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Effects of Trauma-Hemorrhagic Shock on Hepatocellular Signal Transduction Mechanism and Properties of Enzymes Concerned with Glucose Metabolism: Beneficial Effects of ATP-$MgCl_2$ Treatment

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Mohammad S. W. Mahmoud

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EFFECTS OF TRAUMA-HEMORRHAGIC SHOCK ON HEPATOCELLULAR SIGNAL TRANSDUCTION MECHANISM AND PROPERTIES OF ENZYMES CONCERNED WITH GLUCOSE METABOLISM: BENEFICIAL EFFECTS OF ATP-$MgCl_2$ TREATMENT

By

Mohammad S. W. Mahnoud

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ABSTRACT

EFFECTS OF TRAUMA-HEMORRHAGIC SHOCK ON HEPATOCELLULAR SIGNAL TRANSDUCTION MECHANISM AND PROPERTIES OF ENZYMES CONCERNED WITH GLUCOSE METABOLISM: BENEFICIAL EFFECTS OF ATP-$MgCl_2$ TREATMENT

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Severe hemorrhage, leads to subtle alterations that occur in the microcirculation and at the cellular level. Such changes may become progressive and lead to multiple organ failure (MOF). The liver is a key organ involved in MOF. Since the primary insult of all types of shock occurs at the plasma membrane and disturbances in hepatic glucose regulation can contribute to the potentially lethal hypoglycemia during the late stages of shock; This dissertation focused on the study of how trauma-hemorrhagic shock and crystalloid resuscitation (shock) affects

hepatocellular signal transduction mechanism and properties of enzymes concerned with glucose metabolism. The second focus was on the effects of ATP-$MgCl_2$ treatment on the aforementioned processes.

The first hypothesis tested was that shock alters the characteristics of P_2-purinoceptors. *In vitro* radioligand binding assays demonstrated that there was a significant loss in receptors number (B_{max}) with no change in affinity (K_d). ATP-$MgCl_2$ treatment restored B_{max} and enhanced the affinity.

The second hypothesis tested was that shock alters, in addition to receptors dynamics, transmembrane coupling. Hepatocytes were stimulated with receptor-dependent (RD) and receptor-independent (RID) stimuli of both second messengers cAMP and IP_3. Results indicate that following shock RD stimuli failed to elicit cAMP or IP_3 accumulation. In contrast RID stimuli expressed their responses normally. ATP-$MgCl_2$ treatment restored the ability of hepatocytes to respond to RD stimulation.

The third hypothesis tested was that shock alters the properties of enzymes concerned with glucose metabolism. Results revealed that the activity and mRNA levels of glucokinase (GK) were not affected. Shock depressed the activity of phosphoenolpyruvate carboxykinase (PEPCK) and increased the activity and mRNA levels of pyruvate kinase (PK). ATP-$MgCl_2$ treatment alleviated the depression of PEPCK activity and decreased the activity and mRNA levels of PK.

The results presented leads us to conclude that shock alters hepatocellular signal transduction and differentially affects properties of enzymes concerned with glucose metabolism. The mechanism(s) of the beneficial effects of ATP-$MgCl_2$ treatment may include restoration of the signal transduction mechanism and alleviating the adversary effects of shock on glucose pertained enzymes.

This thesis is dedicated to my wife Ghadah M. Ashour, to whom I am deeply and eternally grateful.

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ABBREVIATIONS

ATP = adenosine triphosphate
BSA = bovine serum albumin
cAMP = cyclic adenosine monophosphate
cl = chlorine
DNA = deoxyribonucleic acid
DMSO = dimethylsulfoxide
EDTA = ethylenediamine tetracetic acid
EGTA = ethylene glycol bis (betaaminoethylether)-N,N'-tetraacetic acid
fbs = fetal calf serum
HEPES = N-2-hydroxyethylpiperazine-N'-2 ethanesulfonic acid
IBMX = 3-isobutyl-1-methylxanthine
IDP = inosine 5'-diphosphate
IP_3 = D-myo-inositol 1,4,5-triphosphate
K^+ = potassium
MAP = mean arterial pressure
MEM = meniumum essential media
mRNA = messenger RNA
Na-F = sodium fluoride
Na^+ = sodium
NADH = nicotinamide adenine dinucleotide
NADPH = nicotinamide adenine dinucleotide phosphate
$P_{sq}0_2$ = subcutaneous oxygen tension
$P_{tc}0_2$ = transcutaneous oxygen tension
RNA = ribonucleic acid
TCA = trichloroacetic acid
TNF = tumor necrosis factor

INTRODUCTION

The most frequent causes of death after an injury and severe hemorrhage are sepsis and the failure of various organs including the liver, lungs, kidneys, clotting system, and cardiovascular system (1). Multiple or sequential organ failure continues to be a frequent cause of death in patients who survive the first few hours after an injury with significant blood loss, and who do not have a major central nervous system injury (2). Studies have also shown that the liver is one of the major organs involved in multiple organ failure (3).

Although hepatic failure following hemorrhage, trauma, and sepsis, is well recognized, it is generally thought to be a late complication following pulmonary and renal failure (2). However, it has been recently reported that hepatocellular dysfunction occurs very early following hemorrhage and persists despite crystalloid resuscitation, and may form the basis of hepatic failure observed following severe and prolonged hemorrhage (4). Therefore, the examination of specific hepatocellular functions under such conditions could enhance our understanding of hepatic failure and the failure of other organs observed following trauma-hemorrhagic shock. The information derived from such studies, however, could also be helpful in providing

therapy for trauma-hemorrhagic shock victims when fluid resuscitation alone is unsuccessful.

Hemorrhagic shock leads to acute loss of circulating blood volume and, thus, tissue perfusion is diminished. As a result of prolonged reduction in tissue perfusion, various alterations in tissue metabolism, structure, and function occur at the systemic, cellular, and subcellular levels. It is generally believed that the primary insult on the cell, following shock, occurs at the cell membrane (5). Among the most important functions that the cell membrane performs is signal transduction. This vital cellular process is composed of three different membrane components including membrane receptors, transmembrane signaling elements (coupling elements, e.g., G-proteins), and membrane effectors (e.g., adenylate cyclase and PIP_2 systems). A disruption in the signal transduction mechanism would result in a loss of control of certain vital cellular activities. Examination of the effects of hemorrhagic shock on signal transduction is, therefore, a critical question in terms of understanding alterations in various cellular functions observed following such conditions. Consequently we examined two representative steps of the signal transduction mechanism in rat hepatocytes, in order to determine whether hemorrhagic shock alters this mechanism. The first representative step of the signal transduction mechanism examined in this study was the dynamics of P_2-purinoceptors. These receptors were chosen for the following reasons: they are specific for ATP and they are involved in hepatic glucose homeostasis (6-12).

Furthermore, changes in various receptor characteristics with conditions such as hemorrhage, ischemia, hypoxia, and endotoxemia are well established (13-18). Therefore, it is possible that hemorrhagic shock alters the dynamics of P_2-purinoceptors in the liver and this may be a factor in the altered glucose homeostasis also observed following shock.

The second representative step of the signal transduction mechanism examined in this study was the ability of the second-messengers (cAMP and IP_3) to respond to receptor-dependent as well as receptor-independent stimulation following hemorrhagic shock. These experiments were designed to determine whether membrane effectors and/or transmembrane coupling elements are also altered following shock.

P_2-purinoceptors are coupled to two signal transduction systems leading to inhibition of cAMP generation and to production of IP_3 in rat hepatocytes (10). Alterations in various receptor-transductional coupling are well-recognized following adverse circulatory conditions (19-22). Consequently, if the effectiveness of the second-messenger systems is decreased, a loss of control of certain vital cellular activities could result. Both steps in signal transduction studied would explain why various external signals fail to evoke their intended cellular response following shock and identify which components of the signal transduction mechanism are altered following such a condition.

One may pose the question of why study the key enzymes of glycolysis and glyconeogenesis in this respect? The maintenance of a constant

blood glucose level is essential for normal physiological functions in the body, particularly in the central nervous system. The liver plays a central role in this biological homeostasis. Disturbances in glucose homeostasis are recognized during shock conditions and characterized by initial hyperglycemia changing to hypoglycemia in the terminal stages of shock (23-28). Hemorrhagic shock causes the release of various agents and mediators such as cytokines (29). These agents are known to have profound effects on enzymatic activities such as enzymes of gluconeogenesis (30). However, it is not known whether hemorrhagic shock has any effects on such enzymes. Therefore, studying the key enzymes of glycolysis and gluconeogenesis would provide better understanding of how hemorrhagic shock alters glucose metabolism on the subcellular (enzymatic) level.

There is ample evidence that ATP-$MgCl_2$ administration following hemorrhage, ischemia, and sepsis has a myriad of beneficial effects on organ, cellular, and subcellular functions (5,31). ATP-$MgCl_2$ was also shown to correct various metabolic disturbances observed following shock (5). Furthermore ATP-$MgCl_2$ treatment significantly improved survival of animals subjected to shock (5,31). However, the mechanism(s) of action of this agent is (are) not clearly understood. It is hypothesized that ATP receptors (P_2-purinoceptors) are involved in this mechanism. Therefore, the second aim of this dissertation focused on the determination of whether ATP-$MgCl_2$ administration following shock has any salutary effects on the examined process. This can enhance our understanding of

the mechanism(s) by which ATP-$MgCl_2$ exerts its beneficial effects.

Arrangement of the Dissertation

Following the introduction and literature review, a detailed outline of all the experimental methods used to conduct this research will be presented. The third part presents the results obtained from this work. The fourth and final section will discuss the results presented in part three and provide conclusions resulting from this study as well as shortcomings and future directions.

Resulting Publications

The work presented in this dissertation has led to the following manuscripts being submitted to the American Journal of Physiology for publication.

Papers:

1. Mahmoud, M.S.W., P. Wang, S.R. Hootman, S.S. Reich, and I.H. Chaudry. Effects of hemorrhage and resuscitation on hepatocyte P_2-purinoceptors: I. Down Regulation of Receptor Numbers.
2. Mahmoud, M.S.W., P. Wang, S.R. Hootman, S.S. Reich, and I.H. Chaudry. Effects of hemorrhage and resuscitation on hepatocyte P_2-purinoceptors: II. Increased Affinity and Restoration of Receptor Numbers with ATP-$MgCl_2$-treatment.

Two additional papers are in the process of completion and they will be submitted for publication.

In addition, the following abstract was selected as one of the four finalists in the Young Investigator Award, presented at the Shock Society, fifteenth annual conference on shock. Point Clear, Alabama, June 7-10, 1992.

Abstracts:

1. Mahmoud, M.S.W., S.R. Hootman, P. Wang, and I.H. Chaudry. Mechanism of the Beneficial Effects of ATP-$MgCl_2$ Following Hemorrhage (HEM): Increased Affinity and Restoration of P_2-Receptor (P_2-R) Numbers. *Circ. Shock* 37(1):21, 1992.

LITERATURE REVIEW

Historical Perspective

As the care of injured patients has evolved and improved, various organ systems have in turn been the limiting factor affecting recovery after an injury, severe hemorrhage, or major operation. Although patients after operation or injury with significant blood loss may die from a number of specific or general complications, there has usually been, at any one time in surgical history, a particular organ system that has been the most common and difficult problem. In the 1930s and during the early part of World War II, the major organ system that limited survival after injury was the cardiovascular apparatus (shock). An understanding of blood loss, the requirements of whole blood replacement for hypovolemia, and the nature of traumatic shock, once considered a mysterious entity, provided successful resuscitation and management of the circulation in most injured patients. Shock, although not eliminated, ceased to be a major limiting problem. By the end of World War II, the kidney emerged as the most prevalent limiting organ system, and during the Korean War, acute renal failure after injury or after operation was still a major roadblock to recovery for many. Through development of knowledge about the need for support of the circulation, maintenance of renal blood flow, and urine formation, renal failure has since been prevented more frequently or if not prevented, treated successfully by dialysis. Then in the 1960s the lung became the limiting organ. Lung problems were described as shock lung, pump lung,

and finally by the more general term, posttraumatic pulmonary insufficiency, just as renal problems were called posttraumatic renal insufficiency during the Korean conflict. Now the development of knowledge about lung problems is preventing ventilatory failure in many patients, and treatment is successful in others.

What, then, is the next limiting organ system after severe trauma and prolonged hemorrhage either in the field or in the operating room? Surely it is the central nervous system, and attention is currently being devoted to it. However, it seems that the major limiting factor after trauma-hemorrhagic shock in patients who do not have central nervous system injury is not so much a system, but rather a combination of events that can best be called multiple systems dysfunction and eventually failure. Although not a well defined syndrome, it involves progressive failure of two or more systems after an overwhelming injury, severe blood loss, or an operation.

To provide better means of support for organs that have failed or prevent the failure of various organs following trauma-hemorrhagic shock or operation we must understand the effects of such insults on the cellular and subcellular level. It is clear that shock and all of its ramifications result from a prolonged deficiency of the flow of blood. Simeone in 1964 (32) stated that shock may be defined as a "clinical condition characterized by signs and symptoms which arise when the cardiac output is insufficient to fill the arterial tree with blood under sufficient pressure to provide organs

and tissues with adequate blood flow". A more precise definition of shock is "inadequate blood flow to vital organs or the inability of the body cell mass to metabolize nutrients normally" (Maclean, 1977) (33). Shock and all of its ramifications result from a sustained reduction in perfusion of capillaries and therefore of tissues and organs. Major operations, trauma, myocardial disease, and severe infection all may lead to circulatory failure or shock. Along with these, atherosclerosis, repair or transplantation of an organ or various organs. Thus, what happens to an organ with regard to the function of its component cells and what happens to the cell of an organ with sufficiently diminished flow becomes a critical series of events in terms of survival of the organ and the individual. The acute loss of circulating blood volume with or without mechanical tissue trauma initiates an intense neurohormonal activation. Hemodynamic disturbances occur along with changes in microcirculation, and the release of various vasoactive agents also takes place during shock and trauma. As a result of prolonged reduction in tissue perfusion, various alterations in tissue metabolism, structure, and function occur at the systemic, cellular, and subcellular levels. Although much has been learned about the function and structure of a normal cell, the lesions produced by shock and trauma have not been completely understood. The information derived from such studies, however, could be helpful in providing therapy for the shocked organs when blood flow is interrupted. The cornerstone of treatment of cellular and metabolic defects produced by shock is the restoration of circulation.

Fluids or blood is given first to provide or maintain an adequate vascular volume. This is often sufficient to correct the problem. If not, then an inotropic agent is used to increase cardiac output and improve blood flow. Along with this, various adjunctive agents and approaches may be used such as steroids and buffering agents. If the foregoing regimens are not successful, then direct support of cellular functions and survival may become necessary; increasing cellular energy levels, improving microcirculatory blood flow, improving membrane function, or by helping other aspects of cell function. Therefore, the cellular and subcellular alterations produced by shock and the possible ways by which such lesions could be corrected is an exciting area of research to provide care for the injured when other means of circulatory support have failed.

Effects of Hemorrhagic Shock on Organ and Cellular Function

Organ function: Changes in the various organ systems occur as blood flow decreases to organs and capillaries. In the kidney there is vasoconstriction with decreased renal blood flow and an intrarenal distribution of flow from the cortex, favoring the medulla (31). After hemorrhage and resuscitation there is a decrease in glomerular filtration rate (GFR) (i.e., C_{In}: [^{3}H] inulin clearance), urine flow rate (UFR), and cortical microvascular blood flow (CMBF) (34). In the heart, there is a decreased coronary flow which may contribute to a further decrease in cardiac output (CO). The decrease in CO is dramatic following hemorrhage, and is accompanied by a

decrease in mean arterial pressure (MAP) despite the increase in total peripheral resistance (TPR) (35). Electrocardiographic changes and myocardial ischemia may also be seen. Hepatic function is also altered following hemorrhage (4,36-38). As hepatic blood flow decreases (37) hepatocellular function is depressed, and there is decreased ability to break down lactate and to detoxify other substances. Gut absorptive capacity is depressed early after trauma-hemorrhagic shock (39) and bacterial translocation may occur (40) which can lead to sepsis. In the brain, sensorium and electroencephalographic activities are depressed if hypotension is severe. Initially hyperventilation occurs, probably from decreased central nervous system blood flow, which may produce respiratory alkalosis and further decreases cerebral blood flow by a fall in arteriolar PCO_2. Later, hyperventilation continues, owing to metabolic acidosis from increased lactate production. Decreased blood flow in the lungs may contribute to hypoxia and carbon dioxide retention.

Cellular function: The initiating event in hemorrhagic shock is hypovolemia with inadequate blood flow to the tissues which results in progressive cellular deterioration. What happens to an organ in terms of function of its component cells and what happens to the cells of an organ with a decreased blood flow becomes a critical series of events in terms of survival of the organ and the person. The hemodynamic disturbances in hemorrhagic shock have been extensively studied during the past thirty years. The results of more recent studies have emphasized defining the

disruption of basic cellular functions that occur in the late stages of hemorrhagic shock. The study of the physiopathologic characteristics of shock at the cellular level has been facilitated by the development of techniques that allow direct assessment of cellular function. The evaluation of many aspects of cell function in order to develop a better understanding of the initial and progressive changes that lead to cell malfunction and death will allow the development of therapy. Thus, such approaches now are becoming of practical significance to surgeons in treating and supporting patients in the emergency room after an injury, in the operating room during reparative procedures, and in the intensive care unit.

Work over the years from a number of investigative units have led to the development of a hypothesis about progressive cell injury with insults such as shock and trauma (I.H. Chaudry, 1983)(5). Although hemorrhagic shock is a precise condition, a common denominator of all types of shock appears to be inadequate perfusion of capillaries and, therefore, tissues and organs. Shock, trauma, ischemia, dehydration, or any adverse condition that produces decreased circulatory volume would cause the following abnormalities in cell function. The initial change seems to occur at the cell membrane.

<u>The transmembrane potential:</u> Hepatic cell membrane potential decreased with hemorrhagic shock, perhaps due to catecholamines or other circulating factors, and was partially restored after resuscitation (41-43). With this change in membrane potential, one would expect, according to the

chloride space and Nernst equation, that Na^+ and water enter the cell and K^+ leave the cell. The extent of the membrane potential decrease, on one hand, and tissue increase in lactate or decrease in pH or high energy compounds, on the other, could directly be correlated with the extent of cellular metabolic disturbance.

Membrane permeability: Following shock membrane permeability is altered (43-47). Studies have shown that there is an increased permeability to Na^+ and Cl^- coupled with a decrease in intracellular K^+ concentrations (43-45).

The Na^+-K^+-ATPase system: The Na^+ pump is activated to extrude excess Na^+ (43-49). ATP is used and the mitochondria are stimulated to produce ATP (43). More Na^+ gets into the cell because of altered membrane permeability. ATP further decrease because of a hyperactive Na^+-K^+-ATPase. As ATP levels decrease further (31,41,43,45) the Na^+-K^+-ATPase can no longer keep pace with Na^+ entering the cell. Consequently water accumulates and the cell, the mitochondria, and endoplasmic reticulum swell (31,44,45). The above alterations lead to abnormalities in Ca^{2+}-regulation by the cell and the mitochondria (31,45,50). These changes lead to decreased metabolic capability and eventually lysosomes leak and there is cell destruction. This, then, may be a cycle of deterioration by which one cell and its products can damage adjoining cells.

Mitochondrial function: It is well recognized that mitochondrial electron transport-linked reactions provide almost 95% of the body's energy

needs under normal conditions. To perform this function, however, the mitochondria utilize over 90% of the available cellular O_2. It is thus obvious that any abnormality in mitochondrial function due to lack of O_2, to defects in electron-transport-linked carriers and enzymes, or to lack of substrates would be expected to have deleterious effects on the cellular energy supplies.

Alterations in mitochondrial function during low flow conditions consist of :

1) Decrease in the metabolic capability: Measurement of liver mitochondrial function at various stages of hemorrhagic shock with succinate as a substrate indicated that there is a progressive but very small decrease in the capability of mitochondria to utilize succinate (51). With α-ketoglutarate or β-hydroxybutyrate, however, the magnitude of decrease in respiratory control ratio (RCR) (i.e., the ratio of the stimulated rate to the basal rate) was significantly more (5,52). The different magnitude of changes in shock with different substrates may be related to differences in stability of enzyme systems which metabolize these substrates. Since the succinate system is located in and bound to the inner mitochondrial membrane, it would be expected that this system should contribute to functional stability. To metabolize α-ketoglutarate, however, a complex multienzyme system located in the mitochondrial matrix is required.

2) Alterations in mitochondrial cation contents: Severe hemorrhagic shock in rat liver mitochondria is characterized by a great increase in Na^+

and Ca^{2+}, by a decrease in K^{+}, and by an increase in the lability of Mg^{2+} (5,51-53).

3) Increased mitochondrial free fatty acids (FFA): Studies have shown that hepatic mitochondrial FFA levels increase progressively after ischemia and reflow (54). Addition of bovine serum albumin (BSA) restored the RCR in mitochondria isolated from ischemic livers or hearts (55,56). Since albumin is known to bind FFA, it was suggested that its addition removed the inhibitory effect produced by intracellular FFA which could have been released during shock or ischemia before isolation of the mitochondria (56). Thus increased FFA could also contribute to the inhibition of mitochondrial function during shock.

4) Mitochondrial Ca^{2+} levels: Ca^{2+} has also been found to increase after shock or ischemia (50,57,58,59). Since high levels of Ca^{2+} are known to produce loss of mitochondrial function (52), this may be another factor responsible for the decreased mitochondrial function in shock and ischemia. However, influx of Ca^{2+} may well be a late manifestation of cell swelling resulting in inhibition of mitochondrial production of ATP.

5) Decreased adenine nucleotide translocase activity: Energy-linked shuttle mechanisms exist for the translocation of ATP from the intramitochondrial site of oxidative phosphorylation to the cytoplasm, and ADP and inorganic phosphate must be returned to the mitochondria for resynthesis of ATP. Studies have, in fact, suggested aberrations in shuttles during shock (60-62).

6) Ultrastructural changes: Mitochondrial and cellular ultrastructural changes have been demonstrated after prolonged hemorrhagic shock (53,63-65). Mitochondria from livers of animals in late shock were found to be swollen, less dense, and appear to undergo disruption of intramitochondrial structure (cristae) when compared with mitochondria from control animals.

Reticuloendothelial function: Shock induces a state of reticuloendothelial system (RES) depression which is directly proportional to the relationship between the RES phagocytic activity and survival after shock (66-70). Animals that survive shock manifest only transient RES depression with subsequent recovery and animals that eventually die as a result of shock are characterized by a persistent and progressive depression in RES function (67).

Hepatic gene expression: After shock and resuscitation, with use of complementary DNA (cDNA) prepared from post-shock/resuscitation messenger RNA (mRNA), a library was constructed and subsequently screened for differential gene expression. Of 32/4000 clones initially screened as positive for induction after shock/resuscitation, six were confirmed positive by Northern blot analysis (71). This shows that in response to specific stresses, such as shock or the sequence of hypoxia-reoxygenation, each cell alters gene expression to synthesize or alter the synthesis of a set of proteins that are important for intracellular homeostasis.

Energy production: Since a common feature in all forms of shock is an inadequate circulation with diminished blood flow to tissues, this results in tissue hypoxia. As a result of this decreased oxygen availability to tissues in shock, an increased demand is placed on the anaerobic metabolism, leading to decreased levels of high-energy phosphate compounds such as ATP and creatine phosphate (72-81). Studies (80,81) indicated that although liver, kidney, and diaphragm ATP levels decreased, there was no significant decrease in skeletal muscle ATP levels during early hemorrhagic shock in conscious animals. During late shock, however, ATP levels in the skeletal muscle decreased 50% and creatine phosphate levels decreased 96%. The maintenance of adenine nucleotides and creatine phosphate levels in the early stages of shock is probably due to the fact that skeletal muscle have a highly active glycolytic system, and are therefore able to resist the effects of reduced blood flow and hypoxia. However, skeletal muscle does require energy to maintain resting metabolism and cannot resist decreased blood flow for prolonged periods of time, as was evident from the results in late shock (81). The greater and earlier decrease in the ATP content of liver and kidney than in the skeletal muscle of animals in shock may be indicative not only of their greater metabolic activity, but can also reflect a more critical decrease in blood flow to these organs.

