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Mechanisms regulating plasminogen activators in transformed retinal ganglion cells

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Abstract

Irreversible loss of retinal ganglion cells (RGCs) is a major clinical issue in glaucoma, but the mechanisms that lead to RGC death are currently unclear. We have previously reported that elevated levels of tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) cause the death of RGCs *in vivo* and transformed retinal ganglion cells (RGC-5) *in vitro*. Yet, it is unclear how secreted proteases such as tPA and uPA directly cause RGCs' death. In this study, by employing RGC-5 cells, we report that tPA and uPA elicit their direct effect through the low-density lipoprotein-related receptor-1 (LRP-1). We also show that blockade of protease-LRP-1 interaction leads to a complete reduction in autocrine synthesis of tPA and uPA, and prevents protease-mediated death of RGC-5 cells. RGC-5 cells were cultured in serum-free medium and treated with 2.0 μ M Staurosporine to induce their differentiation. Neurite outgrowth was observed by a phase contrast microscope and quantified by NeuroJ imaging software. Proteolytic activities of tPA and uPA were determined by zymography assays. Cell viability was determined by MTT assays. Compared to untreated RGC-5 cells, cells treated with Staurosporine differentiated, synthesized and secreted elevated levels of tPA and uPA, and underwent cell death. In contrast, when RGC-5 cells were treated with Staurosporine along with the receptor associated protein (RAP), proteolytic activities of both tPA and uPA were significantly reduced. Under these conditions, a significant number of RGC-5 cells survived and showed increased neurite outgrowth. These results indicate that LRP-1 regulates autocrine synthesis of tPA and uPA in RGC-5 cells and suggest that the use of RAP to antagonize the effect of proteases may be a way to prevent RGC death in glaucoma.

Keywords

Retinal ganglion cells; cell surface receptor; RGC-5; tPA; uPA; RAP; LRP-1

Introduction

Although loss of RGCs is a major clinical problem in glaucoma (Nickells, 2004, Osborne, et al., 2004, Osborne, et al., 1999, Quigley, 1996, Weinreb and Khaw, 2004), the mechanisms underlying death of RGCs are still poorly understood. By employing a number of animal models related to glaucoma, we have previously reported that elevated levels of tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) cause the death of RGCs *in vivo* (Mali, et al., 2005, Zhang, et al., 2003). By employing an *in vitro* system, in

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which levels of tPA and uPA can be controlled, we have further reported that elevated levels of tPA and uPA, acting in autocrine fashion, directly cause the death of RGC-5 cells (Harvey and Chintala, 2007), which were derived by transforming postnatal day 1 rat retinal cells with ψ_2 E1A virus (Krishnamoorthy, et al., 2001). Yet, it is unclear how tPA and uPA, which are secreted proteases, directly cause death of RGC-5 cells. A better understanding of these mechanisms will have broader implications to prevent loss of RGCs in glaucoma.

The autocrine effect of proteases observed in RGC-5 cells indicates that these proteases might act on a cell surface receptor to cause cell death. One such receptor is the low-density lipoprotein receptor-related protein-1 (LRP-1). LRP-1, a member of the LDL receptor superfamily, is well-known for its classical role in scavenging chylomicron remnants in the liver (Herz, 2003, Herz and Strickland, 2001). Subsequent studies, however, have demonstrated that LRP-1 also acts as a multifunctional scavenging receptor by transporting a variety of ligands including proteases such as tPA and uPA into endosomes in the cells. In the reduced pH environment of endosomes, ligands including proteases uncouple from LRP-1 and are sorted into lysosomes for degradation whereas LRP-1 recycles back to the cell surface. During this recycling process, the extracellular domain of LRP-1 recognizes a number of “extracellular ligands” including lipoproteins, proteases, protease-inhibitor complexes, bacterial toxins, and extracellular matrix (ECM) proteins such as thrombospondin-1 and thrombospondin-2. The extracellular domain of LRP-1 also recognizes numerous “intracellular proteins” including heat shock protein-96 (HSP-96), HIV-1 tat protein, and receptor associated protein (RAP).

