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Acute and chronic stress alter ecto-nucleotidase activities in synaptosomes from the rat hippocampus

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Abstract

Hyperactivity of the stress response has long been recognized as maladaptive. The hippocampus, a brain structure important in mediating this response, is known to be affected by chronic stress, a situation reported to induce changes in adenine nucleotide hydrolysis in the rat. The enzymes catalyzing the hydrolysis of ATP to adenosine in the synaptic cleft are thought to have a role in modulating and controlling synaptic transmission. This study aimed to investigate the effect of acute and repeated restraint stress on the ATP, ADP and AMP hydrolyses in rat hippocampal synaptosomes. Adult male Wistar rats were submitted to acute or repeated (15 and 40 days) stress, and ATPase–ADPase, and 5′nucleotidase activities were assayed in the hippocampal synaptosomal fraction. Acute stress induced increased hydrolyses of ATP (21%), ADP (21%) and AMP (40%). In contrast, ATP hydrolysis was increased by 20% in repeatedly stressed rats, without changes in the ADP or AMP hydrolysis. The same results were observed after 15 or 40 days of stress. Therefore, acute stress increases ATP diphosphohydrolase activity which, in association with 5′-nucleotidase, contributes to the elimination of ATP and provides extracellular adenosine. Interestingly, increased ecto-ATPase activity in response to chronic stress reveals an adaptation to this treatment.

Keywords: Stress; Chronic stress; ATPase–ADPase activities; 5′-Nucleotidase activity; Hippocampus

1. Introduction

Activation of the stress response is essential for vital functions (McEwen, 1998; Pacák and Palkovits, 2001); however, the deleterious effects of a constant hyperactivity of this response have long been recognized as maladaptive and pathological. Chronic stress may lead to impaired feedback mechanisms (Mizoguchi et al., 2001), cognitive impairments and disturbances of the activity of the limbic–hypothalamus–pituitary–adrenal (LHPA) axis (Sapolsky et al., 1986). Several studies have shown that chronic corticosterone administration may lead to a significant atrophy of CA3 neurons in the hippocampus, structural alterations and volume reduction of the hippocampal formation (Sapolsky et al., 1985; Uno et al., 1989; Woolley et al., 1990; Watanabe et al., 1992; Magariños et al., 1996, 1997; McEween, 1997) and an increased vulnerability to metabolic insults (Uno et al., 1989; Watanabe et al., 1992; Magarinos and McEwen, 1995). The hippocampus has been extensively studied following exposure to chronic stress, because it is one of the most important brain structures mediating the stress response. The high concentration of receptors for glucocorticoids in the hippocampus (McEwen et al., 1968, 1986) modulates glucocorticoid release through a negative feedback loop, exerting inhibitory effects on the LHPA axis (Sapolsky et al., 1986).

Some studies have reported that after the exposure to stressors, such as shear and electrical stimulation, changes occur in the concentrations of extracellular ATP (Kennedy et al., 1997) and adenosine (Latini and Pedata, 2001). Alterations in activities of enzymes involved in nucleotide hydrolysis have also been reported in the spinal cord and serum after repeated restraint stress (Torres et al., 2002a,b).

ATP is recognized as a neurotransmitter in the peripheral, as well as in the central nervous system (CNS; Edwards et al., 1992). In physiological situations, extracellular ATP

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exists at low concentrations; however, in pathological conditions, large quantities of extracellular ATP may cause cell death (Inoue, 2002).

The ATP released in the synaptic cleft can be hydrolyzed to adenosine, a neuroprotective and neuromodulatory agent. The enzymes that promote this conversion are proposed to have a role in modulating and controlling excitatory synaptic transmission (Cunha, 2001a; Cunha and Ribeiro, 2000). Adenosine has several functions within the CNS, including the inhibitory tone of neurotransmission and neuroprotective actions in pathological conditions (Latini and Pedata, 2001), and is particularly well suited to function as a transcellular messenger to signal metabolic imbalance. The neuroprotective actions of adenosine are attributed to the activation of presynaptic A1 receptors, which reduce neurotransmitter release and depress the neuronal activity in the CNS (Phillips and Wu, 1981).

