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ORIGINAL ARTICLE

Determining time of death: temperature-dependent postmortem changes in calcineurin A, MARCKS, CaMKII, and protein phosphatase 2A in mouse

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Abstract While the determination of postmortem interval (PMI) is a crucial and fundamental step in any death investigation, the development of appropriate biochemical methods for PMI estimation is still in its infancy. This study focused on the temperature-dependent postmortem degradation of calcineurin A (CnA), calmodulin-dependent kinase II (CaMKII), myristoylated alanine-rich C-kinase substrate (MARCKs), and protein phosphatase 2A (PP2A) in mice. The results show that MARCKS, CaMKII, and the use of lung tissue do not appear to warrant further study for the determination of PMI in humans. In skeletal muscle, CnA underwent a rapid temperature-dependent cleavage (60→57 kDa) over the first 48 h of postmortem interval. At 21°C, this transformation was completed within 24 h. In contrast, PP2A increased within the first 24 h after which it degraded at 21°C but remained stable for up to 96 h at 5°C and 10°C. The 60→57 kDa postmortem conversion of CnA was inhibited by addition of protease inhibitors and MDL-28170 indicating a calpain pathway mediates this breakdown. Proteasome inhibition (MG-132) and calmodulin antagonism (calmidazolium) also inhibited this conversion suggesting that other protein degradation pathways also are in play. In contrast, all of the protease inhibitors and calmidazolium but not ethylene glycol tetraacetic acid led to increased levels of PP2A. The data are discussed in terms of developing a useable field-based biochemical assay for postmortem interval determination in humans and understanding the protein degradation pathways that are initiated upon death.

Keywords Postmortem interval (PMI) · Protein degradation · Calcineurin A · Protein phosphatase 2A · Skeletal muscle

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Introduction

Postmortem interval (PMI) determination is a critical step in many death investigations [1]. Precisely defining the PMI facilitates the process of identifying the deceased, assists in distinguishing pathology from postmortem artifact, and helps exclude or include suspects based on their whereabouts at the time of death. PMI determination is a complex problem due to an array of factors that alter the course of postmortem change. Intrinsic factors such as the age, sex, physiological, and pathological state of the deceased and extrinsic factors, such as temperature, humidity, and insect or animal activity. For example, Hayashi et al., have shown that different results were obtained for aquaporin-5 from lung tissue after drowning in freshwater versus salt water [2]. Such studies also emphasize that the selection of tissue for postmortem analysis is paramount. Typically, postmortem changes that are used for determining PMI fall into three broad categories: physical, physiological, and biochemical. Physiological changes such as algor mortis, livor mortis, rigor mortis, and supravital activity are mainly used to estimate earlier PMIs. Physical changes such as arthropod activity and physiological changes such as the classical signs of decomposition are regularly employed to estimate longer PMIs. Each of these

methods provides only a rough estimate of PMI and often one method contradicts the results of another [1]. Some progress has been made exploring the biochemical changes of the body as markers for PMI estimation. New lines of research such as protein, RNA, and DNA degradation are starting to be unravelled that may provide more precise methods for PMI determination [3–5]. Other biochemical markers include analysis of postmortem levels of urea, creatinine, glucose, iron, potassium, calcium, insulin in pancreatic β-cells, strontium90, myo-albumin, myofibril proteins, and several enzymes [6–10]. A previous study by Kang et al. [3] showed that PMI has an effect on calmodulin-binding protein degradation patterns in rats revealing that postmortem changes in Ca^{2+}/calmodulin (CaM)-dependent kinase II (CaMKII), myristoylated alanine-rich C-kinase substrate (MARCKS), and calcineurin A (CnA) in lung and skeletal tissue could serve as useful markers in PMI determination [3].

A well-known biochemical change in the early stages after death is an increase in the permeability of the plasma membrane to Ca^{2+} ions [11]. The influx of Ca^{2+} ions results in the activation of many calcium-binding proteins including CaM and calpain [11]. CaM is a ubiquitous intracellular Ca^{2+} sensor protein that is activated by Ca^{2+} subsequently binding to a variety of downstream CaM-binding proteins (CaMBPs) [12]. Calpain has been implicated as the primary enzyme responsible for postmortem protein degradation [13–15]. Calpain belongs to a family of Ca^{2+}-activated cysteine proteases that digest many important proteins including CaMBPs [13, 14]. Studies have shown that addition of calpastatin, a calpain inhibitor, to postmortem tissue greatly reduces degradation of specific proteins including CaMBPs like fodrin [13–15]. A previous study has shown that CaM protects some proteins from degradation by calpain [16]. Overall, these studies suggest a postmortem interaction between CaM, CaMBPs, and calpain.

Calcineurin is a Ca^{2+}/CaM-dependent protein phosphatase that consists of two subunits, a catalytic 60-kDa subunit (CnA) and a regulatory 19-kDa subunit (CnB) [17]. Overactivation of CnA leads to excessive dephosphorylation of critical structural and signaling proteins which can lead to cell death [18, 19]. In addition to activation by the CnB subunit, CnA is also activated by proteolytic cleavage. A 57-kDa cleavage product retains its Ca^{2+}/CaM dependence while 45- and 48-kDa cleavage products become Ca^{2+}/CaM independent [18, 20]. The addition of calpastatin, an endogenous calpain inhibitor, to postmortem tissue greatly reduces the degradation of CnA [18]. Adding to this, CaM can activate certain calpain substrates (i.e., CnA and fodrin) and also play a role in their cleavage, protecting some while making others more vulnerable to fragmentation [20, 21]. This dynamic interplay is known to be important in ischemic and excitotoxic injuries and in the pathogenesis of neurodegenerative diseases such as Alzheimer's but has not been studied in postmortem tissue.

