RESEARCH ARTICLE

Effect of duration of ischemia on myocardial proteome in ischemia/reperfusion injury

Justyna Fert-Bober[1], Rasphal S. Basran[2], Jolanta Sawicka[1] and Grzegorz Sawicki[1]*

[1] Department of Pharmacology, College of Medicine, University of Saskatchewan, Saskatoon, Canada
[2] Department of Medicine, College of Medicine, University of Saskatchewan, Saskatoon, Canada

Received: January 9, 2008
Revised: February 19, 2008
Accepted: February 27, 2008

Ischemia/reperfusion (I/R) injury is a serious problem resulting from clinical setting of coronary revascularization. Despite extensive studies on I/R injury, the molecular bases of cardiac dysfunction caused by I/R are still unknown, but are likely to result from alterations in protein expression. Isolated rat hearts were subjected to 15–30 min of no-flow ischemia without (Ischemia protocol) or with 30 min of reperfusion (I/R protocol). 2-DE analysis of heart proteins from both experimental protocols showed wide-ranging changes in protein levels. In the Ischemia protocol, 39 protein spots were changed in ischemic groups and those changes correlated with duration of ischemia. Ninety percent of the affected proteins were increased. In contrast to increased protein levels, the total messenger RNA (mRNA) level decreased approximately two fold. Compared to the Ischemia protocol, changes in protein levels in the I/R protocol did not correlate with the duration of ischemia and the degree of recovery of mechanical function. The decrease of affected protein from I/R protocol was associated with the increase in total protein level in reperfusate. Our studies show that the protein increase is correlated with the mechanical function of the I/R hearts and the increase is not likely associated with an increase in protein synthesis.

Keywords:
Coronary effluent / Ischemia / Ischemia-reperfusion / Myocardium

1 Introduction

Despite the significant progress that has been made in cardiac surgery since the first cardiac surgical operation in the mid-1950s, the operative mortality is still 1–10%, and around 24% of high-risk patients will die within 3 years after surgery [1]. One of the most important factors involved in cardiac surgery is the induction of global ischemia and the consequent heart arrest. The complete reference on ischemia/reperfusion (I/R) injury as it applies to the science and practice of cardiothoracic surgery is summarized and edited by Beyersdorf [2]. The induction of ischemia is convenient for the surgeon, but it has potentially serious consequences to the heart, particularly when considering the underlying heart disease. Ischemia with following reperfusion is a clinical event that can produce dysfunction of coronary vessels and dysfunction of the myocardium. There are several factors that determine the extent of these dysfunctions, ischemia is one of them and includes the duration and severity [3–5].

Despite extensive research on molecular bases of cardiac dysfunction caused by I/R, the understanding of the mechanism of this injury is far from complete. Much evidence

Correspondence: Dr. Grzegorz Sawicki, Department of Pharmacology, College of Medicine, University of Saskatchewan, 107 Wiggins Road, Saskatoon, Saskatchewan, S7N 5E5, Canada
E-mail: greg.sawicki@usask.ca
Fax: +1-306-966-6220

Abbreviations: I/R, ischemia/reperfusion; **mRNA,** messenger RNA; **RPP,** rate pressure product; **SSP,** standard spot number

* On leave of absence from Department of Clinical Chemistry, Medical University of Wroclaw, Poland.

points to multifactoral etiology of I/R injury such as strong association with the inflammatory response [6, 7]. However, ROS have been also suggested as having a pivotal role in the etiology of I/R injury [8–11].

Because the duration of the ischemia and mechanical recovery of the heart are crucial factors in the cardiac surgical setting, in the present study we focused on the changes of the heart proteome dependent on the duration of ischemia, and the association of these changes with recovery of the mechanical function. Proteomics is a very powerful approach which to date has identified changes in more than 40 proteins involved in heart diseases such as dilated cardiomyopathy, varying degrees of I/R injury, and heart failure [12–17]. However, no one has studied changes in protein expression associated specifically with duration of ischemia. Examination of global changes in protein levels due to the duration of ischemia in the heart could provide new insights into cellular mechanisms involved in cardiac dysfunction. Moreover, it should result in the generation of new therapeutic strategies and the establishment of precise and sensitive diagnostic markers.

2 Materials and methods

This investigation conforms to the *Guide to the Care and Use of Experimental Animals* published by the Canadian Council on Animal Care (revised 1993).

2.1 Animals

Male Sprague–Dawley rats were anesthetized with an injection of sodium pentobarbital (40 mg/kg IP). The hearts were rapidly excised and briefly rinsed by immersion in ice-cold Krebs-Henseleit buffer. Spontaneously beating hearts were placed in a water-jacketed chamber (EMKA Technologies) to maintain their temperature at 37°C. Hearts were perfused in the Langendorff mode at a constant pressure of 60 mmHg with Krebs-Henseleit buffer at 37°C containing (in mM): NaCl (118), KCl (4.7), KH_2PO_4 (1.2), $MgSO_4$ (1.2), $CaCl_2$ (3.0), $NaHCO_3$ (25), glucose (11), EDTA (0.5) and gassed continuously with 95% O_2/5% CO_2 (pH 7.4).

A water-filled latex balloon connected to a pressure transducer was inserted through an incision in the left atrium into the left ventricle through the mitral valve. The volume of the balloon was adjusted to achieve an end diastolic pressure of 10 mmHg. Coronary flow, coronary perfusion pressure, heart rate, and left ventricular pressure were monitored using an EMKA recording system with IOX2 software (EMKA Technologies). Left ventricular developed pressure (LVDP) was calculated as the difference between systolic and diastolic pressures of the left ventricular pressure trace. The rate pressure product (RPP) was calculated as the product of heart rate and LVDP.

2.2 Ischemia and I/R protocols

All hearts were subjected to an initial baseline stabilization period and block randomized to different experimental settings: I/R protocol (Fig. 1A) and protocol for ischemia only (Fig. 2A). I/R hearts (six hearts *per* group) were subjected to 15, 20–25, or 30 min of no-flow global ischemia followed by 30 min of reperfusion and then freeze clamped. Hearts from Ischemia protocol (four *per* group) were subjected to 15, 25, or 30 min of no-flow global ischemia and freeze clamped without reperfusion. Nonischemic control groups of hearts were aerobically perfused either for 25 min (control for Ischemia setting) or for 75 min (control for I/R setting). All hearts were stored at −80°C.

2.3 The coronary effluent collection and protein concentration

Coronary effluents from control and 50% recovery hearts ($n = 3$ *per* group) were collected during perfusion for meas-

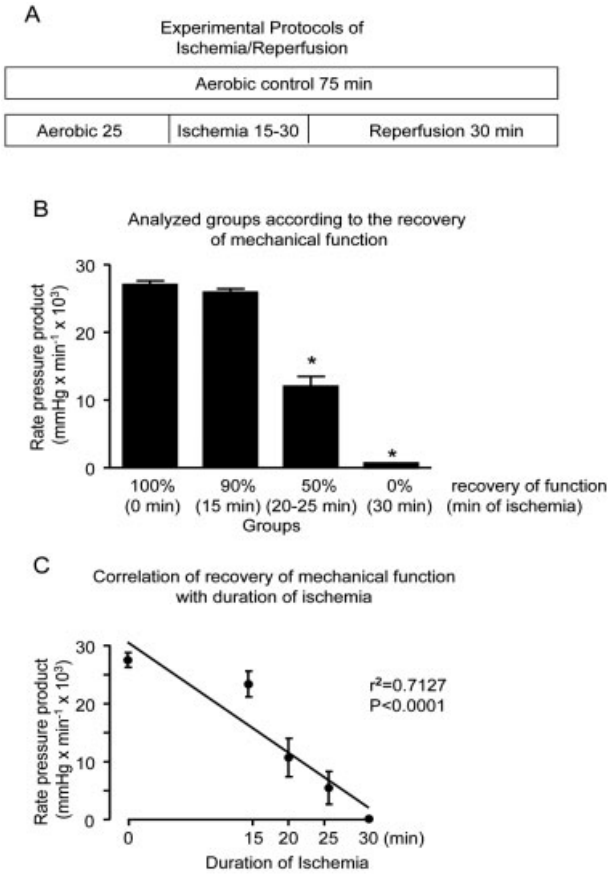

Figure 1. (A) Experimental protocol of I/R injury. (B) Analyzed heart groups ($n = 6$ *per* group) selected according to the level of recovery mechanical function (RPP) of the hearts subjected to no-flow ischemia followed by 30 min of reperfusion. Note that RPP of those hearts subjected to ischemia is decreased. (C) Correlation of RPP with duration of ischemia.

