FABRISIA AMBROSIO

ÉTUDE DE L'INFLUENCE CONTRACTILE ET MÉTABOLIQUE DE L'ANHYDRASE CARBONIQUE III DANS LE MUSCLE SOLEUS ET L'EXTENSOR DIGITORUM LONGUS DE RATS HYPO- ET HYPERTHYROIDIENS

Mémoire
présenté
à la Faculté des Études Supérieures
de l'Université Laval
pour l'obtention
du grade de Maître es Sciences (M.Sc.)

FACULTÉ DE MÉDECINE
PHYSIOLOGIE-ENDOCRINOLOGIE
UNIVERSITÉ LAVAL

Septembre 1998

Résumé

L'anhydrase carbonique III, enzyme retrouvée en grandes quantités dans le muscle squelettique, est maintenant soupçonnée de participer à des fonctions cellulaires beaucoup plus importantes que la simple hydratation du CO_2. De plus, en raison de la nature unique de AC III comparativement aux autres isoformes de cette famille, cette enzyme a donc été d'un intérêt particulier pour plusieurs chercheurs. Un secteur important d'activités reliées à AC III consiste en l'étude de la modulation de son contenu et de son activité par les hormones thyroïdiennes. L'utilisation de ces hormones constitue l'élément clé de nos expériences sur AC III. Dans un premier temps, nous avons comparé la modulation de AC III par les hormones thyroïdiennes dans le muscle à contraction lente soleus (SOL) vs le muscle à contraction rapide extensor digitorum longus (EDL). Nous avons constaté que la modulation de AC III par cette classe d'hormones était qualitativement et quantitativement semblable dans les deux types de muscles. Par la suite, nous avons étudié les caractéristiques contractiles de ces deux mêmes muscles prélevés dans des animaux normaux, hyper- ou hypothyroïdiens. Cette section des travaux nous a amenés à conclure que les différences notées au niveau des propriétés contractiles n'étaient pas souvent directement reliées aux changements observés dans les niveaux d'expression de AC III. Finalement, nous avons démontré qu'il existe une relation linéaire entre les niveaux de AC III musculaire et l'accumulation de glucose-6-P induite par la méthazolamide, un inhibiteur de AC III. Prises dans leur ensemble, ces expériences ouvrent la voie à des études futures sur la fonction spécifique de AC III dans les divers types de fibres musculaires et sur sa fonction possible dans les mécanismes cellulaires du contrôle énergétique.

Résumé

Carbonic anhydrase III, found in high abundance in skeletal muscle, is now known to play a much larger role in muscle metabolism than just the hydration of CO_2, and because of the unique nature of CA III in comparison to the other carbonic anhydrase isoforms, this specific enzyme has been of special interest to researchers. A major area pertaining to CA III studies deals with its content and activity modulation by thyroid hormones. These hormones have been monumental in our experiments concerning CA III research.

Firstly, our studies looked at the modulation of CA III by thyroid hormones in the slow-twitch soleus (SOL) versus the fast-twitch extensor digitorum longus (EDL) . We found that CA III regulation by this class of hormones is quantitatively and qualitatively similar in both types of muscle. Next, we compared the contractile characteristics of thyroid hormone treated or deprived SOL and EDL muscles. In this, we found that the contractile property differences noted in the three thyroid groups, CTR, HYPER, and HYPO, were not directly related to changes in CA III content, but could instead be attributed to fiber type changes. Finally, we showed that there is a linear correlation between the level of CA III activity and the methazolamide induced accumulation of glucose-6-phosphate (G-6-P). These experiments, taken together, pave the way for further studies looking at the specialized function of CA III in specific muscle fiber types and its possible role in energy conservation.

Acknowledgements

This thesis has been compiled with the collaboration of several people who deserve recognition. Firstly, "Professor Côté" has gone above and beyond the common understanding of a graduate studies advisor. Not only has he striven to give me a thorough understanding and to profound my interest of the subjects at hand, but in addition, Professor Claude Côté has been instrumental in my initiation into a new country and culture and has helped me maximize my time and experience in Québec City. I would also like to thank the other graduate students and research assistants in our laboratory for their patience in helping me overcome the initial language barrier and for guiding me through various experimental protocols. I also thank the Centre Hospitalier de l'Université Laval for providing the funding neccessary to make this project a reality. Finally, and above all, I thank God for giving me the opportunity and the capabilities to complete a task as such.

To my dear parents who have not only raised me with a desire and thirst for knowledge, but who have also given me the courage to take on the tasks which may originally seem impossible...

Bear in mind that the wonderful things you learn in your schools are the work of many generations, produced by enthusiastic effort and infinite labor in every country of the world. All this is put into your hands as your inheritance in order that you may receive it, honor it, add to it, and one day faithfully hand it on to your children.

Albert Einstein

Table of Contents

Abbreviations

CA	carbonic anhydrase
CTR	control
D	denervated
EDL	extensor digitorum longus
EDTA	Ethylenediamine Tetra-Acetic Acid
F-6-P	fructose-6-phosphate
FFA	free fatty acid
FG	fast glycolytic
FM1	fast myosin-1
FOG	fast oxidative glycolytic
G-6-P	glucose-6-phosphate
HYPER	hyperthyroidic
HYPO	hypothyroidic
i.p.	intraperitoneal
Lo	optimal muscle length
METH	methazolamide
MgOAC	Magnesium Acetate
MHC	myosin heavy chain
MSV	maximum shortening velocity
PCA	perchloric acid
PFK	phospho-fructo kinase
Po	maximum tetanic tension
Pt	maximum twitch force
PTH	pituitary growth hormone
RBC	red blood cell
RI	reinnervated
1/2 RT	one half relaxation time
SEM	standard error of the mean
SM	slow myosin
SO	slow oxidative fiber
SOL	soleus
SR	sarcoplasmic reticulum
TPT	time to peak twitch
T_3	triiodothyronine

List of Figures and Tables

Chapter 1

Chapter 2

Chapter 3

Chapter 1

General Introduction

Introduction

I. History

In 1928, Henriques made an important discovery that related to the rate of CO_2 transformation in blood. His experiments showed that some catalyst was present for the reaction as seen in physiological conditions (for review, see Roughton 1935). This opened up a new major avenue of studies among scientists, since up until that point, the bicarbonate theory had been accepted as the model of carbon dioxide transport in blood. The bicarbonate theory states that carbon dioxide is carried from systemic circulation to the lungs as bicarbonate. Once it reaches the lungs, blood proteins convert the bicarbonate to carbonic acid. Carbonic acid is then able to dehydrate back into carbon dioxide (Meldrum and Roughton 1933). However, it was becoming increasingly evident that some enzyme must be at work in this reaction. Finally, in 1933, Meldrum and his coworkers made significant discoveries as to the nature of this enzyme (Meldrum and Roughton 1933). Using a primitive enzyme activity technique and crude isolation procedures, the existence of carbonic anhydrase in the "blood corpuscles" first became known to the scientific world. In addition, these researchers identified several characteristics of this new enzyme, including its specific activity, the great extent of its efficiency, and the fact that these enzymes are, in effect, zinc metalloenzymes. They even determined the presence of this enzyme in muscle extracts (Meldrum and Roughton 1933).

Since these initial discoveries, interest has mounted as more and more questions arise as to the precise nature of these enzymes. From the time of Nyman's discovery of three different isozymes (Nyman 1961), this family has grown to include at least seven separate isoforms. These isoforms are characterized based on location, regulation, and metabolic properties. Now, it is known that carbonic anhydrases are found in almost every tissue of the body ranging from endothelial lung cells (Ryan et al 1982) to mammalian erythrocytes (Tashian 1989). Another significant discovery in relation to these enzymes includes the determination of a specific inhibitor. In 1940, an article describing the powerful inhibitory effect of Sulphanilamide, even at extremely low concentrations was first published (Nature, 1940). Since then, increased interest has been shown regarding the characteristics of the various drugs within this family and their possible application. In 1965, studies done by Holder and Hayes (1965) showed that the permeability of these different drugs depended on their aqueous diffusion, plasma binding, lipid solubility characteristics and molecular size.

II. General Functions of Carbonic Anhydrase Isoforms

Table 1. Comparison of Various CA Isoforms in Mammals

Isoform	Activity	Distribution	Ki sulfonamides
CA I	Moderate	-erythrocyte, -epithelium -cornea -salivary glands -adipose cells	10^{-7} M
CA II	High	-erythrocytes -bone tissue, brain -aqueous humor of the eyes -kidney, liver, and lung cytoplasm -salivary glands -pancreas, stomach -CNS neurons	10^{-8} M
CA III	Low	-Type I and IIa skeletal muscle fiber types -liver, smooth muscle -myoepithelial cells (prostate, mammary gland, thymus), lungs, kidney -brain, adipocytes	10^{-4} M
CA IV	High	-membrane-bound form -lung capillary endothelium -membrane of the proximal tubule of the kidney -sarcoplasmic reticulum	10^{-8} M
CA V	Low	-mitochondrial form -lung capillary endothelium -muscle: in pigs, but not in rats	?
CA VI	Variable	-salivary glands secreted form)	?
CA VII	?	-salivary glands (cystolic form)	?
CA VIII	?	-mouse cerebrum* -mouse cerebellum* -mouse embryo	?

Adapted from Tashian (1989) and from Jerome Frenette's thesis (1993); * Lakkis[2] et al (1997)

**Lakkis et al (1997)

The primary function of carbonic anhydrase still appears to be the hydration of CO_2, even though since Meldrum's initial discoveries in 1933 (Meldrum and Roughton 1933), several other functions of this highly diverse family of enzymes have been defined. Gros et al explained that these enzymes can facilitate the transport of CO_2 in muscles (Gros and Dodgson 1988), and it has now been discovered that carbonic anhydrases, more specifically CA V, which is the mitochondrial isoform, plays a role in gluconeogenesis (Tashian 1989) and ureaneogenesis through the synthesis of carbamyl phosphate (Henry 1996; Tashian 1989). In addition, these enzymes appear to play some role in *de novo* lipogenesis, possibly through a pH buffering mechanism associated with fatty acid mobilization and transport (Coulson and Herbert 1984). These findings are very significant considering the fact that CA, specifically CA III, is found in high quantities in adipose tissue (Spicer et al 1990). Also, several studies have been done describing the role of CA IV in calcium reuptake into the sarcoplasmic reticulum (Côté et al 1997). Apparently, this specific isoform may be involved in the removal of calcium from the muscle cytosol during contraction. This is especially significant since accumulation of calcium ions has been associated with the necrosis of muscle fibers. Similarly, CA II has been associated with playing an important role in osteoclastic bone resorption, and a deficiency of this isoform has been related to the onset of osteopetrosis (Sauer et al 1994). It is also possible that CA II functions in mineral formation by maintaining the proper pH during this acid-sensitive reaction (Sauer et al 1994). Overall, considering the great diversity in function, much research remains to be done to more specifically define the undoubtedly significant and vital roles of this enzyme so cautiously conserved throughout evolution.

III. Characteristics of Carbonic Anhydrase III

This present series of experiments focused on one specific carbonic anhydrase isoform, CA III. This isoform is of particular interest to the scientific community because, despite the fact that it is found in very high concentrations in some tissues, its precise physiological role has yet to be determined. Also, several defining characteristics of this enzyme make it highly unique within the family of carbonic anhydrases. For example, as previously stated, sulfonamides have been noted as having a specific inhibitory effect on carbonic anhydrases (Mann and Keilin, 1940). More specifically, this class of inhibitors binds to the zinc and exposes active sites of these enzymes (Deutsch 1987). However, due to the unique organization of amino acid residues of CA

Figure 1. Effect of Acetazolamide on CA Activity

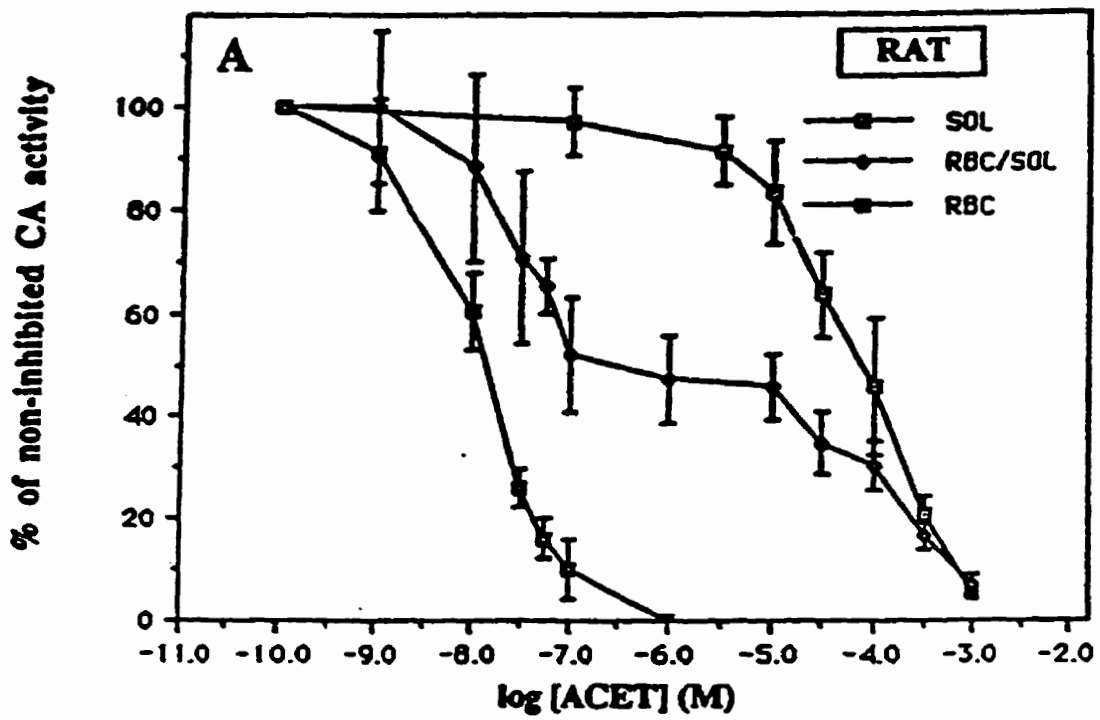


FIGURE 1. Effect of Acetazolamide (CA inhibitor) on CA III (SOL), found in great quantities in the rat soleus muscle, CA II (RBC), found in great quantities in rat red blood cells, and a 50/50 mixture of the two isoforms (RBC/SOL). This is evidence showing CA III resistance to sulfonamide inhibition as compared to other CA isoforms. Taken from Frémont 1990.

As can be seen from this graph, complete inhibition of CA III found in the SOL muscle is only achieved at an inhibitor concentration of 10^{-3}M compared to CA II from RBCs which is completely inhibited at 10^{-6} M (Frémont 1990).

In addition, CA III is in a class by itself because it is the only isoform known to possess a phophatase activity (Deutsch 1987), even though the actual role this enzyme plays with respect to this property remains to be determined.

A. Distribution

CA III is present in several tissues. In addition, the characteristics underlying the quantities of enzyme present are varied from tissue to tissue. For example, in the

hepatocytes surrounding the central vein of the rat liver, CA III expression has been observed to be sexually dimorphic, whereas the concentration of CA III in adipose tissue is directly related to the number of white fat cells. In the adipose cells of lean Zucker rats, CA III is found to be most abundant, making up approximately 24% of the cystolic protein content (Lynch et al 1993). Interestingly enough, Stanton et al (1991) cited that CA III expression is approximately 10 times greater in lean mice as compared to their obese counterparts. It is believed that the decrease in CA III in obese animals is a result of hyperinsulinemia, since obese rats made diabetic with streptozotocin no longer showed this depression. The distribution of CA III also includes tissues such as the lung, the prostate gland, the kidney and skeletal muscle, just to name a few. Globally, however, CA III is found in high concentrations in only three tissues: fat cells, liver and skeletal muscle.

Of particular interest when considering the localization of CA III is its presence in skeletal muscle cells. This enzyme makes up approximately 20% of the total cytosolic protein content of slow-twitch type I muscle fibers (Tremblay et al 1993). CA III concentration is related to muscle fiber type, being most abundant in muscles composed primarily of type I fibers, and least abundant in muscles containing mostly type IIb fibers (Frémont et al 1991). Due to the differences in CA III concentration between the various muscle fiber types, many questions arise as to the alternate roles of CA III besides the hydration of CO_2.

B. Regulation

Another major area of interest when considering CA III concerns its various regulators. The regulation of CA III is fairly diversified and variable from tissue to tissue. In rat liver, for example, where CA III is sexually dimorphic, pituitary growth hormone (GH) has been shown to play a big role in regulating the expression of this enzyme (Tashian 1989). It is believed that in females, because GH is continually secreted, CA III is greatly suppressed. On the other hand, GH is released discontinuously in males, and is not concentrated enough to effect levels of CA III (Tashian 1989). In skeletal muscle, however, other factors appear to control CA III expression. Carter et al (1988) demonstrated that denervation of rabbit and rat type II skeletal muscles actually increased levels of CA III whereas reinnervation had just the opposite effect. Figure 2 (Milot 1995) shows the effect of denervation and reinnervation of rat soleus and EDL muscles on CA III content.

Figure 2. Effect of Denervation and Reinnervation on CA III Activity

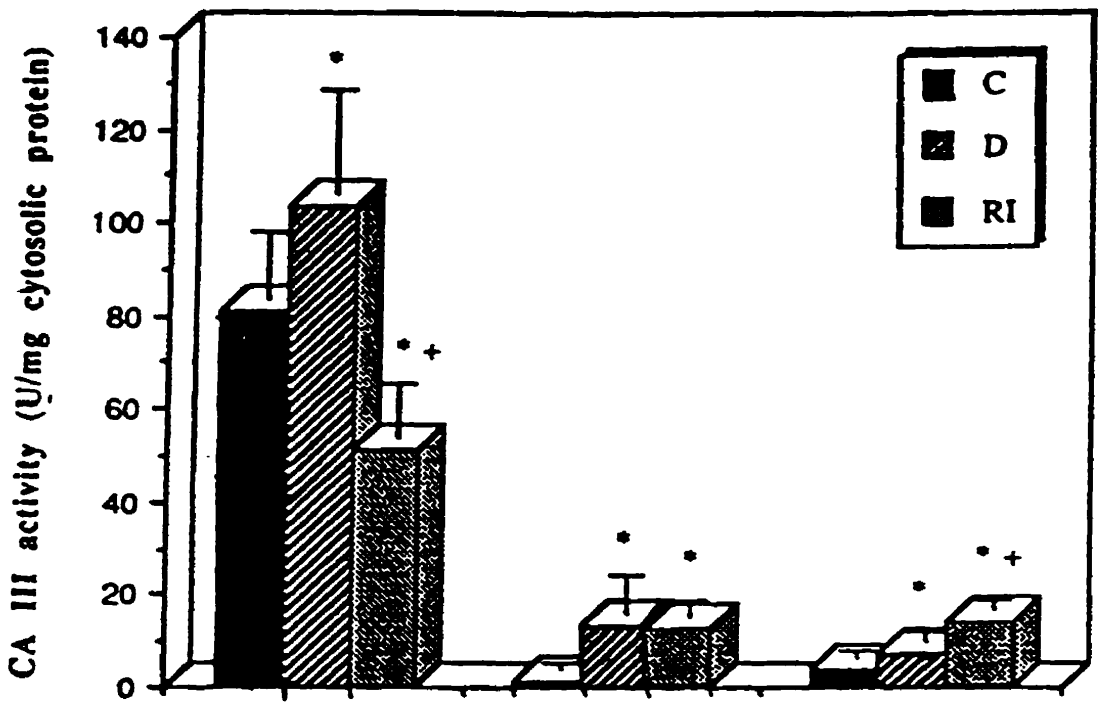


FIGURE 2. Comparison of control (C), denervated (D), and reinnervated (RI) CA III activity in the soleus (SOL), extensor digitorum longus (EDL), and gastrocnemius (GAS) muscles. Muscles were denervated for 9 days and reinnervated muscles were those allowed to recover.

Symbols: * significantly different from control values ($p \leq 0.05$);

+ significantly different from denervated group ($p \leq 0.05$) $5 \leq n \leq 10$ for each group.

Taken from Milot 1995.

It has been proposed that these alterations in CA III concentration of denervated muscles are a result of neuronal modulation of gene expression (Milot 1995). That is, there is evidence of increased CA III synthesis with denervation. These changes may indicate a transition of fast- twitch fibers to a slow-twitch phenotype (Milot 1995) since slow-twitch fibers contain significantly higher quantities of CA III (Frémont et al 1991). Similarly, it is believed that these changes seen with denervation are gradually reversed with reinnervation. This model, however, is still not completely understood and further studies are needed to more clearly describe the mechanisms at work.

Another regulator of CA III in rat skeletal muscle is the thyroid hormones. This has been very useful in experimentation because changes in CA III expression are

brought about very rapidly, possibly as a result of changes in fiber type composition. Fiber type composition is mainly determined by myosin heavy chain (MHC) gene expression (Caiozzo et al 1991). Slow-twitch muscles (composed mainly of type I fibers) express high amounts of slow twitch myosin isoform and this, in turn, is associated with slower contractile velocities and longer relaxation times. More specifically, an increase in slow MHC gene expression leads to a decreased myosin ATPase activity, which in turn leads to a prolonged cross-bridge formation time, consequently resulting in slower contractile properties (Norenberg et al 1996). Gene expression of slow MHC in hypothyroidic rats is increased as compared to control euthyroid rats (Gosselin et al 1996) and this accounts for the slower contractile velocities of these muscles. Also seen in hypothyroidic rats is a decrease in Ca^{2+} pump activity (Larsson et al 1994) and an increase in oxidative capacity (Frémont et al 1987) which also contributes to the increased slow-twitch properties of these muscles. Just the opposite effect is seen in hyperthyroidic rats, where slow MHC gene expression is inhibited and/or fast MHC gene expression is increased (depending on fiber type specificity), resulting in expression of fast-twitch characteristics including faster contractile velocities and shorter relaxation times (Gosselin et al 1996). Another change seen in hypothyroidic muscles includes an increased CA III concentration. This, again, is in line with the fact that CA III is most abundant in slow- twitch muscles. Therefore, it seems that CA III concentration is indirectly affected by thyroid status via changes in muscle fiber type concentration, and more specifically, MHC gene expression.

A time frame for significant phenotypic changes in hyperthyroidic as compared to control rats is dependent on the concentration and frequency of the thyroid treatments as well as the mode of application (i.e. subcutaneous injection versus oral tablets). To better illustrate this, a comparison of two different experiments looking at glycogen content in hyperthyroid versus control rats can be made. In the first experiment, rats were given subcutaneous injections of 10 μg triiodothyronine hormone (T_3)/100 g body weight daily for 10 days (Kudelska et al 1996). In their experiments, there was no significant difference in resting glycogen content between treated and control animals. On the other hand, a second experiment looking at the same variable saw a dramatic difference in resting glycogen content between the two groups. In this experiment, however, animals were subcutaneously injected with 15μg T_3/100g body weight daily for 10 days (Leijendekker et al 1983). Therefore, with even just a small increase in T_3 concentration, significant differences may be seen. However, it is important to note that increasing thyroid hormone levels do generally lead to an increased mortality rate, most likely a result of cardiac arrhythmias (Li et al 1996).

C. Function

The function of CA III is what most intrigues and mystifies researchers. It is evident that this enzyme has been highly preserved throughout evolution, thereby indicating that its role is vital for survival. Yet despite its diverse presence in various tissues, at this point, only theories have been presented speculating the importance of this particular CA isoform.

As is true for all carbonic anhydrases, the most obvious biochemical role as first described over 70 years ago, is the hydration of CO_2. This, is especially significant in skeletal muscle since it has one of the fastest rates of CO_2 production of all organs (Gros and Dodgson 1988). Furthermore, it is known that CA III increases the rate of CO_2 hydration 400 fold (Gros and Dodgson 1988). Studies done by Gros and colleagues suggest a facilitated diffusion of CO_2 out of skeletal muscle by CA III; however it is becoming evident that CA III must possess some other type of activity for various reasons including the fact that it is found in such small quantities in type IIa muscles, which can produce a high amount of CO_2; it is also the only carbonic anhydrase which possesses phosphatase activity, thereby indicating a unique role distinguishing it from other CAs. Additionally, if CA III's sole function was facilitated diffusion of CO_2, then an increased or decreased capacity for oxidative metabolism should be accompanied by an increased or decreased activity of CA III. However, researchers noted just the opposite relationship to be true (Tremblay et al 1993).

