Effect of Lidocaine on Intracellular Calcium Alterations during Energy Depletion and Reperfusion in Glial Cells

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- 국문초록 -

신경교 세포에서 에너지 고갈과 재관류 동안에 생기는 세포내 칼슘 이온 변화에 대한 Lidocaine의 효과

삼성의료원 마취과

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서 론: 국소 마취제로서 lidocaine은 전압 감수성의 칼슘 통로와 소듐 통로를 차단하여 세포막을 안정시킴으로써 세포내의 칼슘 이온 변동을 감소시킨다고 알려져왔다. 이에 본 실험에서는 단 세포(single cell)를 이용한 in vitro 실험에서 허혈성 변화로 인한 세포내 에너지 고갈 및 재관류로 인한 손상(소위, 칼슘 파라독스 현상)을 유도하여 lidocaine의 효과를 알아보고자 하였다.

방 법: 실험 재료로서는 인간의 성상세포종양의 세포를 이용하였다. 세포내 에너지 감소를 유도하기 위해 미토콘드리아 호흡쇄(respiratory chain) 차단제인 amytal/CCCP를 사용하였고, 재관류를 모사하기 위해 일정시간의 저농도 칼슘 완충용액 관류 후 표준 칼슘용액으로 재관류 하였다. 실험 세포내의 칼슘이온 변화를 측정하기 위해서 칼슘 지정 염료인 fura-2를 사용하여 microspectrometry로 측정하였다.

결 과: 세포내 에너지 저하시 생기는 칼슘이온의 증가는 주로 세포내 세포소기관(organelles)으로부터 유리된 것이었고, 세포 소기관으로부터의 칼슘유리는 세포질 내의 소듐 농도에 의존적이었다. 세포내의 에너지 감소시 0.5 mM lidocaine 첨가로 세포내 칼슘이온 증가를 막았다. 또한 0.5 mM lidocaine은 재관류시 칼슘이온 증가의 주된 길인 소듐/칼슘 교환체 작용을 억제하여 세포내 칼슘 농도의 증가를 억제하였다.

결 론: 국소 마취제 lidocaine은 소듐/칼슘 교환체에 작용하여, 허혈 및 허혈 후 재관류시 세포내 칼슘 이온 증가를 막는데 효과적이었다. (Korean J Anesthesiol 1996; 30: 516~522)

핵심용어: 국소마취제, 리도카인, 뇌: 허혈, 세포: 신경교 세포, 이온: 칼슘

INTRODUCTION

Intracellular calcium ($[Ca^{2+}]_i$) overload has often been implicated as a potential cause of cell death after ischemic injury and/or post-ischemic cell damage in the central nervous system (CNS)^(1,2). In this regard, a number of sometimes interdependent pathways including excitatory

amino acid neurotoxicity, energy depletion, voltage-dependent ionic channels and a variety of other insults are capable of producing the degree and duration of $[Ca^{2+}]_i$ elevation necessary to precipitate cell necrosis. In this report, we investigated whether the local anesthetic lidocaine, that is known to alter ionic permeabilities often associated with changes in calcium homeostasis, can block a variety of these potentially deleterious $[Ca^{2+}]_i$ alterations.

As a local anesthetic, lidocaine normally decreases $[Ca^{2+}]_i$ alterations by stabilizing membrane voltage-sen-

sitive calcium or sodium channels and therefore has great therapeutic efficacy as an antiarrhythmic agent^{3,4)}. It also has the potential, as with some general anesthetics⁵⁾, to inhibit $\text{Na}^+/\text{Ca}^{2+}$ exchanger activity and thus prevent sodium-mediated calcium entry during post-ischemic injury⁶⁾. In our present work, we have investigated the possibility that lidocaine, perhaps via these mechanisms, might ameliorate $[\text{Ca}^{2+}]_i$ elevations produced by "ischemic-like" changes (energy depletion) or reperfusion (calcium paradox) injury in vitro⁶⁾.

MATERIALS AND METHODS

1) Cell cultures

Human astrocytoma cell line U1242MG were used for this experiment. The harvesting media for human astrocytoma cells was composed of 10% calf serum in Eagle's B Media (B10media: 500 ml Eagle's MEM, 7.5 ml vitamins, 15 ml essential amino acids, 10 ml non-essential amino acids, 58 ml calf serum, and 4.5 ml Pen strept antibiotics). During harvesting procedures cells were harvested using 0.01% trypsin in 0.02% EDTA for 6 min at 37°C. Single cells were separated from the suspension by pipette trituration and plated in each well on flame-sterilized coverslips at cell densities of 2,000 cells/cm² in 2 ml of B10 media.

