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Evaluation of common housekeeping proteins under ischemic conditions and/or rt-PA treatment in bEnd.3 cells

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ABSTRACT

Thrombolysis with recombinant tissue plasminogen activator (rt-PA) is the only pharmacological approved treatment for ischemic stroke, despite its associated increasing risk of hemorrhagic transformation. Since many of rt-PA effects in blood-brain barrier (BBB) are not well characterized, the study of protein changes in BBB cells after rt-PA administration may help to understand its adverse effects. Our aim was to analyze protein levels of four commonly used housekeeping proteins: β-Actin, α-Tubulin, GAPDH and HPRT in bEnd.3 endothelial cell line subjected to oxygen and glucose deprivation (OGD) conditions and rt-PA treatment to determine their reliability as Western blot loading controls. bEnd.3 monolayers were subjected to 2.5 h of OGD and reperfusion with/without 20 μg/ml of rt-PA. At 3, 6, 24 and 72 h post-OGD, protein levels were analyzed by Western blot using Stain-Free technology. OGD significantly decreased β-Actin, α-Tubulin, GAPDH and HPRT protein levels at 3, 6, 24 and 72 h post-OGD without significant rt-PA treatment effects except for the GAPDH levels increase in control condition at 3 h post-OGD. The present study clearly demonstrated that β-Actin, α-Tubulin, GAPDH and HPRT proteins are not suitable as loading controls for Western Blot analysis in bEnd.3 cells after OGD.

Significance: We reported altered levels of β-Actin, α-Tubulin, GAPDH and HPRT housekeeping proteins in bEnd.3 endothelial cell line after an ischemic insult. Therefore, we demonstrated that these proteins are not suitable as loading controls for Western Blot analysis in our experimental conditions and we recommended the use of Stain-Free gels as an alternative to traditional housekeeping proteins normalization.

1. Introduction

According to the World Health Organization, stroke is a leading cause of death and disability in the world (2012). Despite all clinical trials carried out in recent years, thrombolytic therapy with recombinant tissue plasminogen activator (rt-PA) has been the only drug shown to be effective for the treatment of ischemic stroke when administered within the first 4.5 h of stroke onset [1]. However, its administration is associated with an increasing risk (8%) of symptomatic hemorrhagic transformation (HT) of the ischemic brain. This adverse effect constitutes an important limitation for the generalization of rt-PA therapy which, at the present time, is given to < 5% of patients with ischemic stroke [2, 3].

HT is mostly caused by the disruption of the blood-brain barrier (BBB) after the ischemic insult as a result of the increase of permeability and the subsequent pass of erythrocytes through the structure. BBB is composed of astrocyte end-feet, pericytes, specialized brain capillary endothelial cells (EC) and the extracellular matrix (ECM) components [4, 5]. At the molecular level, it has been shown that rt-PA could affect directly to EC through triggering the degradation of tight junction proteins [6] which are essential for maintaining cerebrovascular homeostasis and regulating the vascular endothelial growth factor (VEGF) expression and the subsequent stimulation of endocytosis, transcytosis and angiogenesis [7]. rt-PA has also been implicated in the degradation of basement membrane components such as collagen IV, laminin and fibronectin through plasmin activation, low density

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lipoprotein receptor associated protein-1 (LRP-1) stimulation, and matrix metalloprotease (MMPs) induction [8].

Since many of rt-PA's pleiotropic interactions are not well characterized, the identification and time profile analysis of endothelial protein levels after ischemic conditions and rt-PA administration may shed light on the molecular mechanisms underlying rt-PA's adverse effects in this important element of neurovascular unit of BBB [9].

The Western Blot or Immunoblot is a widely accepted technic used to determine the expression changes of particular proteins under specific experimental conditions such as ischemia. The most common way to validate the detected differences is performing a loading normalization using high-abundance housekeeping proteins expressed at a constant level and theoretically not affected by experimental conditions. β-Actin, α-Tubulin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), among other housekeeping proteins, are usually chosen as loading controls [10]. Different studies have used these housekeeping proteins to normalize results after *in vitro* and *in vivo* ischemia models [11, 12] but, other studies have reported altered expressions of actin, tubulin and GAPDH due to the effects of the ischemia [13–15]. These controversial results make questionable the use of these loading controls for western blot normalization purposes in ischemic conditions. In this sense, different papers have recently confirmed Stain-Free technology as a more reliable method than housekeeping's labeling, avoiding saturate signals and housekeeping variations due to experimental conditions [16–19]. Moreover, there are no published data analyzing the effect of rt-PA in normoxic and ischemic conditions on expression of these putative housekeeping proteins in bEnd.3 cells. This immortalized mouse cerebral endothelial cell line appears to be a suitable *in vitro* BBB model presenting similar barrier characteristics to the primary brain microvascular endothelial cells [20, 21].

