

A VASOPRESSINERGIC PATHWAY WITHIN THE BRAIN AND ITS ROLE IN  
DRUG-INDUCED ANTIPYRESIS AND PYROGENIC TOLERANCE

By

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B.Sc.(Honours), The University of British Columbia, 1985

M.Sc., The University of British Columbia, 1987

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE  
REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

Department of Physiology

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

September 1990

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Date SEPT. 25, 1990

## ABSTRACT

There is strong evidence which supports a physiological role for arginine vasopressin (AVP) in the negative modulation of the febrile process within the central nervous system (CNS). This evidence arises from a variety of experimental techniques employed in a number of different animal models. The CNS locus of action for AVP-mediated antipyresis is within a rostral diencephalic site called the ventral septal area (VSA). It has become evident that the mechanism by which AVP and aspirin-like drugs transduce changes in febrile body temperature are similar. Moreover, antipyretic drugs and AVP may share a common CNS locus of action. Therefore, investigations were conducted to determine whether antipyretic drugs are functionally linked to the endogenous antipyretic system of the brain. In addition, an examination of the role for centrally acting AVP and the natural suppression of fever during pyrogenic tolerance to endotoxin was conducted.

AVP receptor antagonists of the peripheral V<sub>1</sub> and V<sub>2</sub> sub-type or saline control were microinjected into the VSA of rats rendered febrile by an intracerebroventricular (icv) injection of *E. coli* endotoxin, to assess the effects on the antipyresis elicited by indomethacin. Blockade of central V<sub>1</sub> but not V<sub>2</sub> receptors significantly attenuated the antipyretic effects of indomethacin given intraperitoneally. This effect was even more pronounced when the V<sub>1</sub> antagonist was infused for 30 min before and for 60 min after indomethacin administration. The V<sub>1</sub> analogue alone was without thermoregulatory effects.

In order to determine whether the above effects were applicable to antipyretic drugs in general, central V<sub>1</sub> blockade was performed in the febrile rat subsequently treated with intraperitoneal sodium salicylate or acetaminophen. Salicylate-induced

antipyresis was blocked, in a dose related manner, by VSA administration of the AVP  $V_1$  antagonist. The fever reducing capacity of acetaminophen was unaffected by central  $V_1$  blockade. Collectively these antipyretic drug studies, suggest that some but not all antipyretic drugs activate the endogenous AVP antipyretic pathway within the brain. Moreover, these data suggest that the mechanism of action of antipyretic drugs can no longer be simply explained as an action on prostaglandin biosynthesis.

Endogenous release of AVP from VSA nerve terminals during endotoxin fever and drug-induced antipyresis was examined using the technique of push-pull perfusion. The release of AVP into the perfusion fluid remained unaltered by indomethacin injected into the non-febrile rat. However, during fever indomethacin prompted both an antipyresis as well as a significant increase in AVP release. Acetaminophen injected intraperitoneally also evoked an antipyresis but with no concomitant release of AVP within the VSA. These results are consistent with the antagonist studies.

The effects on central AVP release by indomethacin appear to be related to the pyrogen employed because the drug did not evoke the release of AVP when administered prior to the hyperthermia produced by icv  $PGE_2$ . Indeed,  $PGE_2$  itself stimulated AVP release which was inhibited by indomethacin treatment. These results are not consistent with an antipyretic role for AVP and await further clarification.

Analysis of the release of AVP into the plasma and cerebrospinal fluid (CSF) were conducted during the fever evoked by intravenous endotoxin and subsequent to antipyretic intervention. Intravenous endotoxin was a provocative stimulus for plasma AVP release. Endotoxin-stimulated plasma AVP levels were unaffected by intraperitoneal injections of indomethacin, sodium salicylate or acetaminophen. In non-febrile controls, indomethacin, and to some extent acetaminophen, prompted increases in plasma AVP; although the temporal course of this release was different



between the two drugs. Within the CSF, endotoxin treatment did not alter the normal diurnal rhythm of AVP release. Indomethacin treatment significantly suppressed CSF AVP release in non-febrile animals. A similar but non-significant trend was observed in febrile rats. Collectively, these studies demonstrate the independent regulation of AVP release within three separate biological compartments in response to febrogenic and antipyretic stimuli.

The suppression of fever after repeated daily intravenous injections of bacterial endotoxins was thought to be exclusively a hepatic phenomenon. Experiments were conducted to determine whether a central mechanism involving AVP may also contribute to the antipyretic state observed during pyrogenic tolerance. In endotoxin tolerant animals, administration of a  $V_1$  but not  $V_2$  AVP receptor antagonist within the VSA, resulted in a significant reversal of the tolerant pyrogenic response. These data support the hypothesis that the central endogenous antipyretic system, involving AVP, plays a role in the mechanism of endotoxin tolerance.

Tolerance does not develop following repeated central injections of pyrogens. Further experiments were performed to determine whether tolerance-induced activation of the antipyretic pathway would render an animal hyporesponsive to centrally administered pyrogens. When injected icv, during active endotoxin tolerance, the thermoregulatory responses to  $PGE_2$  or endotoxin were not significantly suppressed from non-tolerant controls. However, analysis of VSA push-pull perfusates performed during a tolerant reaction to intravenous endotoxin revealed that increased AVP activity occurs within the first 30 min after the intravenous injection, well before the time  $PGE_2$  or endotoxin were injected into the cerebral ventricles. This suggests that the antipyretic system is only activated briefly and may explain why centrally evoked fevers were unaffected during active endotoxin tolerance.

In summary, this thesis research has demonstrated a direct functional link between the mechanism of action of antipyretic drugs and the endogenous antipyretic system within the brain. These results call into question the hypothesis whereby the fever reducing properties of antipyretic drugs can be explained exclusively as a result of the inhibition of prostaglandin biosynthesis. In addition, the differential effects on AVP release by antipyretic drugs suggests a number of biological pathways that can be activated by these drugs. Finally, a role for the AVP endogenous antipyretic system in the suppression of fever during endotoxin tolerance was established.

## TABLE OF CONTENTS

ABSTRACT .....	ii
TABLE OF CONTENTS .....	vi
LIST OF TABLES .....	ix
LIST OF FIGURES.....	x
ACKNOWLEDGEMENTS .....	xii
 I. INTRODUCTION .....	 1
A. Fever: Historical Aspects.....	2
Fever in the ancient world .....	2
Fever theory from Galen to the 19th century .....	7
B. Pyrogens: Initiators of the Febrile Response.....	12
Exogenous pyrogens.....	12
Viruses.....	14
Gram positive bacteria .....	14
Endotoxin tolerance.....	18
Endogenous pyrogens .....	20
Interleukin-1.....	21
Tumor necrosis factor $\alpha$ .....	23
Other endogenous pyrogens .....	25
C. The Acute Phase Response .....	28
Serum metallic ions.....	28
Hepatic protein synthesis .....	29
Additional acute phase reactants.....	30
Acute phase and febrile responses: independent regulation.....	31
D. Central Mediators of the Febrile Respinse .....	32

POAH: neuronal substrate for febrile physiology.....	32
Prostaglandin E in febrogenesis.....	33
E. Control of Febrile Body Temperature.....	37
Antipyretic drugs .....	38
Endogenous antipyresis .....	41
Vasopressin and endogenous antipyresis .....	43
F. Rationale for Investigation.....	48
 II. GENERAL METHODS AND MATERIALS .....	 51
A. Animals.....	51
B. Surgical Procedures.....	51
Intra-atrial catheter.....	51
Stereotaxic surgery .....	52
Cerebrospinal fluid withdrawal cannula.....	54
C. Body Temperature Measurements .....	54
D. Vasopressin Radioimmunoassay .....	55
E. Histological Procedures.....	56
F. Drugs.....	56
Pyrogens .....	56
Antipyretics .....	57
Vasopressin receptor antagonists .....	57
G. Treatment of Data.....	58
 III. THE ROLE OF CENTRAL VASOPRESSIN V1RECEPTORS DURING INDOMETHACIN-INDUCED ANTIPYRESIS.....	  62

IV. CENTRAL VASOPRESSIN V1 BLOCKADE PREVENTS SALICYLATE BUT NOT ACETAMINOPHEN-INDUCED ANTIPYRESIS.....	76
V. VASOPRESSIN RELEASE WITHIN THE VENTRAL SEPTAL AREA DURING ANTIPYREIS EVOKED BY INDOMETHACIN AND ACETAMINOPHEN.....	92
VI. VASOPRESSIN RELEASE INTO THE VENTRAL SEPTAL AREA DURING PGE2-INDUCED HYPERTHERMIA AND THE EFFECT OF INDOMETHACIN .....	105
VII. THE EFFECTS OF THREE ANTIPYRETIC DRUGS ON THE RELEASE OF VASOPRESSIN INTO THE PLASMA AND CSF IN FEBRILE AND NON-FEBRILE RATS .....	113
VIII. EVIDENCE SUPPORTING A ROLE FOR ENDOGENOUS VASOPRESSIN IN THE PYROGENIC TOLERANCE TO INTRAVENOUS ENDOTOXIN .....	133
IX. THE THERMOREGULATORY EFFECTS OF CENTREALLY ADMINISTERED PYROGENS DURING ACTIVE PYROGENIC TOLERANCE TO INTRAVENOUS ENDOTOXIN .....	150
X. SUMMARY AND GENERAL DISCUSSION.....	162
XI. REFERENCES .....	170

**LIST OF TABLES**

<b>TABLE</b>	<b>PAGE</b>
I Thermal indices calculated from indomethacin blockade studies.....	66
II Control thermal indices calculated from salicylate blockade studies.....	84

## LIST OF FIGURES

FIGURE	PAGE
1 Hyperthermic response following VSA microinjection .....	59
2 Push-pull perfusion system .....	60
3 Photomicrograph showing VSA and bilateral injection loci.....	61
4 The antipyretic response to indomethacin.....	67
5 The thermoregulatory response to control VSA injections.....	68
6 The effects of Tris buffer during VSA V1 blockade.....	69
7 Microinfusion of V1 antagonist during indomethacin-induced antipyresis .....	70
8 Histological identification of injection loci.....	71
9 The antipyretic response to salicylate during V1 blockade.....	82
10 The antipyretic response to acetaminophen during V1 blockade.....	83
11 The effects of DMSO-saline during V1 blockade.....	85
12 The thermoregulatory response to control VSA injections.....	86
13 Thermal response index: salicylate and acetaminophen .....	87
14 Histological verification of injection loci .....	88
15 VSA push-pull perfusion:antipyretic effects of indomethacin and acetaminophen.....	97
16 VSA push-pull perfusion: effects of indomethacin and acetaminophen in non-febrile rats .....	98
17 AVP levels in VSA push-pull perfusates during antipyresis .....	99
18 Photomicrograph of VSA push-pull perfusion locus.....	100
19 VSA push-pull perfusion: thermoregulatory effects of PGE2 and indomethacin.....	108
20 VSA push-pull perfusion: thermoregulatory controls.....	109
21 AVP levels in VSA push-pull perfusates following PGE2 and indomethacin .....	110

22 The thermoregulatory and plasma AVP responses to intravenous endotoxin and Tris buffer .....	120
23 The thermoregulatory and plasma AVP responses to intravenous endotoxin and indomethacin.....	121
24 The thermoregulatory and plasma AVP responses to intravenous endotoxin and salicylate.....	122
25 The thermoregulatory and plasma AVP responses to intravenous endotoxin and acetaminophen.....	123
26 Cerebrospinal fluid levels of AVP following indomethacin and intravenous endotoxin .....	124
27 Integrated (5h) plasma AVP responses.....	125
28 Thermal response index following antipyretic intervention.....	126
29 The development of endotoxin tolerance .....	140
30 The effect of VSA V1 blockade on the tolerant response to endotoxin.....	141
31 The effect of VSA V2 blockade on the tolerant response to endotoxin.....	142
32 The effect of VSA injections in tolerant controls .....	143
33 Fever indices from tolerance studies .....	144
34 Histological verification of injection loci .....	145
35 The development of pyrogenic tolerance to endotoxin .....	154
36 The effects of PGE2 and endotoxin (icv) during tolerance .....	155
37 Fever indices calculated from naive and tolerant rats .....	156
38 AVP levels in VSA push-pull perfusates in naive and tolerant rats .....	157
39 Hypothetical central pathways activated by antipyretics .....	166



## ACKNOWLEDGMENTS

The preparation of this thesis was generously aided by special efforts from the following people: Mary Forsyth, Zaira Khan, Joey Tay, Dave Phelan, Debbie Yaschuk and John Sanker.

I thank members of my supervisory and examining committees for being merciful. A special acknowledgment is extended to Dr. L.P. Renaud for serving as the external examiner. I would also like to thank Norm Kasting for the style of supervision which allowed me to explore my research interests.

The financial support of the Medical Research Council is gratefully acknowledged. I am also grateful for the donation of the vasopressin V<sub>2</sub> antagonist generously provided by Dr. M. Manning of the Ohio Medical College.

A special thank you is extended to Ray Pederson for his unheard efforts as Graduate Advisor; I suggest a raise in pay is warranted.

## I. INTRODUCTION

Angel of health, have you beheld the Fevers?  
Across pale walls of wards they limp and stumble,  
Like exiles wan, with agues, chills, and shivers.  
Seeking that scanty sun with lips that mumble.  
Angel of health, have you beheld the Fevers?

Baudelaire

This excerpt from "Les Fleurs du Mal" (Baudelaire, transl. by Campbell, 1952) exemplifies the anguish and consternation that was felt by pre-antibiotic society toward febrile illness. The presence of a fever often indicated an imminent death or, at the very least, the patient could anticipate a lengthy illness. While febrile illness still remains clinically relevant today, the presence of fever does not instill the former sense of dread and forboding it once did. In general, fever is recognized as a host mediated reaction to invading pathogens or to localized inflammation. What is truly unique about the febrile reaction is the generalized mobilization of body resources and the priority approach which has evolved to ensure that body temperature becomes and remains elevated at febrile levels following the appropriate stimulus. As will become evident from the following introductory discussion, the physiological mechanisms which result in febrile body temperatures are far from completely understood. However, the mechanisms that are responsible for feedback control of body temperature during fever have received even less attention. Thus, it is the intent of this thesis to present some evidence which will hopefully add, in a meaningful way, to the body of scientific knowledge devoted to understanding the neurobiology of fever in general and to the mechanisms responsible for controlling and reversing the febrile process.

## A. Fever: Historical Aspects

### 1. Fever in the Ancient World

Fever is without a doubt one of the most widely recognized symptoms of illness or disease. Literally thousands of years prior to the invention and use of thermometry, fever and its association with inflammation were recognized. The Sumerians circa 3100 B.C. depicted febrile symptoms using the flaming brazier pictograph. This representation was retained, in modified late Assyrian and Babylonian cuneiform glyphs (600 B.C.). Fever was thought by these ancients to be caused by Gods or spirits who would inflict an illness on an individual for moral reasons. Thus a patient or even family members could be the "cause" of a febrile episode. Interestingly, outbreaks of fever were never the fault of the physician or "asu", who would refuse to "treat" a patient if it was believed to be a lost cause (Majno, 1975). Such as it was, treatment often consisted a confession of sin to a priest, an incantation or the draining of "the white sap" (pus) from an infected wound. Inflammation and fever were inexorably linked and treatment was intended to deal with both issues. However, in ancient Mesopotamia disease was not always a matter of sin or religious manifestations as the link between fever and tooth decay attests (Majno, 1975).

The ancient Egyptians were also aware of the connection between inflammation and fever. Again the hieroglyphic representation was the flaming brazier. Around 1900 B.C. the first known medical text appeared; the contents of which are liberally laced with references to inflammation and fever. In addition, this text contained many magic spells and potions, which like their Mesopotamian predecessors, portrayed the Egyptian belief in supernatural mechanisms of disease. Unlike the Mesopotamians, though, the Egyptian pharmacopia contained some sound treatments for infection and inflammation, which through its association, aided in the prevention of febrile illness. Naturally occurring copper compounds such as  $\text{CuSiO}_4$  and  $\text{CuCO}_3$  were ground to a

fine powder and mixed with animal fat to produce a paste with remarkable antiseptic potency. The use of these compounds, however, was limited to external use because of the toxicity of copper. The oxidized copper resulted in a brilliant green or turquoise color which was exploited in cosmetics by women of the elite classes. Thus for the fortunate few, eye make-up served as an effective preventative measure against the prevalent incidence of eye infections, which persists even today, in these regions. The Egyptian physician also employed honey-containing salves in treatment of inflamed wounds. This served an antiseptic function in two ways. First, the hypertonic honey would draw water out of bacterial cells via an osmotic mechanism and second, the presence of the enzyme glucose oxidase, which catalyzes the aerobic reaction of glucose to gluconolactone and  $H_2O_2$ , would by virtue of the  $H_2O_2$ , be bactericidal even at  $H_2O_2$  concentrations as low as 13% (Majno, 1975). The Egyptians of course had no scientific basis for their treatments and frequently used "magic" and rational medicine in different combinations, not only depending on the wound or symptom, but also depending on the circumstance in which the individual became ill.

Essentially, other ancient societies, such as those of the Indian sub-continent and China, utilized combinations of religion and sound medicine in the treatment of fever and inflammatory illness. The civilization of the Indus river valley (c. 260 B.C.) decreed febrile illness as the lord of all bodily diseases which was common to man and animal alike (Lyons and Petrucelli, 1978). Ancient Chinese medicine mixed the philosophical notion of Yin and Yang to a growing pharmacopia, which remains today a intriguing source of potentially useful pharmaceutical treatments. Interestingly, Chinese philosophers, independent of the Greeks, developed a humoral view of their world, which was the interaction of five basic elements (metal, wood, water, fire and earth) each of which had specific characteristics or qualities. This was remarkably similar to that espoused in ancient Greece, where a four element principle was conceived. The humoral concept in health was essentially that, within the body, these elements,

interacted in proper proportions, resulting in the normal bodily functions of an individual. However, should this ideal proportionality deviate then illness would result. This medical philosophy provided the rationale for the "therapeutic" use of bodily purges such as fasting, vomiting, enemas and bloodletting. However, purges, including bloodletting (Majno, 1975), were frequently used in Egyptian times long before established humoral philosophy.

The cornerstone of medical thinking up until the 19th century was based upon the extraordinary legacy of Hippocrates of ancient Greece (460-380 B.C.). The Hippocratic Collection (probably the remains of the library of his medical school at Cos) contained not only the writings of Hippocrates himself but also anonymous essays and other fragmentary works from other authors. This collection contained volumes of detailed case histories handled by the Greek physicians (the iatros). Indeed such detailed descriptions were recorded that diagnoses of febrile diseases such as malaria could be deduced from them (Dinarello and Wolff, 1980).

The basis of Hippocratic medicine was the humoral theory of disease; which hypothesized that the four humours which normally existed in perfect balance and symmetry, somehow became unbalanced leading to a pathological state. Each of the four humours, blood, phlegm, yellow bile and black bile, had characteristic qualities: hot, dry, moist and cold. Thus, according to this view, fever was the result of an excess of yellow bile since its qualities were hot and dry. Within the Hippocratic Collection frequent descriptions of "bilious bowels", "peripneumonic tongue" (yellow/yellowish white tongue) and other references to bile during febrile illness established a regular association of bile with febrile symptoms (Smith, 1981). The followers of Hippocrates also postulated that the primary sources of disease were derived from the blood, flesh and/or the hypothetical venule. With this theoretical basis, treatment was adjusted accordingly. Thus for the treatment of fever, purges (fasting, emetics, purgatives) and

special diets were commonly employed. Although bloodletting was used for the treatment of inflammatory wounds it is not known whether this procedure was employed for febrile "disease" *per se*. The widespread and documented use of bloodletting as an antipyretic therapy would have to wait another 500 years. It is clear, however, that the Hippocratic approach to medicine and its humoral basis would provide a lasting influence on medical thought up until the 19th century: nearly 2000 years.

As the preeminent medical philosophy the humoral concept regarding health and disease influenced the thinking and the observations of subsequent physician/scientists. A prime example is that of Galen (129-200 A.D.). An influential physician of the highest levels of Roman society and one time physician to the gladiators, Galen had unprecedented opportunities to treat a variety of wounds and make extensive investigations of human as well as animal physiology and anatomy. Because of his belief in humoral physiology his anatomical and physiological observations were often influenced to fit the humoral hypothesis. A prolific writer, Galenic anatomy and physiology were well read and accepted for centuries. Throughout the Dark and Middle ages the religious taboos regarding human dissection further helped to entrench Galenic thought.

Like his predecessors, Galen viewed fever as a disease itself rather than a symptom. Although Galen attempted to address the issue of febrile "disease" in his **De Febrium Differentiis** he left no clear unambiguous definition of fever and it is likely that he assumed in his readers a previous knowledge of the topic (Lonie, 1981). However, throughout his works diffuse comments can be collected in order to establish a Galenic view of febrile illness. Galen believed that "... fever is extraneous heat, kindled in the heart, from which it is diffused to the whole body through the arteries and veins, by means of the spirit and the blood, reaching a heat in the body itself which

is sufficient to injure the natural functions" (Lonie, 1981). Galen treated "heat" not as a consequence of normal physiology but as a separate entity. Thus "...fever occurs through the conversion of innate heat to the fiery" (Lonie, 1981). In an undefined manner, Galen associated putrid humours within the blood as harbouring the extraneous or injurious heat of fever. Because the hypothetical nature of febrile heat was based upon the belief of heat residing in blood\spirits, the rationale for treatment was bodily purging therapy. Indeed, based on the existing physiological hypotheses purging was a logical approach to treatment. Thus, bloodletting was an important component of Galen's therapeutic practice. Of particular interest was his advocacy of bleeding as a means of fever reduction or antipyresis. In 170 A.D. Galen noted that "...up to six pints of blood were removed so that the fever was immediately extinguished and no impairment of strength ensued" (Galen, transl. by Green). However it is likely that bloodletting as an antipyretic therapy preceded Galen as indicated by the testimonials of other physicians (Aurelianus, transl. by Drabkin, 1950) as well as the presumed use of therapeutic bleeding centuries earlier (see p.4 above). However, because of Galen's status, undoubtedly enhanced by his prolific writings, bloodletting as a generally used (as well as antipyretic) therapy persisted up until the middle of the 19th century when the modern concepts of fever and disease, as well as the discovery of quinine and salicin became known. It is indeed testimony to Galen's prominence and the school of thought he represented that bloodletting persisted even after it was shown statistically by Pierre Charles Alexandre Louis (1836) to have no or even detrimental effects as a clinical therapy (cited in Kluger, 1978). However, it is of interest that bloodletting as an antipyretic therapy may have a physiological basis which has recently been reviewed (Kasting, 1990) and the mechanism of which, is the focus of investigation of this thesis.

## 2. Fever theory from Galen to the 19th century

Medicine in the Dark and Middle Ages was characterized by a departure from the Greek method of hypothesis, observation and empirical analysis. In its place was the Biblically derived philosophy of divine intervention, not only in medicine, but in all aspects of life. At no time was this trend more evident than during the great plagues of Europe. The Black Death first appeared in the Crimea during the Crusades and its spread through Europe was facilitated by the returning soldiers (Lyons and Petrucelli, 1978) as well as via the ever increasing commercial traffic between the east and west (Atkins, 1984). The Plague was characterized by febrile symptoms, buboes (black lesions emanating from the lymph nodes), and ultimately death. Contemporary medicine had nothing to offer plague victims and it is likely that some of the methods of treatment, such as draining the buboes, helped to spread the infection. The effect of the Black Death of the mid-14th century was devastating to European society as an estimated 25,000,000 lives (approximately one-third to one-fourth of the population of Europe) were lost to the disease (Atkins, 1984). In keeping with the religious approach to disease the Plague was considered to be a punishment from God. Indeed, support for this contention could be found in many passages from the Bible (Atkins, 1984). This belief of divine direction became firmly entrenched and was actively defended by the clergy (who obviously had a vested interest in this philosophy); the defense of these notions are probably best illustrated by the excesses of the Spanish Inquisition.

Despite the advances in physiology and anatomy which began in the Renaissance the predominant theoretical construct regarding fevers, up until the mid 1800s, remained that espoused by Galen. As a rule, alterations to accepted views in medicine were slow to surface and even slower to be generally accepted. Changes regarding fever etiology were no exception. Certain highlights which pertain to the advancement of fever theory deserve mention. Up until the 16th century, fever was assumed to be



due to an extraneous force (heat) which somehow compromised the body's natural heat. The contention that fever may be a natural response produced by the body to aid in the processes combatting disease was first suggested in Europe by Gomez Pereira (c. 1500-?) (Lonie, 1981). However, Pereira was preceded in this view by the Persian physician Rhazes (c. 850-932) who may also have been the first to believe that the fever *per se* was not a disease (Talbot, 1970). Nevertheless, the etiology of fever and its medical treatment remained essentially that of Galen.

Although this discussion assumes the belief that febrile illness was considered deleterious to health, this was not necessarily a universally accepted view and indeed a febrile reaction was often considered beneficial; but this depended upon the malady which inflicted an individual. For instance, Galen considered fever therapy beneficial in the treatment of epilepsy and asthma (Westphal et al, 1977). Treatment of gonorrhea and syphilis, at least until the 18th century, often employed "fever" therapy. This usually consisted of placing the individual in a specially constructed barrel, which allowed the head to protrude, while heating the interior of the barrel via a fire (or fires) placed to the side or underneath the barrel; heat from the fires was also directed into the barrel using a bellows (Lyons and Petrucelli, 1978). It has subsequently been shown in modern times, that the microorganisms responsible for these venereal diseases are killed at febrile body temperatures of around 41°C (Hellon and Townsend, 1983). Interestingly, fever therapy for the treatment of syphilis was used in the early 20th century using intravenous bacterial vaccines (Greisman, 1983). Thus, debate over the physiological benefits of fever, that was started centuries ago, remains today a controversial and unresolved issue.

Probably the major advance in physiology of the 17th century was Harvey's discovery of the circulation of the blood. The significance of this discovery was not realized as being particularly relevant to the accepted notions of fever. The increasing

tendency towards a mechanistic approach to physiology had little if any influence particularly with regards to treatment. Thus the Galenic doctrine of fevers was not revised, and instead, ironically, received support from Harvey himself, a staunch Galenist. Towards the end of the 17th and beginning of the 18th century Andrew Brown of Edinburgh incorporated Harvey's discovery into a hypothesis whereby fever was caused by a slowing or blockage of the blood due to particles entering the blood from the intestines. As a result the pulse quickened (a hallmark sign of fever) in an attempt to remove the blockage and restore blood flow. This new concept had no effect on treatment, however, as purging and bloodletting were still prescribed to deal with the offending "particles" (Bates, 1981); again a logical therapeutic strategy based upon the existing theory.

Scientific research in virtually all disciplines rapidly progressed during the 18th and 19th centuries. Certain key observations began to reshape or kindle doubt in dogmatic opinions in a variety of topics. With respect to the ideas on fever and temperature regulation, Lavoisier's discovery of oxygen and its role in the process of gas exchange was a major advancement. From his experiments using whole body calorimetry, Lavoisier generated evidence suggesting that the body temperature of an animal was due to a heat process arising from the combustion of oxygen with organic substances within the body (Lavoisier, 1780 cited in Kleiber, 1961). Subsequently, Claude Bernard introduced the hypothesis of *milieu interieur* and established the concept of homeostasis: a central axiom of modern biology. Bernard's experiments in thermoregulation extended earlier works of Chossat (who, in 1820, demonstrated the loss of thermoregulation upon transection of the spinal cord) to show the importance of the autonomic nervous system in controlling the loss of heat through cutaneous blood flow (cited in Bligh, 1973). Concurrent with advances in physiology was the discovery of microorganisms and the subsequent establishment of the germ theory of disease.

Collectively these works established the framework for the development of a modern theory on the maintenance of normal body temperature as well as on the pathogenesis of fever.

The importance of body temperature to physicians played a prominent role in clinical practice as judged by the volumes of material written on the subject of fever. However, body temperature was only qualitatively assessed by physicians until the invention of the thermometer. Prior to this, the hand, patient history and urine and stool "analysis" were the primary means used to judge the presence or absence of fever. Caelius Aurelianus of Rome described his method of diagnosing febrile body temperature in the following way: "...place the hand in hollow concealed parts of the body or over places where the patient has been laying if a considerable amount of burning heat is felt...plus panting, hot breath and desire for cold drink..." then a fever is present (Aurelianus, transl. by Drabkin, 1950).

The thermometer was probably first constructed by Galileo in 1592. His water filled device had no scale, indicated only gross changes in temperature and was influenced by the atmospheric pressure (Lyons and Petrucelli, 1978). A colleague and contemporary of Galileo was Santorio Santorio (1561-1636) who made modest improvements to the device and conducted detailed experiments on metabolism using differences in weight and thermometric measurements to determine, among other things, heat loss through the skin. Santorio also introduced the thermometer to clinical medicine. But because of its large size and slow response time it was not widely accepted. Improvements to the device by Huygens (1665) (introduced the 0-100° scale) and Fahrenheit (replaced water with mercury) paved the way for Herman Boerhaave (1668-1738) and his students to use the thermometer extensively in their clinical practice. These Dutch physicians observed the circadian rhythm of body temperature, noted the increase in temperature associated with shivering and showed the positive

relationship between heart rate and body temperature during febrile illness (Lyons and Petrucelli, 1978). Contemporaneously, James Currie working in the London Fever Hospital, diagnosed fever using the thermometer and temporarily alleviated high body temperatures using cool water baths. It was Currie's experience that the physical lowering of febrile temperature afforded enough temporary relief to enable the patient to eat and rest; ultimately benefitting them to a much greater degree than patients treated with traditional methods (Bynum, 1979). Despite their findings the use of the thermometer was still not widely accepted until well into the 19th century. Probably the most influential person in the clinical use of thermometry was Carl August Wunderlich (1815-1877). Wunderlich's treatise, **On the Temperature During Diseases** (1871) was a compilation of his clinical study of thousands of patients. Wunderlich helped in great part to secure the belief that fever was a symptom of disease and not a disease itself; although he believed erroneously that each disease had its own unique fever curve. He further established the clinical importance of measuring body temperature and, interestingly, was the first to observe the temperature reducing effects of blood loss (both natural and induced).

By the late 1800's the means for quantitative analysis of body temperature along with the accumulation of sufficient scientific knowledge, in a number of disciplines, provided the atmosphere for a major change in the way fever, and disease in general, were conceived. In 1875 Liebermeister hypothesized that febrile increases in body temperature were the result of a pathological process whereby the body temperature is set at a new and higher level. Thus thermoregulation *per se* functioned normally but body temperature, via neural mechanisms, was adjusted and regulated at the higher level (Liebermeister, 1875 cited in Atkins and Bodel, 1979). Thirteen years later William Welch (1888), in consideration of the available scientific evidence, postulated that pyrogenic agents acted on the central nervous system to affect fever. He further believed that fever producing agents arose from areas of inflammation and gained

access to the central nervous system from the blood stream. His poignant comments preceded the endogenous pyrogen concept a notion that we generally believe to comprise at least part of the fundamental mechanism of febrile response today. Welch stated: " It is possible that...various substances which we are ...describing as pyrogenic may produce in the body some common change which gives rise to the real fever producing agent" (Welch, 1888). Thus with the previous statement and his description of the "...liberation of fibrin ferment by...leukocytes", which act on the central nervous system, Welch pioneered the modern concept of the endogenous pyrogen.

## B. Pyrogens: Initiators of the Febrile Response

### 1. Exogenous pyrogens

Pyrogenic or fever causing agents encompass a wide variety of substances that can be both foreign to the host or endogenously produced. However, a discussion of exogenous pyrogenic agents requires that a definition of fever, as we currently understand it, be established. Fever is a pathological process whereby core temperature is elevated as a result of some pyrogenic agent (although there are exceptions to this; neurogenic "hyperthermia" for example). A febrile reaction can be distinguished from hyperthermia on the basis that the elevated temperature observed during fever is due to a functional thermoregulatory response which maintains (regulates) the elevated temperature. Hyperthermia, on the other hand, is an uncompensated disequilibrium of heat balance (Stitt, 1979). Because the temperature elevation during a febrile episode is defended by appropriate thermoregulatory responses, fever is considered to be due to an elevated body temperature set-point (the hypothetical "set" temperature around which body temperature is regulated) (Cooper et al, 1964; Cooper, 1972; Cabanac and Massonet, 1974). Electrophysiological investigations of hypothalamic neurons support this contention (Wit and Wang, 1968; Eisenman, 1969; Boulant and Scott, 1983; Hori et al, 1984; Nakashima et al, 1985), as

do studies involving reptiles and amphibians (Kluger, 1979). If fever is considered as an elevation in the body temperature set-point, then it is possible for both hypothermia (a core temperature below the set-point) or hyperthermia (a core temperature above the set-point) to occur during a febrile episode. Indeed an explanation for the so-called "chill phase" at the onset of fever is that the body temperature set-point, having been elevated, places the subject in a hypothermic state and therefore the preferred temperature is higher. Thus the individual feels subjectively cold. (Stitt, 1979). It should also be mentioned that fever as a syndrome encompasses a wide range of physiological responses in addition to the thermoregulatory changes which are the most widely recognized. These non-thermal aspects of the febrile response are collectively referred to as the acute phase response and will be discussed separately (see page 28).

Having established a working definition of fever it is now possible to discuss the ultimate causes or etiology of the febrile response. The febrile response can be initiated by a number of substances which are foreign to the body. These substances can be collectively referred to as exogenous pyrogens. However, it is unlikely that exogenous pyrogens themselves directly initiate the febrile response. The process, while far from being completely understood, involves a number of endogenous mediators or endogenous pyrogens, which in turn, may act through second messenger molecules to influence the central control of body temperature. The endogenous pyrogens will be subsequently discussed below.

Although exogenous pyrogens have been defined as those substances, foreign to the body, which produce a febrile reaction, it has been further proposed that an exogenous pyrogen be defined as a compound which induces the production of endogenous pyrogen (EP) (Anonymous, 1987). For the sake of this discussion this definition will be adopted. The list of exogenous pyrogens is lengthy but can be reduced to several broad categories which include bacterial pyrogens (both gram

negative and gram positive), viruses, fungi, protozoa and other non-microbial agents. In addition, fever is also a symptom observed in certain malignancies, non-specific and immunological conditions as well as non-infective inflammation (Hellon and Townsend, 1983). The following discussion will focus primarily on those pyrogens most often utilized in the course of fever research.

## Viruses

Viral infections are extremely common in humans, as the frequency of incidence of the common cold and childhood diseases such as mumps and measles, will attest. Not all viruses, however, induce a febrile response in the host. Indeed, the precise biological nature of viruses which render them pyrogenic is not known. It has been determined that rabbits infected with influenza virus develop fever and that plasma from the febrile animals demonstrates EP activity in non-febrile recipients (Dinarello and Wolff, 1982). In addition, human monocytes incubated with influenza virus *in vitro* produce EP as determined following intravenous injections of the supernatant into rabbits (Dinarello and Wolff, 1980). The mechanism by which viral particles induce EP production is not clearly understood but phagocytosis by host cells is important (Dinarello and Wolff, 1982). Viral antigens may play a role in pyrogenicity, however, neutralization of these antigens with specific antisera has yielded equivocal results (Dinarello and Wolff, 1982).

## Gram positive bacteria

Gram positive organisms such as streptococci and staphylococci are common human pathogens. In general, gram positive bacteria induce fever via three pathways: (1) leukocytic phagocytosis of viable organisms; (2) release of soluble exotoxins (e.g. scarlet fever toxin); (3) host responses to cell wall components. While an extensive

discussion of the pyrogenic mechanisms evoked by gram positive organisms or their cellular components is beyond the scope of the present discussion, certain details will be briefly mentioned.

In the rabbit several strains of gram positive microorganisms have been shown to be pyrogenic. This pyrogenicity is not dependent on the establishment of infection as autoclaved cells retain equivalent pyrogenicity compared to viable bacterial cells (Atkins and Freedman, 1963). This study further established that cell filtrates of *Staphylococcus albus*, *Bacillus subtilis* or *Listeria monocytogenes* were not sufficient to evoke a febrile reaction and that plasma from rabbits rendered febrile by these organisms exhibited EP activity in non-febrile recipients. Atkins and Freedman (1963) and others (Brunson and Watson, 1974) also demonstrated that tolerance to the pyrogenic effects of these gram positive bacteria could be induced but the tolerance was specific to each gram positive strain (contrasting that observed during gram negative pyrogenic tolerance). Furthermore, this phenomenon did not exhibit cross tolerance to gram negative strains.

Like gram negative bacteria, the cell walls of gram positive organisms contain peptidoglycan, a polysaccharide peptide polymer. Peptidoglycan imparts rigidity to the cell wall and the biological effects of this polymer are similar to those of gram negative endotoxin. Both these compounds evoke fever, localized inflammatory reactions and induce vascular collapse at high doses (Rotta, 1974). Like the pyrogenic tolerance observed in whole gram positive organisms, pyrogenic tolerance to peptidoglycan is also immunologically (strain) specific (Rotta, 1974).

The chemical structure of peptidoglycan consists of repeating units of N-acetylglucosamine, N-acetylmuramic acid joined by tri- or tetrapeptide bridges. These peptide moieties are highly pyrogenic in rats and produce EP *in vivo* and *in vitro* (Dinarello et al, 1978). Indeed a peptidoglycan moiety consisting of N-acetylmuramyl-



ala-glu, known as muramyl dipeptide (MDP), is a common pyrogen utilized in fever research and is comparable to gram negative endotoxin preparations in its utility. Thus the use of MDP provides a useful model for the study of the acute effects of gram positive infection without the attendant problems of viable bacterial pathogens.

### Gram negative endotoxins

By far the most extensively studied group of pyrogens are the gram negative organisms and their endotoxins. Gram negative bacteria are characterized by the presence of an outer cell wall which is covalently bonded to the inner peptidoglycan layer; this in turn is attached to the cytoplasmic membrane of the cell. This outer layer, which is primarily responsible for eliciting the host response, is what is commonly referred to as the lipopolysaccharide or endotoxin molecule. Endotoxins are ubiquitous in nature because they are constantly shed into the environment by the bacterium. Bacterial endotoxin contains three distinct chemical regions. The outer most portion is composed of hexose and aminohexose sugars which are interspersed between lipopolysaccharides and repeating subunits of oligosaccharides. These polysaccharides (referred to as O-antigens, from the oligosaccharide moiety) protrude with hair-like projections from the surface of the molecule. It is the sequence and composition of the polysaccharides, many of which are unique to gram negative organisms, which impart the serospecificity of each endotoxin preparation (Pearson, 1985). Indeed, the control of the rate of O-antigen variability involves a mechanism which far exceeds the normal frequency of genetic mutation. Thus despite the antigenicity of the O side chains, gram negative bacteria have developed a strategy that allows the cell to escape detection by the immune system (Strominger and Zubay, 1983).

