Available online at www.sciencedirect.com
ScienceDirect
Neuroscience Research 59 (2007) 68–73
Neuroscience Research
www.elsevier.com/locate/neures

S100B modulates IL-6 release and cytotoxicity from hypothermic brain cells and inhibits hypothermia-induced axonal outgrowth

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Received 16 April 2007; accepted 29 May 2007
Available online 2 June 2007

Abstract

Brain protection is essential during neonatal and pediatric cardiac surgery. Deep hypothermia is still the most important method for achieving neuroprotection during cardiopulmonary bypass. Previously, we could demonstrate that deep hypothermia induces substantial cytotoxicity in brain cells as well as increased release of the pro-inflammatory cytokine interleukin-6 (IL-6), which plays an important role in neuroprotection and neuroregeneration. Deep hypothermia is also associated with increased levels of the astrocytic protein S100B in the serum and cerebrospinal fluid of patients. Since S100B may modulate pro-inflammatory cytokines and may stimulate neurite outgrowth, we have tested the hypothesis that nanomolar concentrations of S100B may increase IL-6 release from brain cells and support axonal outgrowth from organotypic brain slices under hypothermic conditions.

S100B administration substantially reduced neuronal and glial cytotoxicity under hypothermic conditions. In the presence of S100B hypothermia-induced IL-6 release in primary astrocytes was significantly increased but reduced in BV-2 microglial cells and primary neurons. Surprisingly, deep hypothermia increased axonal outgrowth from brain slices and – in contrast to our hypothesis – this hypothermia-induced neurite outgrowth was inhibited by S100B.

These data suggest that S100B differentially influences cytokine release and cytotoxicity from distinct brain cells and may inhibit neuroregeneration by suppressing hypothermia-induced axonal outgrowth.
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Keywords: Astrocytes; Neurons; BV-2 microglial cells; Organotypic brain slice; Neuroregeneration; IL-6

1. Introduction

Brain protection is still a major concern during corrective cardiac surgery for children using cardiopulmonary bypass (CPB) (Bellinger et al., 2003; Clancy et al., 2005; Wang et al., 2005). Global or focal cerebral ischemia involving all types of brain cells may occur during non-pulsatile low flow perfusion and periods of total circulatory arrest. Therefore, one of the key protective procedures during pediatric cardiac surgery is systemic cooling, the main rationale being that hypothermia decreases cerebral blood flow and the metabolic rate of O_2 (Bernard and Buist, 2003).

Previously, we could demonstrate that deep hypothermia induces substantial cytotoxicity in brain cells as well as increased release of the pro-inflammatory cytokine interleukin-6 (IL-6) (Schmitt et al., 2006), which protects the brain against neuronal degeneration (Swartz et al., 2001; Penkowa et al., 2003a,b) and which is an important prerequisite for neuronal regeneration (Parish et al., 2002; Cafferty et al., 2004).

The levels of the astrocytic protein S100B are substantially increased after deep hypothermia during cardiopulmonary bypass and extracorporeal circulation in children (Abdul-Khaliq

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doi:10.1016/j.neures.2007.05.011

et al., 1999; Ashraf et al., 1999). S100B is a calcium-binding peptide that exerts paracrine and autocrine effects on neurons and glial cells during brain development and neurodegenerative diseases (reviewed in Van Eldik and Wainwright, 2003). S100B plays a pivotal role in cellular energy metabolism, cytoskeleton modification, cell proliferation, and differentiation (Schafer and Heizmann, 1996; Lehrmann et al., 1998). Secreted glial S100B exerts either trophic or toxic effects depending on its concentration: at nanomolar levels glial S100B stimulates neurite outgrowth and enhances survival of neurons during development; in contrast, micromolar levels of extracellular S100B stimulate the expression of proinflammatory cytokines and induce apoptosis (reviewed in Rothermundt et al., 2003). Elevated levels of S100B are found during many neurodegenerative processes, e.g. Alzheimer's disease, epilepsy, and Down's syndrome (reviewed in Rothermundt et al., 2003). We have shown in animal and clinical studies that hypothermia is associated with astroglial injury and elevated S100B levels (Abdul-Khaliq et al., 2000a,b). Previously, we have used a dynamic time-temperature protocol mimicking the *in vivo* cardiopulmonary bypass situation. Using this model system, we could demonstrate that deep hypothermia stimulates IL-6 release from neuronal and glial cells and induces substantial cytotoxicity (Schmitt et al., 2006).

Therefore, in the present study we have addressed the question whether S100B in nanomolar concentrations may influence cytotoxicity and the release of IL-6, which plays a major role in neuroprotection and neuroregeneration and whether S100B may stimulate axonal outgrowth under hypothermic conditions.

We could show that S100B reduced hypothermia-induced cytotoxicity in neuronal and glial cells. Furthermore, hypothermia-induced IL-6 release was significantly upregulated in primary astrocytes but reduced in BV-2 microglial cells and primary neurons by S100B. Unexpectedly, deep hypothermia increased axonal outgrowth from organotypic brain slices, which was significantly reduced by S100B. These data suggest that S100B differentially modulates cytokine release from hypothermic brain cells and may inhibit hypothermia-induced neuroregeneration by suppressing axonal outgrowth.

