Effect of TNF- α on CD3- ζ and MHC-I in Postnatal Rat Hippocampus

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ABSTRACT: The ζ subunit of the CD3 T-cell receptor complex and the major histocompatibility complex class I (MHC-I) are important not only for the immune response to antigens, they also function as signal molecules in the brain, where they play a role in the postnatal maturation process. The expression of these molecules can be regulated by cytokines. In situations associated with increased cytokine production, such as neonatal hypoxia, the hippocampus is particularly susceptible to permanent damage. This has prompted us to examine the MHC-I and CD3- ζ expression in hippocampus from early postnatal, weanling and adolescent rats and to record the effects of TNF- α and IL-1 β , cytokines commonly increased in neonatal hypoxia, on MHC-I and CD3- ζ expression in the hippocampus. We show that there is a robust postnatal up-regulation of CD3- ζ and MHC-I protein as well as of MHC-I mRNA and that TNF- α down-regulates the expression of CD3- ζ protein and MHC-I mRNA in early postnatal but not in weanling nor in adolescent rats. These results may offer a molecular explanation to the adverse effects of increased circulating levels of cytokines on brain in neonatal hypoxia. (*Pediatr Res* 60: 377–381, 2006)

The brain has long been considered an immune-privileged organ with the blood-brain barrier separating the immune system of the brain from the rest of the body (1,2). During the past decade this concept has been challenged by the discovery of many immune system components and mechanisms in the nervous system (3). It is now generally agreed that the brain responds immunologically to various internal and external injurious stimuli (4,5).

Neurons of several brain areas, including the lateral geniculate nucleus (LGN) and the hippocampus, have been found to express the major histocompatibility protein complex (MHC-I). In addition, neurons of the LGN expresses the ζ subunit of the CD3 T-cell receptor complex (CD3- ζ) (6,7). It was suggested that MHC-I is positioned postsynaptically to transmit retrograde signals to presynaptic CD3- ζ -containing receptors (8). Studies of the LGN have indicated that MHC-I and CD3- ζ also function as signaling molecules involved in postnatal maturation as well as in neuronal function (9). Furthermore it has been reported that CD3- ζ containing receptors might be involved in some of the neuronal functions of MHC class I

molecules (10). Moreover, it was suggested that different cytokines such as Interferon gamma (INF- γ) and tumor necrosis factor-alpha (TNF- α) regulate the MHC-I and the CD3- ζ expression (11,12). TNF- α and IL-1 beta (IL-1 β) are two pro-inflammatory cytokines known to be up-regulated in hypoxic ischemia (13) and to mediate brain injury (14).

The hippocampus is one of the most vulnerable areas in the brain, being responsible for learning and memory (15,16). Hypoxic ischemia affecting mainly the periventricular area of the brain (17,18), is often responsible for learning impairment and developmental amnesia in children (19,20).

This study was designed to get an insight into the possible existence of MHC-I and CD3- ζ in the hippocampus of the developing brain. We studied MHC-I mRNA and CD3- ζ protein expression in hippocampus of early postnatal, weanling and adolescent rats. Furthermore we examined the effect of circulating TNF- α and IL-1 β on the expression of these immune signaling molecules. Our results demonstrate developmental up-regulation in the CD3- ζ and MHC-I, with a down-regulating effect of circulating TNF- α during the early postnatal period in rat hippocampus.

MATERIALS AND METHODS

Animals. All experiments were performed on timed-gestation Sprague Dawley rats (Scanbur B&K, Sollentuna, Sweden). The animals arrived at the laboratory at least 2 d before experiments and were housed in groups, under control conditions of light: darkness (12:12 h, lights on 0800 h). The study was performed on early postnatal (3–8 postnatal days, EPN), weanling (18–25 postnatal days) and adolescent (38–45 postnatal days) rats. Each group was divided into three subgroups composed of animals with equivalent body weight. Two groups were injected with cytokines and one group was injected with vehicle (control). Four days after the last injection all animals were anesthetized, and the brains were dissected after saline perfusion. Hippocampus tissues were extracted and frozen immediately in dry ice to be used in all the experiments. This time point was chosen since it is well documented that this is the peak period for brain insult following injury, such as hypoxia (21). Furthermore it has been shown that intraperitoneal cytokine injection (Interferon gamma) has a maximal effect on MHC-I expression at four days (22). Control and cytokine treated animals had the same weight gain. All experiments were approved by the local committee on Ethics for Animal Experimentation, Stockholm, Sweden.

