Life or death by NFκB, Losartan promotes survival in dy2J/dy2J mouse of MDC1A

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Inflammation and fibrosis are well-defined mechanisms involved in the pathogenesis of the incurable Laminin α2-deficient congenital muscular dystrophy (MDC1A), while apoptosis mechanism is barely discussed. Our previous study showed treatment with Losartan, an angiotensin II type I receptor antagonist, improved muscle strength and reduced fibrosis through transforming growth factor beta (TGF-β) and mitogen-activated protein kinases (MAPK) signaling inhibition in the dy2J/dy2J mouse model of MDC1A. Here we show for the first time that Losartan treatment up-regulates and shifts the nuclear factor kappa B (NFκB) signaling pathway to favor survival versus apoptosis/damage in this animal model. Losartan treatment was associated with significantly increased serum tumor necrosis factor alpha (TNF-α) level, p65 nuclear accumulation, and decreased muscle IκB-β protein level, indicating NFκB activation. Moreover, NFκB anti-apoptotic target genes TNF receptor-associated factor 1 (TRAF1), TNF receptor-associated factor 2 (TRAF2), cellular inhibitor of apoptosis (cIAP2), and Ferritin heavy chain (FTH1) were increased following Losartan treatment. Losartan induced protein expression toward a pro-survival profile as BCL-2 expression levels were increased and Caspase-3 expression levels were decreased. Muscle apoptosis reduction was further confirmed using terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay. Thus, along with TGF-β and MAPK signaling, NFκB serves as an important regulatory pathway which following Losartan treatment promotes survival in the dy2J/dy2J mouse model of MDC1A.

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Congenital muscular dystrophy type 1A (MDC1A) is one of the most common forms of congenital muscular dystrophies (CMDs). Clinical symptoms are severe hypotonia, muscle weakness, and delayed motor milestones. Typically, the children do not achieve independent ambulation and respiratory failure is followed by death in the second or third decade of life. MDC1A is caused by mutations in the LAMA2 gene, encoding the heavy chain of laminin-2. Muscle biopsies are characterized by muscle fiber necrosis, inflammation, apoptosis, and fibrosis. Despite extensive advances in its diagnosis, MDC1A remains an incurable disease. The dy2J/dy2J mouse is a useful model to study the pathophysiology of MDC1A and the effect of various therapeutic agents. This mouse has a mutation in the LAMA2 gene resulting in abnormal splicing of the laminin-α2 polypeptide and a moderate to severe phenotype characterized by development of muscle weakness at about 3 weeks of age, which progressively worsens. The pathology of dy2J/dy2J skeletal muscle is quite similar to children with MDC1A, showing muscle fiber degeneration, necrosis, and apoptosis, followed by inflammation and fibrosis. 

Previous studies among ours have shown that the pathogenesis of muscular dystrophies involves coordinated activation of multiple key signaling pathways. Nuclear factor kappa B (NFκB) has been described as a significant transcription factor that regulates the expression of muscle proinflammatory cytokines. Early studies have shown elevated NFκB levels in skeletal muscle of mdx mice, the mouse model for Duchenne muscular dystrophy (DMD), and in inflammatory myopathies. NFκB activation is thought to contribute to the deterioration of skeletal muscle pathology and muscle loss in DMD. However, NFκB seems to have a multifaceted regulatory role and may show protective activity in different disorders. Baghdiguian et al. have shown that a certain level of NFκB activity is required to protect myofibers from apoptosis in a Calpain-3 mouse model of Limb girdle muscular dystrophies (LGMDs). Several studies indicate that NFκB activation has a positive role in cell survival by inducing transcription of several survival genes. However, there is only limited data regarding NFκB's role in MDC1A.

Losartan, an angiotensin II type 1 receptor antagonist, is a commercially available and extensively used medication for hypertension with a low side effect profile, occasionally used in childhood. In our previous study, we showed that Losartan treatment was associated with significant impressive improvement in muscle strength and amelioration of fibrosis in the dy2J/dy2J mouse model of MDC1A, through inhibition of transforming growth factor beta (TGF-β) and the mitogen-activated protein kinases (MAPK) signaling pathway.
Here, we demonstrate NFkB signaling pathway involvement in the pathophysiology of the dy2J/dy2J mouse model, mediating decreased apoptosis and promoting muscle cell survival following Losartan treatment. Reduced apoptosis and pro-survival NFkB target genes activation following treatment suggest a key regulatory role for the NFkB signaling pathway in this disorder.

