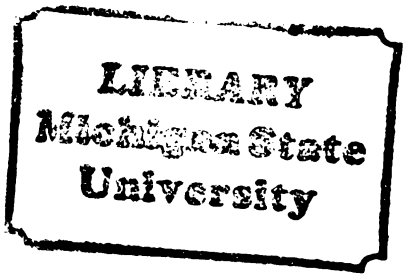

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Investigation of Adenosine and Prostacyclin in Local Hypoxic and Hypercapnic Vasodilation in the Forelimb of the Dog.

presented by

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of the requirements for

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Scott W. Walsh
Major professor

Date August 6, 1982

INVESTIGATION OF ADENOSINE AND PROSTACYCLIN IN LOCAL HYPOXIC AND HYPERCAPNIC VASODILATION IN THE FORELIMB OF THE DOG

By

Maureen Therese Mulrenan

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ABSTRACT

INVESTIGATION OF ADENOSINE AND PROSTACYCLIN
IN LOCAL HYPOXIC AND HYPERCAPNIC VASODILATION
IN THE FORELIMB OF THE DOG

By

Maureen Therese Mulrenan

The role of adenosine and prostacyclin in local hypoxic and hypercapnic vasodilation was investigated in isolated, innervated canine forelimbs. Blood from the femoral artery was pumped at constant flow through an extracorporeal lung obtained from a second dog and then to the brachial artery of the forelimb. Gases ventilating the extracorporeal lung were varied at ten minute intervals: normoxia, 15-20% O_2; mild hypoxia, 5% O_2; severe hypoxia, 0% O_2; hypercapnia, 15% CO_2. After gas tension alterations, blood samples were obtained from the brachial artery, brachial vein, and cephalic vein for adenosine and prostacyclin analysis. Hypoxia and hypercapnia significantly ($p<0.05$ or $p<0.01$) decreased forelimb resistance and perfusion pressure, yet plasma concentrations of adenosine and prostacyclin in the vessels draining the forelimb did not increase. Forelimb levels of adenosine and prostacyclin averaged 0.10uMolar and 1.9ng/ml plasma, respectively. These findings suggest that adenosine and prostacyclin are not involved in the vasodilation associated with local hypoxia or hypercapnia.

DEDICATION

This thesis is dedicated to Dr. Jerry Benjamin Scott whose untimely death did not allow him to see its completion. I am grateful for the opportunity I had to work with such a scholar. But more importantly I am thankful for the sense of honesty and justice he instilled in me with respect to scientific research. His work, words and life touched so many, and he will always be remembered in a special way by those who knew him.

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I. LITERATURE REVIEW

A. Introduction

Since the late 1800's there has been a growing concern and interest in understanding the mechanism by which blood vessel diameter changes. Vessel diameter is an important determinant of resistance which is important in hypertension and vascular disease. Luigi Severini first proposed that both oxygen and carbon dioxide can directly alter vessel diameter (62). This proposal, however, was quickly opposed by W.H. Gaskell who hypothesized that metabolites surrounding the vessel were the main determinants of its contractile state (23). The subject was readdressed occassionally during the following 80 years, but it was not until 1964 that interest in this area surged again. At this time it was reported that isolated arterial segments challenged with various blood oxygen tensions below 100mmHg contracted less than normoxic strips (8). Investigators then began looking for a specific mechanism to determined the mechanism of the decrease in vessel wall activity with lowered PO_2. In addition to oxygen and carbon dioxide, other metabolites have also been proprosed as mediators of the vasoactive state of the blood vessel wall. The following is a brief summary of the current knowledge on various factors known to affect blood vessel diameter. Because there is little information available on skeletal muscle preparations, this review is limited primarily to data obtained from isolated arterial strips.

B. Metabolic Factors in the Local Control of Blood Flow in Skeletal Muscle

1. Direct Effect of Oxygen

This review will begin with the data supporting the direct action of oxygen on the blood vessel wall. The vascular wall needs a constant supply of oxygen to perform work. If the supply is decreased, it seems logical that the amount of work, i.e. contraction, that the vessel could perform would decrease. This has lead many investigators to the hypothesis that vasodilation results directly from a vessel wall oxygen debt. In 1967 Daugherty et. al. (11) noted that a decrease in perfusion pressure occurred when the PO_2 of arterial blood perfusing an isolated skeletal muscle of the dog was lowered by use of an extracorporeal lung. The critical perfusate PO_2 was approximately 40mmHg. Perfusion pressure remained steady as the PO_2 was decreased from 100mmHg to 40mmHg but beyond this a concomitant drop in perfusion pressure was seen with each successive lowering of the oxygen supply to the muscle.

Ross et. al. (54) studied central effects involved with the vasodilation. In a series of experiments, one group of dogs had their spinal cords severed and the spinal cords of the other group remained intact. An isolated hind leg preparation was used. Initally both groups received blood of normal oxygen saturation, 100 percent, for the control period. Then blood from the vena cava was perfused through the hind leg. In both groups of dogs, a decrease in perfusion pressure occurred with this hypoxia and blood flow increased over three times the

resting level. Ross et. al. concluded that the brain was not involved in this autoregulatory mechanism.

A question arose concerning the use of systemic arterial blood PO_2 as an index of the oxygen environment of the vascular smooth muscle cells. Duling et. al. (17) addressed this question using the intact hamster cheek pouch and the cremaster muscle of hamsters and rats. They measured arteriolar and tissue PO_2 and found that the arteriolar wall PO_2 gradient was small, an average of 1.4mmHg different from inside to outside. Thus, the use of arterial blood PO_2 as an index of vessel wall PO_2 seemed appropriate because there did not appear to be a substantial barrier for oxygen diffusion across the vessel wall. Furthermore, the tissue PO_2 was always a few mmHg lower than either the vessel wall measurement or the arterial PO_2. Thus, arterial PO_2 is a better index of vessel wall PO_2 than is tissue PO_2.

Once this was accepted, Duling (16) devised a way to separate the direct action of oxygen on the vessel from the actions of metabolic agents. By superfusing a small vascular portion of a hamster cheek pouch with a micropipet while supplying a different perfusion solution to the tissue he could measure arteriolar diameter under various conditions. With the assistance of PO_2 electrodes placed perivascularly and on the opposite side of the vessel from the superfusion area, PO_2 was measured and correlated with changes in vascular diameter. Several experimental gas changes were performed and in all approaches changes in the perfusate gas tensions to the tissue had a greater influence on vessel diameter than did changes in the PO_2 directly around the vessel. Duling concluded that a vasoactive metabolite may be released by the tissue to control vessel caliber. Because the perivascular PO_2

electrode was placed on the opposite side of the smooth muscle from the lumen, and the blood that perfused the lumen was high in oxygen content, it was not possible to know the oxygen content of the vascular smooth muscle.

Numerous studies have been performed on isolated vascular strips contained in a bath. Tissue baths maintain the strips at a constant body temperature in a physiological salt solution. These in vitro preparations allow central and extravascular mechanisms to be eliminated, so that local control of vascular contraction can be studied. Smith et. al. (63) superfused arterial strips from various animals (cat, guinea pig, rat, pig) with a physiological salt solution with various oxygen contents. Vascular reactivity correlated directly with the oxygen content of the superfusate. This effect was unaltered by hyoscine, phenoxybenzamine, hexamethonium, bromolysergic acid diethylamine or mepyramine indicating that the parasympathetic and sympathetic systems were not involved, nor serotonin or histamine. The direct correlation of vascular reactivity to oxygen has been corroborated by numerous other investigators using different types of arterial segments and species (14, 20, 25, 51, 65). In some studies it was found that vessels responded differently depending on vessel wall thickness. Arterial strips with thick vascular walls had decreased contractile responsiveness at higher oxygen concentrations than thinner vessels, presumably because the core of the thicker vessels became hypoxic sooner due to a larger diffusion gradient (51). In both thick and thin vessels, however, decreased PO_2 resulted in a decreased contractile state.

Two hypotheses have been proposed to explain the relationship

between oxygen and vessel wall diameter. The first states that relaxation occurs because of the decreased ability of the vessel to produce energy (oxidative phosphorylation). The second deals with the liberation of some unknown vasoactive substance.

It is easy to understand how a decreased oxygen supply would interfere with tension development by eliminating a necessary component for oxidative phosphorylation. Fay et. al. (20) isolated ductus arteriosus strips from newborn guinea pigs and placed them in a muscle bath. Inhibitors of oxidative phosphorylation only inhibited the contractile response to oxygen and had little effect on acetylcholine induced contraction. Carbon monoxide also inhibited the oxygen response. This latter inhibition could be reversed by light, most likely due to photodissociation of the cytochrome a_3-CO complex. These data suggest that oxygen stimulates contraction probably by enhancing the rate of oxidative phosphorylation. Namm et. al. (47) studied rabbit aortic strips in an incubation bath. They found that the oxygen concentration in the bath was poorly correlated with ATP levels liberated into the bath by the aortic strips. They could not, however, dismiss the energy-available hypothesis because of possible compartmentalization of ATP. These investigators theorized that ATP pools may be in smooth muscle and it is these small pools that provide the energy for tension development. If this is so, the correlation between ATP and contraction may not be possible to elucidate. Further, the decreased creatine phosphate that they observed may be from the equilibration with this extramitochondrial ATP pool. And furthermore, the nonmuscular cells producing ATP may be responsible for the decrease of ATP that is actually occurring within the contractile compartments.

In addition, Gellai et. al. (25) noted that the decreased responsiveness of strips bathed in an hypoxic medium does not wane with time. Using arterial strips, they demonstrated a 35-40 percent decrease from control tension with a PO_2 change of 100 to 10mmHg. This response was tested for one hour and it was sustained over the entire period.

Chang et. al. (9) measured the PO_2 at the surface of the arterial wall of rabbit thoracic aortas, deep femoral arteries and skeletal muscle arteries using a muscle bath preparation and oxygen-sensitive microelectrodes. From the direct PO_2 measurements, the oxygen tension within the arterial wall was estimated as the oxygen concentration in the muscle bath was reduced. A decreased contraction of the vessel was seen with each reduction below the estimated arterial wall PO_2 of 50mmHg. This hypoxic PO_2 estimation of 50mmHg was the first data to show that vasodilation occurred at physiological oxygen tensions within the muscle strip.

In summary, these data elucidate the role oxygen plays in directly influencing vessel tone. Decreased oxygen causes relaxation of vessels in vitro, as well as, vasodilation in vivo. Decreased contractile state is correlated with decreased oxygen supply. The data indicate that the direct action of oxygen plays an important role in the vasoactive state of blood vessels in the peripheral circulation.

2. Potassium

In 1938, Katz et. al. (36), using Langendorff preparations of dog hearts showed that potassium can produce brief dilation. Increasing the concentration of potassium in the blood to the coronaries from 220mgm. percent to 279mgm. percent dilated the coronary bed, but within a few minutes there was a return to control tone and blood flow. Decreasing

the potassium concentration to the coronary circulation of the dog, in vivo, caused vasoconstriction (29).

When potassium was infused into the arterial blood supply of the forelimb or hindlimb of the dog vasodilation occurred (7). Brace (7) characterized this response by noting that there was a rapid decrease in vessel tone, but the vasodilation waned within the following few minutes.

Many types of vessel strips relax when the concentration of potassium is increased (25, 67). Gellai et. al. (25) showed that inducing relaxation of rabbit coronary and skeletal muscle strips in a bath by addition of potassium (bath concentration = 4mM) only produced a transient relaxation. This response was a 40 percent decrease in contraction from control and the strips recovered to 100 percent of their control tension within five to six minutes.