The cell normally possesses a mechanism for maintaining ATP levels. If, however, the production of ATP is slower than its use, this would result in a decreased nucleotide level. Studies have indicated that there is an

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oxygen deficit in the liver during shock (82,83). A lack of oxygen would cause anaerobic metabolism to predominate, which is not an efficient system for producing ATP. It has also been shown that Na^+-K^+-ATPase activity is increased during shock (43,44,46,48,49) which adds to the ATP deficit. It is also possible that the purine base for the restoration of cellular ATP is lost during shock; thus, the restoration of this nucleotide cannot be accomplished (74).

During shock there is a progressive decrease in the capability of liver mitochondria to use a krebs cycle substrate, α-ketoglutarate, to produce high energy phosphate compound, i.e., ATP (51). However, since shock did not produce any alterations in lung and brain mitochondria, it indicates that central circulation is maintained whereas blood flow to the liver and kidney is depressed. These findings indicate changes that may be produced by decreased blood flow in the energy-producing capability of the subcellular organelles.

Immune function: Numerous studies have been carried out examining the etiology of immunosuppression following mechanical and thermal trauma (84). However, recent studies (29,85,86) have placed more emphasis on examining whether hemorrhage, which occurs in conjunction with accidental injuries as well as a separate pathophysiological entity, plays any major role in producing the immunological alterations. These studies have indeed shown that simple hemorrhage without tissue trauma, produced immunosuppression and enhanced host susceptibility to sepsis.

The depression in various immune functions such as T- and B-cell function as well as macrophage function, was apparent immediately after hemorrhage and persisted for a prolonged period of time despite volume resuscitation. These immunological alterations, following even transient hypotension in the absence of any significant tissue trauma produced a marked depression in both the specific and non-specific cell-mediated immunity. This was evident by the depression of lymphocyte function, macrophage expression of receptors involved in opsonin-mediated phagocytosis, splenic production of interleukin-2, and antigen presentation function by peritoneal and splenic macrophages, as well as Kupffer cells. These alterations in immune function after hemorrhage occur without changes in lymphocyte population and sub-population numbers. Although the precise mechanisms responsible for producing the immunodepression after simple hemorrhage and resuscitation remain unknown, the depression of macrophage antigen presentation, as well as the enhanced capacity of Kupffer cells to produce tumor necrosis factor (TNF), may play an important role in initiating cell and organ dysfunction and in contributing to the host's enhanced susceptibility to sepsis after hemorrhage.

Organ, microvascular blood flow and 0_2 delivery: Cardiac output (CO) decreases dramatically following hemorrhagic shock (35). Although fluid resuscitation restores cardiac output, shortly after resuscitation CO decreases gradually. Therefore, resuscitation alone does not maintain CO following severe hemorrhage. The lack of maintenance in CO following

resuscitation was not due to inadequate resuscitation, since it has been shown (35) that the central venous pressure more than doubled and total peripheral resistance (TPR) was restored to normal. Both of these parameters suggest that adequate resuscitation was provided. Despite this, however, the mean arterial pressure (MAP) did not return to normal. This could result from a decrease in vascular tone following shock, or microvascular depression. Studies have indeed shown that resuscitation of hemorrhagic shock did not restore or maintain the microvascular blood flow in the liver, kidney, spleen, skeletal muscle, and small intestine (87).

A marked depression in CO following hemorrhage results in decreased total organ flow and microcirculation, tissue ischemia, cellular dysfunction and cell death (31). Flow decreases markedly to all organs following hemorrhage except the brain (88). As a result of the decreased total hepatic flow following hemorrhage (88,89) effective hepatic blood flow (EHBF) or nutrient hepatic blood flow (i.e., flow that perfuses functioning sinusoids and is available for metabolic exchange in the liver) was decreased by 71% and correlated with a 65% decrease in MAP (89). Resuscitation significantly improves but does not restore EHBF following hemorrhage. Thus a redistribution of hepatic blood flow occurs after hemorrhage and resuscitation as evidenced by the fact that the fraction of hepatic arterial blood flow increased from 19 to 28% and the fraction of portal blood flow decreased from 81 to 72% of the total hepatic blood flow under such a condition. Along with the changes in EHBF, hepatic microvascular blood

flow (HMBF) decreased significantly during hemorrhage and remained depressed despite resuscitation (89).

During hemorrhage MAP, CO, and transcutaneous as well as subcutaneous oxygen tension ($P_{tc}0_2$ and $P_{sq}0_2$) diminished, although not necessarily in simple proportion to the volume of blood lost. $P_{sq}0_2$ correlates more highly with blood volume lost than $P_{tc}0_2$. Furthermore, $P_{sq}0_2$ is more sensitive to blood loss than either cardiac output or $P_{tc}0_2$ and, also during small volume loss, is more sensitive than mean arterial pressure (90).

Perfusion of bone, skin, intestine, pancreas, and spleen appears to decrease essentially in concert with each other and $P_{sq}0_2$ during progressive blood loss. Yet perfusion of these tissues fell markedly at levels of hypovolemia that interfered little with perfusion of some of the vital organs such as brain, kidney, and liver. Thus, $P_{sq}0_2$ is a very sensitive index of perfusion in peripheral tissues (90). Some organs (e.g., pancreas) appear to lose considerable blood flow with a small loss of blood volume, but their blood flow then stabilizes at a low level despite further hemorrhage. Other organs, notably the kidney, appear to be relatively unaffected by substantial loss of blood volume (20% to 40%) (90), after which, however, their blood flow quite abruptly becomes sensitive to further hypovolemia. This explains why blood flow-related performance of the kidney (e.g., urine volume) may not adequately predict a developing hazard of peripheral perfusion. Some indicators of perfusion are better indexes of blood flow in some organs than in others; cardiac output and $P_{sq}0_2$ correlate more closely with skin, spleen,

and intestinal flows than with vital organ flows.

Oxygen consumption (V_{O2}) is independent of O_2 delivery (DO_2) until DO_2 declines to a critical value (DO_{2c}). Below this value, V_{O2} becomes O_2 supply dependent. Numerous investigators have observed that the distribution of cardiac output among organs changes as whole body DO_2 declines, such that the fraction received by some organs increases at the expense of others (91). Studies have indicated that redistribution of flow, presumably mediated by modulation of regional vasomotor tone, enhances the ability of the whole organism to utilize O_2 efficiently relative to a state wherein fractional distribution of flow among organs remains constant.

During graded progressive hemorrhage, whole body DO_2 ratio declined for liver and kidney and increased for carcass (91). Thus, O_2 delivery is preserved for vital organs at times when tissues are hypoxic. However, as mentioned above, blood flow to these organs reaches a threshold where flow and thus DO_2 becomes abruptly sensitive to further hypovolemia. At such point VO_2 becomes DO_2-dependent (i.e., DO_{2c} is reached). Declining blood hematocrit is a normal response to hemorrhage and can, also, influence O_2 delivery. However, hematocrit 50% of control values were well tolerated and did not influence VO_2, acid-base balance, and mortality following hemorrhagic shock (92).

Metabolism: There is a progressive accumulation of many metabolites during shock, suggesting both increased breakdown and failure of anabolism in the tissues. FFA levels have been shown to increase (93), and

almost all circulating amino acids also increase in concentration (94). Lactate production increases, due to shift to anaerobic metabolism because of the lack of oxygen and/or due to decreased conversion of pyruvate to acetyl-CoA because of decreased cytoplasmic NAD/NADH. Thus, acidosis becomes progressively more severe at the terminal stages of shock.

Especially important to the purpose of this dissertation, is the biphasic change in serum glucose with an early hyperglycemia which progresses to hypoglycemia toward the final phases of the shock period (23-28). In the early phases of shock, increased glycolysis and glycogenolysis have been found in skeletal muscle and liver of animals. Cardiac muscle glycogen increases marginally in the early stages of shock and it decreases at the terminal phases of shock. The glycogen level of the brain also falls towards the terminal phases of shock (5). In contrast to increased glycogenolysis, the rate of oxidation of carbohydrate is decreased after shock (5).

Gluconeogenesis comprises the synthesis of glucose and glycogen from lactate, pyruvate, glycerol, and certain amino acids. The liver is the major site of gluconeogenesis, with the kidney becoming an important site of gluconeogenesis during stress conditions. It would appear that the hyperglycemia which is observed in the early stages of shock results from the increased glycogenolysis and glycolysis. In the later stages of shock, however, the hypoglycemia observed might be related to depressed gluconeogenesis.

In this work an attempt has been made to gain some understanding of the mechanism underlying these two questions namely;

- How glycolysis is increased, thus producing an early hyperglycemia?
- Is the late hypoglycemia due to depressed gluconeogenesis and if so, why gluconeogenesis is depressed?

Low-Flow Conditions and Receptor Characteristics

Since a common feature in all forms of shock is an inadequate circulation with diminished blood flow to tissues, this results in tissue hypoxia. Studies have indicated that there is an oxygen deficit in the liver during shock (82,83). After ischemia and hypoxia, and especially after subsequent reflow, oxygen free radicals are formed and produce tissue injury, in large part through peroxidation of polyunsaturated fatty acids (95-97). Probably during ischemia and hypoxia the efficacy of different protective systems is diminished (98,99), and by the recirculation after ischemia or a recovery time after hypoxia the tissue becomes overloaded with reactive oxygen metabolites, with consequences that membrane transformation through lipid peroxidation causes irreversible damage in the tissue and cell membrane.

Kramer *et al.* (14) have measured glutathione (GSH), superoxide dismutase (SOD), and catalase, also alterations in β-adrenoceptor density under complete ischemia and hypoxia, in the cerebral cortex (homogenate) and the erythrocyte lysate or the erythrocyte membrane of the rat.

They found that, first, during ischemia, parallelism in changes that occur in the central nervous system (cerebral cortex) and the erythrocyte exists; second, the SOD activity became higher and β-adrenoceptor density was decreased in both tissues. However, after hypoxic conditions, a decrease in the number of β-adrenoceptors in the cerebral cortex but an increase in β-adrenoceptor density in the erythrocyte was observed. Furthermore, Beaumont *et al.* (15) showed that 10 min of renal ischemia produced by clamping the renal pedicle reduced the apparent density of thiazide receptors in kidney membranes by 90%, as measured by the binding of [^{3}H]-metolazone, with the release of the clamp and subsequent reperfusion for 10 min, thiazide receptor density returned to within 40% of control levels. It was concluded that renal thiazide receptors undergo a rapid and reversible form of regulation and that the controlling mechanisms are energy dependent. Thus, when metabolic energy is affected such as during low flow conditions (5) changes in receptor characteristics might be expected; as evidenced by the decrease in β-adrenergic receptors in pulmonary and systemic arteries (20), α-adrenergic receptors in cardiac myocytes (21), β-receptors in neonatal ventricular myocytes (22) following hypoxic conditions. Of interest, though, is the observation that during hypoxia in the above mentioned studies, although the density of receptors was affected, no change in receptor affinity was reported. Conversely, endotoxic shock alters both receptor density as well as affinity as reported by several investigators (16-18). Changes in receptor characteristics are also known to occur

following other low flow conditions, such as hemorrhagic shock (13).

The second step in signal transduction, after binding of the ligand to its receptor, is the coupling of that receptor to its second messenger(s). This process involves membrane elements (e.g., G-proteins) that link the receptors to the intramembrane environment, since the cellular damage that result following many adverse circulatory conditions involves alterations in membrane function. One would expect that the coupling of membrane receptors might be affected following such conditions. Indeed, studies (19-22) have indicated that receptor coupling is altered following hypoxia and ischemia. Shaul *et al.* (20) have reported that prolonged hypoxia induces downregulation of β-adrenergic receptors in both systemic and pulmonary arteries of the rat. However, despite the downregulation, prolonged hypoxia has no sustained effect on adenylate cyclase activity in pulmonary arteries, whereas enzyme activity in the systemic arteries is markedly increased. Furthermore, the degree of enhancement observed with hypoxia in the systemic arteries, with stimulation at the various points along the cyclase pathway, suggest that the augmented enzyme activity is mediated by changes at the level of the stimulatory guanine nucleotide-dependent regulatory protein (i.e., G-protein). This explains why prolong hypoxia causes pulmonary hypertension but no change is systemic vasomotor tone. In another study Heathers *et al.* (21) have shown that the enhanced responsiveness to α-adrenergic stimulation in the ischemic myocardia is due to enhanced production of the receptor's second-messenger

(i.e., IP_3), and this could account for the enhanced α-adrenergic responsiveness in the ischemic heart *in vivo*, which is known to facilitate arrhythmogenesis.

A common denominator of all forms of shock is diminished blood flow to tissues and cells which can lead to local hypoxia. Thus, based on the information provided above, changes in receptor characteristics as well as coupling is indeed a possibility following hemorrhagic shock.

PURINERGIC RECEPTORS

For many years, it has been realized that purine nucleotides and nucleosides have widespread and potent extracellular actions on excitable membranes, which may represent a role in physiological regulatory processes (100-102). Subsequently, it was proposed that ATP was released as the principal neurotransmitter from some non-adrenergic, non-cholinergic nerves (103) or as a co-transmitter with noradrenalin, acetylcholine and other substances (104,105).

In 1978, Burnstock suggested a subdivision of purinoceptors based on several criteria (106). At the P_1-purinoceptors an agonist potency order of adenosine > AMP > ADP > ATP was proposed; methylxanthines such as theophylline were proposed to be selective antagonists; and occupation of the P_1-purinoceptor was suggested to lead to change in intracellular cAMP levels (106). At the P_2-purinoceptor an agonist potency order of ATP> ADP> AMP> adenosine was proposed; it was further suggested that

methylxanthines were not antagonists at the P_2-purinoceptors; and finally, occupation of the P_2-purinoceptor was suggested not to lead to changes in intracellular cAMP levels and in some cases to evoke prostaglandin synthesis. However, later studies have shown that occupation of the P_2-purinoceptor by ATP inhibited forskolin-induced accumulation of cAMP in the presence of a phosphodiesterase inhibitor (7,10). Since its proposal this classification has received much support and appears to have been widely accepted.

Subsequently, biochemical, pharmacological and receptor-binding studies have led to a proposed subdivision of the P_1-purinoceptor into A_1 and A_2 receptors (107) or R_i/R_a receptors (108). A_1 receptors appear to be analogous to R_i receptors and A_2 receptors appear to be analogous to R_a receptors (109).

Interestingly, it is becoming apparent that the P_2-purinoceptor also may not form a homogenous group. Subdivision of the P_2-purinoceptor has been suggested previously, although this was based on the action of apamin, a non-specific antagonist of some actions of ATP (110), or on anatomical location (111), and not on the action of agonists and antagonists.

Receptors can be classified on the basis of ligand binding studies or by using a pharmacological approach (i.e., measurement of responses induced by a selected series of agonists and antagonists); for nucleotide receptors the latter method has been mainly employed . Such an analysis can be complicated by several factors: the relation between receptor

occupation and the elicited response is complex; the activity of an agonist reflects both its affinity for the receptor and its efficiency; receptors may interact with each other to give positive or negative cooperativity; agonists (and antagonists) may induce a response by acting at more than one receptor. In addition, the local concentrations of agonists near the receptor site can be difficult to assess, especially where ecto enzymes that metabolize the agonist exist on the cells used for characterization studies.

The design, synthesis, and pharmacology of analogues of ATP have proved extremely useful in the classification of P_2-purinoceptors (111), and provide clear evidence for at least four subtypes (112), which have been termed P_{2x}, P_{2x}, P_{2y}, and P_{2t} (113,114). The P_{2x} purinoceptor is found on mast cells, macrophages, lymphocytes, and epithelial cells; the excitatory P_{2x}-purinoceptor, on visceral and vascular smooth muscle, and sensory neurons; the inhibitory P_{2y}-purinoceptor on visceral and vascular smooth muscle, endothelial cells, hepatocytes, parotid acini, type II alveoli, and pancreatic β cells; and the P_{2t}-purinoceptor, uniquely, on blood platelets (114).

Comparative studies of agonist potency should ideally be carried out in the absence of metabolism and uptake of the agonist. At present the influence of metabolism and uptake on the action of P_2-agonist is unclear since selective potent inhibitors of metabolic enzymes involved in the degradation of ATP and its analogues have yet to be developed. Breakdown products of ATP, therefore, possibly influence its actions. Nevertheless,

many studies have been carried out using ATP and its analogues, mainly α,β-methylene ATP (α,β-meATP); β,α-methylene ATP (β,α meATP; and 2-methylthio ATP). Both α,β-meATP and β,α-meATP have been shown, when tested, to be broken down more slowly than ATP (115,116).

Together with the relative potency of agonists, the use of selective and specific antagonists is necessary in order to fully classify a receptor. Since the proposal of the P_1/P_2 hypothesis in 1978 (106) (when there were no known selective antagonists at the P_2-purinoceptor) two compounds have been proposed to be so. The first, apamin, a toxin from beevenom, has been since shown to be a blocker of K^+-channels and is therefore non-selective for ATP, but has, nevertheless, been of use in the study of purinergic nerves and receptors. Arylazidoamino-propenyl-ATP ($ANAPP_3$), a photolysible analogue of ATP, has also been claimed as a P_2-purinoceptor antagonist, selectively blocks the action of ATP in some tissues, and is irreversible and light-sensitive (114). Unlike the situation with P_{2t}-purinoceptor, where ATP and AMP are competitive antagonists of the action of ADP (117), no competitive antagonists at the P_{2x}, P_{2x}, and P_{2y} subtypes are known (118). Earlier claims that reactive blue is a selective antagonist for the P_{2x} subtype (119) have been discounted (120). In the absence of widespread availability of such an antagonist desensitization of the receptor by repeated administration of agonist has also been employed. Until a competitive antagonist at the P_{2x}, P_{2y}, and P_{2z} purinoceptor subtypes becomes available, it will not be possible to perform classical pharmacology

on their receptors with a variety of adenine nucleotide analogues that are agonists, partial agonists, and antagonists.

Biological actions: Much of Burnstock's work was concerned with ATP and/or adenosine released from 'purinergic' nerves (103) and the term 'purinergic receptors' reflected this emphasis on the neuroeffector junction; as receptors for ATP and adenosine also exist on cells with no neural connections, the term 'purinoceptors' is now generally used. The classification of P_2-receptors is at present based almost entirely on the relative potencies of various agonists (ATP analogues and measurement of responses induced by these agonists [i.e., pharmacological approach]), which is unsatisfactory for reasons discussed above. Classification of these receptors would be facilitated by potent, specific, competitive, reversible antagonists but no such compounds have yet been identified (117,118). Despite these limitations, the pharmacological approach has been used successfully to characterize various P_2-receptors, and their biological actions in a variety of tissues and cells have been elucidated. However, even with current deficiencies of classification, recognition of P_2 subclasses will aid the design of more selective agonists and antagonists, which in turn should lead to better understanding of the biological role of P_2-receptors. The responses mediated by P_2-receptors in different cells, tissues, and organs are individually and briefly discussed below, starting with the platelet, the first cell type in which a P_2-mediated response was described. (For specific references concerning the following topics see reference 117.)

Platelet aggregation: The platelet response observed following stimulation by ADP is a rapid change in shape from biconvex disk to spiny sphere and then aggregation. ADP also inhibits platelet adenylate cyclase; pharmacological evidence indicates that this action of ADP is mediated by the same P_{2t}-receptor. As we have mentioned above, this is the only P_2-receptor subtype that has a known competitive antagonist (namely ATP and AMP). The concentrations of ADP required to produce a half-maximal effect on platelet responses range from 0.2-0.8 μM.

Mast cell secretion: ATP induces calcium-dependent histamine secretion from mast cells via a P_{2z}-purinoceptor. This secretion requires the addition of 100-150 μM-ATP. Mast cells do not respond to other nucleotides, nor to analogues such as APPCP. The concentrations of ATP needed to induce secretion also induce increases in membrane permeability to intracellular metabolites and among the cytoplasmic constituents released by high concentrations (> 100 μM) of extracellular ATP is ATP itself; this generates additional extracellular ATP, making interpretation difficult.

Membrane permeability: A non-lethal 'permeabilizing' effect of high concentrations of ATP has also been studied using cultured cells (e.g., 3T6, SV40-3T3). The responses are not produced by other nucleotides, are greater in transformed than in normal cells, and are increased if the intracellular concentration of ATP falls. Although it has been suggested that the permeabilization resulted from phosphorylation of a 44 KDa membrane protein involved in the formation of an 'aqueous pore' in the membrane,

this protein was subsequently identified as a mitochondrial enzyme.

However, many surface membrane proteins are phosphorylated by ATP under 'non-permeabilizing' conditions (i.e., with ATP concentrations <1μM) which introduces the possibility that receptors for ATP on the cell surface could include kinases. Protein kinases utilizing extracellular ATP have been described on several cell types, including macrophages, hepatocytes, adipocytes and Hela cells. Ecto-nucleoside diphosphate kinase exists on platelets and endothelial cells. Indeed, the platelet enzyme was proposed as the receptor for ADP, although this concept has not found universal support. Which, if any, of the P_2 receptors discussed previously may involve ecto-kinases remains to be determined.

The permeabilizing effect of ATP should be distinguished from its ability to induce a selective increase in K^+ permeability, which is associated with activation of the P_{2y}-receptor by low concentrations of ATP. This effect has been noted in several cell types, including astrocytes, parotid acinar cells and endothelial cells.

Cardiovascular effects

Endothelium-dependent vasodilation: The hypotensive and vasodilatory actions of nucleotides have been noted for many years. In common with some other vasodilators (e.g., acetylcholine), ATP has little or no relaxant effect in many conventional oscillated arterial preparations. The reason for this discrepancy remained obscure until Furchgott and his colleagues discovered that such vasodilators required the presence of

endothelium. This vasodilation is mediated by a substance known as endothelium-derived relaxing factor (EDRF), which is released from the endothelial cells by various stimuli including ATP. EDRF acts by stimulating guanylate cyclase in vascular smooth muscle cells and thus inducing their relaxation. Studies with a range of ATP analogues have established some of the characteristics of the P_{2y}-receptor on endothelial cells that mediates the release of EDRF. These studies have indicated equipotency of ATP and ADP, and the relative ineffectiveness of AMP and adenosine. There is considerable variation in the amount of endothelium-dependent vasodilation that can be induced at different vascular sites, some blood vessels (e.g., the rabbit ear artery) apparently do not have a P_{2y}-receptor on their endothelial cells.

Vasoconstriction: P_{2x} receptors mediating vasoconstriction exist on the smooth muscle of some arteries. If the endothelial cells of the same artery have P_{2y}-receptors that mediate endothelium-derived vasodilation, loss of endothelial cells (or their failure to generate EDRF or prostacyclin) could result in a vasoconstriction rather than vasodilation response to ATP and/or ADP. Such paradoxical vasospasm induced by other stimuli has been demonstrated in the coronary bed. To complicate the situation still further, in some vessels (such as the rabbit portal vein) the smooth muscle apparently exhibits both P_{2x} and P_{2y} purinoceptors, one mediating vasoconstriction and the other vasodilation.

Prostacyclin production: ATP can also induce the release of prostaglandins (including prostacyclin) from perfused vascular beds and isolated blood vessels; and from cultured endothelial cells of pig aorta, bovine aorta and human umbilical vein (117). Responses to ATP and ADP are transient, basal production being restored within about 5 min. The receptor responsible is apparently P_{2y}, like that involved in EDRF production. Prostacyclin may contribute to ATP-induced vasodilation in some vascular beds.

Cerebrovascular effects: There are few studies on P_2-purinoceptors in small blood vessels, but vasodilation in the Pial arteries of the cat was induced by topically applied ATP at concentrations as low as 0.1 nM.

Coronary and cardiac effects: In the coronary bed, purines can induce vasodilation: inosine and hypoxanthine are inactive, but adenosine and ATP are both powerful vasodilators. It has been proposed that adenosine, which is released from the working heart (especially under hypoxic conditions), is an endogenous regulator of coronary flow. ATP is rapidly converted to adenosine in the coronary bed and, therefore, some of its effects may be the result of this conversion; however, ATP is more potent than adenosine as a coronary dilator and the analogue APPCP, which is metabolized much more slowly, is also effective.

