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Alkalinization during re-oxygenation prevents functional damage by hyperglycaemic hypoxia

Ingrid Euchner-Wamser, Peter Grafe,[CA] Elke Sennefelder and Uwe Schneider

Department of Physiology, University of Munich, Pettenkoferstrasse 12, D-80336 Munich, Germany

[CA] Corresponding Author

HYPERGLYCAEMIA impairs recovery from transient cerebral ischaemia: the importance of tissue acidification for this phenomenon has not been clarified in detail. We investigated this issue in a less complex *in vitro* preparation of isolated rat dorsal spinal roots exposed for 30 min to hyperglycaemic hypoxia. Peak height of compound action potentials recovered minimally in 5 mM bicarbonate. However, recovery was greatly improved by addition of the weak base trimethylamine during re-oxygenation. Addition of the weak acid propionate had no such effect. Cytoplasmic alkalinization improved recovery in a brief time window only: application of trimethylamine after 15 min of re-oxygenation was without beneficial effect. These data emphasize the importance of cytoplasmic acidification for neurophysiological recovery from hyperglycaemic hypoxia during the initial period of re-oxygenation.

Key words: Ischaemia; Hypoxia; Reperfusion injury; Metabolism; Acidosis; Peripheral nerve; Diabetic neuropathy

Introduction

It is well known that hyperglycaemia impairs neurophysiological recovery from transient cerebral ischaemia.[1-3] During ischaemia, hyperglycaemia can potentiate tissue acidification, and this has been considered the most likely reason for the deleterious effects of hyperglycaemia during post-ischaemic recovery.[4] However, the importance of tissue acidification has been challenged on the basis of experimental findings summarized in recent reviews.[5,6] In the present study, we analysed the importance of tissue acidosis for neurophysiological recovery from hyperglycaemic hypoxia using a less complex *in vitro* preparation. It is known that hyperglycaemia impairs electrophysiological recovery of isolated peripheral nerves from transient hypoxia.[7-11] Isolated nerves offer methodological advantages over studies *in vivo* and/or experiments using isolated brain slices. For example, cytoplasmic pH can be modified by means of weak acids and bases at different times during and after hypoxia. The interpretation of observations also seems to be less complicated due to the lack of synaptic interactions and/or effects of neurotransmitters. In this study, we made use of these advantages and tested the importance of tissue pH and buffering power for functional recovery from hyperglycaemic hypoxia in isolated rat spinal roots. The results may help provide insights into the pathophysiology of ischaemic and/or reperfusion damage in the central as well as in the peripheral nervous system (for example in diabetic neuropathy).

Materials and Methods

Animals and preparation: Male Wistar rats, weighing 300–400 g, obtained from Thomae, Biberach, Germany, were anaesthetized with urethane (1.5 g kg^{-1}, i.p., supplemented as required) for a laminectomy to expose the cauda equina and the spinal ganglia. Spinal roots were removed in their entire length (from the spinal cord to the spinal nerve) for *in vitro* recording. The anatomical relationship of the isolated roots to the spinal ganglia enabled us to differentiate between dorsal and ventral roots. After preparation and before transfer to the experimental organ bath, the spinal roots were pre-incubated for 3–6 h in a solution containing 25 mM D-glucose.

Solutions: The standard low bicarbonate solution contained (in mM): NaCl 138.0; $NaHCO_3$ 5.0; KCl 3.0; $CaCl_2$ 1.5; $MgCl_2$ 1.0; D-glucose 25 (pH 7.4). Trimethylamine HCl and Na^+ propionate were added to this solution. Hypoxia was induced by changing the equilibrating gas mixture from 20% O_2/1% CO_2/79% N_2 to 1% CO_2/99% N_2. In some of the experiments, the pO_2 in the organ bath was monitored continuously by a Clark style electrode (Diamond Electro-Tech Inc., Ann Arbor, MI, USA) and found to be < 2 mm Hg within 1–2 min after perfusion of the organ bath with the hypoxic solution. The high bicarbonate solution contained 25 mM $NaHCO_3$ and 1.2 mM NaH_2PO_4, equilibrated with 95% O_2/5% CO_2.

