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A PROGRAM FOR THE STUDY OF SKELETAL MUSCLE CATABOLISM
FOLLOWING PHYSICAL TRAUMA

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FINAL REPORT

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FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on the Care and Use of Laboratory Animals in the Institute of Laboratory Animal Resources Commission of Life Sciences. National Research Council (NIH Publication No. 86-23, Revised 1985).

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BACKGROUND

Following moderate to severe injury or infection, there is accelerated net breakdown of skeletal muscle protein associated with negative nitrogen balance. Skeletal muscle proteolysis can be influenced by inactivity and diminished food intake, but when these factors are accounted for in animal or clinical studies, accelerated net proteolysis continues to progress. It is known that the hormonal environment is altered following injury and infection and this may influence the relationship between protein synthesis and breakdown. Catabolic hormones, particularly glucocorticoids, have been associated with a moderate increase in net skeletal muscle catabolism (1). However, the efflux of skeletal muscle amino acids observed during administration of catabolic hormones to normal humans and animals is not comparable to that observed in injured patients (2), and this has prompted investigators to examine other possible causes of the accelerated proteolysis.

This proposal examines the mechanisms which mediate skeletal muscle proteolysis following injury. It is hypothesized that vasoconstriction of skeletal muscle vascular beds initiates local mechanisms which activate proteolysis. Such vasoconstriction would occur by increased sympathetic vasomotor activity in response to neurogenic stimulation and volume adjustments following the traumatic insult. Local norepinephrine release would mediate this change. Vasoconstriction could also initiate local mediators; one such factor could be prostaglandin E_2 (PGE_2) which counteracts sympathetic mediated vasoconstriction in the skeletal muscle bed by causing vasodilation. In addition, this substance has been associated with skeletal muscle proteolysis and altered nitrogen metabolism. The purpose of this contract is to determine the effects of PGE_2 on in vivo skeletal muscle proteolysis. Prostaglandin synthesis will be inhibited and the effects on proteolysis evaluated. Central nervous system narcosis will be utilized to reduce central neurogenic outflow and the effects of this form of blockade will be examined. The effect of stimulation of the sympathetic nerves regulating the vascular bed will also be examined.

THE EFFECT OF IBUPROFEN ON SKELETAL MUSCLE PROTEOLYSIS

Prostaglandin E_2 (PGE_2) has been shown to induce skeletal muscle proteolysis in vitro (3), and indomethacin (a PG synthase inhibitor) administration diminishes nitrogen excretion in normal rats (4) and reduces oxygen consumption in hypermetabolic injured rats (5). This study evaluated the effect of indomethacin administration on posttraumatic nitrogen metabolism.

A) Animal Model

Twelve conditioned mongrel dogs were studied. Their care, preoperative preparation and study procedure have been outlined in detail in last year's annual report. Briefly, on the day of study the animal was anesthetized using pentobarbital anesthesia (30 mg/kg), underwent laparotomy and retroperitoneal dissection and catheter insertion. The catheters were placed in the inferior vena cavae and distal aorta in such a manner that blood flow and hind-half substrate flux could be measured.

Amino acid flux studies were performed 6 hours and 24 hours following operation. All urine was collected and nitrogen excretion measured. The animals were studied in pairs, with one animal receiving Ibuprofen (12.5 mg/kg IV starting before operation and continuing every three hours throughout the study) and the other animal receiving saline injections.

Comparisons in this report were made using the non-paired t-test.

B) Results

The operative procedure lasted approximately 2 hours and all animals were extubated and conscious by 5 hours following induction of general anesthesia.

The average weight of the animals in the control group was 26 kg (range 22-30) and weight in the Ibuprofen treated animals was 23 Kg (range 20-28). The dogs receiving the Ibuprofen excreted less urine volume, less nitrogen, and less urea nitrogen than the control animals. When these values were expressed per Kg body weight, urinary volume remained significantly different between the two groups. Total nitrogen and urea nitrogen approached but did not achieve significance when expressed/kg (see table below, data expressed as mean $\pm$ SEM). Hindlimb flux tended to be reduced in the Ibuprofen-treated animals.

