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Short Communication

Human Astrocytes Express Functional Protease-Activated Receptors that Increase Their Reactive Phenotype Under Certain Culture Conditions

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Abstract

Background: Protease-activated receptors are G protein-coupled receptors with a novel activation mechanism by which proteases cleave the N-terminus revealing a tethered ligand that binds to the second extracellular loop leading to receptor activation. We have previously shown that protease-activated receptor 2 (PAR2) activation impairs hippocampal synaptic transmission and is neuroprotective in *in vitro* excitotoxic assays, both of which are astrocyte-dependent. We have also shown that AC264613, a blood-brain barrier-permeable PAR2 activator, results in behavioural changes associated with depression-like behaviour and recently revealed that AC264613 reduces astrocyte reactivity and amyloid load in a mouse model of amyloid pathology. However, whether functional protease-activated receptors are present in human astrocytes and if they alter their reactivity state is unknown. **Methods:** Commercially available human astrocytes were cultured in serum and serum-free media, and the presence of functional PARs was investigated using standard Ca^{2+} imaging techniques. Immunocytochemistry was used to examine the consequence of PAR activation on their reactive phenotype. **Results:** We reveal that functional PAR1 and PAR2 are present in commercially available human astrocytes. Their activation increased glial fibrillary acidic protein (GFAP) expression only after short-term culture but did not affect complement component 3 (C3) or guanylate-binding protein 2 (Gbp2) expression under any conditions tested. In contrast, no change in reactivity was observed following exposure to inflammatory stimuli. **Conclusion:** Overall, these data highlight that human astrocytes express functional PARs, but their activation may result in different phenotypic changes from those seen in mouse astrocytes.

Keywords: receptors; proteinase-activated; astrocytes; neuroinflammation

1. Introduction

Protease-activated receptors (PARs) are G protein-coupled receptors (GPCRs) that have a unique activation mechanism. In contrast to the usual receptor activation following ligand binding, PARs, of which four subtypes exist (PARs1-4), are activated following proteolytic cleavage of the N-terminus revealing a tethered ligand. This tethered ligand binds to the second extracellular loop, which results in receptor activation that initiates intracellular signalling pathways that are dependent on receptor coupling and location [1].

Within the central nervous system (CNS), PAR1 has been linked to memory formation [2] and astroglia-neuron coupling [3], whereas PAR2 activation, using a blood-brain barrier (BBB) permeable activator, induces behavioural changes associated with depression-like behaviour *in vivo* [4]. Strikingly, these studies indicate that the most abundant glial cell in the CNS, astrocytes, are critically involved in these observations. Furthermore, we recently revealed that PAR2 activation reduces astrocyte reactivity and amyloid load in a mouse model of amyloid pathology [5]. These data indicate that PAR activation may be a novel target for reducing astrocyte reactivity and their contribution to proteinopathies associated with many neurodegenerative disorders.

However, whether our findings have translational relevance is unknown as data regarding the presence of functional PARs on human astrocytes is limited and whether they modulate their reactive phenotype is unknown. Hence, we hypothesised that functional PARs are present on human astrocytes and their activation will alter their reactive phenotype.

2. Methods

2.1 Cell Culture

Human astrocytes (HA, ScienCell #1800, Caltag Medsystems, Buckingham, UK) were cultured and maintained at 37 °C/5% CO_2 in T25 flasks in serum-based astrocyte medium (AM, ScienCell #1801, Caltag Medsystems). Once confluent, the astrocytes were plated on poly-D-lysine (0.1 mg mL^{-1}, A3890401, ThermoFisher, Loughborough, UK) coated 13 mm glass coverslips and maintained in serum AM or serum-free Neurobasal-A culture medium (serum-free NB, neurobasal-A media (12348-017, ThermoFisher) 2% B27 (17504-044, ThermoFisher), 0.5mM L-glutamine (25030-024, ThermoFisher)) previously used for

primary hippocampal cultures, with media changed after 3 days for 7 days post-plating (DPP) experiments. All cell lines were validated by STR profiling and tested negative for mycoplasma as per supplier guidelines.

