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Transient Increase of Lipocortin 1 in Nuclei of the Hippocampal Pyramidal Neurons in Rats Induced by Immobilization Stress

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Changes of lipocortin 1 (LC1) in the brain induced by immobilization stress were investigated in rats. Rats were immobilized for 0, 1, 2, 3, 4, and 5 h, and the brain slices were immunostained with anti-human LC1 antibody (anti-LC1). Immunoreactivity of LC1 (iLC1) was most prominent in neuronal cell bodies and processes of hippocampal CA regions and dentate gyrus. At rest without stress, most of the LC1 in the neuron located in the cytoplasm with the nuclei exhibiting relatively scarce immunoreactivity. Immobilization stress changed this intracellular distribution of LC1 by increasing nuclear LC1. The change was apparent in 1 h and reached the peak by 3 h. However, by 5 h of immobilization, the distribution pattern returned to that of the resting state. This transient nuclear translocation of LC1 was most prominent in CA_1 pyramidal neurons, and was not observed in areas other than the hippocampus. Adrenalectomy abolished this transient translocation of LC1. The roles of hippocampal LC1 as a mediator of glucocorticoid feedback signal and/or as an intracellular stress signaling protein could be suggested.

Keywords: Hippocampus, HPA axis, Lipocortin 1, Stress.

Introduction

LC1 is a 37 kDa member of annexins, a superfamily of multifunctional proteins with calcium- and phospholipid-binding properties, and is found in a wide range of organisms including vertebrates, invertebrates, and plants (Raynal and Pollard, 1994). Synthesis and secretion of LC1 are increased by glucocorticoids (Flower and Rothwell, 1994), although the mechanism of the release is still obscure. Once released, LC1 mediates some of the anti-inflammatory actions of glucocorticoids, which has been evidenced in a range of experimental models such as reduction of rat paw edema (Cirino *et al.*, 1989), fever in rats (Carey *et al.*, 1990; Strijbos *et al.*, 1992, 1993), neutrophil migration (Perretti and Flower, 1993), inhibition of nitric oxide synthase induction (Wu *et al.*, 1995), and in ischemic brain damage in the rat (Relton *et al.*, 1991; Rothwell and Relton, 1993). The inhibition of phospholipase A_2 has been suggested as a mechanism of anti-inflammatory action of LC1 (Kim *et al.*, 1994).

As well as mediating some of the peripheral actions of glucocorticoids, LC1 has been suggested to play some roles in the central nervous system. Firstly, lipocortin 1 is localized in neuronal cells as well as non-neuronal cells in the brain (Strijbos *et al.*, 1991), although the exact localization appeared to be variable depending on the method of tissue processing (McKanna and Zhang, 1997). Secondly, LC1 that is endogenous in the brain is involved in the above-mentioned situations of brain ischemia and febrile response. Increasing evidence also suggests that LC1 plays a key role in the regulation of glucocorticoid secretion by serving as a mediator of the powerful negative feedback actions of the steroids on the hypothaloamo-pituitary-adrenocortical (HPA) activity during stress response (Buckingham, 1996; Buckingham and Flower, 1997).

The hippocampus is the center of stress-response processing and the regulator of HPA axis activity, which is subject to the glucocorticoid negative feedback through two sets of corticosteroid receptors (Jacobson and Sapolsky, 1991; Sapolsky 1992). It is not yet clear if (and how) LC1 plays a role in the glucocorticoid feedback at the level of the hippocampus, although its expression in the hippocampus (but not in other regions of the brain) appears to be dependent on glucocorticoids (Strijbos *et al.*, 1991). Here, we studied the effects of immobilization stress on the distribution of LC1 immunoreactivity in the hippocampus.

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Materials and Methods

Experimental animals Male Sprague-Dawley rats (8 weeks old; 250–300 g) were bred from a closed, specific pathogen-free colony and maintained in the vivarium of Asan Institute of Life Sciences, Seoul, Korea, with *ad libitum* supplies of laboratory chow and tap water. The animal room was kept at 22 ± 1°C, with a 12 h light–dark cycle (lights on during 06:00–18:00 h). All experimental procedures were reviewed and approved by the Animal Care and Use Committee of the University. Animals were randomly divided into two groups. One group of animals was bilaterally adrenalectomized under sodium pentobarbital anesthesia (40 mg/kg, intraperitoneally) and the other group was kept without any manipulation. The adrenalectomized rats were maintained for 7 days before the experiment, with a supply of 0.5% saline solution for drinking water during that period.