The first goal of this research was to compare the results of Kang et al. [3] with another mammalian species to determine which identified proteins might show consistent postmortem changes suggesting that they might serve as potential PMI indicators in humans. The second goal was to extend our understanding by determining the effect of temperature on the degradation of selected proteins. The third goal was to gain insight into postmortem proteolytic events as a way of developing steps to prevent degradation of harvested tissue samples and to gain further insight into the factors that affect protein degradation after death. CnA and CaMKII were chosen because they are potentially useful protein markers for PMI determination [3]. MARCKS was also analyzed by Kang et al. [3] and it was selected to determine if the degradation pattern would be the same across mammalian species. PP2A was selected as a comparison for CnA analysis because it is a Ca^{2+}/CaM-independent protein phosphatase that has an essential role in a diversity of cellular functions. All of these proteins are highly conserved suggesting that results generated in mice and rats should be translatable into human studies [22]. The degradation patterns of CnA, CaMKII, MARCKS, and PP2A were analyzed in mouse lung and skeletal muscle tissue samples at three different PMIs (24, 48, and 96 h) and at three different temperatures (5°C, 10°C, and 21°C). The importance of Ca^{2+}, CaM, calpain, and proteasome in the postmortem breakdown of CnA and PP2A was also investigated. The results of this study should open the door to establishing specific proteins as potential tools for the precise determination of PMI in humans and provide further insight into the postmortem events associated with tissues after death.

Materials and methods

The experimental design for this work is summarized in Fig. 1. This research met the standards and principles of animal care and use as outlined by the Canadian Council on Animal Care (www.ccac.ca). The ethical approval for this research was obtained from the Animal Care Committee of the University of Toronto and the mice were housed in the animal care facility at the University of Toronto at Mississauga. Mice were sacrificed using carbon dioxide asphyxiation then placed in controlled aseptic environmental chambers (Controlled Environments, Winnipeg, Canada) set at 5°C, 10°C, and 21°C. Sets of four mice each were dissected at 0, 24, 48, and 96 h postmortem and the tissues were harvested. Each tissue was dissected out, placed on a glass plate, blotted with tissue paper to remove blood, and macerated with a scalpel. The

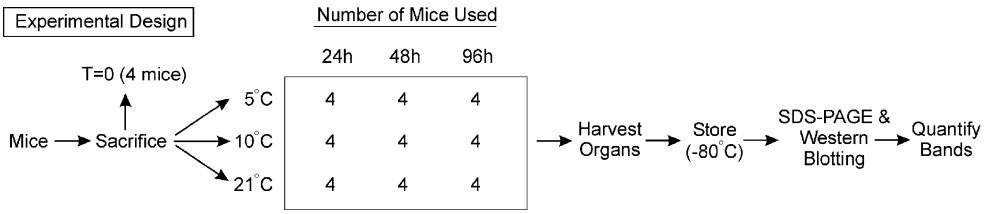

Fig. 1 A flow chart of the experimental design used in this study

tissue was then placed into two separate Eppendorf tubes (1.5 mL tubes) on ice and immediately frozen in liquid nitrogen. The tissue samples were stored at −80°C until homogenization.

Homogenization and sample preparation

Each tissue sample was transferred into a mortar, a small amount of liquid nitrogen was added, and the tissue was ground into a fine powder using a pestle. Homogenization buffer (5 mL containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, and one time complete protease inhibitor cocktail (Santa Cruz Biotechnology, Santa Cruz, CA, USA)) was then mixed with the ground tissue sample [21]. Aliquots (1.5 mL) were transferred into separate Eppendorf tubes which were immediately placed on ice. Samples were then centrifuged twice at 3,500 rpm for 10 min in an Eppendorf 5804R Series Multipurpose Centrifuge (Eppendorf, Hamburg, Germany) with the pellets discarded after each first spin. The resulting supernatant was aliquoted into Eppendorf tubes, frozen in liquid nitrogen, and stored at −80°C until used.

Protein quantification

An aliquot from each tissue sample was thawed and the protein content was quantified using the BioRad Protein Assay technique with a Beckman DU 64 Spectrophotometer (Beckman Coulter, Fullerton, CA, USA). The protein levels were standardized to bovine serum albumin. Equal amounts of protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 12% running gel; 4% stacking gel; Bio-Rad Laboratories, Mississauga, Canada) along with a BenchMark™ prestained protein ladder (Invitrogen, Carlsbad, CA, USA). Gels were then stained with Coomassie blue to verify the loading levels.

