Different effects of the nonsteroidal anti-inflammatory drugs meclofenamate sodium and naproxen sodium on proteasome activity in cardiac cells

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Abstract

The use of nonsteroidal anti-inflammatory drugs (NSAIDs) like meclofenamate sodium (MS), used to reduce pain, has been associated with an increased risk of cardiovascular disease (CVD). Naproxen (NAP), another NSAID, is not associated with increased risk of CVD. The molecular mechanism(s) by which NSAIDs induce CVD is unknown.

We investigated the effects of MS and NAP on protein homeostasis and cardiotoxicity in rat cardiac H9c2 cells and murine neonatal cardiomyocytes. MS, but not NAP, significantly inhibited proteasome activity and reduced cardiac cell viability at pharmacological levels found in humans. Although proteasome subunit gene and protein expression were unaffected by NSAIDs, MS treated cell lysates showed higher ROS proteasome content, while purified proteasomes from MS treated cells had lower proteasome activity and higher levels of oxidized subunits than proteasomes from control cells. Addition of exogenous proteasome to MS treated cells improved cell viability. Both MS and NAP increased ROS production, but the rate of ROS production was greater in MS than in NAP treated cells. The ROS production is likely from mitochondria, as MS inhibited mitochondrial Complexes I and III, major sources of ROS, while NAP inhibited Complex I. MS also impaired mitochondrial membrane potential while NAP did not. Antioxidants were able to prevent the reduced cell viability caused by MS treatment.

These results suggest that NSAIDs induce cardiotoxicity by a ROS dependent mechanism involving mitochondrial proteasome dysfunction and may explain why some NSAIDs should not be given to patients for long periods.

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1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) like naproxen sodium (NAP, also known as Alleve), ibuprofen (Advil), and diclofenac (Cataflam), are commonly used for their anti-inflammatory and analgesic properties in reducing fever and pain [1]. Numerous clinical trials have been reported that suggest an increased risk in the development of CVD due to prolonged exposure to NSAIDs [2]. The adverse effect of NSAIDs is especially prominent in individuals with a history of myocardial infarction and other cardiovascular related disorders [3–5]. Most NSAIDs have been shown to increase the risk of heart failure by up to five times above normal [6]. While NSAIDs have been shown to increase the risk of CVD, NAP has been associated with some cardioprotective effects, possibly due to its aspirin-like property of inhibiting cyclooxygenase platelet thromboxane B2 [6–8]. However, use of NAP increases the risk of stroke by 1.76 fold [9].

Since a previous report showed that aspirin indirectly inhibits proteasome activity in mouse Neur 2a cells [10], it was hypothesized that meclofenamate sodium (MS) and NAP would both inhibit the proteasome but to varying extents. MS, a prescription NSAID, is comparable to other NSAIDs like diclofenac and has demonstrated efficiency in treating autoimmune diseases like chronic and acute osteoarthritis and rheumatoid arthritis [11]. Investigating the effects of NSAIDs on proteasome activity in cardiac cells is important because a recent study utilizing pigs showed that chronic proteasome inhibition led to cardiac dysfunction resulting in cardiomyopathy [12]. Therefore, proteasome inhibition has been suggested as the cause rather than the...
result of CVD [13,14]. Failing human hearts are often characterized by marked accumulation of ubiquitinated proteins and depressed proteasome activity, suggesting that decreased proteasome activity leads to cardiac dysfunction [13,15].

The ubiquitin-proteasome system (UPS) is a major eukaryotic mechanism for protein degradation [16] and proteasomes have been shown to regulate cell proliferation, differentiation, apoptosis, the oxidative stress response, and transcription factors to maintain cellular homeostasis [17–21]. The proteasome is responsible for degradation of >60% of all intracellular proteins in eukaryotes [22,23]. 26S proteasomes consist of one or two outer 19S regulatory caps that recognize and bind the ubiquitin marker and a 20S catalytic core that contains protease active sites [24]. Another form of the proteasome exists in the cell, the 20S proteasome, which lacks the 19S regulatory caps exists [25]. The major difference between the two types of proteasomes is that the 20S proteasomes can degrade oxidized proteins independent of ubiquitination and are known to be upregulated during fever and inflammation [25–27]. The 20S catalytic core contains three distinct catalytic activities for protein degradation: β1 has caspase-like, β2 has trypsin-like, and β5 has chymotrypsin-like activity [13]. Among these three proteolytic activities, β5 has been suggested to be the most important [28].

Apart from proteasome dysfunction, reactive oxygen species (ROS) generation has also been implicated in various pathological conditions including myocardial infarction, inflammatory diseases, diabetes, and hypertension [29–31]. NSAID-induced apoptosis has been shown in several types of cancer cells due to an increase in intracellular ROS levels [32–34]. Low physiological levels of ROS are important for the regulation of numerous biological processes and under normal physiological conditions, a homeostatic balance occurs between the generation of ROS and the antioxidant capacity of the cell. However, under certain pathological conditions this homeostasis can be disrupted resulting in elevated ROS levels which may lead to apoptotic cell death as well as necrosis [35–37].

The H9c2 cell is a commonly used cardiac cell for cardiac cell research and was previously shown to have similar responses to drugs in vitro as primary neonatal cardiomyocyte cells [38]. The phenotype of neonatal cardiomyocytes is highly stable and comparable to in situ heart and therefore provides an efficient cardiac model for studying oxidative stress response in the system [39]. Although the role of NSAIDs in the generation of ROS has been reported in various cancer cells, the effect of NSAIDs on cardiac cell function (or other non-diseased cell types) is not well understood. The possibility that NSAIDs inhibit the proteasome in cardiac cells has not been previously investigated. Using these cell types, MS was found to induce ROS, impair mitochondrial function, reduce proteasome activity, increase oxidation of proteasome subunits, increase 20S proteasome content relative to 26S proteasome content, and reduce cell viability. NAP did not reduce cell viability. The reduced cell viability by MS was prevented by antioxidants and partly restored by addition of exogenous proteasomes. Antimycin A, a mitochondrial complex III inhibitor, reduced cell viability, increased ROS levels, and decreased proteasome activity. Overall, the results suggest that NSAIDs induced cardiotoxicity by ROS dependent mechanism involving mitochondrial and proteasome dysfunction.

