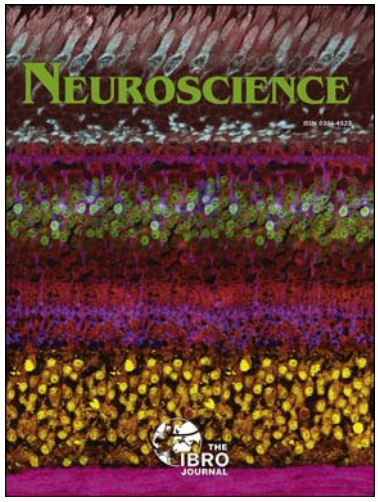

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Tauroursodeoxycholic acid protects retinal neural cells from cell death induced by prolonged exposure to elevated glucose

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Abbreviations

AGEs: advanced glycation end products

AIF: Apoptosis-inducing factor

DCF: Dichlorodihydrofluorescein

DNPH: 2,4-dinitrophenylhydrazine

DPBS: Dulbecco´s PBS solution

ECF: Enhanced chemifluorescence

UDCA: Ursodeoxycholic acid

TUDCA: Tauroursodeoxycholic acid

TUNEL: Terminal transferase dUTP nick end labeling

Abstract

Diabetic retinopathy is one of the most frequent causes of blindness in adults in the Western countries. Although diabetic retinopathy is considered a vascular disease, several reports demonstrate that retinal neurons are also affected, leading to vision loss.

Tauroursodeoxycholic acid (TUDCA), an endogenous bile acid, has proven to be neuroprotective in several models of neurodegenerative diseases, including models of retinal degeneration.

Since hyperglycemia is considered to play a central role in retinal cell dysfunction and degeneration, underlying the progression of diabetic retinopathy, the purpose of this study was to investigate the neuroprotective effects of TUDCA in rat retinal neurons exposed to elevated glucose concentration.

We found that TUDCA markedly decreased cell death in cultured retinal neural cells induced by exposure to elevated glucose concentration. In addition, TUDCA partially prevented the release of apoptosis inducing factor (AIF) from the mitochondria, as well as the subsequent accumulation of AIF in the nucleus. Biomarkers of oxidative stress, such as protein carbonyl groups and reactive oxygen species production, were markedly decreased after TUDCA treatment as compared to cells exposed to elevated glucose concentration alone.

In conclusion, TUDCA protected retinal neural cell cultures from cell death induced by elevated glucose concentration, decreasing mito-nuclear translocation of AIF. The antioxidant properties of TUDCA might explain its cytoprotection. These findings may have relevance in the treatment of diabetic retinopathy patients.

Word count (limit 300 words): 222

Keywords (max 6): Retina, diabetes, diabetic retinopathy, TUDCA, neural apoptosis.

Introduction

Diabetic retinopathy is a leading cause of vision loss and blindness among working age adults. Diabetic retinopathy has been considered a microvascular disease characterized by increased vascular permeability, appearance of macular edema, and neovascularization, which usually occurs at the later stages of the disease and can lead to blindness (Antonetti et al., 2006). However, increasing evidence has shown that the neural components of the retina are also affected, even before the detection of microvascular changes. Alterations in electroretinograms in diabetic patients and animals, and loss of colour and contrast sensitivity are early signs of neuronal dysfunction in the retina (Roy et al., 1986, Daley et al., 1987, Sakai et al., 1995, **Aung et al., 2013**,**Kohzaki et al., 2008**). Apoptosis and increased caspase activation in retinal neurons, as well as retinal thinning have been noted in diabetic animals (Barber et al., 1998, Lieth et al., 2000, Barber et al., 2005, Whitmire et al., 2011, Krady et al., 2005, Gastinger et al., 2006, **Yang et al., 2012**). In postmortem human retinas, an increase in the levels of apoptosis-inducing factor (AIF) was also reported (Abu El-Asrar et al., 2007). Moreover, long-term exposure (7 days) of primary retinal neural cell cultures to elevated glucose concentration, to mimic chronic elevated hyperglycemia, increases cell death in by a caspase-independent mechanism, mediated by the translocation of AIF from the mitochondria to the nucleus (Santiago et al., 2007).

Tauroursodeoxycholic acid (TUDCA) is an endogenous hydrophilic bile acid that is produced at very low levels in humans. TUDCA is formed by the conjugation of ursodeoxycholic acid (UDCA) with taurine. Several reports have demonstrated the hepatic cytoprotective effects of UDCA and TUDCA, with UDCA being widely used in clinical applications such as for the treatment of primary biliary cirrhosis and various other cholestatic disorders (Maillette de Buy Wenniger and Beuers, 2010). Interestingly, it has been proven that TUDCA is neuroprotective in a variety of experimental systems including models of neurodegenerative disorders such as Alzheimer's and Huntington's, as well as against damage induced by ischemia and

hemorrhagic stroke (McLean, 1997, Keene et al., 2002, Rodrigues et al., 2003, Ramalho et al., 2008, Viana et al., 2009, Fernandez-Sanchez et al., 2011). The protective effects of TUDCA are considered to be mainly due to its ability to inhibit apoptosis by reducing Bax translocation to mitochondrial membrane, cytochrome c release from mitochondria to cytoplasm, caspase activation and DNA and nuclear fragmentation (Amaral et al., 2009). Besides these antiapoptotic properties, TUDCA can also function as anti-inflammatory, immunomodulatory and antioxidant agent (Amaral et al., 2009), and activates the PI3-K/Akt-dependent survival pathway (Sola et al., 2003, Amaral et al., 2009). **The molecular mechanisms underlying TUDCA neuroprotective properties appear to be complex and may engage a number of different molecular targets, possibly involving gene regulation, which result in strong anti-apoptotic, anti-inflammatory, immunomodulatory and antioxidant properties (Amaral et al., 2009).**