Western blotting

After proteins were separated, they were electrophoretically transferred onto polyvinylidene difluoride membranes (Pall, Mississauga, Canada) and blocked overnight at 4°C with nonfat dried milk (5%) and Tween 20 (0.1%) in Tris base solution (20 mM Tris base, 150 mM NaCl, pH 7.6). The primary polyclonal antibodies were rabbit anti-CnA (AB1695, Chemicon, Billerica, MA, USA), goat anti-MARCKS (sc-6454, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and rabbit anti-PP2A (sc-15355, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Anti-CnA was diluted 1:400 while the others were diluted 1:200. The bound antibodies were detected with horseradish peroxidase (HRP)-conjugated donkey anti-goat (sc-2020, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or HRP-conjugated anti-rabbit secondary antibodies diluted 1:16,000. The membranes were developed using the ECL Plus detection system (GE healthcare, Piscataway, NJ, USA) and visualized with the STORM Scanner System (Molecular Dynamics, GE healthcare, Piscataway, NJ, USA). The intensity of the resultant bands was quantified using the Image Quant 5.2 program (GE healthcare, Piscataway, NJ, USA). The intensities were standardized to the positive control (0 h) and were expressed as a percentage of the positive control. A model I two-way analysis of variance (ANOVA) and Tukey's test were then used to establish the significance of the results.

Effects of protease inhibitors

Freshly harvested skeletal muscle samples were used. Dissected tissue, as a whole, was placed into sealed tubes and incubated at 21°C with the appropriate treatment for the appropriate time. The following chemicals were diluted in phosphate buffered saline (PBS) and added to the tissue samples: complete protease inhibitor tablet (SantaCruz Biotechnology, Santa Cruz, CA, USA); 1 and 10 μM MDL-28170 (Biomol, Plymouth Meeting, PA, USA); 2 and 20 μM MG-132 (Biomol, Plymouth Meeting, PA, USA); 1 and 10 μM calmidazolium (Sigma, St. Louis, MO, USA); 1 and 10 mM ethylene glycol tetraacetic acid (EGTA; Sigma, St. Louis, MO, USA); and PBS only as a control.

Results

Protein quantification and Western blotting

Standardizing protein levels in postmortem samples poses a problem since the standard markers (e.g., actin, tubulin) that are used can also undergo digestion. We have found that our approach of loading specific amounts of cell extracts based upon predetermined protein concentrations generates protein profiles in SDS-PAGE gels that indicate equivalent protein loading (Fig. 2a). As exemplified by CnA (Fig. 2b), CaMKII (Fig. 2c), MARCKS (Fig. 2d), and

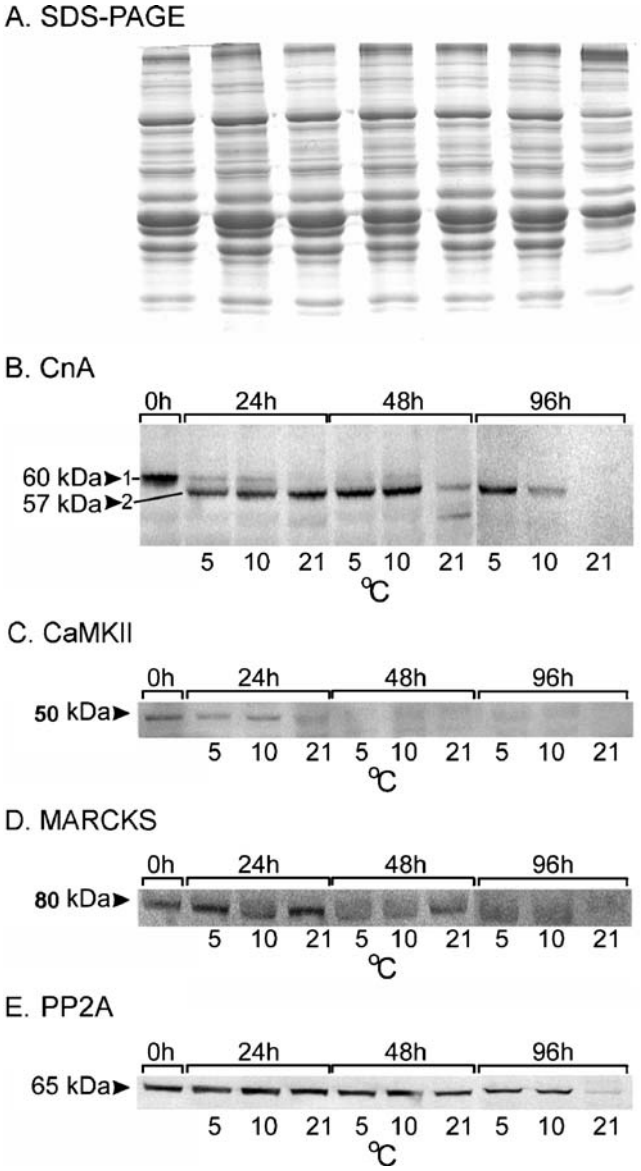

Fig. 2 SDS-PAGE and Western blotting. **a** SDS-PAGE gel demonstrating that each *lane* has an equal amount of total protein. **b** Western blot of CnA postmortem patterns in a skeletal muscle tissue sample. **c** Western blot of CaMKII postmortem patterns in a skeletal muscle tissue sample. **d** Western blot of MARCKS postmortem patterns in a lung tissue sample. **e** Western blot of PP2A postmortem patterns in a skeletal muscle tissue sample

PP2A (Fig. 2e), Western blotting of these separated proteins generates clearly defined bands at appropriate molecular weights that are amenable to scanning and quantification as described in the "Materials and methods" section.

