

Inhibition of Rho-kinase protects cerebral barrier from ischemia-evoked injury through modulations of endothelial cell oxidative stress and tight junctions

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Abstract

Ischemic strokes evoke blood-brain barrier (BBB) disruption and edema formation through a series of mechanisms involving Rho-kinase activation. Using an animal model of human focal cerebral ischemia, this study assessed and confirmed the therapeutic potential of Rho-kinase inhibition during the acute phase of stroke by displaying significantly improved functional outcome and reduced cerebral lesion and edema volumes in fasudil- versus vehicle-treated animals. Analyses of ipsilateral and contralateral brain samples obtained from mice treated with vehicle or fasudil at the onset of reperfusion plus 4h post-ischemia or 4h post-ischemia alone revealed these benefits to be independent of changes in the activity and expressions of oxidative stress- and tight junction-related parameters. However, closer scrutiny of the same parameters in brain microvascular endothelial cells subjected to oxygen-glucose deprivation \pm reperfusion revealed marked increases in prooxidant NADPH oxidase enzyme activity, superoxide anion release and in expressions of antioxidant enzyme catalase and tight junction protein claudin-5. Co-treatment of cells with Y-27632 prevented all of these changes and protected *in vitro* barrier integrity and function. These findings suggest that inhibition of Rho-kinase after an acute ischemic attack improve cerebral integrity and function through regulation of endothelial cell oxidative stress and reorganisation of intercellular junctions.

Introduction

Stroke is the third leading cause of mortality and the primary cause of morbidity in the Western World. Ischemic strokes constitute about 85% of all stroke cases and develop through an interference with blood supply to the brain. Pathologically, ischemic stroke is characterised by initial excitotoxicity and ensuing secondary complications such as inflammation, edema formation, oxidative damage and apoptosis (Moskowitz, 2010). Given the long lasting nature of the secondary mechanisms and their capacity to influence patients' outcome, it is of paramount importance to discover agent(s) that can target most, if not all, of these pathologies. Although to date several compounds with neuroprotective properties have been identified in experimental settings, their translation to clinical practice has been unsuccessful due to associated toxic side effects and/or availability of a limited therapeutic window after an ischemic episode (Sutherland et al., 2012).

Brain edema constitutes the main cause of death within the first week after an ischemic stroke and at molecular level is characterised by dissolution of tight junctions (TJs) that are formed between adjacent endothelial cells via interactions of key transmembrane proteins including claudins and occludin and prevent the entry of blood constituents into the brain parenchyma (Gebel et al., 2002). Bearing these in mind, it is reasonable to suggest that any pathological stimuli affecting the formation of TJs will also affect the permeability of the barrier that exists between the blood and the brain, blood-brain barrier (BBB).

The molecular mechanisms involved in ischemia-evoked BBB damage remain largely unknown (Hacke et al., 1996). However, accumulating evidence have proven Rho-kinase as an important determinant of vascular tone and integrity due to its regulatory roles in cell proliferation, inflammation, stress fibre formation, myosin light chain phosphorylation and nitric oxide bioavailability (Sahai & Marshall, 2002; Van Aelst & D'Souza-Schorey, 1997; Allen et al., 2010; Takemoto et al., 2002). Indeed, increased expression and activity of Rho-

kinase in endothelial cells have recently been shown to account for the hyperpermeability of an *in vitro* model of BBB subjected to oxygen-glucose deprivation (OGD) or hyperglycaemia (Allen et al., 2010, Srivastava et al., 2013).

Oxidative stress emerging from an imbalance between the production and metabolism of free radicals, in particular superoxide anion (O_2^-) during ischemic injury may also contribute to the BBB damage. NADPH oxidase accounts for much of the O_2^- generation in ischemic brain and cerebral cells and is composed of several cytosolic subunits and a membrane-bound cytochrome b_{558} that consists of gp91-phox and p22-phox subunits and is required for enzymatic activity and stability as a whole. Once produced, O_2^- is rapidly converted into H_2O_2 by superoxide dismutases which in turn is further metabolised to H_2O by catalase and glutathione peroxidase (Shao & Bayraktutan, 2013; Bayraktutan U., 2005).

In light of the above, using an animal model of human focal cerebral ischemia, the current study aimed to assess the therapeutic effect of Rho-kinase inhibition on cerebral barrier integrity. It then comparatively examined the efficacies of rapid and slightly delayed Rho-kinase inhibitions in barrier protection. Finally, it aimed to unravel the molecular mechanisms responsible for the putative beneficial effects by using ipsilateral versus contralateral brain slices as well as isolated brain microvascular endothelial cells (BMEC).

Materials and Methods

This study was conducted in accordance with the UK Animals Scientific Procedures Act, 1986 (Project Licence 80/2015) and used adult male C57/BL6 mice (10-12 weeks at the time of surgery) supplied from Charles River (UK).

***In vivo* cerebral ischemia**

In order to induce focal cerebral ischemia, mice were subjected to middle cerebral artery occlusion (MCAO), as described previously (Gibson & Murphy, 2004). A total of 40 mice underwent transient MCAO; 2 mice died following MCAO and 1 mouse was sacrificed for welfare reasons. Anaesthesia was induced by inhalation of 4% isoflurane (in an NO₂/CO₂ 70/30% mixture) and maintained by inhalation of 1.5% isoflurane. Body temperature was maintained at 37±0.6°C using a heated mat and lubricant was applied to the eyes to prevent drying during surgery. Laser doppler flowmetry was used to measure blood flow of animals during MCAO and blood flow had to be reduced by at least 70% in order for animals to be included in analyses. After 45 min of MCAO the animal was re-anaesthetised and the filament was withdrawn in order to allow reperfusion. Mice received either fasudil (Sigma, 10mg/kg dissolved in saline) or vehicle (i.e. saline only) via intraperitoneal injection. Mice were randomly assigned to receiving fasudil at onset of reperfusion and again at 4h post-MCAO, fasudil only at 4h post-MCAO or vehicle treatment. The experimenter was blinded to the treatment the mice had received prior to all subsequent analyses.

Indicators of general well-being and functional outcome

Mice were weighed at 24 h post-surgery as an indicator of their general well-being. Body weights are presented as a percentage change compared with values recorded immediately prior to undergoing MCAO. Survival rates of animals per experimental group are presented as a percentage compared with the number of animals undergoing surgery. At 6 and 24 h post-MCAO each animal was assessed for focal deficits using a 28-point neurological scale

(Clark et al., 1998). In addition, at 24 h post-MCAO all mice underwent the grid test, as previously described (Gibson et al., 2011). Mice were assessed, during locomotion on a grid surface (grid openings of 2.5cm²), for the number of foot faults made by the ipsilateral and contralateral limbs. The foot faults are expressed as the number of errors made by the contralateral limbs as a percentage of the total errors made.