More recently, studies have been done linking CA III to the contractile function of slow- twitch muscles. Côté and his laboratory noticed a significant increase in fatigue resistance in muscles incubated with CA inhibitors (Côté et al 1993). This was rather ironic since CA III is found in most abundance in muscles which are most fatigue resistant. Fatigue has been associated, among other things, with a decrease in glycogen stores. Along these lines, in the fatigue curves derived, they noticed that the effect of CA III inhibition was most noticeable when the muscles changed from anaerobic to aerobic metabolism (Côté et al 1993). This suggested that the role of this enzyme could be incorporated either into glycolysis or glycogenolysis. Further studies done showed that the action of sulfonamides on CA III favored the accumulation of G-6-P in soleus muscles (Côté et al 1993; Côté et al 1997), supporting the previous statement. As seen in figure 3 (Frenette 1993), there is more than one pathway leading to the formation of G-6-P; therefore, CA III may be acting on any one of these, including extracellular glucose taken in by the muscle either by the presence of insulin or by muscle contraction

(Douen et al 1990), the breakdown of muscle glycogen, or CA III could modulate one of the reactions occurring after the formation of G-6-P. Its inhibition would therefore prevent the cycle from continuing, again leading to the accumulation of G-6-P.

Therefore, it stands to reason that, while CA III does play a role in CO_2 hydration, this function is secondary to a more significant role in carbohydrate conservation in skeletal muscles. The precise mechanism by which this happens, as well as the advantage of CA III in such abundance in slow twitch oxidative muscles, have yet to be determined.

IV. Objectives of Experiments

In light of the significantly large quantities of CA III found in fat and muscle, as well as its noted presence in several tissues, it is evident that CA III is of vital importance. Yet, surprisingly enough, the precise function of this enzyme has yet to be defined. Because CA III is found in such large quantities in type I muscle fibers, this was a good basis to begin exploring the many unanswered questions in regards to this unique class of enzymes. In addition, type IIa and IIb fibers provided an excellent example of fibers with minimal CA III content. Therefore, the objectives of these experiments were multiple and centered around the regulation of CA III by thyroid hormones

1st Objective: Because it is a well known fact that CA III is found in the greatest abundance in type I skeletal muscle fibers and in much smaller amounts in type IIa and IIb fibers (Frémont et al 1993), the characterization of its up- and down-regulation in slow twitch muscles by thyroid hormones is of particular significance. And yet, almost no studies have been done in regards to the regulation of CA III in fast-twitch muscles. Therefore, it was our aim to qualitate and quantify the alterations in CA III expression as a result of induced hyperthyroidic and hypothyroidic states in the fast-twitch EDL muscles and compare it to the ones seen with the SOL. This was done using a cytoplasmic extract preparation (Maren 1960) for CA III activity analysis and content quantification by semi-quantitative Western Blotting.

2nd Objective: Based on the present knowledge that CA III inhibition can lead to increased fatigue resistance of slow-twitch muscles, thyroid hormone alteration of fiber type composition was a good means to further explore this phenomenon. Knowing that hypothyroidism leads to slower contraction and relaxation times, while also greatly

increasing CA III, the effects of the inhibition of CA III on muscle fatigue could be more broadly analyzed, giving us a wider range of data for both groups of muscles (fast- and slow-twitch). Similarly, a hyperthyroid state, which induces the exact opposite effects as that seen in hypothyroidism, allowed us to more specifically narrow the score of minimal CA III influence on fatigue resistance. Therefore, our second objective was to test the hypothesis that increases and decreases in CA III activity of the SOL muscle induced by HYPO and HYPER conditions, respectively, would lead to qualitatively similar changes in the influence of methazolamide (METH) on resistance to fatigue.

3rd Objective: The final objective of this series of experiments was to further explore the effect of CA III on glucose metabolism in slow-twitch muscles as well as in fast-twitch muscles. Once again, hypo- and hyperthyroidism were useful in being able to more completely describe the influence of various levels of CA III activity on the accumulation of G-6-P in control muscles as compared to those incubated with a concentration of methazolamide known to fully erase all CA III activity. According to our working hypothesis, variations in the level of CA III activity should impact on the G-6-P accumulation induced by methazolamide-induced CA III inhibition. A secondary objective was also to verify if the accumulation of G-6-P observed with type I muscles could also be observed in type II muscles containing much lower levels of CA III activity.

Figure 3. Glycogenolysis and Extracellular Glucose Uptake: Pathways of Glycolysis

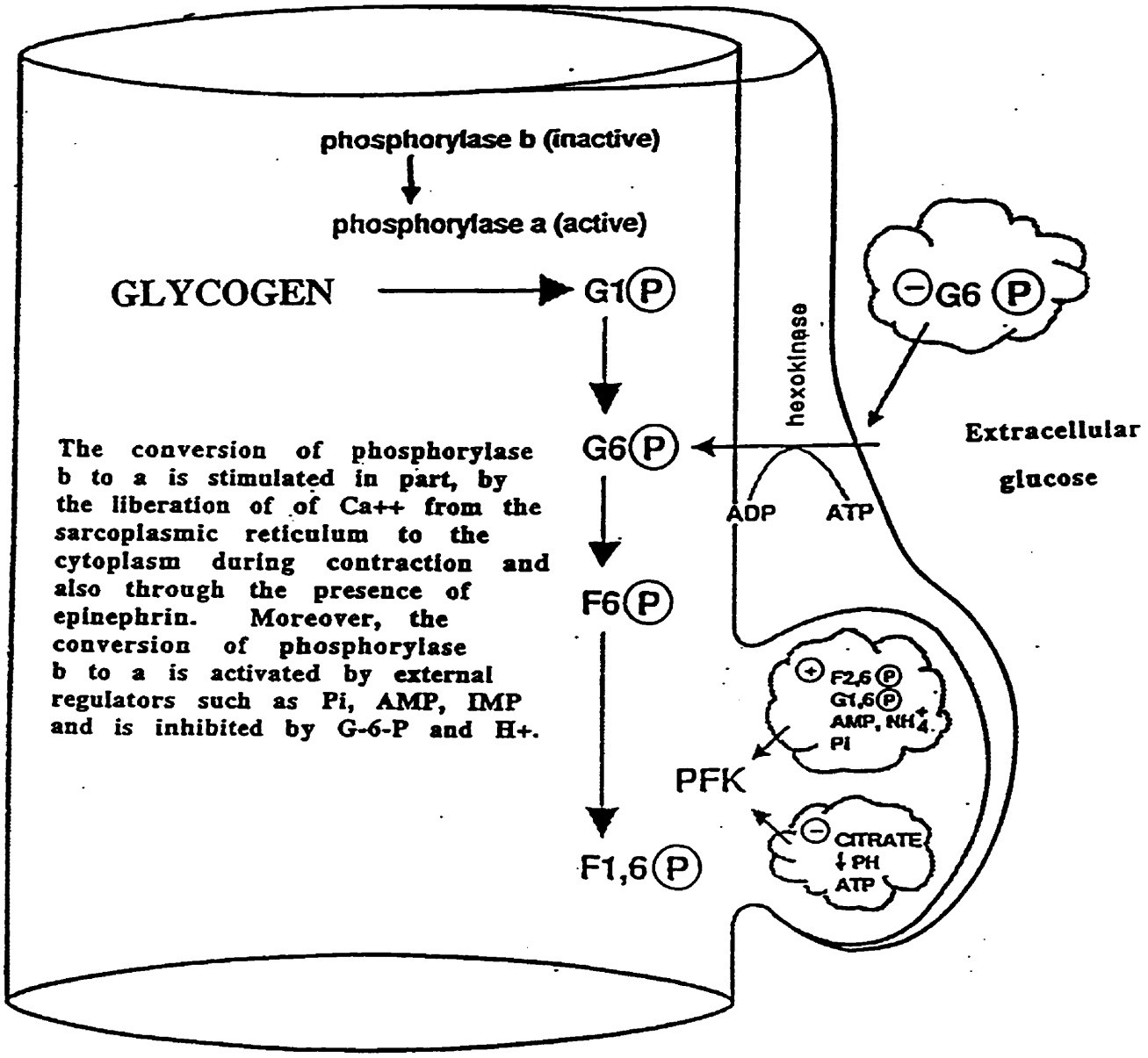


Figure 3. Describes glucose uptake and key enzymes in glycolysis. Taken from Frenette 1993.

Chapter 2

Studies on the Modulating Influence of Thyroid Hormone on CA III Activity and Content in Slow- and Fast-Twitch Muscles

Fabrisia Ambrosio and Claude Côté

CHUL Research Center
Lipid Research Unit
Université Laval

Abstract

It has been well established that thyroid hormones play a role in the modulation of CA III in slow-twitch muscle fibers, however, its influence on CA III in fast-twitch fibers has been poorly described. CA III content was analyzed by a technique using enzyme activity quantification and also Western Blotting. CA III activity and content were assessed in both fast-twitch and slow- twitch skeletal muscles, and within each group, muscles were divided into hyperthyroid (HYPER), hypothyroid (HYPO), and control (CTR) groups, based on thyroid status. Results showed that thyroid status did in fact alter CA III activity in fast-twitch muscles, however to a much smaller degree than was seen in the slow-twitch counterparts. Also, Western Blotting showed that this up- and down- regulation as seen in HYPO and HYPER rats, respectively, is quantitatively similar between the two muscle groups when expressed as percent relative change. That is, HYPER soleus and EDL muscles both showed values for CA III content between 80-86% of their control counterparts. HYPO muscles, by the same token, showed similar results in both soleus and EDL muscles. That is, approximately 188-196% increase in CA III content. These results suggest that CA III content is directly related to thyroid status, and this modulation is seen equally in slow-twitch and fast-twitch skeletal muscle fibers.

Introduction

Carbonic anhydrase (CA III) is found in various tissues of the rat, but in greatest abundance in adipose, liver, and skeletal muscle (Côté et al 1997). In skeletal muscle, CA III is found in the cytosol, sometimes along with an other soluble isoforms such as CA II (Henry 1996). Skeletal muscle is particularly known to contain great amounts of CA III (Shiels 1984). Carbonic anhydrase content in the various types of skeletal muscle fibers differs greatly. Frémont et al (1991) described how the relative CA III content among the three principle types of muscle fibers is type I>IIa>IIb~0. That is, predominantly type I muscles have 5-10 times the CA III content of muscles made up primarily of type IIa, and type IIb contain minimal amounts of this enzyme (Frémont et al 1991). In type I fibers, CA III comprises nearly 20% of the total cystolic protein mass (Côté et al 1997). One way to better study the function of this enzyme, is to promote changes in muscle fiber type proportions through hyper- (HYPER) and hypothyroidism (HYPO). Increases in thyroid hormones are known to decrease the number of slow oxidative fibers in type I muscle, (Nicol and Bruce 1981) while HYPO increases the number of type I fibers in the soleus (SOL) from 85% (Ianuzzo et al 1977) to nearly 100% (Tremblay et al 1993; Caiozzo et al 1991). Along with these changes in type I fiber content, CA III content is also up- and down- regulated in HYPO and HYPER, respectively (Tremblay et al 1993; Gagnon et al 1985) consistent with Frémont's findings (Frémont et al 1988). Therefore, an increase in thyroid hormone is easily associated with a decrease in CA III content in SOL muscles.

Because the SOL muscle is made up primarily of type I fibers, variations in CA III content can be easily determined and several studies showing CA III regulation by thyroid hormone in this muscle have been published (Tremblay et al 1993; Gagnon et al 1985). Studies done on the effects of thyroid hormones on CA III content in fast-twitch muscle fibers, on the other hand, are far less extensive. It is known that tri-iodothyronine (T_3) increases the number of type I fibers in the Extensor Digitorum Longus (EDL) muscle, known to have only about 3% type I fibers (Everts and Clausen 1986), to some extent (Van der Linden et al 1996; Caiozzo 1991; Izumo et al 1986). However, how these alterations in muscle fiber type impact on CA III content in EDL muscle have not until this point, to the best of our knowledge, been studied. Therefore, it was the objective of these experiments to investigate the effects of thyroid hormones on CA III content and activity in fast-twitch muscles, specifically the EDL, and compare these effects with those seen in the SOL muscle.

Materials and Methods

Animal Preparation

Female Wistar rats from Charles River Laboratories, 140-160g were used in these experiments. Rats were divided into three groups: control (CTR), HYPER and HYPO. The control rats (n=40) were normal and not altered in any way. HYPER rats (n=45) received 75μg T_3/100g body weight in a saline solution injected subcutaneously once every two days for two weeks. These rats weighed 170-200g by the end of the protocol. Thyroidectomized rats were ordered from Charles River Laboratories, making up the HYPO group (n=42). A minimum of 14 days post-surgery was allowed before beginning muscle analysis.

Cytoplasmic Extract Preparation and Determination of CA III Activity

Rats were anesthetized through an intraperitoneal injection of 0.1ml sodium pentobarbital/100g (50mg/Kg). The SOL muscle was dissected and homogenized in 10 volumes w:v of Hepes buffer (2.5 mM Hepes, 1.0mM B-mercaptoethanol, 1.0 Magnesium Acetate (MgOAC) and 1.0 mM EDTA $(Na)_2$). Homogenates were then centrifuged at 10,000 rpm for 10 min. The same procedure was used for EDL muscles.

CA III activity of all muscles was measured with the soluble cystolic fraction using a procedure described originally by Maren (1960), and with modifications by Bruns et al (1986). Further modifications by Frémont et al (1988) to double the concentration of Phenol red indicator as well as to use CO_2 saturated water, were also utilized. Total CA activity was quantified by measuring the difference in reaction times for the conversion of $CO_2 + H_2O$ to $HCO_3 + H^+$ with (total CA activity) and without (control values) enzyme extract . CA III activity was then analyzed by adding CA inhibitor at a concentration known to inhibit all CA activity except CA III (5×10^{-6}M). Reaction times were then calculated and compared to the other trials. Sensitive CA activity was then defined as the difference between total CA and CA III activities.

CA III Content Determination by Western Analysis

The proteins of the soluble cytoplasmic fraction of both the SOL and EDL muscles were denatured and analyzed using one-dimensional gel-electrophoresis (Laemmli et al

1970). Proteins were then transferred onto a nitrocellulose film (Towbin et al 1979) and CA III was detected using a CA III specific antibody developed by Milot et al (1991). Goat anti-rabbit antibody was used as a secondary antibody which was visualized using alkaline phosphatase staining NBT and BCIP markers. Relative CA III content among the three thyroid groups was determined using optical densities of the bands. Results were given in percentages relative to control values.

Statistical Analysis

All data are given as mean +/- standard error of the mean (SEM). Statistical comparisons were made using ANOVA or two-tailed student's T-test. Data were considered significantly different at $p \leq 0.05$.

Results and Discussion

CA III Activity

As shown in figure 1, hyperthyroidism, associated with a significant decrease in type I fiber composition (Larsson et al 1994), results in a significant decrease in CA III activity in rat SOL muscles. However, in HYPO muscles, there is an up-regulation of CA III (figure 1). Therefore, alterations in SOL CA III activity in HYPER and HYPO rats as seen in our experiments were in line with expected results, and were similar to those seen in previous studies (Tremblay et al 1993; Frémont et al 1987; Gagnon et al 1985). That is, there is an up- and down- regulation of CA III in HYPO and HYPER rat SOL muscles, respectively. CA III activity in HYPER SOL muscles was 76% of control values, whereas HYPO CA III activity was increased to 216% of control values.

The levels of sensitive CA activity are very similar in all three groups (figure 2) although a slight decrease and increase in HYPER and HYPO muscles, respectively, was seen . In this, it may be postulated that other CA isoforms are not necessarily as much up- and down-regulated by the thyroid hormone as is the case for CA III. Included in this sensitive CA group are CA I, II, IV, and V (Deutsch 1987; Heck et al 1994; Supuran et al 1997). CA I, IV and V, most likely, would not have contributed to the sensitive CA activity in our cystolic fraction since CA I is not found in skeletal muscle (see table 1 of Introduction section). CA IV is a membrane bound isoform (Decker et al 1996) that would have been discarded with the pellet after centrifugation and CA V is mitochondrial CA (Karhukorpi et al 1992) that also would not have been included in the cytosolic fraction. Therefore, CA II is probably responsible for most of the sensitive activity seen, as CA II, being in the cytosol (Henry 1996), would have been isolated in the same fraction as CA III upon homogenization. CA II has a very high level of specific activity (Deutsch 1987) and therefore, even small variations in the amounts of CA II could display significant differences in total activity (Deutsch 1987), yet none were seen among the three different groups. It thus stands to reason that the quantities of CA II were very consistent between the HYPER, CTR and HYPO SOL muscles.

To the best of our knowledge, no studies have been done regarding the modulation of CA III in EDL muscles by thyroid hormones. The EDL has only about 4% type I fibers and the rest is made up of a mixture of type IIa, IIb and a trace quantity of type IId fibers (Hamalainen and Pette 1997). Also, very little CA III activity is present in EDL control muscles, especially when compared to that seen in SOL muscles (Shiels et al 1984; Frémont

et al 1991). Interestingly enough, however, EDL muscles, showed a qualitatively similar regulation of CA III as the SOL in response to varying amounts of T_3, but at much smaller concentrations (see figure 3). That is, there was an up- and down-regulation of CA III activity in HYPO and HYPER EDL muscles, respectively, to the modulation seen in SOL muscles. When these modifications were expressed in percent of control values, HYPER CA III activity was 27% that of CTRs, whereas there was a marked 7 fold increase in enzyme activity in HYPO EDL muscles, these values being considerably different from those seen in SOL muscles. This is most likely due to the fact that EDL muscles have so little CA III to begin with, and therefore, even a small increase or decrease in content makes up a large percentage of the original values. Nonetheless, a pattern of CA III activity modulation by thyroid hormone was evident. Frémont et al (1987) also reported a similar differential modulation of CA III in various muscle fiber types. That is, hyperthyroidism resulted in an deinduction of CA III activity in the SOL muscle (primarily type I), an induction of CA III activity in the superficial vastus lateralus muscle (primarily type IIb), but no significant regulation of CA III in the tibialis anterior muscle, made up mostly of type IIa muscle fiber types. Our results, using the EDL muscle, an intermediate between the vastus lateralis and tibialis anterior muscles are in line with their hypothesis that CA III activity is most affected in muscles containing higher proportions of type I fibers, again supporting the idea of fiber type specific modulation of CA III by thyroid hormones.

Additionally, EDL muscles did not display the same levels of sensitive CA activity among all three groups, as was seen in the SOL muscles (figure 4). Sensitive activity for both HYPER and HYPO muscles was significantly higher than in control muscles, with values of 8.2 U/mg protein in HYPER muscles, 8.7 U/mg protein in HYPO muscles, and only about 3 U/mg protein in CTR muscles. This is probably explained by the fact that all SOL muscles and CTR EDL muscles used here were rinsed in the Kreb's solution for almost two hours while HYPER and HYPO EDLs used in the present study, however, were not. Therefore, blood undoubtedly remained within the muscles upon homogenization. This is significant since red blood cells contain considerable levels of CA II (Dodgson et al 1988). As described earlier, most of the sensitive activity seen in our extracts could probably be attributed to CA II, and this would therefore explain why the sensitive activities of HYPER and HYPO groups were about equal. Most likely, had all three muscles been rinsed, the same pattern would have been seen as was observed in SOL muscles, that is, equal sensitive activity levels among all three groups. Nonetheless, blood in the homogenate would not have thrown off CA III content analysis, making our results very conclusive.

Western Analysis

Semi-quantitative western blotting analysis was used to determine CA III content by measuring relative optical densities of the bands (see figure 2). According to this analysis, CA III content in HYPER SOL and EDL muscles was 82.8% +/- 5.3% and 85.9% +/- 4.09% of CTR values, respectively. HYPO SOL and EDL muscles had a CA III content of 188.4% +/- 9.61% and 195.5% +/- 18.09% of CTR, respectively. SOL values are depicted in figure 5 and EDL in figure 6. There was no statistical difference between up- and down-regulation of CA III in the two muscles according to this data (see figure 7). These results were particularly significant considering CA III activity analysis in SOL muscles showed almost the exact same quantitative results. Therefore, this analysis really shows for the first time how qualitatively and quantitatively similar the regulation of CA III by thyroid hormones is in both slow- and fast-twitch muscles.

Conclusion

It is a well known fact that thyroid hormones are a regulator of CA III content in slow-twitch muscle fibers. Slow-twitch muscles have a very high content of CA III (Frémont et al 1991), and therefore modifications in CA III activity in this muscle can be easily noted. Thyroid hormone changes CA III activity and content of the SOL concomittantly with the changes observed in fiber type composition, that is, with increasing amounts of thyroid hormone, the proportion of type I fibers is decreased. This raises interesting questions on whether CA III is indeed present in levels proportional to the fiber type composition, or if these changes seen in CA III content are a specific effect of modifications in the slow-twitch muscles metabolic activity. Keeping in mind the fact that control SOL muscles contain 85% type I fibers (Tremblay et al 1993), HYPER muscles display a decrease in type I fiber composition to 66-70%, whereas HYPO muscles represent nearly a pure type I tissue sample, which is only a 15% change. Many assumptions may be made regarding the correlation of type I fiber proportion and CA III activity. That is, if CA III modulation was in accordance only with fiber type composition, it is expected that a greater change in CA III concentration would be seen in HYPER soleus muscles versus HYPO muscles. This, however, is not the case and, in fact, HYPO muscles showed a more significant change in CA III content and activity. Therefore, it is more likely that CA III concentration also changes concomitantly with metabolic changes taking place in the muscle. It is therefore obvious that in the HYPO SOL muscles, the increase in CA III content is not

only the consequence of an increased proportion of type I fibers, but also the result of an increased expression of CA III in the already present type I fibers.

From our experiments, using two different methods and three different experimental groups, we were able to demonstrate that the up- and down-regulation of CA III in HYPO and HYPER muscles, respectively, also occurs in type II muscles with very low CA III content. Why CA III is more necessary in muscles with low glycolytic capacites remains to be further analyzed. Looking, for example, at up- and down-regulations of other glycolytic enzymes with increasing and decreasing amounts of thyroid hormone, could help answer this question. Also, while our measurements of the regulation of CA III were remarkably consistent between groups, these data, nevertherless, only look at a short term modulation of this hormone in muscle. However, it is quite likely that in this time frame, the muscles were still in a state of transition and had not reached maximum or minimum CA III potentials. Therefore, it would be interesting to look at a longer time frame. From this, a curve demonstrating relative time span by which thyroid hormone begins to effect CA III concentration, and the point at which CA III levels reach their maximum could be derived. Taken together, these things would also give us a greater idea of how alterations in CA III activity coincides with alterations in fiber type composition. Either way, this enzyme, that has been so carefully conserved throughout evolution and makes up such a large percentage of type I skeletal muscle cytosol, without a doubt plays a much greater role than merely just the hydration of CO_2.

References

1. Bruns, W.; Dermietzel, R.; Gros, G. 1986. Carbonic anhydrase in the sarcoplasmic reticulum of rabbit skeletal muscle. *Journal of Physiology* (London). 371:351-364

2. Caiozzo, V.J.; Herrick, R.E.; Baldwin, K.M. 1991. Influence of hyperthyroidism on maximal shortening velocity and myosin isoform distribution in skeletal muscles. *American Journal of Physiology.* 261: C285-C295

3. Côté, C.H.; Perreault, G.; Frenette, J. 1997. Carbohydrate utilization in rat soleus muscle is influenced by carbonic anhydrase III activity. *American Journal of Physiology.* 273 (*Regulatory Integrative Comp. Physiol.* 42): R1211-R1218

4. Decker, B.; Sender, S.; Gros, G. 1996. Membrane associated carbonic anhydrase IV in skeletal muscle: subcellular localization. *Histochemistry and Cell Biology.* 106(4): 405-411

5. Dodgson, S.J.; Forster, R.E. 2nd; Sly, W.S.; Tashian, R.E. 1988. Carbonic anhydrase activity of intact carbonic anhydrase II-deficient human erythrocytes. *Journal of Applied Physiology.* 65(4): 1472-1480

6. Deutsch, H.F. 1987. Carbonic anhydrases. *International Journal of Biochemistry.* 19(2): 101-113

7. Everts, M.; Clausen, T. 1986. Effects of thyroid hormones on calcium contents and Ca exchange in rat skeletal muscle. *American Journal of Physiology.* 251: E258-E265

8. Frémont, P.; Charest, P.M.; Côté, C.; Rogers, P.A. 1991. Distribution and ultrastructural localization of carbonic anhydrase III in different skeletal muscle fiber types. From: The Carbonic Anhydrases. Edited by Susanna J. Dodgson, Richard E. Tashian, Gerolf Gros and Nicholas D. Carter. Plenum Publishing Corporation.

