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Research article

THE TRANSCRIPTIONAL CASCADE ASSOCIATED WITH CREATINE KINASE DOWN-REGULATION AND MITOCHONDRIAL BIOGENESIS IN MICE SARCOMA

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Abstract: The tissue-specific expressions of creatine kinase (CK) isoforms are regulated by the coordinated action of various transcription factors. The myogenic differentiation factor D (MyoD) family of proteins and the myocyte-specific enhancer binding factor 2 family of transcription factors are important in regulating the muscle-specific expression of cytosolic muscle-type CK (MCK) and mitochondrial CKs. As reported in some related studies, TNF-α mediated degradation of MyoD and myogenin mRNA may lead to severe muscle wasting and cachexia, which is characterized by a low transcript level of MCK and myosin heavy chain proteins. In our previous study, we reported on a complete loss of total CK activity and expression when sarcoma was induced in mouse skeletal muscle (Patra *et al.* FEBS J. 275 (2008) 3236-3247). This study aimed at investigating the transcriptional cascade of CK down-regulation in carcinogen-induced sarcoma in mouse muscle. Both CK deficiency and enhanced nitric oxide synthase (NOS) were known to augment mitochondrial biogenesis, so we also explored the activation of the transcriptional cascade of mitochondrial biogenesis in this cancer. We observed the activation of the

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Abbreviations used: CK – creatine kinase; COX – cytochrome c oxidase; CS – citrate synthase; eNOS – endothelial nitric oxide synthase; iNOS – inducible nitric oxide synthase; MCK – muscle-type CK; Mef2 – myocyte-specific enhancer binding factor 2; mtTFA – mitochondrial transcription factor A; MyHC – myosin heavy chain; MyoD – myogenic differentiation factor D; NM – normal muscle; NOS – nitric oxide synthase; NRF – nuclear respiratory factor; PGC – peroxisome proliferator-activated receptor gamma co-activator; PRC – PGC-related co-activator; sMitCK – sarcomeric mitochondrial CK; ST – sarcoma tissue; TNF-α – tumor necrosis factor α

TNF-α-mediated nitric oxide production pathway with NFκB activation and concomitant degradation of MyoD and myogenin mRNA. Exploration of mitochondrial biogenesis revealed high cytochrome c oxidase activity and mitochondrial DNA content in sarcoma. The PGC-related co-activator seems to have a major role in regulating mitochondrial biogenesis by upregulating nuclear respiratory factors and mitochondrial transcription factor A. From the above findings, it can be concluded that severe muscle degeneration leads to CK down-regulation in sarcoma, and that the stimulation of mitochondrial biogenesis indicated a scenario representing both CK deficiency and NOS overexpression on the one hand, and altered bioenergetic profiling on the other.

Key words: Sarcoma, Creatine kinase, Nitric oxide synthase, Muscle degeneration, Mitochondrial biogenesis

INTRODUCTION

There are three isoforms of creatine kinase (CK) expressed in a tissue-specific manner: muscle-type CK (MCK), brain-type CK (BCK), and mitochondrial CK [1]. In adult muscle, the predominant cytosolic isoform is MCK, and sarcomeric mitochondrial CK (sMitCK) is the mitochondrial isoform. Over the last few decades, extensive research had been done on the transcriptional regulation of CK gene expression [2], the appearance of which is regarded as the terminal point in myogenesis or muscle differentiation. Myogenesis is regulated by different growth factors and regulatory molecules.

Myoblasts, the first of the myogenic lineage, arise from the mesodermal stem cells, are committed to cell cycle arrest, develop into myocytes, and fuse with adjacent myocytes to form multinucleated myofibres. At this stage, the MyoD family of proteins, i.e. MyoD, myogenin, and myogenic factors Myf5 and Myf6, which belong to the basic helix-loop-helix (bHLH) family of transcriptional activators, and myocyte-specific enhancer binding factor 2c (Mef2c) become activated and induce muscle tissue-specific gene transcriptions [3, 4].

Identifying the consensus binding site for the MyoD and Mef2 family of proteins in the promoter region of both MCK and sMitCK [2, 5, 6] led to the understanding of the transcriptional regulation and tissue specificity of these isozymes. These studies convincingly showed the importance of MyoD and Mef2 in CK expression. In our previous study, we observed that in mice sarcoma tissue, CK expression decreased severely [7]. The total CK activity in sarcoma was reduced by 99% in comparison to healthy skeletal muscle. MCK and sMitCK protein expression, when viewed in Western blotting and mRNA expression studies, were found to be greatly reduced or almost absent in a full-grown sarcoma. The histological cross sections showed that upon transformation to sarcoma, the muscle tissue lost the muscle-specific phenotypes. The fiber-like nature of the skeletal muscle gradually disappeared as tumor development advanced, and the tissue became a mass of unorganized cells with no evidence

of the presence of myofibres [7]. These observations suggested that the malignant transformation involved severe degradation of the muscle fibers.

