

The anti-psoriatic drug monomethylfumarate increases nuclear factor erythroid 2-related factor 2 (Nrf2) levels and induces aquaporin-3 (AQP3) mRNA and protein expression

Inas Helwa, Vivek Choudhary, Xunsheng Chen, Ismail Kaddour-Djebbar and Wendy B. Bollag

Charlie Norwood VA Medical Center, Augusta, GA 30904 (VC, XC, IK-D, WBB)

Department of Oral Biology, Augusta University, Augusta, GA 30912 (IH, WBB)

Department of Physiology, Medical College of Georgia, Augusta University, Augusta, GA 30912 (VC, XC, IK-D, WBB)

Department of Medicine (Dermatology), Medical College of Georgia, Augusta University, Augusta, GA 30912 (WBB)

The authors have no conflict of interest to declare.

Address correspondence to:

Wendy B. Bollag, Augusta University, Department of Physiology, 1120 15th Street, Augusta, GA
30912

TEL: (706) 721-0698

FAX: (706) 721-7299

E-mail: wbollag@augusta.edu

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Abbreviations: AQP3, aquaporin-3; EGFR, epidermal growth factor receptor; DCFH-DA, 2', 7'-dichlorodihydrofluorescein diacetate; DMF, dimethylfumarate; DPBS, Dulbecco's phosphate-buffered saline; FAE, fumaric acid esters; GCLC, glutamate-cysteine ligase catalytic subunit, the first rate-limiting enzyme of glutathione synthesis; GSTA, glutathione S-transferase alpha1; HO-1, heme oxygenase-1; KEAP-1, Kelch-like ECH associated protein-1; MMF, monomethylfumarate; NQO1, NAD(P)H quinone dehydrogenase 1; Nrf2, nuclear factor erythroid-derived 2-like 2; PBS, phosphate-buffered saline lacking divalent cations; Prdx6, peroxiredoxin-6; RFU, relative fluorescent units; ROS, reactive oxygen species; SFN, sulforaphane; TBS, Tris-buffered saline

Abstract:

Oxidative stress contributes to inflammatory skin diseases including psoriasis. Monomethylfumarate (MMF) is an anti-psoriatic agent with a poorly understood mechanism of action. In other cell types MMF increases the expression of nuclear factor erythroid-derived 2-like 2 (Nrf2), a transcription factor that regulates cellular anti-oxidant responses, to reduce oxidative stress like that observed in inflammatory disorders such as multiple sclerosis. We tested the hypothesis that MMF enhances Nrf2 activity in keratinocytes, thereby improving their capacity to counteract environmental stresses. We used western analysis, immunofluorescence and qRT-PCR to examine the effect of MMF on the expression of Nrf2 and its targets. We also measured intracellular reactive oxygen species (ROS) levels following MMF treatment. Our data show that MMF increased total and nuclear Nrf2 levels in primary mouse keratinocytes and enhanced mRNA expression of several Nrf2-downstream effectors, including heme oxygenase-1 (HO-1) and peroxiredoxin-6 (Prdx6). Moreover, MMF treatment attenuated the generation of ROS following hydrogen peroxide treatment. On the other hand, the expression and membranous localization of aquaporin-3 (AQP3), a glycerol channel implicated in keratinocyte differentiation, was stimulated by MMF, which also enhanced keratinocyte glycerol uptake. The Nrf2 activator sulforaphane also increased AQP3 levels, suggesting that AQP3 expression may be regulated by Nrf2. We show for the first time that MMF stimulates Nrf2 and AQP3 expression and function/activity in keratinocytes. This effect may account, in part, for the previously observed ability of MMF to inhibit proliferation and inflammatory mediator production and promote differentiation in keratinocytes and to treat psoriasis.

Introduction:

In 1959, the German chemist Schreckendiek successfully treated his own psoriatic lesions with fumaric acid esters (FAE) (Schreckendiek, 1959). Since then, FAE have been known as anti-psoriatic agents despite their unclear mechanism of action. Indeed, Fumaderm[®], a mixture of dimethylfumarate (DMF) and three salts of monoethylfumarate, was licensed in Germany in 1994 for the treatment of psoriasis. Although DMF is the main ingredient of the drug, monomethylfumarate (MMF) is thought to be the bioactive component since FAE are almost completely absorbed in the small intestine where DMF is rapidly hydrolyzed by esterases and converted to MMF (Mrowietz et al., 1999). Fumaderm[®] is not licensed for use to treat psoriasis in many other countries worldwide including the United States (Rostami-Yazdi et al., 2010), perhaps in part because of the unknown mechanism of action. Nevertheless, FAE have gained interest as therapeutic agents in the United States, especially with increased multicenter studies revealing the drug's efficacy and limited safety concerns (Altmeyer et al., 1994; Hodges et al., 2012). MMF has known effects on several cell types including immune cells, retinal pigment epithelial cells and endothelial cells (Ananth et al., 2013; Bozard et al., 2012; Dehmel et al., 2014; Garcia-Caballero et al., 2011; Litjens et al., 2004; Promsote et al., 2014). In contrast, little is known about the effects and mechanism of action of MMF in the epidermis or keratinocytes, despite the crucial role that keratinocytes play in the pathogenesis of psoriasis (Tschachler, 2007).

One of the major difficulties hindering the development of more efficacious and safer anti-psoriatic drugs is the unclear etiology of the disease. Oxidative stress is one of the proposed causative factors for psoriasis, since the skin is constantly exposed to stimuli that generate oxidative insults (Zhou et al., 2009). Nuclear factor, erythroid 2 like 2 or NFE2L2 (Nrf2) is a key transcription factor that regulates several genes encoding reactive oxygen species (ROS)-detoxifying proteins and regulatory enzymes. It has been previously reported that MMF mediates the induction/stabilization of Nrf2 in retinal pigment epithelial cells (Ananth et al., 2013;

Promsote et al., 2014). Moreover, in 2013 the Food and Drug Administration approved a drug (BG-12) with FAE bioactive ingredients as a first-line therapy for adults with relapsing multiple sclerosis. BG-12 is now available in the United States under the trade name Tecfidera® with DMF as its major ingredient (similar to Fumaderm®). One of the major neuroprotective mechanisms of Tecfidera® is thought to be via the induction of Nrf2-dependent pathways (Gold et al., 2012).

Aquaporin-3 (AQP3), an aquaglyceroporin expressed in the epidermis of mammalian skin, efficiently transports glycerol (Hara-Chikuma and Verkman, 2005). Data from our laboratory have suggested a role of AQP3 in keratinocyte differentiation (Bollag et al., 2007; Choudhary et al., 2015). Other investigators have also obtained evidence supporting a role for AQP3 in this process. For example, AQP3 expression/levels can be increased by differentiating agents such as nuclear hormone receptors (Jiang et al., 2011), and siRNA-mediated knockdown of AQP3 in human keratinocytes results in decreased keratin-10 up-regulation in response to differentiation induced by an elevated extracellular calcium concentration (Kim and Lee, 2010). Moreover, AQP3 appears to be dysregulated in psoriasis (Lee et al., 2012; Voss et al., 2011). In addition, retinoic acid, derivatives of which are known anti-psoriatic agents, increases AQP3 gene and protein expression as well as glycerol uptake in *ex vivo* human skin samples and normal human keratinocytes collected from healthy volunteers *in vitro* (Bellemere et al., 2008).

We previously showed that MMF, the active ingredient in Fumaderm® and BG-12, inhibits proliferation and inflammatory cytokine production and stimulates differentiation in keratinocytes (Helwa et al., 2015). In these prior experiments we selected concentrations of 300µM to 1mM, based on studies in other tissues, such as retinal pigment epithelial cells (Ananth et al., 2013; Promsote et al., 2014). Nevertheless, there is a question as to whether such serum concentrations are achieved upon treatment of patients with Fumaderm®. After a single dose of 360mg of fumaric acid esters in patients, the maximum serum concentration reached is reported to be 2.74µg/mL or about 21µM with a coefficient of variance of 33-67%

(http://www.accessdata.fda.gov/drugsatfda_docs/nda/2013/204063Orig1s000ClinPharmR.pdf).