Similarly, Toda (67) induced transient relaxation by increasing the bath concentration of potassium to 5mM. The relaxation was unaffected by tetrodotoxin or propranolol which suggests that the release of neurotransmitters and beta adrenergic mechanisms were not involved.

A potassium-free medium can cause contraction of strips, but relaxation can then be induced with addition of potassium (as low as 0.1mM) (6).

These findings indicate that potassium does not have a role in a sustained vasodilation which would rule out its involvement in hypoxic or hypercapnic vasodilation.

3. Osmolarity

Coronary and deep femoral artery strips from New Zealand white rabbits have been studied in muscle bath preparations to determine the

vasodilation induced by hyperosmotic solutions (25). A bath concentration of 30 milliosmoles/L induced a 20-40 percent relaxation from control tension. However, within 15-60 minutes the strips recovered to control contractile tension. Addition of potassium (bath concentration = 4mM) to this high osmolar solution relaxed strips to 85-95 percent of control tension but recovery was 100 percent complete within 10-15 minutes. Although the additive effect of potassium produced a much larger relaxation than the hyperosmotic medium alone, recovery was still complete within a short time. Other studies (24, 60, 64) have also concluded that sustained vasodilation does not result from a hyperosmolar environment. Thus, osmolarity does not seem to have a role in long term vasodilation as is induced by hypoxia or hypercapnia.

4. Hydrogen Ion and Carbon Dioxide

In 1962, Molnar et. al. (44) infused isoosmotic acids (hydrochloric, nitric, lactic, pyruvic, acetic and citric acids) intra-arterially into the forelimb of the dog to evaluate the resistance changes occurring with decreased pH. Resistance decreased during all infusions, as did arterial and venous pH (mean of 7.36 to 6.72, and 7.39 to 6.85, respectively). They concluded that hydrogen ion was locally vasoactive.

In 1968, Kontos et. al. (39, 40) examined the vasodilation associated with local hypercapnic acidosis and breathing 7 percent CO_2 in the human. Four acid phosphate buffer solutions were infused intra-arterially at successively increasing rates and constant pressure into the intact forearm (40). Venous pH was lowered from 7.34 to 7.24 with a concomitant increase in venous PCO_2 of 42 to 52mmHg. Blood flow to the forelimb increased from 4 to 7ml/min/100g tissue. Hypercapnia induced systemically by 7 percent CO_2 breathing also decreased vascular

resistance in the intact forearm. Phenoxybenzamine and propranolol were given to block sympathetic effects (39). When the subjects breathed the hypercapnic gases and received a sodium bicarbonate infusion to block the acidosis, no change in vascular resistance was noted. Therefore, the authors concluded that the increase in blood PCO_2, not the decrease in pH induced by hypercapnia, was responsible for the vasodilation.

However, Rooke et. al. (53) placed isolated canine coronary arteries and saphenous veins in a tissue bath while altering pH or PCO_2 and noted that extracellular pH influenced the vessel tension, independent of PCO_2. Vessels relaxed when the bath pH was lowered from 7.4 and constricted at pH values above this. Alterations in PCO_2 from 20-56mmHg were not correlated with vessel activity.

The data combined show that the mechanism of hypercapnic vasodilation is still undetermined. Some investigators hypothesize that the increase in the carbon dioxide tension directly effects vessel tone, while others suggest that pH alterations correlate with vessel reactivity.

5. Adenine Nucleotides and Adenosine

The vasodilatory action of the adenine nucleotides were first noted in the general arterial and coronary vasculature in 1929 (15). Since then, adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP) and adenosine have all been recognized as potent vasodilators in most vascular beds (2, 10, 12, 66) including the canine forelimb (22, 28).

Rubio et. al. (55) performed histochemical studies on skeletal muscle from rats and guinea pigs in order to characterize the pathway of adenine nucleotide degradation. Activity of 5' nucleotidase, the enzyme

that produces adenosine from nucleotides, was found to be localized in and near the endothelium of the blood vessels. Other areas of the skeletal muscle degrade nucleotides by the inosinic acid (IMP) pathway. Herlihy et. al. (30) demonstrated that pig carotid strips which had been contracted with norepinephrine and potassium were subsequently relaxed by exogenous adenosine (3×10^{-6}M). Further, pig carotid artery strips incubated during normoxia ($95\%O_2:5\%CO_2$) and anoxia ($95\%N_2:5\%CO_2$) demonstrated a five-fold increase in hypoxanthine and a two-fold increase in inosine during anoxia (68). Tissue ATP levels on the other hand decreased with anoxia. Because adenosine added to the medium was rapidly deaminated to inosine, the authors concluded that adenosine was formed during anoxia by the arterial strips and was rapidly degraded it to inosine.

Hypoxia and hypercapnia were induced in the isolated forelimb of the dog by Kienitz et. al. (37) and the changes in perfusion pressure were observed. Theophylline (2.74mg/min), a blocker of the adenosine receptor, was infused and hypoxia and hypercapnia were again induced. Vasodilation was not inhibited by theophylline. The investigators concluded that adenosine did not appear to be involved in the hypoxic or hypercapnic vasodilation. However, due to the vasodilatory action of theophylline, norepinephrine was needed to raise perfusion pressure to pre-infusion levels.

The data on adenosine in local hypoxia and hypercapnia are inconclusive and contradictory. Some investigators conclude that there is a role for adenosine in local vasodilation, and others that adenosine is not involved. The release of adenosine during local hypoxia and hypercapnia in skeletal muscle has not been investigated.

6. Prostaglandins

Prostaglandins associated with anoxia were first studied in the coronary vascular bed by Block et. al. (3, 4). The coronary beds of isolated rabbit hearts were perfused with a Krebs solution containing 95%O_2:5%CO_2. A prostaglandin, postulated as E_2, increased and its increase could be blocked by indomethacin. However, the coronary vasodilation seen with anoxia was not blocked by indomethacin. Thus, the authors concluded that the prostaglandin released during anoxia did not have a vasodilator role. Criticism arose concerning these studies because the cyclooxygenase enzyme that catalyzes the arachidonic acid cascade needs oxygen to produce prostaglandins. The mechanism behind the increased prostaglandin production during anoxia, therefore, remained a mystery.

Wennmalm et. al. (70) also used isolated rabbit hearts, but induced hypoxia, 5%O_2, rather than anoxia. They found that there was a decreased production of a prostaglandin E-like compound during hypoxia, but production of this compound increased after hypoxia was ended. The investigators concluded that prostaglandins may be involved in reactive hyperemia, but not under conditions of low oxygen.

Two years later in 1976, Kalsner (34) placed isolated bovine coronary artery strips in a muscle bath and induced hypoxia. He found that as the arterial strips were challenged with decreasing bath oxygen tensions (515 to 38mmHg), the release of a prostaglandin E-like substance increased. Contrary to Wennmalm et. al. (70), Kalsner concluded that vascular strips that relaxed when challenged with low oxygen produced a prostaglandin that was correlated with the decreased contractile state of the strip.

Kalsner followed his previous study with another (33) in which the oxygen content of the bath medium surrounding the isolated coronary arterial strips was lowered to 9mmHg. At this low oxygen concentration release of the prostaglandin E-like compound stopped and the strips contracted. Kalsner proposed that vessel strips needed a supply of oxygen to produce a vasodilator prostaglandin, otherwise, contraction occurred.

Detar (13) challenged the work of Kalsner and placed rabbit skeletal and cardiac muscle arteries in a tissue bath and induced hypoxia stepwise from a PO_2 of 60 to 10mmHg. Contraction of the arterial strips was depressed with hypoxia but this depressed activity was not affected by indomethacin (10^{-5}M=bath concentration). No data was provided in the paper and sample size was only two. Detar, nevertheless, concluded that hypoxic vasodilation was due to the direct effect of oxygen and did not involve prostaglandins.

Hypoxia and hypercapnia were also studied in the pial arterioles of the intact cat brain (69). Increasing the perfusing carbon dioxide tension or decreasing the oxygen tension in the artery caused vasodilation. This response was not significantly altered when two cyclooxygenase inhibitors (indomethacin or AHR-5850) were infused. The blockers did, however, substantially inhibit the vasodilator response seen with topical application of arachidonate (100-200ug/ml). The authors concluded that endogenous prostaglandins are not involved in the hypoxic or hypercapnic responses in the pial microcirculation. However, Pickard et. al. (49) obtained data contrary to this. They altered the carbon dioxide tension from 40 to 60mmHg locally to the brain of the baboon and saw an increase in cerebral blood flow from 57 to

110ml/100g/min. Yet, after administration of indomethacin (0.04 to 0.2mg/kg/min) through a lingual artery cather, blood flow did not increase with hypercapnia. However, resting blood flow was lower after pre-treatment with indomethacin, 40ml/100g/min, which constricted the vascular bed prior to hypercapnia.

The vasodilation associated with hypoxia or hypercapnia was studied, in vivo, by Kienitz et. al. (37) in the isolated forelimb of the dog. Vasodilation was observed with both hypoxia and hypercapnia and infusion of indomethacin (1.72mg/min) did not block the vasodilation. The results failed to provide positive support for the involvement of prostaglandins in the vasodilation with hypoxia or hypercapnia, however, prostaglandin levels were not measured.

The literature implicating a role for prostaglandins in local hypoxia or hypercapnia is controversial and not conclusive. Some prostaglandins do produce vasodilation when infused or administered but they do not appear to be necessary for dilation of blood vessels during hypoxia or hypercapnia. Most of the studies in the literature in this area have used isolated vascular strips, and therefore, the in vivo data is very limited. Also the vasodilation associated with local induction of hypoxia and hypercapnia in skeletal muscle needs further investigation.

7. Introduction to Thesis Study

I attempted to uncover more information about the possible role of adenosine and prostacyclin in the vascular response to local hypoxia and hypercapnia. An isolated, innervated forelimb preparation was used similar to that was used by Kienitz et. al. (37) and the forelimb arterial and venous plasma concentrations of adenosine and prostacyclin

during local hypoxia or hypercapnia were measured. Femoral arterial plasma samples were also drawn to compare our prostacyclin concentrations with the results of other investigators (41). Since prostacyclin is the major product synthesized via the cyclooxygenase enzyme from arachidonic acid in the vessel wall (32, 45), we assumed that prostacyclin would increase in the plasma if it was involved in the vasodilation induced by hypoxia or hypercapnia. Also, adenosine is a very potent vasodilator *in vivo* and *in vitro*, and the vasculature of the skeletal muscle can produce adenosine. We assumed that if adenosine was involved in local hypoxia or hypercapnia, it could be measured in the venous plasma of the isolated forelimb.

II. MATERIALS AND METHODS

A. The Isolated Forelimb and the Extracorporeal Lung

1. Preparation

Eighteen mongrel dogs (15-33 kg) of both sexes were anesthetized with sodium pentobarbital (35mg/kg.,iv., Abbot Labortories, North Chicago, IL). Supplemental anesthesia was administered as needed during experimental procedures. Each dog was intubated and the endotracheal tube was connected to a constant volume respirator (Harvard Apparatus Company, Model 613, Millis, MA.) that was supplied with room air and supplemented with 100 percent oxygen to achieve an arterial blood gas tension of 100 mmHg. An acid-base analyzer (PHM 72Mk2, Radiometer Copenhagen, Copenhagen, Denmark) was used to measure PO_2, PCO_2 and pH. The right forelimb of the dog was surgically isolated, except for the major nerves, the brachial artery, and the brachial and cephalic veins (see Figure 1). The right femoral artery and the left femoral artery and vein were exposed. The left femoral artery was cannulated with P.E. 240 tubing (Intramedic Tubing, Clay Adams, Parsippany, NJ) to obtain arterial blood samples and to continuously monitor systemic arterial blood pressure. Blood pressure was measured using a pressure transducer (Statham Laboratory, Model P23Gb, Hato Rey, Puerto Rico) and a Hewlett-Packard eight channel direct writing recorder calibrated daily againsted a mercury manometer (model 7796A, Boston, MA). The left femoral vein was cannulated for drug infusions. The median cubital

Figure 1

Diagram of the isolated forelimb preparation with an extracorporeal lung.

