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Stretch induced endothelin-1 secretion by adult rat astrocytes involves calcium influx via stretch-activated ion channels (SACs)

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ABSTRACT

The expression of endothelins (ETs) and ET-receptors is often upregulated in brain pathology. ET-1, a potent vasoconstrictor, also inhibits the expression of astrocyte glutamate transporters and is mitogenic for astrocytes, glioma cells, neurons, and brain capillary endothelia. We have previously shown that mechanical stress stimulates ET-1 production by adult rat astrocytes. We now show in adult astrocytes that ET-1 production is driven by calcium influx through stretch-activated ion channels (SACs) and the ET-1 production correlates with cell proliferation. Mechanical stimulation using biaxial stretch (<20%) of a rubber substrate increased ET-1 secretion, and 4 μM GsMTx-4 (a specific inhibitor of SACs) inhibited secretion by 30%. GsMTx-4 did not alter basal ET-1 levels in the absence of stretch. Decreasing the calcium influx by lowering extracellular calcium also inhibited stretch-induced ET-1 secretion without effecting ET-1 secretion in unstretched controls. Furthermore, inhibiting SACs with the less specific inhibitor streptomycin also inhibited stretch-induced ET-1 secretion. The data can be explained with a simple model in which ET-1 secretion depends on an internal Ca^{2+} threshold. This coupling of mechanical stress to the astrocyte endothelin system through SACs has treatment implications, since all pathology deforms the surrounding parenchyma.

1. Introduction

The ability of astrocytes to respond to virtually any CNS disturbance with both stereotyped changes (such as GFAP upregulation) and an adaptable repertoire of other components suggest that astrocytes may possess certain *all-purpose sensors* to monitor changes in their local environment [19]. Numerous studies have demonstrated that astrocytes possess mechanosensors and their anatomy, an interconnected meshwork of stellate cells, can integrate the effects of mechanical stress from distant sites. The responses to mechanical stimuli include dynamic cytoskeletal components such as GFAP and vimentin, stretch-activated ion channel (SAC) activation, and second-messenger signaling with Ca^{2+} and IP3 (reviewed in [26]).

ET-1 expression is minimal in quiescent adult astrocytes but robustly upregulated in reactive astrocytes (reviewed in [26]). The expression of ETB receptors is also upregulated in many pathologies [22,28]. ET-1 exerts potent autocrine effects on astrocytes and ET-1 stimulation has been used as an *in vitro* model for reactive astrocytes in culture [7]. The levels of ET-1 following cerebral hypoxia/ischemia and trauma correlate with the degree of astrocyte reactivity *in vivo* [30,34]. Since ET-1 is a strong inhibitor of astrocyte glutamate transporter expression [18], the mechanical stress associated with trauma or disease could potentiate neurotoxicity by downregulating clearance of excess glutamate.

Most *in vitro* studies of the astrocyte ET-system have used cultures from fetal or neonatal animals that may persist in a state of partial reactivity or immaturity [15,37]. Astrocytes from adult rats more closely approximate *in vivo* conditions – upon subculture they recapitulate the cell cycle kinetics, produce neurotrophic factors, and transiently upregulate GFAP and vimentin as seen in reactive gliosis *in vivo*. They then reach a state of proliferative quiescence that can be maintained for months in culture [17,29].

We previously demonstrated that mechanical deformation of adult rat astrocytes by stretching flexible-bottomed culture dishes causes an increase in cytoplasmic Ca^{2+} and inositol triphosphate (IP3), and a substantial increase in ET-1 production and secretion [25]. The trigger for this stretch-induced effect may be the presence of stretch-activated ion channels (SACs), characterized in many cells including neonatal [4,13] and adult rat astrocytes [31]. These channels can be specifically inhibited by a small peptide called GsMTx-4 [5,32].

Here, we demonstrate that the expression of ET-1 by adult rat astrocytes correlates with cell proliferation, becoming negligible in confluent quiescent cultures, akin to the situation in the intact

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brain. The ET-1 production appears driven by internal Ca^{2+} that in turn is coupled to an influx through Ca^{2+} permeable SACs. Production could be reduced by inhibitors of SACs or reduction in the Ca^{2+} influx by lowered extracellular Ca^{2+}.