The Effect of Ibuprofen Treatment on Urinary Excretion of Nitrogen, Urea and Creatinine in Dogs following Laparotomy

	<u>Controls</u>	<u>Ibuprofen</u>	<u>p</u>
n	6	6	
Weight in Kg	26.0 $\pm$ 1.34	23.0 $\pm$ 1.32	
Urine Volume, ml/day	1716 $\pm$ 151	852 $\pm$ 206	0.008
Total Nitrogen, g/day	12.3 $\pm$ 0.8	8.4 $\pm$ 1.2	0.024
Urea Nitrogen, g/day	11.1 $\pm$ 1.0	7.14 $\pm$ 1.0	0.022
Creatinine, g/day	1.24 $\pm$ 0.34	0.89 $\pm$ 0.13	0.37
<u>Values expressed/kg</u>			
Urine Volume, ml/day	66 $\pm$ 5	37 $\pm$ 9	0.02
Total Nitrogen, g/day	0.48 $\pm$ 0.05	0.37 $\pm$ 0.05	0.14
Urea Nitrogen, g/day	0.43 $\pm$ 0.05	0.31 $\pm$ 0.0	0.11
Creatinine, g/day	0.05 $\pm$ 0.02	0.04 $\pm$ 0.01	0.55
<u>Hind Leg Measurements</u>			
Hind leg Blood Flow, ml/minKg	38 $\pm$ 8	37 $\pm$ 9	NS
Hind leg Nitrogen Flux, uM/minKg	-6.67 $\pm$ 3.19*	-2.66 $\pm$ 3.88	NS
<u>24hr Flux Study</u>			
Hind leg Blood Flow, ml/minKg	41 $\pm$ 6	55 $\pm$ 9	NS
Hind leg Nitrogen Flux, uM/minKg	-5.90 $\pm$ 2.10	-3.41 $\pm$ 2.84	NS

*(- denotes release, + denotes uptake)

Blood urea nitrogen and serum creatinine fell in both groups over the 24 hours of study. These changes were comparable in both groups.

C) Conclusions

This data supports the general thesis that proteolysis is diminished following cyclooxygenase inhibition. The sample size is small and the slight variation in the numbers (as when correcting for body weight) will shift the significance of the urinary data. The hind leg flux measurements tend to show a reduction in the rate of amino acid release in the ibuprofen treated animals. However, it is important to note that 3 of 6 ibuprofen treated animals showed positive uptake of amino acids during the study while none of the 6 control dogs showed this response ($X^2=4.1$, $p<0.05$). In this small group of animals, these data demonstrate that protein economy may be affected by cyclooxygenase inhibition.

THE EFFECT OF PGE₂ ON SKELETAL MUSCLE PROTEOLYSIS

A) Animal Model

Six conditioned dogs have been studied by infusing PGE₂ into the arterial tree of one hindlimb. Before the initiation of these experiments, eight animals underwent groin and leg dissections to obtain preliminary data concerning the distribution of the blood flow being perfused by the vessels used for the study and the stability of the animal model. The preoperative preparation of the six dogs utilized in the PGE₂ study was similar to that described in the previous protocol and was described in detail in the annual reports and the initial contract proposal. Using sterile technique, bilateral groin dissections were performed, with exposure of the external iliac artery and vein just above the inguinal ligament. 21-French polyethylene catheters were inserted 1 cm via an arteriotomy in a side branch of both external iliac arteries for infusion of dye and test substances. Catheters were also placed in the external iliac veins via a side branch with their tips located slightly below the bifurcation of the common iliac vein. These catheters were used for venous blood sampling. A catheter was also placed in the carotid artery for arterial sampling and blood pressure monitoring. Rectal temperature was also monitored throughout the study.

Following a period of stabilization, PAH was infused into each femoral artery catheter. Leg blood flow could thus be determined using dye dilution techniques. After at least one hour of infusion, basal samples were drawn simultaneously from each femoral venous catheter and the arterial line in the carotid artery.

PGE₂ infusion (1 ug/kg leg weight/minute) was then infused into one extremity and the diluent was infused into the other (contralateral) leg. The leg selected for infusion of the test substance was alternated throughout the series. Arterial and bilateral femoral vein samples were drawn in triplicate at the end of one, two and three hours of infusion and the experiment terminated. All samples were analyzed for concentration of PAH, glucose, amino acids and PGE₂. Control animals were studied in a similar manner, but received only saline infusion. Blood was measured at 0, 3 and 6 hours, and amino acid flux was determined at 0 and 6 hours.

Blood flow was determined from dye concentrations and mass of dye infused using equations previously described (see initial proposal). The leg receiving PGE₂ infusion was compared to the control leg using the paired t-test. Non-paired analyses were used when comparing animals from the control group with those receiving PGE₂.

B) Results

All animals were hemodynamically stable throughout the study; blood pressure was not greatly altered throughout the three or six hour study period. Rectal temperature rose approximately 1°C over the three hours of study (see Table).

