Korean J Physiol Pharmacol
Vol 8: 207–211, August, 2004

Identification of a Marker Protein for Cardiac Ischemia and Reperfusion Injury by Two-Dimensional Gel Electrophoresis and Matrix-Assisted Laser Desorption Ionization Mass Spectrometry

Youngsuk Lee*, Nari Kim*, Hyunju Kim, Hyun Joo, Youngnam Kim, Daehoon Jeong, Dang Van Cuong, Euiyong Kim, Dae Young Hur, Young Shik Park, Yong Geun Hong, Sangkyung Lee, Joonyong Chung, Daehyun Seog, and Jin Han

2020 Cardiovascular Institute, Department of Physiology and Biophysics, College of Medicine, Inje University, Busan 614–735, Korea

The purpose of the present study was to evaluate the expression of cardiac marker protein in rabbit cardiac tissue that was exposed to ischemic preconditioning (IPC), or ischemiareperfusion injury (IR) using two-dimensional gel electrophoresis (2DE) and matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). We compared 2DE gels of control (uninjured) cardiac tissue with those of IPC and IR cardiac tissue. Expression of one protein was detected in IR heart tissue, however the protein was not detected in the samples of control and IPC tissue. To further characterize the detected protein molecule, the protein in the 2D gel was isolated and subjected to trypsin digestion, followed by MALDI-MS. The protein was identified as myoglobin, which was confirmed also by Western blot analysis. These results are consistent with previous studies of cardiac markers in ischemic hearts, indicating myoglobin as a suitable marker of myocardial injury. In addition, the present use of multiple techniques indicates that proteomic analysis is an appropriate means to identify cardiac markers in studies of IPC and IR.

Key Words: Myoglobin, Ischemiareperfusion injury, Cardiac injury marker, Two-dimensional gel electrophoresis, Matrix-assisted laser desorption ionization mass spectrometry

INTRODUCTION

Ischemic heart disease and its clinical correlate (acute myocardial infarction) have a variety of etiologies, and remain to be the largest cause of death in industrialized societies (Carroll et al, 1999). Ischemic heart disease is characterized by insufficient blood supply to regions of the myocardium, which leads to tissue infarction (Braunwald, 1985). To reduce mortality and improve the effectiveness of therapy, many studies have attempted to identify key biomarkers (molecular 'signposts' of the biological state of cells) for ischemic heart disease (Michael et al, 1999; Penttil et al, 1999; Christian, 2001; Svensson et al, 2003)

The major goal of studies on ischemic heart disease is to detect of cardiac tissue-specific biomarkers of myocardial cell injury, and the use of proteomics appears to be able to simultaneously monitor changes in protein expression and cell signaling pathways in response to conditions such as myocardial infarction and heart failure (Li et al, 1992; Cohen et al, 2001; Robert et al, 2001). The treatment of ischemic heart disease has entered a new era, in which mortality can be reduced by approximately 50% by procedures that allow rapid restoration of blood flow, such as reperfusion, to the ischemic zone(s) of the myocardium. However, reperfusion may lead to such complications as diminished cardiac contractile function and arrhythmias (Braunwald, 1985; Ferdinandy & Schulz, 2003). The recent discovery of an endogenous protective cellular mechanism, known as ischemic preconditioning (IPC), has raised hopes that endogenous intracellular pathways and proteins might be activated to avoid necrosis or apoptosis (Li et al, 1992; Przklenk & Kloner, 1998). In the heart, IPC refers to brief period of ischemia that confers protection against infarction that would otherwise be produced by a subsequent long period of ischemia (Yellon & Bexter, 1995; Robert & Thomas, 2001).

Proteomics, including high-resolution two-dimensional electrophoresis (2DE) and mass spectrometry, has changed the approach to detect the expression of disease markers. The purpose of the present study was to evaluate differences in the expression of proteins in ischemic preconditioned (IPC) and ischemia-reperfusion-injured (IR) rabbit heart tissue, using 2DE and matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS), in an at-

Corresponding to: Jin Han, Department of Physiology and Biophysics, College of Medicine, Inje University, Busan 614-735, Korea. (Tel) +82-51-890-6714, (Fax) +82-51-894-4500, (E-mail) phyhanj@ijnc.inje.ac.kr
*These two authors contributed equally to this work.

