated myocardium, upon reperfusion, the down-regulation of the mitochondrial genes studied was not observed. It is relatively surprising that we found a significant up- and down-regulation so quickly and while the heart is still under ischemic arrest, but this may be natural for mitochondrial-encoded genes since the mitochondrial genetic apparatus is similar to that of procaryotes. As in most other mammals, the rat mitochondrial genome contains only 13 protein coding areas (genes for Cox I–III, ATPase 6, 8, cytochrome B, and NADH 1–4, 4L, 5, 6) [36]. In our studies, the development of reperfusion-induced VF was critically dependent upon the down-regulation of *COXBIII* and *ATPS6*. This finding was directly supported by data obtained in electrically fibrillated myocardium indicating that VF elicits the down-regulation of *COXBIII* and *ATPS6*. The observation in electrically fibrillated myocardium shows that VF is probably responsible for the reduction of signal intensity of these genes, but a fibrillation-induced moderate ischemia cannot be completely ruled out. Although electrical fibrillation-induced coronary vasoconstriction is unlikely to account for the initiation of mitochondrial gene regulation, there seems little doubt that, after 10 min electrical fibrillation, this factor can modify the postfibrillated recovery of hearts or support their maintenance compared to the recovery of ischemic/reperfused fibrillated myocardium. Thus, in comparison to hearts subjected to ischemia/reperfusion protocol, coronary flow failed to recover to its preischemic control value, while in electrically fibrillated hearts, coronary flow recovered to 80–95% of the prefibrillated value. In isolated rat hearts, the relationship and importance between coronary flow and ventricular fibrillation were extensively studied and discussed by Curtis and Hearse [37].

As speculated above, the mechanism underlying the role of mitochondrial mRNA expression in arrhythmogenesis may involve the encoded proteins which may be responsible for the regulation of ion control mechanisms. Our present study might indicate that the heart appears to be able to recover in terms of cardiac function, and *COXBIII* and *ATPS6* down-regulation could be originated from VF. While these results may appear to be in conflict, it may well be that the injury induced by electrical fibrillation is heterogeneous and that irreversible injury is induced in an initially small population of myocardial cells. Thus, on termination of electrical fibrillation, the undamaged or reversible damaged cardiac cells become functionally close to the normal ones but continue to deteriorate in terms of mitochondrial mRNA expression. Such a proposition gains some support from our observations of mitochondrial mRNA expression in fibrillated, nonfibrillated, and electrically fibrillated hearts indicating that ischemia/reperfusion-induced injury in cardiac function is superimposed by VF-induced functional damage in the myocardium.

A puzzling aspect of the mitochondrial gene up- and down-regulation is the issue of coordinated response of nuclear genes for the assembly of multimeric proteins. For instance, if this response was produced by new functional ATP synthase molecules, the expression of nine different genes would be necessary, seven of which are nuclear-encoded genes (subunits α, β, γ, δ, ε, 7, and 9). Although little is known about mitochondrial transcription regulation at present, it is assumed that initiation is under control of a nuclear-derived transcription factor (TF-1) [38], and the mitochondrial genome would thus, complement nuclear gene activity when it is necessary [39]. Our data assume, however, that mitochondrial transcription may precede nuclear transcription of related genes suggesting that some sort of signaling mechanisms may reside at mitochondrial level.

The stimulus that induces enhanced transcription of mitochondrial genes in the ischemic/reperfused heart remains unclear. Many signaling pathways that eventually modulate up- and down-regulation of genes in the myocardium are known to be activated or down-regulated by ischemia and reperfusion, but many studies have so far documented their interaction with promoter elements involved in regulating nuclear genes. A transcription factor

that appears to interact with the promoter region in the D-loop of mitochondrial DNA has recently been described, but it is unclear if it acts only as a permissive factor or is actively modulating transcription in response to some intracellular messengers [35,38].

