Journal of Cellular Biochemistry 86:509–519 (2002)

Altered Expression and Localization of *N*-Myristoyltransferase in Experimentally Induced Rat Model of Ischemia-Reperfusion

Raju V.S. Rajala,[1] Rakesh Kakkar,[1] Rani Kanthan,[1] Jasim M. Radhi,[1] Xintao Wang,[2] Rui Wang,[2] Raju S.S. Datla,[3] and Rajendra K. Sharma[1]*

[1]Department of Pathology and Saskatoon Cancer Centre, College of Medicine, University of Saskatchewan, Saskatoon, Saskatchewan, S7N 4H4, Canada
[2]Department of Physiology, College of Medicine, University of Saskatchewan, Saskatoon, Saskatchewan, S7N 4H4, Canada
[3]Plant Biotechnology Institute, National Research Council, Saskatoon, Saskatchewan, S7N 0W9, Canada

Abstract *N*-myristoyltransferase (NMT) catalyzes the attachment of myristate onto the amino-terminal glycine residue of select polypeptides. In the present study, we investigated the expression and activity of NMT in rat heart after ischemia and reperfusion. Western blot analysis of rat heart samples indicated a prominent immunoreactive band of 66 kDa probed with human NMT antibody. Both the expression and activity of NMT were increased by ischemia-reperfusion. Immunohistochemical studies showed cytosolic localization of NMT in normal rat heart and predominant nuclear localization after ischemia followed by reperfusion. The pre-ischemic perfusion and post-ischemic reperfusion of hearts with a cell-permeable calpain inhibitor (*N*-Ac-Leu-Leu-methioninal) suppressed the increase in calpain expression and reversed the localization of NMT from nucleus to cytoplasm. This is the first study demonstrating the expression and alteration of NMT localization in cardiac ischemia and pertaining to a possible role of co-translational modification of proteins in cardiac functions and injury. J. Cell. Biochem. 86: 509–519, 2002.

Key words: *N*-myristoyltransferase; c-Src; tyrosine kinase; ischemic heart; calpain

Intracellular proteolysis by the calpains, members of the Ca^{2+} activated cysteine protease family, is an ubiquitous yet a poorly understood process. These proteases are implicated in an array of cellular and pathological processes, including long-term potentiation, synaptic remodeling, protein kinase C-mediated and steroid receptor activation, ischemic cellular injury, and apoptosis [Caroall and Demartino, 1991; Saido et al., 1994; Kawasaki and Kawashima, 1996; Stabach et al., 1997]. There are at least two types of calpains (μ- and *m*-calpains) which require a micromolar and a millimolar concentration of Ca^{2+} for activation, respectively. The calpain activity is physiologically regulated by an endogenous calpain specific inhibitor, calpastatin [Murachi, 1997]. Calpains hydrolyze various endogenous proteins [Billger et al., 1988; David et al., 1993] including calmodulin-binding proteins [Kosaki et al., 1983; Wang et al., 1989; Barnes and Gomes, 1995], and components of receptor signaling pathways [Inoue et al., 1977; Magnuson et al., 1993]. Additionally, various transcription factors, including API (c-Fos/c-Jun), AP 2, Pit-1, Oct 1, CP 1a and b, c-Myc, ATF/CREB and AP 3, have been found to be cleaved by *m*-calpain to produce specific partial digestion products [Hirai et al., 1991; Watt and Molloy, 1993].

N-myristoyltransferase (NMT) catalyzes the co-translational transfer of myristate from

Raju V.S. Rajala's present address is Department of Ophthalmology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104.

Rakesh Kakkar's present address is Wyeth Lederlee Vaccines, Pearl River, New York.

Jasim M. Radhi's present address is McMaster University Medical Center Site, Hamilton, Ontario L8N3ZS, Canada.

*Correspondence to: Rajendra K. Sharma, PhD, Department of Pathology and Saskatoon Cancer Centre, College of Medicine, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 4H4. E-mail: rsharma@scf.sk.ca

Received 17 May 2002; Accepted 21 May 2002
DOI 10.1002/jcb.10248

myristoyl-CoA to the amino-terminal glycine residue of selected polypeptides [see reviews, Bhatnagar and Gordon, 1998; Resh, 1999; Rajala et al., 2000]. The catalytic subunit of cyclic AMP-dependent protein kinase was the first protein shown to contain an *N*-myristoyl group [Carr et al., 1982]. *N*-myristoylation was subsequently found in other proteins such as Rous sarcoma virus protein product ($pp60^{v\text{-}src}$), the glycosaminoglycan polyprotein of retroviruses (including human immunodeficiency virus), Gi-subunits and calcineurin [Bhatnagar and Gordon, 1998; Resh, 1999; Rajala et al., 2000]. Myristate appears to be critical for mediating protein–protein interactions or protein–membrane interactions required for expression of the biological activities of many *N*-myristoyl proteins. The catalytic subunit of cAMP-dependent protein kinase and the β-subunit of calcineurin are soluble myristoylated proteins localized in the cytoplasm [Aitken et al., 1982; Carr et al., 1982; Bhatnagar and Gordon, 1998; Resh, 1999; Rajala et al., 2000]. The myristoylation of these proteins is thought to be essential for the interaction between the regulatory and catalytic subunits of cAMP-dependent protein kinase and the β-subunits of calcineurin, respectively [Aitken et al., 1982; Carr et al., 1991]. Studies of the dephosphorylation of the catalytic subunit of myristoylated and non-myristoylated cAMP-dependent protein kinase at Thr-197 by cellular protein phosphatase and protein phosphatase-2A (PP-2A) have indicated that the myristoylated C subunit was more resistant to dephosphorylation than the non-myristoylated enzyme [Liauw and Steinberg, 1996]. The behaviour of cAMP-dependent protein kinase, which contains PEST [polypeptide sequences enriched in proline (P), glutamic acid (E), serine (S), and threonine (T)] sequences, supports the hypothesis that dissociation promotes enzyme degradation. The enzyme dissociates into R_2 and C_2 dimers in the presence of cAMP, the dimers are degraded much faster than the holoenzyme [Steinberg and Agard, 1981]. Both subunits have been reported to have strong PEST sequences, when they are dissociated. The subunits are then fully exposed to proteolytic degradation [Rechsteiner and Rogers, 1996].

