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# PHOSPHOLIPASE $A_2$ INHIBITION IN A TWO -HIT MODEL OF SEQUENTIAL SHOCK

DR. RANDY D. MOORE 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE**

**IN**

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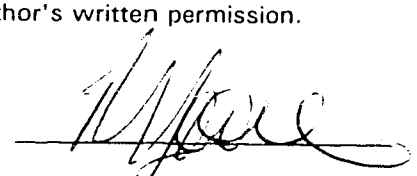

Randy D. Moore

#603 9725-106th St.

Edmonton, Alberta

Canada, T6K 1B5

Date: July 1, 1994

UNIVERSITY OF ALBERTA

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Phospholipase $A_2$ Inhibition in a Two-Hit Model of Sequential Shock** here submitted by **Randy D. Moore** in partial fulfillment of the requirements for the degree of **Master of Science in Experimental Surgery.**

Dr. S. M. Hamilton (Supervisor)

Dr. M. McBurney

Dr. J. A. Talbot

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**ABSTRACT: Phospholipase $A_2$ Inhibition in A Two-Hit Model of Sequential Shock.**

The "two-hit theory" of multi-organ dysfunction (MOD) purports that an initial physiologic insult results in an inflammatory priming effect such that subsequent insults elicit a more profound systemic response, leading to MOD. A prospective trial utilizing a large mammal "two-hit" model was undertaken to assess effects of phospholipase A2 (PLA2) inhibition on responses to sequential shock.

Adult male pigs weighing between 17 and 26 kg were randomized to control (n = 7) or experimental (n = 7) groups, anesthetized, intubated, and had indwelling catheters placed in the internal carotid artery and external jugular vein. Animals underwent sequential 4 hour periods of controlled hemorrhagic (mean arterial pressure (MAP) 40 mmHg) and endotoxic shock (25ucg/kg E.coli O111:B4 lipopolysaccharide) on days 1 and 3. Experimentals received quinacrine hydrochloride 10mg/kg infusion as a PLA2 inhibitor prior to hemorrhage. Controls received equal volumes of normal saline. Baseline measurements of heart rate, MAP, ABG's, sigmoid tonometry (mucosal pHi), and serum PLA2 were taken prior to shock episodes, and determined hourly during each episode.. After shock, animals were awakened, returned to holding, and fed ad lib. Anaerobic and aerobic blood cultures were taken every 12 hours after initiation of hemorrhage, until day 5, when animals were sacrificed, and specimens of kidney, liver, spleen, and mesenteric lymph nodes were obtained for culture. Muscle biopsies for tissue glutamine were obtained prior to hemorrhage, endotoxin administration, and animal sacrifice.

No difference in MAP or sigmoid mucosal pHi was detected between groups at baseline, during hemorrhage, or prior to endotoxic shock. During hemorrhagic shock, control animals demonstrated a significant increase in serum PLA2 activity , while experimentals had PLA2 levels significantly below baseline activity. Controls experienced a significantly greater, and more prolonged hypotensive response, and sigmoid mucosal acidosis ($p < 0.05$) after endotoxin, compared to the quinacrine-treated group. Experimentals demonstrated a trend towards preservation of tissue glutamine after sequential shock, compared to a trend towards slight depletion in controls. Translocation of enteric bacteria was observed in 4% of blood cultures, and 21% of tissue cultures, and occurred with similar frequency ($p > 0.05$)in both groups.

Quinacrine, as a PLA2 inhibitor, when administered prior to a priming hemorrhagic insult, ameliorates hypotension and sigmoid mucosal acidosis seen during subsequent endotoxic shock. This suggests a role for PLA2 activation in the priming phase of MOD. Bacterial Translocation is infrequently observed following sequential shock, and is unaffected by PLA2 inhibition.

## Preface:

Multiple Organ Dysfunction (MOD) remains the leading cause of late mortality in the critically injured patient despite tremendous advances in the support of isolated organ failure. The exact etiology of MOD, and the mechanisms involved, are as yet undefined. The role of systemic inflammatory mediators in the MOD response to critical illness or injury appears paramount. Most of the recent literature in characterizes MOD as a systemic, uncontrolled inflammatory response to severe physiologic insult.

In previous studies, the "two hit phenomena" has been demonstrated, whereby more profound hemodynamic changes, and increased bacterial translocation occur following endotoxic shock if an experimental animal has been previously exposed to a "priming insult" consisting of hemorrhagic shock. The nature of this priming effect has not been well characterized, and is presumed to be due, at least in part, to stimulation of mediator production or release by inflammatory cells. Phospholipase A2 (PLA2) may have a role in this priming event, such that PLA2 inhibition with Quinacrine would lead to an attenuation of the deleterious responses previously reported in the two hit model. The purpose of this experiment is to assess the effect of PLA2 inhibition with quinacrine on the hemodynamic responses, and the bacterial translocation associated with the "two hit" protocol of sequential hemorrhagic and endotoxic shock. Sigmoid mucosal pHi will be followed as a measure of local gut ischemia, and tissue glutamine levels will be assessed in order to provide some indication of the overall nitrogen balance status of the animal model.

A demonstrated beneficial effect of PLA2 inhibition on the aforementioned parameters may ultimately lead to therapeutic interventions in the critically injured patient that could prevent or reduce the incidence of MOD. A review of the pertinent literature follows.

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## LITERATURE REVIEW

### I. Overview of Multi-Organ Dysfunction

The past 30 years has seen incredible advances in the capacity of the medical team to care for, and support the critically ill patient. These advances have taken the form of highly developed systems and techniques for the management of isolated organ dysfunction, such that single organ system dysfunction as a sequela of serious injury is now associated with minimal mortality[1] Tilney et al[2], and Baue[3] in the early 1970's first described a clinical syndrome seen after severe physiological insult that was associated with organ dysfunction distant from the site of initial injury. Multi organ dysfunction (MOD) has now emerged as the leading cause of late morbidity and mortality in the critically injured patient[4]. Mortality due to MOD has been estimated in excess of 60% overall, and has been shown to be directly proportional to the number of dysfunctional organ systems, increasing from 30% with one system involved, to 100% with four systems involved[6,7,15]. In addition, the length of time the patient is in organ failure correlates directly with mortality[15].

Multi-Organ Dysfunction replaces previous descriptions of "Multiple Organ Failure". The term "dysfunction" more appropriately reflects a continuum related to the dynamic, progressive, and potentially reversible nature of the disease process. In the past, difficulty has arisen in the attempt to define a quantifiable "failure" endpoint with respect to a specific organ system. Dysfunction then, more clearly represents the impairment in normal function that is evident in the late phases of severe physiologic insult [1].

Current theories as to the pathophysiology of MOD descibe two phases or components to the development of the disease process: primary MOD and secondary MOD[1]. Primary MOD relates to the organ injury associated with an initial, defineable physiological insult. This insult may take a variety of forms. Severe trauma, major

surgical intervention, massive infection, burn injuries, and severe shock have all been implicated as inciting factors[5].

Common to these initiating events is significant tissue injury or ischemia, or severe inflammatory or metabolic stress. When superimposed on limited organ reserve and/or pre-existing disease, this primary insult is accentuated, and the patient has an even greater predisposition to the development of MOD. The pathophysiologic injury results in direct cellular damage leading to altered cell metabolism, and organ dysfunction.

The primary insult triggers a host systemic stress response that is characterized by the recruitment of a number of physiologic pathways, most notably the inflammatory cascade. A controlled inflammatory response is certainly beneficial to the organism as it bolsters both wound containment or healing, and immune competence. Often however, the primary insult is maintained, or a series of insults may occur, resulting in the persistence of the inflammatory drive. The resultant systemic and uncontrolled inflammatory response results in a hyperdynamic, hypermetabolic state, characterized by fever, leukocytosis, impaired perfusion, and decreased vascular resistance. This is identical to the clinical picture of severe overwhelming sepsis[8]. This uncontrolled inflammatory response constitutes the Secondary phase of MOD, which persists as a result of ongoing local inflammation related to the initial insult, as well as liberation of pan-systemic inflammatory mediators. The self-perpetuating cycle of amplified host inflammation is now felt to be of prime importance in the etiology of MOD[9,10]. This concept, that host inflammatory response is integral to the pathophysiology of MOD, represents a radical departure from earlier theories on pathogenesis that focus primarily on the role of systemic infection or sepsis. The frequent discovery of occult infections in , and the "septic profile" of patients suffering from MOD led, in the past, to the use of broad-spectrum empiric antibiotic therapy, and often diagnostic "blind laparotomy" in an attempt to reverse the advancing process[11,12,16]. Persistent deterioration in these patients despite adequate treatment of septic foci[13,14], as well as the failure to identify

septic foci in many patients with MOD, resulted in the re-evaluation of the role of overwhelming infection. This led to the development of the current theories related to pan-systemic inflammation. A schema has been proposed by Demling and associates[1] to illustrate the interactive nature of MOD:

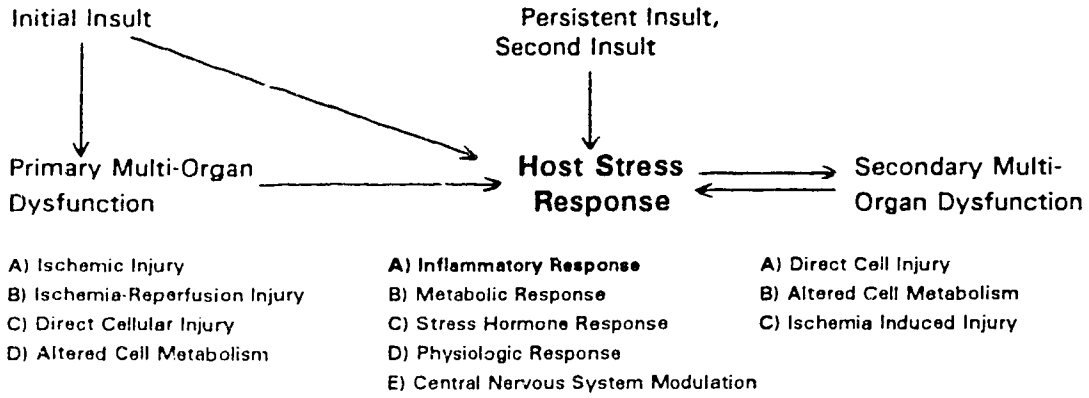



**Figure 1**: Pathophysiologic causes of primary and secondary MOD.

Despite an increased understanding of the potential pathophysiologic mechanisms responsible for the development of MOD, many questions remain unanswered. Much of the current research focuses on interactions at the molecular level, attempting to elucidate the specific systemic mediators responsible for the persistent inflammatory state. Only through the control of the local inciting process and the modulation of the "hyper-inflammatory response" can the course of MOD be ameliorated.

## II. Inflammatory Response: The Role of Cytokines

The current interest in, and understanding of, the massive generalized host inflammatory response has led to the search for the mediators responsible for systemic

inflammation. The *macrophage hypothesis* [4] of MOD proposes that excessive or prolonged activation or stimulation of macrophages results in excessive liberation of cytokines and other reactive intermediates. This initiates the activation of systemic humoral and cellular effector cascade systems (e.g. complement, coagulation systems) to produce the hyper-inflammatory response. Support for the role of cytokine involvement in the inflammatory response is based on the recognition that cytokine release can produce many of the systemic, immunologic, and metabolic changes associated with systemic sepsis response[17-20]. Elevated circulating levels of these small protein mediators have been inconsistently demonstrated in critically ill patients[21-23]. The most important cytokines with respect to the inflammatory response to severe injury appear to be Interleukin-1 (IL-1), Interleukin-6 (IL-6), Tumor Necrosis Factor -alpha (TNF-$\alpha$), Interferon-gamma (INF-$\gamma$), and Platelet Activating Factor (PAF)[1]. TNF-$\alpha$, and IL-1 in particular, have been depicted as being very proximal in the inflammatory cascade, thereby inducing other mediator systems including other cytokines, growth factors, acute phase proteins, arachidonic acid products, and endothelial derived factors. The difficulty in determining the overall contribution of the cytokine response to the development of MOD relates to a number of factors. Cytokinemia following severe injury is not consistently demonstrated[1]. This likely reflects the tremendous biologic potency, and therefore low concentration, of these mediators, as well as the extremely short half life of cytokines in the circulation. The clinical utility of cytokine level assays is further hampered by the complex interactions between cytokines resulting in either synergism, or attenuation of responses.The deleterious effects of TNF-$\alpha$ for example, are enhanced by coadministration with IL-1, endotoxin, or PAF[24]. In addition, cytokines have most of their effects at the local tissue or paracrine level, and cell-associated forms of TNF and IL-1 have been described that differ from the measured circulating forms[17,18,20]. Although a precise understanding of the role of cytokines in the development of MOD does not exist, trials involving cytokine inhibitors have shown promising results.

Recombinant anti-TNF antibodies can increase systemic blood pressure in septic patients[25], and have been demonstrated to improve survival in animals challenged with otherwise lethal doses of bacteria or endotoxin[26,27]. A recently described addition to the cytokine family appears to be a naturally occurring inhibitor: Interleukin-1 Receptor antagonist (IL-1ra)[28]. Clinical trials using this antagonist may demonstrate the ability to limit the early inflammatory response. It is important to remember however, that antagonist trials will be hampered by the fact that physiologic cytokine release is essential for wound healing and immune competency. Low doses of exogenously administered cytokines actually improve survival in experimental models[29,30].

## III. Inflammatory Response: Bacterial Translocation

Central to the *"gut hypothesis"* [4] of MOD is the notion that, as a sequelae to severe injury, viable and non-viable microbes, as well as microbial products such as endotoxin, are able to translocate across a grossly intact, but functionally and/or microscopically impaired, intestinal barrier[31]. This gut mucosal barrier is comprised of both physical and immunological elements. The physical barrier includes epithelial cells and their intercellular tight junctions,as well as the indigenous microflora of the gut. The immunologic barrier is related to secretory IgA present at the level of the mucosal surface, gut associated lymphoid tissue or GALT, and local cell-mediated immunity[43].

According to this hypothesis, the porto-systemic bacteremia and/or endotoxinemia that occurs subsequent to bacterial translocation acts as the signal that initiates, prolongs, or exacerbates the systemic immuno-inflammatory response. An attractive feature of this concept is the explanation it provides for the enteric bacteremia and septic state that often occurs, in the absence of proven infection, in patients succumbing to MOD[44]. Certainly, there is extensive evidence to suggest that bacterial translocation does occur[32-35]. A wide variety of stressors implicated in the development of MOD have been associated with translocation, including intestinal

handling at laparotomy[36,37], thermal injury, endotoxinemia, and hemorrhagic shock. Bacterial overgrowth, seen after the development of the ileus of injury, or after bowel obstruction[38,39], alteration or elimination of indigenous anareobic flora with antibiotics[40], or cessation of enteric feeding[41,42] also appear to have a role in gut mucosal barrier failure. Despite the wide variety of physiologic insults associated with bacterial translocation, the basic pathophysiologic conditions required appear to be:(1) disruption of the balanced distribution of the indigenous microflora, leading to overgrowth with gram-negative enteric bacteria, (2) impaired host immune defences, and (3) actual physical or functional mucosal barrier injury[4].

The precise mechanism by which translocation occurs has not been clearly defined. Using rodent models, and electron microscopy, Wells et al[88,89] have demonstrated translocating organisms within cytoplasmic vacuoles of intact epithelial cells. This has been supported by a study using *Candida*, radiolabelled *E.coli*, and *E.coli* endotoxin, that demonstrated transcellular pinocytotic transport of organisms and endotoxin across the bowel wall[31]. In this study, translocation of whole bacteria most commonly occurred in order of decreasing frequency to the mesenteric lymph nodes(MLN's), spleen, lung, and liver. Maximal bacterial killing however, was detected at the level of the local MLN's. Macrophage associated transport of intraluminal products to regional lymph nodes has also been demonstrated[90]. In addition, it appears that translocation of bacteria may occur at the level of the intestinal epithelial tight junctional complex and intercellular space[91]. Translocation of bacteria to regional mesenteric lymph nodes (MLN's), and distant organs appears to be followed by clearance of the organisms by cells of the reticuloendothelial system (RES)[92] (e.g. Kupffer cell macrophages of the hepatic sinusoids). This clearance process has a protracted time course. *E.coli* that have translocated to MLN's for example, have been shown to survive for up to 4 days after elimination of this organism from the bowel[93]. A failure of the ability of the RES to clear translocated organisms as a result of critical illness has been

postulated as being vital to the development of the hyperinflammatory response characteristic of MOD[83].