2. Materials and methods

2.1. Cell culture

Adult astrocyte cultures derived from stereotactic striatal gelatin implants [17] were obtained from Dr. Robert Plunkett, *Department of Neurosurgery, SUNY at Buffalo*. The cells were grown on six-well, flexible-bottomed culture plates coated with collagen I (Bioflex® Plates, *Flexcell International Corp.,* Hillsborough, NC, USA), maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. After being confluent for 1 week, the cells were incubated in *Starvation Medium* containing 0.1% FBS (instead of 10%) for 24 h. This was then aspirated and replaced with fresh *Starvation Medium* immediately before testing. Experiments performed under "serum starvation" conditions had less variability that those cultured in normal media, possibly due to a smaller percentage of cells that had not reached a quiescent state or possibly bioactive serum components. The qualitative characteristics of the stretch-induced ET-1 responses and the inhibition of this phenomenon were the same in both media [24].

2.2. Evaluation of proliferation by BrdU ELISA

Astrocytes were subcultured in six-well Nunclon™ Surface coated culture plates (*VWR*, West Chester, PA, USA) leaving one well free as a control. Proliferation was evaluated using BrdU Cell Proliferation ELISAs (*Roche* Molecular Biochemicals, Indianapolis, IN, USA). At each time point the wells were incubated with the BrdU labeling solution for 2 h. As a control for nonspecific binding of the anti-BrdU antibody, one well (containing cells) of each plate was processed at each time without being loaded with BrdU.

2.3. ET-1 measurements

In order to correlate ET-1 levels with the proliferative status of the cells, we made duplicate culture plates for the ET-1 assay and the culture medium was changed weekly. Media samples were taken from the wells at specific times and frozen at −80 °C for later analysis. Cell lysates were prepared at the same time points as follows:

(1) Cells were rinsed twice with a protease inhibitor cocktail (PIC): 5 μg/ml Leupeptin, 2 μg/ml Aprotinin, and 0.7 μg/ml Pepstatin (all from *Boehringer Mannheim,* Indianapolis, IN, USA) in phosphate buffered saline (*Gibco–BRL*, Grand Island, NY, USA), pH 7.4.
(2) One milliliter of 0.3% Triton X-100 (*Sigma*, St. Louis, MO, USA) in PIC was added to each well and incubated on ice for 30 min.
(3) Cell scrapers were used to dissociate the monolayers, after which each lysate was passed several times through a 26-gauge needle to disperse any large aggregates, then frozen at −80 °C.

ET-1 was quantified with a Human ET-1 ELISA (*Assay Designs*, Ann Arbor, MI, USA), using a HTS-7000™ BioAssay Reader (*Applied Biosystems*, Foster City, CA, USA).

2.4. Mechanical stretching

A Flexcell FX-3000® Strain Unit (*Flexcell International*, McKeesport, PA, USA) was programmed to produce sinusoidal stretch (0.1 Hz, 0–20% elongation). Culture media was sampled from the individual wells after 24 h of stretching. The sampling occurred during the stretching regimen as previous experiments demonstrated that cessation of stress can serve as an additional stimulus [25].

2.5. Calcium buffering

To buffer the Ca^{2+} concentration in the culture medium, we used nitrilotriacetic acid (NTA, K_d for Ca^{2+} = 99.3 μM at 37.0 °C, pH 7.4, 0.15 N). Since NTA also binds Mg^{2+} (K_d = 0.575 mM), the concentration of Mg^{2+} was augmented to maintain constant free Mg^{2+} as predicted using the software WinMaxC v.2.05 [1].

2.6. GsMtx-4 preparation

GsMtx-4 from three separate purifications of raw *Grammastola spatulata* venom was pooled [31]. The pooled sample was tested on outside-out patches of astrocytes to estimate the concentration of the active peptide: assuming a K_d of 500 nM and single site binding [31], the percent inhibition of the mean SAC current provided the approximate concentration.