Electrophysiological recordings: The organ bath used to record compound nerve action potentials has been previously described (Marsh ganglion bath; Hugo Sachs Elektronik, March-Hugstetten, Germany).[9-12] It consisted of a three-chambered perspex bath divided into compartments by 1 mm vaseline partitions. The length of the spinal roots was about 25–30 mm: 5 mm were located in the central bath and about 10 mm in each of the lateral compartments. The central compart-

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ment of the organ bath was continuously perfused with normoxic or hypoxic solutions by positive gas pressure in buffer flasks. The flow rate was 14 ml min^{-1} (volume of the central compartment: 0.5 ml); bath temperature was adjusted to 36°C. Spinal roots were stimulated by a suction electrode in the left lateral compartment and conducted action potentials were recorded across the partition between the central and the right compartment (pair of Ag/AgCl electrodes embedded in agar). A peak detector enabled continuous measurement of the height of each action potential peak (stimulation rate 0.2 Hz; stimulus strength 2–2.5 times maximal).

Data and statistics: Data were recorded and stored using the AxoTape system (Axon Instruments Inc., Foster City, CA, USA); the software Origin (MicroCal Software Inc., Northampton, MA, USA) was used for subsequent analysis. Since the absolute values of the height of action potential peaks varied between different spinal roots, the digitized data were normalized to the same pre-hypoxic level. Data are expressed as mean ± s.e.m. Statistical analysis was performed by an unpaired two-tailed *t*-test to assess significance of differences.

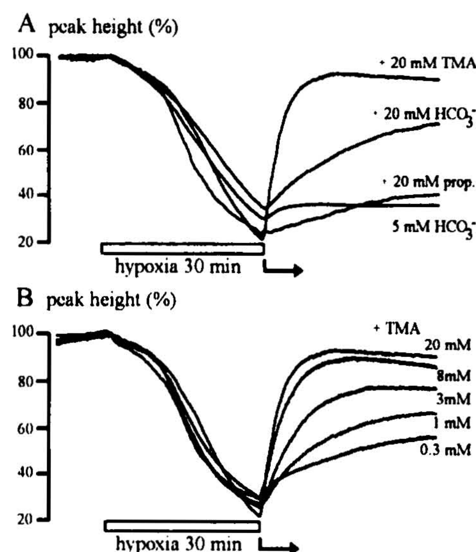

FIG. 1. Recovery from hyperglycaemic hypoxia is modulated by weak acids and bases. Illustrated are averaged changes in the peak height of compound action potentials (mean of at least five different rat dorsal spinal roots in each recording). The bathing solution before and during hypoxia contained 25 mM D-glucose and 5 mM HCO_3^-/1% CO_2. (A) Effects of trimethylamine (TMA, 20 mM), HCO_3^- (20 mM), and propionate (prop., 20 mM) added to this solution at the start of re-oxygenation (arrow). (B) Effects of different concentrations of TMA (0.3–20 mM) added at the start of re-oxygenation (arrow).

Results

Only one exposure to hypoxia was performed on each rat dorsal spinal root and therefore a standardized experimental protocol was used for comparison of data obtained from different spinal roots (we restricted this study to dorsal roots, since sensory fibres are more readily damaged by hyperglycaemic hypoxia[9]). This protocol included the registration of the peak height in compound action potentials for 10 min before hypoxia, during the exposure to hypoxia (30 min), and during the period after re-oxygenation (another 35 min). Modulation of neurophysiological recovery from hyperglycaemic hypoxia by a weak base and weak acids is illustrated in Figure 1A; the corresponding statistics are given in Fig. 3. The peak height dropped to 38% of pre-hypoxic control after 30 min of hypoxia in the standard low bicarbonate solution and recovered only minimally during 35 min of re-oxygenation. An almost complete recovery to 91% was seen when 20 mM trimethylamine (TMA) was added to the standard bathing solution at the start of re-oxygenation. Addition of 20 mM HCO_3^- was less effective (72%) and the effect of 20 mM propionate (41%) did not differ from that of low bicarbonate. The effect of TMA was concentration dependent (Fig. 1B; statistics in Fig. 3).

Addition of TMA improved recovery in a brief time window only (Figs 2, 3). Application 10 and 15 min after the start of re-oxygenation did not result in a recovery of peak height different from that seen in the low bicarbonate solution. This is in strong contrast to the effect of TMA added in the initial period of re-oxygenation.

Discussion

A shift in baseline pH of the brain toward a more alkaline value has been proposed recently as a means to prevent severe brain acidosis.[13] Indeed, in a preliminary report, alkalinization by application of guanidinoethane sulphate was described to be neuroprotective against delayed hippocampal CA1 neuronal death in the gerbil model of forebrain ischaemia.[14] TMA is well known to produce cytoplasmic alkalosis[15,16] with very few other pH-independent effects on electrophysio-

