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ACUTE EFFECTS OF ALL-*TRANS*-RETINOIC ACID IN ISCHEMIC INJURY

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Abstract

All-*trans*-retinoic acid (ATRA) is a vitamin A derivative that is important in neuronal patterning, survival, and neurite outgrowth. Neuroprotective effects of ATRA in ischemia have been demonstrated but its effects on glial swelling are not known. We investigated the relatively acute effects of ATRA on cell swelling in ischemic injury and on key features hypothesized to contribute to cell swelling including increased reactive oxygen species/ reactive nitrogen species (ROS/RNS), depolarization of the inner mitochondrial membrane potential ($\Delta\Psi_m$), and increased intracellular calcium ($[Ca^{2+}]_i$). C6 glial cultures were subjected to 5 hr oxygen-glucose deprivation (OGD). ATRA was added to separate groups after the end of OGD. OGD increased cell volume by 43%, determined at 90 min after the end of OGD, but this increase was significantly attenuated by ATRA. OGD induced an increase in ROS/RNS production in the whole cell and mitochondria, as assessed by the fluorescent dyes CM-H_2DCFDA and MitoTracker CM-H_2-XROS at the end of OGD. The increase in mitochondrial ROS, but not cellular ROS, was significantly attenuated by ATRA. OGD also induced a 67% decline in mitochondrial $\Delta\Psi_m$ but this decline was significantly attenuated by ATRA. OGD-induced increase in $[Ca^{2+}]_i$ was also significantly attenuated by ATRA. Taken together with our previous results where calcium channel blockers reduced cell swelling, the effects of ATRA in attenuating swelling are possibly mediated through its effects in regulating $[Ca^{2+}]_i$. Considering the paucity of agents in attenuating brain edema in ischemia, ATRA has the potential to reduce brain edema and associated neural damage in ischemic injury.

Keywords

• Vitamin A • Ischemia • Brain edema • Mitochondria • Free radical • Calcium

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1. Introduction

Vitamin A (VA) is critical for normal cellular growth, differentiation, and death [1,2]. Within the last 2 decades, a large body of evidence has accumulated which strongly suggests that most of VA's actions in these processes can be explained by the actions of its metabolites, which activate or repress gene transcription through binding Retinoic Acid Receptors (RARs) and/or Retinoid X Receptors (RXR). All-*trans* retinoic acid (ATRA), the predominant metabolite of VA, is a natural, high affinity ligand for RARs (K_d » 1 nM) whereas it binds very weakly to RXR. RAR and RXR form homo- or heterodimers that bind to specific response elements present in the promoters of target genes thereby initiating transcription [3]. Retinoid receptor proteins are widely distributed in the adult nervous system including the cortex, amygdala, hypothalamus, hippocampus, and striatum indicating a role for such proteins in neural functioning (see [4] for review). Disturbances in retinoic acid signaling have been implicated in Alzheimer's disease [5], Parkinson's disease [6], and schizophrenia [7].

Non-transcriptional mechanisms of ATRA have been reported, including stabilization of mRNA, activation of translation, covalent modification of proteins and direct stimulatory and inhibitory effects of ATRA on enzyme systems [8]. ATRA activates STAT1 rapidly, within 5 min to 2 hr [9], cAMP/ protein kinase A (PKA)/CREB [10], Akt/mammalian target of rapamycin (mTOR)/p70 S6 kinase [11], and protein kinase C (PKC) [12] pathways in various cell types, suggesting that some of its effects on cellular growth, differentiation, and death are independent of transcription.

The protective effect of retinoic acid in response to ischemic injury has been reported in PC12 cells [13] and in cultured hippocampal slices [14]. In addition, the neuroprotective effects of ATRA or its isomers or derivatives have been reported in cerebral ischemic injury in animal studies. 9-cis-retinoic acid, reduced cerebral infarction induced by middle cerebral artery ligation in rats [15,16]. ATRA also reduced the infarct volume following middle artery occlusion (MCAO) in rats in the cortex [17] and striatum [18] and in mice [19]. However, the effects of ATRA in ischemia on cell swelling, a component of brain edema, are not known.

Brain edema is a key feature of ischemic injury [20]. Brain edema is defined as an abnormal accumulation of fluid in the brain parenchyma resulting in a volumetric enlargement of the cells or tissue. A consequence of this volume increase is the development of increased intracranial pressure leading to brain herniation, irreversible brain damage, and ultimately, death. Brain edema is generally classified into cytotoxic or vasogenic edema [20]. Cytotoxic edema is defined as a cellular swelling with fluid accumulating within the cell in the brain and astrocyte swelling is a major

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component of cytotoxic edema [21]. Swollen astrocytes following ischemic injury have been reported in cats [22] and rats [23]. Vasogenic edema is characterized by a breakdown of the blood-brain barrier resulting in increased fluid accumulation, originating from blood vessels that amass around cells. Both vasogenic and cytotoxic mechanisms contribute to overall ischemic brain edema [20,24]. Edema further impairs cerebral perfusion and oxygenation, and contributes to additional ischemic injuries.

