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IN VIVO ^{31}P NMR SPECTROSCOPY STUDIES OF HALOTHANE INDUCED PORCINE STRESS SYNDROME. NO EFFECT OF C-PHENYL N-TERT-BUTYL NITRONE (PBN)

PAULA J. GAREAU[a], EDWARD G. JANZEN[a,b,c*], RHEAL A. TOWNER[c], and WENDY A. STEWART[c]

Departments of Clinical Studies[a] and Biomedical Sciences[b] and the MRI Facility[c], Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada, N1G 2W1

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Porcine stress syndrome (PSS) which is an example of malignant hyperthermia (MH) in swine has previously been attributed to oxidative stress primarily due to an inherited antioxidant abnormality in MH susceptible (MHS) animals. C-phenyl-N-*tert*-butyl nitrone (PBN), a free radical spin trap, was selected to investigate whether free radicals are involved in MH. If free radicals cause the MH stress attack, then PBN should alter the time required for the onset of the stress attack, or perhaps protect the animal from experiencing the stress attack. *In vivo* phosphorus-31 (^{31}P) magnetic resonance spectroscopy (MRS) was used to monitor metabolism in three to four week old normal and MHS piglets administered halothane as the stress challenge. Malignant hyperthermia was not reproducibly induced by halothane anesthesia. For those animals which did develop MH a dramatic fall in the level of PCr and a rise in the level of Pi was detected by ^{31}P MRS. Intravenous administration of PBN prior to halothane exposure had no effect on the number of animals experiencing the stress attack. PBN does not appear to prevent, delay or reverse the onset of halothane-induced MH in three to four week old MHS piglets. The primary events leading to the MH syndrome do not appear to be influenced by the intervention of the type of free radicals normally trapped by PBN.

KEY WORDS: PSS, porcine stress syndrome; MH, malignant hyperthermia; MHS, malignant hyperthermia susceptible; ^{31}P MRS, phosphorus-31 magnetic resonance spectroscopy; PBN, C-phenyl N-*tert*-butyl nitrone; PCr, phosphocreatine; Pi, inorganic phosphate.

INTRODUCTION

In various cases of biological stress the intermediacy of free radicals has been postulated. Although the cause for and the effect of the presence of reactive molecules such as oxygen-centered free radicals is difficult to establish in biological systems, these proposals continue to survive at least until better explanations become available.

Oxidative stress has been defined as "a disturbance in the prooxidant-antioxidant balance in favor of the former"[1]. Although this definition does not explicitly identify free radicals as intermediates in oxidative stress it is generally assumed that

*to whom correspondence should be addressed; alternate address: Free Radical Biology and Aging Research Program, Oklahoma Medical Research Foundation, 825 N.E. 13th St., Oklahoma, U.S.A. 73104

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such species may be either the cause or consequence of biological stress. In fact, the effect of adding additional antioxidant to the system (e.g. tocopherol) is often interpreted in terms of support or disproof of a free radical mechanism underlying the disturbance in the system.

The effect of adding C-phenyl-N-*tert*-butyl nitrone (PBN) to a biological system subjected to stress has recently been evaluated in terms of a test for the intermediacy of free reactive radicals. For example, PBN reduces neuronal damage in gerbils subjected to forebrain ischemia[2,3], and reverses brain protein oxidation, produces a decrease in enzyme activity and in the loss of temporal and spatial memory after ischemia/reperfusion injury[4,5], inhibits rat liver edema caused by CCl_4[6], protects against reperfusion-induced arrhythmias[7], reduces muscle fatigue in endurance tests with mice[8], provides long-term survival in Noble-Collip drum-shocked rats[9] and reduces death in rats subjected to septic shock[10]. Although the mechanism of PBN involvement is not known it is assumed that PBN traps reactive free radicals and in this sense PBN is assigned the role of an antioxidant. When PBN has a positive effect on the disturbance which causes the biological stress it is concluded that the stress is caused by or at least aggravated by the intermediacy of free radicals.