The middle layer of the endotoxin molecule consists of a core polysaccharide which bridges the O-side chain and the inner lipid layer (lipid A). The inner lipid layer, referred to as lipid A, derived its name from the initial isolation experiments of Boivin

et al, (1933, cited in Dinarello and Wolff, 1982) who named the lipid soluble fraction, fraction A. This has subsequently been changed to lipid A. This portion of the endotoxin molecule is important for pyrogenicity since lipid A complexed with either bovine serum albumin or human serum albumin results in fever of comparable magnitude to that observed with the parent molecule (Rietschel et al, 1973; Dey et al, 1975). A more rigorous investigation into the pyrogenic properties of this endotoxin moiety were conducted by Kenedi et al (1982). In carefully controlled experiments, these investigators confirmed the significance of lipid A for pyrogenicity but also showed that when separated from the polysaccharide component of the endotoxin, lipid A lost more than 99.9% of its pyrogenicity. It appears then that lipid A is an essential but not exclusive component for imparting pyrogenicity to the endotoxin molecule.

Evidence for the presence of endotoxins dates to the beginnings of research into fever etiology. In 1853, Billroth and Frese observed that the filtrates of autogenous pus were potently pyrogenic in cats and that this pyrogenic factor was not heat sensitive (cited in Welch, 1888). For more than fifty years investigations focused on the apparent cause of "injection fevers"; fevers produced upon the injection of a variety of substances for an equal variety of therapeutic reasons. Bennett and Beeson (1953) finally confirmed that injection fevers were due to endotoxin contamination of the injectates. For some time endotoxins were thought to be the primary causal agents in certain clinical conditions (e.g.typhoid fever). However, the work of Greisman and his coworkers were able to demonstrate the phenomenon of endotoxin (pyrogenic) tolerance during typhoid fever and subsequently ruled out circulating endotoxins as the possible cause of most long term clinical fevers (Hornick et al, 1970). The use of endotoxins has now become an indispensable tool in the study of febrile response because of its ability to evoke the generalized sequelae of infection without the attendant problems of using live bacterial pathogens.

## Endotoxin tolerance

Refractoriness, or tolerance, to the pyrogenic action of endotoxins is a widely recognized phenomenon and has been alluded to several times in the preceding discussions. Pyrogenic tolerance is usually manifest as a reduced or absent febrile reaction following repeated daily intravenous (or intraperitoneal) injections of endotoxin. Pyrogenic tolerance was recognized in the early 1900's by physicians employing fever therapy, utilizing heat inactivated bacterial vaccines, for the treatment of syphilis (Greisman, 1983). In his classic investigations, Beeson (1946, 1947a, 1947b) demonstrated the importance of the reticuloendothelial system (RES) and the apparent lack of importance for humoral factors in the physiological mechanisms involved in the tolerance phenomenon. Thereafter, endotoxin tolerance was studied extensively in the 1950's and 60's and evidence supporting both cellular and humoral mechanisms of action have been described.

One of the most consistent observations in the course of tolerance investigations is the enhanced activity of the RES. This was first observed by Beeson (1947b) using hepatic uptake of colloidal carbon, and was subsequently confirmed applying similar (Greisman et al, 1963; Berry and Smythe, 1965; Greisman and Woodward, 1970) or radioisotopic techniques (Rowley et al, 1958; Cooper and Cranston, 1963; Herring et al, 1963). Although hypertrophy and hyperplasia of tolerant RES cells were thought to be the reason for increased clearance of endotoxin and tolerance in general, it has since been demonstrated that agents which intrinsically hyperactivate the RES (e.g. glucan, zymosan, thorotrast) do not confer tolerance to endotoxin naive animals (reviewed in Dinarello and Wolff, 1982). Alternatively, it has been proposed, and supporting evidence has been provided, for the hypothesis that hepatic RES cells (e.g. Kupffer cells) rapidly remove endotoxin but at the same time fail to secrete endogenous pyrogen, thereby resulting in the diminished febrile reaction (Dinarello et al, 1968;

Greisman and Woodward, 1970). The diminished febrile response that is characteristic of endotoxin tolerance is thought to be the result of RES sequestration of the circulating pyrogen. This action thereby diverts the endotoxin away from non-RES cells such as tissue granulocytes which secrete normal amounts of EP during tolerance (Greisman and Hornick, 1973). The patency of this explanation is, of course, dependent on the legitimacy of the EP hypothesis which remains, as will be discussed below (see p.20), to be unambiguously supported.

The original studies of Beeson (1947b) demonstrated that tolerance could not be passively transferred in serum from tolerant animals to non-tolerant recipients. Since then, however, much evidence has accumulated to suggest that a humoral factor may contribute to endotoxin tolerance. In addition to the observations that RES blockade does not completely reverse the tolerant reaction to endotoxin (Greisman et al, 1963; Wolff et al, 1965a), passive transfer of serum from tolerant donors to non-tolerant recipients has successfully rendered these animals hyporesponsive to a subsequent endotoxin challenge (reviewed in Dinarello and Wolff, 1982; Greisman, 1983). It has been determined that the anti-"O" antibody is unlikely to be involved since circulating titres and the degree of tolerance are not correlated (Mulholland et al, 1965). In addition, tolerance develops despite immunosuppression with 6-mercaptopurine (Wolff et al, 1965b) or in rabbits (and humans) following splenectomy (Greisman et al, 1965; Greisman et al, 1975). More recent studies have identified a circulating endotoxin inactivator substance(s) which has increased activity in tolerant plasma and is enzymatic in nature (Yamaguchi et al, 1986).

The phenomenon of endotoxin tolerance appears to be the result of a multifactorial mechanism of action. The complexity of this tolerant state is further accentuated by the availability of recombinant cytokine products which have many biological actions including effects on tolerance mechanisms (Kawasaki et al, 1987;

Vogel et al, 1988). Thus, nearly 100 years after endotoxin tolerance was first observed the precise mechanism leading to the development and maintenance of the process remains to be precisely elucidated.

## 2. Endogenous pyrogens

The febrile response is a host mediated reaction to infection, inflammation or other immunologically perceived intrusions. Cells of the RES as well as other phagocytes respond to stimulation by producing heat labile proteins referred to as EP. It was previously assumed that EP was one molecule, but extensive research in this area has revealed that EP is actually several intrinsically pyrogenic molecules elaborated from immunologically activated phagocytes. These molecules, collectively referred to as cytokines, have a wide range of biological activities in addition to those affecting thermoregulation. The thermoregulatory effects of EP are generally characterized by the following: monophasic fevers of short onset and duration, which become biphasic with higher doses, the ability to induce central prostaglandin formation and the susceptibility of these fevers to antipyretic drugs (Dinarello et al, 1988). Although it is often convenient in generalized discussions to use the term "EP", it must be born in mind that EP is several compounds, the list of which, continues to grow.

The current working hypothesis for the process of febrileogenesis envisions circulating EP reaching the brain via the cerebral vasculature where its site of action is thought to be at (Wit and Wang, 1968; Eisenman, 1969; Schoener and Wang, 1975a; Dinarello, 1984a) or near (Stitt, 1986) the preoptic and anterior hypothalamus (POAH). To date there is very little evidence supporting the contention that EP (polypeptide molecules) actually crosses the blood brain barrier to affect POAH neurons. Although EP evokes fever when administered intravenously, intracerebrally (i.e. within the POAH) or intracerebroventricularly it has been proposed that circulating EP acts on target cells outside the blood brain barrier probably via the

circumventricular structure called the organum vasculosum of the lamina terminalis (OVLT) (Blatteis et al, 1983; Stitt 1985; Stitt, 1986). The vasculature of the OVLT lacks the typical endothelial tight junctions characteristic of the blood brain barrier. Therefore, the passage of protein molecules, such as EP, is possible within this neural area. The proposed target cell mediating the effects of EP is a mesenchymally derived phagocyte residing within the perivascular space of the OVLT (Stitt, 1986). This hypothesis also suggests the existence of a mediating substance which translates the appropriate signals from the OVLT to the thermoregulatory neurons within the adjacent POAH. This mediator has been suggested to be prostaglandins of the E series. The role of prostaglandins during fever remains a controversial topic and will be discussed separately (see page 33). Although the EP, EP/OVLT hypothesis has assumed a somewhat dogmatic stature (e.g. Dinarello et al, 1988), the evidence often remains inconsistent and at times unsupportive. It should be recognized, therefore, that this hypothesis be considered as a working approach only.

Presently, there are several endogenous pyrogen molecules envisioned plus other candidates that have received comparatively little attention to date. The endogenous pyrogens which have received the most attention are interleukin-1 (IL-1 $\alpha$  and  $\beta$ ) and tumor necrosis factor  $\alpha$  (TNF $\alpha$ ). Other endogenous pyrogens include interferon (IFN $\alpha$ ,  $\beta$  and *gamma*), interleukin-6 (IL-6), macrophage inflammatory protein and lymphotoxin (TNF $\beta$ ).

### Interleukin-1

Both IL-1 $\alpha$  and  $\beta$  are intrinsically pyrogenic molecules (reviewed in Dinarello, 1984a; Dinarello et al, 1988) that are distinguished chemically by their different pI values (5 and 7, respectively), with the IL-1 $\beta$  molecule being the dominant form (Dinarello, 1987). The two forms are virtually identical in their pyrogenic as well as other biological properties despite the limited amino acid homology (26%, IL-1 $\alpha$  to IL-

1 $\beta$ ) (Martin and Resch, 1988). In addition, IL-1 receptors have been demonstrated in a number of peripherally derived cell lines and membrane preparations, and it has been shown that IL-1 $\beta$  binds with an increased affinity to these receptors compared to the  $\alpha$  form (Dinarello et al, 1989). In addition, high affinity IL-1 receptors ( $K_D$  1.0  $\pm$  0.2nM) within the rat hypothalamus have been reported and these receptors reveal a marked preference for IL-1 $\beta$  (Katsuura et al, 1988). The presence of central IL-1 receptors (Katsuura et al, 1988), immunoreactive IL-1 neurons within the hypothalamus (Breder et al, 1988), as well as the dynamic release of IL-1 in response to pyrogens by astrocytes (Fontana et al, 1982; Fontana et al, 1984) and within the cerebrospinal fluid (CSF) (Coceani et al, 1988) lend support for a centrally derived IL-1 mediated fever generating pathway. However, a recent report suggests that, at least for IL-1 $\alpha$ , a bidirectional transport mechanism may be present within the mouse brain that is capable of moving circulating IL-1 into multiple regions of the brain (Banks et al, 1989). If this is substantiated, both peripherally and centrally elaborated IL-1 may participate in fever induction either by a direct action or perhaps through intermediary substances (e.g. prostaglandin E<sub>2</sub>).

Support for separately regulated compartments involved in IL-1 function and fever has come from several independent sources. In order to support a role for peripherally released IL-1 in fever pathogenesis, the cytokine should be measured during the course of pyrogen-induced fever and the levels must precede or at least correlate with febrile rises in body temperature. While the *in vitro* evidence supporting pyrogen mediated IL-1 release is numerous (reviewed in Dinarello, 1984a), these studies fail to deal with the temporal course of cytokine elaboration during an actual febrile episode. Thus cytokine release studies, *in vitro*, fail to address the question of whether peripherally generated cytokines can participate directly in the process of febrileogenesis. Using a specific radioimmunoassay for both IL-1 forms it has been shown in human volunteers receiving intravenous endotoxin, that plasma IL-1 $\alpha$  (Cannon et al,

1990) and IL-1 $\beta$  (Michie et al, 1988) either do not change during the course of the subsequent fever or that plasma IL-1 $\beta$  increases, but only minimally, and well after the onset of the fever (Cannon et al, 1990).

In addition to the studies cited above, investigations utilizing animal models have produced results which suggest similar conclusions. Experiments conducted in rats pre-treated with specific IL-1 $\alpha$  antisera revealed that despite this treatment virtually identical endotoxin-induced febrile responses were observed compared to controls treated with normal serum (Long et al, 1988). Studies investigating the role of corticotropin-releasing factor (CRF) mediation of IL-1 $\beta$  fever, have demonstrated that central administration of a CRF antagonist is capable of blocking the fever induced by IL-1 $\beta$  administered via the same route (Rothwell, 1989). However, similar treatment of rats with the CRF antagonist failed to affect the fever resulting from endotoxin injected into the peritoneal space (Rothwell, 1989). This suggests that peripherally administered endotoxin does not evoke fever via a central IL-1 $\beta$  pathway. Moreover, intravenous endotoxin does not result in changes in CSF IL-1 levels in the cat using a bioassay which does not distinguish between the two IL-1 forms (Coceani et al, 1988). In addition, endotoxin is a potent stimulator of pyrogenic prostaglandin E<sub>2</sub> release from cerebral microvessels whereas IL-1 is not (Bishai et al, 1987). This latter study suggests that IL-1 production may not be necessary for the induction of fever. Thus there is evidence which tends to refute both a peripheral and central febrogenic pathway involving IL-1.

#### Tumor necrosis factor $\alpha$

TNF $\alpha$  is a macrophage derived cytokine that is released in response to infection, immunological reactions, malignancy or inflammatory processes (Maury, 1986). The term TNF was derived from the peptides cytotoxic/cytostatic actions on transformed cells (Dinarello, 1987). The " $\alpha$ " designation is used to distinguish this cytokine from the



closely related  $\text{TNF}\beta$  (also called lymphotoxin) which is the product of activated T-lymphocytes (Bendtzen, 1988). Receptors for TNF have been found on most cells known to respond to the cytokine (Bendtzen, 1988).

$\text{TNF}\alpha$  is considered to be an EP on the basis that intravenous injections of purified or recombinant material produce dose dependent fevers (for e.g., Dinarello et al, 1986; Nakamura et al, 1988; Bibby and Grimble, 1989), acute phase responses similar to those observed after endotoxin or IL-1 (Bendtzen, 1988; Tredget et al, 1988; Bibby and Grimble, 1989; Morimoto et al, 1989) and promotion of putative febrogenic mediators (i.e.  $\text{PGE}_2$ ) both *in vitro* (Dinarello et al, 1986) and *in vivo* (Nakamura et al, 1988) following endotoxin treatment. In addition, detection of circulating levels of the cytokine in the plasma of human volunteers during the course of an endotoxin fever has established a positive correlation between the release of  $\text{TNF}\alpha$  and the febrile response (Michie et al, 1988; Cannon et al, 1990). In the rat, TNF activity in plasma from endotoxin-treated animals was also maximal one hour following the bacterial pyrogen, as measured by bioassay. This response preceded the onset of fever by approximately one hour (Long et al, 1990). In the rabbit, plasma TNF activity, as measured via radioreceptor assay, peaked two hours following a large dose of intravenous endotoxin (Beutler et al, 1985). However, this study did not show the body temperature response data so the relation between TNF release and the apparent febrile reaction could not be assessed.

In order to gain a better understanding of the role of  $\text{TNF}\alpha$  during the fever evoked by endotoxin, experiments have been conducted whereby immunoneutralization of endogenous  $\text{TNF}\alpha$  has been accomplished utilizing specific antisera. In rabbits, treatment of the animals with anti-TNF antisera either concurrently or just prior to endotoxin resulted in the suppression of the second peak of the biphasic endotoxin fever (Nagai et al, 1988; Kawasaki et al, 1989). These studies suggest that  $\text{TNF}\alpha$  acts

primarily in the later stages of the endotoxin fever. How this cytokine functions to affect the febrile response many hours after it has been released (see above) remains to be elucidated, particularly since the half life of the molecule in plasma is 6-7 minutes (Beutler et al, 1985). Based on the evidence that TNF induces the release of IL-1 *in vivo* (Dinarello et al, 1986), it has been suggested that immunoneutralization of TNF prevents the molecule from inducing IL-1 release and in doing so results in the suppression of the second fever peak (Nagai et al, 1988). However, to date, no clear evidence exists demonstrating increased IL-1 release during the late stages of endotoxin fever. A recent report from Long et al (1990) also suggests that TNF has no role in the initiation of fever. However, these investigators found that neutralization of endogenous TNF with TNF antisera enhanced, rather than suppressed, the late stages of endotoxin fever. Thus the role of TNF, like IL-1, remains to be clearly defined with respect to its role in febrileogenesis.

#### Other endogenous pyrogens

Interferon (IFN) is a cytokine protein released by virally infected phagocytes and was initially recognized for its anti-viral activity. There are three forms of IFN depending upon the cell type from which the cytokine was elaborated. Thus, leukocyte-, lymphocyte- or fibroblast-derived IFN have been designated as  $\alpha$ ,  $\beta$  or *gamma*, respectively. In addition to anti-viral activity, however, IFN has many other effects, primarily on immunologically active cells. The interest in IFN as an EP-like compound began after it was observed during clinical trials that fever was a persistent side effect in those patients which had received the cytokine for treatment of malignant tumors (Dinarello et al, 1988). In experimental animals, the profile of IFN-induced fever closely matches that of IL-1 or TNF (Dinarello et al, 1988). The mechanism by which intravenously administered IFN induces fever does not seem to involve circulating EP (Dinarello et al, 1984). However, like IL-1, TNF and endotoxin,

peripheral IFN does promote certain aspects of the acute phase response (Morimoto et al, 1987). When IFN is administered into the cerebroventricular system of conscious cats, the resultant fever may be related to the observed increases in PGE<sub>2</sub> release within the cerebrospinal fluid (Dinarello et al, 1984). A prostaglandin mediated mechanism for central IFN fevers receives additional support from the observations that these types of fevers are sensitive to the action of cyclooxygenase inhibitors (Dinarello et al, 1984; Won and Lin, 1988). The physiological significance of fever evoked by the central administration of IFN remains to be determined since no evidence is available to show that IFN is either present centrally or, if it is present, that endogenous levels change during fever. It is nonetheless true however, that during clinical trials the febrile responses to IFN were attenuated by the administration of antipyretic drugs, which supports animal studies and may indicate a mechanism involving cyclooxygenase products (Dinarello et al, 1984).

Interestingly, there is a rapid development of tolerance to the fevers evoked by peripheral injections of IFN (Dinarello et al, 1984; Morimoto et al, 1987). This tolerance is nearly complete following only three previous intravenous injections and is not due to endotoxin contamination. While this phenomenon has not been reported for IL-1 preparations, pyrogenic tolerance has been observed in some instances following intravenous recombinant TNF (Nakamura et al, 1988; Kawasaki et al, 1989). It is presently unclear whether the pyrogenic tolerance to either IFN or TNF involves mechanisms similar to those observed following repeated challenges with endotoxins.

Like IL-1, IL-6 (also known as IFN- $\beta_2$ ) is secreted from a number of appropriately stimulated macrophages, lymphocytes and fibroblasts to induce, among other things, fever, the acute phase responses and PGE<sub>2</sub> production (Bendtzen, 1988). With regard to acute phase reactants, IL-6 is equal to (Ramadori et al, 1988) or greater than (Castell et al, 1989) IL-1 or TNF in inducing acute phase protein synthesis.

Because IL-1 induces the release of IL-6 it has been suggested that IL-6 may represent a major mediating factor in the biological actions of IL-1, particularly with regard to the induction of acute phase proteins and fever (Helle et al, 1988). In support of this, peripheral (Helle et al, 1988; LeMay et al, 1990) and central (Opp et al, 1989; LeMay et al, 1990) administration of recombinant IL-6 produces dose dependent fevers. The IL-6 evoked fever is also susceptible to the antipyretic action of indomethacin (LeMay et al, 1990). However, the profile of the febrile response is somewhat different from IL-1 or TNF; the latency and duration of IL-6 fevers are longer. It is also of interest that IL-6 activity within the plasma and CSF of endotoxin treated rats is markedly increased and correlates significantly with fever magnitude (LeMay et al, 1990). It would appear, then, that IL-6 mediates febrile increases in body temperature via a mechanism that is similar to that observed following endotoxin, IL-1 or TNF. Thus, there is convincing evidence that IL-6 is another EP that acts both peripherally and centrally in the thermal and non-thermal aspects of fever.

Other endogenously produced cytokines such as lymphotoxin ( $\text{TNF}\beta$ ) and macrophage inflammatory protein-1 (MIP-1) have also been suggested to be EP-like proteins. In rats, lymphotoxin given into the third ventricle increases sympathetic nerve activity to brown adipose tissue, but even at doses as high as 200ng the maximum increase in rectal temperature was only 0.36 °C above baseline (Holt et al, 1989). This is in marked contrast to other endogenous pyrogens where fevers of greater than 1 °C are routinely observed (for e.g. Morimóto et al, 1987; Rothwell, 1988; Rothwell, 1989).

A recent report has suggested that MIP-1 is another endogenous pyrogen. What is particularly novel about MIP-1 is that the fevers evoked by this macrophage derived cytokine are not susceptible to the antipyretic action of ibuprofen (Davatelis et al, 1989). This observation notwithstanding, it should be recognized that in addition to the small sample sizes reported ( $n=3-4$ ) the doses of MIP-1 used to evoke a 1 °C fever are

much greater than that required by IL-1 or TNF administered via the same route (10  $\mu\text{g/kg}$  MIP-1 versus 200-500 ng/kg for IL-1 or TNF). It remains to be seen, therefore, whether the pyrogenic responses observed following lymphotoxin or MIP-1 are pharmacological curiosities or physiologically significant components in the febrile response.

### C. The acute phase response

The febrile response represents only one aspect of the generalized host reaction to immune stimulation, infection, tissue trauma and many other perturbations of normal physiological functions. The cascade of events (including fever) which occur following microbial invasion have been collectively named the acute phase response. In this section the term acute phase response (while technically including fever) will refer to the non-thermal component of the host reaction.

Despite the variety of conditions that can induce the acute phase reaction a remarkably consistent set of changes can be seen to occur. Upon the appropriate stimulation, which can include systemic or localized events, changes in virtually every organ system are known to occur. These changes result in alterations to metabolic, endocrinologic, neurologic, hematologic and many other functions. It has been suggested that in all likelihood virtually every plasma protein undergoes some changes during the acute phase period (Kushner, 1982). Because of this, only a few well studied indices of the acute phase response will be examined.

#### 1. Serum metallic ions

Alterations in serum cation levels are a consistent feature of the acute phase reaction. Zinc and iron levels decrease while plasma copper increases; usually these changes are observed within 8 hours of infection or systemic treatment with endotoxin. The elevation of copper levels is apparently due to the decreased plasma concentration

of the copper binding protein, ceruloplasmin (Dinarelo, 1984b). The increase in the zinc-binding protein, metallothionein, may explain the observed decrease in plasma levels of this cation (Kushner, 1982). While the physiological significance of the changes in plasma zinc and copper are not obvious, the decreases in the availability of free iron has been demonstrated to be beneficial to the host by virtue of its detrimental effect on invading pathogens (Bullen, 1981). Increases in hepatic synthesis of the two iron-binding proteins, transferrin and lactoferrin, reduce the availability of the already limited amount of free plasma iron for bacterial metabolism. These two acute phase proteins are essential for the antibacterial properties of body fluids (Bullen, 1981). In addition, both transferrin and lactoferrin increase their binding in conditions of low pH. Thus for localized infection, the increased presence of lactoferrin (the major iron binding protein in tissue), in the presence of pathogen-induced decreases in pH, ensures increased sequestration of free iron and hence reduced bacterial growth (Bullen, 1981). Further support for the beneficial effect of reduced iron comes from studies demonstrating that the growth of bacterial cultures is diminished at febrile incubation temperatures (41°C) but only in the presence of reduced iron (Greiger and Kluger, 1978). Finally, evidence has been presented repeatedly depicting the enhancement of bacterial virulence by treatment of infected animals with supplemental iron (Bullen, 1981).

## 2. Hepatic protein synthesis

The liver is a major contributor to the acute phase response not only because of its increased enzymatic activity during this time but also because the amount of hepatic protein synthesis is markedly affected (Kushner, 1982). Changes in the synthesis of metallic ion binding proteins have already been mentioned. In addition to these acute phase proteins, increases in normal plasma proteins such as haptoglobin, certain proteases, complement components and others also occur. While the latter proteins

show modest increases, other proteins can increase several hundred fold. It is these hepatic glycoproteins, such as serum amyloid A and C-reactive protein (as well as the metallic ion binding proteins), that are considered to be the true acute phase reactants (Dinarello, 1984b). The role of serum amyloid A in the acute phase period seems to be as a suppressor of immune function; in this capacity its preferential release during tissue injury is likely to limit the amount of immune reactivity to host derived cellular debris (Pepys and Baltz, 1983). C-reactive protein, on the other hand, likely functions as an opsonin by either binding to the bacterial cell or by activating compliment (Mold et al, 1982).

### 3. Additional acute phase reactants

In addition to hepatic protein synthesis, endocrinological effects are also manifest during the acute phase. Increases in insulin, glucagon, ACTH, growth hormone, thyroid-stimulating hormone, vasopressin and oxytocin have all been observed during either bacterial infection or treatment with endotoxins (for e.g. Kasting and Martin, 1982; Dinarello, 1984b; Kasting et al, 1985; Kasting, 1986c; Switala et al, 1988). The contribution of each of these hormones to the acute phase reaction is not entirely understood. However, in the face of these anabolic processes there also exists catabolic mechanisms which contribute to muscle protein breakdown, leading to increases in the mobilization of amino acids. The increased availability of free amino acids may provide additional energy substrates, at a time when appetite is often suppressed, or may simply provide the necessary material for the increases in hepatic protein synthesis.

#### 4. Acute phase and febrile responses: independent regulation

Like the process of febrileogenesis, initiation of the acute phase response is accomplished by host generated mediators. It has been shown repeatedly that administration of IL-1 (or EP preparations) to experimental animals induces many aspects of the acute phase response (for e.g. Sobrado et al, 1983; Dinarello, 1984a,b; Blatteis, 1988; Tredget et al, 1988; Fong et al, 1989; Morimoto et al, 1989a,b). However, other endogenous mediators, such as TNF and IL-6, are also capable of initiating the synthesis of acute phase reactants (for e.g. Ramadori et al, 1988; Tredget et al, 1988; Castell et al, 1989; Fong et al, 1989; Morimoto et al, 1989b). Currently, it is debatable whether the acute phase response mediators, such as IL-1, TNF or IL-6, act entirely on peripheral tissues or within the central nervous system to affect the acute phase response. It is well established that peripheral administration of these mediators evokes a response indistinguishable from that observed during infection or following endotoxin. However, evidence has also been presented to show that EP or recombinant cytokine materials administered into the brain (e.g. POAH or cerebroventricular system) elicit the acute phase reaction as well as fever (Blatteis et al, 1984; Morimoto, et al, 1987a; Morimoto et al, 1988a; Morimoto et al, 1989a,b; Shibata et al, 1989). It is certainly possible that both a central and peripheral mechanism of action contributes to the acute phase response (Morimoto et al, 1987a).

Unlike the mechanism of fever induction there is convincing evidence that the acute phase reactants are not dependent on prostaglandins, as pyrogen-induced aspects of the acute phase response are not reversed by treatment with cyclooxygenase inhibitors (Sobrado et al, 1983; Tocco et al, 1983; Morimoto et al, 1988a). In addition, neither central administration of prostaglandins (Matsumura et al, 1988; Morimoto et al, 1988a) nor thermal stimulation of the POAH (Hunter et al, 1987) are sufficient to evoke components of the acute phase response. From this it is apparent that the febrile



and acute phase responses represent a coordinated reaction aimed at fighting infection or other tissue trauma. It is of interest nonetheless, that while both fever and the acute phase responses involve generalized systemic mobilization, that is evoked by the same endogenous mediators, the lack of importance of fever *per se* for the acute phase response indicates the independent regulation of the two events.

#### D. Central mediators of the febrile response

##### 1. POAH: the neuronal substrate for febrile physiology

The prominent role for the POAH in normal thermoregulation and during fever has been determined from over one hundred years of investigation into this area (see Cooper, 1987). Lesion and thermode studies clearly indicate that the POAH is involved in the control and integration of thermoregulatory processes (reviewed in Simon et al, 1986). Electrophysiological studies of POAH neurons began in 1961, when Nakayama identified hypothalamic neurons which exhibited temperature sensitivity beyond that expected from the equation of Arrhenius. Since that time POAH neurons have been shown to be either warm or cold sensitive or thermally insensitive (Boulant, 1980; Nakayama, 1985). Furthermore, thermosensitive neurons derive this characteristic intrinsically, as temperature sensitivity is retained *in vitro* (Hori et al, 1980; Baldino and Geller, 1982; Hori and Nakayama, 1982; Kelso et al, 1982). The POAH, however, is not the only CNS region that contains thermosensitive neurons. Indeed, the entire diencephalic area (rostral and caudal to the POAH) contains proportions of neurons of equal or greater thermosensitivity to those of the POAH (Dean and Boulant, 1989). It is presently unclear how these additional populations of thermosensitive neurons contribute to normal thermoregulation or to the process of febrileogenesis. However, evidence demonstrating redundant mechanisms involved in the multiple control of fever within the CNS has been presented (Morimoto et al, 1988c).

The role POAH neuronal systems play in the pathogenesis of fever is far from completely understood. Studies employing endotoxins, endogenous pyrogen preparations or recombinant cytokines all tend to show that warm sensitive neurons are inhibited and cold sensitive neurons excited when these pyrogens are applied to POAH neurons *in vitro* (Boulant and Scott, 1983; Hori et al, 1984; Nakashima et al, 1985; Nakashima et al, 1989). Similar results have been obtained from POAH neurons *in vivo* (Wit and Wang, 1968; Eisenman, 1969; Schoener and Wang, 1975a). Thus POAH neuronal responses to pyrogens are consistent with the physiological conditions required to initiate heat production and to limit heat loss. Further, the changes in the sensitivity or gain of the neuronal responses to local temperature changes after exposure to pyrogens provide a physiological correlate for the body temperature set-point and its upward adjustment during fever (Mitchell et al, 1970; Bligh, 1972).

Although electrophysiological studies demonstrate the ability of pyrogenic agents to act directly on central neurons it is more than likely that certain neurotransmitters or other mediating substances participate in central afferent and efferent pathways subserving the febrile increases in body temperature. The research efforts to this end have been extensive but unfortunately are plagued with inconsistencies. Suffice it to say that monamines, acetylcholine, cyclic nucleotides, as yet unidentified protein mediators, as well as  $\text{Na}^+$  and  $\text{Ca}^{2+}$  have all been implicated in the pathogenesis of fever.

## 2. Prostaglandin E: an essential febrogenic mediator?

Probably the most extensively studied, and controversial, centrally acting mediators of the febrile process are the prostaglandins of the E series. The initial observations of Milton and Wendlandt (1970) and Feldberg and Saxena (1971) demonstrated that administration of  $\text{PGE}_1$  into the ventricular system of the cat brain resulted in a potent pyrogenic response. Concurrently, Vane (1971) established the

hypothesis that aspirin-like drugs derived their antipyretic activity by virtue of their ability to prevent prostaglandin (PG) biosynthesis. From these original experiments a compelling case demonstrating an essential role for PGs in the genesis of fever has been built. Microinjection studies have revealed that the POAH is the most sensitive CNS region to the pyrogenic action of PGE<sub>1</sub> (Williams et al, 1977) and PGE<sub>2</sub> (Gollman and Rudy, 1988; Morimoto et al, 1988c). However, sites near but distinct from the POAH are also sensitive to locally applied PGEs (Williams et al, 1977; Gollman and Rudy, 1988; Morimoto et al, 1988b,c). In addition, specific PGE<sub>2</sub> receptor sites are present within various hypothalamic nuclei with the highest concentrations being within the POAH (Watanabe et al, 1988; Watanabe et al, 1989).

Experiments designed to measure endogenous PGs during the course of fever also lend convincing support to the prostaglandin-fever hypothesis. Initial determinations of central PG release during fever utilized a bioassay approach to determine PG activity from samples of cerebrospinal fluid (Feldberg and Gupta, 1973; Feldberg et al, 1973; Dey et al, 1974). Following the administration of pyrogens, PG activity consistently increased in these studies and, furthermore, the febrile body temperature along with the pyrogen-induced increases in CSF PG could be reversed upon treatment of the animals with a number of antipyretic drugs (Feldberg and Gupta, 1973; Feldberg et al, 1973; Dey et al, 1974). This trend has subsequently been confirmed utilizing radioimmunoassay procedures, although PGE concentrations vary from study to study (Bernheim et al, 1980; Coceani et al, 1983; Townsend et al, 1984; Coceani et al, 1988).

Analysis of PG release into the CSF assumes that the tissue of origin of the PG is involved in the febrile process. Thus measurement of CSF PG does not necessarily indicate PG activity within thermoregulatory sensitive areas. A more discrete analysis of central PG release requires direct measurement of the eicosanoids from the

extracellular fluid surrounding neural tissue. This has recently been accomplished by Sirko et al (1989) utilizing the technique of push-pull perfusion within the POAH neuropil. The results of this study are consistent with the hypothesis that hypothalamically derived PGE<sub>2</sub> is essential for the process of febrile response.

However, CNS tissue *per se*, may not be the only source of centrally acting PGs. For example, cerebral microvessels have been demonstrated to elaborate PGE<sub>2</sub> selectively, in response to endotoxin (Bishai et al, 1987). Blood borne PGE<sub>2</sub> also shows a strong correlation with febrile increases in body temperature, whether evoked by intravenous endotoxin or endogenous pyrogen (Rotondo et al, 1988). Intravenous administration of [<sup>3</sup>H]-PGE<sub>2</sub> (Dascombe and Milton, 1979) as well as a tritiated PGE<sub>2</sub> analogue, PGE<sub>2</sub> methyl ester (Eguchi et al, 1988), has revealed that PG molecules can indeed cross the blood brain barrier. This latter effect may be facilitated by an active transport process (Bito et al, 1976). Because PGE<sub>2</sub> is known to be produced following exposure to endotoxin in hepatic Kupffer cells (Decker et al, 1989), skeletal muscle cells (Kettelhut and Goldberg, 1988), monocytes (Hart et al, 1989) and peritoneal macrophages (Kunkel et al, 1988) it is not inconceivable that peripherally derived PGE<sub>2</sub> can act directly within the CNS to initiate the febrile response. This contention is supported by the evidence demonstrating the dose dependent pyrogenicity of intravenously administered PGE<sub>2</sub> as well as the susceptibility of these fevers to blockade by a centrally administered PGE receptor antagonist (Eguchi et al, 1988).

The evidence for a mediatory role for PGE<sub>2</sub> (PGE<sub>1</sub> release during fever has yet to be measured) in the process of fever induction is compelling. Not all investigations into this matter have been supportive, however. Cranston et al (1975) established that increases in CSF PGE levels do not necessarily correlate with febrile increases in body temperature. During the fever evoked by the infusion of endogenous pyrogen these investigators observed increases in PGE levels during the subsequent febrile episode.

However, upon treatment of the animals with a sub-antipyretic dose of sodium salicylate the PGE levels decreased despite the unaltered febrile body temperature (Cranston et al, 1975). In the goat, plasma concentrations of PGE and PGF<sub>2α</sub> are correlated only with the first peak of the biphasic endotoxin fever. During the second and more prolonged phase of an endotoxin fever plasma PG levels had returned to pre-endotoxin levels (Jónasson, 1987; Jónasson et al, 1987). The same circumstance was evident in the plasma of endotoxin-treated sheep (Skarnes et al, 1981). Evidently, correlating PG release with aspects of the febrile response does not necessarily imply a causal relationship.

The use of PG receptor antagonists has also yielded results inconsistent with the PG-fever hypothesis. For instance, central administration of the PG antagonists SC 19220 or HR 546 block PGE<sub>2</sub> but not EP evoked fevers (Sanner, 1974; Cranston et al, 1976). It has also been shown that extensive lesions of the rostral hypothalamus prevents the development of fever to EP but not to PGE<sub>2</sub> (Veale et al, 1977). Studies on the development of fever in newborn lambs have further determined that pyrogenic doses of endotoxin but not PGE<sub>1</sub> or PGE<sub>2</sub> will evoke fever when administered within the POAH of these animals (Cooper et al, 1979).

The integrity of the PG-fever hypothesis also depends heavily on the evidence accumulated from studies employing antipyretic drugs, the cyclooxygenase inhibitors. While the ability of antipyretic drugs to inhibit the synthesis of PGs is unquestioned it has since become recognized that these drugs also exert additional effects which may or may not affect the neuronal networks involved in fever. This topic will receive greater consideration in subsequent chapters.

It should also be mentioned that electrophysiological studies examining the action of prostaglandins on thermosensitive neurons have yielded equivocal results (Stitt and Hardy, 1975; Gordon and Heath, 1980; Ono et al, 1987; Watanabe et al, 1987;

Morimoto et al, 1988b). Finally, incubation of tissue slices containing the preoptic area with EP generates prostaglandin release but only in concentrations far below that required to initiate fever *in vivo* (Scott et al, 1987). Conversely, others have shown that hypothalamic tissue incubated with EP promotes PGE release by 4- to 5-fold (Dinarello and Bernheim, 1981). However, these same investigators also demonstrated the non-specificity of this effect since cortical tissue was even more sensitive to the PGE-inducing effects of EP. Although the PG hypothesis as a mediator in the febrile process is compelling, enough contradictory and inconsistent results exist to indicate that PGs may not be the only mediator in the febrile process. A logical extension to this is the possibility that the PG inhibiting property of antipyretic drugs may also be an insufficient explanation for their febrolytic action.

