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ANOXIA-INDUCED CHANGES IN OPTIMAL SUBSTRATE FOR PERIPHERAL NERVE

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Abstract—Hyperglycemia accentuates the injury produced by anoxia both in the central and peripheral nervous system. To understand whether this is a consequence of changes in metabolic pathways produced by anoxia, the effect of the metabolic substrate used by the rat peripheral nerve on the nerve action potential (NAP) was studied in the presence and absence of anoxia. In the continuously oxygenated state, the NAP was well preserved with glucose, lactate, as well as with high concentrations of sorbitol and fructose but not β-hydroxybutyrate, acetate or galactose. With intermittent anoxia, the pattern of substrate effects on the NAP changed markedly so that low concentrations of fructose became able to support neurophysiologic activity but not high concentrations of glucose. These alterations occurred gradually with repeated episodes of anoxia as reflected by the progressive increase in the time needed for the NAP to disappear during anoxia when using glucose as substrate. This ''preconditioning'' effect was not seen with other substrates and an opposite effect was seen with lactate. In fact, the rate at which the NAP disappeared during anoxia was not simply related to degree of recovery after anoxia. These are distinct phenomena. For example, the NAP persisted longest during anoxia in the setting of hyperglycemia but this was the state in which the anoxic damage was most severe. Correlating the results with existing literature on the metabolic functions of Schwann cells and axons generates testable hypotheses for the mechanism of hyperglycemic damage during anoxia and lead to discussions of the role for a metabolic shuttle between Schwann cells and axons as well as a potential important role of glycogen.

Key words: anoxia, ischemia, peripheral nerve, nerve conduction, glucose, diabetes.

INTRODUCTION

Lack of oxygen delivery is an important mechanism of injury in the peripheral nerve as well as the central nervous system. In vasculitic neuropathy (Collins and Periquet-Collins, 2009), occlusion of the small vessels that supply the peripheral nerve produces anoxia. Similar events contribute to nerve injury in critical illness polyneuropathy (Van den Berghe et al., 2006; Hermans et al., 2009; Mikaeili et al., 2012) where it has also been shown that hyperglycemia may enhance the degree of injury. This creates the possibility that other manipulations of the peripheral nerve metabolic pathways may be effective in ameliorating anoxic injury. This intersection between anoxic injury and metabolic pathways is also important since anoxia (Dyck et al., 1986, 1999; Dyck, 1989) and oxidative stress (Kellogg and Pop-Busui, 2005; Vincent et al., 2007; Russell et al., 2008) may be a contributing factor to diabetic neuropathy, which can be seen in up to 50% of patients with diabetes (Dyck et al., 2011; Callaghan et al., 2012). From a more fundamental standpoint, the peripheral nerve provides a unique window into the effects of anoxia. One measure of its function, the nerve action potential (NAP), is similar in both animal and human nerves and so allows comparison between the results obtained in animal systems to those in humans. Furthermore, small nerves such as the rat sciatic nerve have a sufficiently low metabolic rate that they can be supplied by the diffusion of oxygen from the surrounding medium. This makes well-controlled experiments on *in-vitro* preparations possible and potentially applicable to the human condition.

There have been relatively few investigations of the optimal substrates to support neurophysiologic function in the peripheral nerve. Many older studies (Landowne and Ritchie, 1971; Ritchie and Straub, 1980) used biochemical techniques to determine the rates of oxygen and glucose consumption by the peripheral nerve under various conditions. Subsequent biochemical studies by Greene and Winegrad (1979) suggested that alternate substrates such as β-hydroxybutyrate could be used to maintain cellular supplies of ATP and creatine phosphate for up to 2 h *in-vitro* but did not explore the neurophysiological consequences of these using these substrates. Brown (Brown et al., 2001) studied the NAP in continuously oxygenated optic nerve for 2 h. He found that glucose, mannose, lactate, pyruvate, and to some extent glutamine did support the neurophysiologic function of the optic nerve during that short experiment while β-hydroxybutyrate, octanoate, sorbitol, alanine, aspartate,

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Abbreviations: ANOVA, analysis of variance; AUC, area under the curve; CSR, conditioned stimulus response (paired pulse response); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LDH, lactate dehydrogenase; MCT, monocarboxylate transporter; NAP, nerve action potential; SDH, sorbitol dehydrogenase; T_{50a}, time for NAP to drop to 50% of initial value during anoxia; T_{50r}, time for NAP to attain 50% of eventual value during anoxia.

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and glutamate failed to support function during those brief experiments. None of these studies considered the effects of anoxia on the optimal substrate.

A number of investigators have studied the acute neurophysiologic effects of anoxia but none have explored the long-term effects. Leppanen and Stys (1997) used the rat optic nerve model to show that pyruvate was able to support the membrane potential for up to 2 h and that this was unaffected by the application of metabolic inhibitors such as iodoacetate indicating that most of the energy for the rat optic nerve comes from aerobic metabolism however recovery after anoxia was not studied. In biochemical studies, Stewart et al. (1965) suggested that glycogen is used as a substrate initially during anoxia, a result that was confirmed in neurophysiologic studies of *in-vitro* optic nerve by Brown et al. (2003) who found a linear relationship between the time a nerve can tolerate anoxia and the concentration of glycogen at the time of anoxia onset.