Quantification of infarct volume and brain water content

In order to quantify the area of ischemic damage, mice (fasudil n=8, fasudil + 4h n=8, vehicle n=8) were assessed for lesion volume at 24 h post-MCAO as previously described (Gibson & Murphy, 2004). Infarct volume was calculated using an indirect method as described by Loihl et al. (1999) which avoids overestimation of infarct area due to edema. A group of mice undergoing MCAO (fasudil n=6, vehicle n=7) were sacrificed at 24 h post-MCAO and analyzed for brain water content using the wet-dry method as previously described (Gibson et al., 2005). The percentage of water of each brain hemisphere was calculated as follows: percent brain water = wet weight-dry weight/wet weight x 100.

Cell culture

BMEC (>5x10⁵ cells) and astrocytes (>5x10⁶ cells) isolated from cerebral cortex and cryopreserved at passage 1 were purchased from TCS CellWorks Ltd (Buckingham, UK). Cells between passages 3 and 6 were cultured in their respective specialized media to full confluence before exposure to clinically relevant periods of OGD alone (OGD-R, 4 h) or followed by reperfusion (OGD+R; 4/20 h) or a longer period of OGD alone (20 h). As exposures to longer periods of OGD+R (20/20 h) effectively restored *in vitro* barrier integrity possibly through nascent protein synthesis, the effect of this treatment regimen on the following parameters was not studied (Allen et al., 2010). To investigate the effects of Rho-kinase inhibition on a given parameter, in some experiments the cells were co-treated with a highly specific Rho-kinase inhibitor, Y-27632 (10 µM).

Establishment of an *in vitro* model of cerebral barrier

An *in vitro* model of BBB comprising BMEC and astrocytes was generated using transwell inserts (12 mm diameter, 0.4 μm pore size, Corning). For this, astrocytes were seeded onto the outer surface of inserts directed upside down. Following their overnight adherence to the membranes, the inserts were inverted the correct way and BMEC were seeded onto the inner parts. Both cell layers were cultured to confluence under normal conditions before exposing to the outlined experimental conditions.

Measurements of *in vitro* barrier integrity and function

The integrity and function of BMEC and astrocyte co-cultures were assessed as previously described by measurements of transendothelial electrical resistance (TEER) and paracellular flux of Evan's blue-labelled albumin (EBA; 67 kDa), respectively (Allen & Bayraktutan, 2009; Allen et al., 2010).

Immunoblotting

Equal quantities of total protein samples (10-30 μg) obtained from ipsilateral and contralateral hemisphere slices or cultured BMEC were run on SDS-polyacrylamide gels (10-15%) before transferring onto PVDF membranes. The membranes were then concurrently incubated with primary antibodies raised against β -actin (loading control, Sigma) and gp91-phox, CuZn-SOD, catalase, occludin (Santa Cruz Biotech) or claudin-5 (Abcam). The primary antibodies were detected by appropriate species-specific infrared 700/800-tagged secondary antibodies prior to analyses of bands via Odyssey infrared imaging system (Li-Cor Biosciences).

Measurements of total $\text{O}_2^{\cdot-}$ levels and NAD(P)H oxidase activity

The levels of total $\text{O}_2^{\cdot-}$ were detected by cytochrome *C* reduction assay as previously described (Allen & Bayraktutan, 2009). Briefly, **brain slices or** cell pellets were sonicated in cold lysis buffer containing HEPES buffer (20 mM, pH 7.2), EGTA (1 mM), mannitol (210

mM) and sucrose (70 mM). Equal amounts of homogenate (100 µg) were then incubated with cytochrome C (50 µM) for 60 min at 37°C and absorbances were read 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 free radical-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 min NADH or NADPH (100 µM; Calbiochem) was added to initiate the reaction. The reaction was monitored every minute for 2 h and the rate of reaction calculated. Buffer blanks were also run for both assays and subtracted from the readings.

Statistical analyses

All data are reported as mean±SEM and were analysed using either IBM SPSS 20.0 software or GraphPad Prism version 5.0 for windows (GraphPad Software, San Diego, USA). Data were analysed by comparing the mean using student's t-test or either one-way or two-way analysis of variance followed by *post hoc* analyses, where appropriate. The criterion for statistical significance was $P<0.05$.

Results

Effect of Y-27632 on well-being and functional outcome following ischemic stroke

All experimental groups lost weight following MCAO. However, fasudil treatment initiated at the onset of reperfusion (n=14) significantly reduced the amount of weight lost compared to vehicle treatment (n=15) whereas delayed fasudil treatment had no effect on body weight (n=8). Mice were assessed for neurological function using a 6-point and 28-point neurological score at 6 h and 24 h post-MCAO. Mice that had received fasudil

treatment, either at onset of reperfusion or 4h post-MCAO, performed significantly better on the 6-point neurological score compared to vehicle treatment ($F_{2,32} = 7.809$, $P = 0.0008$) and post-hoc analyses found that both fasudil-treated groups showed improved performance at 6h post-MCAO compared to vehicle treatment. In addition, a significant difference was observed according to treatment ($F_{2,32} = 3.44$, $P = 0.037$) when function was assessed using the 28-point neurological score although post-hoc analyses did not identify significant differences at any individual time points. Following MCAO, there was a significant effect of treatment on the number of foot faults ($F_{2,32} = 10.28$, $P = 0.0004$) and post-hoc analysis revealed that both fasudil treatment initiated at reperfusion, and fasudil treatment initiated at 4h post-MCAO, resulted in significantly fewer foot faults compared with vehicle treatment ($P < 0.01$, Figure 1A-D).