However, doses of Fumaderm® of 1-2 g/day may be required to control psoriasis (Roll et al., 2007), or an amount roughly 5-6 times the 360 mg dose. Since the maximum concentration is linearly related to dose

(<http://www.pharmacytimes.com/publications/issue/2013/June2013/Biogen-Idec-Tecfidera>) and

if a similar coefficient of variance is assumed for the higher doses, the serum concentration of MMF may approach 200µM in certain individuals. Nevertheless, it should be noted that psoriasis requires an extended treatment period, suggesting that the effects of drugs used for its treatment might accumulate over time (even if the serum drug concentration remains steady). Since *in vitro* cells are exposed to treatment over a short time only, a concentration of 200-1000µM to treat mouse keratinocytes in culture does not seem unreasonable. In the current study, we explored the mechanism of action underlying the effects of MMF in this concentration range in mouse keratinocytes. Our results suggest that Nrf2, which may also regulate AQP3 expression, could mediate the effects of MMF on keratinocytes and skin.

Materials and Methods:

Materials:

Commercial keratinocyte serum-free medium (K-SFM) and the appropriate supplements were obtained from Gibco® Invitrogen (Grand Island, NY). MMF, L-sulforaphane (SFN) and primary monoclonal mouse anti- β -actin antibody were from Sigma-Aldrich (St. Louis, MO). Total RNA was reverse transcribed to cDNA using iScript Reverse Transcription Supermix from Bio-Rad Life Sciences (Hercules, CA), and RNA purification kits were from 5 prime (Gaithersburg, MD). AQP3 Taqman probes were from Life Technologies (Grand Island, NY). Primary rabbit anti-Nrf2 antibody was from Santa Cruz Biotechnology, Inc. (Dallas, TX), Novus Biologicals (Littleton, CO) or Abcam (Cambridge, MA). Primary rabbit anti-aquaporin 3 was obtained from LSBio (Seattle, WA). Secondary antibodies used for western blot analysis were: AlexaFluor IRDye 800-conjugated goat anti-rabbit IgG and IRDye 680-conjugated goat anti-mouse IgG secondary antibody from LI-COR (Lincoln, NE). [3 H]Glycerol was from American Radiolabelled Chemicals (St. Louis, MO). Qproteome plasma membrane isolation kit was from Qiagen (Germantown MD).

Methods:

Culture of primary mouse keratinocytes:

Primary murine epidermal keratinocytes were prepared from 1-3 day-old neonatal mice as described in our previous publications (Arun, et al. 2013; Choudhary et al. 2015; Griner, et al. 1999; Helwa et al. 2015). Briefly, the keratinocytes were obtained by dissecting intact sheets of skins followed by an overnight incubation in 0.25% trypsin at 4°C. The epidermis and dermis were then mechanically separated using forceps, and keratinocytes were gently scraped from the underside of the epidermis. Keratinocytes were then collected by centrifugation and plated in Corning tissue culture plates (Corning, NY) in plating medium as previously described (Dodd,

et al. 2005). Cells were incubated overnight in 95% air/5% CO₂ at 37°C at which time the medium was replaced with commercially available K-SFM.

Sample preparation for western blot analysis:

Near-confluent cultures of primary mouse keratinocytes were treated with K-SFM with or without the indicated concentrations of MMF for 24 hours. At the end of the desired time period, cells were harvested by scraping in 0.1875 mM Tris-HCl (pH 8.5), 3% SDS and 1.5 mM EGTA. Protein concentrations were determined using a Bio-Rad protein assay with bovine serum albumin (BSA) as the standard. Sample buffer (30% glycerol, 15% beta-mercaptoethanol and 1% bromophenol blue) was then added to constitute Laemmli buffer (Laemmli 1970). Samples were heated (85-90°C) for 3-5 minutes and stored at -20°C.

Plasma membrane fractionation:

In order to extract plasma membrane from keratinocytes, we used a Qproteome™ plasma membrane protein fractionation kit (Qiagen, Germantown, MD). Near-confluent keratinocytes were treated with 0 and 750μM MMF for 24 hours after which membrane fractionation was performed according to the manufacturer's instructions. Briefly, cells plated in 6 well plates were collected using cell scrapers followed by centrifugation and a single wash with ice-cold phosphate-buffered saline lacking divalent cations (PBS). The pellet was resuspended using 2 mL of the supplied lysis buffer PM without protease inhibitors and the application of gentle pipetting to homogenize the pellet. This step was followed by centrifugation for 5 minutes, discard of the supernatant and resuspension for a second time with 2 mL of the supplied lysis buffer. At the end of two cycles of centrifugation, the pellet was resuspended in 500 μL of lysis buffer PM with protease inhibitors and the cell suspensions were transferred to new microcentrifuge tubes and incubated for 15 minutes at 4°C with brief vortexing every 5 minutes. The supplied lysis solution PL (2.5 μL) was then added to the cell suspension and incubated for

an additional 5 minutes at 4°C. Cell disruption was completed using a syringe and needle with at least 15 strokes. The disrupted cell lysate was then centrifuged at 12,000x g at 4°C for 20 minutes. At this point and according to the manufacturer, the isolated membrane is concentrated in the supernatant, whereas the pellet can be discarded as it mainly contains intact cells, cell debris and major cellular organelles. However, due to the nature of AQP3 and its localization in detergent-resistant lipid rafts in keratinocytes (Zheng and Bollag 2003), we were concerned that the AQP3-containing rafts might not be completely solubilized in the supplied lysis buffer/solution. Therefore, Laemmli buffer (Laemmli 1970) was added to the precipitated pellet and corresponding protein samples and stored immediately at -20°C for later western analysis. Using the isolated supernatant, reconstituted binding ligand (20 µL per sample) was added and samples were incubated with gentle agitation on an end-to-end shaker at 4°C. After 1 hour, a mixture of equilibrated magnetic beads in the supplied lysis buffer was added and incubation continued for 1 hour with gentle agitation on an end-to end shaker followed by placement on a magnetic separator for 1 minute, and then the supernatant was discarded. The previous step was performed twice. After two additional steps of magnetic separation and subsequent washing with the supplied wash buffer, the extracted proteins were eluted using the supplied elution buffer and transferred into fresh tubes. The elution step was repeated 3 times, combining all four eluates in a single tube. A volume of ice-cold acetone equal to four times the eluted volume was used to precipitate the protein fraction. The protein pellet was then resuspended in Laemmli buffer (150 µL) (Laemmli 1970), boiled (5 minutes) and stored (-20°C) until analysis by western blotting.

Western analysis:

Equal amounts of protein were loaded onto graded 4-15% commercially available Mini-PROTEAN® TGX gels (Bio Rad, Hercules, CA), separated by electrophoresis and transferred onto Immobilon-P membranes, which were blocked in Odyssey blocking buffer diluted 1:1 in

Tris-buffered saline (TBS) for 1 hour at room temperature. Membranes were incubated with primary antibodies diluted in Odyssey blocking buffer (1:1 in TBS) at a concentration of 1:10,000 for anti- β -actin, 1:400 for anti-AQP3 and 1:500 for anti-Nrf2 overnight, followed by washing and incubation with secondary Alexa Fluor-conjugated secondary antibodies diluted in Odyssey blocking buffer (1:1 in TBS) at a concentration of 1:10,000 for 1 hour at room temperature. Membranes were then washed, and immunoreactive bands were visualized via an Odyssey[®]SA infrared imaging system from LI-COR and quantified using Odyssey[®]SA internal software according to the manufacturer's instructions. Data were normalized to actin levels and the quantified bands from multiple experiments were compared to the control untreated bands within each experiment.

Glycerol uptake assay:

Near-confluent primary mouse keratinocytes were treated with 0 and 750 μ M MMF in K-SFM for 24 hours at 37°C. After 24 hours, the medium was aspirated, and the cells were incubated at 37°C with 1 μ Ci/mL [³H]glycerol in K-SFM containing 20 mM HEPES for 5 minutes. Extensive washing with ice-cold PBS terminated the reactions and cells were solubilized in 0.3 M NaOH, and aliquots were counted by liquid scintillation spectrometry (Beckman Coulter, Inc. Brea, CA), with values normalized to the protein content in each sample.