9. Frémont, P.; Charest, P.M.; Côté, C.; Rogers, P.A. 1988. Carbonic anhydrase III in skeletal muscle fibers: An immunocytochemical and biochemical study. *The Journal of Histochemistry and Cytochemistry.* 36(7):775-782

10. Frémont, P.; Lazure, C.; Tremblay, R.R.; Chrétien, M.; Rogers, P.A. 1987. Regulation of carbonic anhydrase III by thyroid hormone: opposite modulation in slow- and fast-twitch skeletal muscle. *Biochemistry and Cell Biology.* 65: 790-797

11. Gagnon, J.; Ho-Kim, M.A.; Champagne, C.; Tremblay, R.R.; Rogers, P.A. 1985. Modulation of a major 30-KDa skeletal muscle protein by thyroid hormone. *FEBS Letters.* 180:335-340

12. Gros, G.; Dodgson, S.J. 1988. Velocity of CO_2 exchange in muscle and liver. *Annual Review of Physiology.* 50: 669-694

13. Hamalainen, N.; Pette, D. 1997. Coordinated fast-to-slow transitions of myosin and SERCA isoforms in chronically stimulated muscles of euthyroid and hyperthyroid rabbits. *Journal of Muscle Research and Cell Motility.* 18: 545-554

14. Henry, R.P. 1996. Multiple roles of carbonic anhydrase in cellular transport and metabolism. *Annual Review of Physiology.* 58: 523-538

15. Ianuzzo, D.; Patel, P.; Chen, V.; O'Brien, P.; Williams, C. 1977. Thyroidal trophic influence on skeletal muscle myosin. *Nature.* (London), 270(3): 74-76

16. Izumo, S.; Nadal-Ginard, B.; Mahdavi, V. 1986. All members of the MHC multigene family respond to thyroid hormone in a highly tissue-specific manner. *Science.* 231: 597-600

17. Karhukorpi, E.K.; Lakkakorpi, P.; Carter, N.; Dodgson, S.; Vaananenk. 1992. Mitochondrial carbonic anhydrase in osteoclasts and two different epithelial acid-secreting cells. *Histochemistry Journal.* 24(1):9-14

18. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. *Nature.* 227: 680-685

19. Larsson, L.; Li, X.; Teresi, A.; Salviati, G. 1994. Effects of thyroid hormone on fast- and slow-twitch skeletal muscles in young and old rats. *Journal of Physiology.* 481(1): 149-161

20. Maren, T.H. 1960. A simplified micromethod for the determination of CA and its inhibitors. *Journal of Pharmacological Experimental Therapy.* 130: 26-33

21. McDonough, K.H.; Chen, V.; Spitzer, J.J. 1987. Effect of altered thyroid status on in vitro cardiac performance in rats. *American Journal of Physiology.* 252: H788-H795

22. Milot, J.; Frémont, P.; Côté, C.H.; Tremblay, R.R. 1991. Differential modulation of carbonic anhydrase (CA III) in slow- and fast-twitch skeletal muscles of rat following denervation and reinnervation. *Biochemistry and Cell Biology.* 69:702-710

23. Nicol, C.J.M.; Bruce, D.S. 1981. Effect of hyperthyroidism on the contractile and histochemical properties of fast and slow twitch skeletal muscle in the rat. *Pfluegers Archives.* 390: 73-79

24. Norenberg, K.M.; Herb, R.A.; Dodd, S.L.; Powers, S.K. 1996. The effects of hypothyroidism on single fibers of the rat soleus muscle. *Canadian Journal of Physiology and Pharmacology.* 74: 362-367

25. Sheils, A.; Jeffrey, S.; Wilson, C.; Carter, N. 1984. Radioimmunoassy of carbonic anhydrase III in rat tissues. *Biochemistry Journal.* 218: 281-284

26. Towbin, H.; Staehelin, T.; Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrilamide gels to nitrocellulose sheets: Procedure and some applications. *Proceedings of the National Academy of Science USA.* 76: 4350-4354

27. Tremblay, R.R.; Gaumont, F.; Milot, J.; Candas, B.; Côté, C.H. 1993. Inhibition of carbonic anhydrase III exerts paradoxical influences on the soleus muscle of hypo and hyperthyroid rats. *Basic and Applied Myology.* 3(2): 93-102

28. Winder, W.W.; Holloszy, J.O. 1977. Response of mitochondria of different types of skeletal muscle to thyrotoxicosis. *American Journal of Physiology.* 1(3): C180-C184

Figure 1. CA III Activity in SOL muscles

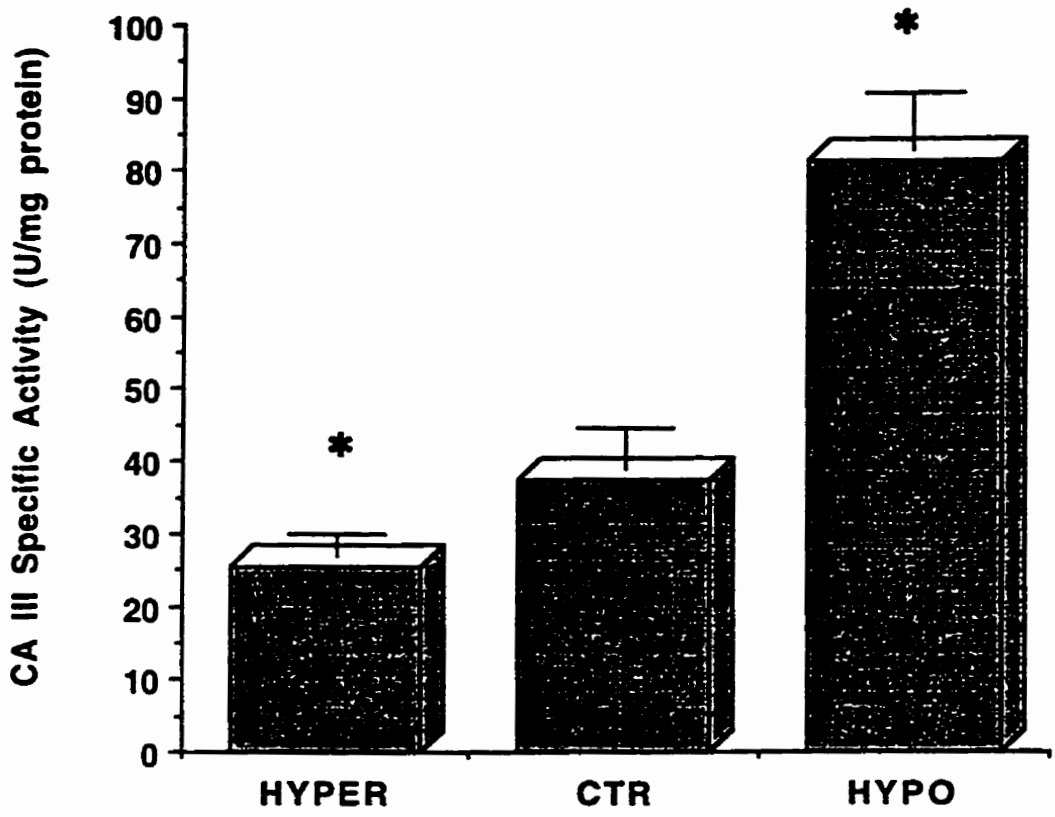


Figure 1. Showing a comparison of CA III activity in HYPER, CTR, and HYPO SOL muscles.
n= 20 for each group.
Symbols: significantly different from CTR values ($p<0.05$).

Figure 2. Sensitive CA Activity in SOL Muscles

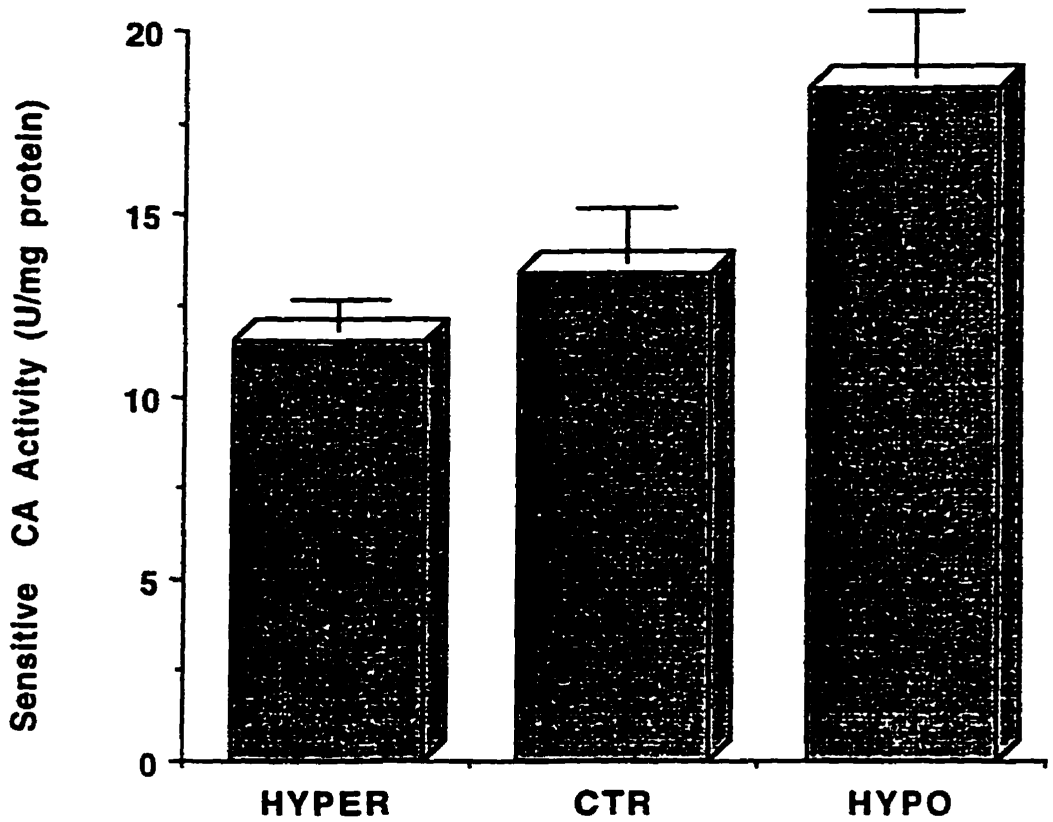


Figure 2. Showing a comparison of sensitive CA activity in HYPER (n= 24), CTR (n= 20), and HYPO (n= 22) SOL muscles.
There was no significant difference between any of the three groups.

Figure 3. CA III Activity in EDL Muscles

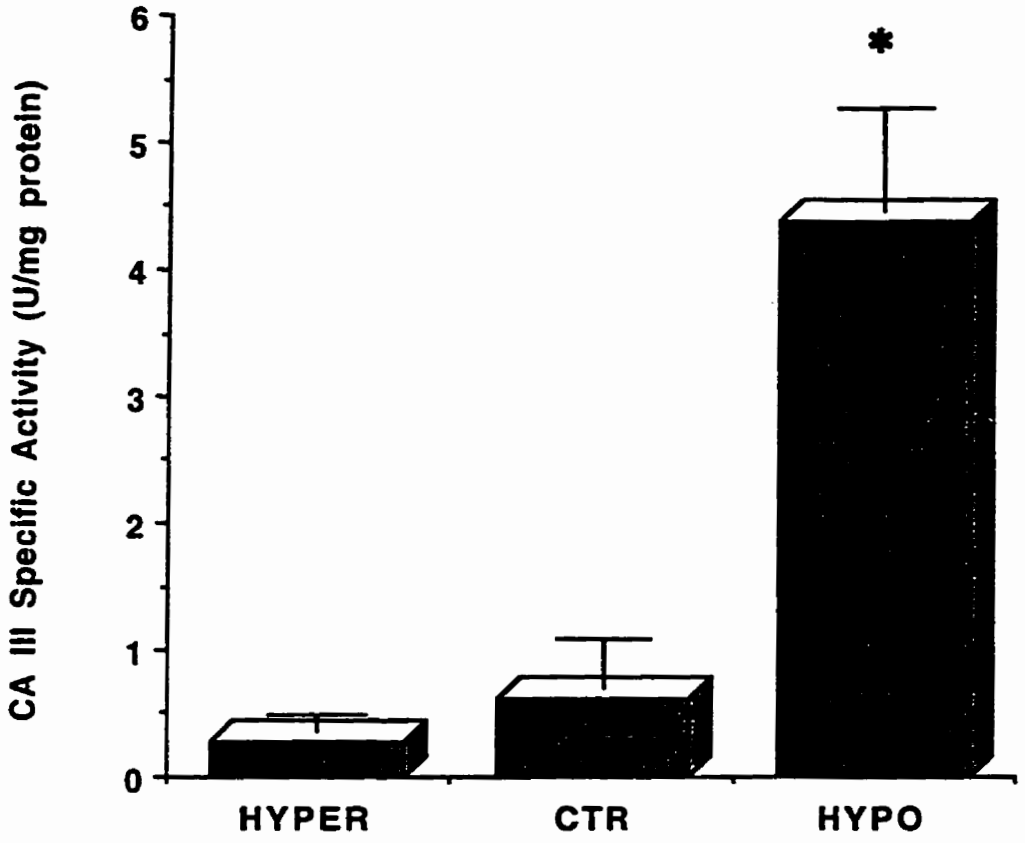


Figure 3. Showing a comparison of CA III activity in HYPER (n= 24), CTR (n=20), and HYPO (n=22) EDL muscles.
Symbols: * significantly different from CTR values ($p < 0.05$).

Figure 4. Sensitive CA Activity in EDL muscles

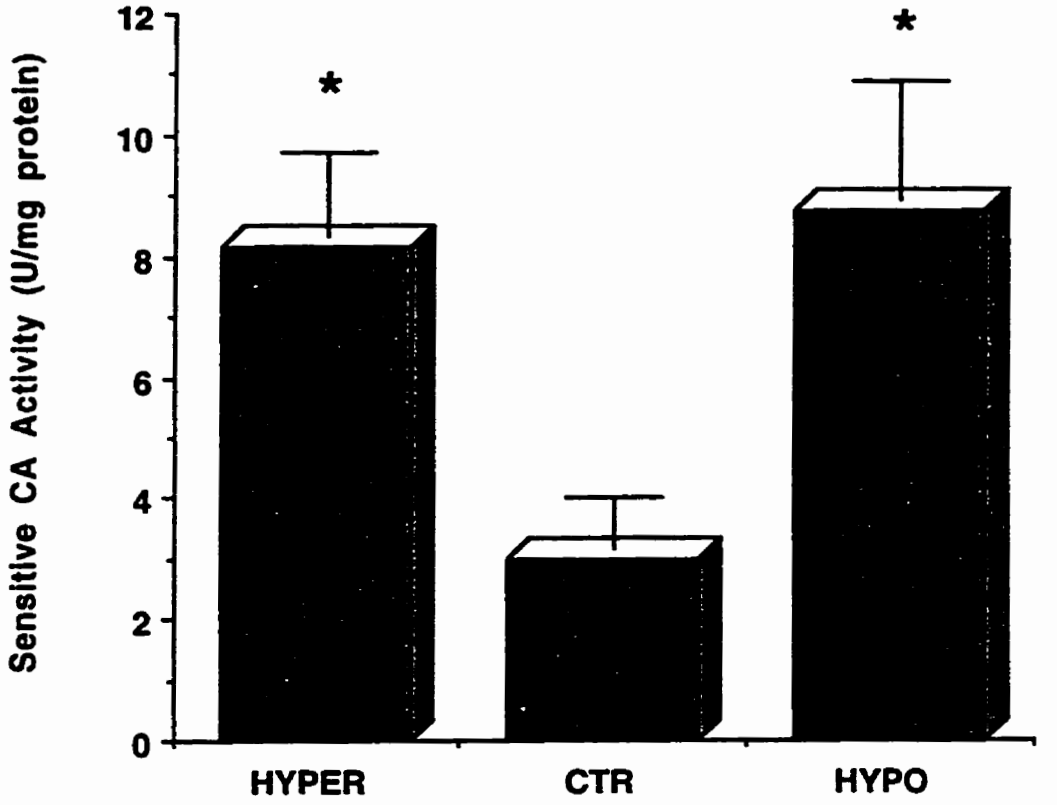


Figure 4. Showing a comparison of sensitive CA activity in HYPER (n=24), CTR (n= 20), and HYPO (n= 22) EDL muscles. Both HYPER and HYPO muscles were significantly different from CTR values ($p < 0.05$).

Figure 5. Relative CA III content in HYPER, CTR, and HYPO SOL Muscles as Determined by Western Analysis.

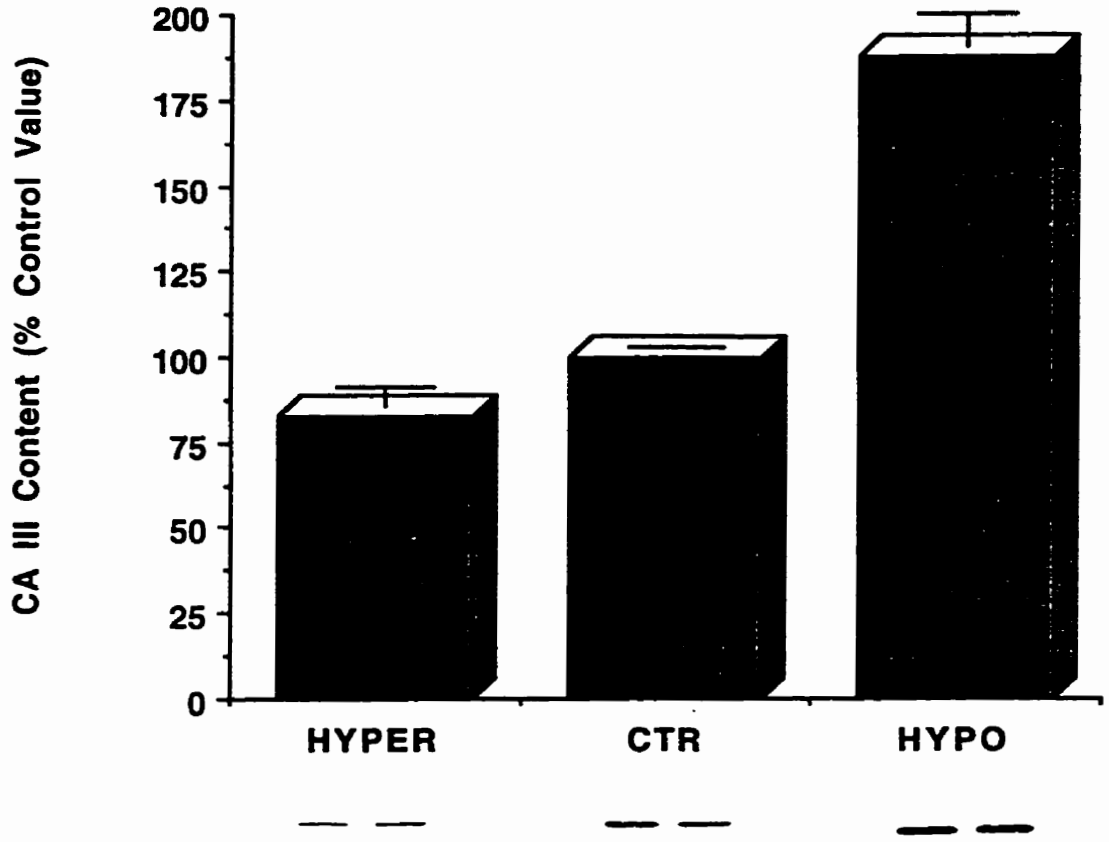


Figure 5. Relative CA III content of HYPER and HYPO SOL muscles compared to CTR values.
n= 20 for each group
Also shown is nitrocellulose film with bands of relative CA III content. Two different muscles are depicted for each thyroid group.

Figure 6. Relative CA III Content in HYPER, CTR, and HYPO EDL Muscles as Determined by Western Analysis

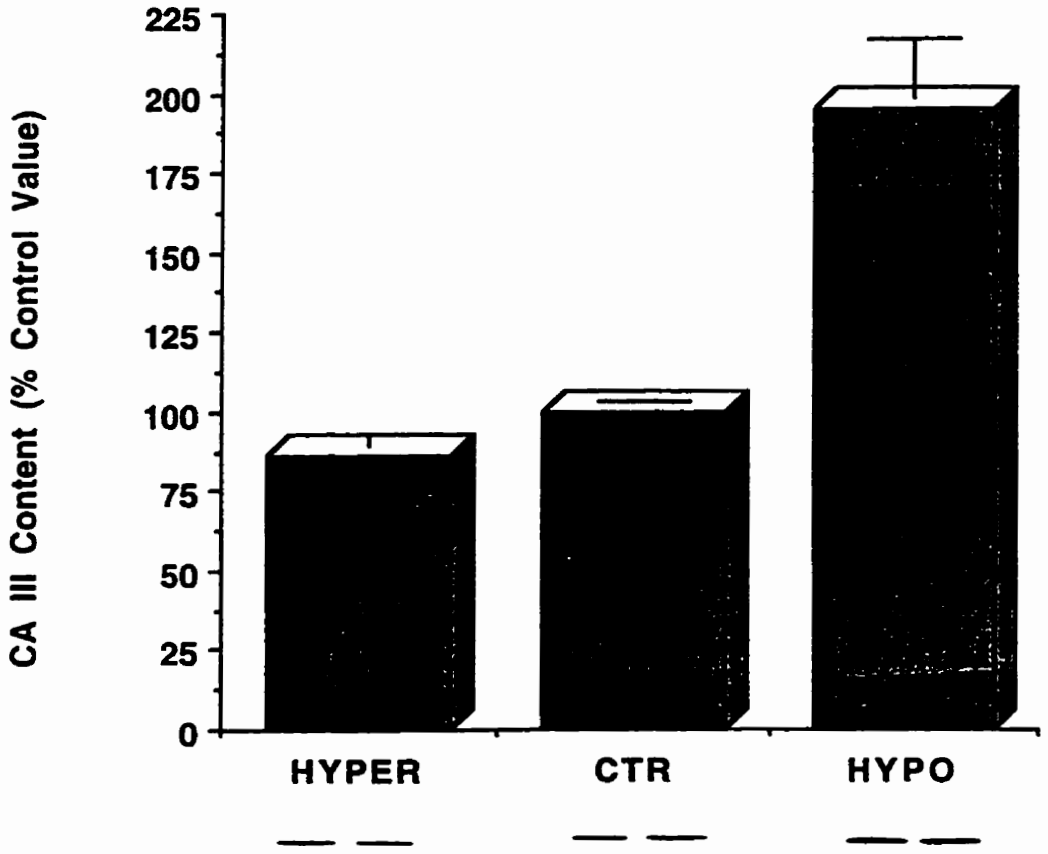


Figure 6. Relative CA III content of HYPER (n=24) and HYPO (n= 22) EDL muscles compared to CTR (n=20) values. Also shown is nitrocellulose film with bands of relative CA III content. Two different muscles are depicted for each thyroid group.

Figure 7. Comparison of CA III Content in Three Different Thyroid Groups of SOL and EDL Muscles as determined by Western Analysis.