2. Materials and methods

Details about the Materials and methods are described in the online data supplement.

2.1. NSAID treatment of cells

NSAIDs were used to treat H9c2 cardiac cells and neonatal cells at the desired concentrations for different incubation periods as described in the online supplement.

2.2. Purified 20S proteasome activity assays

Purified 20S proteasome assays were performed using substrates specific for each of the proteasomal β subunits (n = 5) [25,40–42] as described in the online supplement.

2.3. 26S proteasome activity assays of H9c2 cells, neonatal cells, tissue lysates, and purified proteasomes

H9c2 cells, neonatal mouse cardiomyocytes, heart tissue lysates, and purified proteasomes were treated with different concentrations of MS and NAP, and proteasome activity investigated as described in the online supplement.

2.4. Treatment of H9c2 cells with Antimycin A

H9c2 cells were treated with 200 μM Antimycin A as described in the online supplement.

2.5. Purification of 26S proteasomes from H9c2 cells

Purification of 26S proteasomes from H9c2 cells treated with 100 μM MS was performed using the Rapid 26S Proteasome Purification Kit-L (Ubiquitin-Proteasome Biotechnologies) with slight modifications as described in the online supplement [43].

2.6. Caspase-3 activity measurements

Caspase-3 assay was performed in the presence or absence of 10 μM caspase-3 inhibitor, Ac-DEVD-CHO as described in the online supplement.

2.7. Western blot

Details about Western blotting methods are described in the online supplement.

2.8. MV-151 labeling of proteasome active sites

Proteasome labeling was carried out using MV151 as described in the online supplement.

2.9. OxyBlot analysis for determination of oxidized protein levels

Protein oxidation was determined by pre-derivatization of the carboxyl group of the protein with dinitrophenylhydrazine as described in the online supplement.

2.10. Detection of intracellular reactive oxygen species formation

ROS generation was quantified in H9c2, CV1, CHI10T1/2, and equine skin cells with 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) as described in the online supplement.

2.11. Isolation of functional mitochondria from mouse heart

Mitochondria were isolated from mouse heart tissue as previously described with slight modifications [44] as described in the online supplement.

2.12. Spectrophotometric analysis of mitochondrial respiratory chain complex activities

Fresh or frozen mitochondria (subjected to 3 freeze-thaw cycles) were utilized to measure Complex I (C-I), Complex II (C-II) and Complex III (C-III) activities in the presence of NSAIDs as described in the online supplement.
2.13. Mitochondrial membrane potential assay

Mitochondrial membrane potential (ΔΨm) was studied in intact cardiac H9c2 cells using the mitochondria specific carbocyanine dye JC-1(5.5’-6,6’-tetrachloro-1’,3’,3’-tetraethylbenzimidazolocarbocyanine iodide) as described in the online supplement.

2.14. Transfection of purified proteasomes in H9c2 cells

The exogenous proteasome transfection was carried out using Saint-PHD transfection reagent as described in the online supplement.

2.15. Immunocytochemistry of H9c2 cells transfected with purified proteasomes

The amounts of purified proteasomes transfected into H9c2 cells were analyzed by immunocytochemistry using the anti-β1 proteasome antibody as described in the online supplement.

2.16. Cyclooxygenase activity measurements

Cox activity was measured using the Cox Fluorescent Activity Assay Kit (Cayman Chemicals) as described in the online supplement.

2.17. Statistics

Results are expressed as mean ± SD from at least three experiments. The comparisons were performed using the Student’s t-test or one way ANOVA. Values of P < 0.05 were defined as statistically significant.

3. Results

3.1. Effects of MS and NAP on proteasome activity

Proper functioning of the proteasome system is important for maintaining cardiac homeostasis, and impaired proteasomal function has been implicated in heart diseases [45–50]. Since proteasome impairment has been associated with cardiovascular diseases we investigated the effects of MS and NAP on proteasome activity (Figs. 1 and S1). The amounts of MS used were based upon levels of MS reported in the plasma of horses and humans, and MS levels previously utilized in non-cardiac cells. Peak levels of ~337 μM MS have been reported in horses given MS doses typically utilized for treating muscularskeletal disease [51]. Few studies on the amounts of MS in human plasma are available, but the average to peak pharmacological ranges detected were 10–22 μM MS and 281.4–463.8 μM NAP [52, 53]. The most typical concentration of MS utilized in previous cell based studies on non-cardiac cells was 100 μM–200 μM [54,55]. The relevant levels of MS in animals may be >100 μM based upon results from horse plasma, so a wide range of MS concentrations was investigated. Although lower doses of MS (10 μM) for longer periods of time significantly increased the effects such as cell death and proteasome impairment, it was not feasible to carry out all the experiments for long durations with neonatal cardiomyocytes.

Mouse heart lysates treated with different concentrations of MS (Fig. 1A) showed decreased β1 (peptidylglutamate peptide hydrolyzing) and β5 (chymotrypsin-like) activity (Fig. 1A). The β5 activity of the proteasome is considered to be the most important for protein degradation [56], and a pronounced reduction in the β5 activity (80%) was observed when the mouse heart lysates were treated with 500 μM MS. MG132 which was used as a positive control showed inhibition of all three proteasomal activities. Similar to the mouse heart lysate, β5 proteasome activity in the rat heart lysates was decreased by 50 μM MS (~20%) (Fig. S1B). Since MG132 is not highly specific for the proteasome and could also inhibit cathepsins, experiments were carried out in the presence and absence of the specific proteasome inhibitor bortezomib (Fig. S1D). Fig. S1D shows the difference between the proteolytic activity in the presence of bortezomib subtracted from the activity in the absence of bortezomib which corresponds to the specific proteasome activity. These results suggest that MS inhibits the proteasome activity. Since the heart seems to be more susceptible to NSAIDs than other tissues, the effect of MS on mouse liver and brain lysates was also investigated (Fig. S1C). At 200 and 500 μM concentrations of MS the β5 proteasome activity in heart lysates was considerably lower than in liver or brain lysates suggesting that the cardiac proteasome may be more susceptible to MS than proteasomes from other tissues. In mouse heart lysates, 2 mM NAP inhibited β1 activity (Fig. 1B). At 1 mM, NAP inhibited β5 activity by approx. 30%. Similar results were obtained using rat heart lysates where NAP only inhibited proteasome activity at concentrations of ≥ 1 mM (Fig. S1A). No changes in the β2 activity were detected in mouse or rat heart lysates.