Effect of Y-27632 treatment on ischemic damage and brain water content

Calculation of lesion volume, from TTC staining, revealed via one-way ANOVA, a significant effect ($P=0.016$) of treatment on lesion volume (Figure 2A-B). Post-hoc analysis demonstrated a significant reduction ($P<0.05$) in lesion volume following either fasudil treatment initiated at the onset of reperfusion (16.57 ± 2.31 mm³, $n=8$) or initiated at 4h post-MCAO (17.11 ± 19.93 mm³, $n=8$) compared to vehicle treatment (25.67 ± 2.51 mm³, $n=8$). In addition, analysis of the brain slices showed that treatment had a significant effect on the amount of swelling in the hemisphere ipsilateral to the stroke compared to the contralateral hemisphere ($P = 0.014$). Post-hoc analysis revealed that the amount of swelling in the ipsilateral hemisphere was significantly reduced when fasudil treatment was initiated at 4h post-MCAO compared to animals receiving either fasudil initiated at reperfusion ($P<0.05$) or vehicle only ($P<0.05$). The amount of edematous brain water content was measured by calculating the percentage brain water content. Following MCAO, there was a significant increase in the amount of ipsilateral edema as compared to contralateral oedema ($F_{1,12}$

=11.86, $P = 0.0023$). However, the amount of ipsilateral edematous tissue was significantly reduced following Y-27632 treatment (n=6) compared to vehicle treatment (n=7, $P < 0.01$).

Effect of Y-27632 on cerebral protein expression and activities

To ascertain whether regulations of oxidative stress and TJ protein expressions may in part explain Rho-kinase inhibition-mediated improvements in barrier integrity, the levels of NADPH oxidase activity and O_2^- generation and prooxidant (gp91-phox), antioxidant (CuZn-SOD, catalase) and TJ (occludin, claudin-5) protein expressions were examined in ipsilateral and contralateral brain samples obtained from MCAO mice treated with Y-27632 or vehicle. These revealed similar levels of expression and activity in all outlined parameters in both brain sections of a given animal and between those of Y-27632- versus vehicle-treated animals. Interestingly, no differences in Rho-kinase protein expression were observed in these experiments (Figure 3A-H).

Effect of Y-27632 on oxidative stress-related elements in endothelial cells

As the use of brain slices containing a large number of neurons, astrocytes, glia and pericytes may explain the aforementioned similarities, the molecular causes of Y-27632-mediated barrier protection were investigated using BMEC, the key cellular component of the BBB. Exposures of BMEC to OGD±R led to significant increases in NADPH oxidase activity and O_2^- release. Increases in oxidase activity were mirrored by those seen in gp91-phox expression. Inhibition of Rho-kinase completely abolished all the aforementioned increases (Figure 4A-C).

Effects of Y-27632 on endothelial cell antioxidant and TJ protein expressions

OGD±R did not have any impact on CuZn-SOD protein expression while significantly elevating that of catalase in HBMEC. Inhibition of Rho-kinase effectively neutralised catalase levels but failed to affect CuZn-SOD expression. Although OGD±R did not alter occludin protein expression, exposure to Y-27632 markedly enhanced its expression in all

experimental conditions. In contrast, exposure to OGD+R or longer periods of OGD increased claudin-5 protein levels which were completely neutralised by Y-27632 (Figure 5A-D).

Effects of Y-27632 on *in vitro* cerebral-barrier

OGD±R led to marked decreases in TEER and increases in EBA flux, signifying enhanced physical breakdown and paracellular permeability, respectively. Inhibition of Rho-kinase improved the *in vitro* barrier integrity and function solely after 4h OGD±R (Figure 6A-B).

Discussion

In light of our recent investigation attributing the disruption of an *in vitro* model of human BBB to exaggerated Rho-kinase activation, the current study explored the therapeutic potential of post-ischemic Rho-kinase inhibition in protecting cerebral barrier using an animal model of human focal cerebral ischemia (Allen et al., 2010). Similar to the previous studies displaying neurovascular protection upon pre-ischemic inhibition of Rho-kinase by fasudil (Li et al., 2009; Rikitake et al., 2005), this study has revealed that early (at the onset of reperfusion plus 4h post-ischemia) and slightly delayed (4h post-ischemia alone) inhibitions of Rho-kinase after induction of cerebral ischemia also exerts neurovascular protection by demonstrating significantly improved neurological outcomes and reduced brain water and lesion volumes in fasudil- versus vehicle-treated mice that had been subjected to MCAO. Given that the overwhelming majority of patients receive neuroprotective therapy after, rather than before, an ischemic incidence takes place, this approach was therapeutically more valid. The increase in Rho-kinase expression is known to be closely associated with infarct expansion and worsened neurological outcome and requires several hours to take effect (Rikitake et al., 2005, Yagita et al., 2007). This time-dependence may explain the equal effectiveness of early and delayed treatment regimens in subsiding cerebrovascular

complications. Of course, Rho-kinase-mediated suppression of other time-dependent pathologies such as inflammation and matrix metalloproteinase and inducible nitric oxide synthase activations that develop after an ischemic episode cannot be dismissed in this context (Liu et al., 2011; Li et al., 2009).

The extent of functional recovery after stroke differs enormously amongst patients and is dictated by the concordant concerted activities of a variety of processes like neurogenesis and angiogenesis. Rho-kinase inhibition has previously been implicated in neurogenesis under *in vitro* conditions and proven to be effective and safe in clinical settings even after an extended period of treatment. Indeed, a placebo-controlled clinical trial performed with patients who were able to receive the first injection of fasudil (60 mg, intravenous) within 48h of acute ischemic stroke onset and twice daily for 14 days thereafter exhibited radically improved neurological and clinical outcomes at 2 weeks after the initiation of treatment and at 1 month after the onset of symptoms, respectively (Shibuya et al., 2013; Ding et al., 2010). Intriguingly, a recent translational study has shown that inhibition of Rho-kinase by fasudil or Y-27632 can improve the functional recovery even after irreversible formation of brain lesions following photothrombotic stroke. Like the abovementioned trial, the animals here were also given an extended therapy, namely they received the first dose of Y-27632 or fasudil (both 30 mg) via oral gavage 3 days after the induction of stroke and then twice daily for 4 weeks (Lemmens et al., 2013). The present study unravels the remarkable therapeutic capacity of targeting Rho-kinase during acute phase of an ischemic stroke. Considering the eligibility of a selective subset of patients for thrombolysis and seminal role of Rho-kinase in evoking hemorrhagic transformation, these findings may be of considerable therapeutic importance (Ishiguro et al., 2012).

To investigate the mechanisms involved in Y-27632-dependent benefits, the putative changes in expression and activities of oxidative stress- and TJ-related parameters were

comparatively assessed in extracts of the ipsilateral and contralateral hemispheres obtained from Y-27632- or vehicle-treated mice. Aberrant TJ protein expressions and enhanced oxidative stress emerging from an imbalance between prooxidant and antioxidant enzyme expression and activities are implicated in the initiation and exacerbation of cerebral damage (Allen & Bayraktutan., 2009b). Analyses of NADPH oxidase activity, O_2^- generation and prooxidant (gp91-phox), antioxidant (CuZn-SOD and catalase) and tight junctional (occludin and claudin-5) elements at translational levels revealed similar results in both hemispheres of stroke-induced mice treated with Y-27632 or vehicle. Considering the regulatory role of Rho-kinase in oxidative stress and TJ formation these results were somewhat surprising. However, given the microglial and endothelial localisation of Rho-kinase after an ischemic insult, they were expected (Ding et al., 2010b; Yagita et al., 2007). BMEC and microglia make up a small percentage of the overall cell population that is predominated by glia, astrocytes, neurons and pericytes, a fact supported by similar expression of Rho-kinase in the ipsilateral and contralateral hemispheres of both animal groups.

