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Original Article

CD38 plays key roles in both antioxidation and cell survival of H_2O_2-treated primary rodent astrocytes

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Abstract: CD38 is an ecto-enzyme that consumes NAD^+ to produce cyclic ADP-ribose (cADPR) --- a potent agonist of ryanodine receptors. Recent studies have suggested CD38 may play significant roles in both ischemic brain injury and traumatic brain injury, while the mechanisms underlying the roles of CD38 in neurological diseases remain unclear. Because oxidative stress plays key roles in both ischemic brain damage and traumatic brain damage, in this study we used primary astrocyte cultures as a experimental model to test our hypothesis that CD38 may play significant roles in oxidative stress-induced neural cell death. We found CD38 siRNA-produced decrease in CD38 levels can lead to a significant increase in H_2O_2-induced astrocyte death. Moreover, the CD38 siRNA treatment can significantly aggravate oxidative stress in the H_2O_2-treated cells, as indicated by increases in both superoxide and lipid peroxidation, suggesting that CD38 is required for maintaining the antioxidation capacity of the cells. We also found that H_2O_2 can induce increased CD38 expression. Collectively, our study has obtained novel findings suggesting that CD38 plays a significant role in both antioxidation and cell survival of reactive oxygen species-exposed primary astrocytes, suggesting that CD38 may become a novel target for decreasing the oxidative damage in neurological disorders.

Keywords: CD38, astrocytes, oxidative stress, antioxidation, cell death

Introduction

CD38 is a NAD^+-dependent, multifunctional ecto-enzyme, which can not only generate cyclic ADP-ribose (cADPR) from NAD^+, but also hydrolyze cADPR to ADP-ribose and transport cADPR into cells [1]. cADPR is a potent agonist of ryanodine receptors (RyR), which plays a critical role in modulating intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) [2]. CD38 has been shown to play key regulatory roles in such physiological processes as oxytocin secretion [3]. Our study has also found that CD38 siRNA-induced decrease in CD38 levels can induce apoptosis of BV2 microglia, suggesting an important role of CD38 in the basal survival of the cells [4]. However, the roles of CD38 in the survival of neurons and astrocytes remain unknown.

Two studies using CD38 knockout mice have indicated contrasting roles of CD38 in ischemic brain injury and traumatic brain injury: CD38 knockout mice showed a significant decrease in ischemic brain damage compared to wild type mice [5], while CD38 knockout mice showed exacerbated injury after head trauma compared to wild type mice [6]. It has also been reported that both NAD^+ levels and SIRT1 activity are markedly increased in CD38 knockout mice [7-9]. Therefore, the interpretations of the experimental results from the studies using CD38 knockout mice might be confounded, because both NAD^+ and SIRT1 can affect a large variety of biological functions [10]. Therefore, it is of critical need to apply such approaches as siRNA approach, which does not significantly affect the levels of NAD^+ or SIRT1, to study the roles of CD38 in neural cell death under various pathological conditions.

Because oxidative stress plays key roles in ischemic brain damage and traumatic brain dam-

age [11, 12], in this study we tested our hypothesis that CD38 plays an important role in oxidative cell death by using primary astrocyte cultures as a experimental model. Our study has suggested that CD38 plays significant roles in both antioxidation capacity and survival of primary astrocytes cultures, suggesting that CD38 may become a new target for decreasing oxidative damage in neurological diseases.

Methods

Reagents and animals

Reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) except where otherwise noted. SD rats were purchased from Shanghai SLAC Laboratory Animal Corporation (Shanghai, China).

Cell cultures

Primary rat cortical astrocyte cultures were prepared as described previously [13]. Primary Astrocytes cells were derived from postnatal Day 1 SD rat brains. Briefly, the brain cortices were dissociated in Trypsin-EDTA for 20 minutes at 37°C and cultivated in DMEM supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, USA), 1% penicillin-streptomycin, and 1% L-glutamine (Invitrogen, Carlsbad, CA, USA). The cell cultures became confluent 12 to 15 days, which were treated for 48 hrs with cytosine arabinoside (10 μM). The cultures could be used at least two days after removal of cytosine arabinoside.

