Sheryl L. Gravelle-Camelo THE EFFECTS OF HYPO AND HYPERCAPNIA ON THROMBOXANE AND PROSTACYCLIN LEVELS IN PIGLETS (Under the direction of Gerhard W. Kalmus, Ph.D.) Department of Biology, June 1987.

The purpose of this study was to investigate the effects of hypo and hypercapnic induced changes in cerebral bloodflow on the levels of thromboxane B_2 and prostacyclin in piglets. Cerebral circulation was measured with a bloodflow probe while the animal was exposed to hypo and hypercapnic conditions. Blood samples were withdrawn from the saggital sinus of the piglet, and assayed for thromboxane B_2 and prostacyclin by radioimmunoassay.

Results demonstrated that CO_2 does affect cerebral bloodflow in the piglet. Hypocapnia results in a decrease in cerebral bloodflow, while hypercapnia causes an increase. It was also demonstrated that plasma prostacyclin levels increase under hypercapnic conditions.

These results, while not conclusive, are comparable to studies indicating that prostaglandins affect cerebral bloodflow in neonates to a much greater extent than in adults.

The more complete understanding of cerebral bloodflow in infants may be important in developing a treatment to prevent intraventricular hemorrhage, which results in fatality or neurological impairments in preterm or low birth weight infants.

THE EFFECT OF HYPO AND HYPERCAPNIA ON THROMBOXANE AND PROSTACYCLIN LEVELS IN PIGLETS

A Thesis

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by
Sheryl L. Gravelle-Camelo
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THE EFFECT OF HYPO AND HYPERCAPNIA
ON THROMBOXANE AND PROSTACYCLIN LEVELS IN PIGLETS

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LIST OF ABBREVIATIONS

ABG	Arterial Blood Gas
CBF	Cerebral Blood Flow
CPM	Counts Per Minute
IVH	Intraventricular Hemorrhage
$paCO_2$	Partial Arterial Pressure CO_2
paO_2	Partial Arterial Pressure O_2
PBS	Phosphate Buffer Solution
PG	Prostaglandin
RIA	Radioimmunoassay
RPM	Revolutions Per Minute
TXB_2	Thromboxane B_2

INTRODUCTION

It has been extensively documented that hypercapnic conditions result in increased cerebral blood flow in both infants and adults of many species. Similarly, a decrease in inspired CO_2 levels, such as may develop from hyperventilation, is followed by a decrease in cerebral blood flow. The effects of these conditions on the cerebral vasculature has been extensively studied; what is still unclear is the chemical mediator that links the stimulus to the response.

Prostaglandins are a group of biologically active lipids synthesized from archadonic acid in tissues and blood vessels throughout the body. One of the many physiologically important characteristics of some of the prostaglandins is their vasoactivity. Prostacyclin is a strong hypotensive agent and a dialtor of all vascular tissues. Thromboxane is equally strong as a vasoconstrictor and platelet aggregator.

The role of prostaglandins in cerebral vessel reactivity has not been clearly defined, although it has been the subject of considerable research. Several studies have suggested that prostacyclin synthesis may be the mechanism for vasodilation during hypercapnia. Results of these studies have produced conflicting results, however, and no comprehensive conclusions concerning prostacyclin and hypercapnia have been developed.

This study was developed to further investigate the role of prostacyclin and thromboxane in cerebral blood flow changes that are initiated by altered CO_2 levels.

REVIEW OF LITERATURE

Prostaglandins - History and Nomenclature

The study of prostaglandins was initiated in the early 1930's when scientists working independantly in the U.S. and Europe discovered that lipid soluable fractions of human semen caused contraction of uterine muscle tissue. The Swedish scientist Von Euler named the compounds "Prostaglandins" because he believed the major source of the substances was the prostate gland. The name, although a misnomer, is still used to describe the compounds.

After the inital discoveries, there was little progress made in the studies of prostaglandins until the 1960's. In 1962, Bergstrom and Samuelsson characterized the basic structure of the prostaglandins using mass spectrometry and gas chromatography. Further work in the area was conducted by Vane who isolated thromboxane A_2 and prostacyclin; he also discovered that prostaglandin synthesis is inhibited by aspirin (Vane, 1976). For their advances in this area, Vane, Bergstrom, and Samuelsson were awarded the Nobel prize in 1982 (Nies, 1986). Biochemical studies have elucidated the structures of families of prostaglandins, as well as the structures of related compounds including thromboxanes and leukotrienes.

Prostaglandins, thromboxanes, and their analogues exhibit a wide range of biological activities. Some of the effects of these compounds include: the stimulation or inhibition of C'AMP in certain tissues, constriction or dilation of certain blood vessels, kidney function, gastric secretion, platelet aggregation, bronchial dilation, and

maintenance of pregnancy and parturition.

The prostaglandins have been divided into groups or families on the basis of their chemical activities. The first two groups to be isolated were designated as prostaglandins E and F (PGE and PGF). Prostaglandin E preferentially partioned into ether and PGF partioned into the phosphate (Swedish - fosfat) buffer. Further treatment of PGE with acid resulted in PGA; while treatment with base gave rise to PGB. Other groups of prostaglandins that were isolated later were also given letter names to fill out the alphabetical sequence. There have been nine such groups of prostaglandins (A -I) isolated. It has since been discovered that PGG and PGH are not separately existing families but are short lived intermediates in the formation of other prostaglandins (Roberts and Newton, 1982). Each family of prostaglandins can be divided into three series designated by a numeral 1 - 3. These numerals indicate the number of double bonds present in the side chains.

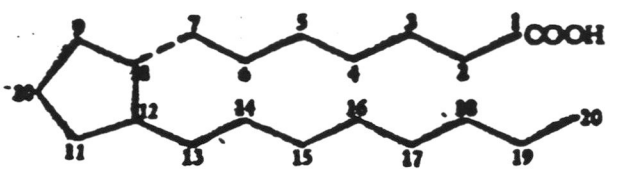
All of the nine prostaglandin familes have the same 20 carbon molecular skeleton - Prostanoic Acid (Figure A). The groups of prostaglandins differ in the substitutions occurring at carbons nine and eleven within the ring structure of prostanoic acid; or with the locations of a double bond within the skeleton (Figure A). All of the prostaglandins have a double bond between carbons 13 and 14, and a hydroxyl group at carbon 15, which seem to be essential for biological activity. One of the prostaglandins of the I group, PGI_2 also knownas prostacyclin; has an oxygen bridge formed between carbons 6 and 9. The thromboxanes, which are closely related to the prostaglandins, contain

an oxycyclohexane ring instead of the cyclopentane ring. In spite of these differences, prostacyclin is still considered to be a "true" prostaglandin; and many scientists also include the thromboxanes within the classification of "true" prostaglandins.

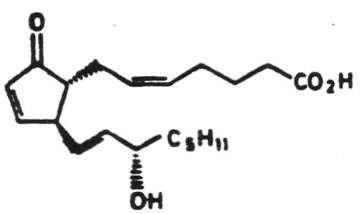
FIGURE A.