Regarding retinal diseases, TUDCA greatly slowed retinal degeneration in light-induced retinal degeneration and rd10 mice. Retinal function was improved in mice treated with TUDCA, and retinas of TUDCA-treated mice had thicker outer nuclear layers, more photoreceptor cells, and more fully-developed photoreceptor outer segments. TUDCA effects were explained by dramatic suppression of apoptosis in both models (Boatright et al., 2006). Subsequent studies, confirmed that TUDCA protects retinal damage induced by light and oxidative stress in animal models of retinitis pigmentosa (Phillips et al., 2008, Fernandez-Sanchez et al., 2011, Oveson et al., 2011). Finally, TUDCA showed neuroprotective effects on photoreceptor cells in an experimental retinal detachment model, which were associated with decreased oxidative stress and caspase activity (Mantopoulos et al., 2011). Recently, TUDCA was shown to reduce endoplasmic reticulum stress, prevented apoptosis, and reduced cone degeneration in a mouse model of Leber congenital amaurosis (Zhang et al., 2000). Interestingly, TUDCA ameliorated the obesity that accompanies retinal degeneration in a mouse

model of Bardet-Biedl syndrome (BBS) type 1, an autosomal recessive ciliopathy that causes severe retinal degeneration in humans (Drack et al., 2011).

Since hyperglycemia, the hallmark of diabetes, is considered to play a central role in retinal cell dysfunction and degeneration, underlying the progression of diabetic retinopathy, the purpose of this study was to investigate for the first time the potential neuroprotective effects of TUDCA in rat retinal neurons against cell death induced by elevated glucose concentration.

2. Experimental Procedures

2.1 Primary cultures of rat retinal neural cells

All procedures involving animals were conducted in accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic and vision research.

Retinal cell cultures were prepared as previously described (Santiago et al., 2006a). Briefly, 3- to 4-day-old Wistar rat pups were decapitated, and the retinas were dissected, using a light microscope, in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (in mM: 137 NaCl, 5.4 KCl, 0.45 KH_2PO_4, 0.34 Na_2HPO_4, 4 $NaHCO_3$, 5 glucose; pH 7.4). The retinas were digested with 0.05% trypsin (w/v) for 10 min at 37°C. After dissociation, the cells were pelleted by centrifugation and resuspended in Eagle's minimum essential medium (MEM) supplemented with 26 mM $NaHCO_3$, 25 mM HEPES, 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 µg/ml). The cells were plated at a density of $2x10^6$ cell/cm^2 on poly-D-lysine (0.1 mg/ml)-coated plates. The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2/air. After 2 days in culture, the cells were incubated with 25 mM D-glucose (yielding a total 30 mM glucose) or with 25 mM D-mannitol (plus 5 mM glucose), which was used as an osmotic control, and maintained for further 7 days. The concentration of glucose in control conditions was 5 mM **(control)**. **These conditions are identical to those described previously (Santiago et al., 2007).** TUDCA (100 µM) was added at culture day two, and every other day.

2.2 Annexin-V FITC staining

The loss of plasma membrane asymmetry is one of the earliest features in the apoptotic program. In apoptotic cells, phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane. Annexin V is a Ca^{2+} dependent phospholipid-binding protein with high affinity for PS. Annexin V staining was performed using a kit from BD Biosciences Clontech (San Jose, CA, USA) following the

instructions provided by the manufacturer. Briefly, cells were rinsed and then incubated with Annexin V-FITC (20 µg/ml in Tris-NaCl) for 10 min in the dark at room temperature. Cells were washed and mounted on a glass slide. The preparations were visualized immediately with a Zeiss Axioshop 2 Plus microscope. For each experimental condition, at least 5 random fields were counted in each coverslip.

2.3 Terminal transferase dUTP nick end labeling (TUNEL) staining

TUNEL, with fluorescein detection, was performed in cultured retinal neural cells, following the instructions provided by the manufacturer (Promega). Briefly, cells were washed in PBS and fixed in 4% paraformaldehyde for 20 min at room temperature. After washing, the cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min. The cells were rinsed and incubated with the biotinylated nucleotides and the enzyme Terminal deoxynucleotidyl Transferase (TdT) for 1 h at 37°C. After rinsing with PBS to remove unincorporated nucleotides, the nuclei were counterstained with DAPI, and the preparations were mounted with Glycergel mounting medium. The preparations were visualized using a Zeiss LSM 710 confocal microscope, and at least 15 random fields were counted in each preparation.

2.4 Preparation of total, mitochondrial and nuclear fractions

Total, mitochondrial and nuclear fractions were prepared according our previous reports (Santiago et al., 2006b, Santiago et al., 2007).

2.4.1. Total extracts

Cells were washed twice with ice-cold PBS and then lysed and homogenized with 150 mM NaCl, 50 mM Tris, 5 mM EGTA, 1% Triton X-100, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS) supplemented with complete-mini protease inhibitor cocktail tablets and 1 mM DTT. The lysates were sonicated and then centrifuged at

16100×g for 10 min at 4°C. The resulting supernatant was collected and stored at -80°C until use.

2.4.2 Mitochondrial extracts

Cells were washed in ice-cold sucrose buffer, containing (250 mM sucrose, 20 mM HEPES/KOH (pH 7.4), 1 mM EGTA, and 1 mM EDTA. Cell extracts were obtained in ice-cold sucrose buffer supplemented with 1 mM DTT, 100 µM PMSF, 1 µg/ml chymostatin, 1 µg/ml leupeptin, 1 µg/ml antiparin, and 1 µg/ml pepstatin A. Lysates were homogenized and centrifuged (4°C) at 500×g for 12 min to pellet the nuclei and cell debris. The supernatant was further centrifuged at 12000×g for 20 min. The resulting pellet (mitochondrial fraction) was resuspended in supplemented sucrose buffer and the samples were stored at -80°C until use.