RAP is a soluble endoplasmic reticulum (ER) protein (39 kDa) that functions as a molecular chaperone by assisting the recycling of LRP-1 to the cell surface. A salient feature of RAP is that while residing in the ER, it prevents the premature binding of “intracellular ligands” to LRP-1, but, when added exogenously, RAP prevents the binding of all “extracellular ligands” including proteases to LRP-1 (Bu, et al., 1995, Bu and Rennke, 1996, Hahn-Dantona, et al., 2001, Wang, et al., 2003, Warshawsky, et al., 1993, Warshawsky, et al., 1993, Willnow, et al., 1996) as shown in embryonic fibroblasts (Hahn-Dantona, et al., 2001), primary human cerebral microvascular endothelial cells (Wang, et al., 2003), astrocytes, and central nervous system neurons (Bu, et al., 1992, Fernandez-Monreal, et al., 2004, Warshawsky, et al., 1993, Zhuo, et al., 2000). However, it is unclear whether LRP-1 regulates expression of tPA and uPA in RGCs. Therefore, using RGC-5 cells, in which expression of tPA and uPA can be induced, we have investigated whether RGC-5 cells express LRP-1 and whether LRP-1 regulates autocrine synthesis of tPA and uPA.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate buffered saline (DPBS), Penicillin, and Streptomycin were obtained from Invitrogen Corporation (Carlsbad, CA). Staurosporine was obtained from Alexis Biochemicals (San Diego, CA). Human plasminogen (Product #410), human fibrinogen (Product #431), and tPA-STOP (2,7-bis-[4-amidinobenzylidene]-cycloheptanone-1-one dihydrochloride; Product #544), 2-chain recombinant tPA (Product #174), and human urokinase (Product #124) were obtained from American Diagnostica (Stamford, CT). MTT was obtained from Sigma Chemical Company (St. Louis, MO). Recombinant rat Receptor Associated Protein (RAP) was obtained from Dr. Guojun Bu (obtained from Dr. Guojun Bu, Washington University School of medicine, St. Louis, MO).

Cell Culture

Transformed retinal ganglion cells (Krishnamoorthy, et al., 2001), RGC-5 (obtained from Dr. Neeraj Agarwal), were routinely cultured in DMEM containing 1g/L glucose, supplemented with 10% fetal bovine serum (FBS), 100 u/mL penicillin, and 100 ug/mL streptomycin. Although RGC-5 cells express neuronal markers characteristic of normal RGCs, RGC-5 cells have some features significantly different from normal RGCs: they are undifferentiated, mitotically active, morphologically similar to fibroblasts, and do not express some ion channels characteristic of RGCs. Therefore, we treated RGC-5 cells with the broad-spectrum protein kinase inhibitor staurosporine (SS), which results in mitotically inactive cells with multiple branched neurites characteristic of a neuronal morphology (Frassetto, et al., 2006, Harvey and Chintala, 2007). Where indicated, RGC-5 cells were either left untreated or treated with indicated concentrations of RAP and Staurosporine in DMEM containing no FBS.

Cell morphology

Morphology of the cells was assessed by using an inverted, phase contrast, and bright-field microscope. Digitized images were obtained using a Nikon digital camera and the images were saved as JPEG files. Neurite outgrowth of RGC-5 cells was assessed by using NeuroJ image software (version 1.36b) downloaded from <http://rsb.info.nih.gov/ij>. By defining neurite as a projection from the cell that was as long as or longer than one cell diameter, neurite outgrowth from a total of 40 cells from each condition were analyzed according to previously published methods (Frassetto, et al., 2006, Harvey and Chintala, 2007). Where indicated, the average length of neurites from three independent experiments was expressed as the mean $\pm$ SEM.

Immunohistochemistry

RGC-5 cells grown in 4-well chamber slides (2×10^3 cells/well) were fixed with 4% Para formaldehyde (prepared in phosphate buffered saline [PBS]) for 15 minutes at room temperature. After permeabilizing the cells with 0.2% Triton-x-100 (in PBS) for 5 minutes, cells were treated with 4% goat serum for 30 min at room temperature. The cells were washed three times (5 min each) with PBS and incubated with polyclonal antibodies against phosphorylated Tau (pS396; 1:100 dilution; Biosource, Camarilla, CA) for 1 hr at room temperature. After washing three times (5 min each) with PBS cells were incubated for 1 hr at room temperature with antibodies conjugated to Alexa Flour 488. Finally, the cells were washed three times with PBS (5 min each), mounted using Fluoromount-G and observed under a microscope equipped with epifluorescence equipment. Where indicated, cells were also counterstained with 1mg/ml 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes, Eugene, OR) and representative results from two independent experiments were shown.