Prostaglandin Structure and Nomenclature

PROSTANOIC ACID



PGA



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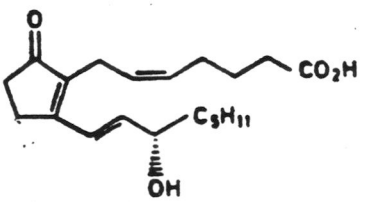

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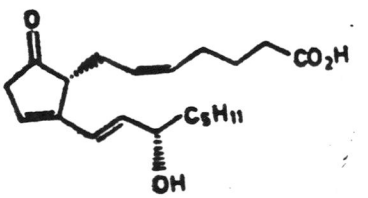
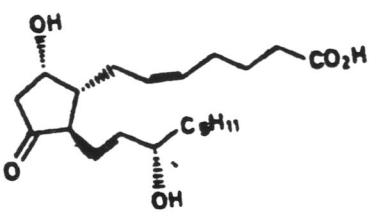
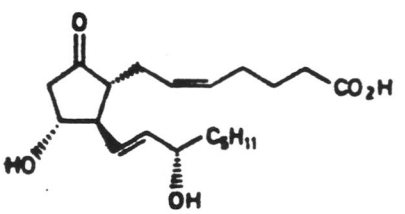


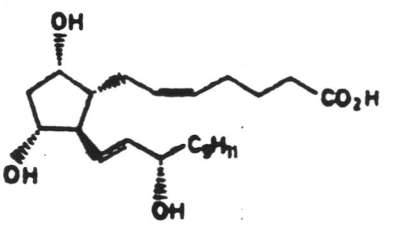
PGD



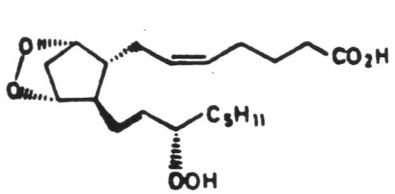
PGE



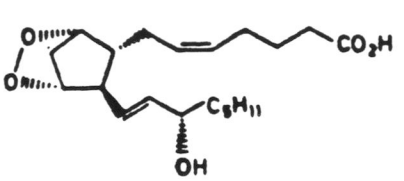
PGF



PGG



PGH



PGI

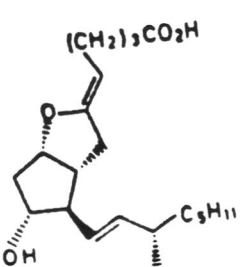
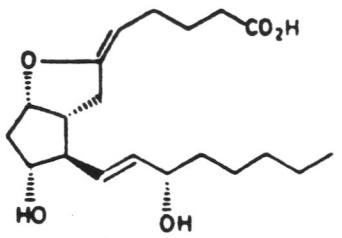
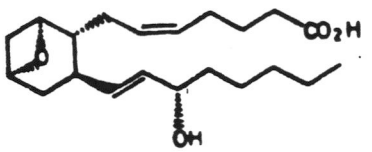


FIGURE A. Continued

PGI_2 - Prostacyclin



Thromboxane



Prostaglandin Synthesis and Metabolism

The synthesis of prostaglandins occurs in almost all tissues, at the level of the cell membrane. The major substrate for prostaglandins, thromboxanes, and leukotrienes is a 20 carbon unsaturated fatty acid, arachadonic acid. Arachadonic acid is esterified in the phospholipid cell membrane, and the rate limiting step in synthesis is the cleaving of the acid from the cell membrane by a phospholipase enzyme. In some tissues, corticosteroids inhibit prostaglandin synthesis by preventing the activation of the phospholipase enzyme, so that no arachadonic acid is freed from the cell membrane.

In the free state, arachadonic acid activates a cyclooxygenase enzyme system. The molecule of arachadonic acid interacts with two molecules of oyxgen to form the endoperoxide intermediate, PGG_2. The cyclooxygenase system is inhibited by aspirin and other nonsteroidal anti-inflammatory drugs. If synthesis is not inhibited at this point, peroxidase activity results in the formation of a second endoperoxide, PGH_2. These two compounds are the precursors of all prostaglandins and thromboxanes and a variety of enzyme systems are responsible for the conversions.

Similar to the biosynthesis of prostaglandins, metabolism and degradation occur rapidly and through various pathways in the tissues. The enzymatic system that is primarily responsible for the degradation of the prostaglandins is most active in the kidneys and the lungs. The oxidation of the C-15 hydroxyl group to a ketone and the reduction of the 13-14 double bond result in the formation of 13,14,dihydro,15 keto-

prostaglandin; which is biologically inactive. Prostacyclin and thromboxane are not enzymatically inactivated, but instead undergo hydrolysis to produce more stable metabolites. Hydrolysis of prostacyclin produces 6-keto PGF_1 alpha and hydrolysis of thromboxane to thromboxane B_2 (TXB_2). Figure B illustrates some of the major steps in the synthesis and metabolism of prostaglandins.

Prostaglandins and the Brain

Prostaglandin F_2 was first identified in ox brain by Samuelsson (1964) and since that time a variety of prostaglandins have been isolated from the brains, cerebral vasculature, and cerbrospinal fluid of a number of species.

Wolfe and coworkers conducted an early study to ascertain the capacity of brain tissue slices and homogenates for synthesis of prostaglandins. They studied rat and cat brains, using a gas chromatographic - mass spectrometric method to analyze the tissues. The predominant prostaglandins isolated were PGE_2 and PGF_2 alpha. The rate of formation of the prostaglandins was inhanced by the addition of norepinenephrine, dopamine, and apomorphine (Wolfe, et al., 1976).

Abdel-Halim studied rat, mice, and rabbit brain tissue; and homogenates of rat cerebral blood vessels to determine the specific prostaglandins formed in each. He found that prostaglandins 6-keto PGF_1 alpha, PGF_2 alpha, PGD_2 and PGE_2, were formed by all of the tissues studied but that the concentrations of the compounds varied in each case. An interesting discovery was the marked differences occuring in the concentrations of prostaglandins present in rat brain tissue and homogenate of cerebral vessels. In the brain, the

predominant prostaglandin was found to be PGD_2, followed by PGF_2 alpha and PGE_2. The amount of 6-keto PGF_1 alpha detected was much lower than any of the other prostaglandins. In rat cerebral blood vessels, however, the profile was completely different. In the vessels, 6-keto PGF_1 alpha was the major prostaglandin produced, in amounts nearly tripling the production of PGF_2 alpha, which was the only other compound dectected. No PGE_2 or PGD_2 were dectected in the cerebral vasculature homgenate (Abdel - Halim et al., 1980).