The complex relationships between endotoxinemia and hemorrhagic shock, and bacterial translocation will be reviewed more extensively herein.

## A. Bacterial Translocation: Hemmorhage

Morbidity and mortality related to absorption of toxic luminal factors as a sequelae of hemmorhagic shock has been described as early as 1959 [50]. A failure of the medical community to appreciate the potential relationship of this phenomenon to MOD following severe injury led to no further investigation of this area until the early 1980's.

An early study of the effect of hypovolemic shock on translocation involved a prolonged (7 hour) period of hemorrhagic shock in a rodent model[51]. Blood cultures in these animals demonstrated enteric flora within 2 hours post shock, and remained positive with progression to a polymicrobial profile over the subsequent 48 hours. This occurred despite the absence of histologically demonstrable lesions at the level of the intestinal epithelium. In contrast to this acute translocation, other studies in swine models[55,69] demonstrated significant translocation only after 6 to 48 hours post insult. Subsequent investigators have demonstrated peak systemic blood levels of enteric bacteria in rodents 24 hours after experimental hemorrhagic shock[52,53]. These investigators did find a correlation between the histological degree of intestinal submucosal and mucosal injury, and translocation. Furthermore, the degree of translocation appeared to be related to the extent and duration of the initial insult: hemorrhagic shock. These early studies also demonstrated delayed clearance, by which the animal eliminated the translocated bacteria over a period of several days following the systemic insult. It is

clear then, that the time course of the process of bacterial translocation has not been definitively characterized.

Studies designed to assess the effect of intervention or resuscitation after hemorrhage to limit translocation have suggested a benefit to using hypertonic saline[54] or crystalloid[55]. Singh et al[56], were able to demonstrate the restoration of gut absorptive capacity to normal pre-shock levels following resucitation with heparan sulfate, a glycosaminoglycan with properties similiar to heparin. The authors propose a relationship between gut function, as measured by absorptive capacity, and the ability of the gut to resist translocation. Further studies are warranted to assess the effect of glycosaminoglycan resuscitation on translocation related to hemorrhagic shock.

The results of the interventional studies have prompted active debate as to the mechanism by which hemorrhagic shock leads to translocation. The "*microcirculatory hypothesis*" of MOD[4] proposes that gut, and systemic organ dysfunction in general, is related to ischemia and /or endothelial injury. The gut is inherently susceptible to the effects of low flow due both to the countercurrent or "hairpin" arrangement of villus capillaries[57], and to the plasma skimming effect, with local hemodilution produced as a result of the perpendicular branching of these capillaries from their arteriolar supply[58]. In addition, the neuroendocrine response to hypovolemia and hemorrhagic shock results in a global reduction in splanchnic perfusion with preferential shunting of flow to vital organ systems[59,60]. Subsequent microcirculatory changes include vascular congestion and sludging, microthrombi formation, interstitial edema, and increased capillary permeability. The net result of this splanchnic hypoperfusion is inadequate tissue and mucosal cellular oxygen delivery. In rodent models, this results in the conversion of intestinal xanthine dehydrogenase to xanthine oxidase, which catalyzes the formation of reactive oxygen metabolites that are directly cytotoxic. This process is enhanced by reperfusion which entails the increased supply of oxygen as substrate - hence the term ischemia reperfusion injury. Activity of xanthine oxidase activity correlates with the area

that shows the greatest damage[61], and reduction of oxygen free radicals through scavengers or inhibitors decreases translocation[62,63]. The applicability of studies related to xanthine oxidase activity in human patients is called into question by the fact that gut concentration of xanthine oxidase is markedly elevated in rodent, as compared to human or larger mammal (e.g. pig) models[75,76]. This genetic variation in xanthine oxidase activity may partially explain the species-specific[94,95] and strain-specific[96] differences in susceptibility to gut injury following endotoxin challenge demonstrated by Deitch's group. Other biolcgically important sources of oxygen radicals include activated leukocytes, mitochondria, prostaglandin synthetase, and catecholamine auto-oxidation. In clinical disease states, it appears that activated leukocytes and xanthine oxidase are of the greatest significance[64,65]. Oxygen free radical production stimulates associated inflammatory cascades, most notably the complement cascade, the products of which have direct effects on vascular permeability[66]. In addition, local ischemia can result in the conversion of endothelium to an inflammatory phenotype with a procoagulant profile[67,68] resulting in the enhancement of focal microvascular thrombosis. Activated endothelial cells appear to express neutrophil binding and activating factors that may be associated with the subsequent development of neutrophil mediated tissue and endothelial injury. Studies utilizing recently developed neutrophil adhesion inhibition antibodies, anti-ICAM-1, and anti-ELAM-1 have demonstrated that ischemia-reperfusion mediated tissue injury can be reduced through the inhibition of neutrophil-endothelium interaction[70-72]. The systemic, activated or primed neutrophil may represent one of the mediators of the distant tissue injury and organ dysfunction seen in MOD. Researchers have demonstrated a significant relationship between gut ischemia-reperfusion, neutrophil activation or priming in the ischemic gut vascular bed, and subsequent distant pulmonary and hepatic injury[73,74]

## B. Bacterial Translocation: Endotoxin

Bacterial cell wall lipopolysaccharide or endotoxin, was one of the first recognized initiators of inflammation-induced disease. Systemic endotoxinemia may serve as one of the primary signals responsible for the maintenance of the inflammatory response leading to MOD. Bacterial endotoxin is released in response to a number of critical illness states associated with the development of MOD including trauma[84]hemorrhage[48,85], and hypovolemic shock[86]. Normally, the endotoxin that is released in these settings from the wound or the gut is cleared by the cells of the (RES). Gut-derived endotoxin is therefore ideally situated to regulate macrophage or Kupffer cell function in the injured patient. Endotoxin is a potent inducer of cytokine release by macrophages, and acts to promote the pro-inflammatory endothelial phenotype previously described[4,77]. In addition, endotoxinemia results in the stimulation and activation of neutrophil proteases and oxidant production, and activates multiple inflammatory cascades including the complement cascade[4]. Endotoxin itself leads to increased permeability of the gut mucosa[78,79], either through activation of inflammatory cascades[80], direct oxidant related injury[81], or through mesenteric ischemia via increasing mesenteric vascular resistance[82]. Cecal and terminal ileal flow appear to be preferentially affected, correlating with the increased mucosal damage in these portions of the bowel described after endotoxin challenge[87]. The local mucosal barrier dysfunction that results allows for translocation to occur, setting the stage for more endotoxin release[83,87]. In addition, the RES dysfunction that often occurs as a sequelae of critical injury due to hypoxia or ischemia results in decreased clearance of endotoxin, with subsequent increased spillage to the systemic circulation and further potentiation of gut barrier failure[4]. These mechanisms may represent a significant component of the self-sustaining phase of MOD that occurs long after the primary insult has been eliminated.

Extensive research into the potential mechanisms of action of endotoxin have revealed a number of biochemical mediators. Xanthine oxidase-associated free-radicals are generated during periods of endotoxinemia[61,104,112], and inhibition of xanthine oxidase activity or free-radical scavenger administration during exogenous endotoxin treatment results in a reduction of mucosal injury and decreased bacterial translocation[81,113]. The same criticism of these results applies, with respect to the previously discussed variability in xanthine oxidase activity among different species.

Tumor necrosis factor (TNF) appears to be one of the most important effectors of endotoxin activity. Endotoxin administration to normal human volunteers is associated with dramatic elevations in serum TNF[97]. TNF administration alone is capable of eliciting most of the pathophysiologic changes associated with endotoxinemia[98-101], and anti-TNF antibodies have been shown to prevent some of the deleterious effects of endotoxin administration[102,103]. Interestingly enough, anti-TNF antibody has not been shown to prevent the alteration in gut mucosal barrier function, and the associated translocation[104]. Platelet activating factor(PAF), liberated through the action of membrane-bound Phospholipase A2 on membrane phospholipids, can be detected at supranormal levels in the serum of endotoxin-treated animals. This mediator appears to have a role in the gut mucosal damage, and increased vascular permeability associated with systemic sepsis[105-107]. Pretreatment with a recently described PAF receptor antagonist can eliminate these effects, as well as the effects of exogenous TNF administration[108], suggesting that PAF represents the mediator by which TNF exerts its effects.

The febrile and neuroendocrine responses to endotoxin administration can be ameliorated through the administration of ibuprofen, a cyclooxygenase inhibitor that prevents the conversion of arachidonic acid to prostaglandins[97]. It appears that the endotoxin-induced reduction in splanchnic blood flow may also be related to arachidonic acid metabolism in that the administration of either a cyclooxygenase inhibitor[109], or a

leukotriene receptor antagonist[110], results in a reversal of splanchnic vasoconstriction. It is important to re-iterate that arachidonic acid is the precursor of the eicosanoid mediators: leukotrienes and prostaglandins . Arachidonic acid release results, in large part, from the action of membrane-bound Phospholipase A2, the same enzyme responsible for the liberation of PAF[111]. This knowledge has stimulated a tremendous interest in the experimental inhibition of Phospholipase A2, and in the effect of this inhibition on the responses to critical illness. This will be extensively discussed in a subsequent section.

## C. Bacterial Translocation: Human Studies

Controversy exists as to the significance of translocation in the development of human MOD. Proponents of the *"gut hypothesis"* claim its importance as paramount, and have described translocation as "the motor of MOD"[8]. Other researchers have described translocation as an epiphenomena occurring co-incident to severe injury; as a method whereby normal antigen-sampling by GALT takes place[45]. These investigators postulate that translocation to lymph nodes is a normal physiologic process that is amplified during pathophysiologic stress such that the RES system is overwhelmed. It is important to note however, that most of the translocation studies have been reported using animal models. Moore et al [46] were in fact unable to demonstrate significant portal venous translocation of bacteria or endotoxin in critically injured patients during the first five post-injury days, despite the eventual 30% incidence of MOD. The authors did acknowledge their lack of assessment of the lymphatic route of translocation, and that mesenteric lymph nodes (MLN's) in sampled patients had a positive culture rate of 25%. Other studies have failed to demonstrate significant MLN infection in critically ill patients undergoing laparotomy[49]. Critics of these studies postulate that bacteria often translocate early in the course of critical illness, but are quickly eliminated by the MLN

tissue. Culture positive translocation therefore occurs only after the GALT system has been weakened or overwhelmed by the persistent inflammatory state.

There does appear to be a relationship between bacteremia associated with presumed translocation and mortality. A study of 147 severely injured patients requiring laparotomy found that those patients with enteric bacteremia in association with shock had a 100% mortality rate[47]. In a study of patients without direct hollow viscus injury and hypotension, Rush and co-workers were able to demonstrate that the highest mortality occurred in the 53% of patients who developed enteric bacteremia[48].

Further investigation is required to provide insight into the pathophysiological importance of translocation in the critically injured patient.

## IV. Inflammatory Response: The Role of Phospholipase A2

Phospholipase A2 (PLA2) is a ubiquitous enzyme with multiple isozymes, and both circulating and membrane-bound forms.[114]. This enzyme has been the subject of intense investigation due to its proximal location in the inflammation cascade. Activation of PLA2 results in the cleavage of membrane bound phosphatidylcholine(PC) and phosphatidylethanolamine(PE) at the sn-2 acyl bond (***Fig. 2***). These membrane phospholipids contain the majority of stored arachidonic acid in the body, as sn-2 esters, and represent basic building blocks of lipid and cell membrane metabolism[114]. PLA2 action on diacyl phospholipid results in the equimolar liberation of free arachidonic acid, and lysophospholipid. Arachidonic acid subsequently serves as the primary substrate for eicosanoid (prostaglandin and leukotriene) synthesis. Lysophospholipid is a potent biological detergent that is directly cytotoxic, and must be either rapidly hydrolyzed by a lysophospholipase, or reacylated to the parent diacylphospholipid.

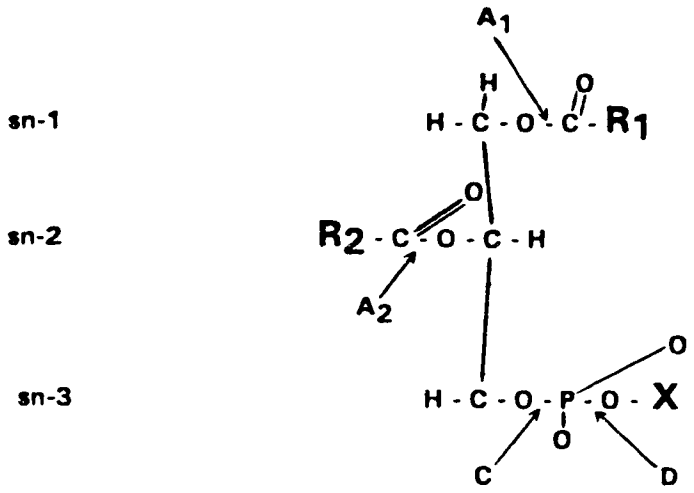



**Figure 2:** Schematic Phospholipid Molecule. Arrows indicate the sites of hydrolysis of the various phospholipases: $A_1$ = Phospholipase $A_1$ etc... The standard nomenclature of the glycerol carbons are indicated (sn). $R_1$ is usually a saturated fatty acid; the $R_2$ position serves as the primary site for stored arachidonic acid (AA). X may be H, choline, serine, ethanolamine, or its various phosphorylated forms, glycerol, or diacylglycerol.[116]

Lysophospholipid produced by the action of PLA2 on PC cannot be degraded further, and instead is acetylated and remodelled into Platelet Activating Factor (PAF), a potent biochemical mediator implicated in the inflammatory response[115].

The inflammatory role of phospholipase action may include not only the production of inflammatory metabolites, as outlined above, but also the effects on lipid bilayer regulating membrane-bound enzymes, and the membrane-bound ionic pumps[116].

Granulocyte-associated free PLA2 can be isolated from inflammatory exudates[125]. Further support for the role of PLA2 in the inflammatory response is provided by the fact that a number of diverse agents associated with critical illness are able to stimulate this enzyme. Bradykinin[117], cerulein[118], renin[119], the macrophage cytokine products IL-1[120] and TNF[121], $\alpha$1-adrenergic receptor stimulation[122], and the increased cytosolic levels of reactive oxygen metabolites, adenosine, and calcium

($Ca^{2+}$) associated with ischemia-reperfusion[115,116] have all been shown to activate the enzyme:

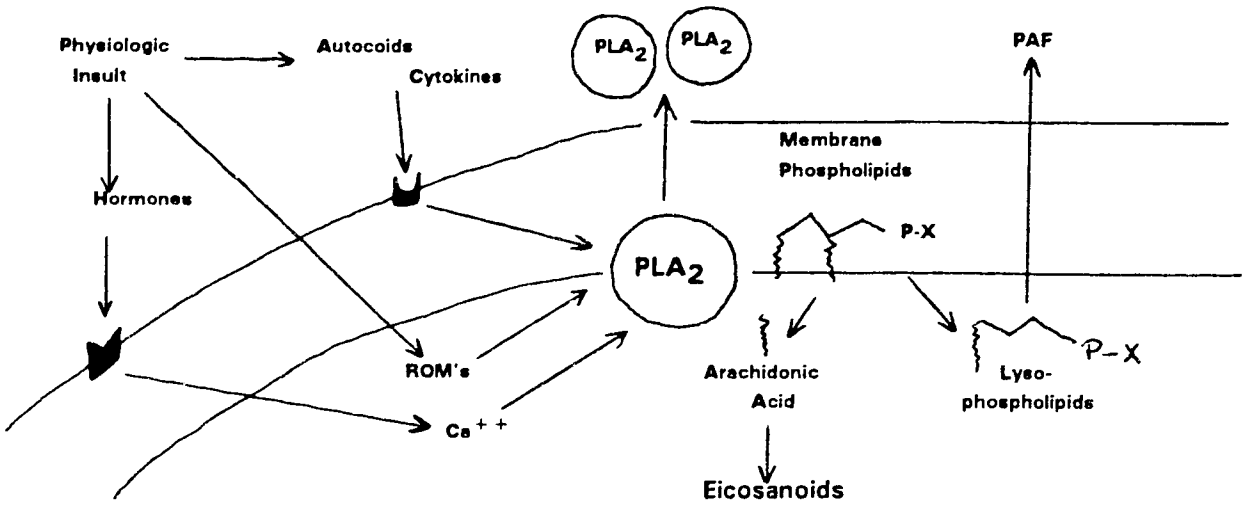



**Figure 3:** $PLA_2$ Activation at the level of the cell membrane. *ROM's*, Reactive oxygen metabolites; *Ca++*, calcium; *PAF*, Platelet-Activating Factor.[115]

The final intracellular transduction pathway by which these signals induce PLA2 activation appears to be either receptor coupling to guanosine triphosphate (GTP) binding proteins, as in the case of $\alpha$1-adrenergic stimulation, or increased cytosolic $Ca^{2+}$ levels[114,116]. This increased cytosolic $Ca^{2+}$ can result from influx of extracellular cation, as seen with bradykinin[117], or from liberation of intracellular stores via a phospholipase C (PLC) - protein kinase C dependent mechanism[116,123]. This interdependence of the PLA2 and the PLC-protein kinase C systems allows for a much greater diversity with respect to stimuli capable of eliciting PLA2 activation after critical injury.