CnA in muscle and lung

Within the first 24 h postmortem, the original band of CnA (band 1; ~65 kDa) decreased markedly in amount as a second faster-migrating band (band 2) appeared just below it (Figs. 2b and 3). At 5°C, the levels of band 1 decreased steadily through the first 48 h to become almost undetectable by 96 h postmortem (Fig. 3a). At 10°C, the level of CnA band 1 was lower at 24 h than at 5°C and lower still at 21°C after which the levels were similar to those seen at 5°C (Fig. 3a). The major statistically significant change occurred within the first 24-h period where the intensity of band 1 decreased drastically, but the consequent decreases in band 1 levels were also found to be significant ($p<0.05$; Tukey). In keeping with the decrease in band 1, band 2 increased steadily at 5°C and 10°C through the full 96 h but after 24 h decreased in amount at 21°C (Fig. 3b). In this case, both PMI and temperature had a statistically significant effect ($p<0.05$; ANOVA) where the greatest amount of protein breakdown was observed within the first 24 h and at the highest temperature, 21°C. The patterns of CnA in lung were not quantified because CnA was broken down almost completely within the first 24-h period.

CaMKII and MARCKS in muscle and lung

MARCKS was undetectable in our mouse muscle tissue preparations but generated distinct quantifiable bands after Western blotting of lung tissue extracts (Fig. 2d). At 5°C, the amount of MARCKS protein decreased to approximately 50% of 0 h levels within the first 48 h then remained essentially unchanged through 96 h (Fig. 4). The pattern of MARCKS degradation was more linear at 10°C with 60% of the band lost by 96 h. At 21°C, there was a great variability in the 24- and 48-h samples but by 96 h, only ~10% of the protein remained (Fig. 4). In general, the banding pattern became more diffuse with increasing PMI and temperature suggesting nonspecific degradation of the protein could be at play. Statistical analysis revealed that PMI had the greatest effect on MARCKS breakdown where the major decrease in MARCKS levels was observed when PMI reached 96 h ($p<0.05$; Tukey). CaMKII was detected in both muscle and lung and showed a similar pattern of postmortem degradation (Fig 2c). In both tissues, CaMKII band was undetectable by 48 h postmortem at all temperatures.

PP2A in muscle and lung

The bands of PP2A were strong and distinct in both muscle and lung but each showed very distinct postmortem patterns (e.g., Figs. 2e and 5). At 5°C, PP2A remained more or less unchanged through 96 h in muscle tissue (Fig. 5a). At the higher temperatures, some interesting variations were seen. The detected levels of PP2A increased at 24 and 48 h to a maximum of 130%, at 10°C, of the 0 h level before decreasing slightly (Fig. 5a). A similar increase was detected after 24 h at 21°C but then the levels of PP2A dropped of markedly to ~10% of 0 h levels by 96 h

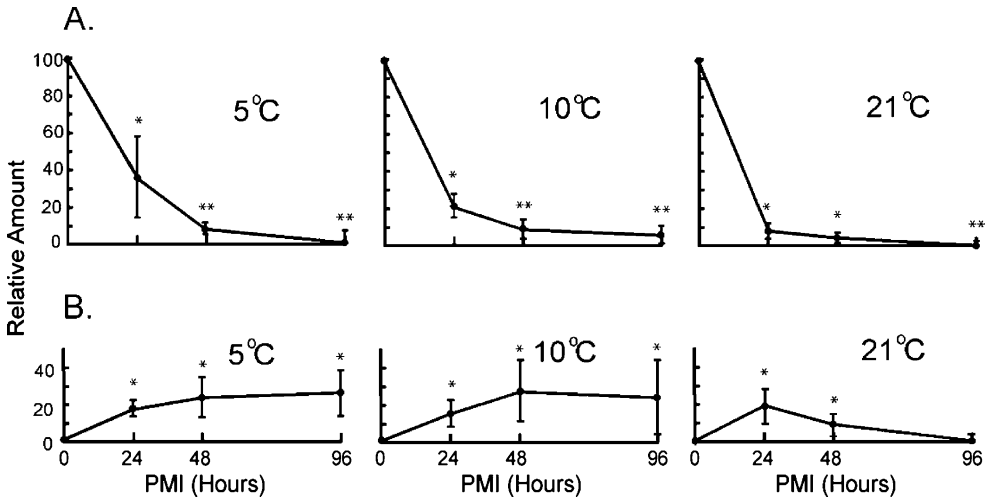

Fig. 3 The temperature-dependent degradation patterns of CnA in skeletal muscle tissue samples. The Western blots were scanned and the amounts of protein present in all postmortem bands were expressed as a percentage of the initial CnA protein band (band 1, 0 h) as detailed in the "Materials and methods" section. Means and standard error of the means are shown. **a** Postmortem changes on full-length CnA (60 kDa band). **b** Postmortem changes of CnA cleavage product (57 kDa band). *$p<0.05$ indicates a significant difference from $T=0$ h. **$p<0.05$ indicates a significant difference from $T=24$ h

postmortem (Fig. 5). There was a significant interaction between the effects of PMI and temperature on the levels of PP2A ($p<0.05$; ANOVA). The combination of high temperature (21°C) and long PMI (96 h) had the most drastic effect on the breakdown of PP2A while protein levels were quite stable otherwise. The initial increase in PP2A levels could possibly be attributed to the production of a more accessible epitope or to postmortem translation. Sanoudou [23] previously suggested that skeletal muscle undergoes a highly active transcriptional and possibly translational phase during the first 46-h PMI. In lung at 5°C, 10°C, and 21°C, the levels of PP2A dropped off significantly ($p<0.05$; Tukey) within the first 24 h to approximately 30% of the 0 h levels after which the fluctuating levels decreased only slightly (Fig. 5b).