Malignant hyperthermia (MH) is a hypermetabolic syndrome which can be triggered in genetically susceptible individuals by potent anesthetic agents, such as halothane. Clinical signs include tachycardia, hyperventilation, acidosis, cyanosis of the skin, muscle rigidity and a rapid rise in body temperature[11]. In swine, MH is also referred to as the porcine stress syndrome (PSS) since it may be triggered by physical, thermal or anoxic stresses as well as by anesthesia. Although stress susceptibility is known to be inherited, the precise mechanism of the disease is unknown. The molecular lesion responsible for MH has been found to be located in the calcium-release channel of muscle sarcoplasmic reticulum (SR) and results in a defect in calcium homeostasis in skeletal muscle[12,13]. This channel has been shown to be hypersensitive to triggering agents such as halothane, caffeine and calcium. It is thought that halothane causes MH indirectly by perturbing the muscle membrane and allowing calcium to diffuse into the cell, increasing calcium concentration to a threshold which opens the hypersensitive, but not the normal calcium channel[12].

A number of studies have pointed to a compromised antioxidant defence system in MH susceptible (MHS) pigs which may be responsible for the perturbation in calcium in skeletal muscle. Duthie *et al.* have shown that cell membrane integrity is impaired in MHS pigs as indicated by a marked increase in plasma pyruvate kinase activity. Plasma concentrations of the byproducts of lipid peroxidation (measured as thiobarbituric acid reactive substances [TBARS]) were also increased implicating free radicals as the species responsible for the membrane damage. Erythrocytes from MHS pigs incubated with hydrogen peroxide produced more TBARS than did those from control pigs[14,16] as did incubations of microsomes prepared from livers of MHS pigs[15]. These data provide evidence of an antioxidant abnormality in MHS pigs which results in an enhanced susceptibility to free radical mediated lipid peroxidation and which can be partly offset by the addition of an antioxidant in the form of an increased dietary intake of vitamin E. Lipid peroxidation could lead to disruption of muscle cell membranes and an increase in myoplasmic calcium which would generate muscle contraction. Schanus *et al.* have also reported that many symptoms of MH are consistent with oxidative damage, stating that MH is likely due to severe oxidative damage initiated by halothane and that the failure to prevent this oxidative damage is the biochemical basis for the disease[17].

Magnetic resonance spectroscopy (MRS) provides a noninvasive method of studying the metabolic state of living tissues. Phosphorus-31 (^{31}P) MRS can be used to detect and follow intracellular changes in the key phosphorus intermediates involved in energy metabolism in skeletal muscle. These are phosphate monoesters (PME), inorganic phosphate (Pi), phosphate diesters (PDE), phosphocreatine (PCr) and three phosphates of adenosine triphosphate (γ, α and β-ATP). This technique has been used to follow the development of MH induced by halothane and succinylcholine in adult pigs. It was shown that the onset of the syndrome is characterized by a rapid and dramatic fall in [PCr] and a corresponding rise in [Pi][18].

This study was undertaken to investigate whether free radicals are involved in the MH syndrome. ^{31}P MRS was used to monitor halothane induced MH in MHS and normal piglets 3 to 4 weeks of age with and without prior PBN administration. In light of the previous reports of a protective role for PBN in cases of oxidative stress, a similar effect was expected in the case of MH if free radicals are to be implicated as the cause of this disease.

MATERIALS AND METHODS

Swine

Permission for these studies was granted by the Animal Care Committee at the University of Guelph. MHS swine from four litters were obtained at three to four weeks of age from the Eramosa Research Farm, Eramosa, Ontario. These were Pietrain × (Yorkshire × Poland China) swine that were inbred to homozygosity for the MH defect. Ten Yorkshire swine were used as controls. These were provided by the Ontario Ministry of Food and Agriculture, from a herd maintained at the Arkell Swine Research Centre.

Animal Preparation

MHS and control piglets were premedicated by intramuscular injection with ketamine hydrochloride (20 mg/kg) for restraint during the insertion of an intravenous line. General anesthesia was induced by intravenous injection of sodium pentobarbital (10 – 15 mg/kg). Piglets were positioned in an imaging cradle and a 2.8 cm diameter four turn ^{31}P-surface coil placed on the skin above the hind leg extensor muscles. A respiratory gating device was set up to monitor breathing, and rectal temperature was measured before and after each experiment to record changes in body temperature.