2.2 Calcium Imaging

HAs (3–4 DPP) were washed with a HEPES-based solution (HBS; 10 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$,10 mM D-glucose, pH 7.4, 310 mOsm) and loaded with Fura 2-AM (5 µM, F1221, Thermofisher) for 1 h at room temperature, following which they were washed 3× with HBS. HAs were placed in a chamber perfused with HBS at 2 mL min^{-1} and images taken every 0.5 Hz with an upright widefield epifluorescence microscope (Olympus BX51WI, Olympus Corporation, Tokyo, Japan) with Fura2 excited using 350/380 illumination (Cairn OptoLED, Faversham, UK). All signals were recorded using the WinFluor imaging software (V4.2.4, University of Strathclyde, Glasgow, UK), with all drugs, PAR activating peptides (APs: TFLLR-NH_2, SLIGKV-NH_2 and AYPGKF-NH_2, Peptide Synthetics, Fareham, UK), the PAR2 small molecule activator (AC264613 (AC), Tocris Bioscience #3370, Bristol, UK) and phospholipase C (PLC) inhibitor (U73122 (U73), Merck, Gillingham, UK) added via the perfusate and changes in fluorescence ratio calculated compared to pre-drug baseline levels using WinFluor V4.2.4.

2.3 Immunocytochemistry

HAs were treated with PAR activators or an inflammatory stimulus (TIC: tumor necrosis factor alpha [TNF-α], 30 ng mL^{-1}, 300-01A, ThermoFisher), interleukin-1 alpha (IL-1α) 3 ng mL^{-1} (PHC0015, ThermoFisher) and complement component 1q (C1q) 400 ng mL^{-1} (204876, Merck) for 24 h, either immediately after plating or for the last 24 h for 7 DPP experiments. Following treatment, HAs were washed 3× with PBS and fixed with ice-cold 4% paraformaldehyde (J61899, ThermoFisher) for 10 mins, washed 2× with PBS and then permeabilised for 10 mins with Triton-X (0.1%, 93443, Merck) diluted in blocking buffer (5% FBS (v/v, 17974731, ThermoFisher) and 1% (w/v) bovine serum albumin (A4737, Merck) in PBS) followed by blocking buffer alone for 1 h. Primary antibodies for glial fibrillary acidic protein (GFAP, 1:500, #MAB360, Merck), complement component 3 (C3, 1:500, #HM1045, Hycult Biotech, Uden, Netherlands) and guanylate-binding protein 2 (Gbp2, 1:500, #11854, Proteintech, Manchester, UK) were diluted in blocking buffer, added to coverslips and incubated overnight at 4 °C in a wetbox. HAs were then washed 3× with PBS and exposed to the relevant secondary antibody (A488, A-21202 or A-31566, ThermoFisher) at room temperature for 1 h. HAs were then washed 3× with PBS and exposed to CellMask™ Orange Stain (1:500, H32713-A, ThermoFisher) for 1.5 h at room temperature. Finally, HAs were washed 3× with PBS and images captured with the same equipment and software used for Ca^{2+} imaging. Non-blinded analysis was carried out using FIJI imaging software v1.53 (https://imagej.net/software/fiji) with cell area analysed by thresholding the CellMask™ staining to identify regions of interest (ROI), with these ROI used to quantify fluorescence intensity in the relevant co-stains for reactive astrocyte markers.

2.4 Statistical Analysis

All data are expressed as mean ± S.E.M. with n = average of at least 10 cells analysed on each individual coverslip, with each coverslip from at least 5 separate culture cycles of HAs. Normality of all data sets from individual coverslips was determined with Shapiro–Wilk tests, with data examined for statistical significance using either one-way ANOVAs with Tukey's post hoc test or a Kruskal-Wallis test where appropriate using GraphPad Prism (v8, GraphPad Software, Boston, MA, USA) with $p < 0.05$ taken as significant.

3. Results

3.1 PAR1 and PAR2 Activation, but not PAR4, Increases Intracellular Ca^{2+} Levels in Human Astrocytes

The PAR1 activating peptide, TFLLR-NH_2 (TF, 1–30 µM) increased intracellular Ca^{2+} levels in a concentration-dependent manner with a EC_{50} of 2.3 µM (n ≥ 5 coverslips per drug concentration, Fig. 1B). Similarly, PAR2 activation with the activating peptide, SLIGKV-NH_2 (KV, 1–30 µM) and the small molecule activator, AC264613 (AC, 1–30 µM) resulted in concentration-dependent increases in intracellular Ca^{2+} levels with EC_{50} values of 4.8 µM and 2.8 µM respectively (n ≥ 5 coverslips per drug concentration, Fig. 1A,C,D). In contrast, the PAR4 activating peptide, AYPGKF-NH_2 (30 µM) was without affect (n ≥ 5 coverslips). We confirmed that increases in intracellular Ca^{2+} were via the PLC pathway as the PLC inhibitor, U73122 (1 µM), abolished intracellular Ca^{2+} increases by PAR1 and PAR2 activators (n ≥ 5 coverslips, Fig. 1E).

