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Changes of ATP and ADP in Cultured Astrocytes Under and After In Vitro Ischemia

Albert Cheung Hoi Yu,[1,2,4] Anson Ming Fung Lau,[3] Ada Wing Yu Fu,[3] Lok Ting Lau,[1,2] and Philip Yeung Lam[3]

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A very large body of evidence from in vivo studies has been accumulated on a link between the change of energy and cell survival/apoptosis. Using an in vitro ischemia model, we have previously shown that ischemia could induce apoptosis in astrocytes. In this study, we utilized the same in vitro model to investigate changes in ATP and ADP levels in cultured astrocytes and attempted to demonstrate an energy–cell death linkage. Astrocytes remained unaltered after 2 hr of ischemia but were moderately or severely damaged after 4 or 6–8 hr, respectively. The astrocytes that survived various lengths of in vitro ischemic incubation retained their ability to produce ATP after ischemia. Both ATP and ADP levels were increased in astrocytes that remained alive under in vitro ischemia for over 6 hr. The largest decline in the percent of viable astrocytes during ischemia corresponded well to the reduction in ATP and ADP levels in these cultures.

KEY WORDS: Astrocytes; culture; in vitro ischemia; energy metabolism; ATP.

INTRODUCTION

We previously employed an anaerobic chamber to study in vitro ischemia-induced apoptosis in astrocytes (1). We showed that astrocyte death is partly due to apoptosis in culture without the presence of neighboring neurons. This implies that astrocytes in primary culture possess all the necessary intracellular components to undergo apoptosis. We have used a similar model to study in vitro ischemia-induced proinflammatory cytokine release (2,3), the protective role of endothelin (4) and the signal transduction pathway involved with cell death in astrocytes (5).

A large body of evidence has been accumulated on changes in energy metabolism associated with ischemia, including the possibility of a link between postischemic ATP and cell survival/apoptosis (6). ATP, the main energy source for all cellular functions, is needed for the proper maintenance of ion homeostasis, translocation of molecules across cells and organelle membranes as well as for many phosphorylation/dephosphorylation reactions. Interestingly, a strong correlation between the percentage decline in ATP and the cell death pathway taken has been observed in kidney tubule cells (7). Apoptosis proceeded when ATP levels are roughly >25% and necrosis when levels are 15% (7). It has been shown previously that the ATP levels in

[1] Neuroscience Research Institute and Department of Neurobiology, Peking University, Beijing, China.
[2] Hong Kong DNA Chips Ltd, Hong Kong SAR, China.
[3] Department of Biology, the Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong SAR, China.
[4] Address reprint request to: Albert Cheung-Hoi YU, Ph.D., Neuroscience Research Institute and Department of Neurobiology, Peking University, 38 Xue Yuan Road, Beijing 100083, P.R. China. Tel (China): (86) 13601644966 or 010 6209 1150; Fax (China): (86) 010 82072207; Tel (Hong Kong): (852) 2111 2123; Fax (Hong Kong): (852) 2111 9762; E-mail: achy@dnachip.com.hk

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astrocytes decline under in vitro ischemia (8,9). No evidence is available on the association of changes in intracellular ATP and ADP concentrations to astrocytic survival under in vitro ischemia. Our goal was to determine the change in ATP and ADP levels in astrocytes under and after in vitro ischemia and correlate these changes with injury and cell death.