2.4.3. Nuclear extracts

Cells were washed in ice-cold PBS and extracts were obtained using buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA) supplemented with 1 mM DTT and 0.4% Nonidet P-40. The lysates were placed on ice for 30 min and then centrifuged at 15800×g for 5 min at 4 °C. The pellet was resuspended in buffer B (20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA), supplemented with 1 mM DTT and 10% glycerol, and incubated for 60 min on ice. Then, the samples were sonicated and centrifuged at 15800×g for 5 min at 4 °C. The supernatant containing the nuclear fraction was collected, and then stored at -80 °C until use.

2.5 Western blot

The protein concentration of each sample was determined by the bicinchoninic acid protein assay (Pierce Biotechnology, USA), following denaturation with 6x concentrated sample buffer (0.5 M Tris-HCl, 30% glycerol, 10% SDS, 0.6 M DTT, 0.02% bromophenol blue) at 95ºC, for 5 min. Equivalent amounts of protein (40 µg or 15 µg

for AIF detection from mitochondrial fraction or nuclear fraction, respectively) were used for **western** blot analysis. Proteins were separated on 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred electrophoretically to PVDF membranes. The membranes were blocked for 1 h at room temperature, in Tris buffered saline (in mM: 137 NaCl, 20 Tris–HCl; pH 7.6) containing 0.1% Tween-20 (TBS-T) and 5% skimmed milk. The membranes were incubated with the mouse anti-AIF antibody (1:1000), overnight at 4°C. After washing for 1 h in TBS-T with 0.5% skimmed milk, the membranes were incubated for 1 h at room temperature with an alkaline phosphatase-linked secondary antibody (rabbit anti-mouse IgG, 1:10000 in TBS-T with 1% skimmed milk). The membranes were processed for detection of AIF using the ECF substrate. Chemifluorescent signal was detected on an imaging system (Typhoon FLA 9000, GE Healthcare, Chalfont St. Giles, UK) and quantification was performed using Image Quant 5.0 software (Molecular Dynamics, Amersham Biosciences, Uppsala, Sweden).

To control for protein loading, the membranes were reprobed with a mouse anti-α-tubulin (1:3000) or with a mouse anti-lamin B (1:60).

2.6 Immunocytochemistry

Cells were washed twice with PBS and fixed with 4% paraformaldehyde with 4% sucrose for 10 min at room temperature. Cells were rinsed twice in PBS and then permeabilized with 1% Triton X-100 in PBS for 5 min. After blocking for 1 h with 3% BSA plus 0.2% Tween 20 in PBS, cells were incubated with the primary antibody mouse anti-AIF (1:100) for 90 min. Cells were rinsed three times with the blocking solution and incubated with Alexa 488-conjugated secondary antibody (goat anti-mouse IgG, 1:200). The nuclei were stained with DAPI (1:2000) for 5 min. The preparations were visualized with a Zeiss LSM 710 confocal microscope.

2.7 Detection of reactive oxygen species (ROS)

The production of ROS was evaluated using 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA). Briefly, cells were rinsed in pre-warmed Dulbecco´s PBS solution (DPBS, in mM: 0.901 $CaCl_2$, 0.493 $MgCl_2$, 2.67 KCl, 1.47 KH_2PO_4, 137.93 NaCl, 8.06 Na_2HPO_4, 5.56 D-Glucose, 0.327 sodium pyruvate; pH 7.4), and then loaded with 5 µM H_2DCF-DA (or DMSO, blank), for 45 min in the cell incubator. Cells were rinsed twice with DPBS, and then incubated for further 20 min in pre-warmed DPBS in the cell incubator. Cells were harvested by scraping, and an aliquot was saved for protein quantification.

Fluorescence measurements were made using a Synergy HT Microplate Reader (BioTek Instruments) set to 37°C. Measurements were made using a 485/20 excitation and a 528/20 emission filter pair and a photomultiplier sensitivity setting of 55. Blank readings were subtracted from loaded sample readings, and values were expressed as fluorescence units per milligram of protein (FU/mg).

2.8 Protein-bound carbonyl determination

For derivatization of carbonyl-containing proteins, equal amounts of proteins (total extracts) were mixed with an equal volume of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 10% trifluoroacetic acid and incubated at room temperature for 15 min. The reaction was stopped by precipitation of proteins with 20% trichloroacetic acid. The pellet was washed with ethylacetate-ethanol (1:1) to remove free DNPH. Next, the pellet was solubilized with Laemmli buffer (15.6 mM Tris-HCl pH 6.8, 2.5% glycerol, 0.5% SDS, 1.25% 2-β-mercaptoetanol, 0.01% bromophenol blue).

The DNP binding sites of the oxidized proteins were specifically detected using an anti-DNP antibody by dot blot. Briefly, 2 µl of the protein carbonyl-DNPH derivatives were loaded on a nitrocellulose membrane at each dot. Membranes were then incubated at 37°C for 40 min, to fix proteins to the membrane. After blocking the membrane with TBS-T containing 5% (w/v) skimmed milk, during 1 h at room temperature, the membranes were incubated with a goat anti-DNP primary antibody (1:2000, in TBS-T

solution with 5% skimmed milk) for 1 h at room temperature. The membranes were washed in TBS-T, and then incubated for 1 h at room temperature, with an alkaline phosphatase-linked rabbit anti-goat IgG secondary antibody (1:10000), in TBS-T containing 1% (w/v) skimmed milk, followed by washing in TBS-T. Protein immunoreactive dots were then visualized using ECF substrate, and fluorescence was detected on an imaging system (Typhoon FLA 9000, GE Healthcare, Chalfont St. Giles, UK). Digital quantification of the densitometry of the bands was performed using Image Quant 5.0 software (Molecular Dynamics, Amersham Biosciences, Uppsala, Sweden).