NMR Studies

^{31}P spectra were recorded at 34.6 MHz using a SISCO 31 cm bore imaging spectrometer (Spectroscopy Imaging Systems Corporation, Fremont CA) operating at 2.0 Tesla. Magnetic field homogeneity was optimized while observing the free induction decay (fid) of the H_2O resonance. Spectra were collected using the depth pulse sequence with 128 transients and an intersequence delay time of 1 sec., giving a total acquisition time of 2.4 min. for each spectrum. A line broadening factor of 15 Hz was applied to the fid before Fourier transformation. The spectra were phased and a common baseline for all peaks was determined. Data analysis was by deconvolution

of the observed spectra into individual Lorentzian line shapes. The following parameters were made available for each line: frequency, height, area and linewidth at half height of the line. The actual spectrum, fitted spectrum and individual fitted components were plotted and the values given for peak heights and peaks area were used to plot changes in [PCr] and [Pi] over time. Four control spectra were obtained from all piglets while pentobarbital-anesthetized. Two experiments were performed.

Halothane Studies

Ten swine susceptible to MH and five control swine were prepared as previously described. Halothane anesthesia (2% in 100% oxygen) was initiated immediately following the collection of control spectra and continued until either signs of the MH syndrome became evident or for 2 hours. ^{31}P spectra were obtained every five minutes as halothane anesthesia progressed to monitor energy state changes within the muscle. The onset of MH was diagnosed by a dramatic decrease in the PCr peak and a corresponding increase in the Pi peak, as observed by ^{31}P MRS. These studies allowed us (a) to determine whether the MH syndrome could be reproducibly induced in 3–4 week old susceptible piglets by halothane anesthesia and (b) to test the reliability of ^{31}P MRS as a sensitive technique for detecting the onset of the syndrome by following the intramuscular changes in the high energy phosphates.

PBN Studies

PBN was obtained from Aldrich or Sigma. This spin trapping agent was chosen because (a) it has previously been established that it can function as an effective free radical spin trapping agent *in vivo*, (b) it has proven to have some type of protective effect on a variety of conditions thought to be due to oxidative stress, and (c) in a separate experiment where one control piglet was administered PBN (3.85 ml/kg) intravenously and phosphorus spectra obtained for one hour, no noticeable changes in phosphorus metabolism were detected by ^{31}P MRS. The intravenous dose of PBN to be used for these experiments was selected to be 3.85 ml/kg body weight, based on a study which used PBN in dogs undergoing myocardial ischemia/reperfusions[7]. The intravenous route of administration was necessary to allow for continuous monitoring by ^{31}P MRS, since the animals had already been positioned inside the magnet. Nine swine susceptible to MH and five control swine were administered PBN (3.85 mg/kg in 1 mL saline) intravenously immediately following collection of control spectra. Four spectra were then obtained allowing 20 minutes to pass before halothane anesthesia was initiated. As in previous experiments, ^{31}P-spectra were collected continuously during the halothane exposure and anesthesia was continued until MH developed or for 2 hours. One MHS piglet was administered twice the original dose of PBN (7.7 ml/kg). These PBN studies were all executed to establish whether PBN could delay or prevent the onset of MH. Two additional experiments were performed to determine if PBN was capable of reversing the effects of the stress syndrome. Two MHS piglets were prepared and exposed to halothane as in previous experiments. Phosphorus spectra were collected continuously during the anesthesia. Immediately upon recognition of the onset of MH, PBN (3.85 ml/kg) was administered intravenously. Spectra continued to be collected for 30 minutes after PBN administration.

