Biochemical Changes of the Nerve Cells of Rats under Restraint Stress

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CONTENTS

I. INTRODUCTION

Historically, stress has been defined biologically as various physiologic changes including an activation of the pituitary adrenal axis. This activation is characterized by the liberation of adrenal steroids triggered by the release of adrenocorticotrophic hormore (ACTH) from the pituitary.[1] The construct of stress may represent the extreme pathologic continuum of overactivation of the body's normal activational or emotional systems and, thus, is linked to the construct of arousal. Such overactivation can produce the psychopathology of anxiety disorders and depression, and with chronic severe stress, actual physical damage can result.[2] Derangements in the stress axis may be induced by a variety of factors in which life events, personality, psychosocial circumstances and gender all may contribute. When such derangements are long-lasting, probably several neuroendocrine modifications are induced, giving rise to many of the symptoms seen in chronic pain syndromes.[3]

Pain is the subject's conscious perception of modulated nociceptive impulses that generate an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage. Functional process of pain can be grossly separated into four categories: transduction, transmission, modulation, and perception. Modulation refers to the ability of the central nervous system to control the pain transmitting neurons. Several areas of the cortex and brain stem have been identified that can either enhance or reduce nociceptive input arriving by way of transmitting neurons.[4] The most of pain disorders in the orofacial area such as temporo-mandibular disorder, trigeminal neuralgia, mucosal erosion or ulcer are mediated by trigeminal nervous system. Especially, the trigeminal ganglion in the peripheral nervous system and the brain stem in the central nervous system play an important role

in the transmission, modulation of pain signals.[4]

Living cells produce macromolecules (proteins, nucleic acids, polysaccharides) that serve as structural components, catalysts, hormones, receptors, or repositories of genetic information. Proteins play a central role in the cell function and cell structure.[5] As stress may cause alterations of proteins of nerve cells which can lead to functional disturbances, the present study was carried out to examine the changes of protein expression of the trigeminal ganglion and the brain stem of rats under restraint stress to inquire the relationship between stress and pathologic changes of peripheral and central nervous system.

II. MATERIALS AND METHODS

1. Experimental animals

Fifteen Sprague-Dawley rats (8-week-old) were purchased from Dae-Han Experimental Animal Research Center, Seoul, Korea. They were maintained at 20-23℃ and fed ad libitum on a normal laboratory diet. The rats were divided into 2 groups: 1) Normal control group; 2) Restraint stress group in which the rats were placed in the stress cage throughout the period of experiment. The animals were then sacrificed at day 0, 1, 3, 5, and 7 of the experiment and the trigeminal ganglion and the brain stem were excised immediately and frozen at -70℃ until use.

2. Tissue preparation

The trigeminal ganglion and the brain stem were homogenized using a homogenizer in ice-cold 0.1 M PBS buffer for 1 min.

3. Protein determination

Protein amount of the homogenized sample was determined by Lowry method.[6] First, standard bovine serum albumin (BSA) solutions of 0, 10, 20, 30, and 40 μg/ 200 $\mu\ell$ were prepared. After preparing

samples and standards of 200 $\mu\ell$/ tube, 1 ml of the reagent solution containing 0.1 ml of 2% Na/K tartrate, 0.1 ml of 1% $CuSO_4$, and 10 ml of 2% Na_2CO_3 in NaOH was added to each tube. They were left at room temperature for 10 minutes. After adding 0.1 ㎖ of 1 N phenol reagent (Sigma Chemical Co., USA), the mixtures were vortexed. After standing at room temperature for 30 minutes, the optical density of the each mixtures was measured at 750 nm in a spectrometer.

4. Immunoblotting

The proteins (30 μg) in the homogenized samples from the trigeminal ganglion and the brain stem from normal and restraint stress rats were subjected to sodium dodecyl sulfate polyacylamide gel electrophoresis and the separated proteins were electroblotted onto nitrocellulose membranes. The membranes were immunostained with monoclonal antibody raised against the purified rat liver P450 2E1 (Oxford Biomedical Research, U.K.) and then incubated with goat anti-mouse IgG conjugated with HRP. The immunostained bands were visualized using HRP color development reagent (Bio-Rad Laboratories Inc, U.S.A.).