There are at least three sites through which ATP could exert effects on coronary flow and cardiac function: coronary smooth muscle cells, endothelial cells, and cardiac myocytes. ATP has little effect on the endothelium-denuded rabbit coronary artery, although the intact artery is

dilated by ATP. This suggests that the major site of action is on the endothelial cells. EDRF and/or prostacyclin could be responsible for the observed vasodilation, but since the doses of ATP required to stimulate prostacyclin production by the perfused heart are greater than those that induce coronary vasodilation, it would appear that the effects are mainly due to EDRF production. The effects of ATP on cardiac function are, in general depressant: decreased heart rate, force of atrial contraction, and (to a lesser extent) force of ventricular contraction also. These effects seem to be due mainly to adenosine formation, although P_2 as well as P_1 receptors have been found in the hearts of various animal species, including amphibians.

Non-vascular smooth muscle: The potent biological actions of purines on smooth muscle of the gastrointestinal tract and other locations have been recognized for many years, and evidence has accumulated indicating that ATP is involved in the physiological regulation of non-vascular smooth muscle tone through liberation from 'purinergic' nerve endings. ATP effects on the gut are usually inhibitory, and it can modulate reflexes that facilitate the movement of material along the alimentary canal. The minimal effective concentration of ATP is around 0.1 μM.

Purinergic control is indicated at several regions of the gastrointestinal tract, including the oesophagus, stomach, small and large intestine and internal anal sphincter. Although most of the gastrointestinal responses mediated by purinoceptors are inhibitory, excitatory responses, possibly

under purinergic control, are also found in the small intestine of several vertebrate species.

Neuromodulation: ATP can exert effects at neuroeffector junctions by acting directly as a neurotransmitter, by increasing or decreasing the release of other neurotransmitters, or by modulating their action. ATP is ubiquitously present in neural tissue, and it is released during nerve stimulation of many tissues including the rabbit ear artery, the guinea pig *taenia Coli* and vas deferens, and the electric organ of Torpedo.

The concept of ATP as a transmitter in 'purinergic' nerves derived from observations indicating that a component of the autonomic nervous system, neither adrenergic nor cholinergic, was associated with smooth muscle in parts of the gastrointestinal tract and elsewhere, including the central nervous system.

Evidence for ATP as a neurotransmitter includes: measurements of ATP release after nerve stimulation; effects of nerve stimulation mimicked by exogenous ATP; presence at neuroeffector junctions of enzymes that metabolized ATP; 'purinergic' nerve endings exhibiting large, opaque characteristic vesicles that contained ATP. ATP is released from some nerves as a co-transmitter, for example, with noradrenalin. Single neurons from cervical ganglion secreted a purinergic transmitter in addition to acetylcholine and noradrenaline. ATP can modulate membrane potentials in autonomic ganglia by depolarizing the membrane through a decrease in the resting K^+ conductance.

Purinergic effects in the central nervous system (CNS) are, in general, inhibitory: e.g., depression of the firing rate of central neurons, and reducing transmitter release from cholinergic and adrenergic synapses. Some neurons in the CNS show biphasic responses to ATP, primary excitation followed by depression, although most show only a reduction in firing rate to ATP. Other studies have revealed nerves excited by ATP in the sensory regions of the brain and in the outer level of the dorsal horn of the spinal cord. Some clinical observations have implicated adenine nucleotides in schizophrenia and in depression.

Sources of extracellular ATP: The effects of ATP discussed above can be seen in proper perspective only in relation to the sources from which such ATP might arise. ATP is an ubiquitous intracellular constituent and, therefore, any cell could potentially serve as a source of extracellular ATP. In practice, three categories might be considered: purinergic nerve terminals and associated cells; cells in or adjacent to the circulating blood, other cells such as cells of the adrenal medulla. We should keep in mind the following questions. What stimuli induce release? What is the mechanism of release? What concentrations of ATP can be achieved extracellularly?

When ATP was measured in the perfusate of the rabbit ear artery after nerve stimulation, it was apparent that about one-third of the ATP released was from the superfused *taenia Coli* on transmural stimulation is from neuronal origin and the rest from the effector cells (i.e., smooth muscle).

Release of purines from brain slices and synaptosomes has been measured in response to depolarizing stimuli. ATP can be released from synaptic vesicles by degranulation, but nucleotides can also be released from other locations during nerve stimulation.

Blood platelets have 'dense granules', these granules are packed with amines and nucleotides. Platelet degranulation *in vivo* usually takes place where platelets aggregate and thus their granule contents are released from platelets that are clustered together, not suspended uniformly throughout the blood. When these aggregated platelets degranulate the pericellular concentration of ATP and ADP will be very high, their concentration within the storage granules is ≈ 1 M.

The adrenal medulla is another cell type that contains nucleotides in storage granules, discharges the contents by degranulation and thus may contribute to the local release of ATP into the plasma. ATP represents about 15% of the dry weight of adrenal granules, and its release accompanies that of catecholamines.

Although degranulation (i.e., exocytosis) is the process by which nucleotides are released from platelets, neurons, and cells of the adrenal medulla, most other cells (which contain ATP in their cytoplasm and mitochondria, but not in storage granules) must release nucleotides by a different mechanism. ATP can be released from vascular endothelial cells and smooth muscle cells in culture; the cells remained viable, and the release was selective in as much as no lactate dehydrogenase was detectable

extracellularly, but there was no evidence that the released nucleotides had been compartmentalized within the cell. ATP was apparently released from the cytoplasm, not from mitochondria, however, the mechanisms involved have not yet been identified. The release process may involve a change in the topography of plasma membrane constituents, resulting in the opening of a pore that allows selective release of some low M_r cytoplasmic constituents. Budding of surface membrane vesicles occurs in some cells. Concentrations of the nucleotides sufficient to affect platelet aggregation were produced in these experiments; the relative amounts of extracellular ATP and ADP depend on their metabolism by ectonucleotidases. Such release from vascular endothelial and/or smooth muscle cells *in vivo* is another potential source of ATP in plasma.

Extracellular ATP has been detected in the blood perfusing working muscle beds, and in plasma flowing through micropunctures of single blood vessels, or from a small incision made in human skin. There were two peaks of ATP release observed following the incision, the first, which appeared only 2-4 S after the incision, reached 0.2-2 μM, and it was suggested that the source of this ATP was cells of the vascular wall. The second peak (≈ 20 μM, 3-5 min after the stimulus) was apparently a consequence of platelet degranulation, stimulated by exposed collagen in the vascular wall and by thrombin formed during clotting.

ATP is also found in solutions perfusing a working heart, especially if made hypoxic. Release is increased by sympathetic nerve stimulation.

Because these solutions were blood-free, the source of the ATP could not be blood cells, but it was not established whether the source was purinergic nerves, myocardium, smooth muscle cells, or endothelial cells. ATP release has, however, been measured from isolated, hypoxic cardiomyocytes in which the intracellular ATP remained approximately constant.

The concentrations of extracellular ATP generated locally in the circulation are not easy to determine. The micromolar concentrations measured in the perfusates of the working hearts and muscle beds underestimate the concentration at the sites of release, because of dilution and because of the efficient ectonucleotidases on vascular endothelial cells.

Cytoplasmic ATP in most cells is > 5 mM in concentration, and a significant proportion can be released with no loss of cell viability; thus the concentrations of pericellular ATP could easily reach the high micromolar range. The local concentrations of ATP will depend on the amount released, the volume of distribution in the extracellular space, and the capacity and accessibility of catabolic enzymes, especially the ectonucleotidases on adjacent cells. Teleological reasoning suggests that this may be why the distribution of ectonucleotidases is widespread.

Metabolism of extracellular ATP: A bolus of ATP is virtually all removed by a single passage through the lung. The rate of removal of ATP is much greater than can be accounted for by enzymes in the blood; metabolism by lungs perfused with blood is, in fact, no greater than in blood-free perfused lung. Metabolism of nucleotides in blood alone is relatively

inefficient: the $t_{½}$ for ATP incubated in samples of cell-free plasma or whole blood at 37°C is around 30 min and 10 min respectively, whereas the $t_{½}$ of nucleotides in the perfused lung is ≈ 0.2 S or less. The efficiency of nucleotide metabolism in the lung is not a peculiarity of the pulmonary vasculature; nucleotides are also efficiently metabolized by the perfused heart, and 99% of a bolus of ATP can be removed on a single passage.

The enzymes responsible for removal of ATP from the extracellular space are ectonucleotidases on endothelial cells. Ectonucleotidases are not restricted to endothelial cells, but have a widespread distribution amongst tissues and isolated cells such as blood platelets and leukocytes. The presence of ectonucleotidases on effector tissues such as skeletal, cardiac, and smooth muscle, and within the central nervous system can complicate the interpretation of experiments involving purinergic nerve stimulation or exogenous nucleotides. Responses to nucleotides in several preparations depend on the susceptibility of the agonist to nucleotidases. Furthermore, responses may be affected by the spital distribution of receptors and nucleotidases on the cell surface, and by factors that regulate this. Membrane topography does not remain constant: transient domain segregation can occur (e.g., in response to stimuli) and this could result in regions rich in purinoceptors but not in ectoenzymes. Topography is altered by changes in membrane fluidity and interactions with the cytoskeleton, which could, therefore, affect responses to stimuli.

HEPATOCYTES PURINOCEPTORS

Characterization: Evidence has been presented for the existence in rat liver of P_2-purinoceptors (6,7). Burnstock & Kennedy (113) have proposed to separate the P_2-purinoceptors into two subtypes, designated P_{2x} and P_{2y}. The P_{2x} subtype shows a specific rank order of agonist potency (PP[CH_2]PA ≈ P[CH_2]PPA > ATP = 2methylthio ATP) and is selectively desensitized by PP[CH_2]PA. The P_{2y} subtype displays different rank order of potency (2-methylthio ATP >> ATP > PP[CH_2]PPA ≈ P[CH_2]PPA), and is only weakly or not desensitized by PP[CH_2]PA. Tentatively, it has been proposed that liver P_2-purinoceptors belong to the P_{2y} subclass (6-8,114).

Isolated rat hepatocytes and purified liver plasma membranes have been used (by Keppens and Wulf, 1986)(8,9) to study the binding of the ATP analogue adenosine 5'-[α-[^{35}S]thio]triphosphate ([^{35}S]αATP) to these postulated P_2-purinoceptors (8). These studies have indicated that specific binding of [^{35}S]αATP to hepatocytes and plasma membranes occurs within 1 min and is essentially reversible on addition of an excess of ATP. Consequently, they performed steady-state binding measurements after an incubation period of 1 min. Because the Scatchard plots of the dose-dependencies data were curvilinear in both cases (hepatocytes and membranes), it could not be adequately described by a Michaelian-type relationship. Therefore, the authors concluded that Scatchard plots can be separated into two independent components. The first represents saturable binding, characterized by a specific dissociation constant (K_d) and maximal binding capacity (B_{max}) and

this component very likely corresponds to the physiological receptor. With cells, the computed K_d and B_{max} values for the high-affinity binding site were 0.23 μM and 5 pmol/10^6 cells. With membranes, the K_d and B_{max} values were 0.11 μM and 30 pmol/mg of protein (8). The other component (low-affinity component) obtained at higher concentrations of [^{35}S]αATP, can be represented by a 'non-saturable' binding component (the authors concluded): Its physiological nature is not readily apparent, but, given the high concentrations, it is probably not of any physiological significance (8).

Later studies have also demonstrated the existence of P_2-purinoceptors on human liver plasma membranes (9). The association of [^{35}S]αATP with purified human liver plasma membranes was rapid (equilibrium being reached within 1 min) and reversible. The binding pattern could not be, either, described by a simple Michaelis-Menten equation, as a curvilinear Scatchard plot was obtained. As with rat liver plasma membranes, there were two independent components of binding. One represents saturable binding, characterized by a K_d of 0.19 μM and a B_{max} of 24 pmol/mg protein. The other, observed at higher concentrations of [^{35}S]αATP, can be considered as non-saturable binding. As this situation is almost identical to that observed with rat liver membrane (where high-affinity sites had K_d of 0.11 μM and B_{max} of 30 pmol/mg protein), the investigators concluded that human and rat liver possess the same class of P_2-purinoceptors.

Signal transduction: In isolated rat hepatocytes, stimulation of P_2-purinoceptors by ATP results in marked production of inositol triphosphate (IP_3) (10). ATP (10^{-6} M) rapidly mobilizes intracellular Ca^{2+} and increases the concentration of free cytosolic Ca^{2+} ($[Ca^{2+}]_i$) within 1-2 s (7). The increase in $[Ca^{2+}]_i$ is maximal (2.5-3 fold) by about 10 s and is dose-dependent, with ATP being half-maximally effective at 8×10^{-7} M. At submaximal concentrations, the rise in $[Ca^{2+}]_i$ is transient due to hydrolysis of the agonist by ectonucleotidases. The increase in $[Ca^{2+}]_i$ in response to ATP can be potentiated by low concentrations of glucagon (10^{-9} M). In addition, the $[Ca^{2+}]_i$ rise can be antagonized in a time and dose-dependent manner by the tumor promoter 4β-phorbol 12β-myristate 13α-acetate. The increase in $[Ca^{2+}]_i$ was preceded by a dose-dependent increase in IP_3 (the putative second messenger for Ca^{2+} mobilization).

ATP also inhibited forskolin-induced accumulation of cAMP in the presence of a phosphodiesterase inhibitor (10). Treatment of hepatocytes with islet-activating protein, pertussis toxin, blocked the nucleotide-induced inhibition of cAMP accumulation, but exerted only a small effect on IP_3 production. Although GTP also inhibited forskolin-stimulated adenylate cyclase, this GTP-induced inhibition of the enzyme was susceptible to islet activating protein (IAP) and dependent on the concentration of ATP. Receptor-mediated inhibition of adenylate cyclase is known to be mediated by the G_i-protein in most tissues, and G_i loses its function as the mediator when it is ADP ribosylated by IAP (10). Thus, ATP inhibition of cAMP

accumulation is mediated via G_i.

Studies have suggested that rat hepatocytes have two types of P_2-purinoceptors: one is linked to adenylate cyclase via an inhibitory guanine nucleotide regulatory protein (G_i) and the other is linked to phospholipase C in a stimulatory fashion (7,10).

Glucose homeostasis: The rapid hormonal regulation of hepatic glycogen metabolism in the rat is mediated by two distinct intracellular effectors, namely cAMP and Ca^{2+}. Glucagon and β-adrenergic agonists (e.g., isoproterenol) bind to receptors which are coupled to adenylate cyclase via the stimulatory guanine nucleotide-binding protein (G_s) and increase the concentration of cAMP (121). On the other hand, α_1-adrenergic agonists (epinephrine and norepinephrine) and vasoactive peptides (vasopressin and angiotensin II) interact with receptors which are coupled to phosphatidylinositol 4,5-bisphosphate hydrolysis and induce a rise in $[Ca^{2+}]_i$ via the second messenger myo-inositol 1,4,5-triphosphate (IP_3) (122).

Exogenous ATP reproduces several effects of the Ca^{2+}-dependent glycogenolytic hormones, such as glucose production (6) at 0.1 mM, Ca^{2+} redistribution at 0.3-0.5 mM (7-10), glycogen phosphorylase (the rate-limiting enzyme of glycogenolysis) activation at 0.8 mM (6-12), hydrolysis of phosphotidyl inositol 4,5-bisphosphate at 0.1 mM (7). Recent studies have shown that, in addition to activating phosphorylase, ATP induces a dose-dependent inactivation of glycogen synthase (7,12). Furthermore, other studies have demonstrated that ATP acts at much lower concentrations

(submicromolar range) and present strong evidence for a common mode of action for ATP and the Ca^{2+}-dependent glycogenolytic hormones (6).

Extracellular ATP was also shown to inhibit the forskolin-induced cAMP production (10,12), thus having opposing action to glucagon and β-adrenergic agonists. This might imply that extracellular ATP increases gluconeogenesis. In this regard, studies (123) have demonstrated that only high, supramaximal concentrations of ATP (10^{-3} M) inhibit gluconeogenesis in isolated hepatocytes. ATP inhibited gluconeogenesis from lactate and pyruvate but not from glycerol or fructose, and this inhibition was associated with an increase in intracellular adenosine content. Furthermore, α,β-methylene-ATP, a slow metabolizable structural analogue of ATP, did not affect the rate of gluconeogenesis. Thus, it was concluded (123) that the inhibition of gluconeogenesis by high levels of extracellular ATP, a situation attainable only under some pathophysiological circumstances (124), may be mediated by adenosine derived from ATP catabolism at the plasma membrane.

Based on the aforementioned information, and the known effects of extracellular ATP to regulate cellular electrolyte homeostasis (125) and to stimulate Na^+-K^+ -ATPase activity (126), several investigators (6-12,123) have concluded that liver metabolism can be regulated by submicromolar concentrations of ATP (10) and it is, indeed under purinergic control (9).

USE OF ATP-$MgCl_2$ FOLLOWING SHOCK

Rational for the use of ATP-$MgCl_2$ following shock: Shock results from a sustained reduction in perfusion of capillaries and, therefore, of tissues and organs. Because of prolonged and sustained reduction in tissue perfusion during shock, various alterations in tissue metabolism, structure, and function occur at the systemic, cellular, and subcellular levels. The numerous alterations in cellular and subcellular function that occur following shock have been dealt with above. Briefly, the major changes during shock occur in the microcirculation, affecting cell membrane transport function, energy metabolism, and mitochondrial function. Such progressive and interrelated events eventually lead to cell swelling. When such swelling has developed, blood flow cannot rapidly return to normal despite fluid resuscitation. Part of the insult under such conditions may be caused by continued ischemia related to endothelial and parenchymal cell swelling along with sledging of blood. Pharmacological interventions which improve blood flow, microcirculation, and cell function after such conditions may be necessary when volume resuscitation and conventional means of circulatory support have failed. In this regard, many pharmacological agents for the treatment of shock and ischemia are being evaluated (5).

Blood or fluid administration to provide or maintain an adequate vascular volume during the early stages of shock is often sufficient to correct the problem. If fluids alone are not successful, then the use of inotropic agents to increase cardiac output and improve blood flow may be

required (5). Along with these, various adjunctive agents and approaches such as administering buffering agents, vasoactive agents, steroids, and other means of circulatory support have been used (5). If the foregoing non-specific treatment of circulation becomes ineffective, then correction of specific alterations in cell function may be necessary.

A common feature of all forms of shock is thought to be an inadequate circulation with diminished blood flow to tissues (5). As a result of this decreased blood flow to tissues, hypoxic conditions prevail and the production of ATP is significantly depressed at a time when more energy is needed to protect the cell against an insult. In addition, with reduced flow and perfusion, the availability and the delivery of substrate to target tissues decreases, and energy production is further compromised.

The essential role of ATP in membrane function, carbohydrate metabolism, tissue respiration, muscle contraction, and supplying energy for various intracellular reactions is well recognized (5). It is clear that in order for ATP levels to be maintained in the tissues, oxidative phosphorylation must continue. This, however, is not the case during low-flow conditions because oxygen delivery as well as substrate delivery to tissues is decreased (5). Moreover, because glycolysis cannot match ATP production in relation to its utilization during shock, the cellular adenine nucleotide levels decrease under such conditions (5).

The provision of substrates such as hypertonic glucose, inosine, and 1,6-diphosphate (127) during shock may be helpful; however, the basic

problem during low-flow conditions is that an adequate amount of substrates cannot reach the target tissues because of reduced blood flow. Moreover, because the metabolism of substrates is decreased during shock (127), it appears unlikely that provision of substrates per se could correct the cellular and metabolic alterations until blood flow increases. Therefore, it could be argued that provision of energy (ATP) that does not have to pass through the glycolytic pathway, the Krebs cycle, and the electron transport chain to produce ATP may be the most advantageous and direct method for the treatment of shock and ischemia when fluid and vasoactive agents are not effective.

Because the resynthesis of ATP is a major rate-limiting factor following adverse circulatory conditions (127), it seems that the most direct approach for raising tissue ATP levels under these conditions would be to infuse ATP rather than administer agents that would synthesize it. Such an approach was used by Talaat and associates (128), who showed that ATP infusion before or during shock improved the survival of animals. Subsequent studies by Sharman and Eiseman (129) showed that ATP was protective if given prior to hemorrhage but that it was not protective if it was administered following severe hemorrhagic shock. Finally, Chaudry and associates (5) extended the work of these investigators (128,129) by introducing one essential difference: they gave $MgCl_2$ along with ATP (5). ATP, which is a biological complexing agent (130), may, when given alone, chelate divalent cations from the vascular system and produce a different

hemodynamic effect. Such undesirable effects may be eliminated by giving an equimolar amount of $MgCl_2$ along with ATP (130). Moreover, studies have shown that magnesium *in vitro* and *in vivo* inhibits the deamination and dephosphorylation of ATP by tissues (130). Thus, if ATP is given along with $MgCl_2$, a higher concentration of ATP could be available to tissues than when ATP is given alone. In addition, because ATP and ADP in the cell exist as magnesium complexes, and because most ATP reactions require not only ATP as substrate but also magnesium as a cofactor, it is possible that parallel changes in the levels of these two substances may contribute to the depression of cellular functions observed following shock and low-flow conditions. Indeed, studies by Chaudry *et al.* (130) have indicated that tissue and mitochondrial magnesium levels decreased significantly following ischemia and reflow and that ATP-$MgCl_2$, but not ATP or $MgCl_2$ alone, after ischemia significantly increased tissue and mitochondrial magnesium levels. Tissue ATP and ADP levels also follow the same trend. Thus, it could be concluded that a significant loss of tissue ATP and ADP occurs, together with decreased tissue and mitochondrial magnesium levels, after ischemia and reflow and that ATP together with $MgCl_2$ treatment is required for increasing cellular and mitochondrial magnesium levels and for improving cellular functions under such conditions (131).

Since mitochondrial calcium levels increase following ischemia and reflow (5,50-53), administration of Mg^{2+} (along with ATP) may also serve as a calcium-entry-blocker, inhibiting calcium uptake across the injured cells.

Mg^{2+} has indeed been shown to behave as a Ca^{2+} entry blocker in vascular muscle (132,133). Studies have, in fact, shown that the elevated mitochondrial calcium levels following hepatic ischemia and reflow were normal with ATP-$MgCl_2$ treatment (134). On the basis of the above information, it can be concluded that ATP together with Mg^{2+} is required for improving cellular functions and the survival of animals following shock and ischemia.

Effects of ATP-$MgCl_2$ on survival and organ function following shock: Studies from a number of laboratories have shown that infusion of ATP-$MgCl_2$ following hemorrhagic shock (5,31,135,136), severe burns (79), sepsis-peritonitis (5), hepatic failure (5), bowel ischemia (137), and endotoxic shock (24,138) significantly improved the survival of animals. The beneficial effects on survival following shock were not observed if ATP or $MgCl_2$ alone or adenosine-$MgCl_2$ were infused (5,139,140). From these results, it could be concluded that ATP together with $MgCl_2$ is required for the treatment to be effective.

ATP-$MgCl_2$ has been shown to accelerate the recovery of hepatic and renal function following acute hepatic (5) and renal failure (141), respectively. Moreover, studies have demonstrated that kidneys subjected to episodes of warm ischemia could be salvaged by the addition of ATP-$MgCl_2$ to the perfusate (142). In addition, ATP-$MgCl_2$ was shown to have beneficial effects on graft survival in ischemically injured kidneys undergoing cold-storage preservation (143). Studies have also suggested that ATP-$MgCl_2$ may be useful in preventing ischemic damage (144).

Finally, infusion of ATP-$MgCl_2$ during reperfusion following hypothermic myocardial ischemia was also shown to ameliorate the reperfusion injury (145).

The effects of ATP-$MgCl_2$ and adenosine-$MgCl_2$ on renal function following ischemia were compared (139). The results indicated that adenosine-$MgCl_2$ treatment failed to provide the same sustained improvement in renal function compared with ATP-$MgCl_2$ treatment. Thus, it was concluded that the salutary effects of ATP-$MgCl_2$ following warm ischemia in the kidney are not mediated by adenosine.