#### E. Control of febrile body temperature

The use of fever reducing or antipyretic drugs has been a common recourse for alleviating febrile symptoms in humans. While the benefits of antipyretic intervention have definite advantages with regard to certain infectious sequelae, such as muscle wasting (Dinarello, 1984a), the benefits of relieving the fever *per se* remain obscure. Thus the debate over the adaptive value of fever continues to be a controversial issue. The ability to respond to infection with fever is a characteristic which is phylogenetically conserved. Invertebrates, fish, reptiles and amphibians all respond to infection by moving to an area in which the ambient temperature raises the body temperature to febrile levels (Kluger, 1979; Reynolds and Casterlin, 1982; Casterlin and Reynolds, 1982). Indeed, experiments conducted by Kluger and his colleagues have determined that in reptiles and amphibians the ability to raise body temperature during infection is critical for survival (Kluger, 1979).

Whether fever is important for survival in mammals is presently unclear. A study in rabbits by Kluger and Vaughn (1978) found that fever height up to 2.25 °C was

correlated with survival. However, febrile temperatures above 2.25 °C were associated with a decrease in survival. Banet (1979) presented evidence from studies in the rat which supports Kluger and Vaughn in that excessively high fevers tended to cause a decline in survival. It has been revealed in bacterially infected rabbits that blunting the febrile response by treatment with sodium salicylate results in 100% lethality compared to 26% lethality in febrile controls (Vaughn et al, 1980).

The benefit of febrile body temperature in enhancing host defense functions is well documented (Roberts, 1979). However, the widespread use of antipyretic drugs in our society would suggest, at least in humans, that febrile body temperatures are not necessary for improved survival or recovery from infection. In contrast to this, there is some indication in elderly patients with bacterial infections, that the lack of ability to mount a febrile response by these patients does indeed lead to increased mortality (Weinstein et al, 1983). Additionally, the risk of febrile convulsion in children indicates the seriousness of high fevers and provides a rationale for antipyretic intervention (Kasting et al, 1982). Thus, the question of using or not using antipyretic drugs remains a matter of both clinical and academic interest.

### 1. Antipyretic drugs

Antipyretics are drugs which decrease febrile temperatures but have no effect on non-febrile body temperatures unless the dose is excessive (Barbour, 1921). The action of these drugs is to alleviate the symptom (fever) but can not eliminate the cause (e.g. infection). Thus antibiotics are not considered to be antipyretic agents (Kasting et al, 1982). Because fever is ultimately considered to be due to the re-setting of the body temperature set-point, antipyretic drugs are similarly thought to act, albeit in an opposite manner, on the same set-point mechanism. Antipyretic drugs come in two general categories, steroidal and non-steroidal. This discussion will focus on the non-steroidal antipyretic drugs.

The earliest use of antipyretic substances dates to the pre-Spanish Incan society of Peru where the bark (the Indian name was 'quina') from the so called "fever tree" was in common use. However, with the arrival of the conquistadors, the Spanish realized the medicinal benefits of the bark and the fever tree eventually became scarce. Thereafter it was discovered that the bark of the chinchona tree was even more potent in its antipyretic property. It was this bark (also known as the Jesuit's bark because they held a monopoly on its trade) which contained the anti-malarial component, quinine (Van Arman et al, 1986). Ultimately it was this monopoly which led to the shortage and prohibitive expense of the Chinchona bark in Europe. The commercial potential for alternatives led to the discovery in 1756, that extracts of willow bark were found to contain antipyretic properties, which was later found to be salicin. From this, salicylic acid and eventually acetylsalicylic acid were derived (Kasting et al, 1982). To date, there are literally scores of antipyretic compounds, derived from many different chemical families. Only a handful, however, receive widespread use.

There are at least 5 possible ways in which antipyretic drugs could act to counter febrile increases in body temperature:

- (1) antipyretics could prevent EP production
- (2) antipyretics could antagonize EP at its neuronal target
- (3) antipyretics could prevent release or synthesis of PGs
- (4) antipyretics could act on thermoregulatory neurons



#### (5) antipyretics could act directly on thermoregulatory effectors

Several studies using *in vitro* as well as *in vivo* techniques have determined that antipyretics have no effect on EP production or release (e.g. Clark and Moyer, 1972; Lin and Chai, 1972; Clark and Cumby, 1975). Thus the validity of point (1) is unlikely. The ability of antipyretics to antagonize EP at the receptor site remains a possible mechanism of action. Sodium salicylate, acetaminophen and indomethacin have all been used to demonstrate parallel shifts in the thermal dose-response curve to EP (Clark and Coldwell, 1972; Clark and Cumby, 1975). These results are suggestive of a competitive interaction between the antipyretic drug and the pyrogen at the receptor level. Additional support comes from observations that antipyretic concentrations of a drug exert effects on body temperature only in the presence of pyrogenic agents (i.e. during fever). This implies that antipyretics may not only compete with the pyrogen but also require the presence of the pyrogen molecule at the receptor in order to affect their antipyretic action. However, the proposed competition between the pyrogen and the antipyretic drug may not fully explain antipyretic drug function since Schoener and Wang (1975b) have demonstrated that aspirin injected on one side of the POAH alters the firing rate of POAH neurons activated by a contralateral injection of EP. It appears most likely that antipyretic drugs affect febrile body temperature via an action on prostaglandin synthesis. However, as discussed previously enough uncertainty in this hypothesis is evident to warrant caution in this regard. Indeed, evidence presented in this thesis will establish an additional possibility for the mechanism of antipyretic drugs, viz., that antipyretic drugs activate an endogenous antipyretic mechanism which mediates the fever reducing properties of these drugs.

As to the other possibilities, the notion that antipyretics may act directly on thermoregulatory neurons or neuronal networks is not inconceivable. Direct application of antipyretic drugs on to pyrogen-altered neurons effectively reverses the

changes in neuronal activity (Wit and Wang, 1968; Eisenman, 1969; Schoener and Wang, 1975b; Nakashima et al, 1985; Nakashima et al, 1989). Moreover, salicylate has rapid and reversible effects on both the potential and input resistance of neuronal membranes, leading to altered output and synaptic input to these neurons (Barker and Levitan, 1971; Levitan and Barker, 1972). Finally, there is overwhelming evidence from experiments utilizing conscious animal models that antipyretic drugs act within the CNS to exert their febrile actions (for e.g. Cranston et al, 1971; Cranston and Rawlins, 1972; Avery and Penn, 1974; Clark and Cumby, 1975; McCain and Mundy, 1987).

The question of whether antipyretics act directly on effector mechanisms has been studied in cold exposed animals. The results would indicate that these drugs do not act directly on effectors (Cranston et al, 1970; Cranston et al, 1975; Pittman et al, 1976; McCain and Mundy, 1987).

## 2. Endogenous antipyresis

Antipyretic drugs are useful in reducing febrile temperatures where the danger of excessively high fevers is possible. Two studies have already been mentioned illustrating the potentially lethal effects of high febrile body temperature (Kluger and Vaughn, 1978; Banet, 1979). In addition, the possibility of febrile convulsions in children warrants the use of antipyretic drugs. However, most febrile episodes associated with inflammation or limited infections present no imminent danger to the patient and are essentially harmless events. What, then, are the mechanisms which control the rise in temperature following the release of pyrogen in the body? It is evident that during endotoxin fevers, pyrogenic mediators remain elevated despite the fact that body temperatures have levelled off (see sections on EP and PGE). Therefore, the process of defervescence is not simply due to the decline or cessation of EP release (or other febrile mediators).

Clinically it has long been observed that fevers in excess of 41 °C are extremely rare (DuBois, 1949). These observations suggest that the febrile rise in body temperature may involve a feedback system to limit deleterious or potentially lethal hyperpyrexias. Indeed, prolonged hyperthermia produces not only neuronal damage but can also lead to convulsions (Stitt, 1979). Therefore circumstantial evidence points to the necessity for and, possibly, the existence of a physiological mechanism designed to limit the height and/or duration of a febrile episode.

With these clinical observations in mind the concept of an endogenous antipyretic mechanism was postulated and received support from experiments on near term ewes and newborn sheep. These studies demonstrated that there was a progressive refractoriness to normally pyrogenic doses of endotoxin which was maximal just prior to delivery (Kasting et al, 1978). This resistance to fever was also present in the newborn 1 to 2 days postpartum (Pittman et al, 1974). This phenomenon has since been observed in other species (Blatteis, 1975; Moraes et al, 1985). It has been suggested that the ability to temporarily suppress the febrile response in the periparturient period confers a significant survival advantage to both the mother and her newborn (Veale et al, 1981). Further clinical evidence has indicated that newborn humans may also exhibit an attenuated febrile response in the face of gastrointestinal (Epstein et al, 1951) or blood borne infections (Smith et al, 1956).

A possible explanation for these observations was that the cells of the RES were not responding to endotoxin with the production of EP. This was tested by Kasting et al (1979a) who verified that leukocytes harvested from both the fetus and ewe were capable of EP production following incubation of the cells with endotoxin *in vitro*. The EP produced in these experiments displayed characteristics that were indistinguishable

from EP derived from non-pregnant controls. It was suggested from these observations that there may be an active inhibition of fever in the periparturient period and that this inhibition may constitute a CNS mechanism.

#### Vasopressin and endogenous antipyresis

A search for a circulating substance which might be involved in fever suppression at or near the term of pregnancy was initiated using three criteria (Kasting et al, 1980). First, the circulating concentration of the substance should increase in both the ewe and fetus. Second, the increase should start about 4 days before term and peak at term, and lastly, the substance should return to normal by about 32 hours postpartum. The substance which best fit these criteria was arginine vasopressin (AVP).

AVP is a nonapeptide which is typically conceived as a peripherally acting pressor or antidiuretic hormone. In this capacity AVP-mediated responses are well studied. Thus decreases in arterial blood pressure or increases in plasma osmolality result in activation of a neuroendocrine reflex pathway leading to the neurohypophyseal release of AVP into the circulation (reviewed in Bisset and Chowdrey, 1988). The endocrine actions of AVP are mediated via 2 basic receptor subtypes. As proposed by Michel et al (1979), the AVP receptor mediating the pressor (or glycogenolytic) responses is labelled the  $V_1$  receptor, whereas the antidiuretic response is mediated by the  $V_2$  receptor. These receptor types are not only functionally distinguishable but also can be classified according to their transduction mechanisms. Thus, activation of the  $V_1$  receptor leads to hydrolysis of phosphatidylinositol and ultimately, increases in intracellular  $Ca^{2+}$ , while  $V_2$  receptor activation is coupled to the adenylate cyclase-cyclic AMP cascade. The cardiovascular effects of AVP are mediated through  $V_1$  receptors located on the vascular smooth muscle (Michel et al, 1979). The antidiuretic effects of AVP occur by binding of the hormone to the  $V_2$  receptors in the renal

collecting ducts (Butlen et al, 1978). The source of peripherally released AVP is primarily the hypothalamic supraoptic nucleus (SON), although the paraventricular nucleus (PVN) also contributes (Sofroniew and Wiendl, 1981). However, other vasopressin-containing nuclei with extensive projections throughout the brain suggest that the neuropeptide may have physiological functions in the manner of a neurotransmitter substance. The discovery of these extrahypothalamic vasopressin pathways provides the neuroanatomical support for a centrally acting AVP-endogenous antipyretic system.

The neuroanatomical work of Buijs (1978) and associates (Buijs et al, 1978; Buijs and Swaab, 1979) have demonstrated extensive intra- and extrahypothalamic vasopressin pathways in the rat brain. In addition to being present in the hypothalamic SON, PVN and suprachiasmatic nuclei (SCN), AVP is found in pathways from the PVN to the lateral and medial septum, substantia gelatinosa, substantia nigra, hippocampus and the nucleus tractus solitarius. Pathways from the SCN project to the OVLT, PVN, SON, septum, diagonal band of Broca and ependymal lining of the cerebral ventricles (Sofroniew and Wiendl, 1981). Furthermore, extrahypothalamic AVP-containing nuclei such as the bed nucleus of the stria terminalis (BST), locus ceruleus and amygdala (Van Leeuwen and Caffé, 1983; DeVries et al, 1985) are important sources for centrally released AVP.

Further investigation has shown that AVP is contained within synaptic vesicles (Buijs and Swaab, 1979) and is released into the CSF in concentrations unrelated to those in the blood (Dogterom et al, 1977; Mens et al, 1982; Landgraf et al, 1988). Moreover, vasopressin released into the neuronal extracellular fluid has been measured *in vivo* in conscious (Cooper et al, 1979; Ruwe et al, 1985a; Landgraf et al, 1988; Neumann et al, 1988) and anaesthetized animals (Demotes-Mainard et al, 1986; Landgraf et al, 1990a) using the technique of push-pull perfusion. The process of

neuronal AVP release has been studied *in vitro* and determined to be both  $K^+$  sensitive and  $Ca^{2+}$  dependent (Buijs and Van Heerikhuize, 1982). The above evidence coupled with the abundant receptor studies (for e.g. Baskin et al, 1983; Biegon et al, 1984; Poulin et al, 1988; Shewey and Dorsa, 1988) provide compelling evidence that AVP, acting via  $V_1$  receptors, has a physiological function within the CNS.

Direct experimental evidence contributes to the notion linking centrally acting AVP and the suppression of the febrile response at the term of pregnancy. Studies conducted in the periparturient guinea pig confirmed the observations made in the sheep in that fever in response to endotoxin was suppressed in near term mothers as well as their newborn (Zeisberger et al, 1981). The link to an AVP function was established in a corollary study in which near term guinea pigs displayed increased AVP-containing neuronal elements in the septo- and amygdalohypothalamic pathways (Merker et al, 1981). These changes lasted until one day postpartum upon which normal AVP staining was observed. As well, increased AVP staining in several key hypothalamic nuclei has been detected in the brains of late term and postpartum rats (Caldwell et al, 1987). In the newborn rat pup the ability to respond to endotoxin with a fever is also suppressed. However, if a specific AVP  $V_1$  receptor antagonist is administered to these animals they are able to respond to the pyrogen by generating a fever behaviorally (Kasting and Wilkinson, 1987). Collectively these studies provide a strong link between centrally acting AVP and the suppression of fever in near term animals and their newborn.

The antipyretic action of AVP is not limited to pregnant or newborn animals. Evidence has accumulated to show that the antipyretic action of AVP may also be manifest during the course of fever in general, perhaps acting as a thermal brake to control the height and/or duration of a febrile episode. This claim is made based on the results from a number of different experimental techniques. Administration of

exogenous AVP into the AVP-sensitive neuronal locus, the ventral septal area (VSA), by continuous infusion or via push-pull cannulae suppresses the onset of fever in virtually every species examined without effecting normal thermoregulation. Thus, suppression of endotoxin-induced fever has been observed in sheep (Cooper et al, 1979; Kasting et al, 1979), rabbit (Naylor et al, 1985), cat (Naylor et al, 1986) and guinea pig (Zeisberger, 1989). In the rat, AVP administration within the VSA has also been employed to suppress the hyperthermic response to centrally applied PGE<sub>1</sub> (Fyda et al, 1990) or PGE<sub>2</sub> (Ruwe et al, 1985). From these studies it has been determined that the antipyretic action of AVP is dose dependent and specific to AVP, since a similar neuropeptide, oxytocin, is ineffective on its own at suppressing fever.

Unlike larger species such as the rabbit (Bernardini et al, 1983; Naylor et al, 1985) or monkey (Lee et al, 1985), intracerebroventricular (icv) administration of AVP in the rat mimics the effects of the perfused or infused peptide. Using this model it has been determined that the antipyretic effect of AVP is dose dependent (Kovacs and DeWied, 1983; Kasting and Wilkinson, 1989), acts via a V<sub>1</sub>-like receptor (Kasting and Wilkinson, 1986, 1987; Naylor et al, 1987) and is specific to AVP (Kovacs and DeWied, 1983; Naylor et al, 1987). Although not completely understood, the mechanism by which AVP acts to counter febrile body temperature may include an action on the body temperature set-point mechanism (Wilkinson and Kasting, 1987a, 1987b), possibly through an interaction with excitatory amino acid neurotransmission (Disturnal et al, 1987).

Although icv administration of AVP does not confine the peptide to a specific neuronal locus, it has been determined, using microdialysis membranes, that ventricularly applied AVP reaches the VSA in physiologically relevant concentrations (Kasting and Wilkinson, 1989). The VSA is currently the only site in the CNS shown to be sensitive to the antipyretic effects of AVP. The VSA is located in and near the

diagonal bands of Broca, bordered medially by and just rostral to the decussation of the anterior commissure. This locus is well suited for the observed effects of vasopressin primarily because it contains many AVP-positive nerve terminals (Sofroniew and Wiendl, 1981; DeVries et al, 1985) as well as a dense population of AVP receptors (Baskin et al, 1983; Lawrence et al, 1988). The major vasopressinergic input to the VSA arises from the bed nucleus of the stria terminalis (BST) (Disturnal et al, 1985,1986). However, these same studies also indicated that afferent input arising from the PVN as well as the central amygdaloid nucleus also converge on the VSA.

The nature of the vasopressin receptor within the VSA has been determined to be similar to the peripheral V<sub>1</sub> (pressor) subtype. This has been demonstrated utilizing *in vitro* binding techniques (Poulin et al, 1988) as well as a number of whole animal studies employing specific AVP receptor agonists and antagonists (Cooper et al, 1987; Naylor et al, 1987; Zeisberger, 1989). In this regard blockade of AVP receptors within the VSA with a V<sub>1</sub> but not V<sub>2</sub> antagonist results in elevated and prolonged fevers evoked by either IL-1 (Cooper et al, 1987) or PGE<sub>1</sub> (Naylor et al, 1988). A centrally administered V<sub>2</sub> agonist is ineffective against IL-1-induced fever (Naylor et al, 1987), whereas a V<sub>1</sub> agonist displays similar antipyretic potency to the natural ligand (Zeisberger, 1989). In addition, suppression of PGE<sub>1</sub> fever can be accomplished via electrical stimulation of the BST, presumably due to AVP release from the BST-VSA pathway (Naylor et al, 1988). This effect can be abolished upon administration of a V<sub>1</sub> receptor antagonist into the VSA (Naylor et al, 1988). These effects of V<sub>1</sub> receptor antagonism on febrile thermoregulation are likely to be physiologically relevant since administration of the antagonist in the absence of a fever evokes no perturbations in normal core temperature. However, it has been shown in the spinal cord that the V<sub>1</sub> antagonist employed in fever studies may exert non-specific



neurodepressant actions (Porter and Brody, 1986); although this appears not to occur within the VSA (Cooper et al, 1987; Naylor et al, 1988; Landgraf et al, 1990b) results opposite to those of Naylor et al (1988) have been published (Fyda et al, 1989).

Additional techniques have been employed to support a role for AVP in the control of febrile body temperature. Lesions of the VSA result in an enhanced thermoregulatory response to PGE<sub>1</sub> (Martin et al, 1988). Whereas castration, which leads to depletion of AVP synthesis within BST neurons, results in an enhanced hyperthermic reaction to PGE<sub>1</sub> (Pittman et al, 1988). Traditional AVP releasing stimuli such as increased plasma osmolality or hemorrhage have been shown to promote central release of the peptide (Ruwe et al, 1985a; Demotes-Mainard et al, 1986; Landgraf et al, 1988). This may provide the basis for the observations that hemorrhage and hypertonic saline injections result in antipyresis in the sheep (Kasting et al, 1981) and rat (Kasting, 1986a). Like the action of antipyretic drugs or that of exogenous AVP, these manipulations are without effect on normal body temperature. Finally, analysis of *in vivo* release of AVP during fever has shown that central release of the peptide in the sheep (Cooper et al, 1979) and rabbit (Malkinson et al, 1987) has fever specific responses that are consistent with a putative antipyretic role for AVP.

#### F. Rationale for Investigation

From the preceding discussion it is evident that vasopressin acting within the VSA possesses similar antipyretic characteristics to that observed following aspirin-like drugs. Both reduce febrile but not afebrile body temperature and thus are thought to affect the as yet undefined set-point mechanism. Centrally acting AVP affects normal and febrile core temperatures in a manner remarkably similar to that observed following indomethacin administered under identical ambient conditions (Wilkinson and Kasting, 1987a). At ambient temperatures at or near thermoneutral both salicylate (Gagalo et al, 1983) and AVP (Wilkinson and Kasting, 1987b) promote antipyresis by

increasing heat loss and not by inhibition of heat production. These few shared attributes suggest the possibility of a functional association between the mechanism of action of antipyretic drugs and that of centrally acting AVP. Indeed this hypothesis receives support from the evidence which shows that both AVP (Ruwe et al, 1985b) and sodium salicylate (Alexander et al, 1987) when administered into the VSA suppress the onset of a prostaglandin-induced hyperthermia. These studies are important because they demonstrate that PG-induced fevers are indeed sensitive to antipyretic drugs if the drug is administered into the VSA. Prior to this, no study had been able to demonstrate a reduction of PG fever by antipyretic drugs (see Clark, 1979). Moreover, the work of Ruwe et al (1985b) and Alexander et al (1987) establish a neuroanatomical and functional basis for the hypothesis that the mechanism of action of antipyretic drugs includes the AVP antipyretic system convergent on the VSA.

As described in periparturient animals there appear to be certain instances where fever is suppressed. A similar occurrence is evident following repeated injections of endotoxin. The mechanism for this endotoxin tolerance remains to be adequately explained. Is it possible that an endogenous antipyretic mechanism contributes to the refractory response to endotoxin? This possibility receives support from a study which demonstrated increased AVP staining within the VSA in pyrogen tolerant guinea pigs in manner similar to that seen in pregnant animals (Cooper et al, 1988). Therefore the following studies were conducted to directly test these two hypotheses:

- (1)  $V_1$  and  $V_2$  receptor antagonists were microinjected into the VSA during endotoxin fever to assess the effects of AVP receptor blockade on the antipyresis evoked by peripherally administered indomethacin.
- (2) A  $V_1$  receptor antagonist were microinjected into the VSA during endotoxin fever to assess the effects of AVP receptor blockade on the antipyretic action of peripherally administered sodium salicylate and acetaminophen.

- (3) The effects of drug-induced antipyresis on the release of AVP into the plasma and CSF were examined utilizing a radioimmunoassay for AVP.
- (4) Utilizing the technique of push-pull perfusion the effects of drug-induced antipyresis on the release of AVP into the extracellular space of the VSA was examined.
- (5) The effects on AVP release into the VSA in response to indomethacin during PGE<sub>2</sub>-induced fever was examined in order to gain some insight as to why indomethacin is effective against endotoxin fevers [experiment (4)] but is ineffective against PGE<sub>2</sub> evoked fevers.
- (6) V<sub>1</sub> and V<sub>2</sub> receptor antagonists were microinjected into the VSA during pyrogenic tolerance to intravenous endotoxin in order to establish a possible role for central AVP in this phenomenon.
- (7) The effects of centrally administered pyrogens (PGE<sub>2</sub> and endotoxin) administered during active pyrogenic tolerance to intravenous endotoxin was assessed in order to determine whether antipyretic mechanisms activated in one condition become functional when prompted by a different pyrogenic stimulus.
- (8) Using push-pull perfusion the extracellular levels of AVP within the VSA neuropil of endotoxin naive and endotoxin tolerant rats was measured.

## II. GENERAL METHODS AND MATERIALS

Many of the studies reported in this thesis utilized similar preparations. Therefore, this section will describe some details relating to the surgical procedures and materials employed in the experiments. Specific experimental protocols will be explained in each chapter as needed.

### A. Animals

Conscious and unrestrained male Sprague Dawley rats (250-320g) were used in all experiments. Surgical preparation of these animals was performed during sodium pentobarbital anaesthesia (65-75 mg/kg). Experiments were performed only on animals that had recovered their pre-operative body weight (5-10 days). Food (standard laboratory rat chow) and water were freely available at all times. Treatment of animals was in accordance with **The Guide to the Care and Use of Experimental Animals**, vol.1-2, Canadian Council of Animal Care, 1980.

### B. Surgical Procedures

#### 1. Intra-atrial catheter

Experiments requiring intravenous injections or withdrawal of venous blood utilized animals prepared with an intra-atrial catheter. This catheter consisted of an 11cm section of bevelled Silastic medical grade tubing (0.025in ID, 0.047in OD; Dow Corning) which was threaded through a small (3mm) cuff of PE-190, such that the cuff was 4cm from the bevelled tip. When inserted into the jugular vein, this cuff provided a measure of depth of the catheter tip and acted as a point, around which, sutures could be tied to secure the catheter in place.

For surgical implantation, the catheter (attached to a 1.0 mL syringe and filled with 40 U/mL of heparinized saline) was passed into the right jugular vein and advanced until the tip lay in or near the atrium. This was usually very close to the maximal depth allowed by the previously placed PE-190 cuff. Rapid pulsing of the catheter indicated that the tip lay against the atrial-ventricular valve; at this point withdrawal of the catheter by approximately 1 cm usually resulted in cessation of the pulsing and placement of the tip in the optimal position for the sampling of blood. This was easily checked by placing the rat in various positions and drawing blood in and out of the catheter. The device was then secured with sutures (placed immediately in front and back of the cuff) and exteriorized subcutaneously to the top of the skull. The free end was then positioned on to a section of 20 gauge stainless steel tubing, bent into a "U", which was further attached to a short piece of PE-100 tubing. The piece of PE-100 was subsequently plugged after filling the catheter line with heparinized saline (1000 U/mL). The animal was then placed in a stereotaxic frame and 4-5 holes were drilled into the exposed skull to accommodate stainless steel screws. Dental acrylic was then applied to secure the intra-atrial catheter firmly to the skull.

## 2. Stereotaxic surgery

Standard stereotaxic surgical techniques were used to direct stainless steel guide cannulae towards a lateral cerebral ventricle and the VSA. All cannulae were secured to the skull by means of stainless steel screws and dental acrylic. Stereotaxic coordinates were obtained from the atlas of Pellegrino et al, (1979). The intracerebroventricular (icv) cannula was constructed from 23 gauge tubing and was implanted using the following coordinates (in mm): AP, -0.4; L,  $\pm$  2.0; depth from skull, -2.5. These coordinates placed the cannula tip such that it lay approximately 2.5mm above the ventricular space. Thus insertion of a 30 gauge injection cannula, of the appropriate length, allowed unobstructed injections into the CSF. When not in use the

guide cannula was sealed with a 30 gauge stylet. Patency and placement of the icv cannula was determined by the ability to administer substances by gravity feed.

For VSA cannula implantation, a laterally oriented, oval shaped hole was trephined into the skull, at the appropriate coordinates. The sagittal sinus was visualized and the cannula assembly was lowered to the dura and adjusted laterally to avoid contact with the sinus upon penetration. The dura was then cut using a needle tip and the guide cannula lowered into the brain. The VSA guide cannulae, constructed from 20 gauge (thin wall) tubing, were implanted bilaterally using the following coordinates (in mm): AP, +2.8; L,  $\pm$  1.0; depth from dura, -3.0. This resulted in placement of the cannulae approximately 5mm above the intended injection site. VSA tissue was then accessed via a 30 gauge injection cannula of appropriate length. Injection loci were verified histologically. Stylets for VSA cannulae were designed in the manner of the injection needles such that they fit snugly within the guide tube but had a 30 gauge portion which rested within the intended injection site. It was observed that the VSA was extremely sensitive to the tissue trauma resulting from an initial microinjection of as little as 0.5  $\mu$ L 0.9% saline from a 30 gauge needle. This sensitivity was manifest as non-specific increases in core temperature. However, this response was essentially abolished if the tissue was desensitized by exposure of the injection site to the 30 gauge stylet during surgical recovery. As a result, animals usually had no response to the control microinjection *per se* (Figure 1).

Cannula used for VSA push-pull perfusion were constructed from 30 and 23 gauge (thin wall) stainless steel tubing. The withdrawal or "pull" cannula (23 gauge TW) was chronically implanted, unilaterally, in a manner identical to the bilateral VSA guide cannulae. However, the pull cannula was inserted such that its tip lay within the VSA (-7.5mm from dura). The infusion or "push" cannula was a 30 gauge device with some modifications to facilitate the two way exchange of fluid, characteristic of this

procedure. The two cannula components were fitted together in situ and sealed with a cuff of PE-50, to form the completed, concentric design push-pull cannula. When fully inserted the tip of the push cannula extended 0.5 mm past the end of the pull cannula. However, when not performing a perfusion experiment the pull cannula was sealed with a stylet (27 gauge). The push-pull cannula and its components are illustrated in Figure 2. Animals prepared for push-pull perfusion were also fitted with a paper clip, bent into an "L" shape, which was positioned on the head such that the vertical segment of the clip lay rostral to the chronically implanted pull cannula. This was then secured with dental acrylic. The paper clip served as a protective device against the inevitable bumping of the push-pull cannula assembly against the top and sides of the cage.

### 3. Cerebrospinal fluid withdrawal cannula

For CSF withdrawal, 21 gauge stainless steel tubes were inserted free hand with the rat's head held in a stereotaxic frame (horizontal position). The cannula was inserted through a midline hole, drilled 1mm rostral to the occipital suture, to a depth of approximately 8mm. Correct placement could be ascertained by the appearance or withdrawal of CSF from the cannula. The device was then secured with screws and dental acrylic. Stylets of 26 gauge tubing were inserted to a length flush with the outer cannula. In addition, the stylet-withdrawal cannula interface was sealed with a cuff of PE-100 tubing to prevent leakage of CSF. Care was taken to avoid contamination of these stylets since this could lead to rapid occlusion of the CSF cannula and the subsequent loss of patency.

### C. Body temperature measurements

Body temperature was measured via individually calibrated and paraffin-coated biotelemetry devices (Mini-mitter) implanted into the peritoneal space at the time of cannulation. Output (Hz) was monitored by a receiver board placed underneath an

individual rats cage and fed into an on-line data acquisition system (Dataquest II, Data Sciences) loaded into an Apple IIe computer. Body temperature was read and recorded at 5 min intervals. To avoid circadian influences on body temperature, experiments were conducted between 0800 and 1700 h.

Unless stated otherwise, body temperature was expressed as changes from baseline. Baseline temperature values before treatment were calculated for each animal and the results were subsequently expressed as the change from this average pre-treatment value.

#### D. Vasopressin radioimmunoassay

AVP concentrations in plasma, CSF and push-pull perfusion fluid were determined using a radioimmunoassay with a sensitivity range of 0.13 - 0.22 pg/tube. Rabbit anti-AVP antiserum was used at a final concentration of 1:810 000. The assay used 50  $\mu$ L of AVP antiserum, 50 - 250  $\mu$ L of sample, 100  $\mu$ L of  $^{125}$ I-AVP (4000 cpm) and 50 - 250  $\mu$ L of assay buffer. The assay buffer was composed of the following : 0.1 M sodium phosphate containing 0.01 M EDTA at pH 7.2, 0.05 M NaCl, 0.02% sodium azide and 0.01% BSA. The incubation schedule consisted of 2 days without label followed by 2 days with label at 4 °C. The double antibody technique was used to precipitate the bound label and the pellet was counted in a gamma counter and calculated using the log-logit method. The range of intraassay (within) variability was 6.3 - 8.3% and interassay (between) variability was 10.5 - 12.2%. Synthetic AVP added to plasma and acid extracts of the neural lobe of the pituitary show curves which were parallel to synthetic AVP whereas arginine vasotocin or oxytocin show non-parallel curves. Cross reactivity with vasotocin and oxytocin was 17 and 0.2% respectively. Cross reactivity with other peptides was <0.01%.



Samples of CSF or push-pull perfusates were assayed directly whereas plasma was extracted using Sep-Pak C<sub>18</sub> cartridges (Waters) prior to assay. The AVP fraction was eluted with 90% ethanol in 4% acetic acid which was subsequently lyophilized and reconstituted with assay buffer for the AVP assay. Recovery has been demonstrated to be > 80% (Kasting et al, 1983; Kasting et al, 1985). CSF or perfusion fluid were not extracted as aliquots ranging from 50 - 250  $\mu$ L resulted in similar AVP values in a pilot assay.

#### E. Histological procedures

All injection loci were verified histologically. Upon completion of an experiment the animal was overdosed with urethane (1.5 g/kg) and the vasculature perfused, via cardiac puncture, with 0.9% saline followed by 10% buffered formalin. The brain was removed and postfixed in buffered formalin containing 20% sucrose. Frozen brain sections were cut at 44  $\mu$ m on a cryostat and thaw mounted onto gelatin coated microscope slides. Sections were stained using Thionin or cresyl violet. Each section was then examined using light microscopy. Figure 3 is a photomicrograph of a typical brain section showing the bilateral injection loci within the VSA.

#### F. Drugs

##### 1. Pyrogens

Fever was induced by an icv or iv injection of an endotoxin derived from a TCA extract of *Escherichia coli* (serotype 026:B6; Sigma L-3755). The endotoxin was dissolved in sterile 0.9% saline at a concentration of 10 ng/ $\mu$ L for icv injection and 50-100  $\mu$ g/kg for iv injection. In some experiments PGE<sub>2</sub> (50 - 200 ng/ $\mu$ L, Sigma) was injected icv to evoke a febrile response. All icv injections were delivered in a volume of 5  $\mu$ L. A stock solution of PGE<sub>2</sub> was initially dissolved in 95% ethanol (10 mg/mL) and

aliquots were subsequently diluted with 0.9% saline such that the final concentration of ethanol was < 2%. These were stored at -70 °C until the day of the experiment.

## 2. Antipyretic drugs

All antipyretic drugs were administered intraperitoneally on a weight basis. These drugs were sodium salicylate, indomethacin (1-[p-Chlorobenzoyl]-5-methoxy-2-methylindole-3-acetic acid), and acetaminophen (N-acetyl-p-aminophenol). Salicylate and acetaminophen were administered at 75 mg/kg and indomethacin at 5 - 7.5 mg/kg. Salicylate and indomethacin were dissolved in 0.2 M tris(hydroxymethyl)aminomethane (Tris) buffer at pH 8. Acetaminophen was dissolved in DMSO:saline (3:2, v/v). All reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

## 3. Vasopressin receptor antagonists

Two vasopressin receptor antagonists were employed for pharmacological blockade of VSA vasopressin receptors. The V<sub>1</sub> receptor antagonist was [1-( $\beta$ -mercapto- $\beta,\beta$ -cyclopentamethylenepropionic acid),2-(O-methyl)tyrosine] arginine vasopressin, abbreviated as d(CH<sub>2</sub>)<sub>5</sub>Tyr(Me)AVP (Bachem). This analogue remains one of the most potent anti-vasopressor AVP antagonists available. At the same time d(CH<sub>2</sub>)<sub>5</sub>Tyr(Me)AVP possesses weak *in vivo* antidiuretic agonist characteristics as well as weak anti-oxytocic activity (Manning and Sawyer, 1986). The V<sub>2</sub> receptor antagonist was [1-( $\beta$ -mercapto- $\beta,\beta$ -pentamethylenepropionic acid),2-D-isoleucine,4-( $\alpha$ -aminobutyric acid)]arginine vasopressin, abbreviated as d(CH<sub>2</sub>)<sub>5</sub>[D-Ile<sup>2</sup>,Abu<sup>4</sup>]AVP (kindly donated by Dr, M. Manning, OH). This V<sub>2</sub> receptor analogue, while exhibiting high antidiuretic potency, remains both a weak anti-vasopressor and anti-oxytocic compound (Manning and Sawyer, 1986). Both compounds were dissolved in sterile physiological saline at the concentrations indicated in each experiment. The AVP analogues were administered into the VSA bilaterally in a volume of 0.5  $\mu$ L.

## G. Treatment of data

Grouped scores were expressed as mean  $\pm$  standard error of the mean ( $\pm$  S.E.M.). Thermoregulatory data was often expressed as an integrated response (the thermal or fever index), such that 1 index unit is equivalent to a 1 °C change in temperature lasting for one hour (Clark and Cumby, 1975). This calculation provides an alternate method for analyzing thermoregulatory responses since it represents both the magnitude and duration of each response within any particular treatment group. The integrated thermoregulatory response was calculated from individual scores and combined for a mean group score. A positive or negative thermal index value was indicative of a sustained pyretic or antipyretic response respectively. A similar method was used to calculate the integrated vasopressin release response. Statistical analysis is described for each experimental series where appropriate.

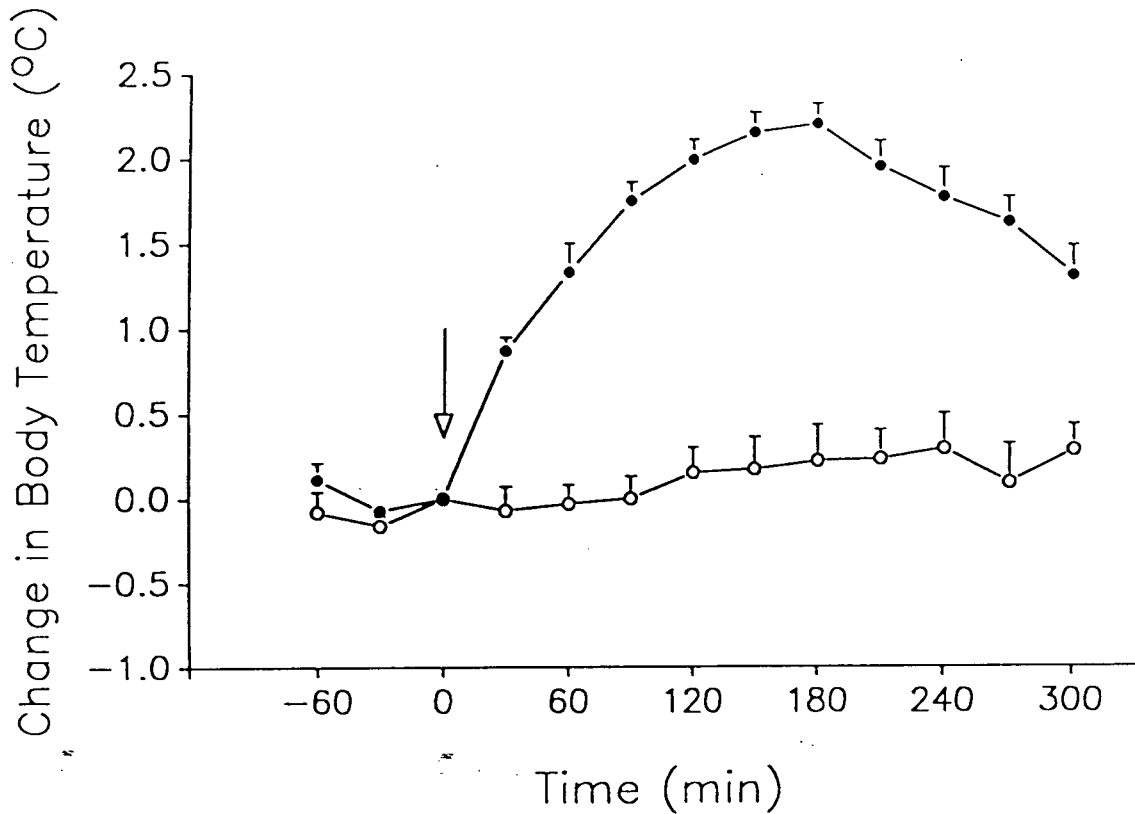


Figure 1. Mean change ( $\pm$  S.E.M.) in body temperature of rats receiving control microinjections ( $0.5 \mu\text{L}$  saline bilaterally) within the VSA, without prior exposure to the VSA stylet during surgical recovery (solid circles,  $n=6$ ). All points were significantly different ( $p < 0.01$ ) from 0 (one way ANOVA). Open circles depict the thermoregulatory response to bilateral VSA microinjection of  $0.5 \mu\text{L}$  saline after surgical recovery with VSA stylets in place ( $n=9$ ). This was not significantly different. Time of microinjection is indicated by the arrow (time 0).