ABBREVIATIONS: IPC, Ischemic preconditioning; IR, Ischemia-reperfusion; 2DE, Two-dimensional gel electrophoresis; MALDI-MS, Matrix-assisted laser desorption mass spectrometry; IPG, Immobilized pH gradient; IEF, Isoelectric focusing; PBS-TB, Phosphate-buffered saline tween-20 buffer.

tempt to identify cardiac tissue-specific markers that reflect ischemiareperfusion injury and/or ischemic preconditioning protection after an ischemic insult.

METHODS

Preparation of isolated heart

Rabbits that had been anesthetized with sodium pentobarbitone were injected with sodium heparin via the femoral vein. Hearts were rapidly excised, placed in cold bicarbonate buffer, cannulated, and then perfused at 37°C with bicarbonate buffer that was gassed with 95% O_2/5% CO_2. Perfusion was carried out in the non-recirculating Langendorff mode at a constant flow of 12 ml/g tissue per min. The bicarbonate buffer contained (in nmol/L): NaCl, 118.5; KCl, 3.1; KH_2PO_4, 1.18; $MgCl_2$, 1.2; $NaHCO_3$, 25.0; $CaCl_2$, 1.4; and glucose, 10.0.

Perfusion protocols

We prepared IPC and IR cardiac tissue according to the perfusion protocols summarized in Fig. 1, while cardiac tissue that was not injured was used as control. In experiments that involved whole-heart high-flow ischemia, ischemia was initiated by terminating perfusion while the heart was maintained at 37°C. Reperfusion was achieved by reinitiating perfusion for a specific period of time. Cardiac tissue that was not injured was used as a control.

Preparation of samples for two-dimensional gel electrophoresis

Frozen cardiac tissue was transferred to a mortar that contained liquid nitrogen, and a precooled pestle was used to homogenize the tissue. The powdered tissue was transferred to a 50-ml tube that contained lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris base, 1% DTT, 0.5% IPG buffer, 0.5% Triton X-100, and protease inhibitor cocktail) and vortexed at 4°C. The homogenate was then subjected to ultracentrifugation (60,000 rpm, 5 h, 4°C in a Beckman 100Ti rotor), after which the supernatant that contained the cytosolic proteins was harvested.

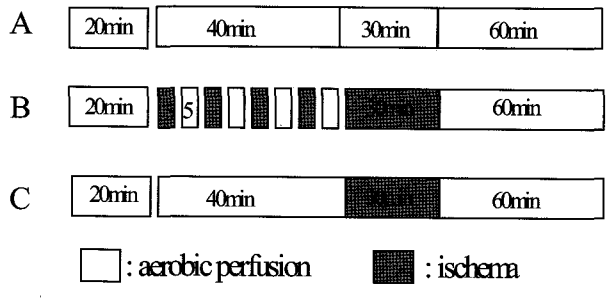

Fig. 1. Perfusion protocols for isolated rabbit hearts used in the present study. A, control; B, ischemic preconditioning; C, ischemiareperfusion injury. Numbers refer to time in minutes.

Two-dimensional gel electrophoresis and image analysis

A 24-cm (pH 3~10) immobilized pH gradient (IPG) strip (Amersham, Uppsala, Sweden) was hydrated overnight at 30 V in 450 μL of isoelectric focusing (IEF) solution that contained approximately 500 μg of the solubilized cytosolic proteins. IEF was carried out at 60,000 Vh at 20°C, as follows: 500 V for 1 h, 1,000 V for 1 h, and finally 8,000 V incremented to 60,000 Vh. The IPG strips were placed in 10 mL of an equilibration solution [50 mM Tris-HCl, pH 8.8, containing 6 M urea, 30% glycerol, 2% sodium dodecyl sulfate (SDS), and bromophenol blue] that contained 1% DTT (v/v) during the first equilibration step and 2.5% iodoacetamide (v/v) during the second equilibration step (15 min per equilibration step).