EXPERIMENTAL PROCEDURE

Primary, Culture of Rat Cerebral Cortical Astrocytes. Primary cortical astrocytic cultures were prepared from the cerebral cortex of newborn Sprague-Dawley rats (Animal Care Center, HKUST) as described previously (1,2,10,11), with minor modifications. Cerebral cortices freed of meninges were cut into small cubes (<1 mm^3) in Minimum Essential Media (MEM) (Gibco BRL, Life Technologies, Inc., NY, USA), then mechanically dissociated by vortexing for 1.5 min. The resulting cell suspension was sieved, respectively, through 70- and 10-μm sterile Mesh® nylon filters (Spectra/Mesh®, Spectrum Medical Industries, Inc., TX, USA). A volume of cell suspension equivalent to approximately one-fiftieth of one cerebrum was plated in each 35-mm Falcon tissue culture dish (Becton Dickinson & Co., Oxnard, CA, USA). Fresh MEM supplemented with 20% fetal calf serum (FCS) (Globepharm Ltd., UK) was added to yield a final volume of 2 mL. All cultures were incubated in a Napco CO_2 incubator (Precision Scientific Inc., Chicago, USA) at 37°C with 95% air/5% CO_2 (v/v) and 95% humidity. The culture medium was changed 3 days after seeding with the MEM containing 20% FCS and subsequently 2 times per week with MEM containing 10% FCS. Cultures were used for experiments after they were 4 weeks old.

In Vitro Ischemia Model. The model used to study in vitro ischemic injury in astrocytes was described previously (1–3). Briefly, the astrocytic cultures were transferred into an anaerobic chamber (Forma Scientific, Inc., Ohio, USA) saturated with a $N_2/CO_2/H_2$ mixture (85/5/10). The cultures were washed 3 times with glucose and serum-free MEM. Glucose- and serum-free MEM (0.8 mL) was added to cover the astrocytes. The medium was degassed for 30 min and saturated with $N_2/CO_2/H_2$ gas mixture for 15 min before being placed in the chamber. All cultures were wrapped with parafilm to prevent evaporation. Post-in vitro ischemia incubation was performed by replacing the glucose-free medium with 2 mL of serum-free normal oxygenated MEM at the end of ischemic incubation, and the cultures were incubated in a CO_2 incubator with 95% air/5% CO_2.

The controls were washed 3 times with normal MEM and incubated in 800 μL of normal MEM in a CO_2 incubator for the corresponding experimental periods. The controls for post-in vitro ischemia were established by refeeding normal cultures with 2 mL of serum-free MEM and incubating in a normal CO_2 incubator.

Cell viability was measured with Live/Dead® Eukolight™ Viability/Cytotoxicity Kit (L-3224) from Molecular Probes, Inc. (OR, USA) (1).

Extraction of Energy Metabolites. Cultures at the end of the incubation were washed twice with ice-cold PBS and subsequently lysed in 0.5 mL of 0.125 M KOH. The extracts were transferred to Eppendorf tubes (Scientific Plastics®, FL, USA) and vortexed immediately. After 5 min on ice, 0.4 mL of 1 M KH_2PO_4 was added to adjust the pH to 6. Following lyophilization, the extracts were stored at −80°C for further High Pressure Liquid Chromatography (HPLC) analysis and protein determination (12).

High Pressure Liquid Chromatography (HPLC). All the chemicals used for HPLC were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

The cellular extracts were resuspended in double-distilled water followed by centrifugation for 5 min at 4°C. The supernatant was utilized for the subsequent adenosine nucleotides determination by HPLC. The HPLC system (Millipore Corporation, Milford, MA, USA) consisted of a Model 600E multisolvent delivery system, a Model 717 autosampler and a Model 996 photodiode array detector. Retention times and peak areas were recorded by a Millennium 2010 chromatography manager system. Separations were performed on a 5-mm Supelcosil LC 18™ column (25 cm × 4.6 mm I.D.) (Supelco, PA, USA) connected to a Supelguard™ guard column. The mobile phases were 0.1 M KH_2PO_4, pH 6 (buffer A) and 0.1M KH_2PO_4, pH 6, with 10% (v/v) methanol (buffer B). The flow rate of the mobile phase was 1.3 mL/min. The chromatogram was developed with the following program: 9 min of 100% buffer A, followed by buffer B increased to 25% gradually for 6 min, 90% buffer B for 2.5 min and 100% buffer B for the final 2 min. Adenosine nucleotide content was quantified by measuring absorbance at 254 nm.