Lindstrom and Brismar (1991) did study the NAP during a brief period of anoxia and recovery finding that the rate of decrease in the NAP with anoxia and the rate of recovery were slowed by ouabain inhibition of the Na-K ATPase. Schneider et al. (1993) studied the effect of different substrates on nerves during anoxia and a brief recovery period although some tests were performed on ventral roots and some on dorsal roots. They demonstrated negative effects of high concentrations of glucose and D-mannose. They also showed that 22.5 mM of D-galactose, L-glucose, D-fructose, and L-fucose did not have such effects and were able to support neurophysiologic function but did not study other concentrations. They also suggested that blockade of glycolysis with iodoacetate may facilitate recovery from hyperglycemic anoxia but not normoglycemic anoxia. None of these studies have studied the response to more than a single period of anoxia and were unable to find anoxia-induced changes in metabolic pathways. This information however is critical to the design of a rational pharmacotherapy since changes in metabolism over extended periods may completely change the effect of any putative therapeutic agent. In addition, all of these neurophysiologic studies use a single marker, such as the amplitude or area under the curve to describe the NAP and do not use additional markers such as velocity and duration that may provide additional insight into the biological processes occurring.

Stecker and Stevenson (2013) confirmed Schneider et al.'s (1993) observations regarding the interaction between oxygen state and glucose concentration but also showed that there were significant changes in the nerve function with multiple periods of anoxia. This illustrates the fact that an understanding of peripheral nerve metabolism in the continuously oxygenated state is not sufficient to understand the pathologic state in which the nerve is subjected to anoxia. This problem is also encountered in the study of cancer cells where the hypoxic environment favors a shift toward anaerobic metabolism (Warburg effect). It has lead to the development of new therapies for cancer (Masoudi-Nejad and Asgari, 2014) and may lead to new therapies for peripheral nerve injury.

In the current study, the ability of various metabolic substrates to sustain the NAP were studied as a function of both the concentration of the substrate and the presence/absence of anoxia. Studying both of these conditions will help to create a unified picture of how peripheral nerve function depends on the nature of the metabolic substrate available. In addition, studies carried out over a more extended time period allow insight into the changes that occur in the peripheral nerve after anoxia exposure.

EXPERIMENTAL PROCEDURES

Experimental setup

A full description of the methods is given in previous papers (Baylor and Stecker, 2009; Stecker and Baylor, 2009; Stecker et al., 2013a,b). Under a protocol approved by the IACUC (Winthrop University Hospital Protocol, WUH-MS#1), a total of 140 sciatic nerves from 70 Sprague–Dawley rats (Hilltop, Scottdale, PA, USA) were studied. The segment studied was the proximal part of the nerve beginning a few millimeters distal to the spine and was roughly 1.2–1.4 cm in total length. The rats were male retired breeders with an average age of 32 weeks with a range of 25–48 weeks. At least four nerves with high quality data in each condition were obtained. Each sciatic nerve was dissected and placed into a perfusion chamber and stimulated using stainless steel subdermal electrodes (Fig. 1). The stimulus consisted of paired unipolar pulses separated by 4 ms, each with a 15-mA peak current, a duration of 0.01 ms and an overall pair repetition rate of 5 Hz. Bipolar recordings of the NAP were made, digitized at 99 kHz/channel, averaged and stored every 4 s. The average distance between the stimulating and recording electrodes was 0.95 cm.

Each experiment (Fig. 1) lasted at least 16 h. In some experiments, termed "stability", the nerve remained continuously oxygenated during the entire experiment. In other experiments (termed anoxia experiments) after a 60-min equilibration period, the nerve was subjected to 90-min periods of oxygenation followed by 90 min of anoxia. The terms phase and cycle are used as convenient surrogate markers for the time during each experiment. The first phase encompassed the first 150 min of the experiment. When $i > 1$ the i'th phase encompassed the time between $150 + 90 * (i - 2)$ and $150 + 90 * (i - 1)$ minutes into the experiment. In all anoxia experiments, phase 1 is the baseline and, in anoxia experiments, phases 2, 4, 6, 8, 10 represent successive periods of anoxia and phases 3, 5, 7, 9, 11 represent the subsequent periods of reoxygenation (Figs. 1 and 2). In the nerves that were continuously oxygenated, the concept of phase is only a convenient method of referring to various time points within the experiment. The term cycle is also used to refer to various time periods during an experiment. Data ascribed to the j'th cycle represent the mean value of a specified parameter during the phase $2j + 1$. In the nerves undergoing intermittent anoxia, these are the data from the period of oxygenation following j cycles