2.9 Statistical analysis

Data are expressed as mean±SEM. Statistical significance was determined by analysis of variance (ANOVA), followed by Dunnett's or Bonferroni's post-hoc tests, as indicated in the figure legends.

3. RESULTS

3.1 TUDCA prevents retinal neural cell death induced by elevated glucose concentration

Previously, **using the same cell culture conditions**, we found that elevated glucose concentration induces cell death by a mechanism independent of caspase activation (Santiago et al., 2007). Since TUDCA has neuroprotective properties, we investigated whether TUDCA could protect retinal neurons from cell death induced by elevated glucose concentration.

Therefore, to quantify cell death by apoptosis at an early stage (Figure 1A), we used annexin V conjugated to fluorescein isothiocyanate (FITC). Exposure of cultured retinal cells to elevated glucose concentration significantly increased annexin-V positive cells as compared to **control cells** (140.1±10.4% of the control; $P<0.01$). When cells exposed to elevated glucose concentration were co-treated with TUDCA, there was a significant decrease in the number of annexin V-positive cells as compared to cells exposed to elevated glucose concentration alone (101.2±7.3% of the control; $P<0.01$).

In cells cultured in 5 mM glucose and treated with TUDCA (TUDCA-treated cells) there was no significant effect as compared control. The incubation of cells with mannitol, in the presence or absence of TUDCA, did not change the number of cells labeled with annexin V as compared to control cells.

TUNEL assay was used to quantify cells with fragmented DNA (Figure 1B). Exposure of cells to elevated glucose concentration significantly increased the number of TUNEL-positive cells (144.6±9.9% of control; $P<0.001$). Co-treatment with TUDCA completely prevented the increase in TUNEL-positive cells induced by elevated glucose concentration (101.9±6.9% of control). The number of TUNEL-positive cells in the mannitol-treated group was not significantly different from control.

3.2 TUDCA prevented AIF mito-nuclear translocation induced by elevated glucose concentration

We have previously found that long-term exposure of retinal neuronal cells to elevated glucose concentration induced the translocation of AIF from the mitochondria to the nucleus (Santiago et al., 2007). Therefore, we investigated whether TUDCA could prevent AIF translocation induced by elevated glucose concentration.

As reported previously, elevated glucose concentration significantly decreased the protein levels of AIF in the mitochondrial fraction (Figure 2A), as compared with **control cells** (34.6±4.2% of control; $P<0.01$). Treatment with TUDCA partially prevented the decrease of AIF in the mitochondrial fraction induced by elevated glucose concentration. In the nuclear fraction (Figure 2B), AIF was significantly increased in cells exposed to elevated glucose concentration (136.3±18.3% of the control; $P<0.05$). The treatment with TUDCA prevented the increase of AIF protein levels in the nuclear fraction of cells exposed to elevated glucose concentration. TUDCA alone did not change AIF protein levels, both in mitochondrial or nuclear fraction, as compared with control cells. By immunocytochemistry (Figure 2C), we observed that AIF immunoreactivity in control cells was mainly found in the perinuclear region, while in elevated glucose-treated cells AIF immunoreactivity was also observed in the nucleus, **corroborating the western data.** When cells exposed to elevated glucose concentration were co-treated with TUDCA, the immunoreactivity of AIF was similar to control, confirming the **western** blot data.

3.3 TUDCA prevented oxidative stress induced by elevated glucose concentration

It is well established that elevated glucose concentration increases oxidative stress (Pennathur and Heinecke, 2004) and it was also reported that TUDCA protects against oxidative-stress induced retinal damage (Mantopoulos et al., 2011, Oveson et al., 2011). We investigated whether TUDCA could protect against oxidative stress induced by elevated glucose concentration in retinal neural cells (Figure 3A). ROS production was assessed by quantifying DCF fluorescence. In elevated glucose concentration-

treated cells, DCF fluorescence significantly increased to 192.6±23.3% of the control ($P<0.01$). This effect was not due to changes in osmolarity, since DCF fluorescence in mannitol-treated cells was not significantly altered compared to **control cells**. Co-treatment with TUDCA significantly decreased DCF fluorescence to 145.9±9.6% of the control ($P<0.05$), as compared to cells exposed to elevated glucose concentration.

Protein carbonyl formation was used as an indicator of oxidized proteins (Figure 3B). We found that oxidative injury to carbonyl residues significantly increased in elevated glucose-treated cells, as compared with control cells (119.9±7.1% of the control; $P<0.05$). The treatment with TUDCA prevented the increase in DNP immunoreactivity, as compared to elevated glucose concentration-treated cells. **In TUDCA-treated** cells there were not significant changes.

4. DISCUSSION

In this study, we demonstrated that TUDCA prevents retinal cell death and mito-nuclear translocation of AIF induced by elevated glucose concentration.