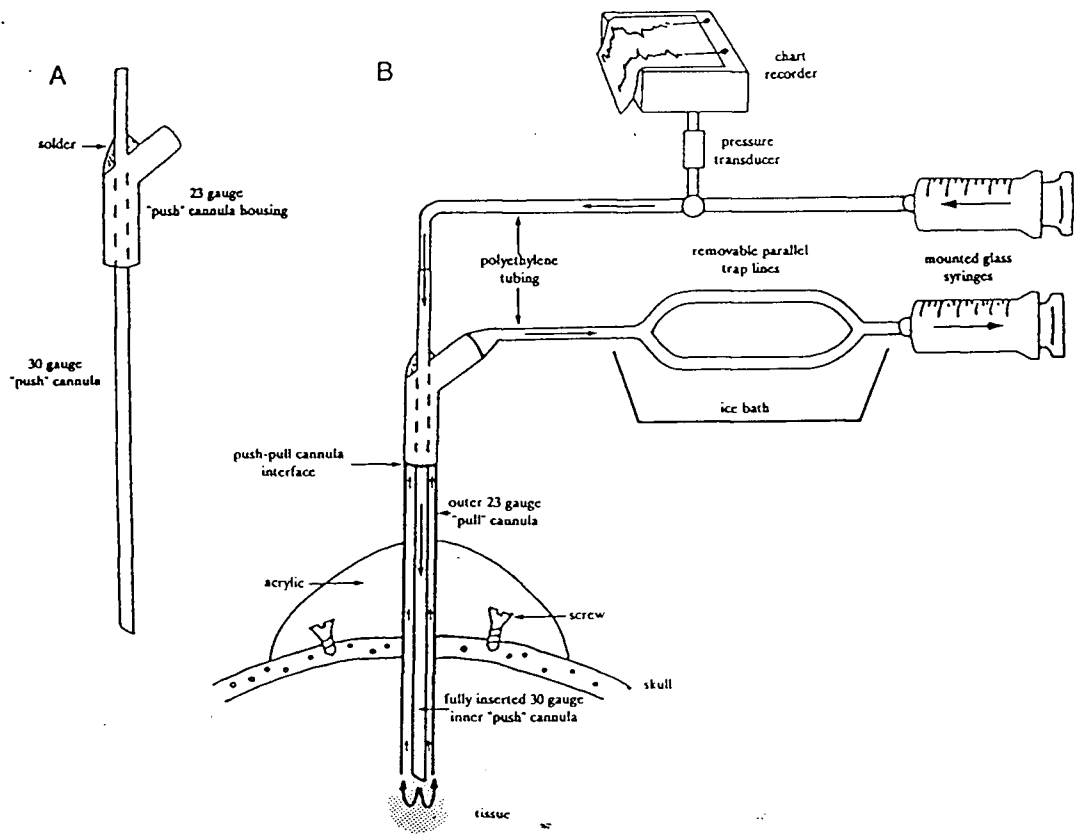


Figure 2. Diagrammatic representation of the push-pull perfusion system. (A) "Push" cannula assembly. A section of 30 gauge tubing was inserted through a small hole placed into an angled portion of 23 gauge TW tubing. The interface was sealed with solder. (B) Intact push-pull cannula and accompanying attachments. The tip of the push cannula extended 0.5 mm past the end of the pull cannula. See text for further details.

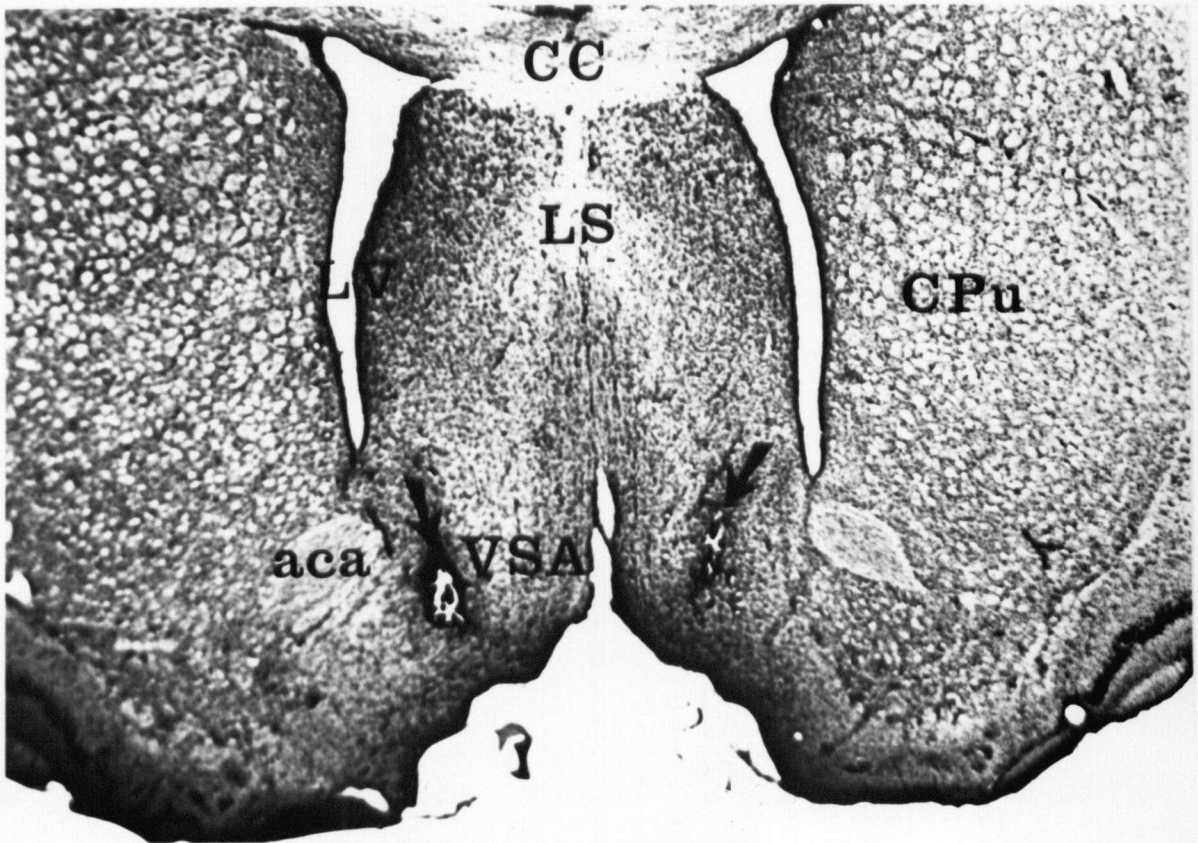


Figure 3. Photomicrograph of cresyl violet-stained coronal brain section (44  $\mu\text{m}$ ) illustrating microinjection loci (arrows) within the VSA. aca, anterior commissure; CC, corpus callosum; CPu, caudate-putamen; LS, lateral septum; LV, lateral ventricle; VSA, ventral septal area.

### III. THE ROLE OF CENTRAL VASOPRESSIN V<sub>1</sub> RECEPTORS DURING INDOMETHACIN-INDUCED ANTIPYRESIS

#### Introduction

The pioneering work of Vane (1971) and colleagues (Flower and Vane, 1972) established the hypothesis that is currently favoured to explain the action of antipyretic drugs. Their research suggested the inhibition of prostaglandin (PG) biosynthesis was responsible for the antipyretic action of non-steroidal analgesic and anti-inflammatory drugs. This hypothesis was attractive because it was advanced at the time that PGs were found to be potently pyretic substances (Feldberg and Saxena, 1971). However, there are also data that are not readily explainable by the PG synthesis inhibition hypothesis (Abdel-Halim et al, 1978; Kantor and Hampton, 1978; Sirén, 1982; Alexander et al, 1987). An alternate explanation for the effects of non-steroidal antipyretic drugs is that they interact with the endogenous antipyretic system of the brain which involves the synaptic release of AVP within the VSA (see page 48). The purpose of the present study was to test this hypothesis by observing the thermoregulatory response of febrile rats after parenteral administration of indomethacin during AVP receptor blockade within the VSA of the rat brain.

#### Methods

Male Sprague-Dawley rats were surgically prepared with a unilateral and bilateral guide cannulae for icv and VSA administration of test substances as well as a radio transmitter for remote monitoring of core temperature as described previously (see chapter II). After surgical recovery, fever was induced by an icv injection of *E. coli* endotoxin (10 ng/ $\mu$ L) in 5  $\mu$ L physiological saline after at least 2h of monitoring

baseline body temperature. After 150 min a robust febrile response was evident at which time the animals received intra-septal injections immediately followed by intraperitoneal indomethacin or Tris buffer according to the following experimental groups: 1) microinjection of an AVP V<sub>1</sub> receptor antagonist, d(CH<sub>2</sub>)<sub>5</sub>Tyr(Me)AVP, or saline followed by intraperitoneal indomethacin; 2) microinjection of an AVP V<sub>2</sub> receptor antagonist, d(CH<sub>2</sub>)<sub>5</sub>[D-Ile<sup>2</sup>,Abu<sup>4</sup>]AVP into the VSA followed by intraperitoneal indomethacin; 3) microinjection of the AVP V<sub>1</sub> antagonist or saline into the VSA followed by intraperitoneal Tris; or 4) microinfusion of the V<sub>1</sub> antagonist or saline into the VSA before and after intraperitoneal indomethacin. A fifth group of animals served as non-febrile controls and received intra-septal V<sub>1</sub> antagonist or saline without subsequent intraperitoneal injections. Body temperature was monitored for 2 h following the injection regimes.

The AVP antagonists (430 pmol) or saline were administered bilaterally in 0.5  $\mu$ L via a 30 gauge injection cannula over 10-15 sec using a 10  $\mu$ L Hamilton syringe. Each rat served as its own control for VSA treatments and received only one antagonist. Thus, separate groups of rats were used for each group of experiments. Treatment order was randomized. For microinfusion experiments, d(CH<sub>2</sub>)<sub>5</sub>Tyr(Me)AVP (430 pmol/ $\mu$ L) or saline were infused at a rate of 1  $\mu$ L/h starting 30 min before and until 60 min after indomethacin treatment. A thermal index was calculated from the data as the cumulative fall in body temperature relative to the temperature at the time of indomethacin or Tris starting 30 min before and until 60 min after indomethacin treatment. A thermal index was calculated from the data as the cumulative fall in body temperature relative to the body temperature at the time of indomethacin or Tris injection. A positive value represents an overall increase in temperature over the 2 h observation period, whereas a negative value is indicative of



a decrease in body temperature or antipyresis. Statistical analysis was performed on the thermal index data using the Student's t test for paired groups. Injection loci were verified histologically.

## Results

Intracerebroventricular administration of endotoxin evoked fevers of similar magnitudes in all experimental groups. When intraperitoneal indomethacin was preceded by bilateral microinjections of saline into the VSA, an antipyresis ensued which was evident within 20 min and persisted for at least 2 h (Figure 4A). However, if the  $V_1$  receptor antagonist,  $d(CH_2)_5Tyr(Me)AVP$ , was microinjected into the VSA not only was the indomethacin-induced antipyresis delayed but there was a prompt hyperthermia that peaked after 20 min. The hyperthermic episode then declined and core temperature leveled off to just below pre-injection values. Thus the effects of indomethacin were inhibited for 80 min, after which body temperature fell parallel to controls. A thermal index was calculated for all experimental groups and presented in Table I. The thermal index following microinjection of  $d(CH_2)_5Tyr(Me)AVP$  into the VSA and intraperitoneal indomethacin ( $-0.24 \pm 0.09$  °C·h) was significantly different compared to the control with saline and indomethacin ( $-1.63 \pm 0.17$  °C·h) ( $p < 0.01$ ; paired t test). Thus indicating a greater degree of antipyresis in the control group. This antagonism of indomethacin was specific to the  $V_1$  receptor since experiments utilizing VSA administration of the  $V_2$  antagonist,  $d(CH_2)_5[D-Ile^2, Abu^4]AVP$  (Figure 4B) resulted in no significant inhibition of indomethacin-induced antipyresis compared with saline controls (Table I). The attenuating effect of  $d(CH_2)_5Tyr(Me)AVP$  on the indomethacin-induced antipyresis was not simply caused by an additive effect of an increase in temperature caused by the antagonist, since the analogue had no effect on core temperature when microinjected into the VSA of non-febrile rats (Figure 5). Furthermore, animals that were treated with saline or the  $V_1$  antagonist followed by

Tris buffer, exhibited neither antipyresis nor a disruption of the normal febrile process (Figure 6, Table I). To further clarify the apparent indomethacin antagonism, saline or d(CH<sub>2</sub>)<sub>5</sub>Tyr(Me)AVP were infused into the VSA for 30 min before and until 60 min after peripheral indomethacin (administered 150 min post-endotoxin).

Bilateral microinfusion of either saline or the V<sub>1</sub> antagonist had no effect on the febrile response compared with the previous experiments. Similarly, 90 min saline infusions had no effect on the antipyresis ( $-1.35 \pm 0.16$  °C·h) produced by the injection of indomethacin. However, infusion of d(CH<sub>2</sub>)<sub>5</sub>Tyr(Me)AVP within the VSA for 90 min completely abolished the antipyretic effect of indomethacin for the 2 h of observation ( $0.13 \pm 0.30$  °C·h) (Figure 7, Table I). Notably absent in this response was the hyperthermic episode that was prominent following the microinjection protocol. Representative histological sections of injection loci are depicted in Figure 8.

TABLE I. Cumulative change in body temperature calculated from the point of indomethacin or Tris administration.

Thermal Index ( $^{\circ}\text{C}\cdot\text{h}$ )

	VSA Microinjection	Thermal Index, $^{\circ}\text{C}\cdot\text{h}$	n	Statistical Significance
<b>A.</b> Intraperitoneal injection				
Tris	Saline	$1.16\pm 0.37$	6	NS
Tris	$V_1$ antagonist	$1.18\pm 0.25$		
Indomethacin	Saline	$-1.63\pm 0.17$	9	$p < 0.01$
Indomethacin	$V_1$ antagonist	$-0.24\pm 0.09$		
Indomethacin	Saline	$-1.04\pm 0.13$	8	NS
Indomethacin	$V_2$ antagonist	$-0.81\pm 0.15$		
<b>B.</b>				
	Saline	$0.50\pm 0.31$	6	NS
	$V_1$ antagonist	$0.30\pm 0.34$		
<b>C.</b> Intraperitoneal injection				
Indomethacin	Saline	$-1.35\pm 0.16$	5	$p < 0.01$
Indomethacin	$V_1$ antagonist	$0.13\pm 0.30$		

n, no. of animals. A: mean thermal indices ( $^{\circ}\text{C}\cdot\text{h}$ ) of febrile rats receiving microinjections of a  $V_1$ , or  $V_2$  receptor antagonist or saline into the VSA followed by intraperitoneal Tris or indomethacin. B: mean thermal indices ( $^{\circ}\text{C}\cdot\text{h}$ ) of nonfebrile rats receiving the  $V_1$  antagonist or saline within the VSA. C: mean thermal indices ( $^{\circ}\text{C}\cdot\text{h}$ ) of febrile rats receiving VSA infusions of saline or the  $V_1$  antagonist prior to and after intraperitoneal indomethacin.

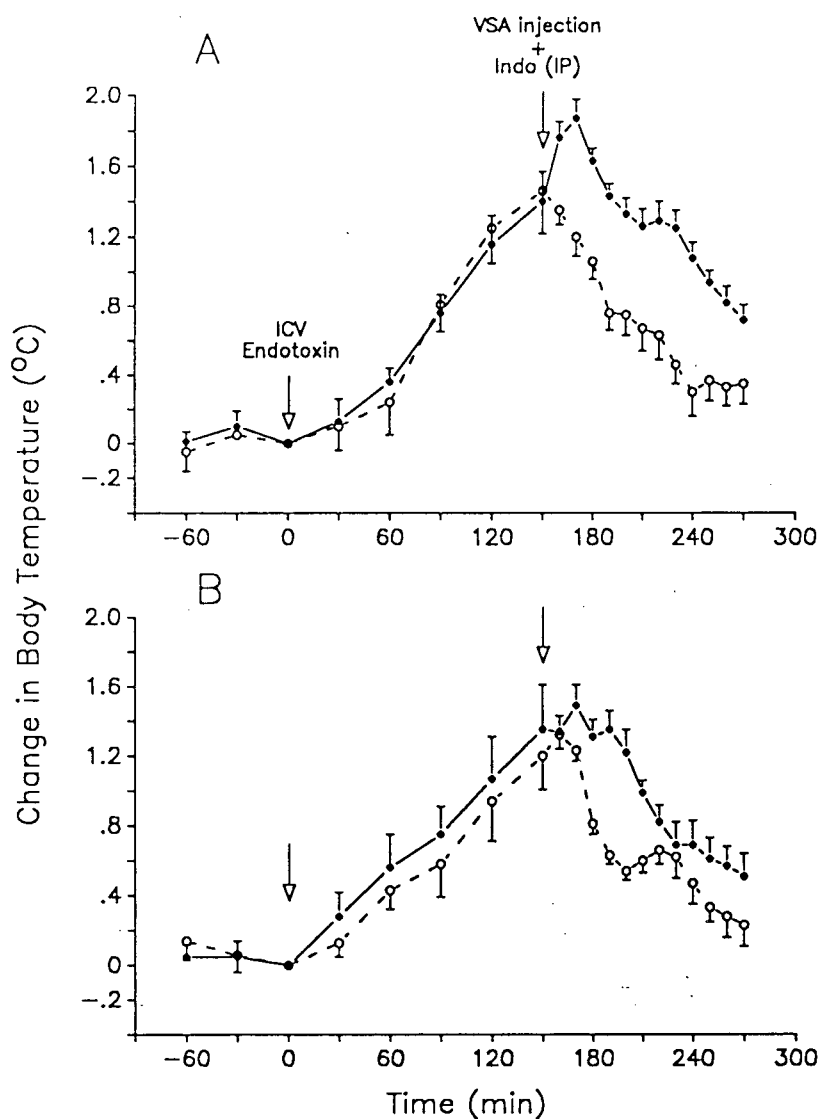


Figure 4. Mean change ( $\pm$  S.E.M.) in body temperature in response to endotoxin icv at time 0 (first arrow). Subsequently (2nd arrow), a V<sub>1</sub> (panel A) or V<sub>2</sub> (panel B) antagonist were microinjected bilaterally into the VSA, immediately followed by intraperitoneal indomethacin. Baseline body temperatures panel A: V<sub>1</sub> antagonist (closed circles),  $37.04 \pm 0.12$  °C; saline (open circles),  $37.17 \pm 0.15$  °C. Panel B: V<sub>2</sub> antagonist (closed circles),  $37.68 \pm 0.25$  °C; saline (open circles),  $37.33 \pm 0.18$  °C. See Table I for statistical analysis of data.

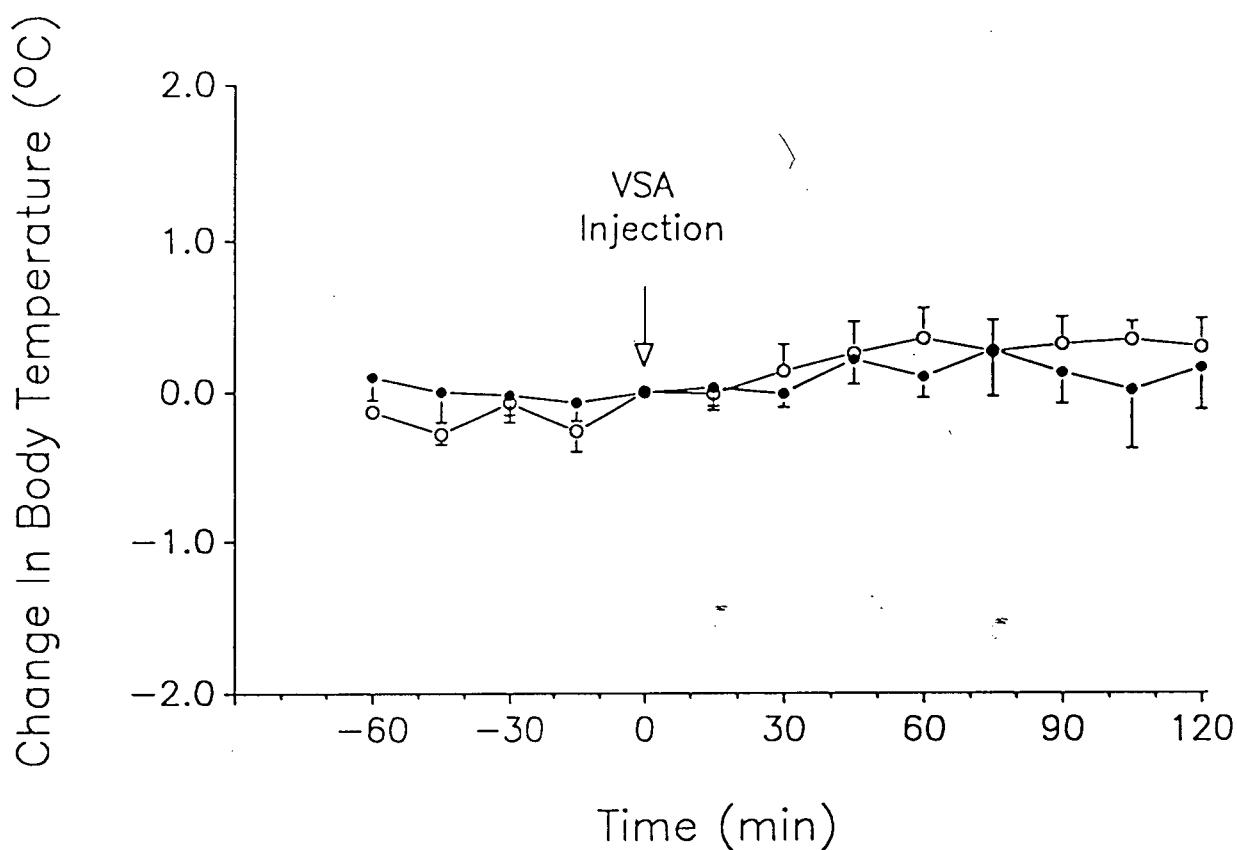


Figure 5. Mean change ( $\pm$  S.E.M.) in body temperature in response to microinjection of a  $V_1$  antagonist (solid circles; baseline body temperature,  $37.46 \pm 0.27$  °C) or saline (open circles; baseline body temperature,  $37.43 \pm 0.31$  °C) into the VSA of nonfebrile rats at time 0. See Table I for statistical analysis.

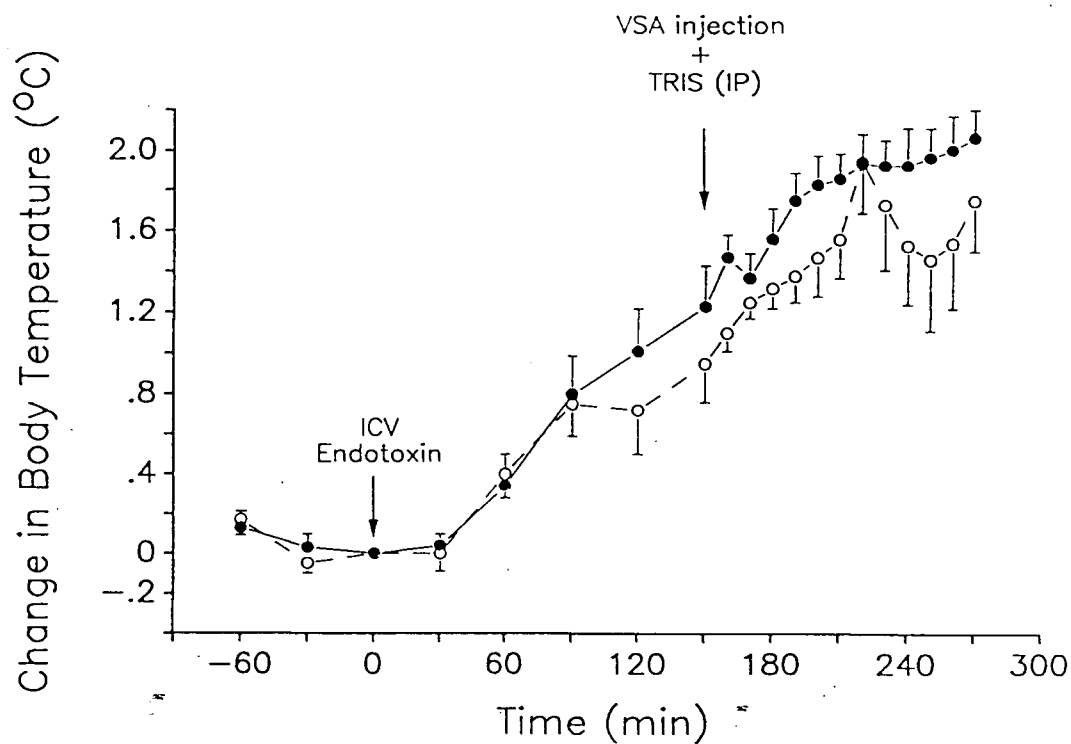


Figure 6. Mean change ( $\pm$  S.E.M.) in body temperature in response to icv endotoxin at time 0 (1st arrow). Subsequently (2nd arrow), a  $V_1$  antagonist (solid circles; baseline body temperature,  $37.13 \pm 0.15$  °C) or saline (open circles; baseline body temperature,  $37.45 \pm 0.25$  °C) were microinjected bilaterally within the VSA, immediately followed by intraperitoneal Tris buffer. See Table I for statistical analysis.

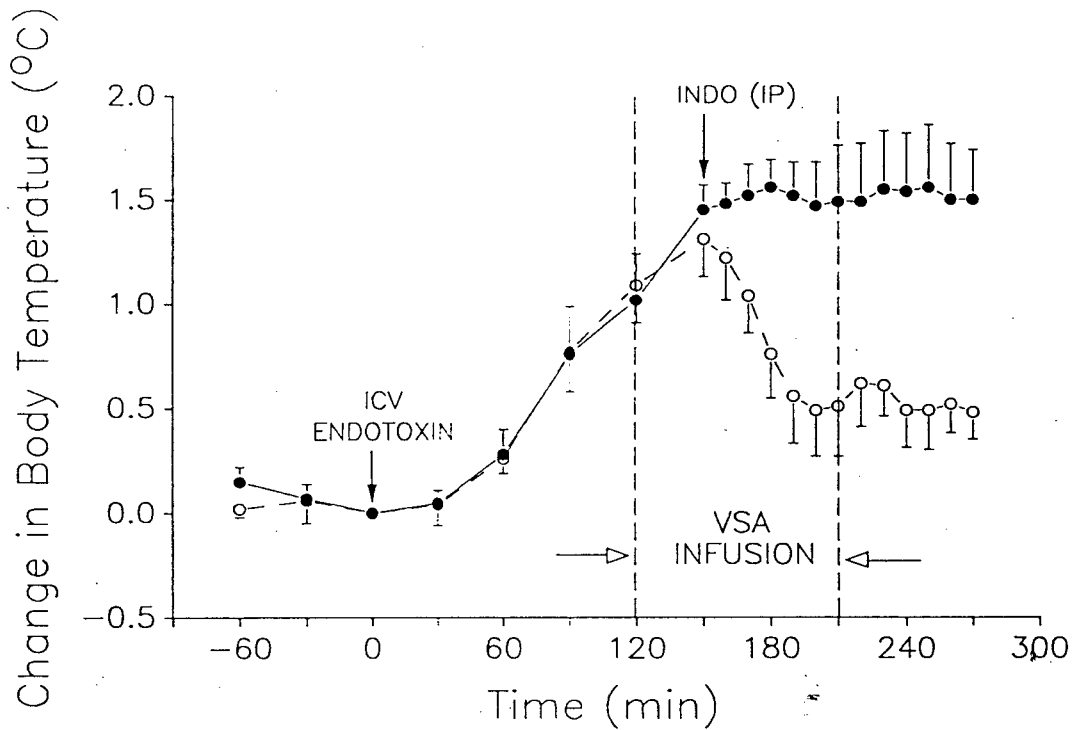


Figure 7. Mean change ( $\pm$  S.E.M.) in body temperature in response to endotoxin at time 0 (1st arrow). Subsequently (2nd arrow), indomethacin was injected intraperitoneally. Dotted vertical lines denote period of microinfusion of either V<sub>1</sub> antagonist (solid circles; baseline body temperature,  $37.60 \pm 0.11$  °C) or saline (open circles; baseline body temperature,  $37.56 \pm 0.26$  °C) within the VSA. See Table I for statistical analysis.

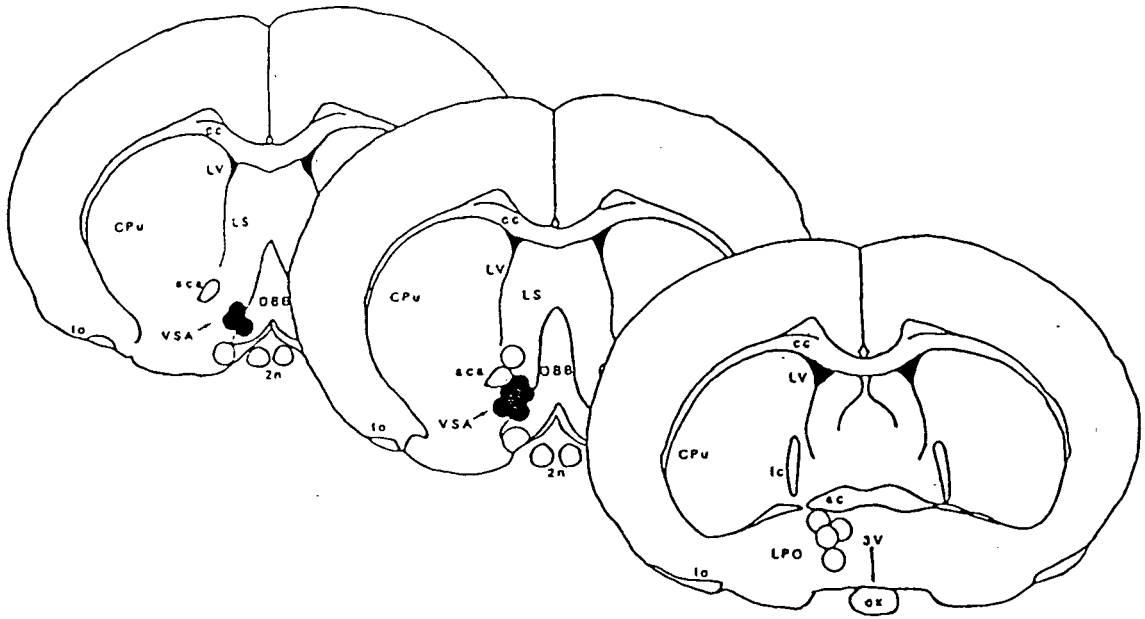


Figure 8. Representative histological sections depicting sites where the  $V_1$  antagonist inhibited or blocked (solid circles) indomethacin-induced antipyresis. Areas where microinjection of the  $V_1$  antagonist were ineffective are also shown (open circles). Loci from one side are shown for clarity. aca/ac, anterior commissure; cc, corpus callosum; CPu, caudate putamen; DBB, diagonal band of Broca; ic, internal capsule; lo, lateral olfactory tract; LPO, lateral preoptic area; LS, lateral septum; LV, lateral ventricle; ox, optic chiasm; VSA, ventral septal area; 2n, optic tracts; 3V, third ventricle.



## Discussion

The prevailing hypothesis that attempts to explain the mechanism of action of the antipyretic effects of non-steroidal analgesic and anti-inflammatory drugs suggests that these drugs inhibit PG biosynthesis (Vane, 1971; Flower and Vane, 1972). Specifically, these drugs act on the cyclooxygenase enzyme (Flower et al, 1980) within the brain to prevent the production of the pyrogenic PGs, therefore mediating the process of defervescence. Two lines of evidence provide compelling evidence for this theory. *In vitro* studies, utilizing brain homogenate preparations, have demonstrated that antipyretic drugs, including indomethacin, inhibit peripheral as well as central PG biosynthesis (Flower and Vane, 1972). These authors also showed that acetaminophen, which is antipyretic but not anti-inflammatory, affected PG synthesis in rabbit brain but not dog spleen homogenates. This suggested a central mode of action for the antipyretic effects of acetaminophen. Rawlins et al (1973) demonstrated that [ $^{14}\text{C}$ ]salicylate, injected subcutaneously, was present in sufficient concentrations within the hypothalamus of the rabbit to inhibit PG biosynthesis. Zeil and Krupp (1974) tested the PG synthesis inhibitory properties of several non-steroidal anti-inflammatory drugs using bovine seminal vesicles. They found that the inhibitory efficacy of PG synthesis *in vitro* closely paralleled the antipyretic activity *in vivo* and concluded that the data was consistent with a central PG synthesis inhibiting action for these drugs.

*In vivo* support of Vane's hypothesis (Vane, 1971), until quite recently (Sirko et al, 1989), relied exclusively on the analysis of cerebrospinal fluid (CSF) levels of PGs before and after antipyretic treatment (Feldberg and Gupta, 1973; Feldberg et al, 1973; Dey et al, 1974). However the role of PGs in fever is controversial (Cranston et al, 1975; Cranston et al, 1976; Laburn et al, 1976). Therefore the role of PGs in antipyresis is controversial as well (Cranston et al, 1976; Mitchell et al, 1986).

The present data indicate that indomethacin, a potent antipyretic and inhibitor of PG biosynthesis, may utilize mechanisms other than, or in addition to, the inhibition of PGs to mediate antipyresis in the rat. In particular, these experiments suggest that the activation of endogenous AVP V<sub>1</sub> receptors within the VSA is an essential neurochemical event in indomethacin-induced antipyresis. The VSA has previously been shown to be an area where exogenously administered AVP suppresses (Cooper et al, 1979; Naylor et al, 1985; Ruwe et al, 1985b; Naylor et al, 1986) and an AVP antagonist (or antiserum) enhances pyrogen elicited fever (Cooper et al, 1987; Malkinson et al, 1987; Naylor et al, 1988). In addition, Alexander et al, (1987) have shown that sodium salicylate infused into the VSA can suppress PGE<sub>1</sub> hyperthermia. It was postulated, therefore, that if the AVP endogenous antipyretic system is activated by antipyretic drugs, such as indomethacin, then the febrile effects of the drug should be inhibited or prevented by AVP receptor blockade within the VSA.

In this study, the antipyresis induced by indomethacin was inhibited for up to 80 min when preceded by microinjection of d(CH<sub>2</sub>)<sub>5</sub>Tyr(Me)AVP into the VSA but not adjacent areas (see Figure 8). This V<sub>1</sub> antagonist is a specific blocker of the peripheral V<sub>1</sub> subtype (Kruzynski et al, 1980), binds to neuronal V<sub>1</sub> receptors (Poulin et al, 1988) and itself has no thermoregulatory effects (Cooper et al, 1987; Naylor et al, 1988; Fyda et al, 1989; see also Figure 5). This is particularly relevant in lieu of the marked hyperthermic response observed in animals receiving intra-septal d(CH<sub>2</sub>)<sub>5</sub>Tyr(Me)AVP followed by indomethacin. Bilateral microinjections into the VSA utilizing the V<sub>1</sub> antagonist or saline followed by intraperitoneal Tris buffer did not result in the rapid hyperthermia described above. Moreover, bilateral microinjections into the VSA with the V<sub>2</sub> antagonist, d(CH<sub>2</sub>)<sub>5</sub>[D-Ile<sup>2</sup>, Abu<sup>4</sup>]AVP (Manning et al, 1984), did not significantly impair the effects of indomethacin compared to saline controls. However, the V<sub>2</sub> receptor analogue did appear to exhibit partial antagonism

of the antipyretic receptor, which may become more apparent with higher concentrations (Figure 4B). The lack of a marked hyperthermic peak observed when  $d(CH_2)_5Tyr(Me)AVP$  was combined with indomethacin suggests that this latter effect may be the principle reason for the observed antagonism of indomethacin by  $d(CH_2)_5Tyr(Me)AVP$ .

To test this possibility, bilateral microinfusions of the VSA with the  $V_1$  antagonist or saline followed by indomethacin were performed. The microinfusion protocol resulted in complete blockade of the fever reducing effects of indomethacin when the infusate contained  $d(CH_2)_5Tyr(Me)AVP$  but not saline. This effect occurred without the hyperthermia observed following the microinjection experiments. It appears therefore, that  $d(CH_2)_5Tyr(Me)AVP$  specifically antagonizes the antipyresis induced by intraperitoneal indomethacin.

The results of this study can not rule out PG biosynthesis inhibition as part of the mechanism of action for antipyretic drugs, such as indomethacin. However, the results derived from this experimental model suggest that other mechanisms are essential. Previous studies have demonstrated that alternative explanations for the antipyretic action of the non-steroidal antipyretic drugs are needed. For instance, antipyretic doses of both aspirin and acetaminophen (administered subcutaneously) failed to affect PG biosynthesis within the rat brain, although indomethacin was effective (Abdel-Halim et al, 1978). A potent inhibitor of PG biosynthesis, floctafenine, has little or no antipyretic properties regardless of whether it is administered centrally or peripherally (Laburn et al, 1980). Cranston et al (1975) reported that sub-antipyretic doses of salicylate suppressed pyrogen evoked PG increases in the CSF while leaving the resultant fever unaffected. In addition, the work of Alexander et al (1987), which showed the lability of PG-mediated fever to VSA administered salicylate, provides a functional and anatomical

link between the endogenous AVP system and a possible mechanism of action for antipyretic drugs such as indomethacin or salicylate.