Mechanisms underlying cell swelling are not clear but several factors have been implicated including increased levels of Ca^{2+} [25], elevated K^+ [21], acidosis, free radicals, release of excitatory neurotransmitters, especially glutamate [26] (see [27] for review) and mitochondrial dysfunction, in particular the opening of the mitochondrial permeability transition (mPT) pore, a megachannel spanning the inner and outer mitochondrial membrane [28-30]. While all the above mentioned factors may play a role in inducing cell swelling in ischemia, the precise role and the interactions between such factors to induce swelling are not clear. Our previous data indicate a more important role for increased $[Ca^{2+}]_i$ in cell swelling when compared to the mPT [31] or reactive oxygen species/reactive nitrogen species [29,31] although mitochondrial dysfunction may contribute to such swelling. We report that ATRA reduced C6 glial cell swelling *in vitro* following oxygen-glucose deprivation (OGD) and such reduction in swelling is possibly mediated by the effects of ATRA in regulating $[Ca^{2+}]_i$.

2. Experimental Procedures

2.1 Materials

All chemicals used were purchased from Sigma-Aldrich (St. Louis, MO) unless noted otherwise. Methyl-D-glucose (3-O-[methyl-^{3}H]-D-glucose) was purchased from American Radiolabel Chemicals (St. Louis, MO). Cell culture supplies, including heat-inactivated serum were purchased from Gibco/Invitrogen (Carlsbad, CA). The fluorescent dyes including tetramethylrhodamine ethyl ester (TMRE), 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA), MitoTracker Red CM-H_2-XROS, Fluo-5F AM, and Hoechst 33258 were purchased from Molecular Probes/Invitrogen (Carlsbad, CA). SR 11237 and BMS 753 were purchased from Tocris Biosciences (Ellisville, MO).

2.2 Cell culture

Rat C6 glioma (CCL-107) was purchased from American Type Culture Collection (ATCC; Manassas, VA). Cultures were grown in F-12 K medium (Gibco/Invitrogen) supplemented with 10% horse serum and 2% fetal bovine serum and maintained at 37C with 5% CO_2/95% air. Cultures were grown to 85% confluency in 75 mm flasks and following trypsinization were subcultivated in 35 mm culture dishes at a density of 0.8 x 10^6; experiments were initiated 24 hr later. All cultures used in the experiment were between passages 22 and 32.

2.3 Oxygen-glucose deprivation (OGD)

OGD was induced in cultures as described by Panickar et al. 2005 [32], but with minor modifications [33]. Briefly, cultures were washed twice with a balanced salt solution (BSS) with the following composition (in mM): NaCl 116, KCl 5.4, $CaCl_2$ 1.8, $MgSO_4$ 0.8, NaH_2PO_4 0.83, $NaHCO_3$ 24 and phenol red 0.001w/v; pH 7.4. Following the washes, BSS was added to the cultures and placed in an airtight container (Billups chamber; Billups-Rothenberg Inc., Del Mar, CA) and continuously flushed with 95% N_2/5% CO_2 for 5 hr. At the end of OGD, BSS was removed and regular medium was added to the cultures and returned to normal conditions to determine cell volume or mitochondrial function. This is similar to the reperfusion phase in *in vivo* ischemic injury.

2.4 Cell volume determination

Cell volume was estimated by measuring the intracellular water space using the method of Kletzien et al. 1975 [34], as described by Norenberg et al. 1991 [35] for glial cultures. Briefly, 1 mM 3-*O*-methylglucose (3-OMG) and 0.5 µCi/ml [^{3}H]-3-OMG were added to the cultures immediately following the end of OGD in regular media and returned to the normal culture incubator. ATRA, as well as ethanol vehicle (100% Etoh, 1:1000), were added immediately after the end of OGD at the same time as [^{3}H]-3-OMG in regular media. Volume assay was conducted at 90 min after the end of OGD. At the end of the incubation period with [^{3}H]-3-OMG, the culture medium was aspirated and an aliquot was saved for radioactivity determination. Cells were rapidly washed with ice-cold buffer containing 229 mM sucrose, 1 mM Tris-nitrate, 0.5 mM calcium nitrate and 0.1 mM phloretin, pH 7.4. Cells were lysed with 400 µl NaOH (1N). Radioactivity in the lysate and medium were determined, and an aliquot of the lysate was used for protein determination with the Bio-Rad bicinchoninic acid kit. Cell volume was normalized to protein level and expressed as µl/mg protein.