Statistical Analysis. All data were analyzed by StatView, Version 4.0, and presented as means ± SEM. Statistical analysis was performed by Student's t test. Unpaired t tests were used and a confidence interval of 95% (i.e., $p \leq 0.05$) was considered to be statistically significant.

RESULTS

Cell, Morphology and Viability. Figure 1A shows representative micrographs of astrocytes stained with Calcein-AM and ethidium homodimer. Astrocytes take up extracellular Calcein-AM and convert it to calcein, which fluoresces green due to the enzymatic activity of intracellular esterases. As a result, Calcein-AM-positive cells with green fluorescence represent live astrocytes. Conversely, ethidium homodimer-positive cells with orange fluorescence represent dead astrocytes. Figure 1A-a shows phase contrast micrographs of astrocytes after 0 hr of in vitro ischemia. Figure 1A-d shows cells in the identical field with green fluorescence, indicating that the astrocytes are alive. Figures 1A-b and 1A-e show cultures at 4 hr of in vitro ischemia. Many astrocyte nuclei were shrunken and the integrity of the culture decreased (Fig. 1A-b). Many cell nuclei were intensively stained red with ethidium homodimer, though many astrocytes still appeared with green fluorescence (Fig. 1A-e). Figures 1A-c and 1A-f show cultures at 10 hr of in vitro ischemia, in which only a few cells were intact and fluoresced green, while most of the nuclei fluoresced red.

Figure 1B summarizes the percent of viable astrocytes remaining attached to the cultures under in vitro ischemic condition. There was no significant decline in viability of astrocytes under in vitro ischemic conditions within the first 2 hr of in vitro ischemia. However,

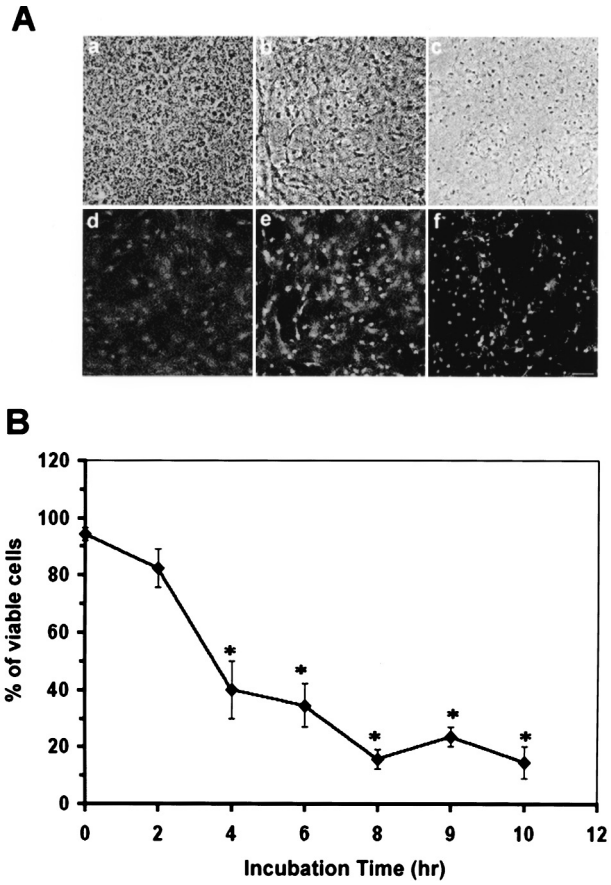

Fig. 1. A. Representative fields of primary astrocytic cultures showing live (Calcein-AM-positive cells with green fluorescence) and dead astrocytes (ethidium homodimer-positive cells with orange fluorescence) under control (a,d), 4 hr (b,e) and 10 hr (c,f) of in vitro ischemia incubation. Photographs were taken with a barrier filter LP 520. Bar size = 50 μm. **B.** The percent of viable primary cultured astrocytes after different periods of in vitro ischemic incubation. Data represent means ± SEM from two separate experiments with $n = 4–5$. (*) $p \leq 0.05$ compared with control at 0 hr incubation.