Effects of ATP-$MgCl_2$ on altered cellular functions following shock: As we have discussed before, when fluid resuscitation along with adjunctive agents and other means of circulatory support have failed following adverse circulatory conditions, then correction of specific alterations in cell function may be necessary following such conditions.

Although the above mentioned studies indicate that ATP-$MgCl_2$ administration improves the survival of animals following shock and ischemia, the question remains whether infused ATP-$MgCl_2$ corrects the abnormalities in cell function after shock or ischemia. Studies have indeed shown that infused ATP-$MgCl_2$ returns the altered membrane permeability toward normal (146,147) and normalizes the transmembrane potential following hemorrhagic shock (148). ATP-$MgCl_2$ infusion was also shown to reverse the inhibition of ornithine metabolism and the change in tissue lactate levels during shock (147). Administration of ATP-$MgCl_2$ following

hemorrhagic shock significantly increased cellular and tissue ATP levels (5,149,150). Moreover, it has been shown that ATP-$MgCl_2$ reverses ischemically induced hepatic cellular edema (151), improves the electrolyte balance (152), and restores the depressed hepatocellular function after ischemia (5). Furthermore, ATP-$MgCl_2$ has been shown to reverse the tissue insulin resistance in skeletal muscle following hemorrhagic shock (153), and to improve the Na^+-K^+ balance in the liver following ischemia (151,154).

Alterations in mitochondrial function following hemorrhagic shock are well-recognized (61-65) and include altered metabolic activity, decreased respiratory control ratio (RCR), increased N^+ and Ca^{2+} contents, and increased free fatty acids (FFA). Treatment of animals after hemorrhagic shock with ATP-$MgCl_2$, however, resulted in restoration of the respiratory control ratio (RCR) (152). Infusion of ATP-$MgCl_2$ after hepatic ischemia and reflow also resulted in progressive and significantly higher adenine nucleotide translocase activity in liver mitochondria, significantly lowered free fatty acid levels, and normalized mitochondrial calcium levels (134). Thus, mitochondrial function can be significantly improved by treatment of animals with ATP-$MgCl_2$ after shock or ischemia. This was also associated with significantly higher hepatic ATP levels and energy charge (54).

Simple hemorrhage with or without tissue trauma, produces immunosuppression and enhances host susceptibility to sepsis (29,85,86). In this regard studies have indicated that administration of ATP-$MgCl_2$ not only restores immunoresponsiveness after burn injury (155) and hemorrhagic

after hepatic ischemia (158), sepsis-peritonitis (5), and hemorrhagic shock (136). More recent studies have also indicated that the decreased interleukin-2 levels following hemorrhage and resuscitation can be restored if ATP-$MgCl_2$ is given to animals following hemorrhagic shock (159). Administration of ATP-$MgCl_2$ is also known to alleviate nephrotoxicity and improve organ allograft survival rate (160) and to reduce drug-induced nephrotoxicity following acute renal failure which can result from ischemic injury (161).

Effects of ATP-$MgCl_2$ on blood flow and cardiac function following shock: Blood flow is depressed following hemorrhagic shock and ischemia to all but certain vital organs (88). As total hepatic blood flow decreases following hemorrhage (88,89), effective hepatic blood flow (EHBF) or nutrient hepatic blood flow is severely depressed following such a condition (89). In this regard, the microcirculation, which is responsible for the flow to cellular components of an organ, is of critical importance. The microcirculation was shown to be depressed in hepatic tissues after ischemia and reperfusion (162) and renal microcirculation was also depressed following trauma and severe hemorrhage (163) as well as during ischemia and reflow (164). The administration of ATP-$MgCl_2$ following shock and ischemia restored hepatic and renal blood flow as well as the microcirculation to vital organs following such conditions (162-165). Thus, ATP-$MgCl_2$ improves microcirculatory blood flow. Furthermore, additional studies in this regard have indicated that postischemic administration of ATP-$MgCl_2$ corrects the

intracellular acidosis, restores the tissue ATP stores, and prevents the deterioration in perfusate flow that occurs following ischemia and reflow (166,167). Thus, it can be concluded that ATP-$MgCl_2$ prevents the reperfusion injury.

Administration of ATP-$MgCl_2$ has been shown to restore the depressed cardiac output (CO) following adverse circulatory conditions (168-171). Although, ATP-$MgCl_2$ administration decreases total peripheral resistance (TPR), the increase in CO produced by ATP-$MgCl_2$ administration offsets the decrease in TPR and thus, the mean arterial pressure (MAP) is maintained (169). ATP is a potent vasodilator, the administration of ATP-$MgCl_2$ produces peripheral vasodilation and increases myocardial efficiency (170). It could be argued, however, that if such an agent is administered during compromised cardiac function, increased myocardial performance plus peripheral vasodilation might create a situation in which O_2 demand exceeds supply (170). In this regard, studies have demonstrated that infusion of ATP-$MgCl_2$ not only decreased total body oxygen consumption (172), but myocardial oxygen consumption during normovolemic as well as hypovolemic conditions (170). These decreases occurred in spite of increased coronary flow and cardiac output (170). Thus, it could be concluded that the use of ATP-$MgCl_2$ during low-flow conditions and during clinical instances of coronary insufficiency is of therapeutic use (173). In support of this notion, other studies have demonstrated that ATP-$MgCl_2$ can be administered safely in awake human volunteers (169).

SPECIFIC AIMS

The work presented in this dissertation focuses on the study of how trauma-hemorrhagic shock and crystalloid resuscitation affect four hepatocellular processes in the rat. These processes include: 1) The binding properties of P_2-purinoceptors; 2) Basal levels of the second messengers (cAMP and IP_3) and their ability to respond to receptor-dependent as well as receptor-independent stimulation; 3) Activities of glucokinase (GK), pyruvate kinase (PK), and phosphoenolpyruvate carboxykinase (PEPCK), key enzymes in glucose metabolism; 4) Expression of mRNA's involved in the translation of the above mentioned enzymes. The second focus will be on the effect of ATP-$MgCl_2$ administration during the resuscitation of hemorrhagic shock on the aforementioned processes. The hypothesis to be tested in this thesis is that hemorrhagic shock will alter the dynamics of P_2-purinoceptors and diminish the ability of hepatocytes to respond to receptor-dependent stimulation, a result of the documented (5,43) primary insult and destabilization of the plasma-membrane following shock. It is also hypothesized that hemorrhagic shock will alter the activities and/or the mRNA levels of GK, PK, and PEPCK; this will correlate with the previously reported altered glucose homeostasis following shock (23-28). The final hypothesis to be tested is that the beneficial effects of ATP-$MgCl_2$ administration during the resuscitation of hemorrhagic shock will include these altered processes.

To test these hypotheses, several diverse experimental strategies were used in this thesis. First, a specific *in vitro* radioligand binding assay was used to measure P_2-receptor binding in hepatocytes from sham-operated, hemorrhaged and saline-treated, as well as hemorrhaged and ATP-$MgCl_2$-treated rats and to answer the first question addressed in this dissertation: Does trauma-hemorrhagic shock alter the binding affinity and/or density of P_2-purinoceptors on rat hepatocytes and, if so, whether ATP-$MgCl_2$ administration during fluid resuscitation have any restorative effects on these parameters. Next, the ability of hepatocytes from sham-operated, hemorrhaged and saline-treated, as well as hemorrhaged and ATP-$MgCl_2$-treated animals, to respond to receptor-dependent and receptor-independent stimuli of the second messengers cAMP and IP_3 was examined. This study was designed to answer the second question posed in this dissertation: Does shock-induced destabilization of the plasma-membrane produce receptor-transductional uncoupling and, if so, whether ATP-$MgCl_2$ treatment reverses this adversity ? Finally, the activities and mRNA levels of glucokinase, pyruvate kinase, and phosphoenolpyruvate carboxykinase were measured in liver homogenates or hepatocytes, respectively, from sham-operated, hemorrhaged and saline-treated, as well as hemorrhaged and ATP-$MgCl_2$-treated rats. This experimental approach was used to address the third question examined in this dissertation: Are the metabolic alterations in glucose homeostasis following shock related to changes in the activities and/or expression of the key glycolytic and/or gluconeogenic

enzymes (GK,PK, and PEPCK) and, if so, whether ATP-$MgCl_2$ treatment have any beneficial effects on these alterations.

CUMULATIVE METHODS

TRAUMA-HEMORRHAGIC SHOCK MODEL

Male Sprague-Dawley rats (280-310g) were fasted overnight (16-18 h), but allowed water *ad libitum*. At the time of the experiment, the rats were lightly anesthetized with ether and a 4-5 cm ventral midline laparotomy was performed in order to introduce trauma prior to hemorrhage. The abdominal incision was then closed in layers and bathed with 1% lidocaine. Polyethylene (PE) 50 tubing was used to cannulate the following vessels: the right carotid artery for monitoring the mean arterial pressure (MAP); the left jugular vein for infusion of crystalloid fluids; the left femoral artery for bleeding the animal; and the left femoral vein for infusion of treatments. All incisions were then closed and bathed with 1% lidocaine. The rats were allowed to recover from the anesthesia (to awaken), then rapidly bled to a MAP of 40 mmHg (within 10 min) and maintained at that pressure, by either withdrawal of more blood or infusion of crystalloid fluid, until 40% of the estimated maximum bleedout volume was returned in the form of Ringer's lactate. The total hypotensive period was 80-90 min. The rats were then resuscitated with three times (3X) the volume of maximum bleedout with Ringer's lactate over 45 min followed by 2X Ringer's lactate

containing either ATP-$MgCl_2$ (50 μmol/Kg BW), $MgCl_2$ (50 μmol/Kg BW), ATP (50 μmol/Kg BW), nitroglycerine (45 μmol/Kg BW), or equal volume of saline over 95 min. Maximum bleedout was the volume of blood withdrawn following which the animal could not maintain the MAP of 40 mmHg unless some fluid (Ringer's lactate) was returned. The time required to reach maximum bleedout was approximately 45 min and the maximum bleedout volume was 60-65% of estimated effective blood volume (174). The shed blood was not returned in this hemorrhage model and the animals were not heparinized throughout the experiment. Sham-operated animals underwent the same surgical procedure, but were not bled. The experiments described here were performed in adherence to guidelines from the National Institute of Health for the use of experimental animals.

Preparation of Isolated Rat Liver Parenchymal Cells

Hepatocytes were isolated according to the method of Berry and Friend (175), at maximum bleedout (MBO), and 0, 4, 17, and 27 h post-resuscitation. The rats were anesthetized with ether, the abdominal cavity was re-opened and the portal vein was cannulated using PE-90 tubing. The vena cava was then severed and the liver perfused *in situ* at 37°C. The perfusion started with Ca^{2+}-free Krebs-Henseleit bicarbonate buffer (pH 7.4) supplemented with 2.5 mM EGTA, at 14 ml/min for 10 min with continuous gassing (95% O_2: 5% CO_2). Perfusion was then changed to Puck's saline, containing collagenase type II, 0.12 mg/ml, and continued for 30 min in an

atmosphere of 95% O_2: 5% CO_2. The digested liver was then removed into a Petri dish containing 10 ml of the same buffer (Puck's saline). It was cut into small pieces with scissors, mashed gently with a spatula, and placed into a 250 ml glass Erlenmeyer flask containing 50 ml of the same buffer. The flask was then shaken at 60 cycles/min for 10 min at 37°C (water bath) with continuous gassing (95% O_2: 5% CO_2).

The flask contents (dispersed liver cells, undigested liver fragments, erythrocytes) were poured through nylon mesh into 50 ml plastic conical centrifuge tube and centrifuged at 50g, 4°C for 2 min (Sorvall RT 6000D). The supernatant fluids including the erythrocyte layer were withdrawn and the sedimented cells were washed (three times) with 30 ml of oxygenated, normal Krebs-Ringer bicarbonate buffer (pH 7.4). The washed cells were then layered over a Percoll cushion and centrifuged for 5 min at 270g (1200 rpm), at 4°C, resuspended in minimum essential medium (MEM) containing 10% dimethyl sulfoxide (DMSO), 40% fetal bovine serum (fbs), and stored at -72°C. Cell viability, 90-95% prior to freezing, and 80-85% after thawing, was measured by trypan blue exclusion.

Percoll cushion was made as follows: first, a Percoll stock was prepared; 90 ml Percoll (sterile), 10 ml 10X PBS (sterile), and 1 ml 1 M Hepes (sterile), the pH was adjusted to 7.4 ± 0.2, stored at 4°C. Second, plating media; 23 mM HEPES, 26 mM sodium bicarbonate, 1 µg/ml pen-strep, 2 mM glutamine, 0.1 U/ml insulin, 10 nM dexamethasone, and 10% fbs; pH was then adjusted to 7.4 , and the media was filter sterilized.

At the time of use, Percoll cushion was prepared by mixing 1:1 Percoll stock and plating media.

Measurement of [^{35}S]αATP Binding to Isolated Hepatocytes

The procedure that was described by Keppens and Wulf (8) for studies of P_2-purinoceptors was used in our experiments. Cells were preincubated for 20 min at 37°C in Krebs-Henseleit bicarbonate medium (pH 7.4) with 10 mM glucose. Hepatocytes (1×10^6) in 1.0 ml aliquots were then incubated for 1 min, at 37°C, in Krebs-Henseleit bicarbonate medium (pH 7.4) with [^{35}S]αATP concentrations ranging from 50 nM to 5 μM. Incubation was terminated by dilution into 4 ml of ice-cold 3 mM Hepes buffer (pH 7.4) containing 0.15 M NaCl, 2 mM $CaCl_2$, 1.3 mM $MgCl_2$ and 1 mM ATP. Diluted samples were immediately filtered on ice-cold Whatman glass fiber filters (GFA) pre-soaked in this buffer and rinsed 3X with 7 ml of ice-cold 3 mM Hepes buffer (pH 7.4) containing 0.15 M NaCl. In preliminary studies, it was determined that binding of [^{35}S]αATP had reached equilibrium by 1 min at the lowest concentration used (50 nM). The presence of non-labeled ATP in the dilution buffer is mandatory to prevent spurious binding to the filters (8). It has been checked that this temperature (0-4°C) avoids disassociation of the bound [^{35}S]αATP. Indeed, the same amount of bound [^{35}S]αATP was detected when samples were treated as described or were kept for 30 s at 0°C in the stopping buffer before filtration. Filters were air dried and the radioactivity determined by

conventional liquid-scintillation spectrometry techniques (LKB, Wallack). Non-specific binding was determined by the inclusion of a 200-fold excess (over each respective concentration) of SαATP over [^{35}S]αATP to duplicates in the respective incubation mixtures. The binding of [^{35}S]αATP to hepatocytes increased linearly with the cell concentration tested up to 10^7 cells/ml. Scatchard plots were performed on the saturating binding curves with the assistance of the nonlinear, least-squares computer curve fitting program Ligand (G.A. Mcpherson Biosoft, London) to obtain the disassociation constant (K_d) and the maximal binding capacity (B_{max}) (176).

MEASUREMENTS OF cAMP

For measurements of cAMP content, hepatocytes were first incubated for 20 min at 37°C (shaking waterbath 120 cycles/min) in an atmosphere of 95% O_2: 5% CO_2, at a density of about 5 x 10^6 cells/ml in Hepes-buffered medium supplemented with 4 mM glucose and 2% bovine serum albumin (BSA). The Hepes-buffered medium consisted of 134 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5mM $CaCl_2$, 5 mM $NaHCO_3$, and 10 mM Hepes (pH 7.4). After this preincubation procedure, 200-µl aliquots of the cell suspension were transferred to plastic tubes containing 50-µl saline fortified with drugs or not (as control). The tube was then shaken in a bath at 37°C at a rate of 180 cycles/min to be analyzed for the response of hepatocytes to added drugs (177-179).

After 5 min incubation, in the presence of a phosphodiesterase inhibitor (1 mM IBMX), saline (for assessment of cAMP basal levels), 10^{-6} M glucagon ± 1 mM ATP, or 50 µM forskolin, reactions were terminated by adding HCl and EDTA to final concentrations of 0.1 N and 5 mM respectively. The incubation tubes were then immediately immersed in a boiling water bath for 3 min to provide an acid extract suitable for the assay of cyclic nucleotides as described by Honma *et al.* (179). Tubes were centrifuged and cAMP in the supernatant was then measured by a sensitive radioimmunoassay procedure (177,179), using a cAMP radioimmunoassay kit (DuPont NEK-033).

Measurements of IP_3

For measurements of IP_3 content, hepatocytes were preincubated for 10 min at 37°C (shaking waterbath 120 cycles/min) in an atmosphere of 95% O_2: 5% CO_2, at a density of about 5 x 10^6 cells/ml in Hepes-buffered medium supplemented with 4 mM glucose and 1% bovine serum albumin (BSA). The Hepes-buffered medium was of the same contents as used for cAMP measurements (see above). LiCl was then added to a final concentration of 10 mM and incubation was continued for another 10 min. Aliquots of cell suspension were then transferred to plastic tubes containing the required concentration of various agents or not (as control) (10,121,122).

After 15 sec incubation with saline (for assessment of IP_3 basal levels), 10^{-7} M vasopressin or 1 mM ATP, or 1 min and 15 sec incubation

with 12.5 mM Na-F, reactions were terminated by adding 0.2 volumes of 100% ice-cold TCA solution. Tubes were then transferred on ice and incubated for 15 min. Following centrifugation for 10 min at 0-4°C at 1000g, the supernatant was removed and the pellet was discarded. The supernatant solution was then incubated at room temperature for another 15 min. IP_3 was measured by a radioreceptor assay procedure following TCA extraction, both of which were carried out utilizing an IP_3 radioreceptor assay kit (DuPont NEK-064).

In both second messenger (cAMP,IP_3) experiments the concentrations of the various drugs used and the times of incubation were based on information obtained from preliminary studies (dose-response curves and time courses) with each individual drug. In addition, when Purine nucleotides were used as agents in these experiments, the same concentration of $MgCl_2$ as that of the nucleotide was added to the reaction mixtures (10).

ANALYTICAL METHODS

Glucokinase activity: Livers were homogenized in 5 volumes of buffer containing 50 mM Tris/HCl, pH 7.0, 0.15 M KCl, 5 mM EDTA, 4 mM $MgCl_2$, and 2 mM dithiothreitol. Homogenates were centrifuged at 100,000 x g_{av} for 60 min, and the clear supernatant was assayed for enzyme activity immediately or after freezing and storage at -80°C. The glucose-6-phosphate formed was coupled with the reduction of $NADP^+$ (nicotinamide-adenine dinucleotide phosphate) by the dehydrogenase of glucose-6-phosphate.

The formation of NADPH was followed at A_{340} in cells of 1-cm light path in Hitachi U-3110 spectrophotometer. The incubation medium, in a total volume of 3 ml, contained (final concentrations) 44 mM sodium glycylglycinates, 28°C, pH 7.5; 0.75 mM $NADP^+$; 7.5 mM $MgCl_2$; 3 mM ATP and glucose-6-phosphate dehydrogenase, 0.625 Kornberg units. In addition, individual cuvettes contained the following: cuvette 1, 0.5 mM glucose; cuvette 2, 200 mM glucose. Glucokinase activity was estimated by subtracting the hexokinase activity measured at 0.5 mM glucose from the activity measured at 200 mM glucose. A unit of glucokinase is defined as the enzyme activity resulting in the formation of 1 µmol of glucose-6-phosphate/min under the conditions of the assay (180-182).

PEPCK activity: Livers were excised and placed in 0.9% NaCl solution at 4°C. Organs were suspended in 4 volumes of buffer, 10 mM triethanolamine, pH 7.5, 0.25 M sucrose, 1 mM EDTA, 1 mM dithiothreitol and dispersed with a polytron operated 10 to 15 s at maximum speed at 4°C. The homogenate was centrifuged at 4°C, 100,000 x g_{av} for 1 h to yield a cytosolic fraction that was used for enzyme assays. Aliquots (100 µl) of liver high-speed supernatant were assayed at 25°C in final volume of 2.0 ml containing 50 mM Hepes, pH 7.2, 1 mM IDP, 2 mM $MnCl_2$, 1 mM dithiothreitol, 0.25 mM NADH, 2 mM phosphoenolpyruvate, and 10 µg of malate dehydrogenase. Background utilization of NADH was monitored by the decrease in A_{340} in a Hitachi U-3110 spectrophotometer. $NaHCO_3$ was added to 50 mM and the new rate of NADH utilization due to PEPCK was

monitored. The oxaloacetate (OAA) produced by PEPCK was quantitatively reduced with NADH by malate dehydrogenase in the coupled enzyme reaction. The rate of utilization of NADH over background was employed to estimate PEPCK activity (1 unit = 1 μmole of OAA formed/min at 25°C) (183-185).

Pyruvate kinase activity: Cytosolic fractions were prepared as described above for glucokinase. Aliquots (100 μl) of high speed supernatant were used to measure the catalytic activity of pyruvate kinase. Assays were performed at 31°C in reaction mixtures containing 100 mM Tris/HCl, pH 7.4, 100 mM KCl, 4 mM $MgCl_2$, 0.15 mM NADH, 5 units of lactate dehydrogenase, 2 mM ADP, 1 mM fructose 1,6-bis-phosphate, and 1mM phosphoenolpyruvate in a total volume of 2.0 ml. The reaction was started by the addition of adenosine diphosphate (ADP) after a 2 min preincubation at 31°C. The pyruvate formed from phosphenol pyruvate (by pyruvate kinase) was coupled to the utilization of NADH by the lactate dehydrogenase reaction. The utilization of NADH was followed spectrophometrically at A_{340}. A unit of pyruvate kinase activity will catalyze the formation of 1 μmol of pyruvate/min under the conditions defined (186-188).

Protein assays: Protein levels used to normalize the data were determined by the spectrophotometric assay of Bradford (189) using bovine serum albumin as a standard. In this regard the Bio-rad protein assay kit II (500-0002) was utilized.

Measurement of mRNA: Hepatocytes isolated from sham-operated, hemorrhaged and saline-treated, as well as hemorrhaged and ATP-$MgCl_2$-treated rats at 4 h post-resuscitation were used for Northern blot analysis. Total RNA was isolated using the methods of Chirgwin *et al.* (190) and Jonas *et al.* (191). Hepatocytes were lysed with a solution of 25 mM Tris containing 4.2 M guanidine isothiocynate, 0.5% sarkosyl and 0.1 M 2-mercoptoethanol, followed by extraction with phenol/chloroform and chloroform/isoamyl alcohol. The extracted RNA was precipitated in alcohol and the pellet was dissolved in TE buffer (Tris 10 mM/EDTA 0.1 mM). RNA concentration and purification were determined by measuring the absorbance at 260 and 280 nm. Total RNA (20 µg-25µg/lane) was fractionated in 1% Agarose/2.2 M formaldehyde gel. RNA was transferred to nitrocellulose membranes (Schleicher & Schuell Inc., Keene, NH) and fixed with ultraviolet light (U.V. Stratalinker, Stratagene, LaJolla, CA). Blots were prehybridized and then hybridized with ^{32}P-labeled cDNA (PEPCK, GK, PK) probes at 57°C in 6X SSC, 1X Denhardt's, 100 µg/ml RNA, 0.05% sodium pyrophosphate and 50 µg/ml polyadenylic acid. Blots were washed with 2X SSC/0.05% sodium pyrophosphate at 57°C. Autoradiography was carried out using Kodak X-OMAT film and an intensifying screen (DuPont NEN, Boston, MA) at -70°C. Blots were then stripped and rehybridized with β-actin as described above. The intensity of the bands on the autoradiograms was evaluated densitometrically by JAVA Image Analysis System (Jandel Scientific, San Rafael, CA).

Materials: Male Sprague-Dawley rats were obtained from Charles River (Wilmington, MA). Collagenase type II from Worthing (Freehold, NJ). Minimum essential media (MEM), fetal calf serum (fbs), and bovine serum albumin (BSA) from Gibco Laboratories (Grand Island, NY). [^{35}S]αATP-NaOH, cAMP radioimmunoassay kit, and IP_3 radioreceptor assay kit from NEN (Bannockburn, IL). SαATP-tetralithium from Boehringer Mannheim (Indianapolis, IN). Malate dehydrogenase was obtained from ICN Biochemicals (Irvin, CA). All other drugs and chemicals were purchased from Sigma Chemicals (ST. Louis, MO) unless otherwise specified. Finally the protein assay kit was obtained from Bio-Rad Laboratories (Richmond, CA).