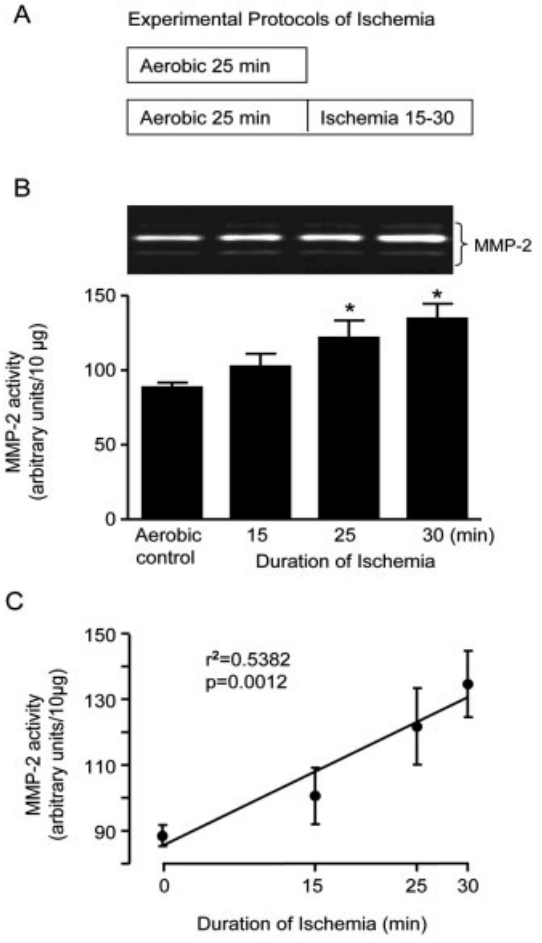

Figure 2. (A) Experimental protocol of Ischemia. (B) Analyzed heart groups ($n = 6$ *per* group) selected according to the increase of MMP-2 activity. Note that the densitometric analysis of activities of MMP-2 in heart subjected to different length of ischemia shows increased level of MMP-2 in ischemic hearts. Representative zymogram of MMP-2 activity is shown above the bar graph. (C) Correlation of MMP-2 activity with duration of ischemia.

uring protein release. Twenty milliliters of coronary effluent were collected for protein measurement at times ending at 25, 45, 50, 55, and 75 min of the perfusion protocol (Fig. 6A). As equal volume samples were collected, the period of time required to collect samples varied during early reperfusion. The time required to collect the coronary effluent samples was between 70–160 s for the perfusion and reperfusion samples. The samples were stored at 4°C and processed on the same day. Protein concentration in the effluent and heart homogenates was determined by the BioRad protein assay using BSA as the standard.

2.4 Preparation of heart extracts for zymography

Frozen hearts were crushed using a mortar and pestle at liquid nitrogen temperature and then homogenized by sonication in ice-cold 50 mM Tris–HCl (pH 7.4) containing 150 mM NaCl, 0.1% Triton X-100, and 10 µL/mL of Protease Inhibitor Cocktail (Sigma, Cat# P8340). The homogenate was centrifuged at 13 000 rpm at 4°C for 10 min and the supernatant was collected and stored at −80°C.

2.5 Measurement of MMPs by gelatin zymography

Gelatin zymography is an assay where the latent and active forms of MMP-2 can be detected and measured. This assay was performed as described previously [18]. Briefly, preparations of samples were applied to 8% polyacrylamide gel copolymerized with 2 mg/mL gelatin. After electrophoresis, gels were rinsed in 2.5% Triton X-100 (3×20 min) to remove SDS, washed twice in incubation buffer (in mM: Tris–HCl (50), $CaCl_2$ (5), NaCl (150), and 0.05% NaN_3) for 20 min each at room temperature and kept in incubation buffer at 37°C overnight. The gels were stained for 2 h with 0.05% CBB R250 (BioRad) in a mixture 2.5:1:6.5 methanol/acetic acid/water and destained 1 h in 2% methanol/4% acetic acid before being scanned. Gelatinolytic activities were detected as transparent bands against the background of CBB-stained gelatin. Enzyme activity was quantified by densitometry analysis of gelatinolytic bands and expressed as arbitrary units *per* microgram protein.

2.6 Criteria of choosing hearts for proteomic analysis

To select I/R hearts for proteomic analysis, the level of mechanical function recovery was used as criteria for grouping hearts instead of length of ischemia. The level of recovery correlates well with short (15 min) and severe ischemia (30 min); however, when 20–25 min of ischemia was used, significant variations in RPP were observed due to individual conditions of analyzed hearts/rats. Four groups of hearts ($n = 3$–4 *per* group) were analyzed. Control group: includes hearts with 100% mechanical function maintained throughout the experiment (no ischemia); 90% recovery group: includes hearts subjected to 15 min ischemia; 50% recovery group: includes hearts subjected to 20 or 25 min of ischemia; 0% recovery group: includes hearts subjected to 30 min of ischemia.

Because data of heart function is not available in the Ischemia setting, we measured the level of MMP-2 as a biomarker of heart injury. Recent clinical studies demonstrate an association between circulating levels of MMP-2 and cardiac dysfunction [19–21]. It has also been shown that elevated MMP-2 level is connected with left ventricular remodeling after myocardial infarction [22] and with poor outcomes in patients with congestive heart failure [23]. This is why MMP-2 can be used, at least in preclinical studies, as a marker of heart injury subjected to acute ischemia.

2.7 2-DE

Selected heart samples, weighing approximately 50 mg, were homogenized by sonication in ReadyPrep 2-D Starter Kit

Rehydration/Sample Buffer (BioRad, 10 mL, 8 M urea, 2% CHAPS, 50 mM DTT, 0.2% Bio-Lyte 3/10 ampholyte, 0.001% Bromophenol Blue). The samples were centrifuged for 10 min at 13 000 rpm at room temperature to remove any insoluble particles, and protein concentration was measured using the BioRad protein assay after suitable dilution of the samples. Protein (0.2 mg) was applied to each of 11 cm immobilized linear pH gradients (5–8) strips (IPG, BioRad), with rehydration for 16–18 h at 20°C. For isoelectrofocusing (IEF), the BioRad Protean IEF cell was used with the following conditions at 20°C with fast voltage ramping: Step 1: 15 min with end voltage at 250 V; Step 2: 150 min with end voltage at 8000 V; Step 3: 35 000 V·h (approximately 260 min). After IEF, the strips were equilibrated according to the manufacturer's instructions. Second dimension of 2-DE was then carried out with Criterion precast gels (8–16%) (BioRad). After separation, proteins were stained for 2 h with 0.05% CBB R250 (BioRad) in a mixture 2.5:1:6.5 methanol/acetic acid/water and destained in 4% methanol with 8% acetic acid.

