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Proteomic analysis of global protein expression changes in the endothelin-1 rat model for cerebral ischemia: Rescue effect of mild hypothermia

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ABSTRACT

Mild hypothermia is a promising neuroprotective therapy in stroke management. However, little is known about its effects on the global protein expression patterns in brain regions affected by ischemic stroke. We investigated protein expression changes associated with the neuroprotective effects of hypothermia via a functional proteomics approach through the analysis of the core (striatum) and the penumbra (cortex) after an ischemic insult in rats induced by endothelin-1 (Et-1). Functional outcome, infarct volume and related global protein expression changes were assessed 24 h after the insult using two-dimensional difference gel electrophoresis. Mild hypothermia, induced 20 min after endothelin-1 infusion, improved the neurological outcome, reflected by a 36% reduction in infarct volume and a significantly better neurological deficit score. Hypothermia was typically associated with opposite protein expression changes inthe cortex to those induced by stroke under normothermic conditions, but not in the striatum. The main cellular processes rescued by hypothermia and potentially involved in the protection of the cortex are cellular assembly and organization, followed by cell signaling, thereby confirming that hypothermia is neuroprotective through multiple molecular and cellular pathways.

1. Introduction

Stroke is one of the most common causes of death and permanent cerebral disability worldwide. The cerebral blood supply reduces severely or is completely abolished, sufficient to cause metabolic and functional dysfunction. A multifunctional cascade of molecular events called the ischemic cascade is initiated (Durukan and Tatlisumak, 2007). So far, thrombolysis with recombinant tissue plasminogen activator (rt-PA) is the only approved stroke therapy. Unfortunately, less than 10% of all stroke patients can be treated with rt-PA, since this treatment has important limitations such as a short treatment window, reperfusion associated injury and hemorrhagic complications (Candelario-Jalil, 2009; Hacke et al., 2008; Kollmar and Schwab, 2009; Zgavc et al., 2011). Besides thrombolysis, a second approach to treat stroke is neuroprotection.Neuroprotective strategies are urgently needed since the neuronal damage often continues even when the cerebral blood flow is restored (Weinberger, 2006). Therapeutic hypothermia is one of the best studied neuroprotective therapies. Small decreases in brain temperature are well tolerated and have been shown to confer a significant degree of neuroprotection in several animal models of cerebral ischemia (Ceulemans et al., 2011; Kobayashi et al., 2008; Maier et al., 1998; Nagel et al. 2012; Yenari and Han, 2012; Zgavc et al., 2011).

Currently, little is known about the effects of hypothermia on global protein expression patterns in the affected brain regions after an ischemic stroke. Most animal studies on cerebral ischemia

Abbreviations: ACN, acetonitrile; AP, anterior–posterior; ANOVA, analysis of variance; BBB, blood brain barrier; BVA, biological variation analysis; CHAPS, 3-cholamidopropyl dimethylammonio 1 propanesulfonate; DTT, dithiothreitol; 2-D DIGE, 2 dimensional difference gel electrophoresis; Et-1, endothelin-1; FA, formic acid; H, hypothermic; HPLC, high performance liquid chromatography; IEF, isoelectric focusing; i.p., intraperitoneal; IPA, ingenuity pathway analysis; IPG, immobilized pH gradient; MCA, middle cerebral arterie; MS, mass spectrometry; N, normothermic; NDS, neurological deficit score; rt-PA, recombinant tissue plasminogen activator; S, sham; SDS, sodium dodecyl sulfate; TBS, Tris buffered saline.

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investigated changes in individual or a small set of proteins (e.g. Bcl-2, caspase-3, GFAP, MMP-9, Mn-SOD; Ceulemans et al., 2011; Hamann et al., 2004; Maier et al., 2002; Yang et al., 2009). In the present study, we investigated the neuroprotective effects of hypothermia and the accompanied protein changes via a functional proteomics approach in which the core (striatum) and the penumbra (cortex) were analyzed after an ischemic insult induced by endothelin-1 (Et-1). The identification of proteins with altered expression following stroke and correlated to the neuroprotective outcome of hypothermia may provide information on potential therapeutic targets towards new neuroprotective therapies.

2. Material and methods

The experiments described in this study were performed according to the National Guidelines on Animal Experimentation and approved by the Ethical Committee for Animal Experimentation of the Faculty of Medicine and Pharmacy of the Vrije Universiteit Brussel.