H9c2 rat cardiocytes treated with either 100 μM MS or 500 μM NAP for 24 h (Fig. 1C) showed that MS decreased β5 26S proteasome activity by >30%, while NAP had no effect on β5 activity. β1 and β2 proteasome activity in the presence of either of the compounds remained unaffected (Fig. S2A and B). Long term treatment of H9c2 cells for 12 days with 10 μM MS decreased β5 activity (Fig. 1C). These results suggest that some NSAIDs can cause proteasome dysfunction in cardiac cells.

3.2. Proteasome chymotrypsin-like activity of neonatal cardiomyocytes was impaired by MS and not by NAP

The β5 proteasome activity in mouse neonatal cardiomyocytes was significantly reduced in the cells treated with 10 μM MS for 5 days (Fig. 1C). However 100 μM NAP did not alter the proteasome activity under the same conditions.

3.3. Purified murine 20S proteasome activity was decreased by MS and NAP

Purified 20S proteasomes incubated with 500 μM MS showed decreased β1, β2, and β5 proteasome activity (Figs. 1D and S1E). At 100 μM MS, the β5 activity of the proteasome was decreased by >20% compared to control purified proteasomes. The β2 proteasome activity was decreased by approx. 80% when 500 μM MS was present. While NAP (up to 5 mM) had no effect on β2 proteasome activity (Fig. S1F), β1 (Fig. S1F) and β5 (Fig. 1D) activities were decreased in the presence of 2.5 and 5 mM NAP.

3.4. MV151 labeling of proteasome active sites in the presence of MS and NAP

To determine if NSAIDs could directly bind the proteasome active sites, the fluorescently labeled reversible proteasome inhibitor MV151 was utilized (Fig. 1E). Although 100 μM MS inhibited the intracellular proteasome β5 activity in H9c2 cells, even 500 μM MS was unable to compete with the MV151 to bind to the proteasome active sites. NAP (2 mM) was also unable to displace the MV151. On the other hand, the proteasome inhibitor MG-132 displaced MV151 from interacting with the proteasome active sites. These results suggest that MS and NAP do not interact directly with the active sites of the proteasome. Although MS does not directly bind to the β1, β2 and β5 active sites it may be binding directly to other sites on the proteasome since MS inhibited purified proteasome activity.

3.5. Cardiac cell viability was decreased by MS but not by NAP in H9c2 and neonatal cells

H9c2 cells treated with 50 μM MS for 24 h showed significant decreases (~30%) in cell viability (Fig. 2A). Hydrogen peroxide (H2O2), which was used as a positive control, also resulted in significant decreases in cell viability. Neonatal cardiac cells treated with either
10 μM MS or 200 μM NAP for 48 h showed decreased cell viability only for the MS treated cardiomyocytes (Fig. 2C). In these latter experiments cells were incubated with media containing the NSAID or H$_2$O$_2$ for 48 h without changing or adding new media after 24 h. These results are consistent with the H9c2 cardiac cell results where 10 μM MS for 48 h reduced cell viability while 200 μM NAP did not [Fig. 2B]. Incubation of H9c2 cells with 10 and 30 μM MS for 6 days resulted in approximately 24% and 37% reductions in cell viability respectively [Fig. 2B]. For all experiments where H9c2 cells were incubated with NSAID or H$_2$O$_2$ for longer than 48 h, media was replaced every 48 h with new media containing freshly made NSAID or H$_2$O$_2$. Higher concentrations of MS (250 and 500 μM) resulted in greater cell death (Fig. 2D). Incubation of cells with 250 μM MS resulted in greater cell death after 24 h when compared to 4 h. These results suggest that both concentration and exposure time are important for NSAID-induced cardiotoxicity. NAP had little effect on the cell viability at all concentrations investigated.

### 3.6. ROS levels in H9c2 cells and mouse neonatal cardiomyocytes were increased by MS and NAP

H9c2 cells were treated with different concentrations of MS (10 μM to 100 μM) and NAP (100 μM to 1000 μM) to measure ROS generation (Fig. 3A). In control cardiac H9c2 cells the small amount of ROS normally generated intracellularly was used as the baseline. ROS formation was significantly increased in the presence of 10 μM MS. Similarly, cells exposed to higher concentrations of NAP (500 μM) showed a significant increase in ROS generation (Fig. 3A). Freshly isolated neonatal cardiomyocytes also showed significant increases in ROS generation in MS and NAP treated cells at all the concentrations utilized (Fig. 3B). Fluorescent microscopy using H$_2$DCFDA showed that H9c2 cells incubated with MS at concentrations of 10–100 μM exhibited a significant increase in fluorescent intensity compared to untreated cells (Figs. 3C and 5A). NAP at 0.5 mM and 1 mM also showed increased ROS formation, but the amount of ROS detected with 1 mM NAP was less than that...
detected at 100 μM MS suggesting that MS was a more potent inducer of ROS than NAP. H$_2$O$_2$ was used as a positive control for ROS formation (Fig. 3C).

The rate of ROS generation in H9c2 cells was also determined, and the initial rate of ROS generation at 1 h in MS or NAP treated cells was significantly higher compared to the control cells (P < 0.001). A gradual decline in the rate of ROS generation was observed in cells treated with either MS or NAP (Fig. 3D). However this decline was more pronounced with NAP than MS. After 3 h and 5 h the rate of ROS generation in MS treated cells was significantly higher when compared to NAP treated cells suggesting that MS was a potent inducer of ROS formation compared to NAP. The rate of ROS generation/hour was significantly higher in neonatal cardiomyocytes treated with MS when compared to NAP treated cells (Fig. 3D), similar to what was observed in H9c2 cells. Since cardiac cells seem to be more susceptible to NSAID induced damage than other cell types, the effect of MS on different cell types was compared. A comparison between H9c2 and monkey kidney CV1 cells showed that ROS production in H9c2 was significantly greater when compared to NAP treated cells suggesting that MS was a potent inducer of ROS formation compared to NAP. The rate of ROS generation/hour was significantly higher in neonatal cardiomyocytes treated with MS when compared to NAP treated cells (Fig. 3D), similar to what was observed in H9c2 cells. Since cardiac cells seem to be more susceptible to NSAID induced damage than other cell types, the effect of MS on different cell types was compared. A comparison between H9c2 and monkey kidney CV1 cells showed that ROS production in H9c2 was significantly greater when compared to NAP treated cells (Fig. 3D), similar to what was observed in H9c2 cells. Since cardiac cells seem to be more susceptible to NSAID induced damage than other cell types, the effect of MS on different cell types was compared. A comparison between H9c2 and monkey kidney CV1 cells showed that ROS production in H9c2 was significantly greater when compared to NAP treated cells (Fig. 3D), similar to what was observed in H9c2 cells.

