[Frontiers in Bioscience 5, a18-23, September 1, 2000]

INTACT SMOOTH MUSCLE METABOLISM: ITS RESPONSES TO CYANIDE POISONING AND PYRUVATE STIMULATION

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1. ABSTRACT

Smooth muscle mitochondria are unique in their compartmentalization of metabolism with the contractile proteins and putative role in cell fate choice. In this study we examine the relative and quantitative differences that smooth muscle mitochondria have with regard to cyanide inhibition. The effect of cyanide poisoning in the mitochondria of smooth muscle was examined in the intact porcine carotid artery and intact guinea pig stomach. The respiratory responses of these tissues were monitored in the presence of cyanide and following the addition of various metabolites. In HEPES buffer with 10 mM glucose as the substrate, it was found that the EC_{50} for cyanide inhibition was 0.11±0.02 and 0.14±0.02 mM in the pig carotid and guinea stomach respectively. We also found that the signaling metabolite, pyruvate could partially reverse this inhibition. With pyruvate (10 mM) as the substrate, the EC_{50} increased significantly to 6.52±0.11 (carotid artery) and 1.95±0.30 (stomach) mM as well as being significantly different between the tissues. This apparent resistance to cyanide inhibition caused by pyruvate was lost when sodium bicarbonate buffer was used (EC_{50} in the presence of pyruvate of 0.13±0.04 and 0.25±0.04 for carotid and stomach respectively). HEPES buffer permitted pyruvate's protection from cyanide poisoning whereas bicarbonate buffer did not. The rate of respiration caused by pyruvate re-stimulation was not significantly different than control in the stomach (2.63±0.77 and 2.69±0.3 μMol O_2/min/g dw respectively) but was significantly greater in the carotid artery. Therefore, smooth muscle with 10 mM pyruvate and 1 mM cyanide had a rate or respiration significantly greater than with 10 mM glucose alone (0.90±0.2 and 0.59±0.06 μMol O_2/min/g dw respectively). Using ^{31}P NMR spectroscopy, we observed a complete normalisation of high energy phosphates and pH in the guinea pig stomach smooth muscle caused by pyruvate after cyanide poisoning. These results suggest that cyanide's toxicity of smooth muscle oxidative metabolism is affected by the buffer (HEPES versus Bicarbonate) and metabolic signalling molecules or substrates (pyruvate versus glucose) in which the tissues are exposed to as well as tissue to tissue variations.

2. INTRODUCTION

There are many homeostatic systems in smooth muscle that require Mg^{++} such as myosin light chain kinase (MLCK), the myosin ATPase and the mitochondria. It has been found that intracellular Mg^{++} concentration is tightly protected to levels of above 4 (moles/g protein). This suggests that the maintenance of intracellular Mg^{++} be tightly controlled and we have recent unpublished information that isolated smooth muscle mitochondria require Mg^{++}. Cytosolic Mg^{++} is a requirement for the activity of MLCK, because Ca^{++} and Mg^{++} -free solutions actually inhibit the kinase. It has been proposed that Ca^{++}-dependent mechanisms for the regulation of smooth muscle that are Mg^{++}-dependent.

Mg^{++} has been postulated to antagonise intracellular and mitochondrial Ca^{++} signalling. Mg^{++} may directly displace Ca^{++} from its binding sites for this antagonistic action. Some reports have suggested competition between Mg^{++} and Ca^{++} ions for binding sites on the MLCK regulatory protein, calmodulin, and entrance into the mitochondria. When Mg^{++} is bound to calmodulin, Ca^{++} is unable to activate MLCK. Thus there is a role for Mg^{++} in the regulation of the smooth muscle function, metabolism and mitochondria. Another unique aspect of smooth muscle is that the mitochondria are compartmented to produce energy for contractile work while glycolytic ATP is used for membrane and homeostatic events. Because of the control requirements needed for this unique relationship in the smooth muscle we decided to determine if the flux through the mitochondrial oxidative phosphorylation was affected by cyanide and pyruvate. This is especially important because pyruvate is an important metabolite and signalling molecule for the control of mitochondrial and oxidative metabolism.