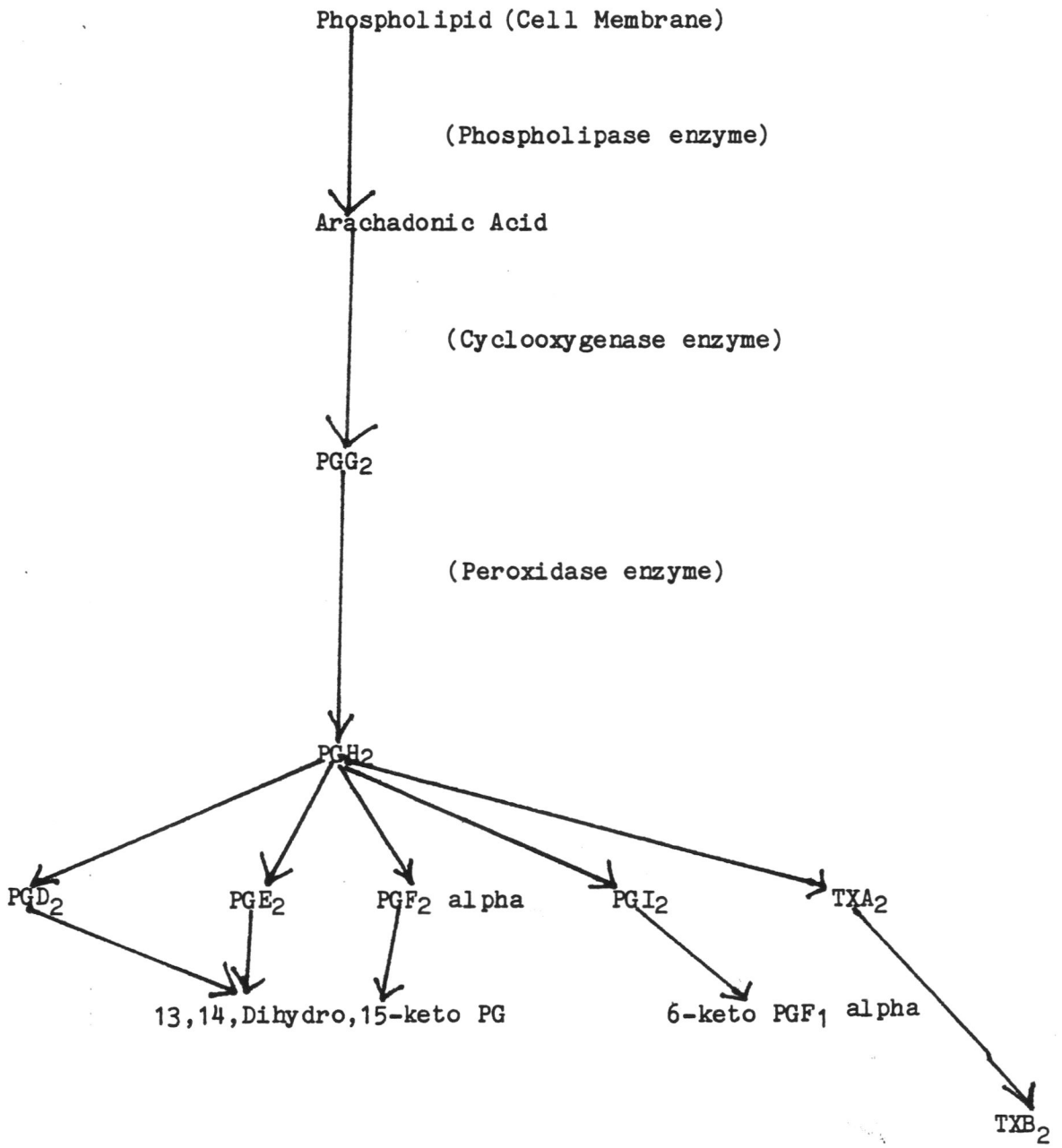
Further studies on bovine cerebral vasculature showed evidence of production of prostaglandins PGE_2, PGF_2 alpha, PGD_2 and 6-keto PGF_1 alpha, as well as, TXB_2. Approximately equal amounts of PGE_2, PGF_2 alpha and 6-keto PGF_1 alpha were produced, while the amount of PGD_2 appeared to be about two thirds that of the others. Thromboxane B_2 was in the lowest concentration, and the possibility that it had been produced by platelets and not the cerebral vessels was considered (Hagen et al., 1979).

These studies indicate that both brain tissue and cerebral vessels possess the necessary enzymes to synthesize prostaglandins from endogenous arachadonic acid. The predominant type of prostaglandin formed, however, appears to vary not only in the type of organism studied, but also in the type of tissues used for the study.

FIGURE B.

Synthesis and Metabolism of Prostaglandins

The major steps in the biosynthesis of prostaglandins and thromboxanes. The more stable and less biologically active metabolites are also illustrated.



The Actions of Prostaglandins on Cerebral Vasculature

Once the prescence of prostaglandins in the brain and cerebral vasculature had been verified and the ability of these tissues to synthesize the compounds had been demonstrated, researchers began to speculate on the physiological implications that these compounds may have on these tissues. Many of the studies undertaken focused on the role of prostaglandins in pathological processes such as cerebral ischemia. Cerebral ischemia is believed to contribute to post ischemic hypoperfusion, brain edema, and irreversible neuronal damage such as occurs in stroke (Chen et al., 1986). Another area of investigation is the possible role of prostaglandins in regulating cerebral blood flow during conditions of altered CO_2 and O_2 levels.

Spagnuolo et al. (1979) measured the levels of vasoconstricting arachadonic acid metabolites, namely thromboxane B_2 and PGF_2 alpha during periods of cerebral ischemia induced by occlusion of the common carotid arteries in gerbils; and of the same metabolites following decapitation. Tissue levels of the compounds were determined with radioimmunoassays (RIA). They discovered that there was no rise in levels of thromboxane B_2 and PGF_2 alpha during cerebral ischemia, but after decapitation the levels were substantially increased. Thus, they concluded that the different metabolite concentrations were due to central nervous system stimulation which occurs with decapitation, but not with carotid artery occlusion. They further theorized that reperfusion, following cerebral ischemia, may be necessary for increased synthesis of thromboxanes and prostaglandins.

Gaudet and Levine (1979; 1980) studied the effects of total cerebral ischemia followed by reperfusion on the production of PGF_2 alpha, PGE_2, TXB_2, PGD_2, and 6-keto PGF_1 alpha in gerbils. During the period of cerebral ischemia, metabolite concentrations as measured by RIA were unchanged from pre-ischemia controls. Upon reperfusion, however, levels increased dramatically. The predominate metabolites detected were PGD_2, PE_2, and PGF_2 alpha. Levels of all metabolites were highest during the initial period of reperfusion, and decreased after two hours of reperfusion to approach control levels. Gaudet and Levine speculated that the reperfusion induced rise in prostaglandins and thromboxanes is due to the re-introduction of oxygen into a system in which there is an exsisting high level of the precursor arachadonic acid for these metabolites. Pretreatment of the animals with cyclooxygenase inhibitors such as aspirin, indomethacin, flufenamic acid or piroxicam greatly reduced or eliminated the post-ischemic accumulation of the metabolites studied. The animals pretreated with inhibitors also recovered quicker with fewer ill effects from the ischemic episode.

