Suppression of sodium pump activity and an increase in the intracellular Ca\(^{2+}\) concentration by dexamethasone in acidotic mouse brain

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Abstract

The effects of dexamethasone on adenosine 5'-triphosphatase (ATPase) activity and the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) were investigated in acidotic mouse brain. Dexamethasone (3 mg / kg, i.p.) or vehicle was administered 3 h before decapitation ischemia, and the brain concentration of adenosine 5'-triphosphate (ATP) was determined 0.5–2 min after ischemia. The effects of dexamethasone (0.3–3 mg / kg, i.p.) on Na\(^+\),K\(^+\)-activated ATPase (Na\(^+\),K\(^+\)-ATPase) and Ca\(^{2+}\)-ATPase activities were evaluated at pH 7.4 and 6.8. Changes in [Ca\(^{2+}\)]\(_i\) in an acidic medium were determined in hippocampal slices by microfluorometry using rhod-2 acetoxymethyl ester as a Ca\(^{2+}\) marker, and the effects of dexamethasone (240 \(\mu\)g/l) was evaluated. Decapitation ischemia for 0.5 and 1 min reduced the brain ATP contents to 32% and 16% of the basal level, respectively. Dexamethasone slightly suppressed the extent of the decrease in the ATP level. Although dexamethasone did not affect Na\(^+\),K\(^+\)-ATPase activity at pH 7.4, the activity was suppressed by dexamethasone (3 mg / kg) to 68% at pH 6.8. The activity of Ca\(^{2+}\)-ATPase was not affected by dexamethasone at either pH 7.4 or pH 6.8. When the pH of the medium of the brain slices was changed from 7.4 to 6.8, almost no increase in [Ca\(^{2+}\)]\(_i\) was observed in the control group. The dexamethasone treatment increased [Ca\(^{2+}\)]\(_i\) in the CA1 field and dentate gyrus immediately after induction of the acidic medium, the effect being significant after 150 s. Because anaerobic glucose metabolism in the early stage of ischemia enhances intracellular lactic acidosis, the findings may suggest a mechanism for the aggravation of ischemic neuronal damage by glucocorticoids.

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1. Introduction

Glucocorticoids are adrenal steroid hormones released in response to stress such as cardiac arrest and cerebral ischemia [12,25]. However, the increase in the plasma concentration of endogenous glucocorticoids may aggra-
level by the ion transport. When the adenosine 5′-triphosphate (ATP) preservation decrease to 13–18% in cerebral ischemia, the membrane depolarizes, and an increase in [Ca^{2+}], occurs, which plays an important role in mediating neuronal death [9,19,35]. In the present study, we examined the effects of dexamethasone, which stimulates glucocorticoid receptors, on energy depletion in ischemia, ion-pump related enzyme activity, and [Ca^{2+}]. Further, since the brain changes to an acidic state after the initiation of cerebral ischemia due to facilitated anaerobic metabolism, the effect of dexamethasone on these changes in the acidic state was also evaluated.

### 2. Materials and methods

#### 2.1. Animals

This study was approved by the Committee on Animal Experimentation at Ehime University School of Medicine, Ehime, Japan. All animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by Ehime University School of Medicine. Male ddY mice were housed in groups in a room controlled at 23±1 °C and maintained in an alternating 12-h light/12-h dark cycle (lights on at 06:00 h). In Expt. 1, the effect of dexamethasone on energy metabolism was investigated by measuring the brain concentrations of adenylates. In Expt. 2, the activities of Na⁺,K⁺-ATPase and Ca^{2+}-ATPase were determined, and the effects of dexamethasone were evaluated. In Expt. 3, the effects of the agent on changes in the [Ca^{2+}], were examined.

#### 2.2. Experiment 1: brain concentrations of adenylates

Forty-eight mice were prepared and assigned to four control groups and four dexamethasone groups (six animals in each) to determine the brain concentrations of ATP, adenosine 5′-diphosphate (ADP), and adenosine 5′-monophosphate (AMP) after various durations of decapitation ischemia. The control group animals were injected with 0.5% Tween-80 (vehicle) and the dexamethasone group were injected with dexamethasone (3 mg/kg, i.p.) emulsified in Tween-80. The animal was anesthetized with 2% halothane under balanced 50% O₂ and 50% N₂O. Anesthesia was maintained under spontaneous ventilation, and the rectal temperature was kept at 37.5 °C. Three hours after drug administration, the animal was decapitated and the head was frozen in liquid nitrogen after 0, 0.5, 1, or 2 min. During decapitation ischemia, the temperature of the temporal muscle was maintained at 37.5 °C with a heating lamp.