The present results demonstrate that a potent PG synthesis inhibitor and antipyretic, indomethacin, utilizes a mechanism that requires vasopressin V<sub>1</sub> receptor activation within the VSA to mediate antipyresis. Whether indomethacin stimulates AVP release within the VSA or binds to all or a portion of the peptide receptor cannot be ascertained. Physiological receptors for indomethacin have been reported on blood platelets (Magous et al, 1985) but currently there is no available evidence to suggest that these receptors exist within the brain or that there is an interaction with AVP receptors. These data do suggest, however, that a simple PG inhibition hypothesis is no longer sufficient to explain the mechanism of action for antipyretic drugs.

#### **IV. CENTRAL VASOPRESSIN V<sub>1</sub> BLOCKADE PREVENTS SALICYLATE BUT NOT ACETAMINOPHEN-INDUCED ANTIPYRESIS**

##### **Introduction**

The ability to inhibit prostaglandin (PG) biosynthesis by non-steroidal antipyretic drugs is generally accepted as the mechanism by which these drugs reduce core temperature during fever. The work of Vane (1971) and colleagues (Flower and Vane, 1972) provided the first evidence that antipyretic drugs inhibit PG formation and that the site of their antipyretic action was the PG synthetase (cyclooxygenase) enzyme within the central nervous system (CNS). While the majority of research in this area has provided convincing support for Vane's hypothesis (see previous discussion p.72), new questions have recently been raised regarding the mechanism of action of antipyretic drugs. Experiments conducted by Alexander et al. (1987, 1989) have demonstrated that fevers elicited by PGE<sub>1</sub> are indeed sensitive to salicylate if the antipyretic is administered within the ventral septal area (VSA) of the brain. Previous to this the lack of effect of antipyretic drugs against PG evoked fever was assumed to be due to the drugs acting at an enzymatic step (cyclooxygenase) in the synthesis of PGs. Thus fevers evoked by exogenous PGs by-pass the antipyretic drug-sensitive enzymatic pathway which renders these fevers insensitive to PG synthesis inhibition. It has been observed further that the antipyretic effect of VSA administered salicylate, during PGE<sub>1</sub>-induced fever, can be blocked if salicylate is given in conjunction with a vasopressin V<sub>1</sub> receptor antagonist (Alexander et al, 1989). Similarly, the antipyretic effects of indomethacin, administered intraperitoneally during endotoxin fever, are also abolished by V<sub>1</sub> blockade within the VSA (see chapter III). Thus, there appears to be a relationship between a central vasopressinergic system and the action of at least two

antipyretic drugs. The present study was conducted to examine the effects of V<sub>1</sub> receptor blockade, within the VSA, on the antipyretic effects of peripherally administered sodium salicylate and the structurally dissimilar, acetaminophen.

## Methods

As previously described, male Sprague-Dawley rats (250-300g) were prepared with cannulae for icv and VSA administration of endotoxin and the AVP V<sub>1</sub> antagonist, d(CH<sub>2</sub>)<sub>5</sub>Tyr(Me)AVP, respectively. The body temperature of each rat was monitored at 5 min intervals using a pre-calibrated transmitter (Mini-mitter), placed into the peritoneal cavity at the time of cannulation. Data were collected and stored on an Apple IIe computer. During the experiments the animals were conscious and unrestrained in their home cage, with food and water available at all times.

After at least 2h monitoring of baseline body temperature, fever was induced by an icv injection of 10 ng *E. coli* endotoxin in 5 µL sterile 0.9% saline. This route of administration enables each animal to serve as its own control since endotoxin tolerance does not develop following icv administration of this pyrogen (Sheth and Borison, 1960). The resultant fever was allowed to progress for 150 min at which time bilateral microinjections (0.5 µL/side) of saline or the AVP V<sub>1</sub> receptor antagonist, d(CH<sub>2</sub>)<sub>5</sub>Tyr(Me)AVP, were performed. Immediately following the VSA injections, sodium salicylate, acetaminophen or the respective solvents, Tris buffer and dimethyl sulfoxide in 0.9% saline (DMSO:saline; 3:2, v/v) were injected intraperitoneally. Core temperature was followed for an additional 2h. Both drugs and the vehicles were injected at 75 mg/kg and 1 mL/kg respectively.

Animals receiving salicylate were divided into two groups: one group received a low dose of d(CH<sub>2</sub>)<sub>5</sub>Tyr(Me)AVP (0.5 µg/side), and the second group received a high dose (5.0 µg/side) of d(CH<sub>2</sub>)<sub>5</sub>Tyr(Me)AVP within the VSA. Animals receiving

acetaminophen were pretreated with the high dose of  $d(CH_2)_5Tyr(Me)AVP$  only. In a separate experiment non-febrile rats received (within the VSA) injections of saline or  $5.0 \mu g/side$  of the  $V_1$  antagonist. All central injections were performed over 10-15 seconds using a 30 gauge injection cannula affixed, via PE-20 tubing, to a  $10 \mu L$  Hamilton syringe. Each animal received only one antipyretic drug or vehicle but served as its own control with respect to the VSA treatment. The order of the VSA microinjections was random. A thermal index was calculated from the data for the 2h following antipyretic drug or vehicle injection as described previously (see chapter II). Statistical analysis was performed on the thermal index data using a one way ANOVA and the Newman-Keuls post-hoc test.

## Results

Figure 9 illustrates the effects of salicylate, when preceded by saline or  $d(CH_2)_5Tyr(Me)AVP$  within the VSA, on the fever evoked by icv endotoxin. The onset of defervescence was prompt in the control experiments with a maximum effect occurring approximately 80 min after salicylate administration. If, however,  $d(CH_2)_5Tyr(Me)AVP$  preceded salicylate, the effects of the antipyretic drug were markedly altered. The effect the AVP antagonist on the actions of salicylate were dose related.

In contrast to that observed following salicylate, bilateral administration of  $5.0 \mu g$   $d(CH_2)_5Tyr(Me)AVP$  into the VSA, did not affect the antipyretic effect of intraperitoneal acetaminophen (Figure 10). In both saline and  $V_1$  antagonist pretreatment trials, acetaminophen elicited a rapid core temperature reduction to slightly below baseline ( $-0.10^\circ C$ ) within 60-70 min after drug administration. Unlike the persistent action of salicylate, however, acetaminophen-treated animals

did not achieve a stable level of antipyresis as body temperature immediately began to rise from the nadir and were at febrile levels by the end of the 2h of observation.

Figure 11 depicts the thermoregulatory response, in febrile rats, to intraperitoneal DMSO:saline, after VSA pretreatment with saline or the high dose of the  $V_1$  antagonist. In addition, the febrile response to icv endotoxin, without subsequent VSA or intraperitoneal injections is included (shaded area). In all three instances the fever plateaued between 180 and 210 min after icv endotoxin. There were no statistical differences in the febrile response to endotoxin in any of the groups. A similar experiment was conducted utilizing saline and both doses of  $d(CH_2)_5Tyr(Me)AVP$ , within the VSA, followed by intraperitoneal Tris buffer with similar results (Table II).

An additional control experiment was performed to assess the effects of VSA administration of saline and  $d(CH_2)_5Tyr(Me)AVP$  ( $5.0 \mu g/side$ ) on the core temperature of non-febrile rats (Figure 12). It is evident that neither saline nor the  $V_1$  antagonist evoked any consistent alterations in body temperature.

Figure 13 illustrates the mean fever indices for the animals receiving salicylate (Figure 13A) or acetaminophen (Figure 13B) following the VSA microinjection protocol. Compared with saline-treated controls ( $-1.91 \pm 0.20 \text{ } ^\circ C \cdot h$ ), pretreatment with  $d(CH_2)_5Tyr(Me)AVP$  significantly attenuated salicylate-induced antipyresis at both concentrations ( $-0.57 \pm 0.13 \text{ } ^\circ C \cdot h$ , low dose versus  $0.17 \pm 0.47 \text{ } ^\circ C \cdot h$ , high dose). In contrast, the antipyretic effects of acetaminophen were not affected by VSA pretreatment with either saline ( $-2.07 \pm 0.24 \text{ } ^\circ C \cdot h$ ) or the high dose of the  $V_1$  antagonist ( $-1.99 \pm 0.34 \text{ } ^\circ C \cdot h$ ).



The neural loci where  $d(CH_2)_5Tyr(Me)AVP$  was effective in antagonizing the antipyretic action of salicylate are depicted in the schematic histological sections of Figure 14.

## Discussion

The ability of antipyretic drugs to inhibit the formation of PG is well established. The action of these drugs is not limited to PG biosynthesis, however. Additional biological effects (see Gryglewski, 1974) often within the therapeutic dose range, have been observed indicating the activation of additional or alternate biochemical pathways by these drugs. Indomethacin, for example, acts as a calcium antagonist (Northover, 1977) and inhibits endogenous protein phosphorylation via a cyclic AMP-mediated protein kinase (Kantor and Hampton, 1978). The variable effects of several antipyretic drugs against arachidonic acid hyperthermia suggest that not all of these drugs act via the cyclooxygenase pathway (Clark and Cumby, 1976). Furthermore, the ability of antipyretic drugs to cause a parallel shift in the log dose-hyperthermic response relationship for leukocytic pyrogen, suggests that these drugs may compete allosterically for pyrogen at the receptor level (Clark, 1979).

The results reported here provide further evidence that alternative mechanisms of action for drug-induced antipyresis must be considered. The present data indicate that peripherally administered salicylate, when preceded by saline within the VSA, was an effective antipyretic with a duration of action of  $> 2h$ . However, when the  $V_1$  antagonist,  $d(CH_2)_5Tyr(Me)AVP$ , was similarly injected into the VSA prior to intraperitoneal salicylate, the effects of the salicylate were attenuated or abolished, depending on the dose of the antagonist. The abolition of salicylate-induced antipyresis was not merely due to a hyperthermic effect of  $d(CH_2)_5Tyr(Me)AVP$ , since the antagonist had no significant effects on core temperature when injected into the VSA of

non-febrile rats. In addition, neither the V<sub>1</sub> antagonist nor saline, administered within the VSA prior to intraperitoneal injections of drug vehicle solutions, significantly altered the normal progress of an endotoxin fever, compared to endotoxin alone. Although these control experiments (see Figure 11) appear inconsistent with previous reports (Cooper et al, 1987; Naylor et al, 1988), this may be related to the observations that vasopressin release within the VSA is lowest during the rising phase of fever (Cooper et al, 1979; Malkinson et al, 1987). Thus blockade of the receptors at this point would have little effect on fever height. However, our observations were only for a two hour period; it is not inconceivable therefore, that fevers in V<sub>1</sub> antagonist-treated animals may have been prolonged compared to saline controls.

The central locus of action of the observed effects of the V<sub>1</sub> antagonist on salicylate-induced antipyresis was within the VSA, but not surrounding tissue. This neuroanatomical site has been shown previously to be endowed with vasopressin axon terminals (DeVries et al, 1985) and receptors (Baskin et al, 1983) resembling the peripheral V<sub>1</sub> vasopressor subtype (Lawrence et al, 1988; Poulin et al, 1988). The VSA is sensitive to the antipyretic effects of exogenous vasopressin in virtually all species tested and the release of endogenous AVP has been detected from push-pull perfusates in a pattern that is consistent with an antipyretic role for the peptide (see page 43). As mentioned previously, the VSA is also the site where d(CH<sub>2</sub>)<sub>5</sub>Tyr(Me)AVP, at a similar dose to that used in this study, elevated and prolonged the fevers elicited by interleukin-1 (Cooper et al, 1987) and PGE<sub>1</sub> (Naylor et al, 1988). Thus a convergent link is evident for the neuronal mechanism(s) of certain antipyretic drugs and the AVP-endogenous antipyretic system.

The present results confirm (Alexander et al, 1989) and extend the work of previous studies (see chapter III). Using a different approach than that adopted here, Alexander et al. (1989) have found that the antipyretic action of direct VSA infusion

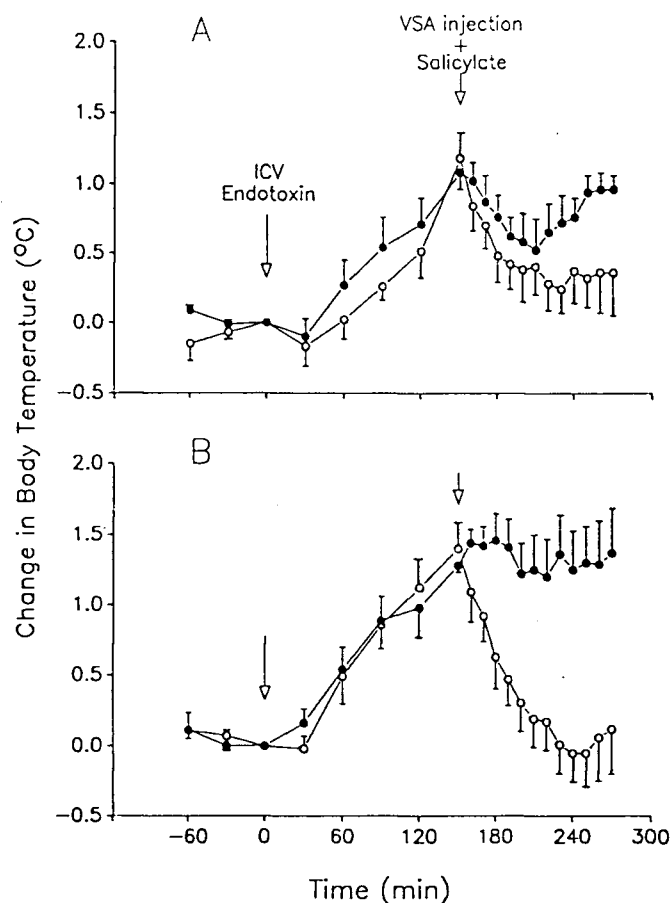


Figure 9. Change in body temperature (mean  $\pm$  SEM) in response to icv endotoxin at time 0 (first arrow). After 150 min (second arrow) a  $V_1$  antagonist (closed circles) or saline (open circles) were microinjected bilaterally into the ventral septal area (VSA), immediately followed by intraperitoneal sodium salicylate. A: The effect of 0.5  $\mu$ g  $V_1$  antagonist or saline administered bilaterally within the VSA, on salicylate-induced antipyresis. The mean baseline temperature for the  $V_1$  antagonist or saline treatment day were  $37.81 \pm 0.28$  and  $37.75 \pm 0.22$  °C respectively ( $n=5$ ). B: The effect of 5.0  $\mu$ g  $V_1$  antagonist or saline bilaterally administered within the VSA, on salicylate-induced antipyresis. The mean baseline temperatures for the  $V_1$  antagonist or saline treatment day were  $37.21 \pm 0.21$  and  $37.40 \pm 0.19$  °C respectively ( $n=7$ ). See Figure 13 for statistical analysis of data.

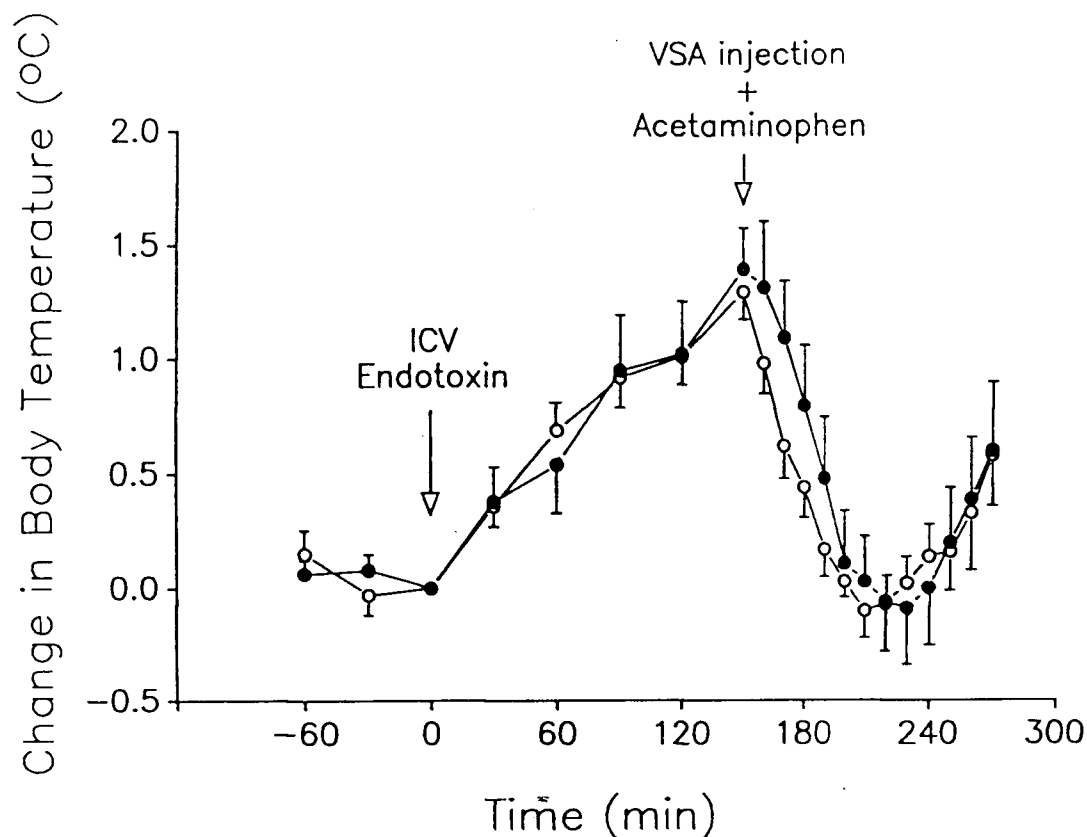


Figure 10. Change in body temperature (mean  $\pm$  SEM) in response to icv endotoxin at time 0 (first arrow). After 150 min (second arrow) 5.0  $\mu$ g of V<sub>1</sub> antagonist (closed circles; baseline body temperature,  $37.66 \pm 0.15$  °C) or saline (open circles; baseline body temperature,  $37.71 \pm 0.21$  °C) were microinjected bilaterally into the ventral septal area (VSA), immediately followed by intraperitoneal acetaminophen ( $n=8$ ). See Figure 13 for statistical analysis of data.

TABLE II. Cumulative change in body temperature (thermal index) for control experiments calculated from point of TRIS or DMSO:saline administration during fever.

THERMAL INDEX ( $^{\circ}\text{C}\cdot\text{h}$ )

V <sub>1</sub> antagonist (VSA, $\mu\text{g}/\text{side}$ )	TRIS (Intraperitoneal)	DMSO:saline	icv endotoxin (no VSA or ip injection) $0.79 \pm 0.21$ (6)
0 (saline)	$0.47 \pm 0.28$ (5)	$0.78 \pm 0.30$ (5)	
0.5	$1.01 \pm 0.28$ (5)	-	
5.0	$0.61 \pm 0.40$ (3)	$0.76 \pm 0.37$ (5)	

Numbers in parentheses denote n for each group. Animals received the VSA injection immediately followed by an intraperitoneal injection of vehicle solution. A separate group of 6 animals received endotoxin but did not receive subsequent VSA or intraperitoneal injections. In this group a thermal index was calculated from the same time as VSA and ip injected groups. There were no cases of statistical significance.

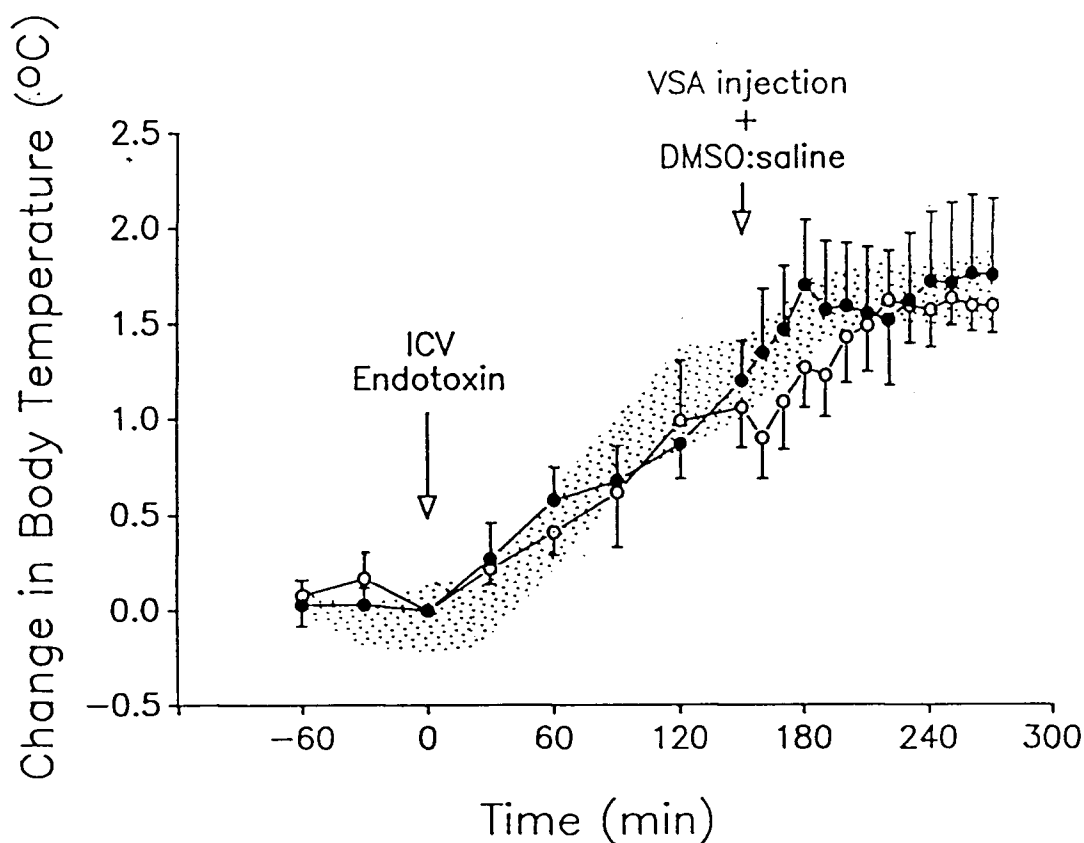


Figure 11. Body temperature response (mean  $\pm$  SEM) to icv endotoxin (first arrow) at time 0. After 150 min (second arrow) 5.0  $\mu$ g of  $V_1$  antagonist (closed circles; baseline body temperature,  $37.33 \pm 0.27$  °C) or saline (open circles; baseline body temperature,  $37.84 \pm 0.19$  °C) were microinjected bilaterally into the ventral septal area (VSA) immediately followed by intraperitoneal DMSO:saline (3:2) (n=5). In addition, a separate group of animals (n=6) received icv endotoxin without subsequent VSA or intraperitoneal injections (shaded area, mean  $\pm$  SEM. Baseline body temperature,  $38.09 \pm 0.17$  °C). See Table II for statistical analysis of data.

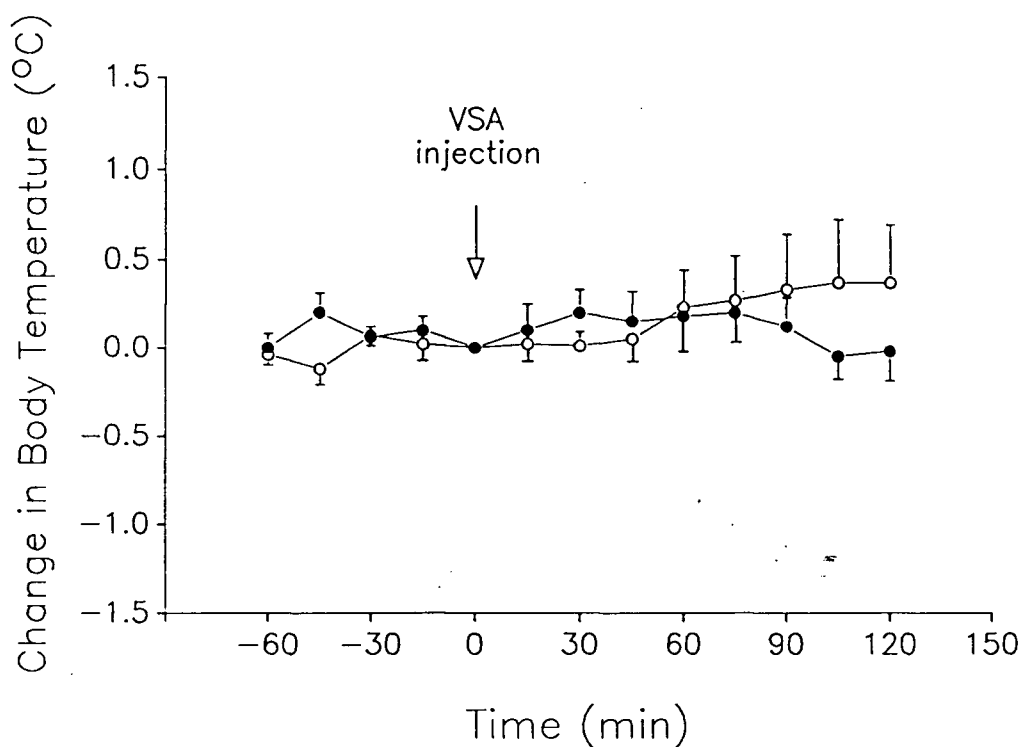


Figure 12. Body temperature response (mean  $\pm$  SEM) of non-febrile animals to bilateral administration of 5.0  $\mu$ g of V<sub>1</sub> antagonist (closed circles; baseline body temperature,  $37.49 \pm 0.14$  °C) or saline (open circles; baseline body temperature,  $37.55 \pm 0.21$  °C) within the ventral septal area (VSA) at time 0 ( $n=7$ ). These were not statistically different (ANOVA).

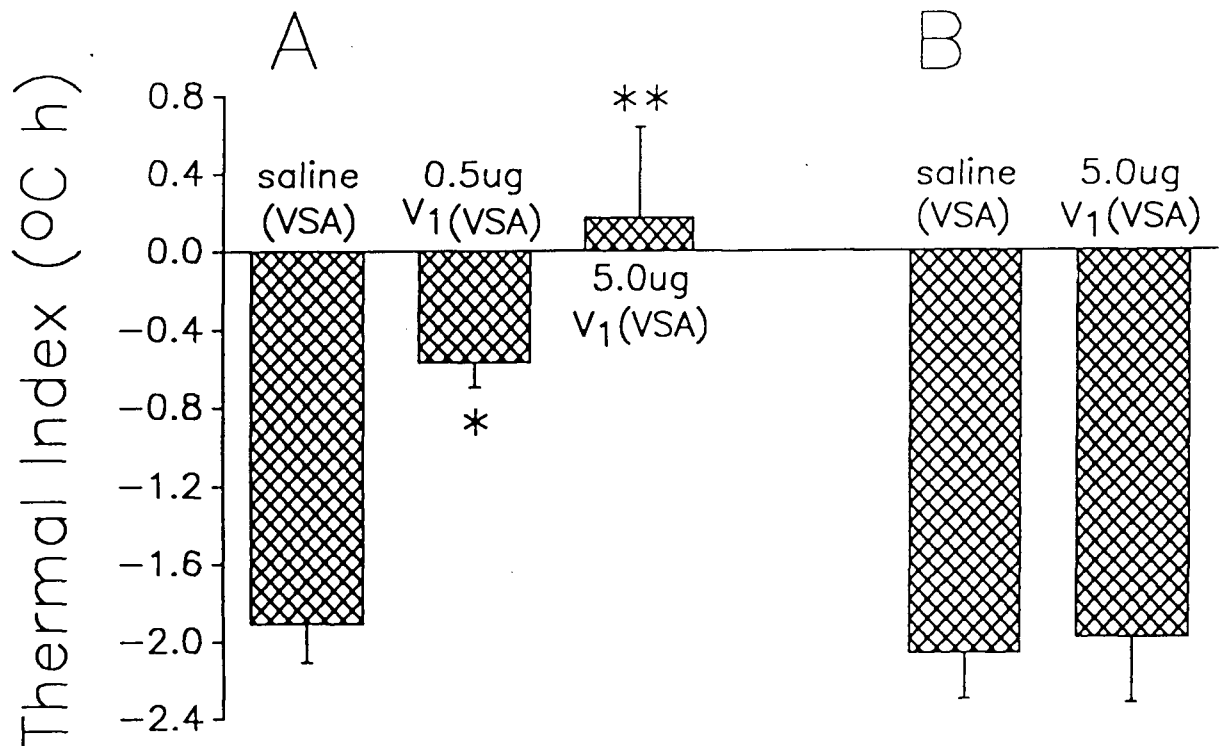


Figure 13. Cumulative 2h thermoregulatory responses (Thermal Index) to sodium salicylate or acetaminophen preceded by saline or V<sub>1</sub> antagonist within the ventral septal area (VSA) during endotoxin fever. A: Mean thermal indices ( $\pm$  SEM) of febrile animals receiving saline ( $n=13$ ), 0.5  $\mu$ g ( $n=5$ ) or 5.0  $\mu$ g ( $n=7$ ) of V<sub>1</sub> antagonist bilaterally into the VSA followed by intraperitoneal salicylate. \*  $p < 0.05$ , \*\*  $p < 0.01$  compared to saline control. ANOVA, Newman-Keuls post hoc test. B: Mean thermal indices ( $\pm$  SEM) of febrile animals ( $n=8$ ) receiving saline or 5.0  $\mu$ g V<sub>1</sub> antagonist bilaterally into the VSA followed by intraperitoneal acetaminophen. These were not statistically different.



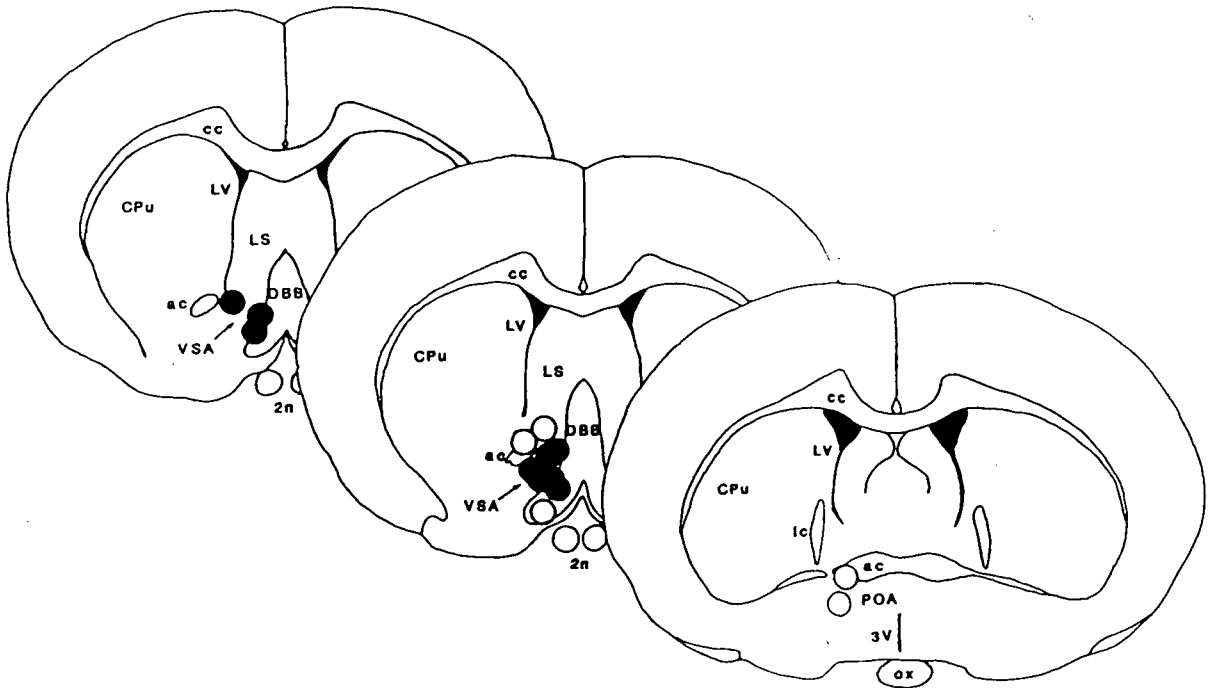


Figure 14. Representative histological sections depicting sites where the  $V_1$  antagonist effectively altered (solid circles) or did not affect (open circles) the antipyresis elicited by sodium salicylate. Loci from one side are shown for clarity. ac, anterior commissure; CPU, caudate putamen; DBB, diagonal band of Broca; ic, internal capsule; LS, lateral septum; LV, lateral ventricle; ox, optic chiasm; POA, preoptic area; VSA, ventral septal area; 2n, optic tracts; 3V, third ventricle.

with sodium salicylate can be abolished if  $\text{d}(\text{CH}_2)_5\text{Tyr}(\text{Me})\text{AVP}$  (or AVP antisera) is present in the infusate. Thus our results strengthen the hypothesis that salicylate acts at a central locus (the VSA) to elicit drug mediated defervescence.

Although it has been reported that antipyretic drugs cross the blood brain barrier poorly (Davison et al, 1961), it is likely that changes in the permeability of this barrier occur during fever, thereby increasing the likelihood of passage of certain small molecules (du Moulin et al, 1985). Indeed, peripherally administered salicylate is readily detectable within the hypothalamus during fever (Grundman, 1969; Rawlins et al, 1973).

In contrast to that observed after salicylate, the antipyresis induced by intraperitoneal acetaminophen, was not affected by vasopressin receptor blockade within the VSA. Although, like salicylate, acetaminophen appears to cross the blood brain barrier poorly (Davison et al, 1961), it seems unlikely that transient changes in the barrier observed during fever (du Moulin et al, 1985), would selectively favor salicylate over acetaminophen since they are both of similar molecular weight. In addition, we cannot claim the effects of the  $V_1$  antagonist as specific for antipyretic versus hypothermic actions since, at this dose, acetaminophen had no appreciable hypothermic effects (data not shown). Thus we have to conclude that the antipyretic mechanism of acetaminophen does not include activation of a central vasopressinergic pathway. It is interesting, however, that several differences exist between acetaminophen and the other antipyretic drugs we have examined. From the present data it is evident that the antipyretic profile of acetaminophen is quite different from both salicylate and indomethacin (see chapter III). The onset of acetaminophen-induced antipyresis had a rapid onset, generally reaching the maximum effect faster than salicylate. Moreover, the return to febrile temperature levels was equally prompt. Thus a marked difference in antipyretic effects was evident between salicylate and acetaminophen.

Acetaminophen also lacks anti-inflammatory activity, whereas both salicylate and indomethacin possess this property. Thus a consistency is apparent relating the possession of anti-inflammatory activity by an antipyretic drug and an AVP receptor interaction within the VSA. It has previously been assumed that anti-inflammatory characteristics of antipyretic drugs are due to the differential effects of these drugs on centrally or peripherally derived cyclooxygenase, this hypothesis, however, fails to adequately explain the results reported here (Flower and Vane, 1972).

From these data the mechanism by which salicylate interacts, or alternatively, why acetaminophen does not interact with VSA vasopressin receptors cannot be described with certainty. The possibility exists that certain antipyretic drugs, such as salicylate or indomethacin, enhance vasopressin release within the VSA. Thus the presence of the V<sub>1</sub> antagonist would negate the post-synaptic effects of the peptide and the action of the drug as well. It is also possible that a coupling of the vasopressin receptor with either a putative antipyretic drug receptor [specific binding sites for indomethacin have been demonstrated (Magous et al, 1985)] or the cyclooxygenase enzyme may occur. The concept of a vasopressin receptor-cyclooxygenase coupling is not a novel one, and indeed this relationship is well established in the kidney (see Abramow et al, 1987). A similar situation has been demonstrated on mononuclear phagocytes (Locher et al, 1983) as well as with vascular AVP receptors (Walker, 1985). In all these cases, vasopressin activation of cyclooxygenase is linked to a V<sub>1</sub> type receptor. Interestingly, the ability of salicylate to displace AVP from VSA receptors has been attempted in an *in vitro* binding assay (Poulin et al, 1988). However, the observed lack of effect maybe related to the fact that salicylate reaches the brain in greater concentrations (80-100  $\mu$ M) (Grundman, 1969; Rawlins et al, 1973) than that tested (1  $\mu$ M) by Poulin et al. (1988). This issue, therefore, remains to be resolved.

The results from this study indicate that the antipyretic mechanism of salicylate, like indomethacin, includes activation of vasopressin  $V_1$  receptors within the VSA. However, this is not the case for antipyretic drugs in general since VSA  $V_1$  blockade had no effect on acetaminophen-induced antipyresis. While the reasons for this discrepancy are unclear, it is apparent that the mode of action of antipyretic drugs is more than just an inhibition of PG biosynthesis and may include one or more parallel neural pathways.

## **V. VASOPRESSIN RELEASE WITHIN THE VENTRAL SEPTAL AREA DURING ANTIPYRESIS EVOKED BY INDOMETHACIN AND ACETAMINOPHEN**

### **Introduction**

From the preceding chapters (III and IV) and the work of others (Alexander et al, 1987, 1989) it is apparent that a central vasopressinergic pathway is an important component to the mechanism by which antipyretic drugs exert their febrile actions. These deductions were based on the observations that  $V_1$  receptor antagonism within the VSA, but not surrounding tissue, has dramatic effects on the antipyretic actions of salicylate and indomethacin. Curiously, this was not the case with acetaminophen-induced antipyresis as the degree of fever inhibition following this drug was the same regardless of whether or not a  $V_1$  blocker was administered within the VSA (chapter IV). However, the use of receptor antagonists does not completely address the question as to the precise mechanism whereby centrally acting AVP contributes to drug-elicited defervescence. At least two possibilities could account for the effects of antipyretic drug inhibition by vasopressin antagonism within the VSA; first, there may be a functional coupling between the vasopressin receptor and the cyclooxygenase enzyme, or, certain antipyretic drugs may stimulate synaptic release of vasopressin. The simplest approach would be to address the latter option first. Thus in order to substantiate the results with the AVP antagonists it was necessary to directly measure the release of the endogenous  $V_1$  receptor ligand. To this end, the technique of push-pull perfusion was utilized and AVP levels within the extracellular fluid of the VSA was measured during fever and drug-induced antipyresis. In this way, it was hoped that a more definitive explanation for the previous observations (see chapter III) could be derived.