High levels of circulating PLA2 have been demonstrated experimentally to cause profound systemic hypotension, and degradation of pulmonary surfactant[124]. Increased

circulating PLA2 levels have also been implicated in the pathogenesis of hypotension in experimental endotoxin shock[125,126]. In a clinical study of patients with gram-negative septic shock, Vadas[124] was able to demonstrate mean plasma PLA2 activity in septic patients that was increased 16-fold over that of non-hypotensive controls. In patients suffering from adult respiratory distress syndrome (ARDS) as a result of gram-negative sepsis, mean PLA2 activity was increased 20.2 fold over that of controls, and was 44% higher than that of septic patients without ARDS. In addition, this investigator was able to demonstrate a return of PLA2 levels to baseline levels in those patients who survived the shock episode. Other investigators have demonstrated increased plasma PLA2 levels in patients with MOD[126]. Researchers postulate that elevated circulating PLA2 levels may represent increased activity of membrane-bound enzyme, either in association with tissue cell membranes, or with the membranes of mobile inflammatory cells (e.g. PMN's)[115]. A number of inflammatory states, including rheumatoid and osteoarthritis[130], and pancreatitis[131] have been associated with release of PLA2 from PMN's after their stimulation.

The increase in intestinal permeability seen after ischemia can be potentiated by lysophosphatidylcholine (LPC), a potent lysophospholipid membrane surfactant produced as a result of PLA2 action on membrane phospholipid. This powerful biological detergent has been shown to induce direct mucosal injury at higher concentrations[127,128]. Otamiri et al[129], in a rodent model of total intestinal ischemia, were able to demonstrate increased PLA2 levels, and decreased lysophospholipase levels, in association with elevated LPC levels, that correlated with morphologic mucosal damage. Furthermore, these investigators were able to prevent the accumulation of LPC, and the associated mucosal damage, through pretreatment with Quinacrine, a PLA2 inhibitor. The role of quinacrine and PLA2 inhibition will be discussed more extensively below. The authors of this study suggest that accumulation of lysophospholipids as a

result of PLA2 activation may be responsible for the mucosal injury seen after intestinal ischemia.

As previously discussed, lysophospholipids produced by the action of PLA2 on membrane phospholipids are either degraded, re-acylated to phospholipid, or remodelled to become platelet-activating-factor (PAF). Initially recognized in 1979 [132] by its capacity to aggregate and degranulate platelets in vivo, PAF is now known to have a diverse array of actions pertaining to the inflammatory response, at concentrations far lower than those required for platelet effects[133]. PAF causes a marked increase in microvascular permeability, significantly lowers arterial blood pressure, and has a negative inotropic effect on the heart[111]. In animal models, PAF is capable of eliciting profound bronchoconstriction and increased airway resistance[134], as well as pulmonary hypertension[135]. Gonzalez-Crussi et al[136], in a rat model, were able to demonstrate a synergistic effect of endotoxin and PAF in the production of ischemic intestinal necrosis. These investigators showed that the pathogenesis of the experimental necrosis was independent of the platelet aggregation effect of PAF. Other researchers have confirmed the role of PAF as a mediator of endotoxin-induced gastrointestinal ulceration[137]. In addition to its action as a platelet aggregating agent, PAF is a potent leukocyte activator[135]. High concentrations of PAF have been shown to stimulate neutrophils to marginate and adhere to endothelial cells, to undergo chemotaxis, to degranulate, and to produce reactive oxygen metabolites[132]. High dose PAF stimulation results in monocyte and tissue macrophage liberation of TNF, IL-1, and eicosanoids[132]. Low dose PAF has been shown to result in a "priming" effect with respect to inflammatory cells. An in vitro study by Vercellotti et al[133] has demonstrated enhanced PMN responses to otherwise-weak neutrophil agonists after preexposure to PAF. These researchers demonstrated that PAF is capable of amplifying neutrophil oxidant free radical and protease production, as well as margination and adhesion to endothelium, and endothelial damage. Furthermore, these effects could be

inhibited through the use of PAF antagonists. Other investigators have suggested a role for PAF, and other products of PLA2 activation, in the control of lysosomal enzyme release from human PMN's[138]. It appears that a component of the PAF-priming process entails activation of PLA2 in an autocrine fashion, thereby upregulating further PAF release[123]. The priming effect of PAF on neutrophil function has been confirmed by a number of investigators[139,140], and may represent a significant component of the "first hit" response pertaining to the "two hit" theory of MOD (vide infra).

PAF antagonists have been part of the Chinese Herbalist's armamentarium to treat a variety of pulmonary disorders for thousands of years[132]. Most of the modern antagonists are derived from these ancient sources, and have been shown to be potent inhibitors of many of the effects of systemic endotoxin. PAF antagonists have been shown to ameliorate endotoxin-induced peripheral vasodilatation and hypotension, as well as mortality post endotoxic- shock[132]. Interestingly, the improved mortality post endotoxic-shock is seen even if the administration of PAF antagonist is delayed until several hours after the insult, suggesting an important inflammatory mediator attenuating effect of these agents. The endotoxin-induced decrease in glomerular filtration rate and renal blood flow, and subsequent oliguric renal failure, can be inhibited by the use of PAF receptor antagonists[142]. PAF antagonists have also been demonstrated to improve survival in animal models after traumatic and hypovolemic shock[141]. The gastrointestinal ulceration seen with endotoxin administration can be attenuated by the use of PAF receptor antagonists, raising the question of their applicability in the limitation of bacterial translocation through this abrogation of mucosal barrier damage[128]. To date, no studies have assessed the effect of PAF antagonists in the septic or severely traumatized human patient.

The proximal location of PLA2 in the aforementioned inflammatory cascade has led to interest in the effects of PLA2 inhibition on clinical and experimental parameters associated with critical illness. A large number of PLA2 inhibitors exist, both

reversible, and irreversible[114]. Quinacrine, or mepacrine, is a synthetic acridine derivative that is structurally similiar to parent compounds chloroquine and primaquine. These agents all have established efficacy with respect to the treatment of malaria, and all have relatively large therapeutic windows, with low toxicity at therapeutic levels. Quinacrine is one of the most commonly utilized PLA2 inhibitors in the experimental setting, and acts rapidly through non-specific interference with substrate-enzyme interaction[143]. In an early study by Blackwell et all[144], both basal and stimulated PLA2 activity of a perfused guinea-pig lung preparation were completely blocked by pretreatment with chloroquine, with subsequent decreased production of rabbit aorta contracting substance (RCS). RCS is now known to be a mixture of prostaglandin endoperoxides and thromboxane A2. A later review by Loffler et al[143] demonstrated that mepacrine inhibited both PLA2 and lysophospholipase activity in plasma membrane, mitochondrial, and cytosolic subcellular fractions of rat liver.

Knowledge regarding the ability of quinacrine to inhibit PLA2 has led to its use in experimental settings designed to mimic the effects of severe injury. Most of the current research has focused on the ability of PLA2 to protect the gastrointestinal mucosa from the deleterious effects of intestinal ischemia-reperfusion. As previously described[127], Otamiri et al were able to demonstrate that luminal lysophosphatidylcholine (LPC) administration, resulted in intestinal mucosal injury and subsequent increased permeability to macromolecules. A follow-up study by the same group, in a rat model, showed that quinacrine pretreatment, in a dose of 10mg/kg body weight, was able to eliminate the increase in PLA2 activity, the accumulation of lipid peroxides, and LPC, as well as the intestinal mucosal damage, and macromolecular permeability associated with total intestinal ischemia and reperfusion[129]. These investigators have also demonstrated that ischemia-reperfusion results in increased mucosal permeability to sodium fluorescein, increased lysosomal enzyme release (N-acetyl-$\beta$-glucosaminidase) from mucosa into the lumen, and elevated levels of mucosal myeloperoxidase, a

neutrophil granulocyte marker. This evidence for mucosal damage was completely eliminated through the use of PLA2 inhibition with quinacrine pretreatment[145]. In addition, these authors were able to demonstrate that a lipoxygenase inhibitor, BW755C, had no effect on the aforementioned parameters, and that a cyclooxygenase inhibitor, indomethacin, actually potentiated the increase in mucosal permeability and mucosal lysosomal enzyme release seen after intestinal ischemia-reperfusion. The use of a specific PAF antagonist (BN 52021) in this model resulted in a decrease in mucosal lysosomal enzyme release, mucosal permeability, and mucosal lipid peroxidation products, compared to ischemic controls. These decreases, however, were not to the same extent as those seen with quinacrine pretreatment. In addition, the PAF antagonist was not able to prevent the increased mucosal myeloperoxidase activity associated with neutrophil infiltration seen after ischemia-reperfusion. In an attempt to determine the importance of oxygen free-radical production in the induction ischemic mucosal damage, Tagesson et al[146] examined the effects of combined free-radical scavengers, superoxide dismutase and catalase, on biochemical indicators of mucosal injury. Once again, pretreatment with quinacrine was demonstrated to inhibit the increase in mucosal permeability, the increase in malondialdehyde (lipid peroxidation product), the increased lysosomal enzyme release, and the increase in mucosal activities of myeloperoxidase and phospholipase A2. Although pretreatment with the combination of free-radical scavengers was able to prevent lipid peroxidation and the accumulation of malondialdehyde, these agents were only able to reduce the other indices of mucosal damage by 50% compared to the quinacrine-treated group. The authors of this study postulate that the failure of free-radical scavengers to inhibit ischemic mucosal damage is likely due to the inability of these agents to prevent ischemic activation of mucosal PLA2. In addition, these investigators suggest that the PLA2-induced neutrophil infiltration and activation at the intestinal mucosal level may represent an important pathway for the development of ischemic intestinal mucosal damage. Liver and lung

injury, in the form of increased $^{125}$I-albumin leak, have been demonstrated to occur co-incident with gut ischemia-reperfusion in the rat[147]. This effect can be prevented by depletion of neutrophils prior to gut ischemia-reperfusion (I/R) with intravenous vinblastine. Moore et al[115], in fact, suggests that neutrophil chemotaxis and activation at the level of the ischemic intestinal mucosal vascular bed represents a "priming" event that ultimately leads to diffuse activated-PMN-mediated tissue injury. This group was able to demonstrate that gut ischemia-reperfusion in a rat model resulted in PLA2 activation, gut PMN influx, and increased $^{125}$I-labelled albumin lung leak. Gut ischemia-reperfusion also resulted in enhanced PMN oxidative burst (superoxide production), compared to that seen in pre-ischemic neutrophils, in response to neutrophil agonist N-formyl-met-leu-phe. All of these effects were completely eliminated by PLA2 blockade with quinacrine, 10mg/kg body weight, administered 30 minutes prior to the onset of ischemia.

The abundance of experimental evidence implicating PLA2 activation in the development of gut mucosal injury, and possibly distant injury, seen during low-flow states, has led to speculation regarding the ability of PLA2 inhibitors to decrease mortality in the critically ill patient. Recently developed monoclonal antibodies to PLA2 have been shown to completely inhibit the PLA2 activity in the plasma from septic patients[148,149]. To date, however, there are no reports of the use of PLA2 inhibitors in the severely injured or critically ill patient. In addition, no data exists regarding the ability of PLA2 inhibitors to prevent bacterial translocation associated with severe metabolic stress and injury.

## V. Tissue Glutamine

The catabolic state associated with critical illness is associated with a tremendous loss of body protein, primarily in the form of skeletal muscle protein, as evidenced by the appearance of muscle atrophy and progressive weakness[154]. This

muscle wasting is accompanied by the increased use of muscle-derived amino acids as substrates for gluconeogenesis, and the other metabolic processes associated with the responses to severe injury. Glutamine is the most abundant amino acid in blood, and in the free amino acid pool of the body. Skeletal muscle serves as the principal site of glutamine synthesis and storage in the body; 75% of skeletal muscle amino acid pool is comprised of glutamine and glutamic acid[151], and glutamine concentration in muscle is 30 times that of the circulating levels[150]. Recently, glutamine has been characterized as a conditionally essential dietary nutrient rather than a non-essential or nutritionally dispensible amino acid[155]. This conceptual change has arisen as a result of the observation that both cellular and circulating levels of glutamine decline markedly following trauma ,systemic infection, or any critical illness[156-160]. Even uncomplicated elective surgical intervention is associated with depressed muscle and circulating glutamine levels[150]. This decrease in glutamine concentrations is greater than that for any of the other amino acids, and generally correlates with the severity of the metabolic insult[155]. In addition, this decrease occurs despite an increased release of glutamine from skeletal muscle and lung (vide infra), suggesting that endogenous synthetic pathways are not able to meet the increased demands of glutamine requiring tissues during critical illness. A return to normal, "pre-insult" glutamine levels does not occur until late in the course of recovery from illness, when full dietary and ambulatory activities have been resumed.

Glutamine has a pivotal role in a number of important metabolic pathways. It is an essential precursor for nucleic acid synthesis in all cells, and has been demonstrated to play a key role in the regulation of amino acid metabolism during catabolic states[150]. Its two readily mobilizable nitrogen groups form the basis for its role as a vehicle for the transfer of nitrogen between tissues[161]. The complex inter-organ glutamine relationships following surgical stress or other critical illnesses has been the subject of intense research. Glutamine serves as the primary source of fuel for renal

tubular cells. During periods of stress, renal glutamine utilization is increased, largely as a result of elevated blood corticosteroid levels[162]. Acidosis, as a result of the accumulation of products of catabolism, also leads to increased renal glutamine consumption in order to support ammoniagenesis[163]. Inflammatory cells involved in the response to critical illness are also important glutamine consumers[164]. Lymphocytes and macrophages metabolize glutamine in order to generate vital cellular intermediates related to the immune and inflammatory responses[165].

Skeletal muscle is considered the primary source of glutamine release during the catabolic state[150,167]. Certainly, profound intramuscular glutamine depletion and massive glutamine efflux is characteristic of any injury[152], and glutamine appears to have a direct role in the regulation of muscle protein balance. Evidence for the importance of intramuscular glutamine to muscle protein balance arises from observations that the rate of protein synthesis in skeletal muscle shows a direct correlation with intracellular glutamine levels[152], and that protein anabolism in an in vitro model of isolated perfused rat skeletal muscle is stimulated by glutamine[153,166]. Smith et al[173] have demonstrated a direct inhibitory effect of glutamine on protein degradation in a muscle cell culture model.

Examination, at the cellular level, of muscle glutamine metabolism has resulted in the identification of a specific skeletal muscle amino acid carrier with unique characteristics. A rise in intramuscular $Na^+$ [153], a fall in blood insulin levels[166], elevated levels of blood glucagon, corticosteroids[154], or catecholamines, or muscle denervation - all of which commonly occur in the clinical setting - results in a net efflux of muscle glutamine, and a fall in the intracellular concentration. Askanazi et al[159] examined muscle biopsies obtained from multiply traumatized patients, and confirmed the marked depletion of intracellular glutamine. Stehle and his group[152], were able to prevent the injury associated depletion of intracellular muscle glutamine in post-operative patients by administration of exogenous parenteral glutamine. A similar study of post-

operative cholecystectomy patients demonstrated that provision of exogenous glutamine by way of TPN ameliorated the decrease in intracellular glutamine content and ribosome concentration seen after surgical stress[172]. The amino acid transporter also appears to be sensitive to live bacteria and bacterial endotoxin, which accelerate the efflux of intracellular glutamine[153]. In addition to the decreased ability of the amino acid transporter system to maintain a glutamine concentration across muscle, it appears that metabolic insult may also lead to a change in the actual "setting" of this system for muscle glutamine, such that a lowered plasma/muscle glutamine ratio occurs, despite the net efflux of glutamine from muscle to plasma[152].