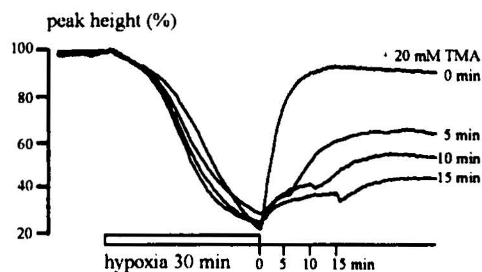

FIG. 2. Trimethylamine (TMA) improved functional recovery in a brief time window only. Illustrated are averaged changes in the peak height of compound action potentials (mean of five different rat dorsal spinal roots in each recording). The bathing solution before and during hypoxia contained 25 mM D-glucose and 5 mM HCO_3^-/1% CO_2. TMA (20 mM) was added to this solution either at the start of re-oxygenation (0 min) or after 5, 10 and 15 min of re-oxygenation in 5 mM HCO_3^-/1% CO_2.

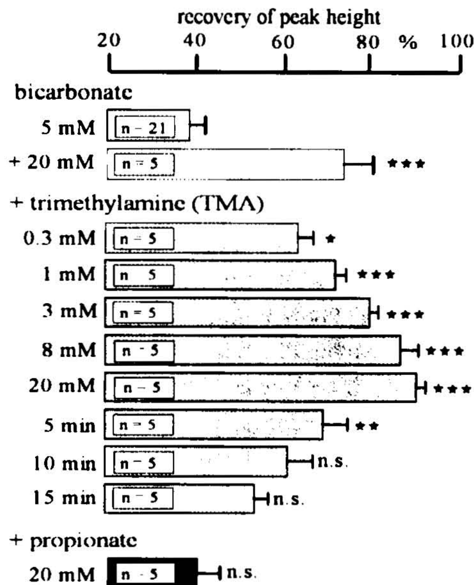

FIG. 3. A statistical analysis of recovery from hyperglycaemic hypoxia. The analysis was performed using the peak height of compound action potentials 35 min after the start of re-oxygenation. Data stem from the recordings illustrated in Figs 1 and 2. Given are means ± s.e.m. (numbers of spinal roots in insets); $*p < 0.05$, $**p < 0.005$, $***p < 0.001$; n.s. = not significant.

logical neuronal parameters.[17] It is therefore extremely likely that an alkaline shift in cytoplasmic pH underlies the great improvement in neurophysiological recovery from hyperglycaemic hypoxia seen in the present study. The beneficial effect of a higher bicarbonate concentration can be explained by an increase in the extra- and/or intracellular buffering power[18] and activation of HCO_3^--dependent transmembrane pH regulating mechanisms. However, both mechanisms have slower effects on cytoplasmic pH than passive alkalinization by means of TMA. In fact, based on observations made in several other cells (for example in snail neurones[19]), an initial transient acid-going shift is to be expected after application of 25 mM HCO_3^-/5% CO_2. The failure of recovery to improve after addition of the weak acid propionate can be explained by its known ability to induce cytoplasmic acidification.[20] This finding is in accordance with recent publications which report that acidosis induced by hypercapnia exaggerates ischaemic brain damage[21] and that persistence of acidosis contributes to post-ischaemic electrophysiological deficits.[22]

Addition of TMA during re-oxygenation was able almost completely to restore functional electrophysiological parameters to their pre-hypoxic values. This indicates that the usual loss of excitability seen after hyperglycaemic hypoxia in low bicarbonate[8-11] is due to electrophysiological damage produced after and not during hypoxia. Reperfusion injury has been described in a variety of tissues, including the peripheral nerve.[23] Protection against such damage by cytoplasmic alkalinization supports the previous suggestion[4] that cytoplasmic acidification may be a key factor in the pathogenesis of this phenomenon. However, a few minutes after the start of re-oxygenation, passive alkalinization by addition of TMA no longer improved functional deficits. This indicates that the importance of cytoplasmic pH for functional recovery is limited to the initial period of re-oxygenation. pH-dependent factor(s), but not pH itself, seem to underlie the failure of recovery if re-oxygenation proceeds in an acidic cytoplasm. Such factors might be oxygen free radicals,[5,6,23] high cytoplasamic calcium[24] and/or pH-induced inhibition of the sodium pump.[25]

Conclusion

This study provides experimental evidence that passive cytoplasmic alkalinization during re-oxygenation improves recovery from hyperglycaemic hypoxia. The importance of acidosis for aggravation of ischaemic damage by hyperglycaemia is therefore supported. However, the data indicate that the role of cytoplasmic pH for electrophysiological recovery is restricted to the initial period of post-hypoxic re-oxygenation. These results indicate that passive cytoplasmic alkalinization might be a useful pharmacological tool to further explore the pathophysiology of ischaemia and/or reperfusion in the central as well as in the peripheral nervous system.

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