Western blot analysis

RGC-5 cells were left untreated or treated with Staurosporine along with indicated concentrations of RAP. After the treatment, cells extracts were prepared, aliquots containing equal amounts (50 ug) of cell extracts were mixed with gel loading buffer, boiled for 5 minutes, and separated on 10% SDS-polyacrylamide gels. After separation, the proteins were transferred to PVDF membranes and incubated for 1 hr in 10% nonfat dry milk prepared in Tris-buffered saline containing 0.1% Tween-20 (TBS-T). PVDF membrane were then probed with polyclonal antibodies against LRP-1 (1:1000 dilution; Orbigen, San Diego, CA), phosphorylated Tau (pS396; 1:2000 dilution), or actin (1:2500 dilution; Sigma, St. Louis, MO). After incubation with primary antibodies overnight, membranes were washed with TBS-T and incubated for 1 h at room temperature with appropriate secondary antibodies conjugated to horse radish peroxidase (HRP). Finally, the proteins were detected using an ECL

chemiluminescence kit (Pierce, Rockford, IL) and exposing the membranes to X-ray film and representative results from two independent experiments were shown.

Cell viability

Cells plated at 4×10^3 cells/mL in 96-well tissue culture plates were left untreated or treated with indicated concentrations of RAP and Staurosporine in DMEM containing no FBS. At forty-eight hours after treatment, cell viability was determined by incubating cells with 1.2 mM MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) for 2 hrs at 37 C. At the end of 2 hrs, the formazan product formed by viable cells was dissolved in 0.01 M HCl, and the optical density was read at 570 nm using an automated spectrophotometer.

Zymography

tPA and uPA proteolytic activity in RGC-5 cells was determined by substrate zymography according to methods described previously (Harvey and Chintala, 2007). Briefly, aliquots containing equal amount of conditioned medium (20 uL) or cell extracts (50 ug), collected at defined intervals after each treatment were mixed with 4× SDS gel-loading buffer and loaded onto 10% SDS polyacrylamide gels containing fibrinogen (5.5 mg/mL) and plasminogen (50 ug/mL). The gels were washed three times with 2.5% Triton-X 100 (15 min each time) to remove SDS from the gels, placed in 0.1M glycine-buffer (pH 8.0), and incubated overnight at 37 C to allow proteolysis of the fibrinogen and plasminogen in the gels. After overnight incubation, the gels were stained with 0.2% Coomassie Brilliant Blue-R250 for 5 min and then de-stained with a solution containing 50% methanol and 10% acetic acid in de-ionized water. Proteolytic activity of tPA and uPA as evidenced by clear bands in the zymograms was scanned and relative protease activity levels were determined by using Scion image analysis software (Scion Corporation, Frederick, MD). Where indicated, the proteolytic activity in the gels from three independent experiments was represented as mean arbitrary densitometric units $\pm$ SEM. Statistical significance was analyzed by using a non-parametric Newman-Keuls analog procedure (GB-Stat Software, Dynamic Microsystems, Silver Spring, MD).

Results

Expression of LRP-1 in RGC-5 cells

Since RGC-5 cells are mitotically active and show fibroblastic morphology, they were treated with Staurosporine to halt cell division and to induce differentiation. Inhibition of cell division was assessed by bromodeoxyuridine (BrdU)-labeling (data not shown) and differentiation was assessed by neurite outgrowth. In our experience, Staurosporine induces differentiation of RGC-5 cells all the time and 75–80% RGC-5 cells become non-mitotic (BrdU-negative) after 48 h treatment with 2 uM Staurosporine.