Second dimensional separation was carried out, using the Ettan DALTtwelve System (Amersham). The IPG strips were loaded onto a 12% SDS-polyacrylamide (SDS-PAGE) gel, running buffer (25 mM Tris, 2 M glycine, 3.5 mM SDS, pH 8.3) was added, and a constant current (5 W/gel) was applied for 6 h. The gels were then stained with silver nitrate. The stained gels were scanned on a flatbed scanner, and the images that were acquired were analyzed, using commercially available software (Image Master 2D Elite; Amersham).

Identificationn of the protein

For mass spectrometry fingerprinting, the portion of the 2D gel that was stained (see above) was excised, and this gel fragment was then destained with 100 mM sodium thiosulfate and 30 mM potassium ferricyanide. The gel fragment was then washed with 50% acetonitrile which was then removed, and the fragment was dried in a vacuum centrifuge. Dried gel fragments were rehydrated in 20 μL of 25 mM NH_4HCO_3 that contained 0.5 μg of sequencing grade trypsin (Promega, Madison, WI), and they were incubated overnight at 37°C. The supernatant was then collected, and peptides were extracted twice with 30 μL of 50 mM NH_4HCO_3: acetonitrile (ACN) (1 : 1) mixture. The combined extracts were evaporated in a vacuum centrifuge. Aliquots of the peptide-containing samples were applied to a target disk, and the aliquots were letf to evaporate. Spectra were obtained using a Voyager DE PRO MALDI-MS (Applied Biosystems, Foster City, CA, USA). Protein databases were searched with MS-Fit (http:/prospector.ucsf.edu/ucsfhtml3.4/msfit.htm) using monoisotopic peaks. A mass tolerance within 50 ppm was allowed at first, after which a recalibration was performed using the list of proteins that was obtained at 20 ppm.

Western blotting

Proteins were separated on 12% SDS-PAGE gels and transferred onto Immobilon membranes (Millipore, Bedford, MA, USA), using a semi-dry transfer blotter. The membrane was blocked for 1 h in a solution (PBS-TB) of phosphate-buffered saline (PBS; 4 mM KH_2PO_4, 16 mM Na_2HPO_4, 115 mM NaCl, pH 7.4) containing 0.1% (v/v) Tween-20 and 1% bovine serum albumin (BSA). The membrane was then incubated for 60 min in the presence of polyclonal anti-myoglobin antibody. After washing (3×10 min) in PBS-TB, the blot was incubated for 45 min in PBS-TB that contained horseradish peroxidase-conjugated

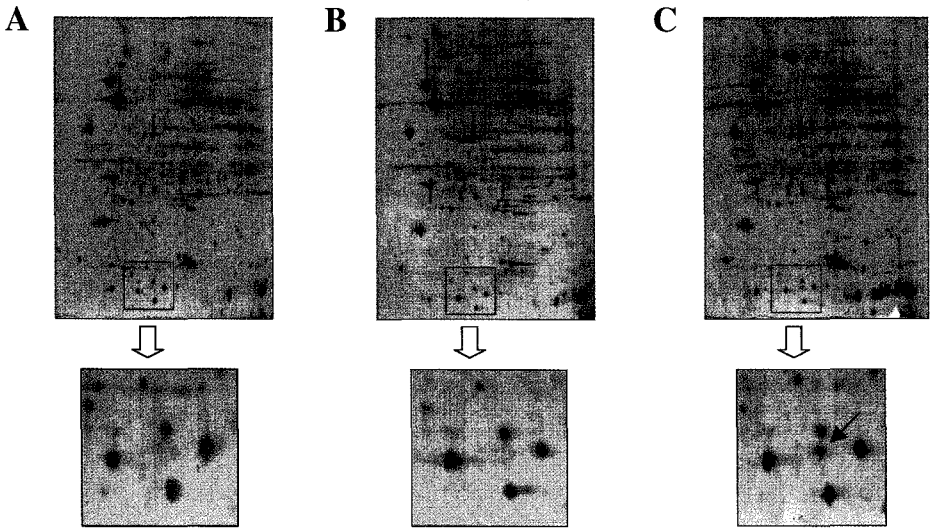

Fig. 2. Two-dimensional gel electrophoretic analysis of cytosolic proteins in rabbit heart tissue. Paired samples of control, ischemic preconditioned, and ischemiareperfusion-injured tissue are shown. Differentially expressed proteins are marked in blue boxes. A, control; B, ischemic preconditioned; C, ischemiareperfusion-injured. The arrow in C indicates the protein spot that corresponds to myoglobin.