There is a large body of research discussing and describing the poisoning of mitochondria by cyanide binding to and inhibiting cytochrome C oxidase. Cytochrome C Oxidase has a bimetallic haem iron-copper reaction centre and catalyses the reduction of O_2 to water simultaneously with the translocation of protons (1). There are two coppers in it, with the two haem planes perpendicular to the membrane plane. It has been the centre of much research using the purified enzyme, isolated mitochondria and mitochondrial particles (1). The interest in cytochrome C oxidase is indeed expanding because its structure down to 2.8 Å has recently been reported (2-5).

The redox state of cytochrome C oxidase is important to the normal function of the enzyme and there is evidence for conformational changes of the enzyme caused by different redox states (6). Under aerobic conditions, the fully reduced enzyme is not normally found (1) and there is little *in vivo* data on the relative toxicity responses of the protein in the different redox states. There are some reports, however, that the reduced form of cytochrome C oxidase *in vitro* is more vulnerable to the action of cyanide (7, 8). When the enzyme is fully reduced, and reduction of O_2 is initiated, there is a 'slip' in proton pumping which may correspond to a short circuit in the reaction scheme (1, 5). The rate of inhibition by cyanide with isolated oxidised cytochrome C oxidase is slow even in the presence of 1 mM cyanide. It has been reported that the oxidised-inhibited enzyme is readily reactivated upon removal of cyanide (7). Therefore, it appears that the ability of cyanide to act on and be removed from cytochrome C oxidase is effected by the redox state of the enzyme. The dissociation of cyanide from the isolated enzyme, compared to isolated mitochondria, is variable depending upon solution conditions. For example, the binding is pH dependent in the mitochondrial fraction but not the isolated enzyme, with the largest changes occurring above pH 7.0. Interestingly, excess ATP causes a 1000 fold slowing of cyanide inhibition and ATP/ADP ratios are implicated in alterations of cytochrome C oxidase efficiency (7, 9). Reversal of cyanide poisoning in this was however, observerable. There is also evidence that certain metabolic and experimental conditions can alter the ability of cyanide to bind to and inhibit cytochrome C oxidase, thus changing its toxicity.

Research into the function of the mitochondria in smooth muscle is behind that of other oxidative tissues because of the well documented difficulties with isolating smooth muscle mitochondria, including very low yield (10, 11). There are also relatively few studies on cytochrome C oxidase in intact tissue due to the difficulty of maintaining tissue viability and homeostasis during metabolic manipulations and poisoning (12). Vascular smooth muscle in particular has a relatively stable high energy phosphate pool during periods of cyanide inhibition (13) and therefore is well suited for studying cyanide inhibition of cytochrome C oxidase. Because of smooth muscle's stability during cyanide poisoning we have therefore performed experiments to study oxidative metabolism in the intact smooth muscle.

This study was performed to determine the tissue specific ability of cyanide to poison the respiration in two metabolically different smooth muscle types. We used intact smooth muscle to examine the role of metabolism in the ability of cyanide to act on cytochrome C oxidase by measuring the muscle's respiration. We see that metabolic conditions alter the tissues' susceptibility to cyanide inhibition, and that there are tissue specific differences.

3. MATERIALS AND METHODS

3.1. Materials

All chemicals were of reagent grade. All solutions were made with double distilled water and pH verified and adjusted accordingly.

3.2. Solutions

Bicarbonate buffer contained (mM) 116 NaCl, 25 $NaHCO_3$, 5.4 KCl, 5 KH_2PO_4, 2.4 $CaCl_2$, 1.2 $MgSO_4$, and 10 glucose. HEPES buffer contained 125 NaCl, 5.3 $KHCO_3$, 2.4 $CaCl_2$, 1.2 $MgSO_4$, 10 glucose and 11.8 mM HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid) with pH adjusted to 7.4 with Tris base.

3.3. Tissue

Porcine carotid arteries were obtained from the abattoir at the time of slaughter and prepared as described previously (13). Rings of intima media were prepared and placed in the oxygraph chamber. Carotid arteries were maintained in the HEPES buffer at 4°C for up to three days (solution freshened daily) prior to use. Guinea pigs were humanely euthanized following accepted guidelines. The stomach muscle was isolated and prepared following the procedures of Nakayama et al., (14, 16) and used within 24 hours of collection.