Previously we have demonstrated that the degradation of cardiac muscle NMT by *m*-calpain in vitro resulted in the abolishment of NMT activity [Raju et al., 1998]. The role for calpains has been suggested in the setting of myocardial ischemia/reperfusion injury [Iizuka et al., 1993; Yoshida et al., 1995a], myocardial stunning [Gao et al., 1996], and cardiac hypertrophy [Arthur and Belcastro, 1997]. Intracellular Ca^{2+} overloading is implicated in the myocardial injury that results during ischemia/reperfusion and is considered to be a pivotal event in cell death [Gao et al., 1996]. An increase in Ca^{2+} influx can activate dormant Ca^{2+}-dependent enzymes, including calpains, causing damage to structural proteins (myocardial troponin, calspectin (fodrin), microtubules) thereby leading to membrane breakdown and eventually cell death [Maki et al., 1990; Yoshida et al., 1995b]. The NMT substrates of Src family tyrosine kinases, such as Src and Lck, play an important role in the genesis of late ischemic preconditioning (IPC) by serving as downstream elements of PKC-mediated signal transduction [Ping et al., 1999]. IPC confers cardioprotection against a prolonged ischemic insult. Tyrosine kinase inhibitors have been shown to attenuate IPC [Fryer et al., 1998]. All Src family members are regulated through N-terminal myristoylation [Bhatnagar and Gordon, 1998; Resh, 1999; Rajala et al., 2000]. Therefore, an attempt was made to study the expression profile of NMT in the experimentally induced ischemia/reperfusion rat model. In this study, we have used a rat model to investigate the effect of ex vivo regional myocardial ischemia and reperfusion on the expression, activity and the localization of NMT and have assessed the protective effect of cell-permeable calpain inhibitor (*N*-Ac-Leu-Leu-methioninal) on NMT during reperfusion myocardial injury.

MATERIALS AND METHODS

Materials

[1-^{14}C]-myristoyl-CoA (54.7 mCi/mmol) was obtained from Amersham International. *Pseudomonas* acyl-CoA synthetase and coenzyme A were obtained from Sigma. Nitrocellulose membranes were purchased from Bio Rad laboratories. General laboratory chemicals were of analytical grade.

Peptide Synthesis

The following peptide was synthesized from Alberta Peptide Institute, Canada: Gly-Ser-Ser-Lys-Ser-Ser-Lys-Pro-Lys-Arg (based on the NH_2-terminal sequence of $pp60^{src}$ tyrosine kinase).

Isolated Heart Preparation and Perfusion

All procedures for animal experimentation were undertaken according to the guidelines of the Canadian Council on Animal Care. Male Sprague Dawley rats (250–275 g) were anesthetized with sodium somnitol (0.1 ml/100 g body weight) and heparinized (500 IU) intravenously. The hearts were excised, washed in ice-cold oxygenated Krebs-Hanseleit (KH) buffer solution, pH 7.4, containing in mM: NaCl 118, KCl 4.8, $CaCl_2$ 1.25, $MgSO_4$ 0.86, KH_2PO_4 1.2, $NaHCO_3$ 2.54, glucose 11.1, EDTA 0.027, and L-ascorbic acid 0.057 and then mounted on a Langendorff heart perfusion apparatus. The hearts were perfused at a constant pressure of 10 kPa (100 cm H_2O) at 37°C with KH buffer. The KH buffer was filtered through a 0.8 μm cellulose acetate membrane (to remove particulate contaminants, which otherwise block the coronary circulation) and was continuously gassed with a mixture of 95% O_2 and 5% CO_2. Left ventricular pressure (LVP) was monitored by a saline-filled latex balloon inserted into the left ventricle by way of the left atrium and connected to a pressure transducer. Epicardial electrogram (EPI-ECG) was recorded with two electrodes positioned one to the aorta and the other to the apex of the heart. The effluent of the perfusate was collected for the determination of coronary flow (CF). Data acquisition and analysis were done using a BioPac System (Biopac System, Inc., Golato) including TC 100 amplifiers and Acknowledge Software (3.01), universal modules and Macintosh Computer.

Experimental Protocol

Regional ischemia was induced by occluding the left anterior descending artery with a ligature positioned around and at a point close to its origin [Wang et al., 1999]. The ligature was first loosely tied over a piece of plastic tubing and sustained regional ischemia was achieved by tying the ligature tightly around the tube. A successful occlusion was confirmed by 40–50% LVP reduction, 60–70% LVDP elevation, and 40–50% reduction in coronary flow, compared with pre-ischemic values. At the end of the ischemic period, reperfusion was achieved by cutting the ligature on the plastic tubing with a scalpel blade and rhythmic disturbances were monitored for the times indicated for the various groups. After an initial 15–20 min of equilibration, the cardiodynamic variables were continuously monitored during the ischemia and reperfusion protocol period. Five experimental groups were studied:

Group 1 (Control group): Hearts were perfused with KH buffer without interference of ischemia or reperfusion (n = 5).

Group 2 and 3: Hearts underwent ischemia for 15 min and reperfusion for 5 or 15 min (n = 4 for each group).

Group 4: Hearts were perfused with calpain peptide inhibitor (100 μM dissolved in 0.1% DMSO) for 5 min before 15 min of ischemia and again during 15 min of reperfusion (n = 5).

Group 5: Hearts were perfused with 0.1% DMSO alone as described in Group 4 (n = 4).

After completion of the experiment for each heart, a small piece of left ventricle tissue sample was taken and stored in 10% buffered formalin and embedded in paraffin for immunohistochemical and studies. The rest of the left ventricle tissue sample was taken for biochemical studies and kept at −70°C until use.

Human Tissue Sample

Human heart samples were collected from the Royal University Hospital, Saskatoon, Saskatchewan at autopsy. To minimize the effect of autolysis on post-mortem tissues, we collected samples within 24 h after death, from bodies kept at 4°C.

Tissue Homogenization

The rat heart tissue samples were homogenized at 4°C in 100 mM Tris, pH 7.4 containing 1 mM EDTA, 10 mM 2-mercaptoethanol, 1 mM benzamidine, and 0.2 mM phenyl methyl sulfonyl fluoride with a Polytron homogenizer four times each for 30 s at the maximum speed with a cooling interval of 15 s. The homogenates were centrifuged at 1000*g* for 20 min. The supernatants were further centrifuged at 100,000*g* for 60 min, the pellet was discarded and the supernatants were used for various biochemical studies.