Ca^{+2}, CaM, calpain, and proteasome in CnA and PP2A degradation

After comparison of CnA Western blots, the breakdown pattern of CnA in dissected whole tissue was almost identical to that found in the whole organism at 21°C. Most of full length CnA was broken down in the first 15 min but low levels remained detectable up to 1 h of incubation (Fig. 6a). The 57-kDa cleavage product first appeared at 30 min with the amount increasing up to 4 h and then beginning to decline and by 48 h, it was almost undetectable. Next, we analyzed the involvement of Ca^{+2}, CaM, calpain, and proteasome in the postmortem changes of CnA and PP2A (Fig. 6b, c). The specific cleavage of CnA into the 57-kDa immunoreactive product still occurred when the complete protease inhibitor

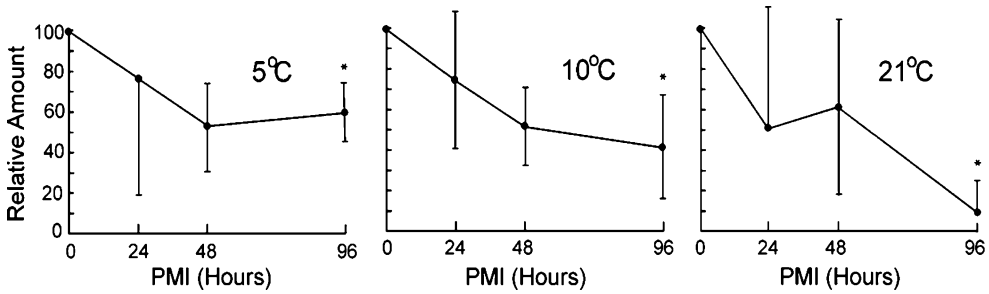

Fig. 4 The temperature-dependent degradation patterns of MARCKS in lung tissue samples. The Western blots were scanned and the amounts of protein present in all postmortem bands were expressed as a percentage of the initial MARCKS protein band (0 h) as detailed in the "Materials and methods" section. Means and standard error of the means are shown. *$p<0.05$ indicates a significant difference from $T=0$ h

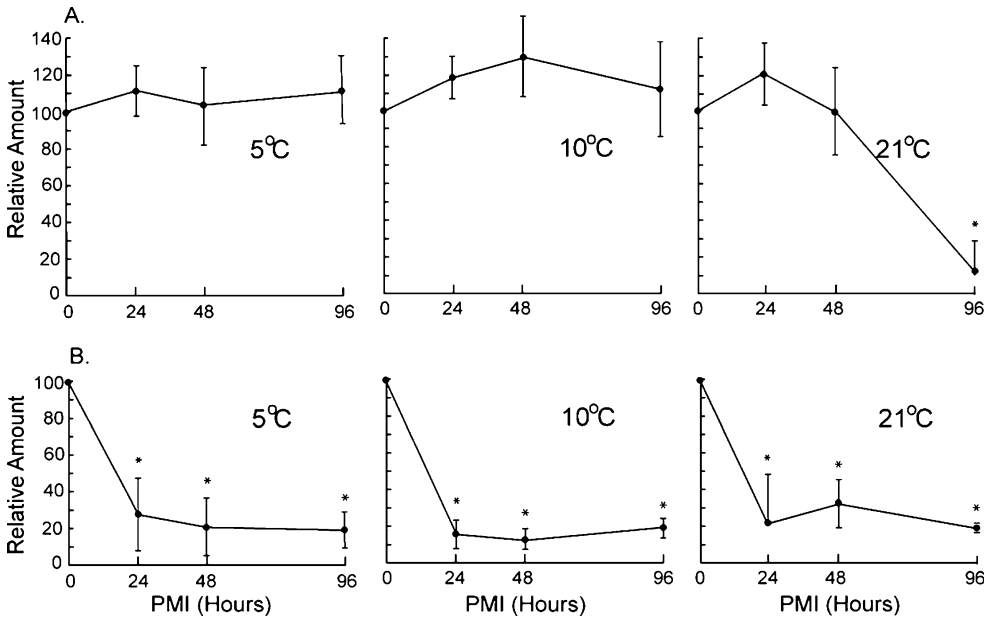

Fig. 5 The temperature-dependent degradation patterns of PP2A in **a** skeletal muscle and **b** lung tissue samples. The Western blots were scanned and the amounts of protein present in all postmortem bands were expressed as a percentage of the initial PP2A protein band (0 h) as detailed in the "Materials and methods" section. Means and standard error of the means are shown. *$p<0.05$ indicates a significant difference from $T=0$ h