Immobilization Stress Acute immobilization stress was given as previously described (Kvetnanstky and Mikulaj, 1970; Cizza *et al.*, 1993). Immobilization stress experiment always started at 09:00 h in the morning to minimize the variation associated with the circadian rhythm. The rats were immobilized for 0 (basal), 1, 2, 3, 4, or 5 h by taping all the limbs of the rat to metal mounts. For the basal group, rats were decapitated just after the removal from the home cage in the morning. For stress groups, animals were decapitated at the end of the designated immobilization periods, and the brains rapidly removed. Trunk blood was collected in plastic tubes and serum was separated and stored at −80°C for later determination of serum corticosterone concentration.

Immunohistochemistry Immediately after the removal of the brains, a 2 mm-thick coronal slice (2–4 mm caudal from the bregma) was cut using a brain matrix (Activational Systems Inc., USA) and fixed for 3 days at 4°C in the fixation buffer (4% paraformaldehyde, 0.25% glutaraldehyde, 0.1 M sodium phosphate buffer, pH 7.4). The fixed brain slices were sectioned at 30 μ thickness using a vibratome (TPI 1000). The sections were incubated with PBS containing anti-human LC1 polyclonal antibody (Anti-LC1; 1:8000) plus 2% normal goat serum (NGS) at 4°C for 36 h. For control sections, the antibody was omitted at this step. After washing 3 times, the sections were incubated with PBS containing biotin labeled goat anti-rabbit IgG (1:400; Vector Laboratory, Burlingame, USA) plus 2% NGS at room temperature for 2 h, washed, immersed in 0.3% hydrogen peroxide solution for 30 min at room temperature to inactivate the endogenous peroxidase activity, and finally incubated for 1 h in streptavidine-conjugated peroxidase solution (1:200, Vector Laboratory, USA). Next, the sections were rinsed and visualized in PBS containing 0.05% diaminobenzidine and 0.01% H_2O_2 as a chromogen for 5 min at room temperature. Sections were then rinsed and mounted onto slides, and observed under the light microscope.

Anti-human LC1 polyclonal antibody production Polyclonal Anti-LC1 was raised in New Zealand rabbits by repeated injections of LC1 (Huh *et al.*, 1990). The rabbits received three subcutaneous injections of recommbinant human LC1 with 3 to 4 week intervals. The serum was obtained and the IgG fraction was separated by protein A-agarose affinity chromatography. The antibody was confirmed by immunodiffusion test and Western blotting using LC1 as an antigen. The specificity of the antibody was also tested on a cultured monocyte cell line.

Biochemical measurements Serum corticosterone concentration was measured by radioimmunoassay using a commercial assay kit (Diagnostic Products Corporation, USA). The detection limit of the assay was approximately 10 ng/ml. Protein concentration in the tissue homogenate was determined by the Bradford method using a commercial assay kit (Bio-Rad Labs., USA; Bradford, 1976).

Results

Serum corticosterone concentration The level of serum corticosterone in intact rats varied diurnally; low in the morning and high at the end of the light period (data not shown). Table 1 shows the changes on serum corticosterone level in a typical experiment. The rise of serum corticosterone level was immediate, reaching a plateau in 1 h of immobilization stress. The maximum level was about ten times that of the basal level (770 ng/ml at 2 h over 76 ng/ml of basal). It started to decrease after 2 h, but was still significantly high after 5 h of the continuous stress. Adrenalectomy abolished the stress-induced changes of the serum corticosterone level, which stayed mostly below the detection limit throughout the period of immobilization.