## Methods

Animals were surgically prepared with a unilateral "pull" cannula, whose tip lay within the VSA, and a contralateral icv cannula as described in chapter II. These cannulae were occluded with snug fitting stylets. Biotelemetry devices were implanted intraperitoneally for the remote monitoring of body temperature. During surgical recovery, the animals were briefly handled each day to minimize stress during an experiment.

The push-pull perfusion technique was employed in two randomly assigned experimental groups: non-febrile and febrile. The perfusion system is illustrated in Figure 2 (page 60). The perfusate was an artificial cerebrospinal fluid (aCSF) with the following composition (in mM): NaCl 124, KCl 4.9, NaHCO<sub>3</sub> 25.9, NaH<sub>2</sub>PO<sub>4</sub> 1.2, MgSO<sub>4</sub> 4.0, glucose 9.9, CaCl<sub>2</sub> 1.9, bacitracin 0.10. By means of the push cannula assembly, both the push and the pull cannulae were connected to an infusion/withdrawal pump (Harvard) by means of polyethylene tubing. The rate of infusion/withdrawal had been calibrated previously to be 20  $\mu$ L/min. Prior to the start of an experiment the stylets were removed from both cannulae. The perfusion procedure began by filling all lines with aCSF and inserting the push cannula assembly into the pull cannula, with the pump running, in order to fill the pull cannula with aCSF. Once this cannula was filled the push cannula assembly was fully inserted into the pull cannula, such that the tip extended 0.5 mm past the pull cannula into the VSA tissue. To obtain an air tight seal the interface was sealed with a cuff of PE-50. A steady flow was ensured prior to collecting perfusion fluid. Partial or complete blockages of the push-pull cannula could be detected immediately via an on-line pressure transducer connected to a chart recorder. Four consecutive 30 min VSA perfusions were carried out in each experiment. Perfusion fluid was collected on ice in trap lines calibrated to hold 600  $\mu$ L. When one line was filled the other could be

opened by means of an adjustable valve. In this way perfusions could progress uninterrupted. The initial 15 min perfusion (300  $\mu$ L) sample was discarded. Samples were stored at - 70 °C until assay for AVP.

Prior to the start of the perfusion, animals in the non-febrile group received 5  $\mu$ L of saline while the febrile group received 10 ng of *E. coli* endotoxin in 5  $\mu$ L of saline icv. In order to obtain a pre-drug sample, the perfusion was started 105 min after the icv injection. Thus after discarding the initial 15 min of perfusate the first 30 min sample was representative of the pre-drug AVP levels. At 150 min post-endotoxin (or saline) indomethacin (7.5 mg/kg), acetaminophen (75 mg/kg) or Tris buffer (1 mL/kg) were administered intraperitoneally. Subsequent perfusion periods would thus reflect peptide release during drug-induced antipyresis in the endotoxin-treated rats while saline injected animals would serve as the non-febrile control.

In order to obtain an estimate of the exchange rate of the push-pull cannula, perfusions were carried out *in vitro* in a 250 mL beaker containing a known amount of synthetic AVP (40 pg/mL). A series of 6 *in vitro* perfusions were performed. The volume of perfusate collected was 250  $\mu$ L. An aliquot of 100  $\mu$ L was removed directly from the beaker prior to the start of each perfusion period. The perfusates and the beaker aliquots were then subjected to AVP assay. The volume removed from the beaker was neglected in the calculation of the exchange rate.

Statistical analysis was performed on the data using a one way analysis of variance, after log transformation, followed by the Newman-Keuls test for multiple comparisons. Significance was considered with  $p < 0.05$ .

## Results

The thermoregulatory responses to the intraperitoneal injection of indomethacin, acetaminophen and Tris buffer in both experimental groups are shown in Figure 15 and 16. Perfusion of the VSA had no apparent effect on the development of fever following endotoxin. In addition, antipyretic intervention with indomethacin or acetaminophen resulted in typical responses to these drugs (see Figure 4 and 10).

AVP concentrations in VSA perfusion fluid following antipyretic drug treatment is shown in Figure 17A and 17B. Prior to the administration of antipyretics, vasopressin levels, as determined from the 30 min interval between 120 and 150 min post-endotoxin, ranged from 0.57 to 2.70 pg/30 min sample in all groups. These levels were consistent within each experimental group but tended to vary between groups. Because of this, statistical evaluation was made on within group values only. Following intraperitoneal indomethacin, AVP levels increased significantly from  $0.85 \pm 0.11$  to  $1.60 \pm 0.22$  pg/sample ( $p < 0.05$ ). AVP levels in subsequent perfusion periods remained elevated but not significantly so. In contrast to this acetaminophen was without effect on central AVP release despite being utilized at a comparable antipyretic dose. Similarly, Tris buffer was without effect on AVP release from VSA push-pull perfusates (Figure 17A).

Figure 16 illustrates the thermoregulatory responses in non-febrile rats during VSA push-pull perfusion and subsequent intraperitoneal injections of indomethacin, acetaminophen and Tris buffer. In Tris-treated animals a significant hyperthermia ( $p < 0.05$ ) developed after 90 min of VSA perfusion. This effect was attenuated by indomethacin but not by acetaminophen. Extracellular vasopressin levels under these conditions is shown in Figure 17B. Despite the marked changes in body temperature, no significant changes in AVP levels were detected.



In order to obtain an estimate of the exchange rate of the push-pull cannula aliquots from a beaker containing a known concentration of AVP were subjected to AVP assay along with push-pull perfusates from the same beaker. Thus when the measured concentration of AVP from the push-pull perfusates was divided by the measured concentration of AVP obtained directly from the beaker the exchange rate was calculated to  $10.5 \pm 2.0\%$ . Therefore it can be assumed that when perfusing living neural tissue, concentrations of AVP in the push-pull perfusates represent approximately 10% of the AVP present in the extracellular fluid in and around the cannula tip.

The neural loci of all reported AVP levels were within the VSA. In 4 animals with perfusion loci located outside the VSA (which included areas rostral and ventral to the VSA) the mean AVP levels were  $0.71 \pm 0.24$  pg/sample before indomethacin to  $0.64 \pm 0.07$  pg/sample after administration of the drug. These were not significantly different. The mean values of the last 2 perfusion periods were 0.56 and 0.79 pg/sample respectively. Figure 18 is a photomicrograph of a 44  $\mu\text{m}$ -thick coronal brain section containing the VSA and surrounding neural structures. The perfusion locus and the lesion resulting from the push-pull perfusion procedure is indicated by the arrow.

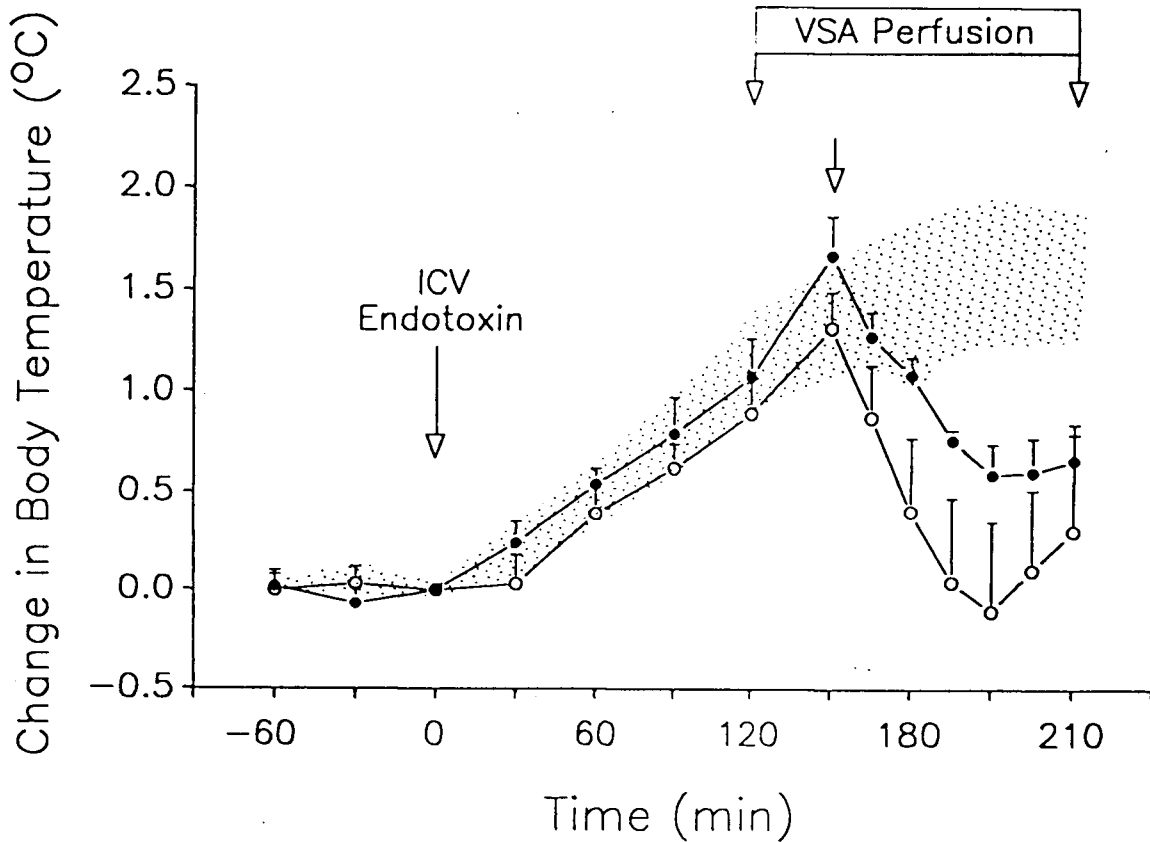


Figure 15. Thermoregulatory response (mean  $\pm$  S.E.M.) to endotoxin (time 0) followed 150 min later by indomethacin (closed circles; baseline body temperature,  $37.44 \pm 0.13$  °C,  $n=8$ ), acetaminophen (open circles; baseline body temperature,  $37.88 \pm 0.23$  °C,  $n=7$ ) or Tris buffer (shaded area; baseline body temperature,  $37.45 \pm 0.11$  °C,  $n=8$ ). The push-pull perfusion period is denoted by the horizontal bar.

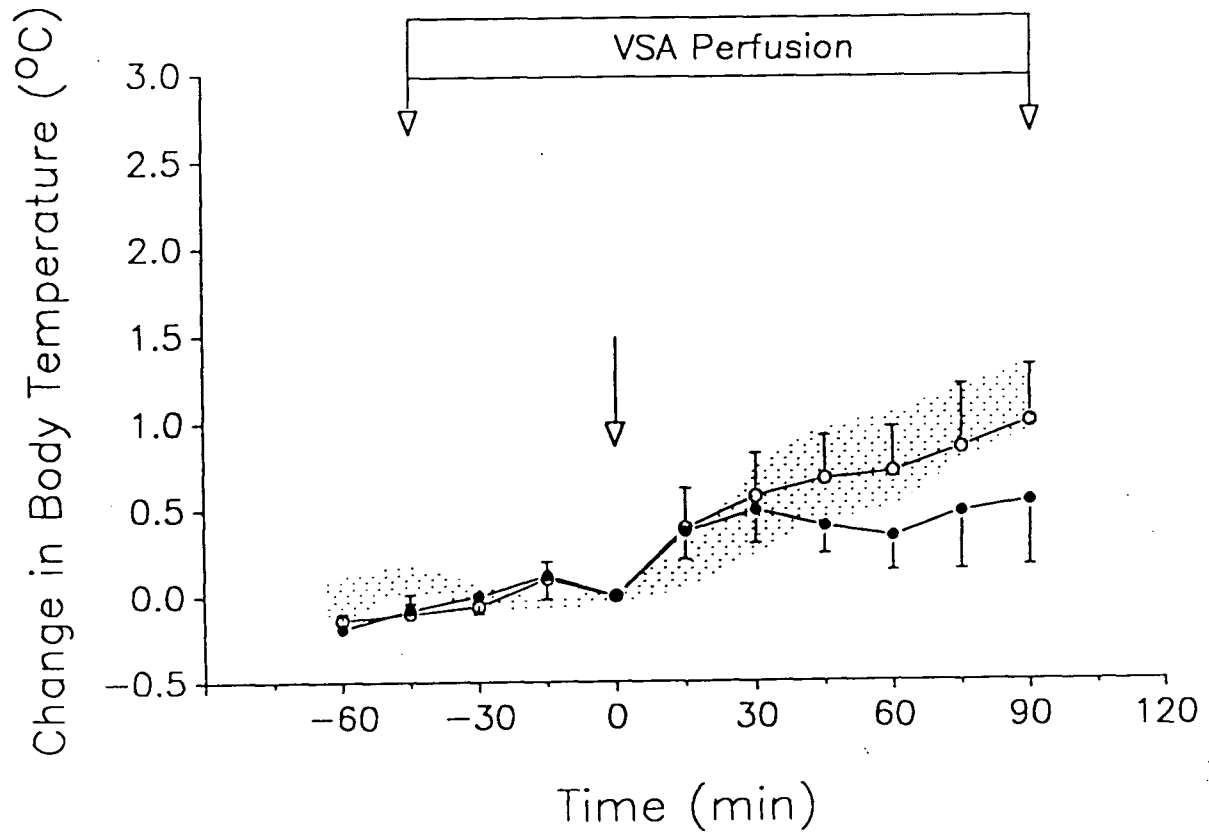


Figure 16. Thermoregulatory response (mean  $\pm$  S.E.M.) in non-febrile rats treated intraperitoneally, at time 0 (arrow), with indomethacin (closed circles; baseline body temperature,  $37.90 \pm 0.25$  °C,  $n=8$ ), acetaminophen (open circles; baseline body temperature,  $37.51 \pm 0.07$  °C,  $n=6$ ) or Tris buffer (shaded area; baseline body temperature,  $37.26 \pm 0.25$  °C,  $n=8$ ). The push-pull perfusion period is denoted by the horizontal bar.

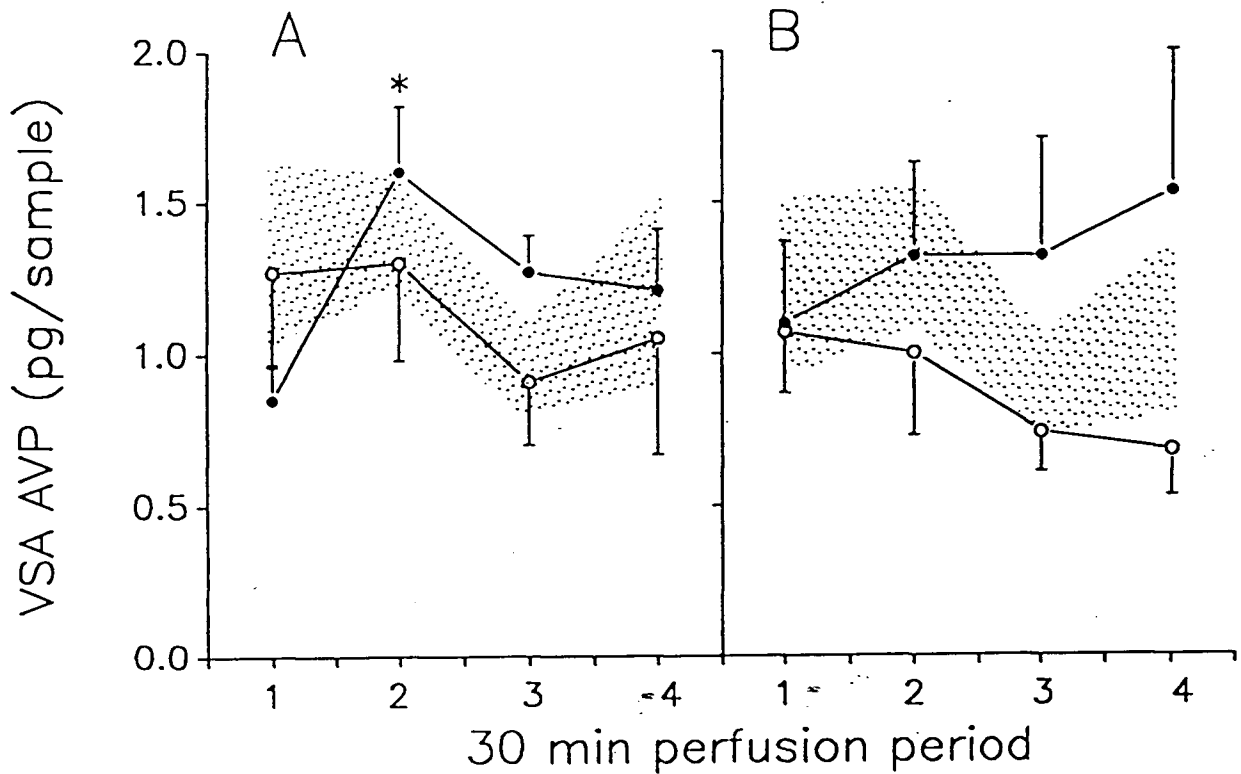


Figure 17. Panel A: Vasopressin levels in 30 min VSA push-pull perfusates of febrile rats before (period 1) and after (periods 2 - 4) indomethacin (solid circles,  $n=8$ ), acetaminophen (open circles,  $n=7$ ) or Tris buffer (shaded area,  $n=7$ ). Panel B: Vasopressin levels in VSA push-pull perfusates of non-febrile rats before and after intraperitoneal administration of indomethacin (closed circles,  $n=8$ ), acetaminophen (open circles,  $n=6$ ) or Tris buffer (shaded area,  $n=8$ ). \*  $p < 0.05$ , ANOVA, Newman Keuls.

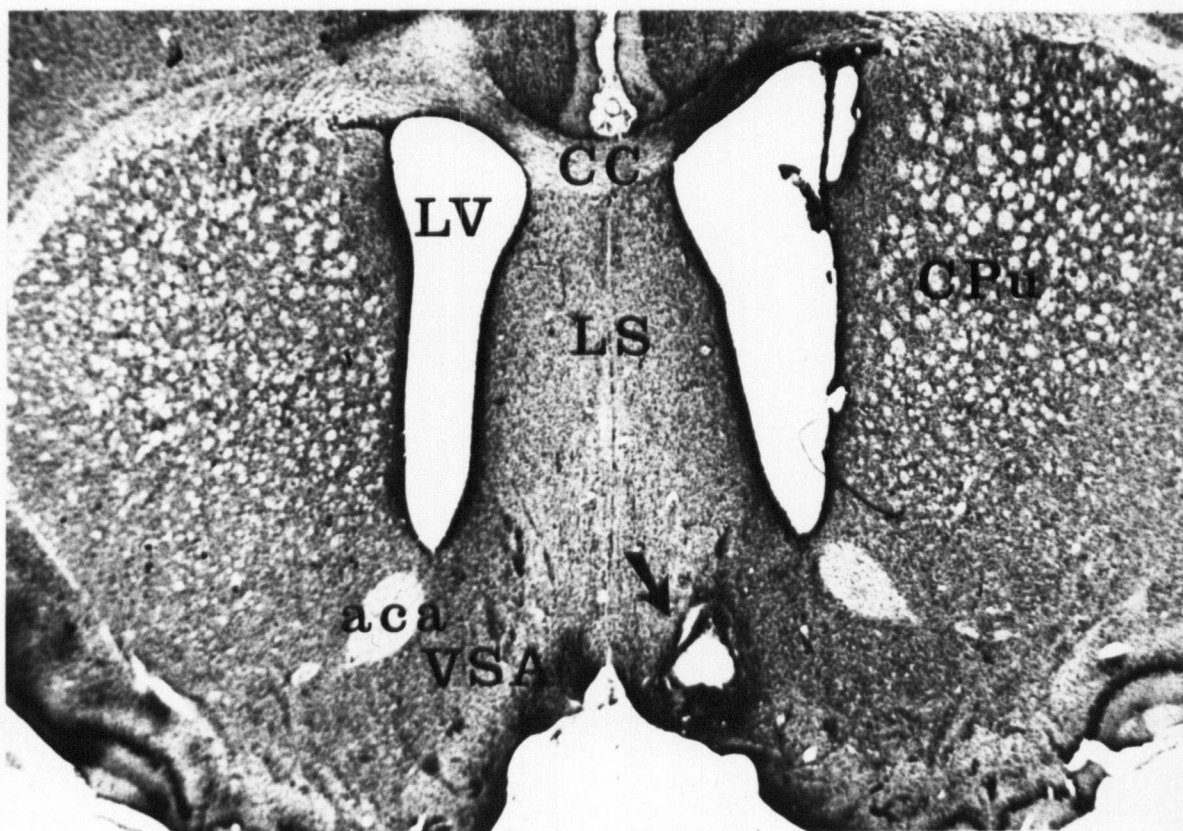


Figure 18. Photomicrograph of a representative coronal section of the VSA and surrounding neural structures. The perfusion locus is indicated by the arrow. aca, anterior commissure; CC, corpus callosum; LS, lateral septum; LV, lateral ventricle; VSA, ventral septal area.

## Discussion

Local release of AVP within the VSA of conscious and unrestrained rats was demonstrated during fever and the antipyresis induced by two different antipyretic drugs. Administration of indomethacin during an endotoxin-evoked fever resulted in an antipyretic response that was accompanied by a significant increase in AVP levels from VSA push-pull perfusates. Unlike that observed following indomethacin, however, the reversal of fever by acetaminophen was not accompanied by significant alterations in VSA AVP levels. Furthermore, intraperitoneal Tris buffer was similarly without effect on vasopressin release. Thus the effect of indomethacin on the release of AVP could not be ascribed to its effects on temperature *per se* or through a non-specific stress reaction due to the injection procedure.

The effects of indomethacin and acetaminophen on the release of vasopressin within the VSA are consistent with previous studies utilizing vasopressin receptor antagonists (chapters III and IV). In these studies VSA V<sub>1</sub> receptor blockade resulted in attenuation of the antipyresis induced by indomethacin but not acetaminophen. Thus the increased levels of AVP following indomethacin indicates that this drug is at least partially responsible for this releasing effect and supports the hypothesis that indomethacin promotes the release of AVP to mediate its antipyretic action.

Both indomethacin and acetaminophen are predominantly characterized by their inhibitory actions on PG biosynthesis. It seems unlikely, however, that this characteristic is sufficient to fully explain the present results. The effects of PGs on peripheral AVP release is largely a stimulatory action. Thus, centrally administered PGE<sub>2</sub> results in increases in plasma AVP (Yamamoto et al, 1976; Hoffman et al, 1982; Hashimoto et al, 1989), while the use of PG synthesis inhibitors tends to suppress peripheral release of the peptide (Hoffman et al, 1982; Walker, 1983a,b). Because

stimulation of peripheral and central AVP are often linked (Demotes-Mainard, 1986; Landgraf et al, 1988) it would seem unlikely that an effect on endogenous PGs could explain the stimulatory action of indomethacin on central AVP release. The lack of effect of acetaminophen on AVP levels in VSA perfusates also supports this argument, since at the concentrations used acetaminophen would have similar inhibitory actions on PG synthesis. Further, it has also been observed that indomethacin and an equipotent PG synthesis inhibitor, sodium meclofenamate, have differential actions on the cardiovascular and thermoregulatory effects of icv delivered PGE<sub>2</sub> (Sirén, 1982). Indeed, the partial antagonism of PGE<sub>2</sub>-induced hyperthermia by indomethacin (Sirén, 1982) is consistent with the results presented here. Collectively, it seems unlikely that the effects of indomethacin on VSA AVP release can be fully explained by PG synthesis inhibition.

The AVP concentrations in the perfusates from non-febrile rats are also supportive of the above conclusions. Indeed, the reduction, by indomethacin, of the hyperthermia that accompanied VSA perfused animals was not a sufficient condition for the promotion of AVP release within the VSA. The presence of the pyrogen (endotoxin) as well as the indomethacin is apparently an essential requirement for the promotion of AVP release. The hyperthermic response observed in non-febrile rats may be related to the perfusion procedure *per se* since administration of Tris buffer did not evoke hyperthermia in rats not undergoing VSA push-pull perfusion (see chapter VI). However, it has been observed in preliminary studies utilizing urethane-anaesthetized rats that hyperthermia does not occur during VSA push-pull perfusion. This has subsequently been reported in the literature (Langraf et al, 1990b). Differences in thermoregulatory responses between anaesthetized and conscious animals is not an uncommon observation (for e.g. McCain and Mundy, 1987; Malkinson et al, 1988) and may explain the present observations.

The differential effect of two cyclooxygenase inhibitors on the perfusion-induced hyperthermia does not support a prostaglandin-mediated mechanism for this response. Other studies have provided evidence which suggests that neuronal damage to thermoregulatory sensitive areas results in hyperthermia by the local release of prostaglandins (Rudy et al, 1977; Quan and Blatteis, 1989). Because the perfusion fluid makes physical contact with neural tissue, increases in body temperature could develop via the stimulation or inhibition of neurally active substances. Alternatively, actual physical stimulation, by the perfusate, of the neural elements may be enough to increase body temperature.

The release of AVP into push-pull perfusates has been demonstrated previously to be due to the dynamic activity of functioning neurons within the perfusion sphere and not the result of non-specific leakage from damaged neurons (Demotes-Mainard et al, 1986; Landgraf et al, 1988). Moreover, vasopressin release into septal perfusates is not due to leakage from the plasma (Mens et al, 1983) or CSF (Landgraf et al, 1988; see also chapter VII). Indeed the perfusion system employed in the present studies is virtually identical to that used by Landgraf et al (1988). The results obtained, therefore, can assumed to be due to the functioning of intact neurons. Although the perfusion process does induce some neuronal damage (see Figure 18) this is apparently not enough to effectively alter normal thermoregulatory functioning during fever. However, this cannot necessarily be claimed for non-febrile thermoregulation.

Although not directly comparable, the amount of AVP released in response to indomethacin seems modest when compared with the concentrations of antagonist required to block this response. It has been reported that oxytocin release often accompanies central AVP release (Landgraf et al, 1988; Neumann et al, 1988; Landgraf et al, 1990a,b). It has also been shown that oxytocin dramatically sensitizes the antipyretic response to exogenously administered AVP (Poulin and Pittman, 1989).



Thus it is possible that oxytocin may play an important role in sensitizing the AVP receptors such that only modest concentrations of the peptide are required to exert a maximal effect. Because the  $V_1$  antagonist employed in these studies (see chapter III and IV) has weak anti-oxytocic activity, larger concentrations of the analogue may be required to effectively attenuate the endogenous activity of both oxytocin and vasopressin. While the present study did not examine oxytocin release during antipyresis it is a distinct possibility that simultaneous release of the two neuropeptides plays an important role in the endogenous antipyretic system. Previous studies have administered oxytocin into the VSA (Naylor et al, 1986; Fyda et al, 1990) or the cerebral ventricles (Kovacs and De Wied, 1983) and found that it is without thermoregulatory effects. Thus it would be of great interest to examine more closely the interactions of AVP and oxytocin during fever and antipyresis by utilizing experimental protocols involving simultaneous administration of these two neuropeptides.

The results of this study provide direct evidence that during endotoxin-induced fever indomethacin increases the endogenous extracellular levels of AVP in the VSA, the AVP-sensitive antipyretic locus. This action is consistent with the previous observations that AVP receptor blockade within the VSA renders animals hyporesponsive to the antipyretic effects of indomethacin. Although these results cannot directly rule out the previously held prostaglandin hypothesis (reviewed in chapter III and IV), it is apparent that mechanisms other than prostaglandin inhibition are utilized by certain antipyretic drugs; in this case indomethacin.

## **VI. VASOPRESSIN RELEASE INTO THE VSA DURING PGE<sub>2</sub>-INDUCED HYPERTHERMIA AND THE EFFECT OF INDOMETHACIN**

### **Introduction**

The case for the inhibition of PG biosynthesis as the mechanism of action for antipyretic drugs is strongly supported by the observations that the hyperthermia evoked by centrally administered PGs is unaffected by non-steroidal antipyretic agents (reviewed in Clark, 1979). Because antipyretic drugs are traditionally thought to reduce fever by blocking the PG synthesis pathway it follows that these compounds should be ineffective against the thermal reaction evoked by exogenously administered PGs. Evidence presented in this thesis and by others (for e.g. Sirén, 1982; Alexander et al, 1987,1989) tends to suggest that alternate explanations are required to fully explain the diverse physiological actions of antipyretic drugs. The previous chapter offered evidence substantiating the studies utilizing vasopressin receptor antagonists, which collectively suggest that a vasopressinergic mechanism is an important central component in the mechanism of action of some antipyretic drugs.

Previous investigations have shown that PG-mediated fevers are sensitive to the central antipyretic action of AVP (Ruwe et al, 1985b; Fyda et al, 1990) and that blockade of VSA V<sub>1</sub> receptors augments PGE<sub>1</sub>-induced hyperthermia in conscious (Naylor et al, 1988) and anaesthetized rats (Landgraf et al, 1990b). These studies suggest that the AVP endogenous antipyretic system is activated during the thermoregulatory responses to PGE<sub>1</sub>, although failure of VSA V<sub>1</sub> antagonism to enhance PGE<sub>1</sub> hyperthermia has been reported (Fyda et al, 1989). Nevertheless, it was of interest to examine the effects of indomethacin on the central release of vasopressin

during PGE<sub>2</sub>-induced hyperthermia. In doing this study, and given the possibility of an active AVP role in indomethacin-induced antipyresis, it was hoped that the results might help to explain why antipyretic drugs are ineffective against PG-mediated hyperthermias.

## Methods

Animals were surgically prepared with cannulae for icv injections of PGE<sub>2</sub> and for push-pull perfusion of the VSA as described in detail previously (chapter II and V). Body temperature was measured via biotelemetry. PGE<sub>2</sub> (200 ng) or saline (5  $\mu$ L) was injected icv, by gravity flow, just prior to collecting the first of four consecutive 30 min VSA push-pull perfusions. Immediately following the icv injections Tris buffer or indomethacin (7.5 mg/kg) was administered intraperitoneally. Perfusion loci were verified histologically as in chapter V. Vasopressin content within the VSA perfusates was measured via radioimmunoassay. Statistical analysis utilized the one way ANOVA followed by Newman-Keuls test. AVP levels were subjected to logarithmic transformation prior to statistical evaluation.

## Results

Intracerebroventricular administration of PGE<sub>2</sub> evoked a brisk monophasic hyperthermia that was maximum ( $1.86 \pm 0.30$  °C) after 30 min ( $p < 0.01$ , compared to time 0). Thereafter, body temperature quickly fell toward baseline (Figure 19). This effect was unaltered during VSA push-pull perfusion or by intraperitoneal administration of indomethacin. In control animals a significant ( $p < 0.05$ ) hyperthermic response occurred during the perfusion period that was attenuated by indomethacin (Figure 20).

Analysis of AVP levels in VSA perfusates revealed a significant ( $p < 0.05$ ) increase in AVP in the first perfusion period following icv PGE<sub>2</sub> (Figure 21A). AVP values increased from  $1.37 \pm 0.34$  to  $7.1 \pm 3.46$  pg/sample. Two of these animals had AVP levels as high as 18.3 and 22.4 pg/sample following the PGE<sub>2</sub> injection. When PGE<sub>2</sub> was immediately followed by indomethacin, vasopressin levels were not significantly different from pre-PGE<sub>2</sub> levels. In control animals AVP release remained unaffected (Figure 21B).

## Discussion

Results presented previously in this thesis have shown that a vasopressinergic pathway converging upon the VSA appears to play an important role in the mediation of indomethacin and salicylate-induced antipyresis. Furthermore, AVP release from VSA push-pull perfusates is enhanced in the first 30 min period following peripherally administered indomethacin but only during a febrile episode. The present experiments were performed in order to assess the ability of intraperitoneal indomethacin to promote AVP release during the hyperthermia evoked by PGE<sub>2</sub>. If indomethacin stimulates the release of an endogenous antipyretic during fever, then the analysis of AVP release during PGE<sub>2</sub>-induced fever may help to explain why peripherally administered antipyretics have no effect on body temperature under these conditions. This is particularly important because exogenous AVP, administered into the VSA suppresses PG-elicited hyperthermias (Ruwe et al, 1985b; Fyda et al, 1990).

The present results confirm the work of Landgraf et al (1990b) who showed, using push-pull perfusion, that PGE<sub>1</sub> administered into the lateral ventricles of anaesthetized rats prompted, significantly, VSA AVP release. Thus in the conscious rat, PGE<sub>2</sub> administered icv, has similar effects on both body temperature and VSA AVP release. However, the present data from experiments utilizing indomethacin,

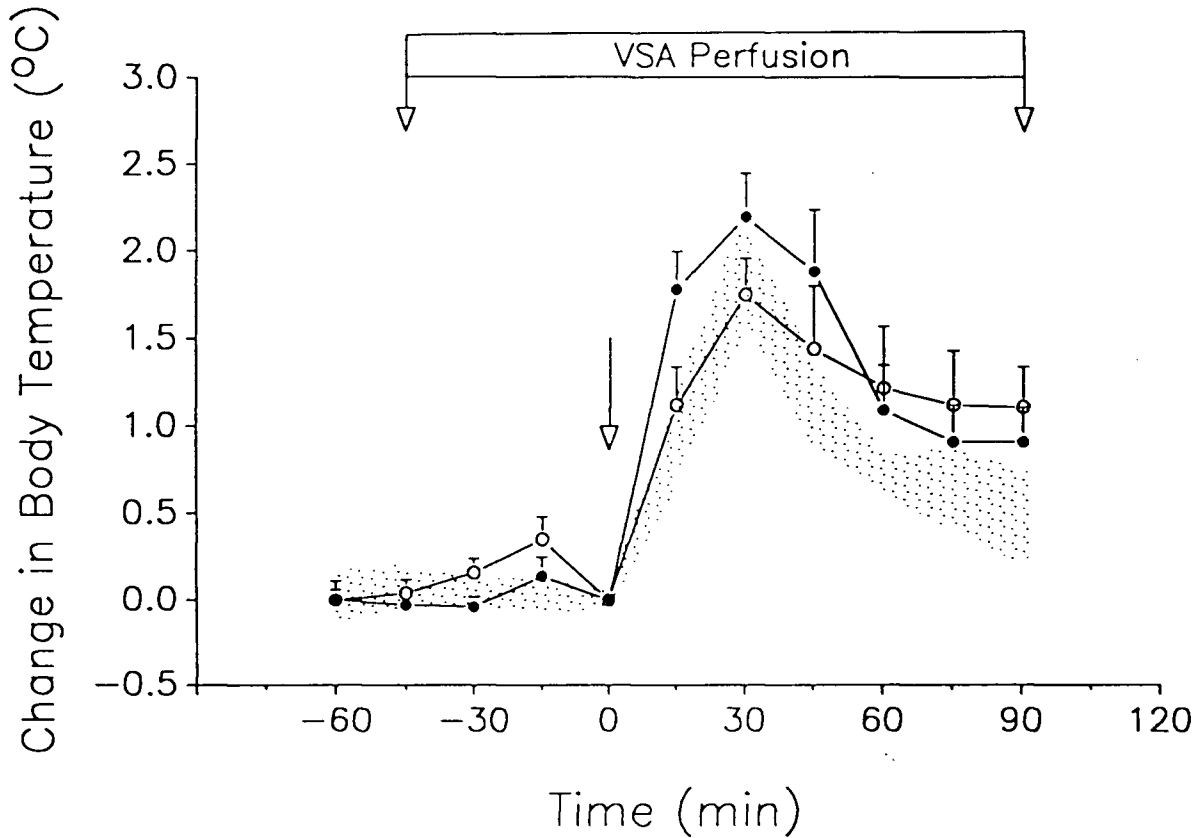


Figure 19. Mean change ( $\pm$  S.E.M.) in body temperature ( $^{\circ}\text{C}$ ) in response to  $\text{PGE}_2$  administered icv at time 0 (arrow) without indomethacin (open circles), with indomethacin (closed circles) or  $\text{PGE}_2$  alone without push-pull perfusion (shaded area). Duration of VSA perfusion is indicated by the horizontal bar. Mean baseline temperatures:  $\text{PGE}_2$  (with perfusion),  $37.80 \pm 0.15$   $^{\circ}\text{C}$ ,  $n=7$ ;  $\text{PGE}_2$  + indomethacin,  $37.59 \pm 0.33$   $^{\circ}\text{C}$ ,  $n=7$ ;  $\text{PGE}_2$  (no perfusion),  $37.50 \pm 0.20$   $^{\circ}\text{C}$ ,  $n=6$ .