Twenty microliters of concentrated effluent samples was mixed with Rehydration/Sample Buffer (BioRad) and applied to 11 cm immobilized linear pH gradients (3–10) strips (BioRad) and equilibrated. The electrophoresis conditions were the same as for heart homogenates. The 2-DE gels were stained with Silver Staining Plus kit (BioRad). To minimize variation in staining, all gels were stained in the same bath. The reproducibility of 2-DE and quality of protein loading has been previously verified by us [24–26].

2.8 Image analysis

Developed gels were scanned using a GS-800 calibrated densitometer (BioRad). Quantitative analysis of MMP-2 bands from zymography and spot intensity from 2-DE gels were measured using QuantityOne 4.6 and PDQuest 7.1 software, respectively (BioRad).

2.9 MS

Selected protein spots demonstrating statistically significant changes in spot size and density were manually excised from the 2-DE gel. They were processed using a MassPrep Station from Micromass using the methods supplied by the manufacturer. Briefly, the excised gel fragment containing the protein spot was first distained, reduced, alkylated, digested with trypsin, and extracted. Mass analysis of the trypsin digest was performed on a MALDI-TOF Voyager DE-Pro from Applied Biosystems. A mass deviation of 0.2 was tolerated and one missed cleavage site was allowed. Resulting values from MS analysis for monoisotopic peaks were used for searches against the NCBInr and Swiss-Prot databases with *Rattus norvegicus* specified. We used the MASCOT (www.matrixscience.com) search engine to search the protein database for protein identification. The MOWSE scoring algorithm [27] was used for justification of accuracy of protein identification and is incorporated in the MASCOT search engine.

2.10 Messenger RNA (mRNA) isolation from ischemic hearts

To evaluate the total mRNA level, only hearts from the Ischemia experimental protocol were used. First, the total RNA was extracted. Hearts were powdered in liquid nitrogen, homogenized ($\leq$30 mg tissue) in RTL buffer (Qiagen, CA) and total RNA was purified with the Qiagen RNeasy kit (Qiagen) using the clean-up step according to the manufacturer's instructions. The total RNA quality was evaluated by measuring the A_{260}/A_{280} ratio and the concentration of total RNA.

In the second step, the mRNA was isolated. Briefly, mRNA was separated from the total RNA with oligo(dT) polystyrene-latex particles (Poly(A) oligotex directly mRNA Isolation Kit, Qiagen). Samples were then incubated at 70°C in a water bath to disrupt the secondary structure of RNA and then were placed at room temperature to allow the mRNA to bind with the particles. The samples were further washed and enriched for mRNA content by spin column using the same protocol.

2.11 Statistical analysis

One-way analysis of variance (ANOVA) was used to compare the differences between the groups. Analysis of spot density of the 2-DE gels was carried out by the *t*-test and the Mann–Whitney *U* test. *Post hoc* analysis was performed using the Tukey–Kramer multiple comparisons test. A *p*-value less than 0.05 was considered statistically significant. Data are expressed as the mean $\pm$ standard error in all figures.

3 Results

3.1 Duration of ischemia and recovery of the mechanical function of the hearts

The recovery of the mechanical function of I/R hearts after 30 min of reperfusion following different durations of global no-flow ischemia was significantly reduced (Fig. 1B). This reduction was correlated with duration of ischemia (Fig. 1C). I/R hearts were not significantly altered by 15 min of ischemia, whereas 20 min or longer ischemia caused severe depression of the function. When hearts were subjected to 20–25 min ischemia, a 50% decrease in mechanical function was observed, and 30 min ischemia caused a 100% inhibition of heart contractility. In Fig. 1C, the straight line is the linear regression between RPP and duration of ischemia, and shows that the period of 15 min of ischemia represents the threshold for the loss of mechanical function of the heart.

3.2 Duration of ischemia and heart MMP-2 activity

The hearts from the Ischemia protocol (Fig. 2A) were found to express an increased activity of MMP-2 (Fig. 2B). Fifteen minutes of ischemia led to a slight increase (~15%) in MMP-2 activity. After 25 and 30 min of ischemia, MMP-2 level was increased by about 40 and 50%, respectively (Fig. 2B). There was a positive correlation between MMP-2 activity and duration of ischemia (Fig. 2C).

3.3 Analysis of myocardial proteins from 2-DE

Our earlier studies with 2-DE have showed that most proteins were localized near the center of the gel, in the mid pH range [24, 26]. Therefore, for 2-DE of heart extracts we used narrow range strips (pH 5–8) for the first dimension and 8–16% gradient gels in the second dimension. We did not analyze by 2-DE the 15 min ischemia group from Ischemia protocol and the 90% recovery group from I/R protocol because MMP-2 activity (Ischemia setting) and mechanical function (I/R protocol) are not significantly different from controls. Figures 3 and 4 show representative gels from Ischemia and I/R experimental protocols. The most profound changes in protein levels *versus* the control group in both experimental protocols were observed in 30 min ischemia and 50% recovery groups, respectively. In the Ischemia protocol, the vast majority of altered protein spots increased and the changed protein spots are marked only in the ischemia gel (Fig. 3). Conversely, in the I/R protocol, the altered protein spots mostly decreased, and altered spots are indicated only on the control gel (Fig. 4).

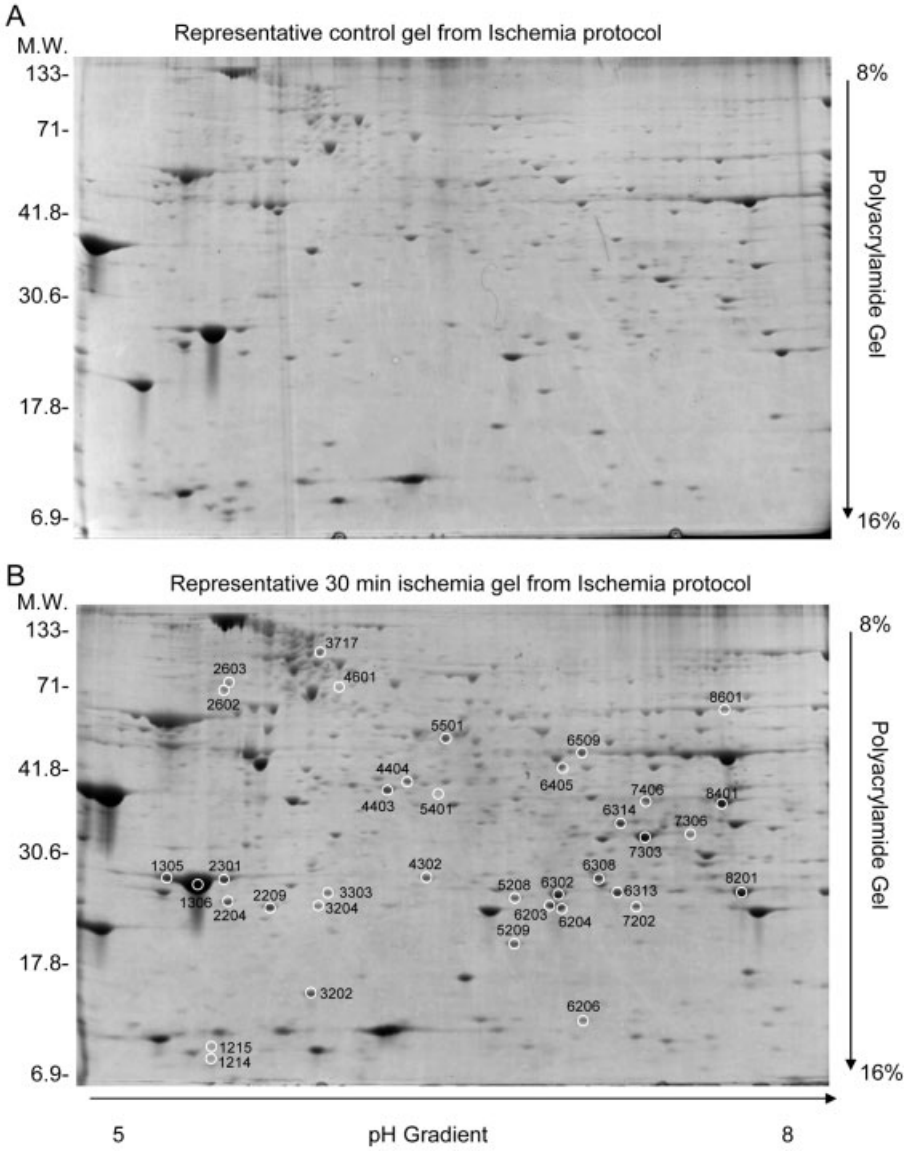

Figure 3. Representative 2-D map of proteins from control heart (A) and from heart subjected to 30 min of ischemia without reperfusion (B). Circles show affected protein spots and the SSPs (next to the circle) indicate the protein spots from quantitative analysis using PDQuest measurement software (BioRad). $n = 4$ *per* group.