FA = femoral artery

BA = brachial artery

BV = brachial vein

CV = cephalic vein

Blood was pumped from the femoral artery to the extracorporeal lung and then to the isolated forelimb. The blood was drained via the intact brachial and cephalic veins.

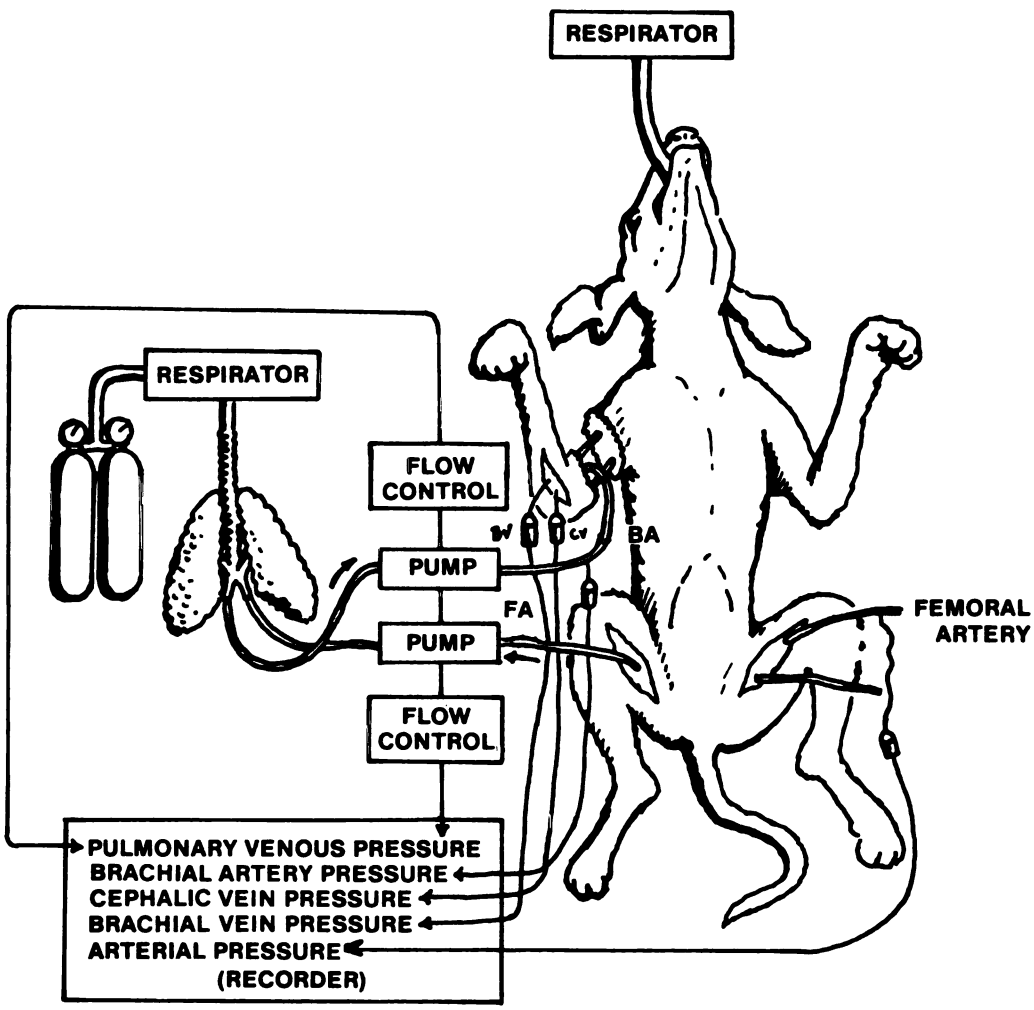
RESPIRATOR
RESPIRATOR
FLOW CONTROL
PUMP
PUMP
FLOW CONTROL
BA
FA
FEMORAL ARTERY
PULMONARY VENOUS PRESSURE
BRACHIAL ARTERY PRESSURE
CEPHALIC VEIN PRESSURE
BRACHIAL VEIN PRESSURE
ARTERIAL PRESSURE
(RECORDER)

Figure 1

vein, which is the major anastomotic connection between the brachial and cephalic veins, was ligated and a cannula was inserted into each forelimb vein via the ligated vessel. These cannulae were used to measure brachial and cephalic venous pressures and to sample venous blood from the forelimb muscle and skin, respectively. Venous pressures were recorded as described above for the left femoral artery.

An extracorporeal lung (ECL) was obtained from a second anesthetized dog (10-12kg) that was injected intravenously with sodium heparin (10,000 USP units, Elkins-Sinn Inc., Cherry Hill, NJ). After ten minutes, 300-500cc of blood was taken to be used later for priming the ECL. A left thoracotomy was performed at the fifth intercostal space. The inferior vena cava was ligated and transected. The pericardial sac was opened and a ligature placed around the pulmonary artery for easy indentification later. Then the heart/lung unit was excised. The lower half of the left heart, as well as the entire portion of the right heart below the pulmonary arterial ligature was removed. The lung unit was rinsed with isotonic saline to remove residual blood. Meanwhile, a reservoir containing the heparinized blood obtained from the donor dog was used to prime a Masterflex blood pump (Cole-Parmer Instrument Co., Chicago, IL) and tubing for the pulmonary arterial cannula (P.E. 380). Sodium heparin (10,000 USP units) was administered to the recipient dog. A 1 $\frac{1}{2}$ inch diameter tubing was inserted into the left atrium of the excised heart and positioned to receive pulmonary venous blood. The trachea from the excised lungs was intubated and ventilated with a second respirator. The blood primed pulmonary arterial cannula was inserted into the pulmonary artery. Blood was pumped from the beaker reservoir, through the pulmonary artery, the lung lobes, the pulmonary

vein and back to the beaker reservoir via a Holter roller pump (Extracorporeal Medical Specialties Inc., King of Prussia, PA).

The right femoral artery of the recipient dog was cannulated (P.E. 240) to supply blood to the pulmonary artery of the ECL unit. The venous cannula from the ECL was connected to the brachial artery. In this way, blood was pumped from the femoral artery of the recipient dog, through the ECL, the isolated forelimb and drained via the brachial and cephalic veins of the forelimb (Figure 1). Pulmonary arterial and venous pressures were continously monitored and recorded as described above. The pump supplying the ECL was regulated by a Leeds-Northrop controller (North Wales, PA) that adjusted flow to maintain a venous pressure of 2-6mmHg. Thus, capillary hydrostatic pressure in the ECL was regulated to avoid damage to the aveoli and to minimize pulmonary edema. Interposing the ECL between the recipient dog and the isolated forelimb made it possible to change gas tensions of the blood supplying the forelimb without altering systemic blood gas tensions.

Before any experimental manipulations were performed, blood flow to the forelimb was adjusted until perfusion pressure approximated mean arterial blood pressure and remained constant throughout the study. The blood pressure, the perfusion pressure and the blood gases of the ECL were allowed to equilibrate for 20-30 minutes. After equilibration, systemic arterial blood gases were as follows: PO_2=123$\pm$6.5, PCO_2=37$\pm$1.1, pH=7.37$\pm$0.01 (mean $\pm$ SEM, n=18).

2. Experimental Protocols

a. Hypoxia and Hypercapnia

Changes in oxygen and carbon dioxide tensions were made to evaluate their vasodilator effects in the isolated forelimb. At ten minute

intervals, gas tensions to the forelimb were randomly altered by changing the gas mixture supplying the ECL. Two levels of hypoxia, one level of hypercapnia, and two control periods were performed in each experiment. The gas mixtures used were as follows: normoxia, 15-20% O_2:5% CO_2:75-80% N_2; mild hypoxia, 5% O_2:5% CO_2:90% N_2; severe hypoxia, 0% O_2:5% CO_2:95% N_2; hypercapnia, 15% O_2:15% CO_2:70% N_2. These experimental manipulations resulted in a brachial arterial blood PO_2 of 49 ± 2.5mmHg for mild hypoxia, a PO_2 of 21 ± 1.7mmHg for severe hypoxia, and a PCO_2 of 93 ± 6.3mmHg for hypercapnia (in this latter case, pH decreased from 7.35 ± 0.01 to 7.08 ± 0.02, mean $\pm$ SEM). During normoxia intervals PO_2 and PCO_2 were adjusted to 100mmHg and 40mmHg, respectively, with a pH of 7.4.

Each experiment began with a ten minute control period, followed by one or two experimental alterations, a control period, and the additional experimental alteration(s). Blood was taken at the end of each ten minute interval from: 1) femoral artery (systemic arterial blood); 2) brachial artery (blood coming from the ECL); 3) brachial vein (forelimb muscle); 4) cephalic vein (forelimb skin). A steady state was usually attained during each ten minute interval with respect to perfusion pressure, systemic blood pressure, PO_2, PCO_2 and pH; however, in some cases up to 13 minutes were required.

b. Forelimb Blood Flow Determination

Pump flow (forelimb inflow) was measured at the end of each experiment with a graduated cylinder and a stop watch in all animals. Before sacrifice in six animals, one of the forelimb veins was cannulated to provide direct collection of venous blood. Venous outflow was measured using a graduated cylinder and stop watch at the end of a

ten minute control period, and after ten minutes of severe hypoxia. Total forelimb inflow minus the directly measured venous outflow gave the flow rate of the other vein. Any redistribution of blood flow between the brachial and cephalic veins could thus be determined. This procedure was completed during severe hypoxia and since no redistribution of blood occured, mild hypoxia was not tested. Hypercapnia was not studied because a previous study demonstrated no redistribution of forelimb blood flow during hypercapnia (52).

B. Adenosine

1. Sample Collection and Preparation

Blood was collected to determine whether adenosine was involved with the vasodilation induced by hypoxia and/or hypercapnia. Blood samples (approximately 2ml) were placed, within 30 seconds, in precooled, preweighed tubes containing 250ul of 3uM erythro-9(2-hydroxy-3nonyl) adenine (EHNA, Burroughs Welcome, Research Triangle Park, NC), 0.26uM dipyridamole (Boeringher Ingleheim, Ridgefield, CT), and five percent methanol in isotonic saline. EHNA and dipyridamole were added to block the breakdown of adenosine and the uptake of adenosine by red blood cells, respectively, so that adenosine concentrations measured in the plasma would be indicative of forelimb adenosine production or uptake. Samples were then centrifuged (IEC Clinical Centrifuge, Needham Hts, MA) at 2800rpm (1360xg) and 4^{o}C.

Four quality control tubes were included in each experiment, that is, a "spiked" tube containing the above collecting solution plus 20-25ul of adenosine (approximately 0.3nmole), and three tubes containing five or 20ul (10 or 1.7mg/ml, respectively) of Type I

adenosine deaminase (Sigma, St. Louis, MO) without EHNA. The adenosine deaminase tubes were used to determine the purity of the unknown samples during analysis on the high pressure liquid chromatograph (HPLC). An adenosine deaminase sample was taken with each experimental alteration and analyzed along with its paired unknown sample taken at the same time and sampling site. Adenosine deaminase samples were set aside at room temperature for ten minutes before centrifugation to allow adenosine deaminase to break down adenosine to inosine and hypoxanthine. Otherwise, they were processed as the other samples.