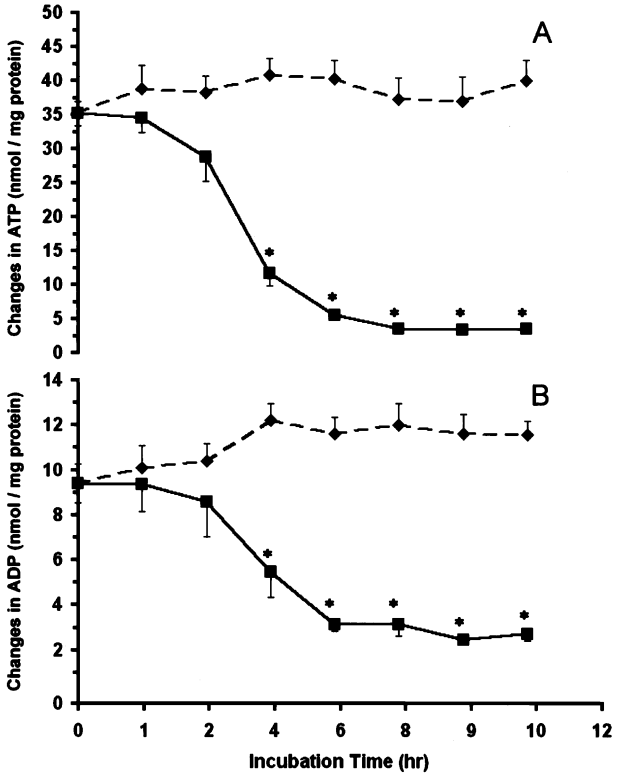

Fig. 2. Changes in ATP and ADP content in primary astrocytic cultures under control and in vitro ischemic incubation. **A.** ATP content in control incubation (—♦—) and in vitro ischemia incubation (—■—) (*) $p \leq 0.05$ compared with the ATP content in the control culture at 0 hr. **B.** ADP content in control incubation (—♦—) and ADP content in cultures in vitro ischemia incubation (—■—). (*) $p \leq 0.05$ compared with the ADP content in the control culture at 0 hr. Data represent the means ± SEM from a minimum of five cultures examined per time point.

after 4 hr of in vitro ischemia, the percent of viable cells was significantly reduced, by more than 50%. It was further reduced by 15% after 8 hr. The number of viable cells was probably overestimated because cells undergoing necrosis appeared to detach from the coverslips and were not taken into account when calculating the percent of viable astrocytes.

ATP and ADP Content during In Vitro Ischemia. The average amounts of ATP and ADP in control astrocytic cultures at 0 hr were 35.10 ± 1.77 and 9.38 ± 0.87 nmol/mg protein, respectively (Fig. 2). The changes in ATP and ADP amounts in astrocytic cultures under control and ischemic incubations are shown in Fig. 2A and B. Within the 10 hr of control incubation, there was no significant change in ATP or ADP contents. In up to 2 hr of in vitro ischemic incubation, both ATP and ADP contents showed a slight and statistically nonsignificant depletion. At 4 hr of in vitro ischemia, ATP and ADP contents were reduced by 65% and 40% of the initial value, respectively. ATP content was further reduced to 15% of the control at 6 hr and to less than 10% at 8 hr of in vitro ischemia. ATP content was 3.46 ± 0.78 nmol/mg protein at 10 hr of in vitro ischemia. ADP content was reduced by 65% at 6 hr of in vitro ischemia and remained at this level for the rest of the in vitro ischemic incubation. The level of ADP was lowered to 2.68 ± 0.32 nmol/mg protein at 10 hr of in vitro ischemia. Both ATP and ADP showed a similar depletion course but the rate of ADP decrease was slower than that of ATP.