Since evaluation of Rho-kinase activity through staining for phosphorylated substrates of Rho-kinase, namely myosin light chain and adducin has shown ipsilateral blood vessels, in particular endothelia, as the main source of enzymatic activity in rats at 3-6 h after induction of ischemic injury by sodium laurate injection or MCAO (Yagita et al., 2007; Yano et al., 2008), *in vitro* studies with BMEC were performed to further investigate the mechanisms that account for Y-27632-mediated barrier protection. In addition to the increases previously reported in Rho-kinase expression and activity, this study shows that exposure of BMEC to pathologically relevant periods of experimental ischemia significantly perturbs *in vitro* cerebral barrier integrity and function as evidenced by Y-27632-preventable decreases in TEER and increases in paracellular flux of EBA, respectively (Allen et al., 2010). This study also shows that alongside the normalisation of nitric oxide levels and restoration of actin

microfilaments concurred with disappearance of stress fibres, the suppression of oxidative stress and reorganisation of TJ proteins also contribute to the barrier-preserving effects of Y-27632 (Allen et al., 2010).

TJ proteins occludin and claudin-5 seal the intercellular gaps between adjacent endothelial cells and thus form a selectively permeable barrier for the circulating molecules. Exposure of BMEC to OGD±R elevated the expression of claudin-5 protein, a hallmark of paracellular hyperpermeability in microvessels, without affecting that of occludin compared to the cells cultured in normoxia (Brooks et al., 2005, Willis et al., 2010). Inhibition of Rho-kinase activity during OGD±R kept claudin-5 protein expression at normal levels but increased that of occludin, a phenomenon has recently been coupled to the protective effects of Rho-kinase inhibitors on BBB and blood-tumor barrier after subarachnoid hemorrhage and treatment with endothelial-monocyte-activating polypeptide II, respectively (Fujii et al., 2012, Xie et al., 2012). It is noteworthy that the possible changes in phosphorylation pattern of occludin after OGD±R may also account for the beneficial effects of Rho-kinase inhibitors. Bearing in mind the correlation between enhanced phosphorylation of claudin-5 and the BBB dysfunction, this suggestion warrants further investigation (Yamamoto et al., 2008).

Enhanced endothelial cell oxidative stress characterised by selective increases in catalase expression, NADPH oxidase activity and O_2^- release may initiate or exacerbate the overall barrier dysfunction. As the inhibition of Rho-kinase activity effectively normalise these increases, it is safe to suggest that Rho-kinase acts upstream to a series of enzymes that determine the level of oxidative injury during or after an ischemic insult. In contrast, treatments of BMEC with Y-27632 failed to elevate the activity of CuZn-SOD that accounts for much of the metabolism of O_2^- to H_2O_2 in BMEC (Allen & Bayraktutan, 2009). Although these data imply that both anabolic and catabolic mechanisms contribute to excessive accumulation of O_2^- in BMEC, they do not rule out the possibility of cell-specific increases

in SOD activity by Rho-kinase inhibition in that immunohistochemical examination of rat brain slices subjected to OGD revealed $\geq 50\%$ of increases in SOD activity in cerebral cortex and hippocampus following treatments with fasudil (Li et al., 2009).

The critical role played by NADPH oxidase in modulating *in vitro* and *in vivo* cerebral barrier function under a variety of pathological phenomena including ischemia is well-documented (Allen & Bayraktutan, 2009; Kahles et al., 2007, Genovese et al., 2011). Once generated, O_2^- appears to compromise barrier integrity in a direct fashion and through induction of BBB-related cell apoptosis orchestrated in part by the components of plasminogen-plasmin system and matrix metalloproteinases, notably urokinase plasminogen activator and MMP-2 (unpublished findings). Although the underlying mechanisms remain to be elucidated, induction of proapoptotic protein Bax and the mitochondrial death pathway appear to account for Rho-kinase-evoked apoptosis in other cell lines such as cardiomyocytes and PC12 cells, a cell line derived from a pheochromocytoma of the rat adrenal medulla (Del Re et al., 2007; Li et al., 2011).

In conclusion, the current study demonstrates that inhibition of Rho-kinase activity, following the onset of cerebral stroke, may represent a viable therapeutic option to neutralise a variety of phenomena that contribute to developing pathology and the associated morbidity.

There are some limitations to this study. Measurements of the systemic Rho-kinase activity, oxidative stress and antioxidant capacity would have yielded additional information relating to the efficacies of early and delayed post-ischemic inhibition of Rho-kinase. The immunohistochemical analyses of ipsilateral and contralateral brain slices may have been useful in revealing if and to what extent the outlined parameters would be affected by OGD and treatments with Y-27632 in other cerebrovascular cells.

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Figure legends

Figure 1. cerebral artery occlusion (MCAO). Mice were treated with fasudil initiated at onset of reperfusion (n = 14), fasudil initiated 4 h post-MCAO (n = 8) or vehicle treatment (n = 15). Body weights were measured as an indicator of general well-being and fasudil treatment, when initiated at reperfusion significantly reduced (*p < 0.05) the amount of weight lost compared to vehicle treatment (a). Fasudil treatment significantly improved functional outcome, as assessed using a 6-point and 28-point neurological score (b, c *p < 0.05, **p < 0.05 versus vehicle treatment). Unilateral deficits, as measured by the number of contralateral foot faults expressed as the percentage of total errors made, were significantly higher in vehicle-treated animals than in fasudil-treated animals (d, **p < 0.01 versus vehicle treatment). Data are expressed as mean \pm SEM.