Recent evidence suggests that the lung is also of critical importance to inter-organ glutamine metabolism. Surgical patients with pulmonary artery catheters have been demonstrated to release large amounts of glutamine from the lungs into the systemic circulation during hyperdynamic sepsis[150], and glucocorticoid hormone level elevation, such as that which occurs during severe metabolic stress, accelerates this release of pulmonary glutamine.

Glutamine, whether absorbed across the basolateral membrane from arterial blood, or transported across the mucosal brush border from the lumen, is metabolized in a similiar fashion, and serves as one of the primary sources of fuel for the enterocyte[150,168]. The tremendous avidity of the gut epithelium for glutamine is felt to be related to both the highly developed mucosal transport system, and to the high concentrations of mucosal glutaminase, the major enzyme in glutamine metabolism[169]. From a physiologic point of view, glutamine metabolism by the enterocyte provides the major source of energy for the gut, and results in the conversion of carbon and nitrogenous intermediates from peripheral tissues into precursors for hepatic ureagenesis and gluconeogenesis[150]. In addition, it appears that glutamine has a trophic or protective effect with respect to gut mucosal integrity. Glutamine-supplemented parenteral formulas reduce the intestinal mucosal atrophy seen during treatment with

glutamine-free formulas[155], and glutamine supplementation appears to accelerate intestinal adaptation following massive bowel resection[150]. The incidence of bacterial translocation is decreased, and the rate of clearance of translocated bacteria from MLN's increased, in animals given glutamine following intestinal damage from radiation[174,175], or 5-fluorouracil[176]. Supplemental glutamine can also increase survival, and abrogate histologic intestinal damage in animals recieving whole abdominal radiation[177] or chemotherapy with methotrexate[178]. Recently, investigators have demonstrated that intestinal blood flow after hemorrhagic shock can be restored with glutamine suffusion[182].

Glutamine consumption by the intestinal tract is greatly increased during systemic injury, or metabolic stress[150,152,170], a response that appears to be largely related to increased synthesis and activity of mucosal glutaminase, and mediated by elevated levels of corticosteroids and glucagon[170,171]. A study of interorgan glutamine flux in 60% enterectomized dogs by Souba et al[179] revealed markedly elevated arterial glutamine levels compared to non-enterectomized controls, suggesting that the increased intestinal consumption of glutamine during stress contributes significantly to the depressed glutamine levels that characterizes these states. In addition, this model clearly demonstrated the important role of the gut in the provision of alanine to the liver to support gluconeogenesis. A subsequent review of septic patients with, and without massive (>80%) bowel resection[183] demonstrated that the decrease in intestinal glutamine uptake following enterectomy is associated with a marked decrease in the peripheral efflux of tissue glutamine, suggesting a strong relationship between peripheral glutamine efflux and intestinal glutamine consumption. These investigators could not demonstrate a significant relationship between corticosteroid levels and the rate of peripheral glutamine efflux. The authors of this study postulate the presence of an unidentified humoral factor or signal, that is normally released or induced by both the stressed and non-stressed intestinal tract, and that is

responsible for sustaining peripheral tissue glutamine balance. This raises the question as to whether support of the gut during critical illness, for example through the use of cytokine inhibitors, may allow for a preservation of skeletal muscle mass, and an improvement in overall nitrogen balance. In addition, the effect of PLA2 inhibition, and subsequent decreased liberation of PAF, on gut and skeletal muscle glutamine metabolism remains to be elucidated.

Gut glutamine uptake from the bloodstream is diminished in septic patients, and in rats treated with endotoxin[180]. This effect appears to be mediated via an endotoxin induced decrease in both mucosal glutaminase activity, and sodium-dependent brush-border transport synthesis[169], despite increased vesicle-mediated brush border transport[181], and is independent of the decrease in mucosal blood flow. The combined effect of endotoxin-induced impairment of skeletal muscle glutamine gradient (vide supra), and decreased gut glutamine consumption results in an elevation of circulating glutamine levels[169]. Endotoxin-induced inhibition of gut glutamine metabolism may therefore represent another component of the sepsis-related impairment of gut mucosal barrier function previously discussed. Reports regarding the ability of other inflammatory mediators or their inhibitors (e.g. PAF, PLA2 inhibitors) to affect gut glutamine metabolism have not been published.

## VI. Two-Hit Theory

The "two hit" theory represents an extension of the concept of primary and secondary MOD previously discussed (vide supra). Specifically, this theory relates to the concept that an initial metabolic insult results in a "priming" effect with respect to the inflammatory potential of the organism, such that a subsequent stimulus, or prolongation of the initial stimulus elicits a more profound systemic response[1,4]. It has been previously demonstrated that the deleterious hemodynamic responses, and the systemic bacterial translocation that occurs as a sequelae of endotoxic shock in a pig

model, can be amplified by a priming insult consisting of hemorrhagic shock[184]. From an experimental point of view, it is well recognized that non-lethal doses of endotoxin can elicit a fatal response in animals subjected to a wide variety of pathophysiologic stimuli including shock, severe trauma, or burn injury[185].

The process of systemic inflammation is initiated early after any severe injury; cytokine release, inflammatory cell sequestration and adherence to endothelial cells, intravascular complement activation, tissue lipid peroxidation - all have been demonstrated to occur after massive local tissue trauma or metabolic insult[1]. Despite these early biochemical responses, physiologic evidence of the systemic inflammatory response is often not present until several days after the initial insult[6]. This phenomenon is now felt to be due to the delayed or "priming" effect of local injury on the systemic inflammatory response, such that a second insult, occurring in the initial post-resuscitative phase, elicits the delayed response, that is a more profound physiologic response than is expected. This insult might take the form of gut or wound derived endotoxin, a vascular catheter-associated bacteremia, a subsequent ischemic event, tissue necrosis, or any inflammatory process[186,187]. This has definite implications for the treatment of the injured patient in that prevention, or limitation, of the second insult, may allow for the limitation of the systemic inflammatory response, and the prevention of MOD.

Most of the current theories as to the mechanism of the priming events implicate the systemic leukocytes; neutrophils and macrophages. Certainly, the priming effect of systemic inflammatory mediators such as PAF on neutrophil responses to normally weak leukocyte agonists has been clearly demonstrated[133]. In addition, it appears that the magnitude of the stimulus required to prime the inflammatory macrophage or neutrophil may be on the order of 1/100th the amount required to induce full activation of these same cells[188]. This may explain why the priming process is usually not manifest clinically[1].

Based on these concepts, a current schemata of the "two hit" theory is as follows:

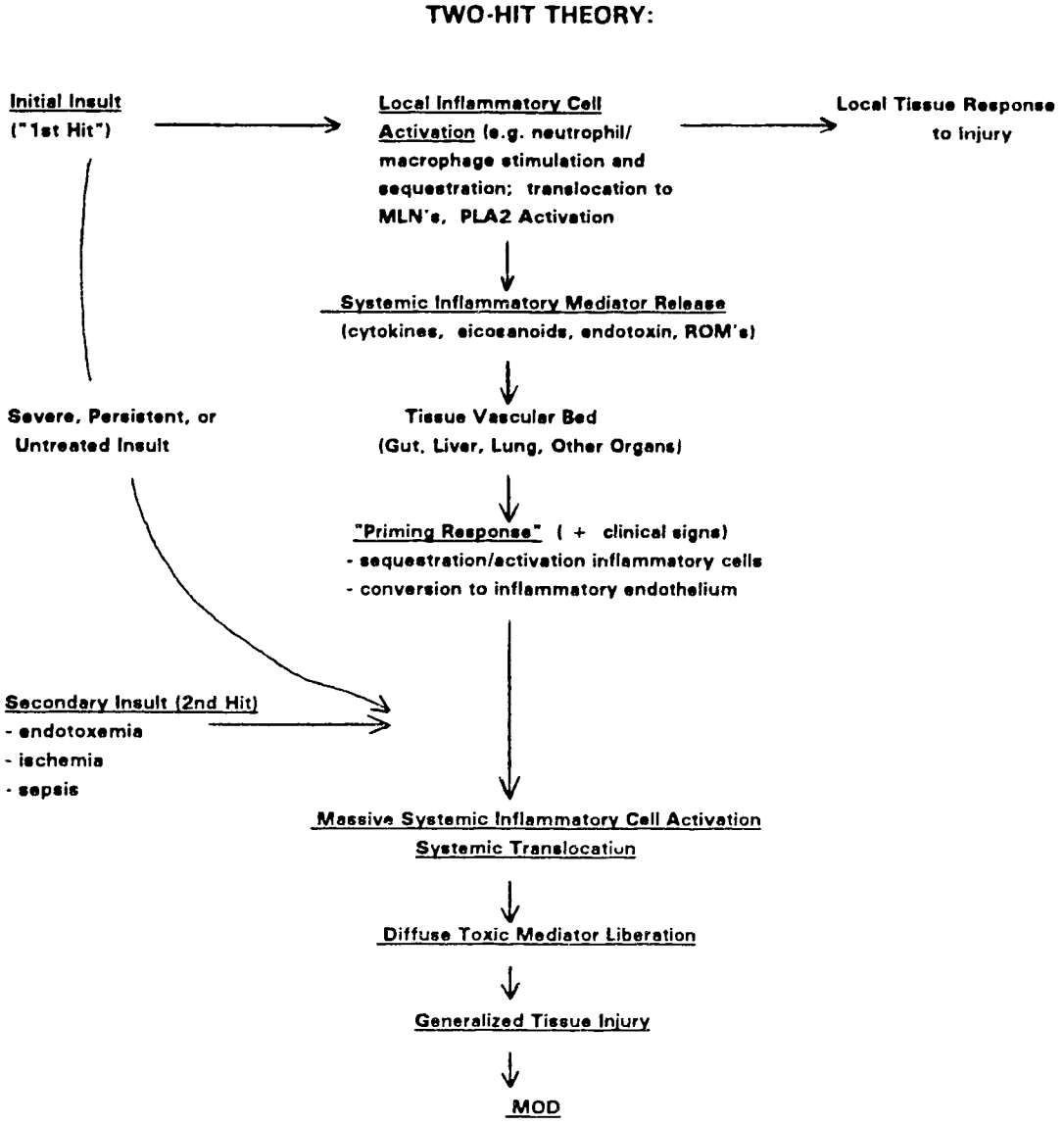



Figure 4: "Two Hit" Model for the Development of MOD. *ROM*, reactive oxygen metabolites[1].

## EXPERIMENTAL DESIGN

The purpose of this experiment is to determine the effect of Phospholipase A2 inhibition, administered prior to a priming insult consisting of hemorrhagic shock, on the subsequent responses to a second hit consisting of endotoxic shock. Specifically, the effect of PLA2 inhibition on hemodynamic changes, mucosal pH, and tissue glutamine will be assessed. The null hypothesis is formulated as: Phospholipase A2 inhibition does not affect the responses to the endotoxic shock, subsequent to a prior stimulus consisting of hemorrhagic shock.

In order to test the hypothesis, the following experiment was carried out over 5 days in a swine model:

Figure 5 : Experimental Protocol - Day 1

| | t = 0 h | t = 1.5 h | t = 2 - 6 h |
|---|---|---|---|
| Control Group | Anesthesia/Intubation<br>Sigmoid Tonometry<br>Surgery<br>Monitoring:<br>- *ABG, MAP*<br>- *mucosal pHi*<br>- *PLA2* | Saline 100ml<br>iv bolus | Hemorrhage<br>MAP = 40mmHg x 4 hours<br>Monitoring q 30 mins<br>Retransfusion/ resuscitation<br><br>Blood Cultures<br>q 12h |
| Study Group | " " | Quinacrine i.v.<br>10mg/kg in<br>100 ml Saline | " " |

Figure 6 : Experimental Protocol - Day 3, Day 5

| | Day 3 | Day 5 |
|---|---|---|
| Control Group | Anesthesia/Intubation<br>Sigmoid Tonometry<br>Monitoring<br>- MAP, ABG<br>- Mucosal pHi<br>E. coli 0111:B4<br>Endotoxin Infusion<br>25 ucg/kg<br><br>Resuscitation LR<br>after 4 Hours | Anesthesia/Intubation<br>Laparotomy: Tissue Cultures<br>- Liver, Kidney, Spleen<br>- Mesenteric Nodes x 3 |
| Study Group | " " | " " |

**Variables Measured:** Mean arterial blood pressure (MAP), heart rate, respiratory rate, arterial blood gases, mucosal pHi, temperature, intravenous fluid rate, blood/regional lymph node/tissue cultures, and oxygen concentration of inspired air ($FiO_2$).

## MATERIALS AND METHODS

### I. Animal Preparation

Approval for the use of experimental animals was obtained from the Health Sciences Animal Welfare Committee at the University of Alberta, and animals were cared for according to guidelines set forth by the Canadian Council on Animal Care[190].

16 male swine were purchased (one at a time) from one supplier (University of Alberta Farms, Edmonton, Alberta). The Landrace - White York cross-bred strain was chosen due to the low incidence of malignant hyperthermia seen in these animals relative to other strains of domestic swine[189]. The animals were housed, and placed on an antibiotic-free diet at the Biomedical Animal Facility for two weeks prior to the experiment, and weighed between 15 and 27 kg on the first day of the protocol. Animals were fasted for 12 hours prior to the experimental protocol, and were randomly assigned to either the control, or the study group.

On the morning of Day 1 of the protocol (Fig.5), each animal was brought to the operating laboratory, and an intramuscular dose of Ketamine Hydrochloride (Ketalean, 25mg/kg, MTC Pharmaceuticals, Cambridge, Ontario) was administered to sedate the animals. This was combined with Atropine Sulfate (Atropine, 0.05mg/kg IM, Abbott Laboratories Limited, Montreal Quebec) in order to reduce respiratory and salivary secretions. Anesthesia was then induced by face mask inhalation with isofluorane (Forane, 3%, Anaquest, Mississauga, Ontario), and the animals were subsequently intubated with a cuffed endotracheal tube (6.5mm internal diameter, Portex). Correct endotracheal tube placement was confirmed with auscultation. Isofluorane concentration was subsequently reduced to 2%, and positive pressure ventilation was commenced at a tidal volume of 10-15ml/kg at 10-15 breaths per minute, inspiratory:expiratory ratio 1:2 (Fraser Harlake anesthesia ventilator; Fraser Sweatman Inc; Orchard Park N.Y.). Ventilator settings were adjusted through the course of the

anesthetic period in efforts to maintain the partial pressure of carbon dioxide ($PCO_2$) at 35 - 45mmHg. The $FiO_2$ was adjusted to maintain the partial pressure of oxygen in arterial blood ($P_aO_2$) between 300 - 400 mmHg.

Electrocardiographic patches (Red Dot , 3M, London, Ontario) were placed on the chest. Leads were connected to a preamplifier (Model 78203C, Hewlett-Packard) equipped with a stripchart recorder (Model 7754A, Hewlett Packard) and heart rate was recorded continuously. A sigmoid tonometer (Tonometrics Inc, Worcester MA, USA) was placed per rectum and introduced to a length of 30cm and secured with tape. Normal saline was instilled into the tonometer balloon and allowed to equilibrate with mucosal gas partial pressures for at least 60 minutes. A digital thermometer (Model 8500-40, Cole Parmer, Chicago, Illinois) was also placed per rectum to continuously monitor the body temperature. Body temperature was maintained between 36.5 - 39.5$^oC$ using a heating/cooling blanket (Aquamatic Thermia Model RK-200, Gorman-Rupp Industries Inc., Bellville,Ohio) placed on the operating table under the animal. Normal body temperature values for the resting pig are reported to be near 39$^oC$ [190].

## II. Surgical Procedures

### A. Aseptic Technique

The nature of this study required aseptic technique at all times, and unless otherwise stated, can be assumed.

Sterilization of the surgical field was attained by way of a 5 minute scrub preparation with chlorhexidine gluconate 4% solution (Hibitane, Ayerst Laboratories, Montreal Quebec) followed by two applications of povidone-iodine 10% (Betadine Solution, Purdue Frederick Inc., Toronto, Ontario). Animals were then draped in a sterile fashion.

All equipment in contact with the animal was sterilized prior to use, either by steam treatment or by exposure to ethylene oxide.