Results

Losartan treatment modifies TNF-α expression. The tumor necrosis factor alpha (TNF-α) serum level was significantly increased in treated dy2J/dy2J mice compared with untreated dy2J/dy2J mice (5.1 ± 0.96 pg/ml versus 2.05 ± 0.58 pg/ml; *P<0.05). TNF-α serum level was also significantly increased in Losartan-treated WT mice (treated: 5.69 ± 0.49 pg/ml versus untreated: 2.42 ± 0.56 pg/ml; **P<0.0005). These results are presented in Figure 1a. As these results were unexpected, we further examined the effect of Losartan on TNF-α transcript levels, using quantitative real-time PCR (TaqMan). The TNF-α mRNA level was unchanged in untreated dy2J/dy2J mice compared with WT groups, but was significantly increased in treated dy2J/dy2J mice hind limb muscles (*P<0.05; Figure 1b). Since TNF-α induces NFkB target gene expression, we analyzed NFkB activity and its downstream effects following Losartan treatment in more detail.

Losartan treatment altered NFkB activation. Using western blot analysis we found significantly decreased expression of the classic NFkB inhibitor, IκB-α protein, in hind limb muscles of both treated and untreated dy2J/dy2J mice compared with WT mice (treated dy2J/dy2J: 0.74 ± 0.07-fold versus untreated WT: 1 ± 0.08-fold; P<0.005, and treated dy2J/dy2J: 0.72 ± 0.05-fold versus treated WT: 0.99 ± 0.13-fold; P<0.005). However IκB-α protein expression was unchanged in dy2J/dy2J mice following Losartan treatment, indicating pre-existing NFkB activation in untreated dy2J/dy2J mice (Figure 2a).

We next measured protein expression of IκB-β, an additional classic NFkB inhibitor. Only following Losartan treatment was IκB-β expression significantly decreased in both dy2J/dy2J and WT mice (treated dy2J/dy2J: 0.32 ± 0.05-fold versus untreated dy2J/dy2J: 0.53 ± 0.07-fold; *P<0.05; treated WT: 0.37 ± 0.04-fold versus untreated WT: 1 ± 0.15-fold; **P<0.005) (Figure 2b). Thus in untreated dy2J/dy2J NFkB activation follows decreased expression of IκB-α, however after Losartan treatment NFkB activation follows decreased expression of both inhibitors; IκB-α and IκB-β.

In the next step, immunofluorescence staining of quadriceps muscles revealed significantly higher accumulation and co-localization of NFkB p65 subunit in the muscle nucleus of treated and untreated dy2J/dy2J mice, compared with WT mice (untreated dy2J/dy2J: 20.57 ± 1.25% versus WT: 16.6 ± 1.06%; P<0.00001 and treated dy2J/dy2J: 20.71 ± 2.59% versus WT: 0.66 ± 0.49%; P<0.00001) (Figure 2c). These findings confirm NFkB activation in dy2J/dy2J mice with and without treatment.
Losartan treatment increases anti-apoptotic protein BCL-2 expression and decreases the pro-apoptotic protein Caspase-3 expression. Next using western blot analysis we examined the hind limb expression of B-cell lymphoma 2 (BCL-2), an anti-apoptotic protein. BCL-2 expression was significantly higher in Losartan treated

![Western Blot Images](image)