2) Experimental solutions and Perfusion protocols

The control perfusate was a Krebs-Hensleit(K-H) buffer containing(in mM): 116.5 NaCl, 4.5 KCl, 0.9 MgSO₄, 1.0 NaH₂PO₄, 25 NaHCO₃, 1.5 CaCl₂, 7.0 glucose. The calcium-free K-H solutions were prepared without adding calcium to the K-H buffer and by adding 1 mM ethyleneglycol-bis-N,N,N',N'-tetraacetic acid (EGTA) to the solution; sodium-free solutions were made with equimolar substitution of choline chloride. To replicate energy depletion conditions, cells were perfused with a respiratory chain inhibitor that blocks the NADH dehydrogenase enzyme (amytal: 3 mM)⁷⁾ and a chain uncoupler, carbonyl cyanide m-chlorophenyl hydrazone

(CCCP, 6 μM)⁸⁾ which rapidly causes ATP to fall to low intracellular levels⁹⁾. Reperfusion injury was produced by exposure of cells to low calcium solutions for 5 min. and then re-exposing them to the standard calcium containing buffer⁶⁾. To see the lidocaine effect, 0.5 mM lidocaine was added during energy depletion and reperfusion protocols. Experimental protocols called for cells to be exposed to normal calcium containing K-H for at least 30 min.

3) Intracellular calcium measurements

U1242MG human astrocytoma cells were seeded at a density(2,000/cm²) that produced a sparse population of single cells when plated overnight. On the morning of an experiment, the cells were subsequently loaded with 4 μM fura-2 for 30 min and post-incubated, after fura-2 removal, for another 90 min. to allow for formation of the active acid moiety⁶⁾. Experimental manipulations were all performed in a chamber system that allowed rapid (<10 sec.) solution changes. Temperature (36 $\pm$ 0.5°C) was maintained by passage of the buffer solutions through a temperature-controlled heat exchanger; pH (7.4 $\pm$ 0.1) in a K-H buffer solutions of standard composition was adjusted with 95% O₂/5% CO₂⁶⁾. Single cells were selected in microscopic fields for fura-2 microspectrofluorometry and ratio estimations (10 sec.) were made of $[\text{Ca}^{2+}]_i$ with a PTI DELTASCAN system coupled to an inverted Zeiss epifluorescence microscope^{6,10,11)}. Ratio estimations of $[\text{Ca}^{2+}]_i$ were made for limited times to avoid bleaching of the dye and eliminate the potential for cell damage from UV irradiation. The calculation of intracellular calcium concentrations was done by adapting the original Grynkiewicz equation as modified by Li et al⁶⁾. and Groden et al.¹⁰⁾

Statistics were done with two way ANOVA (STAT-GRAPHICS program, STSC Inc., 1876) for the comparison between the two groups and students t Test (paired) for the individual $[\text{Ca}^{2+}]_i$ data as indicated (* P<0.05, ** P<0.005). All data are presented as mean $\pm$ standard error in the figures to follow.

RESULTS

1) The source of intracellular calcium increase

During the energy depletion protocol (Fig. 1), $[Ca^{2+}]_i$ rose quickly to greater than 400 nM at 3 min and showed a persistent elevation for up to 30 min. The same concentration of amytal and CCCP during low calcium perfusion (no external calcium and 1 mM EGTA) also produced a comparable change in $[Ca^{2+}]_i$. There was no statistical difference between these two groups in time [ANOVA, $F=2.584$ (2, 149) $P<0.11$]. $[Ca^{2+}]_i$ changes during energy depletion in the two groups were always significant over time when compared to control $[Ca^{2+}]_i$ levels (t Test, $**P<0.005$). Furthermore, cells perfused with low calcium/low sodium after energy depletion showed comparable increases in $[Ca^{2+}]_i$ only transiently (Fig. 2). Thereafter, considerably lower $[Ca^{2+}]_i$ was seen when compared to cells perfused with standard sodium/low calcium buffers. The low calcium group and low calcium/low sodium group were also different [ANOVA, $F=5.907$ (2, 126) $P<0.05$] Individual calcium levels in low

Fig. 1. $[Ca^{2+}]_i$ alterations in the absence of external calcium during energy depletion. $[Ca^{2+}]_i$ increases in the presence of normal extracellular calcium after energy depletion occurred promptly by 3 min. Partial recovery occurred once the next 5 min. followed by a persistent increase in $[Ca^{2+}]_i$. In the absence of extracellular calcium during energy depletion, the pattern and magnitude of $[Ca^{2+}]_i$ increases were similar.

calcium/low sodium perfusion after energy depletion were significantly elevated over control values at 3, 20 and 30 min. (t Test * $P<0.05$).

