BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **234,** 275–277 (1997)
ARTICLE NO. RC976625

Diverse Cytoprotectants Prevent Cell Lysis and Promote Recovery of Respiration and Ion Transport

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Received April 3, 1997

Numerous agents have been reported to prevent cell lysis. However, little information is available concerning the ability of cytoprotectants to promote the return of physiological functions. The goal of this study was to determine whether a diverse group of cytoprotectants prevent cell lysis and promote the recovery of respiration and ion transport following anoxia (60 min)/reoxygenation (60 min) in rabbit renal proximal tubule (RPT) suspensions. Cell lysis (LDH release) was determined immediately following the anoxic and reoxygenation periods. Mitochondrial function (basal respiration) and active Na^+ transport (ouabain-sensitive respiration) was determined after the reoxygenation period. LDH release increased to 75 ± 11% after the anoxic period and did not increase further during the reoxygenation period. LDH release in controls was 6 ± 1% and did not vary over time. Glycine (2 mM), strychnine (1 mM), nifedipine (100 μM) and niflumic acid (100 μM) added immediately prior to the anoxic period completely blocked LDH release. All cytoprotectants increased basal respiration from 39 ± 7% of controls in the anoxic samples to 65–77% of controls. Glycine, strychnine and nifedipine increased ouabain-sensitive respiration from 10 ± 3% of controls in anoxic samples to 51–77% of control. Niflumic acid did not increase ouabain-sensitive respiration. These results demonstrate that glycine, strychnine and nifedipine are 'true' cytoprotectants preventing both cell lysis and promoting the recovery of mitochondrial function and ion transport after an anoxic insult.

Oncosis or cell death characterized by cell and organelle swelling followed by cell lysis is thought to be responsible for organ failure following anoxia/reoxygenation or toxicant exposure (1). In *in vitro* studies, cell death/lysis is routinely measured as the release of intracellular enzymes (e.g. lactate dehydrogenase (LDH)) or the uptake of large vital dyes (e.g. trypan blue) (2). Most *in vitro* studies that have examined the cytoprotective effects of different agents define cytoprotection as the ability of a compound to block cell lysis. On the basis of this definition numerous compounds have been reported to be cytoprotective against anoxia and chemical injury in renal cells. For example, Cl^- channel inhibitors (5-nitro-2-(3-phenylpropylamino)benzoic acid, niflumic acid, indanyloxyacetic acid, diphenylamine-2-carboxylate) and Ca^{+2} channel blockers (verapamil, methoxyverapamil, felodipine, nifedipine) ameliorate cell lysis in rabbit and/or rat renal proximal tubules (RPT) subjected to hypoxia/anoxia or mitochondrial inhibitors (3-7). Chemicals that modulate the neuronal glycine and $GABA_A$ receptors have been reported also to be cytoprotective (8-14). For example, glycine and strychnine prevent cell lysis produced by a diverse group of toxicants with different mechanisms of action (12).

It can be argued that the prevention of cell lysis may not be true cytoprotection because a cell may be dead even though lysis is blocked. The goal of this study was to determine whether a diverse group of cytoprotectants (glycine, strychnine, nifedipine and niflumic acid) with different mechanisms of action not only prevent cell lysis but also allow RPT to regain mitochondrial function and active Na^+ transport. All of the cytoprotectants used in this study have been shown to prevent cell lysis in rabbit RPT subjected to mitochondrial inhibition (3,7,10).

METHODS

RPT were isolated and purified by the method of Rodeheaver et al., (15) from 1.5 - 2 kg female New Zealand White rabbits (Myrtle's Rabbitry, Thompson Station, TN). RPT were suspended at 1 mg RPT protein/ml in an incubation buffer containing (in mM): 1 alanine, 5 dextrose, 2 heptanoate, 4 lactate, 5 malate, 115 NaCl, 15 $NaHCO_3$, 5 KCl, 2 NaH_2PO_4, 1 $MgSO_4$, 10 HEPES (pH 7.4, 295 mOsm/kg). RPT were incubated under 95% air/5% CO_2 at 37° C in a gyrating water bath at 180 rpm. After a 15 min preincubation period, RPT were exposed to a continuous stream of 95% N_2/5% CO_2 (anoxia) for 1 hr in the presence of 0.2 % DMSO (diluent), glycine (2 mM), strych-