**Figure 2** NF-κB signaling pathway activity in dy2J/dy2J and WT mice. (a) Representative western blot gel and densitometry graph of NF-kappa-B inhibitor alpha (IkB-α) expression in WT and dy2J/dy2J mice. Significant reduction in IkB-α was noted in untreated and treated dy2J/dy2J mice compared with WT groups (*P < 0.005). (b) Representative western blot gel and densitometry graph of NF-kappa-B inhibitor beta (IkB-β) expression in WT and dy2J/dy2J mice. Significant reduction in IkB-β was noted in treated compared with untreated dy2J/dy2J mice (*P < 0.05). Losartan treatment was also associated with decreased IkB-β in treated WT mice compared with the untreated group (**P < 0.005). Results of IkB-α and IkB-β levels were obtained from densitometric analysis and expressed as ratio of IkB-α/β/GAPDH and as change fold over control (WT group). These results represent three independent experiments. Each bar represents the mean ± S.E.M. of 12 mice for IkB-α and 11 mice for IkB-β. (c) Intracellular localization of p65 using Immunofluorescence analysis. Expression of p65 was analyzed using anti-Alexa-647 (yellow fluorescence) antibody. Dystrophin staining as a skeletal muscle marker was analyzed using anti-cy2 (red fluorescence) antibody, and DAPI staining (blue fluorescence) was used as nuclear staining. When p65 protein is localized to the muscle nucleus, Alexa-647, cy2, and DAPI are merged. The quadriceps muscle of untreated and treated dy2J/dy2J mice showed nuclear localization of p65 demonstrating NFκB activation. Lack of p65 in the nucleus was illustrated in untreated and treated WT groups. Scale bar, 50 μm. Each bar represents the mean ± S.E.M. of six mice for the WT groups and seven mice in the dy2J/dy2J groups (*P < 0.0001)
compared with untreated dy2J/dy2J mice (treated dy2J/dy2J: 0.90 ± 0.052-fold versus untreated dy2J/dy2J: 0.59 ± 0.071-fold; *P < 0.01). There was no significant difference in BCL-2 expression between treated and untreated WT mice (Figure 4a).

As for the protein expression level of the pro-apoptotic protein Caspase-3, Losartan treatment reduced significantly its expression in treated compared with untreated dy2J/dy2J mice (treated dy2J/dy2J: 3.9 ± 1.3-fold versus untreated dy2J/dy2J: 10 ± 1.9-fold; *P < 0.0001) and in treated compared with untreated WT mice (treated WT: 0.3 ± 0.1-fold versus untreated WT: 1 ± 0.02-fold; **P < 0.0001 Figure 4b). Taken together, these results suggest that Losartan treatment modifies NFκB signaling toward pro-survival/anti-apoptotic pathway.

Losartan reduces TUNEL-positive muscle cells. In order to confirm NFκB involvement in apoptosis signaling, we used in situ DNA nick-end labeling (TUNEL). DNA fragmentation assay TUNEL analysis (Figure 5) showed significant reduction of TUNEL-positive cells in quadriceps muscles of Losartan treated compared with untreated dy2J/dy2J mice, indicating apoptosis (treated dy2J/dy2J: 2.6 ± 0.35% versus untreated dy2J/dy2J: 10.14 ± 1%; *P < 0.0001). Almost no TUNEL-positive cells were found in untreated and treated WT groups.

Discussion

CMDs are genetically heterogeneous diseases, which result in severe disability and premature death. Muscle fibrotic tissue accumulation and progressive skeletal muscle strength reduction characterize both children and the dy2J/dy2J mouse model of MDC1A, one of the most frequent forms of CMD. We previously showed that Losartan treatment significantly increased both fore and hind limb muscle strength, with reduced collagen accumulation and fibrotic markers, in dy2J/dy2J mouse skeletal muscle. This clinical and histological improvement was associated with TGF-β and MAPK signaling pathway inhibition, as Losartan was associated with reduced expression of the regulatory Smad; P-Smad2 and 3 and increased expression of the inhibitory Smad; Smad7. Furthermore, Losartan was associated with significant reduction of the three parallel MAPK signaling pathways P-ERK1/2, P-JNK, and P-p38.

Our current study sheds new light on the NFκB signaling pathway and its involvement in the pathophysiology of the dy2J/dy2J mouse model of MDC1A, mainly through a new insight into the apoptosis pathway. In addition, these data reveal a new role for Losartan with regard to NFκB signaling and apoptosis in this disorder.

In this study, while evaluating Losartan's effect on cytokine levels in mice serum, we found an unexpected significant
increase in the TNF-α level following treatment in both WT and dy2J/dy2J mice (Figure 1a). TNF-α is known to mediate a variety of cellular responses including inflammation, necrosis, fibrosis, and apoptosis. One major role of TNF-α is stimulation of the NFκB signaling pathway. We therefore further investigated the NFκB signaling pathway involvement in MDC1A pathology.

NFκB is a transcription factor that in its resting state binds to inhibitory IκB proteins, keeping it inactive and localized to the cytoplasm. Following stimulation NFκB detaches from its inhibitors. The resulting free NFκB translocates into the nucleus where it activates or represses target genes. Here we demonstrated NFκB activation in both treated and untreated dy2J/dy2J mice through IκB-α protein reduction and p65 (NFκB subunit) transcription factor accumulation in the nucleus of skeletal muscle cells (Figures 2a and c). Losartan-treated mice showed NFκB activity via reduction of an additional inhibitor, IκB-β (Figure 2b). These results may indicate different branches in the NFκB pathway are activated upon Losartan treatment compared with untreated dy2J/dy2J mice.