Quantitative real-time RT- PCR:

Quantitative real-time PCR amplifications were performed using Taqman probes (**Table 1**) and an ABI Step-One Plus Fast Real-Time PCR system (Applied Biosystems, Life Technologies, Grand Island, NY) using 100ng of cDNA per sample according to the reaction parameters recommended by the manufacturer. All primers for the amplification of target sequences were purchased from Applied Biosystems. For all samples a total volume of 20 μ L per well in a 96-well plate was used for gene detection and consisted of 10 μ L 2XFast Reagent Master Mix, 1 μ L

of probe and 9 μ L of cDNA. GAPDH was used as an endogenous control gene and negative controls contained water instead of cDNA to ensure purity of all reagents. Relative gene expression was calculated by the delta-delta Ct ($\Delta\Delta$ Ct) method and basal values were used for normalization. The results were expressed as the fold difference in gene expression relative to the endogenous gene and untreated samples; the Ct values for the sample and basal treatments were determined by subtracting the average Ct value of the transcript of interest from the average Ct values of the endogenous gene for each sample.

Immunofluorescence:

Near-confluent primary mouse keratinocytes plated on coverslips were treated with various concentrations of MMF for 16 hours (in the case of Nrf2) or 24 hours (for AQP3). Cells were then washed with PBS and fixed with 4% paraformaldehyde followed by washing and permeabilization of cells using 0.2% Triton X-100 for 10 minutes. Goat serum (15%) was used for 1-hour-blocking at room temperature, and then slides were incubated with rabbit anti-Nrf2 antibody or with rabbit anti-AQP3 antibody (1:100 in 15% goat serum) at room temperature for 1-2 hours. After washing with PBS, cells were incubated with Cy3-conjugated goat anti-rabbit secondary antibody (Invitrogen, Eugene, OR) (1:400 in 15% goat serum) at room temperature for 45 minutes. Cover slips were then washed with PBS and inverted on slides using Prolong Anti-Fade reagent (Life Technologies, Grand Island, NY) and stored overnight to dry before imaging using a confocal microscope (Carl Zeiss Microscopy, Göttingen, Germany). Immunofluorescent images were quantified using ImageJ.

Intracellular reactive oxygen species assay:

An OxiSelect intracellular ROS assay kit from Cell Biolabs, Inc. (San Diego, CA) was used to monitor ROS levels. This kit is a cell-based assay for measuring ROS activity using the cell-permeable fluorogenic probe 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA). The assay

was performed according to the manufacturer's instructions. Briefly, primary mouse keratinocytes were seeded in black 96-well plates and cultured until near-confluence. Cells were then pre-treated with different concentrations of MMF for 24 hours. Cells were gently washed 3 times with Dulbecco's phosphate-buffered saline (DPBS) after which 100 μ L of 1X DCFH-DA in K-SFM were added and the cells incubated for 1 hour at 37°C. Cells were washed again 3 times with DPBS and then treated with or without 200 μ M H₂O₂ (in the continued presence of the appropriate concentrations of MMF). Relative fluorescence units (RFU) were measured for 1 hour at 10-minute intervals using a plate reader with 480 nm excitation and 530 nm emission. The RFU values were calculated for each group at all time points and compared to all other groups. Note that the fluorescence intensity is proportional to the ROS levels within the cell cytosol.

Statistical analysis:

All experiments were repeated a minimum of three times in duplicate. The values were statistically analyzed by one way-ANOVA, with a Newmann-Keuls post-hoc test, or one sample T-test using Prism software (Graph Pad Software Inc., San Diego, CA), with statistical significance assigned at $p < 0.05$. All quantified data are expressed in the form of bar graphs with values representing means \pm standard error of the mean (SEM).

Results:

MMF increased Nrf2 levels and induces the expression of Nrf2 target genes:

Our previous data have already shown that MMF has anti-proliferative, pro-differentiative and anti-inflammatory effects in mouse keratinocytes (Helwa et al. 2015). We explored the possible mechanisms underlying these effects. Since Nrf2 has recently been mentioned as an important regulator of the oxidative response as well as of differentiation of keratinocytes (Beyer, et al. 2007; Lee, et al. 2014), we determined whether MMF increased Nrf2 levels using a 200 μ M concentration of MMF that has been used in several other systems (Garcia-Caballero et al. 2011; Nibbering, et al. 1993; Thio, et al. 1994), as well as a 300 μ M dose that we have previously shown to inhibit inflammatory mediator production in keratinocytes (Helwa et al. 2015). As shown in Figure 1A, both MMF concentrations increased Nrf2 protein expression. We then determined whether the lower 200 μ M dose of MMF activated Nrf2 in keratinocytes by examining the expression of several target genes of Nrf2. We found that treatment with this low dose of MMF significantly increased the expression of various Nrf2 target genes: heme oxygenase-1 (HO-1), peroxiredoxin 6 (Prdx6), glutamate-cysteine ligase catalytic subunit (GCLC), glutathione S-transferase- α 1 (GSTA) and NAD(P)H quinone dehydrogenase-1 (NQO1) (Figure 1B). However, we noted that the induction of HO1 and Prdx6, although significant, was rather low and were concerned that detection of other changes related to Nrf2 activation might be difficult, particularly with methods less sensitive than qRT-PCR. Based on this consideration, as well as the fact that higher concentrations have been used in several other cell systems (Ananth et al. 2013; Nibbering et al. 1993; Promsote et al. 2014; Thio et al. 1994), we determined the effect of higher concentrations of MMF on Nrf2 levels, as well as HO1 and Prdx6 expression. Consistent with previous reports in other cell types (Ananth et al. 2013; Promsote et al. 2014; Scannevin, et al. 2012), these higher MMF concentrations also increased the protein expression of Nrf2 in keratinocytes (total cell lysates) (Figure 2A). MMF treatment also significantly increased mRNA levels of both the HO1 and Prdx6 target genes, with maximal

expression observed at a 1mM concentration (Figure 2B and C). Based on the greater increase in target gene expression, for subsequent experiments we decided to use the higher doses of MMF to maximize the effect size.

MMF enhances Nrf2 nuclear localization and exerts a cytoprotective effect against oxidative stress:

To confirm that the increase in the total Nrf2 cellular content elicits an increase in transcriptionally active Nrf2 (i.e., Nrf2 located in the nucleus), we stained control and MMF-treated keratinocytes with anti-Nrf2 antibody and Cy3-conjugated goat anti-rabbit secondary antibody (Figure 3A). We performed immunofluorescence analysis using ImageJ software for both total and nuclear Nrf2 levels (Figure 3B and C, respectively). Consistent with our western results, immunofluorescence analysis showed a significant increase in total Nrf2 levels following a 16-hour MMF treatment (Figure 3B). Moreover, a dose-dependent increase in nuclear Nrf2 levels was also observed, suggesting that MMF treatment induced active nuclear Nrf2. Indeed, nuclear Nrf2 levels were significantly greater than the control untreated cells at all MMF concentrations tested (Figure 3C). The ability of MMF to increase the expression of downstream effectors of Nrf2 (Figures 1 and 2) is also consistent with the idea that the induced Nrf2 is active (Beyer et al. 2007; Chowdhury, et al. 2009).

Next, we wanted to determine the ability of this increase in anti-oxidant enzymes to modulate oxidative stress. Primary mouse keratinocytes were pre-treated with different concentrations of MMF for 24 hours. Pretreated cells were then exposed to hydrogen peroxide, to generate reactive oxygen species (ROS) (Wagener, et al. 2013), using a concentration (200µM) that is not cytotoxic to keratinocytes (Loo, et al. 2011), and ROS generation was monitored for 90 minutes at 10-minute intervals using the cell-permeable fluorogenic probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). Pretreatment with all concentrations of MMF produced a cytoprotective effect against oxidative stress, with ROS levels that were significantly

lower in MMF-treated cells than in keratinocytes exposed to hydrogen peroxide alone (Figure 4). These data may explain, in part, the anti-inflammatory effects of MMF on keratinocytes and its utility as an anti-psoriatic agent, since oxidative stress is a known pro-inflammatory factor (Sandireddy, et al. 2014). In addition, these results are consistent with our previous finding that 300 μ M MMF was able to inhibit the induced expression of inflammatory mediators, such as tumor necrosis factor- α and interleukin (IL)-1 α (Helwa et al. 2015).

MMF increases AQP3 expression, membranous localization and glycerol uptake

Previously, we observed an ability of MMF not only to inhibit keratinocyte proliferation and inflammatory mediator production but also to promote differentiation (Helwa et al. 2015); our prior results have suggested a role for AQP3 in mediating keratinocyte differentiation (Bollag et al. 2007; Choudhary et al. 2015). Therefore, we examined the effect of MMF on AQP3 expression and activity. MMF at a concentration of 300 μ M increased AQP3 protein levels (Figure 5A). We further showed that higher concentrations of MMF also dose-dependently increased the mRNA and protein expression of AQP3 (Figure 5B and C). This stimulatory effect included a significant enhancement of unglycosylated AQP3 at a concentration of 750 μ M (Figure 5D). However, although the glycosylated band of AQP3 (presumably representing the active component of AQP3) showed an identical trend, the increase did not achieve statistical significance (Figure 5E).

