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MicroRNA overexpression increases cortical neuronal vulnerability to injury

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ABSTRACT

Previously we reported that several microRNAs (miRNA) are upregulated following experimentally induced traumatic brain injury (TBI) using both *in vivo* and *in vitro* approaches. Specific miRNAs were found to be sensitive to therapeutic hypothermia and may therefore be important targets for neuroprotective strategies. In this study we developed plasmid constructs that overexpress temperature sensitive miRNAs: miR-34a, miR-451, and miR-874. These constructs were transfected into cultured cortical neurons that were subjected to stretch injury using a cell injury controller device. Levels of expression of genes associated with stress, inflammation, apoptosis and transcriptional regulation were measured by qRT-PCR. mRNA levels of cytokines interleukin 1-β (*IL1-β*) and tumor necrosis factor alpha (*TNF-α*) as well as heat shock protein 70 (*HSP70*) and *Caspase 11* were found to be increased up to 24 fold higher than controls in cells overexpressing these miRNAs. After moderate stretch injury, the expression of *IL1-β*, *TNF-α*, *HSP70* and *Caspase 11* all increased over control levels found in uninjured cells suggesting that overexpression of these miRNAs increases cellular vulnerability. miR-34a directly inhibits *Bcl2* and *XIAP*, both anti-apoptotic proteins. The observed increase in *Caspase 11* with over-expression of miR-34a indicates that miR-34a may be inducing apoptosis by reducing the levels of anti-apoptotic proteins. miR-34a is predicted to inhibit *Jun*, which was seen to decrease in cells overexpressing this miRNA along with *Fos*. Over expression of several miRNAs found to be induced by TBI *in vivo* (miR-34a, miR-451 and miR-874) leads to increased vulnerability in transfected neurons. Therapeutic hypothermia blunts the expression of these miRNAs *in vivo* and antisense silencing could be a potential therapeutic approach to targeting the consequences of TBI.

1. Introduction

MicroRNAs are a class of non-coding regulatory RNAs that have wide-ranging effects on the translation of many proteins and cellular functions in general (Cao et al., 2006; Ivey and Srivastava, 2010; Lee et al., 2002; Madathil et al., 2010; Sempere et al., 2004). These non-coding regulatory RNAs therefore provide a mechanism for the regulation of protein expression levels of various targeted genes (Redell et al., 2011; Qureshi and Mehler, 2010). Because microRNAs can suppress the translation of target genes by binding to their mRNAs, their role in gene regulation in health and disease is an area

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of active investigation (Conley and Alexander, 2011; Esau and Monia, 2007; Madathil et al., 2010; Nakanishi et al., 2010; Wang et al., 2010).

Traumatic brain injury (TBI) is a complex and devastating clinical condition that affects approximately 1.5 million U.S. citizens each year. Following TBI, a cascade of pathomechanisms is activated that leads to increased cell vulnerability and long term neurological deficits. These injury processes include excitotoxicity, inflammation, apoptosis and reactive oxygen species (Dietrich and Bramlett, 2010; Truettner et al., 2007). Various studies have investigated the molecular and cellular processes that are activated after TBI including alterations in microRNA expression after experimental TBI (Lei et al., 2009; Liu et al., 2009; Redell et al., 2009, 2013; Truettner et al., 2011). In a study by Lei et al. (2009), alterations in miRNA expression patterns were reported in the cerebral cortex after fluid percussion (FP) brain injury. In another study, Redell et al. (2009) investigated altered expression in the hippocampus after controlled cortical impact injury. In both studies, unique miRNA profiles in vulnerable brain regions after trauma were documented. In a recent study by Truettner et al. (2011), microarrays were also used to evaluate the effects of trauma on 388 rat microRNAs. In that study, 47 microRNAs were significantly different between TBI and sham at 7 h after TBI, including 15 higher in TBI and 31 lower. After 24 h, 15 microRNAs still differed significantly from control values. In a recent study by Redell and colleagues, miR-21 expression was significantly upregulated in the hippocampus peaking at 3 days post injury and returning to near sham levels by 15. In that study, 99 potential target genes were identified that possessed miR-21 binding sites within their 3 prime untranslated regions. Further analysis documented an overrepresentation of genes involved in enzyme-linked receptor signaling, transcriptional regulation and developmental processes. Taken together, these studies emphasize the relative importance of microRNAs in the pathophysiology of TBI.