Significantly changed protein spot levels from the Ischemia protocol are listed in Table 1 (for clarity, the direction of change of protein spots level, is marked only for the decreased protein spots). The SSP numbers correspond to those on the gels in Fig. 3. Tables 2A and 2B show the altered protein spots and the direction of the changes from the I/R protocol (50 and 0% recovery group, respectively). In contrast to Table 1, the direction of change of protein spots level, is marked only for the increased protein spots. In the Ischemia protocol, groups (25 and 30 min of ischemia) showed that 33 altered proteins increased their level but only four protein spots decreased their level (Table 1). In the I/R protocol, the 50% recovery group showed only one protein spot, out of 19, which increased its level (Table 2A). In the 0% recovery group 13 protein spots were altered: six of these increased and seven decreased in their level (Table 2B).

All altered protein spots from both experimental protocols were analyzed for identification by MS. Eighteen protein spots from the Ischemia protocol were identified (Table 3) and nine from I/R protocol (Table 4). The majority of the protein spots that showed altered abundance from Ischemia and I/R protocols are metabolism-related proteins. Also changed are sarcomeric proteins like myosin light and heavy chains, antioxidant proteins like peroxiredoxin 6 and DJ-1, stress proteins like heat shock protein 20 (HSP20), and hypothetical protein LCO365377.

3.4 Duration of ischemia and mRNA level

To test if the protein increase in the Ischemia protocol is associated with an increase in protein synthesis, we evaluated changes of mRNA level as an indirect marker of

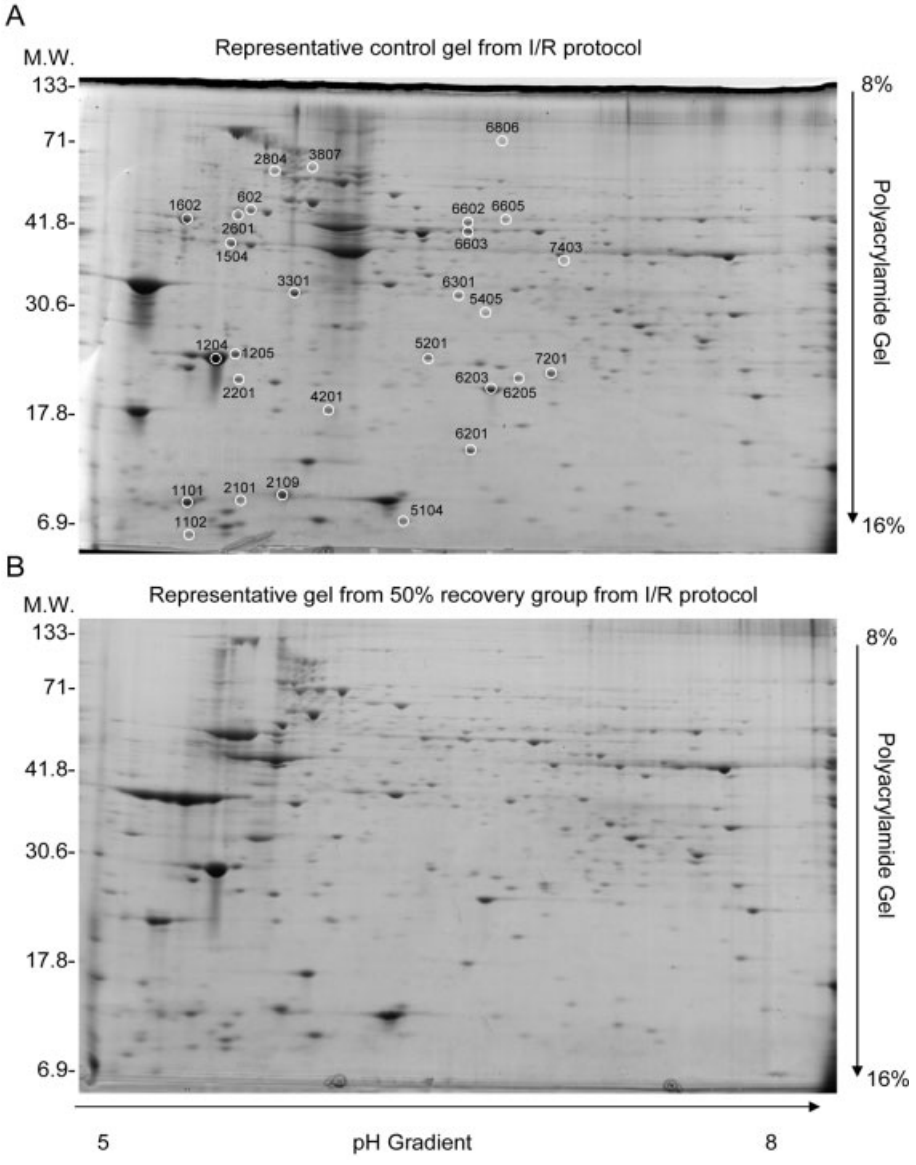

Figure 4. Representative 2-D map of proteins from control heart (A) and heart from 50% recovery group (I/R heart) (B). Circles show affected protein spots and the SSPs (next to the circle) indicate the protein spots from quantitative analysis using PDQuest measurement software (BioRad). $n = 4$ *per* group.

Table 1. List of all protein spots where levels were altered in Ischemia protocol

Protein spot (SSP)	Control		Ischemia 25 min		Ischemia 30 min	
	Mean	±SE	Mean	±SE	Mean	±SE
1214	40.70	6.54	16.52↓	2.23	15.75 ↓	1.22
1215	27.76	3.78	19.13↓	2.93	11.49 ↓	2.09
1305	71.13	12.89	82.51	15.02	113.60	3.67
1306	419.83	63.20	549.94	71.93	758.92	28.74
2204	15.97	5.04	23.89	5.47	36.93	2.24
2209	25.20	1.84	33.38	6.88	54.65	3.91
2301	35.84	4.76	42.09	2.90	65.87	4.91
2602	1.28	0.93	3.66	2.18	11.63	0.95
2603	1.28	0.93	4.42	2.54	12.36	2.18
3202	30.75	8.70	78.12	5.52	63.68	10.24
3204	10.40	1.01	14.51	1.40	16.32	1.26
3303	7.30	2.13	22.82	5.30	25.92	1.63
3717	3.98	1.37	6.32	2.27	13.22	2.40
4302	18.21	2.44	22.13	4.84	29.90	3.15
4403	29.49	5.70	31.69	3.40	49.17	3.43
4404	5.66	1.91	23.29	7.14	21.47	4.22
4601	4.61	1.46	4.417	2.32	9.07	0.77
5208	17.73	2.91	21.28	2.36	27.40	1.87
5209	24.63	3.13	34.39	2.02	40.25	3.37
5401	11.83	4.59	0.49 ↓	0.06	0.38 ↓	0.05
5501	10.42	2.19	32.38	10.91	33.35	6.28
6203	6.40	2.17	35.11	8.81	41.71	0.86
6204	8.27	0.40	40.19	15.61	41.32	3.45
6206	10.17	2.44	16.18	1.19	19.17	1.24
6302	46.13	9.05	95.32	21.46	110.22	5.11
6308	9.35	1.23	60.68	22.28	55.31	4.88
6313	12.83	2.57	41.15	2.66	54.70	3.11
6314	18.74	3.25	34.48	5.27	38.83	6.52
6405	3.95	1.40	16.23	5.01	20.51	2.50
6509	8.34	1.56	22.71	5.75	34.11	3.18
7202	5.66	1.92	22.30	7.83	23.98	2.25
7303	52.98	10.80	176.67	60.01	173.16	9.14
7306	9.68	5.35	35.18	11.71	39.18	1.75
8201	25.60	5.56	69.20	8.34	90.81	17.26
8401	12.21	2.50	90.32	29.51	82.92	11.42
8601	10.57	2.79	3.50 ↓	1.94	1.85 ↓	1.47