Although diabetic retinopathy has been considered a microvascular disease, there is accumulating evidence showing that retinal neurons are also affected by diabetes (Antonetti et al., 2006, Barber et al., 2011, Whitmire et al., 2011). Indeed, retinal neurons have been shown to undergo apoptosis upon diabetes (Barber et al., 1998, Abu-El-Asrar et al., 2004, Barber et al., 2005). **Cultured retinal neural cells were incubated with elevated glucose concentration for seven days to mimic chronic hyperglycemia, which is considered the main trigger for the development of diabetic complications as diabetic retinopathy.**

TUDCA is a bile acid derivative which has been reported to modulate apoptosis and survival signaling (Sola et al., 2003), endoplasmic reticulum-mediated stress (Gao et al., 2011, Xie et al., 2002, Ozcan et al., 2006) and oxidative stress (Oveson et al., 2011) in several models of degenerative diseases. These effects may explain the neuroprotective role of TUDCA (Duan et al., 2002, Rodrigues et al., 2002, Rodrigues et al., 2003, Sola et al., 2003, Viana et al., 2009). In the retina, it was demonstrated that TUDCA prevents photoreceptor degeneration in models of retinitis pigmentosa and of Leber congenital amaurosis (Phillips et al., 2008, Fernandez-Sanchez et al., 2011, Zhang et al., 2000). TUDCA also ameliorated the obesity that accompanies retinal degeneration in a model of Bardet-Biedl syndrome type 1 (Drack et al., 2011).

TUDCA directly inhibits reactive oxygen species production, thus inhibiting the collapse of the transmembrane potential and the disruption of the outer mitochondrial membrane (Rodrigues et al., 2002), inhibiting cytochrome c release, thereby reducing downstream events such as caspase activation and substrate cleavage (Ramalho et al., 2008, Amaral et al., 2009, Viana et al., 2009).

Previously, we demonstrated that prolonged elevated glucose concentration, mimicking chronic hyperglycemia, induces caspase-independent cell death in retinal neural cells,

through the translocation of AIF from the mitochondria to the nucleus (Santiago et al., 2007). In the present study, TUDCA prevented AIF translocation induced by elevated glucose concentration.

The treatment of retinal neural cells exposed to elevated glucose concentration with TUDCA reduced cell death. In our model, high glucose concentration increased nuclear condensation, DNA fragmentation and the exposure of annexin V at the outer leaflet of cell membranes, which are apoptotic features, and these effects were completely prevented by TUCDA. Moreover, in the same experimental model, we demonstrated that exposure of retinal neurons to elevated glucose concentration impairs calcium homeostasis (Santiago et al., 2006b), and it is known that the prolonged increase in intracellular calcium concentration ($[Ca^{2+}]_i$) cause cell dysfunction and death (Duchen, 2004). Moreover, the increase in $[Ca^{2+}]_i$ induces mitochondrial membrane permeabilization, leading to the release of soluble intermembrane proteins, such as AIF, through the outer mitochondrial membrane (Bains et al., 2003). Therefore, it is likely that in our model TUDCA prevents the mito-nuclear translocation of AIF by the stabilization of the mitochondrial membrane. In fact, it was demonstrated that AIF is increased in the retina of postmortem diabetic patients (Abu El-Asrar et al., 2007), and so TUDCA could be viewed as a potential therapeutic agent for the treatment of retinal diabetic complications.

Oxidative stress is believed to play a significant role in the pathogenesis of diabetic retinopathy. The increase in oxidative stress in the retina under diabetic conditions results from a variety of abnormalities, including glucose auto-oxidation, decreased antioxidant enzyme activity, and formation of **advanced glycation end products** (AGEs) and interactions of AGEs with membrane receptors (Giacco and Brownlee, 2010). In fact, it is well established that high glucose induces a sustained increase in ROS production (Pennathur and Heinecke, 2004, Wang et al., 2011), which in turn can contribute to vascular and neuronal damage in diabetes (Tomlinson and Gardiner, 2008). Our results showed that TUDCA partially prevented increased ROS production

in cells exposed to elevated glucose concentration. **Keeping ROS at normal levels prevents activation of protein kinase C, formation of AGEs, sorbitol accumulation, and NF-κB activation, all of which are coupled with diabetes-induced cell damage (Nishikawa et al., 2000). Although we did not assess the formation of AGEs, we can speculate AGEs formation would be reduced in cells exposed to elevated glucose concentration and treated with TUDCA.** Recently, it was demonstrated that TUDCA reduces oxidative stress in a model of prolonged light exposure, protecting retinal degeneration (Oveson et al., 2011). In this model, the predominant effect of TUDCA was to preserve cone cell function and structure. Since increased ROS levels can be triggered by mitochondrial dysfunction leading to an energy deficit (Kowluru and Chan, 2007), it is possible that the antioxidant effect of TUDCA may be due to its ability in suppressing mitochondrial membrane perturbation (Rodrigues et al., 2003). TUDCA was also able to preserve photoreceptors after retinal detachment, and that protective effect was associated with a decrease in oxidative stress and caspase activity (Mantopoulos et al., 2011). In our in vitro model, cell death is independent of caspase activation (Santiago et al., 2007); however, TUDCA was also able to prevent cell death and translocation of AIF from the mitochondria to nucleus. **To our knowledge, this is the first report showing a neuroprotective effect of TUDCA involving the inhibition of AIF translocation to the nucleus.**

In conclusion, we demonstrate that TUDCA is capable of preventing cell death induced by elevated glucose through its anti-apoptotic and antioxidant properties in **cultured retinal neural cells**. **More experiments are needed to take definite conclusions, but considering these and others results, TUDCA could be envisaged as a potential neuroprotective strategy to prevent retinal apoptosis in diabetes.**

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Legends

Figure 1 – TUDCA prevents the increase in apoptosis in retinal neural cells induced by elevated glucose. Cells were incubated in 5 mM glucose (Control), 30 mM glucose (Glucose) or 25 mM mannitol (Mannitol; + 5 mM glucose) for seven days in the presence or absence of 100 μM TUDCA (added every other day). The number of annexin V-FITC positive cells (A) and TUNEL-positive cells (B) was counted. The results are expressed as percentage of control, and represent the mean ± SEM of 4-6 independent experiments. **p<0.01, compared to control, ANOVA followed by Dunnett's post-hoc test; ++p<0.01, +++p<0.001, significantly different compared to glucose, ANOVA followed by Bonferroni's post-hoc test. Arrows indicate TUNEL-positive cells (green). Nuclei were stained with DAPI (blue). Scale bar: 10 μm.