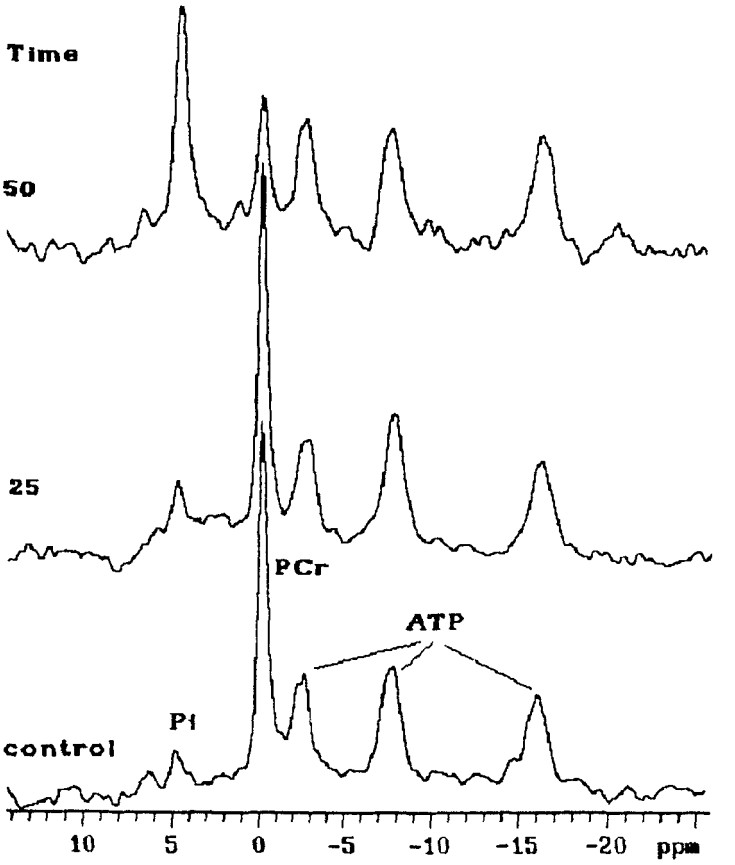

FIGURE 1 A series of phosphorus spectra of pig muscle *in vivo* as a function of the time of halothane exposure. These spectra were obtained from one MH susceptible piglet on a SISCO 2.0 Tesla/31 cm bore imaging spectrometer with a 2.8 cm diameter surface coil and a depth pulse sequence. The control spectrum was obtained while pentobarbital-anesthetized. Time represents minutes of halothane anesthesia. Peak assignments: Pi = inorganic phosphate, PCr = phosphocreatine, ATP = γ, α, and β peaks of adenosine triphosphate. Halothane induced a reduction in the level of PCr and an increase in the level of Pi which characterizes the onset of the MH episode.

RESULTS

The MH syndrome was induced in six out of ten MHS piglets after administration of halothane for 25–50 minutes. Four of the ten piglets did not show any sign of developing MH after 2 hours of halothane anesthesia. For six out of the ten MHS piglets the ^{31}P-spectra showed a dramatic decrease in [PCr] and an increase in [Pi] at the onset of the syndrome (Figure 1). This occurred along with other characteristic symptoms of MH, including respiratory distress, muscle rigidity, blotchy cyanosis of the skin and a rise in body temperature. None of the five age matched control piglets showed signs of developing MH after 2 hours of halothane anesthesia.

In six of seven MHS piglets administered PBN prior to halothane anesthesia, the MH syndrome developed after 30–85 minutes of halothane exposure. ^{31}P MRS

