Intronic Transcriptional Regulation of Intracellular Matrix Metalloproteinase-2 in Skeletal Muscle Ischemia-Reperfusion Injury

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Introduction: Skeletal muscle ischemia-reperfusion injury (IRI) is a serious problem in clinical orthopaedics that can occur from compartment syndrome, crush injuries, iatrogenic consequence of tourniquet use, or vascular injuries. Complications from IRI include muscular weakness, rhabdomyolysis, paraesthesia, paralysis, and systemic tissue damage. The mechanism of IRI in skeletal muscle is not well understood.

Previous work has shown that matrix metalloproteinase (MMP)-2, an important extracellular matrix remodeling enzyme is upregulated in an IRI setting (1). We further demonstrated intracellular activation of MMP-2 in muscle IRI. In a mouse IRI model, we found abundant active MMP-2 remains within myocytes and directly digests important intracellular components, including Troponin after muscle IRI (2).

However, the mechanisms of intracellular activation of MMP-2 in muscle IRI remains unknown. In this study, using in vitro and in vivo models, we demonstrated that intracellular activation of MMP-2 in muscle IRI was due to the expression of a novel N-terminal truncated (NTT) isoform of MMP-2. The expression of NTT-MMP2 is correlated with innate immune response in muscle IRI. Novel information gained from this study may help to develop new treatment for muscle IRI by selectively inhibition of NTT isoform MMP-2 expression.

Methods: In vivo: Three month old male FVB/N mice (N=12) were randomly chosen for unilateral hindlimb IRI or sham treatment using a previously validated model (2). Following anesthesia, ischemia was induced by applying a calibrated 1/8 inch orthodontic latex elastic rubber band (6 ounces) above the greater trochanter of the mouse hindlimb (N=6). The rubber band was removed following three hours to initiate reperfusion. After 24 hours of reperfusion, mice were euthanized and gastrocnemius muscles of the IRI hindlimb were harvested. Gastrocnemius muscles from sham mice (N=6) that underwent anesthesia served as a baseline control. Muscle samples from IRI and sham groups were prepared to extract RNA. Animal care and protocols were approved by the Institutional Animal Care and Use Committee.

In vitro: Murine myoblast C2C12 cells (ATCC) were cultured in DMEM medium with 10% FBS on 6-wells culture plates under normal atmosphere with 5% CO2 at 37°C until they reach 70% confluence. Cells were then transferred into a hypoxia chamber within the same incubator. Oxygen centration was remained at 0.1% within the chamber. In ischemia group, cells were harvested and RNA was extracted immediately after 2, 4, 8, 24, 48 and 72 hours of hypoxia. In ischemia-reperfusion group, cells were removed from hypoxia chamber after 2 hours hypoxia and return to normal atmosphere with 5% CO2 in
the incubator. Cells were harvested and RNA was extracted at 0.5, 1, 2, 4, 8, 16, 24, 48 hours later. Cells in the control group was placed in the same incubator without treatment. (N=6 wells in each condition)

**RT-PCR:** RNA was extracted using Trizol® reagent according to the manufacturer’s instructions. Isolated RNA was quantified and normalized to synthesize cDNA. qRT-PCR was performed to quantify the expression of MMP-2 isoforms and innate immunity markers. Specific primers for full-length (FL) and NTT MMP-2 was designed to distinguish those two isoforms of MMP-2. Amplification reactions were performed with 40 cycles and normalized to β-2-microglobulin. Fold change in mRNA expression was calculated by using ∆∆CT. T-test was used to compare the results between the IRI and sham groups. Significance was defined as a p value of less than 0.05. All data is presented as mean±SEM.

**Results:** In in vivo experiments, although FL-MMP-2 was up-regulated following IRI in gastrocnemius muscles compared to control muscles via qRT-PCR (9.27±5.5), it did not reach significance. NTT-MMP-2 was significantly up-regulated following skeletal muscle IRI, 39.16±10.9 (p<0.05) (Figure 1A). Among the six innate immunity markers evaluated in this study, three of them were significantly up-regulated. They are CXCL-1 (6.03±0.9, p<0.05), OAS1A (6.75±2.5, p<0.05), and IL-6 (1301.44±326.9, p<0.01) (Figure 1B).

In in vitro experiments, NTT-MMP-2 expression in C2C12 cells was up-regulated near 700 fold with 2 hours of hypoxia. NTT-MMP-2 expression was highly up-regulated at all time points of hypoxia. In contrast, the expression of FL-MMP2 was only minimally up-regulated with hypoxia (Figure 2). Following 2 hours’ hypoxia, the expression of NTT-MMP2 peaked at 2 hours after re-oxygenation and remained highly up-regulated till 48 hours after re-oxygenation. The expression of FL-MMP2 showed a similar pattern as NTT-MMP2 with lower fold changes. (Figure 3).

**Discussion:** In this study, we have demonstrated a novel isoform of NTT-MMP-2 in skeletal muscle IRI. Using specifically designed primer with RT-PCR, we proved that the NTT isoform of MMP-2 was transcripted from MMP-2 gene with an alternative promoter within the first intron. Thus, it lacks N-terminal signal peptide as well as pro-enzyme fragment. As a result, this NTT-MMP-2 could not be transported outside the cell as FL-MMP-2. On the contrary, it remains inside the cells. Due to the lack the pro-enzyme fragment, NTT-MMP-2 is an active enzymatic isoform of MMP-2. It could digest numerous intracellular components, including Troponin in the myocytes (2). Thus, it can cause dramatic damage to myocytes during IRI. Our in vitro experiments proved that hypoxia plays a critical role in regulating the expression of this NTT-MMP-2 in myocytes. Future works are needed to further define the transcriptional regulation of this pathological isoform of MMP-2 in muscle IRI.

**Significance:** This study revealed a novel mechanism of MMP-2 mediated muscle injury after ischemia-reperfusion. This information may help to develop new treatment for muscle IRI by selectively inhibition of NTT-MMP-2 expression in the future.
Figure 1. Fold changes of FL-MMP-2, NTT-MMP-2 and innate immunity markers in gastrocnemius muscles following 3 hours ischemia and 24 hours reperfusion. (N=6)

**FL-MMP-2**

![FL-MMP-2 Graph](image)

**NTT-MMP-2**

![NTT-MMP-2 Graph](image)

Figure 2. Expression of FL-MMP2 and NTT-MMP2 in C2C12 cells following hypoxia. Hypoxia significantly up-regulated the expression of NTT-MMP2 in C2C12 cells. However, the expression of FL-MMP2 was only minimally up-regulated with hypoxia.
Figure 3. Expression of FL-MMP2 and NTT-MMP2 in C2C12 cells with re-oxygenation following 2 hours’ hypoxia. Expression of NTT-MMP2 peaked at 2 hours after re-oxygenation and remained up-regulated at 48 hours after re-oxygenation. The expression of FL-MMP2 showed a similar pattern as NTT-MMP2 with lower fold changes.