**B. Carotid Artery and External Jugular Vein Catheterization**

The pig was placed in the left lateral position, and an 8 cm transverse incision was made in the right side of the neck 2 cm cephalad to the shoulder girdle. The external jugular vein, and right internal carotid artery were exposed through dissection between the muscle bellies. Indwelling catheters were prepared by placing intravenous catheters (Flash-Cath, #14 Gauge, Travenol Laboratories Inc.,Deerfield, Illinois) into the lumens of two 55cm lengths of silastic tubing (Silastic Medical Grade Tubing. 0.062 " ID, Dow Corning, Midland, Michigan). These catheters were tunnelled subfascially into the neck incision from a small incision in the dorsal midline between the scapulae. Each of the vessels was ligated, and a catheter introduced proximally, via small venotomy/arteriotomy, and secured in place. The catheters were introduced to a length of 15 cm, corresponding to the level of the aortic arch, and the superior vena cava, in order to minimize clotting during the remainder of the protocol. Each catheter was fitted with a heparin lock cap (PRN Adapter, Becton Dickinson, Sandy, Utah) and flushed with a dilute solution of heparin sodium (Organon Teknika, 1000u/ml, Toronto, Ontario) made to 1 unit/ml with normal saline. The incision was closed in two layers with a deep running suture of 3-0 Dexon Plus (Davis and Geck, Montreal, Quebec), followed by a running suture of 2-0 Surgilon (Davis and Geck, Montreal, Quebec).

The arterial catheter was connected to a pressure transducer (Model 1290A, Hewlett Packard), a preamplifier (Model 78205B, Hewlett Packard), and a stripchart recorder (Model 7754A, Hewlett Packard). Equipment was zeroed according to manufacturer's instructions, and mean arterial pressure was monitored continuously. The venous catheter was connected to an intravenous infusion (Lactated Ringer's Injection, Baxter Corporation, Toronto, Ontario) and maintained at an infusion rate of 20 cc/h for the duration of the protocol by way of an intravenous infusion pump (Imed 960, Imed Let., England).

## III. Hemorrhagic Shock

At this point in the experiment, called T = 1.5 hr, baseline monitoring was initiated. Control animals then received an intravenous bolus infusion of 100 ml of Normal Saline ( 0.9% Sodium Chloride Injection, Baxter Corporation, Toronto, Ontario) over 5 minutes. Animals in the experimental group received the same volume of sterile saline solution containing 10mg/kg Mepacrine ( Quinacrine hydrochloride, Sigma Chemical Co., St. Louis Missouri). After 10-15 minutes of stabilization, animals from both groups underwent a four hour period of hemorrhagic shock during which the MAP was lowered to 40mmHg ( $^{+}/_{-}$ 5mmHg). This was achieved over a 30 minute period by withdrawing blood from the jugular venous catheter, and collecting it in sterile blood bags containing Anticoagulant Citrate Phosphate Double Dextrose Solution with AS-3 (CP2D, Miles Pharmaceuticals Inc., Elkhart, Indiana). Shed blood was re-infused as necessary to maintain desired blood pressure during the shock. At the end of the shock period, blood was re-infused through a blood filter set (2C2141 Y-Type Blood Solution Administration Set and Pressure Pump, Travenol Laboratories Inc., Deerfield, Illinois). Aminals were resuscitated as required with Lactated Ringer's solution via the indwelling jugular venous catheter to restore MAP to baseline levels.

At T = 6hr., anesthesia was discontinued, and the endotracheal tube, thermometer, tonometer, and ECG leads were removed. Incisions were dressed with gauze and tensor bandages. Catheters were disconnected from monitoring and infusion lines, and were flushed with heparinized saline and capped. After awakening, animals were transported to holding cages at the research facility where they were provided food and water ad lib. Arterial blood samples (6 ml) were drawn from the arterial catheter for bacterial culture at T = 12hr, and every 12 hours until the conclusion of the experiment. Vascular catheters were flushed with the heparinized saline solution at the collection intervals in order to maintain patency.

## IV. Endotoxic Shock

At T = 47hr., animals were anesthetized according to the same protocol described for day 1 of the experiment. Endotracheal tube, thermometer, tonometer, ECG, and pressure transducers were all placed as before. Once again, intravenous Lactated Ringer's solution was administered by IMED pump at a rate of 20cc/h. After baseline measurements at T = 47.5 hours, animals from both groups received an intravenous infusion of endotoxin ( E.coli lipopolysaccharide, Serotype 011:B4, Sigma Chemical Co., St. Louis, Missouri) 25ucg/kg over 30 minutes administered via the jugular venous catheter. Animals were again monitored for a four hour period as during the hemorrhagic shock protocol. After the endotoxic shock period, resuscitation was again effected with Ringer's Lactate as required to restore baseline MAP. Anesthesia was then discontinued, all lines disconnected, and the animal returned to the holding cage after awakening.

## V. Tissue Sampling

Forty-eight hours after the second anesthetic period (T = 96hr), animals were brought to the research laboratory to undergo a final anesthetic in the same fashion as described for Day 1 and Day 3 of the protocol. The pig was placed in the supine position, and the abdomen was prepped and draped in the same fashion as that described for the placement of the indwelling cervical catheters. A laparotomy incision was performed, and tissue specimens for bacterial culture measuring approximately $1cm^3$ were obtained from the liver, kidney, spleen, and 3 mesenteric lymph nodes draining the colon.

Tissue glutamine samples consisting of skeletal muscle biopsies measuring $1cm^3$ were obtained at three points during the experimental protocol for each of the animal groups. Biopsy specimens were immediately frozen in liquid nitrogen, placed in labelled containers, and stored at $-80C^o$ until processing. The first specimen was

obtained from the belly of the right sternocleidomastoid muscle during the cervical dissection phase of the protocol on Day 1. The second specimen was obtained on Day 3, immediately prior to administration of endotoxin. A small skin incision was placed adjacent to the vertebral column to allow for a biopsy of the large para-vertebral skeletal muscle mass. The incision was then closed with 3-0 Ethilon in a continuous fashion. The final muscle biopsy was obtained immediately prior to euthanasia through a mirror incision of the second biopsy, placed over the opposing paravertebral muscle mass.

## VI. Euthanasia

Animals were sacrificed after organ sampling with an intravenous infusion of pentobarbital (0.5ml/kg Euthanyl; pentobarbital 240mg/ml, propylene glycol 0.2ml/ml, MTC Pharmaceutical, Cambridge, Ontario). Death occurred within 20 seconds.

## VII. Monitoring During Shock Periods

Baseline measurements were obtained at T = 1.5hr, and at T = 47.5 hr. Animals were monitored during each of the four hour shock periods from T = 2 to T = 6hr., and from T = 48 to T = 52hr. The data sheet used for data collection is illustrated below:

**Table 1: Animal Monitoring During Shock Periods**

Pig #___X___ Weight___X__ kg Group:
Control_____
Q-Pretx_

i.v. rate_____Xcc/h____ Resp.rate___X/min. $FiO_2$___X___ %

| Time | 1.5h | 2h | 3h | 4h | 5h | 6h | 47.5 | 48h | 49h | 50h | 51h | 52h |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| HR | X | X | X | X | X | X | X | X | X | X | X | X |
| MAP | X | X | X | X | X | X | X | X | X | X | X | X |
| Temp $^{o}C$ | X | X | X | X | X | X | X | X | X | X | X | X |
| $pCO_2$ | X | X | X | X | X | X | X | X | X | X | X | X |
| $hco_3^-$ | X | X | X | X | X | X | X | X | X | X | X | X |
| $pO_2$ | X | X | X | X | X | X | X | X | X | X | X | X |
| $SaO_2$ | X | X | X | X | X | X | X | X | X | X | X | X |
| pH | X | X | X | X | X | X | X | X | X | X | X | X |
| Sigm $pCO_2$ | X | X | X | X | X | X | X | X | X | X | X | X |
| $PLA_2$ | X | X 2.5h | | | | | | | | | | |
| pHi | X | X | X | X | X | X | X | X | X | X | X | X |

Tissue Glutamine Specimen #:

Baseline: X Pre-endotoxin: X Pre-Sacrifice: X

Tissue Glutamine:

Baseline: X umol/g Pre-Endotoxin: X umol/g Pre-Scacrifice: X umol/g

A. Weights were measured the morning of surgery, using the same scale each time, and reported as kilograms (kg).

B. I.V. Rate was maintained at a steady rate of 20 cc/h for each of the shock periods as previously described using the IMED infusion pump.

C. Respiratory rate was recorded directly from the ventilator apparatus and was reported as breaths/minute.

D. Fraction of inspired oxygen ($FiO_2$) was recorded directly from the setting of the oxygen blender on the ventilator apparatus, and was reported in per cent.

E. Heart rate was measured as described above utilizing the Hewlett Packard Monitor and ECG leads and was reported as beats per minute.

F. Mean Arterial Pressure was measured directly from the indwelling right internal carotid arterial catheter as displayed by monitoring equipment and reported in mmHg.

G. Temperature was recorded directly from the rectal thermocouple thermometer, and reported as degrees Celsius ($C^o$).

H. Partial Pressure of Carbon Dioxide of arterial blood ($pCO_2$) was measured from samples obtained from the carotid artery catheter, and reported in mmHg. This blood was drawn into a heparinized syringe, and immediately run through an electrode type automated pH and blood gas analyzer (IL 1306 pH/Blood Gas Analyzer, Instrumentation Laboratory, Lexington, Massachusetts).

I. Bicarbonate concentration ($HCO_3^-$) was calculated from the carotid blood sample by the blood gas analyzer and reported in mmol/L.

J. Partial Pressure of Oxygen in arterial blood ($pO_2$) was measured from the same arterial sample, as was the Oxygen Saturation ($SaO_2$), and reported as mmHg and per cent saturation respectively.

K. The pH of arterial blood was also calculated by the blood gas analyzer using the formula: $pH = -\log [H^+]$.

L. Sigmoid Mucosal Partial Pressure of Carbon Dioxide (Sigm $pCO_2$) was measured directly from 3ml saline samples obtained from the rectal tonometer, and reported as mmHg. Sigmoid mucosal pH (pHi) was calculated from the Henderson-Hasselbach equation using the $pCO_2$ of the saline sample according to the techniques set forth by Dr. Fiddian-Green [191,195], and based on the precept that intra-luminal bicarbonate concentration equals arterial bicarbonate concentration, and that the pK of tissue fluid is the same as that of plasma. $CO_2$ is freely diffusible between tissue fluid, cells, the lumen of the sigmoid colon, and the tonometer balloon. Therefore:

$$pHi = pK_a + [HCO_3^-]/ pCO_2 \text{ (tonometer)} \qquad pK_a = 6.10$$

M. Phospholipase $A_2$ Activity ($PLA_2$) was assessed at baseline at T = 1.5hr, prior to the onset of hemorrhagic shock. Repeat levels were assessed for both groups 30 minutes after acheiving target blood pressure (MAP 40mmHg) at T = 2.5hr. Samples(8ml) were obtained from the arterial catheter in vacuum tubes containing Na-citrate as anticoagulant (Vacutainer Cit·Na -0.129M, Silic. (1-10), Becton Dickinson VACUTAINER Systems, Rutherford, NJ). Tubes containing whole blood were subsequently centrifuged at 3500 rpm for 5 minutes, with removal of the top 3ml, representing platelet and cell-free plasma. The specimens were then flash-frozen in liquid nitrogen, and stored at -80C$^o$ until samples were processed in a batch fashion.

The Phospholipase assay was performed according to the technique described by Yokoyama et al (J Biol Chem, 1980 255(15) 7333-39), and involved the use of prepared unilamellar vesicles with tritiated Hydrogen ($^3H$) radiolabel at the SN-2 position of the phospholipid. The plasma samples (50ul) were combined with an incubation mixture consisting of 25ul of prepared vesicle (90ug PC L - $\alpha$ -1 - palmitoyl - 2 - [9,10 -$^3H$]palmitoylphosphatidylcholine), 90ul

of Ca solution (20mM $Ca^{++}$ - pH 7.6), and 135ul of Phosphate buffer system (10mM NaPi, 150mM NaCl, pH 7.4). During incubation for 100 minutes, the radiolabelled lipid was cleaved from the SN-2 position of the vesicle membrane phospholipid in direct proportion to the activity of Phospholipase in the plasma sample. The reaction was terminated, and the free radiolabel extracted with a mixture of 4ml chloroform ($CH_3OH$ 2:1), cold palmitic acid (10mg/100ml), and 700ul $H_2O$. Lipids were separated by thin layer chromatography using developing solvents of hexane/diethyl ether/acetate, (80:20:1,v /v /v /). Each lipid fraction was scraped, and its radioactivity recorded as counts per minute (cpm) in a scintillation counter (Beckman LS600TA Counter, Beckman Corp., USA).

N. Tissue glutamine specimens were processed according to the technique described by Jones et al (J Chromatography, 1983; 266:471-482). Samples were pre-treated by mixing 100ul sample, 100ul saturated $K_2B_4O_7$ solution, and 1.10 ml $H_2O$. 25 ul of this prepared sample was then mixed 1:1 with a fluoraldehyde reagent prior to injection into a high performance liquid chromatograph. Fluoraldehyde reagent consisted of 0.50g of OPA dissolved in 12.5ml methanol, which was subsequently combined with 112ml 0.04M sodium borate buffer (pH 9.5), 0.50ml 2-mercapto-ethanol, and 4ml Brij 35. Separation and quantification of amino acid was accomplished with the use of a Varian 5000 high performance liquid chromato-graph, and a Varian Fluorichrom detector (excitation 340nm, emission 450nm).

The mixed sample was injected into a Supelcosil 3 micron LC-18 reverse phase column (4.6x150mm; Supelco) equipped with a guard column (4.6x50mm) packed with Supelco LC-18 reverse phase packing (20-40um). Chromatographic

peaks were recorded and integrated using a Shimadzu Ezchrom Chromatography Data System, and reported as umol amino acid/g protein.

## VIII. Bacteriologic Monitoring

Arterial blood (6ml) was drawn for culture every 12 hours during the experiment from T = 12h, until just prior to laparotomy at T = 96h. Aliquots of 3ml were injected into aerobic (Bactec NR6A Culture Vial, Becton Dickinson Diagnostic Instruments System, Towson, Maryland), and anaerobic (Bactec NR7A Culture Vial) culture vials.

Culture vials were then transported to the Microbiology Laboratory at the University of Alberta Hospital, and placed through an automated blood culture analyzer (Bactec 860, Becton Dickinson Instruments System, Towson, Maryland). Each vial was sampled to determine the amount of carbon dioxide produced, and if significant, the vial opened and plated on the following media:

1. Blood Agar in Carbon Dioxide
2. Brain Heart Infusion
3. Chocolate Agar

A gram stain was also performed. All unopened vials were sampled twice a day for the first forty-eight hours, and then once a day for the next five days. Lack of significant carbon dioxide production at 5 days was interpreted as no growth.

At T = 96hr., liver, kidney, spleen, and 3 mesenteric lymph nodes draining the colon were sampled under sterile conditions. $1cm^3$ of each tissue sample was placed into a sterile sample jar and transported to the Microbiology Laboratory of the University of Alberta Hospital. Specimens were then ground in a sterile fashion and plated onto the following media:

1. Blood Agar (Carbon Dioxide)

2. McKonkey Agar

3. Brain Heart Infusion (Anaerobic)

4. Phenyl Ethyl Alcohol (Anaerobic)

5. Blood Agar (Anaerobic)

A gram stain was again performed. Plates were observed for two to five days, depending upon the medium. Identification protocol for these tissue cultures was identical to that described for the blood cultures.

Positive cultures were characterized as either true enteric organisms, or as contaminants. This classification was based upon previous work with this model involving a review of the literature [192,193,194], and discussion with a Clinical Microbiologist (Dr. J.A.Talbot - University of Alberta, Department of Medical Microbiology). Organisms considered common swine enteric flora, and contaminants are listed below:

**Table 2: Swine Enteric Flora**

| | |
|---|---|
| Enterococcus | *Enteroc.* |
| Escherichia coli | *E.coli* |
| Clostridia perfringens | *C.perf* |
| Enterobacter cloacae | *E.cloacae* |
| Gram (-) Bacilli | *G.(-) Bac* |
| Klebsiella species. | *Klebs.* |

**Table 3: Contaminant Organisms:**

| | |
|---|---|
| Coagulase (-) Staphylococci | *CNS* |
| Staphylococcus aureus | *S.aureus* |
| Streptococcus viridans | *S.viridans* |
| Diptheroids | *Dipth.* |
| B -hemolytic Streptococcus | B-*strep.* |
| Pasteurella multocida | *P.mult.* |
| Lactobacillus | *Lactob.* |
| non-perfringens Clostridia | *Clost.np* |
| Anaerobic gram(+) cocci | *Ag(+)cocci* |
| Anaerobic gram(+) bacilli | *Ag(+)bacil.* |

## IX. Statistical Method

Descriptive statistics and frequencies were determined for each treatment group by an independent Statistician (Dr. M. Grace). Animals were compared with respect to all variables, at all of the points at which measurements were obtained. The two-tailed T-test was used for all variables measured during the shock periods.