3.7. Effect of MS and NAP on different mitochondrial complex activities

Mitochondria are the major organelles for the production of free radicals in biological systems [57]. To investigate whether NSAIDs target the heart mitochondrial complexes to generate ROS, the effect of MS and NAP on the mitochondrial Complex I, II, and III activities were investigated.

3.7.1. Mitochondrial Complex I activity was decreased by MS and NAP

The effect of NSAIDs on mitochondrial Complex I activity, a major site for ROS generation [58], was investigated using isolated mitochondria. Rotenone (10 μM), an inhibitor of Complex I, significantly decreased the enzymatic activity by ~70% compared to control (Fig. 4A). In mitochondria treated with 100 μM MS, the enzymatic activity was significantly reduced by ~70%, similar to the rotenone induced impairment of Complex I activity. However at lower concentrations of MS (10 μM), there was no significant decrease in Complex I activity. On the contrary, 200 μM and 10T1/2 embryonic and equine skin cells when exposed to MS or H$_2$O$_2$ (Fig. 3F). These results show that MS induces greater ROS production in cardiac cells than in many other cell types investigated.

**Fig. 2.** Viability of H9c2 cells and neonatal cardiomyocytes in the presence of MS and NAP. H9c2 cells were treated with MS for 4, 24 h, 48 h, and 6 days and NAP for 24 h and 48 h at 37 °C and cell viability determined. Neonatal cardiomyocytes were treated for 48 h with MS and NAP. A. Measurement of H9c2 cell viability after 24 h using Alamar Blue. Cells were plated in a 96 well plates and cell viability was measured after 24 h (absorbance of 550 nm). B. H9c2 cell viability after 48 h and 6 days of MS treatment. H9c2 cells were treated with different concentrations of MS for different time periods and cell viability measured. Cells were treated with MS, NAP, or H$_2$O$_2$ once every 48 h. C. Measurement of neonatal cardiac cell viability after 48 h. Neonatal cardiomyocytes (NEO) were treated with MS and NAP for 48 h and cell viability was measured using Alamar Blue. D. CCK-8 assay for determination of H9c2 cell viability. Cells were plated in a 96 well plates and cell viability was measured for different time intervals (absorbance of 450 nm). Values shown are mean ± SD (n = 3 to 9) (* represents P < 0.05, **P < 0.001).
500 μM NAP showed a statistically significant reduction in C-I activity in isolated mitochondria (-50% and -55% reductions respectively).

### 3.7.2. Mitochondrial Complex II activity was unaffected by MS and NAP

To further investigate the effect of NSAIDs on the mitochondrial function, Complex II (C-II) activity was measured in freeze-thawed purified mitochondria in the presence of either MS or NAP. H$_2$DCFDA was used to detect ROS levels in cells. A: control cells; b: 30 μM MS; c: 500 μM NAP and d: 200 μM H$_2$O$_2$. Scale bar represents 100 μm. D. Rate of ROS generation in H9c2 cells and neonatal cardiomyocytes. E. ROS formation in H9c2 and CV-1 cells. * represents difference between MS and NAP treated cells. E. ROS generation in H9c2, CV-1, CH3/10T1/2, and equine skin cells. H$_2$O$_2$ (100–200 μM) was used as a positive ROS control. Levels of ROS in H9c2 cells were significantly higher than in CV-1, CH3/10T1/2, and equine skin cells at all MS concentrations used (P < 0.05). The values represent mean ± SD (n = 5 to 9) (* represents P < 0.05; ** represents P < 0.001).

### 3.7.3. Mitochondrial Complex III activity was impaired by MS but not by NAP

Apart from C-I, C-III has also been shown to be a major site for ROS generation [59]. As such, the potential adverse effect of NSAIDs on mitochondrial C-III activity was investigated. The enzymatic activity observed in control experiments was significantly inhibited by 10 ng/ml antimycin A, an inhibitor of C-III (Fig. 4C). Significant decreases in C-III activity were observed in mitochondria incubated with 10 μM or higher concentrations of MS (Figs. 4C and S6B). However, neither 200 μM nor 500 μM NAP affected mitochondrial C-III activity when compared to mitochondria in the control experiments. These results suggest that only MS is potent at decreasing C-III activity.

### 3.8. Mitochondrial membrane potential was impaired by MS but not by NAP

The effect of varying concentrations of MS and NAP on ΔΨ$_m$ was studied in H9c2 cells using the fluorescent dye JC-1. JC-1 dye accumulates specifically in the mitochondria, and the level of fluorescence gives an indication of the status of ΔΨ$_m$ [60]. Both 10 μM rotenone and 200 μM DNPH substantially decreased ΔΨ$_m$ by ~70% and ~50% respectively in H9c2 cells after 24 h (Fig. 4D). Incubation of H9c2 cells with low concentrations of MS (5 μM and 10 μM) significantly decreased ΔΨ$_m$ (P < 0.05). On the other hand, 200 μM or 500 μM NAP had no effect on ΔΨ$_m$ when compared to the control experiments (Fig. 4D). These results suggest that apart from the adverse effect of MS on the respiratory chain complex of heart mitochondria, MS significantly decreased ΔΨ$_m$ while NAP did not.