ATP and ADP Content during Post-In Vitro Ischemia. During the post-in vitro ischemic recovery period both ATP and ADP levels partially recovered (Fig. 3A, B). For astrocytes under 4 hr of in vitro ischemia,

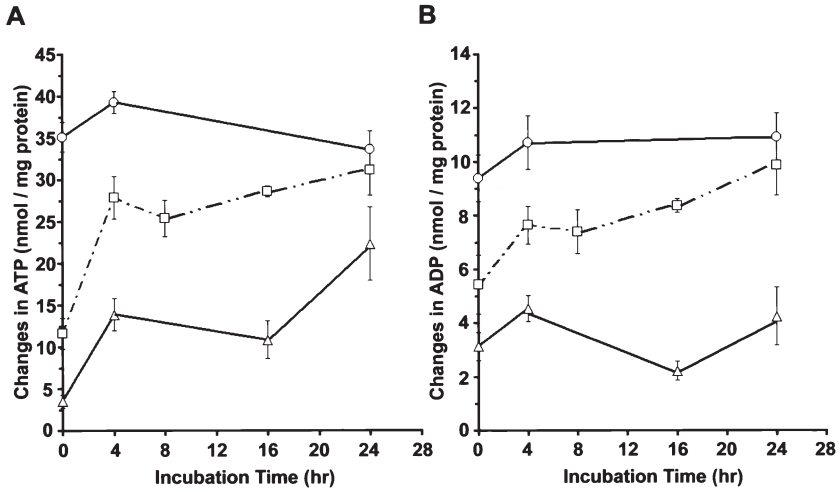

Fig. 3. Changes in ATP and ADP content in primary astrocyte culture under control and post-in vitro ischemic incubation. **A.** Changes in ATP levels during post-in vitro ischemia: (—○—) control astrocytes; (—□—) astrocytes subjected to 4 hr in vitro ischemia; (—△—) astrocytes subjected to 8 hr in vitro ischemia. **B.** Changes in ADP levels during post-in vitro ischemia recovery: (—○—) control astrocytes; (—□—) astrocytes subjected to 4 hr in vitro ischemia; (—△—) astrocytes subjected to 8 hr in vitro ischemia. Each time point represents the means ± SEM from a minimum of five measurements. (*) $p \leq 0.05$ as compared with the ratios under post-in vitro ischemic incubation to the control value at 0 hr. (†) $p \leq 0.05$ as compared with the ratio under post-in vitro ischemic incubations to the value at the end of their corresponding in vitro ischemic incubation. Data represent the means ± SEM from a minimum of five cultures examined per time point.

ATP levels quickly rose in the first 4 hr of post-in vitro ischemia and then gradually returned to a level similar to the control culture (Fig. 3A). For astrocytes incubated under in vitro ischemic conditions for 8 hr, the recovery of ATP to normal levels was incomplete and remained lower than the control culture at 24 hr of post-in vitro ischemic incubation (Fig. 3A). The ADP level of astrocytes undergoing 4 hr of in vitro ischemic incubation rose in the first 4 hr of post-in vitro ischemia and then reached a similar level to the control culture (Fig. 3B). For astrocytes under 8 hr of in vitro ischemia, the pattern of ADP level recovery was similar to that of the ATP recovery in equivalent cultures, which remained significantly lower than in the control at 24 hr of post-in vitro ischemic incubation (Fig. 3B).

DISCUSSION

In this study, the extent of in vitro ischemic damage to astrocytes can be divided into three stages. The first stage occurs between 0–2 hr of in vitro ischemia. The injury induced at this stage was mild, as indicated by the lack of significant changes in the viable astrocytes and the slow decrease in their energy contents, as indicated by the rate of hydrolysis of ATP. The levels of ATP and ADP reflected no significant disturbance in energy metabolism, indicating that the energy stored in cultured astrocytes remained sufficient to support physiological functions. Furthermore, the energy metabolism of astrocytes during post-in vitro ischemic recovery was not affected as the level of energy metabolites in the cultures was maintained at the control level throughout the 24 hr of post-in vitro ischemic incubation. This is consistent with previous investigations (1,3,13,14).