2.5 Measurement of reactive oxygen species/reactive nitrogen species (ROS/RNS)

Changes in cellular ROS and mitochondrial ROS were determined using the fluorescent dyes dichlorofluorescein diacetate (CM-H_2DCFDA) and MitoTracker CM-H_2-XROS respectively, as described by Panickar et al. 2002 [36]. CM-H_2DCFDA detects ROS/RNS in the cytosolic, nuclear, and mitochondrial compartments whereas MitoTracker CM-H_2-XROS detects mitochondrial-specific free radicals [37,38]. For the measurement of cellular ROS/RNS, immediately after the end of 5 hr OGD, BSS was removed and cultures were incubated for 30 min at 37C in 2 ml fresh DMEM (without serum) containing 5 µM DCFDA, and ATRA (or Etoh vehicle) in the normal incubator. Following incubation, cultures were washed quickly with media and cells were lysed with 250 µl of 0.2% Triton-X 100 and collected in centrifuge tubes, triturated and centrifuged at 6000 rpm for 5 min at 4C. An aliquot (200 µl) of the lysate was transferred to a 96-well micro-titer plate for fluorescence measurement (Ex/Em 485/515 nm). Protein content was determined in the remaining sample by the Bio-Rad bicinchoninic acid method and fluorescent values were normalized to protein concentration and expressed as AFU/mg protein (arbitrary fluorescent units/µg protein).

Changes in mitochondrial ROS were assessed using MitoTracker CM-H_2-XROS by fluorescence microscopy. Immediately after the end of

OGD, BSS was removed and cells were loaded with MitoTracker (1 μM) in regular media (but without serum) along with ATRA or vehicle, and returned to the normal culture incubator for 35 min. Cultures were washed with PBS and fluorescence images were captured using a Nikon TE2000 inverted fluorescent microscope (Ex 550/Em 590) and Roper Fast Monochrome cooled camera. Changes in mitochondrial fluorescence between groups were quantitated using the method similar to that used for assessing mitochondrial membrane potential as described previously [39] and described below.

2.6 Measurement of changes in mitochondrial membrane potential ($\Delta\Psi_m$) using the fluorescence dye TMRE

Changes in $\Delta\Psi_m$ were measured with the fluorescent dye TMRE as described previously [39], but with minor modifications. Immediately at the end of OGD, BSS was removed and cells were loaded with TMRE (20 nM) in regular media (but without serum) along with ATRA or vehicle, and returned to the normal culture incubator for 20 min. Cultures were washed with PBS and fluorescence images were captured using a Nikon TE2000 inverted fluorescent microscope (Ex 550/Em 590) and Roper Fast Monochrome cooled camera. At least 10 random image fields, having a similar degree of cell density (nuclei were stained with 1 μM Hoescht 33258; pictures not shown), were analyzed. Exposure time was kept constant within each experiment. Fluorescent intensities were analyzed using a combination of Nikon Elements program and macros written for V++ (Digital Micropotics) [32,33]. In each image field, the total number of pixels was quantified on a gray scale (0-255) and the average intensity (mean pixel value for each cell in an image field) was obtained and expressed as mean ± SEM of average intensity of the total number of cells in each experimental group. The mean pixel value for TMRE was normalized to the total number of nuclei in the respective fields and the percent changes in the fluorescent intensities between control and experimental groups were compared. p-Trifluoromethoxy carbonyl cyanide phenyl hydrazone (FCCP; 5 μM), a mitochondrial uncoupler, when added at the end of the experiment, almost completely diminished TMRE fluorescence in the cells, indicating that the decline in fluorescence observed was not due to dye quenching in the mitochondria.

2.7 Measurement of intracellular calcium ($[Ca^{2+}]_i$)

Changes in $[Ca^{2+}]_i$ were measured with the fluorescent dye Fluo-5F-AM ester, a low affinity binding calcium dye, as described [31]. Immediately at the end of OGD, BSS was removed and cells were loaded with Fluo-5F (1 μM) in regular media (but without serum) along with ATRA or vehicle, and returned to the normal culture incubator for 30 min. Cultures were washed with PBS and fluorescence images were captured using a Nikon TE2000 inverted fluorescent microscope (Ex 488/Em 515) and Roper Fast Monochrome cooled camera. Image analysis to detect changes in fluorescence was conducted as described above for TMRE measurements. Briefly, at least 10 random image fields, having a similar degree of cell density (nuclei were stained with 1 μM Hoechst 33258; pictures not shown), were analyzed. Fluorescent intensities were analyzed using a combination of Nikon Elements program and macros written for V++ (Digital Micropotics) [32]. Percent changes in the fluorescent intensities between control and experimental groups were assessed by comparing the mean pixel value (quantified on a gray scale) for Fluo-5F and normalized to the total number of nuclei in the respective fields

2.8 Statistical Analysis

Each group consisted of 3-4 culture dishes per experiment for the cell volume experiments. Each set of experiments was performed in triplicate from multiple cell seedings. Extent of cell swelling was normalized to protein values and subjected to analysis of variance (ANOVA) followed by Tukey's post-hoc comparisons. At least three cultures dishes per group were used in the fluorescence microscopy experiments. Intensity unit values obtained from imaging experiments were also subjected to analysis of variance (ANOVA) followed by Tukey's post-hoc comparison test and expressed as percent change over control or as mentioned in the figure legends.