2.7. Outside-out patch clamp recording

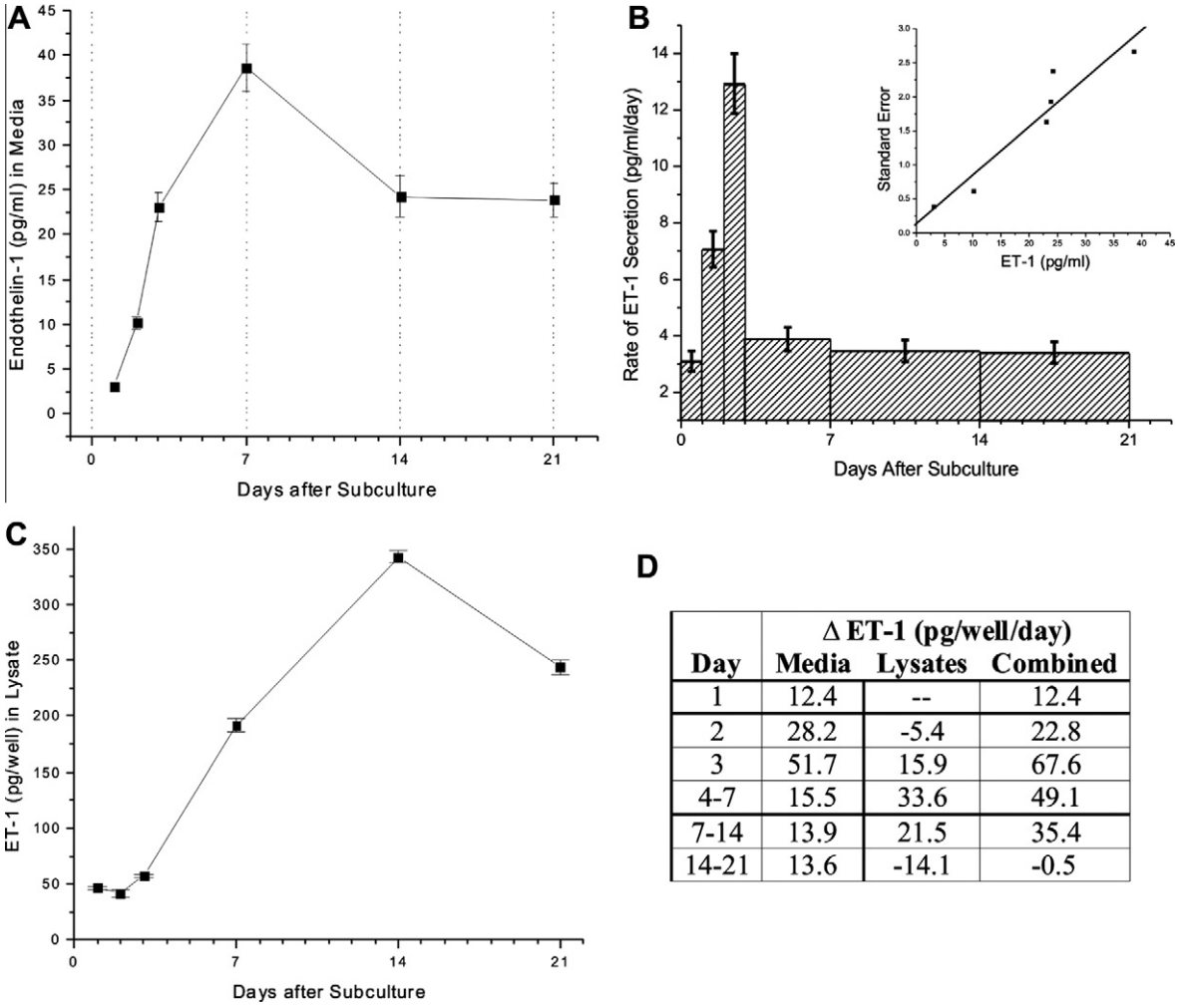
The main goal of the patch clamp recordings was to demonstrate the Ca^{2+} permeability of SACs (Suchyna et al. [31]). The pipette solution contained 140 mM KCl and the bath solution contained 100 mM $CaCl_2$. The patch voltage was hyperpolarized −50 mV to reduce the contribution of voltage-activated Ca^{2+} channels. Currents were sampled at 10 kHz and low-pass filtered at 2 kHz through a four-pole Bessel filter. Electrodes were pulled on a PC-84; (Sutter Instrument Co., Novato, CA, USA), painted with Sylgard-184 (Dow Corning, Midland, MI, USA), and fire polished. Pressure and suction were applied to the pipette by a pressure clamp [2]. Perfusion was handled by a pressurized bath perfusion system (BPS-8; ALA Scientific Instruments, Westbury, NY, USA).