The second stage of in vitro ischemic damage occurred at 4 hr when injury to cell was considered moderate. The number of viable cells decreased by more than 50%. There was a significant decline in ATP and ADP level in these cultures. Cells dying during this stage might be the cell population more vulnerable to ischemia insult. Cell death during this stage of ischemia may be due to apoptosis (ATP $<$ 25%) and necrosis (ATP $\sim$ 15%). The astrocytes surviving after this stage were able to recover their energy within 4 hr after entering post-in vitro ischemia, indicating that ATP could be synthesized by phosphorylating ADP, thus the intracellular components for energy production in these surviving cells was intact and functional. It is at this stage that Annexin V staining of astrocytes was identified in the surviving astrocytes, indicating the commitment of these astrocytes to begin apoptosis (1).

The third stage of in vitro ischemic damage occurred when astrocytes were incubated under in vitro ischemic conditions for more than 6 hr. The injury to as-

trocytes during this period was severe as reflected by the change in morphology and the low levels of ATP and ADP in these cultures. Astrocytes surviving this stage of ischemic insult were severely injured as reflected by their energy level, which could not be recovered throughout the post-in vitro ischemia experimental period.

Yu et al. (8) and Gregory et al. (15) had previously shown a correlation between depletion of ATP and cell death in astrocytes under in vitro severe hypoxia. Cell death was studied by the activity of extracellular lactate dehydrogenase (LDH) in cultured astrocytes after exposure to in vitro ischemic injury and was consistent with the findings of Hertz et al. (16) and Huang et al. (13). In this study, we showed that there was a correlation between ATP depletion and cell death in astrocytes under in vitro ischemia. Cell death of astrocytes began after 2 hr of in vitro ischemia, which corresponded with the time of ATP depletion. After 4 hr of ischemia, the loss of astrocytes was associated with a 65% depletion of ATP. This implied that the energy reserve in astrocytes was exhausted after the first 2 hr of ischemia. The depletion of energy reserve might lead to a deficiency in astrocytic functions, such as maintenance of ionic homeostasis, thus leading to the influx of Na^+, Ca^{2+}, Cl^- and water into astrocytes.

The present study also shows that astrocytes in the same culture might have different tolerance levels to ischemic insult. The variability in tolerance among these astrocytes might be due to differences in the amount of energy reserves and the metabolic stage of the astrocytes during or entering the ischemic insult (17,18). The amount of nutrient storage might also vary from cell to cell. Juurlink and Hertz (18) suggested that the release of cytoplasmic components from dead cells was able to support the metabolism of remaining viable astrocytes under in vitro ischemia. In addition, the cultures might contain subpopulations of astrocytes in younger and less differentiated stages, which are known to be resistant to in vitro ischemia (19) and hypoglycemia (20).

Astrocytes surviving in vitro ischemic insult could resume ATP synthesis during postischemic recovery. The balance of energy metabolism was disturbed during recovery periods for astrocytes exposed to longer in vitro ischemic incubation. Two hours of in vitro ischemia did not cause severe damage to astrocytes, as energy levels were rapidly restored. Four hours caused 50% of cells to die; however, the surviving astrocytes were still able to recover their ATP levels within 4 hr of post-in vitro ischemia. Thus, most of the surviving astrocytes had not yet reached a stage of commitment to death and the changes in ATP synthesis were reversible.

The ischemic incubation between 4–6 hr might be the critical period for astrocytes in response to in vitro ischemic damage as the ability to restore ATP and ADP levels in these astrocytes was largely reduced during postischemic recovery. Although we did not have direct evidence of irreversible damage between 4–6 hr, this is supported by the observed difference in the energy recovery of astrocytes that had less than 4 hr and more than 6 hr of ischemia. This is also supported by the finding of Goldberg and Choi (21), which suggested that cultured astrocytes were irreversibly injured by exposure to 4–6 hr of in vitro ischemia. The disturbance in ATP and ADP synthesis observed in this study may explain the previous observation that astrocytes became apoptotic after 6 hr of ischemia in this model and many of these apoptotic astrocytes died of necrosis (1).