III. RESULTS

Immunoblot

1. Trigeminal ganglion
   A protein band with a molecular mass of approximately 18 KDa was observed in the same intensity in the normal group and the restraint- stress group at day 0, 1, 3, 5, 7 of the experiment. (Fig. 1)

2. Brain stem
   The intensity of the band was was not prominently different between the normal group and the restraint -stress group at day 0, 1, 3, 7 of the experiment. However, the band observed at day 5 was weaker than that observed during the rest of experimental period and that of normal group. (Fig. 2)
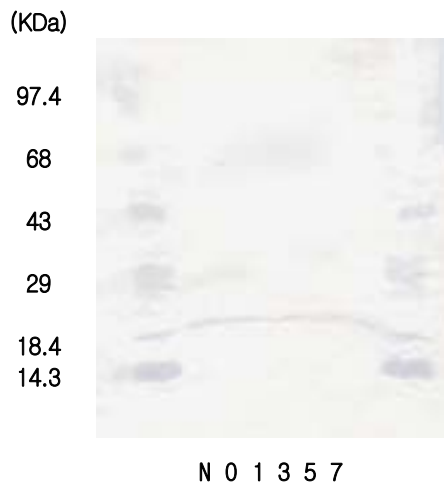
(KDa)

97.4

68

43

29

18.4
14.3

N 0 1 3 5 7

Fig. 1. Western blot of the trigeminal ganglion of the rat
About 18 KDa protein bands were equally observed in the normal group and the restraint- stress group at day 0, 1, 3, 5, 7 of the experiment

(KDa)

68

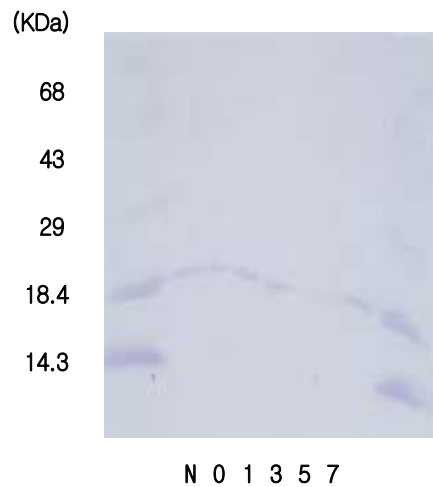43

29

18.4

14.3

N 0 1 3 5 7

Fig. 2. Western blot of the brain stem of the rat
The intensity of the band was was not prominently different between the normal group and the restraint-stress group at day 0, 1, 3, 7 of the experiment. However, the band observed at day 5 was weaker than that observed during the rest of experimental period and that of normal group.

IV. DISCUSSION

As adaptation to persistent social stress is a major requirement for successful living in both animal and human societies, the neural processes by which this occurs is a subject of great interest.[7] Repetition of the same stressor tends to produce some degree of habituation of the acute hypothalamus pituitary adrenal (HPA) response to the same stressor. Even as habitation or adaptation of the HPA is occuring during repetitive stress, there are adaptive changes in neurochemical and neuroanatomical systems that subserve the HPA axis and behavioral stress responses.[8]

The speed of recovery of the HPA axis after its activation by stressors is sensitive to the intensity of the stressors but not to their duration, and that adaptation to a repeated stressor is more apparent during the delayed HPA response.[9]

The neural response to stress begins with immediate early gene induction in neural sites associated with the stress response, and this is followed by other rapid changes such as induction

of corticotropin-releasing hormone (CRH) mRNA in paraventricular nucleus and tyrosine hydroxylase and its mRNA in locus coeruleus.[10] Psychological and/or physiological stress causes NO release in hypothalamic-pituitary-adrenal (HPA) axis and in sympatho-adrenal system.[11]