Cytokines. Recombinant rat TNF- α and recombinant rat IL-1 β were obtained from R&D Systems, UK. Each cytokine was reconstituted in phosphate buffer saline (PBS) and injected IP; 1 μ g for the EPN group, 2 μ g for

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Abbreviations: CD3- ζ , the zeta subunit of the T-cell receptor complex; EPN, early postnatal; MHC-I, major histocompatibility complex class I

the weanling group and 5 μ g for the adolescent group. These doses were selected since they have, when injected IP, been shown to have effect on rat brain (23,24). For each cytokine, three injections were given with 12 h interval. The first injection was given at postnatal day (PD) 3 for the EPN group, at PD 18 for the weanling group and at PD 38 for the adolescent group. The number of animals and experiments are given in the results section. The serum level of TNF- α was found to be 1.2 ± 0.9 pg/mL 24 h after the last injection.

Immunoblotting for CD3- ζ and MHC-I in the hippocampus. Total cell homogenates were prepared from the hippocampus of early postnatal (EPN), weanling and adolescent rats. All tissues were homogenized with 6 volumes 1% sodium dodecylsulfate (SDS). The protein concentration was then measured using Bio-Rad Protein assay Kit, based on the Bradford dye-binding procedure (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein (20 μ g) were loaded onto a 10% SDS-polyacrylamide gel, for electrophoresis, and transferred to PVDF membranes (Amersham Pharmacia Biotech, UK). The blots were blocked with 5% (wt/vol) nonfat dry milk in PBS containing 0.1% tween-20 for one hour at room temperature, incubated with primary polyclonal rabbit anti rat CD3- ζ antibody (FL-163): sc-20919, Santa Cruz Biotechnology, at 1:1,000 dilution for one hour at room temperature; or with primary MAb OX-18, MCA 51 G (Serotec, UK) against rat MHC-I (25), which recognizes an epitope in the α 3 domain of most rat MHC-I α and MHC-I β proteins (26), at [10 μ g/mL], for two hours at room temperature; washed with PBS/0.1% tween-20 (5 times $\times$ 5 min) incubated with Donkey anti-rabbit IgG HRP secondary antibody (Amersham), diluted 1:2,000 or with Goat anti-mouse IgG HRP secondary antibody Star77 (Serotec, UK), at [1 μ g/mL] respectively for one hour at room temperature; washed with PBS/0.1% tween-20 (5 times $\times$ 5 min). Immunosignals were visualized using ECL plus Western blotting detection system (Amersham Biosciences, UK). The resultant protein bands were scanned and quantified using ImageJ, National Institutes of Health (National Institutes of Health), Bethesda, Maryland. This program was further used to calculate the total protein of each sample by scanning and analyzing the amido-black stained membranes. The resultant analysis was then expressed as a ratio between the protein bands and the total protein.

Quantitative analysis of MHC-I mRNA in the rat hippocampus. Hippocampi were used for extraction of total RNA using RNeasy Mini Kit (QIAGEN, Hilden, Germany). RNA samples were further cleaned with RNase-Free DNase Set for DNase digestion during RNA purification (QIAGEN, Hilden, Germany).

Real-time RT PCR. RT PCR and real-time detection of PCR product accumulation was performed using iCycler (Bio-Rad). Each PCR was 20 μ L and consisted of 10 μ L 2 $\times$ iScript One-Step RT PCR Kit with SYBR Green (Bio-Rad), 5 μ L diluted RNA sample and the primer mix (MHC1.U0347:5'-AGGGCGGCTCTCACACCATCC3'&MHC1.L1016:5'CCTCCTCCTCTCACACAACCAC-3'). Primers for real-time RT PCR experiments were designed by PrimerSelect software (DNASTAR Inc, Madison, USA). Rat MHC1 mRNA structure (accession number NM_012645) was used to select MHC1 β specific primers (27).

