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Acid-induced changes of brain protein buffering

Richard P. Kraig¹ and Robert J. Wagner²

¹ Department of Neurology, Cornell University Medical College, New York, NY 10021 (U.S.A.)

² Albany Medical College, Albany, NY 12208 (U.S.A.)

Abstract

Excessive cellular acidosis is thought to enhance destruction of brain from ischemia. Protein denaturation may contribute to such injury although the behavior of brain proteins to acidosis is poorly defined. As a first approach to detect acid-induced changes in brain proteins and to characterize buffer content, homogenates were acidified for 20 min (as low as pH 3.1), returned to baseline pH (6.9), and then titrated. Titration curves show a significant ($P < 0.0001$) and permanent increase in buffer content compared to controls when pH of acid exposure was 4.5–3.7 or less. Since acidity of pH 4.5 is rarely, if ever, achieved in vivo, protein denaturation from acidity alone is unlikely to account for necrosis of brain from ischemia.

Keywords

Protein; Acid-base homeostasis; Ischemia; Brain infarction; Buffer capacity; Acidosis

Acid–base homeostasis of brain is a complex phenomenon which, exclusive of blood-related changes, consists of 3 dynamic and interactive processes: (1) metabolic production and consumption of acids and bases; (2) plasma membrane transport of proton equivalents; and (3) physicochemical buffering of hydrogen ions (H^+)¹⁸. Physicochemical H^+ -buffers consist of bicarbonate (HCO_3^-) and other partially ionized substances that are capable of reversibly combining with excess H^+ . In brain HCO_3^- is the principal H^+ -buffer of the interstitial space while within cells HCO_3^- and histidine-imidazole moieties of proteins are the predominant species which mitigate changes in cellular H^+ concentration¹⁴. Some investigators suggest that the concentration of protein-based H^+ -buffers does not change in biological systems, and thus can be regarded as a constant^{20,21}. Others take a more general view and suggest protein-based H^+ -buffers should be regarded as a variable^{5,7,9} because their precise behavior is poorly understood. The results reported here support this latter approach.

The functional status of proteins depends strongly on their 3 dimensional structure. Physical variables which determine protein structure are temperature, ionic strength, and pH^{3,24}. These variables influence the charge state of proteins. Since histidine-imidazole moieties are the principal charged species of proteins, it follows that protein structure should depend on the degree to which such H^+ -buffers are titrated. This latter concept, termed ‘The α -stat imidazole hypothesis’, states that to survive, cells and tissues strive to keep the charge state of their proteins at some optimum¹⁷. Therefore, under physiologic conditions H^+ -buffer content of cells may be essentially constant.

During pathological conditions such as severe cellular acidosis associated with brain infarction^{8,10,12,13} the number of protein-based H⁺-buffer groups may increase. The latter assumption stems from the fact that denaturation of some proteins exposes previously sequestered histidine-imidazole moieties to the aqueous media. Proteins such as carbonic anhydrase, myoglobin, and hemoglobin contain H⁺-buffer groups which are exposed to aqueous media and thus available for titration of excess H⁺ (ref. ³). In addition, these proteins contain H⁺-buffer groups which are sequestered from aqueous media and consequently are unavailable for titration under normal circumstances. The available buffer *content* of such proteins can be increased by exposure of sequestered buffer groups through acid-induced denaturation (Fig. 1). If similar proteins are present in neurons and glia, their exposure to the severe levels of acidity that can be seen during ischemia, might similarly increase the available buffer content of cell proteins. As a first approach to detect and quantify acid-induced denaturation of brain proteins, we titrated brain homogenates that were previously exposed to varying levels of acid.