Statistics: Statistical analysis were performed using one-way analysis of variance (ANOVA) and Tukey's test. Differences were considered significant at $P \leq 0.05$). The results are expressed as means ± SEM.

RESULTS

HEMORRHAGE AND BLOOD PRESSURE PARAMETERS

Starting blood pressure was 110 ± 4 mmHg. During the first 10 min from the onset of hemorrhage the shed blood volume was 7.6 ± 1.2 ml. The blood pressure of 40 mmHg was reached within 12.6 ± 3.2 min. The time required to reach maximum bleedout from the onset of hemorrhage was 45.6 ± 3.1 min. Maximum bleedout volume was 11.2 ± 2.6 ml (i.e. 61.3 ± 1.8% of estimated circulating blood volume). The total hypotensive period (hemorrhage time) was 86.6 ± 3.3 min, following which resuscitation was commenced. The first part of resuscitation, 3X the volume of maximum bleedout, in the form of Ringer's lactate, was administered over the next 45 min and was the same for all different animal groups. The second part of resuscitation, 2X the volume of maximum bleedout, in the form of Ringer's lactate fortified with saline or ATP-$MgCl_2$, was administered over the following 95 min. The MAP pattern during the entire resuscitation period for saline-treated as well as ATP-$MgCl_2$-treated animals is depicted in Figure 1. ATP-$MgCl_2$ treatment caused a small decrease in MAP (≈10 mmHg) however, the MAP increased gradually and after the completion of ATP-$MgCl_2$ infusion MAP was higher than that in the saline-treated rats.

Survival Rate

There were four survivals in each group. The survival rate in the saline group was 80%, 66%, and 50% up to 4, 17, and 27 h post-resuscitation, respectively. In the ATP-$MgCl_2$-treated group the survival rate was 100%, 80%, and 66% up to 4, 17, and 27 h post-resuscitation, respectively. The sham-operated animals had a 100% survival rate at all time points.

Immediate Effects of Hemorrhage With or Without Resuscitation on the Dynamics of Hepatocyte P_2-Purinoceptors

Hepatocytes were also isolated at the time of maximum bleedout and at the end of resuscitation to investigate whether there are immediate effects on the dynamics of P_2-purinoceptors on rat hepatocytes. Results in Figure 2 and Table 1 demonstrate that there was a significant ($P< 0.05$) decrease in the number of binding sites of the high-affinity component at the time of maximum bleedout, while other binding parameters remained unchanged. At the end of resuscitation, however, all binding parameters were not different from control values.

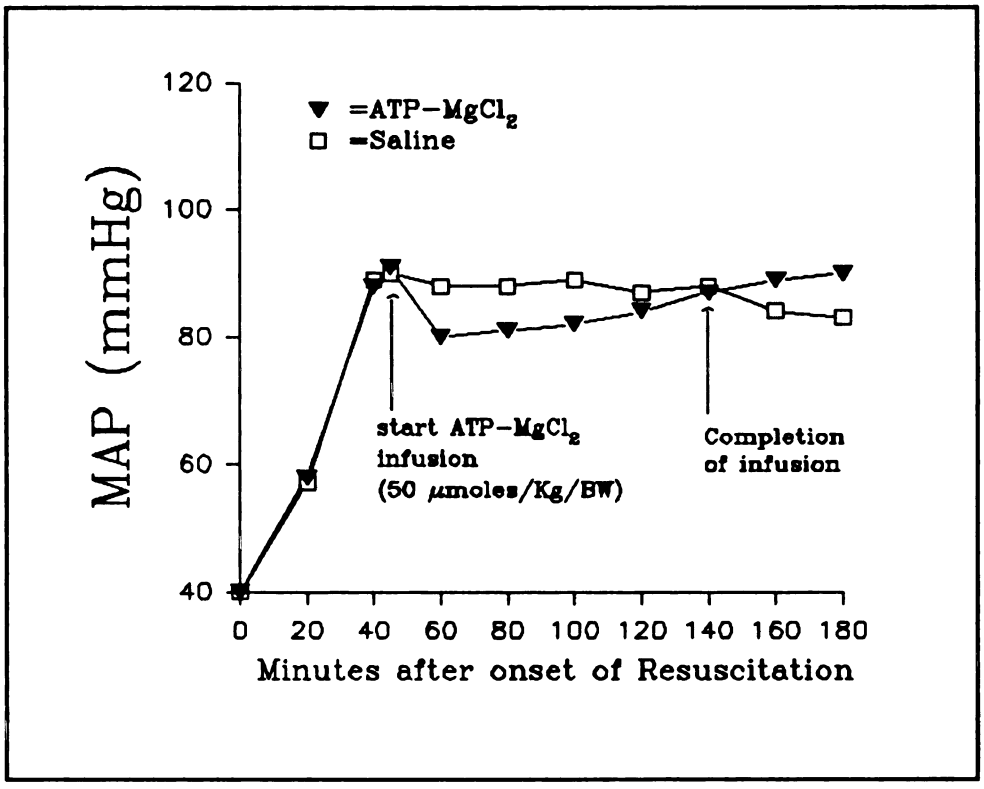

Figure 1. Changes in mean arterial pressure (MAP) in a representative rat with intravenous saline or ATP-$MgCl_2$ infusion during the second part of resuscitation. The rats were hemorrhaged and resuscitated first with 3X the volume of maximum bleedout with Ringer's lactate then, with 2X Ringer's lactate fortified with ATP-$MgCl_2$ (50 μmol/kg BW) or equal volume of saline. ATP-$MgCl_2$ was infused over 95 min. MAP was recorded every minute.

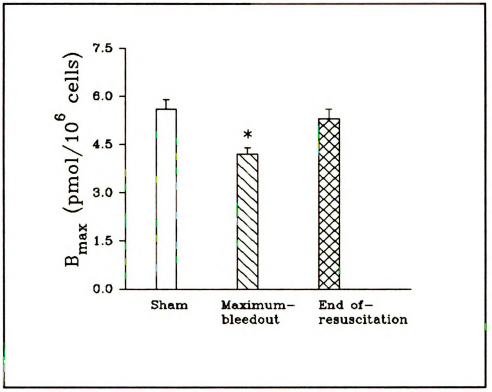

Figure 2. Changes in B_{max} of the high-affinity P_2-purinoceptor binding component at the time of maximum bleedout (i.e., approximately 45 min from the onset of hemorrhage, maximum bleedout volume was 60-65% of estimated effective blood volume, and at the end of resuscitation. Results represent means ± SEM of four animals/group and are compared with one-way ANOVA and Tukey's test: * $P < 0.05$ vs. sham-operated controls.

Table 1. Immediate effects of hemorrhage with or without resuscitation on hepatocyte P_2-purinoceptor binding parameters.

	High Affinity Component		Low Affinity Component	
	B_{max} (Pmol/10^6 cells)	K_d (nM)	B_{max} (Pmol/10^6 cells)	K_d (nM)
Sham-operated	5.2 ± 0.2	232 ± 16	10.8 ± 0.3	1180 ± 33
Maximum-Bleedout	4.2 ± 0.1*	241 ± 18	10.4 ± 0.3	1201 ± 41
End of Resuscitation	5.0 ± 0.2	238 ± 19	10.6 ± 0.2	1197 ± 25

Results represent means ± SEM from binding assays carried out on hepatocytes from four rats in each group and are compared with one-way ANOVA and Tukey's test: * $P < 0.05$ versus sham-operated controls.

Dose-dependency of Specific [^{35}S]αATP Binding to Rat Hepatocytes

Figure 3 illustrates that 4 h following hemorrhage and resuscitation, hepatocytes from saline-treated rats show a significant loss of [^{35}S]αATP specific binding when compared to hepatocytes from sham-operated controls. In contrast to saline-treated rats, hepatocytes from rats treated with ATP-$MgCl_2$ following hemorrhage show significantly higher [^{35}S]αATP specific binding. Results of a single experiment representative of four.

Scatchard Plots of [^{35}S]αATP Specific Binding to Rat Hepatocytes

Scatchard plots for sham-operated, hemorrhaged saline-treated, and hemorrhaged ATP-$MgCl_2$-treated rats, 4 h post-resuscitation are depicted in Figure 4. High and low-affinity binding components of P_2-purinoceptors were observed in all animal groups. Hepatocytes from hemorrhaged saline-treated rats showed dramatically reduced binding of [^{35}S]αATP to both binding components relative to hepatocytes from sham-operated controls, while hemorrhaged and ATP-$MgCl_2$-treated rats showed a significant increase in [^{35}S]αATP binding to both high and low-affinity components compared to saline-treated animals. Results of a single experiment representative of four.

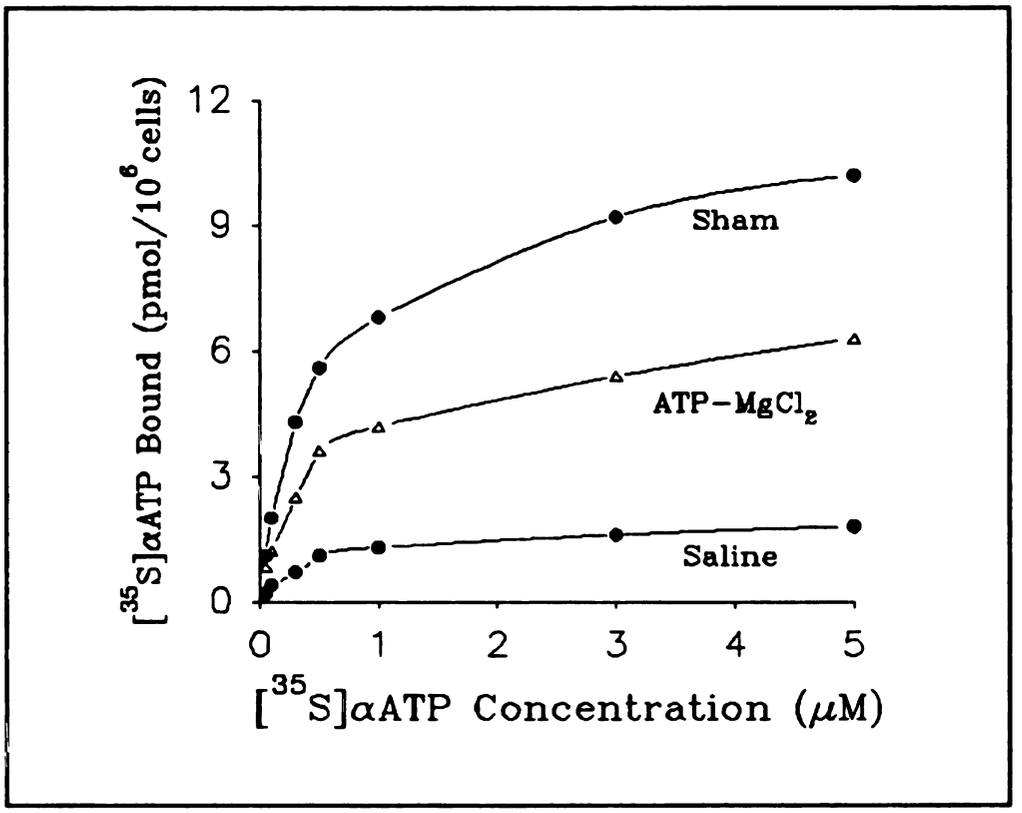

Figure 3. The association of specific [^{35}S]αATP binding to hepatocytes as a function of ligand concentration. 4 h post-resuscitation hepatocytes were isolated from sham-operated, hemorrhaged and saline-treated, as well as hemorrhaged and ATP-$MgCl_2$-treated rats and incubated with increasing radioactive ligand concentrations for 1 min. Results of a single experiment representative of four.

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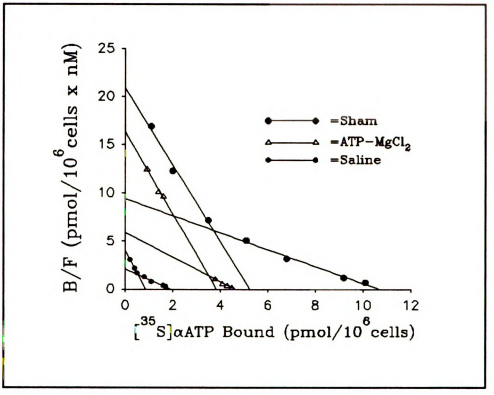

Figure 4. Scatchard plots of [^{35}S]αATP binding to hepatocytes isolated from sham-operated, hemorrhaged saline-treated, and hemorrhaged ATP-$MgCl_2$-treated rats, 4 h post-resuscitation. These data represent specific binding and were taken from Figure 2. Results of a single experiment representative of four.

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B_{max} OF THE HIGH-AFFINITY [^{35}S]αATP BINDING COMPONENT

Data shown in Figure 5 demonstrate that at 4, 17, and 27 h post-resuscitation, hepatocytes from hemorrhaged and saline-treated rats show a dramatic decrease in the number of [^{35}S]αATP binding sites. However, there was a trend toward a recovery in receptor numbers with the progression of time. On the other hand, ATP-$MgCl_2$ treatment ameliorated and subsequently restored the binding capacity of hepatocytes by 17 h post-resuscitation and this was sustained until 27 h post-resuscitation.

B_{max} OF THE LOW-AFFINITY [^{35}S]αATP BINDING COMPONENT

In Figure 6 it is seen that the B_{max} of the low-affinity component of binding was also decreased by hemorrhage and saline treatment throughout the time course. Although there was the same trend toward recovery in receptor numbers with the progression of time, the recovery in this receptor subtype was smaller than that in the high-affinity receptor subtype Figures 5 and 6. ATP-$MgCl_2$-treatment increased but did not restore the number of this receptor subtype.

K_d OF THE HIGH-AFFINITY [^{35}S]αATP BINDING COMPONENT

The K_d of the high-affinity component of binding in Figure 7 was not altered by hemorrhage and saline or ATP-$MgCl_2$ treatment.

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K_d OF THE LOW-AFFINITY [^{35}S]αATP BINDING COMPONENT

At all three time intervals, the K_d's for [^{35}S]αATP binding to hepatocytes from saline-treated rats were not significantly different ($P > 0.05$) from sham-operated controls. On the other hand, K_d's for binding to hepatocytes from hemorrhaged and ATP-$MgCl_2$-treated animals were reduced significantly, reflecting a progressive increase in the affinity ($1/K_d$) of this receptor subpopulation even higher than sham-operated controls at 17 and 27 h post-resuscitation, Figure 8.

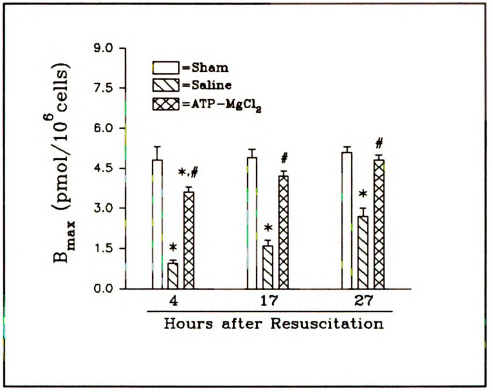

Figure 5. Changes in B_{max} of the high-affinity P_2-purinoceptor binding component at various times following hemorrhage and resuscitation. Results represent means ± SEM of 4 animals/group and are compared with one-way ANOVA and Tukey's test: * P< 0.05 versus sham-operated controls; # P< 0.05 versus saline-treated rats.

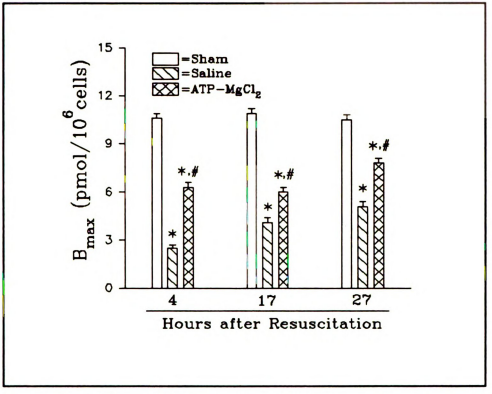

Figure 6. Changes in B_{max} of the low-affinity P_2-purinoceptor binding component at various times following hemorrhage and resuscitation. Results represent means ± SEM of 4 animals/group and are compared with one-way ANOVA and Tukey's test: * P< 0.05 versus sham-operated controls; # P< 0.05 versus saline-treated rats.

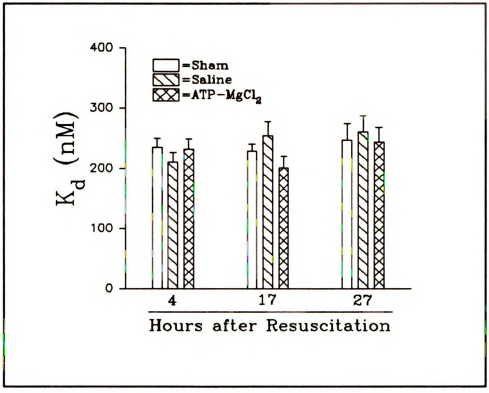

Figure 7. Changes in K_d of the high-affinity P_2-purinoceptor binding component at various times following hemorrhage and resuscitation. Results represent means ± SEM of 4 animals/group and are compared with one-way ANOVA and Tukey's test: * P< 0.05 versus sham-operated controls; # P< 0.05 versus saline-treated rats.

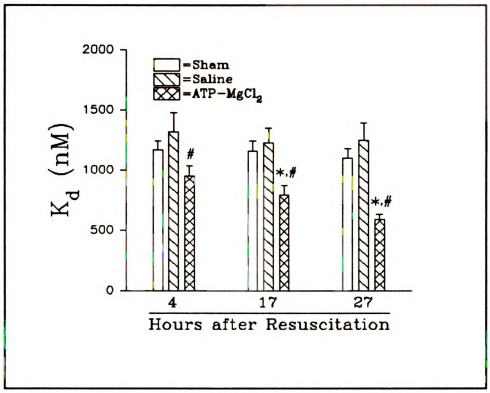

Figure 8. Changes in K_d of the low-affinity P_2-purinoceptor binding component at various times following hemorrhage and resuscitation. Results represent means ± SEM of 4 animals/group and are compared with one-way ANOVA and Tukey's test: * P< 0.05 versus sham-operated controls; # P< 0.05 versus saline-treated rats.

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Effects of ATP, $MgCl_2$, or Nitroglycerine on the Dynamics of P_2-Purinoceptors Following Hemorrhage and Resuscitation

Experiments were also carried out to determine whether treatment during resuscitation of hemorrhagic shock with ATP or $MgCl_2$ alone or with the vasodilator nitroglycerine (N.G) would have any restorative effects on the dynamics of either P_2-purinoceptor binding component, at 4 h after crystalloid resuscitation. Studies were conducted 4 h post-resuscitation because at that time interval the largest difference in B_{max} values between sham-operated controls and saline-treated animals were observed (Figures 5 and 6). Results in Figure 9 and Table 2 show that treatment with any of the three compounds (ATP, $MgCl_2$, and nitroglycerine) did not have any restorative effects on the binding parameter of P_2-purinoceptors when compared to the complex ATP-$MgCl_2$.

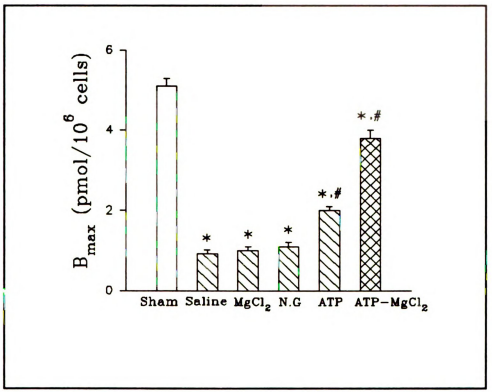

Figure 9. Changes in B_{max} of the high-affinity P_2-purinoceptor binding component. Following hemorrhagic shock, resuscitation was supplemented with saline, $MgCl_2$, nitroglycerine (N.G), ATP, or ATP-$MgCl_2$. 4 h post-resuscitation hepatocytes were isolated and binding assays were performed. Results represent means ± SEM of 4 animals/group and are compared with one-way ANOVA and Tukey's test: * $P < 0.05$ vs. sham-operated controls; # $P < 0.05$ vs. saline-treated animals.

Table 2. Effects of ATP, $MgCl_2$, or nitroglycerine treatment during resuscitation of hemorrhaged rats on hepatocyte P_2-purinoceptor binding parameters.

	High Affinity Component		Low Affinity Component	
	B_{max} (Pmol/10^6cells)	K_d (nM)	B_{max} (Pmol/10^6 cells)	K_d (nM)
Sham-operated	4.7 ± 0.5	228 ± 21	11.0 ± 1.6	1270 ± 160
Saline-treated	0.98 ± 0.05	251 ± 32	3.1 ± 0.3	1380 ± 210
ATP-treated	1.8 ± 0.2*,#	212 ± 18	3.8 ± 0.4*	1210 ± 210
$MgCl_2$-treated	1.2 ± 0.2*,#	229 ± 22	3.3 ± 0.3*	1240 ± 145
Nitro-glycerine treated	1.4 ± 0.3*,#	242 ± 25	3.5 ± 0.5*	1260 ± 128

Results represent means ± SEM from binding assays carried out on hepatocytes from four rats/group, 4 h post-resuscitation, and are compared with one-way ANOVA and Tukey's test: * $P < 0.05$ versus sham-operated controls, # $P < 0.05$ versus saline-treated animals.

cAMP Basal Levels

Figure 10 illustrates that hepatocytes isolated at 4 and 27 h post-resuscitation from saline-treated rats showed a significant decrease in the existing basal levels of the second messenger cAMP when compared to sham-operated controls. In contrast to saline-treated animals, hepatocytes isolated from rats treated with ATP-$MgCl_2$ following hemorrhagic shock showed significantly higher levels of cAMP at 4 h post-resuscitation. Furthermore, cAMP levels were restored to control values by 27 h post-resuscitation in the ATP-$MgCl_2$-treated group.

Effects of Glucagon on cAMP Accumulation

In Figure 11, hepatocytes were isolated at 4 and 27 h post-resuscitation from all three animal groups and stimulated with glucagon. Although hepatocytes isolated from rats following hemorrhage and saline-treatment showed a slight increase in cAMP levels when compared to their basal levels (Figures 10 and 11), their response was dramatically lower than that exhibited by hepatocytes from sham-operated controls. Thus, hepatocytes from saline-treated animals demonstrate a dramatic decrease in their ability to respond to glucagon stimulation. On the other hand, hepatocytes isolated from hemorrhaged and ATP-$MgCl_2$-treated rats showed a significant improvement in their ability to respond to glucagon stimulation; in fact, the levels of cAMP accumulation was near control values in hepatocytes from ATP-$MgCl_2$-treated animals.

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ATP Inhibition of Glucagon-induced cAMP Accumulation

When hepatocytes from saline-treated animals were stimulated with glucagon + ATP, they failed to respond when compared with sham-operated animals, Figure 12. Conversely, when hepatocytes from ATP-$MgCl_2$-treated rats were stimulated in a similar manner they responded by accumulating high levels of cAMP. It is worth noting, however, that the cAMP accumulation by hepatocytes from ATP-$MgCl_2$-treated animals, at 4 h post-resuscitation, was higher than the cAMP accumulation in the sham-operated animals (i.e., ATP inhibition is not fully expressed), but at 27 h post-resuscitation both groups showed similar levels of cAMP in their hepatocytes following glucagon + ATP stimulation.

Effects of Forskolin on cAMP Accumulation

When hepatocytes isolated from all three different animal groups were stimulated with the receptor-independent, potent activator of adenylate cyclase, forskolin, cAMP content increased enormously with no differences observed between the animal groups, Figure 13.