### 3.9. Proteasome expression levels in H9c2 cells remained unaltered by NSAID treatment

To determine if NSAIDs affect the expression of the proteasome, RT-PCR and Western blotting were carried out (Fig. S7). RT-PCR showed that PSMB5 (which codes for the β5 subunit), PSMB6 (β1 subunit), and PSMB8 (β5i subunit) were not significantly changed. PSMB8 showed a trend towards increasing at the 24 h time point (Fig. S7A). Western blotting of PSMA6 and β1 (20S core subunits), and Rpt1 (19S
Fig. 4. Effects of MS and NAP on mitochondrial complex activities. Mitochondrial complex activities were measured in mitochondria isolated from mouse hearts as described in Section 2.1.1. A. Relative mitochondrial C-I activity. Isolated mitochondria were incubated with MS, NAP or the C-I inhibitor ROT (rotenone) and C-I activity measured at an absorbance of 530 nm. B. Mitochondrial C-II activity assay. C-II activity was measured at an absorbance of 595 nm in the presence of MS, NAP, or 500 μM TFA, a C-II specific inhibitor. C. Mitochondrial C-III activity assay. C-III activity was determined by incubating isolated mitochondria with MS, NAP or 0.01 mg/mL Antimycin A. D. Mitochondrial membrane potential assay. H9c2 cells were treated with MS, NAP, ROT and DNPH and mitochondrial membrane potential was determined using 5 μg/ml JC-1 dye at an Ex-490 nm and Em-590 nm. Values are mean ± SD (n = 4 to 6) (* represents P < 0.05, **P < 0.001).

Fig. 5. Effect of MS on proteasome subunit composition. A. OxyBlot and β5 activity of purified 26S proteasome obtained from MS and control cells. Oxidized subunits of the purified 26S proteasome from MS and control treated H9c2 cells were detected by the OxyBlot procedure. 26S proteasome was isolated from 48 h vehicle and MS treated H9c2 cells, and β5 activity was determined. B. Relative proportions of 26S and 20S proteasome complexes in lysates from MS treated, NAP treated and control cells. Native gel electrophoresis was utilized to separate different proteasome complexes. 26S* doubly capped proteasome (19S-20S-19S), 26S, singly capped proteasome (19S-20S). MS treated cells showed a significantly higher proportion of 20S proteasomes than NAP or control cells. Values are mean ± SD (n = 3) (* represents P < 0.05). C. Assignment of proteasome complexes using Western blotting. Proteasome complexes separated on native gels were transferred to nitrocellulose membrane and probed with PSMA6 antibody. Blots were then stripped and reprobed with other proteasome antibodies, β1 and Rpt 6. The figure is representative of 3 technical replicates.
subunit) showed that these three proteasome subunits were similarly expressed under all the conditions tested (Fig. S7B and C). A protein that has been shown to be upregulated by NSAIDs, nonsteroidal anti-inflammatory drug-activated gene (NAG-1) [61], and another protein involved in stress response, heat shock protein 60 (HSP60) [62], were also investigated by Western blotting (Fig. S7C). Neither NAG-1 nor HSP60 expression was affected by MS or NAP.

3.10. MS reduced chymotrypsin-like activity in purified 26S proteasomes isolated from cardiac cells

To determine if MS had any direct effect on 26S proteasome function in cardiac cells, H9c2 cells were treated with 100 μM MS for 48 h, and 26S proteasomes were purified. Proteasomes isolated from MS treated cells showed significant decreases in β5 activity when compared to purified proteasomes from control cells treated with vehicle for 24 h (Fig. 5A). The total amount of 26S proteasome used for the assays was the same suggesting that the proteasomes from MS treated cells contain lower proteasome activity.

3.11. MS increased oxidation of 26S proteasome complexes from MS treated cells

Since purified proteasomes from MS treated cells showed lower activity but the expression of proteasome subunits was unchanged, it is possible that the proteasomes may be undergoing post-translational modification(s). To determine if ROS produced in MS-treated cells oxidized proteasome subunits, proteasomes were purified from H9c2 cells and investigated for oxidized subunits using the OxyBlot procedure (Fig. 5A). While the 19S portion of the proteasome from MS treated cells showed significantly higher levels of oxidized subunits (Fig. 5A), the 20S complex had very little oxidation and similar oxidation patterns between MS treated and control cells (Fig. S7D), suggesting that only some 19S proteasome subunits were being oxidized. Since proteasome activity could also be affected by the relative proportions of 20S and 26S, the amounts of 20S and 26S was determined using native gels and Western blotting (Fig. 5B). The relative proportion of 20S was increased only in MS and not NAP treated cells relative to control cells. The relative proportion of 26S was decreased in MS treated cells. To validate that the 20S proteasome complex observed on the immunoblot is not an individual proteasome subunit, Western blotting of two independent 20S proteasome subunits (PSMA6 and β5) was carried out on the same blot (Fig. 5C). The detection of both proteasome subunits in all the complexes suggest that the bands detected are not from single proteasome subunits. The conditions used for the native gel (3.5% acrylamide gel percentage and 3 h running time) typically only allows proteins >200 kDa to be detected. The 26S complexes were also validated by reprobing these blots with antibody to a 19S subunit, Rpt6, which was only observed in the 26S complexes.

3.12. Improved cell viability of MS treated H9c2 cells in the presence of antioxidants correlates with restored proteasome activity

The role of ROS in the initiation and mediation of cell death has been reported [63,64]. Since MS was found to be a potent inducer of ROS in cardiac cells, the effect of antioxidants on the viability of MS treated H9c2 cells was investigated (Fig. 6). Since no cell death was observed for NAP treated cells, further ROS studies on NAP treated cells were not carried out. When H9c2 cells were pretreated for 24 h with 200 μM ascorbic acid, cell viability was significantly improved relative to the control cells (Fig. 6A). Pretreatment with 100 μM Tempol for 30 min also prevented MS induced cell death (Fig. 6A). Pretreatment with 200 μM ascorbic acid for 24 h and 100 μM Tempol for 30 min fully restored cell viability (Fig. 6B). Pretreatment of the H9c2 cells with ascorbic acid and Tempol also prevented the decrease in β5 proteasome activity observed in MS treated cells (Fig. 6C). Although CCK8 was originally used to determine cell viability, Alamar blue was utilized for all the antioxidant studies because the antioxidants were found to interact with CCK8 (Fig. S8). The antioxidants also interacted with the commonly used MTT reagent (Fig. S8).