The effects of incomplete cerebral ischemia were also studied by Gaudet and Levine (1980) and by Shohami et al. (1982). Gaudet and Levine measured levels of PGD_2, PGE_2, and 6-keto PGF_1 alpha during periods of incomplete cerebral ischemia ranging from 15 minutes to 6 hours, without allowing for reperfusion. In contrast to complete ischemia, the researchers found that the levels of the prostaglandins showed marked increases during the periods of incomplete cerebral ischemia. Gaudet and Levine hypothesized that this increase was due to

the fact that the ischemia was incomplete. During incomplete ischemia the residual blood flow provides enough oxygen for the cyclooxygenation of arachadonic acid, resulting in the synthesis of prostaglandins. In their study, the pretreatment of animals with indomethacin had a similar inhibitory effect on the levels of prostaglandin synthesis.

Shohami, et al. (1982) also studied incomplete cerebral ischemia; but allowed for periods of reperfusion before using RIAs to measure the amounts of PGE_2, 6-keto PGF_1 alpha and TXB_2 synthesized. The levels of PGE_2 and 6-keto PGF_1 alpha increased in a pattern similar to that noted by Gaudet and Levine (1980). The levels of TXB_2, however, increased steadily to a level that was five times greater than the control animals. The researchers speculated that such an increase in the amount of TXB_2 could be creating locally diminished blood flow that was commonly observed during periods of reperfusion.

Prostaglandins and Cerebral Bloodflow Regulation

One of the major mechanisms of regulating cerebral blood flow (CBF) is alteration of CO_2 levels. Although this particular mechanism has been recognized for many years, a detailed knowledge of the chemical mediators that link the altered CO_2 level to the changes in CBF is lacking. A considerable number of studies have been undertaken to investigate the possible role of prostaglandins and thromboxanes in regulation of CBF.

In one of the earliest studies Pickard and MacKenzie (1973) administered the potent cyclooxygenase inhibitor indomethacin to anesthetized baboons. They found that not only was CBF markedly reduced during normocapnic conditions but the response of CBF to

hypercapnia was also reduced. Sakabe and Siesjo (1979) used rats to produce similar results noting that indomethacin did not affect a change in blood flow during hypoxia but reduced hypercapnic induced blood flow changes by as much as 25%. Dahlgren et al. (1981) repeated the results of Sakabe and Siesjo in a follow up study involving greater numbers of animals and more extensive experimentation. Vlahov (1976) found that indomethacin significantly repressed CO_2 induced changes in cerebral blood flow in cats. He noted that the hypercapnic induced blood flow changes in drug treated animals were only 50% of that obtained in control animals.

Eriksson et al. (1983) monitored the effects of indomethacin as well as those of aspirin and naprosyn on CBF in healthy adult humans. They found that administration of indomethacin significantly lowered the increase in CBF that occurred when the volunteers inhaled CO_2. Aspirin and naprosyn, although inhibitors of prostaglandin synthesis, did not affect CBF in any manner.

These studies suggested that PGI_2, also known as prostacyclin, which is a potent vasodialator plays a role in hypercapnic induced CBF changes. Indomethacin inhibits the oxygenation of arachadonic acid which is an essential step in the synthesis of all prostaglandins. It was theorized that the effects of indomethacin on cerebral blood flow were due to the inhibition of prostacyclin synthesis.

Busija and Heistad (1983) conducted a study on the effects of indomethacin on the CBF in cats. They measured CBF with radioactive microspheres so that regional, as well as total CBF could be ascertained. In contrast to Vlahov's results, they concluded that

the inhibition of prostaglandin synthesis by indomethacin has no significant effects on CBF under both normal and hypercapnic conditions. Busija and Heistad suggested that the variation in results could have been due to the fact that Vlahov measured CBF one minute after hypercapnia, which did not allow for a steady state response. These results were similar to an earlier study conducted by Wei *et al.* (1980). Busija (1983) also conducted a study using concious rabbits. Busija's conclusions were similar to those reached in his previous study on cats. He theorized that indomethacin, at doses significant to inhibit prostaglandin synthesis, does not affect CBF.

Jackson *et al.* (1983) used dogs as subjects for a study in which 6-keto PGF_1 alpha production was measured during normal and hypercapnic conditions, and in the same conditions after administration of indomethacin. They found that while indomethacin decreased the amount of 6-keto PGF_1 alpha produced, CBF changes induced by hypercapnia were not altered. The researchers also noted that hypercapnia, while increasing CBF, did not appear to affect the amount of 6-keto PGF_1 alpha produced.

Uyama *et al.* (1983) also measured the production of 6-keto PGF_1 alpha during normal and hypercapnic conditions, but used human volunteers as subjects. None of the volunteers injested any drugs known to interfere with prostaglandin synthesis for two weeks prior to the study. While an increase in cerebral blood flow was noted with mild (CO_2= 44mmHg) hypercapnia, there was no corresponding increase in prostaglandin synthesis.

Based on the conflicting results of these studies, no single theory

could be proposed. Possible suggestions, however, ranged from the amount of time after hypercapnia that CBF was measured (Busija and Heistad 1983) to differences in the administration of indomethacin (Busija 1983). Other researchers felt that the role of prostaglandins in CBF may vary with the species studied (Jackson et al., 1983). No clear conclusions on the role of prostaglandins could be determined.

Recent studies by Stuart et al. (1984), Leffler and Busija (1985), and Leffler et al. (1985) have conjectured that the role of prostaglandins in regulation of CBF may be more critical in the neonate than in the adult of the species. Stuart et al. (1984) exposed umbilical arteries dissected from the umbilical cords of normal full term vaginal deliveries to varying O_2 condtions, while measuring the prostacyclin production of the arterial segments. The study demonstrated a 45% increase in prostacyclin production when the segments were subjected to hypoxic conditions. A similar amount of decrease in prostacyclin production, as measured by RIA, was noted when the arterial segments were exposed to hyperoxic conditions.

Leffler conducted studies using neonatal piglets to ascertain if the role of prostaglandins in CBF regulation was related to age and development (Leffler and Busija, 1985; Leffler et al., 1985). In a study using anesthetized piglets, Leffler and Busija noted a 3 - 5 fold increase in the amounts of 6-keto PGF_1 alpha, TXB_2, and PGE_2 in the subarachnoid cerebrospinal fluid during periods of hypercapnia. In a second study using unanesthetized animals, Leffler et al. found that treatment with indomethacin decreased the amount of 6-keto PGF_1 alpha

and TXB_2 in the plasma to an undetectable level. The drug treated animals also had lower CBF during normal conditions and no increase in CBF during periods of hypercapnia.

The results of these studies further compounded the debate on the role of prostaglandins in cerebral blood flow. No conclusions can be reached in the light of the many conflicting results.