3. Results

3.1. Production and secretion of ET-1 following subculture

Fig. 1A shows the concentration of ET-1 in media at specific times after subculture. Separate replicate wells were used for each time point to maintain equal volumes. The concentration of ET-1 accumulates over time in the media so we normalized the data to *rate of ET-1 secretion per day* as shown in Fig. 1B. We found a linear relationship between the standard error and the ET-1 concentration measured by ELISA, so the standard error values for Fig. 1B were extrapolated from this fit (see figure inset). The rate of ET-1 secretion into the medium was highest during the third day after subculture. It then rapidly declined and plateaued for the remainder of the 3-week time course.

Intracellular ET-1, as inferred from the lysates, remained steady until day 3 and then increased, reaching a peak at day 14 and then started to decline (Fig. 1C). Fig. 1D shows the combined rate of ET-1 *production* (i.e., change in lysates plus media) per culture well. Since the freshly subcultured cells presumably contain some amount of ET-1 even before attaching to the dishes, we called the "Day 1" amounts zero. The lysates showed a slight decrease on day 2 (Fig. 1C) suggesting that production did not begin until



Day	Δ ET-1 (pg/well/day) Media	Lysates	Combined
1	12.4	--	12.4
2	28.2	-5.4	22.8
3	51.7	15.9	67.6
4-7	15.5	33.6	49.1
7-14	13.9	21.5	35.4
14-21	13.6	-14.1	-0.5

Fig. 1. ET-1 production and secretion by adult rat astrocyte cultures. (A) ET-1 measured in media. Media was changed every 7 days (dotted lines) so that measurements on days 1, 2, 3, and 7 represent ET-1 accumulated since subculturing (time 0), whereas measurements on days 14 and 21 represent ET-1 accumulated over the previous week (since the last media change). Separate replicate wells were used for each time-point so media volumes remained constant. (B) The average change in ET-1 concentration per day, over a length of time indicated by the width of the bars. Standard errors were extrapolated from a linear fit of SEs from panel A (see inset). (C) ET-1 measured in cell lysates. (D) The combined rate of change of ET-1 levels in the media and lysates *per well* over the 21-day time course. Each well had 4 ml of media, so multiplying the values in (A and B) by 4 provides pg/well. Error bars = ±SE.

after the first 2 days. The increased ET-1 in the media from days 14–21 is nearly equal to the amount lost from the lysates (Fig. 1D). Apparently pre-formed ET-1 was secreted from the cells during this time, i.e., synthesis of ET-1 had ceased by day 14.

3.2. Correlation between ET-1 production and astrocyte proliferation

The squares in Fig. 2 show the average rate of DNA synthesis per culture well as a function of time. Each data point represents the amount of BrdU incorporated into DNA per well during a 2 h load, and thus reflects both the percentage of cells that are actively proliferating and the total number of cells per well. The proliferation measurement on day 1 was very low – probably because although a high percentage of the cells were in S phase, there were a small *number* of cells in each well. The proliferation rate peaked 2 days after subculture. At day 23 the rate of proliferation was comparable to that on day 1. The open-box data point at day 23 is nearly zero and represents the proliferative activity following a 2-day serum starvation (0.1% serum in medium instead of 10%). The slight discrepancy between this point and the normal media wells suggests that a small percentage of cells were still proliferating in the normal media.

The red bars in Fig. 2 show the *average change in ET-1 concentration per day* over the length of time indicated by the width of the bars (data from Fig. 1D). There is a clear correlation between ET-1 production and cell proliferation. Rates were highest during the first week and then subsided during the next 2 weeks. By the end of the 3-week time course, both proliferation and ET-1 production had essentially ceased. The correlation does not imply causality, but the dependence of ET-1 secretion on Ca^{2+} influx (below) suggests that ET-1 is driving proliferation rather than the opposite.

3.3. Lowering extracellular calcium attenuates stretch-induced ET-1 secretion

If ET-1 secretion is driven by intracellular Ca^{2+} changes from stretch-induced Ca^{2+} influx, it should be inhibited by lowering extracellular Ca^{2+}. We used the 24-h sinusoidal deformation regimen and varied extracellular Ca^{2+}. As shown in Fig. 3B, lowering the extracellular Ca^{2+} concentration decreased the stretch-induced secretion of ET-1 (*squares*) in a nonlinear manner, but it had no effect on ET-1 secretion in the absence of stretch (*triangles*). Therefore, the decrease in ET-1 secretion in the *stretched* wells is not due to other stretch-*independent* secondary effects of lowering external Ca^{2+}.