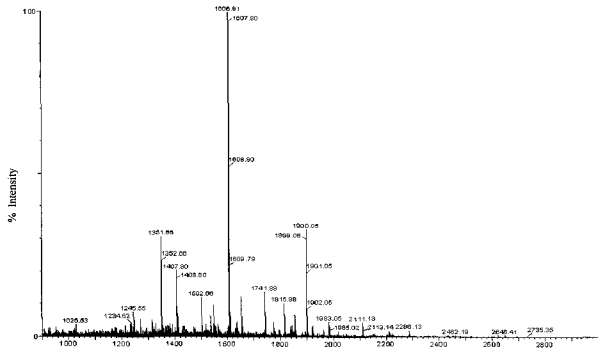

Fig. 3. Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) peptide mass spectrum for myoglobin. Monoisotopic peptide masses were used to search protein databases for matches, which were subsequently used to identify individual proteins. T, trypsin-digested autolytic fragments.

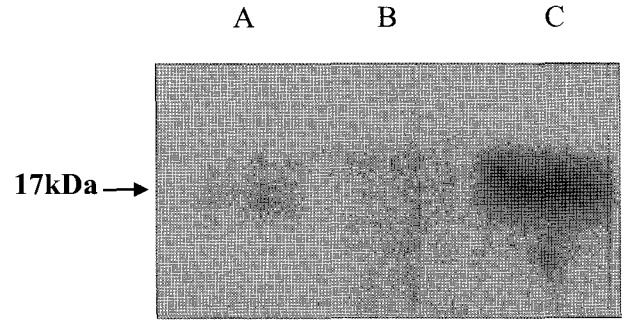

Fig. 4. Western immunoblot with a polyclonal anti-myoglobin antibody. A 17-kDa immunoreactive band was detected only in the ischemiareperfusion-injured tissue samples. A, control; B, ischemic preconditioned; C, ischemiareperfusion-injured.

secondary antibody at a dilution of 1 : 2,000. Goat anti-human IgG s secondary antibody was used to detect myoglobin.

RESULTS

Two-dimensional gel electrophoretic analysis of cardiac tissue samples

In 2DE gels of IPC and IR cardiac tissues, more than 400 protein spots were detected. The protein spots were localized in the pI 3~10, and the range of relative molecular masses was 10~200 kDa. A representative 2DE gel is shown in Fig. 2. Analysis of the silver staining spot patterns of control (a), IPC (b), and IR (c) tissues revealed a spot that was expressed in IR tissue samples in three different 2DE gels, however, it was almost undetectable in the control and IPC cardiac tissues (Fig. 2).

Identification of myoglobin as the cardiac marker

To further elucidate the molecular characteristics of the protein that was detected in the 2DE gels above, the spot in the 2DE gel of the IR cardiac tissue was isolated and subjected to MALDI-TOF analysis. Mass fingerprinting of selected peptide peaks, using low tolerance (< 20 ppm) with recalibration, revealed that this protein was myoglobin (Fig. 3). Myoglobin was identified with score of 1.8e-12 and sequence coverage of 99% (Table 1). The results of the 2DE gel analysis, therefore, suggest that cardiac injury is correlated with myoglobin expression.