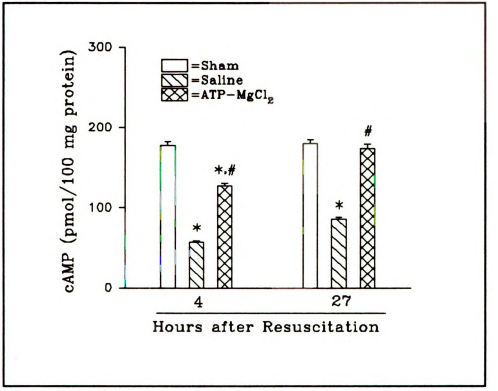

Figure 10. cAMP basal levels in hepatocytes isolated from sham-operated, hemorrhaged saline-treated, and hemorrhaged ATP-$MgCl_2$-treated rats. AT 4 and 27 h post-resuscitation hepatocytes were preincubated with 4 mM glucose as described under "Methods" and were then incubated for 5 min with or without drugs in the presence of 1 mM IBMX. The results are the means ± SEM of four separate experiments and are compared with one-way ANOVA and Tukey's test: * $P < 0.05$ versus sham-operated controls, # $P < 0.05$ versus saline-treated animals. cAMP content is expressed as Pmol/100mg protein. cAMP levels were measured by a radioimmunoassay procedure (NEN Kit #NEK-033).

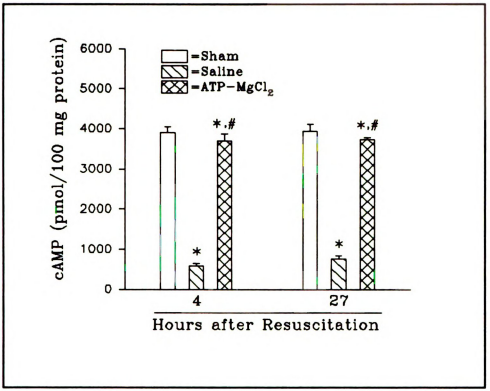

Figure 11. cAMP accumulation as induced by glucagon. After preincubation as described under "Methods," hepatocytes were incubated for 5 min with 10^{-6} M glucagon in the presence of 1 mM IBMX. Results are means ± SEM of four animals/group and are compared with one-way ANOVA and Tukey's test: * $P < 0.05$ versus sham-operated controls, # $P < 0.05$ versus saline-treated animals. For details see Figure 10.

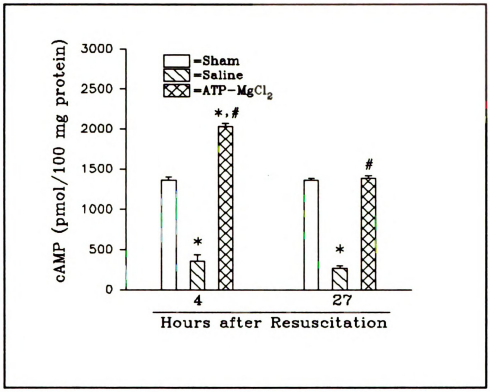

Figure 12. cAMP accumulation as induced by 10^{-6} M glucagon + 1 mM ATP in the presence of 1 mM IBMX. Results are means ± SEM of four animals/group and are compared with one-way ANOVA and Tukey's test: * $P < 0.05$ versus sham-operated controls, # $P < 0.05$ versus saline-treated animals. For details see Figure 10.

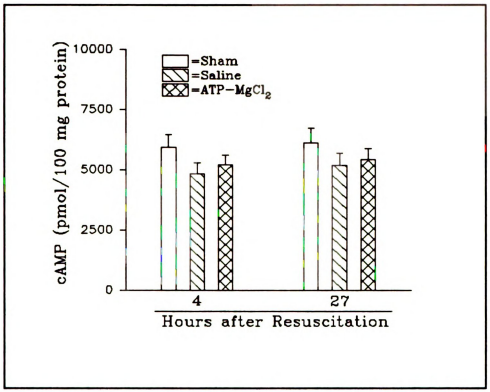

Figure 13. cAMP accumulation following stimulation of hepatocytes with 50 μM foskolin in the presence of 1 mM IBMX. Results are means ± SEM of four animals/group and are compared with one-way ANOVA and Tukey's test: * $P < 0.05$ versus sham-operated controls, # $P < 0.05$ versus saline-treated animals. For details see Figure 10.

IP_3 Basal Levels

Hepatocytes from all three animal groups were isolated at 4 and 27 h post-resuscitation and assessed for their existing basal levels of IP_3. It is demonstrated in Figure 14 that hepatocytes isolated from saline-treated rats following hemorrhagic shock showed an elevated level of IP_3 at 4 and 27 h post-resuscitation. In contrast to saline-treated animals, hepatocytes from rats treated with ATP-$MgCl_2$ showed only a slight increase in their IP_3 basal levels at 4 h post-resuscitation and at 27 h post-resuscitation their IP_3 basal levels were near control values.

Effects of Vasopressin on IP_3 Accumulation

Hepatocytes were isolated at 4 and 27 h post-resuscitation from saline-treated rats following hemorrhagic shock. Then they were stimulated with vasopressin; their accumulation of IP_3 was significantly lower when compared to sham-operated animals. Conversely, when hepatocytes from ATP-$MgCl_2$-treated animals were stimulated in a similar fashion, their response was only slightly lower than control values at 4 h post-resuscitation and by 27 h post-resuscitation the IP_3 accumulation in this group (ATP-$MgCl_2$-treated) was very close to control values, Figure 15.

Effects of ATP on IP_3 Accumulation

As with vasopressin, ATP caused less accumulation of IP_3 in hepatocytes isolated from saline-treated rats, following hemorrhagic shock, 4 and 27 h post-resuscitation, when compared to sham-operated controls. Again ATP-$MgCl_2$-treated animals showed slightly lower levels of IP_3, following ATP stimulation, at 4 h post-resuscitation, and by 27 h post-resuscitation, IP_3 accumulation levels were not different than control values, Figure 16.

Effects of Fluoride-Na on IP_3 Accumulation

Hepatocytes were isolated from animals of the three different groups at 4 and 27 h post-resuscitation and subsequently stimulated with the receptor-independent, activator of PIP_2 hydrolysis, fluoride-Na (F-Na). Hepatocytes from all three animal groups responded to F-Na stimulation with no apparent differences in the levels of IP_3 accumulation, Figure 17, among the animal groups.

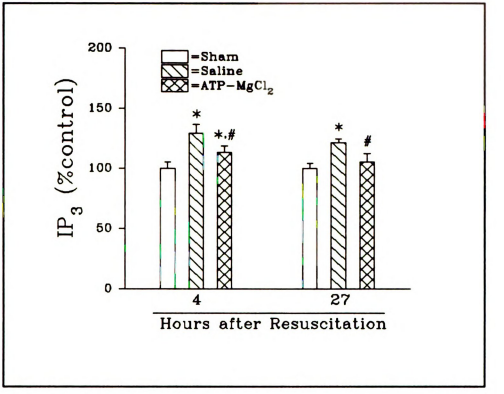

Figure 14. IP_3 basal levels in hepatocytes isolated at 4 and 27 h post-resuscitation from sham-operated, hemorrhaged saline-treated, and hemorrhaged ATP-$MgCl_2$-treated rats. Hepatocytes were preincubated as described under "Methods," and were then incubated with or without drugs in the presence of 10 mM LiCl. The results are means ± SEM of four animals/group, expressed as a percentage of the control value (sham-operated controls are considered 100%) and are compared with one-way ANOVA and Tukey's test: * $P < 0.05$ versus sham-operated controls, # $P < 0.05$ versus saline-treated animals. IP_3 levels were measured by a radioreceptor assay procedure (NEN Kit #NEK-064).

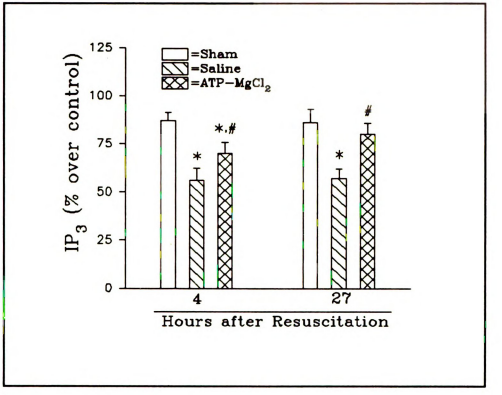

Figure 15. IP_3 accumulation as induced by vasopressin. After preincubation of hepatocytes from all three animal groups as described under "Methods," hepatocytes were incubated for 15 sec with 10^{-7} M vasopressin in the presence of 10 mM LiCl. The results are means ± SEM of four animals/group, expressed as a percentage of the control value (sham-operated controls are considered 100%) and are compared with one-way ANOVA and Tukey's test: * P < 0.05 versus sham-operated controls, # P < 0.05 versus saline-treated animals. For details see Figure 14.

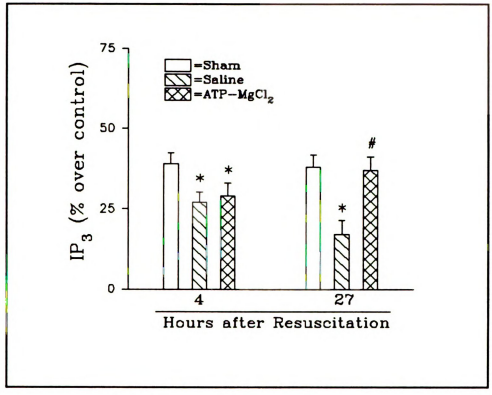

Figure 16. IP_3 accumulation following ATP stimulation of hepatocytes from all three animal groups. After preincubation of hepatocytes, ATP was added to final concentration of 1 mM and incubation was terminated after 15 sec. Incubation was in the presence of 10 mM LiCl. The results are means ± SEM of four animals/group, expressed as a percentage of the control value (sham-operated controls are considered 100%) and are compared with one-way ANOVA and Tukey's test: * $P < 0.05$ versus sham-operated controls, # $P < 0.05$ versus saline-treated animals. For details see Figure 14.

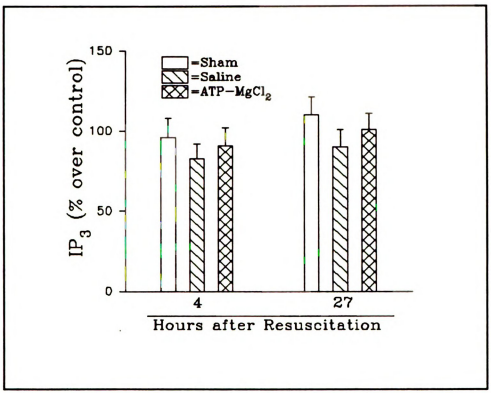

Figure 17. IP_3 accumulation following stimulation of hepatocytes from all three animal groups with 12.5 M Fluoride-Na in the presence of 10 mM LiCl. Incubation was terminated after 1 min and 15 sec (i.e., lag of 1 min needed). The results are means ± SEM of four animals/group, expressed as a percentage of the control value (sham-operated controls are considered 100%) and are compared with one-way ANOVA and Tukey's test: * $P < 0.05$ versus sham-operated controls, # $P < 0.05$ versus saline-treated animals. For details see Figure 14.

GLUCOKINASE (GK) ACTIVITY

Glucokinase activities measured in the cytosol fractions of liver homogenates from all three different animal groups are depicted in Figure 18. It is demonstrated that there were no differences observed in glucokinase activity between sham-operated, hemorrhaged saline-treated, and hemorrhaged ATP-$MgCl_2$-treated rats, at 4 h post-resuscitation.

PHOSPHOENOLPYRUVATE CARBOXYKINASE (PEPCK) ACTIVITY

The activity of the enzyme PEPCK was depressed in the cytosol fractions obtained from liver homogenates of hemorrhaged saline-treated rats, 4 h post-resuscitation, when compared to sham-operated controls, Figure 19. ATP-$MgCl_2$-treatment, however, restored the activity of this enzyme to near control values.

PYRUVATE KINASE (PK) ACTIVITY

Saline treatment following hemorrhagic shock caused an elevation in the activity of cytosolic pyruvate kinase compared to sham-operated animals, Figure 20. Although ATP-$MgCl_2$ treatment did not restore the activity of this enzyme to sham-operated control values, there was a significant decrease in the activity of this enzyme following ATP-$MgCl_2$ treatment of hemorrhagic shock.

GLUCOKINASE, PEPCK, PYRUVATE KINASE mRNA LEVELS

Northern blot analysis revealed that glucokinase was not expressed (bands not detectable) in any of the three animal groups (sham-operated, hemorrhaged saline-treated, or hemorrhaged ATP-$MgCl_2$ treated), 4 h post-resuscitation. Although PEPCK was expressed, there were no differences observed among the animal groups, Figure 21. The average densities of the bands were 257 ± 21, 239 ± 13, and 246 ± 18, for sham-operated, hemorrhaged saline-treated, and hemorrhaged ATP-$MgCl_2$-treated rats, respectively, in arbitrary units (AU), as determined by JAVA Image Analysis System. On the other hand, the expression of pyruvate kinase mRNA was elevated 5-fold in livers of animals hemorrhaged and saline-treated (286 ± 25 AU) when compared to sham-operated controls (56 ± 5 AU). ATP-$MgCl_2$-treatment, however, decreased the expression of pyruvate kinase mRNA to 2.5 fold (147 ± 19 AU) when compared with sham-operated controls, Figure 21.

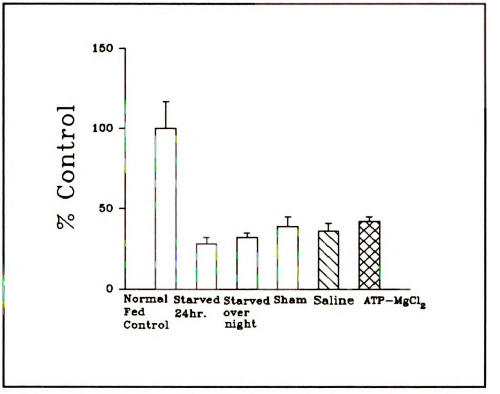

Figure 18. Glucokinase activity measured in the cytosol fractions of liver homogenates from animals 4 h post-resuscitation or as indicated in the Figure. Glucokinase is a glycolytic enzyme and, therefore, mostly expressed during absorptive state, thus, the normal fed group was taken as control. Results represent means ± SEM from four animals/group, expressed as percentage of control, and are compared with one-way ANOVA and Tukey's test: * $P < 0.05$ versus sham-operated controls, # $P < 0.05$ versus saline-treated animals. Control value was 14.3 ± 3.3 milliunits/mg protein. A unit of glucokinase is defined as the enzyme activity resulting in the formation of 1 μmol of glucose-6-phosphate/min under the conditions of the assay.

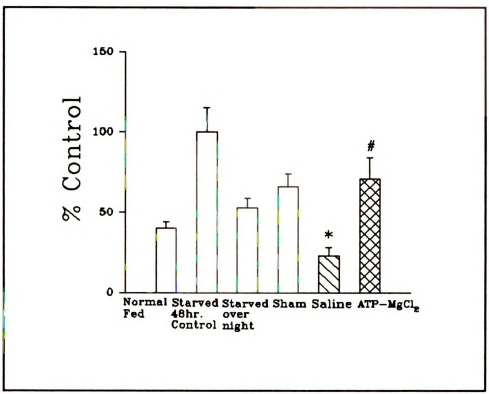

Figure 19. PEPCK activity measured in the cytosol fractions of liver hemogenates from animals 4 h post-resuscitation or as indicated in the Figure. PEPCK is a gluconeogenic enzyme and, therefore, is expressed during post-absorptive state thus, the 48 h starved animals were taken as control. Results represent means ± SEM from four animals/group and compared with one-way ANOVA and Tukey's test: * $P < 0.05$ versus sham-operated controls, # $P < 0.05$ versus saline-treated animals. Enzyme activity is expressed as percentage of control. Control value was 41 ± 4.7 milliunits/mg protein. A unit of PEPCK activity is defined as the enzyme activity resulting in the formation of 1μmol of OAA/min under the conditions of the assay.

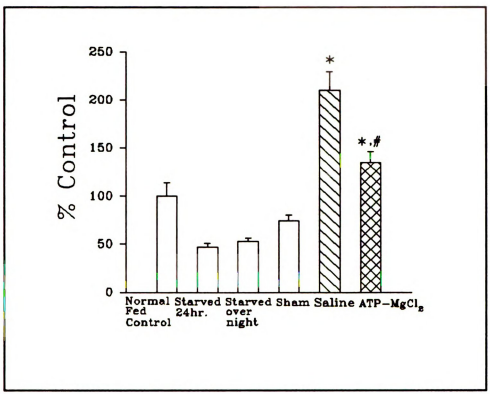

Figure 20. Pyruvate kinase activity measured in the cytosol fractions of liver hemogenates from animals 4 h post-resuscitation or as indicated in the Figure. Pyruvate kinase is a glycolytic enzyme and, therefore, is expressed during absorptive state, thus, the normal fed group was taken as control. Results are represented as means ± SEM from four animals/group and compared with one-way ANOVA and Tukey's test: * P< 0.05 vs. sham-operated controls, # P< 0.05 vs. saline-treated animals. Enzyme activity is expressed as percentage of control. Control value was 74 ± 16 milliunits/mg protein. A unit of pyruvate kinase activity is defined as the enzyme activity resulting in the formation of 1μmol of pyruvate/min under the conditions of the assay.

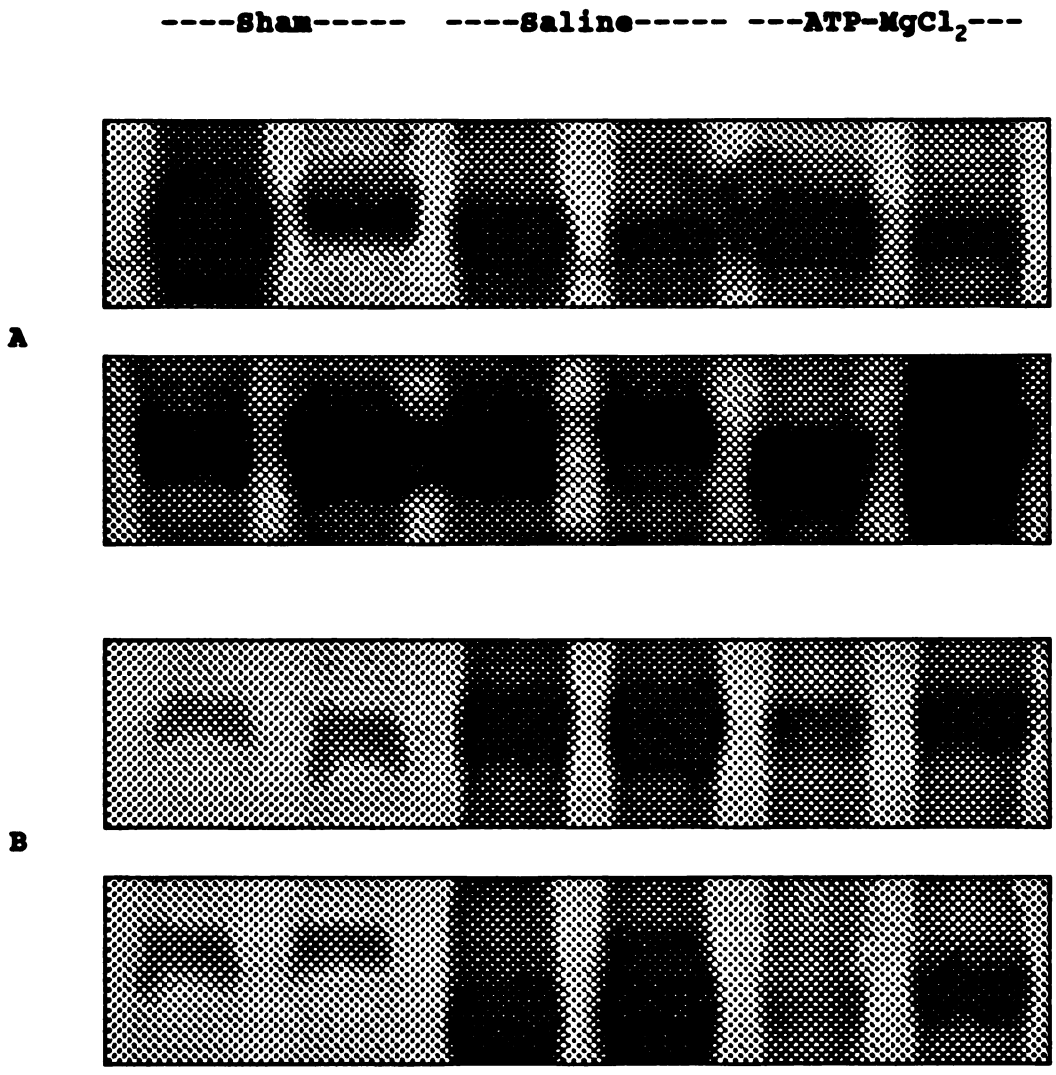

Figure 21. Effects of hemorrhage and resuscitation with saline or ATP-$MgCl_2$ on PEPCK and pyruvate kinase mRNA levels in liver. The procedure for RNA isolation, gel electrophoresis, RNA transfer to nitrocellulose membranes and hybridization to ^{32}P-labeled cDNA (PEPCK,GK,PK) probes were described under "Methods". A, RNA blot probed with rat PEPCK cDNA; B, probed with rat pyruvate kinase cDNA. Intensity of the bands on the autoradiograms were evaluated densitometrically by JAVA Image Analysis System. There were four animals/group.

DISCUSSION

The results presented in Part III indicate that trauma-hemorrhagic shock and crystalloid resuscitation dramatically decreased the number of binding sites for both high and low-affinity components of the P_2-purinoceptor population on rat hepatocytes. In addition, hemorrhagic shock diminished the ability of hepatocytes to respond to receptor-dependent stimulation (ATP, glucagon, and vasopressin). Furthermore, the activity of the gluconeogenic enzyme PEPCK was depressed while the activity and mRNA levels of the glycolytic enzyme pyruvate kinase were elevated following shock. On the other hand, ATP-$MgCl_2$ administration during the resuscitation of hemorrhagic shock significantly increased the number and enhanced the affinity of rat hepatocyte P_2-purinoceptors, restored the ability of hepatocytes to respond to receptor-dependent stimulation, alleviated the depression of PEPCK activity, and decreased the activity and mRNA levels of pyruvate kinase.

In this study, we have used a severe hemorrhagic shock model with late mortality of approximately 50%. This model has been previously established by Chaudry and associates (192). As mentioned above, the hemorrhage model used in this study did not involve resuscitation with

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shed blood. This was done since infusion of stored autologous blood has the disadvantage of containing various activated blood factors. Omission of blood resuscitation in trauma victims, however, would limit the transfusion-related diseases, such as AIDS and hepatitis. In view of this, blood transfusion or the return of shed blood following hemorrhage was not provided in this model in order to alleviate blood-transfusion related problems which occur in the clinical setting. In addition, the availability of stored blood for trauma patients or during major operations is a matter of great debate especially during conflict times (war). Furthermore, we have attempted to keep our model simple so that other treatment modalities can be provided if simple crystalloid resuscitation does not appear to be effective.

The question of which type of fluid to be used during surgery and for shock resuscitation continues to be debated. In the United States, the controversies focused on colloid vs. crystalloid solutions, while in Europe studies more commonly are concerned with comparing different colloid solutions. Studies of crystalloid solutions for resuscitation often use a hemorrhagic shock preparation while colloid studies often involve plasma protein-losing types of shock, such as sepsis and intestinal ischemia. Both groups tend to apply their results more generally to all types of shock and surgical conditions. In this regard, recent studies from our laboratory have shown that crystalloid is as effective as blood in the resuscitation of hemorrhagic shock (193). However, because resuscitation was carried out with Ringer's lactate instead of blood, there was hemodilution which may raise

the concern of a decrease in oxygen delivery. With respect to this, studies have shown that hematocrit of 10-15% in animals subjected to acute hemodilution were well tolerated (194). Other studies have also shown that hemodilution had no adverse effects on O_2 consumption or blood flow autoregulation at low perfusion pressures and may indeed be beneficial during reperfusion following ischemia (195). Furthermore, recent studies from our laboratory have demonstrated that oxygen saturation did not decrease following resuscitation with 2, 3, or 4X Ringer's lactate compared to control values (35). Thus, it would appear that this amount of hemodilution is not necessarily detrimental to the animal. Further support for this notion came from the studies which demonstrated that mortality following hemorrhage and resuscitation in this model was not 100% but approximately 50% (192).