One ml of plasma from each sample was pipetted and placed into 250ul of a 35 percent perchloric acid solution, mixed and centrifuged (Sorvall model RC2-B, Newton, CT) at $4^{o}C$ and 17,500rpm (32,000xg) for 15 minutes. The supernatant was decanted and 900ul transferred to another glass tube. These samples were then neutralized with 110ul of K_2CO_3 (1.0g/ml) solution to a pH of 6.5-7.5, mixed and centrifuged (IEC Centra-7R, International Equipment Co., Needham Hts, MA) at $4^{o}C$ and 2800rpm (1360xg) for ten minutes. The supernatant was decanted into a new tube and frozen ($-20^{o}C$) until the time of assay. The collection tubes were later weighed to calculate the plasma volume that had been collected.

2. Analysis

Adenosine was measured by high pressure liquid chromatography (HPLC) which achieves high selectivity of a compound. An extensive discussion of the procedure and validation of the adenosine assay used can be found in the doctoral thesis by John Paul Manfredi (42). Briefly, samples (100ul) were injected into a reversed-phase column (either uBondapak C18, Waters Associates, Milford, MA, or Partisil-5ODS, Whatman Inc., Clifton, NJ). The support phase was silica and the composition of the

mobil phase changed linearly over a 20 minute analysis period from 100 percent methanol/water (70/30) to 40 percent methanol/water and 60 percent 4mM KH_2PO_4 buffer. Column flow was usually 1.5ml/min and the column was run at ambient temperature. Absorbance of the column eluate was continuously monitored at 254nm (Waters Model 440 Absorbance Detector) and recorded with a Waters data module. This gradient and flow typically eluted adenosine at a retention time of 18.4 minutes.

The adenosine peak in an unknown sample was identified by correspondence of its retention time with that of a known adenosine standard and the absence of an adenosine peak in samples treated with adenosine deaminase to remove adenosine. Sample peak heights were directly measured in mm and compared to the corresponding adenosine standard peak height.

In each experiment a sample "spiked" with adenosine (approximately 0.3nM) and three samples with adenosine deaminase added were collected simultaneously with other blood samples as described in the previous section. These samples were then used to assess the reliability of the assay. The adenosine peak of the "spiked" sample was compared to that of the "unspiked" sample. If the increase in peak height of the adenosine "spiked" sample was less than 80 percent of what it should be, then all the values for that experiment were rejected. Likewise, if the peak heights of any adenosine deaminase samples were greater than 30 percent of the peak height of their corresponding blood samples, all the sample values for that particular experimental alteration were rejected. When an adenosine deaminase sample exhibited a measureable peak less than 30 percent of its paired blood sample, the residual peak was

subtracted from the peak heights of all the samples in that particular experimental alteration.

C. Prostacyclin

1. Sample Collection

Blood was collected to determine whether prostacyclin was involved in the vasodilation associated with hypoxia and/or hypercapnia. Approximately 2ml were collected into precooled 9ml vacutainer tubes containing the potassium salt of the chelating agent ethylenediamine tetraacetic acid, EDTA, and centrifuged for five minutes at 2800rpm (1360xg) and $4^{o}C$. One ml of plasma from each sample was pipetted into a precooled polypropylene tube (12mm by 75mm). Samples were stored at $-20^{o}C$ until assayed.

2. Radioimmunoassay (RIA)

A detailed description and the validation of the prostacyclin RIA are given in the Appendix. Briefly, the stable breakdown product of prostacyclin, 6-keto-prostaglandin F_1 alpha (6-keto-PGF_1 alpha), was analyzed by radioimmunoassay. All reagents were diluted in a 0.1M phosphate buffer. Rabbit antiserum, 100ul (Seragen, Boston, MA), specific for 6-keto-PGF_1 alpha was added to 100ul of unknown sample. Tritiated 6-keto-PGF_1 alpha, 100ul (specific activity 120.0 Ci/mmol, New England Nuclear, Boston, MA), was also added and the tubes were mixed, incubated for one hour at $24^{o}C$ and then incubated at $4^{o}C$ for 18-24 hours. Tubes with known amounts of 6-keto-PGF_1 alpha (0.0048-2.5ng/100ul, U51787, The Upjohn Co., Kalamazoo, MI) were included in each assay. These latter tubes served as the standard curve and were used to calculate the quantity of prostaglandin in the unknown

samples. All standards and samples were run in triplicate.

After incubation, the tubes were placed on ice and 1ml of a precooled charcoal:dextran (0.5%:0.05%) suspension was added to each tube for 12 minutes. Then the tubes were centrifuged at 3000rpm (2000xg) in a refrigerated ($4^{o}C$) centrifuge (Beckman model J-6B, Palo Alto, CA) for 12 minutes. One ml of the supernatant was pipetted, added to 15ml of scintillation fluid (Packard, Downers Grove, IL), and counted for ten minutes or a statistical accuracy of less than 2.0 percent on a Tri-Carb 300C liquid scintillation counter (Downers Grove, IL).

Quality control samples were run in each assay. Within- and between- assay variations were 7.5 percent and 11.9 percent, respectively, for a 100ul plasma volume. Serial dilutions of dog plasma were parallel to the standard curve. Dog plasma stripped of endogenous prostacyclin with a charcoal:dextran suspension (5.0%:0.5%) had no measurable 6-keto-PGF_1 alpha. Known amounts of 6-keto-PGF_1 alpha added to "charcoal stripped" plasma were used to determine the accuracy of the assay.

3. Infusions of Prostacyclin (PGI_2)

To determine the vasodilator effect of prostacyclin, it was administered exogenously. In four animals, prostacyclin (U53217A, The Upjohn Co., Kalamazoo, MI) was infused after the hypoxic and hypercapnic experiments. A 200ng/ml solution of prostacyclin was delivered by an infusion pump (Harvard Apparatus Co., Millis, MA) into the brachial artery at three successive infusion rates (0.494, 1.23, and 2.47ml/min). Steady states, with respect to perfusion and blood pressures, were usually attained within four minutes from onset of each prostacyclin infusion rate. Blood samples were then drawn from all four sampling

sites to determine the concentration of prostacyclin entering and draining the forelimb. Resistances were determined and correlated with prostacyclin concentrations.

D. Statistical Analysis

The data were statistically analyzed by a two-way analysis of variance and the paired Student's t test. Significance was taken at $p<0.05$ or $p<0.01$ (26).

III. RESULTS

A. Perfusion Pressure, Resistance, and Blood Flow

Vascular changes that occurred in the isolated forelimb in response to alterations in gas tensions are shown in Figures 2 and 3. Mild hypoxia, severe hypoxia and hypercapnia all significantly ($p<0.01$ or $p<0.05$) decreased the perfusion pressure and resistance in the vascular bed of the forelimb (Figure 2). Severe hypoxia produced the most dramatic changes: perfusion pressure decreased from a mean of 113mmHg before hypoxia to a mean of 79mmHg after severe hypoxia because resistance decreased from a mean of 1.05mmHg/ml/min to a mean of 0.72mmHg/ml/min. These changes indicate vasodilation in the whole forelimb because there was no redistribution of blood flow between the muscle and skin. This is indicated by consistent blood flows in the brachial and cephalic veins before and after severe hypoxia (Figure 3).

B. Adenosine

The adenosine concentrations measured in the forelimb plasma during alterations in gas tensions are shown in Figure 4. Mild hypoxia, severe hypoxia, and hypercapnia did not change the adenosine production across the vascular bed of the forelimb. Adenosine concentrations were approximately 0.1uMolar in all of the vessels and did not increase from artery to vein with hypoxia or hypercapnia.

Figure 2

Perfusion pressure (inflow pressure minus the average venous pressure) and resistance (perfusion pressure divided by flow) in the forelimb during control (C, 15-20%O_2:5%CO_2:75-80%N_2), mild hypoxia (MH,5%O_2:5%CO_2:90%N_2), severe hypoxia (SH,0%O_2:5%CO_2:95%N_2), and hypercapnia (HPR,15%O_2:15%CO_2:70%N_2). Data represent means $\pm$ SEM, n=18. A paired Student's t test was performed to compare the control period with the experimental alteration. *=$p<0.05$, **=$p<0.01$.

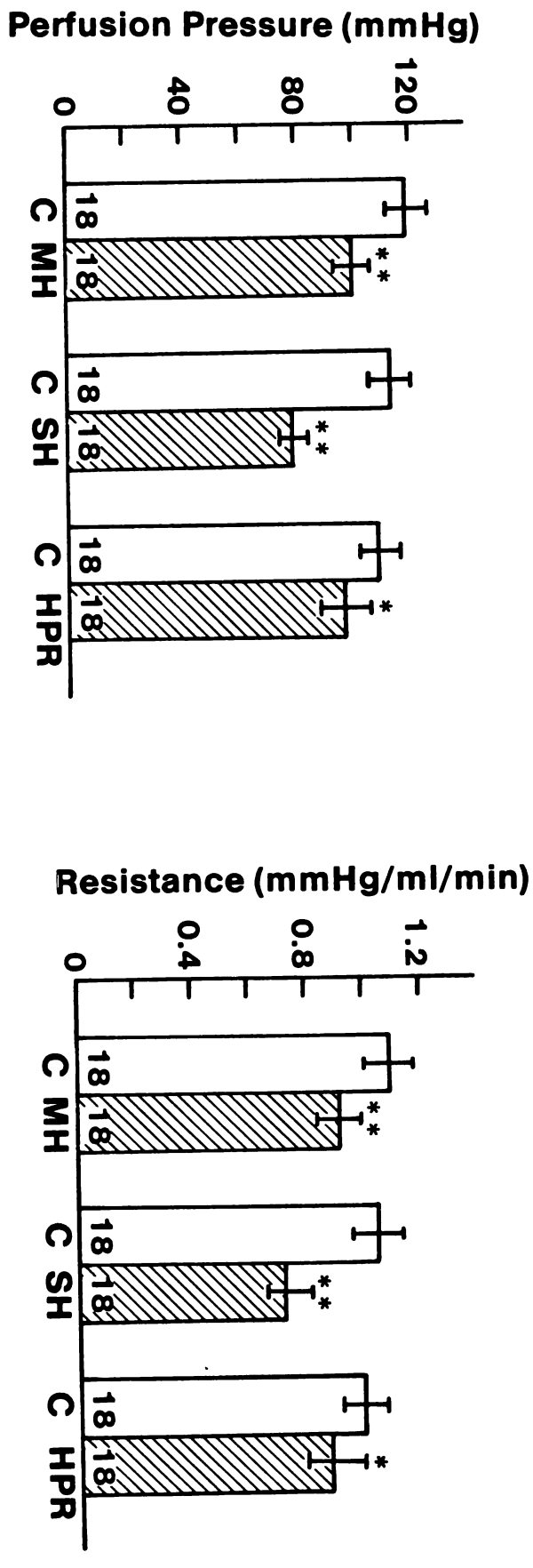
Perfusion Pressure (mmHg)
0
40
80
120
C MH
C SH
C HPR
18
18
*
**
Resistance (mmHg/ml/min)
0
0.4
0.8
1.2

Figure 2

Figure 3

Blood flow through the brachial, BV, and cephalic, CV, veins during control (C,15-20%O_2:5%CO_2:75-80%N_2) and severe hypoxia (SH,0%O_2:5%CO_2:95%N_2). Data represent means $\pm$ SEM, n=6. A paired Student's t test was performed to compare control and severe hypoxia in each vessel.

