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Attenuation of urokinase activity during experimental ischemia protects cerebral barrier from damage through regulations of MMP-2 and NAD(P)H oxidase

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Abstract

Ischaemic injury impairs the integrity of blood-brain barrier (BBB). This study investigates the molecular causes of this defect with regards to the putative correlations amongst NAD(P)H oxidase, plasminogen-plasmin system components and matrix metalloproteinases. Hence, the activities of NAD(P)H oxidase, matrix metalloproteinase-2, urokinase- (uPA) and tissue-type plasminogen activators (tPA) alongside superoxide anion levels were assessed in human brain microvascular endothelial cells (HBMEC) exposed to oxygen-glucose deprivation alone or followed by reperfusion (OGD±R). The integrity of an *in vitro* model of BBB comprising HBMEC and astrocytes was studied by transendothelial electrical resistance and paracellular flux of albumin. OGD±R radically perturbed barrier function while concurrently enhancing uPA, tPA and NAD(P)H oxidase activities and superoxide anion release in HBMEC. Pharmacological inactivation of NAD(P)H oxidase attenuated OGD±R-mediated BBB damage through modulations of matrix metalloproteinase-2 and tPA but not uPA activities. Overactivation of NAD(P)H oxidase in HBMEC via cDNA electroporation of its p22-phox subunit confirmed the involvement of tPA in oxidase-mediated BBB disruption. Interestingly, blockade of uPA or uPA receptor preserved normal BBB function by neutralizing both NAD(P)H oxidase and matrix metalloproteinase-2 activities. Hence, selective targeting of uPA after ischaemic strokes may protect cerebral barrier integrity and function by concomitantly attenuating basement membrane degradation and oxidative stress.

Introduction

Composed of brain microvascular endothelial cells surrounded by a basement membrane and astrocytic endfeet, the blood-brain barrier (BBB) regulates the passage of circulating substances into the brain parenchyma (Hayashi *et al.* 2004). Disruption of blood supply to the brain during ischaemic stroke evokes a cascade of deleterious events, such as depletion of ATP and acidosis, leading to disruption of the BBB (Hayashi et al. 2004, Brouns *et al.* 2009). Ensuing reperfusion of ischaemic tissue accompanied by excessive release of superoxide anion ($O_2^{\bullet-}$), a prominent reactive oxygen species, may exacerbate the damage to the BBB (Margaill *et al.* 2005, Selemidis *et al.* 2008, Walder *et al.* 1997).

Endothelial NAD(P)H oxidase represents the main source of $O_2^{\bullet-}$ in ischaemic vasculature. It contains several cytosolic subunits and a membrane-bound cytochrome b_{558} consisting of gp91-phox and p22-phox, key subunits required for the enzymatic activity and stability as a whole (Kleikers *et al.* 2012, Kleinschnitz *et al.* 2010). While many studies have shown the significance of this enzyme complex in ischaemia-reperfusion injury (Eltzschig *et al.* 2004, Hong *et al.* 2006) the p22-phox subunit has not been studied significantly in this context.

In addition to $O_2^{\bullet-}$, the enhanced activation of serine proteases, namely tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) along with their catalytic product plasmin may also compromise the BBB integrity under ischaemic conditions. The link between these serine proteases and ischaemic stroke is well established with studies showing both beneficial and detrimental roles (Cho *et al.* 2012, Hosomi *et al.* 2001). The activities of plasminogen activators (PAs) are, amongst other mechanisms, controlled by the local availability of their inhibitors, namely plasminogen activator inhibitor-1 (PAI-1) and in the case of uPA also by its specific receptor, uPAR (Irigoyen *et al.* 1999).

PAs have been linked to the levels and activities of MMPs namely MMP-2 and MMP-9 during ischaemic stroke (Barr *et al.* 2010, Rosenberg *et al.* 1994, Thornton *et al.* 2008, Tsuji

et al. 2005). MMPs can be activated during different stages of a stroke, with emerging evidence indicating MMP-9 may have a more severe impact especially during the later stages (Asahi *et al.* 2000, Asahi *et al.* 2001a, Asahi *et al.* 2001b, Lucivero *et al.* 2007). Recent studies have shown that deletion of both MMP-2 and -9 protects against cerebral infarction in mouse models (Suofu *et al.* 2012) and that these MMPs can contribute to BBB damage through degradation of the tight junction protein occludin (Liu *et al.* 2012).

In light of the above, the current study examined whether NAD(P)H oxidase-dependent release of $O_2^{\bullet-}$ might compromise the integrity and function of an *in vitro* model of human BBB under ischaemic conditions through interactions with PAs and/or MMPs.

The study found that inhibition of uPA, NAD(P)H oxidase or MMP-2 exerts barrier-protective effects. It also found that uPA acts upstream to both NAD(P)H oxidase and MMP-2 thereby bestowing a significant therapeutic potential on this enzyme in maintaining BBB integrity and function.

Methods

Cell culture

Human brain microvascular endothelial cells (HBMEC) were purchased from ScienCell and grown to subconfluence in their specialised media before exposure to oxygen-glucose deprivation (OGD; 94.5% N_2, 0.5% O_2 and 5% CO_2) or normoxia (N; 75% N_2, 20% O_2 and 5% CO_2) for 4 h. In some experiments, OGD was followed by 20 hours of reperfusion in that the ischaemic culture medium lacking glucose and FBS was replaced with fresh medium containing 5.5 mM glucose and 10% FBS before exposing cells to normoxic conditions. Similar experiments were carried out with the addition of amiloride (uPA inhibitor, 2.5 μM), apocynin (NAD(P)H oxidase inhibitor, 1 mM) or MMP-2 inhibitor III (2-((Isopropoxy)-(1,1'-

biphenyl-4-ylsulfonyl)-amino)-H-hydroxyacetamide, 100 µM; Calbiochem) during the OGD or reperfusion stages.

***In vitro* model of human BBB**

Human astrocytes (ScienCell) were seeded onto the outer surface of Transwell inserts (0.4 µm pore, Corning Costar) seated upside down and on the following day, HBMEC were seeded onto the inner surface of the same inserts. Both sets of cells were grown to confluence before exposure and/or treatment.

Assessment of BBB permeability

The BBB integrity and function were studied, as previously described, by measurements of transendothelial electrical resistance (TEER) and the flux of Evan's blue-labelled albumin (EBA, 67 kDa) across co-cultures, respectively (Allen *et al.* 2009a). TEER was measured using STX electrodes and an EVOM resistance meter (World Precision Instruments). To measure EBA flux, inserts were washed twice with Hank's Balanced Salt Solution (HBSS) and then transferred to new 12-well plates containing 2 mL of HBSS. EBA (500 µL, 165 µg/mL) was added to the luminal compartments and after 60 minutes samples were taken from both abluminal and luminal chambers. The concentration of dye in each chamber was determined by measuring the absorbance of the sample at 610 nm and flux was calculated (absorbance abluminal x 2000 x absorbance luminal^{-1}).

Immunoblotting

Equal amounts (40-60 µg) of protein were run on 10-15% SDS-polyacrylamide gels before transferring onto Hybond-P PVDF membrane (GE healthcare). The proteins were successively detected by primary antibodies specific for β-actin (mouse; synthetic β-cytoplasmic actin N-terminal peptide; Sigma; cat. no. A5441; internal control; 1:10,000), p22-phox (rabbit (FL-195); full length human p22-phox; Santa Cruz; cat. no. sc-20781; 1:200), PAI-1(rabbit (H-135); amino acids 24-158 of the N-terminus of human PAI-1; Santa Cruz;

cat. no. sc-8979; 1:750), tPA (rabbit (H-90); amino acids 1-90 of human tPA; Santa Cruz; cat. no. sc-15346; 1:300), uPA (rabbit (H-140); amino acids 136-275 of human uPA; Santa Cruz; cat. no. sc-14019; 1:500) or uPAR (rabbit (FL-290); full length human uPAR; Santa Cruz; cat. no. sc-10815;1:500) and infrared dye-tagged appropriate secondary antibodies (goat anti-mouse and anti-rabbit; LI-COR Biosciences; cat. no. 926-68020 and 926-32211, respectively; 1:30,000). The bands were scanned and analysed using the Odyssey Infrared Imaging System (LI-COR Biosciences).

Cell viability

An aliquot of cells mixed with 0.1% Trypan blue was visualised under a light microscope. To calculate percentage viability, 100 cells were counted.