2.1. Experimental and surgical protocols

Experiments were carried out in male Wistar rats weighing 270–300 g (Charles River Laboratories, IFFA-CREDO, Germany) as previously described (Ceulemans et al., 2011; Van Hemelrijck et al., 2003). Twenty-four hours before the induction of the insult, 2 intracerebral guide cannulas were stereotactically implanted under anesthesia (ketamine/diazepam 75:4 mg/kg intraperitoneal (i.p.)). A cannula for Et-1 administration was positioned close to the middle cerebral artery (MCA) (relative to bregma: AP +0.9 mm; L +5.0 mm; V +2.8 mm) and a cannula for the thermocouple probe was positioned in the contralateral prefrontal cortex (AP +3.2 mm; L −3.0 mm; V +2.3 mm) (Paxinos and Watson, 1986). As post-operative analgesia,the rats received 4 mg/kg ketoprofen (i.p.). After surgery, the rats were allowed to recover overnight. On the day of the Et-1 experiment, the rats were anesthetized with 4% sevoflurane (Sevorane®, Abbott, Kent, England) and oxygen was insufflated into a transparent chamber. During the experiments, anesthesia was maintained by 2% sevoflurane with oxygen at 0.8 ml/min via a facemask. The guides were replaced by a microdialysis probe of which the membrane was removed (CMA, 3 mm probe, Stockholm, Sweden) and a thermocouple probe (HYP-O-SLE, Omega Corporation, Stamford, USA). Focal ischemia was induced by infusion of Et-1 (Sigma–Aldrich, St-Louis, MO, USA) dissolved in Ringer's solution (500 pmol/6 μl) through the probe near the MCA at a flow rate of 1 μl/min. In sham experiments, only Ringer's solution was infused. By infusing Et-1 adjacent to the MCA, a reproducible insult can beobtained in which the core is represented by the striatum and the penumbra by the surrounding cortex (Ceulemans et al., 2011; Van Hemelrijck et al., 2003). In normothermic rats, the brain temperature was maintained at 37.0 ± 0.5 °C throughout the experiment using a heating pad and an infrared lamp. In the hypothermic group, the temperature was reduced to 33.0 ± 0.5 °C during 2 h starting with a delay of 20 min after the ischemic insult. Cooling the animal to the target temperature was achieved by spraying alcohol onto the animal and cooling it with a fan. With this method the brain temperature can be decreased from 37.0 to 33.0 °C within 10 min. A heating pad and an infrared lamp were used to re-warm the animal from 33.0 to 37.0 °C in 30 min. Twenty-four hours after the induction of the ischemic insult, the effects on neurological outcome (neurological deficit score; NDS), infarct size and protein expression levels (two-dimensional difference gel electrophoresis; 2-D DIGE) were evaluated.

Rats were randomized into 3 experimental groups. Group 1 were sham animals (S group, $n = 6$), group 2 were normothermic rats (N group, $n = 6$). In both groups, the brain temperature was kept constant at 37.0 ± 0.5 °C. Rats from group 3 received the hypothermic treatment initiated 20 min (H group, $n = 6$) after the ischemic insult.

2.2. Behavioral testing

All rats were subjected to sensory- and motoric evaluation before and 24 h after the administration of Et-1 with the use of a NDS. To estimate the degree of neurological deficit after the induction of the insult, six parameters were scored between 0 and 3 or 1 and 3. Spontaneous activity, symmetry in the movement of the 4 limbs, forepaw outstretching, equality of strength in the forepaws, body proprioception and response to vibrissae touch were observed. The NDS was calculated as the sum of these scores, 18 being the best and 3 the worst possible score (Garcia et al., 1995). The control NDS score is the score of all animals before the start of the experiment. All data are expressed as mean ± standard error of the mean (SEM) and a p-value <0.05 was considered significant. To compare the results between the different groups, the non-parametrical Kruskal–Wallis test and *post hoc* Wilcoxon test were used.

2.3. Infarct volume determination

After the behavioural analysis, 24 h after the insult onset, rats were deeply anesthetized with 6% pentobarbital. The brains were immediately removed and snap-frozen. Coronal sections of 50 μm were cut using a cryostat and stored in the −80 °C freezer. Of every consecutive 3 slices, 1 section was used to determine the infarct volume and 2 sections were used for the protein expression analyses.

The sections used to determine the infarct volume were mounted on gelatine-coated (2%; Fluka, Sigma–Aldrich, St-Louis, MO, USA) glass slides and stained with Cresyl Violet (0.5%; Sigma–Aldrich, St-Louis, MO, USA). In this way the infarct area was visualized every 150 μm. At a magnification of 1.25 and using a Carl Zeiss Axioskop 40 microscope (Zeiss, Belgium), pictures of all stained sections were taken and transferred to the computer programme Image J (NIH, version 1.37) to calculate the infarct volume. After scaling the pictures, the marked infarcted area and hemisphere (in mm^2) could be calculated and was multiplied with the interspace. The influence of oedema on the infarct volume was corrected by applying the following formula: (area of normal hemisphere/area of infarcted hemisphere) × area of infarct (Ceulemans et al., 2011;Weston et al., 2007). All data are expressed as mean ± SEM and a p-value <0.05 was considered significant. To compare the results between the different groups, a one-way ANOVA with Bonferroni *post hoc* test was used.

2.4. Tissue and sample preparation

The 50 μm sections used to determine the protein expression changes were mounted on baked slides and the cortex (penumbra) and the striatum (core) were consistently collected from the infarcted hemisphere (12 sections/animal), using a razor blade.

For 2-D DIGE, brain tissue, separately from each animal ($n = 6$ for each of the 3 conditions) was transferred to 100 μl ice-cold lysis buffer, containing 7 M urea, 2 M thiourea, 4% w/v CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1 propanesulfonate; Sigma–Aldrich, Steinheim, Germany), 1% w/v dithiothreitol (DTT) (Serva, Heidelberg, Germany), 40 mM Tris base (ICN, Aurora, Ohio, USA) and Complete Protease Inhibitor Cocktail (Roche Diagnostics, Basel,