Bacteriologic data, and positive enteric cultures were analyzed using the Fisher's Exact Test. The level of significance was considered to be $<0.05$ unless otherwise stated.

# RESULTS

## I. Exclusion / Dropout Criteria and Mortality

Data for animals was dropped from the study only if the animal died prior to completion of the full five days. This occurred in two animals. The first animal became profoundly cyanotic, hypoxic, and bradycardic immediately after anesthesia induction on day 1 of the protocol, and expired due to cardiac dysrhythmia. Post-mortem study revealed severe congenital pulmonic valve stenosis, and associated lung changes consistent with pulmonary hypertension. The second death occurred in an animal after completion of Day 3 of the protocol. This animal had rubbed off the tape used to fixate the indwelling catheters, and subsequently pulled the catheters and transfixing sutures free from the vascular structures. When the animal was re-assessed 5 hours after completion of the protocol, it was in a moribund state due to profound hemorrhagic shock. The animal was subsequently euthanized with Euthanyl as previously described.

One other animal was excluded after completion of the experiment based on bacteriologic, and autopsy findings consistent with infection derived from a cause other than the experimental shock periods. This animal demonstrated profuse mucopurulent bronchial secretions, and pathologic lung findings consistent with bronchopneumonia. In addition, blood cultures remained persistently positive for Pasteurella multocida, a common etiologic agent in swine pneumonia.

## II. SHOCK PERIODS

### A. Comparability Between Groups

Both groups of animals were comparable with respect to weight, respiratory rate, and oxygen concentration of inspired air (**Table 4**). In addition, due to the fact that IV rate and fluids administered were strictly controlled, both animal groups received identical volumes of fluid during the course of the experimental protocol.

**Table 4: Set Variables For Control and Experimental Groups: (mean with S.D.)**

| | GROUP | | |
|---|---|---|---|
| Variable | Control | Quin-Tx | P-Value |
| **Weight (kg)** | 20.00 (4.16) | 19.75 (3.96) | **0.91** |
| **Respiratory Rate** | 12.57 (2.70) | 12.33 (4.23) | **0.90** |
| **$FiO_2$ (%)** | 57.14 (13.80) | 50.83 (7.36) | **0.34** |
| **IV Fluid Rate (cc/h)** | 20.00 (0.00) | 20.00 (0.00) | **1.00** |

### B. Heart Rate

There were no significant differences ($P > 0.05$) observed in heart rate between the two groups during either of the two shock periods. Profound tachycardia occurred in both groups in response to hemorrhage (**Table 5**), demonstrating that there was no, or minimal blunting of cardiovascular reflexes as a result of anesthesia.

**Table 5: Time vs Heart Rate(bpm) for T = 1.5h to T = 6h: Hemorrhagic Shock (mean with S.D.)**

| Time (hours) | GROUP | | P value |
|---|---|---|---|
| | Control | Quin - Tx | |
| 1.5h | 94.00 (14.93) | 94.43 (11.28) | **0.95** |
| 2.0h | 128.57 (20.68) | 122.86 (19.95) | **0.61** |
| 3.0h | 159.14 (40.84) | 153.29 (21.41) | **0.74** |
| 4.0h | 157.14 (34.98) | 161.14 (24.42) | **0.81** |
| 5.0h | 151.86 (38.68) | 163.00 (27.60) | **0.55** |
| 6.0h | 163.43 (35.04) | 168.71 (27.15) | **0.76** |

Minimal deviation in heart rate from baseline was observed for both groups after exposure to endotoxin (**Table 6**), despite a significant difference between the two groups with respect to MAP (**Figure 7**).

**Table 6: Time vs Heart Rate(bpm) for T = 47.5h to T = 52h: Endotoxic Shock (mean with S.D)**

| Time (hours) | GROUP | | P value |
|---|---|---|---|
| | Control | Quin - Tx | |
| 47.5h | 97.00 (12.86) | 92.86 (12.39) | **0.55** |
| 48.0h | 102.00 (13.86) | 100.14 (14.05) | **0.81** |
| 49.0h | 103.71 (10.53) | 95.00 (14.86) | **0.23** |
| 50.0h | 101.14 (16.39) | 96.86 (7.80) | **0.54** |
| 51.0h | 102.00 (15.30) | 92.57 (9.64) | **0.19** |
| 52.0h | 105.43 (15.47) | 94.71 (13.97) | **0.20** |

### C. Mean Arterial Pressure (MAP)

Mean arterial pressure was strictly controlled during the hemorrhagic shock period through removal or re-infusion of blood to maintain pressure at 40mmHg $^{+}_{-}$ 5mmHg. As such, MAP did not differ significantly between the two groups during the first shock period (**Table 7**).

**Table 7: Time vs MAP(mmHg) for T = 1.5h to T = 6h: Hemorrhagic Shock (mean with S.D )**

| Time (hours) | GROUP | | P value |
|---|---|---|---|
| | Control | Quin - Tx | |
| 1.5h | 94.00 (14.93) | 94.43 (11.28) | **0.95** |
| 2.0h | 40.86 (.90) | 39.71 (1.70) | **0.14** |
| 3.0h | 39.71 (0.76) | 41.71 (0.76) | **0.06** |
| 4.0h | 39.43 (2.15) | 39.43 (2.37) | **1.00** |
| 5.0h | 40.29 (1.11) | 40.71 (1.38) | **0.53** |
| 6.0h | 41.14 (1.46) | 41.14 (3.24) | **1.00** |

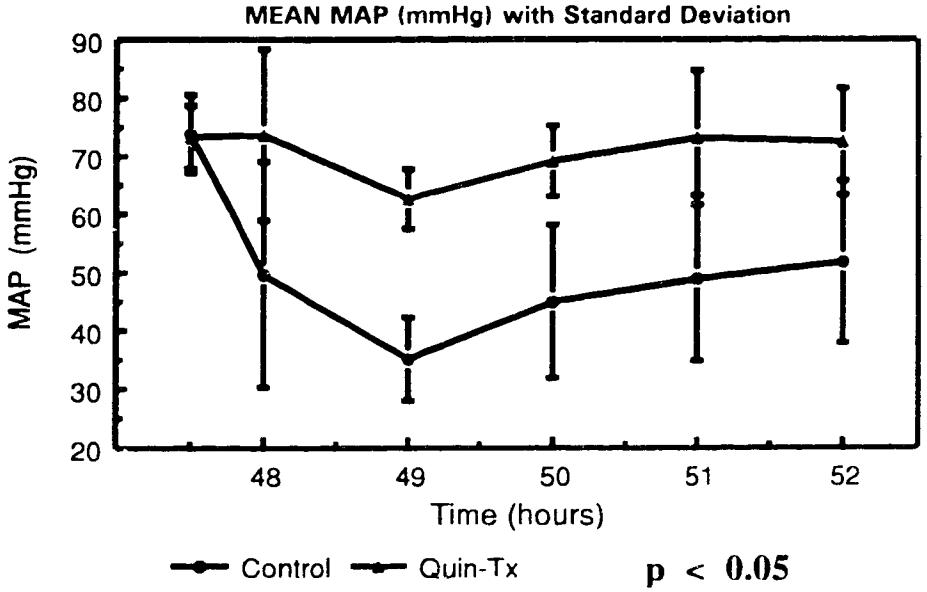
There was a significant difference in MAP between the control and quinacrine-treated groups after exposure to endotoxin, despite the fact that the level of shock induced during hemorrhage was identical, and that the baseline MAP at T = 47.5h prior to endotoxin administration did not show any significant difference (**Table 8**).

**Table 8: Time vs MAP(mmHg) for T = 47.5h to T = 52h: Endotoxic Shock (mean with S.D.)**

| Time (hours) | GROUP | | P value |
|---|---|---|---|
| | Control | Quin - Tx | |
| 47.5h | 73.86 (6.15) | 75.43 (7.44) | **0.67** |
| 48.0h | 50.57 (17.86) | 75.29 (14.09) | **0.01** |
| 49.0h | 38.29 (10.52) | 65.14 (7.71) | **0.000** |
| 50.0h | 48.00 (14.40) | 70.71 (6.92) | **0.003** |
| 51.0h | 51.71 (14.81) | 73.86 (10.73) | **0.008** |
| 52.0h | 55.57 (16.10) | 72.86 (8.45) | **0.03** |

Both animal groups demonstrated hypotension within 60 minutes of exposure to endotoxin (25ucg/kg). The control animals however, demonstrated a more profound hypotension that was persistent throughout the endotoxic shock period (**Figure 7**). Quinacrine-treated animals developed mild, transient hypotension, and recovered to baseline MAP well before the conclusion of the endotoxic shock period.

**Figure 7: Time vs MAP(mmHg) for T = 47.5h to T = 52h: Endotoxic Shock**



### D. Temperature

There were no significant rectal temperature differences between animal groups during either of the two shock periods (**Table 9, Table 10**). In addition, neither group demonstrated a significant febrile response to the endotoxic challenge, despite the obvious biological effect of endotoxin on mean arterial pressure (**Figure 7**).

**Table 9: Time vs Temperature( $C^0$) for T = 1.5h to T = 6h: Hemorrhagic Shock (mean with S.D.)**

| Time (hours) | GROUP | | P value |
|---|---|---|---|
| | Control | Quin - Tx | |
| 1.5h | 37.74 (0.84) | 37.94 (0.69) | **0.64** |
| 2.0h | 37.77 (0.81) | 38.09 (0.78) | **0.48** |
| 3.0h | 37.56 (0.98) | 38.19 (0.73) | **0.20** |
| 4.0h | 37.53 (1.13) | 38.20 (0.60) | **0.19** |
| 5.0h | 37.79 (1.24) | 38.07 (0.47) | **0.58** |
| 6.0h | 37.59 (0.92) | 38.23 (0.48) | **0.13** |

**Table 10: Time vs Temperature($C^0$) for T = 47.5h to T = 52h: Endotoxic Shock (mean with S.D)**

| Time (hours) | GROUP | | P value |
|---|---|---|---|
| | Control | Quin - Tx | |
| 47.5h | 37.61 (0.42) | 37.69 (0.78) | **0.84** |
| 48.0h | 37.49 (0.55) | 37.59 (0.78) | **0.79** |
| 49.0h | 37.36 (0.43) | 37.63 (0.77) | **0.43** |
| 50.0h | 37.53 (0.25) | 37.63 (0.64) | **0.71** |
| 51.0h | 37.94 (0.71) | 37.50 (0.80) | **0.30** |
| 52.0h | 38.17 (0.67) | 37.90 (0.80) | **0.51** |

### E. Arterial Partial Pressure of Carbon Dioxide ($PaCO_2$)

Arterial blood gases were monitored every 30 minutes in an attempt to maintain acid-base status of the animals within defined parameters. Respiratory rate and tidal volume were adjusted in order to maintain $PaCO_2$ betweeen 35 and 45 mmHg. Animals from both groups demonstrated a remarkable stability with respect to arterial partial pressure of $CO_2$ (**Table 11, Table 12**). There were no significant differences between the two groups with respect to $PaCO_2$ except at one measurement point during

endotoxic shock (T = 51h). This difference in $PaCO_2$ was appropriately compensated, since arterial pH did not show significant differences between the two groups (**Table 17, Table 18**).

**Table 11: Time vs $paCO_2$(mmHg) for T = 1.5h to T = 6h: Hemorrhagic Shock (mean with S.D.)**

| Time (hours) | GROUP | | P value |
|---|---|---|---|
| | Control | Quin - Tx | |
| 1.5h | 36.71 (8.01) | 40.36 (15.90) | **0.60** |
| 2.0h | 38.97 (6.30) | 38.36 (9.67) | **0.89** |
| 3.0h | 35.87 (4.40) | 40.60 (7.41) | **0.17** |
| 4.0h | 37.73 (4.38) | 40.24 (5.62) | **0.37** |
| 5.0h | 37.07 (6.44) | 39.44 (5.09) | **0.46** |
| 6.0h | 41.94 (5.17) | 37.14 (6.89) | **0.17** |

**Table 12: Time vs $paCO_2$(mmHg) for T = 47.5h to T = 52h: Endotoxic Shock (mean with S.D.)**

| Time (hours) | GROUP | | P value |
|---|---|---|---|
| | Control | Quin - Tx | |
| 47.5h | 43.56 (10.15) | 36.64 (5.64) | **0.14** |
| 48.0h | 41.00 (7.55) | 36.20 (3.70) | **0.16** |
| 49.0h | 41.46 (6.54) | 36.83 (1.32) | **0.09** |
| 50.0h | 41.10 (6.34) | 40.51 (6.66) | **0.87** |
| 51.0h | 44.86 (6.16) | 38.37 (2.58) | **0.03** |
| 52.0h | 42.94 (7.78) | 38.20 (5.34) | **0.24** |

### F. Arterial Bicarbonate ($hCO_3^-$) Concentration

Arterial bicarbonate concentration did not show any significant differences between the two groups of animals during the course of the experimental protocol, with the exception of the small differences recorded at T = 47.5h (**Table 13, Table 14**). Once again, due to the controlled fluid administration, and to the comparability with respect to arterial pH, this difference in arterial bicarbonate (**Control** 27.73 vs **Quin - x** 24.04) was not likely of clinical significance. In addition, both bicarbonate values at this timepoint reflected base excess, and not base deficit, as one might find in an under-resuscitated, hypovolemic animal.

**Table 13: Time vs Arterial $hCO_3^-$(mmol/L) for T = 1.5h to T = 6h: Hemorrhagic Shock (mean with S.D.)**

| Time (hours) | GROUP | | P value |
|---|---|---|---|
| | Control | Quin - Tx | |
| 1.5h | 26.90 (2.23) | 26.79 (2.41) | **0.93** |
| 2.0h | 27.03 (1.49) | 26.26 (2.43) | **0.49** |
| 3.0h | 25.27 (2.64) | 25.21 (1.48) | **0.96** |
| 4.0h | 25.56 (3.24) | 25.44 (2.64) | **0.94** |
| 5.0h | 24.97 (3.82) | 25.33 (3.46) | **0.86** |
| 6.0h | 23.94 (5.49) | 24.54 (3.48) | **0.81** |

**Table 14: Time vs Arterial $hCO_3^-$(mmol/L) for T = 47.5 to T = 52h: Endotoxic Shock (mean with S.D.)**

| Time (hours) | GROUP | | P value |
|---|---|---|---|
| | Control | Quin - Tx | |
| 47.5h | 27.73 (0.99) | 24.04 (3.25) | **0.01** |
| 48.0h | 25.86 (3.72) | 25.03 (5.24) | **0.74** |
| 49.0h | 24.87 (3.03) | 24.94 (4.43) | **0.97** |
| 50.0h | 25.46 (3.91) | 26.56 (5.47) | **0.67** |
| 51.0h | 26.00 (5.60) | 26.73 (3.96) | **0.78** |
| 52.0h | 25.70 (3.49) | 26.33 (6.41) | **0.82** |

## G. Arterial Oxygen Saturation ($SaO_2$)

The fractional concentration of inspired oxygen ($FiO_2$) was carefully regulated during the course of the shock periods to maintain the arterial oxygen saturation at >95% (**Table 15, Table 16**) corresponding to the flat, top portion of the oxygen-hemoglobin dissociation curve. As such, there were no clinically significant differences in arterial oxygen saturation betweeen the two groups of animals during the shock periods.