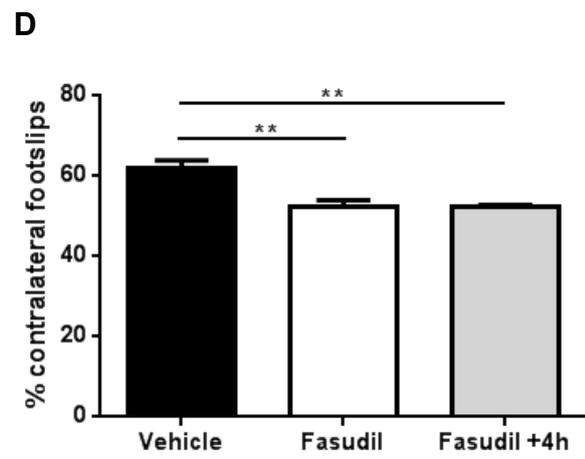
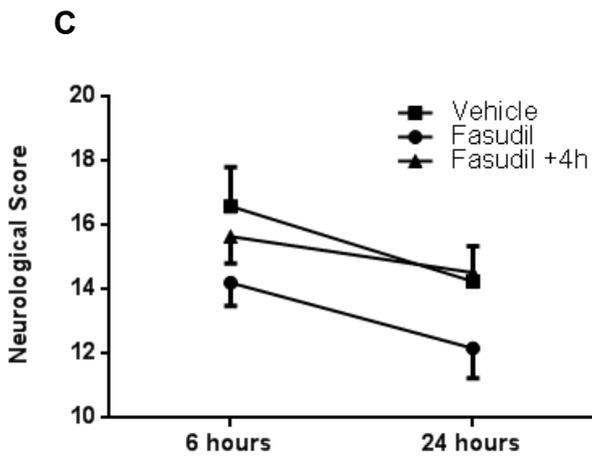
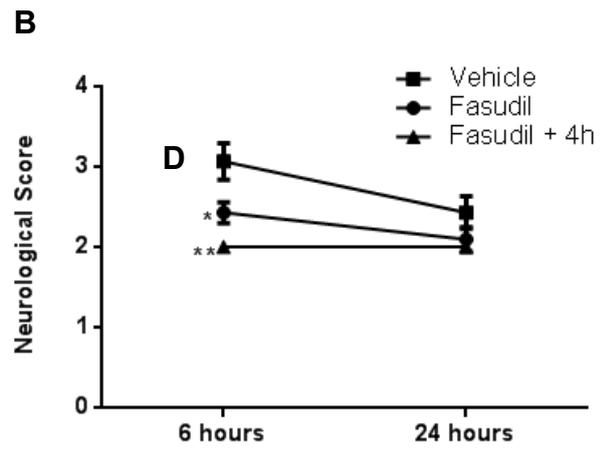
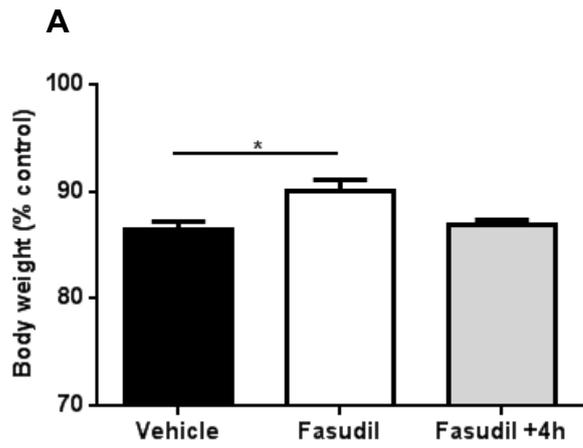
Figure 2. Representative images of slices stained with 2,3,5-triphenyltetrazolium chloride following either fasudil or vehicle treatment (a). Measurements of lesion volume revealed a significant reduction in lesion volume following fasudil treatment initiated either at the onset of reperfusion (*p < 0.05) or 4 h following the onset of middle cerebral artery occlusion (MCAO) (*p < 0.05) compared to vehicle treatment (b, n = 8/group). Fasudil treatment initiated at 4 h post-MCAO also significantly reduced the amount of swelling in the hemisphere ipsilateral to the stroke (c) compared to fasudil treatment at reperfusion onset (*p < 0.05) or vehicle treatment (*p < 0.05). Wet-dry analysis of brain water content revealed a significant effect of fasudil treatment 24 h following MCAO (d). Ischaemia resulted in a significant increase in ipsilateral water content compared to contralateral brain water content (p < 0.01). However, fasudil treatment (n = 6) significantly reduced the ipsilateral brain water content compared to vehicle treatment (n = 7, *p < 0.05). Data are expressed as mean \pm SEM.

Figure 3. Comparative evaluation of (a) NADPH oxidase activity, (b) total O₂^{·-} levels and (c) gp91-phox, (d) CuZn-SOD, (e) catalase, (f) occludin, (g) claudin-5 and (h) Rho-kinase protein expressions in homogenized contralateral (c) and ipsilateral (i) brain slices of mice subjected to middle cerebral artery occlusion prior to treatments with vehicle or fasudil. Data are expressed as mean \pm SEM from six animals in each group.

Figure 4. Assessment of NADPH oxidase activity (a), total O₂^{·-} levels (b) and gp91-phox protein expression (c) in brain microvascular endothelial cells exposed to oxygen–glucose deprivation alone or followed by reperfusion (OGD \pm R) in the absence or presence of Y-27632 (10 μ M). Data are expressed as mean \pm SEM from four different experiments. *p < 0.05 compared to the controls.

Figure 5. Analyses of CuZn-SOD (a), catalase (b), occludin (c) and claudin-5 (d) protein expressions in brain microvascular endothelial cells subjected to oxygen–glucose deprivation alone or followed by reperfusion (OGD \pm R) in the absence or presence of Y-27632 (10 μ M). Data are expressed as mean \pm SEM from four different experiments.*p < 0.05 compared to the controls.

Figure 6. Measurements of cerebral barrier integrity (a) and function (b) by transendothelial electrical resistance (TEER, Ω cm²) and flux of Evans blue labelled albumin (EBA, IL) across an in vitro model of blood–brain barrier subjected to oxygen– glucose deprivation alone and followed by reperfusion (OGD \pm R) in the absence and presence of Y-27632 (10 μ M). Data are expressed as mean \pm SEM from four different experiments. *p < 0.05 compared to the controls, #p < 0.05 compared to short OGD \pm R.