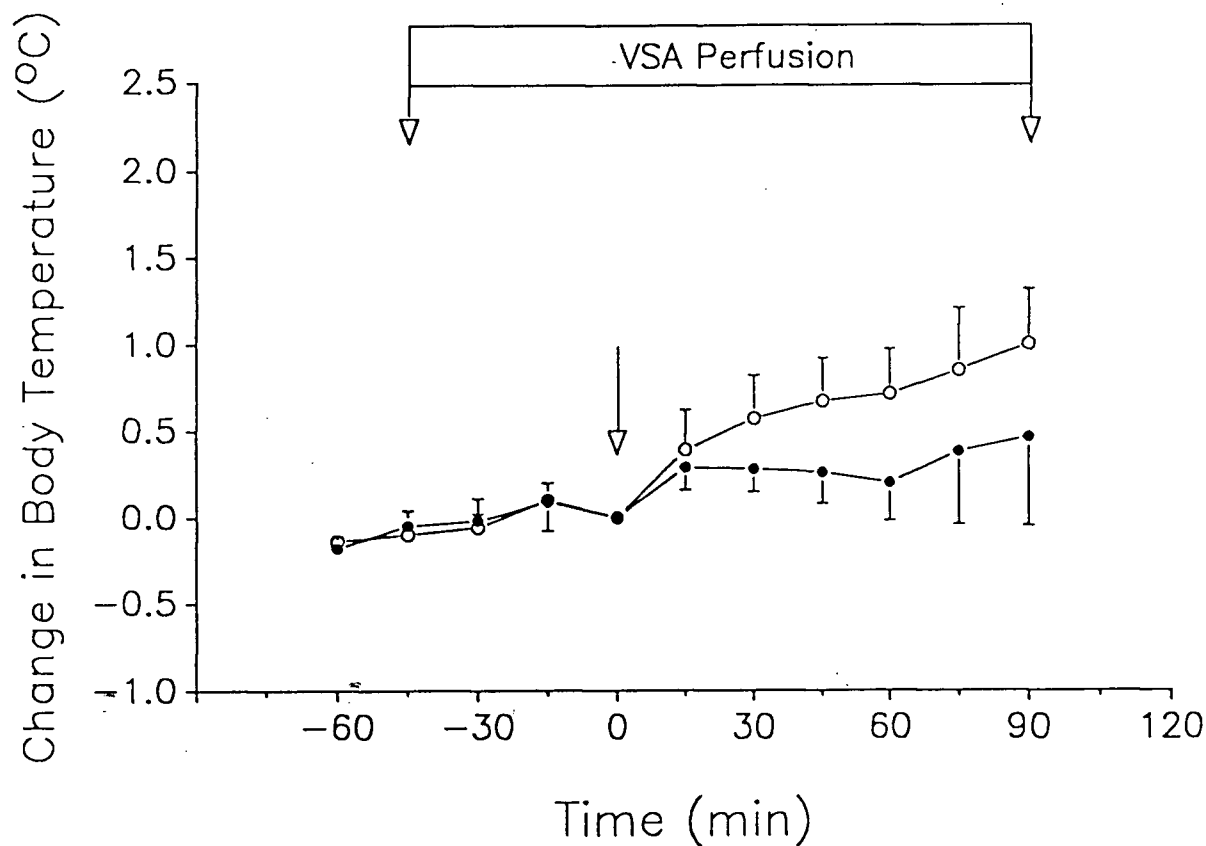


Figure 20. Mean change ( $\pm$  S.E.M.) in body temperature ( $^{\circ}\text{C}$ ) in response to saline administered icv at time 0 (arrow) followed by indomethacin (closed circles) or Tris buffer (open circles). Duration of VSA perfusion is indicated by the horizontal bar. Mean baseline temperatures: indomethacin,  $37.65 \pm 0.23$   $^{\circ}\text{C}$ ,  $n=6$ ; Tris buffer,  $37.26 \pm 0.25$   $^{\circ}\text{C}$ ,  $n=8$ .

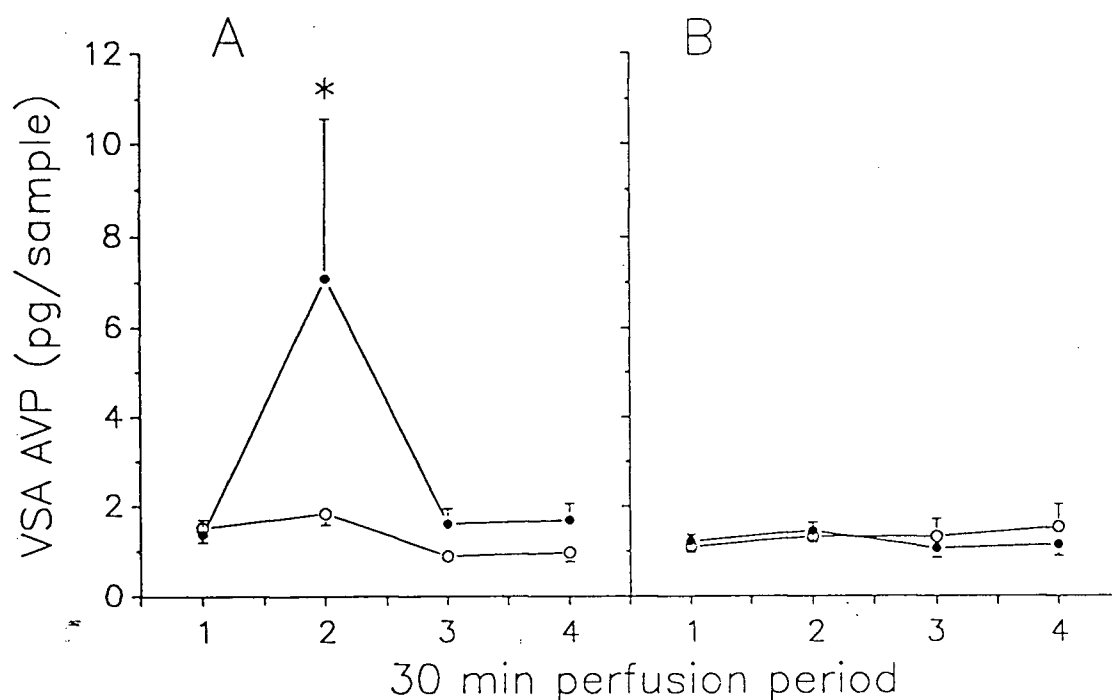


Figure 21. Mean ( $\pm$  S.E.M.) AVP concentrations in push-pull perfusates of the VSA before icv injection (period 1) and 90 min after (period 2 - 4). Panel A: AVP levels in VSA push-pull perfusates following icv administration PGE<sub>2</sub> + Tris (closed circles, n=6) and PGE<sub>2</sub> + indomethacin (open circles, n=7). Panel B: AVP levels in VSA push-pull perfusates following icv administration of saline followed by Tris (closed circles, n=8) and icv saline followed by indomethacin (open circles, n=7). \*  $p < 0.05$ , ANOVA, Newman-Keuls, compared to sample 1.

were somewhat surprising in that the drug suppressed the PGE<sub>2</sub> stimulated AVP levels. This AVP suppression occurred without significant effect on the thermoregulatory response to PGE<sub>2</sub>. This suggests that the AVP released in response to centrally administered PGE<sub>2</sub> is not involved in the regulation of a PGE<sub>2</sub>-mediated fever and is inconsistent with studies showing enhancement of PG-mediated fever during VSA V<sub>1</sub> blockade (Naylor et al, 1988; Landgraf et al, 1990b). Indeed, one would expect an elevation of PG hyperthermia if the AVP released in response to PGE<sub>2</sub> is acting to negatively modulate febrile body temperature.

In addition to studies utilizing vasopressin antagonists (Naylor et al, 1988; Landgraf et al, 1990b), the existing evidence suggests an active involvement of the VSA antipyretic system during the thermoregulatory responses to PGE<sub>1</sub>. For example, castration markedly affects the synthesis of AVP in the vasopressinergic neurons of the bed nucleus of the stria terminalis (BST) (DeVries et al, 1984; DeVries et al, 1985). The BST is a major source of vasopressinergic nerve fibres to the VSA (DeVries et al, 1985; Disturnal et al, 1985). Thus it has been observed that in castrated rats PGE<sub>1</sub> induces a markedly enhanced hyperthermic response (Pittman et al, 1988). Furthermore, kainic acid lesions of the VSA result in similar thermoregulatory responses when lesioned animals are challenged with PGE<sub>1</sub> (Martin et al, 1988). The present results are not consistent with these reports. However, these data are consistent with the results of Fyda et al (1989) who failed to demonstrate augmentation of PGE<sub>1</sub> hyperthermia with AVP V<sub>1</sub> antagonism within the VSA. Clearly, more studies into this problem will be required to clarify these discrepancies.

The observation that central administration of PGE<sub>2</sub> stimulates AVP release within the VSA is similar to that seen in the plasma (Yamamoto et al, 1976; Hoffman et al, 1982; Hashimoto et al, 1989). However, leakage of vasopressin from the periphery into the brain is an unlikely explanation for the present results (DeMotes-Mainard et al,



1986; Landgraf et al, 1988; Landgraf et al, 1990a,b). This contention receives additional support from push-pull perfusion studies in sheep where intravenous endotoxin results in suppression of VSA AVP (Cooper et al, 1979) despite elevated levels of the peptide peripherally (Kasting et al, 1983). Furthermore, AVP levels from VSA push-pull perfusates in the rat are not significantly changed during the fever evoked by intravenous endotoxin (see chapter IX). This occurs despite significant levels of plasma vasopressin following intravenous endotoxin (Kasting et al, 1985; see also chapter VII).

An alternative explanation for these results is that the neural pathways involved in the febrile process are different depending on the pyrogen used. Thus the neural mechanism involved in endotoxin fevers may differ from those involved in PG-mediated fevers. By the same token, PGE<sub>1</sub> may differ from PGE<sub>2</sub>-induced fevers. These mechanisms may indeed display different sensitivities to antipyretic therapy, possibly due to differences in the vasopressin response. In this way, the differential effects of indomethacin on central AVP release during febrile body temperatures may be rationalized. While the differences in the mechanisms involved in the production of fever between endotoxin and PGE<sub>2</sub> seem tenable, this argument is unlikely to explain why PGE<sub>1</sub> fevers apparently activate the AVP antipyretic pathway whereas PGE<sub>2</sub> fevers are unaffected by AVP suppression within the VSA.

In summary, PGE<sub>2</sub> administered icv in conscious unrestrained rats evoked a significant hyperthermic response which occurred simultaneously with increased AVP levels from VSA push-pull perfusates. Treatment with intraperitoneal indomethacin had no effect on PGE<sub>2</sub>-induced hyperthermia but suppressed the release of vasopressin from VSA perfusates. These results are not consistent with the AVP endogenous antipyretic hypothesis but may indicate differences in the activation of central AVP release by different pyrogenic substances.

## VII. THE EFFECTS OF THREE ANTIPYRETIC DRUGS ON THE RELEASE OF VASOPRESSIN INTO THE PLASMA AND CSF IN FEBRILE AND NON-FEBRILE RATS

### Introduction

The neurohypophyseal peptide arginine vasopressin (AVP) is widely recognized as a pressor or antidiuretic hormone. Many studies have examined the physiological and pharmacological basis for vasopressin secretion in relation to osmotic or volume control. AVP release in response to either decreases in arterial blood pressure or elevations in plasma osmolality involves a complex neuroendocrine reflex pathway incorporating many neurotransmitters or neuromodulating constituents (Bisset and Chowdrey, 1988). Stimuli for plasma AVP release, however, are not limited solely to changes in body fluid osmolality or blood pressure. For example, perturbations in thermoregulation resulting in hyperthermia lead to increases in vasopressin release in the rat (Itoh, 1954; Kasting et al, 1981), pig (Forsling et al, 1976) and dog (Szczepanska-Sadowska, 1974). Whereas acute cold exposure results in AVP suppression, the so called "cold diuresis" (Morgan et al, 1983), cold adaptation, on the other hand, elicits increases in circulating AVP (Zeisberger et al, 1988).

Pyrogenic compounds affect normal thermoregulation by eliciting the febrile response as well as vasopressin release. Putative pyrogenic mediators such as interleukin-1 $\beta$  (IL-1 $\beta$ ) (Christensen et al, 1989) and PGE<sub>2</sub> (Ishikawa et al, 1981) promote AVP release from the *in vitro* rat neurohypophysis. *In vivo*, centrally administered PGE<sub>1</sub> (Leskell, 1976) and PGE<sub>2</sub> (Vilhardt and Hedqvist, 1970; Hoffman and Schmid, 1979) stimulate vasopressin release as determined by bioassay or

radioimmunoassay (Yamamoto et al, 1976; Hoffman et al, 1982). Consistent with this are the results demonstrating the pyrogenic and vasopressin releasing properties of bacterial endotoxin (Kasting et al, 1985; Kasting, 1986b).

The use of PG synthesis inhibitors has been an invaluable tool for assessing the role of endogenous PGs in the release of vasopressin (reviewed in Sklar and Schrier, 1983). While the doses used have been ascertained to have no effect on either blood pressure or osmolality (Kamoi et al, 1983; Brooks et al, 1986), no study has apparently confirmed the dose of the inhibitors as one of measurable therapeutic effect. Typically, cyclooxygenase inhibiting drugs are utilized for their analgesic, anti-inflammatory or antipyretic actions. In this study, therefore, measurement of body temperature and plasma AVP in the conscious, unrestrained rat were conducted during endotoxin-stimulated fever and the antipyresis induced by three structurally dissimilar PG synthesis inhibitors. In this way an assessment of the effects of endogenous PGs on vasopressin secretion could be made utilizing cyclooxygenase inhibitors at doses of measurable therapeutic value. In addition, it was of particular interest to compare the effects of indomethacin on plasma and CSF vasopressin release since this drug had such dramatic effects on the release of AVP within the VSA (see chapter V and VI).

## Methods

Male Sprague-Dawley rats were surgically prepared with indwelling intra-atrial Silastic catheters for blood sampling and intravenous injections of *E. coli* endotoxin (100  $\mu$ g/kg) or sterile 0.9% saline. The catheter was exteriorized to the top of the head, secured to the skull and sealed with a heparin lock (1000 U/ml) as described in chapter II. Body temperature was measured via biotelemetry.

In addition to the intra-atrial catheter, animals in a separate group were also implanted with a 21 gauge stainless steel cannula whose tip lay within the cisterna

magna for withdrawal of cerebrospinal fluid (CSF). The CSF cannula was fitted with a 26 gauge obturator and the cannula-obturator junction sealed with a sleeve of PE-100 tubing. The surgical procedure followed the details outlined in chapter II.

Upon recovery of pre-operative body weight (5-7 days) baseline body temperatures were recorded and endotoxin or saline administered (between 0830 and 0930h) after the removal of a 1.0 mL sample of blood. Blood was subsequently sampled hourly for 7h. Each 1.0 mL aliquot of whole blood was dispensed into ice cold polypropylene tubes, immediately centrifuged and the plasma stored at -70 °C until assayed for AVP. After each sample the erythrocytes were re-suspended in heparinized saline (40 U/mL) and returned to the animal at the time of the next sample. This technique has been demonstrated to have no effect on haemodynamic or other physiological parameters (Kasting et al, 1985).

For CSF sampling, blood-free CSF was slowly withdrawn from the cannula via a small length of pre-calibrated PE-100 attached to a 1.0 mL syringe. The sample (120  $\mu$ L) was dispensed into chilled polypropylene tubes and stored at -70 °C until assayed for AVP. After surgical recovery, a basal sample (always taken between 0830 and 0900h) was collected and endotoxin or saline injected intravenously. Subsequent samples of CSF were collected every 2.5h for 7h. These animals were not used for blood sampling.

Two hours after intravenous administration of saline or endotoxin, animals received an intraperitoneal injection of indomethacin (5 mg/kg), sodium salicylate (75 mg/kg), acetaminophen (75 mg/kg) or vehicle (1 ml/kg). Each rat was used for two experiments, saline and endotoxin (on alternate days) followed 2h later by intraperitoneal administration of vehicle or one of the three PG synthesis inhibitors. This timing was chosen in order to observe the possible relation between vasopressin

release and the therapeutic (as opposed to prophylactic) actions of the test drugs. Administration of each drug at 2 hours into the experiment was chosen because this was the stage in which the rate of increase in body temperature due to endotoxin was maximal. For experiments involving the sampling of CSF, indomethacin or vehicle were administered 30 min after the intravenous injections due to the limited number of CSF samples which can be withdrawn within the duration of the experiment.

Indomethacin and sodium salicylate were dissolved in Tris buffer and acetaminophen was dissolved in DMSO:saline (3:2, v/v). Because both vehicles were found to have no effect on AVP release in saline- or endotoxin-treated animals (saline,  $F = 0.738$ ,  $p > 0.5$ ; endotoxin,  $F = 1.22$ ,  $p = 0.27$ ), the data were combined and collectively referred to as "vehicle" experiments. Each cyclooxygenase inhibitor was used at a dose which was determined to have little or no effect on normal core temperature but to have a marked effect on the febrile response to intravenous endotoxin.

Plasma and CSF samples were assayed for AVP in duplicate by radioimmunoassay. CSF was assayed unextracted whereas plasma was extracted on Sep-Pak C<sub>18</sub> cartridges (Waters) prior to assay. Recovery has been demonstrated to be >80% (Kasting et al, 1983; Kasting et al, 1985). Reported vasopressin levels are uncorrected for recovery. Sensitivity of the assay was 0.18 pg/tube and the intra- and interassay variability was 6.3 and 12.2% respectively.

The integrated vasopressin and thermoregulatory responses were calculated from the point of drug or vehicle administration as previously described (chapter II). Statistical analysis was performed using a one way ANOVA with and without repeated measures followed by Newman-Keul's *aposteriori* analysis. When data did not meet the

homogeneity of variance assumption a logarithmic transformation was performed. CSF data was assessed utilizing Students' t-test (unpaired). Significance was considered at  $p < 0.05$ .

## Results

Intravenous endotoxin resulted in significant increases in plasma vasopressin from the basal value of  $3.07 \pm 0.44$  pg/mL to a maximum of  $32.6 \pm 7.35$  pg/mL one hour later ( $p < 0.01$ ; Figure 22B). These levels remained significantly elevated for up to 4h. After a 1h latency, body temperature began to rise dramatically during the second hour after endotoxin and exhibited a characteristic biphasic febrile response with temperature peaks at 3 and 6h (Figure 22A). Intravenous 0.9% saline did not evoke significant alterations in plasma AVP concentrations or body temperature (Figure 22A,B). In both endotoxin- and saline-treated groups intraperitoneal administration of vehicle had no apparent effect on vasopressin output or body temperature.

Endotoxin followed by indomethacin, significantly attenuated the onset of fever (Figure 23A, Figure 27) but had no effect on endotoxin-stimulated vasopressin release (Figure 23B). In animals treated with intravenous saline, followed by indomethacin, there was a small but insignificant decrease in body temperature (maximum decrease,  $-0.30 \pm 0.19$  °C). At the same time a steady increase in vasopressin release was observed which reached significance ( $p < 0.01$ , compared to time 0) 5h after indomethacin administration (Figure 23B).

Figure 24 depicts the thermoregulatory and plasma AVP response to sodium salicylate following previous intravenous saline or endotoxin. Sodium salicylate markedly suppressed the febrile response to endotoxin but was without effect on normothermic core temperature (Figure 24A). Intraperitoneal salicylate did not alter

the endotoxin-stimulated AVP response compared to vehicle or indomethacin treatment groups. Similarly, there were no significant alterations in plasma AVP following intraperitoneal salicylate in non-febrile animals, although this response was somewhat variable (Figure 24B). It should also be noted that, like indomethacin, sodium salicylate administered to endotoxin-treated rats, produced an initial hypothermic response (i.e. a reduction in core temperature which was approximately 1 °C below baseline temperature after 2h). Interestingly, this response is not observed if antipyretics are given as a pre-treatment (Kasting et al, 1985) or if administered at the height of the fever (unpublished observations). It is evident, however, that plasma AVP release seems unaffected by these thermoregulatory perturbations.

The body temperature and vasopressin response following endotoxin or saline and subsequent treatment with acetaminophen are summarized in Figure 25. Although acetaminophen significantly suppressed endotoxin-induced fever, it was less potent when compared to the other antipyretics (Figure 28). In saline-treated animals acetaminophen prompted both a fall and a rise in core temperature. These responses, however, were not significantly different from vehicle-treated animals (Figure 28).

Acetaminophen had no effect on the endotoxin-stimulated vasopressin release (compared to endotoxin + vehicle). In saline controls, acetaminophen treatment resulted in variable increases in plasma AVP, that were not significantly different from pre-injection values. However, when the total 5h AVP response following acetaminophen was compared to vehicle this was found to be significant ( $p < 0.05$ ; Figure 27).

Figure 26 illustrates the effects of intravenous endotoxin or saline, followed by indomethacin or vehicle, on the AVP release into the CSF. In the absence of indomethacin, the circadian release of AVP was evident in both control and endotoxin

groups with peaks approximately 5h from time 0 (between 1330 and 1400h). Whereas indomethacin had no apparent effect on CSF AVP release in endotoxin-treated animals, in saline controls the drug significantly inhibited ( $p < 0.05$ ) AVP release at 5 and 7.5h.

The integrated AVP response (total AVP release over 5h) in all experimental groups are shown in Figure 27. There were no cases of significance following endotoxin regardless of the PG synthesis inhibitor employed (compared to vehicle). However, in saline controls both indomethacin and acetaminophen significantly enhanced vasopressin release compared to vehicle ( $p < 0.05$ ).

## Discussion

The present study examined the effects of cyclooxygenase inhibition on basal and endotoxin-stimulated AVP release. The protocol employed three different PG synthesis inhibitors in an attempt to match the therapeutic (antipyretic) action of these drugs with their effects on vasopressin release. The drugs were administered at a time when the rate of change in body temperature (due to endotoxin) was highest and when plasma AVP was elevated and fairly constant. As expected, indomethacin, sodium salicylate and acetaminophen were potent inhibitors of the febrile response to endotoxin. However, at the doses employed these drugs had no significant effect on normothermic core temperature. In addition, indomethacin, the most potent cyclooxygenase inhibitor we examined (Van Arman et al, 1985) has repeatedly been demonstrated to have no effect on haemodynamic or osmotic parameters (Walker, 1983a; Kasting et al, 1985; Brooks et al, 1986; Manner et al, 1989). Thus it can be confidently assumed that the drugs elicited specific therapeutic effects that were not secondary to alterations in blood pressure or osmolality.



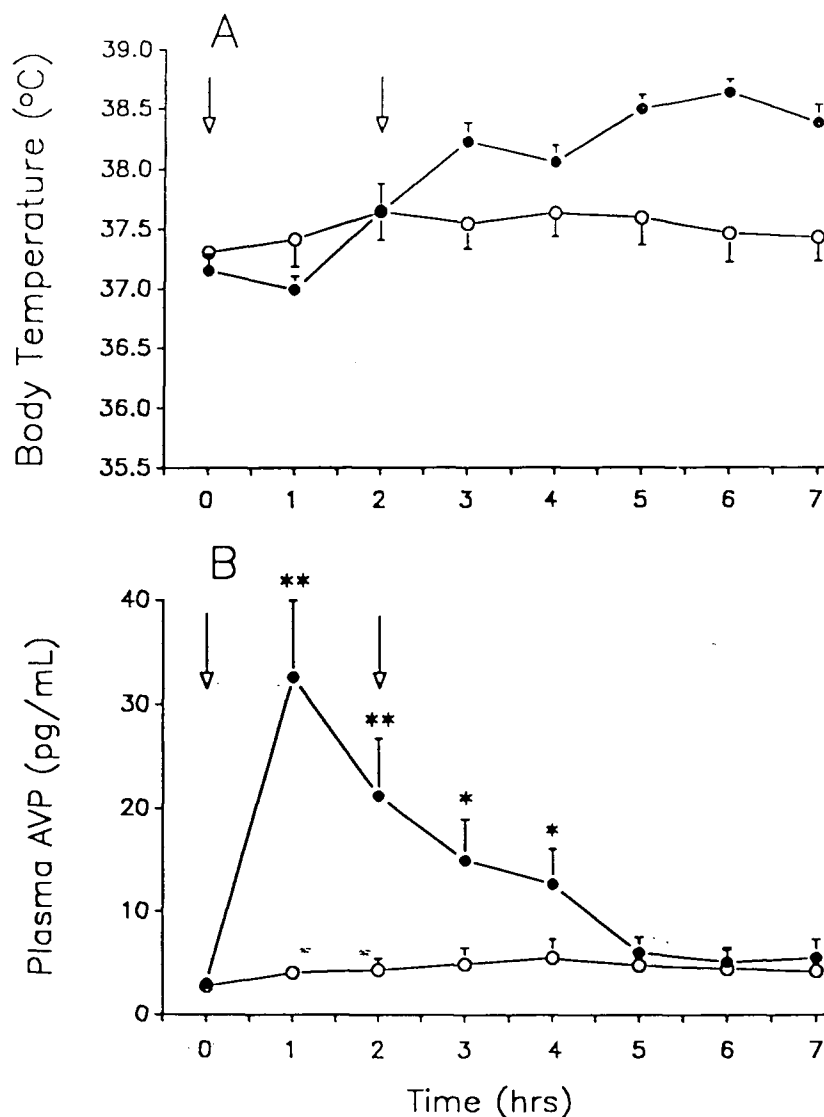


Figure 22. The thermoregulatory (panel A;  $n = 10$ ) and plasma vasopressin (panel B;  $n = 9$ ) responses to intravenous endotoxin (filled circles) or saline (open circles) administered at time 0 (first arrow). Two hours later, vehicle (Tris buffer or DMSO-saline) was administered intraperitoneally (second arrow). Statistical analysis of thermoregulatory data in this and subsequent figs. are shown in Figure. 28. For plasma AVP, within groups responses were assessed with a one way ANOVA followed by Newman-Keuls test. \*\*  $p < 0.01$ ; \*  $p < 0.05$  (compared to time 0).

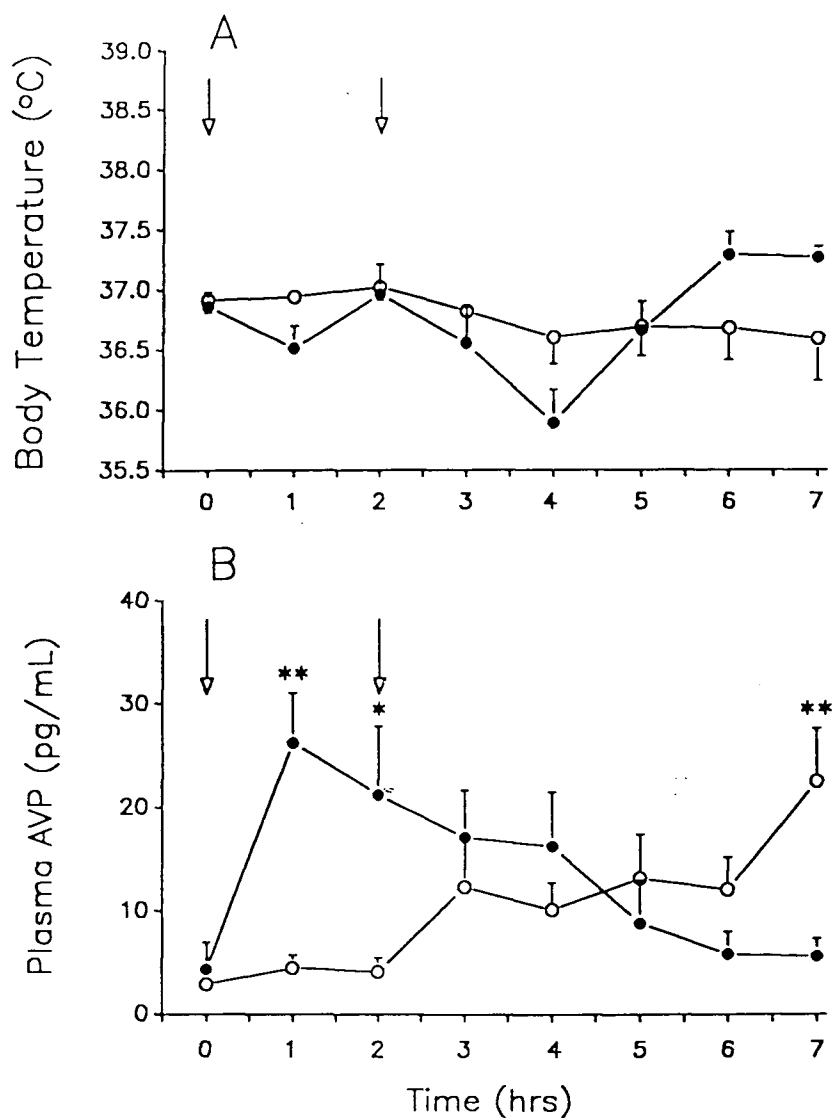


Figure 23. The thermoregulatory (panel A;  $n = 10$ ) and plasma vasopressin (panel B;  $n = 10$ ) responses to intravenous endotoxin (filled circles) or saline (open circles) administered at time 0 (first arrow). Two hours later indomethacin (5 mg/kg) was administered intraperitoneally (second arrow). \*\*  $p < 0.01$ ; \*  $p < 0.05$  (compared to time 0).

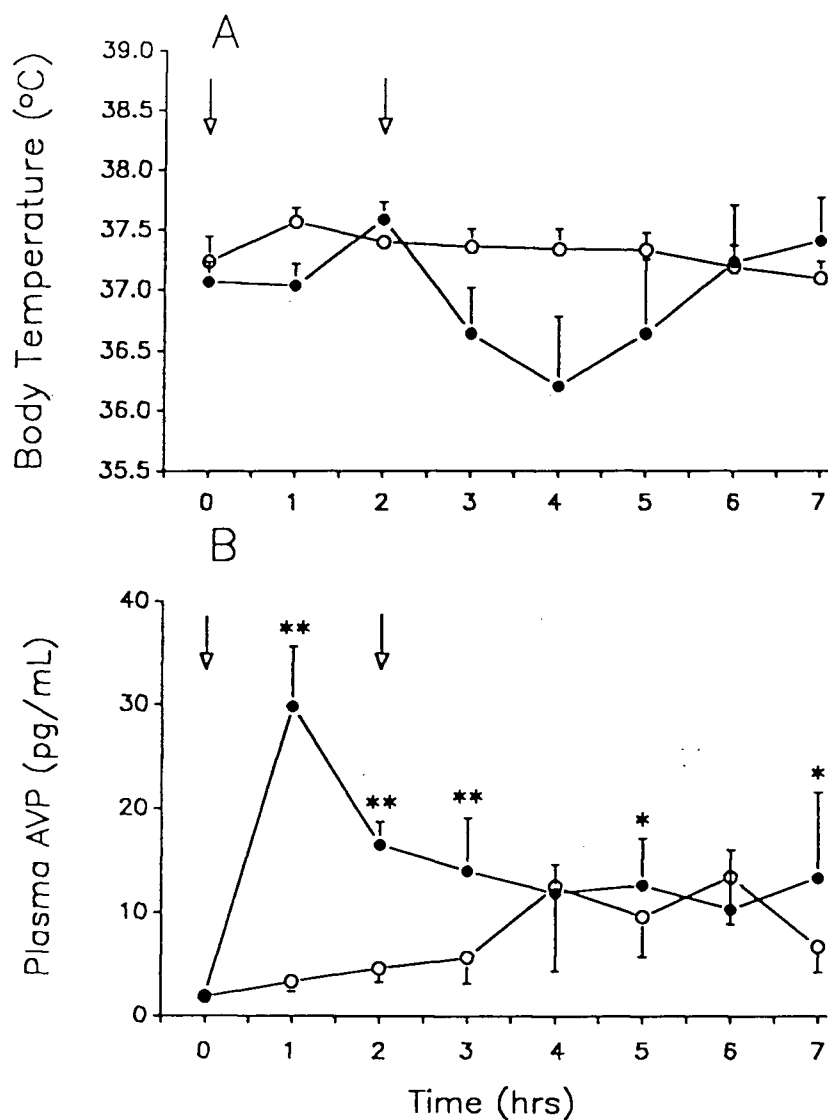


Figure 24. The thermoregulatory (panel A;  $n = 9$ ) and plasma vasopressin (panel B;  $n = 7$ ) responses to intravenous endotoxin (filled circles) or saline (open circles) administered at time 0 (first arrow). Two hours later sodium salicylate (75 mg/kg) was administered intraperitoneally (second arrow). \*\*  $p < 0.01$ ; \*  $p < 0.05$  (compared to time 0).

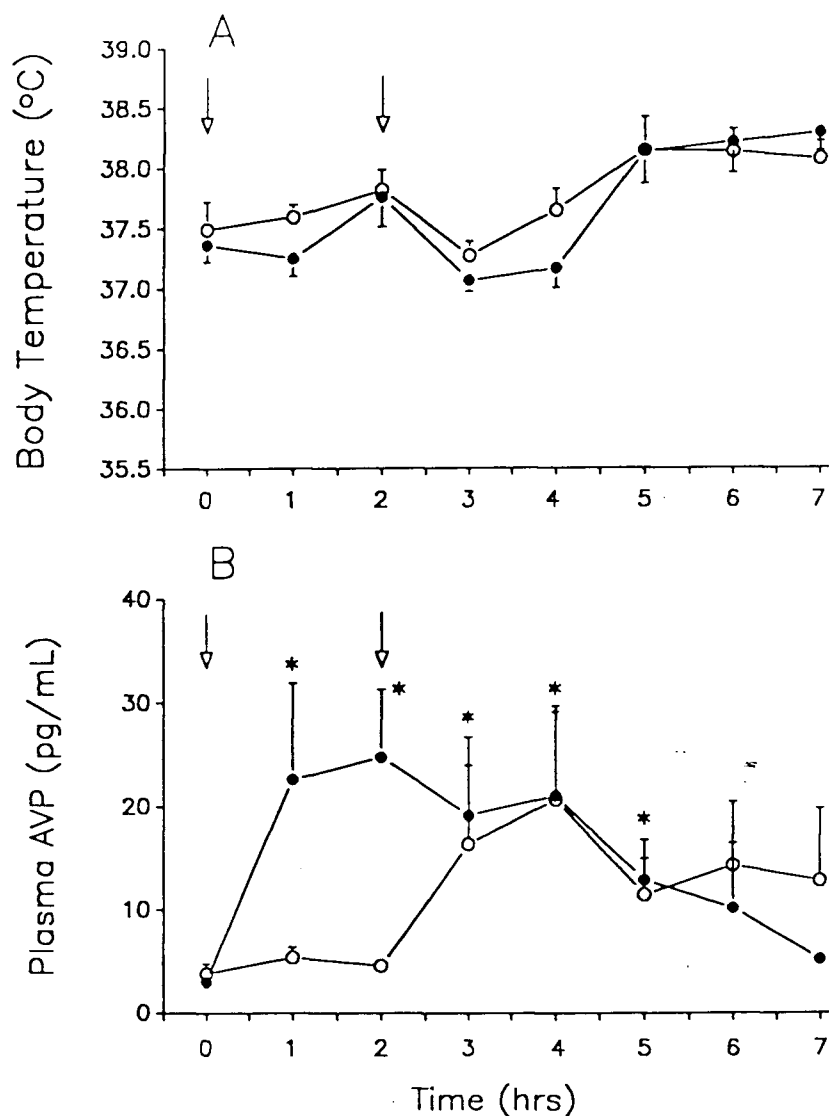


Figure 25. The thermoregulatory (panel A;  $n = 9$ ) and plasma vasopressin (panel B;  $n = 7$ ) responses to intravenous endotoxin (filled circles) or saline (open circles) administered at time 0 (first arrow). Two hours later acetaminophen (75 mg/kg) was administered intraperitoneally (second arrow). \*\*  $p < 0.01$ ; \*  $p < 0.05$  (compared to time 0).

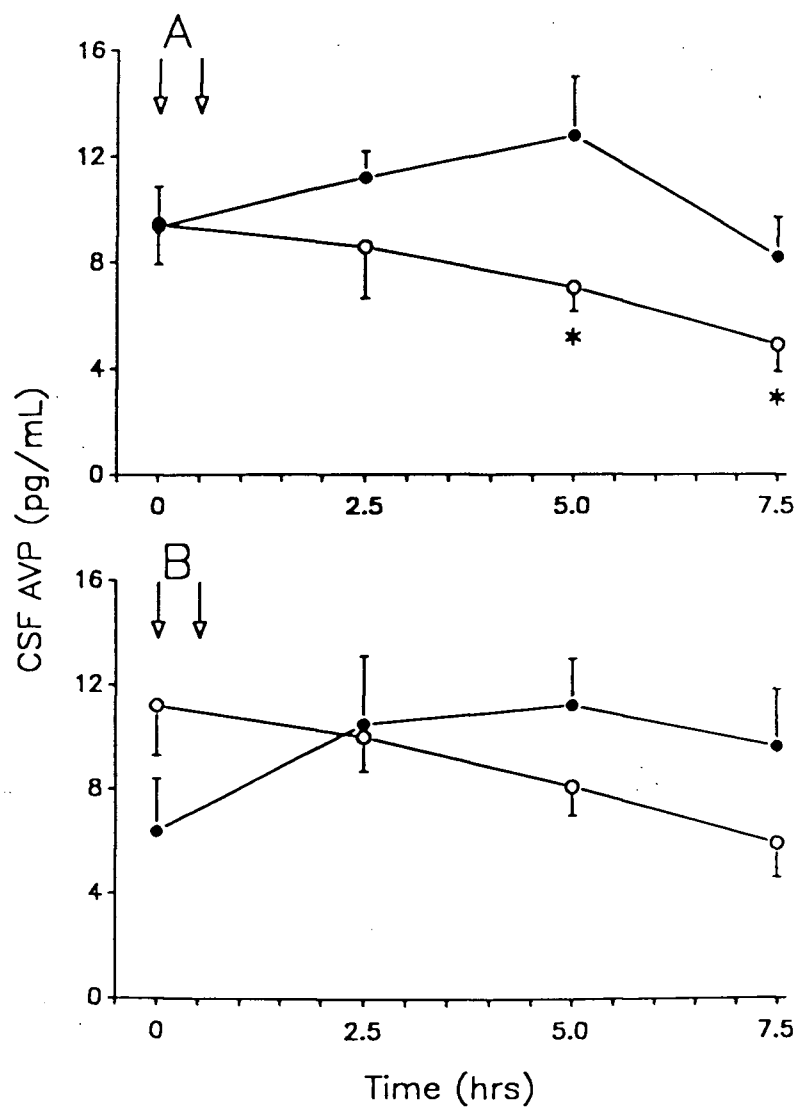


Figure 26. The effect of intravenous saline (panel A) or endotoxin (panel B) followed 30 min later by Tris buffer (closed circles) or indomethacin (open circles) on the release of vasopressin into the cerebrospinal fluid. \*  $p < 0.05$ , unpaired t-test.