Transfection experiments

HBMEC (~$5x10^6$) were re-suspended in 500 µl of media without FBS and antibiotics and stored on ice in a pre-chilled sterile cuvette. Anti-PAI-1 or anti-uPAR antibody (each 3 µg) or double-stranded p22-phox cDNA (50 ng) was added to the cell suspension and the mixture was electroporated at 1.8kV with an Easyjet Prima Electroporator (Equibio). Cells electroporated with equal volumes of vehicle (distilled H_2O), control cDNA (28S rRNA) or a rabbit anti-IgG served as controls. Since electroporation reduced the viability rates by approximately 40%, the cell numbers were adjusted accordingly before seeding reasonably high numbers in flasks or on inserts to ensure all experimental groups reach confluence simultaneously. Cells were exposed to OGD and harvested within 3 days of transfection.

Pyrogallol experiments

In some experiments, HBMEC alone or in co-culture with human astrocytes were exposed to pyrogallol ($O_2^{\bullet-}$ generator, 2 µM) for a period of 3 days and fed twice a day with fresh medium containing pyrogallol.

Measurements of total $O_2^{\bullet-}$ levels and NAD(P)H oxidase activity

Total $O_2^{\bullet-}$ levels were detected using the cytochrome *C* reduction assay as previously described (Allen et al. 2009a). Briefly, cell pellets were sonicated in cold lysis buffer (20 mM HEPES buffer, pH 7.2, 1 mM EGTA, 210 mM mannitol, 70 mM sucrose). Equal amounts of homogenate (100 µg) were incubated with cytochrome *C* (50 µM) for 60 minutes at 37°C before absorbances were measured at 550 nm.

NAD(P)H oxidase activity was measured with the lucigenin chemiluminescence assay. Briefly, samples of homogenates (~100 µg) were incubated at 37°C in assay buffer (50 mM potassium phosphate buffer (pH 7.0), 1 mM EGTA, 150 mM sucrose, 5 µM lucigenin) containing the specific inhibitors for other reactive oxygen species-generating enzymes i.e. nitric oxide synthase (NG-nitro-L-arginine methyl ester, 100 µM), mitochondrial complex I (rotenone, 50 µM), xanthine oxidase (allopurinol, 100 µM) and cyclooxygenase (indomethacin, 50 µM). After 15 minutes NADH or NADPH (100 µM; Calbiochem) was added to initiate the reaction. The reaction was monitored every minute for 2 hours and the rate of reaction calculated. Buffer blanks were also run for both assays and subtracted from the data.

Determination of PA activities

uPA and tPA activities were measured using an ELISA-based assay. Streptavidin coated plates (Thermo Scientific) were coated with biotinylated PAI-1 (300 ng/mL; Abcam) prior to addition of equal volumes of cell culture media or uPA and tPA standards (Calbiochem). Bound active uPA or tPA was then detected by successive incubations with the respective primary (see above; 1:50) and horseradish peroxidase-linked secondary antibodies (donkey anti-rabbit; Santa Cruz; cat. no. sc-2313; 1:5000). A colourimetric substrate, 3,3',5,5'-tetramethylbenzidine (Thermo Scientific), was added to the reaction before stopping it with

H_2SO_4. Absorbances were read at 450 nm and normalised against the respective standards and total protein concentrations.

Gelatin Zymography

Total cell protein (20 µg) or culture media devoid of FBS used to grow HBMEC were run on 9% SDS-polyacrylamide gels containing 0.1% gelatin. MMP-2 and MMP-9 positive controls were also run on each gel. The gels were washed 3 times for 15 minutes in 2.5% Triton X-100 before incubating overnight at 37°C in buffer consisting of 50 mM Tris (pH 7.5), 10 mM $CaCl_2$, 150 mM NaCl and 0.05% Brij-35 (Calbiochem). Gels were then successively stained for 2 hours with 0.1% Coomassie blue dye in 25% methanol and 5% acetic acid and destained for 1 hour in 50% methanol and 10% acetic acid and then overnight in water. MMP activity was detected as clear bands and gels were scanned and analysed with the Odyssey Imaging System.

Reagents

All reagents unless specifically stated were from Sigma.

Statistical analyses

Data are presented as mean±SEM from a minimum of 3 independent experiments. Statistical analyses were performed using one way ANOVA followed by a Tukey post hoc analysis or, where appropriate, by an independent Student's *t*-test or a two way ANOVA followed by a Tukey post hoc test. $p<0.05$ was considered as significant.

Results

Effects of OGD and reperfusion on the BBB integrity and function

OGD alone and followed by reperfusion (OGD±R) impaired BBB integrity and function as confirmed by decreases in TEER and concurrent increases in EBA flux, respectively. The magnitudes of these changes were greater during the reperfusion phase. While inhibitions of

uPA and NAD(P)H oxidase restored barrier function in OGD±R, the inhibition of MMP-2 appeared to be only effective during the reperfusion stage (data were analysed using two way ANOVA with a Tukey post hoc analysis: $n \geq 3$, $F_{4,71}=2.79$, $p<0.05$; Figure 1A and $n \geq 3$, $F_{4,36}=5.37$, $p<0.03$; Figure 1B).

Effects of OGD±R on NAD(P)H oxidase

Exposures of HBMEC to OGD±R led to substantial increases in NAD(P)H oxidase activity and $O_2^{\bullet-}$ levels which were effectively suppressed by attenuations of uPA and NAD(P)H oxidase but not MMP-2 activities (data were analysed using two way ANOVA with a Tukey post hoc analysis: $n \geq 3$, $F_{4,112}=1.68$, $p<0.05$; Figure 2A and $n \geq 3$, $F_{4,116}=1.84$, $p<0.05$; Figure 2B). These imply that uPA and MMP-2 may act upstream and downstream to the oxidase, respectively. The increases in oxidase activity were mimicked by those observed in p22-phox protein expression, confirming the regulatory role of this particular subunit in determining overall enzyme activity (data were analysed using one way ANOVA with a Tukey post hoc analysis: $n \geq 3$, $F_{2,6}=9.65$, $p<0.05$; Figure 2C). The difference in p22-phox expression between OGD and OGD+R groups cannot be attributed to reintroduction of glucose during the reperfusion phase as longer exposures to hyperglycaemia (≥3 days) are required to affect NADPH oxidase isoform protein expressions in HBMEC (Allen *et al*. 2009a, Shao *et al*. 2013).

$O_2^{\bullet-}$ impairs BBB integrity and increases tPA protein expression

To ascertain the contribution of $O_2^{\bullet-}$ to BBB dysfunction, the co-cultures were treated with an $O_2^{\bullet-}$ generator, pyrogallol which impaired the BBB integrity and function as evidenced by marked decreases in TEER (data analysed using one way ANOVA with Tukey post hoc: $n \geq 3$, $F_{2,12}=18.7$, $p<0.05$; Figure 3A) and concomitant increases in paracellular flux (data analysed using one way ANOVA with Tukey post hoc: $n \geq 3$, $F_{2,12}=41.0$, $p<0.05$; Figure 3B), respectively. Again, inhibition of uPA activity abolished $O_2^{\bullet-}$-mediated barrier damage (data

analysed using Student's *t*-test: n≥3, p<0.05; Figure 3C) while pyrogallol enhanced tPA protein expression in HBMEC (data analysed using Student's *t*-test: n≥3, p<0.05; Figure 3D). Taken together, these imply the presence of a differential crosstalk between specific PAs and $O_2^{\bullet-}$.

Increased NAD(P)H oxidase activity amplifies tPA protein levels and BBB disruption

Having established the pivotal role of $O_2^{\bullet-}$ in barrier damage, it was critical to unravel the involvement of NAD(P)H oxidase in this phenomenon. Hence, NAD(P)H oxidase activity was augmented in some HBMEC through electroporation of double-stranded p22-phox cDNA which significantly increased p22-phox protein levels and produced similar quantities of $O_2^{\bullet-}$ as pyrogallol where the electroporation of control cDNA had no impact on NADPH oxidase activity or $O_2^{\bullet-}$ release. Inhibition of uPA activity with amiloride or by an anti-uPAR antibody normalised $O_2^{\bullet-}$ levels and NAD(P)H oxidase activity. Electroporation with an anti-IgG antibody did not reduce $O_2^{\bullet-}$ levels or NAD(P)H oxidase activity (data analysed using one way ANOVA with Tukey post hoc: n≥3, $F_{5,14}$=19.0, p<0.05; Figure 4A and n≥3, $F_{5,15}$=14.3, p<0.05; Figure 4B and data analysed using Student's *t*-test: n≥3, p<0.05; Figure 4C). Specific increases in NAD(P)H oxidase activity also substantially increased tPA protein expression (data analysed using Student's *t*-test: n≥3, p<0.05; Figure 4D). Inhibitions of uPA and uPA:uPAR binding through treatments with amiloride and an anti-uPAR antibody, respectively abolished all barrier-disruptive effects induced by p22-phox overexpression and ensuing NAD(P)H oxidase overactivity while electroporations of control cDNA or non-specific isotype-matched antibody had no effect on barrier integrity or function (data analysed using one way ANOVA with Tukey post hoc: n≥3, $F_{2,15}$=68.5, p<0.05; Figure 4E, n≥3, $F_{2,6}$=0.92, p=ns; Figure 4G, n≥3, $F_{2,11}$=85.77, p<0.05; Figure 4H and n≥3, $F_{2,6}$=0.27, p=ns; Figure 4J and data analysed using Student's *t*-test: n≥3, p=ns; Figure 4F and I).