Leg blood flow was comparable in both study groups during the basal period. With the initiation of the PGE₂ infusion, flow in the perfused extremity rose dramatically to three times basal concentrations and maintained this level of perfusion throughout the three hour study period (see Table). Little change in flow to the other extremity was observed throughout this time period, although there was a tendency for flow to the control limb to gradually rise. This change could be attributed to inadequate clearance of PGE₂ by the lungs or stimulation of PGE₂ synthesis by the lungs. However, arterial PGE₂ concentrations did not change throughout the study (157±75 at the start of the study to 220±12 at study completion, NS). Alternatively, the increase in body temperature which occurred in the animals receiving PGE₂ might have accounted for the slight increase in blood flow which was observed in the contralateral extremity. However, studies in the control animals showed a fall in blood flow with time and stabilization of amino acid release over a six hour period and these animals demonstrated similar changes in core temperature.

The Effects of PGE₂ Infusion on Blood Flow and Nitrogen Flux in 6 Dogs, Compared to 6 Control Animals (Mean±SEM)

<u>Time in Hours</u>	0	1	2	3
Mean Arterial Blood Pressure (mm Hg)	139±14	147±13	135±14	132±13
Rectal Temperature (°C)	38.2±0.3*	38.6±0.3	38.8±0.3	39.1±0.3
Control Leg:				
Blood Flow (ml/min·kg)	7.7±1.1*	10.1±1.9	12.2±1.7	13.7±2.6
Nitrogen Flux (uM/min·kg)	-1.19±0.49*	-1.51±0.46	-2.97±0.63	-4.09±0.56
PGE ₂ Infused Leg:				
Blood Flow (ml/min·kg)	7.2±1.2*	28.5±5.2*	31.3±3.9*	30.7±2.2*
Nitrogen Flux (uM/min·kg)	-1.10±0.35*	-1.76±0.16	-3.99±0.86	-4.57±1.52
Control Animals:				
Blood flow (ml/min·kg)	10.0±0.6	---	---	7.8±2.3**
Nitrogen Flux (uM/min·kg)	-1.64±0.45	---	---	-1.63±0.42**

* Different from control by p<0.05

+ Measurements increased or decreased significantly with time

** Values obtained at 6 hours, flow measurements observed at 3hr were 6.0±0.3

Thus, it appears that the PGE₂ infusion induced a catabolic state which was comparable in both extremities; no differences in amino acid release were observed between the two extremities. This systemic effect could be due to a metabolite of PGE₂, the induction of a substance from another site, possibly the lungs (?cytokine or other prostanoid) or alteration of the hormonal milieu. While further studies will be needed to determine this effect, it is important to realize that these results are consistent with the trends observed in the first study and indicate that prostaglandins may play an important role in the protein catabolic response following injury. The significance of this observation is that the investigator has never elicited a protein catabolic response of this magnitude to date. This response is greater than the administration of catabolic hormones, cytokines or endotoxin.

EFFECT OF SYMPATHETIC NERVE STIMULATION ON SKELETAL MUSCLE PROTEOLYSIS

A) Background

We initially hypothesized that norepinephrine mediated vasoconstriction would stimulate local PGE₂ production. This catabolic prostanoid would then stimulate skeletal muscle protein catabolism. This thesis was tested by stimulating the sympathetic lumbar chain to one leg of the dog, and comparing this response in amino acid release to the opposite or control leg.

B) Animal Model

Conditioned mongrel dogs were fasted overnight but allowed access to water and underwent general anesthesia in the early morning. They were intubated, prepped and draped as previously described in Section 2 and, in a sterile manner, arterial and venous catheters were inserted in the groins bilaterally. The dogs then underwent laparotomy and the sympathetic chain on the right side was dissected in the lumbar area. The chain was divided at this level so as to prevent reflex changes from occurring following nerve stimulation, and a shielded electrode was placed on the chain between L5-L6 level, distal to the transection. The abdomen was closed loosely and a carotid artery catheter was inserted in the right neck. A rectal temperature probe was also inserted.

A PAH infusion was started in both of the arterial groin catheters and the animal was allowed to stabilize for at least 1 1/2 hours. At this time (indicated as time 0), blood was drawn simultaneously from the venous catheters in both groins and the arterial catheter in the neck. Triplicate samples were drawn for concentrations of PAH, glucose and amino acids.

Nerve stimulation was then initiated (10 Hz, square wave form with repeated stimulation for 10 seconds every minute) and the stimulations repeated continuously for three hours. (Preliminary studies performed in three animals determined the type and extent of stimulation that should be utilized.) The blood samples were repeated at hourly intervals. At the end of the third hour, the stimulator was turned off and a recovery sample was obtained one hour later.