The aim of this study was to analyze the levels of four common housekeeping proteins, β-Actin, α-Tubulin, GAPDH and Hypoxanthine-guanine phosphoribosyltransferase (HPRT) in the bEnd.3 endothelial cell line subjected to oxygen and glucose deprivation (OGD) conditions and/or treatment with rt-PA to determine whether they are reliable as loading controls to Western blot or not in these experimental conditions during a time period from 3 to 72 h post-OGD.

2. Material and methods

2.1. Cell culture

Immortalized mouse brain endothelial cell line (bEnd.3) were purchased from ATCC (CRL-2299), seeded in 60 mm Petri dishes (Corning, USA) and grown in DMEM high glucose (HG) medium with 1% glutamine (Gibco, USA), 10% fetal bovine serum (Gibco, USA) and 1% Penicillin/Streptomycin (HyClone Laboratories, USA). All bEnd.3 cells used for these experiments were cultured between 25 and 30 passages, which have been shown to maintain excellent BBB characteristics *in vitro* [20].

2.2. OGD performance and rt-PA treatment

After overnight starvation in DMEM HG with 1% fetal bovine, bEnd.3 monolayers were subjected to OGD. Briefly, the medium was replaced with glucose-free DMEM without FBS (Gibco, USA) previously perfused with N_2 to purge the oxygen. Then, the cells were placed into a 37 °C humidified hypoxic chamber with a constant N_2 flow of 1 L/min and 0.15 bar pressure for 2.5 h. This OGD period was selected after confirm that a viable cell population remains until 72 h though with ionic and paracellular permeability as well as tight junction protein levels differences between control, OGD and OGD + rt-PA treated cells (unpublished results). Regarding the control (CTR) group, the same procedure was carried out with the difference that the glucose-free medium was supplemented with glucose (5.5 mM) and incubated at 37 °C with 5% of CO_2. At the end of the OGD period, media were removed and replaced with DMEM HG medium containing 10% FBS and with or without rt-PA at a concentration of 20 μg/ml. As reported in previous publications, we used a dose of 20 μg/ml of tPA, based on the finding that such a concentration can be observed in blood [22].

2.3. Cell viability

At 0, 24, 48 and 72 h of reperfusion, with or without rt-PA treatment, cell viability was assessed with 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide (MTT) assay (5 mg/ml, Sigma). Absorbance was measured at 570 nm using a SpectraMax 340PC384 Microplate Reader and results were expressed as a percentage of the value in the control group.

2.4. Western Blot analysis

At 3, 6, 24 and 72 h post-OGD, cells were collected and protein was isolated using Lysis Buffer (Cell Signaling, The Netherlands). Protein concentration was measured using BCA method (Thermo fisher scientific, USA). Protein samples (10 μg) were loaded and separated by electrophoresis on Criterion™ TGX Stain-Free™ Precast Gels (Bio-Rad) at 120 V for 80–90 min. Then, proteins were transferred to PVDF membranes at 30 V overnight at 4 °C. After 1 h of blocking with TBST 5% BSA (EMD Millipore, USA), membranes were incubated 1 h at room temperature with primary antibodies: anti-β-Actin (sc-47,778), 1:1000; anti-α-Tubulin (sc-5286), 1:1000 from Santa Cruz Biotechnology and anti GAPDH, 1:20000 (MAB374) from EMD Millipore, USA and overnight at 4 °C with anti-HPRT (sc-376,938), 1:100 from Santa Cruz Biotechnology, in TBST 3% BSA. HRP-conjugated secondary antibodies were used for 1 h at room temperature. Protein bands were revealed using Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore, USA) and quantified with Alpha Innotech software (AlphaEaseFC™).

2.5. Stain-Free total protein staining and quantification

The Criterion Stain-Free gels (BioRad) were used as a loading control in Western Blot since they contained a trihalo compounds that allowed rapid fluorescent detection of total protein loaded in each lane and after the transfer in PVDF membranes. Once the gel had run, it was placed to the transilluminator and exposed 5 min with UV light in order to activate the fluorescence signal. Then, each lane was quantified using Alpha Innotech software (AlphaEaseFC™). The space between lanes was taken as a background according to Aldridge GM et al. [10] as shows in Fig. 1.

2.6. Statistical analysis

SPSS software (IBM SPSS Statistics 22) was used to perform the statistical analysis. Since variables were not normally distributed, non-parametric Mann-Whitney test was used to perform the analysis. P value < 0.05 was considered as significant.

3. Results

OGD decreased significantly the viability of bEnd.3 cells from 0 to 72 h while rt-PA administration at reperfusion only induced a significant decrease of viability at 72 h in the OGD rt-PA group (Fig. 2).

Fig. 3 shows a representative gel with total protein staining of bEnd.3 murine endothelial cells subjected to 2.5 h of OGD and the subsequent reperfusion, in which it is possible to observe differences in protein profile between OGD and CTR samples. The most remarkable differences were seen between 3 and 24 h post-reperfusion, while at 72 h the profiles were similar again. Regarding rt-PA treatment, the possible differences between groups were much more attenuated and therefore, practically unobservable.