3.2 PAR Activation Increases GFAP Expression in HAs 1 Day but not 7 Days Post-Plating

Having established that functional PAR1 and PAR2 are present in HAs, we next examined whether PAR activation, using the EC_{50} values obtained above, modulated the expression of the reactivity markers, GFAP, C3 and Gbp2, in HAs. No significant differences in marker expression or cell area were observed between serum AM and serum-free NB at both 1 and 7 DPP (Fig. 2B–I). However, when maintained in the presence of serum based AM, both TF (3 µM, 24 h n = 5, $p = 0.003$ vs vehicle control) and KV (5 µM, 24 h, n = 5, $p = 0.024$ vs vehicle control) significantly increased GFAP expression when compared to vehicle control at 1 DPP, whereas AC (3 µM, 24 h, n = 5) was without effect (Fig. 2A,B). However, when HAs were maintained in serum-free NB, AC (3 µM, 24 h) significantly increased

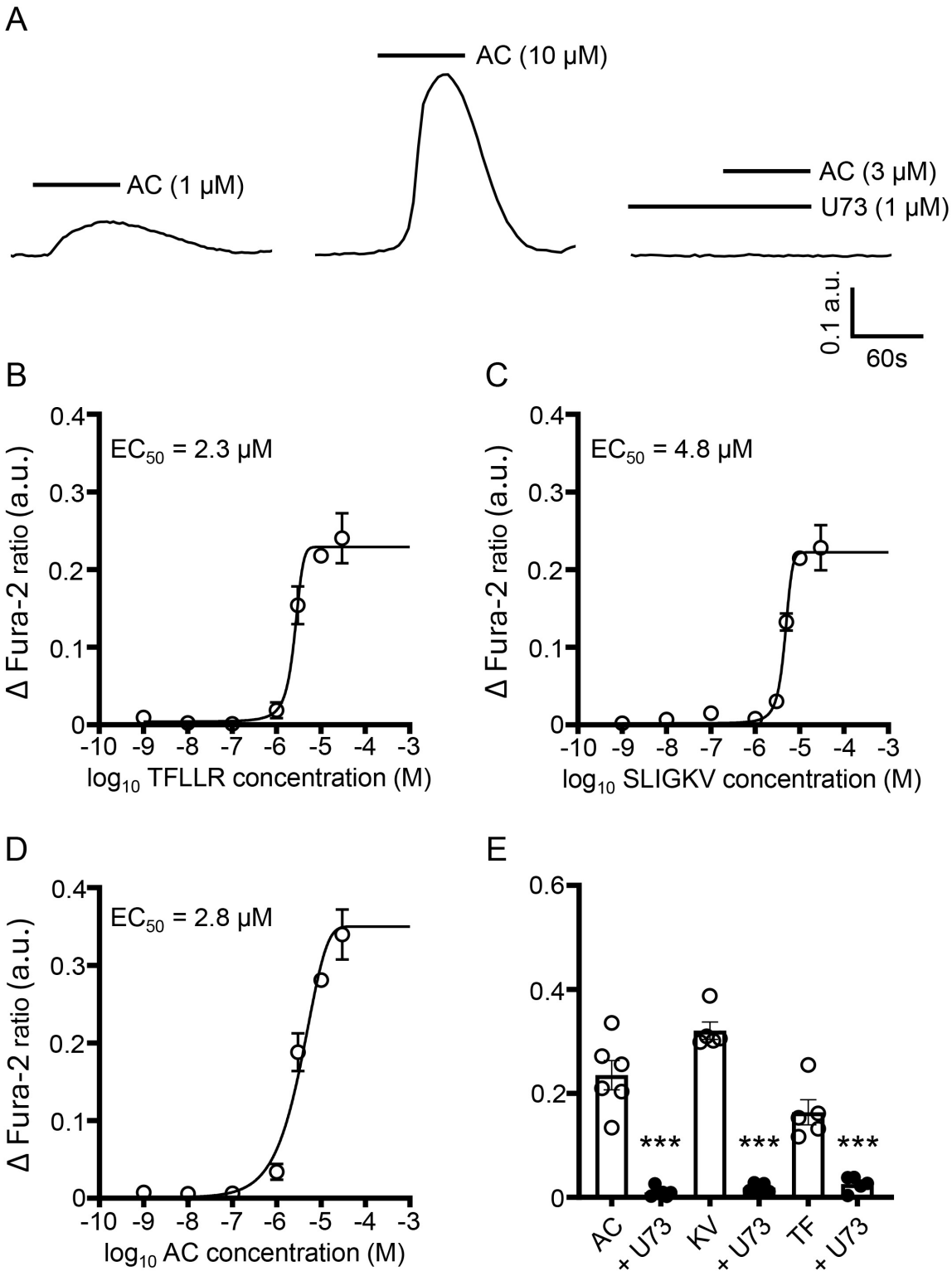

Fig. 1. Functional PAR1 and PAR2 are present on HAs. (A) Representative traces of increases in intracellular Ca^{2+} elicited by AC in HAs in the absence and presence of U73122 (U73). (B–D) Concentration-response curves for TFLLR-NH_2 (TF), SLIGKV-NH_2 (KV) and AC264613 (AC). (E) The PLC inhibitor, U73122 (1 µM) inhibits PAR activator induced increases in intracellular Ca^{2+}. n ≥ 5 coverslips per drug concentration or treatment with *** = $p < 0.001$, one-way ANOVA with Tukey's post hoc test.

GFAP expression (n = 5, $p = 0.007$ vs vehicle control) at 1 DPP whereas both TF and KV were without effect (Fig. 2B). In contrast, PAR activation did not affect HA GFAP expression in both serum AM and serum-free NB media