2) Lidocaine effect on the energy depletion

Addition of local anesthetic 0.5 mM lidocaine in energy depleted cells, totally blocked the $[Ca^{2+}]_i$ increase even after long time periods. [ANOVA, $F=74.816$, (2, 185) $P<0.005$] (Fig. 3) Interestingly, 0.5 mM lidocaine addition to the low calcium buffer during amytal/CCCP exposure only slightly modified the increases of intracellular calcium [ANOVA, $F=4.474$, (2, 149) $P<0.05$] (Fig. 4). Therefore, data in Fig. 3 and 4 support the hypothesis that the local anesthetic lidocaine prompted $[Ca^{2+}]_i$ efflux by the forward mode of the surface Na^+/Ca^{2+} exchanger.

3) Lidocaine effect on the reperfusion-mediated calcium increase

Initially, we studied the effects of lidocaine on $[Ca^{2+}]_i$ changes in the absence of extracellular calcium. In the absence of extracellular calcium, 0.5 mM lidocaine caused a small but significant increase ($P<0.05$) in $[Ca^{2+}]_i$ probably through effects on intracellular stores (Fig. 5).

Fig. 2. $[Ca^{2+}]_i$ alterations in the absence of external calcium and sodium during energy depletion. $[Ca^{2+}]_i$ changes in the calcium free/sodium free perfusate during energy depletion were not different at 3 min.; compared to that of calcium free alone, thereafter $[Ca^{2+}]_i$ levels decreased and began to increase slightly after 20 min with energy depletion.

Fig. 3. Lidocaine effects on $[Ca^{2+}]_i$ changes during energy depletion. Lidocaine addition (0.5 mM) to the calcium perfusate during energy depletion blocked the $[Ca^{2+}]_i$ alterations.

Fig. 4. Lidocaine effects on $[Ca^{2+}]_i$ changes during energy depletion in the absence of external calcium. Lidocaine addition to the calcium free perfusate could not block the $[Ca^{2+}]_i$ alterations during energy depletion.

Fig. 5. Lidocaine effects on internal calcium stores. When lidocaine was added to the calcium free perfusate, lidocaine released a small amount of calcium from internal stores

Fig. 6. Effects of lidocaine on the surface Na^+/Ca^{2+} exchanger during reperfusion. An addition of lidocaine during the calcium reperfusion period after low calcium perfusion completely blocked the $[Ca^{2+}]_i$ alterations.

The addition of 0.5 mM lidocaine to the calcium perfusate after low calcium perfusion, however, produced an effective blockade of the reperfusion increases in $[Ca^{2+}]_i$ [ANOVA, $F=4.399$, (2, 75) $P<0.005$] (Fig. 6). As shown in Fig. 6, calcium entry during the reperfusion period occurred rapidly after cessation of the low calcium exposure. In addition, at all times after the start of reperfusion in which $[Na^+]_i$ was previously elevated during low calcium perfusion⁶, $[Ca^{2+}]_i$ was elevated above control values in quiescent cells.

DISCUSSION

A series of pathogenic events occurs during ischemic and post-ischemic injury in central nervous system. Here we have only attempted to mimic the effects of two of these factors: energy depletion and reperfusion injury.

In experiments designed to mimic in vivo ischemia, a variety of pathogenic events involving $[Ca^{2+}]_i$ changes are possible. Insufficient ATP production in the mitochondria may result in membrane depolarization and calcium entry via activated voltage-dependent and agonist-dependent

calcium channels. Calcium may also be released from intracellular organelles (i.e., mitochondria and endoplasmic reticulum)¹²⁻¹⁴⁾. Subsequently, lack of ATP production blocks energy-dependent calcium extrusion through the plasma membrane and prevents sequestration of calcium into the intracellular organelles¹⁵⁾.