Clinical Importance of Hypercapnia in Neonates

Changes in cerebral blood flow become particularly important when dealing with neonates, especially preterm or low birth weight infants. Disorders of cerebral circulation may be responsible for many of the effects of asphyxia or other respiratory diseases that produce permanent damage or fatality in infants (Cooke et al., 1979). In particular hypercapnic effects on CBF merit attention due to the fact that hypercapnia is often present for the first several hours after birth in normal deliveries and longer in infants with respiratory distress (Cooke et al., 1979). Hypercapnia may also result from seizures in the neonate, which often follow an earlier hypoxic or ischemic insult (Hill and Volpe, 1982).

Hypercapnia can be deleterious to infants by causing tissue acidosis, focal ischemia, and hemorrhages (Hill and Volpe, 1982). Intraventricular hemorrhage (IVH) and bleeding into the germinal matrix due to increased cerebral blood flow are the major causes of neonatal death and neurological disability in preterm and low birth weight infants (Lou et al., 1979; Ment et al., 1985).

Lehy et al. (1980) measured CBF in otherwise healthy preterm infants and found that CBF increased significantly with inhalation of

CO_2. The increase in CBF was twice that noted in adults exposed to the same amount of CO_2. This increased sensitivity to CO_2 in preterm infants could be an important factor in determining which infants would be at risk for intraventricular hemorrhage.

These results were also demonstrated in a 1984 study by Hansen et al. using newborn piglets. This study demonstrated that CBF increased significantly with exposure to CO_2 in the newborn animals. Hypocapnia, or exposure to low levels of CO_2, did not result in decreased CBF until very low levels (CO_2 less than 15mmHg) were reached. They felt that there may have been a gradual decline in brain blood flow throughout the hypocapnic testing, but that this decline was not statistically significant until the very lowest levels of hypocapnia had been reached.

Based on previous studies which indicated that prostaglandins may regulate hypercapnic induced changes in CBF Ment et al. (1985) administered indomethacin to low birth weight infants. In the study, the infants treated with the drug had significantly lower serum levels of TXB_2 and 6-keto PGF_1 alpha than did the control infants. Of the 24 control infants, 58% experienced intraventricular hemorrhages, while only 25% of the experimental group experienced them. They discussed the possibility that prostaglandins may in fact be an important mediator for hypercapnic changes in CBF. Also this mechanism may be present in the neonate, as indicated in earlier animal studies but not adults (Leffler and Busija, 1985; Leffler et al., 1985).

The Pig Model

Pigs have been used as models in biomedical research for approximately 25 years, and the incidence of researchers using pigs has been increasing rapidly. The pig is similar to the human in many aspects including renal, cardiovascular, and digestive morphology and physiology. In addition, the pig is an ideal animal model for immunlogical studies. The pig is also readily available at reasonable cost to many researchers (Pond and Houpt, 1978).

Pigs have been used as models for a wide variety of studies. They have served as excellent models in the study of atherosclerosis, cerbrovascular ateriosclerosis, and cardiac failure and infarcts (Liedbtke et al. 1975). Pigs have also been used in the studies of alcoholism, because they develop a preference for alcohol and will voluntarily consume it (Dexter et al., 1976). A third area where pigs have served as very useful models is in studies of obesity. The pig has a greater propensity for fattening than any other domestic animal and also can be genetically selected for fattening (Gurr et al., 1974).

Pigs are also an excellent choice for neonatal studies. The piglet and the human neonate have very similar digestive physiology and nutritional requirements. Piglets have been used to develop formulas for human infants and to study malnutrition (Book and Bustad, 1974). The maturation of the piglet brain is comparable to the human infant of 36 - 38 weeks gestation and for this reason is ideal to use when studying responsiveness to CO_2 (Hansen et al., 1984). The piglet is also apparently more sensitive to changes in CO_2 as reflected in blood flow changes than the adult pig, which is similar to the sensitivity

noted in the human neonate (Lehy et al., 1980).

Summary

From the conflicting results presented in the above mentioned studies, it should be evident that no definite conclusions concerning the role of prostaglandins in CBF have been reached. It seems that the extent of the prostaglandins as a mediator of CBF varies not only with the species but with the developmental state as well.

Hypercapnic induced changes in CBF are related to intraventricular hemorrhages in preterm and low birth weight infants. The outcome of this event may be permanent neurological damage or fatality. For these reasons, it is clear that a more complete understanding of the cerebral blood flow mechanism is needed.

The present study was undertaken to better understand the role of prostaglandins in the phenomenon of hypercapnic induced changes in CBF in neonatal piglets. By exposing the animals to altered CO_2 conditions and measuring the 6-keto PGF_1 alpha and TXB_2 produced, it is hoped that some further knowledge of the role of these metabolites in cerebral blood flow may be elucidated.

MATERIALS AND METHODS

Animals

The piglets used were farm bred domestic swine of both sexes. The weights ranged between 2.3 and 3.0 Kg, with a mean of 2.61 Kg. The age of the piglets was 5 - 8 days, mean age was 7 days. The animals were delivered the morning of the experiment from Gaskins Pig Farm, Black Jack, North Carolina.

Surgical Procedure

The piglets were anesthetized using Alpha Chloralose delivered IP (100 mg/Kg). A tracheotomy was performed and the piglet was placed on a Edco Scientific small animal ventilator at a rate of 30 cycles per minute, tidal volume 15 cc/Kg. A gas mixture of 60% nitrous oxide-40% O_2 (National Welders Supply Company) was used to maintain anesthesia throughout the surgical procedure. Tubocuarine (Lilly) at 0.1 cc/Kg was administered IM as needed for relaxation and Demerol (Winthrop) at 5 mg/Kg was used for analgesic purposes.

The saggital sinus of the skull was entered with PE 50 tubing (Fisher), to allow for blood sampling. The femoral artery was cannulated with a 5 French umbilical catheter (Argyle). The artery was used for blood pressure readings which were obtained with a Hewlitt - Packard arterial pressure monitor; and for obtaining blood samples to monitor the arterial blood gases (ABGs). The ABGs were analyzed using a Corning Blood Gas Analyzer. The femoral vein was also cannulated with a 5 French umbilical catheter and attached to an IV drip of $D_5 .2$ saline (Abbott) running at rate of 4cc/Kg/hr. The right external carotid

artery and the right occipital artery were isolated and occluded with anerysm clips. The right common carotid was also isolated and blood flow to the brain was monitored with the Square Wave Electro - Magnetic Flow Meter Probe (Carolina Medical Electronics). At the conclusion of the surgical procedure, the 60% nitrous oxide-40% O_2 was discontinued and the animal was placed on room air.

Experimental Procedure

After a ten minute stabilization period on room air a blood sample was obtained via the femoral artery to monitor the ABGs. The results of the blood gases established the phsiological baseline (limits paO_2 80 - 90 mmHg, $paCO_2$ 35 - 45mmHg, pH 7.35 - 7.45)for each individual animal. If the ABG was not within these limits, the respiratory rate was adjusted accordingly. A stabilization period was allowed, and a second ABG was obtained.

