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Dexamethasone enhances necrosis-like neuronal death in ischemic rat hippocampus involving μ-calpain activation

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ABSTRACT

Transient forebrain ischemia (TFI) leads to hippocampal CA1 pyramidal cell death which is aggravated by glucocorticoids (GC). It is unknown how GC affect apoptosis and necrosis in cerebral ischemia. We therefore investigated the co-localization of activated caspase-3 (casp-3) with apoptosis- and necrosis-like cell death morphologies in CA1 of rats treated with dexamethasone prior to TFI (DPTI). In addition, apoptosis- (casp-9, casp-3, casp-3-cleaved PARP and cleaved α-spectrin 145/150 and 120 kDa) and necrosis-related (calpain-specific casp-9 cleavage, μ-calpain upregulation and cleaved α-spectrin 145/150 kDa) cell death mechanisms were investigated by Western blot analysis. DPTI expedited CA1 neuronal death from day 4 to day 1 and increased the magnitude of CA1 neuronal death from 66.2% to 91.3% at day 7. Furthermore, DPTI decreased the overall (days 1–7) percentage of dying neurons displaying apoptosis-like morphology from 4.7% to 0.3% and, conversely, increased the percentage of neurons with necrosis-like morphology from 95.3% to 99.7%. In animals subjected to TFI without dexamethasone (ischemia-only), 7.4% of all dying CA1 neurons were casp-3-immunoreactive (IR), of which 3.1% co-localized with apoptosis-like and 4.3% with necrosis-like changes. By contrast, DPTI decreased the percentage of dying neurons with casp-3 IR to 1.4%, of which 0.3% co-localized with apoptosis-like changes and 1.1% with necrosis-like changes. Western blot analysis from DPTI animals showed a significant elevation of μ-calpain, a calpain-produced necrosis-related casp-9 fragment (25 kDa) and cleavage of α-spectrin into 145/150 kDa fragments at day 4, whereas in ischemia-only animals a significant increase of casp-3-cleaved PARP, cleavage of α-spectrin into 145/150 and 120 kDa fragments was detected at day 7. We conclude that DPTI, in addition to augmenting and expediting CA1 neuronal death, causes a shift from apoptosis-like cell death to necrosis involving μ-calpain activation.

Introduction

The use of glucocorticoids (GC) in cerebral ischemia remains controversial. Clinical studies could not show improvement of functional outcome and mortality by the use of GC following stroke (Sandercock and Soane, 2011). Although vasogenic edema is halted effectively in animal studies by GC (Barbosa-Coutinho et al., 1985; Fenske et al., 1979), GC do neither in animal models of focal ischemia (Barbosa-Coutinho et al., 1985; Hartmann et al., 1990; Lee et al., 1974) nor in patients suffering from stroke (Shaikh et al., 2011) reduce infarct size. Similarly, in models of global cerebral ischemia, GC prevent local edema (Dux et al., 1990) but aggravate neuronal death in the highly ischemia-susceptible hippocampal CA1 sector (Koide et al., 1986; Sapolsky and Pulsinelli, 1985). Augmented NMDA-receptor activation (Armanini et al., 1990; Takahashi et al., 2002) and increased intracellular calcium concentrations [Ca^{2+}] (Adachi et al., 1998; Namba et al., 2002), both of which are linked to excitotoxicity, appear to play key roles in GC enhanced ischemic CA1 neuronal death. Excitotoxicity, defined by excessive glutamatergic stimulation due to elevated extracellular glutamate and overstimulation of its receptors, is a major pathogenic mechanism in focal and global cerebral ischemia (Benveniste et al., 1988; Siesjö et al., 1989). In ischemic CA1 prolonged NMDA-receptor activation causes drastic elevation of [Ca^{2+}], which in turn causes mitochondrial dysfunction (Racay et al., 2009), formation of radical oxygen species (Frantseva et al., 2001), and μ-calpain activation (Roberts-Lewis et al., 1994; Sahara and Yamashima, 2010) all of which have traditionally been linked to necrosis. In parallel apoptosis-related events, such as activation of the c-Jun N-terminal kinase (JNK) pathway (Carboni et al., 2004; Ozawa et al., 1999), mitochondrial release of cytochrome c (Ouyang et al., 1999; Sugawara et al., 1999), caspase-9 (casp-9) (Cao et al., 2002) and caspase-3 (casp-3) (Chen et al., 1998a) activation play a role in ischemic CA1 death. Despite of casp-3 activation, which among other effector caspases brings about characteristic morphologic changes recognized as classical apoptosis (Lockshin and Zakeri, 2004), morphology-based