To determine whether RGC-5 cells express LRP-1, RGC-5 cells were left untreated or treated with 0.5 uM and 2.0 uM Staurosporine for 16–18 hrs. At the end of treatment, cells were collected and lysed in RIPA buffer. Aliquots containing equal amount of cell extracts were then subjected to western blot analysis using antibodies against LRP-1. Results presented in Fig. 1 indicate that undifferentiated RGC-5 cells (left untreated) constitutively express low levels of LRP-1. In addition, both immunohistochemical (**A**) and western blot analysis (**B**) results indicate that differentiated RGC-5 cells (treated with Staurosporine) express increased levels of LRP-1.

Effect of RAP on synthesis of tPA and uPA

We have previously shown that compared to undifferentiated RGC-5 cells, differentiated cells synthesize and secrete elevated levels of tPA and uPA into the conditioned medium (Harvey

and Chintala, 2007). To determine whether synthesis of these proteases is mediated by LRP-1, RGC-5 cells were left untreated or treated with 2.0 μ M Staurosporine along with varying concentrations of rat RAP. At forty-eight hours after the treatment, conditioned medium and total proteins from RGC-5 cells were collected, and plasminogen/fibrinogen zymography assays were performed to determine tPA and uPA proteolytic activity in the conditioned medium (Fig. 2A,B,C, extracellular) and cell extracts (Fig. 2A,B,C, intracellular). Results indicate that RGC-5 cells left untreated synthesize and secrete low levels of uPA, but not tPA. RGC-5 cells treated with Staurosporine (2 μ M) synthesized elevated levels of uPA as well as tPA, and secreted both the proteases into the conditioned medium, consistent with our previous report. In contrast, RGC-5 cells treated with 2 μ M Staurosporine and increasing doses of RAP synthesized reduced levels of tPA and uPA in a dose-dependent fashion. RGC-5 cells treated with RAP also secreted reduced levels of tPA and uPA into the conditioned medium. A similar reduction in tPA and uPA synthesis was observed when RGC-5 cells were treated with recombinant human RAP obtained from Dr. Dudley Strickland of American Red cross, Rockville, MD (data shown). These results suggest that LRP-1 regulates synthesis of tPA and uPA in RGC-5 cells.

Effect of RAP on survival of RGC-5 cells

To determine whether RAP, which inhibited the synthesis and secretion of tPA in RGC-5 cells (figure 2), also prevents protease-mediated cell death, RGC-5 cells were left untreated or treated with 2 μ M Staurosporine (SS) along with varying doses of recombinant rat RAP. At forty-eight hours after treatment, cell viability was determined by incubating cells with 1.2 mM MTT. The results presented in Fig. 3 indicate that compared to RGC-5 cells left untreated, viability of cells was significantly decreased when treated with Staurosporine. In contrast, compared to cells treated with Staurosporine alone, viability of RGC-5 cells treated with RAP and SS increased significantly. These results along with results presented in figure 2 indicate that RAP not only inhibits the autocrine synthesis of tPA and uPA, but it also prevents tPA- and uPA-induced cell death.

Effect of RAP on Tau phosphorylation and neurite outgrowth

We have previously shown that elevated levels of tPA and uPA significantly reduces neurite outgrowth in RGC-5 cells. To determine whether RAP-mediated inhibition of tPA and uPA synthesis observed in figure 2 also attenuates neurite degeneration in RGC-5 cells, RGC-5 cells were left untreated or treated with 1 μ M RAP and 2 μ M Staurosporine for 48 hrs. Neurite outgrowth was then analyzed by phase contrast microscopy (Fig. 4) and by immunolocalization for phosphorylated Tau (Fig. 5A), used a marker for neurite outgrowth. In addition, phosphorylation of Tau was assessed by western blot analysis (Fig. 5B). RGC-5 cells left untreated did not express detectable levels of p-Tau. Although RGC-5 cells treated with Staurosporine expressed low levels of p-Tau, a majority of the cells had smaller neurites. In contrast, RGC-5 cells treated with RAP and Staurosporine expressed increased levels of p-Tau (Fig. 5A and B) and, under these conditions, RGC-5 cells showed extended neurites. These results indicate that RAP prevents tPA and uPA-mediated cell death and increases neurite outgrowth in RGC-5 cells.