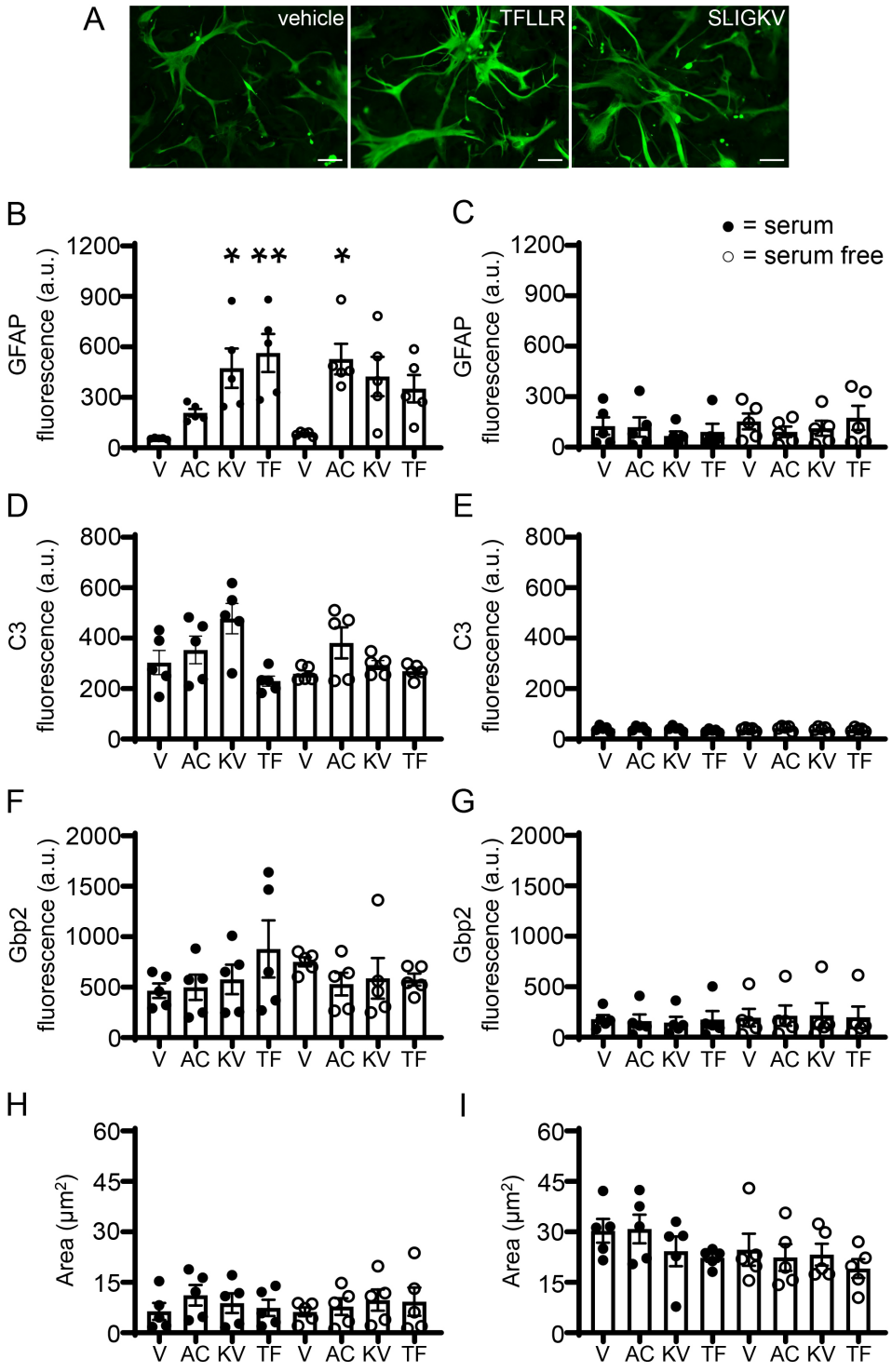

Fig. 2. PAR activation increases GFAP expression in HAs. (A) Representative images of GFAP expression in HAs maintained in serum AM following PAR activation at 1 DPP. Scale bar = 10 μm. (B,C) PAR activation increases GFAP expression at 1 DPP but not 7 DPP. (D–G) PAR activation did not affect C3 or Gbp2 expression at both 1 & 7 DPP. (H,I) PAR activation did not affect cell area at both time points tested. n ≥ 5 coverslips per drug treatment with * = $p < 0.05$, ** = $p < 0.01$, Kruskal-Wallis test. GFAP, glial fibrillary acidic protein; AM, astrocyte medium; DPP, days post-plating; Gbp2, guanylate-binding protein 2; C3, complement component 3.

when examined at 7 DPP (Fig. 2C). Whilst there is no clear consensus on reactive astrocyte markers, we also examined whether PAR2 activators affected C3 and Gbp2 expression in HAs both at both 1 and 7 DPP. Under both serum AM and serum-free NB conditions, PAR activators did not alter either C3 or Gbp2 expression at either 1 or 7 DPP (Fig.