tablet was used at 1/10th of the recommended dosage (Fig. 6b, lane 2). This cleavage was completely inhibited at the appropriate concentration of the inhibitor (Fig. 6b, l ane 1). Treatment with MDL-28170, a potent and selective cell permeable calpain inhibitor, showed a concentration-dependent effect on the breakdown of CnA (Fig. 6b, lanes 3, 4). The cleavage of CnA to produce the 57-kDa band was almost completely inhibited at 10 μM MDL-28170 indicating calpain is involved in CnA cleavage. Lanes 5 and 6 (Fig. 6) show a concentration-dependent effect of MG-132, a potent and selective proteasome inhibitor. CnA degradation was completely inhibited with the addition of 20 μM MG-132, as seen in lane 6. The absence of the 57-kDa band suggests that there may be an interplay between the proteasome and the calpain systems. In this case, inhibition of proteasome could have led to accumulation of calpastatin, which would have inhibited calpain from cleaving CnA. The concentration-dependent disappearance of the 57-kDa band in lanes 8 (10 μM calmidazolium) and 10 (10 mM EGTA) also suggests that CaM and Ca^{+2} have functions during the

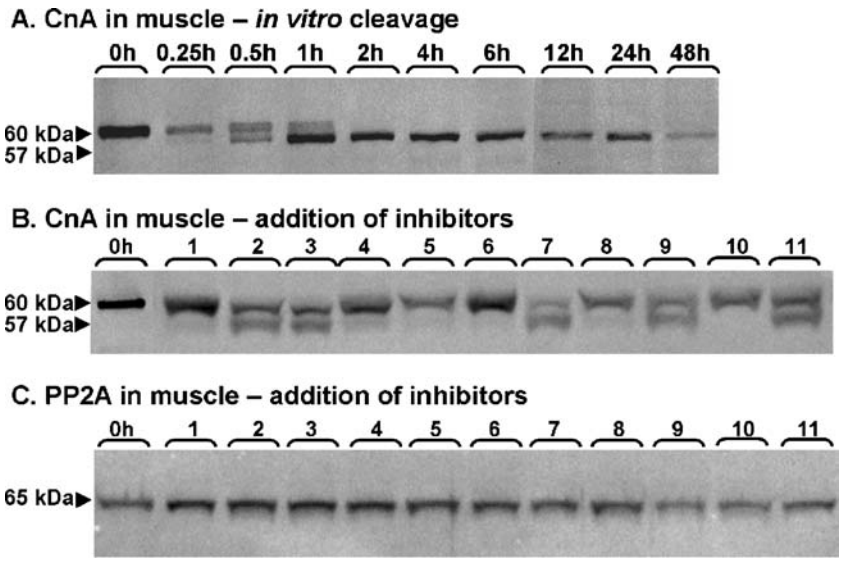

Fig. 6 The importance of calcium, calpain, calmodulin, and the proteasome in postmortem degradation of CnA and PP2A in mouse skeletal muscle of mice. **a** Postmortem CnA cleavage in the first 48 h at 21°C. **b** The effect of inhibitors on CnA levels after 1 h PMI. **c** The effect of inhibitors on PP2A levels after 1 h PMI. *1* complete protease inhibitor tablet, *2* 1/10 complete protease inhibitor tablet, *3* 1 μM MDL-28170, *4* 10 μM MDL-28170, *5* 2 μM MG-132, *6* 20 μM MG-132, *7* 1 μM calmidazolium, *8* 10 μM calmidazolium, *9* 1 mM EGTA, *10* 10 mM EGTA, *11* PBS only (control)

cleavage of CnA. Proteasomal degradation of CnA is also suggested by inhibitor treatment. As seen in Fig. 6/C, PP2A levels increased in the presence of the complete protease tablet (lanes 1 and 2), MDL-28170 (lanes 3 and 4), MG-132 (lanes 5 and 6), and calmidazolium (lanes 7 and 8). These increases could be due to postmortem translation or inhibition of proteolysis of PP2A or of proteins involved in PP2A stabilization or translation. In contrast, EGTA had no effect on PP2A levels (lanes 9 and 10). This suggests that Ca^{+2} is involved in postmortem increase in PP2A levels.

Centrifuged versus crude tissue extracts

Samples prepared using previously established techniques involving centrifugation to clear cell debris revealed that muscle CnA breaks down to two bands as detected by Western blotting, after which the original band decreases and the second band undergoes temperature dependent changes (Figs. 2b, 3, and 7a). When the time-consuming centrifugation step was removed, the resulting Western blots for CnA showed conserved band patterns compared to the centrifuged control (Fig. 7b). Full-length CnA was converted to 57-kDa cleavage product within 24 h of PMI at all temperatures.