**Table 15: Time vs Arterial $O_2$ Saturation ($SaO_2$[%]) for T = 1.5h to T = 6h: Hemorrhagic Shock (mean with S.D.)**

| Time (hours) | GROUP | | P value |
|---|---|---|---|
| | Control | Quin - Tx | |
| 1.5h | 99.96 (0.05) | 99.79 (0.39) | **0.28** |
| 2.0h | 99.81 (0.36) | 99.91 (0.11) | **0.50** |
| 3.0h | 99.77 (0.36) | 99.69 (0.49) | **0.71** |
| 4.0h | 99.81 (0.25) | 99.63 (0.63) | **0.48** |
| 5.0h | 99.70 (0.42) | 99.71 (0.41) | **0.95** |
| 6.0h | 99.50 (0.60) | 99.71 (0.34) | **0.43** |

**Table 16: Time vs $SaO_2$(%) for T = 47.5h to T = 52h: Endotoxic Shock (mean with S.D.)**

| Time (hours) | GROUP | | P value |
|---|---|---|---|
| | Control | Quin - Tx | |
| 47.5h | 99.94 (0.05) | 99.89 (0.04) | **0.04** |
| 48.0h | 99.16 (1.61) | 99.84 (0.16) | **0.28** |
| 49.0h | 99.71 (0.46) | 98.96 (2.50) | **0.45** |
| 50.0h | 99.66 (0.49) | 99.89 (0.04) | **0.24** |
| 51.0h | 99.57 (0.60) | 99.89 (0.04) | **0.19** |
| 52.0h | 99.66 (0.37) | 99.84 (0.11) | **0.23** |

## H. Arterial pH

Arterial pH was measured every 30 minutes and recorded every 60 minutes during each of the shock episodes. Although the shock episodes resulted in hypotension (**Table 7, Table 8**) and profound mucosal acidosis (**Table 19,Table 20, Figure 8**) in both groups of animals , neither group demonstrated any significant arterial pH changes. The marked difference observed in sigmoid mucosal pH during endotoxic shock (**Figure 8**) did not show a correlation with arterial pH, indicating a low sensitivity of arterial pH to regional hypoperfusion.

**Table 17: Time vs Arterial pH( - Log [$H^+$] for T = 1.5h to T = 6h: Hemorrhagic Shock (mean with S.D.)**

| Time (hours) | GROUP | | P value |
|---|---|---|---|
| | Control | Quin - Tx | |
| 1.5h | 7.48 (0.12) | 7.46 (0.16) | **0.81** |
| 2.0h | 7.45 (0.06) | 7.45 (0.10) | **0.90** |
| 3.0h | 7.45 (0.04) | 7.40 (0.07) | **0.10** |
| 4.0h | 7.43 (0.03) | 7.40 (0.05) | **0.19** |
| 5.0h | 7.43 (0.05) | 7.41 (0.05) | **0.51** |
| 6.0h | 7.35 (0.15) | 7.43 (0.06) | **0.24** |

**Table 18: Time vs Arterial pH( - Log [$H^+$]) for T = 47.5h to T = 52h: Endotoxic Shock (mean with S.D.)**

| Time (hours) | GROUP | | P value |
|---|---|---|---|
| | Control | Quin - Tx | |
| 47.5h | 7.41 (0.09) | 7.42 (0.05) | **0.80** |
| 48.0h | 7.40 (0.07) | 7.43 (0.08) | **0.49** |
| 49.0h | 7.38 (0.09) | 7.41 (0.08) | **0.49** |
| 50.0h | 7.40 (0.10) | 7.42 (0.07) | **0.64** |
| 51.0h | 7.37 (0.13) | 7.44 (0.07) | **0.21** |
| 52.0h | 7.39 (0.11) | 7.43 (0.09) | **0.55** |

**I. Sigmoid Mucosal pH**

Hemorrhagic shock resulted in the development of mucosal acidosis in both groups of animals (**Table 19**) due to gut mucosal hypoperfusion. Since the level of shock, and therefore the level of mucosal hypoperfusion was controlled, there were no significant differences in mucosal pH between the two groups during hemorrhage. During the subsequent endotoxic shock period, the control group demonstrated the most severe mucosal acidosis (**Table 20**). Both groups of animals developed marked mucosal acidosis within 30 minutes of exposure to endotoxin (**Figure 8**). The control group, however, had a significantly greater mucosal acidosis, and this difference in mucosal pH compared to the quinacrine-treated group persisted for the duration of the endotoxic shock period.

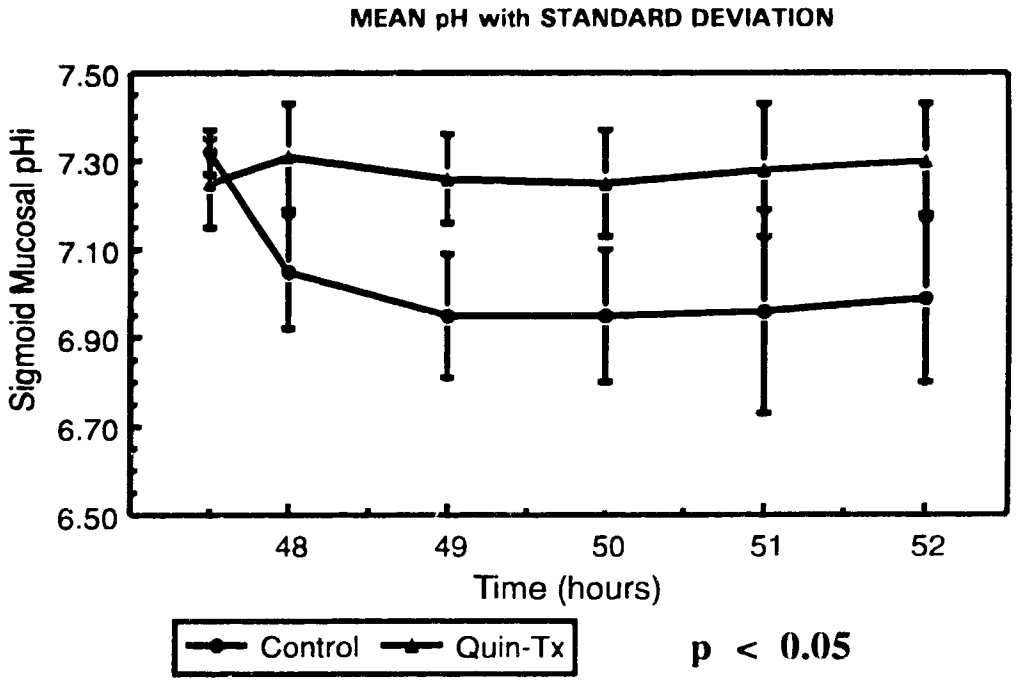
**Table 19: Time vs Mucosal pH( - Log [$H^+$]) for T = 1.5h to T = 6h: Hemorrhagic Shock (mean with S.D)**

| Time (hours) | GROUP | | P value |
|---|---|---|---|
| | Control | Quin - Tx | |
| 1.5h | 7.32 (0.05) | 7.26 (0.16) | **0.32** |
| 2.0h | 7.12 (0.10) | 7.02 (0.10) | **0.08** |
| 3.0h | 7.07 (0.14) | 6.99 (0.12) | **0.28** |
| 4.0h | 7.05 (0.12) | 6.99 (0.14) | **0.39** |
| 5.0h | 7.02 (0.13) | 6.98 (0.14) | **0.55** |
| 6.0h | 6.96 (0.14) | 6.98 (0.14) | **0.85** |

**Table 20: Time vs Mucosal pH( - Log [$H^+$]) for T = 47.5h to T = 52h: Endotoxic Shock (mean with S.D.)**

| Time (hours) | GROUP | | P value |
|---|---|---|---|
| | Control | Quin - Tx | |
| 47.5h | 7.32 (0.05) | 7.24 (0.09) | **0.16** |
| 48.0h | 7.07 (0.13) | 7.25 (0.19) | **0.05** |
| 49.0h | 6.99 (0.16) | 7.23 (0.13) | **0.01** |
| 50.0h | 6.97 (0.15) | 7.20 (0.17) | **0.02** |
| 51.0h | 6.99 (0.23) | 7.25 (0.16) | **0.03** |
| 52.0h | 7.01 (0.18) | 7.25 (0.19) | **0.03** |

**Figure 8: Time vs Sigmoid Mucosal pH for T = 47.5h to T = 52h: Endotoxic Shock**



## J. Phospholipase $A_2$

Prior to the onset of hemorrhagic shock, there were no significant differences in the serum phospholipase activity between the two groups of animals (**Table 21**). At 30 minutes after achievement of the target blood pressure (MAP 40mmHg) during the hemorrhagic shock period, significant differences in enzyme activity were demonstrated (**Figure 9**). The control group developed a marked elevation in activity in response to hemorrhage. Quinacrine-treated animals however, demonstrated a significant fall in phospholipase activity to levels below even those at baseline (prior to hemorrhage).

**Table 21: Phospholipase Data: CPM (counts per minute) $^3H$ • PCholine**

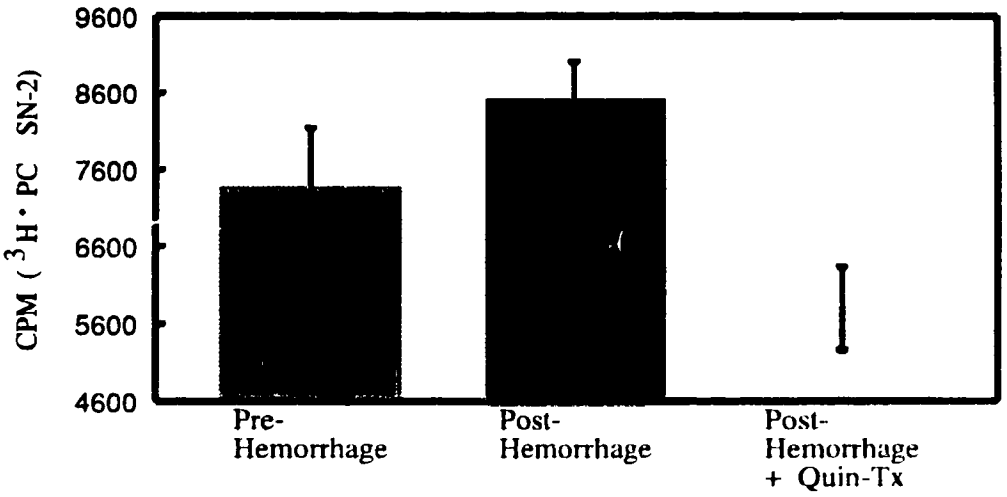
| Pre-Hemorrhage | Post-Hemorrhage (Control Group) | Post-Hemorrhage (Study Group) |
|---|---|---|
| 7019.0 | 8705.0 | |
| 6748.0 | 7719.0 | |
| 6876.0 | 8870.0 | |
| 6632.0 | 8638.0 | |
| 8889.0 | | 5206.0 |
| 8183.0 | | 5613.0 |
| 7189.0 | | 5900.0 |
| 7256.0 | | 6478.0 |

**MEAN CPM WITH STANDARD DEVIATION**

**7349.0 (785)** **8483.0 (519)** **5799.0 (535)**

$P < 0.05$ with t-test

**Figure 9: Phospholipase Activity Before and After the Onset of Hemorrhagic Shock in Control and Experimental Animals (Mean CPM with S.D.).**




## K. Tissue Glutamine

Tissue glutamine levels were assessed prior to both shock periods, and prior to the sacrifice of the animal on Day 5 of the protocol. Control animals demonstrated a net depletion of muscle glutamine during the course of the protocol (**Table 22**), while quinacrine-treated animals showed a modest increase in glutamine levels, suggesting preservation of overall nitrogen balance. Unfortunately, the trend towards higher glutamine levels in the quinacrine-treated group did not achieve statistical significance when compared to the levels observed in the control animals.

**Table 22: Tissue Glutamine Data: umol amino acid/g tissue**

| Animal # | SPECIMEN | | |
|---|---|---|---|
| | Control (pre-OR) | Pre-Endotoxin | Pre-Sacrifice |
| CONTROLS | | | |
| #3 | 2.885 | | 0.029 |
| #5 | 1.745 | | 3.936 |
| #7 | 1.268 | | 1.124 |
| #9 | 2.610 | 0.499 | 1.837 |
| #11 | 1.858 | 1.636 | 1.675 |
| #13 | 2.825 | 3.039 | 1.488 |
| #16 | 1.515 | 2.393 | 2.095 |
| MEAN (WITH S.D.) | 2.10 (0.66) | 1.89 (1.09) | 1.74 (1.18) |
| QUIN - TX | | | |
| #4 | 2.098 | | 2.110 |
| #6 | 1.802 | | 3.218 |
| #8 | 2.313 | 5.540 | 6.257 |
| #10 | 3.043 | 1.213 | 1.494 |
| #12 | 1.384 | 3.739 | 1.988 |
| #14 | 1.651 | 3.175 | 1.927 |
| #15 | 1.146 | 3.770 | 3.664 |
| MEAN (WITH S.D.) | 1.92 (0.64) | 3.49 (1.55) | 2.95 (1.65) |
| | | | |
| P - VALUE | 0.61 | 0.11 | 0.14 |

## III. BACTERIOLOGY

All positive cultures were categorized as either enteric organisms (**Table 2**), or as culture contaminants (**Table 3**). The positive enteric cultures, and the organism cultured for each animal are depicted below (**Table 23**). There was no statistically significant difference observed between the two groups in the rate of enteric bacterial translocation to blood, regional lymph nodes, or tissue (**Table 24**). The most common organism cultured was E.coli, followed by non-characterized coliform bacteria. Anaerobic bacteria were cultured with slightly higher frequency in the control group (C.perfringens X 3) as compared to the quinacrine-treated group (Bacteroides X 1). The small numbers render this data difficult to interpret. The only Enterococcal culture was detected in the control group. The overall rate of translocation of enteric organisms for both groups to blood was 4%, to regional nodes ,21%, and to tissue, 21%.

**Table 23: Bacteriology Summary Table: Positive Enteric Organism Cultures**

| Animal #: (Group) | Blood | Tissue | Regional Nodes |
|---|---|---|---|
| **CONTROL** | | | |
| #3 | 0/8 | 0/3 | 0/3 |
| #5 | 1/8 (C. perfringens) | 1/3 (E.coli) | 0/3 |
| #7 | 0/8 | 1/3 (Enterococcus) | 2/3 (E.coli x 1, Coliforms x 1) |
| #9 | 0/8 | 0/3 | 0/3 |
| #11 | 0/8 | 0/3 | 2/3 (E.coli x 1 Coliforms x 1) |
| #13 | 2/8(C.perfringens x2) | 0/3 | 2/3 (E.coli x 1 Coliforms x 1) |
| #16 | 0/8 | 2/3 (E.coli x 2) | 0/3 |
| **% (+) CULTURE** | **3/56 = 5.36%** | **4/21 = 19.05%** | **6/21 = 28.57%** |
| **Quin Tx:** | | | |
| #4 | 1/8 (G(-) bacillus) | 1/3 (Bacteroides sp.) | 2/3 (E.coli x 2) |
| #6 | 0/8 | 0/3 | 0/3 |
| #8 | 0/8 | 1/3 (E.coli) | 0/3 |
| #10 | 0/8 | 0/3 | 0/3 |
| #12 | 0/8 | 0/3 | 2/3 (E.coli x 1, Coliforms x 1) |
| #14 | 0/8 | 0/3 | 1/3 (E.coli) |
| #15 | 0/8 | 3/3 (E.coli x 3) | 0/3 |
| **% (+) Culture** | **1/56 = 1.79%** | **5/21 = 23.8%** | **3/21 = 14.29%** |

**Table 24: Bacteriologic Data Analysis: % Positive Enteric Cultures**

| Culture | Control | Quin - Tx | P-Value* |
|---|---|---|---|
| **Blood** | 5.36% | 1.79% | **0.31** |
| **Regional Nodes** | 28.57% | 14.29% | **0.26** |
| **Tissue** | 19.05% | 23.80% | **0.71** |

*Fisher's Exact Test

## DISCUSSION

### I. Experimental Design

Bacterial translocation is not an acute event in the swine model [69], and can be most reliably demonstrated after an interval of 48 hours from a systemically stressful event [92]. This explains the rationale for the design of the experiment, with a second physiological hit administered 48 hours after the priming insult, and tissue cultures obtained 48 hours after that. The nature of the priming, and subsequent insult (hemorrhagic shock, and endotoxic shock respectively) was determined by previous research [184] demonstrating the effectiveness of this regimen in eliciting the two-hit response. In addition, this regimen closely mimics the clinical situation in which the traumatized, hemorrhagic patient is resuscitated, only to develop sepsis ( e.g.pneumonia, catheter infection, wound infection) in the critical care setting. The main interest of the study was related to the priming effect, and the potential to limit this response by way of PLA2 inhibition. The Wigger's model of hemorrhagic shock[196] with profound hypotension (MAP 40mmHg) was utilized in order to maximize the physiologic priming response, and thereby maximize any differences in the control vs the treated groups with respect to observed variables. The minimal nature of the surgical intervention (simple neck dissection) reduced the potential for "experimental impurity" related to post-operative pain, impairment of appetite and fluid intake, and manipulation of the bowel. It has been clearly demonstrated in the literature that diminished oral food intake[41,42], and bacterial overgrowth associated with post-operative ileus[38,39], as well as simple manipulation of the bowel during laparotomy[36,37], are themselves promoters of the translocation response.