3.4. Oxygen Consumption

The rates of respiration were determined by a Clark oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH, USA) in an oxygraph cell with a 4 ml volume containing carotid rings or stomach strips with the experiments performed at 35°C. The solubility of oxygen

Table 1. Effects Of Cyanide Poisoning In The Presence And Absence Of Pyruvate

	EC_{50} HEPES -pyr	EC_{50} HEPES +pyr	EC_{50} Bicarb -pyr	EC_{50} Bicarb +pyr
Carotid	0.11±0.022	6.52±0.11 #	0.13±0.044	0.19±0.002*
Stomach	0.14±0.024	1.95±0.31 #	0.18±0.032	0.26±0.039*

±: SEM, EC50 for Cyanide in mM, *: Significantly different from HEPES without Pyr, #: Significantly different from all other groups.

was assumed to be 0.2 μMol/ml and the results are expressed as oxygen consumed in μMol/min/g dw.

The EC_{50} for cyanide was determined by sequential additions of sodium cyanide to the oxygraph chamber. [Sodium cyanide was used, as opposed to potassium cyanide, to prevent stimulation due to potassium depolarisation.] The ability of pyruvate to re-stimulate respiration was determined by sequential additions of sodium pyruvate to the tissue in the presence of 1 mM cyanide. These data were plotted and fit to determine the inhibitory or stimulatory parameters. EC_{50} is the effective concentration of cyanide to inhibit 50% of the respiration. K_m is the apparent K_m for pyruvate stimulation of respiration. V_{MAX} is the maximal rate of respiration obtained by addition of pyruvate in the presence of 1 mM cyanide. V_{BAS} is the basal rate of respiration in the presence of glucose alone.

3.5. NMR Experiments

To demonstrate tissue viability during the cyanide treatment the stomach muscle was prepared and perfused according to the methods of Nakayama *et al* (14) and Boehm et al., (15). After removing the mucosa of the stomach, the smooth muscle was mounted in a 10 mm diameter NMR tube and superfused (12 ml/min) at 35°C. Radio-frequency pulses corresponding to a 30° pulse angle were repeated every 0.6 sec. Intracellular concentrations of metabolites and pH were estimated according to the methods described by Nakayama et al. (14, 16). The ^{31}P NMR spectra were collected in blocks of 5 including 25 minutes of summed spectra. From the NMR spectra the pHi, as well as the concentration of Pi, PCr and total nucleotide (NTP) were obtained.

3.6. Statistical analysis

The test for significance was the Student's T test. Results are reported as ± SEM.

4. RESULTS

Table 1 demonstrates the inhibitory constants of cyanide in the two smooth muscles with glucose or pyruvate as the substrate. Pyruvate significantly increased the EC_{50} for both tissues, but the carotid's EC_{50} value was significantly greater than the stomach. There was no significant difference in the EC_{50} values for the two tissues in the absence of pyruvate (N is a minimum of four for all values).

There was no significant difference in the basal rate of respiration (V_{BAS}) between bicarbonate buffer and HEPES buffer and no significant difference between V_{BAS} with glucose *versus* pyruvate as the substrate (data not shown). The rate of respiration by pyruvate re-stimulation was not significantly different than control in the stomach (2.63±0.77 and 2.69±0.3 μMol O_2/min/g dw respectively) but significantly greater in the carotid artery (Figure 1). Therefore, 10 mM pyruvate and 1 mM cyanide, in the porcine carotid artery, had a rate or respiration significantly greater than with 10 mM glucose alone (0.90±0.2 and 0.59±0.06 μMol O_2/min/g dw respectively).

Figure 1 demonstrates the results for pyruvate *re*stimulation of respiration in the smooth muscle in the presence of 1 mM cyanide. The apparent K_m for stimulation between the two tissues was not significantly different. Before cyanide addition there was however, a significantly greater rate of V_{BAS} (unstimulated) respiration in the stomach smooth muscle. V_{MAX} is the rate of oxygen consumption in the presence of glucose but with pyruvate restimulating respiration after 1 mM cyanide inhibition. The V_{MAX} of pyruvate restimulation, obtained for the stomach muscle is not significantly different than the basal rate V_{BAS}. In the porcine carotid artery the EC_{50} for cyanide inhibition was significantly greater than the stomach smooth muscle in the presence of pyruvate when HEPES buffer was used (Table 1). Interestingly, upon addition of pyruvate to the carotid arteries, there was an acceleration of respiration, as determined by the V_{MAX} for these conditions, that was significantly greater than the original basal rate (Figure 1).