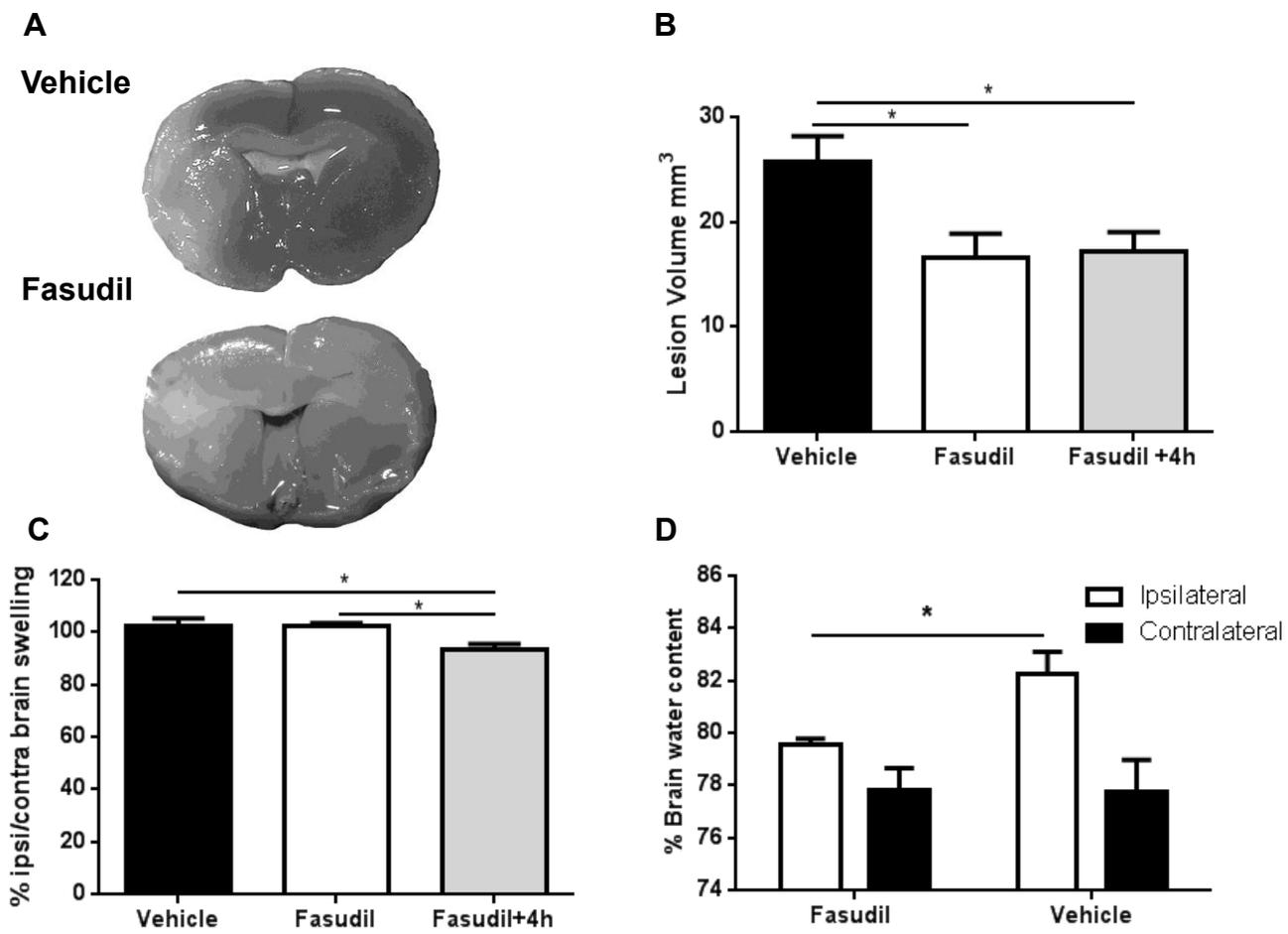
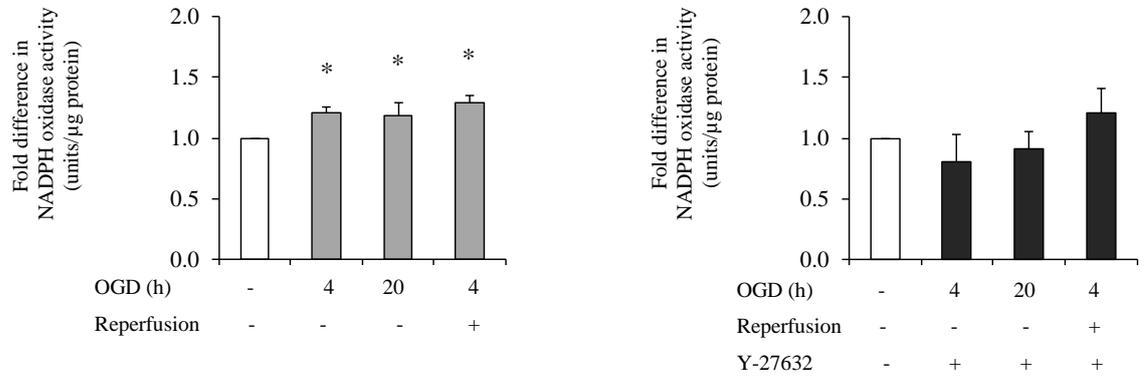


Figure 3

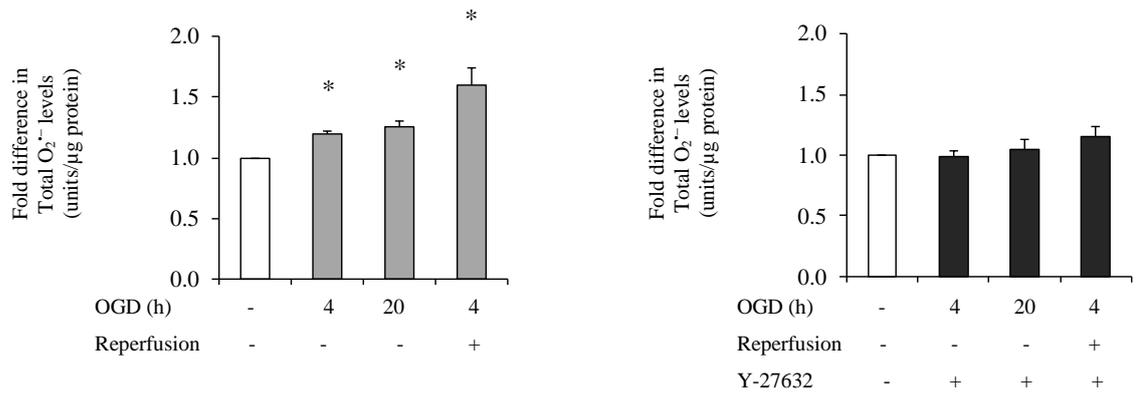
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Figure 4