LC1 immunoreactivity (iLC1) in hippocampus Positive iLC1 was distributed widely in the brain including the neuronal cell bodies, processes, and ependymal epithelia. Among these, pyramidal cells of CA_{1-3} regions and granular cells of dentate gyrus of the hippocampus showed the most prominent iLC1 (Fig. 1). Adrenalectomy did not affect the overall intensity of iLC1 in most regions of the brain section including the hippocampus (data not shown).

Changes of hippocampal iLC1 in response to immobilization stress At the basal, unstressed state, a high density of iLC1 was present in the cytoplasm of cell

Table 1. Serum corticosterone concentration during the immobilization stress (ng/ml). The data is from a typical experiment which corresponds to the animals in Fig. 2. ND: not detectable because the concentration is below the detection limit.

Immobilization Duration (h)	Normal	Adrenalectomized
0	76	29
1	719	22
2	770	ND
3	657	ND
4	483	ND
5	547	ND

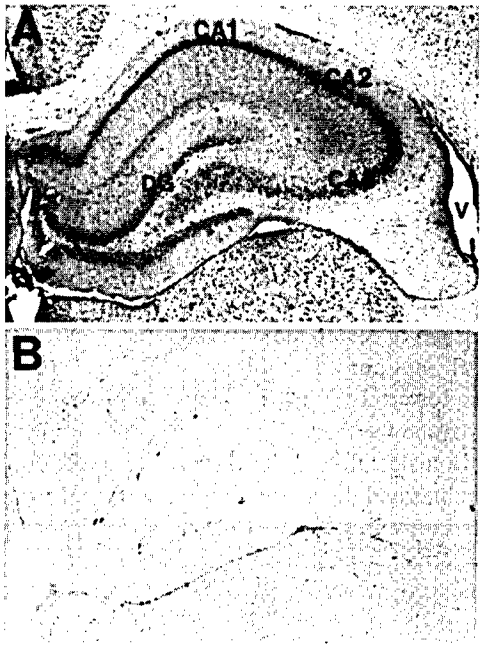

Fig. 1. Microphotograph of lipocortin 1 immunoreactivity (iLC1) in coronal sections of the hippocampus of the intact rat. A: iLC1 appears in all the regions of hippocampus including gyrus dentatus (DG), CA_1, CA_2, and CA_3 regions. Strong positive iLC1 is observed in cell bodies of hippocampal neurons as well as in the cells lining the ventricle (V). B: the same as A except that anti-lipocortin 1 antibody was omitted (control section).

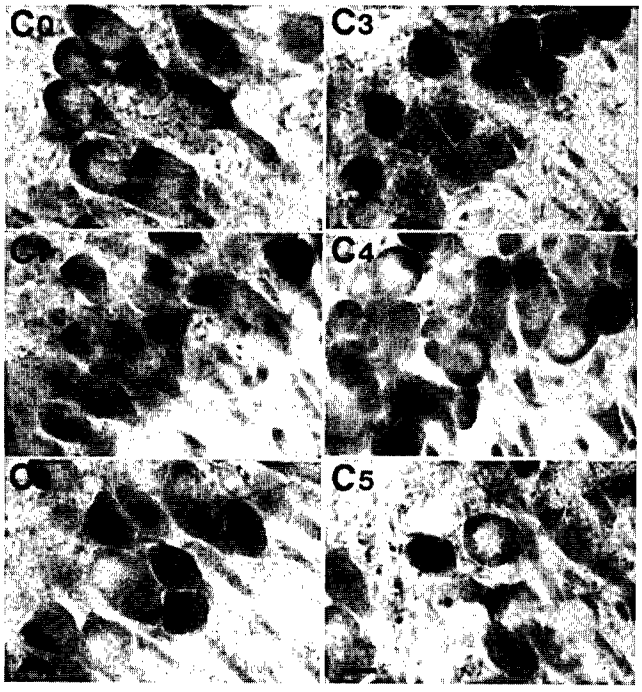

Fig. 2. Microphotograph of iLC1 in cell bodies of hippocampal pyramidal cells in normal rats. Rats were immobilized for 0 (before the immobilization), 1, 2, 3, 4, and 5 h before the sacrifice and immunostaining with anti-LC1 (C0 to C5). The scale bar is 20 μM.