RNA silencing

The small interfering RNA (siRNA) duplexes were all commercially synthesized by Invitrogen (Shanghai, China), for rat CD38 (NM_013127.1) at nucleotides (sense: 5'-C GGACCCAAAUAAGGUUCAUTT-3' and antisense 5'-AUGAACCUUAUUUGGGUCCTT-3'), (sense: 5'-GCAUCCAUCAUGUAGACUUTTAUTT-3' and antisense 5'-AAGUCUACAUGAUGGAUGCTT-3'), or (sense: 5'-CCAAGAAUCCUUGCAACAUTT-3' and antisense 5'-AUGUUGCAAGGAUUCUUGGTT-3'), respectively; For controls, scrambled RNA oligonucleotides were used. For each well, a combination of 33.3 nM of each of the three oligos was transfected into the cells by using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After incubation for 5 hrs, the media was replaced by DMEM containing 5% fetal bovine serum.

Western blot

As described previously [14, 15], cells were harvested and lysed in RIPA buffer (Millipore, Temecula, CA, USA) containing Complete Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany) plus 1 mM PMSF. Lysates were centrifuged at 12,000 g for 20 min at 4°C. After quantification of the protein samples using BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA), 30 μg of total protein was electrophoresed through a 10% SDS-polyacrylamide gel, and then transferred to 0.45 μm nitrocellulose membranes (Millipore, CA, USA) on a semi-dry electro transferring unit (Bio-Rad Laboratories, CA, USA). The blots were incubated overnight at 4°C with a goat polyclonal anti-CD38 antibody (1:350 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), then incubated with a rabbit anti-goat polyclonal HRP-conjugated secondary antibody (EPITOMICS, Hangzhou, Zhejiang Province, China). Protein signals were detected using an ECL detection system (Pierce Biotechnology, Rockford, IL, USA). An anti-β-actin antibody (1:1000 dilution, Santa Cruz Biotechnology, CA, USA) was used to normalize sample loading and transfer. The intensities of the bands were quantified by densitometry using Gel-Pro Analyzer.

Extracellular and intracellular lactate dehydrogenase (LDH) assays

As described previously [4], extracellular LDH assay was performed to determine cell death. In brief, 100 μl of extracellular media was mixed with 150 μl potassium phosphate buffer (500 mM, pH7.5) containing 1.5 mM NADH and 7.5 mM sodium pyruvate. Subsequently changes of the $A_{340\ nm}$ of the samples were monitored over 90 sec. Intracellular LDH assay was also conducted to determine cell survival, as described previously [16]. Briefly, cells were lysed for 20 min in lysing buffer containing 0.04% Triton X-100, 2 mM HEPES, 0.2 mM dithiothreitol, 0.01% bovine serum albumin, and 0.1% phenol red (pH 7.5). Fifty μl cell lysates were mixed with 150 μl potassium phosphate buffer (500 mM, pH 7.5) containing 1.5 mM NADH and 7.5 mM sodium pyruvate. Subsequently changes of the $A_{340\ nm}$ of the samples were monitored over