Bacterial endotoxin was used to mimic the effect of a large bacterial load, without the complications related to managing an actual infection. The decision to administer the endotoxin by way of the indwelling venous catheter was based primarily

on the observation that bacteria translocating from the intestinal lumen are first observed in the regional lymphatics[92]. This lymphatic load of organisms and/or endotoxin must subsequently re-enter the systemic circulation by way of the lymphatico-venous anastomoses present in the neck (thoracic duct). Direct venous administration most closely represented the clinical situation.

The swine model was chosen based upon previous research in our laboratory demonstrating the ability of this model to withstand the sequential shock protocol[184]. In addition, the swine model is similiar to the human patient with respect to protein and carbohydrate metabolism, as well as cardiovascular responses to systemic stress[197]. Human and pig levels of gut-associated xanthine oxidase, an enzyme thought to play a role in free-radical related mucosal damage, are also closely related[75,76].

The anesthetic agents employed in the protocol were chosen based upon their short duration of action, as well as their minimal effects on cardiovascular reflexes, cardiac sensitization to catecholamines, and hepatic metabolism[198]. Althought the animals never became completely apneic, intubation and ventilation were required to maximize systemic oxygenation, and prevent complications related to aspiration during recovery from anesthesia.

## II. Hemorrhagic Shock

Hemorrhagic shock was induced over a period of 30 minutes using the Wigger's technique[196] to acheive a target MAP of 40mmHg. A total of 800 - 1000ml of blood was removed in order to acheive the shock state, and blood was removed, or re-transfused in order to stabilize the animal at the target level. The main function of the shock period was to elicit a maximal priming response. Although the target arterial blood pressure chosen was lower than that commonly encountered in the clinical situation, it was felt that this would represent a maximal insult. Tachycardia developed in both animal groups only after at least 30 minutes at target MAP, likely representing a

period of occult, or compensated shock. Both groups of animals demonstrated a significant sigmoid mucosal acidosis early after the onset of shock, that persisted for the duration of the observation period. This is best explained by reflex splanchnic vasoconstriction in response to massive systemic hypoperfusion[59,60]. Despite the prolonged hypotensive state, and the sigmoid mucosal acidosis, arterial pH and blood gas values did not move significantly from baseline in either group, suggesting a low sensitivity to regional hypoperfusion. This has important implications for the management of the critically ill patient, who may be fully resuscitated according to classical parameters (e.g. ABG's, urine output, central venous pressure) and still suffer from splanchnic hypoperfusion. A number of studies have confirmed the utility of tonometry both in the experimental setting, in a swine model of mesenteric ischemia[201], and in the clinical setting to asses bowel viability after abdominal aortic aneurysm repair[199,200]. These studies have also demonstrated a poor correlation between arterial, and mucosal, acid-base assessment. Gastric mucosal acidosis, present in the critically injured patient on admission to the ICU, along with persistent mucosal acidosis despite full resuscitation based on conventional parameters, has been shown to correlate with patient mortality[202]. A number of authors have suggested routine tonometric assessment of the critically ill patient, as a necessary feature of optimal management[203]. The degree of mucosal acidosis and tachycardia elicited during the hemorrhagic insult served to validate this shock period as a significant systemic, physiologic insult.

Phospholipase A2 (PLA2) levels in the serum were significantly elevated, compared to baseline, in the control group after the achievement of target MAP. This corresponds with a large body of literature demonstrating an early rise in PLA2, and accumulation of PLA2 products of activation (e.g. PAF) after virtually any stimulus related to critical illness[115-123]. The quinacrine-treated animals demonstrated a significant inhibition of PLA2 levels below baseline, despite the hemorrhagic stimulus for

enzyme activation. The dose of quinacrine utilized (10mg/kg) was chosen primarily based upon rodent studies demonstrating efficacy with respect to PLA2 inhibition[145]. In addition, dose-response curves for quinacrine and PLA2 have been developed in *in-vivo* studies confirming near complete inhibition of PLA2 activity at this dose[144,204]. No series are reported that have assessed this dosage regimen in a large mammalian model. There were no significant side effects noted with administration of quinacrine by way of bolus infusion. A mild, transient drop in MAP (<5mmHg X 5-10 minutes) was observed in some of the animals from each group. A potential criticism of our protocol is the lack of a dose-response curve with respect to quinacrine and PLA2 activity in the swine model. The degree of remaining PLA2 activity was not assessed - It is clear that complete inhibition was not achieved. This may have resulted in less difference between groups with respect to measured variables than might have been achieved through complete inhibition. In addition, the decision regarding the early (30 minutes) measurement of PLA2 activity after hemorrhage was arbitrarily based upon the fact that quinacrine is not an irreversible inhibitor of PLA2[143]. Construction of a dose response curve for quinacrine on PLA2 activity, the use of a continuous quinacrine infusion, or multiple measurement points for PLA2 activity during the protocol would have provided a better representation of PLA2 activity during priming. Financial constraints however, restricted assay determination to one timepoint. With respect to measured variables in our protocol, PLA2 activity represented the only significant difference between the two groups of animals prior to the onset of endotoxic shock.

### III. Endotoxic Shock

Baseline measurements obtained prior to the administration of endotoxin did not differ significantly from those observed prior to hemorrhage, either within, or between, each group. Both groups of animals demonstrated a hypotensive response to endotoxin within 30 minutes of adminstration. The quinacrine-treated animals however, developed

only mild hypotension compared to that observed in the controls, and were able to recover to baseline MAP early during the endotoxic shock episode. Thus, PLA2 inhibition prior to the 1st insult appeared to protect the experimental animals from the hypotensive response to endotoxin. High PLA2 levels, and associated elevations of platelet-activating-factor (PAF) have been implicated in the pathogenesis of hypotension during endotoxic shock[124,125,132]. Despite the severe hypotension that occurred in the control group, there was no significant tachycardic reponse in these animals after exposure to endotoxin. This suggests an inhibition, or a blunting of the cardiovascular reflexes responsible for shock compensation, and may represent an important component of the lethality of endotoxin in the critically ill patient. PAF, one of the primary metabolites of PLA2 activation, demonstrates a negative inotropic effect on the heart[111], and may also have played a role in the blunted chronotropic response to hypotension. It is important to note that previous research in our laboratory has demonstrated that swine receiving the same dose of endotoxin, in the absence of a prior priming insult, develop a massive tachycardia in association with a hyperdynamic circulatory state[184]. This dichotomy after endotoxin exposure in our swine model implicates the priming phenomenon in the subnormal response to the secondary insult evident in the control animals. Although not well characterized, this priming response is felt to represent a turning on of the inflammatory machinery of the cell, possibly related to conversion of endothelium to an inflammatory phenotype with pro-coagulant activity[67,68], and expression of neutrophil binding/activating factors[70-71]. PAF, produced by way of PLA2 activation, is a potent priming agent with respect to inflammatory cell function[132,133,135], and lysosomal enzyme release[138]. Furthermore, elevated PAF levels are themselves capable of up-regulating PLA2 activity in an autocrine fashion[123], possibly leading to a cascade effect. In addition, low concentrations of PAF have demonstrated synergism with endotoxin and tumor necrosis factor, on hypotension, and gut mucosal ischemia[136]. Elevated PLA2 levels in the

control animals after hemorrhagic shock would have resulted in a priming of the inflammatory cell mass, with subsequent magnification of the deleterious responses noted after endotoxin. This represents the essence of the two-hit theory.

Sigmoid mucosal acidosis developed early after administration of endotoxin in the control group, and persisted for the duration of the observation period. This was significantly different from the response observed in the quinacrine-treated group, where no mucosal acidosis occurred. Once again, a poor correlation was observed between arterial blood gas values, which remained stable during endotoxemia in both groups, and mucosal pH values. The acidosis observed in the control group may have been largely related to the degree of hypotension elicited by endotoxemia, and subsequent reflex splanchnic vasoconstriction. A role for the priming response in the development of the profound mucosal acidosis is also likely. Endotoxin is itself capable of eliciting splanchnic vasoconstriction, independent of its effects on arterial blood pressure. This effect can be clearly blocked by administration of either a cyclo-oxygenase inhibitor[109], or a leukotriene receptor antagonist[110], suggesting a role for metabolites of arachidonic acid in this response. Most of the arachidonic acid release from membrane-bound phospholipid results from the activation of PLA2[111]. Both PAF receptor antagonist[108], and PLA2 inhibitor (quinacrine)[127,127,145] have been demonstrated to eliminate the deleterious effects of elevated PLA2 on gut mucosal ischemia and vascular permeability.

Due to the absence of specific biochemical determinations in this study, with the exception of a limited PLA2 activity assay, the specific biochemical mechanisms responsible for the prevention of hypotension and gut mucosal acidosis in the quinacrine-treated animals can only be postulated. It is clear from this study that PLA2 inhibition prior to a priming hemorrhagic insult, affords protection for the animal from the deleterious responses to subsequent endotoxemia. This obviously suggests a role for PLA2 activation in the priming response to major physiologic insult.

## IV. Tissue Glutamine

Tissue glutamine levels were assessed prior to hemorrhagic shock, prior to endotoxic shock, and prior to animal sacrifice. The second assessment point reflected the effects of hemorrhagic shock on intracellular glutamine. This assessment point was added only after completion of 5 animals, in order to provide some information regarding the effect of hemorrhagic shock on amino acid flux. As such, information regarding this effect was absent for the first 5 animals. Despite the fact that no significant differences were observed with respect to tissue glutamine, either within, or between groups, there was a trend towards preservation of levels in the quinacrine-treated animals after hemorrhagic and endotoxic shock, that was not observed in the controls. The lack of significance may have been related to the small numbers, and possibly also to the lack of complete PLA2 inhibition. This trend towards preservation of glutamine in quinacrine-treated animals may be interpreted as indicative of improved intestinal mucosal "health" in this group.

A number of studies, for example, have demonstrated intramuscular glutamine depletion following physiologic injury[155-160] . The degree of this depletion seems to correlate with the severity of the metabolic insult. There is also abundant evidence implicating the gut as a primary site of glutamine utilization[150,152,170], and a primary source of the impetus for skeletal muscle glutamine mobilization[179,183], during metabolic stress. Massive enterectomy is associated with a marked decrease in peripheral skeletal muscle glutamine efflux. Gut-mediated tissue glutamine depletion may also be inhibited by exogenous administration of glutamine[152,172]. This may represent another explanation for the overall lack of significant depletion of tissue glutamine observed in our study. Animals from both groups were fed ad lib, in an oral fashion, between each shock period, with a regular formula containing glutamine. Such a situation is rarely encountered in the clinical setting. Enteral glutamine, as well as other luminal nutrients may therefore have diminished the mobilization of peripheral muscle glutamine. In

addition, enteral nutrition has been demonstrated to exhibit a trophic, or protective effect on bowel mucosal integrity after stress[150,155,174-178], and restoration of intestinal blood flow after hemorrhagic shock[182]. This has obvious implications with respect to prevention of bacterial translocation, discussed below.

## V. Bacterial Translocation

Previous research utilizing the swine model in our laboratory has demonstrated significant rates of enteric bacterial translocation as a result of the two-hit, sequential shock protocol[184]. In these historic studies, translocation of enteric organisms to blood was demonstrated in 50% of blood cultures, and a high proportion of tissue and regional node cultures. In addition, there was a significant number of positive Enterococcal cultures, an organism known to be associated with increased organ failure rates, and mortality in the critically ill patient[205,206]. Translocation of enteric bacteria was markedly increased in animals subjected to the sequential shock protocol, compared to those animals recieving only a single physiologic insult[55,69]. This provided direct evidence of the two-hit phenomenon with respect to bacterial translocation. Translocation of virulent enteric bacteria has also been frequently demonstrated in rodent models of physiologic stress[51,88,89,93]. The rodent studies, however, have been criticized for their failure to recognize the significant differences between rodent, and large mammal (including human) levels of gut mucosal xanthine oxidase[61-64,75,76]. Elevated xanthine oxidase activity enhances production of oxygen free-radicals during ischemia, and therefore enhances gut mucosal injury during critical low-flow states. Human studies, for example, have not convincingly, or consistently demonstrated translocation, despite the subsequent development of MOD, and a "septic" state[46,49].

Translocation of enteric bacteria to blood, regional lymph nodes, and tissue was not observed with the same frequency in our study, as in the historical studies from our

laboratory. Positive cultures for enteric organisms were demonstrated overall in only 4% of blood, 21% of nodal, and 21% of tissue samples. In addition, despite the marked differences between the two study groups with respect to mucosal acidosis, and systemic arterial blood pressure during endotoxemic shock, there were no significant differences in the rates of translocation between groups. The types of organisms encountered also did not demonstrate study group specificity. In contrast to previous observations, virulent bacteriologic cultures such as anaerobes, and Enterococcus were infrequently demonstrated.

One possible explanation for the low level of translocation observed in the sequentially shocked animals relates to sampling. Blood cultures were assessed at q12 hourly intervals, with attendant risk of missing significant bacteremic episodes. Previous studies, however, have demonstrated bacteremia using this protocol[184]. A more important contributing factor relating to the low levels of translocation observed may be the relative decrease in the number of promoters of translocation present. Our previous studies have utilized a laparotomy prior to hemorrhage, with the associated features of bowel manipulation, post-operative ileus, and post-operative pain and decreased food intake. All of these factors have been shown to promote translocation in experimental models. The cumulative effect of these factors, in a sequentially shocked animal, may have led to the increased translocation rates observed. Our current protocol did not involve major surgical intervention, and no analgesia was required post-operatively. Animals were fed within 4 hours of surgery, and demonstrated no evidence of ileus, or impaired appetite. The role of luminal nutrition on translocation in our protocol has been discussed, and may also have contributed to the low rates of translocation observed. The 21% rates of positive nodal and tissue cultures were not associated with a similiar frequency of positive blood cultures (4%). This has important clinical implications, and confirms our previous observations that blood cultures obtained from a critically ill patient may not adequately represent the bacteriologic basis of a septic state.

The fact that translocation rates between groups was similiar, despite the clear differences in mucosal and systemic perfusion status (mucosal pH, MAP) during endotoxemia, brings into question the significance of translocation during physiologic injury. It may be that translocation in a large mammal, as some literature suggests[45], represents a normal physiologic process, or epiphenomenon associated with injury, by which the organism samples antigenic load in order to upregulate immunologic function. It is also possible that during physiologic stress, the presence of promoters of translocation such as laparotomy/intestinal handling, or loss of enteral nutrition, impart a much greater significance to the translocation of enteric bacteria with respect to the development of subsequent organ dysfunction.

## CONCLUSIONS

The purpose of this study was to examine the effect of PLA2 inhibition, administered prior to a priming insult consisting of hemorrhagic shock, on the previously observed deleterious responses to subsequent endotoxemia. Specifically, the effects of PLA2 inhibition on hemodynamics, tissue glutamine flux, and bacterial translocation were assessed.

We have provided evidence for the important role of PLA2 activation in the responses to severe physiologic insult. PLA2 levels demonstrate a marked elevation in response to hemorrhagic shock, and this elevation can be inhibited with quinacrine hydrochloride pretreatment. PLA2 inhibition prior to priming results in a protection of the organism from severe hypotension and mucosal acidosis observed during subsequent endotoxemia, implicating PLA2 activation as an important feature of the priming response. In the absence of other promoters of bacterial translocation such as post-operative ileus, decreased oral intake, and intra-operative bowel manipulation, sequential shock alone is not associated with significant rates of translocation. This calls into question the significance of observed translocation during a single physiologic insult. Although PLA2 activation appears to play a role in systemic and mucosal perfusion changes after severe metabolic insult, there does not seem to be a relationship between PLA2 activation and either bacterial translocation, or peripheral tissue glutamine flux. Further biochemical studies focussing on inflammatory mediator release during critical injury are required to provide a more specific profile of the priming, and secondary responses. The development and use of more specific, irreversible PLA2, and PLA2 product inhibitors, and assays for the products of PLA2 activation (e.g. PAF) is ongoing, and will enhance our understanding of the pathophysiology and treatment of MOD.

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