ATP is hydrolyzed to adenosine by an extracellular chain of ecto-nucleotidases (Cunha et al., 1992; Sarkis and Salto, 1991). This chain of enzymes includes ecto-ATPases (NTPDase2, EC 3.6.1.3), ATP diphosphohydrolase (ecto-apyrase, NTPDase1, CD39, EC 3.6.1.5) and ecto-5́-nucleotidase (CD73, EC 3.1.3.5). While NTPDase1 hydrolyzes ATP and ADP equally well, NTPDase2 prefers ATP as a substrate (the ratio ATPase/ADPase for this enzyme is approximately 10). The enzyme 5′-nucleotidase is not able to promote the hydrolysis of ATP or ADP. This enzyme promotes the hydrolysis of nucleosides monophosphated, like AMP.

It has been recently demonstrated that ATP is hydrolyzed to adenosine by the conjugated action of an ATP diphosphohydrolase and a 5′-nucleotidase (Sarkis and Salto, 1991; Battastini et al., 1995), both in the central and peripheral nervous systems. The concerted action of ecto-nucleotidases controls the availability of ligands (ATP, ADP, AMP and adenosine) for both nucleotide and nucleoside receptors, and consequently, the duration and extent of receptor activation (Chen and Guidotti, 2001). Therefore, this ecto-nucleotidase pathway has a double function of removing one signal (ATP) and of generating a second one (adenosine). This cascade may play a role in the effective regulation of several processes, because it shows considerable plasticity in different pathophysiological situations (Agteresch et al., 1999; Cunha et al., 2001; Bonan et al., 2000). These enzymes may also have a protective function by keeping extracellular ATP/ADP and adenosine at physiological levels (Agteresch et al., 1999).

Because adenine nucleotides, as well as adenosine, are involved in the modulation of several physiological and pathological processes, and their levels may be altered by stress in some situations, we hypothesized that the enzymes involved in the degradation of ATP to adenosine may have their activities altered after chronic stress as an adaptation to protect the tissue against an excessive excitatory transmission induced by this treatment. Therefore, we investigated the effect of acute and repeated restraint stress on the ATP diphosphohydrolase and 5′-nucleotidase activities via ATP, ADP and AMP hydrolyses in hippocampal synaptosomes of adult male Wistar rats.

2. Materials and methods

2.1. Subjects

Adult male Wistar rats (60 days at the beginning of the treatment, weighing 200–230 g) were used. Experimentally naive animals were housed in groups of five in home cages made of Plexiglas material (65 × 25 × 15 cm) with the floor covered with sawdust and maintained on a standard dark–light cycle (lights on between 7 a.m. and 7 p.m.) at a room temperature of 22 ± 2 °C. The rats had free access to food (standard lab rat chow) and water, except during the period when restraint stress was applied. The restraint procedure was always performed between 10 and 12 a.m. All animal treatments were in accordance with the institutional guidelines and according to the recommendations of the International Council for Laboratory Animal Science (ICLAS) and all efforts were made to reduce the number of animals used in the experiment.

2.2. Acute stress procedure

The animals were divided into two groups: stressed and control. Restraint stress was applied by placing the animals in a 25 × 7 cm plastic bottle, and fixing it with plaster tape on the outside so that the animal was unable to move. There was a 1-cm hole at one far end for breathing. The animals were stressed for 1 h, 22 h before the assay. Control animals were kept in their home cages.

2.3. Chronic restraint stress procedure

The animals were divided into two groups: stressed and control. Restraint was applied using the same procedure as described above. The animals were stressed 1 h/day, 5 days a week for 15 or 40 days (Ely et al., 1997). Control animals were kept in their home cages.

2.4. Subcellular fractionation

Approximately 22 h after the last stress session (chronic stress) or the single session (acute stress), the animals were killed by decapitation and the hippocampi were rapidly removed and gently homogenized in 5 vol. of ice-cold medium, consisting of 320 mM sucrose, 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 5 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethane-sulphonic acid], pH 7.5, with a motor-driven Teflon-glass homogenizer. Synaptosomes from hippocampus were prepared as described previously (Nagy and Delgado-Escueta, 1984). Briefly, 0.5 ml of the crude mitochondrial fraction was mixed with 4.0

ml of an 8.5% Percoll solution and layered onto a Percoll/sucrose discontinuous gradient (10/16%). The synaptosomes that banded at the 10/16% Percoll interface were collected with wide tip disposable plastic transfer pipettes. Synaptosomal fractions were then washed twice at 12,000 × *g* for 20 min with the same ice-cold medium to remove the contaminating Percoll. The synaptosome pellet was then resuspended to a final protein concentration of approximately 0.5 mg/ml. The material was prepared fresh daily and maintained at 0–4 °C throughout preparation.