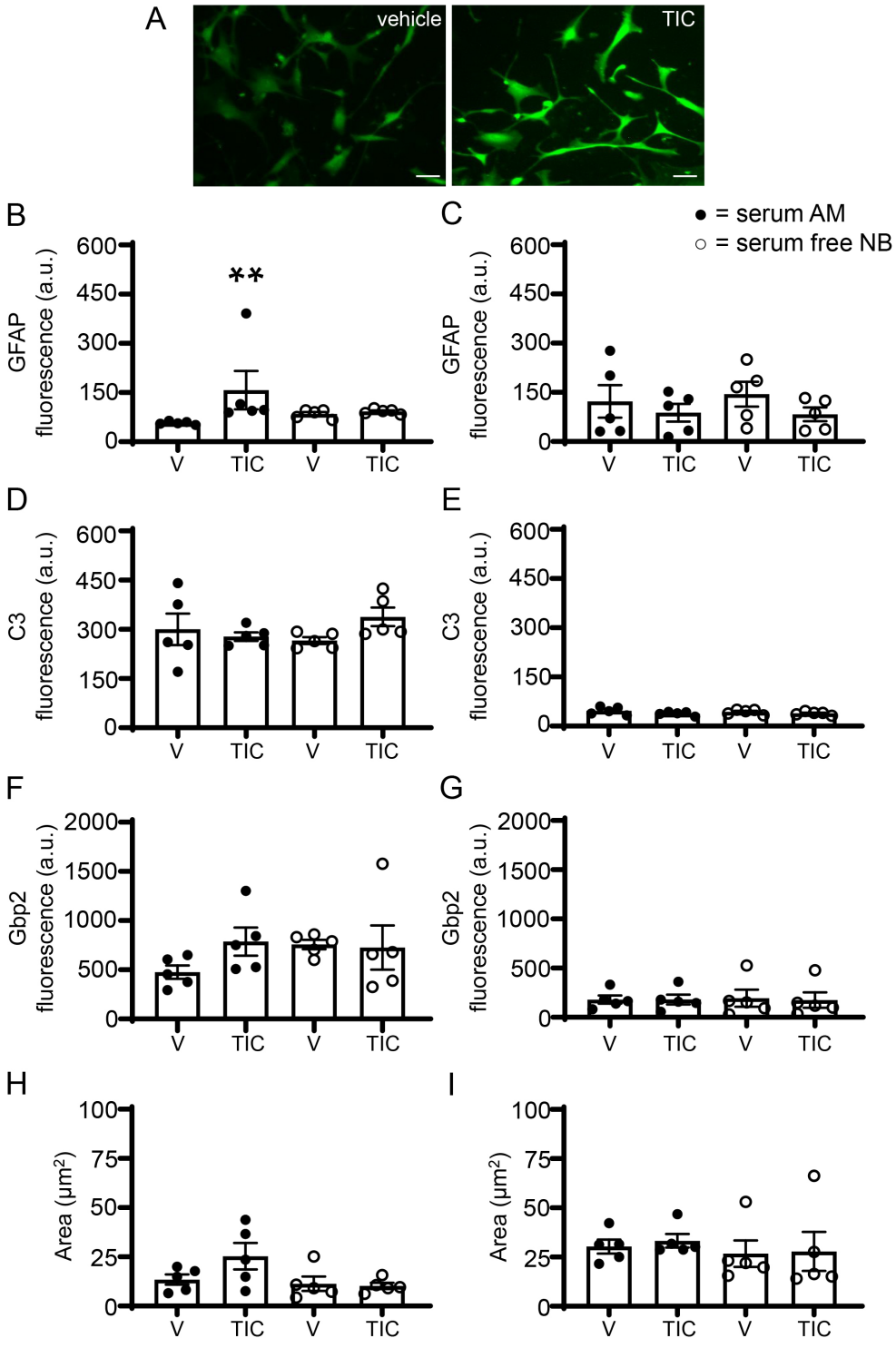

Fig 3. TIC-induced increases in GFAP expression are dependent on culture conditions. (A) Representative images of GFAP expression in HAs maintained in serum AM following TIC exposure at 1 DPP. Scale bar = 10 μm. (B,C) TIC increases GFAP expression at 1 DPP in serum media but not 7 DPP. (D–G) TIC did not affect C3 or Gbp2 expression at both 1 & 7 DPP. (H,I) TIC did not affect cell area at both time points tested. n ≥ 5 coverslips per drug treatment with ** = $p < 0.01$, Kruskal-Wallis test. TIC, TNF-α, IL-1α and C1q; TNF-α, tumor necrosis factor alpha; IL-1α, interleukin-1 alpha; C1q, complement component 1q.

2D–G). We also examined cell area given that changes in morphology are also associated with astrocyte reactivity. However, no changes in cell area were observed following PAR2 activation when examined under serum AM and serum-free NB conditions at both 1 and 7 DPP (Fig. 2H,I).

3.3 Astrocyte Reactivity Was not Induced by an Inflammatory Stimulus in the Presence or Absence of Serum

The inflammatory stimulus, TIC (TNF-α, IL-1α and C1q), which mimics the microglial cytokine release during neuroinflammatory episodes, is used to induce reactivity in induced pluripotent stem cells (iPSC)-derived astrocytes. Hence, we examined whether TIC increased the expression of reactivity markers in the HAs used to examine PAR function. TIC exposure (24 h) significantly increased GFAP expression in serum AM at 1 DPP (n = 5, $p = 0.004$ vs vehicle control, Fig. 3A,B) but did not affect GFAP expression in serum-free NB (Fig. 3B). However, TIC has no effect on GFAP expression in both serum AM and serum-free NB media when examined at either 7 DPP (Fig. 3C). Similarly, TIC had no effect on C3 or Gbp2 expression when examined at 1 and 7 DPP (Fig. 3D–G). Similarly, HA cell area did not change following exposure to TIC at both 1 and 7 DPP (Fig. 3H,I).