Previous studies have addressed Losartan’s role in inhibition of NFκB inflammatory processes. They showed Losartan reduces and inhibits NFκB activity in muscle cells from porcine coronary artery, and suppresses inflammation in aged rat kidney. However, NFκB signaling regulates transcriptional...
programs that are essential for the development and maintenance of the skeletal system, epithelium, and immune system, which in turn impacts differentiation, proliferation, cell death, and survival. Therefore, NFκB signaling pathways have a multifaceted regulatory role that can either mediate apoptosis or anti-apoptotic routes. NFκB signaling can also engage the Caspase signaling pathway to mediate cell apoptosis. On the other hand, it can activate TNF-α inhibition of TNF-α signaling.

In Duchenne muscular dystrophy, NFκB activation is perceived as contributing to the deterioration of skeletal muscle pathology and skeletal muscle loss. For example, deletion of a single allele of NFκB (RelA/p65 subunit) was sufficient to considerably reduce infiltration of macrophages, fiber necrosis and calcification in dystrophic muscle in mdx mice (DMD mouse model). In addition, NFκB inhibition augmented the regeneration of mdx mice myofibers. Furthermore, overexpression in skeletal muscle of A20 protein, a potent negative regulator of NFκB, reduced chronic inflammation and muscle degeneration in mdx mice.

However, in the current study, NFκB activity following Losartan treatment maintained survival and anti-apoptotic effects in dy2J/dy2J mice. Losartan treatment was associated with increased mRNA expression of pro-survival genes TRAF1, TRAF2, CIAP2, and FTH-1 (Figures 3a and d). These findings support other studies findings showing gene expression of TRAFs and CIAPs following NFκB activation, leading to inhibition of TNF-α induced cell death. Cells lacking c-IAPs through genetic ablation or treated with IAPs antagonists have been shown to be more sensitive to TNF-α induced cell death through decreased NFκB survival mechanism.

Losartan’s role in apoptosis inhibition was further demonstrated by a significant increase of anti-apoptotic BCL-2 protein expression in skeletal muscle of treated dy2J/dy2J mice (Figure 4a). BCL-2 has been shown to have an important role in increasing lifespan, growth rate, and reducing apoptosis following muscle-specific overexpression in Lama2 null mice. Furthermore, overexpression of BCL-2 was found to ameliorate muscle weakness and reduce apoptosis in oculopharyngeal muscular dystrophy (OPMD) mouse model. As for DMD, transgenic overexpression of BCL-2 did not improve muscle pathology in mdx mice, and indeed the effect of Losartan in mdx mice is less pronounced than in dy2J/dy2J mice.

We further investigated the anti-apoptotic effect of Losartan. Losartan treatment significantly decreased the pro-apoptotic protein Caspase-3 in dy2J/dy2J and WT mice (Figure 4b), and overall apoptosis was significantly decreased in Losartan-treated dy2J/dy2J mice as demonstrated by TUNEL-positive skeletal muscle cell reduction (Figure 5).

All of the above data together propose an important role for NFκB signaling in the pathophysiology of MDC1A, mainly in terms of its contribution to the apoptosis process. On the basis of current findings, we suggest that Losartan treatment shifts NFκB signaling to favor the survival route versus inflammation, fibrosis and apoptosis/damage in the dy2J/dy2J mouse model of MDC1A.

We therefore suggest a model regarding NFκB signaling activity following Losartan treatment. Losartan treatment results in increasing TNF-α, which in turn activates NFκB through IκB-α and IκB-β degradation, p65 nuclear accumulation and upregulation of pro-survival genes and proteins to mediate the anti-apoptotic effect of NFκB in the dy2J/dy2J mouse model of MDC1A (Figure 6).

In muscular dystrophies there are indications that apoptosis, beside necrosis, may contribute to muscle loss and dysfunction. In human and mouse models of muscular dystrophy signs of muscle cell death by apoptosis have been documented but not discussed in detail. In this study, we show for the first time that apoptosis has an important role in dy2J/dy2J muscle pathology. Therefore, it seems that therapies designed to include apoptosis inhibition might be beneficial for patients with congenital muscular dystrophy. These new findings support our previous data that demonstrated significant improvement in animal muscle strength and reduced fibrosis following 12 weeks of Losartan treatment. A future more prolonged Losartan study will provide additional information regarding long-term benefit and survival in dy2J/dy2J mice. This trial provides further support for a Losartan therapeutic trial in children with MDC1A.