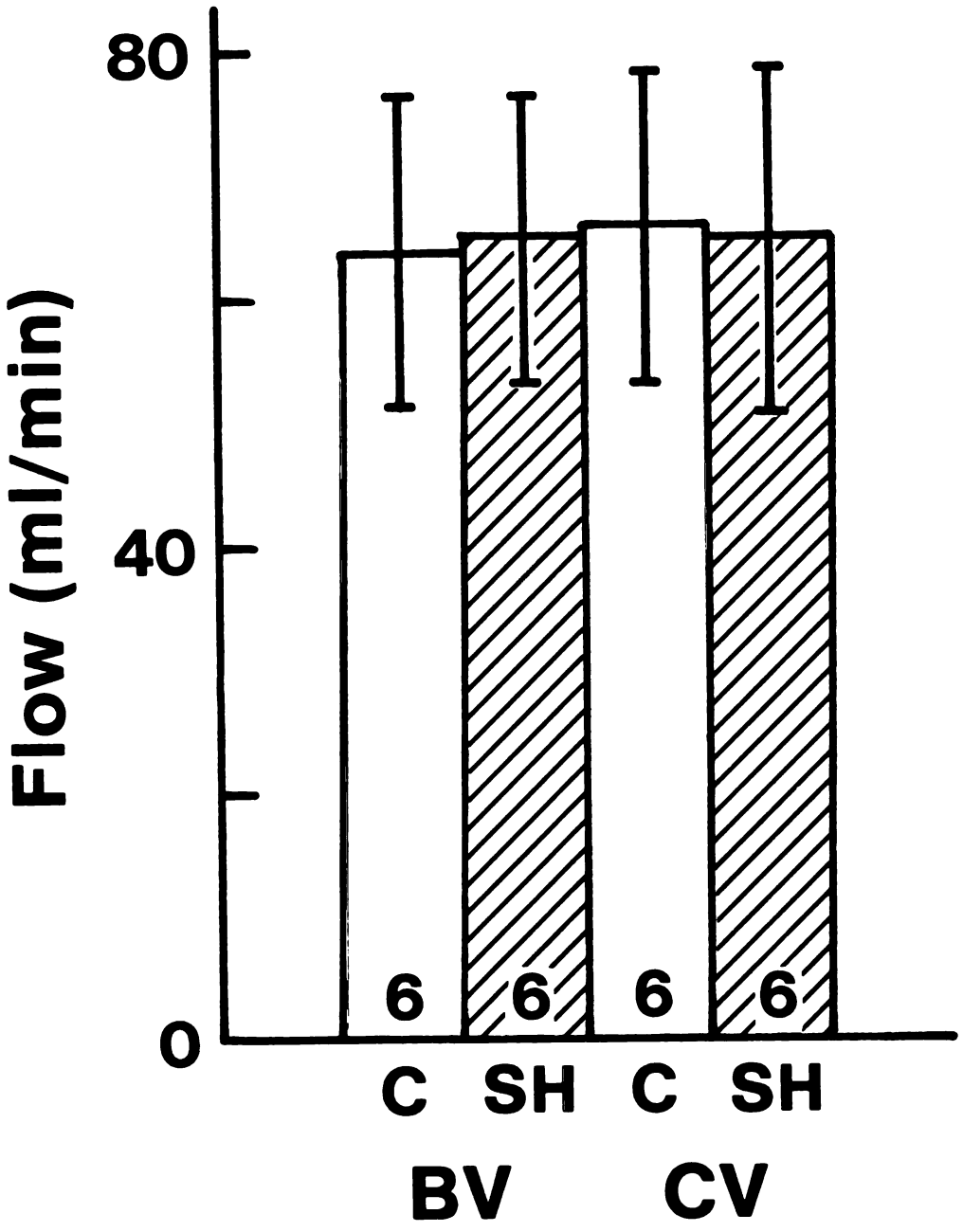

Figure 3

Figure 4

Concentrations of adenosine in plasma water (uMolar) during control (C,15-20%O_2:5%CO_2:75-80%N_2), mild hypoxia (MH,5%O_2:5%CO_2:90%N_2), severe hypoxia (SH,0%O_2:5%CO_2:95%N_2), and hypercapnia (HPR,15%O_2:15%CO_2:70%N_2).

BA = brachial artery

BV = brachial vein

CV = cephalic vein

Data represent means $\pm$ SEM, the number at the bottom of each bar represents sample size. A paired Student's t test was performed to compare the adenosine concentration in the control vs. the experimental alteration in like vessels.

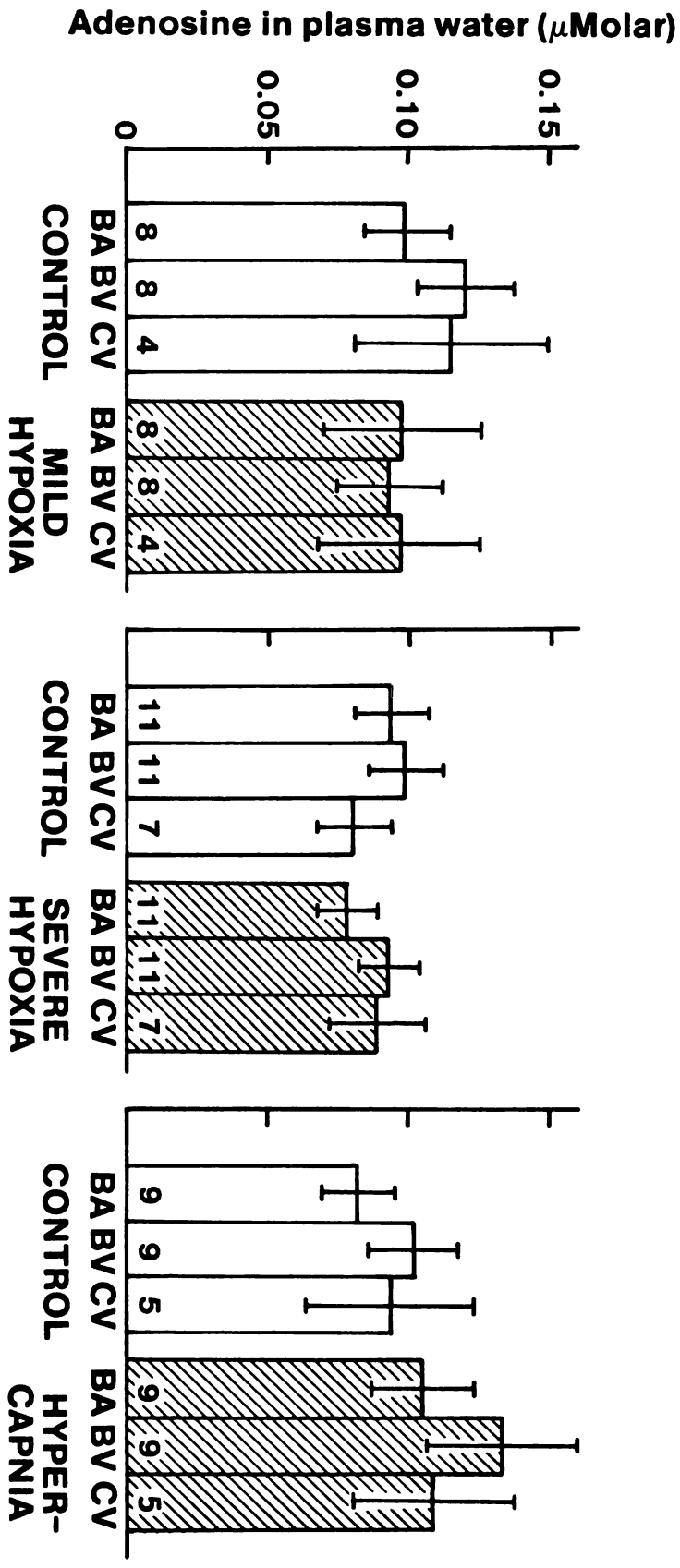
Adenosine in plasma water (μMolar)
0
0.05
0.10
0.15
BA
BV
CV
CONTROL
MILD HYPOXIA
SEVERE HYPOXIA
HYPER-CAPNIA
8
8
4
8
8
4
11
11
7
11
11
7
9
9
5
9
9
5

Figure 4

C. Prostacyclin (6-keto-prostaglandin F_1 alpha)

The systemic and forelimb plasma concentrations of 6-keto-prostaglandin F_1 alpha (6-keto-PGF_1 alpha), the major breakdown product of prostacyclin, are shown in Figure 5 before and after mild hypoxia, severe hypoxia and hypercapnia. Systemic levels of 6-keto-PGF_1 alpha, as estimated by femoral arterial concentrations, are significantly lower ($p<0.01$) than the concentrations in the vessels supplying and draining the forelimb (approximately 0.8 vs. 1.9ng/ml plasma). Concentrations of 6-keto-PGF_1 alpha in the vessels draining the forelimb (i.e. brachial and cephalic veins) were not significantly higher ($p>0.05$) than the concentrations in the vessel supplying the forelimb (i.e. brachial artery). The increase in plasma 6-keto-PGF_1 alpha concentrations from the femoral artery to the forelimb vessels represent a constant production of prostacyclin by the lungs (21). Average concentrations in the forelimb veins ranged from 1.69 to 2.18ng/ml plasma.

Prostacyclin was infused into the brachial artery of the forelimb and plasma samples were drawn to determine the concentrations of 6-keto-PGF_1 alpha at the four sampling sites (Figure 6). The concentrations measured in the systemic circulation, as well as in the veins draining the forelimb, were significantly different from the level of 6-keto-PGF_1 alpha of the brachial artery in all groups ($p<0.05$ or $p<0.01$). With infusion, the mean values in the veins rose from a control concentration of 1.88ng/ml plasma to 3.32, 5.14, 8.81ng/ml plasma with increasing infusion rates of prostacyclin (98.8, 162.8 and 494ng/ml, respectively). The changes in systemic blood pressure and forelimb perfusion pressure and resistance during prostacyclin infusion

Figure 5

Concentrations of 6-keto-prostaglandin F_1 alpha (ng/ml plasma) during control (C,15-20%O_2:5%CO_2:75-80%N_2), mild hypoxia (MH,5%O_2:5%CO_2:90%N_2), severe hypoxia (SH,0%O_2:5%CO_2:95%N_2), and hypercapnia (HPR,15%O_2:15%CO_2:70%N_2).

FA = femoral artery

BA = brachial artery

BV = brachial vein

CV = cephalic vein

Data represent means $\pm$ SEM, n=10. A 2-way ANOVA was performed to compare the 6-keto-PGF_1 alpha concentrations across vessels in each group. **= $p<0.01$.