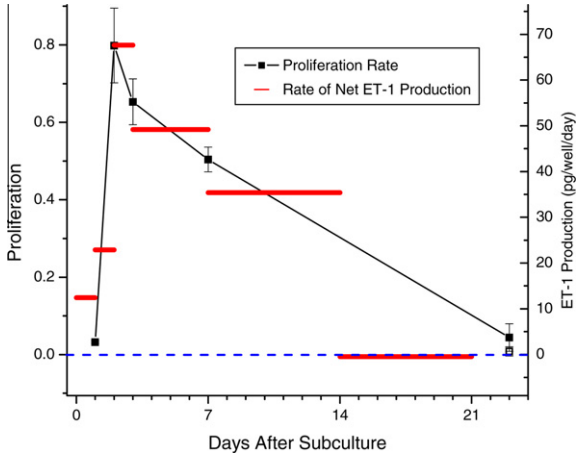


Fig. 2. ET-1 production rate correlates with proliferation (DNA synthesis). The open-box data point at day 23 represents DNA synthesis following 2-days of serum starvation (0.1% serum). The left *Y*-axis has been normalized from zero to one. The horizontal red bars are the values from the "Combined" column in (D) of Fig. 1, the *average change in ET-1 concentration per* day averaged over the length of time indicated by the width of the bars. Error bars = ±SE. (For interpretation of the references to colors in this figure legend, the reader is referred to the web version of this paper.)

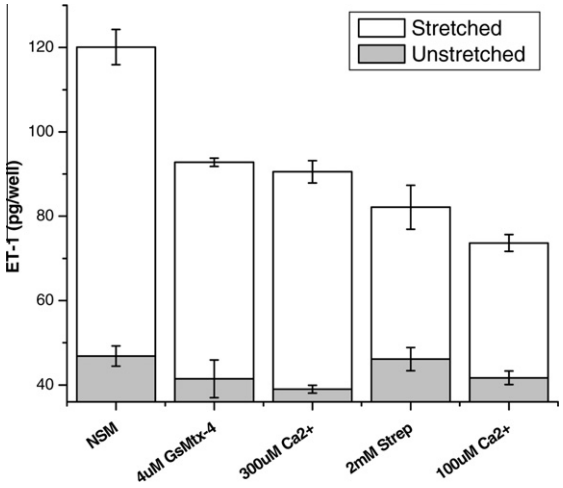


Fig. 4. ET-1 in the media increases with stretch and Ca^{2+} influx. NSM = "normal starvation media" that has 1.8 mM Ca^{2+}. The gray bars are from unstretched controls. Error bars = ±SE. The data show stimulation by the mechanical input and inhibition by changes that reduce Ca^{2+} influx (24 h of cyclic stretch, 0.1 Hz, 0–20% strain).

This also indicates that the Ca^{2+}-chelator NTA did not alter ET-1 secretion through nonspecific mechanisms. There was a slight, but statistically insignificant decrease of ET-1 in the unstretched wells, but a background effect is expected since the probability of an ion channel being closed is always greater than zero.

To demonstrate that the SACs conduct Ca^{2+} in response to stretch, we perfused outside-out patches with saline where Ca^{2+} was the only cation and demonstrated inward single currents with stretch (Fig. 3A). The kinetics were similar to those previously published where the channel conducted monovalent ions [31].

3.4. Inhibition of stretch-induced ET-1 secretion by GsMtx-4 and streptomycin

In adult rat astrocytes, GsMtx-4 blocks SACs with a K_d of ~500 nM while exhibiting minimal effects on voltage-sensitive currents [31]. The mechanism of action is as a gating modifier that involves both the channel and its lipid environment [32]. Adding 4 μM GsMtx-4 to the culture media caused a 23% reduction in the amount of ET-1 detected in the media following a 24-h cyclic deformation regimen ($P < 0.003$). It had no significant effect on secretion of unstretched controls (Fig. 4). This corresponds to a ~30% inhibition of the *stretch-induced* ET-1 secretion (i.e., after subtracting the unstretched baseline controls). Streptomycin, a nonspecific inhibitor of cationic SACs (2 mM), caused somewhat larger inhibition of stretch-induced secretion of 32% ($P < 0.0001$), while again having no effect on unstretched controls. This corresponds to ~50% inhibition of *stretch-induced* ET-1 secretion.