Once the baseline was established, a 2 - 3 cc sample of blood was withdrawn from the saggital sinus for RIA purposes. All samples for the RIA were drawn into cold syringes pretreated with heparin and indomethacin. These samples were immediately placed into a refrigerated centrifuge (Clay Adams) and spun to separate the formed elements from the plasma. The plasma was drawn off and stored at -4 ^{o} C until the time of the assay.

After the baseline ABG and saggital sinus blood samples were obtained, the ventilator rate was increased to induce hypocarbia. Two preset levels of hypocarbia were used: $paCO_2$ 25 - 35 mmHg and $paCO_2$ 15 - 25 mmHg. The animal was allowed to stabilize for ten minutes at the more rapid rate of ventilation, before ABGs were checked. If the ABGs

were within either of the hypocarbic ranges, a saggital sinus blood sample for RIA was also obtained. If the ABG was not within either of the desired ranges, the ventilator was adjusted accordingly and the animal was allowed to restabilize before further arterial blood gases were tested. The second level of hypocarbia was reached by further adjustment of the ventilator rate. The ABGs and the saggital sinus blood samples were obtained in the same manner as the earlier samples. After achieving the preset levels of hypocarbia, the ventilator rate was returned to the baseline level, and there was a ten minute stabilization period, to return the blood gases to a physiological range. A second base line ABG and saggital sinus sample were obtained before initiating the hypercarbic conditions. Levels of hypercarbia were preset at $paCO_2$ 55 - 70 mmHg and $paCO_2$ greater than 70 mmHg. Hypercapnia was achieved by removing the piglet from room air and introducing different gas mixtures through the ventilator. There were two mixtures of gases used: tank one contained 25% O_2 and 75% N_2, tank two contained 25% O_2, 15% CO_2, and 60% N_2 (National Welders Supply Company). By varing the gas mixture ratios, it was possible to achieve blood gases within each of the two levels of hypercarbia. The blood sampling procedures were the same as those in the earlier stages of the experiment. At the conclusion of the experiment, the piglet was euthanized with 1 cc of Beuthanasia - D (Schering Corporation) injected directly into the heart.

A complete experiment would result in a total of six ABG readings and six saggital sinus blood samples for RIA. The six samples would include: baseline, two hypocarbic readings, a second baseline and two hypercarbic readings. The complete set of samples were obtained for

five piglets. There were three additional animals that had sets of samples that were incomplete. These incomplete sets of samples were included with the others when analyzing the data.

RIA Method - Extraction

The technique for extraction and purification of the samples for assay was similar to that of Allen (1980). The sample size was 200 ul of serum per tube. The samples were treated with 2.0 cc of cold 100% ethanol and centrifuged at 2500 rpm for 15 minutes. The supernatant, which contains the lipid fraction, was drawn off and saved.

The samples were then dried down completely, using a nitrogen stream. Following dehydration, the samples were resuspended in 1 cc of phosphate buffer solution (PBS), and vortexed for 10 minutes. After vortexing, aproximently 1500 cpm (10 ul) of tridiated 6-keto PGF_1 alpha or TXB_2 was added to the tubes. This small amount of radioactivity was used to monitor recoveries after the extraction procedures. After a 30 minute incubation period at room temperature 2.0 cc of petroleum ether was added to extract all neutral lipids. The samples were centrifuged for 10 minutes at 2500 rpm and a super cooled methanol bath was used to extract the lower phase which contained the 6-keto PGF_1 alpha and TXB_2.

After the extraction process the samples were acidified. For the samples used in the assay for 6-keto PGF_1 alpha, 200 ul of 2 M citric acid was added to each tube. To this, 2 cc of ethyl acetate were added, the samples were vortexed for 10 minutes and centrifuged for 15 minutes at 2500 rpm. The supernatant was saved and the step involving the addition of the ethyl acetate, vortexing and centrifugation was repeated, and the two sets of supernatant were

combined. The process for the samples assayed for TXB_2 was slightly different. Instead of citric acid, 1 cc of acetate buffer was added to the samples; replacing the ethyl acetate was 1.5 cc of a 1:1 by volume solution of cyclohexane and ethyl acetate. The vortexing and centrifugation was unchanged. This step was also repeated and the two sets of supernatant combined. All of the samples were dried down using a nitrogen stream. The extracts were then resuspended in 300 ul of phosphate buffer solution (PBS). Of this 300 ul, 30 ul were used for recovery estimation.

RIA Method - Assay

Standard curves of both 6-keto PGF_1 alpha and TXB_2 ranged from 0 - 500 picograms (0, 5, 10, 25, 50, 100, 250, and 500 pg). Standards (UpJohn Company) were dissolved in 100% ethanol and stored at - 20 ° C. Antibody for the 6-keto PGF_1 alpha assay (Institute Pasteur, Paris) was used at a final dilution of 1:1000 while the antibody for TXB_2 (Institute Pasteur, Paris) was used at a final dilution of 1:5000. The assay procedure consisted of the addition of 100 ul of the appropriate antibody and 100 ul PBS to the samples and standard curves. The samples were briefly vortexed and incubated at room temperature for 30 minutes. Following the incubation period, 100 ul of tridiated 6-keto PGF_1 alpha or TXB_2 and an additional 100 ul of PBS was added to the samples. After vortexing for five minutes the samples and standard curves were incubated over night at 4 ° C.

Separation of the antibody bound and free radioactivity was accomplished by the addition of 0.25 cc cold dextran coated charcoal. The samples then underwent seven minutes cold incubation followed by

five minutes centrifugation at 2500 rpm. The supernatant was poured into scintillation vials, 5 cc of scintillation fluid (Scintiverse II, Fisher Scientific) was added and the samples were counted in a Beckman liquid scintillation counter.

Statistical Analysis of the Data

For statistical analysis of the data, the readings obtained during the two levels of altered CO_2 were combined; instead of two levels of hypocapnia and two of hypercapnia, there was one reading for each condition. This allowed for a larger sample size. The baseline values were also combined, so there was one set instead of two. The values for hypercapnia and hypocapnia were then subtracted from the corresponding baseline readings, to eliminate individual variations. The resulting values were used for all statistical operations.

Using the T-test for a single mean, blood flow readings were compared to CO_2 levels, to ascertain that altering the level of CO_2 did have an effect on brain blood flow. This was done for both hypercapnic and hypocapnic conditions. A T-test for a single mean was also conducted on the following sets of data: prostacyclin levels during hypocapnic and hypercapnic conditions and thromboxane B_2 levels during the altered CO_2 conditions.

Pearson's correlation coefficient was also conducted on the data. The data was considered as two groups, hypocapnic and hypercapnic conditions. The correlation coefficient compared each variable to all of the other variables in that data group. The Pearson's correlation coefficient, while not as sensitive as the T-test, was used to demonstrate relationships between variables.