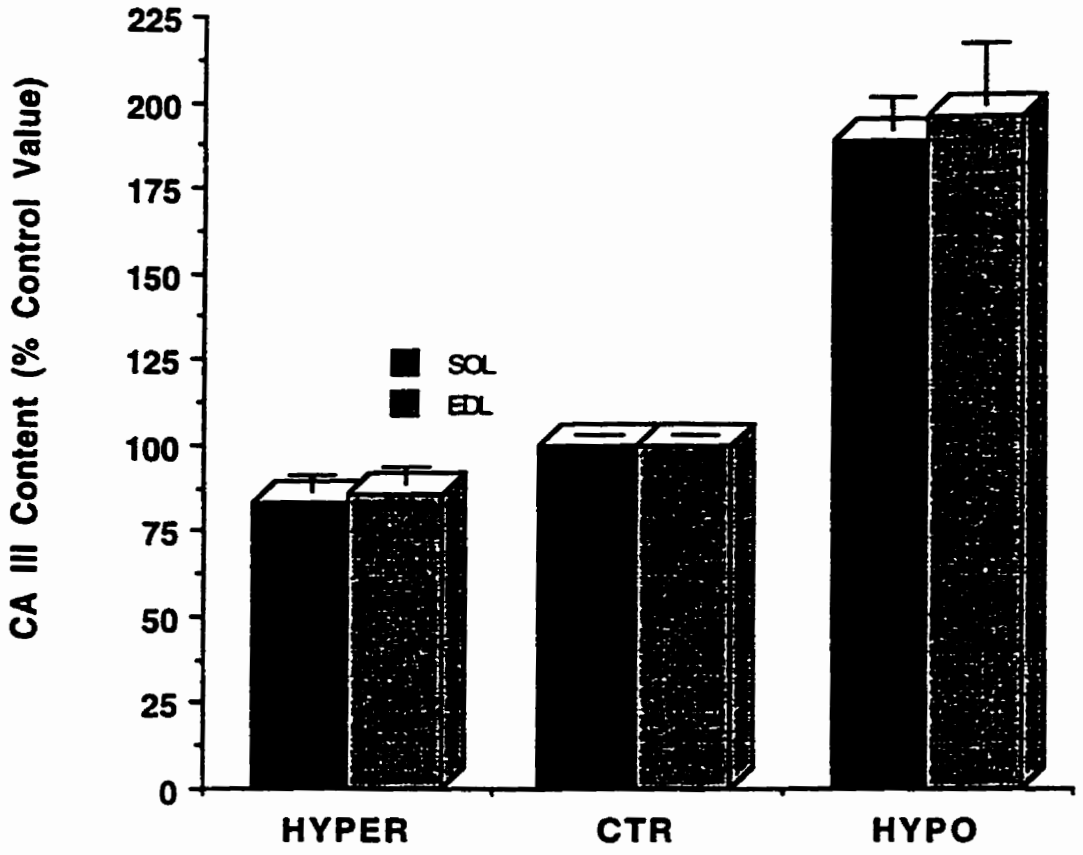


Figure 7. Relative CA III content in HYPER, CTR, and HYPO SOL and EDL muscles. Modulation of CA III content by thryoid hormone is qualitatively similar in slow- and fast-twitch muscles.

Chapter 3

The Influence of Carbonic Anhydrase III Inhibition on Contractility and Fatigability is Dependent on its Level of Expression in Type I Muscle

Fabrisia Ambrosio and Claude Côté

CHUL Research Center
Lipid Research Unit
Université Laval

Abstract

Contractile properties of skeletal muscle are mainly determined by myosin heavy chain (MHC) gene expression (Caiozzo 1991). With increased slow MHC expression, there is an increase in slow oxidative fiber type expression, and therefore slower contractile properties. Just the opposite is seen with an increase in fast MHC expression. In addition, CA III is relatively high in slow twitch fibers as compared to fast twitch fibers (Tremblay 1993). It was the objective of this experiment to determine the effect of thyroid hormone on the contractile properties and fatigueability of fast-twitch muscles. Because of the known modulation of MHC expression by thyroid hormones (Caiozzo 1991), this was an elegant model to study the effects of thyroid hormone on the contractile properties of slow twitch versus fast twitch muscles, while at the same time correlating these phenotypic changes to alterations in CA III content and activity. Rats were made hyperthyroidic (HYPER) by injections of triiodothyronine (T_3) hormone every other day for two weeks. Hypothyroidism was surgically induced for the HYPO group. Contractile properties of control, HYPER and HYPO groups and fatigue tests of 20 and 30 minutes for soleus and EDL muscles, respectively, were performed on each thyroid group of muscles to further examine the already noted effect of CA III activity/inhibition in skeletal muscle. Results indicated significant differences in the contractile properties of HYPER and HYPO soleus muscles, however, not as solid a modification between the EDL muscles groups was seen. Fatigue protocols showed that with increasing amounts of T_3, muscles became less fatigue resistant, and raised speculation about CA III's role in this mechanism, since the effect of CA III inhibition on fatigue resistance of the muscle was clear in control soleus muscles, however, less apparent in the other experimental groups. It is therefore concluded that the influence of CA III on the fatigue resistance of skeletal muscle is dependent on the concentration of this enzyme, as well as the contractile status of the muscle to begin with.

Introduction

For several years now, the influence of variation in the circulating levels of thyroid hormone on the contractile properties of skeletal muscle has been known. For example, soleus (SOL) muscles from thryoidectomized rats undergo a conversion from 85% type I fibers to nearly a completely pure type I tissue (Tremblay et al 1993). In comparison, treatment with tri-iodothyronine (T_3) makes the normally slow-twitch muscle SOL take on fast-twitch muscle characteristics. Caiozzo et al (1991) describe this effect graphically as seen in figure 1.

Figure 1. Effect of T_3 Hormones on SOL Rat Muscle Isometric Twitches

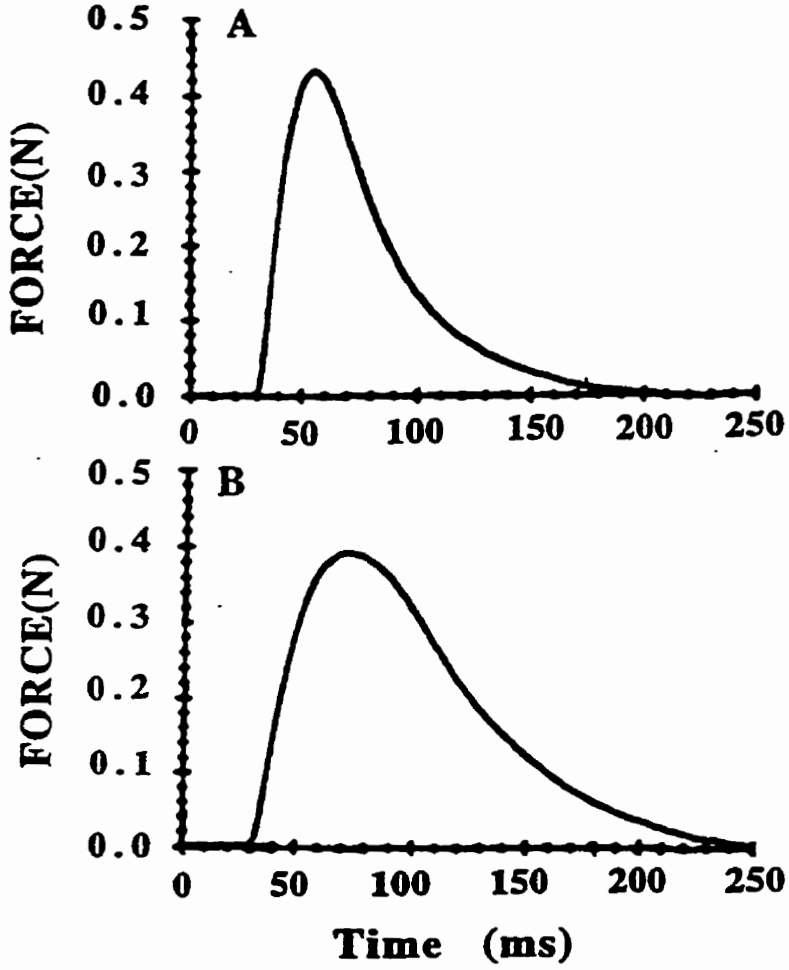


Figure 1a. and 1b. represent hyperthyroid (HYPER) versus control (CTR) SOL muscle isometric twitches, respectively. HYPER contractile properties were significantly different from CTR values. HYPER was induced by 300g i.p. injections of T_3 every other day for 20 weeks.

For each group, n=8.

The changes as seen in the above figure are believed to occur because T_3 is a very potent regulator of myosin heavy chain (MHC) expression, which directly relates to the maximum shortening velocity (MSV) (Larsson et al 1994). The head portion of the myosin heavy chain is responsible for cross bridge cycling via the binding to actin during contractions (Sieck et al 1996). The contractile apparatus appears to be controlled by two main factors, MHC and the intrinsic properties of the sarcoplasmic reticulum (SR)(Larsson et al 1994). In slow-twitch fibers, the slow myosin isoform (SM) predominantly exists, whereas in fast twitch muscles, fast myosin-1 (FM1) dominates (Caiozzo et al 1991) and it is the degree to which each of these isoforms is expressed that determines MSV and also twitch contractile speed. However, SR can also control the speed of contraction of the isometric twitch (Larsson et al 1994; Li et al 1996), mainly by its Ca^{2+} release and uptake properties. Thyroid hormones influence the contractile properties of skeletal muscles through its regulation of myosin isoform composition and SR properties (Li et al 1996). Studies done by van der Linden and colleagues (1996) showed that in both SOL and extensor digitorum longus (EDL) muscles, increased T_3 levels induced a full conversion to a fast MHC expression in one half of the fibers that had been slow twitch before the treatment and the remaining fibers were described as having a mixed phenotype. Also, they observed an increased SR Ca^{2+}-ATPase activity pumps causing faster relaxation times (Kragie and Smeihorowski 1993). On the other hand, it has been proven that HYPO results in a fiber type shift from fast to slow fibers in SOL muscles (Norenberg et al 1996; Caiozzo et al 1992; Ianuzzo et al 1977) and a concomitant decrease in myosin ATPase activity (Caiozzo et al 1992). It has also been suggested that the calcium containing ability of HYPO SR is reduced, leaving less available for contractions (Norenberg et al 1996; Nwoye et al 1982).

Keeping in mind the mechanical consequences of increasing and decreasing amounts of circulating T_3, it is also important to look at alterations occurring at the metabolic level. That is, thyroid hormones have been shown to have a significant effect on carbohydrate metabolism in skeletal muscles (Kudelska et al 1995). For example, increases in T_3 levels augment glucose uptake and glycolysis, and also has an effect on glucose oxidation and glycogen synthesis in rat skeletal muscles (Kudelska et al 1996; Dimitriadis et al 1988). More importantly for our purposes, T_3 has been shown to have a significant role in the regulation of carbonic anhydrase (CA), specifically CA III. This enzyme is found in extremely high quantities in the cytosol of type I skeletal muscle (Côté et al 1993). Tremblay et al (1993) described the up- and down-regulation of CA III in HYPO and HYPER rats, respectively. With increasing amounts of thyroid hormones, as the muscle becomes faster and more glycolytic, CA III content diminishes.

Since inhibition of CA III activity in the type I SOL muscles has been shown to improve the resistance to fatigue under specific conditions, our goal here was to test the hypothesis that increases and decreases in CA III activity of the SOL muscle induced by HYPO and HYPER conditions, respectively, would lead to qualitatively similar changes in the influence of methazolamide (METH) on resistance to fatigue. Secondly, as shown in the previous chapter, we also described a regulation of CA III by thyroid hormones in the fast-twitch EDL, that is, a decrease in CA III concentration in HYPER muscles and an increase in HYPO muscles. Therefore, as our second objective, we aimed to determine if CA III inhibition in the type II EDL muscle under normal, HYPO, and HYPER conditions could influence fatiguability and/or contractility.

Materials and Methods

Treatment of Animals

Female Wistar rats (130-150 g) ordered from Charles River Laboratories were divided into three groups. In the CTR group, rats (n= 40) were left unmanipulated and were fed a normal diet. The HYPER group (n=35) was subcutaneously injected with 75µg/100g body weight of tri-iodothyronine (T_3) every other day for two weeks. These rats were also given a normal laboratory diet *ad libidum*. The HYPO group consisted of thyroidectomized rats (n=45) ordered from Charles River Laboratories. These rats were allowed a minimum of two weeks in the HYPO condition before beginning manipulations.

Surgical Procedures

Rats were anesthetized i.p. using 5mg sodium pentobarbital/100g body weight. The SOL or the EDL muscle was isolated and removed. Next, the muscle was fixed in a vertical position between two platinum field electrodes in a brace attached at the bottom to a rigid support, and on top to an isometric force transducer (GRASS). The brace was placed inside a 150ml bath filled with Kreb's Ringer Bicarbonate (with 2mg/ml glucose and 20 µg/ml curare), and maintained at 25°C. Half of all muscles were incubated with 1mM METH (a concentration known to inhibit all CA III activity) added to the Kreb's solution immediately as the muscle was fixed in place. METH was chosen because of its high permeability and potency (Holder 1965). Muscles were then allowed 30 minutes to equilibrate. The optimal length (Lo) of the muscle, that is, the length at which maximum twitch tension is achieved, was determined at this time.

Contractile Properties

Immediately after the equilibration period, contractile measurements began. Stimulation was done with supramaximal 0.5 ms pulses. The maximum twitch force (Pt), time to peak twitch (TPT), and 1/2 relaxation time (1/2 RT) were determined first. Shortly after, a force frequency curve was obtained. For the SOL, these measurements included frequencies of 10, 20, 35, 80, and 100 Hz with each tetanic contraction lasting 700 ms. For the EDL, stimulation frequencies used were 35, 50, 80, 100, 120Hz and

140Hz for 200ms duration. Intervals of 60s were allowed for both muscles in between consecutive tetanic stimulations. At the end of this procedure, 10 minutes of rest were given in order for muscles to recover before beginning the fatigue tests.

Fatigue Tests

From the force frequency curves obtained for each group of muscles, the frequency of stimulation producing 60% of the maximum tetanic tension was determined for SOL muscles. Muscles were stimulated at that specific frequency for 500 ms, every 5 seconds for 30 min. For EDL muscles, 75% of the maximum tetanic tension was calculated. Muscles were stimulated at 200 ms every 5 seconds for 20 min.

Statistical Analysis

All results are given as means +/- the standard error of the mean (SEM). Data were considered significantly different at $p \leq 0.05$. ANOVA and Student's T-test were used to analyze data.

Results

Contractile Properties

In the SOL muscle, T_3 induced variations in contractile properties were, for the most part, significant. In the EDL muscle, however, drastic differences were often hard to see. Values for TPT for both HYPER and HYPO SOL muscles were statistically different from CTR values (see figure 2). Values for TPT in the EDL, on the other hand, showed a less consistent pattern between the three groups. Values for TPT in HYPER muscles were significantly faster than CTR values, but no difference could be found between control and HYPO EDLs (see figure 3). There was also no significant different between muscles incubated with and without methazolamide.

Figures 4 and 5 describe the force frequency curves of the three groups for SOL and EDL muscles, respectively. Values at each frequency point are expressed as percentage of maximum force produced. As seen for both muscles, with increasing amounts of T_3, the muscle reached its maximum output at a higher frequency of stimulation. Values for HYPER and HYPO SOL muscles were significantly different from CTR values at all points. HYPO EDL muscles were significantly different from control values only in the beginning of the curve, whereas HYPER EDL muscles were significantly different at all points.

Values for relaxation times (1/2 RT), maximum twitch tension (Pt), maximum tetanic tension (Po), and the ratio between the two (Pt/Po) are presented in tables 1a and 1b for the SOL and EDL, respectively. While the SOL muscle showed significant differences for nearly every variable, variations in the EDL contractile properties were basically limited to HYPO muscles. Differences in both groups of muscles with and without METH were minimal, with the exception of 1/2 relaxation times of control soleus and EDL muscles. This effect was also seen in previous studies done by our laboratory (Côté et al 1997).

Fatigue Protocol

Since thyroid state affected the work load of muscles at given frequencies, we decided to use a stimulation frequency leading to similar work load as determined by tension output based on the force frequency curves we had previously derived. For SOL muscles, fatigue tests were initiated at 60% of the muscle's Po. CTR muscles were stimulated at 10 Hz during the 30 minute protocol, but for HYPER SOL to work at a

comparable workload, 21 Hz was used. For HYPO muscles, the frequency had to be decreased to 8Hz. Figure 6 shows distinctly that in SOL muscles, lower amounts of thyroid hormone increase the resistance to fatigue of the muscle when such a test is used. However, a clear significant difference was not observable between HYPER and CTR groups except in the first 5 minutes of the protocol, where HYPER muscles were slightly more fatigue resistant. Significant differences in fatiguability were not noticed between muscles incubated with and without METH in any but the CTR group (see figure 7a), where a significant difference in fatigue was noticed almost immediately after the fatigue test began. In the HYPER group, a slight tendency toward improved fatiguability with METH could be seen, however minimal and not statistically significant (see figure 7b). Finally, in HYPO muscles, both fatigue curves were remarkably similar and there was no significant difference between the two (figure 7c).

For CTR EDL muscles 75% of maximum tetanic force was elicited at 35 Hz, while the frequency had to be increased in HYPER muscles to 40 Hz with and without METH, respectively. In HYPO muscles, decreased frequencies of 30 Hz were used. Using these adjusted stimulation frequencies, fatigue curves for the three groups could be better compared. Once again, differences among the three different thyroid states were less drastic than was seen in SOL muscles, and no significant difference was seen between the three curves (figure 8). Similarly, there was no significant difference between EDL muscles incubated with and without METH in any of the three groups of muscles. Again, this is most likely due to the extremely small quantities of CA III present in this muscle.

Discussion

Treatment of Animals:

Final values for body weights for the three different groups of animals were about equal, as also observed in experiments done by Sillau (Sillau et al 1984). Others, however, have found that HYPER causes decreases in body weight and/or muscle weight (Li et al 1996; Pernitsky et al 1996) because of an increase in metabolic rate. Such differences can be attributed to variations in experimental design, such as time allowed to induce the thyroidic condition and dosages used. In Pernitsky's experiments, mice were injected with 2μg T_3/g body weight/day for four weeks (Pernitsky et al 1996), and in Li's experiments, subcutaneous injections of 300μgT_3/kg body weight were administered every other day for four weeks to male albino rats. We, however, used a two week time frame and only 75μgT_3/kg body weight to introduce changes in thyroid state and muscle properties based on our observations that this time frame was sufficient to bring the muscles to a transitional state, and significant differences between the three groups of both fast and slow type muscles could be observed (see previous chapter). This was of special importance considering the fact that the onset of change in muscle fiber type proportion in the two different types of muscle is not equal, according to Gambke et al (1983) who observed a delay in certain fibers of EDL muscles.

Contractile Properties

For the SOL muscle, our results mainly confirmed previous studies done on the effect of T_3 on contractile properties. Contractile speed (TPT) was significantly different between the three thyroid groups. There are several theories on the actual mechanism by which thyroid hormone effects SOL muscle properties. Some say the faster speed of contraction with increasing thyroid concentration is a result of atrophy of type I fibers and a resulting increase in fast twitch characteristics due to the remaining type IIa fibers (Lomax and Robertson 1992). Studies done by Lomax and Robertson (1992) showed that HYPO SOL muscles showed a decrease in type II fiber composition from 8.0% type II fibers to 0.8% type II fibers. In HYPER SOL muscles, just the opposite was true, and muscles contained approximately 33% type II fibers (Lomax and Robertson 1992). Others propose that changes in HYPER muscles are a result of modified MHC isoform expression (Gosselin et al 1996). In fact, it is probably a combination of the two mechanisms considering 1.) atrophy of muscle fibers is probably happening since an

increased rate of protein degradation in HYPER muscles can be observed (Brown and Millward 1983) and 2.) several have found solid evidences supporting the idea of modulation of MHC gene expression by thyroid hormones (Gosselin et al 1996; Li et al 1996; Larsson et al 1994). However, despite all the studies done on the regulation of MHC expression by thyroid hormones, there is still discrepancy in this area. Some authors say HYPER results in a down regulation of slow MHC isoform expression (Izumo et al 1986), whereas others describe a coordinated conversion of slow MHC to a fast twitch phenotype (van der Linden 1996). The latter of the two seems most likely since there is also evidence that HYPER induces an increase in the percentage of fibers expressing fast type SR Ca^{2+}-ATPase activity (Li et al 1996). On the other hand, it appears to be generally agreed that the HYPO state induces an increase in slow MHC gene expression (Sieck et al 1996; Gosselin et al 1996).

In contrast, significant differences were not as obvious in the EDL muscle. This, as well, is in line with previous studies done (Fitts et al 1994; Sillau 1984). In 1984, Sillau stated that muscles made up primarily of fast twitch fibers, as is the case with EDL, do not show a great response to T_3, as compared to the response seen in SOL muscles. That is, in the SOL, there is a drastic conversion from slow oxidative (SO) to fast oxidative glycolytic fibers (FOG), whereas in the EDL, the change is primarily from fast glycolytic (FG) to FOG (Nicol and Bruce 1981). Since EDL already contains mostly FOG fibers, the observable change in speed is minimal. Similarly, Caiozzo et al (1991) saw that thyroid hormones exerted little influence over fast-myosin pools. However, since EDL is made up of 96% type II fibers (Hamalainen and Pette 1997), drastic differences would have been expected in a HYPO state, since a majority of the fibers are available to be converted to slow twitch, and this was not the case in our experiments. One possible explanation is that, as stated before, studies show that certain fibers in the EDL take longer to respond to thyroidal influences as compared to SOL muscles (Gambke et al 1983). Therefore, perhaps the treatment duration selected for these experiments was not sufficient to see a more dramatic difference in MHC gene expression in the EDL muscles.

In regards to other contractile characteristics (1/2 RT, Pt, Po, Pt/Po), once again, the SOL muscles showed a greater responsiveness than the EDL muscle, which is most likely due to the drastic alteration in fiber type composition. SOL muscles showed an increased relaxation time in the HYPO state while just the opposite effect was seen in the HYPER state. This was in agreement with studies done by Kim et al (1982). Kim and colleagues noted that HYPER led to an increased rate of Ca^{2+} uptake by the sarcoplasmic reticulum and an increase in Ca^{2+}-ATPase activity. Leijendekker saw similar results in

SOL and EDL muscles, and also attributed this to an increase in Ca^{2+}-ATPase activity (Leijendekker et al 1993). Everts and Clausen also noted that pretreatment with T_3 increased the accumulation of Ca^{2+} content in the intracellular pools (Everts and Clausen 1986). Finally, it is possible that the changes as noted in SOL muscles in the absence or in excess of thyroid hormone may involve alterations in CA IV content. CA IV is involved in the Ca^{2+} uptake process into the SR, and relaxation times are strongly influenced by the rate of this uptake (Côté et al 1997). Therefore, because relaxation times were shorter in HYPER muscles, CA IV content may have been increased. This suggests there could be a differential and opposite regulation of CA IV and CA III. However, this is all fairly speculative, and deserves musch more experimentation in order to be able to hypothesize anything solidly.

Interestingly enough, no difference was seen in contractile properties either between SOL muscles with and without METH, or between EDL muscles with and without METH. This tells us that CA III is not controlling the contractile characteristics of these muscles, or their MHC expression and that METH is not interfering with the contractile process.

Fatigue Protocol

SO fibers (type I) are known to contain low SR and myofibrillar ATPase activities. This explains their slower contractile velocities. Also, because of their relatively high mitochondrial content and oxidative capacity, these muscles are more fatigue resistant than FG (IIb) fibers, which have a lower mitochondrial content, and are highly dependent on anaerobic metabolism (Fitts 1994). Therefore, fatigue curves as shown in our experiments were in line with these statements, SOL being more fatigue resistant than EDL, and HYPO SOL muscles more resistant than HYPER and CTR muscles. Along these lines we were able to more completely analyze the effects of CA III inhibition on the fatigue resistance of the muscles in each of the three different groups for SOL and EDL muscles. Côté et al (1993) stated that CA III inhibition in the SOL led to a significant increase in fatigue resistance during tetanic protocols. Studies with the inhibition of CA III in the three groups were, however, less conclusive. In CTR SOL muscles, inhibition of CA III with 1mM METH did indeed induce a significant increase in resistance. However, similar results could not be seen in HYPER or HYPO muscles. In the HYPER group, the lack of significant difference between the two curves (with and without METH) is understandable and in agreement with our hypothesis since HYPER leads to a decrease in CA III content, which should result in a less important effect of

METH than in CTR muscles. However, the two groups of HYPO muscles were remarkably similar, leaving much room for speculation. One point of interest which deserves attention is the fact that the two curves appear to begin to separate as they reached the 50% mark. Because of the increased fatigue resistance of the HYPO muscles with respect to the CTR group, it is possible that in order to see a significant effect of METH on these muscles, the length of the fatigue protocol would need to be increased. Perhaps, the effect of CA III inhibition is only noticeable when fatigue reaches a certain point. This appears possible since a significant difference between the HYPO and CTR groups is also only obvious in the later part of the fatigue protocol.

EDL muscles, by the same token, showed almost no variation either between the three groups, or between muscles with and without METH. This is most likely explained by the fact that even in the most extreme case, HYPO EDL CA III content is still only approximately 1/10th of that seen in CTR SOL muscles (refer to figure 1 and 2 of previous chapter), making it very difficult to see a significant influence. Thus, once again, it appears that CA III works within a specific range by which it can influence muscle fatiguability.