Production of Polyclonal NMT Antibodies

New Zealand white rabbits were immunized by subcutaneous injection of 100 μg of *E.coli* expressed recombinant human NMT and the

antisera were purified according to the method described earlier [Raju et al., 1997].

Western Blot Analysis

The protein expression of NMT in heart tissues of different groups was determined by the immunoblotting method [Towbin et al., 1979]. Equal amount of protein from normal and ischemic heart supernatants (100,000*g*) of different groups were separated on 10% SDS–PAGE, electroblotted onto nitrocellulose membrane and probed with NMT antibody at a dilution of 1:1000. The immunoreactive proteins were visualized by chemiluminescence reagent (NEN Life Science Products, Boston) and employing Kodak autoradiography film.

Immunohistochemistry

Immunohistochemistry studies were performed on human ischemic myocardium obtained from autopsy material and also on rat ischemic samples from different groups as described above. A polyclonal NMT antibody was applied with appropriate negative controls following the exclusion of primary antibody [Hsu et al., 1981]. Approximately 5 μm thick sections of rat heart from different groups and human ischemic samples were cut and subjected to the avidin-biotin complex method as previously described [Hsu et al., 1981]. The NMT antibody was used as the primary antibody at a concentration of 14 μg/ml.

Assay for *N*-Myristoyltransferase

NMT activity was carried out according to the method described [King and Sharma, 1991; Rajala and Sharma, 1999]. For the standard enzyme assay, the reaction mixture contained 50 μM [1-^{14}C]myristoyl-CoA, 225 mM Tris-HCl, pH 7.4, 0.5 mM EGTA, 0.45 mM 2-mercaptoethanol, 1% Triton X-100, 500 μM pp 60^{src}—derived peptide and heart tissue lysates as NMT source in a total volume of 25 μl. The transferase reaction was initiated by the addition of radiolabeled myristoyl-CoA and was incubated at 30°C for 10 min. The reaction was terminated by spotting aliquots of incubation mixture onto P81 phosphocellulose paper discs and drying them under a stream of warm air. The P81 phosphocellulose paper discs were washed in three changes of 40 mM Tris-HCl, pH 7.3, for 90 min. The radioactivity was quantified in 7.5 ml of Beckman Ready Safe Liquid Scintillation mixture in a Beckman Liquid Scintillation Counter. One unit of NMT activity was expressed as one pmol of myristoylated peptide formed per min.

Other Methods

Polyacrylamide gel electrophoresis in the presence of SDS was carried out according to the procedure [Laemmli, 1970]. Protein concentration was determined as described [Bradford, 1976] using bovine serum albumin as a standard.

RESULTS

The polyclonal antibody raised against human NMT recognized an immunoreactive band of 66 kDa (Fig. 1). We examined levels of protein expression in normal and ischemic/reperfused heart tissues. Western blot analysis of normal and ischemic heart supernatants indicated a low level of NMT expression in normal heart tissue; however, an increase in protein expression was observed with the duration of ischemia and repurfusion (Fig. 1A,B). In Group 4, cell-permeable calpain inhibitor did not alter the expression of NMT which was similar to Group 2 and 3, respectively (Fig. 1A,B).

Since we have shown an increase in protein expression during ischemia and reperfusion (Fig. 1A,B), we also compared NMT activity. Figure 1C shows the activity of NMT, which was found to be higher in Group 2, 3, and 4 compared to normal heart tissue, Group 1. These findings suggest that NMT expression correlates well with NMT activity (Table I). In Group 4, the calpain inhibitor appears to decrease the activity of NMT. It is interesting to note that rat hearts perfused with DMSO alone (Group 5) has a slightly stimulatory effect on NMT activity without any effect on the gene expression at protein level (Fig. 1A). Theoretically, the concentration of DMSO we used in this study (0.1%) should not exert any stimulatory effect on NMT activity in vitro. Very recently, we have reported that 10% DMSO increased human NMT activity about 2.0-fold in vitro towards serine containing peptides such as pp60^{SRC}, myristoylated alanine-rich C kinase and M2 gene segment derived peptide substrates [Pasha et al., 2002]. However, it may be possible that the lower concentration of DMSO in vivo may have stimulatory effect on cardiac NMT activity. The NMT activity in Group 4 was found

to be reduced compared to Group 5 suggesting that DMSO has no effect on the cell-permeable calpain inhibitor mediated reduction of NMT activity.

Immunohistochemical analysis performed on normal rat hearts of ischemia or reperfusion, NMT expression was found to be localized in cytosol (Fig. 2, Group 1). Previously, we have also reported the cytosolic localization of NMT in normal human cardiac muscle [Raju et al., 1998]. Nuclear membrane staining starts appearing in Group 2 and the staining becomes darker in Group 3 with predominant nuclear and little cytoplasmic staining (Fig. 2). In Group 4, calpain inhibitor treatment reversed the nuclear staining to cytosolic (Fig. 2). In addition to rat ischemic/reperfusion samples, human ischemic samples were also studied for NMT expression. The results indicated predominant nuclear staining and less cytosolic staining in ischemia further attested the alteration of NMT localization in response to regional ischemia (Fig. 3).

Dysfunction of reperfused ischemic heart varies with the duration of ischemia. To explore whether the altered expression of NMT during ischemia/repurfusion correlated with altered myocardial function, we examined the left ventricular developed pressure (LVDP), heartbeat (HB), and coronary flow (CF) during the stabilization period (pre-ischemia), ischemia and post-ischemia reperfusion (Table I). Reperfusion after 15 min ischemia induced an arrhythmia with 100% ventricular fibrillation (Group 2 and 3 total number = 8). In Group 4, LVDP and CF rate decreased to 44.7 and 49.6% of control values during ischemia, but HB remained unchanged. Reperfusion with calpain inhibitor following ischemia for 15 min significantly improved the recovery of LVDP and CF rate. The ventricular fibrillation incidence in this group only occurred in 40% of hearts, which was quite lower than 100% incidence in the absence of calpain inhibitor under the same ischemia-reperfusion scheme (Group 3). Since DMSO was the solvent used to dissolve the