Effects of OGD±R on MMP-2 and MMP-9 activities

Although dramatic increases in intracellular and secreted MMP-2 activities were obtained selectively during reperfusion phase of the ischaemic insult, the MMP-9 activity stayed below the level of detection in both OGD±R. As expected the MMP-2 inhibitor III normalised increases observed in MMP-2 activities (data analysed using one way ANOVA with Tukey post hoc: $n \geq 3$, $F_{4,55}=15.94$, $p<0.05$; Figure 5A and $n \geq 3$, $F_{5,79}=6.96$, $p<0.05$; Figure 5B). Inhibitions of uPA and NAD(P)H oxidase activities reversed the increases observed during reperfusion and affirmed the notion that both enzymatic systems operate upstream to MMP-2 (data analysed using one way ANOVA with Tukey post hoc: $n \geq 3$, $F_{4,58}=17.58$, $p<0.05$; Figure 5C and $n \geq 3$, $F_{5,83}=7.27$, $p<0.05$; Figure 5D). Interestingly, apocynin reduced the MMP-2 activities in OGD alone-treated cells below the levels seen in control cells (data analysed using one way ANOVA with Tukey post hoc: $n \geq 3$, $F_{4,59}=32.71$, $p<0.05$; Figure 5E and $n \geq 3$, $F_{5,83}=12.93$, $p<0.05$; Figure 5F). As serum evokes an increase in secreted MMP-2 activity and was present during reperfusion phase of the experiments, two different controls were used to normalise secreted MMP-2 activities. N4h lacked serum and served as controls for OGD experiments while N20h containing equal amounts of serum served as controls for OGD+R groups. In order to illustrate the actual fold differences in enzymatic activities, both N4h and N20h values were normalised to "1".

Effects of OGD±R on plasminogen-plasmin system components

Similar to p22-phox protein, OGD±R also increased uPA, tPA and PAI-1 protein expressions without affecting that of uPAR in HBMEC (data analysed using one way ANOVA with Tukey post hoc: $n \geq 3$, $F_{2,23}=10.94$, $p<0.05$; Figure 6A, $n \geq 3$, $F_{2,33}=0.70$, p=ns; Figure 6B, $n \geq 3$, $F_{2,24}=10.46$, $p<0.05$; Figure 6C and $n \geq 3$, $F_{2,12}=4.82$, $p<0.05$; Figure 6E). The increases in uPA and tPA activities were somewhat reflective of those observed in the relevant protein expressions. To determine the putative regulatory effects of NAD(P)H

oxidase and MMP-2 on PA activities, the uPA and tPA activities were measured in HBMEC subjected to OGD±R in the absence or presence of apocynin or MMP-2 inhibitor III. While the inhibition of MMP-2 had no effect on either PA activities, the inactivation of NAD(P)H oxidase selectively suppressed the tPA activity. As expected, amiloride diminished OGD±R-mediated increases seen in uPA but not tPA activity to the levels recorded in control cells (data analysed using one way ANOVA with Tukey post hoc: $n \geq 3$, $F_{8,18}=14.55$, $p<0.05$; Figure 6D and $n \geq 3$, $F_{8,19}=8.56$, $p<0.05$; Figure 6F).

PAs contribute to BBB damage during 20 h of OGD

To consolidate the specific contributions of PAs to the barrier damage, co-cultures were established using HBMEC transfected with anti-uPAR or anti-PAI-1 antibody to quench uPA activity and enhance overall PA activities, respectively. Electroporation of anti-PAI-1 antibody impaired barrier integrity and function under normoxic and 20 h OGD compared to corresponding anti-uPAR antibody and control groups. In contrast, electroporation of anti-uPAR antibody did not affect BBB integrity under normoxic and ischaemic conditions compared to controls (data analysed using one way ANOVA with Tukey post hoc: $n \geq 3$, $F_{7,26}=40.18$, $p<0.05$; Figure 7A and $n \geq 3$, $F_{7,24}=35.49$, $p<0.05$; Figure 7B). To confirm tPA and uPA were being affected the PA activities were also measured after the above transfections. As expected electroporation with anti-PAI-1 antibody increased both tPA and uPA activities in both control and 20 h OGD groups. Transfection with anti-uPAR antibody had no effect on tPA activity but did decrease uPA activity in control and 20 h OGD groups. The use of an anti-IgG control had no effect on PA activities (data analysed using one way ANOVA with Tukey post hoc: $n \geq 3$, $F_{7,25}=28.34$, $p<0.05$; Figure 7C and $n \geq 3$, $F_{7,26}=16.46$, $p<0.05$; Figure 7D).

Discussion

The BBB serves as a highly selective, yet permeable, barrier between systemic circulation and the central nervous system. Several pathologies, including ischaemic injury, compromise its integrity therefore allowing leakage of circulating molecules into the brain and triggering formation of vasogenic oedema as a consequence, the main cause of death within the first week after a stroke (Hayashi et al. 2004). Microvascular endothelial cells constitute the main cellular component of the BBB due to their ability to express many tight junction proteins, notably occludin and claudin-5, to prevent vascular permeability. Since endothelial cells express and retain these proteins only when co-cultured with astrocytes (Persidsky *et al.* 2006, Hayashi *et al.* 1997), a contact co-culture model of human BBB comprising HBMEC and human astrocytes was used during the current study. This model showed normoxic TEER values which are consistent with similar endothelial cell models reported previously and mimicked the characteristics of the BBB and displayed significant physical and functional impairments when subjected to OGD±R as evidenced by marked decreases in TEER and simultaneous increases in paracellular flux, respectively (Allen et al. 2009a, Allen *et al.* 2010).

As well as being the primary cells to form cerebral barrier, endothelial cells can act as key generators of $O_2^{\bullet-}$ in acute ischaemic settings to initiate or worsen cerebrovascular injury (Raat *et al.* 2009, Chrissobolis *et al.* 2008). Indeed, exposure of HBMEC to OGD±R in the present study led to substantial increases in $O_2^{\bullet-}$ production which was attributed to the enhanced NAD(P)H oxidase activity during a clinically-relevant period of experimental ischaemia, 4 hours OGD alone or followed by reperfusion (Hacke *et al.* 2004). Although high concentrations of peroxidisable lipids, low antioxidant capacity and reactions involving glutamate oxidation may further explain the susceptibility of cerebral barrier to prolonged

oxidative injury, putative contributions of mechanisms functionally intertwined with NAD(P)H oxidase cannot be fully dismissed in this context (Allen *et al.* 2009b).

Recent studies with matrix-degrading proteolytic enzymes have implicated PAs in cerebral barrier disruption because of their ability to digest the basal lamina around the capillaries or modulate MMP-2 activity via their end-product, plasmin (Baramova *et al.* 1997). Consistent with previous studies reporting higher proteolytic activity in mice after induction of focal cerebral ischaemia, significant increases have also been observed in the expressions and/or activities of tPA, uPA and PAI-1 in HBMEC following exposures to OGD±R (Ahn *et al.* 1999, Hosomi et al. 2001, Wang *et al.* 1998). Similar levels of uPAR expression in HBMEC before and after application of ischaemic insult imply adequate existence of this receptor on cerebral endothelial cells to realise full uPA proteolytic activity (Wang *et al.* 2003) and the continuing high level of uPA activity despite high PAI-1 levels in the same conditions may be due to its resistance to inhibition once complexed with uPAR (Higazi *et al.* 1996).

In terms of MMP activities, while dramatic increases were recorded in intracellular and secreted activities of MMP-2 only after induction of reperfusion, the activity of MMP-9 stayed below the detection threshold of gelatin zymography throughout (Tahanian *et al.* 2011, Cavdar *et al.* 2010). Similar to recent studies (Suofu *et al.* 2012) attenuation of MMP-2 activity with MMP-2 inhibitor III showed improvement in both barrier integrity and function during the reperfusion stage. Since MMP-2 activity was not found to be increased during OGD alone it is not surprising that inhibition of MMP-2 during OGD did not have a beneficial impact. A dose dependence study was conducted with MMP-2 inhibitor III and 100 nM selected, as further increasing the dose had no effect in reducing MMP-2 activity further during OGD±R (data not shown). To determine the actual relevance of NAD(P)H oxidase activity to the OGD±R-mediated activations of MMP-2 and PAs, in some experiments the oxidase activity was quenched by apocynin, which has previously been shown to reduce

NAD(P)H oxidase activity in endothelial cells (Bayraktutan, 2004). Apocynin significantly suppressed MMP-2 and tPA activities without affecting that of uPA. Taken together these data suggest that NAD(P)H oxidase operates downstream to uPA and upstream to tPA and MMP-2. The severity of BBB damage during reperfusion, where MMP-2 activity and $O_2^{\bullet-}$ levels are at their highest, further confirm the existence of a close link between MMP-2 and NAD(P)H oxidase. Furthermore, the attenuation of constitutive MMP-2 activities in cells exposed to OGD alone, may indicate the $O_2^{\bullet-}$-independent beneficial effects emerged from targeting of NAD(P)H oxidase (Chen *et al.* 2011, Chen *et al.* 2009).