Table 1. Summary of matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) masses and partial amino acid sequence of myoglobin. Result of the database search using MS-Fit. Myoglobin was identified with score of 1.8e-12 and sequence coverage of 99%

Masses submitted	MH+ matched	Position	Proposed peptide sequence
1351.71	1352.54	32~42	(R)LFHTHPETLEK(F)
1407.83	1408.69	64~77	(K)HGNTVLTALGAILK(K)
1502.67	1503.64	119~133	(K)HPGDFGADAQAAMS(K)
1606.85	1607.81	17~31	(K)VEADLAGHGQEVLIR(L)
1741.89	1742.98	32~45	(R)LFHTHPETLEKFDK(F)
1814.94	1816.08	1~16	(-)GLSDAEWQLVLNVWG(K)
1899.03	1900.24	103~118	(K)YLEFISEAIIHVLHAK(H)
1982.05	1983.25	79~96	(K)KGHHEAEIKPLAQ-SHATK(H)
2119.11	2120.39	80~98	(K)GHHEAEIKPLAQSH-ATKHK(I)
2939.54	2941.34	17~42	(K)VEADLAGHGQE-VLIRLFHTHPETLEK(F)

PPM, parts per million.

Immunoblotting

When the tissue homogenates were examined with an anti-myoglobin antibody by Western blot, a single immunopositive bandwas detected that corresponding to a protein with a molecular mass of ~17 kDa, being consistent with the molecular weight of myoglobin. The myoglobin- positive band was observed only in the IR cardiac tissue sample.

DISCUSSION

Recent advances in genomics and proteomics provide a great potential for diagnostic, prognostic, and therapeutic applications (Srinivas et al, 2001). Comparisons of gene transcription and corresponding protein expression have revealed that the levels of mRNA and protein expression are not necessarily closely correlated (Charlwood et al, 2000; Larsson et al, 2000). Therefore, proteomic methods have been devised to detect the functional units of expressed genes using protein fingerprints (Wilkins et al, 1996; Wilkins & Mann, 2000). Such proteomic methods use biomarkers to reveal both the intrinsic genetic changes within a cell and the impact of environment of the cell. For example, the molecular events that occur during neoplastic progression are complex and diverse, and they have not fully been characterized. The identification, quantification, classification, and functional assignment of proteins are essential to fully understand such molecular events (Ryu et al, 2003).

In the present study, we investigated the differential expression of proteins in ischemia-injured cardiac tissue by using proteomics. One protein was found to be expressed in IR, but not in IPC tissue, and this protein was identified as myoglobin. Myoglobin is a 17,800-Da heme protein that is present in the cytosol of skeletal and cardiac muscle. It transports oxygen from hemoglobin to the terminal mitochondrial oxidase, and is released from damaged muscle (Antonini & Brunori, 1971; Wittenberg & Wittenberg, 1989; Jannie et al, 1995; Esquerra et al, 1998; Alderton et al, 2003). Myoglobin that is released from damaged muscle has been suggested to exacerbate the vascular and tissue injury that is associated with myocardial ischemia, rhabdomyolysis, and crush syndrome (Galaris et al, 1989; Moore et al, 1998; Holt & Moore, 2000; Alaya et al, 2001; Agnillo & Alayash, 2002). Because of its small size, myoglobin is released rapidly from the areas of ischemic injury for a limited time, and is transferred to serum (Klocke et al, 1982). Furthermore, myoglobin has been shown to be a very early marker of myocardial necrosis, and the expression of myoglobin precedes the release of creatine kinase MB isoenzyme (CK-MB) by 2~5 h (Drexel et al, 1983; Grenadiere et al, 1983). Therefore, the early increase in myoglobin expression in the patients with acute myocardial infarction has the potential to be an ideal biomarker for early detection and treatment of thrombosis (Jannie et al, 1995).

The results in the present study are consistent with those of previous studies in which the expression of cardiac ischemia associated biomarkers was examined. Although myoglobin has been well characterized as a biomarker, it has nevertheless been considered to be not an ideal marker of cardiac injury, because of lack of specificity (Hudson et al, 1999). The present results challenge this notion: Our finding that myoglobin was specifically expressed in IR, but not in IPC cardiac tissue, suggests that myoglobin is a suitable biomarker of myocardial injury. Moreover, our present aforementioned observation suggests that ischemic preconditioning protects the heart from ischemic insult. Our present successful use of multiple techniques, including 2DE, MALDI-MS, and Western blot, demonstrate that proteomic analysis can be an appropriate means of identifying cardiac biomarkers of ischemia-induced cardiac injury.