2. Materials and methods

2.1. Cell culture

Tissue culture material was obtained from Becton Dickinson (Heidelberg, Germany). DMEM high glucose medium, phosphate-buffered salt solution and fetal calf serum were from GIBCO (Karlsruhe, Germany), all other reagents were from Sigma–Aldrich (Munich, Germany).

Primary neurons were prepared from newborn mice using a modification of a technique described previously (Giulian and Baker, 1986). The brain tissue was dissociated with 0.25% trypsin, centrifuged at 470 × *g* for 10 min, resuspended in neurobasal medium, plated on poly-L-lysin-coated 24-well plates, and incubated at 37 °C in a humified atmosphere with 5% CO_2. After confluence of the cell layer, adherent glia cells were mechanically dissolved and the proliferation of the remaining glia cells was inhibited by 8 μM cytosine arabinoside for 3 days.

Primary astroyctes were prepared from newborn mice by modifying a technique described previously (Giulian and Baker, 1986). In brief, after removal of the meninges, cerebral cortices were dissociated with a glass pipette in HBSS containing 4 mg/ml trypsin and 0.5 mg/ml DNase (Worthington Biochemical Corporation, Lakewood, NJ). After centrifugation (165 × *g*, 10 min, 4 °C), cells from four cortices were pooled and plated into 75 cm tissue culture flasks containing DMEM with 4.5 g/l glucose supplemented with 10% fetal calf serum, 1% L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. After 10 days *in vitro*, the flasks were shaken gently to remove adherent microglia. The primary astrocyte cell cultures obtained were >95% pure (tested via double-immune histochemistry) (Heppner et al., 1998; Ullrich et al., 2001).

BV-2 microglial cells are primary mouse microglial cells immortalized by stable transfection with the *c-myc* oncogene using a J2-retrovirus (Blasi et al., 1990), leading to a phenotype functionally identical to native primary microglia (Laurenzi et al., 2001).

2.2. Time temperature protocol

We applied a standard time-temperature protocol used during pediatric cardiac surgery as described previously (Schmitt et al., 2006). Briefly, two hours before the study period, the culture medium was replaced with 2 ml serum-free DMEM supplemented with 5 mM glucose, 0.06 g/l penicillin, and 0.1 g/l streptomycin. The cells were re-incubated in the specially developed incubator for 2 h at 37 °C in a humified atmosphere with 5% CO_2. Primary astrocytes, BV-2 microglial cells and primary neurons were treated according to the following protocol (Fig. 1A): Time point 1 (t1) indicates the end of phase 1 (deep hypothermia for 2 h at 17 °C), time point 2 (t2) the end of phase 2 (rewarming for 2 h up to 37 °C = 0.17 °C/min), and time point 3 (t3) the end of phase 3 (normothermia for 20 h at 37 °C). The temperature of the culture medium was continuously monitored using a thermocouple paratrend probe inserted in one petri dish. At every time point one 6-well plate was removed and the supernatant was collected. One culture dish was examined throughout the incubation protocol to monitor cell morphology microscopically.

2.3. S100B application

Protein S100B was measured manually with a sandwich-type immunoluminometric assay kit (BYK Sangtec, Dietzenbach, Germany) using a LB952 luminometer (Berthold Technologies, Bad Wildbad, Germany) (Nagdyman et al., 2003).

Under normothermic and hypothermic conditions, endogenous S100B levels of all brain cells and organotypic slices were below detection levels (data not shown). After extensive pilot studies a final concentration of 40 ng/ml (~2 nM) S100B (Calbiochem, San Diego, CA) in the medium was used in all experiments because it was the lowest concentration needed to influence IL-6 release from neuronal and glial cells. This concentration was not significantly altered by endogenous S100B release (data not shown).

2.4. Cell viability assay by Trypan blue exclusion

All cells were grown over night on 24-well tissue culture plates (4 × 104 cells/well) coated with 1% gelatine. After adherence of cells medium was removed and replaced by fresh medium (essential medium).

After timepoint 1, 2 and 3 cells in culture suspension and adherent cells were exposed to Trypan blue dye (0.004% in PBS), which stains damaged (leaky) cells. Cells were placed on a hemocytometer and unstained (=living) cells were counted under a light microscope (Leica). Cells were counted after each treatment and the percentage of viable, Trypan blue-negative cells was expressed.

2.5. Quantification of lactate dehydrogenase (LDH) release

LDH release into cell culture supernatants was quantified using a Cytotoxicity Detection Kit (Roche, Mannheim, Germany) following the manufacturer's instructions. At the end of the experiments, supernatants were collected and the LDH content was measured. LDH release is expressed as a percentage of the total content, determined by lysing an equal number of cells with 1% Triton X-100.

2.6. ELISA

Supernatants from treated cells was tested for IL-6 using a commercially available sandwich enzyme-linked immuno-sorbent assay (ELISA) according

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