Having deduced that uPA may sit at the helm of oxidative stress-related BBB damage that emerge during ischaemic injury, the effects of uPA inhibition with amiloride on barrier integrity, MMP-2 and NAD(P)H oxidase activities and $O_2^{\bullet-}$ generation, were tested in OGD±R. A dose dependence study was conducted and 2.5 μM was observed to be effective in normalising uPA activity under OGD±R (data not shown). At lower concentrations, amiloride acts as a specific inhibitor of uPA and displays no effect on tPA expression and activity despite being able to inhibit angiogenesis, capillary morphogenesis and Na^+/H^+ exchange pathway at considerably higher concentrations, ≥130 μM (Haworth *et al*. 1993). Similar to treatments with apocynin, amiloride selectively suppressed $O_2^{\bullet-}$ release and NAD(P)H oxidase activity in cells exposed to OGD±R, affirming the supposed regulatory effect of uPA on NAD(P)H oxidase activity. Again, like apocynin, it attenuated reperfusion-mediated increases in intracellular and secreted MMP-2 activities. Amiloride also improved the physical and functional aspects of the BBB in the same settings.

To discover the specific contributions of PAs to OGD-evoked BBB damage, the intracellular levels of both PAs and uPA alone were manipulated by electroporation of anti-PAI-1 or anti-uPAR antibodies into HBMEC before establishing and exposing co-cultures to a condition that yields considerable barrier damage i.e. 20 hours of OGD alone (Allen *et al.*

2010). These revealed that selective increases in total PA activity but not tPA activity alone led to severe impairments in the BBB integrity and function under both normoxic and ischaemic conditions. Part of the modulation of uPAR function involves the internalisation of the uPA-PAI-1- uPAR complex. Once internalised, the uPA-PAI-1 complex dissociates from uPAR and is transferred to the lysosomes while the free uPAR returns to the plasma membrane. To suppress this recycling which regulates uPAR signalling, in these experiments the uPAR antibody was introduced into the cells. The use of a relatively high, but nontoxic, concentration of anti-uPAR IgG (3 μg) ensured concurrent neutralisation of uPAR on the plasma membrane as well.

Despite exaggerated release of $O_2^{\bullet-}$, treatments of acute ischaemic stroke patients with free-radical scavengers produced somewhat contradictory results -beneficial versus neutral- in terms of lesion volume regression and clinical outcomes (Shuaib *et al.* 2007, Nakase *et al.* 2011). To explore the specific involvements of NAD(P)H oxidase and $O_2^{\bullet-}$ on barrier integrity and function and also to assess the therapeutic role of interventions with uPA activity, the levels of enzyme activity and $O_2^{\bullet-}$ availability were elevated in HBMEC through electroporation of p22-phox cDNA and treatments with pyrogallol, respectively (Ear *et al.* 2001). Both applications produced pathologically relevant quantities of $O_2^{\bullet-}$, similar to the levels observed after 4 OGD±R, and evoked substantial impairments in the BBB integrity and function. Similar to ischaemic insult, elevations in NAD(P)H oxidase activity and ensuing $O_2^{\bullet-}$ release also increased the protein expressions of tPA. Attenuation of uPA proteolytic activity by amiloride or cessation of its binding to uPAR, a prerequisite for enzyme activity (Fuhrman *et al.* 2008) completely abolished these changes.

In conclusion, the data presented in the current study suggest that suppression of uPA activity may protect cerebral barrier against ischaemia-reperfusion injury by subduing MMP-

2 activity in a direct fashion or through attenuation of NAD(P)H oxidase-dependent $O_2^{\bullet-}$ generation.

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None

Abbreviations

BBB, Blood-brain barrier; EBA, Evan's blue-labelled albumin; HBMEC, Human brain microvascular endothelial cells; MMP, MMP-2, MMP-9, matrix metalloproteinases, -2, -9; MMP-2 inhibitor III, 2-((Isopropoxy)-(1,1'-biphenyl-4-ylsulfonyl)-amino)-H-hydroxyacetamide; $O_2^{\bullet-}$, superoxide anion; OGD, oxygen-glucose deprivation; OGD±R, OGD±reperfusion; PAs, plasminogen activators; PAI-1, plasminogen activator inhibitor-1; TEER, transendothelial electrical resistance; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor.

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Figure legends

FIG. 1. The effects of OGD±R on the blood-brain barrier integrity and function. Human brain microvascular endothelial cell-human astrocyte co-cultures were exposed to 4 hours of oxygen-glucose deprivation alone (OGD) and followed by 20 hours of reperfusion (OGD+R) with and without inhibitors for urokinase-type plasminogen activator, amiloride; NAD(P)H oxidase, apocynin and matrix metalloproteinase-2, MMP-2 inhibitor III. OGD alone and OGD+R presented a significant decrease in transendothelial electrical resistance (TEER) and concurrent increase in Evan's blue albumin (EBA) flux. Treatments with amiloride and apocynin increased TEER values (A) and decreased EBA flux volumes (B) in all conditions compared to untreated groups. Treatment with MMP-2 inhibitor III increased TEER values and decreased EBA flux volumes during the reperfusion stage only. Data are expressed as mean±SEM from $n \geq 3$ and were analysed using a two way ANOVA followed by a Tukey post hoc test. *, † and ‡ $p<0.05$ vs normoxia (N), 4 hours of OGD and corresponding untreated cells, successively.

FIG. 2. The effects of OGD±R on NAD(P)H oxidase system. Human brain microvascular endothelial cells were exposed to 4 hours of oxygen-glucose deprivation alone (OGD) and followed by 20 hours of reperfusion (OGD+R) with and without inhibitors for urokinase-type plasminogen activator, amiloride; NAD(P)H oxidase, apocynin and matrix metalloproteinase-2, MMP-2 inhibitor III. NAD(P)H oxidase activity (A), total $O_2^{\bullet-}$ levels (B) and p22-phox protein expression (C) were significantly increased after OGD±R compared to controls. Treatments with amiloride and apocynin normalised total $O_2^{\bullet-}$ levels and NAD(P)H oxidase activity during ODG±R while treatments with MMP-2 inhibitor III proved to be ineffective. Data are expressed as mean±SEM from $n \geq 3$ and were analysed using a two way ANOVA followed by a Tukey post hoc test for (A-B) and a one way ANOVA followed by Tukey post

hoc test for (C). *, † and ‡ $p<0.05$ vs normoxia (N), 4 hours of OGD and corresponding untreated cells, successively.

FIG. 3. The effects of pyrogallol on TEER, EBA flux and tPA protein levels. Human brain microvascular endothelial cell-human astrocyte co-cultures were exposed to pyrogallol with and without and inhibitor for urokinase-type plasminogen activator, amiloride. Exposure to pyrogallol significantly impaired BBB integrity as shown by decreases in transendothelial electrical resistance (TEER) and increases in Evan's blue albumin (EBA) flux volumes which were normalised by amiloride (A-B). Pyrogallol significantly increased total superoxide anion ($O_2^{\bullet-}$) levels (C) and tissue-type plasminogen activator (tPA) protein levels (D) in endothelial cells. Data are expressed as mean±SEM from $n\geq3$ and were analysed using a one way ANOVA followed by Tukey post hoc test for (A-B) a Student's *t*-test for (C-D). *$p<0.05$ vs control and †$p<0.05$ vs pyrogallol-treated cells.