ACKNOWLEDGMENTS

This work was supported by Korea Research Foundation Grant (KRF-2002-042-E00006).

REFERENCES

Agnillo FD, Alayash AI. A role of the myoglobin redox cycle in the induction of endothelial cell apoptosis. *Free Radical Biology & Medicine* 33: 1153-1164, 2002

Alaya AI, Patel RP, Cashon RE. Redox reactions of hemoglobin and myoglobin: biological and toxicological implications. *Antioxid Redox Signal* 3: 313-327, 2001

Alderton AL, Faustman C, Liebler DC, Holl DW. Induction of redox instability of bovine myoglobin by adduction with 4-hydroxy-2-nonenal. *Biochemistry* 42: 4398-4405, 2003

Antonini E, Brunori M. Hemoglobin and myoglobin in their reactions with ligands. *Elsevier, New York*, 1971

Braunwald EK. Myocardial reperfusion: a double- edged sword? *J Clin Invest* 76:1713-1719, 1985

Carroll R, Walker JM, Yellon DM. Myocardial protection: The role of the KATP channel. *J Clin Basic Cardiol* 2: 8, 1999

Charlwood J, Skehel FM, camilleri P. Analysis of N-linked oligosaccharides released from glycoproteins separated by two-dimensional gel electrophoresis. *Anal Biochem.* 284: 49-59, 2000

Christian WH. Cardiac biomarkers for rapid evaluation of chest pain. *Circulation* 104: 1454-1456, 2001

Cohen MW, Baines CP, Downey JM. Ischemic preconditioning: from adenosine receptor to K_{ATP} channel. *Annu Rev Physiol* 62:79-109, 2000

Drexel H, Dworzak E, Kirchmair W. Myoglobinemia in the early

phase of acute myocardial infarction. *Am Heart J* 105: 642–651, 1983

Esquerra RM, Goldbeck RA, Kim-shapiro DB, Kliger DS. Spectroscopic evidence for nanosecond protein relaxation after photodissociation of myoglobin-CO. *Biochemistry* 37: 17527–17536, 1998

Ferdinandy P, Schulz R. Nitric oxide, superoxide, and peroxynitrite in myocardial ischemia-reperfusion injury and preconditioning. *British Journal of Pharmacology* 138:532–543, 2003

Galaris D, Eddy L, Arduini A, Cadenas E, Hochstein P. Mechanisms of reoxygenation injury in myocardial infarction implications of a myoglobin redox cycle. *Biochem Biophys Res Commun* 160: 1162–1168, 1989

Grenadier E, Keidar S, Kahana L. The role of serum myoglobin, total KCP and CK-MB isoenzyme in the acute phase of myocardial infarction. *Am Heart J* 105: 408–416, 1983

Holt S, Moore K. Pathogenesis of renal failure in rhabdomyolysis: the role of myoglobin. *Exp Nephrol* 8: 72–76, 2000

Hudson MP, Christenson RH, Newby LK, Kaplan AL, Ohman EM. Cardiac markers: point of care testing. *Clinica Chimica Acta* 284: 223–237, 1999

Jannie W, Felocotas LL, Robert RS, David L, John BM. Is myoglobin useful in the diagnosis of acute myocardial infarction in the emergency department setting? *Am J Clin Phathol* 103: 725–729, 1995

Jannie W, Felocotas LL, Robert RS, David L, John BM. Is myoglobin useful in the diagnosis of acute myocardial infarction in the emergency department setting? *Am J Clin Phathol* 103: 725–729, 1995

Katrin M, Dorian I, Joachim S, Helmut EM. Identification of platelet proteins separated by two-dimensional gel electrophoresis and analyzed by matrix-assisted laser desorption/ionization-time tyrosine-phosphorylated proteins. *Electrophoresis* 21: 2622–2636, 2000