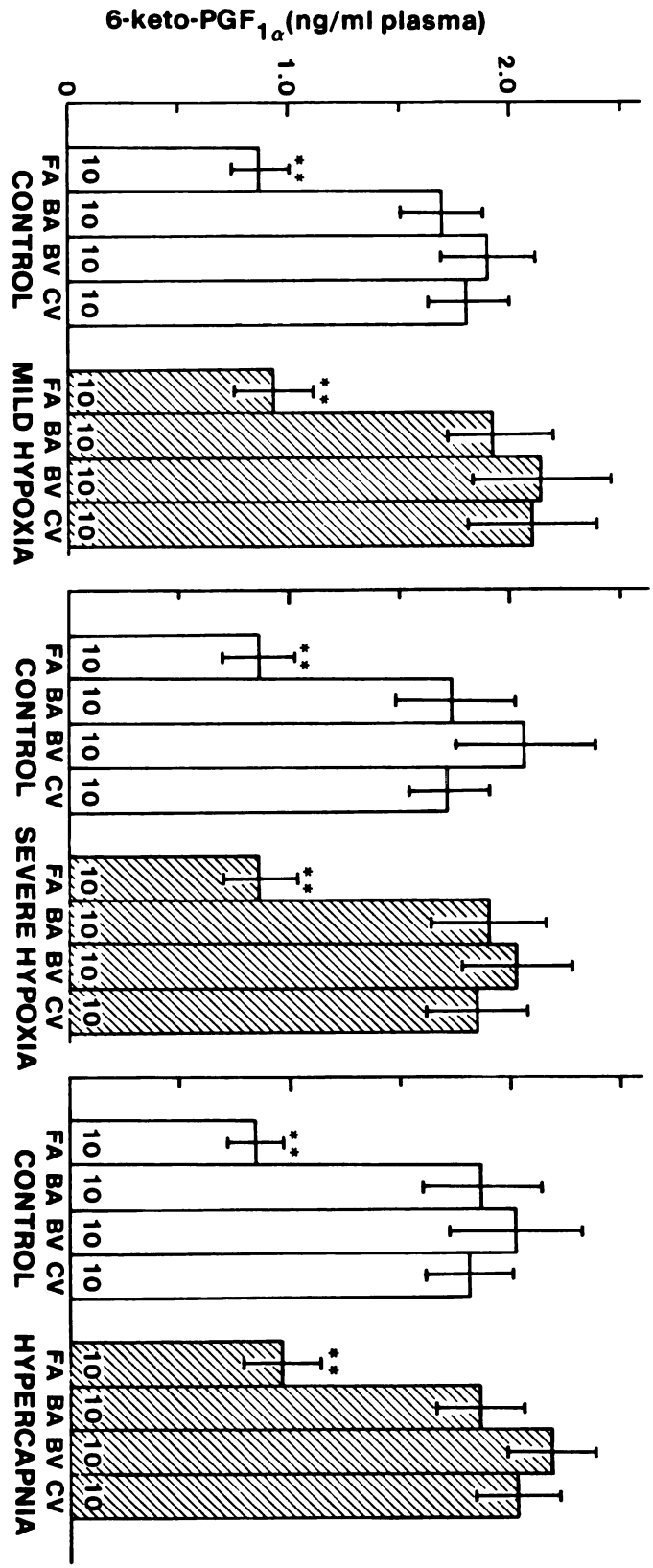
6-keto-PGF$_{1\alpha}$(ng/ml plasma)
0
1.0
2.0
FA BA BV CV
CONTROL
MILD HYPOXIA
SEVERE HYPOXIA
HYPERCAPNIA
10
**

Figure 5

Figure 6

6-keto-prostaglandin F_1 alpha measurements during prostacyclin infusion into the isolated forelimb.

FA = femoral artery

BA = brachial artery

BV = brachial vein

CV = cephalic vein

Data represent means $\pm$ SEM, the number at the bottom of each bar represents sample size. A paired Student's t test was performed to compare the control and each of the infusion groups. *= $p<0.05$, **= $p<0.01$.

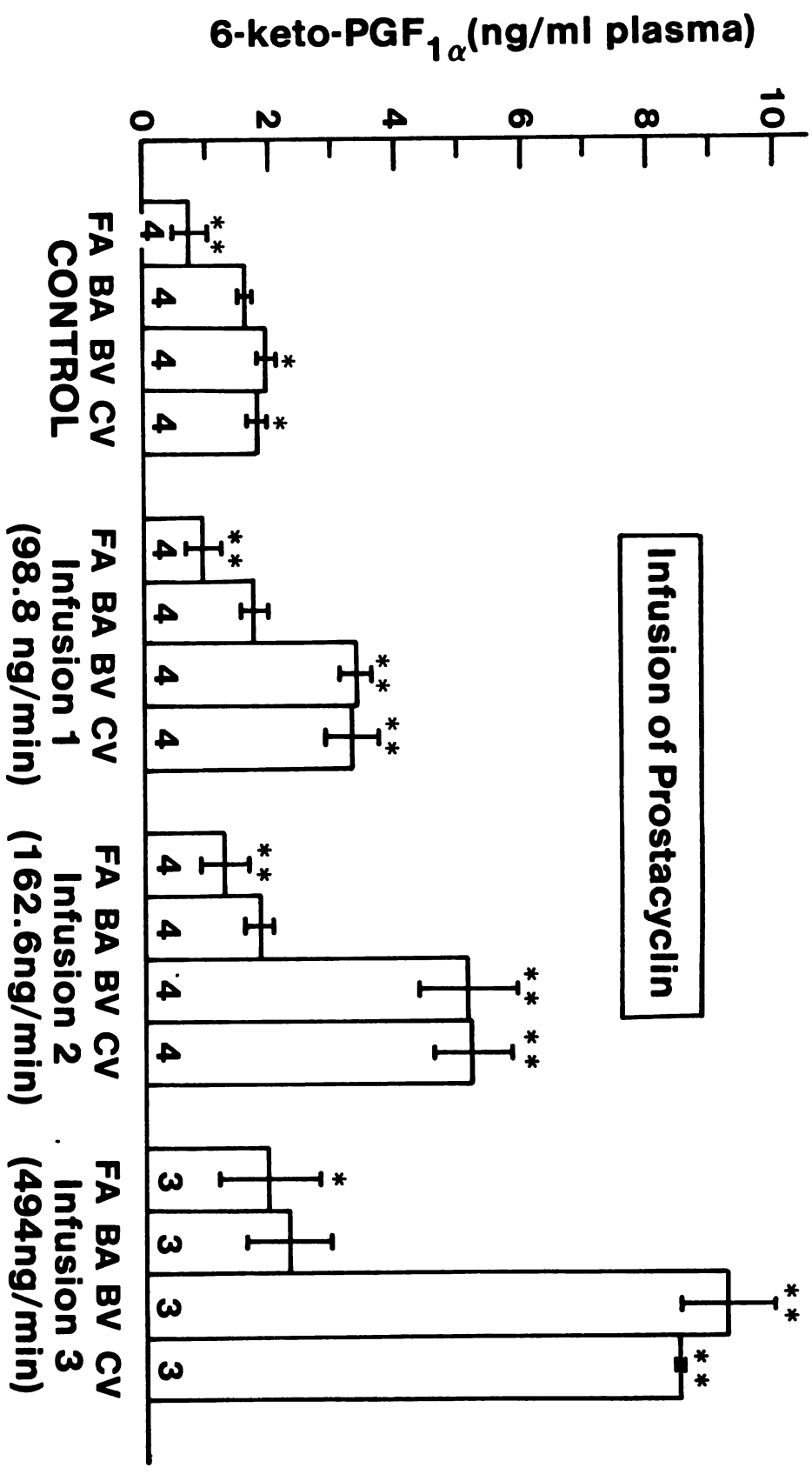
6-keto-PGF$_{1\alpha}$(ng/ml plasma)
0
2
4
6
8
10
Infusion of Prostacyclin
FA BA BV CV
CONTROL
FA BA BV CV
Infusion 1
(98.8 ng/min)
FA BA BV CV
Infusion 2
(162.6ng/min)
FA BA BV CV
Infusion 3
(494ng/min)

Figure 6

are shown in Figure 7. The mean systemic blood pressure decreased from 83 to 62mmHg with increased concentrations of prostacyclin. Although not statistically significant, due to the small sample size, this hypotensive effect has been documented in the literature (1). Similarly, perfusion pressure and resistance in the forelimb declined with increased prostacyclin infusion rates. Perfusion pressure decreased from 135mmHg perfusion pressure to 101mmHg and resistance declined from 1.04 to 0.662mmHg/ml/min.

Figure 7

Systemic blood pressure, forelimb perfusion pressure (inflow pressure minus average venous pressure) and resistance (perfusion pressure divided by inflow) during infusion of prostacyclin into the isolated forelimb.

C = control

Infusion 1 = 98.8ng/min

Infusion 2 = 162.6ng/min

Infusion 3 = 494.0ng/min

Data represent means $\pm$ SEM, the number at the bottom of each bar represents sample size. A 2-way ANOVA was used to compare values within each group.

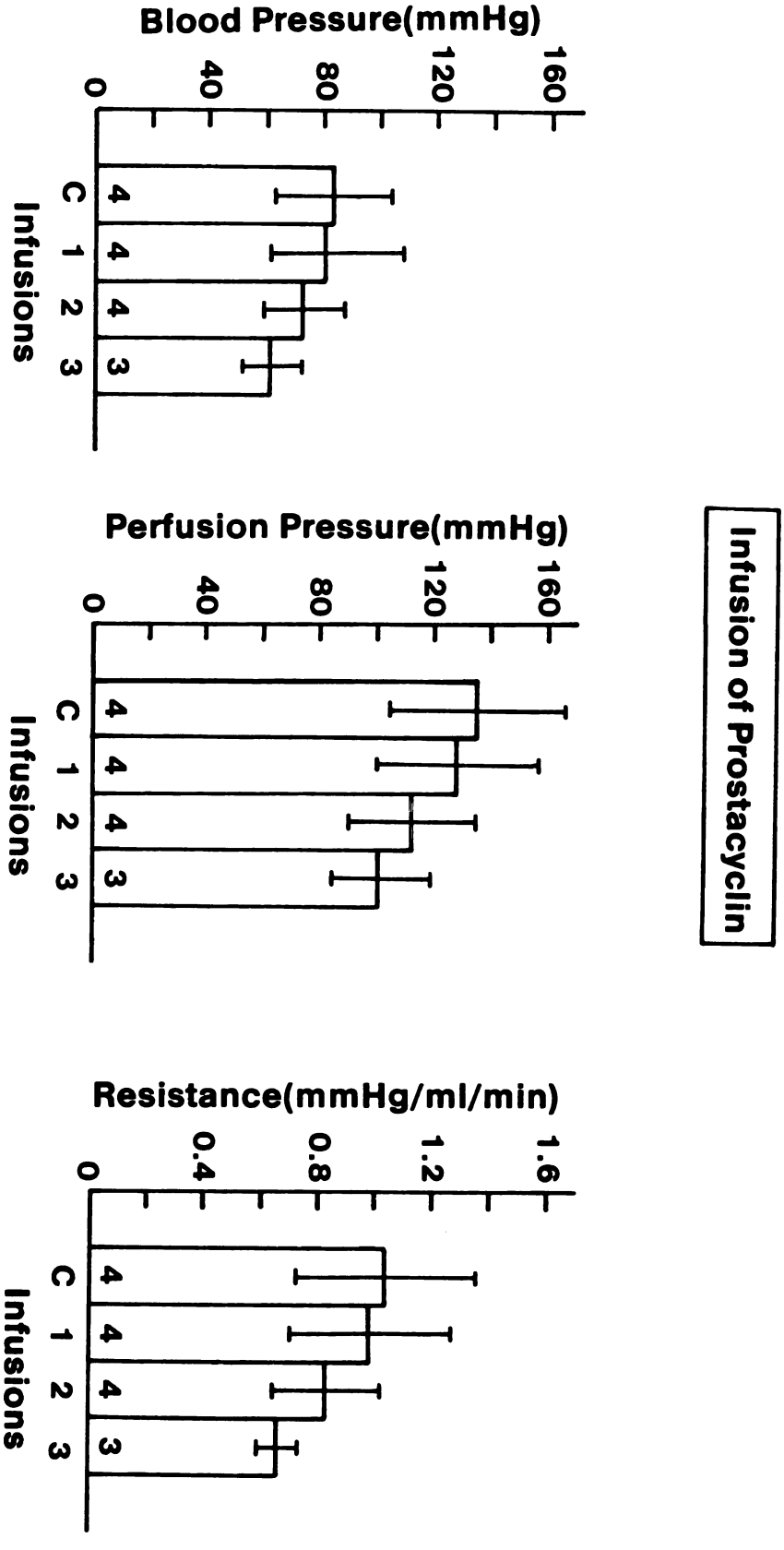
Blood Pressure(mmHg)
0
40
80
120
160
C
1
2
3
Infusions
4
4
4
3
Perfusion Pressure(mmHg)
0
40
80
120
160
C
1
2
3
Infusions
4
4
4
3
Infusion of Prostacyclin
Resistance(mmHg/ml/min)
0
0.4
0.8
1.2
1.6
C
1
2
3
Infusions
4
4
4
3

Figure 7

DISCUSSION

Mild hypoxia, severe hypoxia and hypercapnia induced locally in the isolated forelimb of the dog caused the resistance and the perfusion pressure to decrease (Figure 2). Systemic arterial blood PO_2, PCO_2 and pH did not change during ventilation of the extracorporeal lung with hypoxic or hypercapnic gases. Other investigators have also found that the use of an extracorporeal lung is a good way of locally inducing blood gas changes in a vascular bed without affecting the systemic circulation (11). Thus, the vasodilation observed in this study was mediated by local rather than central mechanisms. Factors considered most likely to be involved in the local vasodilation were: direct effects of O_2 or CO_2 and increased production of adenosine and/or prostacyclin.