3.13. Mitochondrial Complex III inhibitor, Antimycin A, increased ROS and decreased proteasome activity

To determine if inhibition of C-III affected ROS, cell death, and/or proteasome function, H9c2 cells were treated with 200 μM of the C-III inhibitor Antimycin A. The amount of Antimycin A utilized was based upon amounts used in a recent publication [65]. Antimycin A significantly increased ROS levels in H9c2 cells and decreased cell viability (Fig. 6D, E and F). Additionally, a significant decrease in proteasome β5 activity was observed in cells treated with the inhibitor for 24 h (Fig. 6G). These results strongly suggest a cross-talk between mitochondrial function and proteasome activity, where mitochondrial C-III inhibition leads to increased ROS, proteasome dysfunction and cardiac cell death.

3.14. MS increased polyubiquitinated protein levels in H9c2 cells

Since decreased proteasome activity usually leads to increased ubiquitinated protein levels, the ubiquitination levels were investigated. Although 200 μM MS did not cause any significant increase in the ubiquitinated protein levels, 500 μM MS significantly increased the levels of ubiquitinated proteins (Fig. 7A) with decreased free ubiquitin levels in H9c2 cells (Fig. 7B) suggesting proteasome dysfunction. Antioxidant treatment reduced polyubiquitination levels in the MS treated cells. Additionally, caspase-3 activity was significantly increased in cells treated with 200 or 500 μM MS (Fig. 7C). However, at 30 μM MS, a concentration at which cell death and lower proteasome activity occurred, no caspase-3 activation was detected, suggesting that apoptosis was not responsible for the cardiac cell death at these concentrations (Fig. S3C).

3.15. MS induced H9c2 cell death is reversed by transfection with purified proteasomes

To further investigate the involvement of the proteasome in MS induced cardiotoxicity, H9c2 cells treated with 100 μM MS were transfected with purified 26S proteasome. Transfection of 0.05 μg 26S proteasome significantly increased the 26S β5 chymotrypsin-like proteasome activity compared to the control cells (Fig. 8A). Proteasome transfected MS treated cells showed a significantly higher cell viability compared to the transfection reagent treated cells (approximately 50% higher cell viability than MS treated cells) (Fig. 8B). Proteasome transfection of cells not exposed to MS did not show any change in cell viability. The use of very small amount (2 nM) of a well-established reversible inhibitor of the proteasome, bortezomib, decreased cell viability (Fig. 8B). Proteasome transfection of the bortezomib treated cells did not improve the cell viability.

Transfection of MS treated cells with 20S proteasome (instead of 26S proteasome) also resulted in a significant increase in cell viability (Fig. 8C). 20S proteasome transfection of non-MS treated cells did not affect cell viability relative to control cells transfected with the transfection reagent. These results all suggest the involvement of the proteasome in H9c2 cell death.

To verify that the proteasome transfected into cells actually entered the cells, transfected H9c2 cells were washed, fixed and probed with the β5 proteasome polyclonal antibody, and the fluorescence intensity of a secondary antibody against rabbit IgG was measured to determine proteasome levels in the cells. The cells transfected with 0.05 μg 26S or 0.1 μg 20S proteasomes showed a significant increase in fluorescence intensity (~65% and ~40%) relative to the control cells (Figs. S8D and S5A). These results suggest that the proteasome is being transfected into the cell and not just interacting with the outer plasma membrane of the
H9c2 cell. The results are also in agreement with the β5 proteasome activity and cell viability measurements where 0.1 μg of 26S did not further increase the proteasome amount in the H9c2 cell relative to 0.05 μg of 26S. Increased 20S was only detected in cells transfected with 0.1 μg 20S (Fig. S5A). These results are in agreement with the results obtained for cell viability. For the 26S proteasome it may be that higher amounts of proteasome resulted in aggregation, as several more brightly fluorescent spots were observed in cells transfected with 0.1 μg 26S relative to lower concentrations.

3.16. Oxidation of proteins is increased by MS but not by NAP in H9c2 cardiac cells

H9c2 cells treated with either 100 μM MS or 500 μM NAP did not show any increase in the level of oxidized proteins (Fig. S5A). However a significant increase in the oxidized proteins levels was observed in cells treated with 200 μM MS (Fig. S5A). The increased NSAID induced oxidative stress observed in cardiac cells at higher MS levels is likely to be prevented at lower levels due to increased expression of oxidative stress related enzymes. This possibility was investigated by using RT-PCR to measure the expression of heme oxygenase 1 (HO1), Glutathione S-Transferase Omega (GSTO), catalase 1 (CAT1) and thioredoxin reductase (TRXR1) in H9c2 cells exposed to either 100 μM MS or 500 μM NAP (Fig. S5B). HO1, GSTO and CAT1 were all upregulated, while TRXR1 was not affected in MS treated cells relative to control cells. NAP did not affect the expression of the four genes investigated.

3.17. Inhibition of cyclooxygenase activity in response to antioxidants

It is well documented that the main mechanism through which NSAIDs exert their anti-inflammatory and analgesic effects is through the inhibition of the enzyme cyclooxygenase (COX) [66]. Cell death induced by MS (due to ROS generation) could be reduced or prevented with antioxidants like ascorbic acid and Tempol. However, it is unclear what effects these antioxidants will have on COX activity. Interference with the anti-inflammatory and analgesic properties of NSAID by antioxidants would reduce the effectiveness of the NSAIDs. The COX activity of purified ovine COX-1 was inhibited ~90% by 200 μM MS (Fig. S10). MS is an inhibitor of both COX-1 and COX-2. Interestingly, different concentrations of the antioxidants alone reduced COX-1 activity by ~50%. Pre-incubation of the purified enzyme with varying concentrations of antioxidants did not seem to alter the inhibitory effect of MS on COX activity (Fig. S10). These results suggest that antioxidants would not affect the analgesic and anti-inflammatory properties but would reduce NSAID induced cell death in cardiac cells.
4. Discussion

Although increased occurrences of CVDs have been reported in NSAID users [9], the mechanism(s) associated with the adverse effect of NSAIDs on the cardiovascular system has not been reported. As a first step towards understanding the role of NSAIDs in the cardiovascular system we conducted an extensive investigation to determine if MS and NAP affect protein homeostasis and cardiotoxicity in cardiac cells directly. Our experiments demonstrate that NSAIDs are potent inducers of ROS generation in cardiac cells. Cardiac toxicity to various drugs as well as ROS induced myocardial damage have been long studied in neonatal cardiomyocytes [39]. A significant amount of ROS generation by MS and NAP was evident not only in the H9c2 cell line but also in the primary neonatal cardiomyocytes. Although both MS and NAP increased ROS production, MS was a more potent inducer of ROS in cardiac cells compared to NAP.