2.5. Enzyme assays

The reaction medium used to assay ATP and ADP hydrolyses in synaptosomal preparation was as previously described (Battastini et al., 1991) and contained 5.0 mM KCl, 1.5 mM $CaCl_2$, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose and 45 mM Tris–HCl buffer, pH 8.0, in a final volume of 200 μl. The reaction medium used to assay 5′-nucleotidase activity contained 10 mM $MgCl_2$, 100 mM Tris–HCl, pH 7.5 and 0.15 M sucrose in a final volume of 200 μl (Heymann et al., 1984).

The synaptosomal fraction (10–20 μg protein) was added to the reaction mixture, preincubated for 10 min and then incubated for 20 min at 37 °C. The reaction was initiated by the addition of ATP, ADP or AMP to a final concentration of 1.0 mM, and was stopped by the addition of 0.2 ml 10% trichloroacetic acid (TCA). The samples were chilled on ice for 10 min and 100-μl samples were taken for the assay of released inorganic phosphate (Pi). Pi released was determined as previously described by Chan et al. (1986).

The incubation times and protein concentration were chosen to ensure the linearity of the reactions (results not shown). The Chan method is largely used to evaluate the ATP diphosphohydrolase and 5′-nucleotidase activities and presents an adequate sensitivity to the purpose of our work (Bonan et al., 2000; Sarkis and Salto, 1991; Torres et al., 2001, 2002b); these enzyme activities are well established and characterized in synaptosomal preparations (Battastini et al., 1991, 1995). Controls with the addition of the enzyme preparation after addition of TCA were used to correct for nonenzymatic hydrolysis of substrates. All samples were assayed in triplicate. Enzyme activities are expressed as nanomoles of Pi released per minute per milligram of protein.

2.6. Protein determination

Protein was determined by the Coomassie Blue method, according to Bradford (1976), using bovine serum albumin as standard.

2.7. Statistical analysis

Data are expressed as mean ± standard deviation and analyzed by Student's *t* test.

3. Results

3.1. Effect of acute restraint stress on ATPase–ADPase and 5′-nucleotidase activities in rat hippocampal synaptosomes

ATPase and ADPase activities, as well as 5′-nucleotidase activity, in synaptosomes from hippocampus were increased 22 h after exposure to restraint stress (Student's *t* test, ATP [$t(8)=3.23$]; ADP [$t(8)=3.39$]; AMP [$t(8)=4.87$]; $P<.05$ in all cases; test for equality of variances, $P>.05$ in all cases), as compared to controls (Fig. 1). The ATPase/ADPase ratio was the same in both groups (mean ± S.D.: control, 2.64 ± 0.31; after acute stress, 2.64 ± 0.39).

3.2. Effect of chronic restraint stress on ATPase–ADPase and 5′-nucleotidase activities in rat hippocampal synaptosomes

Repeated restraint stress caused an increase in ATPase activity in synaptosomes from hippocampus of around 20% (in relation to controls), 22 h after the last exposure to stress [Student's *t* test, $t(4.07)=4.82$, $P<.01$, for 15 days of restraint and $t(11)=2.45$; $P<.05$, for 40 days of restraint; test for equality of variances, $P>.05$ for 40 days of treatment; $P<.05$ for 15 days of treatment; therefore, in this case, *t* test was performed for equal variances not assumed]. The same effect was observed after both 15 and 40 days of stress. On the other hand, no effect was observed on ADPase activity [Student's *t* test, $t(7)=0.29$ for 15 days of stress and $t(10)=0.73$ for 40 days; $P>.05$ in both cases]. In addition, 5′-nucleotidase activity was also not altered in synaptosomes from repeatedly stressed rats [Student's *t* test, $t(7)=1.25$ for 15 days of stress and $t(11)=0.30$ for 40 days; $P>.05$ in both cases; test for equality of variances, $P>.05$ in all these cases; Fig. 2]. The ATPase/ADPase ratio was significantly different in the repeated stressed animals