After the induction of hypoxia or hypercapnia, plasma samples were obtained for adenosine and prostacyclin analysis to determine if the release of these compounds increased with the decreased perfusion pressure and resistance. The concentrations of adenosine and prostacyclin in the blood draining the forelimb, however, did not change with hypoxia or hypercapnia (Figures 4 and 5).

Because plasma concentrations were assayed, questions may arise concerning the reliability of using such samples as an index of compounds formed within the smooth muscle cell of the vessel. The data obtained on the release of adenosine and prostacyclin in myocardial and

muscle strip preparations subjected to hypoxia and hypercapnia address this issue. First, adenosine was found in the coronary effluent of isolated cat and guinea pig hearts subjected to anoxia and severe hypoxia after pretreatment with an inhibitor of adenosine deaminase, 8-azaquanine (35). The increased adenosine release corresponded with a decreased coronary vascular resistance. Mustafa et. al. (46) in 1975, incubated cultured cardiac cells from 16-day-old chick embryos and found that hypoxia produced a two-fold increase in adenosine production and its metabolites inosine and hypoxanthine. In 1977, Schrader et. al. (56) prelabeled cardiac nucleotides with ^{14}C-adenine in the isolated guinea pig heart. During hypoxia, there was an increased release of labelled adenosine, inosine and hypoxanthine. The authors concluded that the major part of the changes in coronary vascular resistance during hypoxia were due to adenosine, the concentration of which was in the range known to produce vasodilation. Lastly in 1979, Scott et. al. (58) bioassayed coronary sinus blood in an isolated autologus kidney of the dog during hypoxic dilation. Large increases in renal vascular resistance were induced by the blood leaving the hypoxic heart. This change in resistance was blocked by theophylline and decreased 40 percent with adenosine deaminase. Adenosine, which causes vasodilation in the heart while inducing vasoconstriction in the kidney, was suggested to be involved in the myocardial hypoxic vasodilation. These data combined show that adenosine is released during coronary vasodilation induced by hypoxia in sufficient concentrations to be measured or exert a physiological effect. Therefore, if adenosine is involved in vasodilation of the forelimb it should be possible to measure it in the venous plasma. However, in the experiments reported

in this thesis, vasodilation occurred in the isolated forelimb but no changes in the concentrations of adenosine in the venous effluent were detected. This suggests that if adenosine in the venous plasma is a good index of adenosine release, then we can conclude that adenosine is not involved in the vasodilation induced by hypoxia or hypercapnia in the forelimb of the dog.

The information available implicating prostacyclin in vasodilation in vivo is much more limited than that for adenosine. There are, however, many facts known about prostacyclin and how it differs in its vasodilator effects from prostaglandin E_2. Until 1976 (32) prostacyclin was unknown and all hypotensive actions were attributed to prostaglandin E_2. Prostaglandin E_2 concentrations were determined with bioassay techniques that used rat stomach strips and chick rectum to measure contractile responsiveness. Yet, after the discovery of prostacyclin, it was noted that effects resulting from prostaglandin E_2, prostacyclin and its stable metabolite, 6-keto-prostaglandin F_1 alpha (6-keto-PGF_1 alpha), could not be separated using these bioassays because all three arachidonate products had similar contractile effects on the vascular strips used (1). Thus, effects caused by release of prostacyclin from the vascular smooth muscle cells had been erroneously defined as effects due to prostaglandin E_2. Because of this, new bioassay techniques were developed in 1977, to differentiate between the vasodilator prostaglandins. By using bovine coronary artery, rabbit transverse stomach strip, rabbit celiac artery and rat stomach strip it was possible to differentiate among prostaglandin E_2, prostacyclin, and 6-keto-PGF_1 alpha.

Prostacyclin effects were also investigated in animal preparations.

Prostacyclin infused into the coronary circulation of rabbit hearts produced vasodilation, whereas, prostaglandin E_2 had no effect (1). Exogenous arachidonic acid, PGH_2, and prostacyclin relaxed coronary artery strips (19). The relaxation induced by arachidonic acid and PGH_2 could be inhibited by 15-hydroperoxy arachidonic acid, which inhibits prostacyclin synthetase. Also, aortic wall homogenates converted only one to two percent of ^{14}C-arachidonate to prostaglandin E_2, but lamb ductus arteriosus and fetal calf arteries produced 6-keto-PGF_1 alpha as the major product (1). Schror et. al. (57) perfused isolated guinea pig hearts with arachidonic acid and noted coronary vasodilation. This same response was seen when prostacyclin was added to the perfusate. Further, chromatographic studies showed 6-keto-PGF_1 alpha present in the effluent and coronary vasodilation was blocked by 15-hydroperoxy arachidonic acid. These findings lead the investigators to conclude that prostacyclin was the major product formed from arachidonic acid and produced coronary vasodilation in the guinea pig heart. In vivo, as well as, in vitro data have led investigators to believe that local metabolism of arachidonic acid by the vascular smooth muscle produces prostacyclin and not prostaglandin E_2.

In our preparation, infusion of successively increasing concentrations of prostacyclin produced successively decreasing perfusion pressures and resistances in the forelimb (Figures 6 and 7), the concentrations of prostacyclin infused were all vasodilator. In addition, the concentrations of prostacyclin infused at the highest rate (494ng/min to achieve a total venous plasma concentrations of 8.8ng/ml) produced a resistance change similar to that seen with induction of severe hypoxia (1.04 to 0.66mmHg/ml/min vs. 1.05 to 0.72mmHg/ml/min,

respectively). Therefore, if prostacyclin was involved in the vasodilator response associated with hypoxia or hypercapnia, an increase should occur.

The study performed by Kienitz et. al. (37) was very similar to our study except that they did not measure adenosine or any prostaglandins. The preparation involved an isolated, innervated canine forelimb and local severe hypoxia and hypercapnia were induced in this system. The investigators noted a decreased perfusion pressure with hypoxia or hypercapnia. However, when indomethacin was infused into the forelimb, hypoxia and hypercapnia still produced vasodilation. The authors concluded that prostaglandins did not appear to play a role in the local vasodilator mechanism(s) associated with hypoxia and hypercapnia.

In conclusion, it appears from our data and the data of others that neither prostacyclin nor adenosine is involved in the local hypoxic and hypercapnic vasodilation in the isolated forelimb. It seems highly probable in view of the mounting data against adenosine and prostacyclin that the direct effect of oxygen and carbon dioxide (or pH) upon the vasculature induces dilation. Hypoxic vasodilation is thought to occur through the depression of the cytochrome a_3 oxidative phosphorylation chain, however, a mechanism for hypercapnic vasodilation has not been postulated. More evidence is needed to elucidate mechanisms through which hypoxia and hypercapnia induce vasodilation.

V. SUMMARY AND CONCLUSIONS

(1) Induction of local hypoxia and hypercapnia in the isolated forelimb decreases perfusion pressure and resistance. Severe hypoxia causes the largest alterations.

(2) Redistribution of blood flow does not occur between the brachial and cephalic veins during severe hypoxia.

(3) Venous plasma concentrations of adenosine do not change during local induction of hypoxia or hypercapnia.

(4) Venous plasma concentrations of prostacyclin do not change during local induction of hypoxia or hypercapnia.

(5) Prostacyclin infused into the arterial circulation of the forelimb can be recovered in the venous circulation.

(6) Successively increasing concentrations of prostacyclin decrease local perfusion pressure and resistance and also cause systemic hypotension. Prostacyclin can produce vasodilation in the isolated forelimb, but it is apparently not the mechanism responsible for vasodilation during hypoxia and hypercapnia.

(7) Therefore, because there is little evidence involving potassium, hyperosmolarity, adenosine or prostaglandins, we conclude that the direct effect of oxygen or the direct effect of carbon dioxide (or pH) is apparently responsible for the local hypoxic or hypercapnic vasodilation, respectively, in the forelimb of the dog.

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APPENDIX

Prostacyclin Radioimmunoassay (RIA)

Plasma prostacyclin concentrations were determined by measuring the stable breakdown product, 6-keto-prostaglandin F_1 alpha (6-keto-PGF_1 alpha). Prostacyclin converts to 6-keto-PGF_1 alpha nonenzymatically within approximately 2.5 minutes within the sample tube (1). Disposable polypropylene tubes (12mm x 75mm) were used for the RIA. Phosphate buffered saline (0.1M phosphate buffered, 0.154M saline with 0.1 percent gelatin), PBSG, was used as dilutant for the assay reagents (Table 1). It was made fresh before each assay; sodium azide (0.01 percent) was added as preservative. Plasma samples (100ul) or varying amounts of pure 6-keto-PGF_1 alpha standard (The Upjohn Co., Kalamazoo, MI; 4.88 pg - 2.5ng/100ul buffer) were placed in each tube. Standards were made fresh every three months. Antibody (Seragen, Boston, MA) specific for 6-keto-PGF_1 alpha, Table 2, was diluted with buffer to a titer of 1:12,000. Radioactive 6-keto-PGF_1 alpha (New England Nuclear, Boston, MA, NET-615 keto-prostaglandin-F_1 alpha, 6-[5, 8, 9, 11, 12, 14, 15-$^3H(N)$], 120.0 Ci/mmol) was diluted to yield a concentration of approximately 10,000dpm/100ul. The original 0.25ml of acetonitrile: water (9:1) containing 0.025 mCi of radioactive 6-keto-PGF_1 alpha obtained from New England Nuclear was diluted with an additional 1.0ml of acetonitrile:water (9:1) to decrease evaporation and adsorption of the radioactive hormone on the New England Nuclear vial. At the time of assay, 80ul was taken and diluted to 22ml with PBSG to yield a

Table 1

0.1M Phosphate Buffer

2.76 grams $NaH_2PO_4 \cdot H_2O$

21.44 grams $Na_2HPO_4 \cdot 7H_2O$

(11.35 g anhydrous)

9.0 grams NaCl

0.1 grams Na azide

1.0 grams gelatin

1000.0 ml distilled water

Charcoal Solution

2.5 grams Norit A charcoal

(Fisher Scientific Co., Fair Lawn, NJ)

0.25 grams Dextran T_{70}

(Pharmacia Fine Chemicals, Uppsala, Sweden)

500.0 ml of 0.1M PBSG

Table 2 6-keto-PGF_1 alpha

Antibody Cross Reactivity

6-keto PGF_1 alpha	(100.0%)
PGF_2 alpha	9.5%
PGE_2	3.0%
PGA_2	< 0.2%
PGD_2	< 0.1%
Thromboxane B_2	< 0.1%

concentration of approximately 10,000dpm/100ul. Antibody (100ul) and the radioactive hormone (100ul) were added to each assay tube to yield an incubation volume of 300ul. Additional tubes were prepared containing only 200ul of buffer and 100ul of the radioactive hormone to assess non-specific binding (NSB) in the assay and total amount of radioactivity (TCT) added to each tube (Table 3). All tubes were vortexed and incubated at room temperature (25°C) for one hour, followed by incubation at 4°C for 18-24 hours.