RESULTS

RIA

The plasma samples were seperately assayed for prostacyclin and thromboxane B_2. Percent binding (cpm of bound/cpm of total radioactivity) was 40.29% for prostacyclin and 38.42% for thromboxane B_2. Recovery percentages were calculated by counting 10% of the plasma sample, correcting for the percentage of sample taken, and dividing by total cpm. This resulted in values of 69.25% for prostacyclin and 66.90% for thromboxane B_2. Correction factors for the assays were calculated at 14.44 for prostacyclin and 14.93 for thromboxane B_2. Standard curves for both assays are illustrated in Figure C.

Data

The results of the Pearson's correlation coefficient test are illustrated in Table 1 A and B. As stated previously, the data was divided into two groups, hypocapnic and hypercapnic conditions. In the hypocapnic group, the Pearson's coefficient was not significant for any of the pairings tested. In the hypercapnic group, a significant correlation exsisted between prostacyclin levels and CO_2 levels, blood flow readings and prostacyclin levels, blood flow readings and thromboxane B_2 levels, and prostacyclin and thromboxane B_2 levels.

Table 2 shows the results of the T-test for a single mean. The values that were significant were the blood flow readings for both hypercapnic and hypocapnic conditions. This demonstrates that altering CO_2 level does effect blood flow; and blood flow changes in the same

manner as CO_2. That is, if CO_2 levels are increased, blood flow also increases, and if CO_2 is decreased, blood flow to the brain also decreases. The only other variable that was significant was prostacyclin levels during hypercapnic conditions. The T-test demonstrated that as CO_2 levels increased, prostacyclin levels also increased. This was not the case for prostacyclin levels during hypocapnic conditions. The T-test was also not significant for thromboxane B_2 levels during both hypercapnic and hypocapnic conditions.

Figure C:

Radioimmunoassay Standard Curves for Prostacyclin and Thromboxane B_2

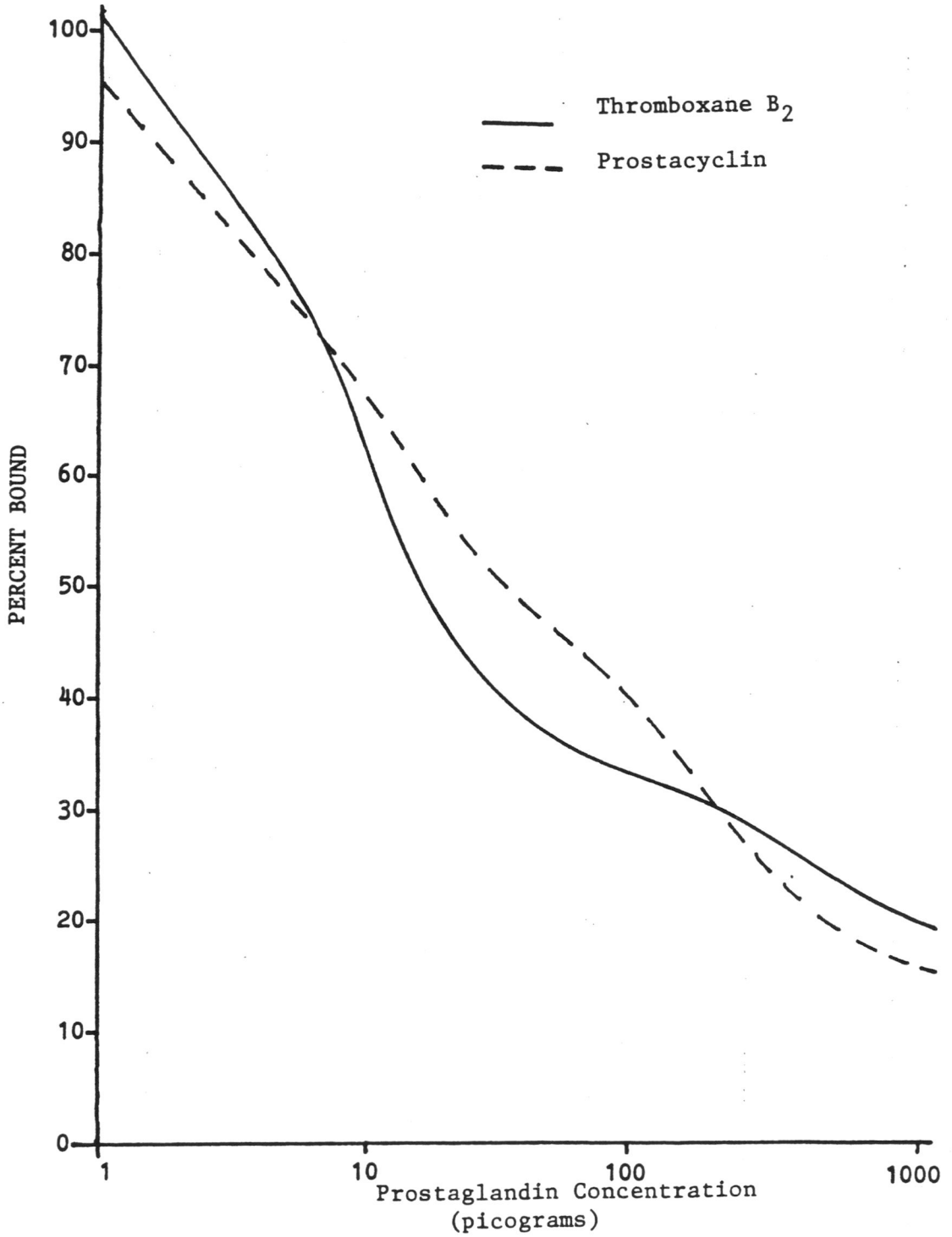


TABLE 1A:

Pearson's Correlation Matrix for Hypocapnic Conditions

	CO_2	Blood flow	Prostacyclin	Thromboxane B_2
CO_2	1	-.4295	-.1766	.2451
Blood flow	-.4295	1	.2148	.0498
Prostacyclin	-.1766	.2148	1	.3634
Thromboxane B_2	.2452	.0498	-.3634	1

* Significant at P .05

TABLE 1B:

Pearson's Correlation Matrix for Hypercapnic Conditions

	CO_2	Blood flow	Prostacyclin	Thromboxane B_2
CO_2	1	-.5139	.6072	-.0859
Blood flow	-.5139	1	-.5609	.6523
Prostacyclin	.6072*	-.5609*	1	-.6399
Thromboxane B_2	-.0859	.6523*	-.6399*	1

* Significant at P .05

TABLE 2:

T-test of a Single Mean

VARIABLE TESTED	VALUE OF T
Blood flow at hypocapnic CO_2 levels	2.2684*
Blood flow at hypercapnic CO_2 levels	-5.2272*
Prostacyclin at hypocapnic CO_2 levels	-.8440
Prostacyclin at hypercapnic CO_2 levels	2.6759*
Thromboxane at hypocapnic CO_2 levels	.2640
Thromboxane at hypercapinc CO_2 levels	.4461

* Significant at P .05

DISCUSSION

Cerebral Bloodflow Changes and the Neonate

As noted previously, intraventricular hemorrhage (IVH) is a major neurologic problem of preterm and low birth weight neonates. Studies place the incidence of intraventricular hemorrhage at 40 to 50 percent for these infants, although some estimates reach as high as 90% (Ment, 1985; Ment *et al.*, 1985). Consequences of IVH include post-hemorrhagic hydrocephalus, seizures, and a higher rate of mortality when compared to neonates that do not suffer IVH. It is also noted that infants surviving intraventricular hemorrhage have a higher incidence of developmental handicaps (Hill and Volpe, 1982; Ment, 1985).