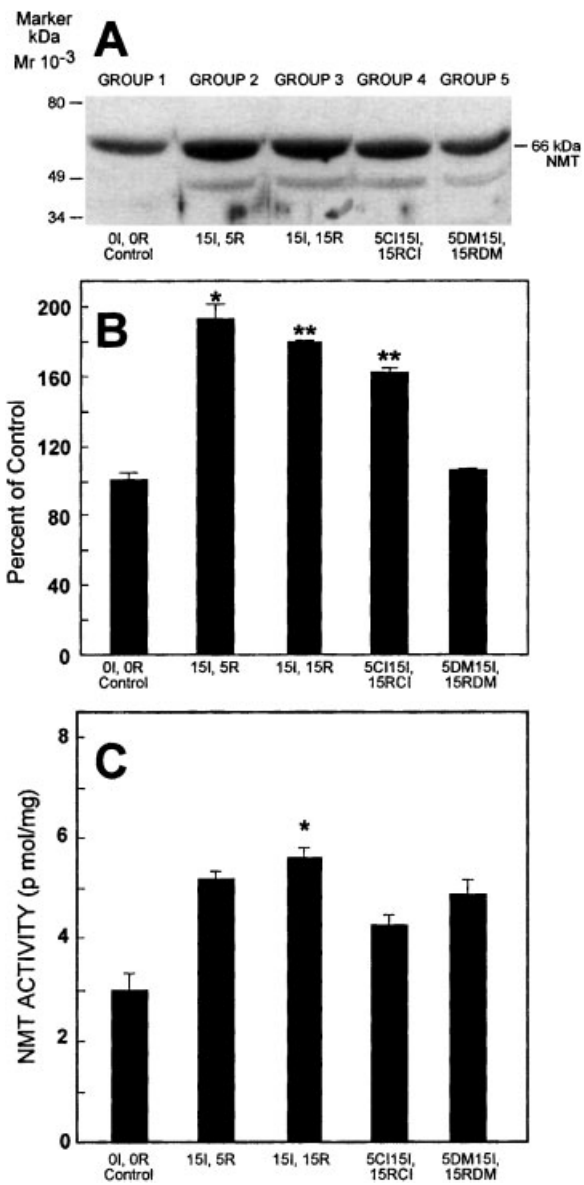

Fig. 1. (**A**) Immunoblot of NMT from normal and ischemia/reperfused rat heart tissue supernatants. Twenty five microgram of protein was loaded on to each lane, subjected to SDS–PAGE, transferred to nitrocellulose membrane and probed with NMT antibody as described in Methods. Five ischemia/reperfusion experimental groups were analyzed: Group 1, control (no ischemia or perfusion); Group 2, 15 min ischemia, 5 min reperfusion (15I, 5R); Group 3, 15 min ischemia, 15 min reperfusion (15I, 15R); Group 4, hearts were perfused with 100 μM calpain inhibitor in 0.1% DMSO for 5 min before 15 min of ischemia and again during 15 min of reperfusion (5CI15I, 15RCI); and Group 5, with 0.1% DMSO alone as described in Group 4 (5DM15I, 15RDM). (**B**), Quantitative analysis of bands of respective Western blots was carried out using imaging software; (NIH at http://rbs.info.nih.gov/nih-image/download.html). (**C**), Effect of ischemia/reperfusion of rat heart samples on NMT activity. Rat heart supernatant (20–40 μg) was used to carry out NMT activity in the presence of 500 μM $pp60^{src}$-derived peptide. Transferase activity was initiated by the addition of 50 μM [1-^{14}C]myrisotyl-CoA as described under methods. Results are expressed as specific activity. The data were expressed as the mean ± SD of two samples in each group and level of significance was assessed by Student's *t*-test. The critical level of significance was set at $P < 0.05$ (*) and $P < 0.01$ (**). The *P* value denote significantly different from control.

TABLE I. Cardiodynamic Parameters With NMT, Protein Expression NMT Activity and NMT Localization in Various Experimental Groups

Various Groups	Left ventricular developed pressure: Stabilization 10 min	Ischemia	Reperfusion	Heart beat: Stabilization 10 min	Ischemia	Reperfusion	Coronary flow rate: Stabilization 10 min	Ischemia	Reperfusion	Fibrillation (%)
A. Cardiodynamic parameters										
Group 1 Control	Hearts did not undergo any ischemia or reperfusion									
Group 2 15 I, 5 R	105.8 ± 10.6	33.3 ± 14.2	—	287.5 ± 25	220 ± 31.6*	—	14.3 ± 2.6	5.0 ± 0.3	—	100
Group 3 15 I, 15 R	100.4 ± 9.9	34.2 ± 11.6*	—	265.8 ± 28.0	220 ± 16.3*	—	11.4 ± 1.2	6.7 ± 2.2*	—	100
Group 4 5 CI 15 I, 15 RCI	98.5 ± 13.5	45.4 ± 15.9*	64.4 ± 25.5*	260 ± 16.3	240 ± 12.5	240 ± 43.5	14.3 ± 1.8	7.1 ± 0.8*	8.1 ± 3.2*	40
Group 5 5 DM 15 I, 15 RDM	108.9 ± 21.1	33.0 ± 17.1*	—	290 ± 20.0	200 ± 15	—	11.5 ± 2.0	3.8 ± 0.0*	—	100

B. NMT parameter	NMT protein expression %	NMT activity, P mol/mg (%)	NMT localization: Cytosolic	Nuclear
Group 1 Control	100 ± 3.53	3.2 ± 0.35 (100)	↑	▲
Group 2 15 I, 5 R	190 ± 7.07	5.1 ± 0.113 (159)	↑ - - -	▲ + + +
Group 3 15 I, 15 R	180 ± 1.41	5.5 ± 0.21 (172)	↑ - - -	▲ + + +
Group 4 5 CI 15 I, 15 RCI	160 ± 3.53	4.1 ± 0.176 (128)	↑	▲
Group 5 5 DM 15 I, 15 RDM	110 ± 1.41	4.7 ± 0.28 (146)	↑	▲

*Significant from stabilization period values at $P < 0.05$. I, ischemia; R, reperfusion: 5 and 15 time intervals (min); CI, calpain inhibitor; DM, DMSO.