Further experiments should be done to better determine how CA III influences fatigue resistance. Recent evidence suggests CA III can inhibit glucose uptake in muscles (Côté et al 1997). Glycogen depletion and altered glucose metabolism in skeletal muscles are associated with muscle weakness and fatigue (Fitts 1994). From the fatigue curves as derived from our experimentation, it is evident that the effect of the inhibition of CA III (particularly in CTR SOL muscles) begins to be noticeable a couple of minutes after the fatigue protocol has begun, more specifically, when the muscles switch from anaerobic to aerobic metabolism. This suggests that CA III plays some role in the utilization of glucose/glycogen stores and opens a wide avenue for further investigation for an alternative role for CA III in skeletal muscle. This, in essence, is the basis behind the experimentation described in the following chapter.

References

1. Brown, J.G.; Millward, D.J. 1983. Dose response of protein turnover in rat skeletal muscle to triiodothyronine treatment. *Biochimica et Biophysica Acta.* 757:182-190

2. Caiozzo, V.J.; Herrick, R.E.; Baldwin, K.M. 1991. Influence of hyperthyroidism on maximal shortening velocity and myosin isoform distribution in skeletal muscles. *American Journal of Physiology.* 261: C285-C295

3. Caiozzo, V.J.; Herrick, R.E.; Baldwin, K.M. 1992. Response of slow and fast muscle to hypothyroidism: Maximal shortening velocity and myosin isoforms. *American Journal of Physiology.* 263: C86-C94

4. Côté, C.H.; Perreault, G.; Frenette, J. 1997. Carbohydrate utilization in rat soleus is influenced by carbonic anhydrase III activity. *American Journal of Physiology.* 273 (*Regulatory Integrative Comparative Physiology.* 42): R1211-R1218

5. Côté, C.H.; Riverin, H.; Barras, M.; Tremblay, R.R.; Frémont, P.; Frenette, J. 1993. Effect of carbonic anhydrase III inhibition on substrate utilization and fatigue in rat soleus. *Canadian Journal of Physiology and Pharmacology.* 71: 277-283

6. Dimitriadis, G.D.; Leighton, B.; Vlanchonikolis, I.G.; Party-Billings, M,J.; Challiss, R.A.J.; West, P.; Newsholme, E. 1988. Effects of hyperthyroidism on the sensitivity of glycolysis and glycogen synthesis to insulin in the soleus muscle of the rat. *Biochemistry Journal.* 253:87-92

7. Fitts, R.H. 1994. Cellular Mechanisms of Muscle Fatigue. *Physiological Reviews.* 74(1): 49-94

8. Fitts, R.H.; Brimmer, C.J.; Troup, J. P.; Unsworth, B.R. 1984. Contractile and fatigue properties of thyrotoxic rat skeletal muscle. *Muscle and Nerve.* 7: 470-477.

9. Gambke, G.; Lyons, G.E.; Haselgrove, J.; Kelly, A.M.; Rubinstein, N.A. 1983. Thyroidal and neural control of myosin transitions during development of rat fast and slow muscles. *FEBS Letters.* 156: 335-339

10. Gosselin, L.E.; Zhan, W.; Sieck, G.C. 1996. Hypothyroid-Mediated Changes in Adult Rat Diaphragm Muscle Contractile Properties and MHC Isoform Expression. *Journal of Applied Physiology.* 80(6): 1934-1939

11. Hamalainen, N.; Pette, D. 1997. Coordinated fast-to-slow transitions of myosin and SERCA isoforms in chronically stimulated muscles of euthyroid and hyperthyroid rabbits. *Journal of Muscle Research and Cell Motility.* 18: 545-554

12. Holder, L.B.; Hayes, S.L. 1965. Diffusion of sulfonamides in aqueous buffers and into red cells. *Molecular Pharmacology.* 1: 266-279

13. Ianuzzo, D.; Patel, P.; Chen, V.; O'Brien, P.; Williams, C. 1977. Thyroidal trophic influence on skeletal muscle myosin. *Nature* (London). 270(3): 74-76

14. Izumo, S.; Nadal-Ginard, B.; Mahdavi, V. 1986. All members of the MHC multigene family respond to thyroid hormone in a highly tissue-specific manner. *Science.* 231: 597-600

15. Kragie, L.; Smiehorowski, R. 1993. Measures of skeletal muscle calcium channels and acetycholine receptors in thyroidectomized rats. *Endocrine Research.* 19(2&3): 207-219

16. Kudelska, G.; Gorski, J.; Swiatecka, J.; Gorski, M. 1996. Effect of exercise on glycogen metabolism in muscles of triiodothyronine-treated rats. *European Journal of Applied Physiology.* 72:496-501

17. Larsson, L.; Li, X.; Teresi, A.; Salviati, G. 1994. Effects of thyroid hormone on fast- and slow-twitch skeletal muscles in young and old rats. *Journal of Physiology.* 481(1): 149-161

18. Li, X.; Hughes, S.M.; Salviati, G.; Teresi, A.; Larsson, L. 1996. Thyroid hormone effects on contractility and myosin compostition of soleus muscle and single fibres from young and old rats. *Journal of Physiology.* 494(2): 555-567

19. Lomax, R.B.; Robertson, W.R. 1992. The effects of hypo- and hyperthyroidism on fiber composition and mitochondrial enzyme activities in rat skeletal muscle. *Journal of Endocrinology.* 133: 375-380

20. Norenberg, K.M.; Herb, R.A.; Dodd, S.L.; Powers, S.K. 1996. The effects of hypothyroidism on single fibers of the rat soleus muscle. *Canadian Journal of Physiology and Pharmacology.* 74: 362-367

21. Nwoye, L.; Mommaerts, W.; Simpson, D.R.; Seraydarian, K.; Marusich, M. 1982. Evidence for a direct action of thyroid hormone in specifying muscle property. *American Journal of Physiology.* 242: R401-R408

22. Pernitsky, A.N.; McIntosh, L.M.; Anderson, J.E. 1996. Hyperthyroidism impairs early repair in normal but not dystrophic mdx mouse tibialis anterior muscle. An in vivo Study. *Biochemistry and Cell Biology.* 74: 315-324

23. Sieck, G.C.; Wilson, L.E.; Johnson, B.D.; Zhan, W. 1996. Hypothyroidism alters diaphragm muscle development. *Journal of Applied Pysiology.* 81(5): 1965-1972

24. Sillau, A.H. 1985. Capillarity, oxidative capacity and fibre compostion of the soleus and gastrocnemius muscles of rats in hypothyroidism. *Journal of Physiology.* 361: 281-295

25. Tremblay, R.R.; Gaumont, F.; Milot, J.; Candas, B.; Côté, C.H. 1993. Inhibition of carbonic anhydrase III exerts paradoxical influences on the soleus muscle of hypo and hyperthyroid rats. *Basic and Applied Myology.* 3(2): 93-102

26. van der Linden C. G.; Simonides, W. S.; Muller, A.; van der Laarse, W.J.; Vermeulen, J.L.; Zuidwijk, M.J.; Moorman, A.F.; van Hardeveld, C. 1996. Fiber-specific regulation of Ca(2+)-ATPase isoform expression by thyroid hormone in rat skeletal muscle. *American Journal of Physiology.* 271(6 Pt 1): C1908-C1919

Figure 2. Time to Peak Twitch Tension in HYPER, CTR, and HYPO SOL Muscles

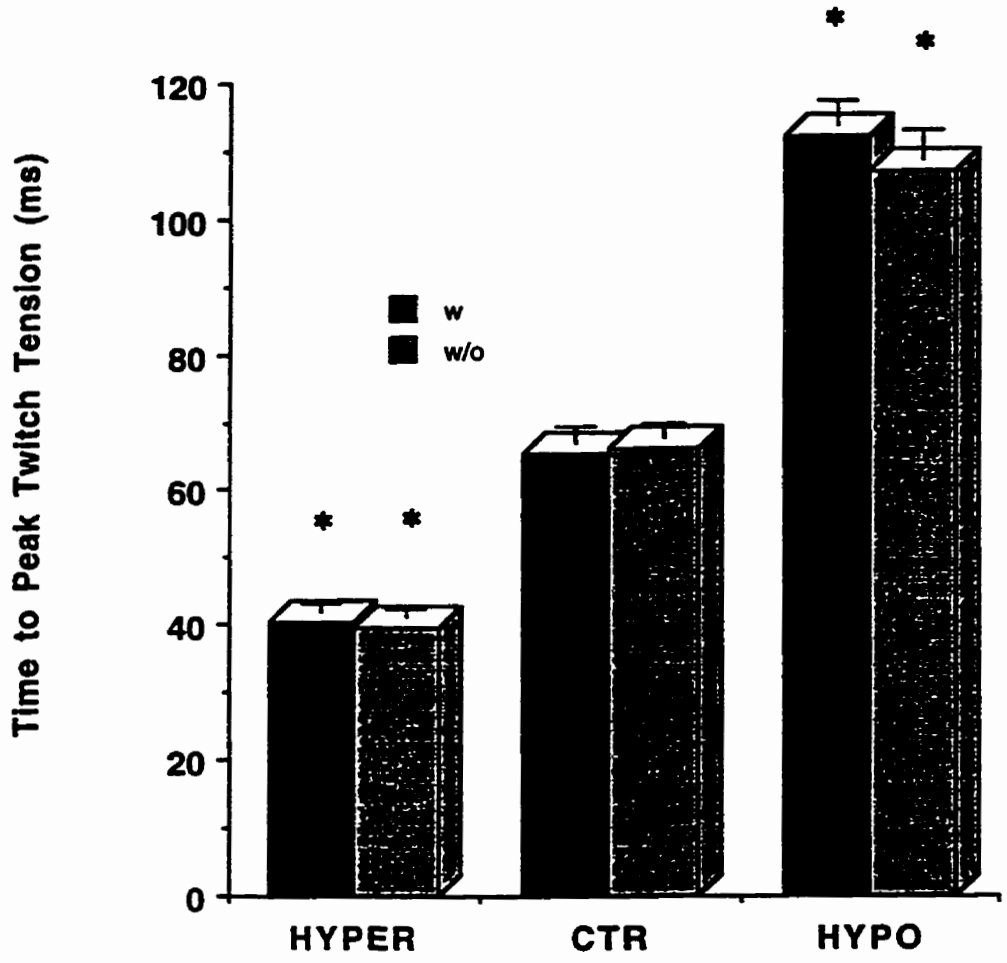


Figure 2. Represents time to peak twitch tension (TPT) in SOL muscles. There was no change in TPT between muscles incubated with METH (w/) and those that were not (w/o)
w/: HYPER (n= 10); CTR (n= 17); HYPO (n= 13)
w/o: HYPER (n= 11); CTR (n= 12); HYPO (n= 12)
Symbols: * significantly different from control values ($p<0.05$).

Figure 3. Time to Peak Twitch Tension in HYPER, CTR, and HYPO EDL Muscles

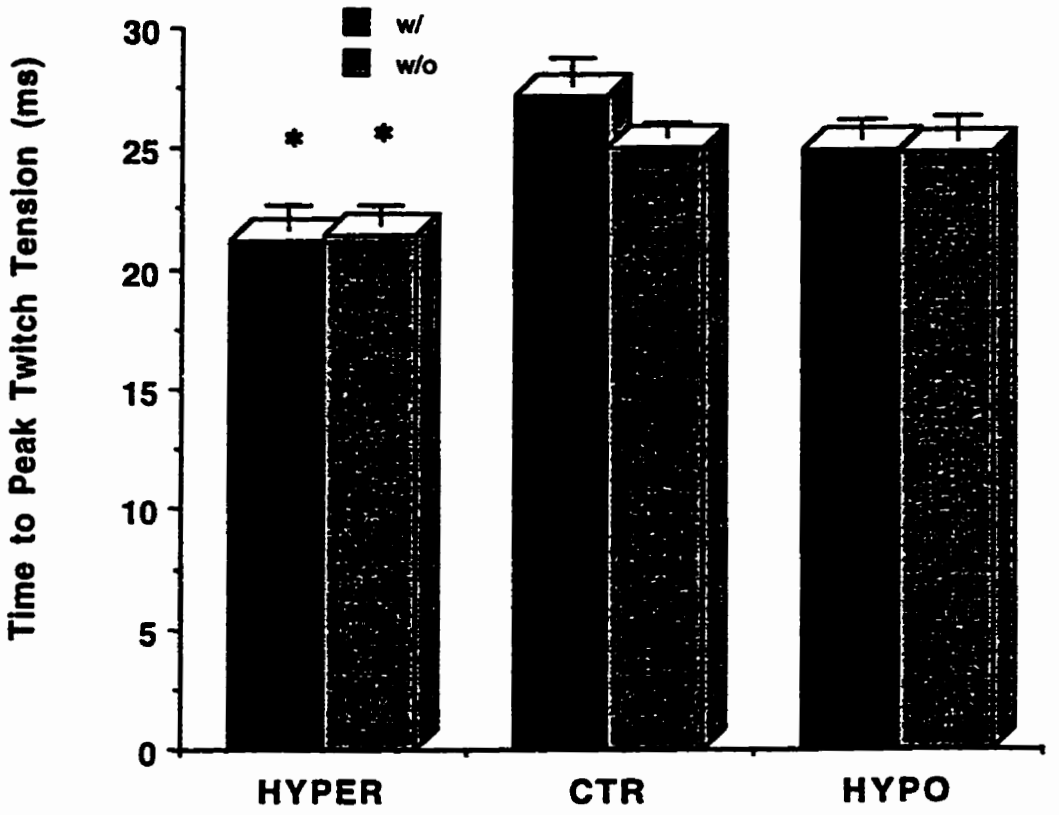


Figure 3. Represents time to peak tension (TPT) in EDL muscles.
w/: HYPER (n= 6); CTR (n= 5); HYPO (n= 9)
w/o: HYPER (n= 5); CTR (n=16); HYPO (n= 9)
Symbols: * significantly different from CTR values.

Figure 4. Force Frequency Curves of HYPER, CTR, and HYPO SOL muscles

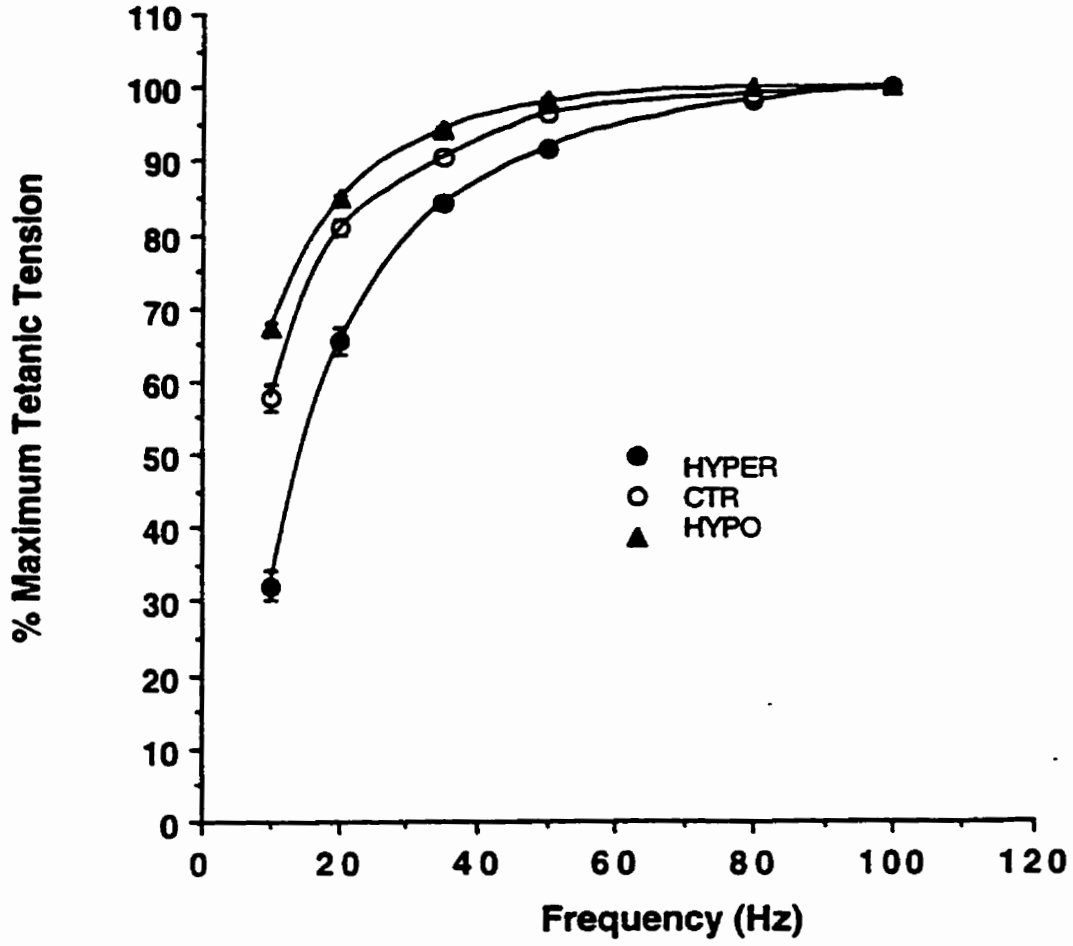


Figure 4. Describes the left-shift in the force frequency curves in HYPO SOL and the right-shift in HYPER SOL muscles relative to CTR. HYPER (n= 21); CTR (n= 29); HYPO (n= 25)
HYPER and HYPO muscles were significantly different from CTR values at all points except 80 and 100 Hz, as well as at 50 Hz for HYPO muscles ($p < 0.05$).

Figure 5. Force Frequency Curves of HYPER, CTR and HYPO EDL Muscles

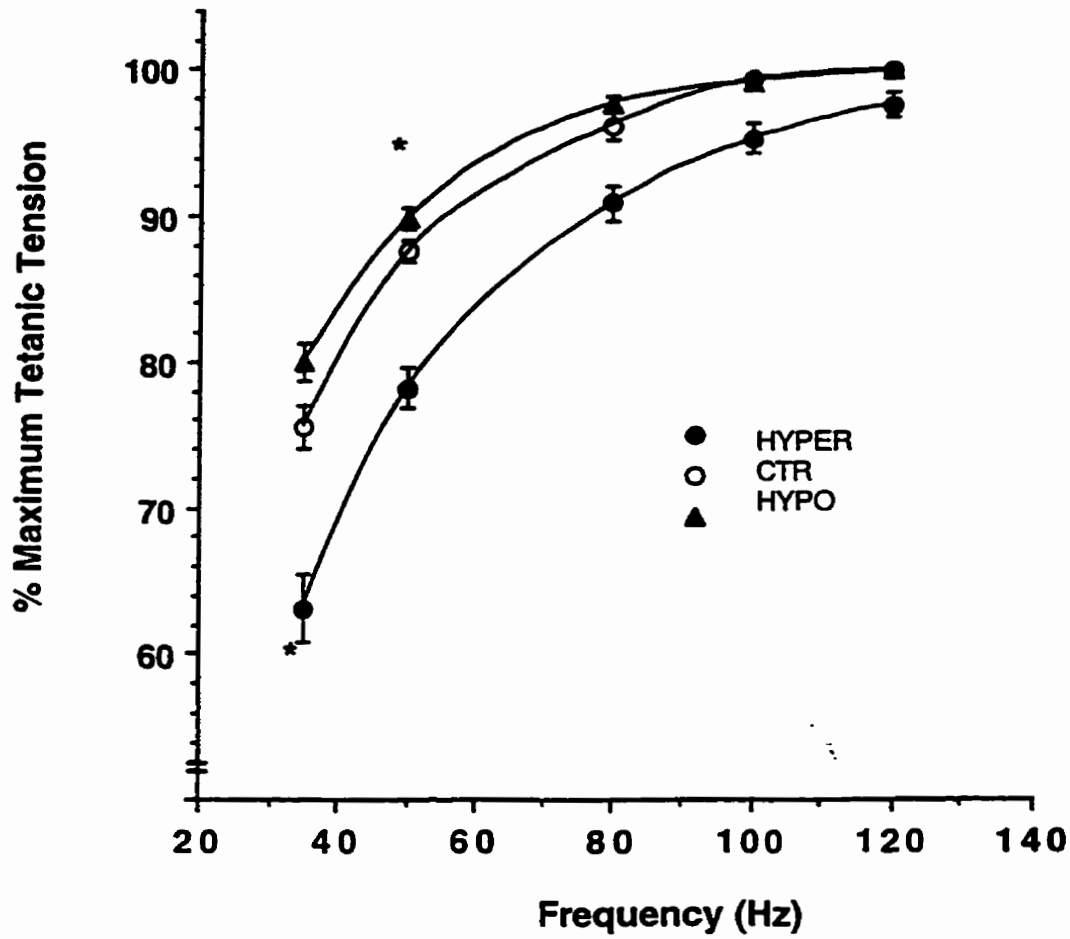


Figure 5. Describes the left-shift in force frequency curve of HYPO EDL muscles and the right-shift in HYPER EDL muscles relative to CTR values.
HYPER (n= 11); CTR (n= 21); HYPO (n= 18)
Symbols: * significantly different from CTR values ($p < 0.05$)
HYPER data were significantly different from CTR values at every point.

Figure 6. Soleus HYPER, CTR, and HYPO Fatigue Curves

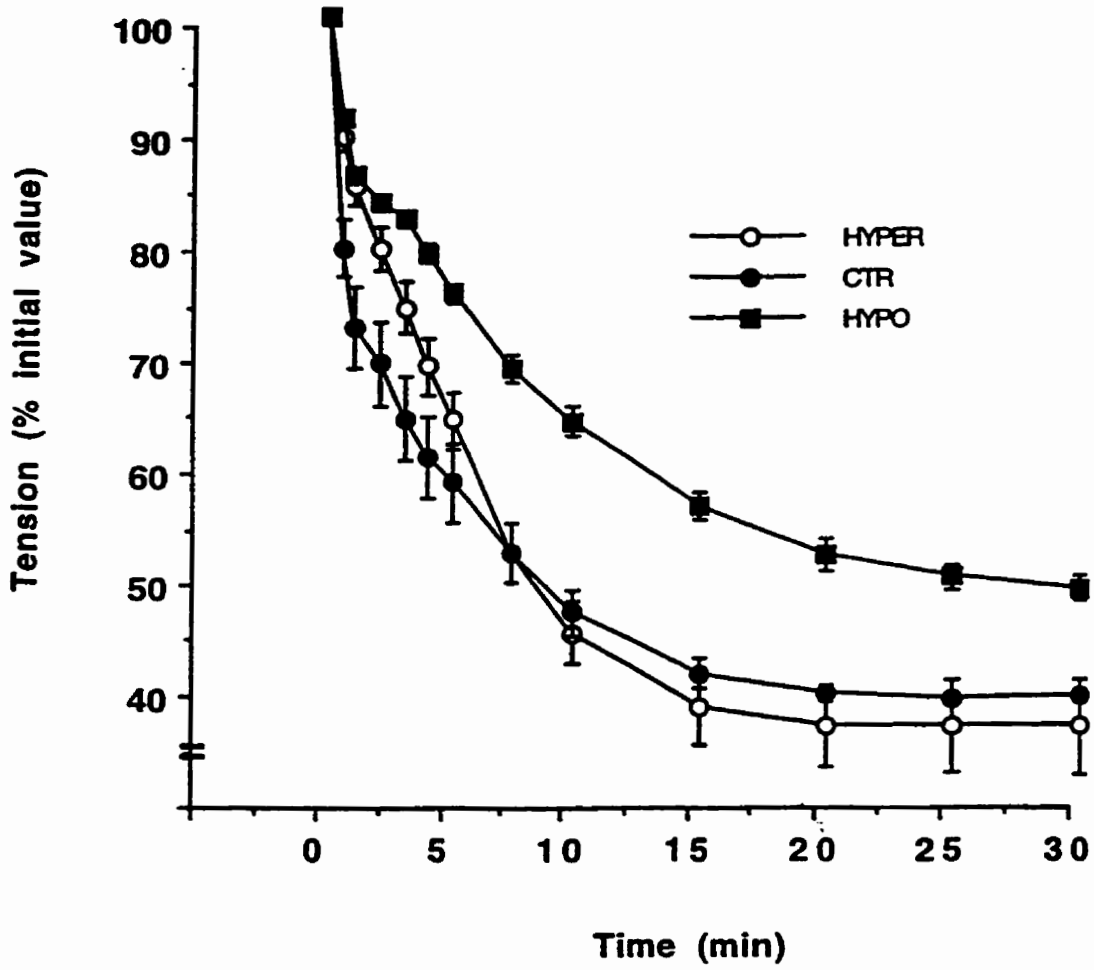


Figure 6. Shows a comparison between the fatigue resistance of three groups of SOL muscles.
Distinction between the three groups becomes evident after approximately 3 min. Muscles with increased oxidative capacity (HYPO) compared to CTR show an improved resistance to fatigue.
HYPO data was significantly different from the CTR group at every point beginning with t= 4 min. HYPER data was significantly different at t= 0.5 min and t= 3 min.
HYPER (n= 6); CTR (n= 6); HYPO (n=8).