Figure 2 – TUDCA prevents translocation of AIF from the mitochondria to the nucleus induced by elevated glucose. Cells were incubated in 5 mM glucose (Control) or 30 mM glucose (Glucose) for seven days in the presence or absence of 100 μM TUDCA (added every other day). Mitochondrial (A) and nuclear (B) extracts were prepared and assayed for AIF by **western** blot. Representative **western** blots presented above each graph. Subcellular localization of AIF was analyzed by immunocytochemistry (C) using an antibody against AIF (green). Nuclei were stained with DAPI (blue). Scale bar: 10 μm. The results are expressed as percentage of control, and represent the mean ± SEM of 3-9 independent experiments. *p<0.05, **p<0.01 compared to control, ANOVA followed by Dunnett's post-hoc test; +p<0.05, ++p<0.01 compared to glucose, ANOVA followed by Bonferroni's post-hoc test.

Figure 3 – TUDCA prevents oxidative stress induced by elevated glucose concentration. Cells were incubated in 5 mM glucose (Control) or 30 mM glucose (Glucose) for seven days in the presence or absence of 100 μM TUDCA (added every

other day). (A) Generation of ROS was assessed by using the H_2DCF probe. The results represent DCF fluorescence in arbitrary units (AUF) per mg protein and are expressed as percentage of control, of at least four independent experiments. (B) Oxidized proteins were detected by dot blot using an anti-DNP antibody. A representative dot blot is shown above the graph. Data are presented as percentage of control and represent the mean ± SEM of 4-6 independent experiments. *$p<0.05$, **$p<0.01$ compared to control, ANOVA followed by Dunnett's post hoc test; +$p<0.05$, +++$p<0.001$ compared to glucose, ANOVA followed by Bonferroni's post-hoc test.

Figure 1A

A

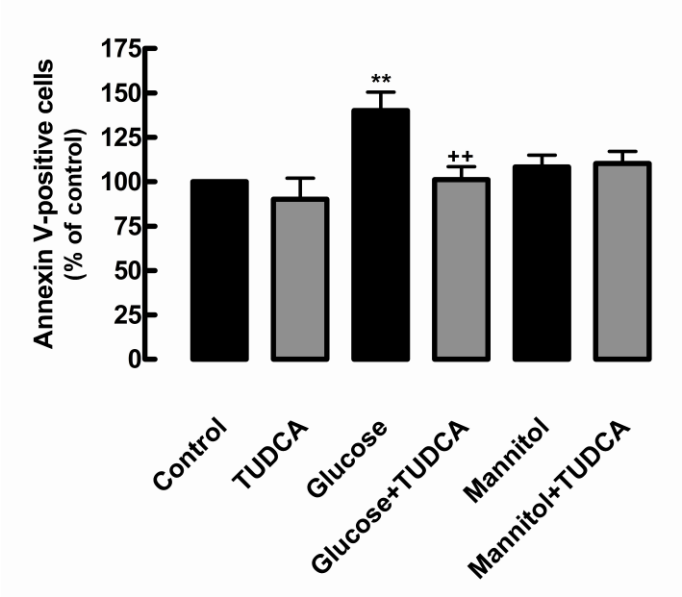

Annexin V-positive cells
(% of control)
175
150
125
100
75
50
25
0
**
++
Control
TUDCA
Glucose
Glucose+TUDCA
Mannitol
Mannitol+TUDCA