Although not specifically studied in the present investigation, it is of interest to note the findings of Wang and Semenza [40], and Semenza [41] with hypoxia-inducible nuclear factor (HIF-1). Their studies demonstrated that HIF-1 is present and functions in cell lines and tissues including HeLa cervical carcinoma cells, LTK^- fibroblast, C_2C_{12} skeletal myoblasts, cardiac and vascular development, suggesting that this transcription factor plays a more general role in the hypoxic/ischemic response of mammalian tissues and may regulate and control the expression of other hypoxia/ischemia-induced genes. Indeed HIF-1 binding sites were identified in other functional classes of hypoxia-inducible genes such as inducible nitric oxide synthase [42]. Given the potential significance in changes of mitochondrial mRNA expression in ischemic/reperfused myocardium, the identification of mitochondrial genes as a target for HIF-1 regulation may lend further support to an additional hypothesis that changes in mitochondrial mRNA expression could contribute to the coordination of postischemic dysfunction via HIF-1.

In summary, it has been published that diabetic hearts respond on different ways to the susceptibility of the myocardium in comparison with the nondiabetic myocardium [43–46]. However, in our studies, significant changes, regarding the arrhythmogenic mechanism in connection with *COXBIII* and *ATPS6* mRNA expression, were not observed between ischemic/reperfused diabetic and nondiabetic myocardium, indicating that these two mitochondrial genes may play a crucial role in arrhythmogenesis under diabetic as well as nondiabetic conditions. Thus, *COXBIII* and *ATPS6* were up-regulated after 30 min ischemia in both nondiabetic and diabetic hearts. Upon reperfusion, the down-regulation of these mRNAs was only observed in fibrillated myocardium. In nonfibrillated and reperfused hearts, the down-regulation of *COXBIII* and *ATPS6* was not observed either in nondiabetic or diabetic myocardium. In electrically fibrillated hearts, the down-regulation of *COXBIII* and *ATPS6* was observed indicating that VF elicits the down-regulation of these mitochondrial genes both under diabetic and nondiabetic conditions. Thus, our findings suggest that pharmacological stimulation of the expression of *COXBIII* and *ATPS6* during reperfusion could prevent the development of VF, and these genes could regulate the mechanism(s) of arrhythmogenesis in both ischemic/reperfused nondiabetic and diabetic myocardium.

Additional studies are needed to resolve the links in the apparent cascade of the up- and down-regulation of ischemia/reperfusion-induced mitochondrial gene expression and related protein synthesis in fibrillated and nonfibrillated hearts.

Acknowledgments

This study was supported in part by the grants from DAAD-MOB (33), Germany, NATO (LST.CLG.977254) the USA and Hungary, ETT (62/2001) and OTKA (T-32008), Hungary.