Figure 7a. Influence of 1mM METH on the Resistance to Fatigue of CTR Soleus Muscles

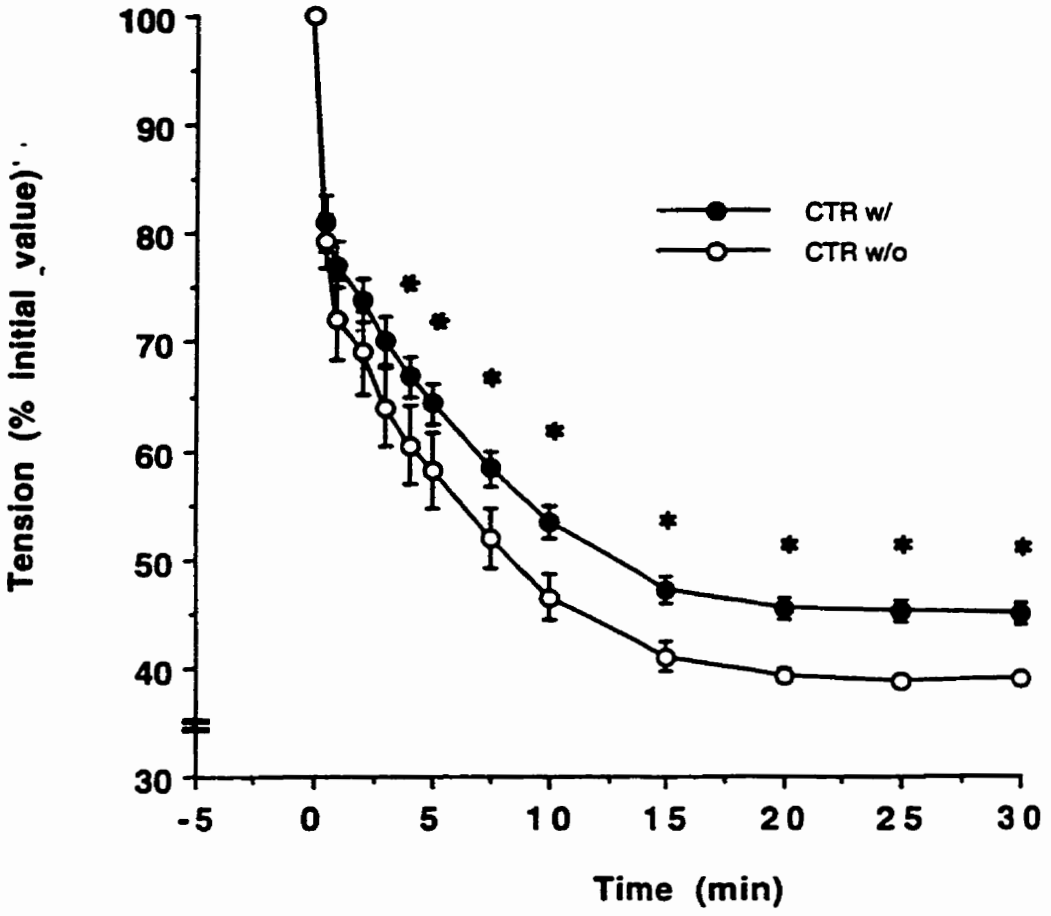


Figure 7a. Shows a comparison of fatigue resistance of CTR SOL muscles with (w/) and without (w/o) METH.
Symbols: * significantly different from CTR values ($p<0.05$).
n= 10 for CTR w/; n= 6 for CTR w/o

Figure 7b. Influence of 1mM METH on the Resistance to Fatigue of HYPER SOL Muscles

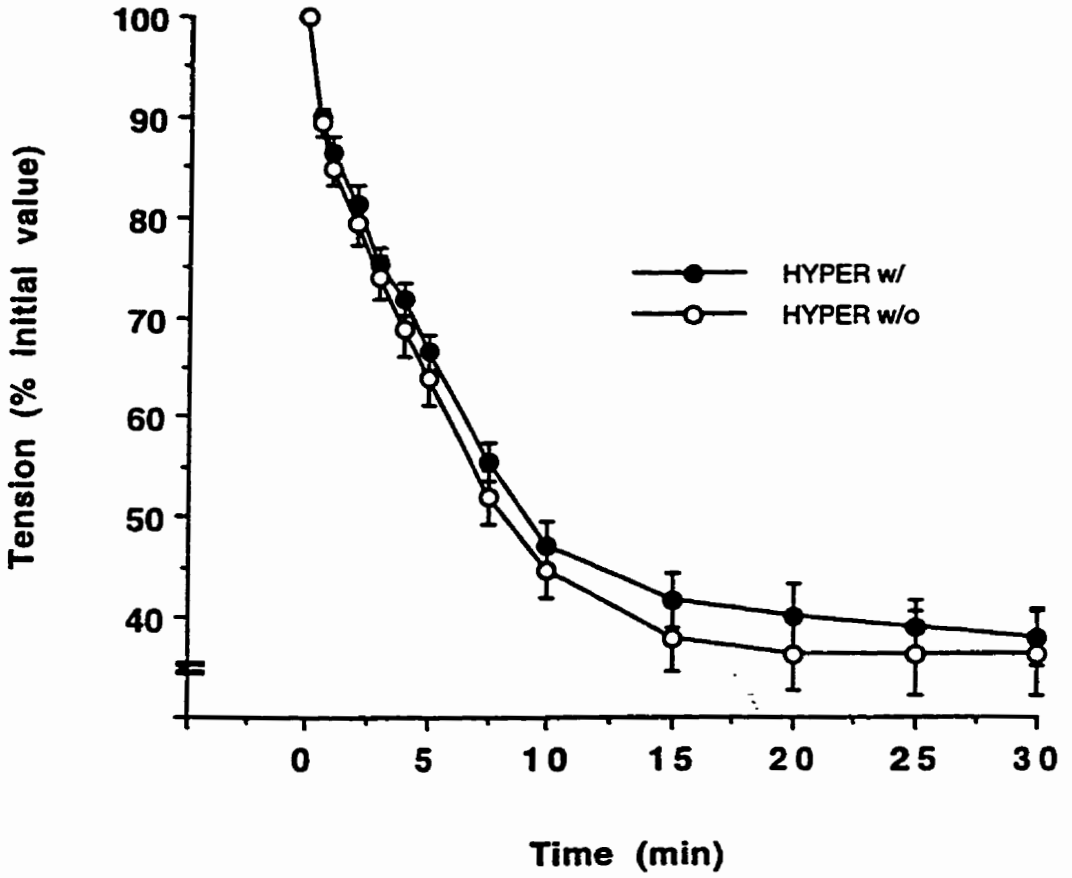


Figure 7b. A comparison of fatigue resistance of HYPER SOL muscles with (w/) and without (w/o) METH.
There was no significant difference between the two curves.
n= 7 for HYPER w/; n= 6 for HYPER w/o

Figure 7c. Influence of 1mM METH on the Resistance to Fatigue of HYPO SOL Muscles

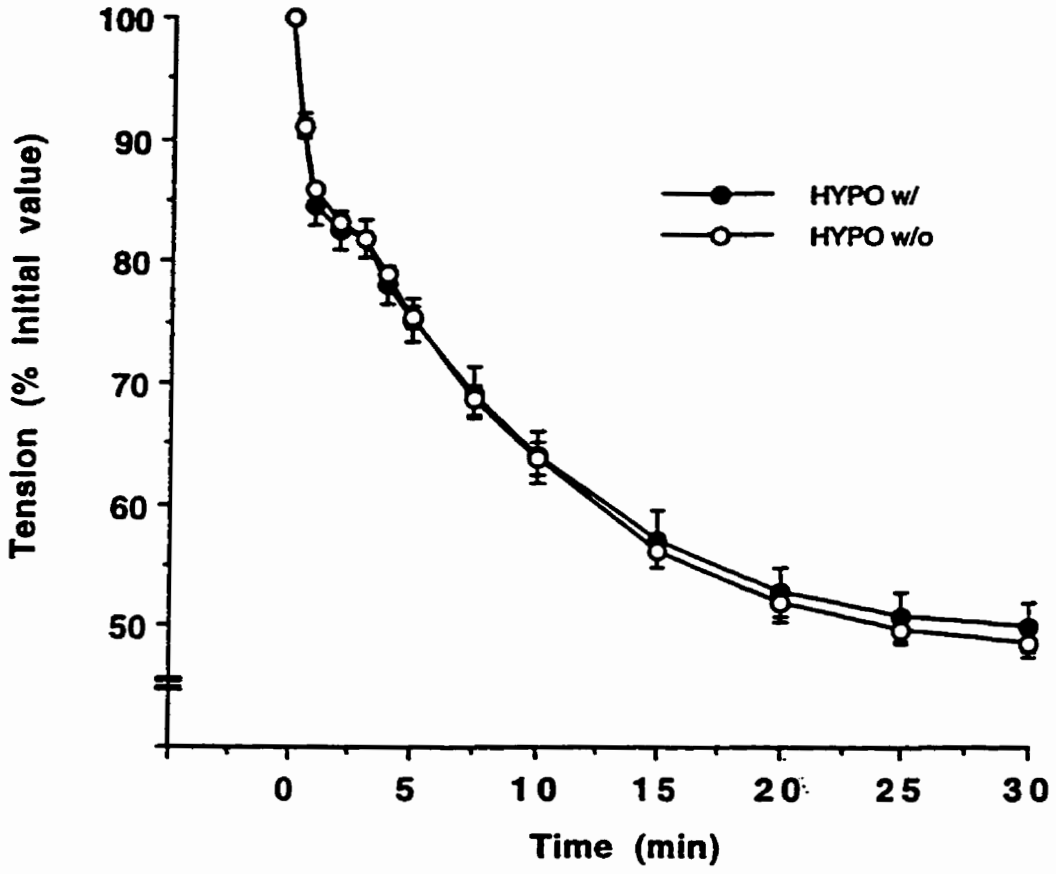


Figure 7c. Comparison of fatigue resistance of HYPO SOL muscles with (w/) and without (w/o) METH.
There was no significant difference between the two curves.
n= 7 for HYPO w/; n= 8 for HYPO w/o

Figure 8. EDL HYPER, CTR, and HYPO Fatigue Curves

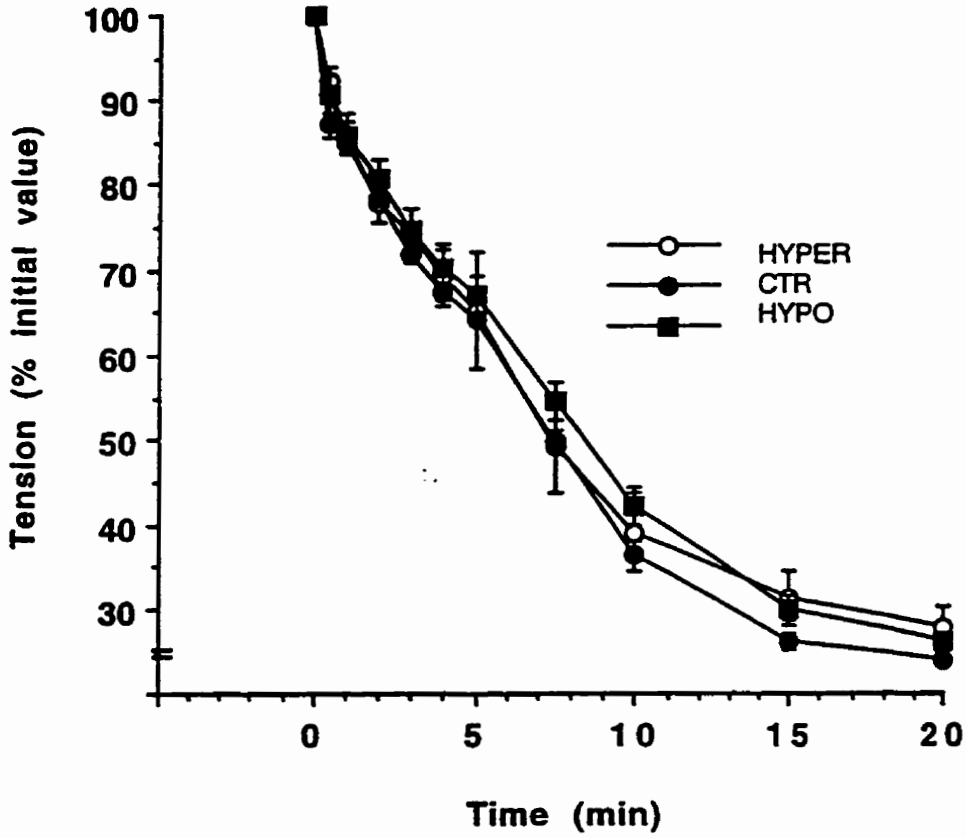


Figure 8. Shows a comparison of three thyroid groups of EDL muscles. There was no significant difference between any of the three curves. In addition, there was no significant difference in any of the three groups between muscles incubated with and without METH.

Table 1a. Contractile properties of HYPER, CTR, and HYPO SOL muscles

	TPT	1/2RT	Pt	Po	Pt/Po
HYPER n= 24	*40.3 +/-1.28	*60.1 +/-2.21	*15.4 +/-.84	93.7 +/-3.33	*.17 +/-.01
CTR n= 23	65.8 +/-2.22	85.8 +/-5.16	22.3 +/-.92	97.0 +/-4.86	.24 +/-.01
HYPO n=20	*109.8 +/-3.98	*172.7 +/-7.62	*28.7 +/-2.23	*113.2 +/-3.10	*.26 +/-.01

Table 1a. Describes the contractile properties of HYPER, CTR, and HYPO SOL muscles. Overall, HYPO data showed increased contraction and relaxation times, while just the opposite was true for HYPER data. Data from muscle groups with and without METH were pooled since there was no significant difference between the two.
Values are means +/-SEM

Symbols: * significantly different from CTR values ($p \leq 0.05$)

Table 1b. Contractile properties of HYPER, CTR, and HYPO EDL muscles

	TPT	1/2RT	Pt	Po	Pt/Po
HYPER	*21.3	30.0	33.8	151.2	*.23
n= 11	+/-0.95	+/-3.18	+/-4.70	+/-17.5	+/-.01
CTR	26.2	27.4	42.0	145.7	.30
n= 18	+/-0.86	+/-2.19	+/-6.64	+/-12.4	+/-.02
HYPO	*24.9	23.1	*51.5	144.6	*.37
n= 17	+/-0.84	+/-1.17	+/-1.73	+/-7.02	+/-.02

Table 1b. Describes the contractile properties of HYPER, CTR, and HYPO EDL muscles. The same change in TPT, Pt, and Pt/Po as was seen in SOL muscles also existed in this group of muscles. That is, HYPER and HYPO data decreased and increased relative to CTR values, respectively. Once again, data from muscle groups with and without METH were pooled since there was no significant difference between the two.
Values are means +/-SEM

Symbols: * significantly different from CTR values ($p \leq 0.05$)

Chapter 4

The Modulation of CA III Content by Thyroid Hormones has a Direct Effect on CA III Influence on Carbohydrate Metabolism

Fabrisia Ambrosio and Claude Côté

CHUL Research Center
Lipid Research Unit
Université Laval

Abstract

Studies have shown that inhibition of carbonic anhydrase III (CA III) can result in an increased fatigue resistance in soleus (SOL) muscles (Côté et al 1993). More recently, Côté and his colleagues showed that in resting muscle, CA III inhibition also leads to an increased glucose-6-phosphate (G-6-P) accumulation in SOL muscles (Côté et al 1997). These studies, together, suggest a role for CA III in carbohydrate utilization. It was therefore the objective of these experiments to further explore this role for CA III in SOL as well as in the extensor digitorum longus (EDL) muscles (SOL representing a high concentration of CA III and EDL muscles containing only trace quantities). Using the already well known mechanism of thyroid hormone up- and down-regulation of CA III in hyper- (HYPER) and hypothyroidic (HYPO) rats, respectively, we were able to more broadly scope the effect of CA III on G-6-P accumulation and determine a relationship with its presence and fatigue resistance of muscles. Our studies show that in SOL muscles, muscles with increasing CA III activity show a greater increase in G-6-P concentration upon inhibition of this enzyme. This supports the hypothesis that inhibition of CA III plays some role in carbohydrate utilization. Similarly, EDL muscles with minimal CA III content, even under HYPO conditions, showed no significant difference in G-6-P concentration, further emphasizing specificity of the effect of CA III on glucose metabolism. This leads us to believe that CA III favors some other mechanism of ATP production, therefore allowing the muscle to preserve its glycolytic supply.

Introduction

Muscle fatigue is defined as "the failure to maintain a required level of force or power output" (Edwards et al 1981). It has been suggested that this is related to a decrease in ATP availability associated with energy expenditure or to modifications in metabolic or ionic factors (Chin et al 1997). Along these lines, we know that different skeletal muscle fiber types respond differently to muscle fatigue (Fitts 1994). That is, muscles composed primarily of type I fibers being more resistant to fatigue than fast-twitch muscles composed primarily of type IIa and/or IIb fibers. This variation is associated with differences in the metabolic machinery of the various muscle fibers. Muscles with a greater oxidative capacity show a higher resistance to fatigue partly due to their increased number of mitochondria and increased mechanical efficiency; these muscles show a smaller depletion in glycogen stores upon exercise as compared to glycolytic muscles. This is biologically significant since glycogen depletion is known to correlate with muscle weakness and fatigue. One mechanism by which glycogen sparing may occur was first introduced in 1963 by Randle who stated that free fatty acid (FFA) utilization, which contributes ATP through the Kreb's cycle, indirectly inhibits glycolysis. That is, citrate, a Kreb's cycle intermediate, inhibits the action of phosphofructokinase (PFK), causing an accumulation of glucose-6-phosphate (G-6-P) and fructose-6-phosphate (F-6-P), for example, and decreases the glycolytic flux or rate. Along these lines, it was shown in previous studies that the inhibition of CA III significantly increased the fatigue resistance of soleus (SOL) muscles and allowed for a greater rate of carbohydrate utilization (Côté et al 1993). Furthermore, accumulation of G-6-P, a glycolytic intermediate, in the muscle was noticed under resting conditions, suggesting a role for CA III in carbohydrate metabolism. However, the precise mechanism by which CA III acts, and the reason behind its high level of expression in type I muscle fibers as compared to type IIa or IIb (Frémont et al 1991) is still speculative. The objective of these experiments was to look at the influence of CA III on carbohydrate metabolism, more specifically G-6-P accumulation, in various types of muscle fibers with very different CA III content. Because it has been established that thyroid hormones regulate fiber type composition (Caiozzo et al 1991) and CA III content, hyperthyroidic (HYPER) and hypothyroidic (HYPO) rats were used in these experiments to give a broader understanding as to the role of CA III.

Materials and Methods

Animal Preparation

Female Wistar rats from Charles River Laboratories (140-160 g) were used in these experiments. Rats were divided into three groups based on thyroid status. The control group, CTR, were unmanipulated rats (n=12). HYPER (n=12) received .1ml subcutaneous injections of 75μg/100g tri-iodothyronine (T_3) solution every other day for two weeks. HYPO (n=14) were ordered from Charles River Laboratories who had performed thyroidectomies. HYPO rats were allowed a minimum of two weeks post-surgery before being used.

Each group was then subdivided into two groups, in one, muscles were incubated with 1mM methazolamide (METH) during contractile measurements, and the other as a control group without METH.

Surgical Procedures and Contractile Measurements

Rats from all three groups were anesthetized using 5 mg sodium pentobarbital/100g body weight. The hindleg skin was carefully pulled away from the fascia, and the biceps femoris muscle was cut away to expose the SOL or extensor digitorum longus (EDL) muscle. Whole muscles were carefully dissected and immediately placed in a Kreb's Ringer Bicarbonate bath (with 11mM glucose) maintained at 25°C. The muscle was suspended by a glass hook and attached vertically to an isometric force transducer. The pH of the bathing solution was maintained at 7.4 using a 95% O_2 and 5% CO_2 gas mixture. The methazolamide (METH) concentration used (1mM) is sufficient to inhibit all CA activity, including CA III, which is known to be more sulfonamide resistant than other CA isoforms (Tashian 1989). Thirty minutes were allowed for the muscle to equilibrate before beginning any contractile measurements. During this time, the optimal length (Lo) was determined. This is the length at which the muscle produces maximum isometric twitch tension. Muscles were stimulated with 25V pulses of 0.2 ms duration. Using a twitch contraction, time to peak twitch (TPT), maximum twitch tension (Pt), and 1/2 relaxation time (1/2 RT) were recorded. Maximum tetanic tension (Po) was then measured, followed by 10 minutes of rest. During this time, frequencies required for the SOL muscles to work at 60% of the maximum tetanic tension, and EDL muscles to work at 75% of the maximum tetanic tension were calculated from their force frequency curves as described in Chapter 3. One group of SOL muscles, however, were immediately frozen with liquid nitrogen after the

ten minutes of rest, just prior to the onset of the fatigue test, and never underwent any fatigue protocol (t= 0). This was to establish the influence of methazolamide on G-6-P content in muscles exposed only to minimal contractile activity.

Fatigue Protocol

The fatigue test consisted of one stimulation every five seconds for 30 minutes in SOL muscles, and 20 minutes in EDL muscles with train durations of 500 ms and 200 ms, respectively. As in previous experiments which defined this fatigue protocol, (Côté et al 1993; Frémont et al 1988), the purpose of these tests were to induce a gradual decrease in force production over an extended period of time of continued stimulation. This allows for the recruitment of aerobic metabolism (Côté et al 1997). SOL CTR muscles were stimulated at 10Hz, while HYPER were stimulated at 21 Hz and HYPO at 8Hz. EDL CTR muscles worked at 35Hz, whereas HYPER and HYPO muscles were stimulated at ~40 Hz and ~30 Hz, respectively. Variations in frequencies of stimulation for muscles with and without METH were not significant. Finally, at the end of the fatigue protocol, muscles were quickly frozen using liquid nitrogen, and stored at -80°C until they could be homogenized for G-6-P determination.

Glucose-6-Phosphate (G-6-P) Determinations

Since glucose undergoes glycolysis at an extremely fast rate, and yet is stopped at extremely low temperatures, all muscle samples were homogenized at 0°C, in order to preserve quantities of glycolytic intermediates found immediately after the fatigue test. Samples were homogenized in 5 volumes (w/v) of 0.6M perchloric acid (PCA), and were then centrifuged at 10,000rpm for 10 minutes. The supernatant was then isolated from the pellet, and neutralized with 2M $KHCO_3$. This was the same protocol originally established by Lowry and Passonneau (Lowry and Passonneau 1972). G-6-P concentrations were then obtained spectrofluorometrically (Lowry and Passonneau 1972).

Statistical Analysis

All data are presented as means +/- standard error (SEM). Statistical differences were determined using an analysis of variance or a student's two-tailed test, and were considered statistically different at $p \leq 0.05$.

Results and Discussion

Data for contractile measurements in our experiments were in line with those seen in previous studies (Sieck et al 1996; Norenberg et al 1996) showing that deficiency of thyroid hormone causes an increase in the proportion of type I fibers in both fast- and slow-twitch muscles and the opposite was true for muscles supplemented with T_3, the HYPER group (refer to tables 1a and 1b in Chapter 3).

Figure 1 shows the results for the measurement of G-6-P in the three different groups of SOL muscles at the end of the test. In both CTR and HYPO groups, there was an increased G-6-P concentration in muscles with METH. This is in line with previous results reported by Côté et al (1997). Along these lines, figure 2 (t= 30) illustrates the linear relationship that exists between the relative increase in G-6-P content in muscles incubated with METH versus CA III content, as established earlier by calorimetric determination.

SOL muscles which never underwent a fatigue protocol told somewhat the same story as muscles that did, with the exception of HYPO muscles. In this group, no difference at all was noticeable between muscles with and without METH (see figure 3).

For the EDL, no clear trend in G-6-P concentration between the three groups of muscles was observable. By the same token, there was no correlation in Δ G-6-P in EDL muscles incubated with and without METH as was seen in SOL muscles.