$n = 4$ in each group. The arrows (↓) indicate only decreased protein levels, all other unmarked protein spots are increased. SSP, standard spot number.

changes of protein synthesis in relation to duration of ischemia. The total mRNA level was measured as a ratio of mRNA to total RNA (see Section 2). The mRNA levels from 25 and 30 min groups in the Ischemia protocol were similar and decreased approximately two-fold in comparison to the control group (Fig. 5).

3.5 Protein release into the coronary effluent during I/R

We analyzed the total amount of protein released from hearts into the effluent at different time points of perfusion. For this analysis we chose the 50% recovery group, as this is where the most profound protein changes were observed. The time points of effluent collection are shown in Fig. 6A. During the first minute of reperfusion, the protein level in the effluent of I/R hearts was significantly increased in comparison to control (0.186 ± 0.019 μg/mL in I/R *vs.* 0.014 ± 0.003 μg/mL in control; $p<0.05$, $n = 4$). The protein level remained higher than the control in the 50th and 55th min and returned to the preischemia value at the 75th min of reperfusion (the end of perfusion, Fig. 6B).

The proteins released into the effluent were also analyzed by 2-DE (Fig. 7). The 2-D gel of protein from effluent collected during the first minute of reperfusion (45th min) showed that effluent from the I/R heart has several times

Table 2A. List of all protein spots where levels were altered in 50% recovery group in I/R protocol

Protein spot (SSP)	Control		50% Recovery	
	Mean	±SE	Mean	±SE
1101	77.02	9.36	44.45	3.88
1204	436.21	7.82	286.65	33.11
1205	40.03	6.57	23.06	2.89
1504	51.90	4.34	1.86	0.86
1602	93.37	46.63	2.48	2.36
2109	78.92	4.31	52.96	7.90
2201	16.61	1.58	7.15	3.35
2601	14.52	1.44	0.10	0.02
2602	11.25	5.55	0.10	0.02
3301	43.44	2.56	21.81	4.57
3807	1.96	1.14	0.10	0.02
5104	4.66	1.98	8.38 ↑	0.61
5201	9.40	1.45	7.33	1.08
6201	23.36	4.10	15.25	1.38
6203	80.90	10.27	66.06	4.69
6205	9.89	1.52	6.26	0.41
6301	5.88	1.15	2.72	0.28
6602	10.85	2.87	4.45	0.61
6603	26.28	1.29	10.78	5.42

$n = 4$ in each group. The arrow (↑) indicates only increased protein level, all other unmarked protein spots are decreased.

Table 2B. List of all protein spots where levels were altered in 0% recovery group in I/R protocol

Protein spot (SSP)	Control		0% recovery	
	Mean	±SE	Mean	±SE
1102	7.82	0.69	2.99	0.79
1204	448.42	34.18	636.57 ↑	
19.51				
2101	9.28	1.31	2.40	1.15
2804	6.60	1.46	0.15	0.02
3301	36.27	2.45	45.12 ↑	0.74
4201	12.28	2.01	3.96	1.17
5104	14.75	0.93	4.68	1.19
5405	1.32	0.06	1.82 ↑	0.09
6603	31.83	0.86	24.96	2.15
6605	2.38	0.33	4.19 ↑	0.06
6806	0.61	0.09	1.33 ↑	0.15
7201	17.94	2.64	8.49	0.94
7403	13.45	0.92	15.67 ↑	1.89

$n = 4$ in each group. The arrow (↑) indicates only increased protein level, all other unmarked protein spots are decreased.

more measurable protein spots (226 spots) than the control 2-D gel (26 spots). The protein spots from effluent also were analyzed by MS. Only four protein spots were matched with protein spots from the heart subjected to I/R injury: two spots represented alpha enolase, one spot was MLC1, and one was ATP synthase. In contrast to heart tissue, where the alpha subunit of ATP synthase was identified, in effluent we found the beta subunit. Results for protein identification of protein spots from effluent are shown in Table 5.

4 Discussion

In this study, we found that during 25 min global no-flow ischemia in the Ischemia protocol, levels of many proteins are increased and this increase is more pronounced when ischemia is prolonged up to 30 min. In contrast, in the I/R protocol the corresponding duration of ischemia decreased the vast majority of affected protein spots and increased the level of total protein in early reperfusate. Longer ischemia in the same experimental protocol caused a mixed effect in the changes of protein spot levels (decrease and increase). These results suggest that the processes responsible for increasing protein spot levels are triggered during ischemia. The decrease in protein spot levels in the I/R protocol could be explained by an increased release of proteins from the myocardium which can be noticeable only during reperfusion. This explanation is supported by the detection of enolase, ATP synthase, and MLC1 in early reperfusate (Fig. 7, Table 5), and the decrease of these proteins in I/R hearts (Table 4). Because both protein spot increase and protein release are synchronized, the changes of protein spot levels are not so comprehensible like in the Ischemia setting.

One of the first studies showing that ischemia may induce protein synthesis was done by Das and Maulik [31]. They showed an increase in mRNA for three HSP genes (27, 70, and 89), two antioxidant enzyme genes (Mn-superoxide dismutase (SOD) and catalase), and two oncogenes (c-fos and c-myc). Furthermore, this study showed that activities of four major antioxidant enzymes (SOD, catalase, glutathione peroxidase, and glutathione reductase) were increased by ischemic preconditioning of the heart [28]. In other studies, where ischemic preconditioning was induced by temporary middle cerebral artery occlusion [29], or by occlusion of the left anterior descending coronary artery [30], blocking of protein synthesis with cycloheximide was associated with the reduction of ischemic tolerance. However, what is important is that all research which implicates induction of protein synthesis during ischemia is based on measurements of mRNA, inhibition of protein synthesis or measurement of enzymatic activity (for review see ref. [31]). Despite that none of these studies directly measure protein changes, the explanation of protein increase as effect of protein synthesis could be easily accepted. However, in our experiments, hearts are perfused with Krebs-Henseleit buffer which does not contain the amino acids necessary to support protein synthesis. Of course, it is possible that myocardial cells may use intracellular or preexisting amino acids for protein synthesis. Additionally, instead of an increase in mRNA, we observed a dramatic decrease of total mRNA in both groups of ischemic hearts (25 and 30 min of ischemia, Fig. 5). We realize that the