The heart has been shown to be one of the most susceptible organs to the adverse effects of NSAIDs compared to other tissues of the body [67]. This may be due to NSAIDs inducing greater ROS generation in cardiac cells than in other cell types. MS induced ROS generation was substantially greater in cardiac cells compared to kidney cells (CV1), skin fibroblasts or mouse embryo cells (CH3/10T/1/2). Apart from MS and NAP increased ROS production, MS was a more potent inducer of ROS in cardiac cells compared to NAP.

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Cell death by apoptosis has been shown to be induced by elevated levels of ROS [68]. Our results suggest that exposure of cardiac cells to MS for 24 h significantly increased cell death. Since caspase-3 activity was not increased in cells with pharmacological levels of MS it is unlikely that apoptosis is involved in MS induced cell death in cardiac cells. However, cell death caused by MS treatment was prevented by the antioxidants Tempol and ascorbic acid, suggesting that ROS is the major contributing factor for NSAID induced cardiac cell death. Exposure of cardiac cells to NAP (500 μM) did not affect cell viability.

The protein expression levels of two proteins, NAG-1 (nonsteroidal anti-inflammatory activated gene 1), which induces apoptosis in certain cell types [69,70], and the stress related protein HSP60, which is upregulated under conditions of glucose deprivation, exposure to ROS, and tissue injury [71], were not affected by NSAIDs in cardiac cells. The NAG-1 results are consistent with previous reports that suggest NAG-1 is not upregulated by all NSAIDs or in every cell type [72,73] and suggest that cardiac cells have different physiological changes when exposed to NSAIDs compared to other cell types.

Mitochondria are the major intracellular producers of ROS, and mitochondria themselves are the primary organelles in the cellular system to be impaired by ROS generation [57]. C-I and C-III of the mitochondrial electron transport chain are the major sites for the generation of ROS [74]. Indomethacin, naproxen, aspirin and piroxicam caused mitochondrial dysfunction by the inhibition of C-I and CII + C-III in rat liver [75]. In rat hepatic cells, diclofenac increased mitochondrial transition permeability leading to mitochondrial swelling and membrane depolarization [76]. The direct effect of NSAIDs on cardiac mitochondria has not been previously investigated. MS (10 μM and 30 μM) was a potent inhibitor of C-III activity, while higher concentrations of MS (100 μM) decreased C-I activity in isolated heart mitochondria. These results suggest that MS induced ROS generation in cardiac cells may be due to the impairment of C-III activity and to a lesser extent the C-I activity of the mitochondrial electron transport chain. NAP impaired C-I activity but not...
C-III activity suggesting that NAP may cause mitochondrial ROS generation mainly through impairment of C-I. The observation that MS is a more potent longer-term inducer of ROS formation compared to NAP may be due to MS acting on two different steps of the electron transport chain (C-I and C-III) while NAP acts only on C-I.

Although, mitochondrial C-II has been recently associated with ROS generation [77], no effect of MS or NAP on C-II activity was detected at the concentrations utilized. Aside from mitochondria, other non-mitochondrial sources like NADPH oxidase, xanthine oxidase, lipoxygenase, and nitric oxide synthases may also contribute to ROS generation [78]. It remains to be determined if any of these non-mitochondrial sources of ROS are affected by NSAIDs, but it is unlikely that they are the major contributors to ROS generation since mito-Tempol was able to prevent proteasome dysfunction (Fig. S3B), suggesting that the mitochondria produces most of the ROS related to proteasome dysfunction.

MS induced ROS generation was associated with significant decreases in cell viability of neonatal cardiomyocytes as well as H9c2 cells, while naproxen did not cause any cell death after 48 h treatment. Analysis of ROS formation using microplate analysis suggested higher levels of ROS formation using 10 μM MS relative to 100 μM NAP, but similar levels of ROS were detected using 100 μM MS relative to 500 μM NAP. However fluorescence microscopy results suggest that MS was even a more potent inducer of ROS compared to NAP than the microplate analysis suggested. Relative ROS levels detected using fluorescence microscopy suggest 50 μM MS produced comparable amounts of ROS to that produced by 1 mM NAP, and at higher concentrations, MS (100 μM) produced significantly greater amounts of ROS compared to NAP (1 mM).

The reduced cell viability in MS treated cells relative to NAP treated cells may be due to the lower levels of intact 26S proteasomes in MS treated cells. It is also possible that the higher ROS levels and/or the prolonged relatively high levels of ROS production in MS treated cells (higher rate of ROS produced by MS relative to NAP) may be associated with the lower cell viability. These results may explain the more severe adverse effects of MS on proteasome function as well as the more severe adverse effects of MS on mitochondrial function.

The mitochondrial membrane potential is an important indicator of mitochondrial health and the general health of the cell. The synthesis of ATP by ATP synthase (Complex V) in the mitochondria is driven by ΔΨ_m and any disruption in ΔΨ_m could lead to cell death due to apoptosis or necrosis [79]. In the present study, MS not only decreased mitochondrial

![Figure 8](image-url)

**Fig. 8.** Effects of purified 26S and 20S proteasome transfection on H9c2 cell viability and 26S proteasome activity in the presence of MS. To investigate the effect of additional proteasomes on cell death induced by the NSAID, vehicle treated and MS treated H9c2 cells were transfected with different concentrations of 26S proteasome using Saint PhD transfection reagent in serum free media for 4 h at 37 °C. This was followed by an overnight incubation in normal media at 37 °C after which a cell viability assay was performed using Alamar Blue. Bort, Bortezomib (highly specific proteasome inhibitor). A. 26S β5 proteasome activity was determined in H9c2 cells after transfection with purified 26S proteasome. B. Cell Viability of MS treated cells after 26S proteasome transfection. C. Cell viability of cardiac cells transfected with purified 20S proteasome in the presence of MS. D. Immunocytochemistry of H9c2 cells transfected with 26S purified proteasomes. H9c2 cells were transfected with purified 26S proteasomes as described in Section 2.14. After transfection, cells were fixed, permeabilized, and probed with β1 antibody. The β1 antibody was detected using the fluorescently labeled Dylight 549-conjugated goat anti-rabbit antibody. DAPI (blue), was used to stain the nuclei of the cells. Each image is representative of 3 or more independent experiments. a: control cells; b: 0.05 μg 26S proteasome; c: 0.1 μg 26S proteasome. Scale bar (white) represents 20 μm. The values are mean ± SD (n = 3) (*) represents P < 0.05; ** represents P < 0.001.)
C-I and C-III activities, but also impaired cardiact $\Delta V_{m}$, NAP however did not alter $\Delta V_{m}$. These results suggest that the NAP induced generation of ROS may have been counteracted by the cell mitochondrial antioxidant defense mechanism, while the higher rate of ROS production in MS treated cells may be greater than cardiac cells are able to counteract, and therefore the effects of NAP was less severe compared to MS. Consistent with this possibility was the observation that several antioxidant enzymes in cardiac cells were upregulated in MS treated cells but not NAP treated cells.