Muscle wasting and degeneration are very common in several pathological conditions including cancer. Many factors, such as the bHLH transcription factor family, which plays a great role in maintaining the typical nature of the differentiated myofibers, might be affected. It had been reported earlier that TNF-α induces muscle degradation and wasting through NFκB-dependent inhibition of muscle differentiation [8-10]. Loss of MyoD mRNA in muscle cells through TNF-α- and IFN-γ-induced activation of NFκB was also studied [8, 11]. In a separate study, it was also shown that TNF-α mediates the loss of myogenin mRNA by inhibiting insulin-like growth factor I (IGF-I) expression [12].

Vaarmann *et al.* [13] observed that MCK knockout mouse muscle showed enhanced mitochondrial biogenesis. Mitochondrial biogenesis involves an increased rate of mtDNA replication and transcription of both mitochondrial and nuclear DNA-coded mitochondrial proteins with several interplaying transcriptional regulators [14]. Identifying the nuclear respiratory factors (NRFs) such as NRF-1 and NRF-2 led to a better understanding of the transcriptional circuits controlling mitochondrial biogenesis [14, 15]. NRF binding to the promoter region of mitochondrial transcription factor A (mtTFA) initiates mitochondrial replication and transcription [14]. Upstream of the NRFs, the peroxisome proliferator-activated receptor (PPAR) gamma co-activator (PGC-1) family of co-activators, which includes PGC-1α, PGC-1β and PGC-related coactivator (PRC), play a vital role in mitochondrial biogenesis [14]. Hence, we found it interesting to explore whether the loss of CK activity and expression upregulates the transcriptional pathways of mitochondrial biogenesis in sarcoma. In this study, we attempted to identify in mouse sarcoma tissue the interplaying action of various cytokines, transcription factors and co-activators leading to a loss of muscle phenotypes and to mitochondrial remodeling. The expression levels of the various transcription factors and co-activators were studied by RT-PCR. Electrophoretic mobility shift analysis (EMSA) was done to study the nuclear translocation and enhanced DNA-binding activity of NFκB. Mitochondrial biogenesis was assessed by studying COX enzyme activity and the expression level of the mitochondrial DNA-coded COX I subunit and several transcription factors related to mitochondrial biogenesis.

MATERIALS AND METHODS

Cytochrome c, acetyl CoA, oxaloacetic acid, 5,5'-dithiobis-(2-dinitrobenzoic acid), 3-methylcholanthrene (3MC), nitrocellulose membrane (0.45 μM pore size) and peroxidase-conjugated anti-goat IgG (whole molecule) were obtained from the Sigma Chemical Co., USA, and goat polyclonal COX I IgG and luminol reagent were obtained from Santa Cruz Biotech, USA. M-MLVRT, Taq polymerase, dNTP, random hexamer, Trizol reagent and the DNA isolation kit were from Invitrogen, USA. The CK assay kit was obtained from Bayer

Diagnostics India (Baroda, India). The other chemicals were of analytical grade and obtained from local manufacturers.

Growth of tumors

The animal experiments were carried out in accordance with the guidelines of the institutional ethics committee for animal experiments. Appropriate measures were taken to minimize the pain and discomfort to the animals.

Sarcoma was induced as described earlier with 3MC in the hind legs of 45-day old Swiss albino female mice with a body weight of 20-22 g [7]. A stock solution of 3MC was prepared by dissolving 2 mg per 1 ml of hot olive oil, and cooling to room temperature. 0.1 ml of this solution was injected into the hind leg of each mouse. This procedure was repeated twice with a one-week interval between the injections. When the tumor weight reached approximately 5-7 g, it was considered a full-grown sarcoma. Malignancy was confirmed by histological examinations as described earlier [7]. Sarcoma tissue or unaffected normal muscle was excised from the hind legs and immediately placed in the respective pre-chilled buffers for the preparation of the mitochondrial or nuclear fractions.

Isolation of mitochondria

Mitochondria were isolated from normal muscle and sarcoma as described earlier [7]. Briefly, normal muscle or sarcoma tissue was washed and finely minced in a preparation buffer (250 mm sucrose, 1 mm EDTA, 0.1% BSA and 10 mm Tris-Cl, pH 7.4) and homogenized in a Potter-Elvehjm homogenizer. The homogenate was centrifuged at 650 x g for 10 min, and the supernatant, termed 'total homogenate', was again centrifuged at 14,000 x g for 10 min. The final supernatant, termed post-mitochondrial supernatant (PMS), was collected for the assay of creatine kinase activity. The pellet containing the mitochondria was suspended in the preparation buffer and washed twice by centrifuging at 14,000 x g for 10 min. The final mitochondrial suspension was made in a minimum volume of the preparation buffer. The mitochondrial purity was checked with the succinate dehydrogenase and glucose-6-phosphate dehydrogenase assay.