Abbreviations: DGC, dentate granule cells; DPTI, dexamethasone prior to ischemia; GC, glucocorticoids; TFI, transient forebrain ischemia.

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studies found that the vast majority of ischemic CA1 neurons display necrosis-like features (Colbourne et al., 1999; Müller et al., 2004) and only a minority show morphologic changes akin to apoptosis (Müller et al., 2004; Zeng and Xu, 2000).

Although excitotoxic mechanisms may mediate GC enhanced damage in cerebral ischemia, the influence of GC on cell death programs and resultant cell death morphology remain to be elucidated. To investigate the influence of the potent synthetic GC dexamethasone on apoptosis and necrosis in cerebral ischemia, we investigated its effect on ischemic neuronal death in the hippocampal CA1 sector. In contrast to neurons throughout an infarct, CA1 pyramidal cells are a homogenous neuronal population. Moreover, the fact that all CA1 neurons are equally affected by an ischemic insult allows to depict a temporal profile of morphologic and biochemical changes related to ischemic neuronal death. Shortly, rats were pretreated with dexamethasone and subjected to 10 min transient forebrain ischemia (TFI). Previously established light microscopic criteria for morphologic classification of ischemic CA1 cell death subtypes (Müller et al., 2004) were used in combination with casp-3 immunohistochemistry (IHC). In addition, Western blot analysis was used to characterize the relative importance of apoptotic (formation of pro-apoptotic casp-9 fragments, casp-3 cleavage and casp-3-cleaved PARP, α-spectrin cleavage into 145/150 and 120 kDa fragments) and necrotic (μ-calpain expression and calpain-mediated pro-necrotic cleavage of casp-9, α-spectrin cleavage into 145/150 kDa fragment) cell death mechanisms. The combination of morphologic criteria and biochemical evidence (Western blot) was applied in compliance with previously suggested guidelines for molecular definitions and nomenclature of cell death (Galluzzi et al., 2012). Importantly, morphologic criteria were included in this study a) to shed light on discrepant data from molecular- and morphology-based studies on ischemic cell death (Chen et al., 1998a; Colbourne et al., 1999; Müller et al., 2004) and b) to assess the final histo-morphologic outcome which may have clinical and functional relevance for patients.

Materials and methods

Animal care

All animal experiments were performed in accordance with the guidelines of the Danish Animal Experiments Committee (#2001/561-478) and the European Council Directive #86/609 for the Care of Laboratory Animals.

Induction of transient forebrain ischemia

The study was conducted on adult male Wistar rats (Taconic M&B, Ry, Denmark) weighing 300–350 g. Ten minutes of TFI was induced by transiently occluding both common carotid arteries during systemic hypotension (mean arterial blood pressure, 55 mm Hg) as described previously (Bering et al., 1995). Briefly, rats were fasted overnight with free access to water. The animals were then anesthetized with a mixture of 1% halothane in O_2/N_2O (30%/ 70%). The left femoral artery was cannulated for continuous control of blood pressure, induction of hypotension, as well as for intermittent blood sampling. Both common carotid arteries were gently exposed under anesthesia, which then was discontinued for 2 min before both common carotid arteries were ligated for 10 min. Systemic hypotension was induced immediately before ligation by withdrawing blood into a heparinized syringe and was maintained throughout 10 min. Reperfusion was established by infusion of the extracted blood immediately after the ligation was removed. Animals were allowed to survive for 1 (n = 5), 2 (n = 4), 4 (n = 6), 5 (n = 7) and 7 (n = 5) days, respectively; control rats (n = 4) were sham-operated, kept under identical conditions and allowed to survive for 7 days. Additional rats, processed for Western blots, were subjected to ischemia (survival 2, 4, 7 days; n = 4 in each group) or sham-operated (n = 4). Animals subjected to ischemia without precedent dexamethasone treatment were termed 'ischemia-only'.