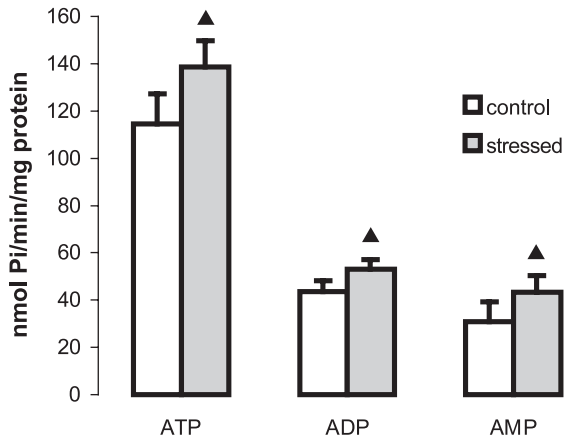

Fig. 1. Effect of acute stress on ATP and ADP hydrolyses, and on 5′-nucleotidase activity in synaptosomes from rat hippocampus measured 22 h after stress exposure ($n=4–5$ animals/group). Values are mean ± standard deviation of specific activity (nmol of phosphate production/min/mg protein). ▲: Different from control group (Student's *t* test, $P<.02$).

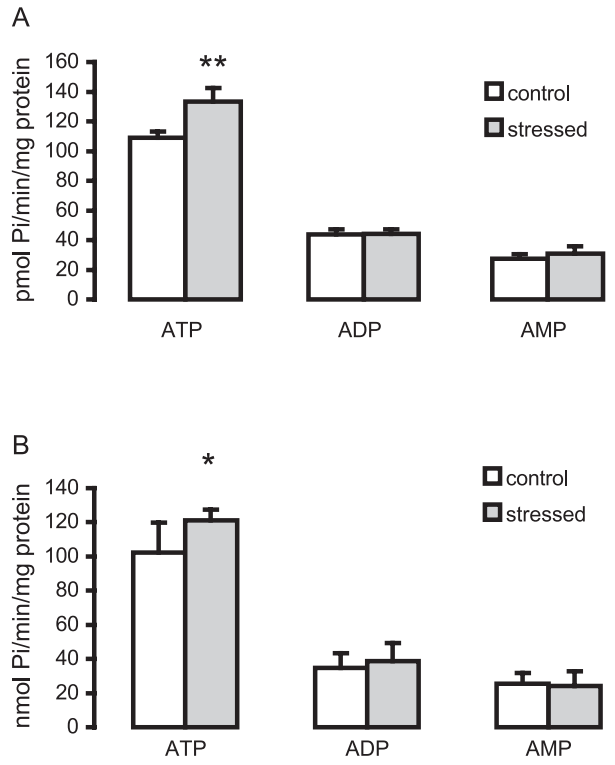

Fig. 2. Effect of 15 days (A) or 40 days (B) of repeated stress on ATP and ADP hydrolyses, and on 5′-nucleotidase activity in synaptosomes from rat hippocampus ($n=4-6$ animals/group). Values are mean ± standard deviation of specific activity (nmol of phosphate production/min/mg protein). * Different from control group (Student's t test, $P<.05$). * * Different from control group (Student's t test, $P<.01$).

compared to controls: 2.50 ± 0.13 (mean ± S.D.) for the control group, and 3.01 ± 0.17 after 15 days of repeated stress [Student's t test, $t(7)=5.04$; $P<.005$]. After 40 days of treatment, although the stressed group presented a higher ratio, there was no significant difference compared to controls ($P>.05$).