Enzyme analysis

The total CK (TCK) and CK isoform activities were estimated, respectively in the total homogenate and in the PMS and mitochondrial fractions. CK activity was estimated as per the assay kit manufacturer's instructions. The reaction medium contained 25 mM Tris-Cl, pH 7.2, 2.5 mM magnesium acetate, 5 mM N-acetyl-l-cysteine, 0.5 mM ADP, 1.25 mM AMP, 0.5 mM NADP, 5 mM D-glucose, 2.5 mM diadenosine pentaphosphate, 0.5 mM EDTA, 7.5 mM phosphocreatine, 8.5 units hexokinase and 5 units glucose- 6-phosphate dehydrogenase. After 2 min incubation at 30ºC, tissue samples were added and the formation of NADPH at 340 nm was monitored for 5 min.

COX activity was assayed according to Moyes *et al.* [16]. The assay medium contained 50 μM reduced cytochrome c, 0.5% Tween-20 and 20 mM Tris-Cl

(pH 8.0). Cytochrome c was reduced with ascorbate, and dialyzed overnight in Tris-Cl buffer (pH 8.0) to remove unreacted ascorbate. The concentration of reduced cytochrome c was determined with an extinction coefficient of 28.5 mM^{-1} at 550 nm. Mitochondria were added to the assay medium and incubated for 5 min. The reaction was started by adding reduced cytochrome c and the change in absorbance was recorded at 550 nm for 3 min.

Citrate synthase (CS) activity was estimated in the mitochondrial preparations according to Leek *et al.* [17]. Briefly, CS activity was determined by measuring the formation of the CoA-SH at 412 nm for 5 min. The assay medium contained 20 mM Tris-Cl (pH 8.0), 0.15 mM acetyl-CoA, 0.1 mM DTNB, and 0.5 mM oxaloacetate. The mitochondrial samples were added, after waiting 1 min to eliminate any false readings from the side reaction of DTNB with CoA-SH. The enzyme activity was calculated from the rate of formation of mercaptide ion with an extinction coefficient of 17.6 mM^{-1} at 412 nm.

mRNA expression study

Total cellular RNA was isolated with Trizol reagent as per the manufacturer's instructions. 1 μg of total RNA was used for cDNA synthesis by using M-MLV reverse transcriptase and oligo-dT primer. The cDNA sequence was amplified with a specific primer set (Tab. 1) by PCR. The general PCR conditions were 30 sec denaturation at 94ºC, 30 sec annealing at the respective temperatures given in Tab. 1 followed by 30 sec extension at 72ºC. Cycling was started by 5 min denaturation at 94ºC and terminated by 7 min incubation at 72ºC. All the PCR experiments were performed using a Thermocycler (Applied Biosystems 2720). 18s rRNA was chosen as the housekeeping gene for normalization, as its expression did not differ between the tissue types. The reaction cycles of PCR were performed in the range that demonstrated a linear correlation between the amount of cDNA and the yield of the PCR products, which is generally 25-40 cycles. PCR-amplified DNA fragments were run on 1.5% agarose gel stained with ethidium bromide and visualized and photographed by irradiation with UV light. The band intensities were calculated with Quantity One 1-D analysis software (Bio-Rad, USA).

Determination of the mtDNA content

Total cellular DNA, which includes both nuclear and mtDNA, was isolated from both normal muscle and sarcoma tissue with a DNA isolation kit as per the manufacturer's instructions. The mitochondrial DNA contents of normal muscle and sarcoma tissue were estimated by PCR amplification of a 129-bp fragment of mtDNA that codes a COX I subunit using the primer set shown in Tab. 1 and total cellular DNA as the template. Absolute COX I DNA copies were normalized to the DNA copy of the nuclear gene, 18s rRNA, which was also amplified as described above. The PCR conditions were similar to those detailed for the mRNA expression analysis. The band intensities were calculated as before.

Tab. 1. Primers used for RT-PCR.