Figure 1B

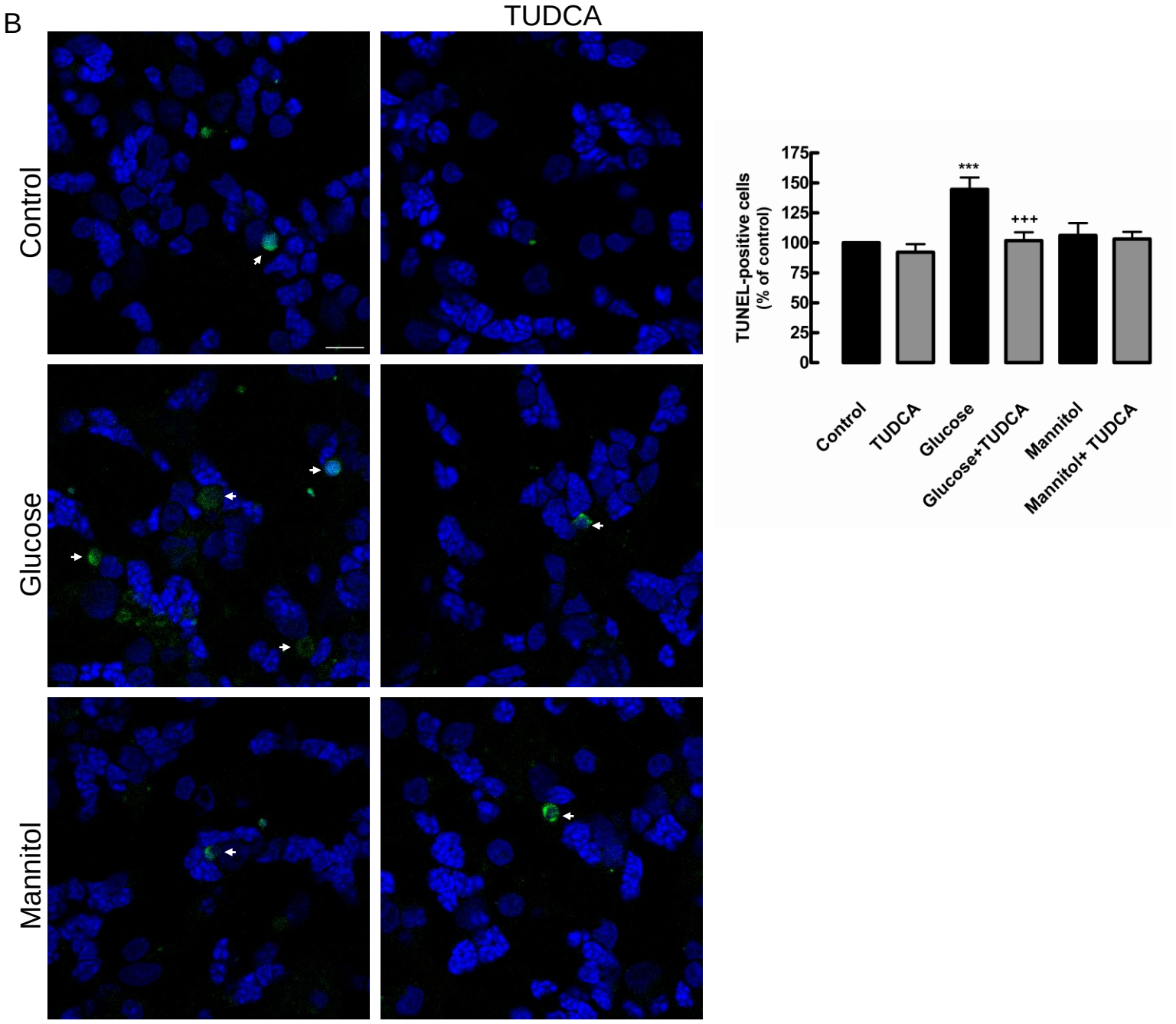
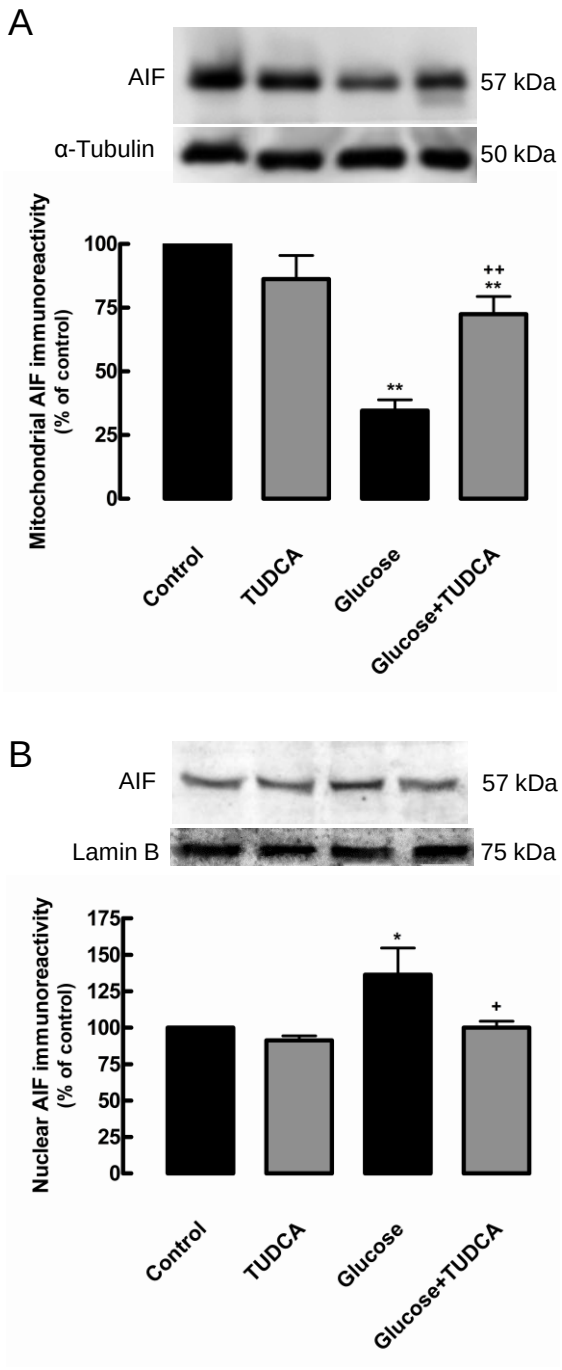

B
TUDCA
Control
Glucose
Mannitol
TUNEL-positive cells
(% of control)
175
150
125
100
75
50
25
0
***
+++
Control
TUDCA
Glucose
Glucose+TUDCA
Mannitol
Mannitol+ TUDCA

Figure 2


A
AIF
57 kDa
α-Tubulin
50 kDa
Mitochondrial AIF immunoreactivity (% of control)
100
75
50
25
0
**
++
**
Control
TUDCA
Glucose
Glucose+TUDCA
B
AIF
57 kDa
Lamin B
75 kDa
Nuclear AIF immunoreactivity (% of control)
175
150
125
100
75
50
25
0
*
+
Control
TUDCA
Glucose
Glucose+TUDCA