FIG. 4. The effect of p22-phox cDNA transfection on NAD(P)H oxidase system, tPA protein levels and blood-brain barrier. Human brain microvascular endothelial cells transfected with p22-phox or control cDNA cultured alone or with human astrocytes. Cells were then exposed to amiloride, a urokinase-type plasminogen activator (uPA) inhibitor, or concurrently transfected with an anti-uPA receptor (uPAR) or anti-IgG antibody. Transfection markedly increased NAD(P)H oxidase activity and total superoxide anion ($O_2^{\bullet-}$) levels compared to controls which were normalised by treatments with amiloride or anti-uPAR antibody. Transfection with vehicle, control cDNA or anti-IgG antibody had no effect on NAD(P)H oxidase activity or $O_2^{\bullet-}$ levels (A-B). p22-phox cDNA transfection led to significant increases in both p22-phox and tissue-type plasminogen activator (tPA) protein expressions (C-D). Treatments with amiloride and transfection of anti-uPAR antibody

abolished p22-phox cDNA transfection-mediated changes in the blood-brain barrier integrity (E-G) and function (H-J). Data are expressed as mean±SEM from n≥3 and were analysed using a one way ANOVA followed by Tukey post hoc test for (A-B, E, G-H and J) and a Student's *t*-test for (C-D, F and I). $^{*}p<0.05$ vs vehicle and control cDNA and $^{\dagger}p<0.05$ vs p22-phox cDNA-transfected cells.

FIG. 5. The effects of OGD±R on intracellular and secreted MMP-2 activities. Human brain microvascular endothelial cells were exposed to 4 hours of oxygen-glucose deprivation alone (OGD) and followed by 20 hours of reperfusion (OGD+R) with and without inhibitors for urokinase-type plasminogen activator, amiloride; NAD(P)H oxidase, apocynin and matrix metalloproteinase-2 (MMP-2), MMP-2 inhibitor III. In the normoxia groups cells were exposed to cell culture media for either 4 or 20 hours to correspond to 4 hours in OGD or 20 hours of reperfusion. OGD+R, but not OGD alone, caused significant elevations in intracellular and secreted MMP-2 activities in brain microvascular endothelial cells (A-F). While treatments with apocynin significantly reduced intracellular and secreted MMP-2 activities in all conditions (E-F), the inhibitory effects of amiloride were confined to OGD+R groups (C-D). Data are expressed as mean±SEM from n≥3 and were analysed using a one way ANOVA followed by Tukey post hoc test. *, † and ‡ $p<0.05$ vs normoxia groups (N4h or N20h), 4 hours of OGD and corresponding untreated cells, successively.

FIG. 6. The effects of OGD±R on plasminogen-plasmin system components. Human brain microvascular endothelial cells were exposed to 4 hours of oxygen-glucose deprivation alone (OGD) and followed by 20 hours of reperfusion (OGD+R) with and without inhibitors for urokinase-type plasminogen activator, amiloride; NAD(P)H oxidase, apocynin and matrix metalloproteinase-2, MMP-2 inhibitor III. OGD±R evoked significant increases in

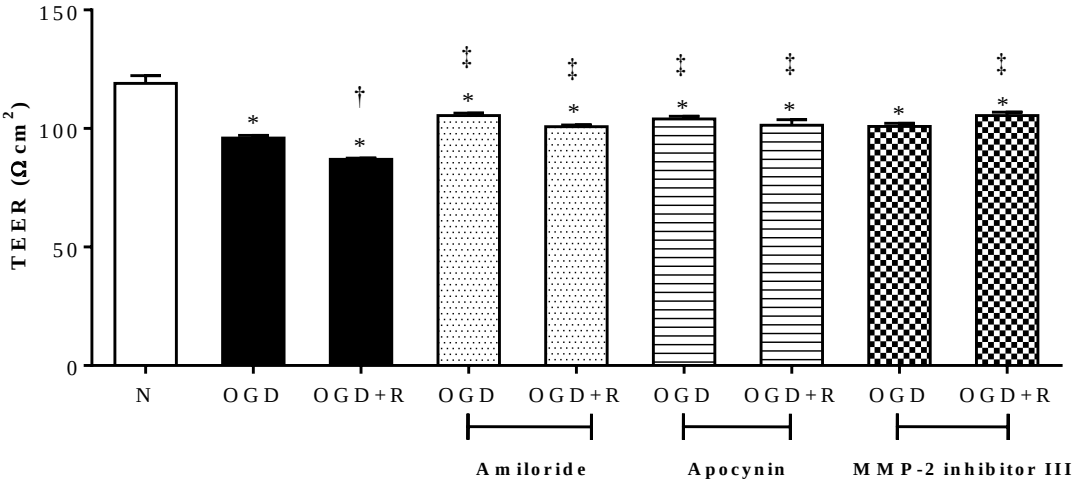
plasminogen activator inhibitor-1 (PAI-1), tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) protein levels and/or activities without affecting that of uPAR, uPA receptor (A-F). Treatments with apocynin normalised tPA activity but failed to affect uPA activity. MMP-2 inhibitor III did not affect tPA and uPA activities. uPA but not tPA activity was effectively suppressed by amiloride confirming the ability of this drug to attenuate uPA activity alone (D and F). Data are expressed as mean±SEM from n≥3 and were analysed using a one way ANOVA followed by Tukey post hoc test. *, † and ‡ $p<0.05$ vs normoxia (N), 4 hours of OGD and corresponding untreated cells, successively.

FIG. 7. The effects of anti-PAI-1 and anti-uPAR antibody transfection on blood-brain barrier. Human brain microvascular endothelial cells transfected with anti-plasminogen activator inhibitor-1 (PAI-1) or anti-urokinase-type plasminogen activator receptor (uPAR) antibodies were co-cultured with human astrocytes and exposed to 20 hours of oxygen-glucose deprivation (OGD). Exposures to extended periods of OGD led to significant disruption of the blood-brain barrier as evidenced by decreases in TEER, transendothelial electrical resistance (A) and increases in Evan's blue albumin (EBA) flux (B). The magnitudes of these changes were greater in co-cultures established with anti-PAI-1 antibody-transfected endothelial cells. In contrast, co-cultures established with anti-uPAR antibody-transfected endothelial cells displayed similar TEER or EBA flux when compared to vehicle-transfected (distilled H_2O) controls (A-B). Both tPA and uPA activities were increased after 20 h OGD and further increased in endothelial cells transfected with anti-PAI-1 antibody. Transfection with anti-uPAR antibody had no effect on tPA activity but reduced uPA activity in normoxic and 20 h OGD exposed cells. Transfection with an anti-IgG antibody had no effect on PA activities (C-D). Data are expressed as mean±SEM from n≥3 and were analysed using a one way ANOVA followed by Tukey post hoc test. *, †, ‡ and §

$p<0.05$ vs vehicle transfected normoxia (N), vehicle transfected 20 hours OGD, anti-PAI-1 transfected normoxia and anti-PAI-1 transfected 20 hours OGD, successively.

Figure 1

A


150
100
50
0
TEER (Ω cm²)
N
OGD
OGD+R
OGD
OGD+R
OGD
OGD+R
OGD
OGD+R
Amiloride
Apocynin
MMP-2 inhibitor III