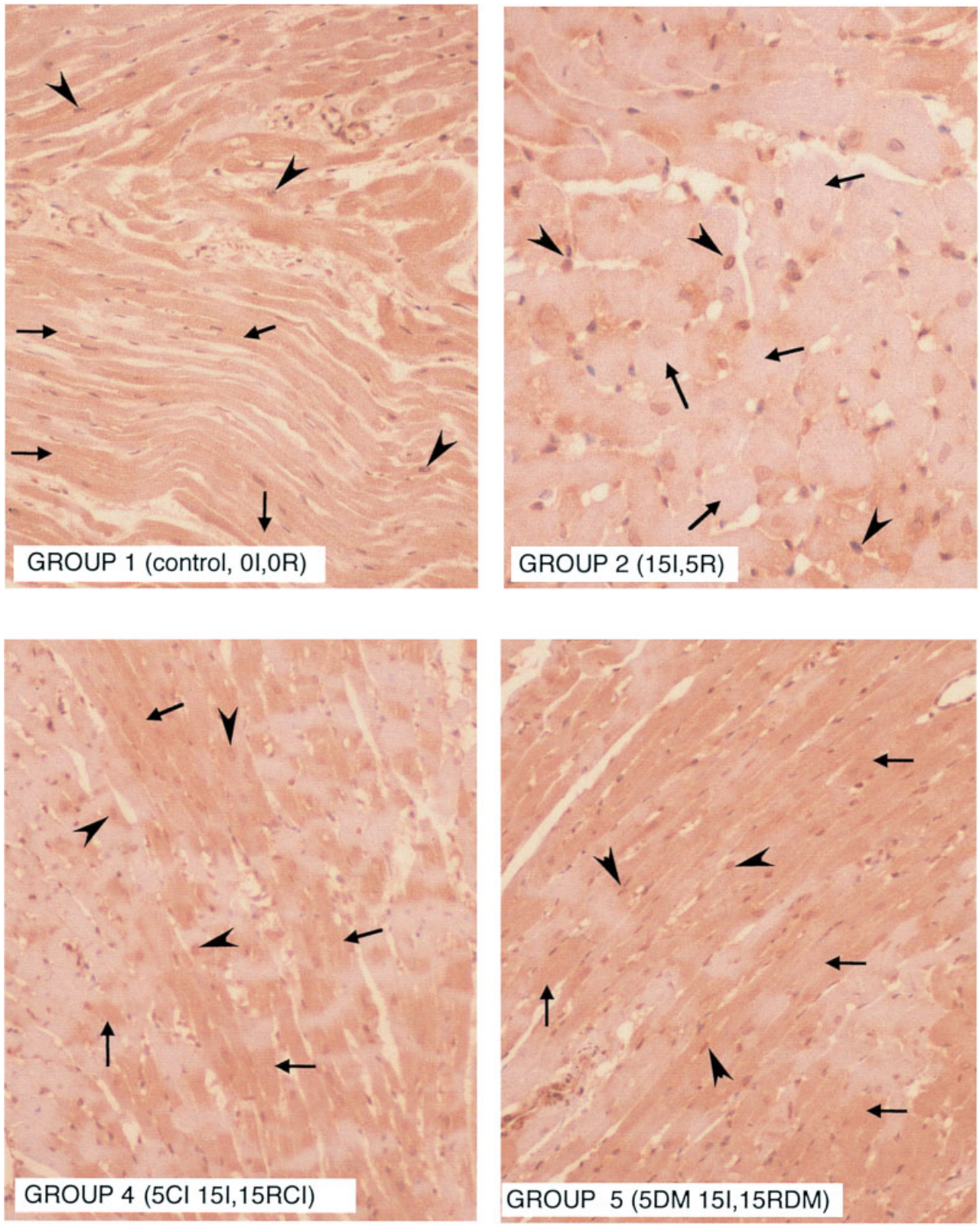

Fig. 2. Immunohistochemical expression of NMT in ischemia/reperfused rat heart tissues (Magnification × 10). Details for various groups see Figure 1 (Arrow and arrowheads indicate cytosolic and nuclear staining, respectively).

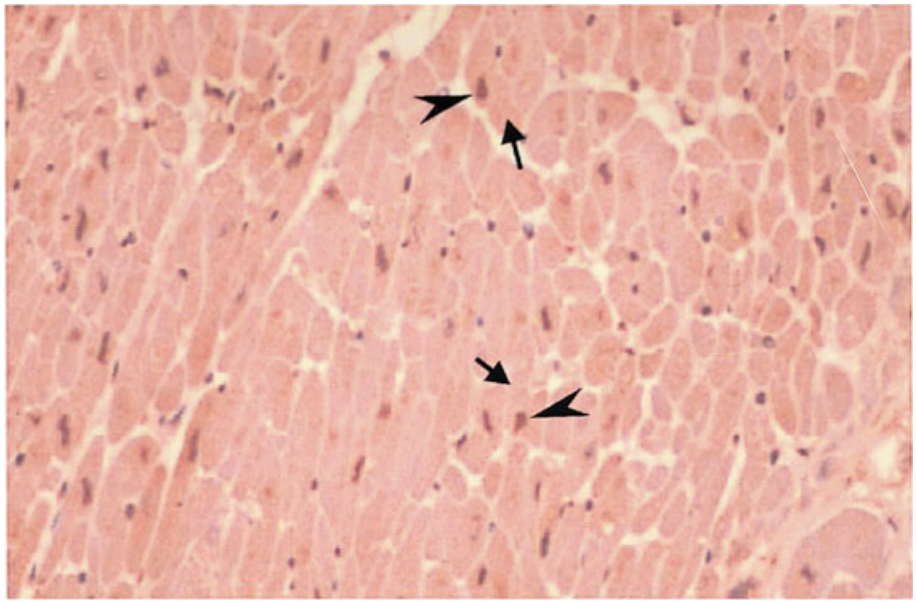

Fig. 3. Immunohistochemical expression of NMT in human ischemic samples. Magnification × 20. (Arrow and arrowheads indicate cytosolic and nuclear staining, respectively).

calpain inhibitor, it is crucial to determine the effect of DMSO alone on the cardiodynamic changes in Group 5. As shown in Table I, after reperfusion with DMSO containing perfusate 100% of hearts underwent ventricular fibrillation in Group 5. Thus, the reduced ventricular fibrillation after reperfusion (40%) in Group 4 was specifically attributed to the effect of calpain inhibitor. In addition, 100% ventricular fibrillation in Group 2, 3, and 5 after reperfusion made it impossible to measure LVDP, HB and CF rate after reperfusion (Table I). The aforementioned cardiodynamic changes induced by ischemia-reperfusion are consistent with the increased expression of NMT and NMT activity (Fig. 1 and Table I; Group 2 and 3). For example, 100% fibrillation was observed in Group 2 and 3, which correlates to a higher NMT expression and NMT activity (Table I). In addition, NMT was trans-located from cytoplasm to nucleus (Fig. 2, Table I; Groups 1, 3). Treatment with the calpain inhibitor in Group 4 not only decreased the incidence of ventricular fibrillation but also altered the localization of NMT from nuclear to cytosolic (Fig. 2, Table I).