C) Results

The six animals studied weight 22.5 ± 1.8 kg. Preparation of the model was carried out without the incident and was complete after approximately 2 hours. The animals remained stable throughout the study, although the temperature tended to increase approximately 1.5°C. Additional vital signs were stable and are presented in a previously submitted annual report. Blood flow data from five of the animals behaved in a similar manner. The response to sympathectomy was comparable in the sixth animal but the control leg showed a highly variable blood flow. This animal was considered an outlier and was excluded from the remainder of the analysis.

With sympathectomy, hind limb blood flow increased significantly from 4.5 ± 0.6 ml/min·kg to 14.0 ± 0.6 , $p < 0.015$. With nerve stimulation, the flow to the sympathectomized limb dropped, but was still greater than that observed in the control extremity (see Table). With cessation of the stimulus, leg blood flow remained below postsympathectomy values.

Amino acid efflux was increased following sympathectomy, but with nerve stimulation and time the efflux returned to near control rates. When nerve stimulation was discontinued, blood flow was reduced from initial values, and amino acid nitrogen efflux rate in both limbs was approximately 2 μ M/kg·min, or approximately one half of the observed values seen in the PGE_2 infusion experiments.

The Effect of Sympathetic Nerve Stimulation
on Hindlimb Amino Acid Release (Mean $\pm$ SEM)

<u>Time in hours</u>	<u>NONSTIMULATED LIMB</u>		<u>STIMULATED LIMB</u>	
	<u>Blood Flow (ml/min·kg)</u>	<u>Amino Acid Nitrogen Flux (μM/min·kg)</u>	<u>Blood Flow (ml/min·kg)</u>	<u>Amino Acid Nitrogen Flux (μM/min·kg)</u>
0	4.5 ± 0.6	-1.38 ± 0.20	$*14.0 \pm 0.6^*$	$-5.43 \pm 0.39^*$
1	4.6 ± 0.6	-1.78 ± 0.09	$^{**}8.8 \pm 1.5^*$	-4.01 ± 1.31
2	4.1 ± 0.7	-1.49 ± 0.32	$^{**}7.2 \pm 1.1^*$	-1.92 ± 0.56
3	4.3 ± 0.7	-2.61 ± 0.50	$^{**}8.3 \pm 2.0^*$	-1.44 ± 0.67
4	4.1 ± 1.1	-2.21 ± 0.80	$6.4 \pm 0.8^*$	-2.09 ± 0.45

* post sympathectomy

* $p < 0.05$ when compared to control limbs

** period of nerve stimulation

THE EFFECT OF HIGH-DOSE FENTANYL ANESTHESIA ON POSTOPERATIVE SKELETAL MUSCLE AMINO ACID METABOLISM

A) Introduction

High-dose narcotic anesthesia has been proposed as a mechanism for suppressing the stress response to surgery as evidenced by the absence of characteristic glucocorticoid, catecholamine, and ADH elaboration (7). Narcotics may act by blocking peripheral input to subcortical areas of the brain such as the hypothalamus, or affect signaling via circulating substances such as prostaglandins, thus affecting sympathetic nervous system outflow. Fentanyl has an effective, short-acting (half-life of elimination = 2 hours) narcotic which does not cause hypotension or histamine release as is seen with morphine. This experiment evaluates the effect of high dose narcotic anesthesia and muscle relaxation on the catabolic response to stress.

B) Methods

Six conditioned mongrel dogs weighing between 20-35 kg were obtained and prepared for study as previously described. The animals initially received fentanyl (50 micrograms/kg IV) administered over four minutes, and the anesthesia was maintained by continuous infusion of fentanyl (0.3 micrograms/kg · minute IV) for 6 hours. Muscle relaxation was provided by giving pancuronium bromide 0.25 mg/kg IV initially and at hourly intervals. Laparotomy was performed and aortic and vena caval catheters were implanted and exteriorized via the left flank. Ventilation was assisted in all animals during the course of the operation using a volume ventilator (Harvard Instrument Co.) (15 breaths/minute and a tidal volume equal to 15 ml/kg inspired oxygen = 60%). Arterial blood gases were obtained during the period of ventilation and the ventilator was adjusted to maintain blood pH between 7.38-7.42, pCO₂ between 35-40, and paO₂ 90 mm Hg. Body temperature was maintained >98.0°F. Following the operation, the anesthesia was discontinued and the animals were allowed to breathe spontaneously and extubated.