Trauma, in the form of a 4-5 cm midline laparotomy, was introduced to simulate the clinical arena since hemorrhagic shock is usually associated with tissue trauma. This trauma is not insignificant, since studies have shown that such a procedure in itself produces a marked depression in cell-mediated immunity (196). Moreover, results from our laparotomy indicated that, whereas the mortality was only 10% following hemorrhage and resuscitation in the absence of such trauma, it was 60% if the laparotomy was performed prior to hemorrhage (I.H. Chaudry *et al.*, unpublished observation). Other investigators have also shown that, even a 1-cm skin incision before or after hemorrhage increases infection in the rat (197). Thus, our

present model involved not only hemorrhage but also significant tissue trauma (5-cm ventral midline laparotomy), which does play a major role in the outcome of the animals after hemorrhage and resuscitation.

Although the beneficial effects of ATP-$MgCl_2$ after hemorrhage have been tested in a model of hemorrhagic shock which involves preheparinization of animals (136,168,198,199), recent studies in our laboratory, however, indicated that heparinization prior to hemorrhage per se significantly improves active hepatocellular function, renal function and cardiac output (200) as well as it has protective effects on the microvasculature (201), following severe hemorrhage and crystalloid resuscitation. Therefore, the model used in the present study did not involve any heparinization of the animals to determine whether or not the beneficial effects were due to ATP-$MgCl_2$ treatment alone. In addition trauma victims are not heparinized prior to their accidents.

Studies by other investigators have suggested that ATP-$MgCl_2$ has a profound vasodilatory effect following hemorrhage and resuscitation (202). They reported no beneficial effects of ATP-$MgCl_2$ on cardiovascular responses following hemorrhage and resuscitation with shed blood and crystalloid in the dog. Since a large dose of ATP-$MgCl_2$ (100 μmol/Kg BW) was given rapidly (over 30-40 min), MAP of such animals was approximately 70 mmHg during and following ATP-$MgCl_2$ infusion in that study (202). In contrast, Wang *et al.* (165) have used a smaller dose of ATP-$MgCl_2$ (50 μmol/Kg BW) and it was infused more slowly over 95 min; they found

MAP was approximately 90 mmHg in ATP-$MgCl_2$-treated rats after completion of ATP-$MgCl_2$ infusion. Furthermore, they reported (165) that, MAP was 9-10 mmHg higher in ATP-$MgCl_2$ treated than in saline-treated rats at 1.5 and 4 h after hemorrhage and resuscitation. Since ATP-$MgCl_2$ is a potent vasodilator, these findings emphasize the necessity of administering ATP-$MgCl_2$ at a rate which does not produce a secondary significant hypotension following hemorrhage and resuscitation. In addition, I.H. Chaudry (203) has also established that a 50 μmol/Kg BW of ATP-$MgCl_2$ dose was most beneficial for animals following hemorrhagic shock. Based on the aforementioned information, we have used the same model described by Chaudry *et al.* (192) and we have used the same dose of ATP-$MgCl_2$ (50 μmol/Kg BW) also described by I.H. Chaudry (203). Furthermore, the rate of infusion was also slow (over 95 min) to avoid a secondary significant hypotension as suggested by Wang *et al.* (165). In this regard, our observations were in agreement with those of Wang *et al.* (165), in that, although ATP-$MgCl_2$ produced a small decrease in MAP (≈ 10 mmHg) during constant slow infusion, MAP was improved after completion of ATP-$MgCl_2$ infusion. Thus, with the slow administration of ATP-$MgCl_2$ following shock to prevent the significant decrease in blood pressure, beneficial effects on various organ functions are observed (165,203). In this regard, the nitroglycerine dose (i.e., 45 μmol/Kg BW) used in this study was chosen to simulate the same hypotensive effects produced by ATP-$MgCl_2$ at 50 μmole/Kg BW (i.e., ≈10 mmHg). Finally, it is worth mentioning that ether

was our choice of an anesthetic because it is short lived and, thus, would not significantly affect hemodynamics or metabolism.

Scatchard analysis (Figure 4) revealed high and low-affinity components of [^{35}S]αATP binding to rat hepatocytes. For almost every ligand-receptor system studied, some investigators report findings inconsistent with the predictions of the law of mass action for the simplest model of a reversible, biomolecular interaction between the ligand and a single set of identical, independent receptors. The Scatchard Plot (204) of the ratio of bound ligand to free ligand against the amount (or concentration) of bound ligand has a curvilinear appearance, instead of the predicted straight-line relationship. The most common pattern is for the Scatchard Plot to appear progressively less steep as it approaches the abscissa axis (upwardly concave); this is interpreted as evidence of some non-uniformity in the interaction between ligand and receptor, such as the presence of a heterogenous population of receptors. This concept of functional receptor heterogeneity is widely accepted in the receptor field (205); much work is now focused on its likely nature and biological relevance. The term "functional heterogeneity" is used here in its broadest sense to encompass differences in behavior between individual receptors (due, for example, to the influence of neighboring molecules) as well as any actual molecular differences between receptors.

Despite the numerous reports of curvilinear Scatchard Plots, many questions about the explanation for the curvature remain unanswered. One

reason for this is that, although some investigators appear to find clear evidence of a curvilinear plot, with the same receptor systems other investigators often obtain a linear plot. Thus, for essentially every receptor system, the case for receptor hetrogenity is best regarded as unproven (205). In this regard, however, the association dose-dependency patterns (Figure 3), the curvilinear Scatchard plot (Figure 4), B_{max} values (Figures 5 and 6), and K_d values (Figures 7 and 8) in our control animals are in close agreement with reports by most investigators in this field (6-12,113,117,118,206, 207).

Theoretical analysis has indicated that a number of experimental artifacts may explain the curvilinear nature of the Scatchard plot without the need to invoke receptor heterogeneity (205). These experimental artifacts include: imprecise estimate of non-specific binding, poor procedure for data analysis, affinity difference between labeled and unlabeled ligands, impure labeled ligand, effect of membrane microenvironment, contamination of bound by free, incomplete recovery of bound fraction, irreversible binding and internalization, ligand degradation, receptor degradation, and non-equilibrium binding conditions. In our studies we have attempted to minimize these artifacts by observing the following: we have used the nonlinear, least-square computer curve fitting program Ligand (by G.A. Mcpherson Biosoft, London) (176) to analyze our data; we used [^{35}S]αATP as labeled ligand and SαATP as unlabeled ligand to minimize difference in affinity; intact hepatocytes were utilized to avoid effects of membrane

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microenvironment and attain equilibrium and reversible binding; the highest grade purity labeled and unlabeled ligands were used; and, finally the assay procedure design eliminated ligand or receptor degradation as well as internalization by immediate decrease in temperature to 4°C, which was checked to avoid dissociation and degradation of the bound ligand.

B_{max} of the high-affinity P_2-purinoceptor binding component on rat hepatocytes decreased by 20% at the time of maximum bleed out and rebounded at the end of resuscitation (Figure 2 and Table 1). B_{max} of both binding components, however, decreased again following resuscitation in the saline-treated animals at 4, 17, and 27 h post-resuscitation (Figures 5 and 6). Likewise, various organ, cellular and subcellular functions, such as cardiac output and hepatocellular function were restored, but failed to be maintained following fluid resuscitation alone (4,35,36,39). The lack of maintenance of the various organ, cellular, and subcellular functions with fluid resuscitation may be a result of tissue injury (13-18,22,98,99,125,208). These studies, among others, indicate that after hypoxia, but especially after subsequent recovery, oxygen free radicals are formed and produce tissue injury, in large part, through peroxidation of polyunsaturated fatty acids (95,96,209,210). Since a common feature in all forms of shock is an inadequate circulation with diminished blood flow to tissues, this results in tissue hypoxia. Probably during hypoxia the efficacy of different protective systems is diminished, and by recovery time after hypoxia, the tissue will be overloaded with reactive oxygen metabolites, with consequences that

membrane transformation through lipid peroxidation causes irreversible damage in the tissue (14). It is also apparent in Figures 5 and 6 that hepatocytes from hemorrhaged and saline-treated animals displayed a gradual gain in receptor numbers with the progression of time. This gain is most likely to be transient or it's an indication of survival (i.e., only animals who eventually will survive display this trend). On the other hand, administration of ATP-$MgCl_2$ during fluid resuscitation of hemorrhagic shock ameliorated and subsequently restored the B_{max} of the high-affinity binding component and significantly improved B_{max} of the low-affinity component of binding.

The affinity ($1/K_d$) of both components of binding was not altered by trauma-hemorrhagic shock (Figures 7 and 8). In this regard, studies have shown that low-flow conditions such as hemorrhage and ischemia usually affect the density of various receptors without any effects on the affinity of such receptors (13-15). Other conditions such as endotoxemia affect both density as well as affinity of various receptor systems (16-18,211). Therefore, such studies indicate that low-flow conditions affect receptor populations via destabalization of the plasma membrane without direct effects on the receptor molecule; conversely during endotoxemia, *Escherichia coli* seems to affect receptor population and affinity by acting directly on these receptor molecules. Although the affinity of P_2-purinoceptors was not altered by hemorrhagic shock, ATP-$MgCl_2$-treatment, however, enhanced the affinity of the low-affinity binding site progressively even higher than

sham-operated controls at both 17 and 27 h post-resuscitation. It is worth renoting that intact hepatocytes were chosen over hepatic plasma membranes for three reasons: first, as we have mentioned above, to avoid effects of membrane microenvironment; second, to attain equilibrium, since equilibrium was not attainable with plasma membranes; third, non-specific binding was too high when plasma membranes were used.

The beneficial effects of ATP-$MgCl_2$ treatment on survival following shock were not observed if ATP or $MgCl_2$ alone or adenosine-$MgCl_2$ were infused (5). It was reported that ATP-$MgCl_2$ improves cellular function not simply through vasodilation (ATP is a potent vasodilator) as produced by dopamine or papaverine but directly through long-term improvement in the microcirculation (5). Consequently we have carried out further studies to determine whether the beneficial effects of ATP-$MgCl_2$-treatment on the density and affinity of P_2-purinoceptors on rat hepatocytes can be duplicated by the treatment of hemorrhagic shock with ATP or $MgCl_2$ alone or with the vasodilator nitroglycerine. Results of these studies (Figure 9 and Table 2) demonstrate that neither $MgCl_2$ nor the vasodilator nitroglycerine had any beneficial effects on these receptors. Although ATP increased B_{max} of the high-affinity component of binding, this was significantly lower when compared with the complex ATP-$MgCl_2$ (Figure 9). Furthermore, ATP treatment had no beneficial effects on the affinity of these receptors; in contrast ATP-$MgCl_2$-treatment progressively enhanced the affinity of P_2-purinoceptor (the low-affinity subtype) (Figure 8). Therefore, the beneficial effects on

P_2-purinoceptor dynamics observed here are due to the complex ATP-$MgCl_2$ and not to the physiological ligand of these receptors, ATP.

The precise mechanism responsible for the hemorrhage-induced reduction in the number of P_2-purinoceptors on rat hepatocytes is unclear. Nonetheless, there may be several factors responsible for this depression. Tissue and cellular ATP levels are dramatically decreased following hemorrhagic shock (72-81). Studies have shown that ATP depletion can result in a rapid, duration-dependent, disassociation of the Na^+-K^+-ATPase from the actin cytoskeleton and redistribution of Na^+-K^+-ATPase within the membrane. These studies concluded that ATP depletion causes profound alterations in cell polarity by inducing major changes in the actin cytoskeletal architecture (212). In a similar manner the P_2-receptor protein molecule could undergo the same disassociation from the cytoskeleton and redistribution within the membrane following hemorrhagic shock thus leading to loss of receptor numbers. In addition, ATP depletion was also shown to cause efflux of protons from acidic vesicles, which contributes to cytosolic acidification and thus, inhibition of protein synthesis (213). Such an inhibition could curtail the turnover of the P_2-purinoceptor population and decrease the number of receptors available for binding at a specific time. Furthermore, ATP depletion by metabolic inhibition with KCN and iodoactate (chemical hypoxia) leads hepatocytes to form surface-membrane blebs within 10 to 20 min after chemical hypoxia and most cells lose viability within an hour (214). Alteration in the physical state of the membrane

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lipid bilayer of this nature and severity might affect several biochemical processes within the membrane. These processes include conformational changes in protein molecules impeded in the membrane lipid bilayer. Since the P_2-purinoceptor is an intrinsic plasma-membrane protein, the conformation of this protein molecule could be altered under these conditions. This alteration could affect the functional state of the receptor by masking its cryptic binding site and, therefore, rendering the molecule unavailable for binding.

Among the membrane-biochemical processes that also could be affected by the nature of this change in the physical state of the membrane is phospholipid methylation. Phospholipids are a major component of biomembranes and provide the fluid matrix for protein movement and organization. Membrane fluidity has been closely related to the biological and biochemical processes such as transport of chemicals, cell fusion, and protein rotation and diffusion. Genetic, nutritional and temperature manipulation have been used to alter the fluidity of the cell membrane, but physiological and biochemical events that regulate membrane fluidity are still poorly understood. However, enzymatic methylation of phosphatidylethanolamine in the membrane induces a marked increase of the bulk membrane fluidity (215). An increase in membrane-lipid fluidity was shown to cause a substantial increase in the average number of available transferrin receptors per bone marrow cell (216). In addition, stimulation of phosphatidylcholine synthesis by the methyl donor s-adenosyl-l-methionine,

increased the number of β-adrenergic receptor binding sites in reticulocyte ghosts (217). These studies have also suggested that the appearance of β-adrenergic binding sites was dependent on the formation of phosphatidyl-choline by the enzyme that converts phosphatidyl-N-monomethylethanola-mine to phosphatidyl choline and that, both the synthesis of phosphatidyl-choline and the unmasking of cryptic receptors did not occur in the presence of the methyltransferase inhibitor, S-adenosyl-L-homocysteine (217). In addition to unmasking of cryptic sites, phospholipid methylation can affect receptor density by other membrane-biochemical processes such as vertical displacement (positioning) of the membrane receptor-proteins via changes in membrane fluidity (microviscosity) (216), and translocation of protein molecules from the inside of the membrane to the outer surface, a process that is facilitated by step-wise methylation of phospholipids (215). The severe change that takes place in the plasma-membrane of hepatocytes following hemorrhagic shock and ATP depletion could, therefore, affect the density of P_2-purinoceptors by altering or inhibiting processes such as phospholipid methylation in the membrane, via, for example, a loss or inhibition of a methyltransferase. In this regard it is generally believed that lipid peroxidation is an important contributor to the inhibition of several enzymes located both in the endoplasmic reticulum and in the plasma membrane (210), thus curtailing the unmasking of the P_2-purino-ceptor cryptic site, preventing displacement and positioning of P_2-purino-ceptors protein molecules, or disfacilitating their movement to the outer

surface of the membrane. Finally, hemorrhagic shock causes immunosuppression and release of cytokines interleukin-1,6 (IL-1,IL-6) and tumor necrosis factor (TNF) (29,85,86); these agents were shown to affect the density of some receptors (211).

The beneficial effects of ATP-$MgCl_2$ treatment following hemorrhagic shock on P_2-purinoceptor numbers and affinity demonstrated here could be attributed to its restoration of tissue and cellular ATP levels (5,149,150), thus, alleviating the adverse effects of ATP-depletion mentioned above. In addition, ATP was demonstrated to have stabilizing effects on the plasma membrane (146,147,218) and to stimulate cellular synthesis of more ATP (150). Furthermore, studies have shown that ATP plays an important role in the normal assembly and turnover of cytoskeletal elements essential for preserving membrane integrity (147,212). ATP was also shown to phosphorylate various membrane proteins (219), thus, regulating membrane functions. Moreover, ATP-$MgCl_2$ administration during the resuscitation of hemorrhagic shock has been shown to restore hepatic blood flow and hepatic microcirculation (162-164), thus, increasing oxygenation and removal of waste products and preventing lipid peroxidation, which was shown to cause membrane transformations (14). Finally, ATP-$MgCl_2$ was shown to ameliorate the immunodepression following hemorrhagic shock, thus, preventing the release of cytokines and their adverse effects on the plasma membrane (157).

The basal levels of the second messenger cAMP were dramatically depressed 4 and 27 h following hemorrhage and saline treatment (Figure 10). In addition, when glucagon was used to stimulate cAMP production in hepatocytes obtained from hemorrhaged and saline-treated animals, their response was significantly lower when compared to sham-operated control animals (Figure 11). Moreover, when ATP was used to inhibit the glucagon-induced cAMP accumulation (Figure 12), again hepatocytes from hemorrhaged and saline treated animals did not respond. However, when the receptor-independent activator of adenylate cyclase, forskolin, was used to stimulate cAMP production, there were no apparent differences between all of the animal groups in terms of their cAMP accumulation (Figure 13).

On the other hand, ATP-$MgCl_2$ administration following hemorrhagic shock increased cAMP basal levels at 4 h post-resuscitation and cAMP basal levels were restored to control values by 27 h post-resuscitation (Figure 10). ATP-$MgCl_2$ treatment also restored the ability of hepatocytes to respond to glucagon stimulation (Figure 11). Although ATP-$MgCl_2$ treatment restored the ability of hepatocytes to respond to ATP inhibition of the glucagon-induced cAMP accumulation at 27 h post-resuscitation, this inhibition was not fully expressed at 4 h post-resuscitation (Figure 12). One possible explanation of this observation is that ATP receptors (P_2-purinoceptors) were increased at 4 h post-resuscitation with ATP-MgC_2 treatment, but they were not restored until 27 h post-resuscitation (Figures 5 and 6).

Figure 14 illustrates that hemorrhage and saline-treatment caused an elevation of the basal levels of the second messenger IP_3 at both 4 and 27 h post-resuscitation. Moreover, when vasopressin or ATP were used to stimulate IP_3 production, the response of hepatocytes isolated from hemorrhaged and saline-treated animals was significantly lower than that of hepatocytes isolated from sham-operated control animals at 4 and 27 h post-resuscitation (Figures 15 and 16). However, similar to the situation with cAMP, when the receptor-independent activator of PIP_2 hydrolysis, fluoride-Na, was used to stimulate IP_3 accumulation, there were no significant differences observed among the animal groups (Figure 17).

IP_3 basal levels were decreased at 4 h post-resuscitation and they were returned to control values by 27 h post-resuscitation with ATP-$MgCl_2$ treatment (Figure 14). The ability of hepatocytes to respond to vasopressin or ATP stimulation of IP_3 production was also restored with ATP-$MgCl_2$ treatment (Figures 15 and 16). However, similar to cAMP, the ATP stimulation of IP_3 production was only increased at 4 h post-resuscitation, but was fully restored at 27 h post-resuscitation (Figure 16). Again, this might be due to the fact that P_2-receptors were not fully restored in number until 27 h post-resuscitation (Figures 5 and 6).

Several investigators have reported that cAMP levels decrease following hemorrhagic shock in several organs such as liver, kidney, muscle and brain (220-224). However, Rutenburg *et al.* (220) concluded that the decrease in cAMP was due to a defective glucagon receptor site,

when glucagon was used as a stimulator of cAMP production, and not due to abnormalities in the second messenger system itself; this is because, when fluoride-Na was used as a receptor-independent non-specific stimulator of the adenylate cyclase enzymatic activity, cAMP production was similar in hemorrhaged as well as control animals (220).

In line with these studies, our results indicate that hepatocytes isolated from hemorrhaged and saline-treated animals failed to respond to receptor-dependent stimuli (ATP, glucagon, and vasopressin) of both second messengers (cAMP and IP_3), (Figures 11, 12, 15, and 16) when compared to cells obtained from sham-operated animals. Conversely, when receptor-independent, non-specific stimuli of the second messengers were used (forskolin and fluoride-Na), no difference in response was observed between hemorrhaged and control animals (Figures 13 and 17). Thus, it can be concluded that hemorrhage and saline-treatment caused defects in these receptor systems or uncoupling between receptors and their transmembrane signaling elements.

The nature of the uncoupling in the signal transduction process is not clear. However, it may involve alterations at one or more of the elements constituting transmembrane signaling. Cell-surface receptors, coupling proteins (e.g., G proteins), and membrane effectors (e.g., adenylate cyclase and inositol phospholipids). The results in Figures 13 and 17 may exclude membrane effectors, in view of the fact that these systems responded normally when receptor-independent stimuli were used. Therefore, the

defect could be in the different receptor systems, as the case with P_2-purinoceptors in our studies or with glucagon receptors in the studies of Rutenburg *et al.* (220) and/or in the coupling proteins. In this regard, studies have shown that changes in phospholipid methylation affect membrane microviscosity and thus the lateral movements of receptors and coupling with elements in the transmembrane signaling process (225).

The mRNA of the glycolytic enzyme, glucokinase, was not detectable by Northern blot analysis in sham-operated, hemorrhaged and saline-treated, or hemorrhaged and ATP-$MgCl_2$-treated rats, 4 h post-resuscitation. Moreover, there was no significant difference in the activity of glucokinase among all three animal groups, 4 h post-resuscitation (Figure 18).

Although the mRNA levels of the gluconeogenic enzyme, PEPCK, were similar in all three animal groups, 4 h post-resuscitation (Figure 21), the activity of PEPCK (Figure 19) was depressed with hemorrhage and saline-treatment, 4 h post-resuscitation, when compared to either sham-operated or hemorrhaged and ATP-$MgCl_2$-treated animals. Thus, hemorrhage and saline-treatment altered the activity of PEPCK without any effect on the transcription or translation of the enzyme. In this regard, the activity of PEPCK was shown to be depressed by high circulating levels of the tumor necrosis factor (TNF) (30), and hemorrhagic shock was demonstrated to induce an increase in serum TNF without the presence of endotoxins (29). ATP-$MgCl_2$-treatment following hemorrhagic shock, in this respect, was shown to ameliorate the immunosuppression and prevent the

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release of inflammatory cytokines following shock (155-159). This might account for the depression in the activity of PEPCK by hemorrhage and saline-treatment and the restoration of this enzyme activity following ATP-$MgCl_2$ treatment of hemorrhagic shock.

Hemorrhage and saline treatment caused an increase in the mRNA levels of the enzyme pyruvate kinase, 4 h post-resuscitation, as detected by Northern blot analysis (Figure 21), when compared to sham-operated animals. ATP-$MgCl_2$ treatment, however, significantly decreased the mRNA levels of pyruvate kinase to near control values, 4 h post-resuscitation (Figure 21). The activity of pyruvate kinase, when correlated with the mRNA levels of this enzyme (Figures 20 and 21), demonstrates that hemorrhage and saline-treatment resulted in an elevation of the activity of the glycolytic enzyme pyruvate kinase apparently due to the elevation in the mRNA levels of the enzyme. Likewise, the decrease in the activity of pyruvate kinase with ATP-$MgCl_2$ treatment, apparently was due to the decrease in mRNA levels of the same enzyme. Therefore, it might be concluded that, unlike PEPCK which is altered by hemorrhagic shock at a post-translational level, alteration in pyruvate kinase by hemorrhage and saline treatment, apparently was at the transcriptional level.

The L-type pyruvate kinase gene, encoding the liver-specific glycolytic enzyme is transcriptionally regulated, positively by glucose and insulin and negatively by glucagon, through its second messenger cAMP (226). The inhibition of the L-pyruvate kinase gene requires precise interaction

between cAMP and the nucleoprotein complex built up at sites L4 and L3 of the L-pyruvate kinase transcription initiation complex (226). Our results in Part III and those of other investigators (220-224) demonstrate that cAMP levels are dramatically depressed following hemorrhagic shock. Furthermore, the studies of Rutenburg *et al.* (220) along with our results, indicate that there are some abnormalities in the glucagon receptor site and/or in its transmembrane signaling process. Consequently, the significant increase in the expression of pyruvate kinase observed following shock is apparently due to the early hyperglycemic response (elevated glucose and decreased insulin) observed in the early stages of hemorrhagic shock. During the later stages of shock, however, glucagon levels are increased, and regardless of this increase, there is a lack of inhibition of pyruvate kinase transcription which can be attributed to the absence of the inhibitory element (cAMP) in sufficient concentrations required to interact with the transcription initiation complex on the pyruvate kinase gene. ATP-$MgCl_2$ treatment decreased the expression of pyruvate kinase (Figure 21). This can be accounted for considering that ATP-$MgCl_2$ treatment returned cAMP levels following shock towards control values (Figure 10) and restored the ability of hepatocytes to respond to glucagon stimulation (Figure 11).