Dexamethasone treatments

Dexamethasone treatment prior to transient forebrain ischemia ('DPTI') was conducted according to a previously used protocol (Koide et al., 1986). Rats treated identically and in parallel to ischemia-only animals as mentioned above, were injected with dexamethasone (2 mg/kg) intraperitoneally 3, 24 and 48 h before induction of TFI. Rats, processed for histology, were allowed to survive 1 (n = 4), 2 (n = 4), 4 (n = 8), 5 (n = 4) and 7 (n = 4) days, respectively. For Western blots, rats were subjected to treatment with dexamethasone prior to ischemia ('DTPI') and allowed to survive 2, 4, and 7 days, respectively (n = 4 in each group). Additional sham-operated rats, treatment with dexamethasone, were allowed to survive 7 days (n = 4) and used for Western blots. Five animals, all from DPTI groups, were found dead in their cages. Previous observations have assumed generalized seizures as a cause of death in DPTI animals (Koide et al., 1986).

Intracerebroventricular colchicine administration

Dentate granule cell (DGC) apoptosis following colchicine toxicity served as a positive control for classical casp-3-mediated apoptosis in neurons of the adult central nervous system (Müller et al., 2006). Briefly, rats were anesthetized and stereotaxic surgery was performed as described previously (Müller et al., 2006). Ten micrograms colchicine, dissolved in 10 μL sterile saline, were injected into the right lateral ventricle and animals were allowed to survive for 7 days (n = 4). Sham-operated animals (n = 4) received 10 μL sterile saline instead and were allowed to survive 7 days. Intracerebroventricular (icv) injection of 10 μg colchicine produces a 30% loss of DGC in the dorsal hippocampus 48 h following injection (Müller et al., 2006).

Histology

For histological studies, brain sections from ischemia-only animals, used in previous studies (Müller et al., 2004, 2007), were included in the current study. Brain sections from animals, which were subjected to DPTI in parallel, have not yet been used for publication. For histological processing, animals were deeply anesthetized with 1% halothane in O_2/N_2O (30%/ 70%) and perfused transcardially with 4% paraformaldehyde in 0.15 M phosphate buffer (PB, pH 7.3 at 4 °C). Brains were then fixed in the same fixative for 4 h at room temperature before embedding into paraffin. Series of coronal sections (3 μm thickness) from the dorsal hippocampus (−3.5 mm caudal to Bregma in ischemic animals and −3.0 mm caudal to Bregma in colchicine-treated animals and their respective controls) were cut on a microtome and processed for hematoxylin/eosin (H/E) staining, Nissl staining and IHC, respectively.

Immunohistochemistry

IHC was performed using a rabbit polyclonal antibody (CM1 antibody, kindly provided by IDUN Pharmaceuticals, CA) against the p17 subunit of casp-3, which originates during casp-3 activation. Briefly, after deparaffinization the antigen was retrieved by boiling the mounted sections for 60 min in 10 mmol/L citrate buffer, pH 6.0, in a domestic kitchen steamer (MultiGourmet FS 20, Braun, Kronberg/Taunus, Germany). When the sections had cooled, they were transferred to 10% fetal calf serum (FCS) in 0.12 M phosphate buffered saline (PBS, pH 7.3) for 30 min at room temperature to block background staining before applying the primary antibody. The primary antibody was applied 1:1500 in 10% FCS/PBS overnight at 4 °C. Primary antibody binding was visualized using a standard avidin–biotin–peroxidase method with 3,3-diaminobenzidine tetrahydrochloride (DAB) as chromogen. Control sections (of treatment and control groups) were incubated