References

[1] Hagar JM, Hale SL, Kloner RA. Effect of preconditioning ischemia on reperfusion arrhythmias after coronary artery occlusion and reperfusion in the rat. Circ Res 1991;68:61–8.
[2] Hearse DJ. Reperfusion-induced injury: a possible role for oxidant stress and its manipulation. Cardiovasc Drugs Ther 1991;5:225–36.
[3] Berger PB, Ruocco NA, Ryan TJ, Frederick MM, Podrid PJ, and the TIMI investigators. Incidence and significance of ventricular tachycardia and fibrillation in the absence of hypotension or heart failure in acute myocardial infarction treated with recombinant tissue-type plasminogen activator: results from the thrombolysis in myocardial infarction (TIMI) phase II trial. J Am Coll Cardiol 1993;22:1773–9.
[4] Bissel JP, Castaigne A, Mercier C, Lion L, Leizorovicz A, and EMIP group. Ventricular fibrillation following administration of thrombolytic treatment. Eur Heart J 1996;17:213–21.
[5] Lincoff AM, Topol E. Illusion of reperfusion: does anyone achieve optimal reperfusion during acute myocardial infarction? Circulation 1993;87:1792–17105.
[6] Mark DB, Sigmon K, Topol EJ, Kereiakes DJ, Pryor DB, Candela RJ, Califf RM. Identification of acute myocardial infarction patients suitable for early hospital discharge after aggressive interventional therapy: results from the thrombolysis and angioplasty in acute myocardial infarction registry. Circulation 1991;83:1186–93.
[7] McComb JM, Gold HK, Leinbach RC, Newell JB, Ruskin JN, Garan H. Electrically induced ventricular arrhythmias in acute myocardial infarction treated with thrombolytic agents. Am J Cardiol 1988;62:186–91.
[8] Behar S, Boyko V, Reicher-Reiss H, Goldbourt U. Ten-year survival after acute myocardial infarction: comparison of patients with and without diabetes. Am Heart J 1997;133:290–6.
[9] Pierce GN, Ramjiawan B, Dhalla NS, Ferrari R. Na^+–H^+ exchange in cardiac sarcolemmal vesicles isolated from diabetic rats. Am J Physiol Heart Circ Physiol 1990;258:H255–61.
[10] Khandoudi N, Bernard M, Cozzone P, Feuvray D. Intracellular pH and role of Na^+/H^+ exchange during ischemia and reperfusion of normal and diabetic rat hearts. Cardiovasc Res 1990;24:873–8.
[11] Heyliger CE, Prakash A, McNeill JH. Alterations in cardiac sarcolemmal Ca^{2+} pump activity during diabetes mellitus. Am J Physiol Heart Circ Physiol 1987;252:H540–4.
[12] Ganguly PK, Pierce GN, Dhalla KS, Dhalla NS. Defective sarcoplasmic reticular calcium transport in diabetic cardiomyopathy. Am J Physiol Endocrin Metabol 1983;244:E528–35.
[13] Cave AC, Ingwall JS, Friedrich J, Liao R, Saupe KW, Apstein CS, Eberli FR. ATP synthesis during low-flow ischemia: influence of increased glycolytic substrate. Circulation 2000;101:2090–6.
[14] Esumi K, Nishida M, Shaw D, Smith TW, Marsh JD. NADH measurements in adult rat myocytes during stimulated ischemia. Am J Physiol 1991;260:H1743–52.
[15] Neubauer S, Newell JB, Ingwall JS. Metabolic consequences and predictability of ventricular fibrillation in hypoxia: a ^{31}P- and ^{23}Na-nuclear magnetic resonance study of the isolated rat hearts. Circulation 1992;86:302–10.
[16] Keating MT, Sanguinetti MC. Molecular genetic insights into cardiovascular disease. Science 1996;272:681–5.