3. Results

3.1 ATRA attenuated the OGD-induced cell swelling in C6 glial cultures

Cell swelling was assessed at 90 min after the end of 5 hr OGD. ATRA (100 nm and 1 μM) was added to the cultures in normal media after the end of OGD for 90 min. There was a 43.3% increase in cell volume in the OGD group when compared with control ($p<0.05$; Figure 1). The OGD-induced increase in cell swelling was significantly diminished in the presence of both doses of ATRA tested (both $p<0.05$ vs OGD; Figure 1). ATRA (100 nM) attenuated cell swelling by 60% while the higher dose (1 μM) reduced swelling by 55%. However, there was no reduction in OGD-induced cell swelling with SR11237 (100 nm and 1μM), an RXR agonist without any RAR activity, or with BMS 753 (100 nm and 1 μM), an RARα agonist (data not shown). Ethanol (100%; 1:1000; vehicle) did not affect cell volume in either control or OGD groups (data not shown).

3.2 ATRA attenuated the OGD-induced increase in mitochondrial ROS but not cellular ROS/RNS in C6 glial cells

Increased ROS is hypothesized to induce cell swelling in glial cultures following ischemia *in vivo* [40] and in ammonia neurotoxicity in astrocyte cultures [41]. To determine whether ATRA displayed anti-oxidant effects, cellular ROS/RNS production was assessed immediately at the end of 5 hr OGD by measuring DCFDA fluorescence. ATRA (100 nm and 1 μM) was added after the end of OGD along with DCFDA for 30 min at 37C in the incubator. The rationale for assessing ROS/RNS at 30 min and not 90 min after the end of OGD was two-fold. Firstly, we hypothesized that increased ROS is an early event that contributes to cell swelling and 30 min was the time taken for the dye to optimally load the cells. Secondly, ROS increases rapidly and subsequently decreases well before cell swelling [30,42]. When compared to controls, OGD increased ROS/RNS production by 106% (1160.1

± 112.3 vs. 2393.6 ± 716.73; p<0.05; Figure 2). ATRA (100 nm and 1 μM)) did not significantly decrease the OGD-induced ROS/RNS although a small decrease in ROS/RNS was observed with the higher dose (1 μM) (Figure 2).

Mitochondrial ROS was assessed at 30 min after the end of OGD. ATRA (100 nm and 1 μM) was added to the cultures along with MitoTracker Red CM-H_2-XROS for 30 min at 37C in the incubator. Cultures were visualized using fluorescence microscopy. OGD increased mitochondrial ROS, as assessed by the increase in fluorescence, by 84% when compared to control (P<0.05; Figure 3A and B). This increase was completely blocked by both concentrations of ATRA when compared to OGD (p<0.05; Figure 3A and B).

3.3 ATRA attenuated the depolarization of the mitochondrial inner membrane potential ($\Delta\Psi_m$) following OGD

Induction of the mPT has been hypothesized to contribute to glial swelling in cultures in traumatic injury [30] and ammonia neurotoxicity [41]. We examined the effects of ATRA on attenuating the OGD-induced decline in ($\Delta\Psi_m$). Following 5 hr OGD, ATRA (100 nm and 1 μM) was added to the cultures and the change in $\Delta\Psi_m$ was assessed 20 min later with TMRE (20 nM) using a fluorescence microscope. OGD induced a 66.7% decline in $\Delta\Psi_m$ when compared with controls (p<0.05, Figure 4). This decline in $\Delta\Psi_m$ was significantly prevented by both concentrations of ATRA (both p<0.05 versus OGD). While the highest dose of ATRA (1 μM) significantly prevented the decline in $\Delta\Psi_m$ by 65% when compared to OGD, the lower dose (100 nM) prevented the decline by 56% (Figure 4). In addition, both concentrations of ATRA were also significantly different from controls (p<0.05). FCCP (5 μM), a mitochondrial uncoupler, added at the end of the experiment to the cultures almost completely diminished TMRE fluorescence within 10 min (data not shown).

3.4 ATRA attenuated the OGD-induced increase in intracellular calcium ([Ca2+]$_i$) in C6 glial cells

We have previously demonstrated an increase in $[Ca^{2+}]_i$ following OGD and blocking such increase reduced OGD-induced cell swelling [31]. Also, an increase in $[Ca^{2+}]_i$ induced cell swelling in lactacidosis-induced glial swelling [43,44], and hypo-osmotic swelling in cultured astrocytes [45]. To examine whether ATRA regulates [Ca2+]$_i$, we assessed changes in [Ca2+]$_i$ in C6 cultures immediately at the end of 5 hr OGD using Fluo-5F AM, a low affinity binding calcium dye. OGD increased [Ca2+]$_i$ by 190%, as assessed by Fluo-5F fluorescence, when compared with controls (p<0.05, Figure 5). This increase in OGD-induced fluorescence was significantly attenuated by both concentrations of ATRA tested (100 nm and 1 μM). Both doses of ATRA attenuated the increase in [Ca2+]$_i$ by > 65 % (100 nm by 66% and 1 μM by 68%; both p<0.05) when compared to OGD. However, the effect of both concentrations of ATRA was also significantly different from control (p<0.05, Figure 5).