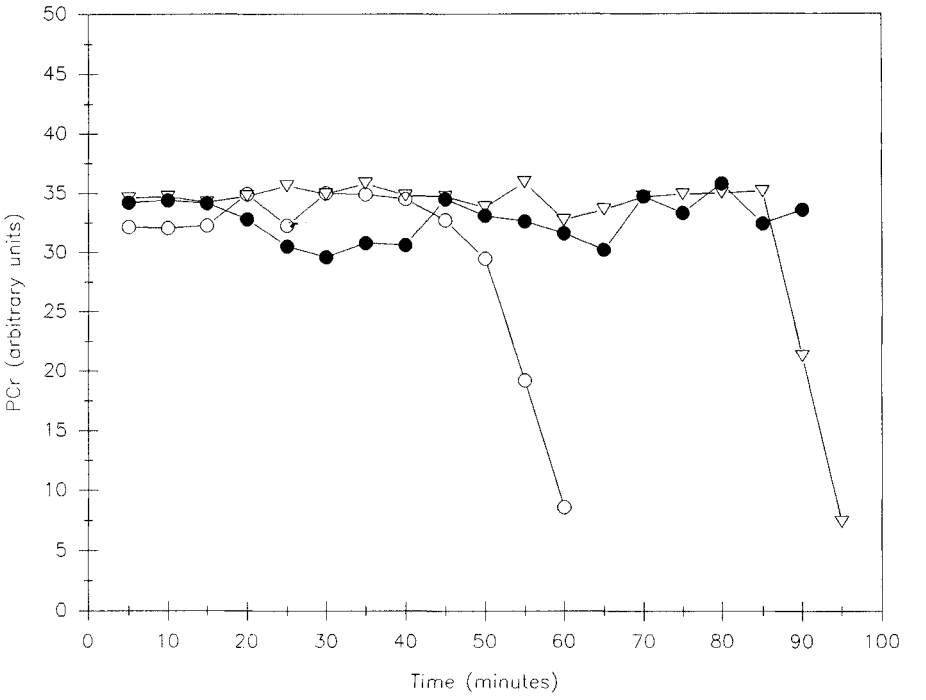

FIGURE 2 Variation in pig muscle phosphocreatine (PCr) over time for one representative control piglet (solid circles) and one representative MH susceptible piglet exposed to halothane initiated at 20 minutes (hollow circles) and for one MH susceptible piglet administered PBN at 20 minutes with halothane initiated at 40 minutes (hollow triangles). These results represent experiments from a SISCO 2.0 Tesla/31 cm bore imaging spectrometer with a 2.8 cm diameter surface coil and a depth pulse sequence. All PCr peak height values were divided by their individual vertical scale values to normalize the data for accurate comparisons between spectra. For both MH susceptible piglets a dramatic fall in the level of PCr occurs marking the onset of the MH episode.

revealed a dramatic fall in the PCr peak along with a rise in the Pi peak, at the onset of the syndrome, which was previously observed in MHS piglets administered halothane alone. Variations in the levels of PCr over time for one control piglet and for one MHS piglet exposed to halothane and one MHS piglet exposed to halothane plus PBN are shown in Figure 2. Only one of these seven piglets did not develop MH after exposure to halothane for 2 hours. None of the five control piglets administered PBN displayed signs of developing MH after 2 hours of halothane anesthesia. One MHS piglet administered twice the original dose of PBN (7.7 ml/kg) prior to halothane exposure showed signs of the MH syndrome after approximately 30 minutes of halothane anesthesia. In two additional MHS piglets PBN was administered upon recognition of the onset of the stress syndrome. As the syndrome developed in these pigs ^{31}P MRS revealed the characteristic changes in skeletal muscle metabolism associated with halothane-induced MH. After administration of PBN the level of PCr continued to decline and the level of Pi continued to climb. As this disturbance progressed the PCr reserve was completely depleted and eventually ATP reservoirs began to deteriorate.

DISCUSSION

^{31}P MRS proved to be a reliable method for determining biochemical information about the levels of phosphorus intermediates in 3–4 week old piglets and provided a phosphate-metabolic profile for normal and MHS skeletal muscle. The MH was characterized by a rapid fall in phosphocreatine and an elevation of inorganic phosphate, changes which consistently marked the onset of the syndrome. These characteristic changes in the phosphorus metabolites have been documented previously in ^{31}P NMR spectroscopy studies of the MH syndrome in MHS swine and humans[18–22]. However the youngest piglets used in these studies have been 8 to 10 weeks of age. Three of these studies[19–22] on muscle biopsies from MHS pigs indicated a decrease of PCr concentration after exposure to halothane.

An *in vivo* study[18] which used a combination of halothane and succinylcholine also indicated a decrease of PCr concentration; however, only three pigs were examined. More recently, Geers *et al.* reported results which indicated that the PCr decrease within muscle from MHS pigs exposed to halothane, as determined by *in vivo* ^{31}P NMR spectroscopy, was 100% reliable with respect to homozygous MHS pigs[21]. Only six of ten MHS pigs administered halothane and six of seven MHS pigs administered PBN plus halothane showed signs of the MH syndrome which indicates that MH cannot be reproducibly induced in these young piglets by halothane anesthesia alone. The fact that four of ten MHS piglets did not show signs of MH with halothane exposure may be explained by the fact that MH susceptibility is not expressed to the same extent in all pigs. It has been suggested that the MH gene is not fully expressed before roughly eight weeks of age[23]. There is a full spectrum in the expression of the MH trait ranging from very mild to very severe and depending on the nature of the primary genetic defect and also on the degree of exposure to MH triggers[12]. Stress plays an important role in the genesis of MH reactions and it appears that individual piglets can tolerate different amounts of "stress" induced by halothane alone or in combination with other stressors.

PBN presumably acts by trapping free radical species thus preventing oxidative damage. Results from these studies indicate that PBN (3.85 ml/kg) does not appear to protect against or reverse halothane-induced MH in young pigs. Despite the studies which report evidence of an antioxidant abnormality in MH pigs, our data do not provide additional support for free radical involvement in the onset of the MH syndrome in MHS piglets. Further studies are underway to investigate the following postulate: (a) the factors leading up to and the influences driving the animal into the actual PSS attack are not significantly affected by free radical intermediates; and (b) the tissue damage caused by the PSS event is caused by free radical oxidative stress.

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