Male Wistar rats (250–400 g) were anesthetized with halothane and their brains frozen by the method of Pontén¹⁵. Brains were removed, weighed, and placed into a 20-ml homogenizer at –20 °C. Three ml of a solution that contained 145 mM sodium chloride and 5 mM sodium fluoride was added to the homogenizer tube followed by 3 ml of light mineral oil. Brain samples (100–300 mg) were rapidly homogenized, warmed to 37 °C in a water bath, and constantly stirred. A glass pH electrode (Thomas 4094-L15) was used to monitor homogenate pH. The initial pH of all homogenates was adjusted (pH < 0.1) with either sodium hydroxide or lactic acid to a range of 6.90–6.95. Homogenates were acidified with 50–100 µl aliquots (50–70 per titration) of 0.1–1.0 M lactic acid (Fig. 3). Hydrochloric acid was used to acidify homogenates below pH 4 since the ionization equilibrium constant for lactic acid is 3.89 (at 37 °C)¹. For this same reason, carbonic anhydrase (Fig. 1) was acidified with hydrochloric acid. Each titration curve shown represents a polynomial regression analysis of data from three separate experiments. Polynomial regression analysis was used so as to produce a most representative line for each set of titration data. However, linear regression analysis was used for statistical comparison of titration curves in Fig. 3 since the curves were nearly linear over the reduced pH range of 6.9–4.8.

Titration of brain homogenates (Fig. 2) shows that brain also contains proteins with H⁺-buffer groups that normally are unavailable for titration. Acidification of homogenates to pH 2–3 caused an irreversible increase in H⁺-buffer content for the range of pH 3.5–5.0. However, above approximately pH 5.2, buffer content of homogenates was less than normal after severe (pH 2–3) acidification. Loss of buffer content between pH 5.2–7.0 may be a result of protein destruction and precipitation. Such an extreme level of acidification is unknown in vivo^{12,13}.

We next exposed homogenates to less extreme acidic levels which more closely approximate conditions in vivo associated with brain necrosis. We acidified homogenates to a specific level of acidity for 20 min since this duration of near complete ischemia in rat can result in infarction under hyperglycemic conditions¹⁶. In addition injection of sufficiently acidic sodium lactate solutions directly into rat neocortex for 20 min will result in necrosis of all brain cells¹¹. Finally, titrations were carried out to a level equivalent to 100 mmol lactate per kg of brain. This level of lactate was taken as a liberal upper limit of potential cellular lactate levels that might be attained in vivo during ischemia. Lactate content between 10 and 50 mmol/kg can be generated in brain during various types of ischemia¹⁹. Since acid content is now thought to be greater in glia than the rest of brain (at least during complete ischemia and perhaps during other forms of ischemia)^{10,12,13}, initial lactic acid production in glia might be greater than the 10–50 mmol/kg seen for whole brain.

Acidification of homogenates had no effect on the buffer content of brain proteins until such exposure reached pH 4.5–3.7 (Fig. 3). Homogenates exposed to pH 6.0, 5.5, or 5.0 for 20 min had titration curves that were analogous ($P < 0.0001$) to control homogenates. (Control homogenates were stirred at 37 °C for 20 min before titration.) On the other hand, homogenates that were exposed to acidity of pH 3.7 or 3.1 had significant ($P < 0.0001$) increases in their H^+ -buffer content. Homogenates acidified to pH 4.5 produced variable results with curves that ranged from controls to ones shown for pH 3.7. This variability to acid-induced denaturation at pH 4.5 might indicate that pH 4.5 (for 20 min) represents a threshold for denaturation of lumped brain proteins. Other physical forces that are capable of denaturation such as heat, stirring, or other more subtle effects could variably add to the denaturing influence of pH 4.5 to produce the range of titration curves seen at this pH.

Stewart has most recently suggested the use of formalisms of solution chemistry to define the physicochemical behavior of acid-base interactions in biological systems²³. Others, to varying degrees, have used a similar format^{2,6,19–22}. According to Stewart, dependent and independent variables should first be clearly defined. Dependent variables include H^+ , hydroxyl ions, carbonate ions, HCO_3^- , anions of weak acids, and undissociated weak acids, all of which are defined in relation to a particular system (i.e. interstitial or intracellular space) as well as by externally imposed independent variables. Independent variables are imposed on a particular system from the outside. Independent variables do not influence one another but do determine the values of dependent variables. Independent variables include the partial pressure of carbon dioxide, the total weak acid concentration (A_{tot}), and the strong ion difference (SID). The latter term is defined as the sum of the strong (i.e. fully ionized) cations minus the sum of the strong anions. In the interstitial space SID represents the HCO_3^- concentration while in cells SID represents the sum of bicarbonate concentration plus the concentration of anions from partially ionized weak acids. The titration results reported here (Fig. 3) show that brain contains proteins which increase A_{tot} through denaturation from acidosis. If similar physicochemical behavior occurs in vivo, these results suggest that A_{tot} should not be treated as a constant^{20,21}, especially under conditions of severe brain acidosis associated with brain infarction^{10,12,13}.