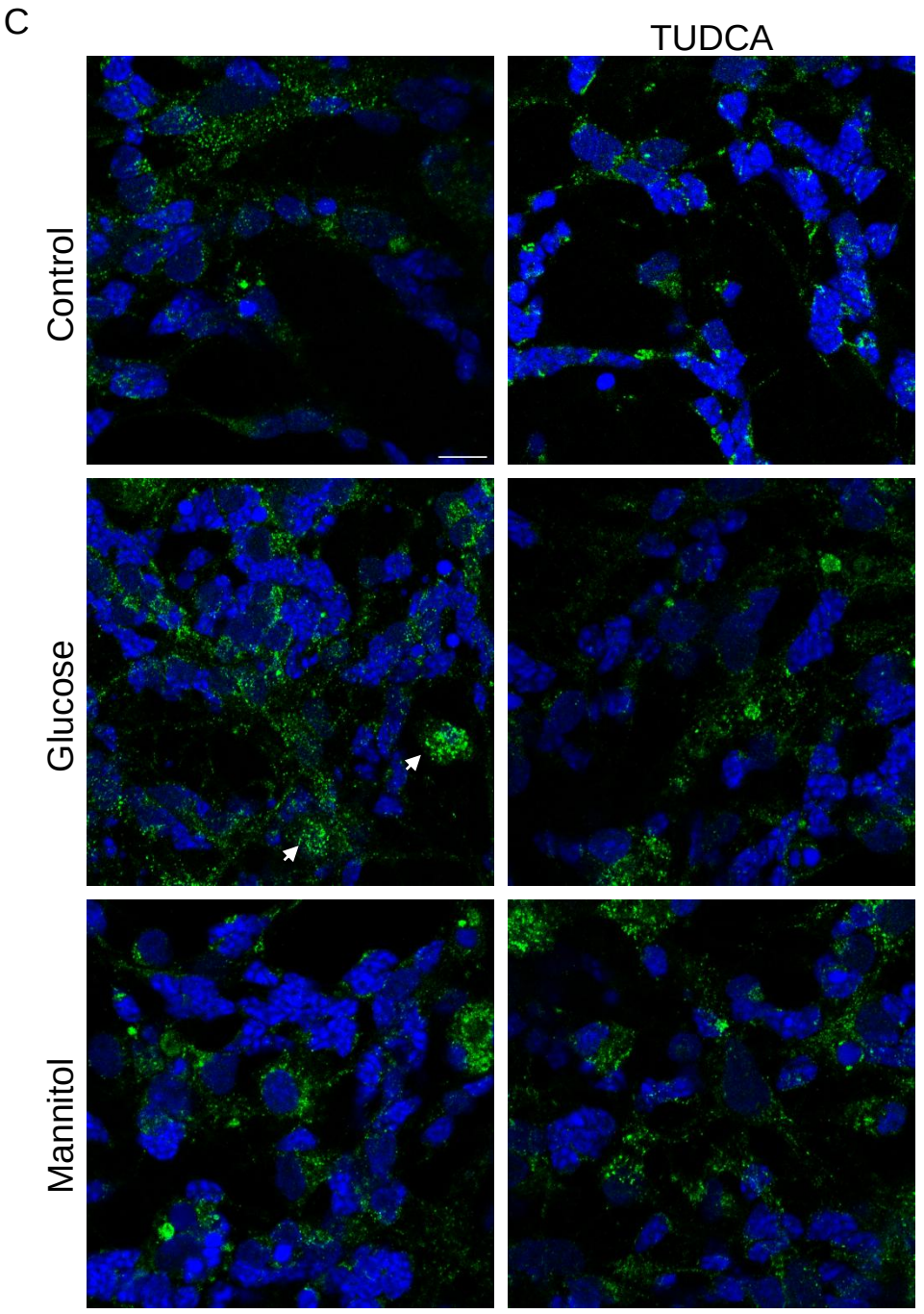
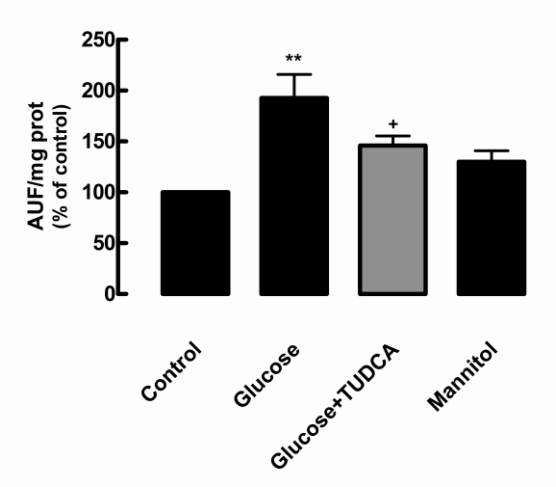
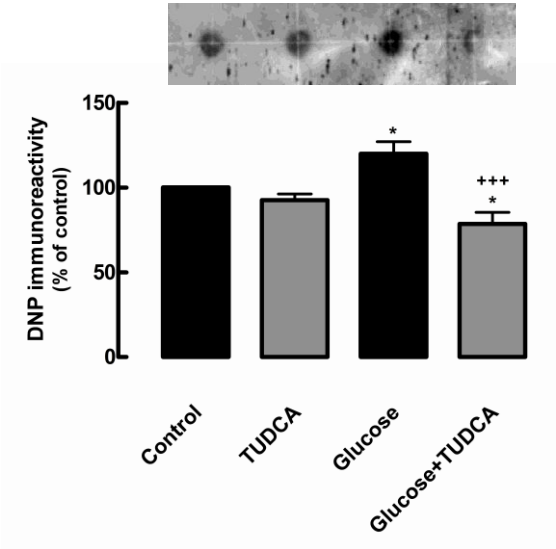

C
TUDCA
Control
Glucose
Mannitol

Figure 3

A


AUF/mg prot
(% of control)
250
200
150
100
50
0
**
+
Control
Glucose
Glucose+TUDCA
Mannitol

B


DNP immunoreactivity
(% of control)
150
100
50
0
*
+++
*
Control
TUDCA
Glucose
Glucose+TUDCA

Manuscript “Tauroursodeoxycholic acid protects retinal neural cells from cell death induced by prolonged exposure to elevated glucose”

Highlights

1. TUDCA prevented cell death induced by high glucose concentration
2. TUDCA prevented AIF translocation induced by high glucose concentration
3. TUDCA prevented oxidative stress induced by elevated glucose concentration