Table 3. Identification of protein spots from Ischemia protocol

Protein spot (SSP)	MOWSE score[a)]	Queries matched	Sequence coverage (%)	MW (kDa)	Protein identification
4403 ↑[b)]	80	8	28	36.9	Lactate dehydrogenase B
5401 ↓	88	11	34	39.6	Isocitrate dehydrogenase 3 alpha
5501 ↑	128	17	38	49.5	Myosin H
7303 ↑	66	9	15	49.5	Myosin H
6509 ↑	102	22	21	141.8	Myosin binding protein C
7406 ↑	65	9	36	36.1	Glyceraldehyde 3-phosphate-dehydrogenase
8401 ↑	68	8	30	36.1	Glyceraldehyde 3-phosphate-dehydrogenase
6314 ↑	101	15	51	36.5	Delta(3,5)-delta(2,4)-dienoyl-CoA isomerase
8201 ↑	117	18	63	25.1	ATP synthase, alpha subunit
6313 ↑	84	9	42	25.1	ATP synthase, alpha subunit
6302 ↑	88	15	26	25.1	ATP synthase, alpha subunit
6203 ↑	83	11	33	25.1	ATP synthase, alpha subunit
6204 ↑	69	10	31	25.1	ATP synthase, alpha subunit
5208 ↑	96	10	69	20.2	DJ-1 protein
5209 ↑	84	7	50	17.5	HSP20
4302 ↑	123	11	58	24.9	Peroxiredoxin 6
6206 ↑	67	6	33	24.9	Peroxiredoxin 6
3303 ↑	52	7	19	53.9	Ubiquinol-cytochrome c reductase

a) $-10 \log(P)$ where P is the probability that the observed match is a random event. Individual ions scores >61 indicate identity or extensive homology ($p<0.05$).
b) The arrows (↓↑) indicate how the protein level is changed.

Table 4. Identification of protein spots from I/R protocol

Protein spot (SSP)	MOWSE score[a)]	Queries matched	Sequence coverage (%)	MW (kDa)	Protein identification
6605 ↓[b)]	82	11	28	53.8	Hypothetical protein LOC365377
6602 ↓	148	17	43	53.8	Aldehyde dehydrogenase
6603 ↓	76	12	35	47.4	Enolase 1, alpha
7403 ↓	77	9	26	46.9	Isovaleryl coenzyme A dehydrogenase
5405 ↑	64	6	25	33.2	3-Mercaptopyruvate sulfurtransferase
4201 ↓	76	7	41	22.3	Myosin, light chain 1 (MLC1)
5201 ↓	74	6	33	24.8	Peroxiredoxin 6
6203 ↓	76	9	66	18.8	ATP synthase, subunit d
6205 ↓	64	8	55	20.2	DJ-1 protein

a) $-10 \log(P)$ where P is the probability that the observed match is a random event. Individual ions scores >61 indicate identity or extensive homology ($p<0.05$).
b) The arrow (↓↑) indicate how the protein level is changed.

measurement of mRNA can be used only as indirect marker of protein synthesis. This is why the decrease of total mRNA cannot be used as an ultimate proof excluding *de novo* synthesis of individual proteins.

Two possible explanations for these observed alterations in protein levels are as follows. The first and most risky hypothesis is that during ischemia some proteins are synthesized at the expense of other proteins (*i.e.*, some proteins are degraded into their constituent amino acids and these amino acids are used for *de novo* synthesis of other protein). However, we did not observe a marked decrease in very many protein spots, only four low abundant protein spots decreased their levels in both ischemic groups. This theory is very interesting, but there is no evidence available in the literature supporting this notion and it remains to be determined if enough amino acids are provided to support the observed increase in protein levels.

The second hypothesis is based on the idea that protein precursors are being processed protelytically. The maturation of proteins/peptides by proteolytic cleavage of protein precursors is well established, for example amyloid cleavage in Alzheimer's disease [32] or release of kinins from kinino

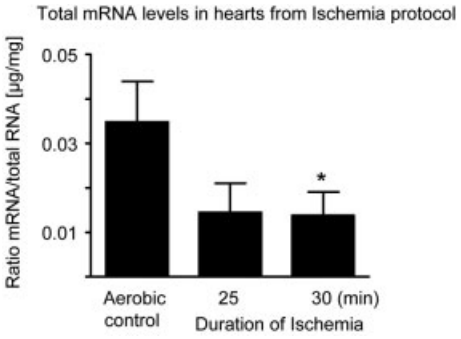

Figure 5. Quantitative analysis of total mRNA level in the heart tissues from Ischemia protocol. The analyzed hearts are from control, ischemia 25 min, and ischemia 30 min group. $n = 4$ *per* group.

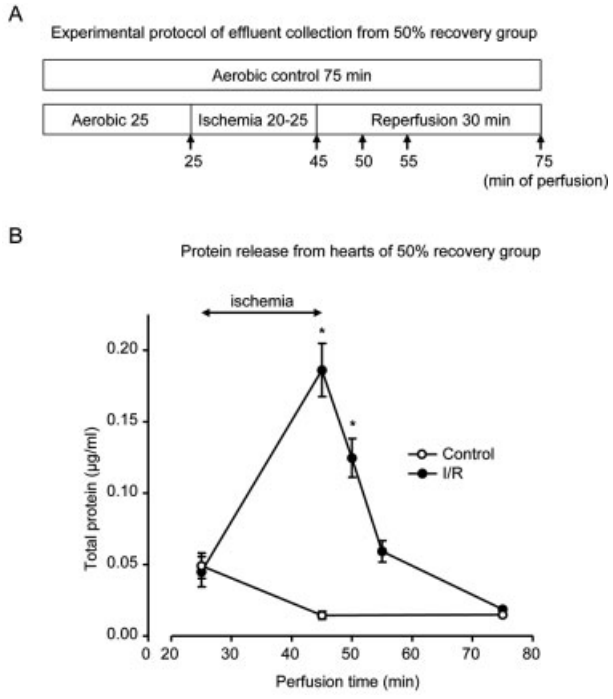

Figure 6. (A) Experimental protocol for coronary effluent collection from I/R protocol. (B) Total protein release into coronary effluent from (○) control hearts and (●) hearts subjected to I/R from 50% recovery group (20–25 min of ischemia). $n = 3$ *per* group.

gens by kalikrein [33]. However, despite several studies on lower molecular weight protein/peptide maturation, there is no evidence that suggests that such a concept can be used to elucidate the massive increase of protein spots observed in our study. The molecular range of increased protein spots vary from 10 kDa to more then 100 kDa. Consequently, the molecular weight must be higher than can be detected by 2-DE used in our experiments. The limitations of 2-DE are reviewed in ref. [34]. The concept of high molecular weight

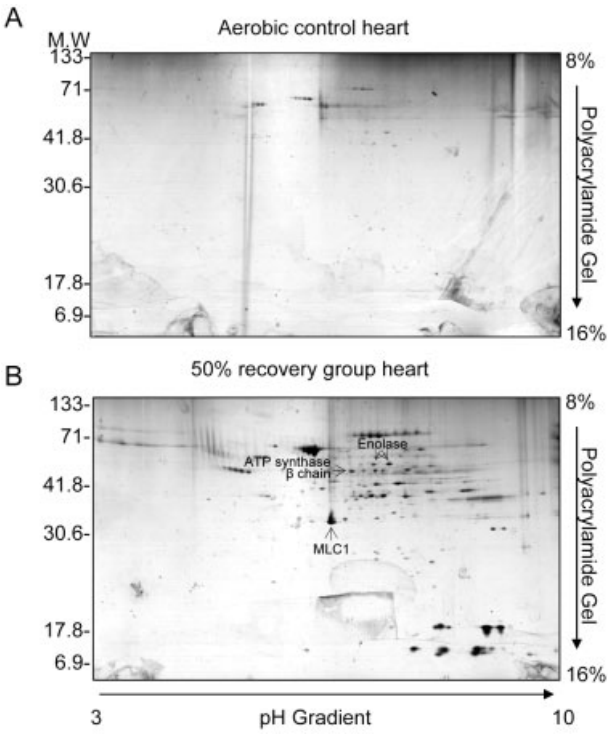

Figure 7. 2-D map of proteins of coronary effluent from control heart (A) and heart from 50% recovery group (I/R heart) (B) after 45 min perfusion.

protein processing can be additionally supported by the increase in MMP-2 activity which was observed in our study and was positively correlated with duration of ischemia (Fig. 2). MMP-2 is best known for its degradation of extracellular proteins and remodeling of the extracellular matrix. However, studies from the last decade on the intracellular action of MMP-2 introduced a new paradigm pertaining to the site of MMP-2 activity. We have shown that MMP-2 degrades MLC1 in I/R injury [24], and in the present study, we found a decrease of MLC1 in the I/R protocol, and an almost a three-fold increase in two protein spots corresponding to degradation products of myosin H in the Ischemia protocol. The novel aspects of the action of MMP-2 in heart injury have been reviewed by Schulz [35] and the role of MMPs in cardiac diseases has been reviewed by Spinale [36].