Discussion

The mechanisms by which secreted proteases, tPA and uPA, directly cause death of RGCs are unclear, although previous studies from our laboratory (Zhang, et al., 2003) and other laboratories (Kumada, et al., 2005, Kumada, et al., 2004) have provided evidence elevated levels of these proteases cause death of RGCs *in vivo* (Mali, et al., 2005) and RGC-5 cells *in vitro* (Harvey and Chintala, 2007). In this study, we provide further evidence that tPA and uPA dictate the survival of RGC-5 cells, in part, by interacting with LRP-1.

The findings presented in this study are significant for two reasons. First, these results provide a potential mechanism by which tPA and uPA directly cause death of RGCs. Historically, plasminogen activators, tPA and uPA, are known to regulate cell death by activating plasminogen to plasmin. Recent studies, however, indicate that these proteases can also cause loss of RGCs independent of plasminogen activation. For example, we have previously reported that, when death of RGCs is induced by ligating the optic nerve in CD-1 mice, elevated levels of tPA and uPA caused RGC death by activating plasminogen to plasmin. On the other hand, when RGC death is induced by injecting glutamate analogues into the vitreous humor, elevated levels of the same proteases caused RGC death independent of plasminogen activation (Mali et al., 2005, (Kumada, et al., 2005, Kumada, et al., 2004). Furthermore, the results presented in this study show that tPA and uPA cause death of RGC-5 cells, independent plasminogen activation, through tPA and uPA interaction with LRP-1. Currently, the downstream events of LRP-1 that regulate death of RGC-5 cells are unclear. It is plausible that tPA and uPA can activate certain intracellular signaling pathways in an autocrine fashion or induce the expression of proteases such as matrix metalloproteinases (MMPs) in a paracrine fashion (Wang, et al., 2003). In support of the later possibility, we have shown that elevated levels of tPA, expressed by RGCs, associate with elevated levels of uPA and MMP-9 expression in astrocytes in glaucoma-related animal models (Chintala, et al., 2002, Mali, et al., 2005, Zhang, et al., 2003, Zhang, et al., 2004).

Second, our results provide evidence that LRP-1 is an important regulator of tPA and uPA expression in RGC-5 cells. Although, the detrimental role of tPA and uPA in death of RGCs has been well documented, the mechanisms that regulate the synthesis of these proteases in the retina unclear. By employing antibodies against LRP-1 or by using recombinant RAP, previous studies have suggested that blocking of tPA interaction with LRP-1 results in a complete inhibition of tPA synthesis (Hardy, et al., 1997). In this study we showed that RAP inhibits the synthesis and secretion of not only tPA, but also of uPA in RGC-5 cells.

Previous studies on the central nervous system have shown that hypo- or hyper-phosphorylation of microtubule-associated protein, Tau, might be one of the key factors that regulates survival of neuronal cells in neurodegenerative diseases such as Alzheimer's. In addition, Tau has been shown to control the length of ganglion cell axons in the retina (Mercken, et al., 1995) and neurite outgrowth in cultured neuronal cells (Caceres and Kosik, 1990, Caceres, et al., 1991). However, the role of Tau in RGC loss is unknown. In this study, we show that elevated levels of tPA and uPA associate with dephosphorylation of Tau and a decrease in neurite outgrowth of RGC-5 cells, whereas inhibition of tPA and uPA interaction with LRP-1 reverses these events. There is one caveat associated with this study. We have studied the role of LRP-1 in synthesis of uPA and tPA in transformed retinal ganglion cells (RGC-5 cells), and not in normal RGCs. Thus, the results presented in this study apply only to RGC-5 cells. Although RGC-5 cell line continue to be used as an *in vitro* model system to study a variety of biochemical and pharmacological mechanisms involved in the death of normal RGCs, this cell line has both advantages and disadvantages. The advantages are that RGC-5 cells are readily available and can be differentiated by treating them with a non-specific kinase inhibitor, Staurosporine. The disadvantage is that RGC-5 cells cannot be considered as a model system of normal adult RGCs because RGC-5 cells are derived from neonatal retinal ganglion cells and transformed with a viral antigen. Yet, a number of recent studies, including our own, have shown that these cells are useful for studying the mechanisms involved in death of RGCs *in vitro*. For example, RGC-5 cells have been used to investigate the mechanisms involved in glutamate-induced cytotoxicity (Aoun, et al., 2003), hydrostatic pressure-mediated cell death (Agar, et al., 2006), ischemia-induced cell death (Ji, et al., 2007) and in axon and dendrite formation (Lieven, et al., 2007).