4. Discussion

In the present study, we observed changes in adenine nucleotide hydrolysis after the induction of acute and repeated stress. Additionally, the modifications observed in enzyme activities were distinct in relation to acute and chronic stress, suggesting the involvement of different enzymes in both situations studied. Animals submitted to acute stress showed a significant increase in ATP, ADP and AMP hydrolyses when compared to their respective controls. Because ADP is considered to be a substrate marker for ATP diphosphohydrolase activity (Battastini et al., 1991), this result suggests increased activity of this enzyme after exposure to acute stress. Moreover, the activation observed in ATP (21%) and ADP (21%) hydrolyses, was very similar, and the parallelism in kinetic behavior (similar profile for both substrates) is also characteristic of ATP diphosphohydrolases described in the literature (Sarkis and Salto, 1991; Frassetto et al., 1993).

AMP hydrolysis was also significantly increased after acute stress, indicating an increase in the 5′-nucleotidase activity and probably in adenosine levels resulting from the extracellular ATP hydrolysis cascade. It has been proposed that the reaction catalyzed by 5′-nucleotidase is the rate-limiting step in this extracellular pathway from ATP to adenosine (for a review, see Cunha and Ribeiro, 2000). It should be noted that this enzyme is inhibited by ATP and/or ADP (Cunha and Sebastião, 1991). Thus, only when ATP and ADP levels decrease below the threshold of inhibition of 5′-nucleotidase will adenosine be formed in a significant amount. Because both ATPase and ADPase activities are increased in the case of acutely stressed rats, these nucleotides will be rapidly converted to AMP. Therefore, ATP and ADP levels will decrease and AMP, the substrate to 5′-nucleotidase, will be increased, as well as this enzyme activity. These results could suggest that, after a single exposure to restraint stress, a long-term (at least 22 h) effect on enzymes involved in extracellular ATP breakdown will result in increased extracellular adenosine in the hippocampus.

Several reports have documented an increase in the extracellular concentration of adenosine upon stressful metabolic challenges, including hypoxia and ischemia (Latini and Pedata, 2001; Pearson et al., 2001), as well as glucoprivation by 2-deoxy-D-glucose administration (Minor et al., 2001). The term retaliatory metabolite has been used for this homeostatic role of adenosine, which occurs in virtually all cell types (for a review, see Cunha, 2001b). Although adenosine may act on A_2 receptors causing increased neurotransmitter release in hippocampus (Cunha et al., 1992), most reports of adenosine action in this structure have shown inhibition of hippocampal neurons. A_1 agonists appear to act presynaptically, inhibiting the release of neurotransmitters or postsynaptically reducing neuronal excitability (Hass and Greene, 1988; Lamber and Teyler, 1991). Because the hippocampus is well documented as being particularly sensitive to cellular injury (Inoue, 1998), this result suggests the presence of a physiological response to protect this structure from damage caused by exposure to stressors.

In contrast to the effects observed after acute stress, repeatedly stressed rats presented a significant difference only in ATP hydrolysis, with no significant changes in ADPase or 5′-nucleotidase activities in synaptosomes from hippocampus. This pattern of activity for these enzymes was already observed after 15 days of repeated restraint, and was still present after 40 days of stress.

The apparent dissociation observed between the two substrates (ATP and ADP) in this case, may be due to the simultaneous presence of at least two different ectoenzymes involved in ATP hydrolysis, an ecto-ATP diphosphohydrolase (NTPDase3) and an ecto-ATPase (NTPDase2; Kegel et al., 1997; Zimmermann, 1996). Conversely, only one

enzyme, an ecto-ATP diphosphohydrolase (NTPDase3), is involved in ADP hydrolysis. When we have similar changes in ATP and ADP hydrolyses, it is tentative to think in the enzyme ATP diphosphohydrolase, but when we have changes only in ATP hydrolysis, the more probable enzyme involved is an ecto-ATPase. Therefore, the increase observed in ATP hydrolysis, and not in ADP hydrolysis, suggests the participation of an ecto-ATPase in modulation of the response to chronic stress. The presence of different populations of nucleotidases is also supported by the increased ATPase/ADPase ratio observed in synaptosomes from chronically stressed rats. The presence of an ecto-ATPase was demonstrated in the CNS, where it is coexpressed with the ATP diphosphohydrolase (Kegel et al., 1997). These enzymes have been identified in molecular terms and differ in their preference for their substrates (Zimmermann, 1996).