Previous work has reported the beneficial effects of therapeutic hypothermia in models of TBI (Dietrich and Bramlett, 2010; Tomura et al., 2012). Indeed, therapeutic hypothermia is one of the most powerful cytoprotective strategies demonstrated in models of cerebral ischemia and trauma. In models of TBI, posttraumatic hypothermia has been reported to protect against irreversible neuronal injury, reduce overall contusion volumes and axonal injury and improve behavioral outcome. Additional studies have indicated that mechanisms underlying the hypothermic effects include a number of injury cascades including excitotoxicity, free radical generation, apoptosis and inflammatory processes. In terms of inflammation and apoptosis, posttraumatic hypothermia differentially regulates the expression of inflammatory cytokines, pro-apoptotic proteins and stress response genes after TBI (Kinoshita et al., 2002; Truettner et al., 2005a, 2005b; Vitarbo et al., 2004). Currently it is unclear exactly how hypothermia may have a regulatory control over gene expression.

Limited data are available regarding the effects of posttraumatic hypothermia on patterns of miRNA expression. In a previous study from our group (Truettner et al., 2011) the effect of posttraumatic hypothermia on expression of a variety of microRNAs was investigated following fluid percussion brain injury. Interestingly, some microRNAs that were differentially regulated by TBI showed a temperature sensitivity to hypothermia verified by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). These findings emphasize that early hypothermia treatment could be affecting traumatic outcome by targeting temperature-sensitive microRNAs involved in basic cell processing events.

Because of the beneficial effects of hypothermia in preserving neuronal viability after TBI, we sought in the present investigation to determine whether the overexpression of selective microRNAs that are affected by therapeutic hypothermia would alter the vulnerability of cultured cortical neurons subjected to stretch injury. Several of the miRNAs sensitive to hypothermic treatment following TBI identified in our previous study (miR-34a, miR-874 and miR-451) were targeted for analysis in the present study. We report that overexpression of these microRNAs induced by TBI leads to increased stress and vulnerability of transfected cells following an *in vitro* stretch injury that mimics some of the biophysical characteristics of a TBI.

2. Results

2.1. *Effect of overexpression of miRNAs*

Primary neuronal cultures were transfected with plasmid constructs to over express miR-34a, miR-451 and miR-874, plus the vector alone as a control and plated. Three days after transfection, many cells were seen to be expressing GFP (**Supplemental** Fig. 1). In contrast, neurons cultured for 14 days and then transfected had very low transfection rates, $<0.1\%$, and were therefore not used for further experiments (data not shown). One day after transfection, the media was changed and 3–5 days later conditioned media was removed and media and cells were harvested for RNA extraction. Quantification of miRNA expression in non-transfected as well as transfected cells, including an empty vector control was performed by Real Time RT-PCR and normalized to reference small molecule U6. Cultures that were transfected with the empty vector had undetectable levels of the 3 miRNAs miR-34a, miR-451, and miR-874 (Table 2). The cells transfected with the overexpressing constructs all showed elevated levels of these miRNA as compared to the empty vector. Levels of microRNAs were elevated in both the cells and the conditioned media at various levels but higher in the cells. miR-34a showed a very robust overexpression of over 1800 fold with miR-451 and miR-874 lower but still highly expressed (7.78 fold and 46.85 fold increase, respectively). The amount of miRNAs present in the media as a proportion of the amount in the cells is indicative of the levels of secretion of these miRNAs. miR-34a was the least secreted microRNA with 150 fold greater amounts in the cells than the media. miR-451 was present at a higher level in the media with the cells measuring about 7 fold more miR-451 than the media. miR-874 was present at the highest levels in the media with the cells having only a 4.35 fold excess in miRNA levels (Table 2).

To estimate the consequences of miRNA over expression in the absence of injury, were causing cellular stress or dysregulation, the expression of genes known to be upregulated in response to different types of stress or dysregulation were measured by qRT-PCR. *IL1-β* and *TNF-α* are inflammatory molecules that are frequently upregulated in response to many cellular pathologies including trauma and ischemia