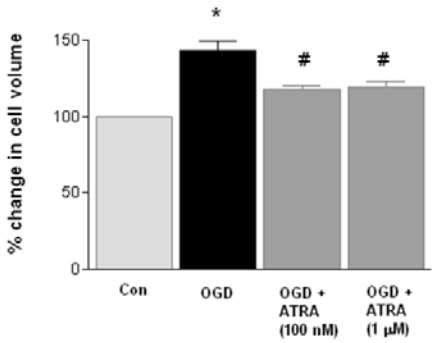

Figure 1. All-*trans*-retinoic acid (ATRA) significantly attenuated cell swelling after the end of 5 hr oxygen-glucose deprivation (OGD) in C6 glial cultures. ATRA was added after the end of OGD and cell volume was assessed 90 min later. *p<0.05 versus control (Con); # p<0.05 versus OGD. Each group consisted of at least 8-12 culture dishes taken from multiple cell seedings. Data are presented as mean ± SEM.

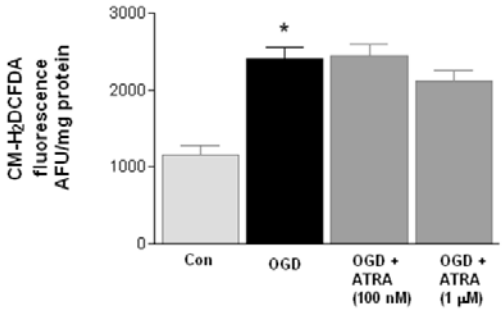

Figure 2. ATRA did not significantly attenuate the OGD-induced increase in reactive oxygen species/reactive nitrogen species (ROS/RNS). ROS/RNS were measured using the fluorescent probe CM-H_2DCFDA. Immediately after the end of OGD, DCFDA and ATRA were added to the cultures and incubated for 30 min. Fluorescence intensity was measured at the end of the 30 min after incubation with DCFDA using a fluorescent plate reader (see text for details). Each group consisted of 5 culture dishes and taken from multiple seedings. Fluorescence values (Arbitrary fluorescent units (AFU) were normalized to protein concentration and expressed as mean ± SEM. *p<0.05 vs control (Con).

4. Discussion

We demonstrate a protective effect of ATRA on cell swelling in ischemic injury in C6 glial cultures. In addition, ATRA also attenuated key features associated with ischemic injury including a reduction in mitochondrial ROS production, dissipation of mitochondrial $\Delta\Psi_m$, and increased $[Ca^{2+}]_i$. These results are important in ischemic injury since the acute effects of ATRA on mitochondrial ROS, mitochondrial $\Delta\Psi_m$, and $[Ca^{2+}]_i$ are protective but generally in contrast to the effects observed

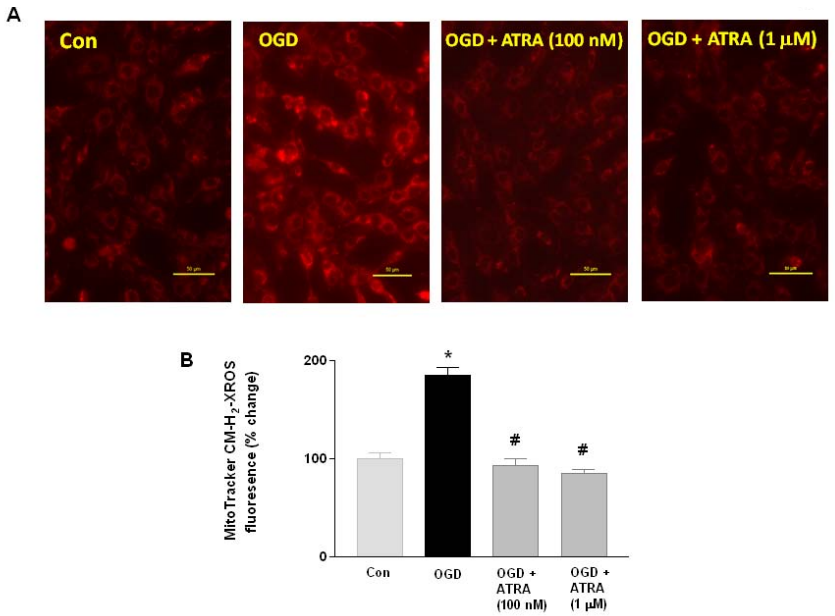

Figure 3. Oxygen-glucose deprivation (OGD)-induced increase in mitochondrial ROS, as assessed by MitoTracker CM-H_2-XROS fluorescence, is significantly attenuated by ATRA (A) Sample photomicrographs of MitoTracker red fluorescence after 5 hr OGD in control (Con), OGD, and OGD + ATRA. Immediately after the end of 5 hr OGD, cells were loaded with MitoTracker (1 μM) and ATRA in regular media (but without serum) and returned to the normal culture incubator for 35 min before visualization. (B) Quantitative measurement of MitoTracker fluorescence normalized to Hoechst (sample micrographs not shown) and the average fluoresence intensities in cells from each experimental group were converted to percentages. Each group consisted of 3 culture dishes. Data are presented as mean ± SEM. *p<0.05 vs control (Con). # p<0.05 versus OGD.