In conclusion, under in vitro ischemia, a correlation between the depletion of ATP and cell injury in astrocytes was observed. The degree of ATP metabolism alteration during in vitro ischemia would determine the fate of astrocytes under post-in vitro ischemia. This might explain our previous finding (1) that astrocytes became apoptotic under ischemia might be related to the level of energy metabolites in cells.

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REFERENCES

1. Yu, A. C. H., Wong, H. K., Yung, H. W., and Lau, L. T. 2001. In vitro ischemia-induced apoptosis in primary cultures of astrocytes. Glia 35:121–130.
2. Yu, A. C. H. and Lau, L. T. 2000. Expression of interleukin-1 alpha, tumor necrosis factor alpha and interleukin-6 genes in astrocytes under in vitro ischemic injury. Neurochem. Int. 36: 369–377.
3. Lau, L. T. and Yu, A. C. H. 2001. Astrocytes produce and release interleukin-1, interleukin-6, tumor necrosis factor alpha and interferon-gamma following traumatic and metabolic injury. J. Neurotrauma 18:351–359.
4. Ho, M. C. Y., Lo, A. C. Y., Kurihara, H., Yu, A. C. H., Chung, S. S. M., and Chung, S. K. 2001. Endothelin-1 protects astrocytes from in vitro ischemic/in vitro ischemic injury. FASEB J. 15: 618–626.
5. Jiang, Z., Zhang, Y., Chen, X. Q., Lam, P. Y., Yang, H., Xu, Q. Y., and Yu, A. C. H. 2002. Activation of Erk ½ and Akt in astrocytes under ischemia. Biochem. Biophys. Res. Commun. 294: 726–733.

6. Maeda, K., Hata, R., Gillardon, F., and Hossmann, K. A. 2001. Aggravation of brain injury after transient focal ischemia in p53-deficient mice. Brain Res. Mol. Brain Res. 88:54–61.
7. Lieberthal, W., Menza, S. A., and Levine, J. S. 1998. Graded ATP depletion can cause necrosis or apoptosis of cultured mouse proximal tubular cells. Am. J. Physiol. 274:F315–F327.
8. Yu, A. C. H., Gregory, G. A., and Chan, P. H. 1989. In vitro ischemia-induced dysfunctions and injury of astrocytes in primary cell cultures. J. Cerebr. Blood Flow Metab. 9:20–28.
9. Sonnewald, U., Muller, T. B., Westergaard, N., Unsgard, G., Petersen, S. B., and Schousboe, A. 1994. NMR spectroscopic study of cell cultures of astrocytes and neurons exposed to in vitro ischemia: compartmentation of astrocyte metabolism. Neurochem. Int. 24:473–483.
10. Yu, A. C. H., Chan, P. H., and Fishmen, R. A. 1986. Effects of arachidonic acid on glutamate and gamma-aminobutyric acid uptake in primary cultures of rat cerebral cortical astrocytes and neurons. J. Neurochem. 47:1181–1189.
11. Yu, A. C. H., Lee, Y. L., and Eng, L. F. 1993. Astrogliosis in culture: I. The model and the effect of antisense oligonucleotides on glial fibrillary acidic protein synthesis. J. Neurosci. Res. 34:295–303.
12. Lowry, O. K., Rosebrough, W. J., Farr, A. L., and Randall, R. J. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
13. Huang, R., Shuaib, A., and Hertz, L. 1993. Glutamate uptake and glutamate content in primary cultures of mouse astrocytes during anoxia, substrate deprivation and simulated in vitro ischemia under normothermic and hypothermic conditions. Brain Res. 618:346–351.
14. Huang, R. and Hertz, L. 1994. Effect of anoxia on glutamate formation from glutamine in cultured neurons: dependence on neuronal subtype. Brain Res. 660:129–137.
15. Gregory, G. A., Welsh, F. A., Yu, A. C. H., and Chan, P. H. 1990. Fructose-1,6-bisphosphate reduces ATP loss from in vitro ischemic astrocytes. Brain Res. 516:310–312.
16. Hertz, L., Code, W. E., Huang, R., Juurlink, B. H., Peng, L., Sochocka, E., Zhong, Z., and Yu, A. C. H. 1992. Glutamate and anoxic-in vitro ischemic cell death in neurons and astrocytes. Clin. Neuropharmacol. 15:126A–127A.
17. Peng, L., Hertz, L., Huang, R., Sonnewald, U., Petersen, S. B., Westergaard, N., Larsson, O., and Schousboe, A. 1993. Utilization of glutamine and of TCA cycle constituents as precursors for transmitter glutamate and GABA. Dev. Neurosci. 15:367–377.
18. Juurlink, B. H. and Hertz, L. 1993. In vitro ischemia-induced death of astrocytes and neurons in primary culture: pitfalls in quantifying neuronal cell death. Brain Res. Dev. Brain Res. 71: 239–246.
19. Yager, J. Y., Kala, G., Hertz, L., and Juurlink, B. H. J. 1994. Correlation between content of high-energy phosphates and in vitro ischemic damage in immature and mature astrocytes. Dev. Brain Res. 82:62–68.
20. Hertz, L., Yager, J. Y., and Juurlink, B. H. J. 1995. Astrocyte survival in the absence of exogenous substrate: comparison of immature and mature cells. Int. J. Dev. Neurosci. 13:523–527.
21. Goldberg, M. P. and Choi, D. W. 1993. Combined oxygen and glucose deprivation in cortical cell culture: calcium-dependent and calcium-independent mechanisms of neuronal injury. J. Neurosci. 13:3510–3524.