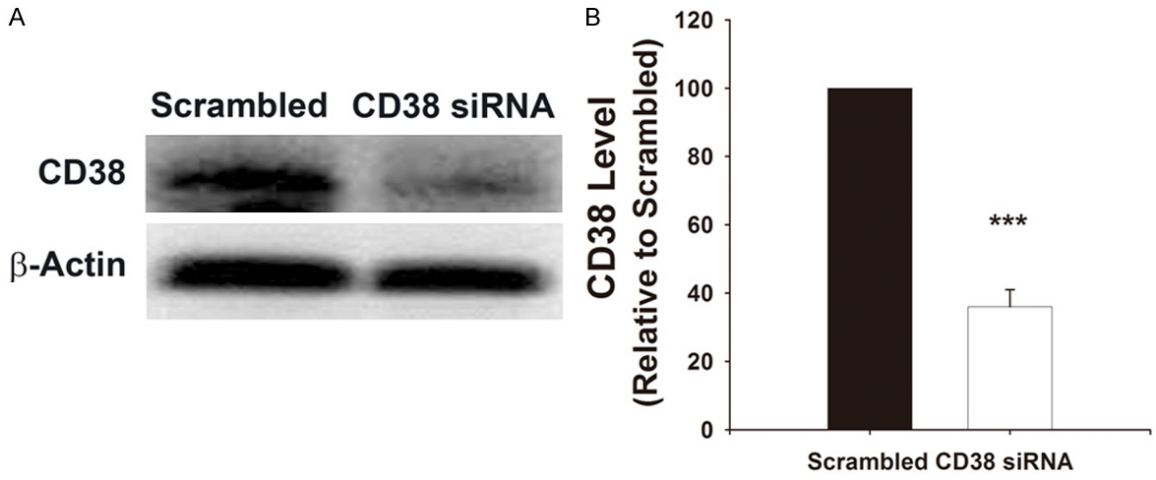

Figure 1. CD38 siRNA treatment led to a significant decrease in the CD38 levels of astrocytes. A. Western blot assay showed that CD38 siRNA treatment led to decreased CD38 levels in astrocytes. For controls, scrambled RNA oligonucleotides were used. B. Quantifications of the Western blots showed that CD38 siRNA significantly decreased the CD38 levels by approximately 60%. Thirty μg of total protein was loaded on the gels. N = 6. ***$p < 0.001$. Data were representative of 3 independent experiments.

90 sec. Percentage of cell survival was calculated by normalizing the LDH activity of the lysates of a sample to the LDH activity of the lysates of controls.

TBARS assay

The levels of thiobarbituric acid reactive substances (TBARS) in the primary rat astrocytes were determined by using a commercially available kit (Cayman Chemical, Ann Arbor, MI). The assay was performed using a plate reader according to the manufacturer's protocol.

Dihydroethidium (DHE) assay

The primary rat astrocytes were incubated with 5 μM DHE (Beyotime, Jiangsu, China) for 30 min at 37°C. Subsequently the cells were washed once with PBS, and the fluorescence signals were observed under a Leica fluorescence microscope at excitation wavelength of 545 nm and emission wavelength of 605 nm.

NAD^+ cycling assay

NAD^+ concentrations were measured by enzyme recycling assay [13]. Briefly, C6 glioma cells were extracted in 0.5 N perchloric acid. The lysates were centrifuged at 12,000 rpm for 5 min and the supernatants were neutralized to pH 7.2 using 3 N potassium hydroxide and 1 M potassium phosphate buffer. After centrifugation at 12,000 rpm for 5 min, the supernatants were mixed with a reaction medium containing 1.7 mg 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), 10.6 mg phenazine methosulfate, 1.3 mg alcohol dehydrogenase, 488.4 mg nicotinamide, and 2.4 mL ethanol in 37.6 mL Gly-Gly buffer (65 mM, pH 7.4). The A560 nm was determined immediately and after 10 minutes, and the readings were calibrated with NAD^+ standards. Results were normalized to protein contents as determined by the BCA assay.

Statistical analyses

Statistical analysis was performed by using the two-tailed unpaired Student's t-test or one-way ANOVA, followed by Student-Newman-Keuls post hoc test. *P* Values less than 0.05 were considered statistically significant. The data are presented as Means ± SEM.