So far, evidence for the mind-body interaction paradigm has been collected with regard to the role of nerve fibres in lymphatic tissues, the effects of brain lesions on the immune system (IS), the interplay of neurotransmitters, hormones and immunotransmitters in a network of bidirectional feedback loops between the brain and the IS, the effects of ontogeny, learning and conditioning on the development of the IS, the impact of experimental and naturally occurring stressors on the IS, the possible immune modulating effects of personality characteristics, life style and psychodynamic processes and the role of the IS in disease.[12] HPA axis disturbances are clinically related to stress-

related disorders including fibromyalgia, although there is little direct information as to how the specific HPA axis perturbations can be related to the symptomatic manifestations of pain, fatigue, sleep disturbance, and psychological distress.[13] The responsiveness of the HPA axis to psychological stress is sufficient to alter disease progression.[14] Limbic-hypothalamic-pituitary-adrenal (LHPA) stress circuit is a complex system with multiple control mechanisms and that these mechanisms are altered in pathological states, such as chronic stress and depression.[15]

Stress can give rise to changes of the protein including neurotransmitters, hormones, metabolic substances or structural components of cells, adhesion molecules as well as mRNAs preceding their corresponding proteins.

Psychological and/or physiological stress causes NO release in hypothalamic-pituitary-adrenal (HPA) axis and in sympatho-adrenal system. And that NO may modulate a stress-induced activation of the HPA axis and the sympatho-adrenal medullary system.[16]

Stress stimulates the sympathoadrenal system, causing activation of the catecholamine biosynthetic enzymes. The changes of gene expression of tyrosine hydroxylase, the initial enzyme of catecholamine biosynthesis occured with stress.[17] Dunn et al. found that secretion of ACTH at least partially mediates the stress-induced changes of [^{3}H]-lysine incorporation into brain and liver proteins.[18]

Giaume et al. reported that cerebral protein synthesis was altered in response to acute and chronic immobilization stress in the rat and this stress-induced specific molecular changes in brain are also associated with changes in more general molecular components of cellular metabolism.[19] It has been shown that acute immobilization of rats resulted in a decrease of total protein content (per 1 cell) in neurones of the supraoptic nucleus of the hypothalamus and in their glial satellite cells.[20] Fujita reported that cold stress changed the lipid composition of cellular membranes, and suppressed the rate of protein synthesis and cell proliferation.[21]

It was found that the immune activation that accompanies acute psychologic stress might be sufficient to alter the expression of certain cellular adhesion molecules.[22]

The effects of acute physical and psychological stressful stimuli have been reported to increase proopiomelanocortin mRNA levels in the anterior and neurointermediate lobes of the pituitary gland.[23] It was reported that repeated stress in rats produced changes in IL-6 and IL-6R mRNAs in the midbrain and hypothalamus that are different than those of a single stress episode.[24]

In addition to the changes of proteins in central nervous system in response to stress, the expression of heat shock proteins in the trigeminal ganglion under thermal stress may occur[25], which is the change of protein in the peripheral nervous system related to stress.

Stress may also affect the enzymes including cytochrome P450. Restraint stress significantly alters several enzymatic systems differently at a basal level. In addition, stress was found to significantly interfere with the expression processes of cytochrome P450 2A1 and cytochrome P450 2A5.[26]

The reticular formation of brain stem plays an extremely important role in monitoring impulses that enter it. The reticular formation controls the overall activity of the brain by enhancing or inhibiting the impulses to the brain. This portion of the brain stem has an extremely important impact on pain and other sensory input. The pons also has centers for reflexes mediated by the fifth, sixth, seventh, and eighth cranial nerves. Two important midbrain structures are the red nucleus and substantia nigra. Each of these consists of clusters of cell bodies of neurons involved in muscular control.[4]

Cytochrome P450s are broadly classified into two types: one that metabolizes endogenous substrates; the other that metabolizes exogenous substrates (xenobiotics). Unusually high or low levels of a particular cytochrome P450s are associated with some disease states.[27] It has been reported about the role of brain cytochrome P450 in the synthesis

and degradation of neurosteroids.[28]

Therefore, we performed the study on the change of the protein of the trigeminal ganglion and the brain stem of rats on the basis of that cytochrome P450 is involved in the synthesis and degradation of the neurosteroid which may probably be related to nerve cell damage. In the immunoblot of the trigeminal ganglion, a protein band with a molecular mass of approximately 18 KDa was observed in the same intensity in the normal group and the restraint-stress group at day 0, 1, 3, 5, 7 of the experiment. In the immunoblot of the brain stem, the intensity of the band was not prominently different between the normal group and the restraint-stress group at day 0, 1, 3, 7 of the experiment. However, the band observed at day 5 was weaker than that observed during the rest of experimental period and that of normal group.