Gene	Forward primer Reverse primer (5'-3')	Annealing temperature (ºC)
MyoD	GAT GGC ATG ATG GAT TAC AGC GAC TAT GTC CTT TCT TTG GGG	56
Myogenin	GCT CAG CTC CCT CAA CCA G ATG TGA ATG GGG AGT GGG GA	60
Mef2c	GGG ATC CAA CAC GGG GAC TAT GGG GAG GGC CAT GGT GCG GCT CGT TGT ACT CGG	65
MyHC-II	CCG TGA TAT ACA GGA CAG TG GTT CCG TAA GAT CCA GCA CG	65
IGF-I	GCT CTT CAG TTC GTG TGT GGA C CAT CTC CAG TCT CCT CAG ATC	65
TNFα	TCT CAT CAG TTC TAT GGC CC GGG AGT AGA CAA GGT ACA AC	58
IFN-γ	GCT CTG AGA CAA TGA ACG CT AAA GAG ATA ATC TGG CTC TGC	58
p65	ATC AAT GGC TAC ACA GGA CCC GTG AAA TAC ACC TCA	60
iNOS	TGC ATG GAC CAG TAT AAG GCA AGC GCT TCT GGT CGA TGT CAT GAG CAA	58
eNOS	GGC TCC CTC CTT CCG GCT G TCC CGC AGC ACG CCG AT	65
COX I	CAC TAA TAA TCG GAG CCC CA TTC ATC CTG TTC CTG CTC CT	65
NRF-1	GGA GCA CTT ACT GGA GTC C CTG TCC GAT ATC CTG GTG GT	60
NRF-2α	AGG TGA CGA GAT GGG CTG C CGT TGT CCC CAT TTT TGC G	58
mtTFA	GCT GAT GGG TAT GGA GAA G GAG CCG AAT CAT CCT TTG C	56
PGC-1α	CAC CAA ACC CAC AGA GAA CAG GCA GTT CCA GAG AGT TCC ACA	65
PGC-1β	TGG AAA GCC CCT GTG AGA GT TTG TAT GGA GGT GTG GTG GG	60
PRC	AAG GAG CGT GCA ATA GAA GA TCT TCT GGG CCT GTT TCA AC	60
18sRNA	CAC GGC CGG TAC AGT GAA AC CCC GTC GGC ATG TAT TAG CT	58

EMSA

The DNA-binding activity of NFκB was determined by EMSA using NFκB consensus oligonucleotide sequences containing NFκB-binding sites present in the murine iNOS promoter. The oligonucleotide sequences were 5'-AGTTGAGGGGACTTTCCCAGGC-3' (upper band in Fig. 2B) and 5'-GCCTGGGAAAGTCCCCTCAACT-3' (lower band in Fig. 2B), which were radiolabeled using [γ^{32}P] dATP and T4 polynucleotide kinase (Roche Molecular Biochemicals). Nuclear extracts from both normal muscle and sarcoma tissue were prepared according to Cox and Emili [18]. Nuclear proteins (10 μg) were incubated with radiolabeled nucleotides for 40 min on ice. The protein-DNA

complexes were resolved on a 7.5% non-denaturing polyacrylamide gel at 300 V for 4 h. The gels were dried, exposed to X-ray films, and photographed.

Western blot

Mitochondrial protein was used for the Western blot analysis of sMitCK and COX I, and PMS was used for that of MCK. The protein samples were resolved on 7.5% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was blocked with 5% skim milk in 50 mM sodium-phosphate buffer containing 0.9% NaCl (PBS) and incubated overnight with a primary antibody solution in PBS following 2 h incubation with the appropriate peroxidase-conjugated secondary antibody solution in PBS. The immunoreactive bands were detected using Luminol reagent, exposed to X-ray films, and photographed. The primary antibody dilutions used for immunoblot were 1:5000 for MCK, and 1:1000 for sMitCK and COX I. The secondary antibody dilutions were 1:10,000 peroxidase-conjugated anti-rabbit IgG for MCK and sMitCK, and 1:1000 peroxidase-conjugated anti-goat IgG for COX I. Equal protein loading was confirmed with a parallel gel stained with Coomassie blue.

Estimation of protein content

The protein content was estimated with bovine serum albumin (BSA) as a standard using the method of Lowry *et al.* as outlined by Layne [19].

Statistical analysis

The data is presented as means ± standard deviation (s.d.) for n = 3 animals. A comparison between the experimental groups was done using Student's two-tailed t test. Values of $p \leq 0.05$ were considered significant.

RESULTS

CK activity and expression analysis

We recently reported a drastic reduction in CK activity and expression in mouse sarcoma tissue induced by the chemical carcinogen 3MC [7]. Similar results were obtained in this study with about a 99% reduction in the total CK activity in 3MC-induced mouse sarcoma. Cytosolic CK activity, which is mainly represented by MCK, and mitochondrial CK activity, which is represented by sMitCK, followed a similar trend of reduction by about 95-99%, as shown in Fig. 1A. The MCK and sMitCK protein expression study by Western blot (Fig. 1B) and the mRNA expression study by RT-PCR analysis (Fig. 1C) clearly demonstrated that the reduction is at the transcriptional level. These results and those from the previous study [7] prompted us to explore the status of the transcriptional cascade responsible for CK gene expression in this tissue.