Consequently, we performed immunofluorescence analysis to determine whether MMF stimulated plasma membrane localization of AQP3, an integral membrane protein. Indeed, immunofluorescence analysis demonstrated that the AQP3 induced by MMF was located in the plasma membrane at all tested concentrations (Figure 6A). To further support this hypothesis, we isolated plasma membrane proteins from keratinocytes treated with 750 μ M MMF, the concentration that showed the greatest potency in inducing AQP3 expression as well as activity in terms of glycerol transport (see below). There is evidence that AQP3 is localized in

keratinocyte lipid rafts (Zheng and Bollag 2003); since these membrane microdomains are known to be resistant to certain detergents, we were concerned that the lysis buffer used for plasma membrane isolation would not solubilize the AQP3 from these rafts. Thus, we isolated both the initial precipitated membrane pellet (containing intact cells, organelles and insoluble membranes) as well as the supernatant as explained in the Methods section. We prepared protein lysates from both fractions and probed for AQP3 protein. Our data showed that AQP3 was more concentrated in the isolated supernatant and that MMF treatment increases the expression of both glycosylated and non-glycosylated AQP3 in this fraction (Figure 6B). This result, together with, the immunofluorescence quantification suggests that MMF not only stimulated the expression of AQP3, but also increased its membrane localization, at which site it should show increased glycerol transport activity. To confirm this hypothesis, we performed a [³H]glycerol uptake assay to measure AQP3 transport activity. For this experiment, we selected the concentration (750μM) that stimulated a significant increase in AQP3 protein expression and induced obvious membrane localization by fractionation/western blotting and immunofluorescence analysis, respectively. Near-confluent keratinocytes were incubated for 24 hours with 0 and 750μM MMF before measurement of [³H]glycerol uptake. MMF treatment significantly increased glycerol uptake as compared to control untreated cells (Figure 6C).

The Nrf2-stimulator sulforaphane increases protein expression of AQP3 in keratinocytes

At this point, we had shown a stimulatory effect of MMF on Nrf2 and AQP3 levels and activities, which may explain, in part, how MMF stimulates keratinocyte differentiation and inhibits inflammation and proliferation (Helwa et al. 2015). The question still remained: does Nrf2 play a role in AQP3 up-regulation? To test this idea, we investigated the effect of sulforaphane (SFN), a known inducer of Nrf2 (Lee et al. 2014; Xu, et al. 2006), on AQP3 protein expression. We performed western blot analysis using primary mouse keratinocytes treated with various doses of SFN (1μM, 5μM and 10μM) for 24 hours. Our results showed that SFN

treatment dose-dependently stimulated the protein expression of both the unglycosylated and glycosylated forms of AQP3 (Figure 7A), with a significant increase in both forms at a concentration of 5 μ M (Figure 7B and C). *In silico* analysis of the murine AQP3 promoter region identified two putative Nrf2 consensus sites (Figure 8A), providing further evidence for a potential direct effect of Nrf2 on AQP3 expression. Taken together, our data suggest that MMF's anti-psoriatic effects may be mediated, in part, via stimulating the expression and activity of AQP3, a glycerol transport channel that plays a role in regulating keratinocyte proliferation and differentiation ((Bollag et al. 2007; Choudhary et al. 2015) and reviewed in (Qin, et al. 2011)). MMF also increases the expression/levels of Nrf2 and induces its activity, i.e., nuclear localization. The Nrf2 activator SFN, alone, can stimulate an increase in AQP3 levels as well. These results suggest that Nrf2 might be involved in the differentiation of keratinocytes via its induction of AQP3, as shown in the schematic in Figure 8B.

Discussion:

Although the actions of MMF in other cell types have been investigated extensively (Bozard et al. 2012; Litjens et al. 2004; Nibbering, et al. 1993; Promsote et al. 2014), this report is one of few that have explored the effects of the anti-psoriatic agent MMF in keratinocytes. We have recently reported that MMF has anti-proliferative, pro-differentiative and anti-inflammatory actions on keratinocytes (Helwa et al. 2015). In light of the aforementioned study, we investigated the possible mechanism by which MMF exerts its effects, examining the anti-oxidative stress regulator Nrf2 and the pro-differentiative mediator AQP3 (Choudhary et al. 2015). Nrf2 is a crucial modulator of cellular oxidative stress, especially in skin which is constantly being exposed to environmental challenges (Schafer and Werner 2015). Our results show that treatment with MMF, the bioactive ingredient of the anti-psoriatic drug Fumaderm[®], significantly increased Nrf2 cellular expression, nuclear localization and transcriptional activity, measured as target gene expression, resulting in protection against oxidative stress. Since a role for Nrf2 in inducing keratinocyte differentiation and regulating skin homeostasis has recently been suggested (Beyer et al. 2007; Lee et al. 2014), these results provide a possible justification for greater use of MMF in the treatment of psoriasis. This applicability seems particularly obvious because of the recent proposal of a role for oxidative stress as a causative factor for several inflammatory disorders including psoriasis (Okayama 2005; Sandireddy et al. 2014; Wagener et al. 2013; Zhou et al. 2009). Hence, Nrf2 may be a potential therapeutic target for the treatment of many skin diseases including those affecting keratinocyte function and increasing oxidative stress.

The exact mechanism of action of MMF is not well understood; however, the effect of MMF on Nrf2 has been previously explored in other cell types (Ananth et al. 2013; Promsote et al. 2014). In these cells, Keap1 (Kelch-like ECH associated protein 1) binds to and physically confines Nrf2 to the cytosol where it is inactive. Glutathionylation of Keap1 induces the dissociation of Nrf2 from Keap1 and allows Nrf2 to enter the nucleus and activate the

transcription of target anti-oxidant cytoprotective genes (Beyer et al. 2007). *In vitro* MMF is thought to covalently modify active cysteines in Keap1, thereby releasing and stabilizing Nrf2 (Ahuja, et al. 2016). The alkylating capacity of MMF is reflected in its ability to form adducts with glutathione (Ahuja et al. 2016). Since adduct formation increases over time (Ahuja et al. 2016), the ability of MMF to activate Nrf2 should also be time-dependent. Therefore, a lower concentration over a prolonged period of time, such as occurs *in vivo* with the treatment of a chronic disease such as psoriasis, will likely result in a similar change in alkylated Keap1 and free Nrf2 as a higher concentration over a shorter time, as occurs with treatment of cells *in vitro*. Indeed, studies using similar concentrations to those employed here indicate that MMF mediates the induction/stabilization of Nrf-2 in retinal pigment epithelial cells *in vitro* (Ananth et al. 2013; Promsote et al. 2014). Our immunofluorescence results showing a significant MMF-induced increase in both total (cytosolic and nuclear) Nrf2 and nuclear Nrf2 levels suggests that MMF not only elevates total cellular Nrf2 levels but also increases its localization in the nucleus, where it is able to induce the transcription of target genes, consistent with results in retinal pigment epithelial cells (Ananth et al. 2013) and astrocytes (Scannevin et al. 2012). We further confirmed the transcriptional activity of Nrf2 by testing the effect of MMF on several downstream targets, including the anti-oxidative enzymes HO-1 and Prdx6. Using qRT-PCR, we showed a significant increase in mRNA expression of both HO-1 and Prdx6 after a 24-hour treatment with MMF. Additional target genes GCLC, GSTA and NQO1, the first two of which are involved in glutathione homeostasis, were also up-regulated after a 24-hour incubation with MMF.

In order to examine whether this up-regulation of anti-oxidant enzyme expression exerted a functional effect in keratinocytes, we monitored intracellular ROS. MMF significantly attenuated ROS generation in primary mouse keratinocytes at all concentrations as compared to keratinocytes treated with H₂O₂ only. The H₂O₂ concentration used (200μM) is considered a high yet non-cytotoxic oxidative stress challenge, and the ability of MMF to counteract its effect likely reflects its ability to induce the expression of cellular anti-oxidant systems. Interestingly, a

formulation of FAE (mainly DMF) has been licensed in the US under the trade name Tecfidera® and is now being used as a first-line treatment for multiple sclerosis (Salmen and Gold 2014). One of the main mechanisms by which MMF (the bioactive ingredient of Tecfidera®) is thought to improve multiple sclerosis is via its cytoprotective and antioxidant effects, which seem to be primarily mediated via Nrf2 (Ahuja et al. 2016). Thus, for the first time, we are showing a similar effect of MMF on Nrf2 expression and activation in keratinocytes.