DISCUSSION

The PEST regions are considered to be recognized by specific proteases, particularly calpain, that has been flagged as a potentially key candidate for the degradation of PEST-containing proteins [Rogers et al., 1986; Rechsteiner and Rogers, 1996]. We have demonstrated previously in vitro that the cloned cardiac muscle NMT is a substrate of *m*-calpain [Raju et al., 1998]. NMT activity is important for the myristoylation of various cellular proteins in cardiac muscle [Bhatnagar and Gordon, 1998; Resh, 1999; Rajala et al., 2000]. The involvement of calpains has been implicated in myocardial ischemia/reperfusion injury [Iizuka et al., 1993; Yoshida et al., 1995b], myocardial stunning [Gao et al., 1996], and cardiac hypertrophy [Arthur and Belcastro, 1997]. Intracellular Ca^{2+} overloading is implicated in the myocardial injury during ischemia/reperfusion and is considered to be a pivotal event in cell death [Gao et al., 1996]. An increase in Ca^{2+} influx can activate dormant Ca^{2+}-dependent enzymes, including calpains, causing damage to structural proteins (myocardial troponin, calspectin (fodrin), microtubules) leading to membrane breakdown and eventually cell death [Maki et al., 1990; Yoshida et al., 1995a]. We have also demonstrated the generation of active calmodulin-dependent phosphodiesterase by *m*-calpain [Kakkar et al., 1998] and also recently reported the altered expression of high molecular weight calmodulin binding protein in human ischemic myocardium [Kakkar et al., 2000]. Since NMT plays an important role in the

regulation of various signal transduction proteins such as cAMP-dependent protein kinase, calcineurin, an attempt was made to study the expression profile of NMT in a rat model of experientially induced ischemia/reperfusion.

NMT is a group of enzymes broadly classified as Type I and II enzymes with long N-terminal extensions and truncated versions [Rajala et al., 2000]. It has been previously shown that the N-terminal extension containing poly lysine residues are involved in targeting NMT to poly-ribosomes [Glover et al., 1997]. It is interesting to note that the polyclonal antibody directed against 50 kDa human NMT had cross-reacted with 66 kDa rat NMT (Fig. 1A). The primary structures of various NMTs are highly conserved in various species [Bhatnagar and Gordon, 1998; Resh, 1999; Qi et al., 2000; Rajala et al., 2000]. Western blot analysis of normal and ischemia/reperfused rat heart samples indicated an increase in expression of NMT in Group 2 and 3 (Fig. 1A,B). In addition, the NMT activity was found to be higher in ischemia/reperfusion compared to control Group 1 (Fig. 1C). Furthermore, a strong cytosolic NMT staining was previously reported in normal human cardiac muscle [Raju et al., 1998], whereas human ischemic tissues showed a marked nuclear staining with less cytosolic staining (Fig. 3). It is interesting to note that in control Group 1, NMT was found to be predominantly cytosolic and upon the onset of ischemia/reperfusion NMT was found to be localized to the nucleus (Fig. 2; Group 3). Calpain inhibitor reversed the localization of NMT from nucleus to cytoplasm (Fig. 2; Group 4). The precise role of NMT in nucleus is not known at present, whether NMT, myristoylates any nuclear protein(s) in a post-translational manner in cardiac muscle needs to be investigated. Post-translational myristoylation has been reported for a 68 kDa protein *D. discoideum* [da Silva and Klein, 1990]. Myristoylation of *P. syrinagae* effector protein occurs after the proteins enter the plant host cell and serves to target the bacterial proteins to the host plasma membrane [Nimchuk et al., 2000]. Cleavage of the pro-apoptotic BID protein by caspase 8 generates a post-translational myristoylation site that enables the BID p15 fragment to localize at the mitochondria and induce cell death [Zha et al., 2000].

Previous studies from our laboratory have demonstrated that the expression levels of μ-calpain and *m*-calpain were higher in ischemia/reperfused heart tissues as compared to normal [Kakkar et al., 2001]. Calpains are widely distributed in myocytes and are implicated in myocardial injury [Iizuka et al., 1993; Yoshida et al., 1995b; Gao et al., 1996]. Earlier, we have observed that cardiac NMT is proteolyzed by calpain [Raju et al., 1998]. Therefore, it is possible that the altered localization of NMT from cytoplasm to nucleus could be due to the cleavage of NMT by calpains. Calpain inhibitor (Group 4) altered the localization of NMT from nucleus to cytoplasm (Fig. 2).

In isolated rat hearts, a bell-shaped relationship occurs between the incidence of reperfusion-induced arrhythmias and the duration of preceding ischemia, with maximal fibrillation incidence (100%) at 10–15 min of regional ischemia [Manning and Hearse, 1984]. Accordingly, 15 min of ischemia was chosen for our experiments. These functional changes induced by ischemia/reperfusion are consistent with the altered expression of NMT localization. Treatment with calpain inhibitor of Group 4 having 15 min ischemia decreased the incidence of ventricular fibrillation from 100–40% and resulted in recovery of cardiodynamic parameters. At the same time, there was no change in the expression of NMT (Fig. 1A). However, we have observed that the majority of NMT altered from the nucleus to the cytoplasm (Fig. 2, Group 4).