B

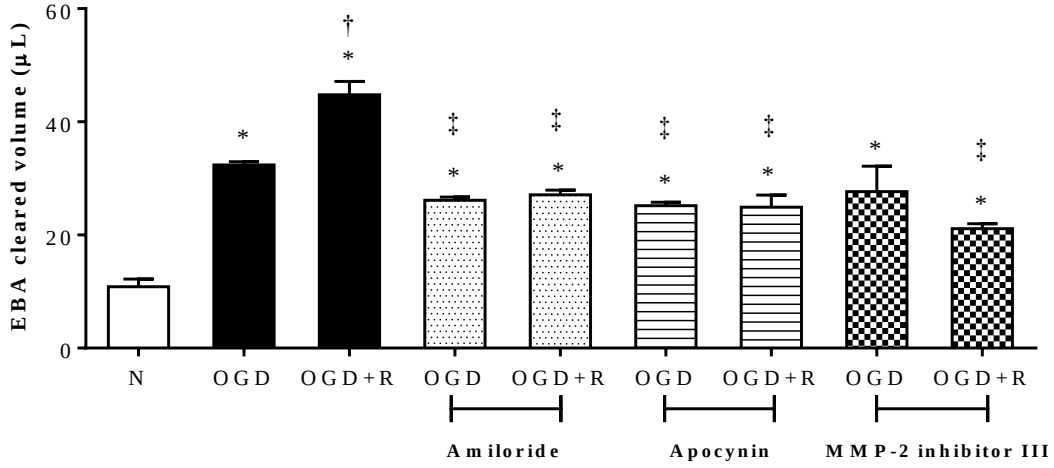
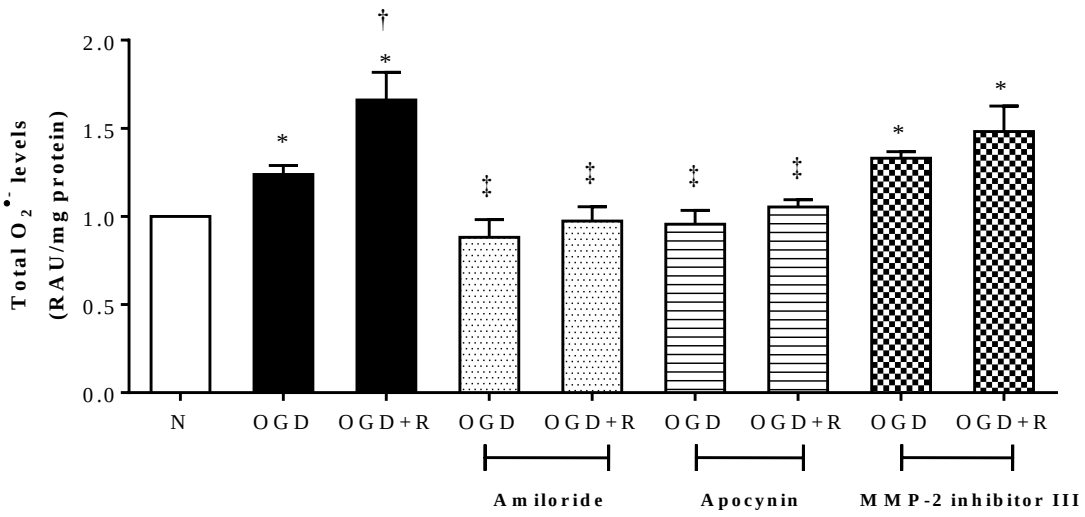
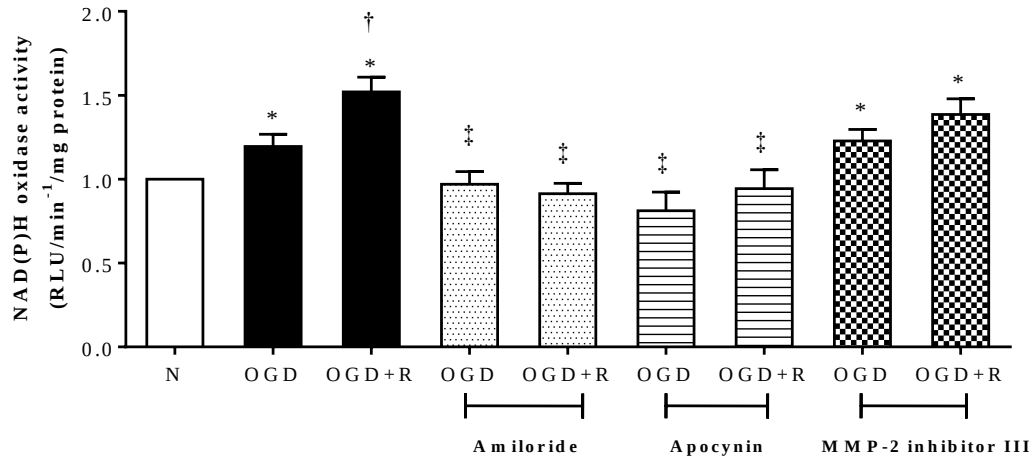

60
40
20
0
EBA cleared volume (µL)
N
OGD
OGD+R
OGD
OGD+R
OGD
OGD+R
OGD
OGD+R
Amiloride
Apocynin
MMP-2 inhibitor III

Figure 2

A


Total $O_2^{\bullet-}$ levels (RAU/mg protein)
2.0
1.5
1.0
0.5
0.0
N
OGD
OGD+R
OGD
OGD+R
OGD
OGD+R
OGD
OGD+R
Amiloride
Apocynin
MMP-2 inhibitor III

B


NAD(P)H oxidase activity (RLU/min^{-1}/mg protein)
2.0
1.5
1.0
0.5
0.0
N
OGD
OGD+R
OGD
OGD+R
OGD
OGD+R
OGD
OGD+R
Amiloride
Apocynin
MMP-2 inhibitor III

C

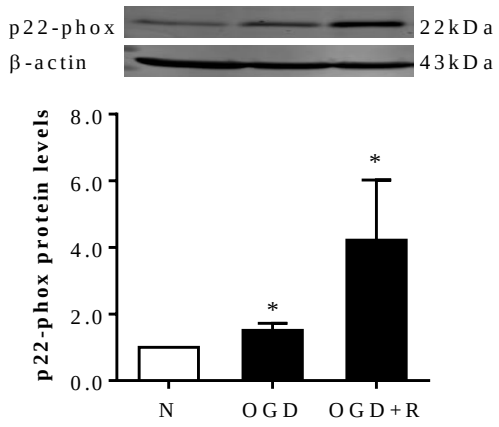

p22-phox
22kDa
β-actin
43kDa
p22-phox protein levels
8.0
6.0
4.0
2.0
0.0
N
OGD
OGD+R

Figure 3

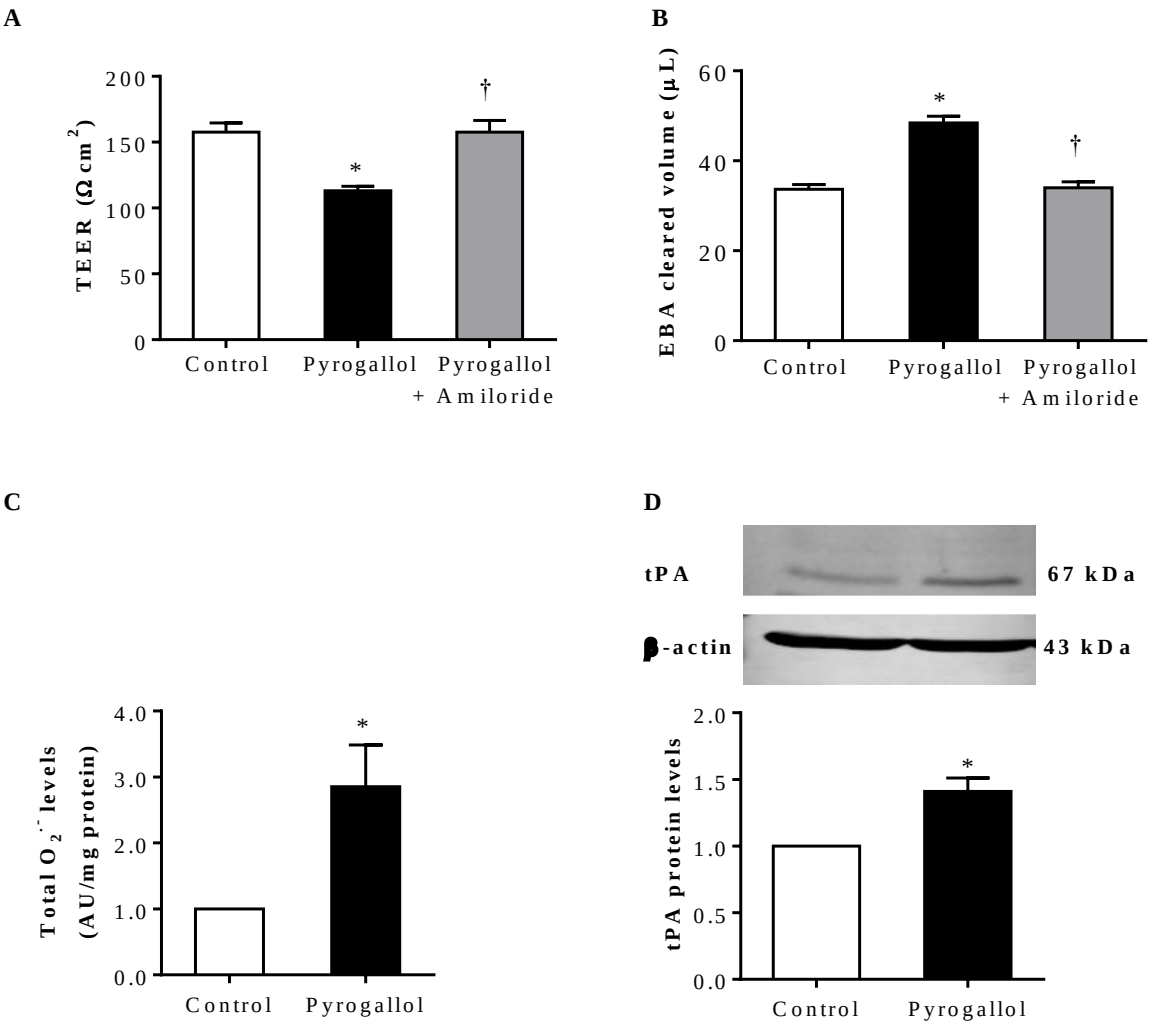

A
TEER (Ω cm²)
200
150
100
50
0
*
†
Control
Pyrogallol
Pyrogallol + Amiloride
B
EBA cleared volume (µL)
60
40
20
0
*
†
Control
Pyrogallol
Pyrogallol + Amiloride
C
Total O₂·⁻ levels (AU/mg protein)
4.0
3.0
2.0
1.0
0.0
*
Control
Pyrogallol
D
tPA
67 kDa
β-actin
43 kDa
tPA protein levels
2.0
1.5
1.0
0.5
0.0
*
Control
Pyrogallol