Klocke F, Copley DP, Krawczyk A, Leichlin M. Rapid renal clearance of immunoreactive canine plasma myoglobin. *Circulation* 65: 1522–1528, 1982

Larsson T, Bergstrom J, Nilsson C, Karlsson KA. Use of an affinity proteomics approach for the identification of low-abundant bacterial adhesions as applied on the Lewis (b)-binding adhesin of Helicobacter phlori. *FEBS Lett* 469: 155–158, 2000

Li Y, Whittaker P, Kloner RA. The transient nature of the effect of ischemic preconditioning on myocardial infarct size and ventricular arrhythmias. *Am Heart J* 23:346–353, 1992

Mark PM, Ben RH, Martin BS, Thierry R, Amanda SN, Keith LW, Andrew AG. Proteomic analysis of *Escherichia coli* outer membrane. *Eur J Biochem* 267: 2871–2881, 2000

Michael PH, Robert HC, Newby LK, Andrew LK, Ohman EM. Cardiac markers: point of care testing. *Clinica Chimica Acta* 284: 223–237, 1999

Moore KP, Holt SG, Patel RP, Svistunenko DA, Zackert W, Goodier D, Reeder BJ, Clozel M, Anand R, Cooper CE, Morrow JD, Wilson MT, Darley-Usmar V, Roberts LJ. A causative role for redox cycling of myoglobin and its inhibition by alkalinization in the pathogenesis and treatment of rhabdomyolisis-induced renal failure. *J Biol Chem* 273: 31731–31737, 1998

Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: A delay of lethal cell injury in ischemic myocardium. *Circulation* 75:1124–1136, 1986

Park KS, Kim HG, Kim NG, Cho AY, Choi KH, Seong JK, Paik YK. Proteomic analysis and molecular characterization of tissue ferritin light chain in hepatocellular carcinoma. *Hepatology* 35: 1459–1466, 2002

Penttil K, Koukkunen H, Kemppainen A, Halinen M, Rantanen T, Pyrl K, Penttil I. Myoglobin, creatine kinase MB, troponin T, and troponin I- rapid bedside assays in patients with acute chest pain. *Int J Clin Lab Res* 29: 93–101, 1999

Przklenk K, Kloner RA. Ischemic preconditioning: Exploring the paradox. *Pro Cardiovasc Dis* 40: 516–547, 1998

Robert AK, Thomas S, Chrisyopher PC, and Karin P. Ischemic preconditioning: Implications for the geriatric heart. *AJGC* 10: 145–151, 2001

Robert AK, Thomas S, Chrisyopher PC, Karin P. Ischemic preconditioning: Implications for the geriatric heart. *AJGC* 10:145–151, 2001

Ryu JW, Kim HJ, Lee YS, Myong NH, Hwang CH, LeeGS, Yom HC. The proteomics approach to find biomarkers in gastric cancer. *J Korean Med Sci* 18:505–509, 2003

Srinivas PR, Kramer BS, Srivastava S. Trends in biomarker research for cancer detection. *Lancet Oncol* 2: 698–704, 2001

Svensson L, Axlsson C, Nordlander R, Herlitz J. Elevation of biochemical markers of myocardial damage prior to hospital admission in patients with acute chest pain or other symptoms raising suspicion of acute coronary syndrome. *Journal of Internal Medicine* 253: 311–319, 2003

Wilkins A, Mann M. Proteomics to study genes and genomes. *Nature* 405: 837–846, 2000

Wilkins MR, Sanchez JC, Gooley AA, Appel RD, Humphery-Smith L, Hochstrasser DF, Williams KL. Progress with proteome projects: why all proteins expressed by a genome should be identified and how to do it. *Biotechnol Gen Eng Rev* 13: 19–50, 1996

Wittenberg BA, Wittenberg JB. Transport of oxygen in muscle. *Annu Rev Physiol* 57: 857–878, 1989

Yellon DM, Bexter GF. A 'second window of protection' or delayed preconditioning phenomenon: Future horizons for myocardial protection. *J Mol Cell Cardiol* 27: 1023–1034, 1995