4. Discussion

In this study, we show that functional PAR1 and PAR2 are present in HAs as evidenced by their activation leading to concentration-dependent increases in intracellular Ca^{2+} levels. Whilst evidence exists for PAR1 and PAR2 expression in astrocytes in human tissue [6,7], to the best of our knowledge, this is the first time that functional PARs have been shown to be present in HAs but given the lack of specific PAR antibodies, their expression could not be confirmed using other techniques. Furthermore, whilst we reveal the presence of functional PARs in commercially available astrocytes, this should be extended to human iPSC-derived astrocytes and human organotypic brain slice cultures to confirm this is not an artifact of the HAs used in this study.

Having established the presence of functional PARs in HAs, we then examined the consequence of PAR activation on recognised markers of astrocyte reactivity under serum and serum-free conditions. Our data indicate that PAR activation can alter astrocyte reactivity depending on the culture conditions and the activator used. Much of our knowledge regarding human astrocyte reactivity comes from iPSC-derived astrocytes, which are standardly maintained in serum-free media to reduce reactivity [8]. However, the commercially available HAs used in the present study are recommended to be maintained in serum media, which is proposed to induce astrocyte reactivity [9,10]. Hence, we cultured HAs in both serum and serum-free media but no difference in reactivity markers or cell area was observed in vehicle treated HAs at either 1 or 7 DPP under both conditions. This indicates that at these time points, reactivity levels are unaffected by culture conditions. This is in contrast to a previous study where it has been suggested that cells in serum-free media for 7 days have a morphology similar to astrocytes *in vivo* and higher reactivity markers than those cultured in serum media [10]. However, it should be noted that in the previous study, reactivity markers were examined using mRNA expression whereas in our study we are measuring protein expression using immunocytochemistry.

When exposed to PAR activators, increases in the reactivity marker, GFAP, were observed at 1 DPP under both culture conditions, with these being significant for PAR APs in serum and AC in serum-free conditions. No changes were observed for GFAP at 7 DPP or for C3 and Gbp2 at any time point. Exposure to the inflammatory cytokine stimulus, TIC, also increased GFAP expression at 1 DPP but only in HAs cultured in serum media. This is consistent with previous studies where exposure to FBS increased GFAP expression but was without effect on other reactivity markers both in rodent and human astrocytes [10,11] but is in contrast to iPSC-derived astrocytes where TIC increases the expression of several reactivity markers including those examined here [8,9]. Hence, our data agree with previous studies that reveal these commercial HAs can become reactive with certain stimuli, but it should be noted that the observed changes in GFAP only may represent an early change in reactive phenotype rather than inducing a fully reactive phenotype. Future experiments should examine the signalling pathways associated with this, including whether increases in intracellular Ca^{2+} levels or other signalling pathways previously shown to be induced by PAR activation [1] are involved. In addition, our data agree with the heterogeneity in astrocyte phenotype that exists both *in vitro* and *in vivo* studies and between the species used [12]. This difference in effect being dependent on experimental environment and species is also highlighted as we recently showed that the blood-brain barrier-permeable PAR2 activator, AC, reduces, rather than increases, astrocytic reactivity, in a mouse model of amyloid pathology [5].

5. Conclusion

Our findings reveal that functional PAR1 and PAR2 are present on commercially available human astrocytes and, based on the consequence of their activation on reactive phenotype, they support previous studies that indicate functional differences exist between rodent and human astrocytes. However, to determine the role of PARs and their therapeutic potential going forward in human astrocytes, use of iPSC-derived astrocytes, organoids containing multiple cell types or human organotypic brain slice cultures may help decipher their true role in neurodegenerative disorders.

Availability of Data and Materials

All data reported in this paper will also be shared upon reasonable request by the corresponding author.

Author Contributions

AM and TB designed the research study. AM performed the research. AM and TB analysed the data. TB supervised the project. TB wrote the manuscript. Both authors contributed to editorial changes in the manuscript. Both authors read and approved the final manuscript. Both authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

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Conflicts of Interest

The authors declare no conflicts of interest.

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