Because the CYP2E1 used in the study recognizes only one epitope on the protein, it might have interacted with cytochrome P450 in a negligible level but cross-reacted with an unknown protein. Whatever it may be, it is for sure that CYP2E1-cross reactive protein is weakly expressed at day 5 of the experiment in the brain stem.

The overall results suggest that stress may cause the change of a certain protein with an apparent molecular mass of 18 KDa in the brain stem and depending on the type of tissue, stress may induce cellular protein change.

V. CONCLUSIONS

Stress may cause alterations of protein of nerve cells which can lead to functional disturbances. The present study was carried out to examine the changes of protein expression of the trigeminal ganglion and the brain stem of rats under restraint stress to inquire the relationship between stress and pathologic changes of peripheral and central nervous system.

Eighteen Sprague-Dawley rats (8-weeks old, 323 -367 g/bw) were used for the experiment and the rats were divided into 2 groups: 1) Normal control group; 2) Restraint stress group : the rats were placed in the stress cage throughout the period of experiment. All the animals were then sacrificed at day 0, 1, 3, 5, and 7 day of the experiment and the trigeminal ganglions and the brain stems were excised immediately and stored at −70℃ until used. The results were as follows.

1. In the immunoblot of the trigeminal ganglion, a protein band with a molecular mass of approximately 18 Kd was observed in the same intensity in the normal group and the restraint-stress group at day 0, 1, 3, 5, 7 of the experiment.
2. In the immunoblot of the brain stem, the intensity of the band was not prominently different between the normal group and the restraint-stress group at day 0, 1, 3, 7 of the experiment. But, the band observed at day 5 was weaker than rest of the experimental period.

The overall results suggest that stress may cause change in the expression of a 18 KDa protein in the brain stem. The change seems to depend on types of tissue. Further studies are required to identify the 18 KDa protein.

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국문초록

구속스트레스에 의한 백서 신경세포의
생물학적 성상의 변화

경희대학교 치과대학 구강내과학 교실[1], 구강미생물학 교실[2]

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스트레스가 인체의 항상성에 영향을 미쳐 다양한 질병 및 동통을 일으킬 수 있다는 것은 선학들의 연구에 의해 증명되어 왔다. 특히 정서적으로 중요한 구강안면영역에는 스트레스와 관련된 질병 및 동통이 많이 존재하는데, 이에대한 병리적 기전은 아직 뚜렷하게 밝혀져 있지 않다. 이에 저자는 스트레스와 말초 및 중추신경과의 병리적 관계를 알아보고자, 구속스트레스하에서 구강안면영역의 감각과 운동에 중요한 역할을 하는 삼차신경의 집합체인 삼차신경절과 뇌간조직의 단백질 변화를 western blot을 통해 살펴보았다.

실험동물은 생후 8주된 Sprague-Dawley계 웅성 백서 (323-367 g/bw)를 대조군 3마리, 실험군 15마리로 배정하였고 실험군은 실험 전기간에 걸쳐 구속스트레스를 부여하였다. 실험동물의 삼차신경절과 뇌간은 희생 즉시 적출되었으며, western blot을 시행하여 다음과 같은 결론을 얻었다.

1. 삼차신경절의 모든 군에서 약 18 KDa의 단백질이 균일하게 나타났다.
2. 뇌간의 모든 군에서 약 18 KDa 단백질이 발현되었으며, 정상 대조군, 실험 즉일, 1일, 3일, 7일에서는 뚜렷한 변화가 없었으나 실험 5일군에서는 현저한 감소를 보였다.

구속스트레스에 의해 뇌간의 5일군에서 약 18 KDa의 특수한 단백질이 뚜렷이 감소된 후 7일 군에서 다시 증가되었던 것은 스트레스에 의한 뇌간세포의 반응으로 생각되며, 뇌간에서와 다르게 삼차신경절에서는 그 변화가 나타나지 않았던 것은 스트레스에 대한 조직의 반응 차이라고 생각된다. 따라서 이를 명확하게 확인하기 위해서는 향후 수종의 스트레스와 관련된 단백질 변화에 대한 추가적인 연구가 필요하리라 사료된다.