[17] Keating MT, Sanguinetti MC. Molecular and cellular mechanisms of cardiac arrhythmias. Cell 2001;104:569–80.
[18] Rosen MR, Long QT. Syndrome patients with gene mutations. Circulation 1995;92:3373–5.
[19] Tosaki A, Braquet P. DMPO and reperfusion injury: arrhythmia, heart function, electron spin resonance, and nuclear magnetic resonance studies in isolated working guinea pig hearts. Am Heart J 1990;120:819–30.
[20] Chomczynski P, Sacchi N. Single step method of RNA isolation by acid guanidinium thiocyanate–phenol–cloroform extraction. Anal Biochem 1987;162:156–9.
[21] Feldman AM, Weinberg EO, Ray PE, Lorell BH. Selective changes in cardiac gene expression during compensated hypertrophy and the transition to cardiac decompensation in rats with chronic aortic banding. Circ Res 1993;73:184–92.
[22] Gubler U, Hoffman BJ. A simple and very efficient method for generating cDNA libraries. Nucl Acids Res 1983;15:6304–8.
[23] Okayama H, Berg P. High-efficiency cloning of full-length cDNA. Mol Cell Biol 1982;2:161–8.
[24] Rhyner TA, Biguet NF, Berrard S, Borberly AA, Mallet J. An efficient approach for the selective isolation of specific transcripts from brain mRNA populations. J Neurosci Res 1986;16:167–81.
[25] Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 1977;74:5463–8.
[26] Pearson WR, Lipman DJ. Improved tools for biological sequence analysis. Proc Natl Acad Sci USA 1982;85:2444–8.
[27] Brand T, Sharma HS, Fleischmann KE, Duncker DJ, McFalls EO, Verdouw PD, Schaper W. Proto-oncogene expression in porcine myocardium subjected to ischemia and reperfusion. Circ Res 1992;71:1351–60.
[28] Yasojima K, Kilgore KS, Washington RA, Lucchesi BR, McGeer PL. Complement gene expression by rabbit heart: upregulation by ischemia and reperfusion. Circ Res 1998;82:1224–30.
[29] Barrett TD, MacLeod BA, Walker MJ. A model of myocardial ischemia for the simultaneous assessment of electrophysiological changes and arrhythmias in intact rabbits. J Pharmacol Toxicol Methods 1997;37:27–36.
[30] Gray RA, Pertsov AM, Jalife J. Spatial and temporal organization during cardiac fibrillation. Nature 1998;392:75–8.
[31] Janse MJ, Wilms-Schopman FJ, Coronel R. Ventricular fibrillation is not always due to multiple wavelet reentry. J Cardiovasc Electrophysiol 1995;6:512–21.
[32] Witkowski FX, Leon LJ, Penkoske PA, Giles WR, Spano ML, Ditto WL, Winfree AT. Spatiotemporal evolution of ventricular fibrillation. Nature 1998;392:78–82.
[33] Chen Q, Kirsch GE, Zhang D, Brugada R, Brugada J, Potenza D, Moya A, Borggrefe M, Breithard G, Ortiz-Lopez R, Wang Z, Antzelevitch C, O'Brien RE, Schulze-Bahr E, Keating MT, Towbin JA, Wang Q. Genetic basis and molecular mechanism for idiopathic ventricular fibrillation. Nature 1993;392:293–6.
[34] Priori SG, Barhanin J, Hauer RN, Haverkamp W, Jongsma HJ, Kleber AG, McKenna WJ, Roden DM, Rudy Y, Schwartz K, Schwartz PJ, Towbin JA, Wilde AM. Genetic and molecular basis of cardiac arrhythmias: impact on clinical management III. Circulation 1999;99:674–81.
[35] Attardi G, Schatz G. Biogenesis of mitochondria. Annu Rev Cell Biol 1988;4:289–333.
[36] Bay Y, Attardi G. The mtDNA-encoded ND6 subunit of mitochondrial NADH dehydrogenase is essential for the assembly of the membrane arm and the respiratory function of the enzyme. EMBO J 1998;17:4848–58.
[37] Curtis MJ, Hearse DJ. Reperfusion-induced arrhythmias are critically dependent upon occluded zone size: relevance to the mechanism of arrhythmogenesis. J Mol Cell Cardiol 1989;21:625–37.
[38] Shade GS, Clayton DA. Mitochondrial transcription initiation. J Biol Chem 1993;268:16083–6.
[39] Boore JL. Survey and summary: animal mitochondrial genomes. Nucl Acids Res 1999;27:1767–80.
[40] Wang GL, Semenza GL. General involvement of hypoxia inducible factor 1 in transcriptional response to hypoxia. Proc Natl Acad Sci USA 1993;90:4304–8.
[41] Semenza GL. Regulation of mammalian O_2 homeostasis by hypoxia-inducible factor 1. Annu Rev Cell Dev Biol 1999;15:551–78.
[42] Jung F, Palmer LA, Zhou N, Johns RA. Hypoxic regulation of inducible nitric oxide synthase via hypoxia inducible factor-1 in cardiac myocytes. Circ Res 2000;86:319–25.
[43] Beatc GN, McNeill JH. Ventricular arrhythmias following coronary artery occlusion in the streptozotocin diabetic rat. Can J Physiol Pharmacol 1988;66:312–7.
[44] Kusama Y, Hearse DJ, Avkiran M. Diabetes and susceptibility to reperfusion-induced ventricular arrhythmias. J Mol Cell Cardiol 1991;24:411–21.
[45] Chattou S, Coulombe A, Diacono J, Le Grand B, John G, Feuvray D. Slowly inactivating component of sodium current in ventricular myocytes is decreased by diabetes and partially inhibited by known Na^+/H^+ exchange blockers. J Mol Cell Cardiol 2000;32:1181–92.
[46] Ravingerova T, Neckar J, Kolar F, Stetka R, Volkova K, Ziegelhoffer A, Styk J. Ventricular arrhythmias following coronary artery occlusion in rats: is the diabetic heart less or more sensitive to ischemia? Basic Res Cardiol 2001;96:160–8.