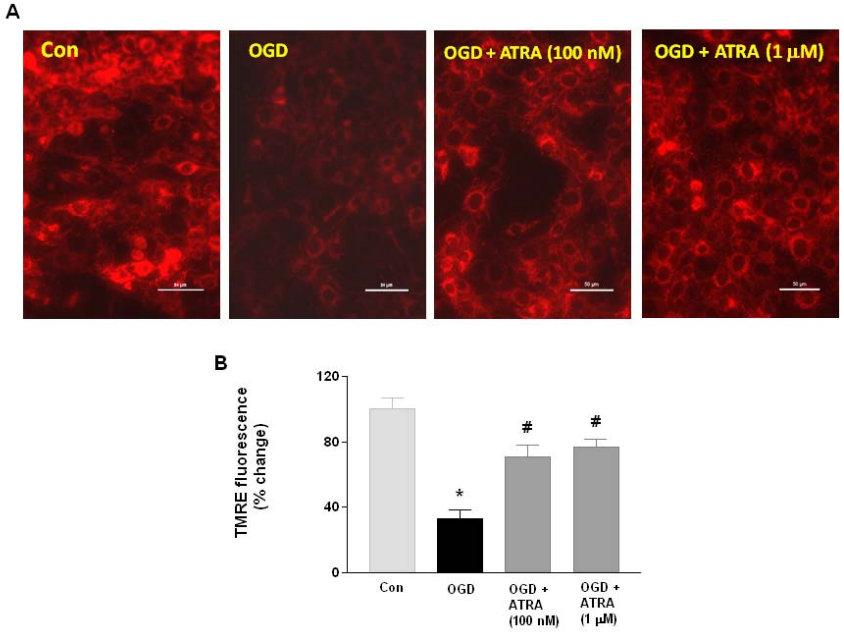

Figure 4. Oxygen-glucose deprivation (OGD)-induced depolarization of the mitochondrial membrane potential ($\Delta\Psi_m$) is significantly attenuated by ATRA (A) Sample photomicrographs of TMRE fluorescence after 5 hr OGD in control (Con), OGD, and OGD + ATRA. Immediately after the end of 5 hr OGD, cells were loaded with TMRE (20 nM) and ATRA in regular media (but without serum) and returned to the normal culture incubator for 20 min before visualization. (B) Quantitative measurement of TMRE fluorescence normalized to Hoechst (sample micrographs not shown) and the average fluoresence intensities in each experimental group were converted to percentages. Each group consisted of 3 culture dishes. Data are presented as mean ± SEM. *p<0.05 vs control. # p<0.05 versus OGD.

following longer exposure of cells to ATRA in other stresses or conditions.

Results from our study support the hypothesis that ATRA exerts such protective effects via a non-transcriptional mechanism. The well-known effects of ATRA including differentiation and neuronal patterning and neural plasticity generally require a prolonged exposure to ATRA [4]. Given that our cultures were exposed to ATRA from times ranging from 20 min to 90 min, it is unlikely that the protective effects of ATRA were mediated by a genomic effect involving transcription. Agonists of RXR and RARα also did not reduce cell swelling. In addition, given ATRA's abilities to attenuate mitochondrial dysfunction and reduce $[Ca^{2+}]_i$, factors that are hypothesized to contribute to cell swelling, it is possible that the protective effects of ATRA in reducing cell swelling are predominantly due to its relatively acute effects on such cellular functions. Nongenomic effects of RA (by binding to non-nuclear receptors) have been reported and the direct binding of RA to the C2 domain of protein kinase C (PKCα) [46] indicates a potential for modulating PKCα activity. Other nongenomic effects of ATRA include inhibition of transmitter release at developing motoneurons in *Xenopus* cells *in vitro* [47] and activation of CREB and ERK in primary cultures of cerebrocortical cells and of dorsal root ganglia neurons from rat embryos [48]. While it remains to be established which cellular receptors were involved in the rapid effects of ATRA on C6 glial cells, our results show a robust protection of cell swelling by ATRA.

The mechanism by which ATRA reduced OGD-induced cell swelling appears to be through its ability predominantly to regulate $[Ca^{2+}]_i$ although the role of other factors including ROS/RNS and mPT cannot be ruled out. Several lines of evidence indicate an important role of increased $[Ca^{2+}]_i$ in cell swelling over increased ROS/RNS and the induction of the mPT. Firstly, ATRA reduced the rise in $[Ca^{2+}]_i$ following ischemic injury. We have previously demonstrated that such rise in $[Ca^{2+}]_i$ after OGD is associated with cell swelling [31]. Further, such swelling is blocked effectively by L-type calcium channel blockers including nifedipine, nimodipine, verapamil, as well as by regulators

of $[Ca^{2+}]_i$ including BAPTA-AM and dantrolene [31]. The downstream pathways following increased $[Ca^{2+}]_i$ that cause cell swelling are not clear and need to be investigated. The effect of ATRA on decreasing Ca^{2+} influx is not entirely surprising since RA inhibited Ca^{2+} currents in a B-lymphocyte cell line [49] and ATRA also reduced the angiotensin-induced increase in $[Ca^{2+}]_i$ in cultured rat cardiac myocytes [50]. It should, however, be noted that prolonged exposure of ATRA generally increases $[Ca^{2+}]_i$ in cells and such increases in Ca^{2+} influx play an important role in various RA-mediated functions including Ca^{2+}-dependent protein phosphorylation in PC12 cells [51], induction of calcium channel expression in human NT2N neuronal cell line [52], and the growth cone turning response in mollusc neurons *in vitro* [53].