The frozen brain was removed from the skull on dry ice, weighed, and quickly homogenized in 4 ml of ice-cold 0.4 M perchloric acid. After centrifugation at 18 000×g for 30 min, the supernatant was injected into a high-performance liquid chromatography (HPLC) system to determine the brain concentrations of ATP, ADP, and AMP. The HPLC system consisted of a pump (L-7100; Hitachi, Tokyo, Japan) used to deliver the mobile-phase, a model L-7250 sample injector (Hitachi) with a 100-μl sampling loop, two separation columns (GL-W510-S, 7.8×300 mm inside diameter; Hitachi), and an ultraviolet (UV) detector (L-7400; Hitachi). The mobile phase was 0.2 M NaH₂PO₄, with a pH of 3.5 adjusted with 0.2 M H₃PO₄, and the flow rate was 0.6 ml/min. The absorption intensity (peak height) was measured at a wavelength of 270 nm.

#### 2.3. Experiment 2: ATPase activity

Twenty-four animals were assigned to the control and three dexamethasone groups to evaluate the effect of dexamethasone on Na⁺,K⁺-ATPase activity (n=6 in each). Each animal was injected with Tween-80 or dexamethasone (0.3, 1, or 3 mg/kg, i.p.). Three hours after the administration, the animal was decapitated. The brains were rapidly dissected and quickly homogenized in 5 ml of ice-cold 0.32 M sucrose containing 1 mM ethylenediaminetetraacetic acid and 100 mM Tris–HCl (pH 7.4) using a Teflon–glass homogenizer. The homogenate was centrifuged first at 900×g for 10 min followed by centrifugation at 12 000×g for 20 min. The supernatant has been reported to contain microsomal membrane particles and a soluble cytosol fraction [31]. Fifty microliters of the supernatant was added to each of two test tubes containing 900 μl of either 120 mM NaCl, 5 mM KCl, 5 mM MgCl₂, 0.5 mM ouabain, 100 mM Tris–HCl (pH 7.4) or the same medium without ouabain. Because ouabain inhibits Na⁺,K⁺-ATPase activity, the difference in the enzyme activity represents Na⁺,K⁺-ATPase activity.

The medium was preincubated at 37 °C for 10 min, and the reaction was started by adding 50 μl of 50 mM ATP in buffer to each tube to give a final ATP concentration of 2.5 mM. After 10 min, the reaction was stopped by the addition of 50 μl 60% perchloric acid and stabilized for 15 min. Then, the pH was increased to 8.5 with 70 μl 8 M NaOH to prevent the spontaneous degeneration of ATP in acid. The suspensions were centrifuged at 18 000×g for 20 min. The supernatant was injected into the HPLC system to determine the amounts of ADP and AMP according to the method described above. The remaining precipitate was used for protein assay using bovine serum albumin as the standard [26]. The activity of Na⁺,K⁺-ATPase was determined as the difference in the enzyme activity measured in the absence or the presence of ouabain, and expressed as the produced ADP and AMP per hour per mg of protein. To evaluate the effect of dexamethasone on the Na⁺,K⁺-ATPase activity in an acidic buffer, an identical procedure was performed using an incubation medium adjusted to a pH of 6.8.

Another set of 24 animals was assigned to the control
and dexamethasone (0.3, 1, or 3 mg/kg, i.p.) groups to evaluate the effect of dexamethasone on Ca\(^{2+}\)-ATPase activity \((n=6\) in each). Ca\(^{2+}\)-ATPase activity was determined as the difference in the enzyme activity measured in the absence or the presence of Ca\(^{2+}\). The brains were homogenized in 5 ml of ice-cold 0.32 M sucrose containing 100 mM Tris–HCl (pH 7.4). The homogenate was centrifuged, and the supernatant was reacted in each of two test tubes containing either 120 mM NaCl, 5 mM KCl, 5 mM MgCl\(_2\), 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), and 100 mM Tris–HCl (pH 7.4) or the same medium with 5 mM CaCl\(_2\).