A report by Lee et al. (Lee et al. 2014) has also elucidated a role for Nrf2 in epidermal differentiation. These authors showed that Nrf2 activation increases the promoter activity of the epidermal differentiation marker loricrin. Accordingly, we investigated a possible mediator of the Nrf2-mediated pro-differentiative effect, AQP3. AQP3 is the most abundant aquaglyceroporin in mammalian epidermis (Hara-Chikuma and Verkman 2005; Matsuzaki, et al. 1999; Sougrat, et al. 2002; Sugiyama, et al. 2001), and AQP3 null mice exhibit a skin phenotype of decreased stratum corneum hydration, reduced epidermal water and glycerol permeability, dry skin and delayed wound healing and barrier recovery (Hara and Verkman 2003; Ma, et al. 2002). This phenotype reflects the importance of AQP3 in providing adequate stratum corneum hydration to maintain skin plasticity and barrier integrity. Moreover, data from our laboratory have suggested a role for AQP3 in inducing keratinocyte differentiation (Bollag et al. 2007). Thus, co-expression studies have shown that AQP3 inhibits keratin-5 promoter activity and increases keratin-10 promoter activity (an effect characteristic of the switch from proliferative to a differentiative phenotype) (Bollag et al. 2007). Furthermore, re-expression of AQP3 in AQP3 knockout keratinocytes induces the expression of several markers of keratinocyte differentiation (Choudhary et al. 2015). Interestingly, Voss et al. (Voss et al. 2011) have reported abnormal AQP3 levels and/or aberrant localization in human skin diseases including psoriasis, as compared to normal epidermis. Lee et al. (Lee et al. 2012) also demonstrated reduced AQP3 levels in psoriatic patients, whereas another report (Bellemeire et al. 2008) indicated that retinoic acid increased AQP3 expression and glycerol uptake in *ex vivo* human

skin samples and normal human keratinocytes collected from healthy volunteers. Retinoids are known regulators of keratinocyte proliferation and differentiation and have been used for decades as a topical treatment for psoriatic lesions (Menter, et al. 2009). Thus, the ability of MMF to increase AQP3 levels through Nrf2 might be related to the efficacy of this drug in treating psoriasis.

Our data showed that MMF treatment significantly increased mRNA and protein expression of AQP3. An increase in unglycosylated and total levels was observed, especially at 750 μ M, which is the concentration that also increased glycerol uptake, a measure of AQP3 activity. Although the increase in the glycosylated form of AQP3 (considered to be the active protein) did not achieve statistical significance, this may be attributed to the technical difficulty of quantifying this diffuse band as well as the biphasic regulation of AQP3, the expression of which is decreased as differentiation advances (Zheng and Bollag 2003). Immunofluorescent analysis suggested that MMF enhances AQP3 membrane localization and hence presumably activity, since as a transmembrane glycerol transporter, AQP3's activity should be closely associated with its membranous localization (Kim and Lee 2010; Zheng and Bollag 2003). Cell fractionation experiments also indicated that MMF increased plasma membrane-localized AQP3. Interestingly, however, the majority of AQP3 protein was found in the lysis solution-insoluble fraction of cells, although whether this AQP3 was localized in debris, organelles, intact cells or lipid rafts (as in (Zheng and Bollag 2003)) is unclear.

FAEs like MMF are used to treat psoriasis and multiple sclerosis, both of which are thought to be immune-mediated diseases. However, recent data have suggested the likely importance of immune cell-keratinocyte crosstalk in the psoriatic disease process (Brotas, et al. 2012; Lowes, et al. 2013; Sabat and Wolk 2011). Indeed, keratinocytes both produce and respond to pro-inflammatory cytokines (Nestle, et al. 2009). We have previously shown that MMF can inhibit the expression of several pro-inflammatory cytokines in keratinocytes (Helwa et al. 2015), which may be one mechanism through which MMF demonstrates efficacy in these

immune-mediated disorders. In addition, MMF has proven to have effects on immune cells (Litjens, et al. 2004; Nibbering et al. 1993), which may amplify its effects *in vivo*. Thus, the ability of MMF to act on multiple cell types for prolonged periods of time may account for its effectiveness *in vivo* at low doses, despite the fact that access to cells is more direct *in vitro*.

Finally, we investigated whether Nrf2 might be involved in the MMF-induced increase in AQP3 expression. We used the Nrf2 activator sulforaphane to show for the first time that Nrf2 stimulation is associated with an increase in AQP3 protein levels. This result suggests that Nrf2 may regulate the expression of AQP3 in keratinocytes, and indeed, we identified two likely Nrf2 transcription sites in the AQP3 promoter. SFN is a natural dietary agent abundant in cruciferous vegetables. It has been previously reported that SFN suppresses normal human keratinocyte proliferation (Chew, et al. 2012), an effect similar to what we observed with MMF (Helwa et al. 2015). This result further supports our idea that the mechanism of action of MMF in keratinocytes (similarly to SFN) is Nrf2-dependent.

In conclusion, this is the first report to detect an increase in the expression of Nrf2 and downstream effectors (HO-1 and Prdx6) in keratinocytes following MMF treatment. Our data are consistent with a recent clinical study that reported the induction of glutathione and Nrf2 pathway genes in lesional skin of patients with psoriasis following 12 weeks of treatment with FAE (Onderdijk, et al. 2014). Moreover, we demonstrated that MMF stimulated AQP3 gene and protein expression and glycerol transport capacity. We also suggest a possible relationship between the transcription factor Nrf2 and AQP3 expression, and this effect may underlie, in part, the action of MMF as an anti-psoriatic agent.

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Author Contributions:

Participated in research design: Helwa, Choudhary, Kaddour-Djebbar and Bollag.

Conducted experiments: Helwa, Choudhary, Chen and Kaddour-Djebbar .

Performed data analysis: Helwa, Choudhary, Chen and Bollag.

Contributed to the writing of the manuscript: Helwa, Choudhary, Kaddour-Djebbar and Bollag.

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Footnotes:

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Figure Legends:

Figure 1: MMF increased the protein expression of Nrf2 and the mRNA expression of Nrf2

target genes. (A) Keratinocytes were treated for 24 hours with or without 200 or 300 μ M MMF followed by western analysis for Nrf2 levels using rabbit anti-Nrf2 antibody (Abcam) and quantified using the LiCor Odyssey system. A representative blot is shown in the left panel; multiple experiments (n=3) were quantified and expressed relative to the loading control Hsp90 (right panel). Results were statistically analyzed and values presented as the means \pm SEM; *p \leq 0.05 versus control (untreated) cells. (B) Keratinocytes were treated for 24 hours with or without 200 μ M MMF and RNA isolated for qRT-PCR (n=4-6). Results were statistically analyzed using a one-sample two-tailed t-test relative to a hypothetical mean value of 1.0 (the control). Values represent the means \pm SEM; *p \leq 0.05, **p \leq 0.01 versus control (untreated) cells.

Figure 2: Higher concentrations of MMF enhanced cellular Nrf2 protein levels and

activity. (A) Near-confluent keratinocytes were treated with 0, 500 μ M, 750 μ M and 1mM MMF for 16 hours. Cells were then harvested and analyzed by Western blotting using rabbit anti-Nrf2 antibody (Santa Cruz; note that this was a different antibody from that used in Figure 1) and the LiCor Odyssey system with quantification shown in the right panel. Statistical analysis of the results of 4 separate experiments with values normalized to actin levels and shown as the means \pm SEM; *p \leq 0.05 versus the control. (B and C) Keratinocytes were treated with 0, 500 μ M, 750 μ M or 1mM MMF for 24 hours. mRNA levels were determined using qRT-PCR for (B) heme oxygenase-1 (HO-1) (n=4) and (C) peroxiredoxin-6 (Prdx6) (n=6). Values represent the means \pm SEM; *p \leq 0.05, ***p \leq 0.0005 versus control (untreated) cells.