Various studies have demonstrated the modification of proteasome subunits by phosphorylation, glycosylation, and ubiquitination, but the physiological roles of most of these post translational modifications on proteasome functions have not yet been determined [80,81]. Whether the inhibitory effect of MS on the proteasomes is due to a modification(s) such as phosphorylation or other post-translation processes of proteasomes remain unclear. High levels of ROS could potentially oxidize proteasomes, and oxidized proteasomes have been previously shown to have reduced proteasome activity [82]. Consistent with this, purified proteasomes from MS treated cells show higher levels of oxidized subunits and lower proteasome activity compared to proteasomes purified from control treated cells. The decrease in proteasome activity was not due to changes in protein expression levels of proteasome subunits since the levels of the 20S catalytic core subunit (PSMA6/α1) and the 19S regulatory subunit (Rpt 1) were unaffected by MS. The gene expression of several proteasome subunits (PSMB5 (β5), PSMB6 (β1), and PSMB8 (β5i)) were also not significantly altered by MS, but PSMB8 levels showed a trend towards increasing after 24 h of MS treatment. These results suggest that the ROS induced by MS may result in increased oxidation of 19S subunits resulting in lower proteasome activity.

An important finding was that addition of proteasome to MS treated cells improved cell viability. Transfection of either 26S or 20S purified proteasomes into H9c2 cells pretreated with MS significantly restored cell viability. This may be due to the ability of proteasomes to degrade oxidized proteins, reducing oxidative stress. Since addition of proteasome to bortezomib-treated cells did not restore cell viability while addition of proteasome to MS treated cells improved cell viability, this suggests that the decrease in cell viability may be due to MS-induced ROS generation in the cardiac cells. Improvement in H9c2 cell viability was not observed in the presence of bortezomib because transfection of new proteasomes into the cell system leads to the binding of the free bortezomib to the transfected proteasomes, making them inactive. On the other hand, most ROS generation in cardiac cells by MS is transient and occurs for a limited period of time (3–5 h) and therefore the addition of new proteasomes is able to reverse cell death induced by the NSAID.

It is possible that the proteasome inhibitory effect of MS may not be important for the initial cardiomyocyte toxicity. However, the transfections of purified proteasomes in cardiac cells improved cell viability after treatment by MS, suggesting that proteasomes are important in MS induced cardiotoxicity. Recent studies have reported the ability of lysosomes to degrade polyubiquitinated proteins by switching from the ubiquitin pathway to the lysosomal pathway. At 100 or 200 μM MS, it may be possible that the lysosomal pathway partly degrades the tagged proteins which otherwise would have been broken down by the proteasomes [83]. If lysosomal degradation of ubiquitinated proteins occurs then little or no accumulation of polyubiquitinated proteins would occur in the cell. It may be that at higher concentrations of MS, both the pathways are significantly impaired leading to significant accumulation of polyubiquitinated proteins.

Impaired proteasome function has been linked to mitochondrial dysfunction in different biological systems like Caenorhabditis elegans, yeast cells and mammalian cells [84]. ROS induction in respiration-deficient yeast mutants (Δsoi) caused proteasome dysfunction due to increased dissociation of 20S core particle from the 19S regulatory subunit [84]. Similar results were observed in yeast cells and mammalian cells treated with either Antimycin A or H2O2 [84]. We observed increased dissociation of the 20S core particle from the 19S regulatory subunit, which will alter proteasome function. Significant cross talk between mitochondrial function and proteasome function has been described, where the malfunctioning of either one of the systems can negatively affect the function of the other [85]. In the present study Antimycin A increased ROS generation and cell death, and impaired proteasome function in cardiac cells, suggesting that decreased proteasome activity may be associated with increased mitochondrial ROS generation in H9c2 cells. Since β5 proteasome activity was significantly inhibited by MS in both the neonatal and H9c2 cardiac cells and antioxidants could prevent this, these results suggest a possible association between MS-induced mitochondrial dysfunction leading to excessive ROS generation and subsequently causing proteasome impairment. Antioxidants were able to counteract the effect of MS on the induction of cell death and proteasome dysfunction, possibly by inhibiting mitochondrial ROS generation in the cardiac cells. Interestingly, the extent of the inhibitory effect of MS on proteasomes was more predominant in heart tissue relative to either brain or liver suggesting that heart proteasomes are more susceptible to MS than other tissues.

Previous reports suggest that compounds with antioxidant properties reduce pain through the inhibition of COX-1 and 2 [86]. Since NSAIDs exert their analgesic effect through COX inhibition, the role of antioxidants on COX activity was determined. Tempol and ascorbic acid inhibited COX-1 activity (~50%) [Fig. S10], while MS inhibited COX-1 activity by about 90%. Therefore it may be possible that antioxidants could reduce the side effect of NSAIDs (ROS production and cell death) without interfering with their analgesic properties.

5. Conclusion

In an effort to determine if NSAIDs directly affect cardiomyocytes and if so then by what mechanisms, it was found that MS inhibited mitochondrial complexes, induced ROS, increased the oxidation of proteasome subcomplexes, decreased proteasome activity, and increased the amount of 20S proteasome relative to 26S, leading to a reduction in cell viability. NAP on the other hand inhibited mitochondrial C-I and induced ROS but had less effect on the mitochondria and did not affect proteasome activity at pharmacological levels. These results suggest that MS would have a significantly more deleterious effect on the heart than NAP, consistent with the clinical data which suggest that MS increases the risk of CVD while NAP does not.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yjmcc.2016.03.016.

References