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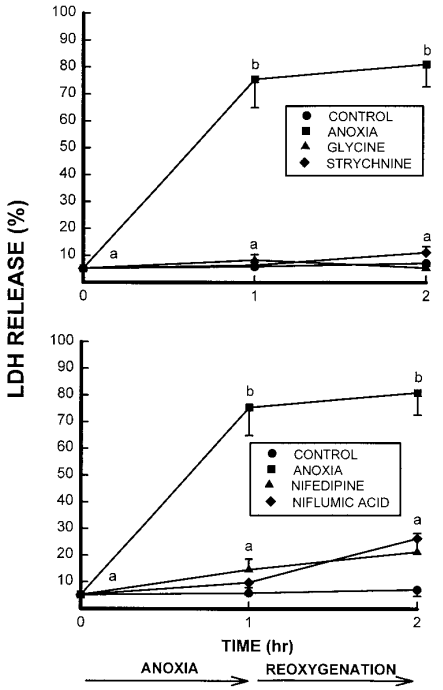

FIG. 1. The effect of glycine and strychnine (upper panel) and nifedipine and niflumic acid (lower panel) on LDH release following anoxia and reoxygenation. Glycine (2 mM), strychnine (2 mM), nifedipine (100 μM) or niflumic acid (100 μM) was added immediately prior to the 1 hr anoxia exposure. Symbols are means ± SE. N = 4-5. Means with different superscripts are significantly different from one another ($P < 0.05$).

nine (1 mM), nifedipine (100 μM) or niflumic acid (100 μM). Immediately after the anoxic period, aliquots were removed for the determination of LDH activity release. The RPT suspensions were reoxygenated with 95% air/5% CO_2 for 1 hr. Following reoxygenation, aliquots of RPT were removed for the determination of LDH activity release and oxygen consumption.

Mitochondrial function was determined by measuring oxygen consumption with a Clark-type oxygen probe as previously described (16). After basal oxygen consumption was obtained, the Na^+/K^+-ATPase inhibitor ouabain (0.1 mM) was added to obtain ouabain-insensitive oxygen consumption. Ouabain-sensitive oxygen consumption, active Na^+ transport, was determined by taking the difference between basal oxygen consumption and ouabain-insensitive oxygen consumption. Na^+/K^+-ATPase activity in RPT membrane fragments was determined by the method of Schwartz et al., (17). Cell lysis was measured by the release of LDH activity as previously described (2). Protein content was quantified by the method of Lowry et al., (18). Data are presented as means ± SE and were analyzed by ANOVA. Multiple means were tested using the Student-Newman-Keuls test and a P value of 0.05.

RESULTS

RPT underwent extensive cell lysis during the anoxic period (75%) and did not increase further during the reoxygenation period (Fig. 1). The addition of glycine, strychnine, nifedipine or niflumic acid to RPT at the onset of anoxia completely blocked LDH release following the anoxic and reoxygenation periods (Fig. 1). These results show that glycine, strychnine, nifedipine and niflumic acid prevent cell lysis in this anoxia/reoxygenation model.

RPT subjected to anoxia/reoxygenation showed a 60% decrease in basal oxygen consumption (Fig. 2). In the presence of glycine, strychnine, nifedipine or niflumic acid RPT basal oxygen consumption increased 1.5-1.9 fold. Basal oxygen consumption can be separated into two components, ouabain-senstive and ouabain-insensitive. Ouabain-sensitive oxygen consumption is directly linked to Na^+/K^+-ATPase activity and active Na^+ transport while ouabain-insensitive oxygen consumption is associated with all other cellular oxygen consuming processes (16). Anoxia/reoxygenation decreased ouabain-insensitive oxygen consumption 45% (Fig. 2). Glycine, strychnine, nifedipine or niflumic acid treatment increased ouabain-insensitive oxygen consumption 1.3-1.6 fold. Anoxia/reoxygenation produced a 90% decrease in ouabain-sensitive oxygen consumption (Fig. 2). Glycine, strychnine or nifedipine treatment increased ouabain-sensitive oxygen consumption 5-8 fold while niflumic acid had no effect.