Figure 3: MMF enhanced nuclear Nrf2 levels. (A) Keratinocytes were stained with primary

anti-Nrf2 antibody (red) and DAPI nuclear stain (blue). Shown are micrographs typical of three separate experiments. The upper panel shows Nrf2 staining only, the middle panel shows DAPI

nuclear staining only and the lower panel shows the merged image. The scale bar in the top right panel represents 20 μm . Quantification and statistical analysis of total and nuclear Nrf2 levels (B and C, respectively) using ImageJ analysis software. Quantification and statistical analysis of nuclear Nrf2 levels considered 10 individual keratinocytes from 3 micrographs representing 3 separate experiments performed in duplicate. Statistical analysis was performed for the 3 separate experiments, and results are shown as the means \pm SEM of arbitrary fluorescence units reflecting the intensity of fluorescence; * $p \leq 0.05$ versus the control.

Figure 4: MMF decreased hydrogen peroxide-induced ROS generation. Near-confluent primary mouse keratinocytes pre-treated for 24 hours with the indicated doses of MMF were incubated with DCFH-DA (100 μM) for 1 hour followed by treatment with 200 μM hydrogen peroxide (H_2O_2). Fluorescence intensity was measured as relative fluorescence units (RFU) and expressed as fold change relative to cells treated with H_2O_2 alone; * $p \leq 0.05$ relative to control, $\$ \leq 0.05$ relative to cells treated with H_2O_2 only.

Figure 5: MMF enhanced AQP3 mRNA and protein expression. (A) Keratinocytes were treated for 24 hours with or without 200 or 300 μM MMF followed by western analysis for Nrf2 levels. A representative blot is shown in the left panel; multiple experiments ($n=6$) were quantified (with both glycosylated and unglycosylated AQP3 quantified together) and expressed relative to the loading control Hsp90 (right panel). Results were statistically analyzed and values presented as the means \pm SEM; * $p \leq 0.05$ versus control (untreated) cells. (B) mRNA was purified and reverse transcribed from keratinocytes treated with 0, 500 μM , 750 μM and 1mM MMF for 24 hours. AQP3 mRNA levels were detected by qRT-PCR using GAPDH as a housekeeping gene. Values represent the means \pm SEM of 6 separate experiments performed in duplicate. Data are presented as fold change where * $p \leq 0.05$ versus the control. (C) Keratinocytes were treated with 0, 500 μM , 750 μM and 1mM MMF for 24 hours. AQP3 levels

were determined by Western blotting using rabbit anti-AQP3 antibody (LSBio) and the LiCor Odyssey system. Quantification and statistical analysis of (D) unglycosylated AQP3 bands and (E) glycosylated AQP3 bands are shown. Values represent the means \pm SEM of 5 separate experiments performed in duplicate and normalized to actin levels; * $p \leq 0.05$ versus the control.

Figure 6: MMF enhanced AQP3 cellular membrane localization and glycerol uptake by keratinocytes. (A) Primary mouse keratinocytes were stained with primary anti-AQP3 antibody (red) and DAPI nuclear stain (blue), and results shown are representative of 3 separate experiments. The upper panel represents AQP3 only, the middle panel shows DAPI nuclear staining only and the lower panel illustrates a merged image. (B) Near confluent keratinocytes were treated with 0 and 750 μ M MMF for 24 hours. Plasma membrane isolation was performed with protein lysates prepared from both the lysis solution-soluble isolated membrane fraction as well as the lysis solution-insoluble pellet as indicated; AQP3, aquaporin-3; EGFR, epidermal growth factor receptor. (C) Near-confluent keratinocytes were pretreated for 24 hours with 0 or 750 μ M MMF. Cells were then incubated for 5 minutes with [3 H]glycerol in K-SFM, as previously described (Bollag et al. 2007; Zheng and Bollag 2003). Results represent the means \pm SEM of 6 separate experiments. Values of [3 H]glycerol uptake were normalized to the protein content of each sample. Analysis was performed using a one-sample unpaired t-test and plotted as percent of control with * $p \leq 0.05$.

Figure 7: The Nrf2-stimulator sulforaphane (SFN) enhances AQP3 protein expression. (A) Near-confluent primary mouse keratinocytes were treated with 0, 1 μ M, 5 μ M and 10 μ M sulforaphane (SFN) for 24 hours. Proteins were harvested and analyzed by western blotting. Shown is a representative blot. Quantification and statistical analysis of the cumulative results for (B) the unglycosylated band (left panel) and (C) the glycosylated band (right panel) of AQP3

from 4 separate experiments are illustrated. Data are presented as -fold over the control of the AQP3 bands normalized to their corresponding actin bands and represent the means \pm SEM; * $p \leq 0.05$, ** $p \leq 0.01$ versus the control and \$ $p \leq 0.05$ versus 1 μ M SFN.

Figure 8. The AQP3 promoter possesses putative Nrf2 consensus sites, consistent with a possible direct effect of MMF-activated Nrf2 on AQP3 expression, as indicated in the proposed model. (A) An approximately 800-bp fragment of the murine AQP3 promoter was analyzed using the Tfsitescan program to identify two putative Nrf2 consensus sequences, supporting the idea that Nrf2 may directly induce AQP3 expression. (B) A schematic illustrating the proposed mechanism of action of MMF through activation of Nrf2 to upregulate antioxidant systems, thereby reducing oxidative stress and inhibiting inflammatory mediator production (Helwa et al. 2015). Nrf2 also induces AQP3 expression to inhibit proliferation and promote differentiation of keratinocytes.

Table 1. Sequences of Taqman probes used for quantification of gene expression by real-time quantitative RT-PCR

Gene Symbol	Assay ID	Sequence (5'-3')
Aqp3	Mm01208559_m1	CATCCTTGTGATGTTTGGCTGTGGC
Hmox1	Mm00516005_m1	AGGCTTTAAGCTGGTGATGGCTTCC
Prdx6	Mm00725435_s1	AACAAAGATGCCTGGCTTGAGCATG
Gclc	Mm00802655_m1	TCATTCCGCTGTCCAAGGTTGACGA
Gsta	Mm01233706_m1	TTGGCTGCAGCTGGTGTGGAGTTTG
Nqo1	Mm01253561_m1	GACAACGGTCCTTTCCAGAATAAGA
Gapdh	Mm99999915_g1	GGTGTGAACGGATTTGGCCGTATTG