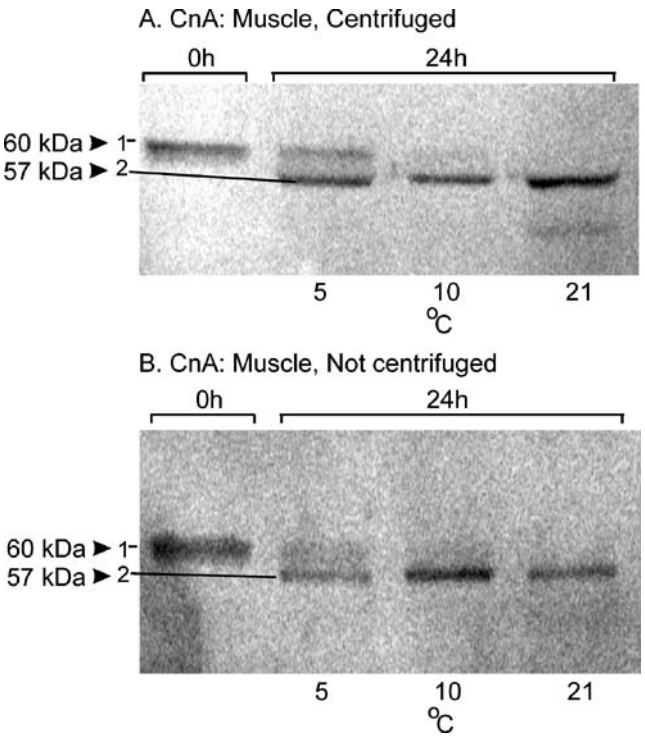

Fig. 7 CnA degradation pattern in the skeletal muscle tissue samples, **a** after centrifugation and **b** with no centrifugation prior to SDS-PAGE and Western blotting

Discussion

Postmortem interval is defined as the time between physiological death and the examination of the deceased. Current methods of PMI estimation do not always accurately or reproducibly estimate the time since death. Approaches that are used to estimate PMI fall into three broad categories: physical, physiological, and biochemical. Recent technological advances have made the detection and quantification of changes at the biochemical level possible. Studies in mammalian brain, myocardium, skeletal muscle, and lung have identified specific proteins that are altered time dependently postmortem [3, 14, 24–29]. In the rat brain, Fountoulakis et al. [24] found reduced amounts of neurofilament, α-internexin, glial fibrillary acidic protein, and heat shock protein 70 at 48 h postmortem. Sorimachi et al. [14] showed that α-fodrin, a calmodulin-binding protein, undergoes rapid cleavage from 240 to 150/145 kDa products within 24 h PMI in the rat brain. Studies in lamb skeletal muscle demonstrated that α-actinin and myosin show little degradation even after 56 days postmortem but the proteins nebulin, titin, vinculin, dystrophin, desmin, and troponin T are degraded during this time [25]. Sabucedo and Furton [26] examined the breakdown pattern of cardiac troponin 1 in human postmortem samples and found that it could be a very useful marker for estimating PMIs of 1–5 days. Xiao and Chen [27] examined the breakdown patterns of actin and tubulin in the liver tissue extracts of rats and found that actin was completely degraded by 10 days postmortem while β-tubulin could not be detected after 4 days postmortem. This research was later extended to include several rat tissues to establish tubulin as a potential marker for shorter PMIs and actin as a marker for longer periods [28, 29]. The lack of stability of the housekeeping proteins such as actin and tubulin make them unsuitable as markers for controlling protein loading for SDS-PAGE.

Clearly, differences in sequence, function, stability, and protease cleavage sites can drastically alter the pattern of postmortem degradation in specific proteins, a difference that can be exploited in the development of biochemically based methods to estimate PMI. Selecting appropriate proteins with specific patterns of degradation and different sensitivities to environmental (e.g., temperature) events should provide a group of proteins that could be employed together to generate specific information about time since death. Thus, proteins that show consistent patterns of change in specific tissues between different mammalian species would likely present the most promising candidates for determination of PMI in humans.

The results of Kang et al. [3] indicated that specific calmodulin-binding proteins could serve as estimators of short-term PMIs (e.g., CnA) while others could be used for

longer-term PMI determination (e.g., CaMKII). CnA undergoes a dramatic shift in molecular weight, from 60 to 57 kDa, within the first 24 h, in both mouse and rat muscle tissue. In mouse tissue, the rapidly migrating cleavage product undergoes temperature-dependent changes in amount while the initial band decreases at all temperatures. Since mouse and human CnA share 99% sequence identity, it is likely that these results will reflect human postmortem changes. PP2A could be useful for longer-term PMI determination as, after initial increases, it is very stable at 5°C and 10°C for up to 96 h while undergoing temperature-dependent changes at 21°C. The initial increase in PP2A levels could possibly be attributed to the production of a more accessible epitope or to continued postmortem protein synthesis. Sanoudou [23] previously indicated that skeletal muscle undergoes a highly active transcriptional and possibly translational phase during the first 46-h PMI. A diversity of antibodies are commercially available against both of CnA and PP2A; therefore, it should be possible to study these proteins for PMI determination in humans. The work presented here suggests that centrifugation of tissue samples is not an essential step indicating that it would also be possible to develop field-based assays for the immunological estimation of PMI using crude muscle tissue extracts. Alternatively, the addition of certain protease inhibitors to tissues harvested at crime scenes would ensure that samples used for later PMI determination in a laboratory setting would remain unchanged.