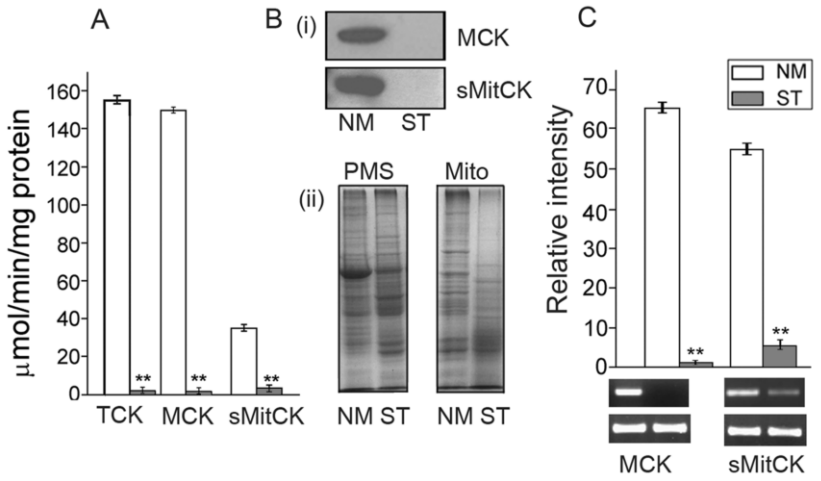

Fig. 1. CK activity and expression. A – The CK activity in the total homogenate (TCK), PMS (MCK) and mitochondrial fractions (sMitCK). B – (i) An immunoblot of MCK and sMitCK in PMS and mitochondria, (ii) A parallel gel stained with coomassie blue to confirm equal protein loading in PMS and mitochondria (Mito). C – The mRNA levels of MCK and sMitCK. A densitometric analysis of the amplified PCR fragments and a representative agarose gel of the amplified DNA fragments are shown. The bottom panel in the gel images represents 18s rRNA. NM – normal muscle, ST – sarcoma tissue. The values are the means ± sd, n = 3; **p < 0.001 versus NM.

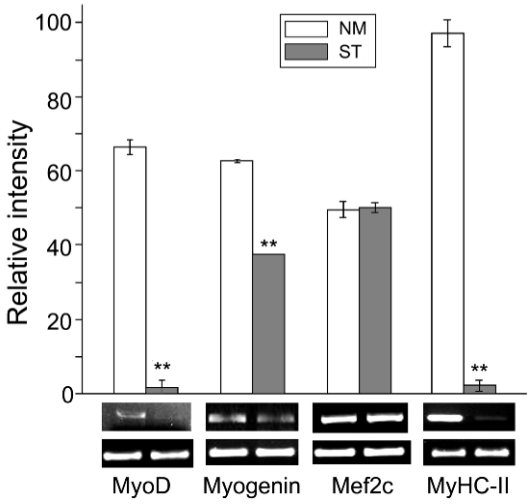

Fig. 2. The mRNA levels of different regulatory factors for muscle differentiation. A densitometric analysis of the amplified PCR fragments and a representative agarose gel of the amplified DNA fragments of MyoD, myogenin and Mef2c and the MyHC-II subunit. NM – normal muscle, ST – sarcoma tissue. The bottom panel in the gel images represents 18s rRNA. The values are the means ± sd, n = 3; **p < 0.001 versus NM.

PCR analysis of muscle differentiation factors

Very low expressions of MyoD and myogenin, two important transcriptional regulators of muscle cell differentiation, were observed in the sarcoma tissue (Fig. 2), whereas Mef2c expression was found to be unaltered. To establish the fact of muscle degeneration, the myosin heavy chain-II (MyHC-II) subunit mRNA level was also estimated, and was also found to be very low in sarcoma. As shown previously [7] and in this study, the loss of CK expression in sarcoma is associated with severe muscle degeneration occurring upon transformation.

Activation of the pathway for TNF-α/IFN-γ-induced NFκB activation and iNOS overexpression

Fig. 3A clearly indicates that both the TNF-α and IFN-γ expressions were upregulated in sarcoma. Moreover, the p65 subunit, the most abundant of all the NFκB subunits, was found to be overexpressed (Fig. 3B, ii). Increased nuclear