A



B



C

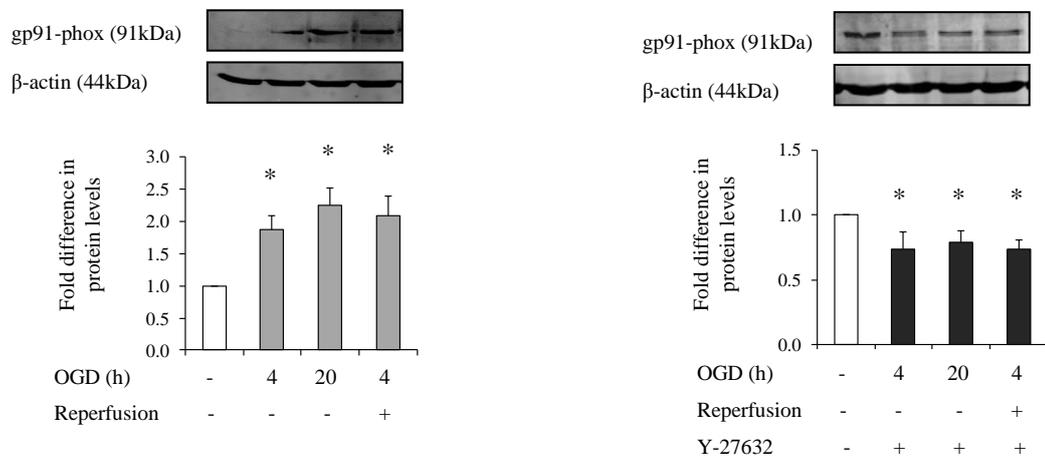
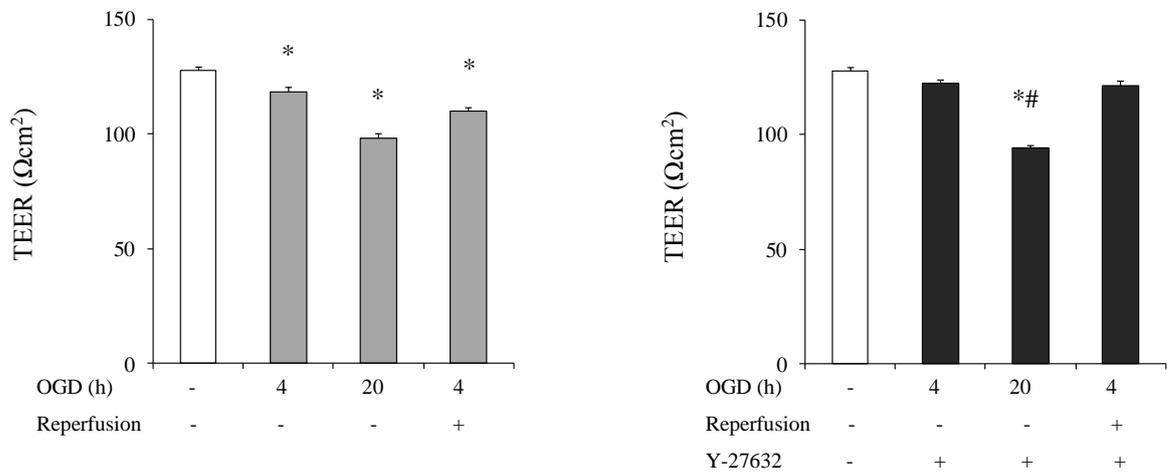