One of the events that causes IVH is hypercapnic induced changes in cerebral bloodflow. The fact that hypercapnic conditions result in increased cerebral bloodflow is certainly not new, and has been demonstrated in numerous studies, including this one. Leahy *et al.*, (1980) indicated that human infants may be more sensitive to alterations in CO_2 than adults. A 1984 study conducted by Hansen *et al.* demonstrated a greater sensitivity to hypercapnia in newborn piglets. Several recent studies have indicated that prostaglandins, particularly prostacyclin, may be important in these hypercapnic induced changes in infants, although this may not be the case in older children and adults.

Levels of prostacyclin in urine and plasma of infants have been shown to be significantly higher than those of older children and adults. Using RIA, Kaapa *et al.* (1982) measured the plasma prostacyclin levels in children ranging from 1 day to 16 years of

age, and in adults. Prostacyclin levels were the highest in one day old children and dropped dramatically between the ages of 6 - 8 days to about one third of the earlier level. Following this sudden decrease, prostacyclin levels decreased gradually throughout childhood and adolescence. The concentration in adult plasma was approximately one half that of the adolescent. This finding is similar to that of Fischer et al. (1982). Using gas chromatography - mass spectrometry and RIA, urine levels of prostacyclin were measured in neonates and adults. Fischer et al. measured prostacyclin levels in 3 and 5 day old infants, as well as in adult volunteers. The amount of prostacyclin was markedly higher in infants, although there was a decline by as much as 50% between the 3 day and 5 day old infants.

Findings in several studies involving newborn piglets (Leffler and Busija, 1985; Leffler et al., 1985) beagle puppies (Ment et al., 1983) and human infants (Ment et al., 1985) have produced similar results concerning the role of prostaglandins in cerebral circulation. Leffler and Busija (1985) noted a dramatic rise in the levels of PGE_2, 6-keto PGF_1 alpha and TXB_2 in the cerebrospinal fluid of neonatal piglets when the animals were exposed to a mixture of 9% CO_2, 10% O_2 and 81% N_2. In a similar study Leffler et al. (1985) found that plasma levels of the prostaglandins decreased to nondetectable levels after the administration of indomethacin. Indomethacin also eliminated the increase in cerebral bloodflow that usually occurred under hypercapnic conditions.

In a study using infants defined as "high-risk" for intraventricular hemorrhage, Ment et al. (1985) demonstrated that

infants treated with indomethacin had serum levels of 6-keto PGF_1 alpha and TXB_2 that were one third of those infants in the control group. Of the infants in the treatment group only 25% were identified as having IVH compared to 58% of the control group. These results were similar to those obtained by in an earlier study using beagle puppies (Ment et al.,1983). The animals underwent the treatment of hemorrhagic hypotension followed by volume re-expansion, which is known to induce IVH. Following this treatment, indomethacin was adminstered. Eighty percent of the control animals suffered IVH, while only 9% of the drug treated beagles did so.

This consistency of results in infant studies presents a sharp contrast to the variation noted among studies using adults of various species. It seems that prostaglandins play a more important role in the regulation of cerebral bloodflow in the neonate than the adult. The results of the present study, while far from conclusive, would appear to agree with these findings. This study demonstrated that altering the level of CO_2 does affect the cerebral bloodflow in the test animal. It was also shown that prostacyclin levels in the plasma of the piglets show an increas during periods of hypercapnia. This relationship, however, was not noted with thromboxane B_2.

Limitations of the Study

Although this study does agree with the conclusions reached in several similar studies, it is not without limitations. Had some of these factors been altered, the outcome of this study may have been changed, or more conclusive results obtained.

The first factor which may have affected the outcome of this

study was the age of the piglets that were used. The piglets used ranged between 5 - 8 days of age, which while quite young, is older than animals used in similar studies (Hansen et al., 1984; Leffler and Busija, 1985). Studies using human infants have indicated that both urine and plasma levels of 6-keto PGF_1 alpha have declined markedly by the fifth day of life (Fischer et al., 1982; Kaapa et al., 1982). Although it was noted that 6-keto PGF_1 alpha did increase during hypercapnia, this change may have been more profound if younger animals had been used for the study.

A second aspect of the study which may have limited the quality of the results was the choice of using RIA to analyze the plasma. Radioimmunoassays are less time consuming and less expensive than other types of assays, and are quite sensitive. Some RIAs, however, have problems with cross-reactivity (Fischer et al., 1982). When dealing with prostaglandins, though, specifically TXB_2 Delvos et al., (1985) and Kuzuya et al., (1985) have indicated that cross-reactivity can be minimal if samples are extracted carefully. Fischer et al., (1982) suggest that data obtained via RIA should be validated with gas chromatography - mass spectrometry because of the high selectivity of this method.

Care must be taken in the collection, extraction, and processing of all samples for RIA. The sample size should be sufficently large to allow for the vigorous extraction procedure. A 200 ul sample was used in this study, which is smaller than that used in other RIA studies (Kaapa et al., 1982; Kuzuya et al., 1982). The sample size of 200 ul allowed for maximum number of replicates per sample;

perhaps, however, the number of replicates should have been sacrificed for the sake of a larger sample size. The large number of replicates per sample greatly increased the number of samples per assay, to the point that the assay procedure was quite unwieldy. Decreasing the number of samples would have resulted in a more manageable assay.

Conclusions and Speculations

This study has demonstrated that alterations in CO_2 produce alterations in the cerebral bloodflow of the 5 - 8 day old piglet. Also, plasma levels of the vasodialator prostacyclin were shown to increase under the hypercapnic conditions.

The role of prostaglandins in the control of cerebral circulation has been extensively studied, with conflicting results. Recent studies, however, particularly those of Leffler and Busija (1985) and Ment *et al.*, (1985) suggest that prostaglandins represent an integral component in the cerebral bloodflow regulation of the neonate of several species. This information may be crucial in preventing or lessening the occurance of death and developmental handicaps in preterm and low birth weight infants due to IVH. Studies involving indomethacin have indicated that treatment with this drug may provide protection from intraventricular hemorrhage, possibly by preventing prostaglandin mediated changes in cerebral bloodflow. Future studies should further investigate the protective actions of indomethacin, or the role of prostaglandins in recovery from IVH.

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