body and neuronal processes, while the nuclei appeared clear (Fig. 2, C0). This pattern of intracellular distribution of iLC1 was changed with the immobilization stress. The nuclei started to show dense iLC1 after 1 h of the immobilization stress (Fig. 2, C1), and almost all nulcei were filled up with iLC1 by 3 h of the stress (Fig. 2, C3). With the continuous immobilization, the nuclei started to be cleared of iLC1 and returned to the basal pattern by 5 h (Fig. 2, C5). This transient redistribution of iLC1 into nuclei upon immobilization stress was most prominent with pyramidal neurons in the CA_1 region. Adrenalectomy abolished the transient, immobilization-induced translocation of iLC1. The basal pattern of intracellular distribution of LC1 (with clear nuclei without iLC1) in the hippocampal neuron persisted throughout the 5 h period of immobilization stress (Fig. 3).

Discussion

LC1 mediates some of glucocorticoid actions through the autocrine mechanisms after being released from the inctracellular depot by an unconventional mechanism other than exocytosis (Frey *et al.*, 1991; Goulding and Guyre, 1993). Translocation of LC1 from the cytosol to the plasma membrane might be a part of the release process, as implied in a human leukocyte cell line (Solito *et at.*, 1994). The translocation of LC1 is also dependent on the differentiation of the cells (Kang *et al.*, 1996), which makes the relationship between glucocorticoids and LC1

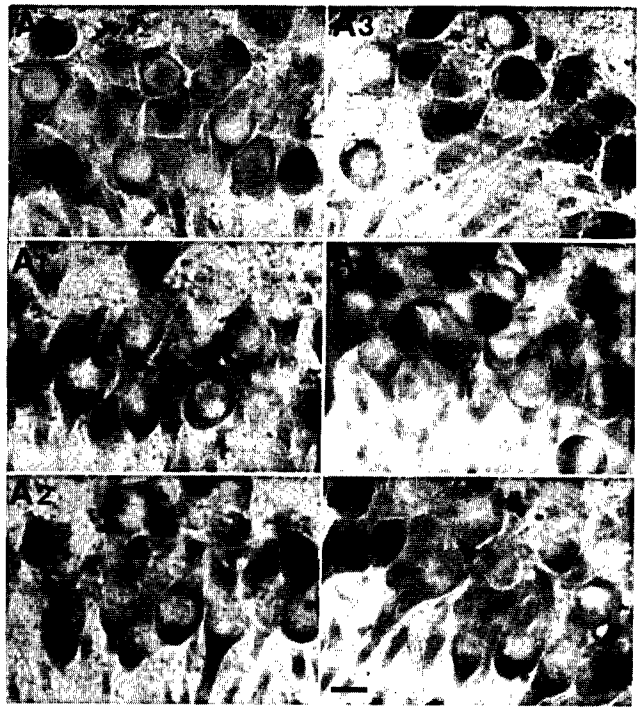

Fig. 3. Microphotograph of iLC1 prepared in the same way as for Fig. 2 in adrenalectomized rats (A0 to A5).

more complicated. Unlike the immunohistochemical study, the transient increase of LC1 in the nuclear fraction was not observed on the western blot. Adrenalectomy did not change the Western blot pattern either (data not shown). This might be due to the fact that the CA1 region is only a small portion of the whole hippocampus with which the Western blot was performed.

The transient nuclear translocation of LC1 in this study is the first to be reported as far as we are aware of. It was observed in the hippocampal neurons of rats that were subjected to the immobilization stress. The physiological meaning of this nuclear translocation is not clear yet, but it is possible that LC1 has other functions than the mediation of glucocorticoid anti-inflammatory actions. The stress from immobilization is purely neurogenic without pain or inflammatory changes of the tissue, activating the adrenergic system and the HPA axis dramatically (Kvetnanasky and Miulaj, 1970; Graessler *et al.*, 1989). The nuclear translocation of LC1 in the hippocampus appeared to be dependent on the glucocorticoids, since the same protocol of immobilization stress on the adrenalectomized rats did not induce the translocation. However, effects by other factors that could be eliminated by adrenalectomy such as adrenal medullary catecholamines and peptides could not be ruled out.