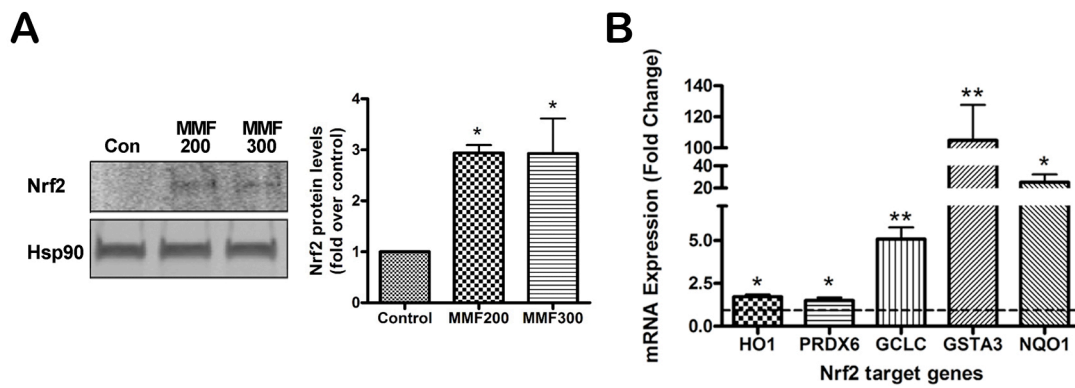


Figure 1

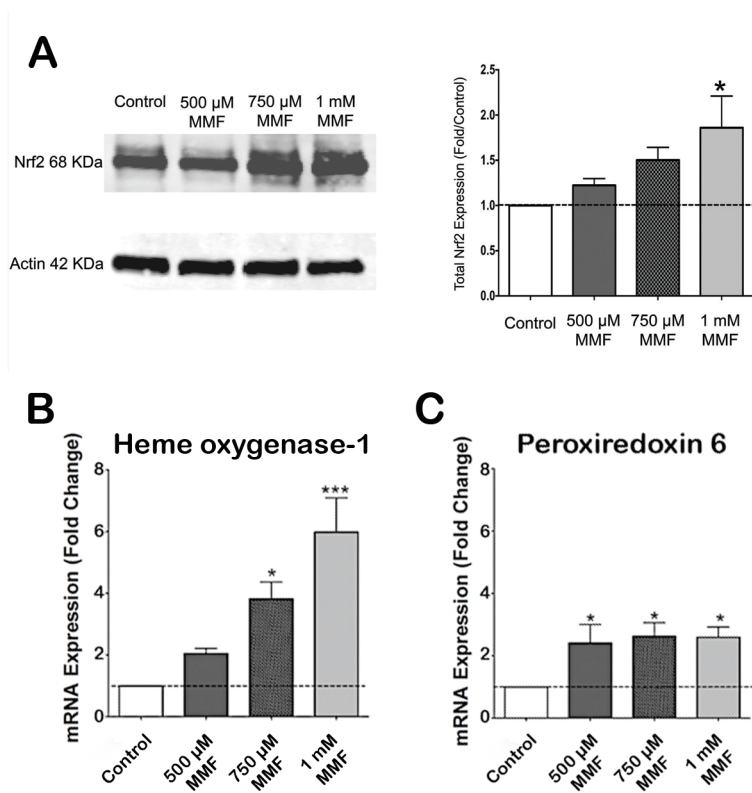


Figure 2

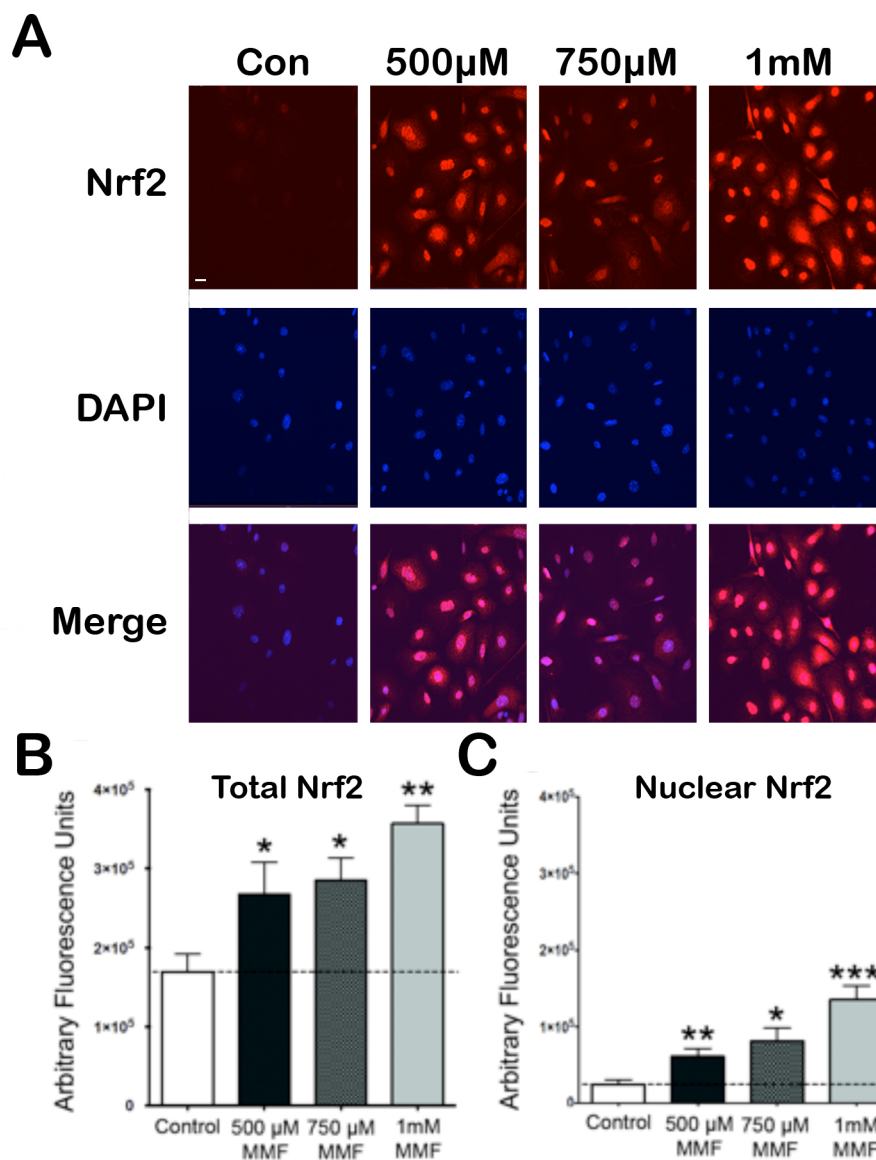


Figure 3

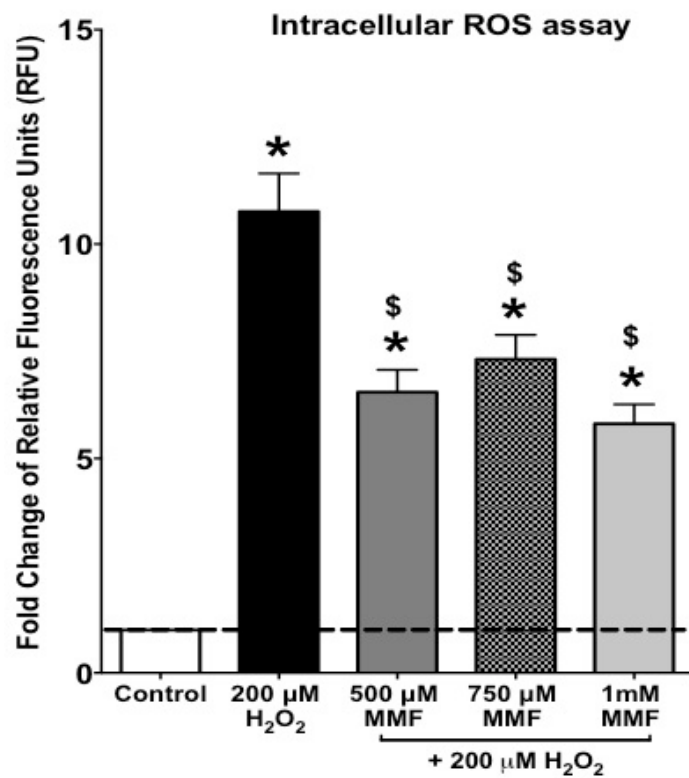


Figure 4

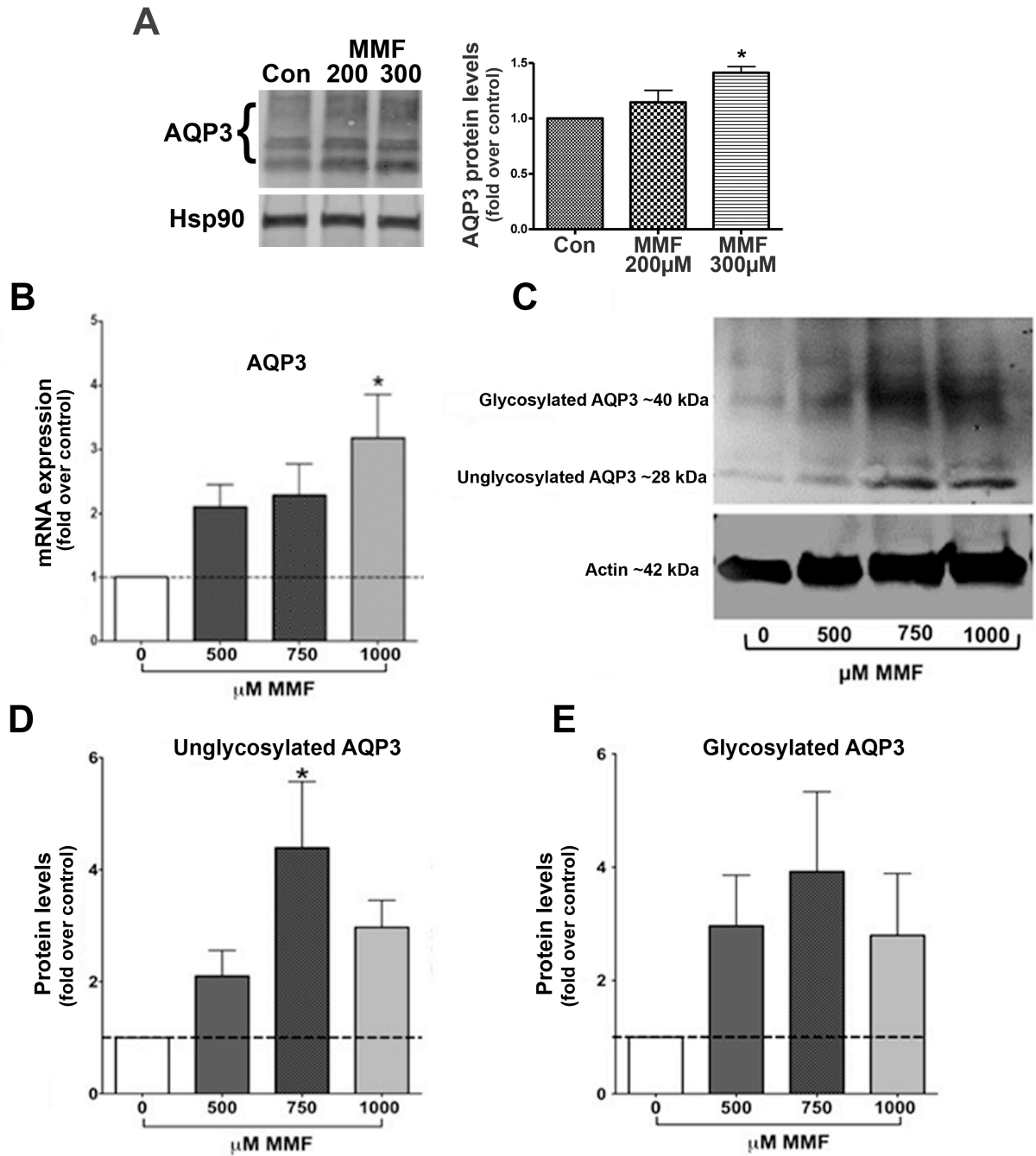


Figure 5

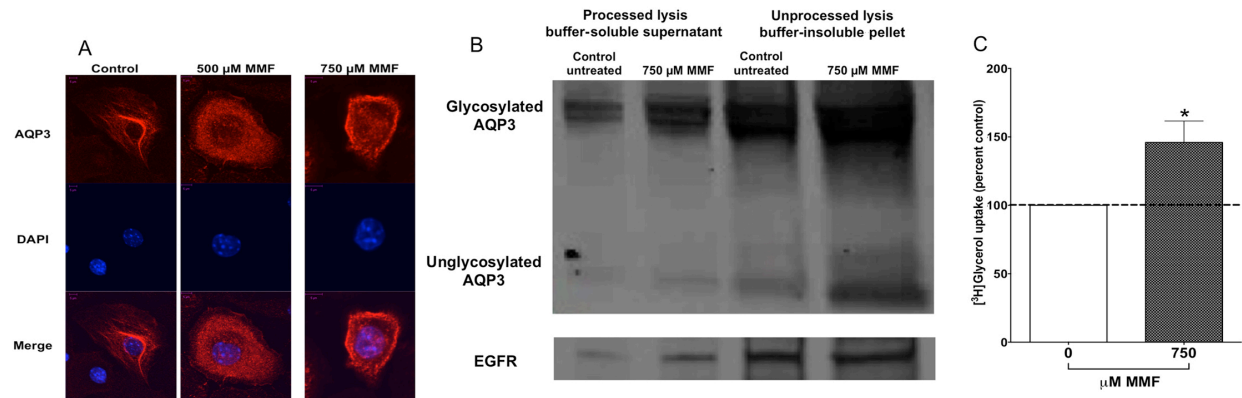


Figure 6

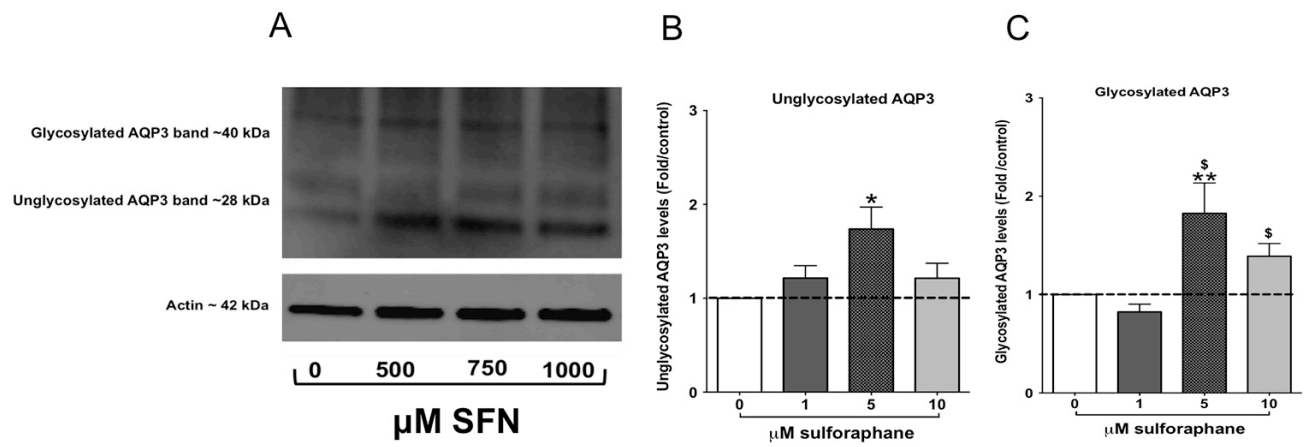


Figure 7

A

***Mus musculus* AQP3 Promoter Sequence**

Accession #NM_016689.2 and #NC_000070.6 (mouse C57BL/6 chromosome #4)

AATGATGATATACATGTGGGCACATGCACTGCTATGAACGTGTGGAGTTGGTTCTTTCTCTTCGGGTGGG
TTTCAGGGATTGAATTCGGGTGTGTCAGTCTGCCCTGGCAAGC**GCTCTTCCTGT**TGAGCCTACTCCCGGGGC
CCCAGCTTAATTACTTAAAGAGTAGATTTACATCGATGGGAGTGGGAAAGGAGGAGTTAAAAACAGCTG
GAATTCACATGATGTAGCATGTGACATGTCAAGACAACAGCAGCTCAGCCAAGGGGGAAGGTCCACAAGG
AGAAAGCCCAGGTATCCCGGGCCGCACGTGGCGCGTCCAGACGGTGTGACCAGGTCACAGGACATATACCA
TGCTAATCAACACATGCAACATGTGACATGTTCCCAGGGCACACGAAGCGCTGGTGAATTCTGGTTAGAAC
TCTGCATACACCTGCCTACCAGGTCGTCACCTCTGAGGTCTCTTCCCACTAGAGGTTTATAGCCTCCTTTA
AAAATCAGCCGCCCCGATTGAAGACCTGTTCTATCCAGTCATCGCAGCAAGCTGGTTCCCTCACTCCTAGCGT