Soleus Muscles:

In this series of experiments, we used the well known model of HYPO and HYPER animals knowing that a deficiency in thyroid hormone increases the amount of type I fibers in muscle tissue (Caiozzo et al 1992), making the muscle even more fatigue resistant. On the other hand, treatments with thyroid hormone have just the opposite effect and appear to make the muscle take on more fast-twitch characteristics (Izumo et al 1986). Furthermore, levels of thyroid hormones are negatively correlated with CA III levels in the muscle, and this holds true in both fast- and slow-twitch muscles as was shown in our studies described in Chapter 2. Based on this known effect of thyroid hormones on CA III concentration, we decided to use this model to further examine the effect of CA III on the accumulation of glycolytic intermediates in skeletal muscle. By using HYPO and HYPER rats, and examining two different types of muscle: the SOL, representing high concentrations of CA III, and the EDL, with close to no CA III activity at all, we set out to investigate the role of CA III in carbohydrate metabolism.

Very little research has been done on the influence of CA III on glycogen and glucose metabolism. In previous studies, our laboratory found that CA III inhibition influences G-6-P content in SOL muscles (Côté et al 1997). It was also proposed that the increased resistance to fatigue associated with CA inhibition was at least partially related to the accumulation of glycolytic substrates in the period preceding the fatigue protocol. Our results are in line with previous studies done by Côté and colleagues on the effect of CA inhibition on G-6-P concentrations in SOL muscle. That is, in the presence of METH, G-6-P levels were found to be increased in the three groups of SOL muscles tested. Additionally, we saw that there was a rather linear correlation between the increase in G-6-P content seen in the three types of SOL muscles and their respective CA III activity even though a better curve fit could be obtained with a second order polynomial equation. This suggests that a) the effect of METH on G-6-P accumulation is really due to the inhibition of CA III activity and b) CA III most likely plays a role in hindering glucose utilization. As seen in figure 3 of Chapter 1, glucose for glycolysis is either supplied as a result of glycogen breakdown (glycogenolysis), or is taken into the cell by means of facilitated transport. External glucose molecules move with the concentration gradient with the aid of transporters. In skeletal muscle, two major isoforms of transporters have been found, GLUT1 and GLUT4 (Etgen et al 1993). While GLUT1 is active during resting conditions (Dombrowski et al 1996), GLUT4 is stimulated either by the presence of insulin or by contraction (Douen et al 1990). Interestingly enough, GLUT4 concentration is found in greater concentrations in muscles containing high amounts of type I fibers as compared to mainly fast-twitch muscles (Philips et al 1996). Côté et al (1997) showed that in the absence of external glucose, the effect of the inhibition of CA III on G-6-P concentration disappeared. They also noted that the effect of CA III inhibition on G-6-P accumulation disappeared when inhibitors of glucose transport were placed in the bath. Therefore, it stands to reason that the presence of CA III may inhibit the uptake of extra cellular glucose via inhibition of these GLUT transporters. Along these lines, it is possible that CA III favors some other means of ATP production, such as the utilization of free fatty acids (FFA) (Côté et al 1993). It was found that the increased fatigue resistance associated with CA III inhibition in muscle was removed when the bath was supplemented with glucose and FFA (Côté et al 1993). Also interesting is the fact that CA III is found in extremely high quantities in adipose tissue, up to 25% of the soluble protein extract (Spicer et al 1990). Coggan (1997) stated that by postponing the depletion of glycogen stores, the muscle is more capable of prolonged exercise. Therefore, CA III either by inhibiting some step in the glycolytic pathway, and thereby favoring FFA utilization, or by stimulating FFA

metabolism directly, could be indirectly favorable for muscle fatigue resistance. Thus, the possible role of CA III in FFA utilization is one that definitely deserves further attention.

In regards to G-6-P measurements taken before the fatigue test was even performed, obviously, concentrations were lower than muscles which had worked for the duration of the fatigue protocol. During exercise, muscle contraction is known to stimulate the action of GLUT 4 glucose transporters (Etgen et al 1993) as well as glycogenolysis and glycolysis, thus, increased levels of glucose intermediates in the muscle were to be expected. However, during resting conditions, extra cellular glucose uptake is primarily the responsibility of GLUT 1 transporters, present at the plasma membrane (Dombrowski et al 1996). Since we did not see the same pattern of increased G-6-P concentration with increased inhibition of CA III in resting conditions as was seen after exercise, it is possible that CA III is working to indirectly inhibit the action of GLUT 4 transporters, however, this action is not as consequential in GLUT 1 transporters. To further look at this phenomenon, it would be helpful to look at GLUT 4 and GLUT 1 concentration in HYPER and HYPO muscles and determine if there is and up- and down- regulation as is seen with CA III. Also, the effects of GLUT inhibitors in HYPER and HYPO muscles would aid in our understanding in the mechanism at work under these conditions.

EDL Muscles:

The effect of CA III inhibition, as expected, was hardly noticeable in the CTR EDL muscle since the EDL muscle is used as an example of a virtually CA III free muscle. CA III inhibition had no effect on G-6-P content even in HYPO muscles. This is significant considering the fact that in the EDL, the HYPO state is the extreme case for CA III concentration, that is, where CA III content is the highest, with HYPER being just the opposite. Even so, HYPO EDL muscles still contained less that 5 U/mg protein CA III activity compared to the lowest value observed for SOL HYPER muscles, 25 U/mg protein. Therefore, it is safe to say that the influence of CA III is only visible within a certain range of enzyme activity, and at small quantities, the effect is practically none. It would be interesting to look at different intermediate ranges of CA III content to examine at what concentration the effect of this enzyme actually becomes noticeable. This could be done using different muscles, with CA III content somewhere between that of SOL and EDL muscles, the two extremes. One such muscle would be the gastrocnemius. Also, more time could be allowed for the rats to adjust to a HYPER or

HYPO state, 4 weeks for example. This could demonstrate further the range at which CA III is effective.

In summary, we confirmed previous results stating that inhibition of CA III leads to an increased quantity of G-6-P in SOL skeletal muscle. More importantly, this was found to be linearly related to the level of CA III. The greater the CA III inhibition, the greater the apparent glucose utilization. Also, we demonstrated that the effect of CA III on carbohydrate utilization in fast-twitch muscle was insubstantial due to its relatively small quantities. Finally, questions were raised about the difference in role of CA III in basal and stimulated glucose uptake via GLUT-1 and GLUT-4 glucose transporters, respectively.

References

1. Bergemeyer, H. Methods of Enzymatic Analysis. New York: Academic, 1965

2. Caiozzo, V.J.; Herrick, R.E.; Baldwin, K.M. 1991. Influence of hyperthyroidism on maximal shortening velocity and myosin isoform distribution in skeletal muscles. *American Journal of Physiology.* 261: C285-C295

3. Caiozzo, V.J.; Herrick, R.E.; Baldwin, K.M. 1992. Response of slow and fast muscle to hypothyroidism: Maximal shortening velocity and myosin isoforms. *American Journal of Physiology.* 263: C86-C94

4. Chin, E.R.; Balnave, C.D.; Allen, D.G. 1997. Role of intracellular calcium and metabolites in low- frequency fatigue of mouse skeletal muscle. *American Journal of Physiology.* 272: C550-C559

5. Coggan, A.R. 1997. Plasma glucose metabolism during exercise: Effect of endurance training in humans. *Med. Sci. Sports Exerc.* 219(5): 620-627

6. Côté, C.H.; Perreault, G.; Frenette, J. 1997. Carbohydrate utilization in rat soleus is influenced by carbonic anhydrase III activity. *American Journal of Physiology.* 273(Regulatory Integrative Comparative Physiology) 42: R1211-R1218

7. Côté, C.H.; Riverin, H.; Barras, M.; Tremblay, R.R.; Frémont, P.; Frenette, J. 1993. Effect of carbonic anhydrase III inhibition on substrate utilization and fatigue in rat soleus. *Canadian Journal of Physiology and Pharmacology.* 71: 277-283

8. Dombrowsi, L; Roy, D.; Marcotte, B.; Marrette, A. 1996. A new procedure for the isolation of plasma membranes T-tubules, and internal membranes from skeletal muscle. *American Journal of Physiology.* 270: E667-E676

9. Douen, A.G.; Ramlal, T.; Rastogi, S.; Bilan, P.J.; Cartee, G.D.; Vranic, M.; Holloszy, J.O.; Klip, A. Exercise induces recruitment of "Insulin-responsive glucose transporter". Evidence for distinct intracellular insulin- and exercise-recruitable transporter pools in skeletal muscle. *Journal of Biological Chemistry.* 265(23): 13427-13430

10. Edwards, R.H.T. Human muscle function and fatigue. In: Human Muscle Fatigue: Physiological Mechanisms, edited by R. Porter and J. Whelan. London: Pitman Medical, 1981, p 1-8

11. Etgen, G.J.; Memon, A.R.; Thompson, G.A.; Ivy, J.L. 1993. Insulin- and contraction stimulated translocation of GTP-binding proteins and GLUT4 protein in skeletal muscle. *Journal of Biology and Chemistry.* 268(27): 20164-20169

12. Fitts, R.H. 1994. Cellular Mechanisms of Muscle Fatigue. *Physiological Reviews.* 74(1): 49-94

13. Frémont, P.; Charest, P.M.; Côté, C.; Rogers, P.A. 1991. Distribution and ultrastructural localization of carbonic anhydrase III in different skeletal muscle fiber types. From: The Carbonic Anhydrases. Edited by Susanna J. Dodgson, Richard E. Tashian, Gerolf Gros, and Nicholas D. Carter. Plenum Publishing Corporation.

14. Frémont, P.; Charest, P.M.; Côté, C.; Rogers, P.A. 1988. CA III in skeletal muscle fibers: an immunocytochemical and biolchemical study. *Journal of Histochemistry and Cytochemistry.* 8: 775-782

15. Frémont, P.; Lazure, C.; Tremblay, R.R.; Chrétien, M.; Rogers, P.A. 1987: Regulation of carbonic anhydrase III by thyroid hormone: opposite modulation in slow- and fast-twitch skeletal muscle. *Biochemistry and Cell Biology.* 65: 790-797

16. Gosselin, L.E.; Zhan, W.; Sieck, G.C. 1996. Hypothyroid-mediated changes in adult rat diaphragm muscle contractile properties and MHC isoform expression. *Journal of Applied Physiology.* 80(6): 1934-1939

17. Gros, G.; Dodgson, S.J. 1988. Velocity of CO2 exchange in muscle and liver. *Annual Review of Physiology.* 50: 669-694

18. Izumo, S.; Nadal-Ginard, B.; Mahdavi, V. 1986. All members of the MHC multigene family respond to thyroid hormone in a highly tissue-specific manner. *Science.* 231: 597-600

19. Kudelska, G.; Górski, J.; Swiatecka, J.; Górska, M. 1996. Effect of exercise on glycogen metabolism in muscles of triiodothyronine-treated rats. *European Journal of Applied Physiology.* 72: 496-501

20. Larsson, L.; Li, X.; Teresi, A.; Salviati, G. 1994. Effects of Thyroid Hormone on Fast- and Slow-Twitch Skeletal Muscles in Young and Old Rats. *Journal of Physiology.* 481(1): 149-161

21. Leijendekker, W.J.; van Hardeveld, C.; Kassenaar, A.A.H. 1983. The influence of the thyroid state on energy turnover during tetanic stimulation in the fast-twitch (mixed type) muscle of rats. *Metabolism. Clinical and Experimental.* 32(6): 615-621

22. Li, X.; Hughes, S.M.; Salviati, G.; Teresi, A.; Larsson, L. 1996. Thyroid hormone effects on contractility and myosin composition of soleus muscle and single fibres from yound and old rats. *Journal of Physiology.* 494(2): 555-567

23. Lowry, O.H.; Passonneau, J.V. 1973. Preparation of Tissues for Analysis. In: A Flexible System of Enzymatic Analysis. Academic Press, New York. 123-129,164-172.

24. Norenberg, K.M.; Herb, R.A.; Dodd, S.L.; Powers, S.K. 1996. The effects of hypothyroidism on single fibers of the rat soleus muscle. *Canadian Journal of Physiology and Pharmacology.* 74: 362-367

25. Philips, S.M.; Han, X-X.; Green, H.J.; Bonen, A. 1996. Increments in skeletal muscle GLUT-1 and GLUT 4 after endurance training in humans. *American Journal of Physiology.* 270: E456-E462

26. Randle , P.J.; Garland, C.N.; Hales, C.N.; Newsholme, E.A. 1963. The glucose-fatty acid cycle: Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet.* 1: 785-789

27. Sieck, G.C.; Wilson, L.E.; Johnson, B.D.; Zhan, W. 1996. Hypothyroidism alters diaphragm muscle development. *Journal of Applied Pysiology.* 81(5): 1965-1972

28. Sillau, A.H. 1985. Capillarity, Oxidative Capacity and Fibre Compostirion of the Soleus and Gastrocnemius Muscles of Rats in Hypothyroidism. *Journal of Physiology.* 361: 281-295

29. Stanton, L.W.; Ponte, P.A.; Coleman, R.T.; Snyder, M.A. 1991. Expression of CA III in rodent models of obesity. *Molecular Endocrinology.* 5: 860-866

30. Tashian, R.E. 1989. The carbonic anhydrases: Widening perspectives on their evolution, expression, and function. *BioEssays.* 10(6): 186-192

Figure 1. Post-fatigue Comparisons Between G-6-P Concentrations in HYPER, CTR, and HYPO SOL Muscles Incubated With and Without METH

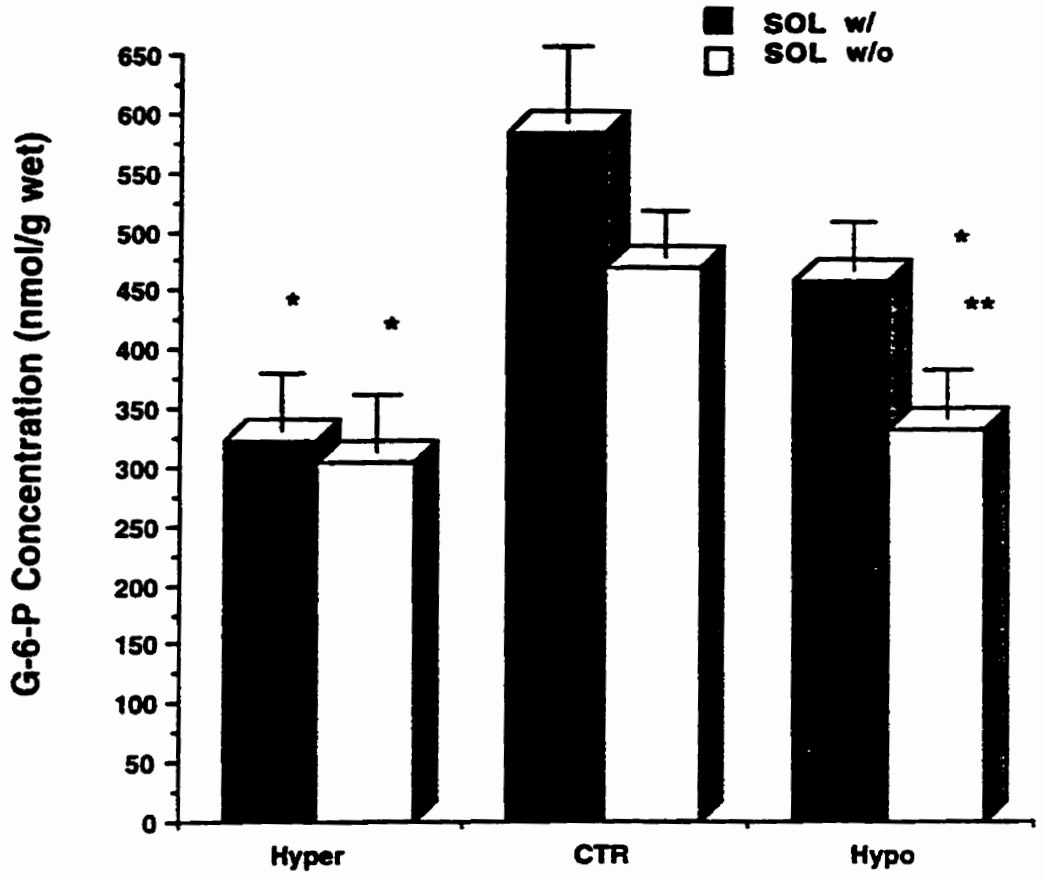


Figure 1. A comparison of G-6-P concentration in HYPER, CTR, and HYPO rat SOL muscles incubated with (w/) and without (w/o) CA inhibitor.
Symbols: * significantly different from CTR values ($p<0.05$).
** significantly different from values with METH

Figure 2. Linear Relationship Between the Difference in G-6-P Concentration for Muscles Incubated With and Without METH and the Level of CA III Activity.

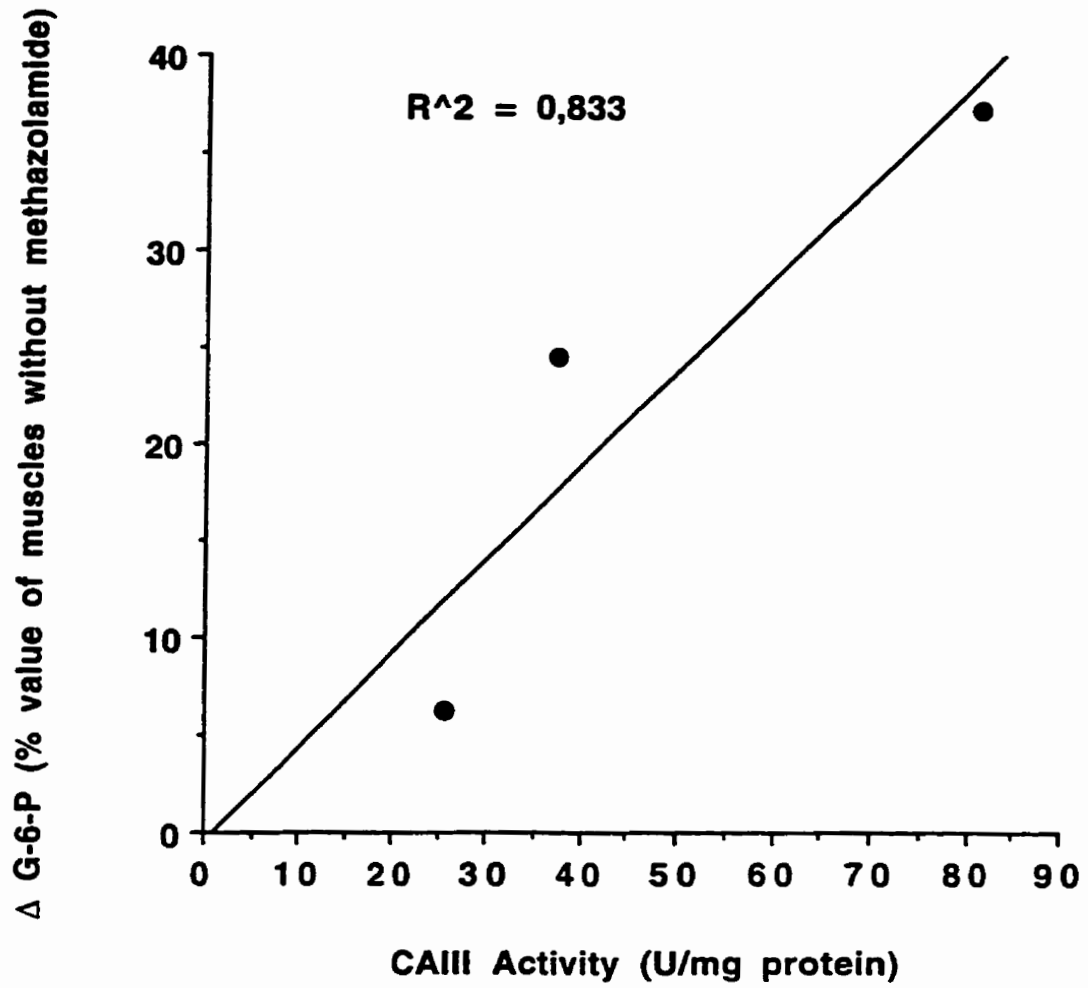


Figure 2. Shows the linear relationship between the difference in G-6-P concentration with respect to CA III Activity in HYPER, CTR, and HYPO SOL muscles at T= 30 min.

Figure 3. Comparisons Between G-6-P Concentrations in HYPER, CTR, and HYPO SOL Muscles Incubated With and Without METH Under Resting Conditions

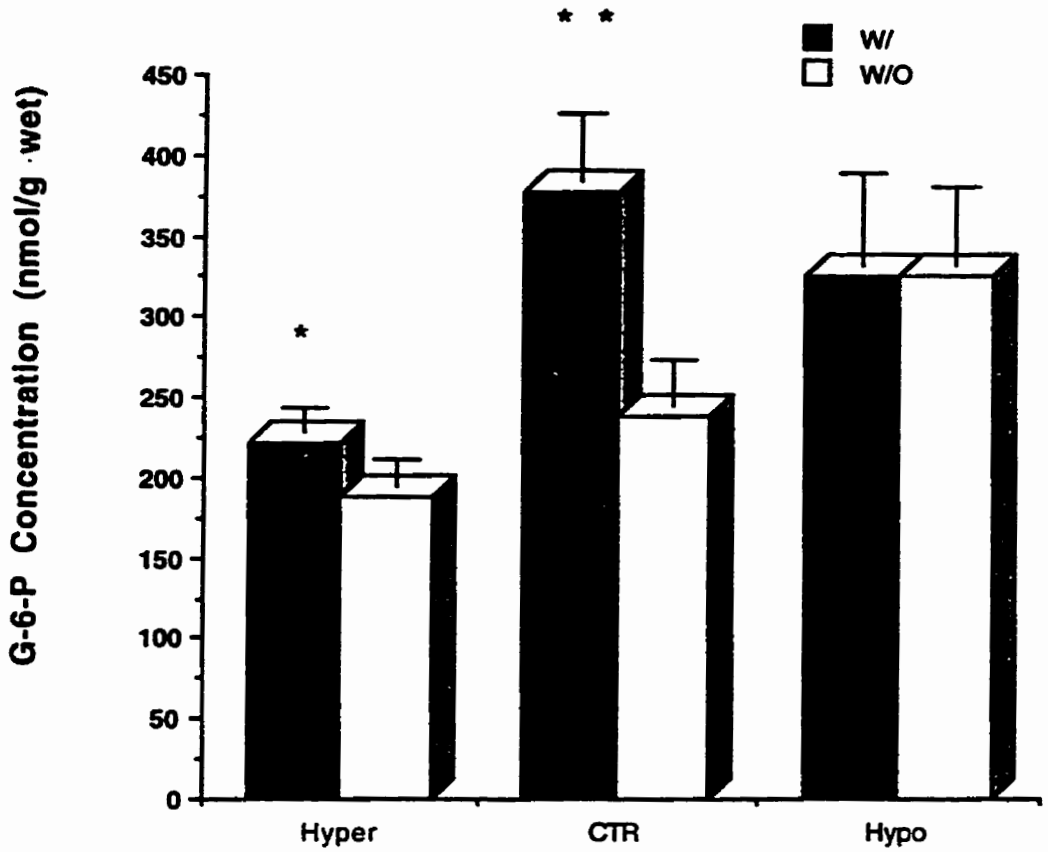


Figure 3. A comparison of G-6-P concentration in HYPER, CTR, and HYPO SOL muscles under resting conditions.
Symbols: * significantly different from CTR values ($p < 0.05$)
** significantly different from values with METH ($p<0.05$).

Figure 4. Post-Fatigue Comparisons Between EDL Muscles Incubated With and Without METH.