The increase of ATP hydrolysis in repeated stress may constitute a protective mechanism, due to the potential neurotoxic effect of this nucleotide (Schulze-Lohoff et al., 1998). The activation of ATP hydrolysis may be a protective mechanism, because ATP acts as an excitatory neurotransmitter, and a number of neurodegenerative events are associated with increased excitatory neurotransmission (Danbolt, 2001). Hence, the activation of the ecto-ATPase could be an adaptive response of the chronically stressed rats. The mechanisms through which repeated stress may modulate ecto-ATPase activity still remain unclear. However, because this is a chronic situation, it is possible that this mechanism involves an increase in expression of mRNA for this enzyme.

The differences here observed in enzyme activities after acute and chronic stress suggest an adaptation. Chronically stressed animals do not experience all the hormonal consequences that animals exposed to one single-stress episode do (Hashiguchi et al., 1997; Torres et al., 2001), and this phenomenon of adaptation to chronic stress may be reflected in several biochemical and physiological processes. Glucocorticoid levels are also different after acute and chronic stress: the increase in corticosterone is smaller when the animals are submitted to repeated stress, as compared to the increase observed after acute stress (Torres et al., 2001).

Glucocorticoids might be involved in the changes of activities of adenine nucleotide hydrolysis observed in the present study. These hormones have already been reported to alter the activity of other enzymes involved in the hydrolysis of ATP. High levels of glucocorticoids or exposure to acute stress have been reported to increase Na^+, K^+-ATPase activity in brain and other tissues (Shaheen et al., 1996; Rodrigo et al., 2002), while decreasing Ca^{2+}-ATPase activity (Bhargava et al., 2000, 2002), although other nonglucocorticoid-dependent factors appear also to be involved in the response of these enzymes to stress (Bhargava et al., 2000). The enzymes cited above are involved in the intracelular degradation of ATP, and, therefore, in the energetic demands of the cell. The enzymes studied herein, on the other hand, are ectoenzymes and are involved in the modulation of neurotransmission by controlling adenine nucleotides/nucleoside.

Other mediators of the stress response could possibly be involved in the effects observed herein. Several data provide evidence that the transcription factor, cAMP response element-binding protein (CREB), and the neurotrophin, brain-derived neurotrophic factor (BDNF) levels, are changed in response to stress (D'Sa and Duman, 2002; Rossant et al., 1999; Hatalski and Baram, 1997; Duman et al., 2000), and suggest that chronic stress may be associated with a disruption of mechanisms that govern cell survival and neural plasticity in the brain (Duman et al., 2000). For example, CREB binding is critical for basal expression of 5′-nucleotidase; however, it is not presently known how tightly this enzyme expression is regulated (Synnestvedt et al., 2002). Conversely, adenosine protective effects are suggested to be mediated by protein kinase C, and protein kinase A activation by means of cAMP and activation of CREB (Lee and Emala, 2002). In addition, extracellular ATP co-operates with growth factors in different tissues (Wagstaff et al., 2000; Huwiler et al., 2000). Therefore, altered extracellular levels of ATP and adenosine may produce changes in multiple signalling pathways.

In previous studies, using synaptosomes from other CNS structures, no effect was observed after repeated stress, either upon ATP or ADP hydrolysis in hypothalamus and cerebral cortex (Torres et al., 2002b); however, a decrease in ADPase activity was observed in the spinal cord (Torres et al., 2002a). Therefore, the increased ATPase activity after repeated stress, observed in this study in synaptosomes from hippocampus, is structure specific. Such alterations that can be observed in particular tissues or structures may reflect the different functions of the nucleotides/nucleosides and enzymes in different regions of the CNS.

In conclusion, the results reported herein demonstrate a significant increase in ATP, ADP and AMP hydrolyses in response to acute stress, suggesting the participation of an ATP diphosphohydrolase which, in association with 5′-nucleotidase, contributes to the elimination of ATP and also provides a source of extracellular adenosine as a neuroprotective agent. On the other hand, repeated stress elicits an increase in ecto-ATPase activity alone, possibly representing a protective adaptation of this treatment. Furthermore, the differences observed in altered ecto-nucleotidase activities in response to both kinds of stresses studied may be used as biochemical tools to characterize chronic and acute stress in hippocampal synaptosomes.

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