Results

We determined the roles of CD38 in H_2O_2-induced death of astrocytes. We used CD38 siRNA to decrease the levels of CD38 in astrocytes. Western blots showed that CD38 siRNA decreased the CD38 levels by approximately 60% (**Figure 1**). We found that CD38 siRNA treatment exacerbated H_2O_2-induced cell death, as assessed by extracellular LDH assay (**Figure 2A**). Intracellular LDH assay also

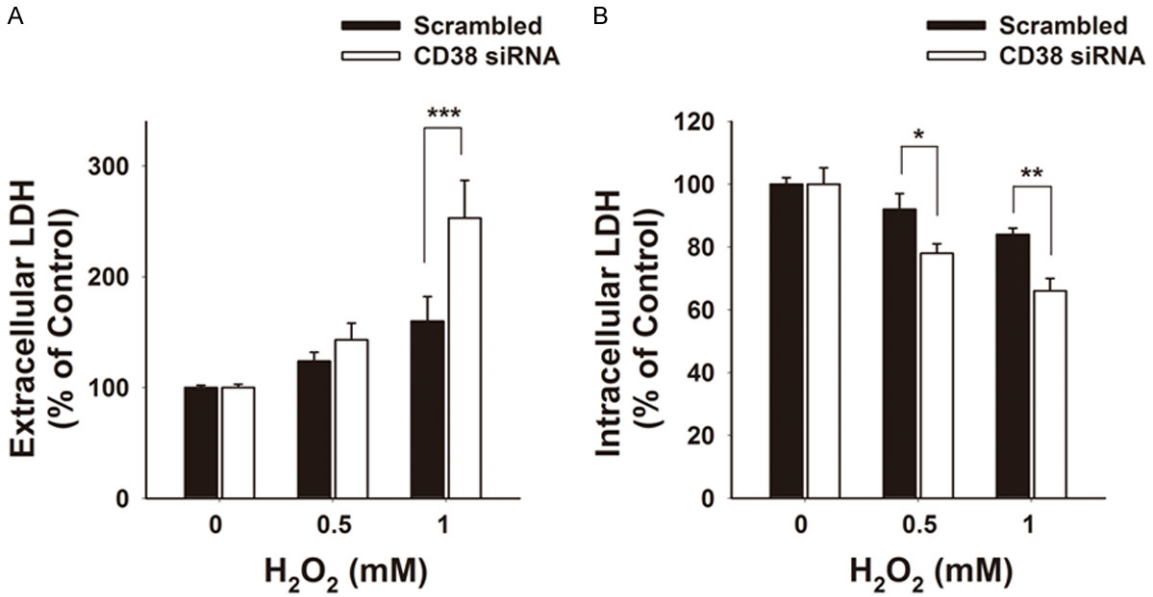

Figure 2. The roles of CD38 in H_2O_2-induced death of astrocytes. A. CD38 siRNA treatment exacerbated H_2O_2-induced increase in astrocyte death, as assessed by extracellular LDH assay. B. CD38 siRNA treatment exacerbated H_2O_2-induced decrease in the number of surviving astrocytes. Astrocytes were transfected with CD38 siRNA for 24 hrs, followed by treatment with 0.5 mM or 1 mM H_2O_2 for 1 hr, then the medium was replaced with DMEM. After 23 hrs, both of the extracellular LDH levels and the intracellular LDH levels of the cells were determined. N = 12-16. *p < 0.05; **p < 0.01; ***p < 0.001. Data were collected from 3 - 4 independent experiments.