Figure 4

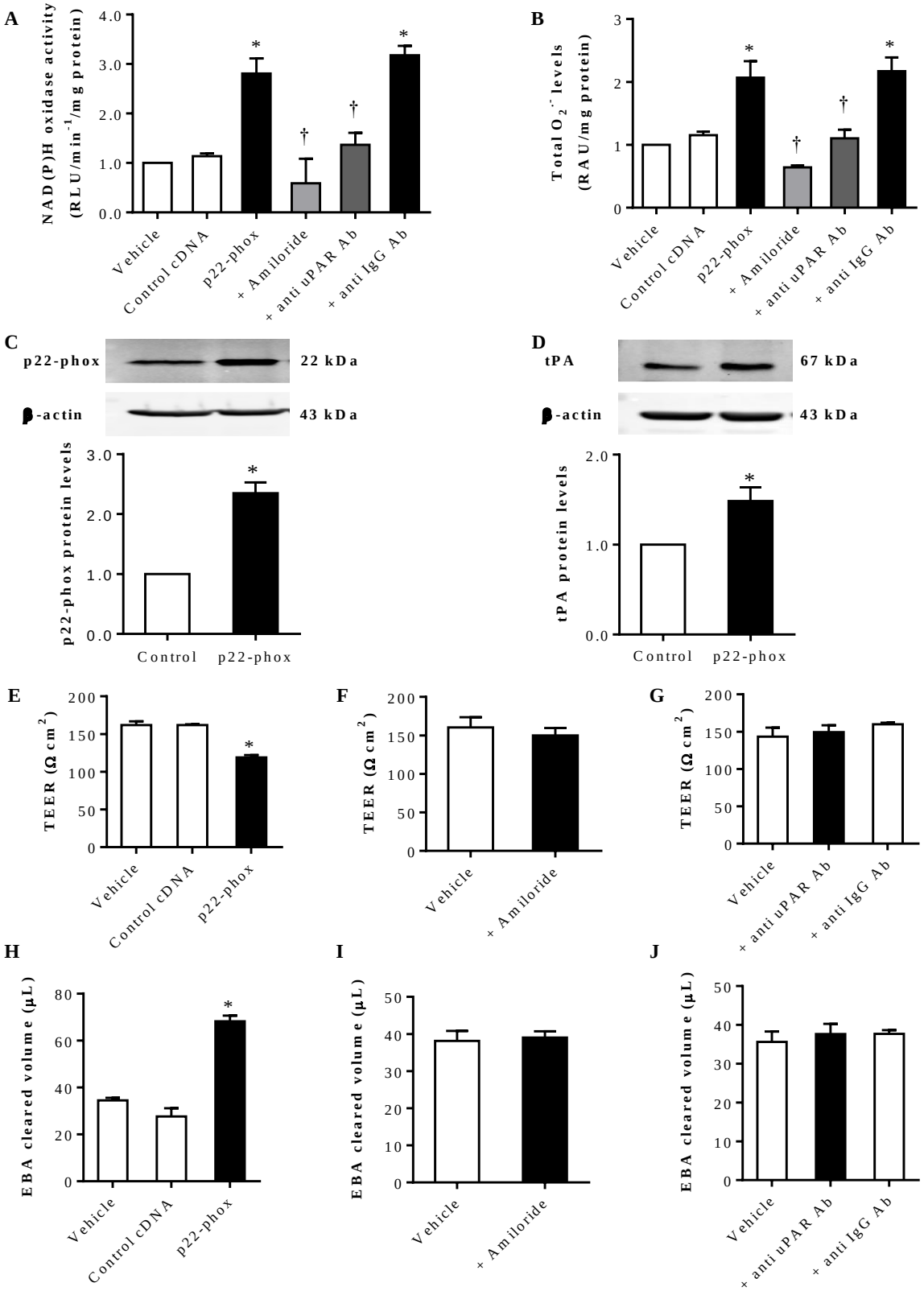

A
NAD(P)H oxidase activity (RLU/min-1/mg protein)
4.0
3.0
2.0
1.0
0.0
Vehicle
Control cDNA
p22-phox
+ Amiloride
+ anti uPAR Ab
+ anti IgG Ab
B
Total O2.- levels (RAU/mg protein)
3
2
1
0
C
p22-phox
22 kDa
β-actin
43 kDa
p22-phox protein levels
Control
p22-phox
D
tPA
67 kDa
β-actin
43 kDa
tPA protein levels
E
TEER (Ω cm2)
200
150
100
50
0
F
G
H
EBA cleared volume (µL)
80
60
40
20
I
50
40
30
10
J

Figure 5

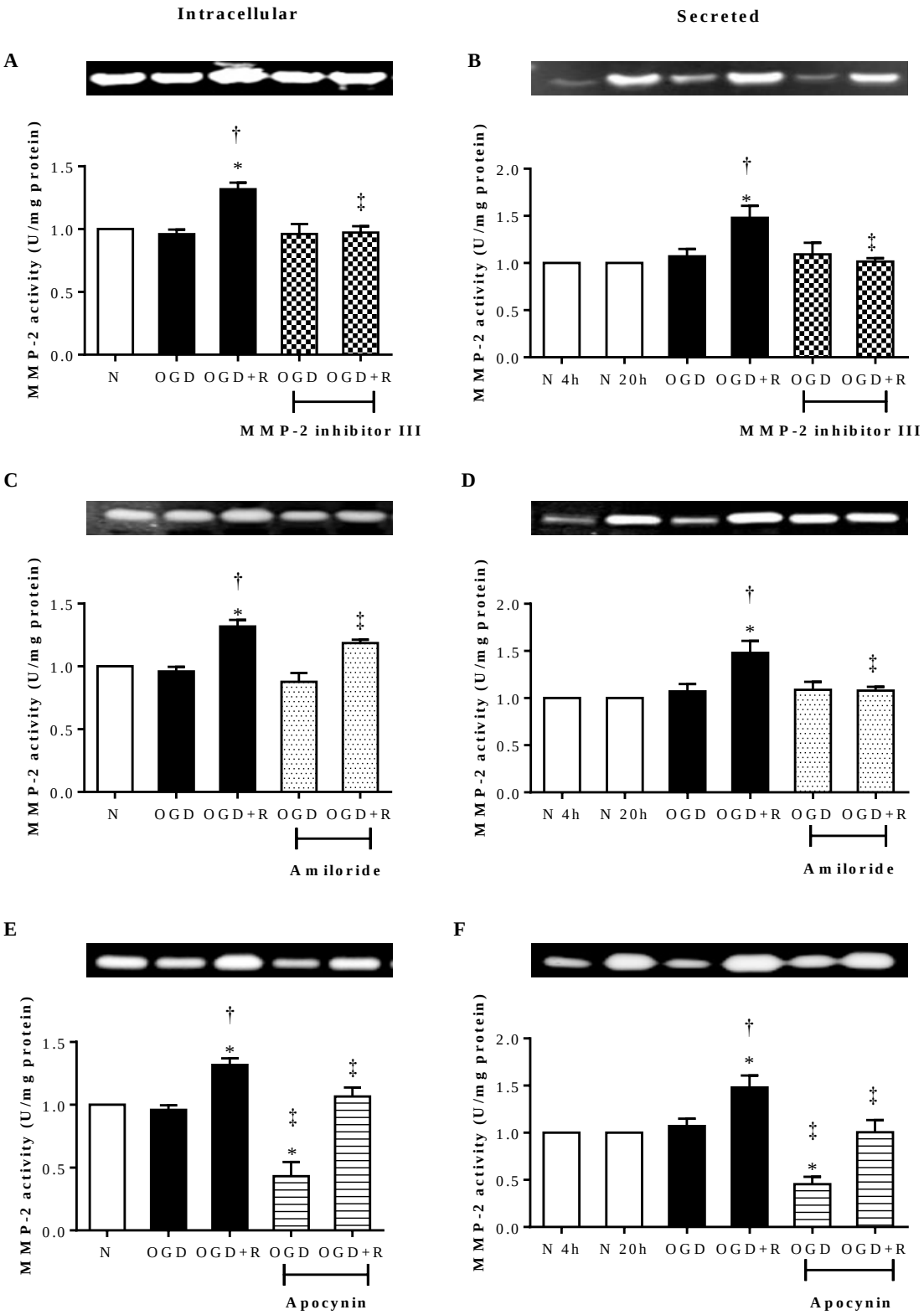

Intracellular
Secreted
A
B
C
D
E
F
MMP-2 activity (U/mg protein)
N
OGD
OGD+R
N 4h
N 20h
MMP-2 inhibitor III
Amiloride
Apocynin