There are a couple of possibilities as to the physiological roles of the hippocampal neuronal LC1. Firstly, the dependence of LC1 translocation on the intact adrenal glands implies that LC1 might be involved in the feedback modulation of HPA activity. In this case, the LC1-mediated modulation of the HPA axis should be at the hippocampal level, and more specifically at the CA_1 pyramidal cells. The similar translocation of LC1 was not observed in the hypothalamus (data not shown), where the glucocorticoid feedback signal is mediated by LC1 (Taylor *et al.*, 1995).

The modulation of HPA activity in the presence of processive stressors (stressors requiring interpretation with respect to previous experience) by the neuronal circuit is extremely complicated, comprising the cortex and the limbic system, while the systemic stressors activate the HPA axis via more or less simple pathway through the hypothalamus and the brain stem (Herman *et al.*, 1996). The hippocampus sits just below the cortex in the hierarchy of the neuronal circuit modulating the HPA activity, and its functional deterioration is believed to be the source of many neuroendocrinological impairments associated with aging (Jacobson and Sapolsky, 1991; Seckle and Olsson, 1991). The dichotomy of corticosteroid receptors in the hippocampus that are involved in the HPA modulation makes the situation even more complicated. Two sets of corticosterone receptors are involved in the feedback modulation along the hippocampus and HPA axis; at least in the rat, the hippocampus is the only brain region expressing both the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR), while the rest of the brain regions, including the hypothalamus, express only GR (Reul and de Kloet, 1985; McEwen *et al.*, 1986). MR and GR have different roles in the feedback regulation of the HPA activity. MR is involved in the regulation of the basal circadian level of corticosterone, while the stress-induced rise in corticosterone level is under the feedback control via GR (Sapolsky, 1992). MR and GR also influence the secretion of ACTH secretagogues, such as corticotropin releasing factor, vasopressin, and oxytocin, in different manners (Sapolsky, 1992). If the nuclear translocation of LC1 in the hippocampus is involved in corticosterone feedback control, it is probably via MR: the nuclear translocation of LC1 was observed only in the hippocampus and the MR is the corticosteroid receptor that is located only in the hippocampus.

An alternative and more probable interpretation of the stress-induced translocation of LC1 is that LC1 might be the primary intracellular stress-signaling molecule in the hippocampal neurons, like heat shock proteins (HSP's). Acute or repeated immobilization stress induces HSP70/90 in peripheral organs and the brain (Vamvakopoulos *et al.*, 1993), with the hippocampus showing the strongest signal. The temporal aspect of the LC1 translocation in our study is compatible to the single immobilization-induced HSP increase which is apparent within 1 h. The HSP expression appears to be glucocorticoid-independent at least in peripheral tissues. Other stress modes also induce the HSP's in the hippocampus and other brain regions; such as seizure (Gass *et al.*, 1995), alcohol (Holownia *et al.*, 1995), subarachnoid hemorrhage (Matz *et al.*, 1996), ischemia (Higashi *et al.*, 1995), and heat shock (Marcuccilli *et al.*, 1996). Moreover, LC1 appears to share some aspects of chaperon proteins (Kim *et al.*, 1997). The dependence of LC1 translocation on the intact adrenal gland may be explained by other than the mediation of glucocorticoid feedback control. The survival and well-being of the hippocampal neurons is dependent on the low level of corticosterone, probably through the MR (Sapolsky *et al.*, 1991; Woolley *et al.*, 1991). The changes in hippocampal neurons which occur as early as 3 days after the adrenaleclomy (Gould *et al.*, 1990) may explain the absence of stress-induced LC1 change which was observed within 7 days of adrenalectomy in this study.

In conclusion, the transient nuclear translocation of LC1 in pyramidal neurons in the hippocampal CA_1 region suggests two possible roles of hippocampal LC1: either the meditation of glucocorticoid feedback signal, or primary intracellular stress signaling. Studies on the LC1 translocation in the cultured hippocampal neurons by various stimuli, in the absence or presence of corticosteroids, are in progress to define the physiological meaning.

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