To evaluate the effect of dexamethasone on the Ca\(^{2+}\)-ATPase activity in an acidic buffer, an identical procedure was performed using an incubation medium adjusted to a pH of 6.8.

### 2.4. Experiment 3: intracellular Ca\(^{2+}\) concentration

Mice were anesthetized with halothane and decapitated. The brains were rapidly removed and placed in an ice-cold physiological medium containing 124 mM NaCl, 5 mM KCl, 2 mM CaCl\(_2\), 2 mM MgCl\(_2\), 1.25 mM NaH\(_2\)PO\(_4\), 26 mM NaHCO\(_3\), and 10 mM glucose (pH 7.4). Hippocampal transverse slices approximately 300 µm thick were cut with a vibrating slicer (DTK-1000; Dosaka, Kyoto, Japan); 2–3 slices were obtained from each hippocampus. The slices were incubated in physiological medium equilibrated with a 95% O\(_2\), 5% CO\(_2\) gas mixture for 60 min at 26°C. The slices were preloaded with a fluorescent indicator, rhod-2 acetoxymethyl ester (Dojin, Kumamoto, Japan), which was diluted to 20 µM in the physiological medium and equilibrated with a 95% O\(_2\), 5% CO\(_2\) gas mixture for 45 min at 26°C. After the loading incubation, the slices in the dexamethasone group were incubated in physiological medium containing 240 µg/l water-soluble dexamethasone (Sigma) for 60 min at 26°C. The control slices were further incubated in the physiological medium for 60 min.

The [Ca\(^{2+}\)]\(_i\) levels were measured using an inverted fluorescence microscope, a high-performance digital charged coupled device (CCD) camera, and an image-processor setup. A low-magnification objective lens \((×4)\) and an illumination system were used to visualize the fluorescent image of the slice. The slice was transferred to a flow-through chamber \((volume \sim 0.2\ ml)\) mounted on the fluorescence microscope (IMT2; Olympus, Tokyo, Japan) and perfused at 3 ml/min with the appropriate medium at 36.5°C. The temperature of the medium in the chamber was monitored using a thermocouple needle probe \((0.4\ mm\ diameter;\ TN-800;\ Unique\ Medical,\ Tokyo,\ Japan)\) and a thermocouple meter \((TME-300;\ Unique\ Medical).\) The slice was excited with 550-nm light produced by a UV lamp \((L5697;\ Hamamatsu\ Photonics,\ Hamamatsu,\ Japan)\), filtered by an interference filter \((550\ nm,\ band\ width<16\ nm)\). The fluorescence signals \((>580\ nm)\) were captured on a digital CCD camera \((C4742-95;\ Hamamatsu\ Photonics)\) and processed using an image processor \((Aquacosmos;\ Hamamatsu\ Photonics).\)

Before the measurement of [Ca\(^{2+}\)]\(_i\), the slice loaded with rhod-2 was excited with 550-nm light, and the picture was examined to confirm that the dye was uniformly distributed throughout the slice. After placement of the slice into the chamber, the slice was perfused with a physiological medium \((pH 7.4)\) equilibrated with a 95% O\(_2\)/5% CO\(_2\) gas mixture for 15 min, and the basal level of the [Ca\(^{2+}\)]\(_i\) was measured. Then, the medium was changed to an acidic medium, with a pH of 6.8 adjusted with HCl, equilibrated with a 95% O\(_2)/5% CO\(_2\) gas mixture. The fluorescence intensity in each pixel was divided by the fluorescence intensity of the corresponding basal pixel that had been taken in the basal physiologic medium. Thus, the ratio of fluorescence intensity was obtained every 30 s.

### 2.5. Statistical analysis

The biochemical data were analyzed by analysis of variance with Bonferroni tests. The data from microfluorometry were analyzed by repeated measures analysis of variance to detect differences among groups. When differences were found, the Scheffe’s test was used post hoc to compare each value with that in the control group. A P value of 0.05 was considered significant.