The NADA dehydrogenase inhibitor(Amytal) and the mitochondrial uncoupler(CCCP) produced immediate and sustained elevations of intracellular calcium. Amytal/CCCPCP also caused a reduction in ATP concentration in other cell line¹⁶⁾. In the current study, the Amytal/CCCPCP application both in the presence and absence of external calcium produced statistically significant $[Ca^{2+}]_i$ changes over control levels. However, in response to Amytal/CCCPCP in calcium free buffer a $[Ca^{2+}]_i$ changes were not different from that of in the presence of external calcium. $[Ca^{2+}]_i$ values in astrocytoma cells of this experiment were not much modified by the extracellular calcium. It is clear that the rise of $[Ca^{2+}]_i$ during energy depletion is mostly due to internal release of calcium. As a protonophore, CCCPCP was observed to cause the release of calcium from mitochondria¹⁷⁾. To investigate the specific response during energy depletion, sodium free/calcium free buffer solution was perfused. The size of intracellular calcium increase with calcium free/sodium free was much less than calcium free alone. The elevation of $[Ca^{2+}]_i$ that occurs during energy depletion is also associated with $[Na^+]_i$ alterations that stimulate the Na^+/Ca^{2+} exchanger on the mitochondrial membrane and thus prompt it to release calcium release^{18,19)}.

In the reperfusion-paradox protocol, we attempted to replicate the extracellular ionic milieu that might occur during post-ischemic injury in vivo. The $[Ca^{2+}]_i$ elevations associated with such injury paradigms are possible after a variety of noxious insults (i. e., persistently low extracellular calcium) known to occur during CNS injury²⁰⁻²²⁾. Our previous study showed that $[Ca^{2+}]_i$ increases were also produced by low calcium exposure and subsequent 1.5 mM calcium reperfusion⁶⁾. Previous sodium loading of the cells during low calcium perfusion may precipitate calcium entry via reversal of the Na^+/Ca^{2+}

exchanger on plasma membrane. The entry of calcium under such conditions has been shown to not involve the voltage-activated calcium channels. The magnitude of calcium entry during the reperfusion was dependent on the transmembrane sodium gradient which mediates the reverse mode of the Na^+/Ca^{2+} exchanger activity⁶⁾. Generally, the $[Ca^{2+}]_i$ increase occurred within 2-3 min. after reperfusion began.

To minimize ischemia or reperfusion induced $[Ca^{2+}]_i$ overload, many drugs and interventions have been investigated. Lidocaine is generally accepted as an anti-arrhythmic agent, which directly interfere with the cell membrane depolarization. Thus it generally reduce membrane ion permeability, such as Na^+ or Ca^{2+} influx and K^+ efflux^{4,23-25)}. Because of very high solubility in cellular membrane and blocking action of ionic movements, lidocaine is considered as a membrane stabilizing agent^{26,27)}. Also lidocaine was observed to reduce the products by lipid peroxydation during the reperfusion²⁸⁾. Several other authors have reported that lidocaine can inhibit ischemia and reperfusion mediated injury in animal model. In addition, lidocaine had an effect of preservation of ATP content during ischemia and reperfusion²⁹⁾.

The major aim of the present study was to determine if the addition of lidocaine could prevent ischemia or reperfusion induced calcium accumulation. Our results have demonstrated that 0.5 mM lidocaine addition in the presence of extracellular calcium during energy depletion inhibited $[Ca^{2+}]_i$ changes. However, 0.5 mM lidocaine addition in the absence of extracellular calcium during energy depletion did not protect $[Ca^{2+}]_i$ accumulation. This results may be looked very paradoxical. But if we consider the Na^+/Ca^{2+} exchanger has been associated with transmembrane calcium flux, this finding suggest that the Na^+/Ca^{2+} exchanger may be able to work forward mode to pump out increased intracellular calcium during energy depletion. Since both calcium pumps on plasma membrane and on endoplasmic reticulum are energy dependent, it is possible that lidocaine acts on the Na^+/Ca^{2+} exchanger activity significantly.

The absence of a $[Ca^{2+}]_i$ change during reperfusion with

lidocaine is probably explained by the ability of lidocaine to inhibit sodium and calcium conductance. The effect of lidocaine on free calcium alterations in this particular experimental setting, however, may indicate a unique effect of lidocaine on exchanger properties mediating this event. When we added 0.5 mM lidocaine during low calcium perfusion, it evoked transient calcium increase. We assume that this was probably due to the collapse of mitochondrial membrane potential and uncoupling of oxidative phosphorylation in the mitochondria^{8,30,31}. If we consider the effect of lidocaine itself on the calcium release from the internal calcium stores, the blocking effect during the reperfusion phase might also be explained by the forward mode of exchanger.

Therefore, we propose that lidocaine has the capacity to promote the forward activity of the surface membrane $\text{Na}^+/\text{Ca}^{2+}$ exchanger rather than merely blocking the reverse mode of exchanger, thus eliminating calcium from the cytosol. Such effects might be particularly beneficial for use during short-duration ischemic and post-ischemic episodes when the lowering of $[\text{Ca}^{2+}]_i$ would be advantageous. This could prevent a variety of the deleterious calcium-mediated sequelae occurring after cellular injury.

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