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word word	#	Close up to normal space
word word	⁀	Close up
cap	cap	Capitalize
lc	lc	Set lower case
,	,	Insert comma
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sp	sp	Spell out
#	#	Insert space
tr	tr	Transpose letters [or words]
f	f	Substitute letter written in margin for letter[s] struck out in text
lc	lc	Set lower case after initial capital
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insert, see copy	insert, see copy	Insert omitted material, see manuscript [or attached typescript]
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Experimental Results

We found very insignificant variations in ~~in~~ the proportions of segmented neutrophilos, lymphocytes, and monocytes. We were unable to find any definite relationships in view of the small variations. In some cases an increase in the proportion of monocytes was found after a course of intra-arterial procaine infusions. for instance, whereas before treatment a Monocyte count of between 1 and 65 percent was observed in 62 patients after treatment this range of monocyte counts was observed in 40 patients similarly, monocyte counts of between 7 and 12% were found before treatment in 87 patients and aftertreatment in 100 patients.

As an additional measure, in order to obtain a deeper understanding of the nature of the processes taking place in the patient after intra-arterial infusion of procaine, G. N. UØINTSEV and V. B. Blank [1957] undertook an investigation with the object of studying possible changes in the morphological composition of the blood in patients with peptic ulceration.

This investigation was in direct relationship to our own, and helps in the solution of problems concerning the reflex regulation of the blood system in general, and during intra-arterial $C_{13}H_{21}ON_3$ infusion in particular. ¶ Changes in the leucocyte count of the peripheral blood and in the ~~monocyte~~ formula were studied. A study of the morphological changes in the immediately after infusion and for some time ~~threafter~~ was also thought to be of interest.

The patients investigated were divided into two groups. The patients of the first group were investigated as follows: 1) before infusion, 5) on the 3rd day after the second infusion, and 6) 5 days after the last intraarterial procaine infusion.

In order to study the course of these changes to repeated intra-arterial procaine infusions and the reaction of the body for a longer period of time, the second group of patients was investigated (1) before infusion, (2) 10 minutes after infusion, 3) 1 hour after infusion, and (4) the day after infusion. The same investigations were also repeated on the 3rd and 5th day and terminated in a final investigation 5 days after the last infusion, i.e., on the 12th to 13th day after the patients first infusions. Seventy peptic ulcer patients—forty males and thirty females were examined.

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