### 3. Results

#### 3.1. Brain concentrations of adenylates

There were no differences between the control and dexamethasone groups in the value of ATP, when the brains were frozen immediately after decapitation \((Fig.\ 1A)\). Decapitation ischemia for 0.5 and 1 min produced marked decreases in the brain ATP contents, the values in the control group being 32% and 16% of those in brains frozen immediately after decapitation, respectively. In the dexamethasone group, the extent of the decrease was slightly suppressed, and the values were 38% and 20% of those of the basal level, respectively. Although 2 min of ischemia further decreased the ATP content in both groups, no difference was found between the two groups in the ATP value.

The brain ADP content decreased after decapitation ischemia in a manner similar to that of the ATP level \((Fig.\ 1B)\). The extent of the decrease was smaller than that of the ATP level. Dexamethasone also suppressed the extent of the decrease, the effect being significant after 1 min. In contrast, the brain AMP content increased after ischemia, and there were no differences between corresponding control and dexamethasone groups \((Fig.\ 1C)\).
Fig. 1. Effects of dexamethasone (3 mg/kg, i.p.) on ischemic changes in brain concentrations of ATP (A), ADP (B), and AMP (C). Each value represents the mean±S.E.M. from six animals. *P<0.05, **P<0.01 compared with each corresponding control group. Control (C) and dexamethasone (D) groups.

3.2. ATPase activity

The activities of Na⁺,K⁺-ATPase at pH 7.4 were 3.1, 3.1, 3.2, and 3.3 μmol ADP+AMP/h per mg protein in the control and dexamethasone (0.3, 1, 3 mg/kg, i.p.) groups, respectively, and there were no differences among the four groups (Fig. 2A). When the pH of the medium was 6.8, the activity in the control group was suppressed to 71% of that at pH 7.4. The activity of Na⁺,K⁺-ATPase in the dexamethasone groups were suppressed as the dose of dexamethasone increased, and the values were 95%, 86%, and 68% of that in the corresponding control group.

The activity of Ca²⁺-ATPase at pH 7.4 was 2.6, 2.4, 2.4, and 2.1 μmol ADP+AMP/h per mg protein in the control and dexamethasone (0.3, 1, 3 mg/kg, i.p.) groups, respectively (Fig. 2B). The dexamethasone treatments did not affect the Ca²⁺-ATPase activity at any doses. When the pH of the medium was 6.8, the activity was decreased in all groups. Dissimilar to Na⁺,K⁺-ATPase, there were no differences in Ca²⁺-ATPase activity among the groups.

3.3. Intracellular Ca²⁺ concentration

When the medium of the brain slices was changed to acidic medium, almost no increase in [Ca²⁺]ᵢ was observed in the control group within 150 s (Fig. 3). Thereafter, a gradual increase in [Ca²⁺]ᵢ was shown in the hippocampal CA1 field and dentate gyrus, the ratio of fluorescence intensity attained 108% and 110% after 1200 s, respectively. On the other hand, the dexamethasone treatment began to increase the ratio of [Ca²⁺]ᵢ in the CA1 field immediately after induction of the acidic medium, and significant differences were found after 150 s between the two groups (Fig. 4). Similar to the increase in the ratio in the CA1 field, the ratio in the dentate gyrus was...
increased to a greater extent in the dexamethasone group than in the control group, the values being significant after 150 s.

4. Discussion

Dexamethasone decreased the consumption of ATP at the early stage of ischemia. The agent also suppressed the activity of Na⁺,K⁺-ATPase and increased [Ca²⁺], in an acidic medium at pH 6.8. A control hippocampal slice (A), and a dexamethasone-treated slice (B) 10 min after changing the pH of the medium from 7.4 to 6.8. Open rectangles represent measured areas.

been shown to delay the initiation of ischemic depolarization of the neuronal membrane and suppress the consumption of ATP in the brain [2,5,13,21,23,30,32], whereas agents that aggravate ischemic neuronal injury facilitate the ATP consumption [3]. Dexamethasone suppressed the depletion of ATP during decapitation ischemia in the present and our previous studies [4]. However, the magnitude of the suppression seems to be too small to show
functional and morphological consequences. Dissimilar to our results, dexamethasone has been reported to enhance the consumption of ATP in cultured astrocytes when they are exposed to hypoxia and glucose deprivation, or metabolic insults [20,33]. Considering that the decrease in ATP depletion was observed in whole brain homogenates in our study, the decrease in ATP depletion may occur primarily in other cells such as neurons. Taken together with these findings, the damaging properties of glucocorticoids, which have been reported in various models of cerebral ischemia [1,18,27,28,34,36], may not be explained by the difference in energy consumption.