Figure 6

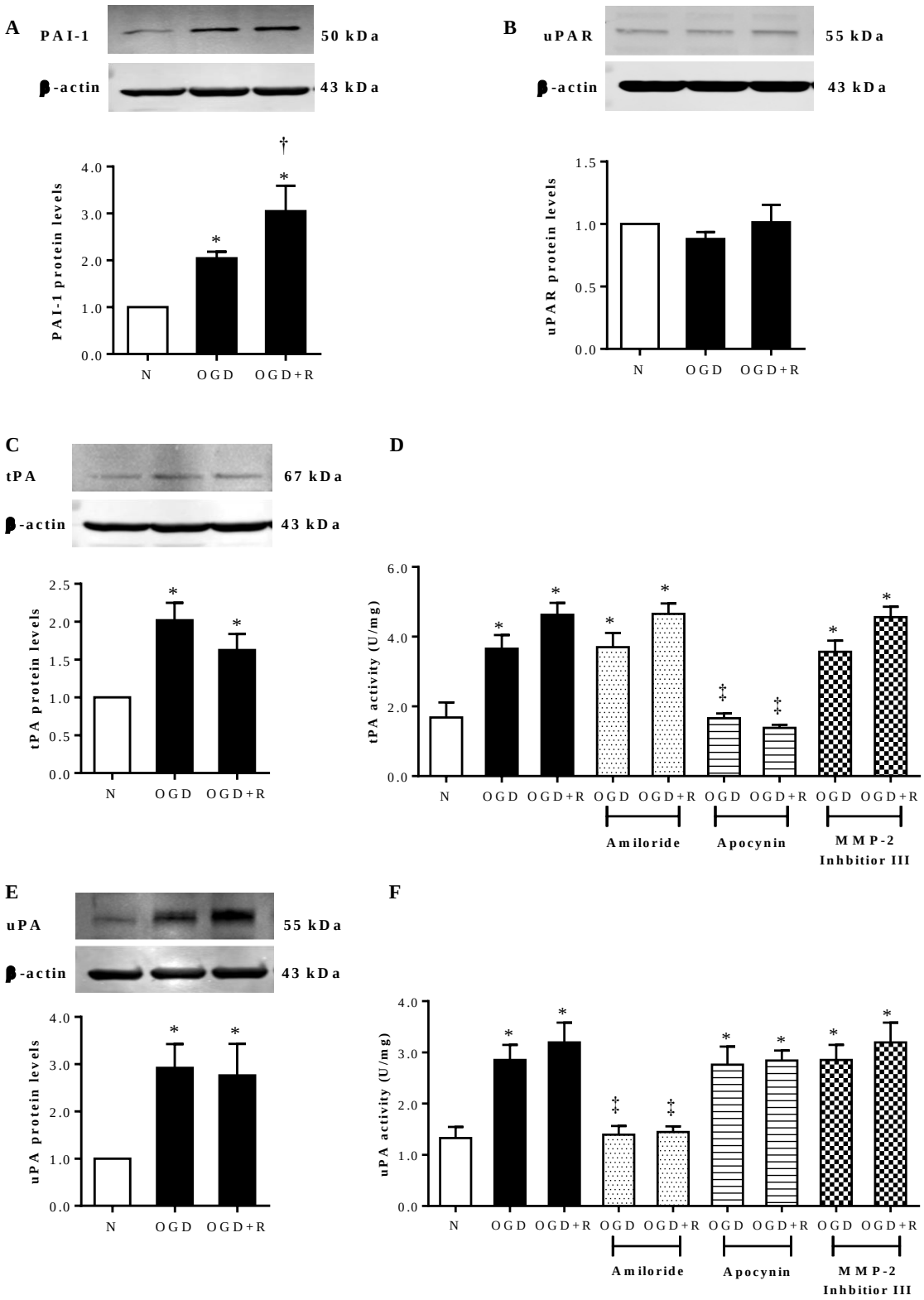
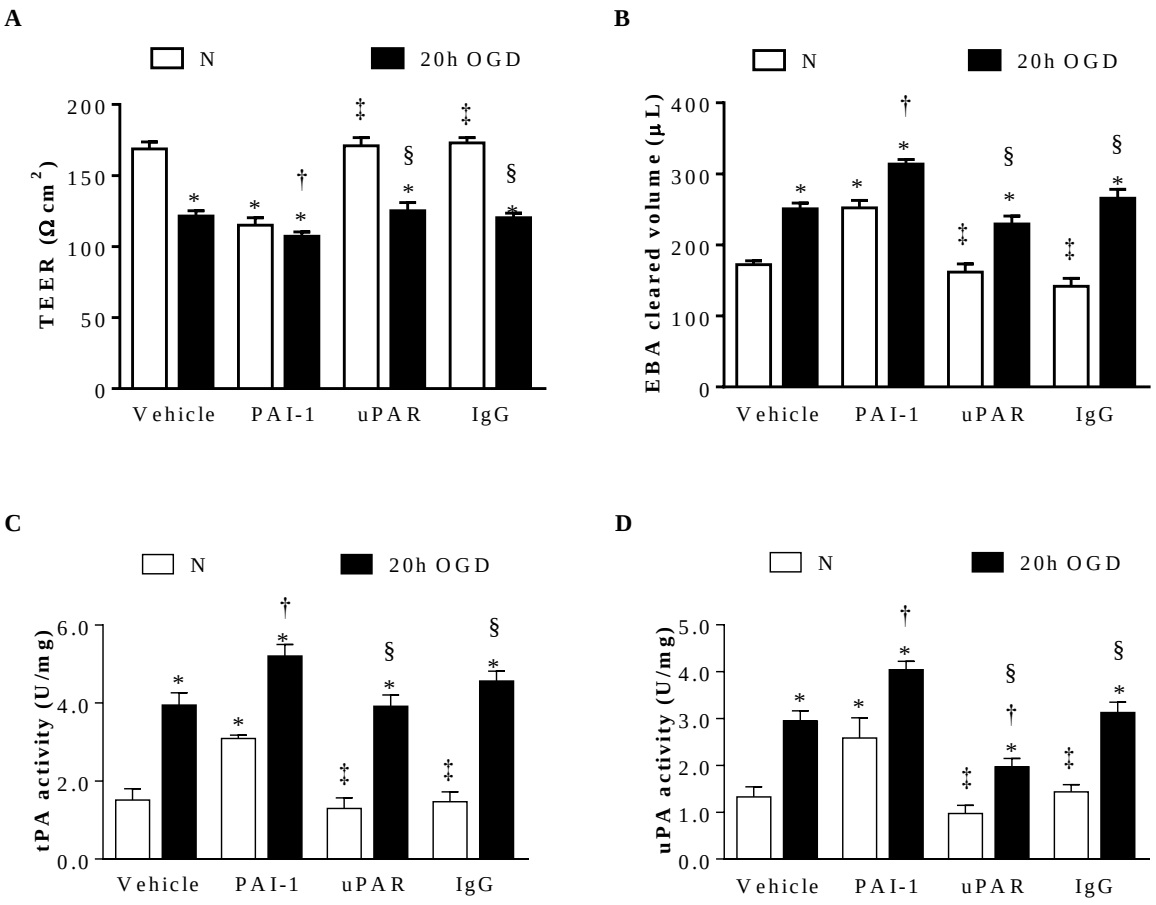

A
PAI-1
50 kDa
β-actin
43 kDa
PAI-1 protein levels
4.0
3.0
2.0
1.0
0.0
N
OGD
OGD+R
B
uPAR
55 kDa
β-actin
43 kDa
uPAR protein levels
1.5
1.0
0.5
0.0
C
tPA
67 kDa
β-actin
43 kDa
tPA protein levels
2.5
2.0
1.5
1.0
0.5
0.0
D
tPA activity (U/mg)
6.0
4.0
2.0
0.0
Amiloride
Apocynin
MMP-2 Inhbitior III
E
uPA
55 kDa
β-actin
43 kDa
uPA protein levels
F
uPA activity (U/mg)

Figure 7


A
N
20h OGD
TEER (Ω cm²)
200
150
100
50
0
Vehicle
PAI-1
uPAR
IgG
B
N
20h OGD
EBA cleared volume (µL)
400
300
200
100
0
Vehicle
PAI-1
uPAR
IgG
C
N
20h OGD
tPA activity (U/mg)
6.0
4.0
2.0
0.0
Vehicle
PAI-1
uPAR
IgG
D
N
20h OGD
uPA activity (U/mg)
5.0
4.0
3.0
2.0
1.0
0.0
Vehicle
PAI-1
uPAR
IgG