After incubation, a chilled 0.5% charcoal:0.05% dextran suspension (Table 1) was added to all tubes except the total count tubes. Charcoal was used to separate the radioactive hormone that was not bound to antibody from that bound to antibody. The charcoal was kept on ice and mixed continuously. A rack with 12 tubes was placed in an ice bath (2-4°C) and one ml of the charcoal:dextran suspension was added to each tube within 1.5 minutes. At the end of 12 minutes, the tubes were centrifuged at 3000rpm (2000xg, Beckman Model J-6B, Palo Alto, CA) for 12 minutes. One ml of the supernatant was pipetted into a vial containing 15ml of scintillation fluid. This procedure was repeated until all tubes were processed. One ml of PBSG was added to the total count tubes so that quenching would be equivalent to standard and sample tubes. All vials were then counted in a Tri-Carb 300C liquid scintillation counter (Packard, Downers Grove, IL).

Standards were plotted as a percentage of the zero dose tubes (B_o). Percentage binding of each sample was then used to obtain the sample concentration from the graph of the standard curve (Figure 1). Most samples were between 40-60 percent binding (B/B_o).

Table 3

Tube*	Buffer (ul)	Tracer (ul)	Antibody (ul)	Receive Charcoal on second day of assay
Total count tube (TCT)	200	100	---	No
Zero dose tube (B_o)	100	100	100	Yes
Non-specific binding (NSB)	200	100	---	Yes
6-keto-standards (100ul)		100	100	Yes
Samples (100ul)		100	100	Yes

*Tubes run in triplicate

Figure 1

Representative binding of 6-keto-prostaglandin F_1 alpha standards. Plotted are the percentage of total binding for each of the ten 6-keto-prostaglandin F_1 alpha standards, mean $\pm$ SD. Also graphed are the serial plasma dilutions: P_{100}=100ul, P_{50}=50ul, and P_{25}=25ul.

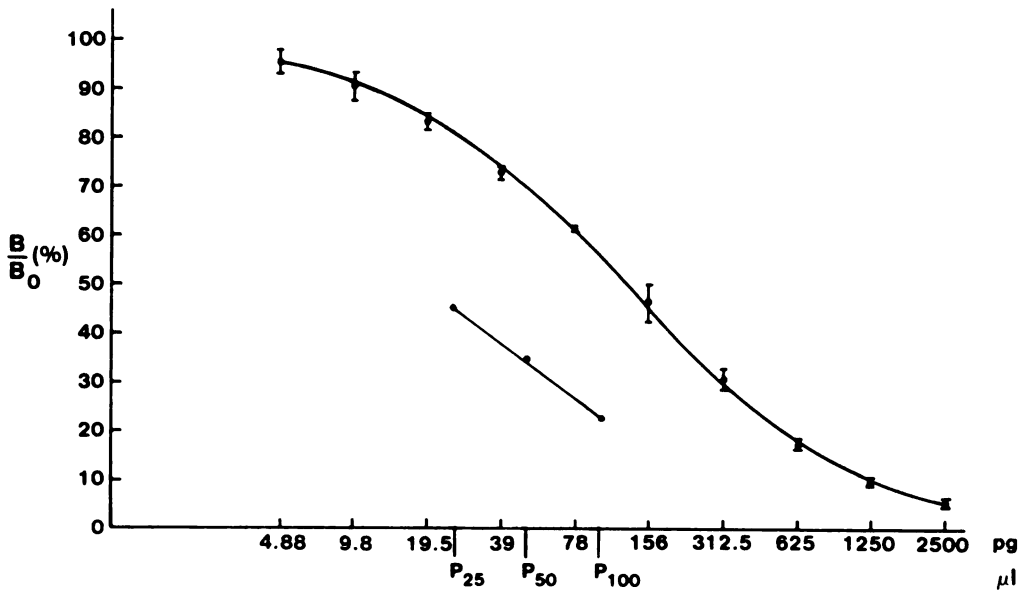
100
90
80
70
60
50
40
30
20
10
0
B/B0 (%)
4.88
9.8
19.5
39
78
156
312.5
625
1250
2500
pg
P25
P50
P100
µl

Figure 1

Reliability of the Assay

Specificity: The antibody used in this assay is highly specific for 6-keto-PGF_1 alpha (Table 2). Therefore, sample extraction was not necessary and plasma was assayed directly.

Test of parallelism: Three serially diluted dog plasma samples were processed in each assay. They were plotted along with each standard curve and were visually parallel to each standard curve (Figure 1).

Sensitivity: The least detectable concentration (LDC, the concentration two standard deviations away from the zero dose level) was 20 ± 14.9pg (mean $\pm$ SD, n=8).

Precision of the assay: Within- and between- assay variations were calculated according to the methods of Rodbard (ref. 48, p. 224-230) for each of the three serial dog plasma dilutions (Table 4). Within- assay variation ranged from 7.5% to 10.2% and between- assay variation ranged from 11.9% to 13.0%.

Accuracy: To determine the accuracy of the assay, plasma was stripped of endogenous prostaglandins by adding a charcoal suspension (2.5g charcoal and 0.25g dextran in 50ml PBSG) to 70ml of dog plasma. The solution was mixed and centrifuged at 2800rpm (1360xg,IEC Clinical Centrifuge, Needham Hts., MA) at $4^{o}C$ for five minutes. Varying concentrations of 6-keto-PGF_1 alpha (0.03, 0.1, and 0.4ng, Figure 2) were added to the charcoal stripped plasma (CSP). Unspiked charcoal stripped plasma was also run in each assay and was equivalent to a zero dose response (mean $\pm$ SEM=107.4 $\pm$ 1.8%, n=7). Recovery values for the charcoal stripped plasma were as follows: CSP + 0.4ng, 83.75%; CSP +

Table 4 Precision of the 6-keto-PGF_{1} alpha Radioimmunoassay

Volume (ul) of plasma standard assayed	Mean (pg)	df	Within-assay SD	Within-assay variation = CV	Between-assay SD	Between-assay variation = CV
25	139	8	14	10.2%	18	13.0%
50	230	8	22	9.7%	28	12.4%
100	433	8	33	7.5%	52	11.9%

Figure 2

Test of accuracy of 6-keto-prostaglandin F_1 alpha assay. Known amounts of 6-keto-prostaglandin F_1 alpha added to phosphate buffer are plotted against amount recovered from the assay, mean $\pm$ SD.

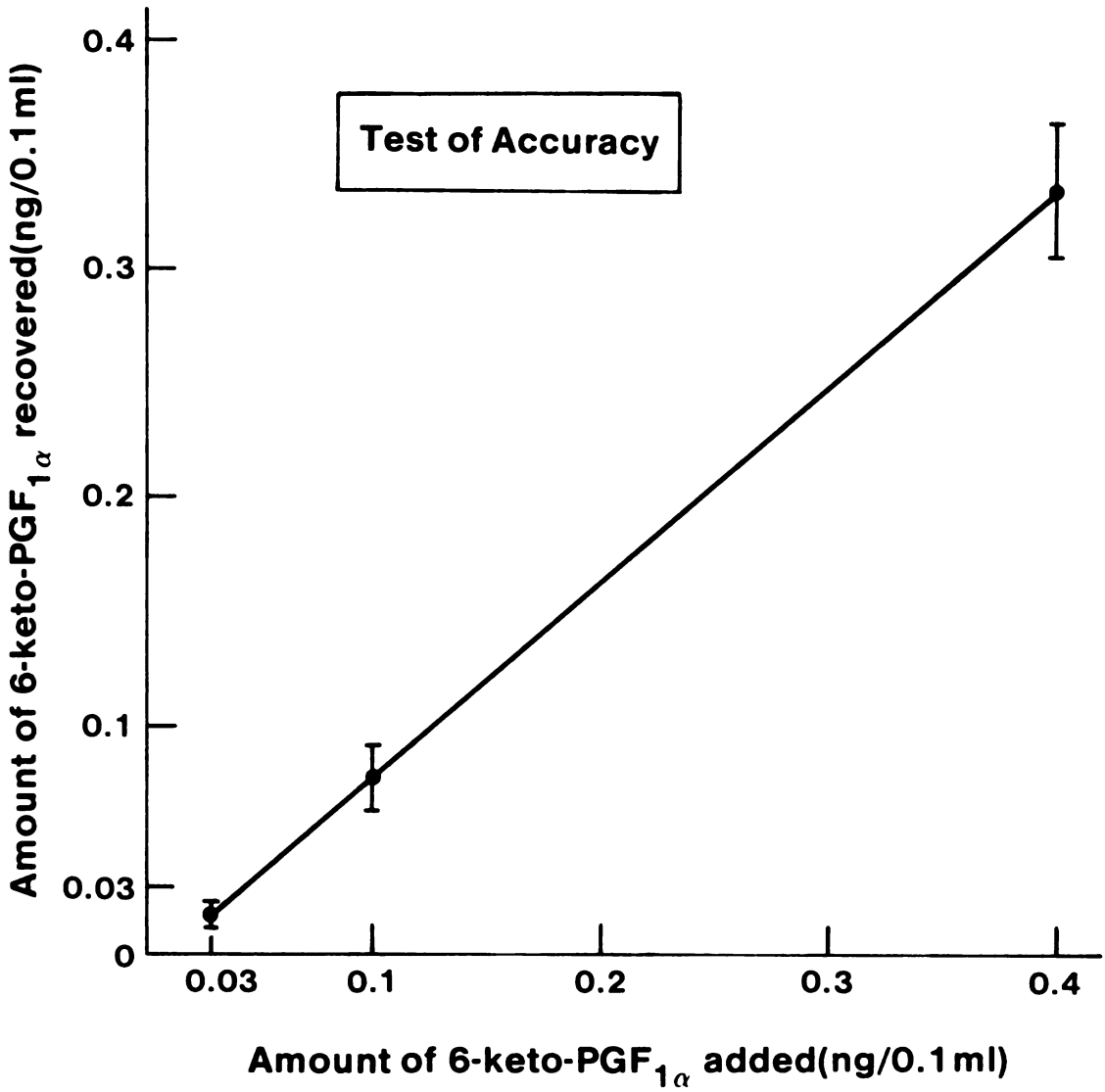
Test of Accuracy
Amount of 6-keto-PGF$_{1\alpha}$ recovered(ng/0.1 ml)
0.4
0.3
0.2
0.1
0.03
0
0.03
0.1
0.2
0.3
0.4
Amount of 6-keto-PGF$_{1\alpha}$ added(ng/0.1 ml)

Figure 2

0.1ng, 76.0%; CSP + 0.03ng, 61.33%. Most sample values were between 40-60 percent binding, which gives approximately 80 percent recovery.