We also observed the degradation of c-Src protein from 60 kDa to a 52, 49, 34, 29 kDa species, respectively, during the course of ischemia/reperfusion (data not shown). A similar pattern has also been observed with $pp60^{src}$ in human blood platelets in response to calpain treatment [Oda et al., 1993]. The calpain cleavage also resulted in the reduction of kinase activity [Oda et al., 1993]. The substrate of NMT, Src tyrosine kinases has been shown to mediate cellular responses to stress in non-cardiac cells [Ping et al., 1999]. However, the effect of myocardial ischemia on Src tyrosine kinases is unknown. Recently, it has been shown that two specific members of the Src family of tyrosine kinases, Src and Lck, play an important role in the genesis of IPC by serving as downstream elements of PKC-mediated signal transduction [Ping et al., 1999]. IPC confers cardioprotection against a prolonged ischemic insult. Tyrosine kinase inhibitors have been shown to attenuate IPC [Fryer et al., 1998]. It has also been shown that activation of

tyrosine kinase is involved in the initiation, but not the maintenance of IPC in the rat myocardium [Fryer et al., 1998]. In addition, it has also been shown that c-Src is a major cytosolic tyrosine kinase in vascular tissues [Oda et al., 1999].

The Src signaling may be impaired in ischemia/reperfusion due to the altered localization of NMT from cytoplasm to nucleus. The complex role of NMT, c-Src, and calpains may be involved in cardioprotection. However, further studies are required to understand the role of co-translational modification of proteins in cardiac functions and cardiac injury.

ACKNOWLEDGMENTS

This work was supported by the Heart and Stroke Foundation of Saskatchewan, Canada. Dr. R. Kakkar was a recipient of a Research Fellowship from the Health Services Utilization and Research Commission of Saskatchewan, Canada. The authors thank Dr. M.D. Hollenberg, Department of Pharmacology and Therapeutics, University of Calgary for his helpful suggestions and critical evaluation of the manuscript. The authors also thank the technical assistance of Dr. Xuguang Bi.

REFERENCES

Aitken A, Cohen P, Santikarn S, Williams DH, Calder AG, Smith A, Klee CB. 1982. Identification of the NH_2-terminal blocking group of calcineurin B as myristic acid. FEBS Lett 150:314–318.

Arthur GD, Belcastro AN. 1997. A calcium stimulated cysteine protease involved in isoproterenol induced cardiac hypertrophy. Mol Cell Biochem1 76:241–248.

Barnes JA, Gomes AV. 1995. PEST sequences in calmodulin-binding proteins. Mol Cell Biochem 149(150):17–27.

Bhatnagar RS, Gordon JI. 1998. Understanding covalent modification of proteins by lipids: Where cell biology and biophysics mingle. Trends Cell Biol 7:14–20.

Billger M, Wallin M, Karlsson JO. 1988. Proteolysis of tubulin and microtubule associated proteins 1 and 2 by calpains. II. Difference in sensitivity of assembled and dissembled microtubules. Cell Calcium 9:33–44.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle protein-dye binding. Anal Biochem 72: 248–254.

Caroall DE, Demartino GN. 1991. Calcium-activated neutral protease (calpain) system: structure, function and regulation. Physiol Rev 71:813–447.

Carr SA, Biemann D, Shozo S, Parmelee DC, Titani K. 1982. n-Tetradeconyl is the NH_2 terminal blocking group of the catalytic subunit of cyclic AMP-dependent protein kinase from bovine cardiac muscle. Proc Natl Acad Sci USA 79:6128–6131.

Carr DW, Stofko-Hann RE, Fraser IDC, Bishop SM, Acott TS, Brennan RG, Scott JD. 1991. Interaction of regulatory subunit (RII) of cAMP-dependent protein kinase with RII-anchoring proteins occurs through an amphipathic helix binding motif. J Biol Chem 266:14188–14192.

da Silva AM, Klein C. 1990. A rapid posttranslational myristoylation of a 68 kD protein in *D.discoideum*. J Cell Biol 111:401–407.

David LL, Shearer TR, Shih M. 1993. Sequence analysis of lens B-crystallins suggests involvement of calpains in cataract formation. J Biol Chem 268:1937–1940.

Fryer RM, Schultz JELJ, Hsu AK, Gross GJ. 1998. Pretreatment with tyrosine kinase inhibitors partially attenuates ischemic preconditioning in rat hearts. Am J Physol 275:H2009–H2015.

Gao WD, Lu Y, Melgren R, Marban E. 1996. Intrinsic myofilament alterations underlying the decreased contractility of stunned myocardium: A consequence of $Ca2^{+-}$dependent proteolysis? Cir Res 78:455–465.

Glover CJ, Hartman KD, Felsted RL. 1997. Human N-myristoyltransferase amino-terminal domain involved in targeting enzyme to the ribosomal subcellular fraction. J Biol Chem 272:28680–28689.

Hirai S, Kawasaki H, Yaniv M, Suzuki K. 1991. Degradation of transcription factors, c-Jun and c-Fos, by calpains. FEBS Lett 287:57–61.

Hsu SM, Raine L, Fanger H. 1981. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem 29:577–580.

Iizuka K, Kawaguchi H, Kitabatake A. 1993. Effects of thiol protease inhibitors on fodrin degradation during hypoxia in cultured myocytes. J Mol Cell Cardiol 25:1101–1109.

Inoue M, Kishimoto A, Takai Y, Nishizuka Y. 1977. Studies on cyclic nucleotide independent protein kinase and its proenzyme in mammalian tissues. II proenzyme and its activation by calcium-dependent protease from rat brain. J Biol Chem 252:7610–7616.

Kakkar R, Raju RVS, Sharma RK. 1998. In vitro generation of an active calmodulin-independent phosphodiesterase from calmodulin-dependent phosphodiesterase (PDE1A2) by *m*-calpain. Arch Biochem Biophys 358:320–328.

Kakkar R, Radhi JM, Rajala RVS, Sharma RK. 2000. Altered expression of high molecular weight calmodulin binding protein in human ischaemic myocardium. J Pathol 191:208–216.

Kakkar R, Wang X, Radhi JM, Rajala RVS, Wang R, Sharma RK. 2001. Decreased expression of high molecular-weight calmodulin-binding protein and its correlation with apoptosis in ischemia-reperfused rat heart. Cell Calcium 29:59–71.

Kawasaki H, Kawashima S. 1996. Regulation of the calpain-calpastatin system by membranes. Mol Mem Biol 13:217–224.