Assuming a 1:1 stoichiometry of GsMTx-4 binding to SACs, and a K_d of ~500 nM [31], 4 μM of GsMtx-4 should block ~90% of SACs, but it resulted in only a ~30% inhibition of stretch-induced ET-1 secretion, implying a nonlinear relationship between the Ca^{2+} influx through SACs and ET-1 secretion. This is not surprising, as astrocytes express several other ion channels capable of conducting Ca^{2+}, including voltage- and ligand-gated channels [36] that are known to be stretch-sensitive (Ca^{2+} [6], Na^+ [3], and K^+ channels [10]). Voltage dependent channels may be indirectly activated by stretch when SACs cause depolarization.

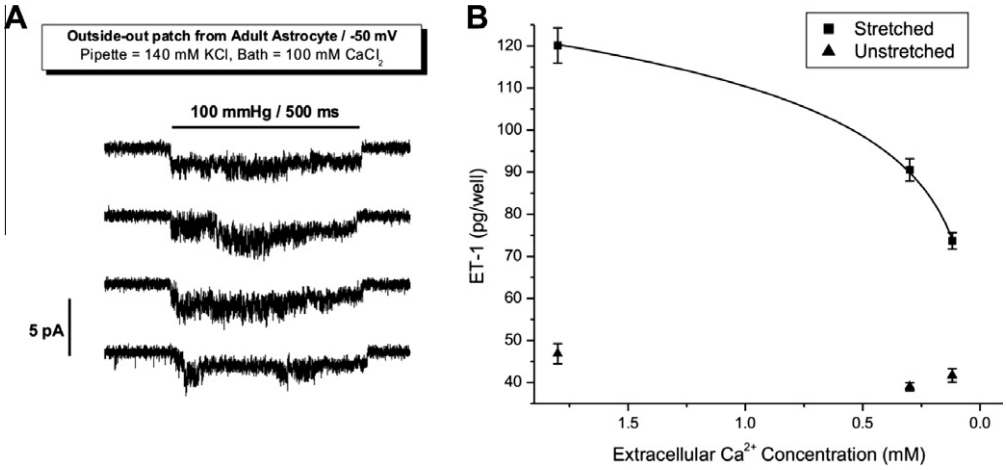


Fig. 3. Stretch-induced ET-1 secretion is dependent on extracellular calcium. (A) Adult astrocyte SACs can conduct Ca^{2+} in response to mechanical stress. An outside-out patch was exposed to 500 ms pressure pulses (100 mm Hg as noted with the horizontal bar) with 1.5 s between pulses. The holding potential was −50 mV and a downward deflection denotes Ca^{2+} influx. (B) ET-1 concentration measured in media following a 24-h sinusoidal stretching regimen (squares, 0.1 Hz, 20% linear strain) and in unstretched control cultures (triangles). Extracellular Ca^{2+} levels in the media were lowered by buffering with NTA. The curve is a fit of the data to a 2-parameter function (see Supplementary Material for derivation): $Y = P1 * \ln[Ca^{2+}]_{out} + P2 - 42.26$, P1 = 16.06 ± 0.3, P2 = 152.7 ± 0.5.

4. Discussion

Following subculture, both ET-1 production and cell proliferation peaked during the first week and then declined over the next two, becoming negligible by ~3 weeks. This time course is similar to the expression of GFAP and vimentin described by Langan et al. [17] for the adult rat astrocytes – the expression of these markers of the reactive phenotype was upregulated during the first 2 weeks following subculture and declined to baseline during the third week, and this paralleled the reactive glial reaction *in vivo* adjacent to an explant. The cessation of proliferation and ET-1 production after the cells became confluent suggests that astrocyte cultures derived from *adult* rats may enter a quiescent phenotype comparable to their *in vivo* behavior, as opposed to neonatal/fetal cells that may persist in a state of partial reactivity or immaturity.