The other question is if the increase of protein levels during ischemia is a part of a defense mechanism or a response to the stress? The changes in the level of proteins involved in energy metabolism can be explained by the shift of energy metabolism. Under normal aerobic conditions, the heart preferentially metabolizes fatty acids, which contribute between 60 and 80% of the required ATP [37]. However, energy metabolism shifts from using free fatty acids as a substrate to using glucose within a few minutes after the induction of ischemia. Therefore, during ischemia glycolysis predominates as the source of energy and becomes the primary source of ATP [38, 39].

Table 5. Identification of protein spots from coronary effluent

MOWSE score[a)]	Queries matched	Sequence coverage (%)	MW (kDa)	Protein identification
74	7	14	47.4	Enolase 1, alpha
102	6	32	22.3	Myosin, light chain 1 (MLC1)
168	16	33	51.1	ATP synthase, subunit beta

a) $-10\log(P)$ where P is the probability that the observed match is a random event. Individual ions scores >71 indicate identity or extensive homology ($p<0.05$).

The induction of peroxiredoxin 6, a novel member of the peroxiredoxin family, has a protective effect against oxidative stress by scavenging ROS [40, 41]. Also, the induction of myosin binding protein C protects the myocardium from ischemic injury by maintaining thick filament spacing and its structure [42]. Detection of DJ-1 protein in the heart deserves special attention. This is the first time that this protein has found in the heart and its up-regulation in other tissues in ischemia has also not been reported before. It may be that DJ-1, like peroxiredoxin 6, plays a role in the protection against oxidative stress as an atypical peroxiredoxin-like peroxidase [43, 44].

The role of small HSPs (sHSPs), like HSP20, which was found to be elevated in this study, is not clear. They are largely spread out in living cells. In mammals, nine sHSPs have been identified to date, with a molecular mass ranging from 15 to 30 kDa. All sHSPs, except αA-crystallin, are abundant in the skeletal muscle and the heart (for review see [45]).

Protein changes in hearts subjected to I/R injury were also reported by White and coworkers. However, in contrast to their work, we did not detect a massive increase of protein spot levels in our I/R protocol. The difference is most likely due to the fact that they used different experimental animals and the hearts were subjected to low flow ischemia and to twice as long reperfusion [46, 47].

In summary, increasing duration of global ischemia prior to reperfusion in the isolated rat heart is very likely to be a major determinant of postischemic contractile dysfunction. Because I/R injury is a serious problem resulting from the clinical setting of coronary revascularization, whether pharmacological, mechanical, or from cardiopulmonary bypass, understanding the consequences of heart proteome changes is critical for the development of new diagnostic strategies and new therapies for the protection and treatment of I/R injury. This is why the examination of global changes in protein levels due to duration of ischemia in the heart is important and can provide new insights into cellular mechanisms involved in cardiac dysfunction.

We thank Dr. J. S. Richardson for help with manuscript preparation. This work was supported by grants from the Canadian Institutes of Health Research, and from the Heart and Stroke Foundation of Canada.

The authors have declared no conflict of interest.

5 References

[1] Flack, J. E., Cook, J. R., May, S. J., Lemeshow, S. *et al.*, Does cardioplegia type affect outcome and survival in patients with advanced left ventricular dysfunction? Results from the CABG Patch Trial. *Circulation* 2000, *102*, 84–89.

[2] Beyersdorf, F., *Ischemia-Reperfusion Injury in Cardiac Surgery*, Landes Bioscience, U.S., Texas 2001.

[3] Coudray, C., Boucher, F., Pucheu, S., De Leiris, J., Favier, A., Relationship between severity of ischemia and oxidant scavenger enzyme activities in the isolated rat heart. *Int. J. Biochem. Cell Biol.* 1995, *27*, 61–69.

[4] Tarantini, G., Cacciavillani, L., Corbetti, F., Ramondo, A. *et al.*, Duration of ischemia is a major determinant of transmurality and severe microvascular obstruction after primary angioplasty: A study performed with contrast-enhanced magnetic resonance. *J. Am. Coll. Cardiol.* 2005, *46*, 1229–1235.

[5] Landesberg, G., Mosseri, M., Zahger, D., Wolf, Y. *et al.*, Myocardial infarction after vascular surgery: The role of prolonged stress-induced, ST depression-type ischemia. *J. Am. Coll. Cardiol.* 2001, *37*, 1839–1845.

[6] de Groot, H., Rauen, U., Ischemia-reperfusion injury: Processes in pathogenetic networks: A review. *Transplant. Proc.* 2007, *39*, 481–484.

[7] Thurman, J. M., Triggers of inflammation after renal ischemia/reperfusion. *Clin. Immunol.* 2007, *123*, 7–13.

[8] Beckman, J. S., Koppenol, W. H., Nitric oxide, superoxide, and peroxynitrite: The good, the bad and ugly. *Am. J. Physiol.* 1996, *271*, C1424–C1437.

[9] Schulz, R., Dodge, K. L, Lopaschuk, G. D., Clanachan, A. S., Peroxynitrite impairs cardiac contractile function by decreasing cardiac efficiency. *Am. J. Physiol.* 1997, *272*, H1212–H1219.

[10] Schulz, R., Panas, D., Catena, R., Moncada, S. *et al.*, The role of nitric oxide in cardiac depression induced by interleukin-1 and tumour necrosis factor. *Br. J. Pharmacol.* 1995, *114*, 27–34.

[11] Panas, D., Khadour, F. H., Szabo, C., Schulz, R., Pro-inflammatory cytokines depress cardiac efficiency by a nitric oxide-dependent mechanism. *Am. J. Physiol.* 1998, *275*, H1016–H1023.

[12] Arrell, D. K., Neverova, I., Fraser, H., Marban, E., Van Eyk, J. E., Proteomic analysis of pharmacologically preconditioned cardiomyocytes reveals novel phosphorylation of myosin light chain 1. *Circ. Res.* 2001, *89*, 480–487.

[13] Jager, D., Jungblut, P. R., Muller-Werdan, U., Separation and identification of human heart proteins. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 2002, *771*, 131–153.

[14] Corbett, J. M., Why, H. J., Wheeler, C. H., Richardson, P. J. *et al.*, Cardiac protein abnormalities in dilated cardiomyopathy detected by two-dimensional polyacrylamide gel electrophoresis. *Electrophoresis* 1998, *19*, 2031–2042.

[15] Foster, D. B., Van Eyk, J. E., In search of the proteins that cause myocardial stunning. *Circ. Res.* 1999, *85*, 470–472.