Figure 5
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Figure 6

A



B

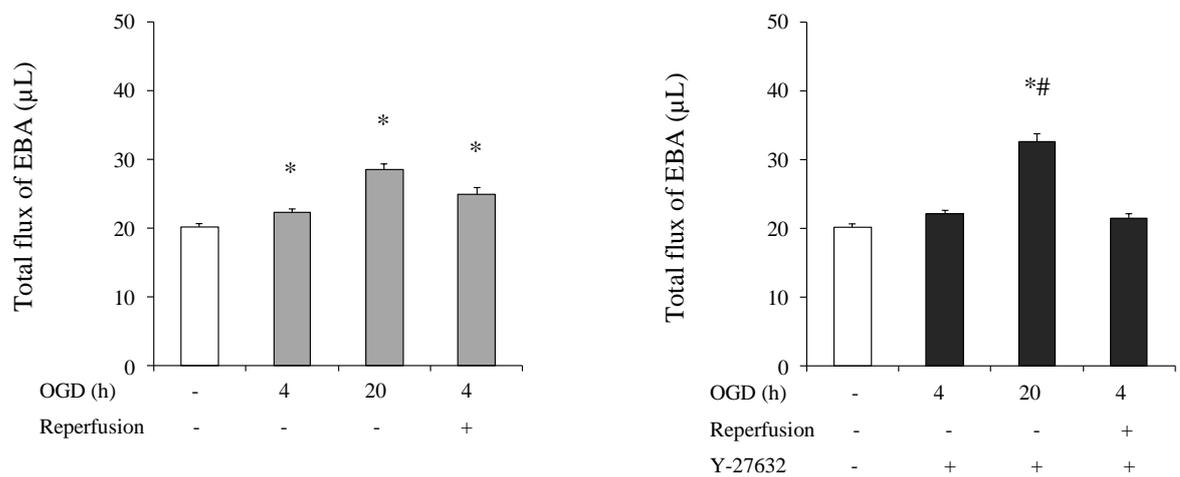


Figure 3

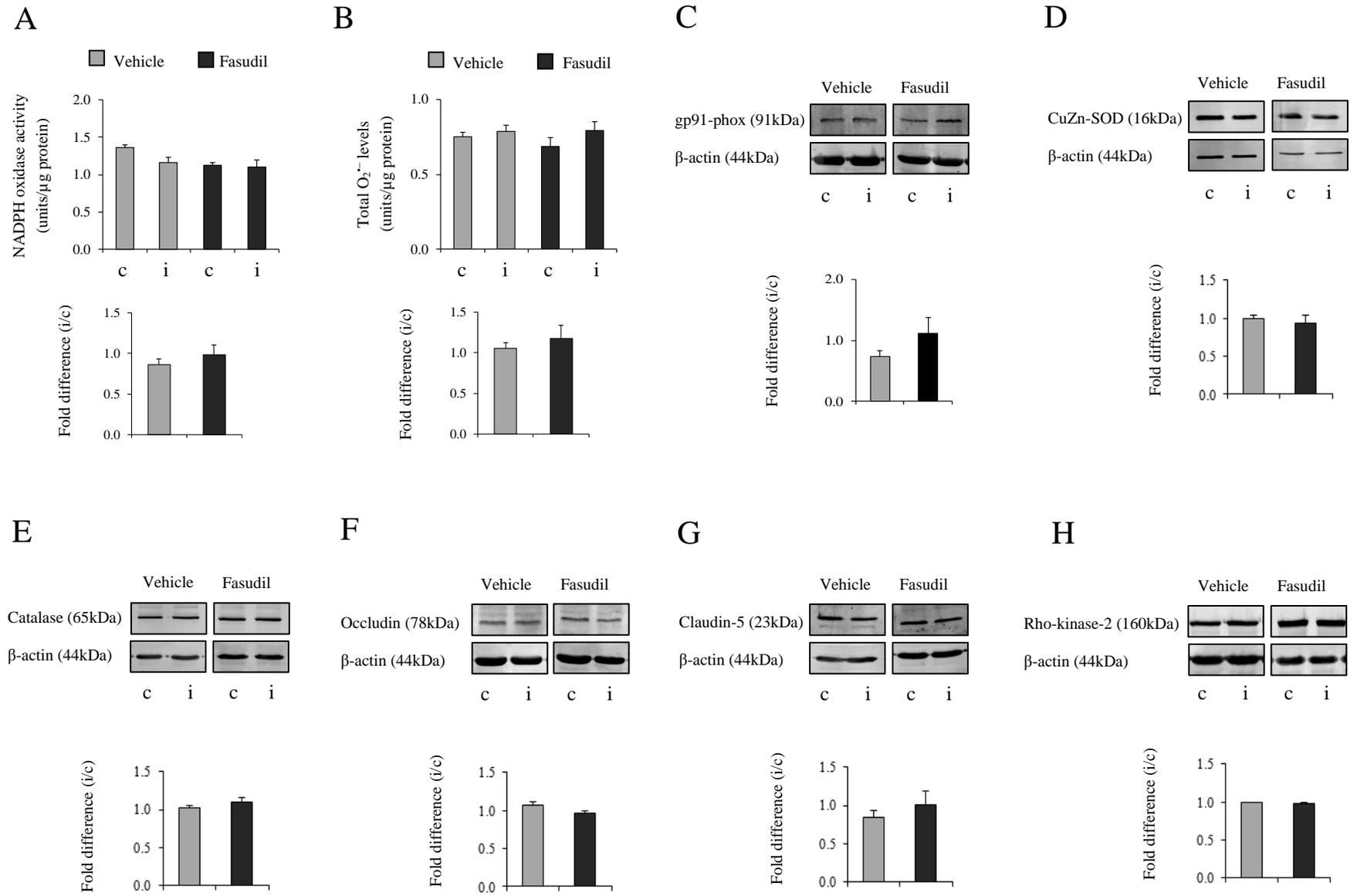
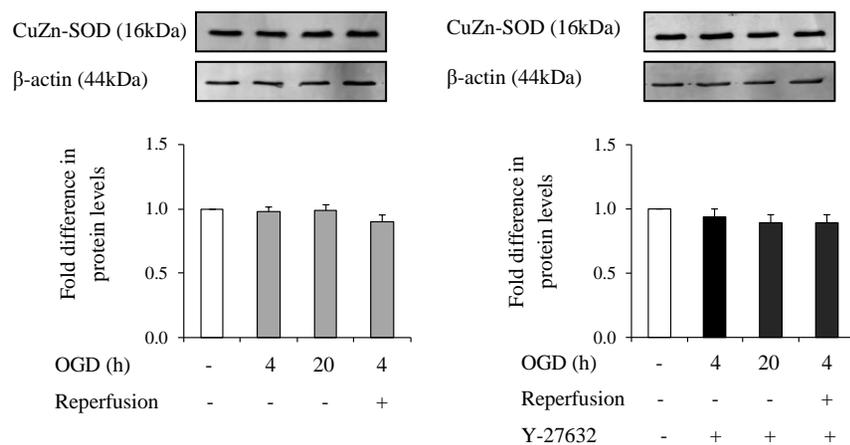
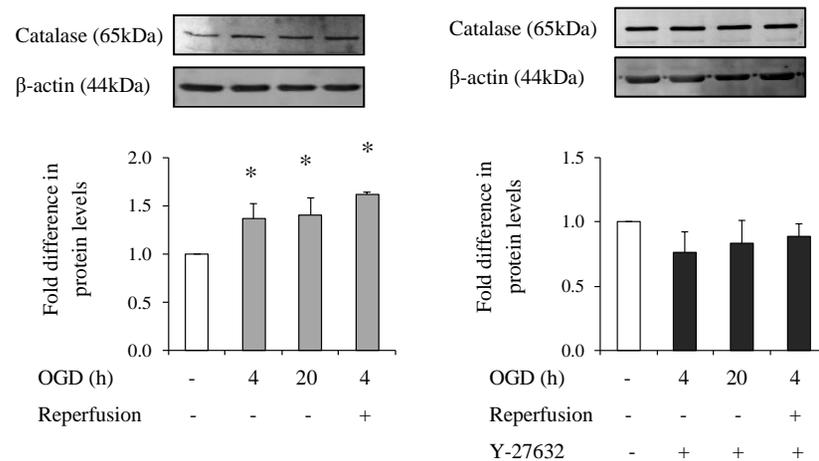


Figure 5

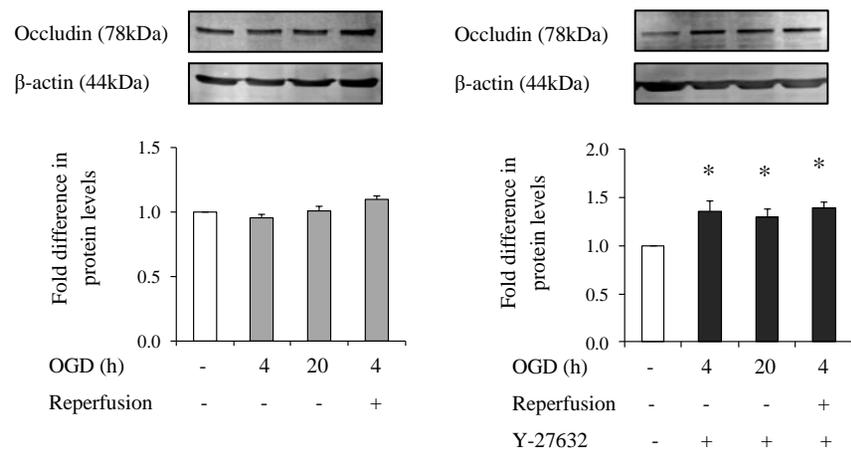
A



B



C



D