Quantum RNA 18S Internal Standards (Ambion) were used as the "house-keeping" gene. 18S stands for a ribosomal 18S RNA. It was used to normalize for variations in RNA quality and starting quantity, and random tube-to-tube variation in RT and PCR reactions. According our experience commonly used "invariant" standard controls, such as β -actin, might vary from tissue to tissue, between cell types, or in response to the experimental treatments. The use of ribosomal 18S RNA, as endogenous control, overcome all these problems since majority of RNA is rRNA (28). MHC-I values were normalized to 18S Classical II values.

Statistical analysis. Data are expressed as means $\pm$ SEM. Differences between means were evaluated by *t*-test and ANOVA analysis of variance. Statistical significance was accepted at *p* < 0.05.

RESULTS

Developmental pattern of CD3- ζ protein in the hippocampus of rats. Immunoblot analysis of hippocampal homogenates revealed a band at approximately 37 kD, which corresponds to CD3- ζ dimers. The CD3- ζ chain, one of the invariant subunits of the T-cell receptor (TCR), is known to form disulphide-linked dimers in SDS-polyacrylamide gel, where the two monomers associate via their transmembrane domains. These proteins share the ability to form disulfide-linked dimers with themselves and with other members of the family (29).

The relative level of CD3- ζ protein abundance increased two-fold from the EPN to the weanling period. The abundance of the CD3- ζ protein was measured using Western blotting of homogenates of hippocampus dissected from EPN, weanling and adolescent rats. Figure 1 illustrates a collective data quantification of material from 7–8 animals in each group, studied in 4 independent experiments. In each experiment, the protein expression in the EPN hippocampus was assigned a value of 100%. A representative immunoblotting of CD3- ζ is also shown. There was no significant difference in CD3- ζ expression between the weanling and the adolescent periods. Analysis of variance showed a significance level of *p* < 0.001.

MHC-I expression in the hippocampus of rats. The abundance of the MHC-I protein was measured by Western blot of homogenates of hippocampus dissected from EPN, weanling and adolescent rats. We compared whole-cell homogenates to a membrane preparation of the hippocampus of weanling rats. Two distinct bands, corresponding to 37- and 53-kD were found in the lane loaded with whole cell lysate. The membrane preparations were enriched with 53-kD species (Fig. 2A), similar in size with MHC-I protein found by Corriveau in membrane fractions prepared using brain tissues (8).

MHC-I genes are divided in two subgroups, the classical (MHC-I α) and non-classical (MHC-I β) classes of MHC-I. Both types of MHC-I are expressed as soluble, as well as membrane-bound proteins. It was also been shown that an intact MHC-I molecule with size 37–45 kD on SDS-polyacrylamide gel may form aggregates with high molecular weight (26).

The MHC-I immunoreactivity in whole-cell homogenate taken from EPN, weanling or adolescent rat hippocampus was compared. Five studies were performed. In each of these studies two rats from each age group were used. (Figure 2B) demonstrates the immunoblot of a representative study. The

Figure 1. Western immunoblotting of the CD3- ζ . Hippocampal tissue from rats of three different age groups, EPN, weanling and adolescent, were used for Western blotting for CD3- ζ . Quantification of the optical densitometry for four studies is demonstrated in the bar diagram. The protein expression of the EPN hippocampus was assigned a value of 100%, and the values are shown as percentage of EPN (% signal of EPN). (**p* < 0.001). Error bars refer to the SEM.