King MJ, Sharma RK. 1991. N-myristoyltransferase assay using phosphocellulose binding. Anal Chem 199:149–153.

Kosaki G, Tsujinaka T, Kambayashi J, Morimoto K, Yamamoto K, Yamagami K, Sobue K, Kakiuchi S. 1983. Specific cleavage of calmodulin-binding proteins by low Ca^{2+}-requiring form of Ca^{2+}activated neutral protease in human platelets. Biochem Int 6:767–775.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 227:680–685.

Liauw S, Steinberg RA. 1996. Dephsophorylation of catalytic subunit of cAMP-dependent protein kinase at Thr-197 by a cellular protein phosphatase and by purified protein phosphatase-2A. J Biol Chem 271:258–263.

Magnuson A, Haug LS, Walaas SI, Ostvold AC. 1993. Calcium induced degradation of the inositol (1,4,5)-triphosphate receptor/Ca^{2+}-channel. FEBS Lett 323:229–232.

Maki M, Hatanaka M, Takano E, Murachi T. 1990. Intracellular calcium-dependent proteolysis. Mellgren RL, Murachi T, editors. Boca Raton, Florida: CRC Press Inc. pp 37–54.

Manning AS, Hearse DJ. 1984. Reperfusion-induced arrhythmias: Mechanism and prevention. J Mol Cell Cardiol 16:497–518.

Murachi T. 1997. Calpain and calpastatin. Trends Biochem Sci 8:167–169.

Nimchuk Z, Marois E, Kjemtrup S, Leister RT, Katagiri F, Dangl JL. 2000. Eukaryotic fatty acylation drives plasma membrane targeting and enhances function of several type III effector proteins from *Pseudomonas syringae*. Cell 101:353–363.

Oda A, Druker BJ, Ariyoshi H, Smith M, Salzman EW. 1993. pp60Src is an endogenous substrate for calpain in human blood platelets. J Biol Chem 268:12603–12608.

Oda Y, Renaux B, Bjorge J, Saifeddine M, Fujita DJ, Hollenberg MD. 1999. c-Src is a major cytosolic tyrosine kinase in vascular tissue. Can J Physiol Pharmacol 77:606–617.

Pasha MK, Dimmock JR, Hollenberg MD, Sharma RK. 2002. Enhanced activity of human *N*-myristoyltransferase by dimethylsulfoxide and related solvents in the presence of serine containting paptide substrates. Biochem Pharmacol (in press).

Ping P, Zhang J, Zheng YT, Li RC, Dawn B, Tang XL, Takano H, Balafanova Z, Bolli R. 1999. Demonstration of selective protein kinase C-dependent activation of Src and Lck tyrosine kinase during ischemic preconditioning in conscious rabbits. Cir Res 85:542–550.

Qi Q, Rajala RVS, Anderson W, Jiang C, Rozwadowski K, Selvaraj G, Sharma RK, Datla R. 2000. Molecular cloning, genomic organization and biochemical characterization of myristoyl-CoA:protein *N*-myristoyltransferase from arabidopsis thaliana. J Biol Chem 275:9673–9683.

Rajala RVS, Sharma RK. 1999. Preparation and assay of myristoyl-CoA:protein–myristoyltransferase. Methods Mol Biol 116:193–211.

Rajala RVS, Datla RSS, Moyana TN, Kakkar R, Carlsen SA, Sharma RK. 2000. N-myristoyltransferase. Mol Cell Biochem 204:135–155.

Raju RVS, Moyana TN, Sharma RK. 1997. *N*-myristoyltransferase overexpression in human colorectal adenocarcinomas. Exp Cell Res 235:145–154.

Raju RVS, Kakkar R, Datla RSS, Radhi JM, Sharma RK. 1998. Myristoyl-CoA:protein–myristoyltransferase from bovine cardiac muscle. Molecular cloning, kinetic analysis, and in vitro proteolytic cleavage by *m*-calpain. Exp Cell Res 241:23–35.

Rechsteiner M, Rogers S. 1996. PEST sequences and regulation by proteolysis. Trends Biochem Sci 21:267–271.

Resh MD. 1999. Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins. Biochem Biophys Acta 1451:1–16.

Rogers S, Wells R, Rechsteiner M. 1986. Amino acid sequences common to rapidly degraded proteins: The PEST hypothesis. Science 234:364–369.

Saido TC, Sorimachi S, Suzuki K. 1994. Calpains: new perspectives in molecular diversity and physiological-pathological involvement. FASEB J 8:814–822.

Stabach PR, Cianci CD, Glantz SB, Zhang A, Morrow JS. 1997. Site-directed mutagenesis of alpha II spectrin at codon 1175 modulates its calpain susceptibility. Biochemistry 36:57–65.

Steinberg RA, Agard DA. 1981. Turnover of regulatory subunit of cAMP-dependent protein kinase in S49 mouse lymphoma cells. Regulation by catalytic subunit and analogs of cyclic AMP. J Biol Chem 256:10731–10734.

Towbin H, Staehelin T, Gordon J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354.

Wang KK, Villalobo A, Roufogalis BD. 1989. Calmodulin-binding proteins as calpain substrates. Biochem J 262: 693–706.

Wang X, Wu L, Aouffen M, Mateescu MA, Nadeau R, Wang R. 1999. Novel cardiac protective effects of urea: from shark to rat. Br J Pharmacol 128:1477–1484.

Watt F, Molloy PL. 1993. Specific cleavage of transcription factors by the thiol protease *m*-calpain. Nucleic Acids Res 21:5092–5100.

Yoshida K, Inui M, Harada K, Saido TC, Sorimachi Y, Ishihara T, Kawashima S, Sobue K. 1995a. Reperfusion of rat heart after brief ischemia induces proteolysis of calspectin (nonerythroid spectrin or fodrin) by calpain. Cir Res 77:603–610.

Yoshida K, Sorimachi Y, Fujiwara M, Hironaka K. 1995b. Calpain is implicated in rat myocardial injury after ischemia and reperfusion. Jpn Cir J 59:40–48.

Zha J, Weiler S, Oh KJ, Wei MC, Korsmeyer SJ. 2000. Posttranslational *N*-myristoylation of BID protein as a molecular switch for targeting mitochondria and apoptosis. Science 290:1761–1765.