[16] Schwertz, H., Langin, T., Platsch, H., Richert, J. *et al.*, Two-dimensional analysis of myocardial protein expression following myocardial ischemia and reperfusion in rabbits. *Proteomics* 2002, *2*, 988–995.

[17] Jiang, L., Tsubakihara, M., Heinke, M. Y., Yao, M. *et al.*, Heart failure and apoptosis: Electrophoretic methods support data from micro- and macro-arrays. A critical review of genomics and proteomics. *Proteomics* 2001, *1*, 1481–1488.

[18] Cheung, P. Y., Sawicki, G., Wozniak, M., Wang, W. *et al.*, Matrix metalloproteinase-2 contributes to ischemia-reperfusion injury in the heart. *Circulation* 2000, *101*, 1833–1839.

[19] Noji, Y., Shimizu, M., Ino, H., Higashikata, T. *et al.*, Increased circulating matrix metaloproteinase-2 in patients with hypertrophic cardiomyopathy with systolic dysfunction. *Circ. J.* 2004, *68*, 355–360.

[20] Tziakas, D. N., Chalikias, G. K., Papaioakeim, M., Hatzinikolaou, E. I. *et al.*, Comparison of levels of matrix metalloproteinase-2 and -3 in patients with ischemic cardiomyopathy versus nonischemic cardiomyopathy. *Am. J. Cardiol.* 2005, *96*, 1449–1451.

[21] Yamazaki, T., Lee, J. D., Shimizu, H., Uzui, H., Ueda, T., Circulating matrix metalloproteinase-2 is elevated in patients with congestive heart failure. *Eur. J. Heart Fail.* 2004, *6*, 41–45.

[22] Matsunaga, T., Abe, N., Kameda, K., Hagii, J. *et al.*, Circulating level of gelatinase activity predicts ventricular remodeling in patients with acute myocardial infarction. *Int. J. Cardiol.* 2005, *105*, 203–208.

[23] George, J., Patal, S., Wexler, D., Roth, A. *et al.*, Circulating matrix metalloproteinase-2 but not matrix metalloproteinase-3, matrix metalloproteinase-9, or tissue inhibitor of metalloproteinase-1 predicts outcome in patients with congestive heart failure. *Am. Heart J.* 2005, *150*, 484–487.

[24] Sawicki, G., Leon, H., Sawicka, J., Sariahmetoglu, M. *et al.*, Degradation of myosin light chain in isolated rat hearts subjected to ischemia-reperfusion injury, a new intracellular target for matrix metalloproteinase-2. *Circulation* 2005, *112*, 544–552.

[25] Sawicki, G., Dakour, J., Morrish, D. W., Functional proteomics of neurokinin B in the placenta indicates a novel role in regulating cytotrophoblast antioxidant defences. *Proteomics* 2003, *3*, 2044–2051.

[26] Sawicki, G., Jugdutt, B. I., Detection of regional changes in protein levels in the *in vivo* canine model of acute heart failure following ischemia-reperfusion injury. Functional proteomics studies. *Proteomics* 2004, *4*, 2195–2202.

[27] Perkins, D. N., Pappin, D. J., Creasy, D. M., Cottrell, J. S., Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* 1999, *20*, 3551–3567.

[28] Das, D. K., Engelman, R. M., Kimura, Y., Molecular adaptation of cellular defences following preconditioning of the heart by repeated ischaemia. *Cardiovasc. Res.* 1993, *27*, 578–584.

[29] Barone, F. C., White, R. F., Spera, P. A., Ellison, J. *et al.*, Ischemic preconditioning and brain tolerance: Temporal histological and functional outcomes, protein synthesis requirement, and interleukin-1 receptor antagonist and early gene expression. *Stroke* 1998, *29*, 1937–1950, discussion 1950–1951.

[30] Matsuyama, N., Leavens, J. E., McKinnon, D., Gaudette, G. R. *et al.*, Ischemic but not pharmacological preconditioning requires protein synthesis. *Circulation* 2000, *102*, 312–318.

[31] Das, D. K., Maulik, N., Cardiac genomic response following preconditioning stimulus. *Cardiovasc. Res.* 2006, *70*, 254–263.

[32] Ling, Y., Morgan, K., Kalsheker, N., Amyloid precursor protein (APP) and the biology of proteolytic processing: Relevance to Alzheimer's disease. *Int. J. Biochem. Cell Biol.* 2003, *35*, 1505–1535.

[33] Imamura, T., Potempa, J., Travis, J., Activation of the kallikrein-kinin system and release of new kinins through alternative cleavage of kininogens by microbial and human cell proteinases. *Biol. Chem.* 2004, *385*, 989–996.

[34] McGregor, E., Dunn, M. J., Proteomics of the heart: Unraveling disease. *Circ. Res.* 2006, *98*, 309–321.

[35] Schulz, R., Targets of matrix metalloproteinase-2 in cardiac disease: Rationale and therapeutic approaches. *Annu. Rev. Pharmacol. Toxicol.* 2007, *47*, 211–242.

[36] Spinale, F. G., Myocardial matrix remodeling and the matrix metalloproteinases: Influence on cardiac form and function. *Physiol. Rev.* 2007, *87*, 1285–1342.

[37] Stanley, W. C., Recchia, F. A., Lopaschuk, G. D., Myocardial substrate metabolism in the normal and failing heart. *Physiol. Rev.* 2005, *85*, 1093–1129.

[38] Opie, L. H., Myocardial ischemia – Metabolic pathways and implications of increased glycolysis. *Cardiovasc. Drugs Ther.* 1990, *4*, 777–790.

[39] Honda, H. M., Korge, P., Weiss, J. N., Mitochondria and ischemia/reperfusion injury. *Ann. N. Y. Acad. Sci.* 2005, *1047*, 248–258.

[40] Nagy, N., Malik, G., Fisher, A. B., Das, D. K., Targeted disruption of peroxiredoxin 6 gene renders the heart vulnerable to ischemia-reperfusion injury. *Am. J. Physiol. Heart Circ. Physiol.* 2006, *291*, H2636–H2640.

[41] Cesaratto, L., Vascotto, C., D'Ambrosio, C., Scaloni, A. *et al.*, Overoxidation of peroxiredoxins as an immediate and sensitive marker of oxidative stress in HepG2 cells and its application to the redox effects induced by ischemia/reperfusion in human liver. *Free Radic. Res.* 2005, *39*, 255–268.

[42] Sadayappan, S., Osinska, H., Klevitsky, R., Lorenz, J. N. *et al.*, Cardiac myosin binding protein C phosphorylation is cardioprotective. *Proc. Nat. Acad. Sci.* 2006, *103*, 16918–16923.

[43] Bonifati, V., Rizzu, P., van Baren, M. J., Schaap, O. *et al.*, Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. *Science* 2003, *299*, 256–259.

[44] Andres-Mateos, E., Perier, C., Zhang, L., Blanchard-Fillion, B. *et al.*, DJ-1 gene deletion reveals that DJ-1 is an atypical peroxiredoxin-like peroxidase. *Proc. Natl. Acad. Sci* 2007, *104*, 14807–14812.

[45] Mounier, N., Arrigo, A. P., Actin cytoskeleton and small heat shock proteins: How do they interact? *Cell Stress Chaperones* 2002, *7*, 167–176.

[46] White, M. Y., Cordwell, S. J., McCarron, H. C., Prasan, A. M. *et al.*, Proteomics of ischemia/reperfusion injury in rabbit myocardium reveals alterations to proteins of essential functional systems. *Proteomics* 2005, *5*, 1395–1410.

[47] White, M. Y., Tchen, A. S., McCarron, H. C., Hambly, B. D. *et al.*, Proteomics of ischemia and reperfusion injuries in rabbit myocardium with and without intervention by an oxygen-free radical scavenger. *Proteomics* 2006, *6*, 6221–6233.