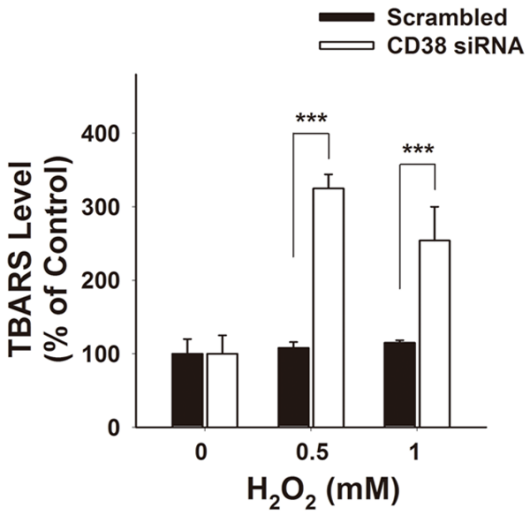

Figure 3. CD38 siRNA treatment significantly increased the TBARS levels in H_2O_2-induced astrocytes. Astrocytes were transfected with CD38 siRNA for 24 hrs, followed by treatment with 0.5 mM or 1 mM H_2O_2 for 1 hr, which was the replaced with DMEM. The TBARS levels of the cells were determined at 23 hrs after the media replacement. N = 12-16. ***p < 0.001. Data were representative of 3 independent experiments.

showed that the CD38 siRNA treatment aggravated H_2O_2-induced decreases in the number of surviving astrocytes (**Figure 2B**).

To study the mechanisms underlying the roles of CD38 in H_2O_2-induced death of astrocytes, we assessed the effects of CD38 siRNA treatment on the oxidative stress levels in H_2O_2-treated astrocytes. By using DHE assay that detects superoxide levels in cells, we found that H_2O_2 induced significant increases in the DHE level of astrocytes, which was exacerbated by the CD38 siRNA treatment (Supplemental Figure 1). Our study also showed that H_2O_2 induced significant increases in the TBARS level of astrocytes - an index of lipid peroxidation, which was also exacerbated by the CD38 siRNA treatment (**Figure 3**).

Under our experimental condition, we found that CD38 siRNA led to an approximately 60% decrease in the CD38 levels of astrocytes, as shown in **Figure 2**. Because previous studies have shown that NAD^+ levels are markedly increased in the tissues from CD38 knockout mice [17], we assessed if CD38 siRNA may affect the intracellular NAD^+ levels of astrocytes in our experimental model: Our study did not find that CD38 siRNA can significantly affect the intracellular NAD^+ levels of astrocytes (Supplemental Figure 2).

We also determined the effects of H_2O_2 on the CD38 levels of primary astrocytes. We found