The α-keto acid, α-ketoglutarate (10 mM final concentration added to 1 mM cyanide) was able to restimulate respiration in both tissues, but oxaloacetate, another α-keto acid, did not however, restimulate respiration. Other metabolic intermediates (all at 10 mM) that did not restimulate respiration were; β-hydroxybutyrate, acetate, lactate, citrate, and succinate (N of four or more). None of these metabolites prevented pyruvate's action either. Acidosis, (pH 6) did, however, prevent pyruvate's stimulation of respiration (Table 2) but did not damage the tissue, whose oxygen consumption remained recoverable (data not shown). Indeed, under all conditions tested, the inhibition by cyanide was reversible after thorough rinsing. Table 2 summarises the qualitative responses of the smooth muscles to restimulation of respiration by pyruvate addition.

Table 2. The qualitative responses for re-stimulation of cyanide inhibited respiration by Pyruvate*

SUBSTRATE	RESPONSE
pyruvate#	restimulation
pyruvate (pH 6)	no restimulation
oxaloacetate	no restimulation
α-ketogluterate	restimulation
acetate	no restimulation
β-hydroxybutyrate	no restimulation
succinate	no restimulation
lactate	no restimulation
acetoacetate	no restimulation
citrate	no restimulation

* N=3 for all experiments in the stomach and carotid. # All pyruvatre concentrations are 10 mM and 1 mM for cyanide.

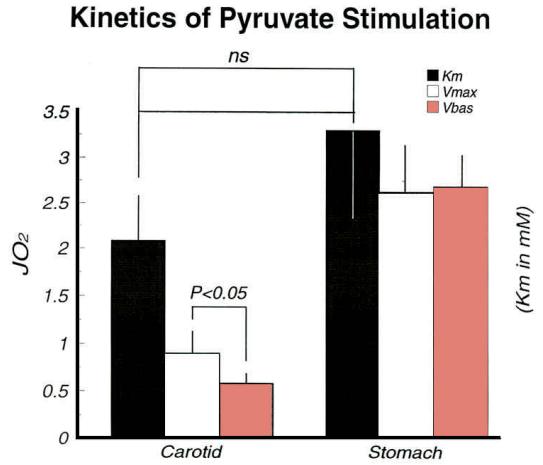

Figure 1. Bar graph comparing the responses of the carotid and stomach smooth muscle respiration under basal conditions (V_{BAS}) or to re-stimulation by pyruvate. The K_m (mM) for pyruvate to stimulate respiration was not significantly different in the two tissues (black). There were significant differences in the basal rates of respiration prior to cyanide addition (white) between the two tissues. In the carotid artery, the V_{MAX} (μMol O_2/min/mg dw) obtained by sequential pyruvate additions (red) was significantly greater than the basal rate of respiration (V_{BAS}).

Apart from acidosis preventing pyruvate's action, 25 mM bicarbonate buffer had a similar effect. Table 1 also summarises the data from these experiments (N = a minimum of 4 for each value). We see that the EC_{50} for cyanide is not significantly different between HEPES and bicarbonate buffer in the absence of pyruvate. In the presence of pyruvate however, the bicarbonate buffer had a slight but significant increase in EC_{50}. Pyruvate's action was therefore, greatly diminished in the presence of 25 mM bicarbonate.

Table 3 presents the pooled data from a series of NMR experiments (N = 4) where the guinea pig stomach was exposed to a series of metabolic manipulations similar to those used above. The results show that there is a decrease in the PCr concentration and acidosis during cyanide administration which is completely reversible with the administration of pyruvate. The NTP peaks contain the combined resonances of all the nucleotides, but the peak compositions are dominated by ATP. Table 3 shows the changes in Pi, PCr, and pHi, with no significant change in total nucleotide concentration (N = 4). All parameters returned to values not significantly different than starting values following the administration of 10 mM pyruvate to the cyanide containing solution.