SIGNIFICANCE

The biphasic response in serum glucose observed following hemorrhagic shock starts with hyperglycemia in the early stages of shock and

changes to progressive hypoglycemia in the later stages of the shock period (23-28,227,228). This condition is characterized by alterations in glucose metabolism, which include increased glycogenolysis, accelerated glycolysis and depressed gluconeogenesis (5). It is clear that three principal peripheral organs including the liver, the pancreas, and the adrenal glands are closely interrelated to maintain glucose homeostasis under the control of the autonomic nervous system (ANS). Indeed, since the original observation that a puncture of the floor of the fourth ventricle triggers hyperglycemia, abundant studies have demonstrated the important role played by the autonomic nervous system in the liver, the endocrine pancreas, and the adrenal gland to control blood glucose level. The liver, however, is the main site for gluconeogenesis and glycogen storage (229) and hepatic glucose metabolism is controlled in a complex fashion by sympathetic and parasympathetic efferent fibers to the liver, pancreatic insulin and glucagon, adrenal catecholamines, and sensory nerves, the capsaicin-sensitive afferent C-fibers, conveying sensory signals of blood glucose concentration from hepatic glucoreceptors to the central nervous system (229).

It is likely that hemorrhagic hypotension increases sympathoadrenal activity by unloading the arterial baroreceptors and cardiopulmonary receptors resulting in increase in adrenal catecholamine secretion (230), although the functional role of the carotid baroreceptors in hemorrhage-induced hyperglycemia still remains unclear (231). Hepatic sympathetic nerves are also stimulated in response to hemorrhage, resulting in the local

release of neural noradrenalin in the liver (230). The increase in local sympathetic nerve activity in the liver and circulating catecholamines of adrenal origin may thus be of prime importance in the immediate response of hepatic glucose mobilization observed during the early phase of hemorrhagic shock.

Although the removal of both adrenal glands and hepatic sympathetic nerves eliminated the early phase of hemorrhage-induced hyperglycemia, a small response with slow onset still persisted following hemorrhage (231). This residual glucose response to hemorrhage could be due to an increase in pancreatic glucagon secretion, because the residual increase in hepatic venous glucose and arterial glucagon concentrations observed in dogs with regional hepatic denervation combined with functional adrenalectomy were virtually abolished in dogs receiving additional surgical pancreatectomy (232). This study also rules out the potential implication of extra-pancreatic sources of circulating glucagon. Adrenergic mechanisms involving both peripheral sympathetic nerves and adrenal catecholamines have been implicated in stimulating the secretion of glucagon from the endocrine pancreas (233). In addition, the ability of glucagon to stimulate hepatic glycogenolysis under various experimental conditions is well documented (234). Thus, it may be concluded that glucagon plays a role in the early hemorrhage-induced hyperglycemic response.

The secretion of pancreatic insulin is also under the control of peripheral sympathetic nerves, and the ability of adrenaline and noradrenaline to

inhibit insulin secretion has been well documented (235). Indeed, the inhibition of insulin secretion following hemorrhage-induced hypotension is believed to be due to the increased sympathetic nerve activity and catecholamine secretion associated with various types of stress so far studied (236). Consequently, the potential implication of increased sympathetic nerve activity in the pancreas may become a likely mechanism, as in the case of glucagon secretion in response to hemorrhage. It should be pointed out that hemorrhagic hyperglycemia results from a glucagon and adrenalin induced increase in hepatic glycogenolysis and not from secondary effect of an inhibition of insulin secretion which, in turn, depresses peripheral glucose utilization. However, the possibility that the inhibitory effect of adrenalin on overall glucose utilization *in vivo* constitutes an important component of its hyperglycemic action (237) cannot completely be ruled out (238). In this context, hemorrhagic stress markedly reduces glucose clearance despite significantly increased insulin levels, suggesting an important role for insulin resistance in causing hyperglycemia after experimental hemorrhage (239).

Many other neurohormonal factors, such as cortisol, growth hormone, vasopressin, oxytocin, angiotensin II, and enkephalins, are known to be released in response to hypovolemic hypotension (240). It is plausible that some of these endogenous substances may contribute either singularly or in combination to the hyperglycemic response to hemorrhage, because vasopressin, angiotensin II, and oxytocin have been shown to stimulate the

hepatic glycogenolysis (241). While catecholamines and glucagon play the dominant role, these endogenous substances other than catecholamines and glucagon may only play a minor role in the early phase of hyperglycemia induced by acute hemorrhage.

The hemorrhage-induced early hyperglycemic response is therefore, seems, largely caused by stimulation and increased sympathetic outflow which, in turn, causes the release of adrenal catecholamines, local hepatic noradrenalin, pancreatic glucagon, and inhibition of insulin release. Blood glucose level, however, must be maintained within a relatively narrow range despite wide variations of glucose influx and efflux (242). The hyperglycemic response induced by hemorrhage would accordingly trigger a glucose counterregulatory mechanism to return blood glucose to normoglycemia.

Insulin is the physiologically dominant glucose regulatory factor and plasma glucose concentration is the major regulator of insulin secretion. Insulin depresses hepatic glucose production by inhibiting glycogenolysis and gluconeogenesis, stimulates peripheral glucose utilization, and therefore, decreases the plasma concentration of glucose. Indeed, Hiebet *et al.* (243) found a large and progressive decrease in insulin secretory-rate with shock, which changes to an increase in arterial concentrations of insulin in late shock.

During the late stages of shock, however, glucose levels decrease. The hypoglycemic response in late shock stimulates the

sympathoadrenal-endocrine system through the activation of hepatic (capsaicin-sensitive) afferent sensory nerves coupled with hepatic glucoreceptors, resulting in enhanced sympathetic outflow towards the target organs including the adrenal gland, pancreas, and the liver. However, it appears likely that hypoglycemia per se in the brain also plays a critical role in controlling particularly the secretion of glucagon and hepatic glucose production. There may exist an interrelationship between hepatic and central glucoreceptor functions, but their precise interactions remain unknown. This activation of the sympathoadrenal-endocrine system will trigger the secretion of glucose counterregulatory hormones. They usually involve glucagon, adrenalin, growth hormone, and cortisol. The effects of cortisol and growth hormone usually require several hours to occur and, therefore, the release of these hormones is unlikely to be critical to rapid glucose counterregulation (244). However, cortisol secretion becomes critical when the catecholamine response is impaired (245). Cortisol may also play an important counterregulatory role during hypoglycemia by enhancing glucose production, depressing peripheral glucose utilization, and accelerating lipolysis in humans (246). Further, the synergistic interactions among glucagon, adrenalin, and cortisol in their hyperglycemic effects are potentially relevant to glucose counterregulation (237). In contrast to cortisol and growth hormone, however, glucagon and adrenalin are potent simulators of hepatic glycogenolysis and glucose production within minutes. Adrenalin also limits peripheral glucose utilization. Thus, it appears that glucagon

and adrenalin are the most important factors, in terms of their hypoglycemic counterregulatory role.

In this context, however, our results and results reported by other investigators indicate that there are several abnormalities observed following hemorrhagic shock that might interfere in this hypoglycemic counterregulatory process. These abnormalities include: a) progressive decrease in glucagon circulating levels following shock, which was reported by Koc *et al.* (228); b) nonfunctional glucagon receptor sites were also documented following shock (220); c) our results (Figure 11) show that hepatocytes failed to respond to glucagon stimulation, and this is due to either alterations in the receptor itself or uncoupling of the receptor from its transmembrane signaling elements (Figures 11 and 13); d) studies have shown that glycogen levels in cardiac muscle, skeletal muscle, brain, and liver decrease at the terminal phase of shock (5), and with respect to adrenalin in this regard; e) there is down-regulation of hepatic adrenergic receptors following hemorrhagic shock (247); f) adrenalin transmits its signal on hepatocytes via the intramembrane phosphoinositide-system. Our results indicate that receptors that utilize this system, such as vasopressin and ATP, are perturbed in their function following hemorrhagic shock (Figures 15 and 16). Like the situation in the adenylate cyclase system-utilizing agonists (glucagon), this alteration is due to either change in the receptor itself or uncoupling of the receptor from it transmembrane signaling components (Figures 15,16, and 17); g) furthermore, on the subcellular level, our results indicate that this

hypoglycemic counterregulatory process might be interrupted, because the activity of the key gluconeogenic enzyme, PEPCK, is depressed following shock (Figure 19). Glycogen stores are depleted and, therefore, glucose production has to rely on gluconeogenesis; h) our results also indicate that the activity and mRNA levels of the glycolytic enzyme pyruvate kinase are elevated following shock (Figures 20 and 21). This elevation will shift the glycolytic pathway towards glycolysis and glucose production will decrease; i) finally, hepatic P_2-purinoceptors share a common mode of action with the Ca^{2+}-dependent gluconeogenic and glycogenolytic hormones angiotensin, vasopressin, and α_1-agonists. Thus, the down regulation of P_2-purinoceptors following hemorrhagic shock, as reported in this study (Figures 5 and 6), might be an important factor in the late hypoglycemic response.

Koc *et al.* (228) have reported that animals treated with ATP-$MgCl_2$ following hemorrhagic shock show progressively increasing levels of glucagon when compared to animals treated with saline. Our results, in this regard, show that ATP-$MgCl_2$ treatment restored the ability of hepatocytes to respond to glucagon (Figure 11) as well as to various receptor-dependent stimuli (ATP and vasopressin) of both second messengers cAMP and IP_3 (Figures 11-13,15-17). ATP-$MgCl_2$ treatment also alleviated the depression of PEPCK activity (Figure 19) and decreased the mRNA and activity levels of pyruvate kinase (Figure 20 and 21). Furthermore, ATP-$MgCl_2$ treatment has restored the density (Figures 5 and 6) and enhanced the affinity (Figure 8) of P_2-purinoceptors on hepatocytes. Thus, it is reasonable to

conclude that treatment of hemorrhagic shock with ATP-$MgCl_2$ has beneficial effects that are potentially related to glucose metabolism.

SUMMARY AND CONCLUSION

It is well established that adverse circulatory conditions, such as hemorrhagic shock, trigger a sequence of events of progressive cell injury and it seems that the initial insult occurs at the cell membrane (5). Since a common feature in all forms of shock is an inadequate circulation with diminished blood flow to tissues, this results in tissue hypoxia. After hypoxia, but especially after subsequent recirculation (reperfusion), oxygen free radicals are formed and produce tissue injury, in large part, through peroxidation of polyunsaturated fatty acids, which results in membrane transformations (95,209,210). Membrane transformations following such conditions result in alterations in various receptor characteristics (13-18) as well as signal transduction through the membrane (19-22). In addition, various forms of shock are known to cause alterations in glucose homeostasis and metabolism (23-28,228).

It is also well documented that ATP-$MgCl_2$ treatment following culatory conditions produces a myriad of beneficial effects including survival of animals (5,31,135,136), membrane stabilization (146,147, 218), and restoration of many metabolic abnormalities observed following ischemia (5). This thesis has addressed the issue of whether trauma-hemorrhagic shock alters P_2-purinoceptor characteristics, transmembrane coupling and/or

membrane effectors, and enzymatic properties associated with glucose metabolism, in an attempt to understand how hemorrhagic shock alters, at the biochemical and molecular level, signal transduction and glucose metabolism. In addition, it's sought to deduce possible mechanism(s) by which ATP-$MgCl_2$ treatment produces the various beneficial effects following such conditions.

The first hypothesis tested in this thesis was that trauma-hemorrhagic shock alters the binding characteristics of rat hepatocytes P_2-purinoceptors. This hypothesis was proposed for four reasons. First, as a representative of the initial step in signal transduction (receptor binding). Second, ATP-$MgCl_2$ treatment following shock conditions would presumably involve these receptors. Third, shock is known to affect membrane functions. Fourth, P_2-purinoceptors are involved in hepatic glucose homeostasis. The results of the association-binding assays in Part III support the proposed hypothesis in regard to the change in receptor density (Figures 5 and 6). Receptor affinity was unaffected (Figures 7 and 8). This latter result refutes the hypothesis that shock affects receptor affinity. In addition, ATP-$MgCl_2$ treatment increased receptor density (Figures 5 and 6) and enhanced receptor affinity (Figure 8), thus, supporting the hypothesis that ATP-$MgCl_2$ treatment involves these receptors and might, in fact, have stabilizing effects on the plasma membrane.

The second hypothesis tested in this thesis was that hemorrhagic shock alters, in addition to receptor systems, transmembrane-signaling

process. This hypothesis was proposed and tested for two reasons. The first reason was that alterations in receptor coupling to transmembrane signaling elements are recognized following ischemic and hypoxic conditions. The second reason for testing this hypothesis was that such uncoupling of receptors would explain the observations that external signals failed to express their intended cellular responses following hemorrhagic shock (220). The results (Figures 10-17) revealed that hemorrhagic shock does indeed affect signal transduction, in view of the fact that receptor-independent stimuli of both second messengers (cAMP and IP_3) (Figures 13 and 17) expressed their effects normally, while receptor-dependent signals (stimuli) of both second messengers (cAMP and IP_3) (Figures 11,12,15, and 16) failed to evoke the expected response.

The third hypothesis tested in this thesis was that hemorrhagic shock alters glucose homeostasis via alterations of enzymes concerned with glucose metabolism. The rational behind this hypothesis is based on the fact that hemorrhagic shock causes the release of various agents and mediators of cell-immunity such as cytokines (29), and these agents are known to have profound effects on enzymatic activities such as enzymes of gluconeogenesis (30). The results (Figure 19) demonstrate that the activity of the gluconeogenic enzyme PEPCK was indeed depressed following hemorrhagic shock. Furthermore, the results in Figures 20 and 21 demonstrate that enzymatic activities are deferentially affected by hemorrhagic shock and enzyme expression can also be affected by shock.

Three conclusions can be drawn from the results of this thesis. One conclusion is that hemorrhagic shock produces multiple alterations in the plasma-membrane including characteristics of P_2-purinoceptors and other receptors (glucagon, vasopressin) and/or their transmembrane-signaling, but not membrane effectors (e.g., adenylate cyclase and PIP_2-system). This may lead to loss of various membrane and cellular functions thus, contributing to many cellular abnormalities observed following hemorrhagic shock. The second conclusion is that hemorrhagic shock alters the expression and/or activity of PEPCK and pyruvate kinase, enzymes involved in glucose metabolism, and this might be a significant factor in the altered glucose homeostasis following hemorrhagic shock. The depression of PEPCK activity coupled with the increase in mRNA levels as well as the activity of pyruvate kinase will shift the glycolytic pathway towards glycolysis and contributes to the hypoglycemic condition observed at the terminal stages of shock.

The third conclusion is that ATP-$MgCl_2$ is a promising adjunct to crystalloid resuscitation in the treatment of hemorrhagic shock, because of its direct beneficial effects on various membrane and enzymatic functions; furthermore, the results in Figure 9 and Table 2 demonstrate that ATP complexed with $MgCl_2$ is required for the treatment to be effective.

In Figures 22-24 schematic presentations of hepatocellular changes observed following hemorrhagic shock, hemorrhage and saline treatment, and the restorations produced following ATP-$MgCl_2$ treatment of

hemorrhagic shock, respectively, are provided. The changes indicated are deduced from the results of this thesis with emphasis on glucose homeostasis.

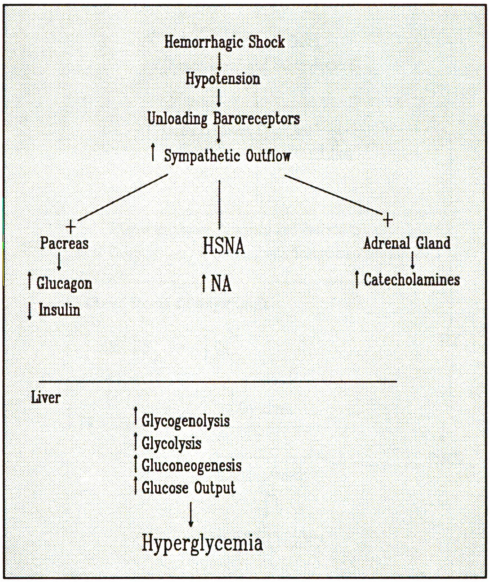

Figure 22. Schematic presentation of the changes in hepatocellular glucose homeostasis in response to hemorrhagic shock. HSNA= hepatic sympathetic nerve activity, NA= noradrenalin.

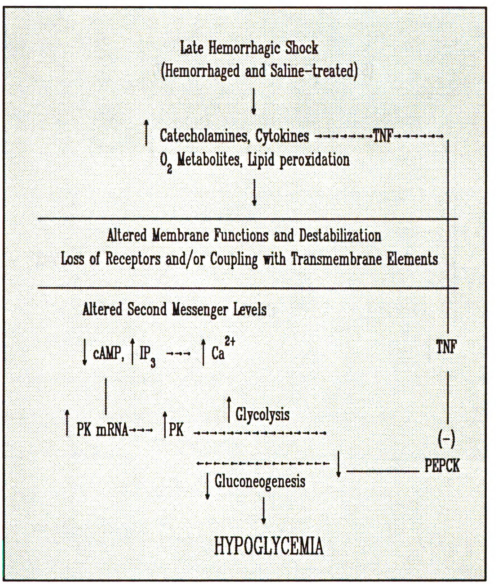

Figure 23. Schematic presentation of the changes in hepatocellular glucose homeostasis in response to late hemorrhagic shock and saline treatment. PK= pyruvate kinase, PEPCK= phosphoenolpyruvate carboxykinase, TNF=tumor necrosis factor.

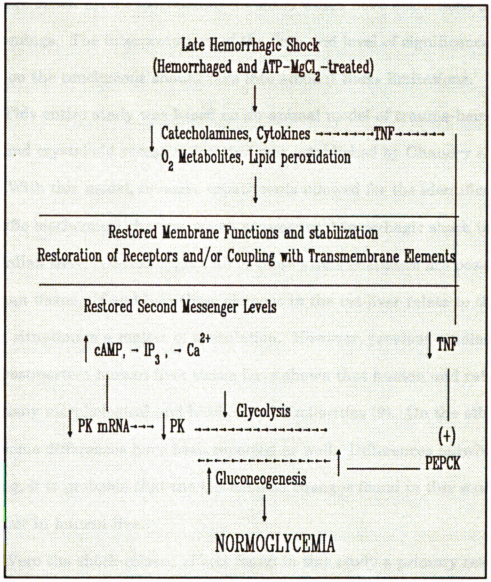

Figure 24. Schematic presentation of the changes in hepatocellular glucose homeostasis in response to late hemorrhagic shock and ATP-$MgCl_2$ treatment. PK= pyruvate kinase, PEPCK= phosphoenolpyruvate carboxykinase, TNF=tumor necrosis factor.

LIMITATIONS AND SHORTCOMINGS

As with all studies, the results and conclusions presented in this thesis are based upon experimental protocols which have limitations and shortcomings. The interpretations of the data and level of significance placed on the conclusions should take into account these limitations.

This entire study was based on an animal model of trauma-hemorrhage and crystalloid resuscitation that was established by Chaudry *et al.* (192). With this model, invasive experiments allowed for the identification of specific biochemical changes which accompany hemorrhagic shock in the mammalian liver. Obviously, not all of these kinds of studies are possible in human tissue. If and how these changes in the rat liver relate to the human situation is a matter of speculation. However, previous studies using postmortem human liver tissue have shown that human and rat livers show many morphological and biochemical similarities (9). On the other hand, some differences have been reported as well. Differences notwithstanding, it is probable that the biochemical changes found in this study also occur in human liver.

Were the shock-related effects found in this study a primary result of hemorrhagic shock per se, or were they a secondary effect of disease? Sprague-Dawley rats are fairly healthy and free of disease as granted by Charles River Co. (Wilmington, MA). Furthermore, the differences (results) were compared to control (sham-operated) animals, which were randomly selected from the same pool of animals that hemorrhaged and saline-treated

as well as hemorrhaged and ATP-$MgCl_2$-treated rats were selected from. All of the experiments performed were exclusively on survivors. Animals who died before the time-point at which their livers were to be utilized for the various experiments were excluded from the study. This might be a factor that influenced the results in one way or the other. For example, in the receptor density results (Figures 5 and 6), we believe that the gradual recovery in the number of receptors displayed by hemorrhaged and saline-treated animals throughout the time course, was a result of this variation (i.e., if all animals were used, probably this trend would have disappeared?). Additionally, there might hade been a reversal of this trend at a later time (post 27 h). This was a severe hemorrhagic shock model with late mortality rate of about 50%, thus, some animals were obviously expected to die. However, some animals died as a result of surgical failure, but those animals were excluded entirely (i.e., not even included in the mortality calculations).

In the receptor binding study, the effects of hemorrhagic shock on the affinity and density were examined in relation to previously reported data (8,9) which are all in close agreement with the values in our control animals. It should be pointed out, however, that a specific antagonist is necessary in order to fully classify a receptor. In the absence of wide spread availability of such an antagonist, the classification of P_2-purinoceptors is at present based almost entirely on the relative potencies of various agonists (ATP analogues). Until a competitive antagonist at the P_{2x}, P_{2y}, and

P_{2x}-purinoceptor subtypes becomes available, it will not be possible to perform classical pharmacology on these receptors with a variety of adenine nucleotide analogues that are agonists, partial agonists, and antagonists.

The data in the receptor-binding studies as well as in the second messenger experiments were normalized to the number of viable cells only. The contribution of non viable cells, therefore, might have affected the interpretation of these results. However, the number (percentage) of viable cells was always within a narrow range (80-85%) in all of the experiments.

In the second messenger experiments, the information that we obtained from these results cannot exclusively imply that the defect was in the intramembrane coupling elements. While the results show that membrane effectors (e.g., adenylate cyclase) were not affected by hemorrhagic shock, the results cannot identify the site of defect, it can be in the receptor system (glucagon and vasopressin) itself as well as in the transmembrane signaling elements (e.g., G-proteins).

Another limitation is that other important receptors in terms of glucose homeostasis and metabolism were not studied (e.g., insulin and glucagon). The study of receptor dynamics was not directed to answer a question concerning glucose but, it was designed to examine the initial step in the signal transduction process. While the second messenger experiments imply that glucagon did not stimulate hepatocytes, this could be due to down regulation of glucagon receptors as in the case with P_2-purinoceptors, and it could be due to transmembrane-uncoupling. In this

regard, however, it should be pointed out that P_2-purinoceptors were chosen in particular for the following reason: the beneficial effects of ATP-$MgCl_2$ treatment following shock and ischemia most probably would involve these receptors.

Future Directions

The results of this dissertation have extended our knowledge and understanding of how trauma-hemorrhagic shock alters the biochemistry of P_2-purinoceptors, membrane signal transduction, and enzymatic properties of glucose metabolism. In addition, possible mechanisms by which ATP-$MgCl_2$ treatment produces its beneficial effects are also deduced. However, several questions remain unanswered and additional experiments are needed to answer them.

One important question which needs to be addressed is how universal are the changes which were found in this study? Experiments need to be repeated using additional strains of rats such as Wistar and Fischer, in order to show that the results of this study are not strain-specific for the Sprague-Dawley rats. In addition, receptor binding studies should be conducted in postmortem human liver tissue in order to verify that the receptor changes found in the rat are relevant to humans.

Receptor binding studies should be carried out using agonists of other receptors, particularly those receptors involved in glucose homeostasis (e.g., glucagon); in addition such studies should be correlated with studies of the

second messengers to infer whether the defect is in the receptor system itself or in the transmembrane signaling elements.

Other enzymes involved in glucose metabolism also should be studied, to build a complete picture of how this pathophysiological condition is affecting glucose metabolism. The role of P_2-purinoceptors in glucose homeostasis deserves attention.

Finally, although the liver is the main site of gluconeogenesis and glycogen storage, the kidney becomes an important site of gluconeogenesis during stress conditions (229). Therefore, renal enzymes concerned with glucose metabolism should be studied under hemorrhagic shock conditions.

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