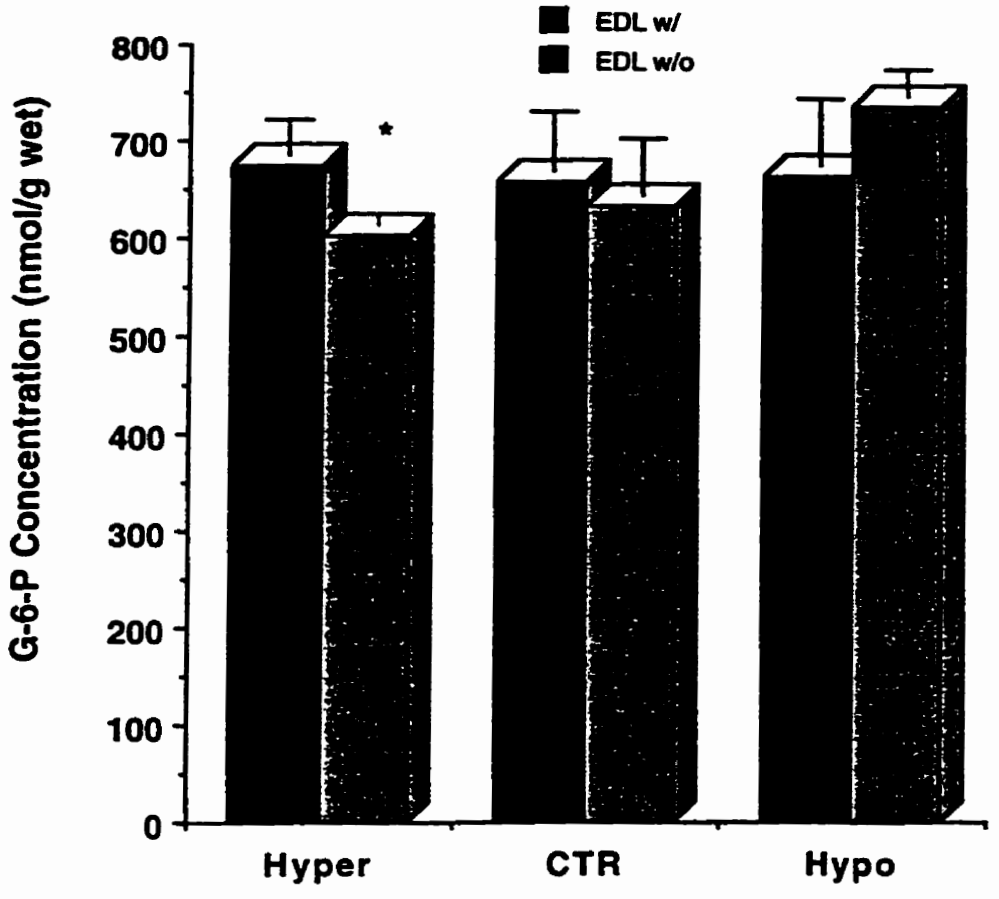


Figure 4. A comparison of G-6-P accumulation in HYPER, CTR, and HYPO rat EDL muscles incubated with (w/) and without (w/o) CA inhibitor. No significant correlation between the G-6-P concentration and CA inhibition was observeable. Symbols: * significantly different from values with METH according to one-tailed student's t-test ($p<0.05$).

Chapter 5

General Conclusion

Even though the first CA enzyme was discovered more than 70 years ago, CAs in general still boggle the mind of many scientists. And yet, progress made to our understanding of this protein has been significant, especially within the last decade or two. The discovery of thyroid hormones as modulator of CA III has provided us with an elegant model to study the regulation and consequently, the function of this isoform. Because of its relative resistance to sulfonamides, as compared to other isoforms within the carbonic anhydrase family, we were further able to isolate the specific effect of CA III activity and its inhibition.

CA III is known to be regulated by various factors, most importantly neuronal and hormonal influences (Frémont et al 1988; Milot 1995). More specifically, CA III levels increased in fast-twitch muscles in the absence of neuronal influence, while not as dramatic effect is seen in slow-twitch muscles (Milot 1995). Similarly, in the absence of thyroid hormones, CA III levels have been known to be increased, however, the effect has mostly been looked at in slow-twitch muscles (Frémont et al 1988). Therefore, in this series of experiments, one of our main goals was to compare the known thyroidic regulation of CA III in slow-twitch muscles to its unexplored modulation in fast-twitch muscles. Using CA III activity measurements as well as semi-quantitative blotting techniques, we were able to determine the relative up- and down-regulation of CA III by tri-iodothyronine (T_3). Results were conclusive as CA III activity and content of SOL were increased, as a result of an induced hypothyroidic state, by 86.0 and 88.4% (relative to control values), respectively. For EDL muscles, western analysis gave similar results, with HYPO values reaching 195.5% of control and HYPER values at 85.9%, therefore suggesting the same trend in up- and down-regulation as was seen in SOL muscles. Overall, we were able to quantify, within a fairly tight range, the modulation of CA III by this family of hormones.

Our next objective, as stated in chapter 1, was to examine the effect of thyroid hormone on the contractile and fatigue properties of both the soleus and EDL muscles. As expected, there were obvious variations in contractile properties of the muscles supplemented or deprived of thyroid hormones and this is mainly explained by differences in slow and fast MHC expression as described by Gosselin et al (1996). HYPO muscles could be compared to denervated muscles once again in that denervation also induces a fast to slow phenotypic transformation of contractile properties in both type I and type II muscles (Finol et al 1981). The changes in contractile properties brought about by both thyroid hormones and muscle innervation are because both result in a dynamic adaptation of myofibers by modulating differential gene expression (Muscat et al 1995). Moreover, thyroid hormones

and neuronal influences regulate contractile protein gene families in rat skeletal muscle, more specifically Myo/D (prevalent in fast-twitch muscles) and myogenin (prevalent in slow-twitch muscles) (Hughes et al 1993) and proportions of each are directly related to proportions of fast glycolytic and slow oxidative muscle fibers, respectively (Hughes et al 1993). Comparison of contractile properties of the three different groups of muscles (control, HYPO, and HYPER), with muscles incubated with methazolamide revealed that inhibition of CA III has no effect on the force production of any of the different groups of muscles. This leaves us to suspect that CA III plays no role in the contractile process or in Myo/D gene expression.

Muscle fatigue tests opened several avenues for further studies. In our experiments, soleus HYPO muscles showed a significant increase in fatigue resistance as compared to control muscles while HYPER muscles did not. This once again, was to be expected in light of the alterations in fiber type composition resulting from differing thyroid hormone levels. That is, soleus muscles, composed of approximately 85% type I fibers (Ianuzzo et al 1977) is converted to an almost pure type I tissue in the absence of thyroid hormones (Tremblay et al 1993; Caiozzo et al 1991). EDL muscles, on the other hand, showed no significant difference in terms of fatigue resistance between the three different groups of muscles. This wasn't surprising for HYPER considering Li and Larsson (1997) subjected rats to 0, 4, and 8 weeks of T_3 treatment and none of the three groups showed significant differences in myosin isoform composition or contractile speed of the EDL.

Muscles were also tested with methazolamide at concentrations known to fully inhibit CA III activity. This was done to observe the effect of CA III (at differing concentrations) on muscle. In control soleus muscles, those incubated with methazolamide were significantly more resistant to fatigue than muscles not incubated with any inhibitor. This was in line with previous studies (Frémont et al 1991). Data for HYPER soleus muscles were not surprising in that the difference between the two fatigue curves (CTR vs METH incubated) were minimal. This is understandable since HYPER muscles contain less CA III as compared to their control counterparts. Data obtained from fatigue tests with HYPO soleus muscles, were more unexpected in that differences in these curves were also minimal, which is surprising since HYPO muscles contain considerably more CA III than untreated animals. It may be that the test intensity was not high enough to fully "stress" or "recruit" the metabolic pathways on which CA III may exert its modulating influence. The glycolytic capacity of these HYPO soleus muscles may be so low that the chances of seeing an effect are probably minimal. Once again, however, this is all very speculative and requires further experimentation to be able to make any solid statements. All three groups of fatigue curves

for EDL muscles incubated with and without methazolamide were virtually the same. This is understandable since even in HYPO muscles, CA III levels are minimal relative to soleus.

Results from the fatigue protocols paved a pathway for further research concerning glucose utilization in the presence or absence of CA III inhibitor, since significant differences in the fatigue curves of control soleus with and without methazolamide were more important during the initial 5 min when glycolysis is predominant. Previous experiments had shown that thyroid hormone treatments resulted in a rapid change in carbohydrate metabolism of skeletal muscle upon just a single injection of thyroid hormones (Chen-Zion et al 1995). Also, glycogen content is also significantly decreased in HYPER muscles (Chen-Zion et al 1995). Interestingly enough, it has also been proven that denervation results in a decreased activity of several glycolytic and oxidative enzymes (Evans 1983). In addition, Côté et al (1997) have shown that CA III does play some role in muscle energy metabolism, more specifically, carbohydrate utilization. Therefore, the final objective in these experiments was, for the soleus, to determine if the methazolamide-induced accumulation of G-6-P was proportional to the level of CA III activity found in the three groups of muscles. As for the EDL, the goal was primarily to determine is such an effect of methazolamide could be observed. When comparing soleus muscles with and without methazolamide, for all three groups, inhibition of CA III resulted in higher concentrations of G-6-P, and the difference was directly related to CA III concentration (see figure 2 of Chapter 4). EDL muscles, most likely because of their overall minimal CA III content, showed very little variation either among the three thyroid groups, or between muscles with and without methazolamide. Therefore, if indeed the presence of CA III inhibits the utilization of glycogen in working muscles, as is possible from the results seen, then there must also be a minimal concentration of CA III necessary to be able to influence glycolysis. Also, if indeed CA III is limiting glucose utilization, by inhibiting some step of glycolysis, for example, then it is only to be expected that this same enzyme favors, either directly or indirectly, another, more efficient mechanism of energy production. One possibility includes the utilization of FFA. Energy coming from these sources would then spare glycogen stores, and favor fatigue resistance. This would make sense considering CA III is found in highest abundance in those muscles which are most energy efficient.

In conclusion, much research remains to be done concerning several different aspects of the cellular biology of CA III. Yet, from our experiments, we clarified the modulation of CA III in fast twitch muscle fibers by thyroid hormones, established an effect of CA III inhibition on fatigue resistance, and proposed a mechanism for glycogen sparing as a result of the presence of this enzyme. Most importantly, several avenues of future research were highlighted. Overall, it is evident that carbonic anhydrase III is of an intriguing and yet

puzzling character, and one which will undoubtedly remain the focus of attention of many scientists for years to come.

Chapter 6

References for Chapter 1

References

1. Caiozzo, V.J.; Herrick, R.E.; Baldwin, K.M. 1991. Influence of hyperthyroidism on maximal shortening velocity and myosin isoform distribution in skeletal muscles. *American Journal of Physiology.* 261: C285-C295

2. Carter, N.D.; Wistrand, P.J.; Isenberg, H.; Askmark, H. Jeffery, S.; Hopkinson, D.; Edwards, Y. 1988. Induction of carbonic anhydrase III mRNA and protein by denervation of rat muscle. *Biochemistry Journal.* 256:147-152

3. Chen-Zion, M.; Bassukevitz, Y.; Beitner, R. 1995. Rapid changes in carbohydrate metabolism in muscle induced by triiodothyronine; the role of glucose 1,6-biphosphate. *Biochemistry and Molecular Medicine.* 56(1): 19-25

4. Côté, C.H.; Perreault, G.; Frenette, J. 1997. Carbohydrate utilization in rat soleus is influenced by carbonic anhydrase III activity. *American Journal of Physiology* 273 (Regulatory Integrative Comparative Physiology. 42: R1211-R1218

5. Côté, C.H.; Riverin, H.; Barras, M.; Tremblay, R.R.; Frémont, P.; Frenette, J. 1993. Effect of carbonic anhydrase III inhibition on substrate utilization and fatigue in rat soleus. *Canadian Journal of Physiology and Pharmacology.* 71: 277-283

6. Coulson, R.A.; Herbert, J.D. 1984. A role for carbonic anhydrase in intermediary metabolism. *Annals of the N.Y. Academy of Sciences.* 429:505-515

7. Deutsch, H.F. 1987. Review: Carbonic Anhydrases. *International Journal of Biochemistry.* 19(2): 101-113

8. Douen, A.G.; Ramlal, T.; Rastogi, S.; Bilan, P.J.; Cartee, G.D.; Vranic, M.; Hollosky, J.O.; Klip, A. 1990. Exercise induces recruitment of "Insulin-responsive glucose transporter". Evidence for distint intracellular insulin- and exercise-recruitable transporter pools in skeletal muscle. *Journal of Biological Chemistry.* 265(23): 13427-13430

9. Evans, O.B. 1983. Muscle pyruvate oxidation following denervation and reinnervation. *Muscle & Nerve.* 6(8): 557-560

10. Finol, H.J.; Lewis, D.M.; Owens, R. 1981. The effect of denervation on contractile properties of rat skeletal muscle. *Journal of Physiology* (London) 319:81-92

11. Frémont, P. 1990. L'Anhydrase carbonique musculaire (AC III): Modulation par les hormones thyroidiennes. Distribution et rôle physiologique. Thesis: Université Laval. p80.

12. Frémont, P.; Charest, P.M.; Côté, C.; Rogers, P.A. 1991. Distribution and ultrastructural localization of carbonic anhydrase III in different skeletal muscle fiber types. From: The Carbonic Anhydrases. Edited by Susanna J. Dodgson, Richard E. Tashian, Gerolf Gros, and Nicholas D. Carter. Plenum Publishing Corporation.

13. Frémont, P.; Charest, P.M.; Côté, C.; Rogers, P.A. 1988. CA III in skeletal muscle fibers: an immunocytochemical and biolchemical study. *Journal of Histochemistry and Cytochemistry.* 8: 775-782

14. Frémont, P.; Lazure, C.; Tremblay, R.R.; Chrétien, M.; Rogers, P.A. 1987: Regulation of carbonic anhydrase III by thyroid hormone: opposite modulation in slow- and fast-twitch skeletal muscle. *Biochemistry and Cell Biology.* 65: 790-797

15. Frenette, J. 1993. Rôle(s) possible(s) de l'anhydrase carbonique comme modulateur des sentiers métaboliques énergétiques chez le muscle soleus de rat. Thesis: Université Laval. p11

16. Gosselin, L.E.; Zhan, W.; Sieck, G.C. 1996. Hypothyroid-mediated changes in adult rat diaphragm muscle contractile properties and MHC isoform expression. *Journal of Applied Physiology.* 80(6): 1934-1939

17. Gros, G.; Dodgson, S.J. 1988. Velocity of CO_2 exchange in muscle and liver. *Annual Review of Physiology.* 50: 669-694

18. Henry, R.P. 1996. Multiple roles of carbonic anhydrase in cellular transport and metabolism. *Annual Review of Physiology.* 58:523-538

19. Holder, L.B.; Hayes, S.L. 1965. Diffusion of sulfonamides in aqueous buffers and into red cells. *Molecular Pharmacology.* 1: 266-279

20. Hughes, S.M.; Taylor, J.M.; Tapscott, S.J.; Gurley, C.M.; Carter, W.J.; Peterson, C.A. 1993. Selective accumulation of MyoD and myogenin mRNAs in fast and slow adult skeletal muscle is controlled by innervation and hormones. *Development.* 118(4) 1137-1147

21. Ianuzzo, D.; Patel, P.; Chen, B.; O'Brien, P.; Williams, C. 1977. Thyroidal trophic influence on skeletal muscle myosin. *Nature* (London) 270(3):74-76

22. Kudelska, G.; Górski, J.; Swiatecka, J.; Górska, M. 1996. Effect of exercise on glycogen metabolism in muscles of triiodothyronine-treated rats. *European Journal of Applied Physiology.* 72: 496-501

23. Lakkis, M. M.; Bergenhem, N. C.; O'Shea, K.S.; Tashian, R.E. 1997. Expression of the acatalytic carbonic anhydrase VIII gene, Car 8, during mouse embryonic development. *Histochemistry Journal.* 29(2): 135-141

24. Lakkis[2], M.M.; O'Shea, K.S.; Tashian, R.E. 1997. Differential expression of carbonic anhydrase genes for CA VII (Car 7) and CA-RP VIII (Car 8) in mouse brain. *Journal of Histochemistry and Cytochemistry.* 45(5):657-662

25. Larsson, L.; Li, X.; Teresi, A.; Salviati, G. 1994. Effects of Thyroid Hormone on Fast- and Slow-Twitch Skeletal Muscles in Young and Old Rats. *Journal of Physiology.* 481(1): 149-161

26. Leijendekker, W.J.; van Hardeveld, C.; Elizinga, G. 1987. Heat Production During Contraction in Skeletal Muscle of Hypothyroid Mice. *American Journal of Physiology.* 253: E214-E220

27. Li, X.; Hughes, S.M.; Salviati, G.; Teresi, A.; Larsson, L. 1996. Thyroid hormone effects on contractility and myosin composition of soleus muscle and single fibres from young and old rats. *Journal of Physiology.* 494(2): 555-567

28. Li, X.; Larsson, L. 1997. Contractility and myosin isoform compositions of skeletal muscles and muscle cells from rats treated with thyroid hormone for 0, 4, and 12 weeks. *Journal of Muscle Research and Cell Motility.* 18(3): 335-344

29. Lynch, C.J.; Brennan, W.A.; Vary, T.C.; CArter, N.; Dodgson, S.J. 1993. Carbonic Anhydrase III in Obese Zucker Rats. *American Journal of Physiology.* 264: E621-E630

30. Mann, T.; Keilin, D. 1940. Sulphanilamide as a Specific Inhibitor of Carbonic Anhydrase. *Nature.* 8692(46): 164-166

31. Meldrum, N.V.; Roughton, F.J.W. 1933. Carbonic anhydrase. Its preparation and properties. *Journal of Physiology* (London) 80: 113-142

32. Milot, J. 1995. Influence neuronale sur l'expression de l'anhydrase carbonique III dans les muscles squelettiques de type I et de type II chez le rat. Thesis: Université Laval. p87

33. Mourrieras, F.; Foufelle, F.; Foretz, M.; Morin, J.; Bouche, S.; Ferre, P. 1997. Induction of fatty acid synthase and S14 gene expression by glucose, xylitol, and dihydroxyacetone in cultured rat hepatocytes is closely correlated with glucose-6-phosphate concentrations. *Biochemical Journal.* 326 (P 2): 345-349

34. Muscat, G.E.; Downes, M.; Downhan, D.H. 1995. Regulation of vertebrate muscle differentiation by thyroid hormone: a role of the myoD gene family. *Bioessays.* 17(3): 211-218

35. Norenberg, K.M.; Herb, R.A.; Dodd, S.L.; Powers, S.K. 1996. The effects of hypothyroidism on single fibers of the rat soleus muscle. *Canadian Journal of Physiology and Pharmacology.* 74: 362-367

36. Nyman, P.O.; Lindskog, S. 1960. *Biochemica et Biophysica Acta.*

37. Roughton, F.J.W. 1935. Recent work on carbon dioxide transport by the blood. *Physiology Review.* 15: 241-296

38. Ryan, U.S.; Whitney, P.L.; Ryan, J.W. 1982. Localization of carbonic anhydrase on pulmonary artery endothelial cells in culture. *Journal of Applied Physiology.* 53(4): 914-919

39. Sauer, G.R.; Gengi, B.R.; Wu, L.N.Y.; Donachy, J.E. 1994. A facilitative role for carbonic anhydrase activity in matrix vesicle mineralization. *Bone and Mineral.* 26: 69-79

40. Spicer, S.S.; Ge, Z.H.; Tashian, R.E.; Hazen-Martin, D.J.; Schulte, B. 1990. Comparative distribution of carbonic anhydrase isozymes III and II in rodent tissues. *American Journal of Anatomy* 187: 55-64

41. Stanton, L.W.; Ponte, P.A.; Coleman, R.T.; Snyder, M.A. 1991. Expression of CA III in rodent models of obesity. *Molecular Endocrinology.* 5: 860-866

42. Sudo, Y.; Mariash, C.N. 1996. Lowering glucose depletes a thapsigargin-sensitive calcium pool and inhibits transcription of the S14 gene. *Endocrinology.* 137(11): 4677-4684

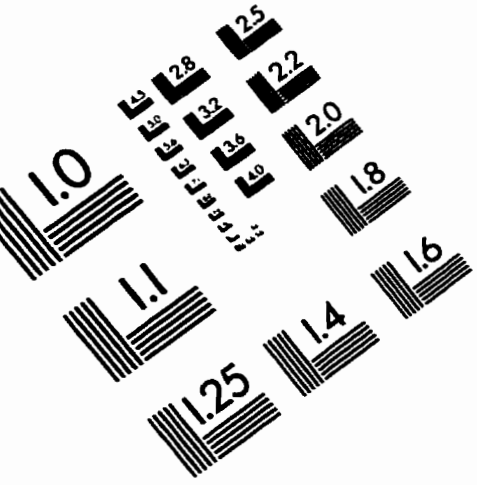
43. Sudo, Y.; Mariash, C.M. 1993. The thyroid hormone receptor can unmask a glucose response in the S14 gene. *Endocrinology.* 133(1): 129-134

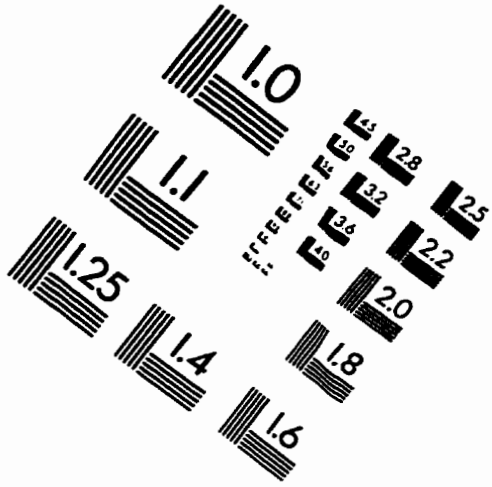
44. Supuran, C.T.; Scozzafaua, A. 1997. Novel aromatic/heterocyclic sulfonamides and their metal complexes as inhibitors of carbonic anhydrase isozymes I, II, and IV. *Journal of Enzyme Inhibition.* 12(1): 37-51

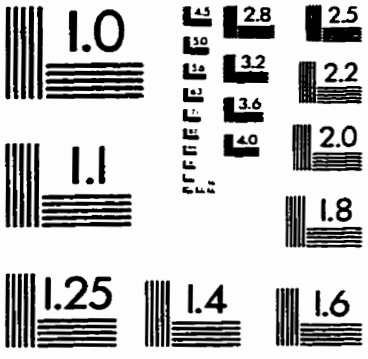
45. Tashian, R.E. 1989. The carbonic anhydrases: Widening perspectives on their evolution, expression, and function. *BioEssays.* 10(6): 186-192

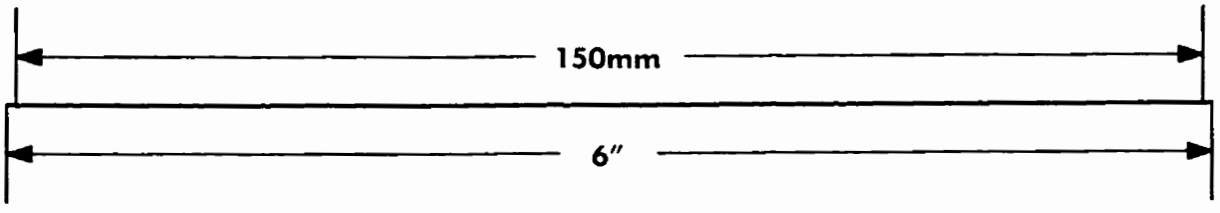
46. Tremblay, R.R.; Gaumont, F.; Milot, J.; Candas, B.; Côté, C.H. 1993. Inhibition of carbonic anhydrase III exerts paradoxical influences on the soleus muscle of hypo and hyperthyroid rats. *Basic and Applied Myology.* 3(2): 93-102

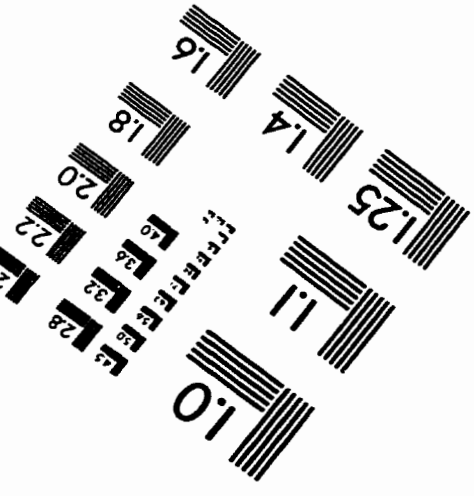
IMAGE EVALUATION
TEST TARGET (QA–3)

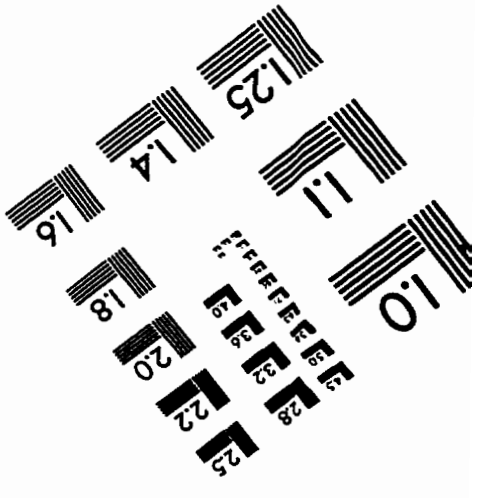












APPLIED IMAGE . Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989