In summary, our results suggest that further investigations into the role of LRP-1 in animal models of glaucoma may have broader implications to prevent protease-mediated loss of RGCs in glaucoma patients.

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Abbreviations used

PAs, plasminogen activators; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator; LRP-1, low-density lipoprotein-related receptor-1; RAP, receptor associated protein.

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Figure 1.

RGC-5 cells express LRP-1. RGC-5 cells (2×10^3 cells/mL) were left untreated or treated with indicated concentrations of Staurosporine (SS) in serum-free medium for 16–18 hrs. Cells were fixed and LRP-1 expression was assessed by immunohistochemistry and counter stained with DAPI (A). In addition, LRP-1 expression was also assessed by western blot analysis (B) by using aliquots containing equal amounts of cellular protein extracts (50 ug). Results indicate that differentiated RGC-5 cells express increased level of LRP-1 compared to undifferentiated cells. Scale bar: 100 um.

Figure 2.

RAP-treated RGC-5 cells synthesize reduced levels of tPA and uPA. RGC-5 cells (4×10^3 cells/mL) were left untreated or treated with 2.0 uM Staurosporine (SS) in serum-free medium. At 48 hrs after treatment, conditioned medium (CM) was collected, cell extracts were prepared, and proteolytic activities of tPA and uPA were determined by zymography assays by using equal amounts of conditioned medium (20 uL) or cell extracts (50 ug). Compared to cells left untreated, cells treated with Staurosporine synthesized (**A, intracellular**) and secreted (**A, extracellular**) elevated levels of tPA and uPA. In contrast, cells treated with increased doses of RAP synthesized and secreted reduced levels of both tPA and uPA. Semi-quantitative analysis of proteolytic activity by densitometry indicate that RAP significantly inhibits the

synthesis and secretion of both uPA (**B**) and tPA (**C**) *P<0.05, compared to untreated cells; #P<0.05, compared to SS-treated cells.

Figure 3.

RAP-treatment attenuates death of RGC-5 cells. RGC-5 cells (2×10^3 cells/mL) were left untreated or treated for 48 h with 2.0 μ M Staurosporine (SS) and indicated concentrations of receptor associated protein (RAP). Quantification of cell survival by MTT assay indicates that viability of RGC-5 cells is significantly decreased after treating them with Staurosporine. In contrast, RAP-treatment attenuated a significant number of RGC-5 cells, in a dose-dependent fashion. # $P < 0.05$, compared to untreated cells; * $P < 0.05$, compared to Staurosporine-treated cells.

Figure 4.

RAP-treatment increases neurite outgrowth. RGC-5 cells (2×10^3 cells/mL) were left untreated or treated for 48 h with 2.0 μ M Staurosporine (SS) or with 1.0 μ M RAP. (A) Staurosporine-treated cells differentiated, but had reduced neurite length. In contrast, RGC-5 cells treated with 2.0 μ M Staurosporine along with 1.0 μ M RAP, differentiated and showed increased outgrowth, compared to cells treated with 2.0 μ M Staurosporine alone. Scale bar, 40 μ m. * $P < 0.005$, compared to SS treated cells.

Figure 5.

RAP-treatment induces phosphorylation of Tau. RGC-5 cells (2×10^3 cells/mL) were left untreated or treated for 48 h with 2.0 uM Staurosporine (SS) or with 1.0 uM RAP and 2.0 uM Staurosporine (SS) and phosphorylation of Tau was assessed by immunohistochemistry (A) and western blot analysis (B). Compared to cells left untreated, RGC-5 cells treated with Staurosporine differentiated and showed low levels of phosphorylated Tau. In contrast, RGC-5 cells treated with RAP expressed increased levels of phosphorylated Tau. Scale bar: 40 um.