Secondly, blockade of the mPT using the immunosuppressant cylosporin A (with the inability of FK506 to block the mPT) generally suppresses cell swelling in cultures induced by ammonia toxicity [54], traumatic brain injury [29,30], and ischemic injury [28,33]. However, we have recently reported that quercetin, a polyphenol, significantly reduced OGD-induced cell swelling but did not block the dissipation of the $\Delta\Psi_m$, an indicator of the mPT [31]. A similar phenomenon was also observed in a separate study where lower doses of a cinnamon polyphenol fraction that significantly blocked cell swelling did not block the OGD-induced depolarization of the $\Delta\Psi_m$ [31]. In addition, we have demonstrated that 3 hr OGD (instead of 5 hr OGD) produced a modest but significant decline in $\Delta\Psi_m$ but did not induce significant cell swelling [33]. In the current study ATRA significantly attenuated the OGD-induced dissipation of mitochondrial $\Delta\Psi_m$. Nevertheless, when taken together with our earlier study it appears that the relation between the mPT and cell swelling is not clear. It is possible that blockade of the mPT may be sufficient in some stresses to block cell swelling whereas in stresses including ischemic injury it may be more important to block a downstream event from the mPT. Regardless, the acute effects of ATRA in the current study include preventing the decline in mitochondrial $\Delta\Psi_m$ in ischemic injury which may be important in attenuating further cellular damage as prolonged dissipation of the $\Delta\Psi_m$ affects ATP production [55]. As mentioned previously, prolonged treatment with ATRA increased the dissipation of the mitochondrial $\Delta\Psi_m$, as assessed for its role in apoptosis [56]. But el-Metwally et al. 2005 [57] reported a normal $\Delta\Psi_m$ in human pancreatic adenocarcinoma cell lines in the first two days when treated with an apoptotic concentration of ATRA (10 μM), indicating that the dissipation of $\Delta\Psi_m$ even in such conditions require either a prolonged exposure or a higher concentration.

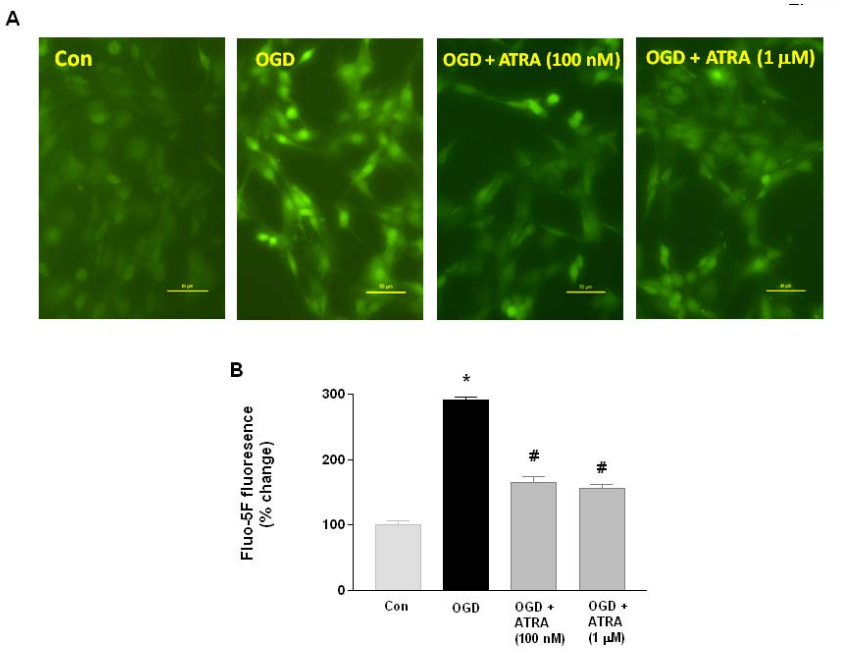

Figure 5. Oxygen-glucose deprivation (OGD)-induced increase in intracellular calcium ($[Ca^{2+}]_i$) is significantly reduced by ATRA (A) Sample photomicrographs of Fluo-5F AM fluorescence after 5 hr OGD in control (Con), OGD, and OGD + ATRA. Immediately after the end of 5 hr OGD, cells were loaded with Fluo-5F AM (1 μM) and trimer 1 in regular media (but without serum) and returned to the normal culture incubator for 30 min before visualization. (B) Quantitative measurement of Fluo-5F fluorescence where the average fluoresence intensities in each experimental group were converted to percentages. Each group consisted of 2-3 culture dishes. Data are presented as mean ± SEM. *p<0.05 vs control. # p<0.05 versus OGD.