GAGACGATGCCACAAAGGCTCTCTGTGAAGGGGTTGGGGTAGGGGGGTGGGGGGGGTGGCGCTCGGG
AGACCGCT**GCTCTTCCCGT**TTTCACAGTATCCAGTCCTCTCAGTACAACACTCACTCCCCTAAGAATCCT
GCAGAGCCAACCCACCTTAAAGCTCCACAGGACCCTCCTTACAACGTGACCCCGCGTCCCCACCAGCGCC
TCCAGGCTAGGTGGAGGGTGGTGGCCGCACTCCGCCCCCGGGGTTCCGAGGTTGGGGCTCAGCAGGCT
GAGCGTATAAAAGGGCGCCACCTGCGTCCGGGCCaccgctctcggtgccttcgctagctactttgcactcgtacgcccgccgac
ctcgccgctgcctgcctgcgcgc**atgg**gtcgacagaaggagttgatgaatcgttggtgggagatgcttcacatccgctaccggctgcttcgccag
gcgctggcggagtgccctggggaccctcatccttggtGTGAGTGCAGGGTAGTGAGCAGTCCTATCTATTTACAGCCCCGT
GGTCCCCAAC

ATG: Start codon (start of translation in the first exon)

Exon 1

Nrf2 binding site

Nrf2 binding site sequence*	Position upstream of start codon (ATG)	Position upstream of exon start site	Position upstream of TATA box	Score (Gaps)	Exp Value
GCTCTTCCCGT	314	240	211	8 (0)	1.42e-02
GCTCTTCCTGT	846	772	743	8 (0)	1.42e-02

*Tfsitescan searches Sites dataset sequences in ooTFD, a descendant of the TFD (Transcription Factors Database) initiated in 1990 as in (Ghosh, 2000).

B