To determine whether the decrease in Na^+/K^+-ATPase activity observed following anoxia/reoxygenation was the result of a direct effect on the enzyme, Na^+/K^+-ATPase activity was determined in RPT membrane fragments following the reoxygenation period. There

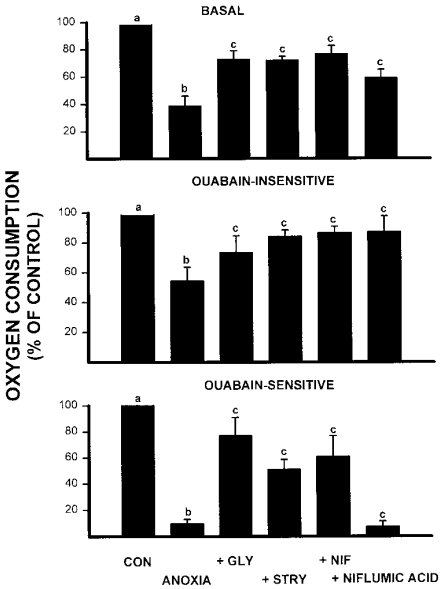

FIG. 2. The effect of glycine, strychnine, nifedipine and niflumic acid on basal, ouabain-sensitive and ouabain-insensitive oxygen consumption. Oxygen consumption was measured following anoxia/reoxygenation. Glycine (2 mM), strychnine (2 mM), nifedipine (100 μM), or niflumic acid (100 μM) was added immediately prior to anoxia. Bars represent means ± SE. N= 5. Bars with different superscripts are significantly different from one another ($P < 0.05$). Basal oxygen consumption in oxygenated controls was 31 ± 2 nmol O_2/mg protein/min. Ouabain-insensitive oxygen consumption in oxygenated controls was 20 ± 1 nmol O_2/mg protein/min.

was no significant difference in Na^+/K^+-ATPase activity between oxygenated controls (237 ± 34 munits/mg cellular protein) and anoxia/reoxygenation samples (259 ± 43 munits/mg cellular protein).

DISCUSSION

Glycine, strychnine and nifedipine prevented cell lysis and promoted the return of RPT mitochondrial function and active Na^+ transport. Thus, these compounds are 'true' cytoprotectants. Niflumic acid was less effective than the other agents in that it prevented cell lysis and promoted partial return of mitochondrial function but did not promote the return of active Na^+ transport. The difference between niflumic acid and the other agents may reflect differences in their mechanism of cytoprotection (see below). These results are consistent with the studies of Mandel et al., (8) and Weinberg et al., (9) who showed that glycine promoted the return of ATP and K^+ contents following anoxia/reoxygenation. From a clinical perspective, these studies are important because agents that are capable of blocking oncosis and allow cells to regain physiological functions may have the ability to promote the return of renal function.

The temporal sequence of events following anoxia or mitochondrial inhibition in RPT has been examined (3,7,11). In rabbit RPT, mitochondrial inhibition leads to respiratory arrest within 1 min and the loss of ATP over the next 10 min. In concert with the loss of ATP, there is K^+ efflux, Na^+ influx, and the loss of the normally negative membrane potential. In the late phase of cell injury Ca^{+2} influx occurs followed by Cl^- influx which leads to cell swelling and lysis (3,7,11). Glycine, strychnine and nifedipine block extracellular Ca^{+2} influx and Cl^- influx, while niflumic acid inhibits Cl^- influx. Thus, intervention in the very late phase of cell injury can still result in cytoprotection. However, it appears that the critical point in which mitochondrial function and ion transport does not return is between the Ca^{+2} influx and Cl^- influx.

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