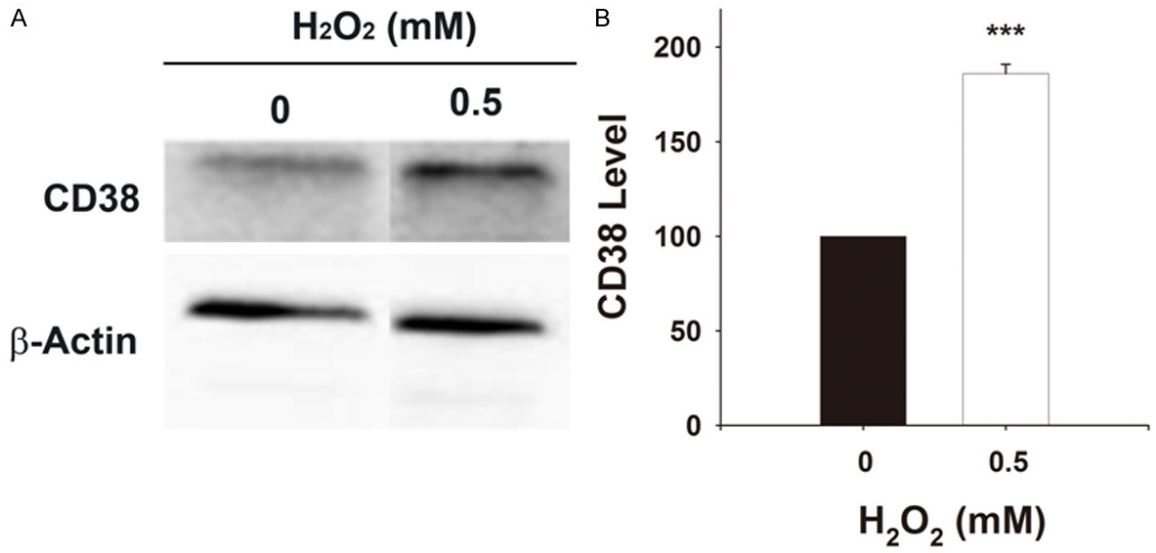

Figure 4. Effects of H_2O_2 on the CD38 levels in primary astrocytes. A. Western blot assay showed that H_2O_2 treatment induced the increases expression of CD38 in astrocytes. The astrocytes were treated with 0.5 mM H_2O_2 for 1 hr, then the medium was replaced by DMEM, the expression of CD38 was assayed at 23 hrs after the treatment by Western blotting. B. Quantifications of the Western blots showed that 0.5 mM H_2O_2 significantly increased the CD38 levels by approximately 90%. Thirty μg of total protein was loaded on the gels. N = 6. ***$p < 0.001$. Data were representative of 3 independent experiments.

that treatment of the cells with 0.5 mM H_2O_2 induced increased expression of CD38 in astrocytes, as assessed by Western blotting (**Figure 4**).

Discussion

The major findings from our current study includes: First, CD38 siRNA-induced decrease in CD38 levels can lead to a significant increase in H_2O_2-induced astrocyte death; second, CD38 siRNA treatment can lead to a significant increase in oxidative stress, as indicated by significant increases in both superoxide and lipid peroxidation in H_2O_2-treated cells; and third, H_2O_2 can induce increased CD38 expression. Collectively, our study has provided the first evidence suggesting that CD38 plays significant roles in both antioxidation capacity and cell survival of H_2O_2-treated astrocytes. Because oxidative stress plays crucial roles in multiple neurological disorders, our study has suggested that CD38 may become a novel therapeutic target for decreasing oxidative stress in neurological disorders.

CD38 is a multifunctional ecto-enzyme that can generate cyclic ADP-ribose (cADPR) from NAD^+ [1]. cADPR is a potent endogenous agonist of ryanodine receptors (RyR), which can modulate $[Ca^{2+}]_i$ [2]. Recent studies have suggested that CD38 may play significant roles in ischemic brain injury and traumatic brain injury. However, the mechanisms underlying the roles of CD38 in the neurological diseases remain unclear. Oxidative stress plays significant roles in multiple neurological diseases including ischemic brain damage, Parkinson's disease and Alzheimer's disease [18]. It is of both critical theoretical and clinical significance to further investigate the mechanisms underlying oxidative stress-induced neural cell death. Therefore, in this study we tested our hypothesis that CD38 plays a significant role in oxidative cell death by using primary astrocyte cultures as a cellular model.

Our current study has suggested that CD38 plays an important role in H_2O_2-induced death of astrocytes: CD38 siRNA can significantly exacerbate H_2O_2-induced death of astrocytes, as assessed by both exatrcellular and intracellular LDH assays. In other words, these observations have suggested CD38 produces protective effects on H_2O_2-induced death of astrocytes. Because our study has also suggested that H_2O_2 treatment can induce increased CD38 levels, our observations have collectively suggested that the H_2O_2-induce increase in

CD38 levels can lead to a decrease in the oxidative stress-induced death of the cells.

Our study has also suggested that CD38 is directly involved in affecting the intracellular oxidative stress and oxidative damage of H_2O_2-treated astrocytes: CD38 siRNA can significantly increase both superoxide and lipid peroxidation of the H_2O_2-treated cells. Because increased ROS levels can lead to increased lipid peroxidation, resulting in increased cell death, our findings in the current study have collectively suggested that the H_2O_2-induced increase in CD38 produces protective effects on H_2O_2-induced cell death at least partially by decreasing the superoxide and lipid peroxidation of the cells.