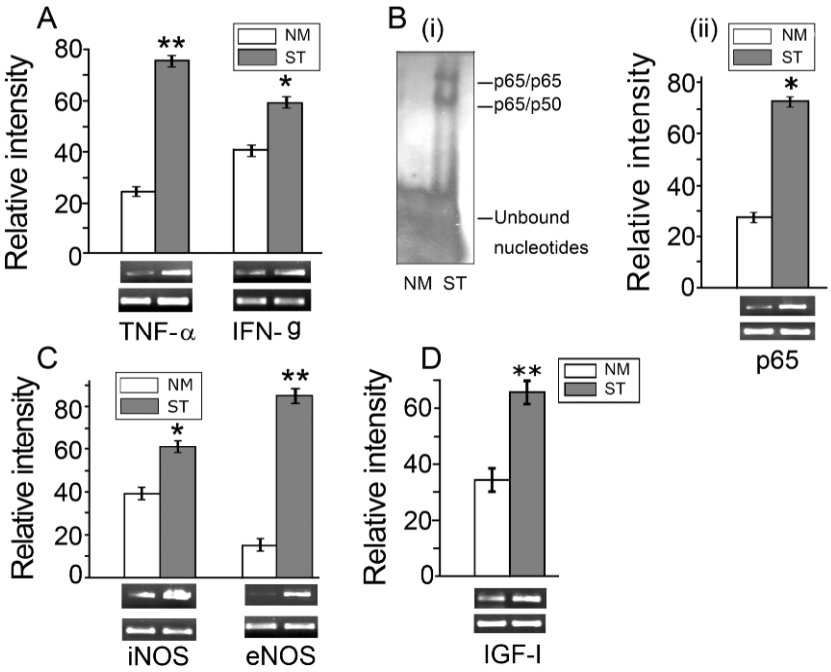

Fig. 3. The mRNA levels of TNF-α, IFN-γ, NFκB (p65 subunit), iNOS, eNOS and IGF-I. A densitometric analysis of the amplified PCR products and a representative agarose gel of the amplified DNA fragments are shown. A – The mRNA expressions of TNF-α and IFN-γ. B – (i) An electrophoretic mobility shift analysis (EMSA) to assess the nuclear translocation and DNA binding of NFκB. 10 μg of protein from the nuclear extracts of both normal muscle and sarcoma were loaded on each lane, (ii) The mRNA expressions of NFκB (p65 subunit). C – The mRNA expressions of iNOS and eNOS. D – The mRNA expression of IGF-I. NM – normal muscle, ST – sarcoma tissue. The bottom panel in the gel images represents 18s rRNA. The values are the means ± sd, n = 3; **$p < 0.001$ versus NM, *$p < 0.05$ versus NM.

accumulation and binding of both the p65 and p55 subunits of NFκB protein to the consensus oligonucleotide sequences containing NFκB binding sites in the iNOS promoter were also evident in sarcoma (Fig. 3B, ii). Fig. 3C shows the increased transcript level of iNOS, confirming that TNF-α/IFN-γ-mediated activation of NFκB activity might be one of the reasons for enhanced iNOS expression. In addition, eNOS was also overexpressed.

Mitochondrial enzyme activity and expression

Mitochondrial protein expression was assessed by measuring the activities of two mitochondrial activity marker enzymes, cytochrome c oxidase (COX), and citrate synthase (CS). It is evident from Fig. 4A that there was a 5-fold increase in COX activity, whereas CS activity was reduced by 2.5-fold in sarcoma when compared with normal muscle. COX I mRNA expression was also increased (Fig. 4B, i) indicating the increased rate of mitochondrial protein synthesis in sarcoma. Western blot analysis revealed overexpression of the COX I subunit in sarcoma in comparison to normal muscle mitochondria (Fig. 4B, ii).

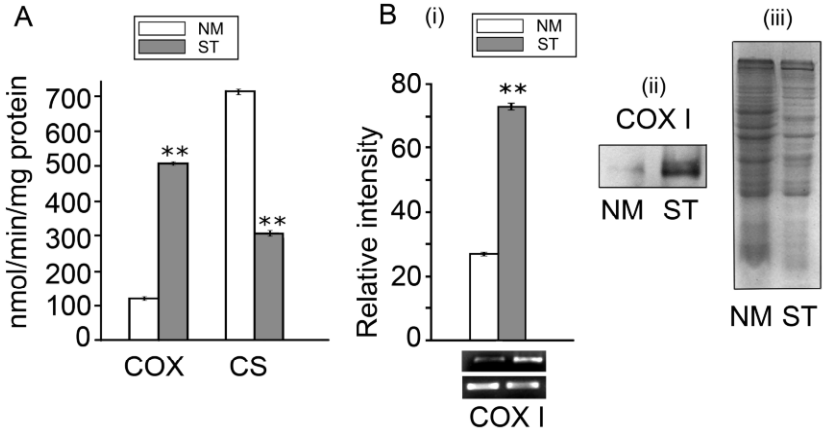

Fig. 4. Mitochondrial enzyme activity and expression. A – COX and CS activity. B – (i) The mRNA expression and densitometric analysis of the mitochondrial COX I subunit. The bottom panel in the gel images represents 18s rRNA, (ii) Immunoblot of COX I in the mitochondrial fraction, (iii) A parallel gel stained with coomassie blue to confirm equal protein loading. 25 μg of protein was loaded on each lane. The values are the means ± sd, n = 3; **p < 0.001 versus NM where applicable. NM – normal muscle, ST – sarcoma tissue.