Figure 2. Immunoblotting of MHC-I. (A) Western blot analysis of membrane preparations (lane I) and whole cell lysate (lane II) of weanling rat hippocampus. Left markers show positions of 53 kD and 37 kD of protein according to low range standards (Bio-Rad Laboratories, Hercules, CA). (B) *Upper panel:* Analysis of MHC-I in whole-cell homogenate from rat hippocampus of EPN, weanling and adolescent rats. Western blot of MHC-I, two samples for EPN, weanling and adolescent rats. Left markers show positions of 53 kD and 37 kD of protein according to low range standards (Bio-Rad Laboratories, Hercules, CA). *Lower panel:* Summary of results of the densitometry analysis for the immunoblots of five studies. In each study two rats of EPN, weanling and adolescent were used ($n = 10$). The protein expression of the EPN hippocampus was assigned a value of 100%, and the values are shown as percentage of EPN (% signal of EPN). Error bars refer to the SEM.

immune signal of the 53 kD MHC-I, was significantly lower in the hippocampus of the EPN rats, than in the weanling and adolescent rats (Analysis of variance $p < 0.001$). The optical densitometry for the 5 studies is shown after normalizing the protein expression at EPN hippocampus to 100.

The expression level of MHC-I mRNA was studied in the hippocampus dissected from rats of the EPN, weanling and adolescent age groups. Two studies were performed. In each of these studies six rats from each age group were used. MHC-I mRNA significantly increased from the EPN to the weanling period while no significant increase occurred between weanling and adolescent (Analysis of variance $p < 0.005$) as shown in Fig. 3.

Effect of circulating TNF- α on CD3- ζ and MHC-I expression. For each age group, four studies were performed to investigate CD3- ζ protein expression. Two control, and two injected rats were used in each of these studies. The Western immunoblotting analysis indicates an effect of the TNF- α on the expression of CD3- ζ in the hippocampus. In the EPN group, TNF- α caused approximately 40% reduction in the level of protein expression of CD3- ζ in the hippocampus ($p < 0.001$). A representative immunoblot of CD3- ζ is shown in

Figure 3. Real-time RT PCR quantification of the MHC-I mRNA expression. Hippocampal tissue from rats of the three age groups EPN, weanling and adolescent, were used for real-time RT PCR analysis. Values are presented as the ratio of MHC-I/18S RNA ($*p < 0.001$). Error bars refer to the SEM.

(Fig. 4A), together with quantification of the optical densitometry for the 8 animals where the hippocampal protein expression of vehicle treated rats was assigned a value of 100%.

In the weanling and adolescent groups, TNF- α had no significant effect on the level of protein expression of CD3- ζ in the hippocampus ($p = 0.31$ and 0.07 , respectively). A representative immunoblot of CD3- ζ is represented in Figures 4B,C, respectively, together with quantification of the optical densitometry for the 6 animals, where the hippocampal protein expression of vehicle treated rats was assigned a value of 100%.

To study the effect of TNF- α and IL-1 β on the mRNA expression levels in the hippocampus, two real-time RT PCR studies were performed using six control animals, six TNF- α -injected animals, and six IL-1 β -injected animals from each age group. TNF- α caused significant reduction of the MHC-I mRNA expression in the hippocampus of EPN rats ($p < 0.001$; Fig. 5A). In the weanling and adolescent groups, TNF- α caused no significant effect on the level of mRNA expression of MHC-I in the hippocampus ($p = 0.12$ and 0.65 , respectively) as illustrated in Figure 5B,C, respectively. The effect of TNF- α on the protein levels of MHC-I could not be estimated due to changes in the antibody batch.

IL-1 β did not have a significant effect on the MHC-I mRNA levels in rat hippocampus in any of the age groups.

DISCUSSION

Emerging evidence suggests that MHC-I and CD3- ζ play important roles not only as defense molecules in the immune system but also as regulators of development and plasticity in the CNS. Neurons in the LGN (Lateral Geniculate Nucleus), and hippocampus express MHC-I and β 2-microglobulin. In addition they showed that CD3- ζ is also expressed in neurons of the LGN (8). Studies performed on knockout mice have shown that MHC-I and CD3- ζ are important for activity-driven structural remodeling and synaptic plasticity (9). *In situ* hybridization studies have indicated that the expression of MHC-I mRNA increases postnatally in the hippocampus (8). In the present study we quantified MHC-I mRNA expression