Hindquarter amino acids and PAH A-V differences were obtained at 6 hours following the operation. Urine was collected by indwelling catheter and aliquots taken at 6 and 24 hours post-op for determination of total nitrogen, urea, creatinine, and NH₃. The results were compared to the responses observed when animals underwent the same operation but received pentobarbital anesthesia (30 mg/kg).

C) Results

The six animals weighed 26.3±1.5 kg. Anesthesia induction, paralysis, operation and anesthesia reversal were performed without difficulty. Vital signs were stable throughout the study, although urine output was decreased. This occurred despite an identical infusion of intravenous fluid in both groups of animals. Nitrogen loss, urea nitrogen excretion and, to a lesser extent, creatinine excretion all tended to be attenuated in the narcotized animals. Hind leg blood flow was reduced (p=0.06) and nitrogen efflux tended to be attenuated.

Thus, these studies demonstrate that central nervous system narcosis achieves effects similar to prostaglandin inhibition in attenuating catabolic responses to injury.

The Effect of Narcotic Anesthesia on the Catabolic
Response to Operation (Mean+SEM)

	<u>Pentobarbital Anesthesia</u>	<u>Narcotic Anesthesia</u>	<u>p</u>
Urine Output ml/kg 24hr	66±5	14±2	0.000
Nitrogen Loss g/kg 24hr	0.48±0.05	0.39±0.02	0.13
Urea Nitrogen g/kg 24hr	0.43±0.05	0.32±0.02	0.10
Creatinine Excretion g/kg 24hr	0.05±0.02	0.03±0.01	0.25
Blood Flow ml/min·kg	38±8	17±3	0.06
6-hour Hindquarter Flux, uM/kg·min	-6.67±3.19	-4.70±1.82	NS

SUMMARY

Four different experiments were performed within the context of this contract. Each study can be considered a pilot trial with minimal numbers of animals studied. Moreover, all data were analyzed by parametric means using two sided t-test. This approach should be viewed as a conservative application of statistical analysis. Despite the ability to achieve statistical significance in all studies, the important points revealed by these investigations should not be overlooked. These salient points are summarized below:

<u>Manipulation</u>	<u>Body Nitrogen</u>	<u>Muscle Blood Flow</u>	<u>Muscle Amino Acid Flux</u>
Injury ± Prostaglandin Inhibition	↓ 25% with indomethacin	no change	↓ ≈ 60% with indomethacin
No Injury--- PGE ₂ Infusion	---	300%↑ in infused leg 70%↑ in noninfused leg	at least 100% ↑ in both legs when compared to noninfused controls
Sympathetic Nerve Stimulation	---	200% ↑ with sympathectomy ≈ 40% ↓ with nerve stimulation	No Effect
Injury ± Narcotic Anesthesia	≈ 23% ↓	≈ 50% ↓	≈ 30% ↓

↑ = increase ↓ = decrease

From this information, we can conclude the following:

- 1) It is reasonable to think that prostaglandins are important mediators of the catabolic responses to injury. Ibuprofen blockade decreased amino acid release from skeletal muscle and nitrogen excretion, and PGE₂ infusion increased the response.
- 2) Host responses to prostaglandins appears to be central and not local. Note that there was no differential response between the PGE₂ infused leg and the control, despite exposure to markedly different concentrations of the test substance. However, there was a catabolic response in both legs when compared to the non-PGE₂ infused animals. Was this due to a breakdown product of PGE₂ present in the circulation which exerts catabolic effects? Is there a second mediator, possibly released by the lung that signals the muscle? Is this due to PGE₂ stimulation of the central nervous system?
- 3) Skeletal muscle blood flow does not determine skeletal muscle protein breakdown. Note that amino acid flux was independent of the large variations in blood flow that occurred with PGE₂ infusion and sympathectomy and nerve stimulation.

4) The central nervous system must play some role in controlling the protein catabolic rate of the body. Body nitrogen loss and skeletal muscle nitrogen loss decrease with high dose narcotic anesthesia; possibly the effects of ibuprofen are mediated by central mechanisms.

Implication for Care of Military Injuries - Recommendations

Because of these findings, and the safety of agents which block the formation of prostaglandins, more effort should be directed toward evaluating the pharmacological effects of those agents on reducing catabolic responses to injury. Animal studies should be performed with large numbers of animals studied emphasizing dose response characteristics of the inhibition, effect of time of administration of the drug following the traumatic insult and evaluating side effects. Following these studies, clinical trials should be initiated.

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