The transcriptional cascade of mitochondrial biogenesis

By amplifying the mtDNA-coded COX I subunit gene, it was observed that the mtDNA content increased significantly in sarcoma, indicating an increased rate of its synthesis and replication (Fig. 5A). mtTFA is one of the main regulatory factors for mitochondrial DNA replication and transcription. In sarcoma, mtTFA expression increased significantly (Fig. 5B) in accordance with the increased expression of COX I mRNA and protein (Fig. 4) and increased mtDNA content. mtTFA expression is regulated by NRFs, and our study revealed that both NRF-1

and NRF2α expression increased significantly in sarcoma (Fig. 5B). In this transcriptional cascade upstream of the NRFs, the PGC-1α transcriptional co-activator family plays a major role in mitochondrial biogenesis, and PGC-1α is often regarded as the 'master regulator' of mitochondrial biogenesis. However, we observed decreased, rather than increased, expression of PGC-1α in sarcoma (Fig. 5C). PGC-1β showed little change in expression and PRC expression increased significantly. It can be assumed that mitochondrial biogenesis in sarcoma is stimulated mainly by the PRC protein.

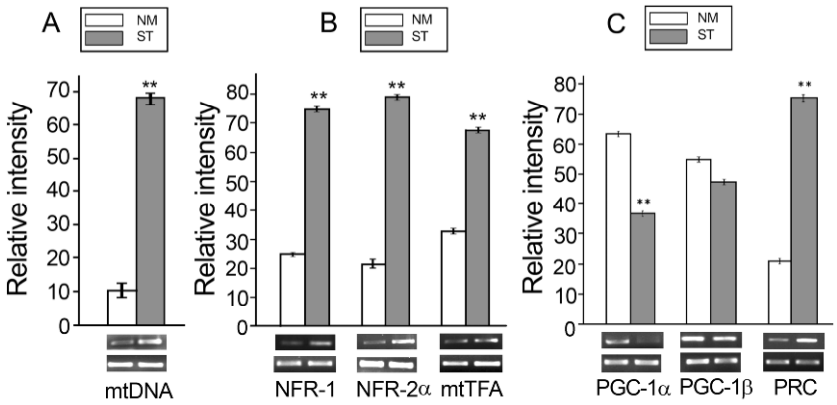

Fig. 5. The mtDNA content and mRNA expression of transcription factors for mitochondrial biogenesis. A densitometric analysis of the amplified PCR products and a representative agarose gel of the amplified DNA fragments. A – mtDNA content. The bottom panel represents an amplified nuclear DNA copy corresponding to the 18s rRNA gene. B – The mRNA expressions of NRF-1, NRF-2α and mtTFA. C – The mRNA expressions of PGC-1α, PGC-1β and PRC. NM – normal muscle and ST – sarcoma tissue. The bottom panel in the gel images represents 18s rRNA. The values are the means ± sd, n = 3; **p < 0.001 versus NM.

DISCUSSION

Continuing from our previous study [7], this study explores the causal events for CK down-regulation in mouse sarcoma. In vertebrates, during embryogenesis, muscle cells are derived from mesodermal stem cells that give rise to committed myogenic cells, which become the skeletal muscle of the head, trunk, and limbs. Terminally differentiated muscle cells express muscle-specific genes such as MCK and MyHC. Muscle degradation is characterized by a reduced transcript level of these muscle markers.

We used 3MC, a polycyclic aromatic hydrocarbon, to induce fibrosarcoma in mouse leg muscle. The members of this class of compounds are known to give rise to DNA adducts, mutations of oncogenes and tumors [20]. Subcutaneous injection of 3-methylcholanthrene in the mice's legs resulted in inflammation

and tissue damage at the site of injection. The inflammatory signals produced during tumor development are mainly mediated by pro-inflammatory cytokines like TNF-α and IFN-γ. The physiological relevance of TNFα in muscle degeneration has been studied extensively [8, 21]. Following muscle tissue damage, the tissue levels of TNFα increase significantly due to its enhanced expression by injured muscle fibers, as well as due to macrophage infiltration [21]. As mentioned earlier, enhanced TNFα inhibits myogenesis by activating NFκB, leading to a stabilization of iNOS mRNA, increased NO production, and a loss of MyoD expression [11].

The 5' flanking regions of both the MCK and sMitCK genes contain binding elements for MyoD and myogenin. Fig. 2 shows that both the MyoD and myogenin mRNA levels reduced significantly in sarcoma, leading to severe degeneration of the muscle phenotypes, as indicated by the degradation of two muscle markers, the CK and MyHC proteins. Consistent with our hypothesis, we also found stimulation of the TNFα-iNOS pathway, which triggers MyoD down-regulation (Fig. 3). Myogenin expression during myogenesis is mainly regulated by IGF-I [22]. Also, IGF-I is overexpressed and has a profound role in the growth and proliferation of the tumor tissue itself [23]. As expected in sarcoma, we observed upregulation of IGF-I expression (Fig. 3). This result indicates that the myogenin down-regulation observed in sarcoma occurs in a pathway independently of IGF-I. Interestingly, TNF-α was also shown to suppress IGF-I mRNA expression [24], leading to muscle degeneration. However, a high level of TNF-α is unable to suppress IGF-I expression in sarcoma, as evident in this study. Further investigation will disclose the role of IGF-I in sarcoma. It appears that IGF-I functions mainly in promoting tumorigenesis rather than modulating muscle degeneration in sarcoma.