Figure 4. Effects of TNF- α *in vivo* injections on CD3- ζ protein expression. A representative Western blot and quantification of the optical densitometry for the effect of TNF- α on the CD3- ζ protein expression in the (A) EPN, (B) weanling, and (C) adolescent hippocampus. Bar graphs show quantification of the optical densitometry for 6 animals. (* $p < 0.001$). The hippocampal protein expression of vehicle treated rats was assigned a value of 100%, and the values are shown as percentage of control (% signal of control). Error bars refer to the SEM.

levels by use of real-time RT PCR. We found a robust increase of the MHC-I mRNA expression in the hippocampus of rats between the EPN period and the end of the weanling period. Furthermore Western blot analysis suggests a parallel increase in the expression of MHC-I in the hippocampus during the same period (Fig. 2B). We also demonstrated that the expression of CD3- ζ protein in hippocampus increases from the EPN period until the end of the weanling period, whereas this developmental pattern for the expression of CD3- ζ protein in hippocampus has not been reported previously. However, we found no change in the expression of MHC-I mRNA and protein, nor in the CD3- ζ protein between the weanling and the adolescent period. Important developmental events take place in the hippocampus during the first three weeks of age (30). In the early postnatal period, growth cones start to appear; dendrites grow and become covered with spines (31). Migration of neurons occurs throughout this interval and the layer differentiation of the hippocampus is not completed until the end of this period (32,33).

Perinatal hypoxia is a major cause of neurologic and intellectual impairment in children (17,19), where the hippocampus is highly vulnerable to the consequences of brain hypoxia (34). Furthermore, cytokines have been implicated in the cause of hypoxia-triggered brain damage (14,35). The extent of posthypoxic brain lesions, as evaluated with magnetic resonance images, has been found to be paralleled by an

increase in blood levels of several cytokines (36), including TNF- α (14,37,38). Here we observed that EPN rats exposed to TNF- α via IP injections displayed a robust down-regulation of both MHC-I and of CD3- ζ . TNF- α is known to penetrate the blood-brain barrier (39). In contrast, no effects of TNF- α on MHC-I and of CD3- ζ expression were detected in weanling nor in adolescent rats. The regulatory effect of TNF- α on CD3- ζ protein is similar to that found in T-cell hybridoma treated with TNF- α (12). The effect of cytokines on the expression of CD3- ζ in the brain has not been studied to date. In contrast to our findings, MHC-I has been reported to be up-regulated in a neuroblastoma cell line exposed to TNF- α (40). It was recently suggested by Boulanger and colleagues (6) that the regulation of MHC-I expression in culture environment may be different from that found *in vivo* (6). The effect of TNF- α on MHC-I in cells from the immune system has not been reported. The down-regulation of CD3- ζ and MHC-I in response to circulating TNF- α might have adverse effects on neuronal development. Moreover there is evidence that TNF- α is a silencer of survival signals in neurons (41). MHC-I and CD3- ζ are considered to be important for development and plasticity of a subpopulation of neurons within the brain (9). We propose a specific role for TNF- α in down-regulating MHC-I and CD3- ζ expression during the period immediately following birth. TNF- α is elevated in hypoxia, thus it will be an important topic for further studies to examine

Figure 5. Effects of TNF- α *in vivo* injections on the MHC-I mRNA expression. The effect of TNF- α on the MHC-I mRNA expression was measured in the hippocampus of EPN (A), weanling (B), and adolescent (C) rats. Values are presented as the ratio of MHC-I/18S RNA (* $p < 0.001$). Error bars refer to the SEM.

changes in hippocampal MHC-I and CD3- ζ expression in an experimental hypoxia model. Whether the effect of TNF- α in the hippocampus is direct or via other molecules needs to be further studied.

In conclusion, our results indicate that important developmental changes occur in the hippocampus with regard to the expression of MHC-I and CD3- ζ . During certain stages of development, MHC-I and CD3- ζ expression can be down-regulated by circulating TNF- α . Taking into account the dual roles of the MHC-I and CD3- ζ molecules and the fact that down-regulation might be risky for brain neuronal development, it will be of importance to further explore the role of MHC-I and CD3- ζ for postnatal brain development as well as the possible adverse effects of TNF- α -induced down-regulation of these molecules.

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