As in our prior experiments [25], a 2 day mechanical stretching regimen produced significant ET-1 compared to unstretched controls. The response to mechanical stress seems to be the result of SAC activation. Inhibition of the channels by GsMtx-4 and streptomycin inhibited stretch-induced ET-1 secretion but did not alter levels in unstretched controls. Coupling between channel activation and ET-1 production seems to be via Ca^{2+} influx through the channels since lowering extracellular Ca^{2+} decreased the *stretch-induced* ET-1 secretion without affecting levels in unstretched controls. GsMTx-4 is a gating modifier whereas streptomycin is an open channel blocker [11,16] so while they both inhibit the channels they should have different side effects and the K_ds are quite different. The lack of specificity of streptomycin may account for the greater inhibition compared to GsMtx-4, as it inhibits voltage-gated Ca^{2+} channels [21,27] which may be indirectly activated by depolarization caused by stretch-induced Ca^{2+} influx.

The coupling of Ca^{2+} influx to ET-1 expression may follow that seen for Ca^{2+} waves and IP3 [26]. As intracellular Ca^{2+} stores are released, the intracellular Ca^{2+} concentration remains elevated for some period of time. If the amount of ET-1 secreted into the media ($[ET\text{-}1]_{media}$) depends on the probability of reaching a threshold for release of Ca^{2+} stores, then ET-1 secretion should be linearly related to total Ca^{2+} influx (i.e., through all *influx pathways* assuming no changes in Ca^{2+} efflux). If the flux of Ca^{2+} varies logarithmically with the ion concentration (the driving force of the Nernst potential), the relationship between $[ET\text{-}1]_{media}$ and $[Ca^{2+}]_{out}$ can be modeled with a two-parameter function:

$$[ET\text{-}1]_{media} = P1 * \ln[Ca^{2+}]_{out} + P2 - 42.3\ pg/well$$

where P1 and P2 are constants that can be obtained by curve fitting (see Supplementary material for detailed derivation).

The (limited) data in Fig. 3B is well fit by the function (black curve) yielding P1 and P2 (16.06 ± 0.3 and 152.7 ± 0.5, respectively). From this we estimate the intracellular Ca^{2+} concentration ($[Ca^{2+}]_{in}$) to be ~75 nM (see Supplementary material). This average intracellular Ca^{2+} concentration during the 24-h stretching regimen represents a steady state between the stress-enhanced Ca^{2+} influx, intracellular storage and efflux. Using Fura-2 (*Molecular Probes*, Eugene, OR, USA), Niggel et al. measured the basal $[Ca^{2+}]_{in}$ for the adult rat astrocytes ~30–40 nM [23]. Other labs have estimated a basal $[Ca^{2+}]_{in}$ of 50–90 nM in neonatal rat astrocytes [8,35]. Therefore, 75 nM obtained from the model seems reasonable.

There was measureable ET-1 in the media from the unstretched cells under all conditions (Figs. 3B and 4). We previously demonstrated that ET-1 in media from unstretched controls remains constant from as early as one hour after the initial media change [28] so that the act of changing media appears to induce (via fluid shear stress) a rapid transient release of ET-1. Macarthur et al. [20] observed a similar transient secretion of ET-1 in both stretched *and unstretched* endothelial cell cultures, which they attributed to the mechanical disturbance of changing media. Tong et al. showed that HEK-293 cells exhibit an increase in Ca^{2+} when culture medium is changed with aspiration [33]. However, they also showed that if the medium was changed by an "overflow" method with no shear stress there was no observable calcium change.

These experiments have significant implications for *in situ* pathology. CNS trauma and diseases deform the surrounding parenchyma (and thus the astrocyte network). The effects may be quite general. Recent studies suggest that ET-1 inhibits astrocyte gap-junctional communication [12], thus potentially providing feedback regulation of mechanically induced intercellular Ca^{2+} and IP3 signaling [26]. The upregulation of astrocyte ET-1 observed in many pathologies and its potent effects on astrocytes, neurons and the cerebral vasculature suggests that mechanical induction of the astrocytes' endothelin system may be common to many disorders. GsMtx-4 provides a unique tool to study SAC activity in the CNS and the utility is illustrated by this work and by the stimulatory effect for GsMTx-4 on neurite growth [9,14]. SAC pharmacology may therefore provide a new class of therapeutic agents for nervous system pathology.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.05.109.

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