It is also important to understand the events that drive postmortem changes in protein levels. Previous work has shown that postmortem degradation of the calmodulin-binding protein fodrin in the rat brain is reminiscent of vital signaling events observed in living cells and in the pathology of several diseases [14, 30]. Here, we saw that the postmortem degradation of CnA produced a cleavage product of 57 kDa in molecular weight. This event is likely mediated by μ-calpain as the cleavage product appears to be the same 57-kDa product observed in the brains of Alzheimer's disease patients and after ischemic injury in several organs of several model systems [18, 20, 21]. One common link between postmortem tissues, Alzheimer's disease, and ischemic/reperfusion injuries is the deregulation of calcium homeostasis caused by membrane leakiness. Increased intracellular calcium levels can lead to activation of calpain, CaM, and other calcium-binding proteins. Calpain 1 (μ-calpain) is the major proteolytic agent that functions in mouse muscle tissue during the first 3-day postmortem, after which its activity diminishes [13]. Calpain 1 is known to cleave CnA but this study is the first to reveal its role in postmortem CnA cleavage. Furthermore, the data indicate that calcium, CaM, and calpain all play a role in postmortem CnA cleavage. Calcium activates calpain which cleaves and activates CnA. CaM appears to be necessary for this cleavage but how remains unknown. In normal cell function, CaM binding alters the conformation of CnA activating its phosphatase activity. This conformational change in CnA could also open epitopes leading to digestion by calpain to specifically generate the 57-kDa cleavage product. Shioda et al. [21] also found that CaM is necessary for the generation of constitutively active forms of CnA (45 and 48 kDa) by calpain since DY-9760e, a novel calmodulin antagonist, inhibited calpain-mediated CnA cleavage in mouse brains. If the 57-kDa cleavage product has increased phosphatase activity, which was demonstrated in Alzheimer's disease brains, this in turn would mediate other postmortem changes (e.g., dephosphorylation of critical proteins) [31]. Our data suggest that the proteasome is also responsible for postmortem degradation of CnA which fits with the results of other research. Atrogin-1 is a skeletal muscle and cardiac muscle-specific component of SCF E3 ubiquitin ligase complex. Within this $SCF^{atrogin-1}$ E3 ligase complex, atrogin-1 is responsible for substrate recognition. Atrogin-1 is capable of interacting with CnA, which is ubiquitinated through the $SCF^{atrogin-1}$ E3 ligase complex and thus targeted for proteasomal degradation. This proteasomal degradation has been shown to regulate CnA protein levels and activity in cardiomyocytes and skeletal muscle in vitro [32]. Our data suggest its involvement in postmortem events as well. As mentioned, PP2A levels appear to increase in the initial stages of PMI. Whether there is an increase in the phosphatase activity associated with increased band intensity of PP2A remains to be determined. Studying calcium-induced postmortem-mediated increases in PP2A levels could provide insight into its increased levels as seen in diabetic hearts and other pathological conditions [33].

The results reported here indicate that certain proteins from muscle tissue are prime candidates for PMI determination in humans. Tissue accessibility, postmortem stability, and asepsis are all important attributes afforded by muscle tissue. In contrast, lung tissue is less useful since it possesses none of these attributes. Calcineurin A appears to be a prime candidate for PMI estimations within the first 24 h as during that time, it undergoes a dramatic shift in molecular weight, from 60 to 57 kDa, after which the second more rapidly migrating band undergoes temperature-dependent changes in amount while the initial band decreases at all temperatures. Protein phosphatase 2A appears useful for longer-term PMI determination as it is very stable at 5°C and 10°C for up to 96 h while undergoing temperature-dependent changes at 21°C. CaMKII was stable for up to 96 h postmortem in rat muscle and lung but in mouse tissues, it was less stable becoming undetectable by 48 h postmortem at all temperatures [3]. Since the pattern of CaMKII degradation did not translate from rat to mouse samples, CaMKII is likely not a good marker to pursue in human studies. In rats, MARCKS levels in the lung decrease steadily after death at 21°C until they were almost un-

detectable at 96 h [3]. Here, we have shown a similar pattern in mice with slower degradation rates at lower temperatures. However, unlike the results of Kang et al. [3], there was greater variability between samples making the use of MARCKS questionable as a useful indicator of PMI. In both studies, MARCKS was not clearly detectable in postmortem skeletal muscle samples.

Conclusion

Skeletal muscle has the most potential for use in PMI determination because it is easily accessible, there are multiple sites to select from based upon the specific cause of death, and it is essentially sterile, removing the issues of bacterial and fungal influence on protein degradation. Previous studies have also shown that the degradation of certain cytoskeletal proteins (e.g., actin and myosin) occur slowly while others (e.g., nebulin, titin, troponin T) degrade more quickly [25]. Here, we have added two additional proteins to this complement: CnA for determining PMIs within 24 h and PP2A for those longer than 96 h. While controlled temperature had a predictable effect on the degradation patterns, under field conditions, significant temperature fluctuations are common. Further studies could define appropriate correction factors that would be applied to compensate for the effect of temperature changes due to environmental conditions such as clothing, water suspension, leaf and other covering, air movement, and surface properties [34]. Since antibodies are commercially available against all of these human proteins, it should be possible to study these proteins further in this regard as potential estimators of PMI. Once these studies are completed, it should be possible to develop a biochemical PMI determination kit that consists of a device much like a pregnancy test kit in which various antibodies are embedded at specific places. Data from initial work presented here suggests that centrifugation of tissue samples is not an essential step indicating that it should be possible to develop field-based assays for the immunological estimation of PMI employing proteins such as CnA and PP2A using crude tissue samples. This implies that PMI could be determined directly at the scene of the crime. Addition of a crude muscle extract to the device would reveal the presence of the specific proteins which would appear as bands. Comparing those bands which are present with those that are absent would allow a precise determination of PMI.

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