Materials and Methods

Mice population and treatment. Muscle tissues for this study were obtained from the mice used in a previous Losartan study as follows; C57BL6/J Lady2dy-2J heterozygote mice (Jackson Laboratories, Bar Harbor, ME, USA) were bred at the Hebrew University specific pathogen-free animal housing facility. The joint ethics committee of Hebrew University and Hadassah Medical Center (accredited by AAALAC) approved the study protocol for animal welfare (permit number: 122.03-04). Mice were maintained under standard conditions, 23 ± 1 °C, 12:12 h light cycle (0700–1900 h), with ad libitum access to food and drink. Delineation between the Lady2dy2J/J (dy2J/dy2J) affected mice, heterozygous for the LAMA2 gene mutation, and wild-type C57BL6/J (WT) mice was detected by PCR.

Losartan treatment. Losartan mediates NFκB in MDC1A mouse model. M Elbaz et al

Results and discussion. Final analyses were conducted in triplicates, and standard deviation was used to calculate error bars.
Immunofluorescence. Quadriceps muscles were isolated and fixed in acetone for 1 h. The quadriceps muscle tissues were sliced into 8 μm cross sections after being embedded in OCT. The mounted sections were washed three times in phosphate-buffered saline (PBS), blocked with 1% bovine serum albumin and probed with anti-p65 rabbit antibody (Abcam, Cambridge, UK) and with anti-dystrophin mouse antibody (Santa Cruz Biotechnology). After three washes in PBS, the sections were incubated with Alexa fluor 647-conjugated affini-pure donkey anti-rabbit IgG (H+L) and with Cy2-conjugated affini-pure donkey anti-mouse IgG (H+L) (Jackson ImmunoResearch, West Grove, PA, USA). After three more washes in PBS, coverslips were mounted on glass slides with a DAPI-containing mounting medium (Vector Laboratories). The apoptotic fluorescent cells were counted under a fluorescent microscope, and the numbers were expressed as the percentage of total P65 area cells ± S.D. A negative control without anti-p65 rabbit antibody (Abcam) was also performed.

Tunel staining. Quadriceps muscles sections were assessed by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay with an In Situ Cell Death Detection Kit (Roche Diagnostic, Indianapolis, IN, USA). Muscles were isolated and fixed in acetone for 1 h; the tissues were sliced into 8 μm cross sections after being embedded in OCT. Afterwards washed twice in PBS. Each slice was embedded with 100 μl permeabilisation solution, 0.1% Triton X-100 for 2 min, washed twice in PBS and the tissue sections were labeled and stained with the TUNEL reaction mixture (label plus enzyme solutions) for 60 min at room temperature and washed twice with PBS. Then the slices were probed with anti-dystrophin mouse antibody (Santa Cruz Biotechnology), and after three more washes in PBS the sections were incubated with Alexa fluor 647-conjugated affini-pure donkey anti-mouse IgG (H+L) (Jackson ImmunoResearch, Jena, Germany). The P65-positive fluorescent cells were counted under a fluorescent microscope, and the numbers were expressed as the percentage of total P65 area cells ± S.D. A negative control without anti-p65 rabbit antibody (Abcam) was also performed.

**Figure 6** Proposed model for NFκB as a key regulator in the survival path of muscle following Losartan treatment. Losartan treatment increases TNF-α, which in turn activates NFκB through IκB-α and IκB-β degradation, p65 nuclear accumulation and upregulation of the pro-survival genes: TRAF1, TRAF2, CIAP2, and FTH1 in addition to anti-apoptotic BCL-2 protein, to mediate the anti-apoptotic effect of NFκB in skeletal muscle of the dy2J/dy2J model mouse of MDC1A. In addition, Losartan treatment results in decreased expression of the pro-apoptotic protein Caspase-3

**Statistical analysis.** All data are expressed as mean and standard error of the mean. Statistical analysis for direct comparison between two groups was performed by unpaired Student’s t-test. Significance was set at P<0.05 for all comparisons.

**Conflict of Interest**
The authors declare no conflict of interest.

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