The combination of the two observed phenomena, loss of CK expression and NOS over-expression, both of which were known to promote mitochondrial biogenesis [13, 25], also prompted us to investigate the status of mitochondrial biogenesis and the transcriptional cascade behind it in sarcoma. Mitochondrial biogenesis is well studied in different human and rodent systems, including in several forms of cancerous tissues [26, 27].

Mitochondrial biogenesis is mainly characterized by an increased rate of mitochondrial DNA replication and transcription. We observed a high level of mtDNA content in sarcoma when compared to normal muscle (Fig. 5A). An increase in the COX activity and COX I mRNA expression (Fig. 4) also specifies the increased mitochondrial biogenesis in sarcoma. Interestingly, the enzymatic activity of CS, which is considered a mitochondrial marker, is reduced in the same tissue (Fig. 4A). CS promoter analysis by Kraft *et al.* [28] provided information about the differential regulation of CS gene expression, which, unlike COX, is independent of NRFs. The elements between -559 bp and -257 bp in the CS promoter were shown to contain the MyoD-binding region. The low level of MyoD in sarcoma might be one of the reasons for the reduced CS activity in this tissue.

The process of mitochondrial biogenesis is well controlled by a set of transcription factors, and their strict co-ordination is well studied in various subjects, including in several forms of cancerous tissues [14, 26, 27]. Mitochondrial transcription factor A (mtTFA) regulates mtDNA replication and transcription, and it is found to be highly upregulated in sarcoma (Fig. 5B), which justifies the increase in mtDNA content in this tissue. The increased transcript level of the mitochondrially coded COX I subunit and the increased COX activity rationalized the role of mtTFA in stimulating mitochondrial biogenesis in sarcoma. Also, NRF-1 and NRF-2α expression was observed to be up-regulated in sarcoma (Fig. 5B). Both of these transcription factors act upstream of mtTFA and participate in mitochondrial biogenesis. Again, upstream of the NRFs, the PGC-1α family of transcriptional co-activators controls mitochondrial biogenesis [29]. This family of co-activators includes PGC-1α, PGC-1β and PRC, with PGC-1α considered the 'master regulator' of mitochondrial biogenesis in several studies [14, 29]. As evident from Fig. 5C, PGC-1α expression reduced drastically in sarcoma, which contradicts its stimulating role in this regard. However, the bHLH family of proteins, like MyoD, was known to activate PGC-1α expression by binding to its promoter [30]. Hence, the reduced level of MyoD may impart a negative regulation, and justified the reduced expression of PGC-1α. The role of other members of the PGC-1 family (i.e. PGC-1β and PRC) in mitochondrial biogenesis has been studied extensively. Both can promote mitochondrial biogenesis independently of PGC-1α [14, 29]. PGC-1β showed no significant change in expression, whereas PRC was significantly upregulated in sarcoma (Fig. 5C). PRC has almost the same potentiality to regulate mitochondrial function as PGC-1α. PRC exerts its function by directly interacting with NRF-1 and NRF-2, which in turn activates several genes of the mitochondrial respiratory chain [31]. PRC up-regulation signifies that enhanced mitochondrial biogenesis in sarcoma is independent of PGC-1α, and indicates that the so-called role of PGC-1α as the 'master regulator' might not be applicable in all systems.

Our findings implied that loss of CK activity is a phenomenal event in muscle transformation, which has a significant resemblance to cancer cachexia. The mitochondrial remodeling observed in this tissue might be due to CK deficiency or enhanced NO production. Hence, it would be interesting to study the interrelations between muscle degeneration and mitochondrial biogenesis in sarcoma.

Rapidly growing tumors need more ATP for their growth and proliferation, and this excess need for energy may drive their energy production units to be hyperactive. As indicated in some earlier studies, CK deficiency led to an increased oxidative capacity of the concerned tissue [32, 33]; we also observed increased mitochondrial COX activity although CS activity was reduced. This is important since a decrease in CS activity will certainly affect the mitochondrial energy metabolism. Hence, investigation of the status of the oxidative metabolism in sarcoma will definitely throw some light on the possible cause(s)

of altered mitochondrial function in relation to CK deficiency in sarcoma. Also, the susceptibility of CK knockout mice to develop sarcoma induced by the carcinogen and the status of mitochondrial biogenesis in established sarcoma cell lines could be studied to understand the interrelations between tumorigenesis and mitochondrial biogenesis.

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