Protein denaturation has been suggested as a potential molecular mechanism for brain destruction from excessive acidosis during ischemia^{4,20}. Levels of cellular acidity (pH 4.5–5.5)^{10,12,13} far in excess of those previously thought to be possible during ischemia^{19–21} are now known to occur in glia during hyperglycemic and complete ischemia^{10,12,13}. These conditions could result in brain infarction if reperfusion occurred¹⁶. However, if protein denaturation is a primary cause of brain cell death from ischemia, it seems unlikely that such loss of protein conformation can occur from acidosis *alone* since sufficient acidity (pH 4.5–8.7) rarely or never is achieved^{12,13}.

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Fig. 1.

Acid–base titration of carbonic anhydrase. Carbonic anhydrase is a protein that contains H^+ -buffer groups which are exposed to aqueous media as well as ones which are sequestered from aqueous media under normal circumstances. The latter buffer moieties can be exposed, and thus made available for H^+ -buffering, through denaturation. Such behavior is shown above. When HCl is added to a solution of carbonic anhydrase (upward arrow) a curvilinear response (line a) is seen. If NaOH is then used (line b) to return the pH (downward arrow) of the carbonic anhydrase solution to its original level, a curvilinear response is seen which is more *alkaline* compared to initial levels of added acid. Such hysteresis indicates the H^+ -buffer content of the carbonic anhydrase solution is increased by exposure to acid of pH 2–3. Here 30 mg of carbonic

anhydrase (E.C.4.2.1.1; Sigma) was titrated at 37 °C in 145 mM NaCl–5 mM NaF under mineral oil with constant stirring.

Fig. 2.

Acid–base titration of rat cerebral cortex homogenate. Upper line (a) shows pH response of homogenate titrated with HCl. As acid was added (upward arrow) pH fell steadily until an inflection occurred above 250 mmol/kg brain to indicate maximum H^+ -buffering occurred at approximately pH 3.5. When base (NaOH) was added (downward arrow) so as to return homogenate pH to its initial level, a curvilinear response (b) was seen which was more alkaline when compared to initial acid levels (line a) of added acid. Thus, brain proteins, like other selected proteins (Fig. 1), contain H^+ -buffer groups which are normally sequestered but can be made available for H^+ -titration through exposure of parent proteins to extreme (i.e. pH 2–3) acidosis. (Titration between pH 2 and 3 is not shown.)

Fig. 3.

Acid-induced increases in brain H^+ -buffer content. Rats were anesthetized and their brains frozen in liquid nitrogen by the method of Pontén¹⁵. Samples of cerebral cortex were homogenized in 145 mM NaCl–5 mM NaF, covered with mineral oil, and warmed to 37 °C. Next homogenates were acidified (with lactic acid) to pH 6.0, 5.5, 5.0, 4.5, 3.7, and 3.1 for 20 min and then titrated with lactic acid. Three homogenates were titrated at each specific pH and the resultant data fitted by polynomial regression to produce the above curves. Controls (pH 6.9) were left for 20 min before titration. The 3 curves at the top (a) are data from normal homogenates and ones acidified to pH 6.0 and 5.0, respectively. The curve from homogenates acidified to pH 5.5 is analogous to the curve from homogenates acidified to pH 5.0 and thus

is not shown. When compared by regression analysis curves from control homogenates and ones acidified to pH 6.0, 5.5, and 5.0 were statistically similar ($P < 0.0001$). On the other hand, curves produced by acidification to pH 3.7 (b), and pH 3.1 (c) were significantly different from controls and from each other ($P < 0.0001$). Homogenates acidified to pH 4.5 (not shown) produced variable results with curves which ranged from controls to ones shown for pH 3.7 (b). Therefore, acidification to pH between 4.5 and 3.7 for 20 min caused a permanent increase in H^+ -buffer content of rat brain cortex proteins.