5. DISCUSSION

It has been proposed that the ability of pyruvate to increase the LD_{50} of cyanide is due to a reaction mechanism caused by the production of a non-toxic cyanohydrin (17). The evidence for this *in vivo* and *in vitro* is incomplete. Indeed, isolated cytochrome C oxidase is only reactivated to 30% of control levels by 48 mM pyruvate (17). This lack of complete reactivation, compared to our complete and supra-basal reactivation, is indicative of striking differences in the ability of cyanide to bind to cytochrome C oxidase *in vivo* compared to *in vitro*. Differences in *in vivo* and *in vitro* enzyme activities have been demonstrated previously (18). Our data supports an hypothesis that metabolism has a contributory role in the ability of pyruvate to re-stimulate oxygen consumption. The apparent lack of protective action by pyruvate in the bicarbonate buffer provides two important pieces of scientific information. **1)** It may help solve some of the controversial results seen in previous studies concerning the reversible inhibition of cyanide by pyruvate and other α-keto acids (17 and references therein). **2)** It is indicative of the importance (and complexity) of metabolism in pyruvate's action as well as the differences in metabolism between smooth muscle types and possibly between different tissues. Because of our interest in smooth muscle metabolism and the poisoning of metabolism, the remainder of this discussion will focus on the metabolic information gained from this study, though we realise that more work needs to be done to determine the bioenergetic implications of these data.

Isolated smooth muscle are relatively hard to perform these experiments because they are very sensitive to the isolation conditions, require adequate concentrations of Mg^{++}, and are relatively unstable once isolated. There are difficulties with studying mitochondrial function in intact tissue and frequently, the mitochondria must be made more accessible before study by isolation or membrane permeabilisation (19, 20). These procedures have many difficulties in characterizing effects on the tissues' normal metabolic control systems. In this study however, we have been able to use intact smooth muscle, which is stable for the duration of the experiments, and make observations regarding the steady state conditions of the mitochondrial function. We have previously shown that smooth muscle can attain and maintain a steady state metabolic turnover during prolonged cyanide exposure (13) which makes this tissue well suited to the study of cytochrome C oxidase in the intact muscle.

The robust metabolism of the smooth muscle is demonstrated in Table 3 where the ATP concentration is better maintained during cyanide administration. Despite the fall in PCr and pHi, these metabolites completely recover with the addition of 10 mM pyruvate. This is also an important observation regarding the interpretation of these data because the return of the PCr concentration

Table 3. Summary of ^{31}P NMR Data

	pH	Pi*	PCr*	ATP*
Control	7.07±0.03	0.51±0.04	0.92±0.04	1±0.04
CN	6.86±0.11#	1.32±0.31#	0.25±0.07#	0.83±0.02#
CN + Pyruvate	7.02±0.06	0.73±0.12	0.77±0.01	0.93±0.07

***** Arbitrary units obtained from the NMR spectrometer. # Significantly different from control and CN + Pyruvate. N=4 for all values.

shows that the cyanide action is being reversed by pyruvate.

There are well characterised differences in enzyme function *in vivo* and *in vitro* (11), and this study partially addresses these differences. *In vitro*, under fully reduced conditions, oxygen may displace cyanide from cytochrome C oxidase (21), although evidence for this *in vivo* is lacking (22). Delhumeau et al, (17) proposed that oxygenation might effect cyanide binding in isolated mitochondria, but oxygenation in their experiments was performed by exposure to ambient air with the risk of altering the solution pCO_2 as well as pO_2. They were unable to distinguish the toxicity of cyanide with varying pCO_2 and pO_2. Therefore, more work needs to be done to determine the importance of O_2/CO_2 to the displacement of cyanide off cytochrome C oxidase *in vivo*. Indeed, hyperbaric treatment of cyanide poisoning is less than satisfactory (17) and not routinely used. It is interesting, however, to speculate that there may be some importance in the production of CO_2 within the mitochondrial matrix ascribed to the two α-keto acids in that this is the next step for both of these Krebs cycle intermediates. The intact tissue conditions used here may also explain the slightly lower affinity for cyanide inhibition that we have obtained (Table 1) compared to other reported values (7, 12).