Thirdly, ROS/RNS have been hypothesized to induce glial swelling in cultures following ammonia toxicity [41] and TBI [30]. However, we have reported that anti-oxidants including L-NAME, vitamin E, and resveratrol (an anti-oxidant polyphenol), do not reduce cell swelling following OGD in cultures [33]. In addition, lower concentrations of a cinnamon polyphenol fraction that effectively prevented swelling following OGD *in vitro*, did not reduce ROS/RNS production. Further, although donors of nitric oxide (NO) robustly increase cell swelling in cultures [41], a polyphenol-enriched cinnamon extract with anti-oxidant effects also increased NO production following OGD in glial cultures yet prevented cell swelling [33]. These studies indicate a complex relationship between increased oxidative stress and cell swelling in ischemic injury. It is possible that several species of ROS/RNS are activated in ischemic injury and they all have to be blocked together to reduce swelling. The relationship between ROS/RNS production and $\Delta\Psi_m$ to cell swelling, while important, is complex and needs to be investigated in future studies. Taken together, it is possible that the reduction in OGD-induced cell swelling by ATRA is predominantly due to its effects on $[Ca^{2+}]_i$ but its effects on reducing mitochondrial ROS and the decline in $\Delta\Psi_m$ may also be important.

In the current study ATRA significantly reduced mitochondrial ROS but not cellular ROS following OGD. Reduction in mitochondrial ROS by ATRA is consistent with that reported by Ahlemeyer and Krieglstein in 1998 [58], who reported a reduction in mitochondrial ROS by ATRA in response to staurosporin in chick embroyonic cultures. In addition, Choudhary et al. in 2008 [59] also reported a reduction in mitochondrial ROS by ATRA in cardiac myocytes subjected to stretch- and angiotensin II stress. However, the mechanism underlying this difference in action of ATRA in reducing mitochondrial ROS but not cellular ROS is not clear although several possibilities exist. One possibility is that ATRA reduces mitochondrial oxygen consumption thus potentially reducing the production of ROS. Such a phenomenon has been observed in a myeloid cell line where ATRA decreases mitochondrial respiration [60]. Another possibility is the direct binding of ATRA to the adenine nucleotide translocator, a mitochondrial protein that is part of the protein complex that constitutes the mPT and which transports the adenosine diphosphate (ADP) into the mitochondria [61]. Whether such an interaction of ATRA with a mitochondrial protein reflects an increased affinity for ATRA to mitochondria over other cellular organelles and thus an increased efficacy to reduce mitochondrial ROS is not clear but needs to be investigated. Yet another possibility is that ATRA can increase mitochondrial uncoupling [62], most likely by increasing uncoupling protein (UCP) activity as reported in yeast mitochondria [63] and in avian UCP over expressed in yeast [64]. Mild uncoupling can reduce mitochondrial ROS [65]. This possibility seems a bit unlikely since increased uncoupling activity would result in reduced inner mitochondrial membrane potential. But in the current study ATRA significantly attenuated the decline in mitochondrial $\Delta\Psi_m$ although the decline was not completely restored to control levels. It is possible that mild uncoupling in ATRA-treated cells was reflected by a reduced $\Delta\Psi_m$ (below control levels) but this might have also contributed to a concomitant reduction in mitochondrial ROS. Lastly, it should be noted that mitochondrial fluorescence of MitoTracker CM-H_2-XROS is not uniquely indicative of mitochondrial oxidant generation, as ROS that spills into the mitochondria from sources in the cytoplasm may also be detected by this dye [66]. However, if such were the case one would expect ATRA to reduce cellular ROS as well.

Cytotoxic edema, together with vasogenic edema, can be fatal if unresolved, and may also contribute to secondary damage to cells surviving the primary ischemic insult [67]. Cytotoxic edema in ischemia is generally an early event when compared to vasogenic edema [20]. Glial swelling also impairs the ability of astrocytes to effectively clear glutamate and K^+ from the extracellular regions and may further contribute to excitotoxicity [68] (see [69] for review). A reduction in edema may also improve neurological outcome [70] and cerebral microcirculation [71,72]. Interventions that reduce edema and associated secondary damage may be of considerable importance in diminishing the severity of ischemic injury [73]. While several studies have examined the neuroprotective effects of ATRA in ischemia as mentioned above, the effects of ATRA on cytotoxic brain edema are not known. A protective effect of Am80 (tamibarotene), an RAR agonist, in brain edema associated with intracerebral hemorrhage in mice was recently reported [74], but the edema assessed was most likely vasogenic edema. The protective effects of ATRA in normalizing the coagulation/bleeding syndrome in ischemic stroke in a patient has been reported [75].

In conclusion, ATRA attenuated cell swelling following ischemia-like injury and also reduced mitochondrial ROS/RNS production and the dissipation in $\Delta\Psi_m$, as well as the rise in $[Ca^{2+}]_i$. Given that glial swelling is a major component of cytotoxic brain edema in ischemia, which can contribute to further neuronal injury, our results indicate a potential for testing ATRA in attenuating *in vivo* brain edema not only in ischemic injury but also in other neural conditions including TBI and hepatic encephalopathy.

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