Cerebral ischemia decreases the intracellular pH in the brain. The decrease is speculated to be a result of lactic acidosis associated with enhanced anaerobic glucose metabolism. In this study, dexamethasone suppressed Na$^+$.K$^+$-ATPase activity in an acidic medium. When the intracellular Na$^+$ concentration is increased by suppressing Na$^+$.K$^+$-ATPase activity, the Na$^+$-Ca$^{2+}$ exchange carrier reverses its resting mode of operation, which leads to an increase in [Ca$^{2+}$]. This is in good agreement with the result of microfluorometry that the dexamethasone treatment increased [Ca$^{2+}$], in an acidic medium. In our previous study on brain slices, dexamethasone facilitated the onset of an increase in [Ca$^{2+}$], produced by a hypoxic and glucose-free condition [1]. The increase in [Ca$^{2+}$], by dexamethasone in an acidic condition may contribute to the facilitation of the abrupt increase in [Ca$^{2+}$], in ischemia [10,11]. In an animal model of cardiac arrest, the extracellular pH has been shown to be 6.8 after 9 min of ischemia [37]. Since the development of acidosis is faster in the intracellular space than in the extracellular space, glucocorticoids may aggravate ischemic neuronal damage in animals subjected to even a short duration of ischemia.

Two mechanisms are conceivable for the removal of cytosolic Ca$^{2+}$ in addition to the Na$^+$–Ca$^{2+}$ exchange system. One is the transportation of Ca$^{2+}$ to the extracellular space by Ca$^{2+}$-ATPase on the cytoplasmic membrane, and the other involves the uptake of Ca$^{2+}$ into the intracellular Ca$^{2+}$ stores such as the endoplasmic reticulum. The membrane fraction that we obtained in the present study has been shown to contain both cytoplasmic and endoplasmic membrane fractions [31]. Therefore, individual changes in the Ca$^{2+}$-ATPase activity could not be evaluated in the present experimental procedure. However, since both of them concern the removal of Ca$^{2+}$ from the cytosol, dexamethasone does not seem to affect the total amount of Ca$^{2+}$ transported by the Ca$^{2+}$ pump. Thus, the reduced sodium pump activity in the acidic state may be a predominant mechanism for the facilitation of ischemic increase in [Ca$^{2+}$], in acidosis. Dexamethasone has been shown to suppress mRNA levels of the plasma membrane Ca$^{2+}$ pump in cultured astrocytes [7]. This effect of dexamethasone is not in agreement with our present finding on Ca$^{2+}$-ATPase. Because the agent was administered 3 h before measurements in our study, the genomic effect by modifying nucleic acids through steroid receptors in the nucleus may not be relevant. Likewise, suppression of Na$^+$.K$^+$-ATPase activity by dexamethasone may be due to non-genomic effects.

Neurons in the CA1 field are the most sensitive to degeneration due to ischemia, whereas neurons in the dentate gyrus are relatively resistant. In this study, an increase in [Ca$^{2+}$], by dexamethasone in the acidic medium was observed in both the CA1 field and dentate gyrus. The lack of correlation between an increase in [Ca$^{2+}$], and the vulnerability may be explained by the following: the level of [Ca$^{2+}$], elevation in the dentate gyrus is not sufficient to provoke enzymatic process to irreversible neuronal injury, or the Ca$^{2+}$-induced enzymatic process in the dentate gyrus may be triggered in a manner different to that in the CA1 field.

Although the molecular mechanisms of dexamethasone for these actions were not clarified in this study, a chemical structure similar to that of digitalis might be conceivable. Digitalis glycosides have the chemical structure of a cyclopentanoperhydrophenanthrene nucleus, which has been shown to play a role in positive inotropic action by inhibiting Na$^+$.K$^+$-ATPase activity in the myocardium [15]. A chemical structure of glucocorticoids similar to that of digitalis might suppress Na$^+$.K$^+$-ATPase activity in acidic conditions. In conclusion, the facilitation of the increase in [Ca$^{2+}$], with the suppression of sodium pump activity may be contributing factors in the aggravation of ischemic neuronal damage by glucocorticoids.

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