However, it remains unclear how increased CD38 may lead to decreased ROS levels and lipid peroxidation in the cell. Because a major biological function of CD38 is to generate cADPR - a potent endogenous agonist of ryanodine receptors, our study has implicated that the cADPR-induced activation of ryanodine receptors might contribute to the decreases in oxidative stress and oxidative damage. Future studies are warranted to further investigate the mechanisms underlying the roles of CD38 in the H_2O_2-induced death of astrocytes.

Based on the finding that the NAD^+ level is markedly increased in the tissues of CD38 knockout mice [7-9], which can decrease oxidative damage [19], a previous study tested their hypothesis that the cells from CD38 knockout mice are resistant to oxidative stress, showing that H_2O_2-induced death of mouse embryonic fibroblasts (MEFs) from CD38 knockout mice was lower compared with H_2O_2-induced death of wild-type MEFs [20]. As discussed above, the interpretations of the experimental results from the studies using CD38 knock mice might be confounded, because both NAD^+ and SIRT1 can affect a large variety of biological functions [10]. Therefore, it is necessary to apply other approaches such as siRNA approach, which do not significantly affect the levels of NAD^+ or SIRT1, to investigate the roles of CD38 in neural cell death. Under our experimental conditions, the CD38 siRNA-produced decrease in the CD38 levels did not significantly affect the intracellular NAD^+ level of astrocytes.

Our study is the first that investigates the roles of CD38 in oxidative stress-induced death of cells that have normal NAD^+ levels. Our study has provided novel evidence suggesting that CD38 plays a critical role in the antioxidation capacity and cell survival of H_2O_2-treated astrocytes, which suggests that CD38 may become a new target for defending oxidative damage in neurological diseases.

Acknowledgements

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Disclosure of conflict of interest

There is no conflict of interest.

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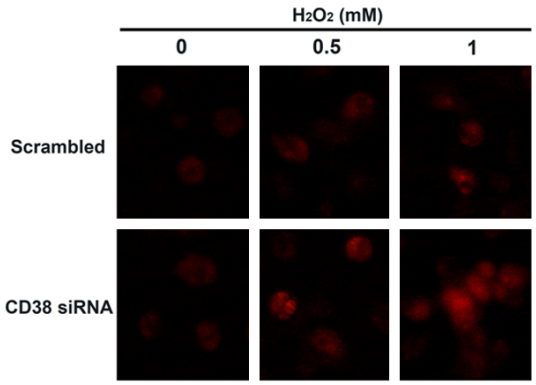
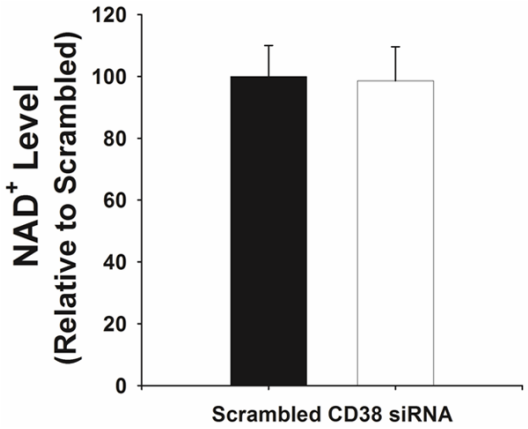
Supplementary Material



Supplemental Figure 1. CD38 siRNA treatment exacerbated oxidative stress in H_2O_2-induced astrocytes. CD38 siRNA treatment significantly increased the DHE levels in H_2O_2-induced astrocytes. Astrocytes were transfected with CD38 siRNA for 24 hrs, followed by treatment with 0.5 mM or 1 mM H_2O_2 for 1 hr, which was the replaced with DMEM. The DHE levels of the cells were determined at 15 hrs after the media replacement. N = 9. Data were representative of 3 independent experiments.



Supplemental Figure 2. CD38 siRNA treatment did not affect the intracellular NAD^+ levels in astrocytes. Astrocytes were treated with CD38 siRNA for 48 hrs, then the intracellular NAD^+ levels were determined. N = 12. Data were representative of 3 independent experiments.