The supra-basal recovery of the carotid's respiration by pyruvate in presence of 1 mM cyanide is striking (Figure 1) and the mechanism for this recovery is unknown. The binding of cyanide to cytochrome C oxidase (isolated enzyme or enzyme in the mitochondria) is decreased when the enzyme is oxidised (7) and the ability of the bicarbonate buffer to prevent pyruvate's protection from poisoning could be explained if it increased the percentage of oxidised enzyme. Furthermore it is possible that pyruvate causes alterations in intracellular metabolites resulting in a change in the efficiency of some mitochondrial enzymes (9). We have no evidence that CO_2 or HCO_3^- can alter cyanides' interactions with pyruvate.

There is, however, a growing body of evidence that muscle mitochondria are capable of changing their inner membrane potential with concomitant effects on oxidative phosphorylation (6, 23, 24). This may also effect the enzyme's redox state. What role, if any, CO_2 or HCO_3^- may play in mitochondrial membrane potential is yet to be determined in smooth muscle. Despite reports that pH can alter the ability of pyruvate to be metabolised in the mitochondria (25) we do not believe that our results are simply due to a change in pH, because the extracellular pH was maintained at 7.4 and verified after the experiments.

Furthermore, NMR experiments on the guinea pig stomach showed a normalisation of pH with the addition of pyruvate (See Table 3). Low extracellular pH (pH of 6) however, did prevent pyruvate's action (Table 2).

Smooth muscle metabolism is quite variable depending upon the smooth muscle type (26). Significant differences exist between vascular smooth muscle compared to stomach smooth muscle (26, 27). In this paper we report striking differences between the tissues' inhibition by cyanide under various conditions, which may be partially explained by their distinct metabolisms. In the absence of pyruvate, the EC_{50} for cyanide was not significantly different between the two tissues, but in the presence of equivalent amounts of pyruvate the carotid had a significantly higher EC_{50}. This could be explained by a greater amount of oxidised cytochrome C oxidase in the presence of pyruvate. Indeed this is likely because the V_{max} for pyruvate re-stimulation is greater than the basal rate of oxygen consumption. Therefore, the redox state of the enzyme may have changed between the beginning basal conditions and post cyanide/pyruvate additions. This is also consistent with the mitochondria in vascular smooth muscle having a novel control system for energy metabolism; for example, the presence of aerobic lactate production (13). Recently it has been proposed that smooth muscle mitochondria may play a potent role in controlling or determining cell fate choices (28) This unique signalling role may be mediated by interactions which occur at the electron transport chain and could respond to intracellular signals via pyruvate or other metabolites (28). It is very doubtful that the ability of pyruvate to increase the EC_{50} in these tissues is due solely to the production of the non-inhibitory cyanohydrin complex because its net formation in HEPES *versus* bicarbonate buffer should be similar. Moreover, the formation of cyanohydrin cannot explain the differences in V_{max} for the two tissues. If we assume an equivalent amount of cyanohydrin produced, and remembering the very similar EC_{50} values for the tissues, then the two conditions should have similar EC_{50} values under these conditions. Because of these large differences, we conclude that the carotid artery smooth muscle mitochondria has a different susceptibility to cyanide poisoning and that cyanide's toxicity is also effected by the buffer used. Much more work needs to be done to characterise the toxicological implications of these differences in cyanide poisoning and how different smooth muscles have different responses to its inhibition.

6. ACKNOWLEDGMENTS

This work was supported by a grant from the Japanese Society for the Promotion of Science. JFC is supported by the Medical Research Council, and a Lecturer

of St. Anne's College Oxford. Thanks go out to Prof. Kenji Kuba for his support of this work and Prof. Shigeru Kobayashi for assistance with the animal experiments. The authors are grateful to S.J. Ferguson, D.A. Harris, G. Pyne, and J.M. Grey for proof-reading this manuscript.

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Key Words: Toxicity, Poisoning, Pyruvate, Glucose, Stomach, Carotid Artery, Mitochondria, Cytochrome, Cytochrome C Oxidase, Respiration, Metabolism, Keto acid, Guinea Pig, Pig, carbon dioxide, Bicarbonate

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