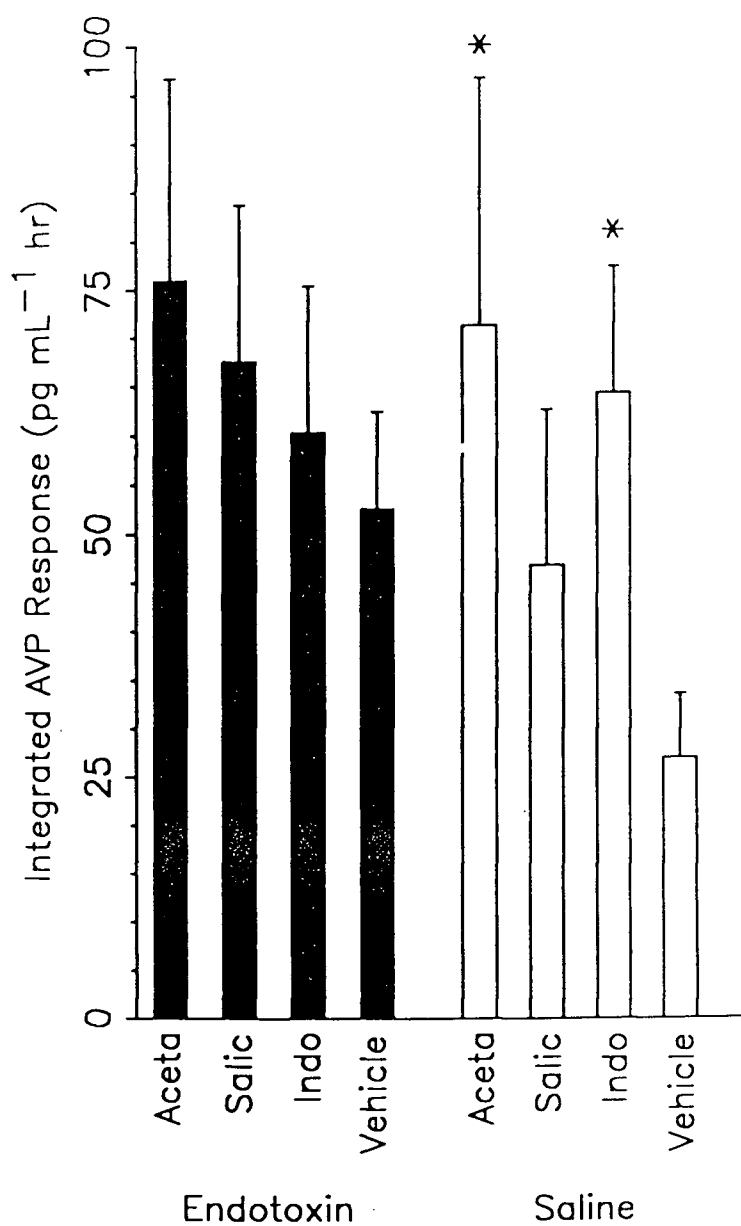


Figure 27. The integrated (5h) vasopressin response following intravenous endotoxin (dark bars) or saline (open bars) calculated from the point of intraperitoneal vehicle, indomethacin, sodium salicylate or acetaminophen. \*  $p < 0.05$ . ANOVA, Newman-Keuls (compared to vehicle).

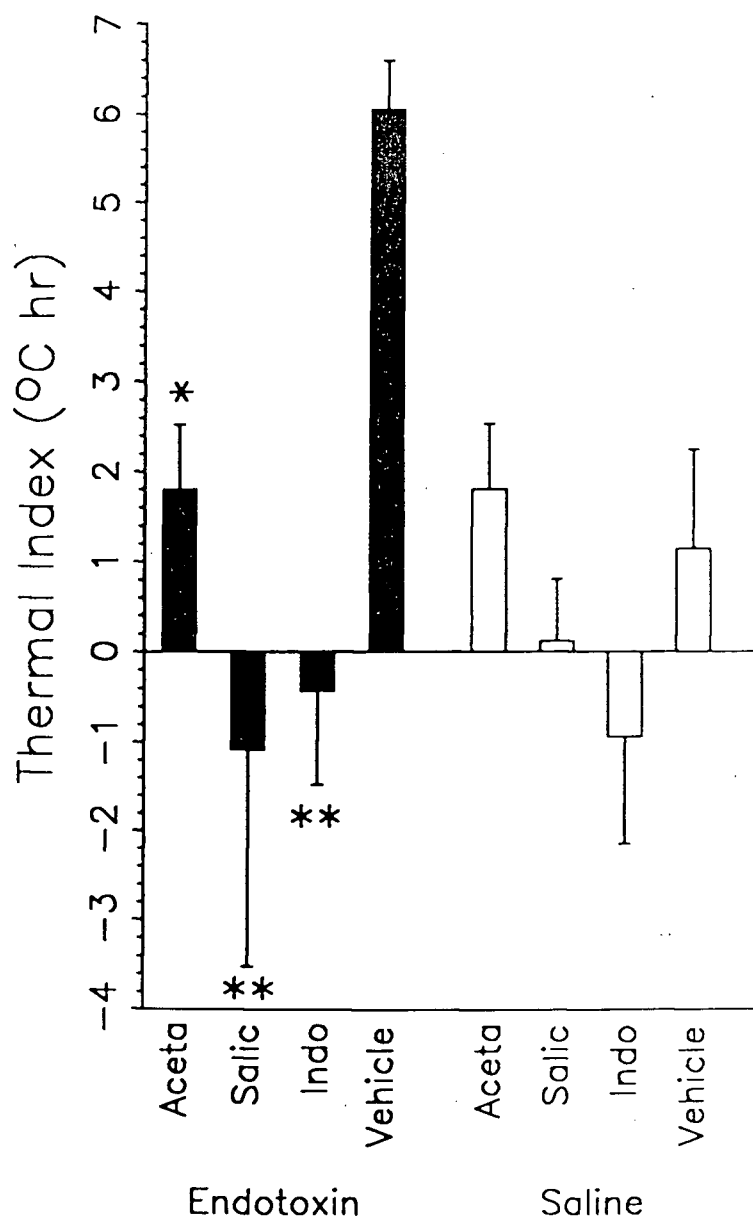


Figure 28. The cumulative change in body temperature (thermal index) calculated from the point of intravenous endotoxin (dark bars) or saline (open bars) in vehicle and drug-treated groups. \*\*  $p < 0.01$ ; \*  $p < 0.05$ . ANOVA, Newman-Keuls (compared to vehicle).

The ability of non-hypotensive doses of endotoxin to evoke fever as well as AVP release has been demonstrated previously to be unrelated to changes in blood pressure or osmolality (Kasting et al, 1985). Interestingly, endotoxin promotes both central (Sirko et al, 1989) and peripheral (Rotondo et al, 1988) PGE<sub>2</sub> release and these increases are positively correlated with the febrile rise in core temperature. In addition centrally administered PGE<sub>1</sub> and E<sub>2</sub> are not only potently pyrogenic (Gollman and Rudy, 1988) but also stimulate plasma AVP release (Leskell, 1976; Yamamoto et al, 1976; Hoffman and Schmid, 1979; Hoffman et al, 1982; Manner et al, 1989). Although there is some controversy as to whether PGE<sub>2</sub> evokes AVP release by a central or peripheral action there is convincing evidence that PGE<sub>2</sub> is of importance for both basal and stimulated vasopressin levels (Sklar and Schrier, 1983). The endotoxin-induced promotion of AVP release, however, cannot be explained by a PGE<sub>2</sub> hypothesis because peripheral pre-treatment with indomethacin was without effect on AVP release while suppressing the febrile response (Kasting et al, 1985). Endotoxin-induced increases in circulating AVP levels may occur via endogenous pyrogens such as the cytokines tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) or IL-1 $\beta$  (Michie et al, 1988). This is supported by the evidence demonstrating the vasopressin stimulatory ability of IL-1 $\beta$  *in vitro* (Christensen et al, 1989).

The present results indicate that neither indomethacin, sodium salicylate or acetaminophen significantly alter endotoxin-stimulated increases in plasma AVP. This strongly suggests a non-prostaglandin mediated mechanism for AVP secretion following pyrogenic doses of endotoxin and is consistent with previous results where a pre-treatment approach to indomethacin administration was employed (Kasting et al, 1985). A non-prostaglandin mechanism of action for indomethacin may also explain the enhancement of vasopressin release observed during hemorrhage in the anesthetized dog (Brooks et al, 1984). In contrast, osmotic (Hoffman et al, 1982) or hypoxic



(Walker, 1983b) stimuli have been found to be dependent on endogenous PGs acting either peripherally or centrally. It should be noted, however, that the role of PGs in osmotic stimulation of AVP remains controversial (Kamoi et al, 1983; Manner et al, 1989).

To control for the effects of endotoxin, experiments were conducted utilizing intravenous saline followed by one of the three cyclooxygenase inhibitors. In non-febrile animals indomethacin did not significantly affect body temperature but prompted a significant increase in AVP 5h after the injection. There was a tendency for vasopressin to be elevated 1h following the administration of indomethacin but this was found not to be significant. However, the total vasopressin release over the 5h subsequent to the intraperitoneal injection was significant compared to the integrated response following vehicle (Figure 27). These observations agree with previous work from this laboratory (Kasting et al, 1985) but are at odds with a number of other reports. Peripherally injected indomethacin has been shown to have either no effect (Manner et al, 1989) or to suppress basal AVP secretion (Yamamoto et al, 1976; Walker, 1983a), while infusion of indomethacin into the cerebral ventricles has no effect on basal AVP levels (Yamamoto et al, 1978; Hoffman et al, 1982; Brooks et al, 1984). A number of procedural differences exist which may help to explain the disparity between our results and those of others. These include state of anaesthesia, dose and route of drug administration and, possibly, the species tested. In preliminary experiments, it was observed that indomethacin, administered icv, over a wide dose range, did not exert an antipyretic effect during an endotoxin fever (unpublished observations). Thus the physiological significance of plasma AVP alterations following centrally administered indomethacin must be questioned. However, the most notable difference in this study was the time course of the AVP

response following indomethacin injection. Vasopressin levels were still increasing after 5h, whereas previous investigations measured hormone levels for 2h at most. Therefore, any late increase in AVP would not have been detected.

As to the mechanism for indomethacin-induced AVP release it is tempting to suggest that endogenous PGs exert a tonic inhibition on the basal secretion of the peptide. However, the majority of experimental evidence suggests the opposite, viz. endogenous and exogenous PGs have a stimulatory influence on vasopressin release (Sklar and Schrier, 1983). Furthermore, indomethacin produces a >80% reduction of endogenous PGs within the rat brain 30 min after a peripheral injection (Abdel-Halim et al, 1978). A similar degree of inhibition would be expected within peripheral tissues. Thus the late increase in plasma AVP cannot be explained by the notion that an effective inhibitory concentration of indomethacin requires several hours to accumulate within an active site. It is possible that indomethacin may affect the metabolism of vasopressin thereby resulting in a steady rise in circulating levels. Alternatively, indomethacin, acting independent of its action on PG biosynthesis (Northover, 1977; Kantor and Hampton, 1978) may have a direct effect at the hypothalamic or pituitary level.

Analysis of vasopressin release into the CSF may provide insight as to the locus of action of indomethacin. Vasopressin neurons of the suprachiasmatic nucleus (SCN) are the major source of AVP within the CSF (Schwartz and Reppert, 1985). Thus an effect on the release of AVP into the CSF must be construed as a central action. In the non-febrile rat, indomethacin resulted in a significant reduction in concentrations of vasopressin within CSF compared to control levels. Interestingly, this inhibitory effect reached statistical significance nearly 5h after intraperitoneal indomethacin. Thus, both peripheral stimulation and central inhibition of AVP requires several hours to become manifest. In animals receiving intravenous endotoxin, the CSF AVP release profile was

virtually identical to that in animals receiving intravenous saline. Because endotoxin has been shown to markedly enhance PGE<sub>2</sub> production within the hypothalamic neuropil (Sirko et al, 1989) and the CSF (Coceani et al, 1988), it seems unlikely that endogenously produced PGs are affecting SCN vasopressinergic neurons or their terminals to any great extent. The effect of indomethacin on CSF AVP in endotoxin-treated rats was qualitatively similar to the non-febrile condition but was not significantly different at any point. The difference in baseline values, while not statistically significant ( $p = 0.07$ ), may have obscured a similar trend observed in non-febrile controls. Thus it is not inconceivable that indomethacin may be acting independent of its action on PG biosynthesis within the central nervous system to evoke differential effects on two subpopulations of vasopressinergic neurons.

In the non-febrile rat, the effects of sodium salicylate and acetaminophen on plasma AVP release were also studied. Sodium salicylate evoked no consistent change in plasma vasopressin concentrations despite being utilized at a dose which inhibits both peripheral (Abdel-Halim et al, 1978) and central (Rawlins et al, 1973) PG biosynthesis. These data do not support a role for endogenous PGs in basal AVP release. Indeed the tendency was to increasing AVP levels. The results from acetaminophen-treated animals also support this position. However, in acetaminophen experiments there was a variable but distinct increase in vasopressin release. This response was significant ( $p < 0.05$ ) only when the total (integrated) vasopressin response was compared with vehicle. Unlike the effects of indomethacin, however, the maximal effect of acetaminophen was observed within the first 2h. It is noteworthy that acetaminophen has been shown to exert an antidiuretic effect in humans within 2h of oral administration without increases in serum osmolality (Nusynowitz and Forsham, 1966).

The differential effects on AVP release by three different cyclooxygenase inhibitors appear unrelated to their antipyretic action. A similar lack of correlation has been observed in plasma glucocorticoid release following the oral administration of a number of antipyretics, including indomethacin, in the rabbit (Switala et al, 1988). However, the results observed following indomethacin or acetaminophen in our study warrant additional comment. Whereas both indomethacin and acetaminophen are antipyretic, acetaminophen lacks the antiinflammatory characteristic of indomethacin. This action has been explained as due to the differential activity of acetaminophen on the peripheral and central cyclooxygenase enzyme (Flower and Vane, 1972). However, acetaminophen crosses the blood brain barrier poorly (Davison et al, 1961) and when administered peripherally has little effect on brain PG production (Abdel-Halim et al, 1978). Therefore, an explanation for the promotion of vasopressin release by acetaminophen must include a mechanism outside the blood brain barrier. Whether acetaminophen acts to increase vasopressin release through, or independent of its action on PGs, a direct action on the pituitary is probable in light of the high levels of the drug which accumulate within this structure (Davison et al, 1961).

The present results have shown that PG synthesis inhibition utilizing 3 different drugs, at therapeutic concentrations, has no effect on stimulated levels of vasopressin induced by intravenous endotoxin. In contrast, non-febrile animals receiving intraperitoneal indomethacin or acetaminophen showed increases in circulating AVP levels while those treated with sodium salicylate exhibited no significant change in basal hormone release. Because all three test agents are inhibitors of PG biosynthesis (Van Arman et al, 1985) these data do not favour a role for endogenous PGs in the stimulation of vasopressin release. Furthermore, the observations of the release of AVP into the CSF lead to the conclusion that indomethacin affects AVP neurons of the hypothalamo-neurohypophyseal system in an opposite manner to the vasopressinergic

neurons of the SCN. The temporal pattern of these effects was similar, however. These data also underscore the need to recognize that experimental results arising from the use of PG synthesis inhibitors may involve mechanisms independent of the effects on PGs alone.

## **VIII. EVIDENCE SUPPORTING A ROLE FOR ENDOGENOUS VASOPRESSIN IN THE PYROGENIC TOLERANCE TO INTRAVENOUS ENDOTOXIN**

### **Introduction**

The neuropeptide arginine vasopressin (AVP) is widely distributed in the central nervous system (Buijs et al, 1978; Sofroniew and Wiendl, 1981; DeVries et al, 1985) as well as in many peripheral tissues (for e.g. Kasson et al, 1985). While the actions of AVP as a blood-borne hormone are well recognized, the function of this peptide outside the hypothalamo-hypophyseal system is less well understood. Centrally, AVP may function as a neurotransmitter or neuromodulator during fever. Specifically, vasopressin acts within a circumscribed neuronal locus as an inhibitory component in the fever regulating pathway, and as such, fulfills a role as an endogenous antipyretic.

Evidence supporting the antipyretic action of AVP has been obtained from many species including rat, cat, rabbit, sheep and guinea pig (reviewed in chapter I). The AVP-sensitive locus, the ventral septal area (VSA) is common between these species and contains a network of AVP containing axons, terminals (DeVries et al, 1985) and AVP receptors (Baskin et al, 1983; Lawrence et al, 1988) resembling the peripheral V<sub>1</sub> vasopressor subtype (Lawrence et al, 1988; Poulin et al, 1988).

Central release of AVP during fever has been measured from VSA push-pull perfusates in sheep (Cooper et al, 1979) and rabbits (Malkinson et al, 1987) and the results are consistent with an endogenous antipyretic function. Evidence suggests that the source of this endogenously released AVP is the bed nucleus of the stria terminalis (DeVries et al, 1985; Disturnal et al, 1985; Naylor et al, 1988) or the paraventricular

nucleus (Disturnal et al, 1985). There is strong evidence, therefore, that endogenous vasopressin acts within the VSA to limit fever height and duration and/or participate in the process of defervescence.

Evidence has also accumulated that AVP may be responsible for the refractory state to pyrogen in the newborn lamb (Kasting et al, 1979) and rat (Kasting and Wilkinson, 1987) as well as near-term sheep (Kasting et al, 1978) and guinea pigs (Merker et al, 1980; Cooper et al, 1988). Although unrecognized as such, the refractory state to endotoxin after one or more intravenous administrations may also represent a condition of endogenous antipyresis. Endotoxin tolerance, among other characteristics, is a phenomenon whereby the febrile response to iv endotoxins is reduced or absent after as little as one previous iv injection. Prior to this and another report (Cooper et al, 1988) pyrogenic tolerance was thought to be primarily due to hepatic mechanisms (Greisman, 1983). However, Cooper et al (1988) have observed enhanced immunoreactive AVP staining in the septal area of guinea pigs rendered tolerant to the pyrogen Poly I:Poly C, similar to that seen in pregnant animals with suppressed fevers (Merker et al, 1980). It was of interest, therefore, to examine the role of AVP during tolerance to *E. coli* endotoxin. In this study specific AVP V<sub>1</sub> and V<sub>2</sub> receptor antagonists, administered within the VSA, were utilized to assess the role of centrally acting AVP during endotoxin tolerance.

## Methods

Male Sprague-Dawley rats (250-300g) were surgically prepared with a bilateral stainless steel guide cannula directed toward the VSA, a Silastic intra-atrial catheter and an intraperitoneal radio transmitter for monitoring of body temperature as described in chapter II. After at least 2h of monitoring baseline body temperature rats received 50  $\mu$ g/kg of *E. coli* endotoxin intravenously and body temperature was

followed for 360 min. All groups received a second *E. coli* injection 24h later. Tolerance to the pyrogenic effects of the endotoxin was usually evident by this time. Subsequent to this second endotoxin treatment, tolerant rats were divided into four experimental groups: 1) a third and fourth endotoxin treatment separated by 24h; 2) saline (0.5  $\mu$ L) or an AVP V<sub>1</sub> receptor antagonist (0.43 - 4.3 nmol) bilaterally administered into the VSA 15 min prior to the third or fourth endotoxin injection; 3) saline or an AVP V<sub>2</sub> receptor antagonist (0.43 nmol) bilaterally administered into the VSA 15 min prior to a third or fourth endotoxin treatment; 4) saline or the V<sub>1</sub> antagonist into the VSA without endotoxin injection. Each rat served as its own control with 24h separating each experiment. Treatment order was random. That is, some animals received antagonist on day 3 and saline on day 4, while others had the opposite.

VSA microinjections were delivered via 30 gauge injection cannula affixed by PE-20 tubing to a 10  $\mu$ L Hamilton syringe. Injection volume was 0.5  $\mu$ L administered bilaterally over 10-15 s. All injected materials were dissolved in 0.9% sterile saline. The V<sub>1</sub> antagonist was d(CH<sub>2</sub>)<sub>5</sub>Tyr(Me)AVP (Bachem). The starting dose was chosen as one that previously augmented fever (Cooper et al, 1987; Naylor et al, 1988) as well as blocked the antipyresis evoked by exogenous AVP (Kasting and Wilkinson, 1986). The V<sub>2</sub> antagonist was d(CH<sub>2</sub>)<sub>5</sub>[D-Ile<sup>2</sup>,Abu<sup>4</sup>]AVP (kindly supplied by Dr. M. Manning, OH). Upon completion of the experiments injection loci were verified histologically.

Fever indices were calculated as areas under the fever curves ( $^{\circ}$ C $\cdot$ h) as previously described (chapter II). Statistical analyses were performed on these data using the one way analysis of variance (ANOVA) with Newman-Keuls test or Student's t-test where appropriate.



## Results

Intravenous administration of *E. coli* endotoxin (50  $\mu\text{g/kg}$ ) on the first day evoked a biphasic febrile response (Figure 29A). After a 90 min latency core temperature quickly rose to a first fever peak ( $1.15 \pm 0.09$  °C) at 150 min and a second fever peak ( $1.25 \pm 0.13$  °C) at 300 min post endotoxin. Just 24h later the same group of animals displayed considerable refractoriness to the endotoxin (Figure 29B). Initially, there was an enhanced sensitivity to the endotoxin as body temperature began to rise 60 min earlier than the previous day and peaked at 120 min ( $1.04 \pm 0.12$  °C). However, temperatures then fell toward baseline and no second peak arose. Thus tolerance to the pyrogenic effects of intravenous endotoxin was apparent after only one previous challenge. The febrile reaction to a subsequent third and fourth injection of endotoxin (Figures 29C and 29D) did not significantly alter the fever profile compared to day 2 (Figure 29B).

When tolerant animals (intravenous endotoxin for 2 days prior to intraseptal microinjection) were administered saline into the VSA (0.5  $\mu\text{L}$  bilaterally) 15 min prior to a third or fourth endotoxin injection, a monophasic febrile response was observed (Figure 30A and 30B). This response was similar to a third or fourth injection of endotoxin alone. However, the thermoregulatory response to endotoxin was markedly enhanced when the pyrogen was preceded by VSA administration of the  $V_1$  antagonist,  $\text{d(CH}_2)_5\text{Tyr(Me)AVP}$ . The magnitude of this effect was dependent upon the concentration of  $\text{d(CH}_2)_5\text{Tyr(Me)AVP}$  utilized (0.43 - 4.3 nmol, bilaterally). The peak effect following  $V_1$  antagonist treatment occurred between 90 and 150 min in both conditions. However, animals receiving the 4.3 nmol dose not only achieved higher core temperatures but maintained this response in excess of 6 hours (Figure 30B). The small difference in baseline body temperatures in the experiments depicted in Figure 30A were not statistically significant ( $p = 0.096$ , paired t-test). In 2 additional animals,

saline or  $d(CH_2)_5Tyr(Me)AVP$  (0.43 nmol) injected into areas dorsal to the VSA (lateral septum), did not affect the resultant tolerant febrile reaction to endotoxin (data not shown).

The same injection regime was employed to examine the effects of the  $V_2$  antidiuretic antagonist,  $d(CH_2)_5[D-Ile^2, Abu^4]AVP$  on endotoxin tolerance. Bilateral administration of  $d(CH_2)_5[D-Ile^2, Abu^4]AVP$  (0.43 nmol) within the VSA did not alter the body temperature profile to intravenous endotoxin compared to paired saline controls (Figure 31). Indeed the core temperature responses were indistinguishable from animals receiving a third or fourth endotoxin treatment alone (Figures 29C, 29D).

Figure 32 shows the thermoregulatory response of tolerant rats after bilateral administration of saline (0.5  $\mu$ L) or two doses of  $d(CH_2)_5Tyr(Me)AVP$  into the VSA without intravenous endotoxin. No significant changes in core temperature were observed following either saline ( $F=0.54$ ,  $p=0.87$ ) or either concentration of  $d(CH_2)_5Tyr(Me)AVP$  (0.43 nmol,  $F=0.22$ ,  $p=1.0$ ; 4.3 nmol,  $F=0.36$ ,  $p=0.96$ ).

Figure 33 illustrates the mean fever indices in each experimental group. The fever indices from animals treated with endotoxin alone are depicted in Figure 33A. After the first endotoxin treatment the mean fever index declined 50% from  $5.0 \pm 0.42$   $^{\circ}C \cdot h$ , day 1 to  $2.53 \pm 0.49$   $^{\circ}C \cdot h$  day 2 ( $p < 0.05$  ANOVA, Newman-Keuls). The mean fever indices for day 3 and 4 of endotoxin were  $2.51 \pm 0.53$   $^{\circ}C \cdot h$  and  $2.76 \pm 0.52$   $^{\circ}C \cdot h$  respectively (data not shown). The fever indices for day 3 and 4 were combined since experimental groups contained data from days 3 and 4 as well. The mean fever index for days 3 and 4 combined was  $2.63 \pm 0.36$   $^{\circ}C \cdot h$  ( $p < 0.05$  ANOVA, Newman-Keuls, compared to day 1). Because animals receiving intraseptal injections served as their

own controls each mean fever index represents the data from the third or fourth endotoxin injection. Thus we could directly compare them with control data from days 3 and 4 combined.

Pretreatment with both concentrations of  $d(CH_2)_5Tyr(Me)AVP$ , within the VSA, significantly enhanced the febrile response to endotoxin, compared to saline controls in tolerant rats (Figure 33B). Animals pretreated with  $d(CH_2)_5[D-Ile^2, Abu^4]AVP$  within the VSA were not significantly different from paired saline controls (Figure 33C) or day 3 or 4 time controls.

The neural loci where  $d(CH_2)_5Tyr(Me)AVP$  was effective in enhancing the febrile response during tolerance are depicted in Figure 34. Closed circles denote positive sites and open circles denote injection loci that did not affect tolerance to iv endotoxin.

## Discussion

Tolerance to the pyrogenic effects of *E. coli* endotoxin were observed in the rat after only one previous intravenous injection. This response was not diminished further following a third or fourth daily injection of endotoxin. The characteristics of this tolerance were similar to that seen in the rabbit in that the initial biphasic febrile reaction was reduced to a monophasic response within 24 h (Beeson, 1947a; Greisman and Woodward, 1970). Tolerance of this type is referred to as early-phase endotoxin tolerance and is characterized by rapid induction, transient nature, lack of association with anti-endotoxin antibodies and enhanced hepatic phagocyticity (Greisman, 1983). It is generally believed that early-phase tolerance is due to the enhanced uptake of endotoxin by hepatic Kupffer cells which have lost their ability to either release and/or

produce endogenous pyrogen (Dinarello et al, 1968; Greisman and Woodward, 1970). However, other serum soluble factors may also contribute (Greisman et al, 1963; Dinarello et al, 1968).

Studies designed to test the hypothesis that the hepatic Kupffer cell is the primary target for endotoxin and thus an integral component in tolerance induction, were conducted utilizing "blockade" of hepatic reticuloendothelial system (RES) function (Beeson, 1947b, Greisman et al, 1963; Greisman and Woodward, 1970). This blockade was accomplished by treatment of animals with a colloidal suspension of thorium dioxide (Thorotrast) designed to saturate RES phagocytosis. Initially, results from Thorotrast studies seemed to confirm the essential role for the hepatic RES in tolerance induction (Beeson, 1947b). However, Greisman and co-workers (1963) later demonstrated that 81% of tolerance activity is retained following Thorotrast treatment. Moreover, Thorotrast alone causes fever in tolerant and non-tolerant rabbits and RES blockade rendered tolerant and non-tolerant animals more reactive to endotoxin. These effects of Thorotrast remain to be adequately explained (Greisman, 1983).

Continued study by Greisman and Woodward (1970) on the hepatic mechanisms during pyrogen tolerance led these investigators to conclude that the initial portion of a biphasic endotoxin fever as well as the monophasic febrile response during tolerance were due to endotoxin acting on extra-hepatic structures, possibly the central nervous system (CNS). Indeed, monophasic fevers are produced by small doses of centrally acting endotoxin (see previous chapters), and the febrogenic mediators interleukin-1 (IL-1) (Dascombe et al, 1989) and tumor necrosis factor (TNF) (Rothwell, 1988), often these fevers are characterized by both higher magnitude and duration than observed after intravenous endotoxin during tolerance. Therefore it is possible that a CNS mechanism may contribute to endotoxin tolerance. Support for this contention has recently been provided by Cooper et al (1988). This group observed increased staining

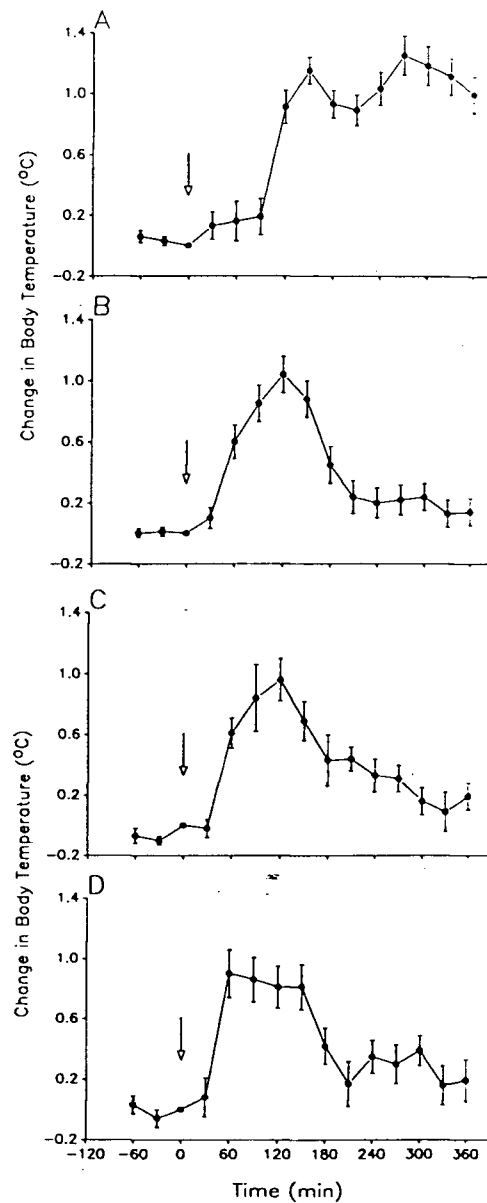


Figure 29. Mean change  $\pm$  SEM in body temperature ( $^{\circ}\text{C}$ ) in response to intravenous endotoxin at time 0 (arrow). A: day 1 of endotoxin, baseline body temperature  $37.28 \pm 0.07$   $^{\circ}\text{C}$  ( $n=29$ ). B: day 2 of endotoxin, baseline body temperature  $37.37 \pm 0.09$   $^{\circ}\text{C}$  ( $n=29$ ). C: day 3 of endotoxin, baseline body temperature  $37.04 \pm 0.06$   $^{\circ}\text{C}$  ( $n=6$ ). D: day 4 of endotoxin, baseline body temperature  $37.09 \pm 0.16$   $^{\circ}\text{C}$  ( $n=6$ ).

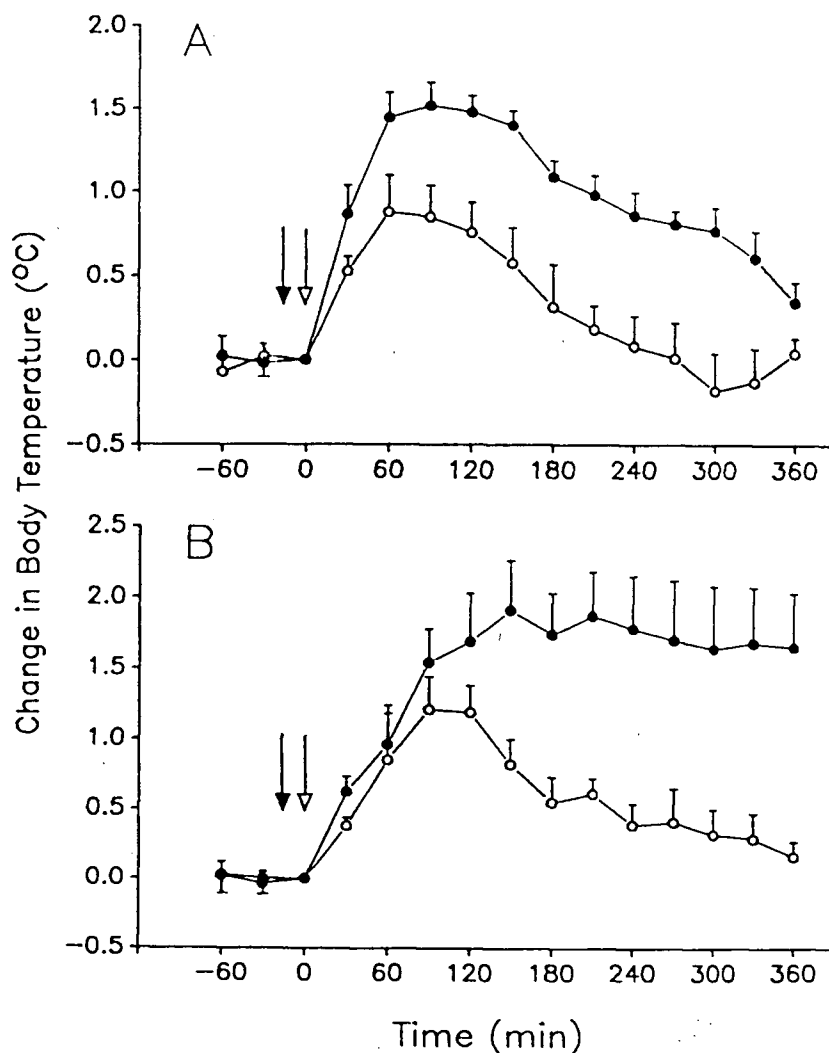


Figure 30. Mean change  $\pm$  SEM in body temperature ( $^{\circ}\text{C}$ ) in response to intravenous endotoxin (open arrow) in endotoxin tolerant rats. 15 min prior to this  $0.5\ \mu\text{L}$  of saline (open circles) or the  $V_1$  antagonist,  $\text{d}(\text{CH}_2)_5\text{Tyr}(\text{Me})\text{AVP}$ , (closed circles) were bilaterally injected into the VSA (closed arrow). Panel A: The effect of  $0.43\ \text{nmol}$   $V_1$  antagonist or saline administered bilaterally within the VSA on the body temperature response to endotoxin. The mean baseline body temperatures were: saline(VSA) + endotoxin,  $37.73 \pm 0.18\ ^{\circ}\text{C}$  ( $n=8$ );  $0.43\ \text{nmol}$   $V_1$  antagonist(VSA) + endotoxin,  $37.41 \pm 0.11\ ^{\circ}\text{C}$  ( $n=8$ ). Panel B: The effect of  $4.3\ \text{nmol}$   $V_1$  antagonist or saline administered within the VSA on the body temperature response to endotoxin. The mean baseline body temperatures were: saline(VSA) + endotoxin,  $37.37 \pm 0.08\ ^{\circ}\text{C}$  ( $n=6$ );  $4.3\ \text{nmol}$   $V_1$  antagonist(VSA) + endotoxin,  $37.32 \pm 0.09\ ^{\circ}\text{C}$  ( $n=6$ ).

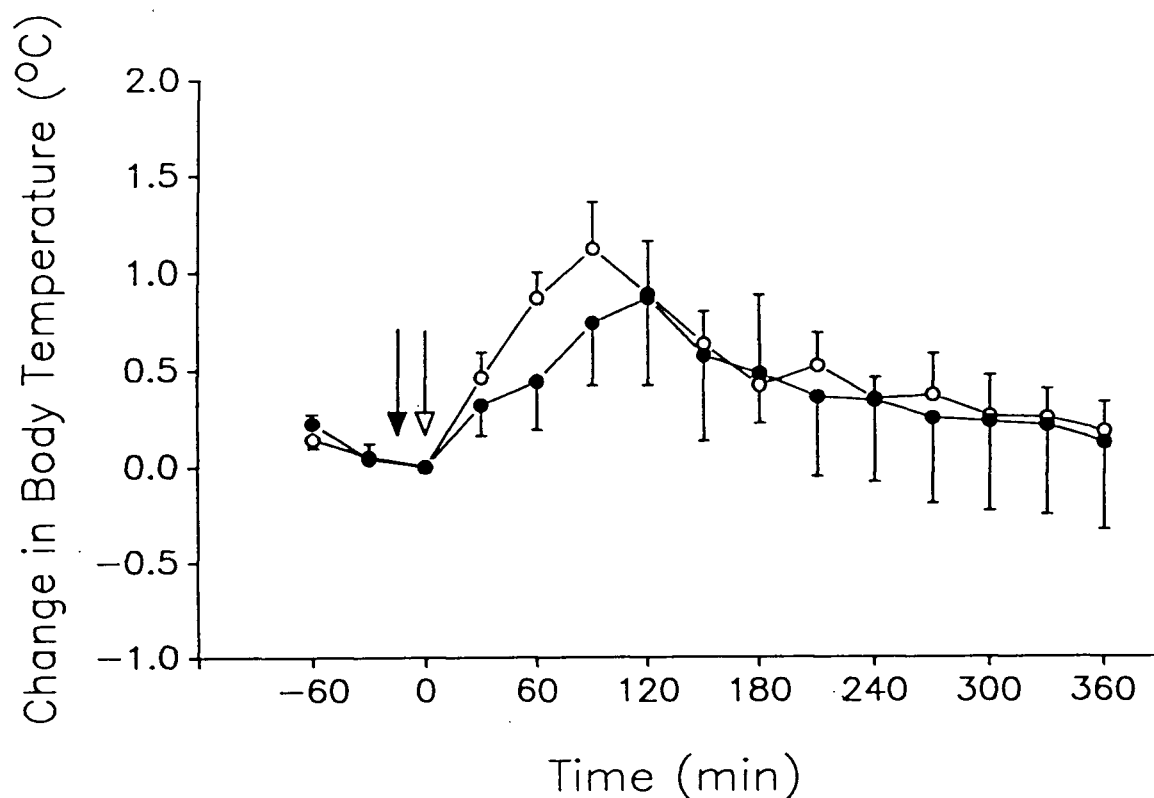


Figure 31. Mean change  $\pm$  SEM in body temperature ( $^{\circ}\text{C}$ ) in response to intravenous endotoxin (open arrow) in endotoxin tolerant rats. 15 min prior to this 0.5  $\mu\text{L}$  of saline (open circles) or 0.43 nmol of the  $\text{V}_2$  antagonist,  $\text{d}(\text{CH}_2)_5[\text{D-Ile}^2, \text{Abu}^4]\text{AVP}$  (closed circles) were bilaterally injected into the VSA (closed arrow). Baseline body temperature: saline(VSA) + endotoxin,  $37.69 \pm 0.15$   $^{\circ}\text{C}$  ( $n=6$ );  $\text{V}_2$  antagonist(VSA) + endotoxin,  $37.91 \pm 0.16$   $^{\circ}\text{C}$  ( $n=6$ ).

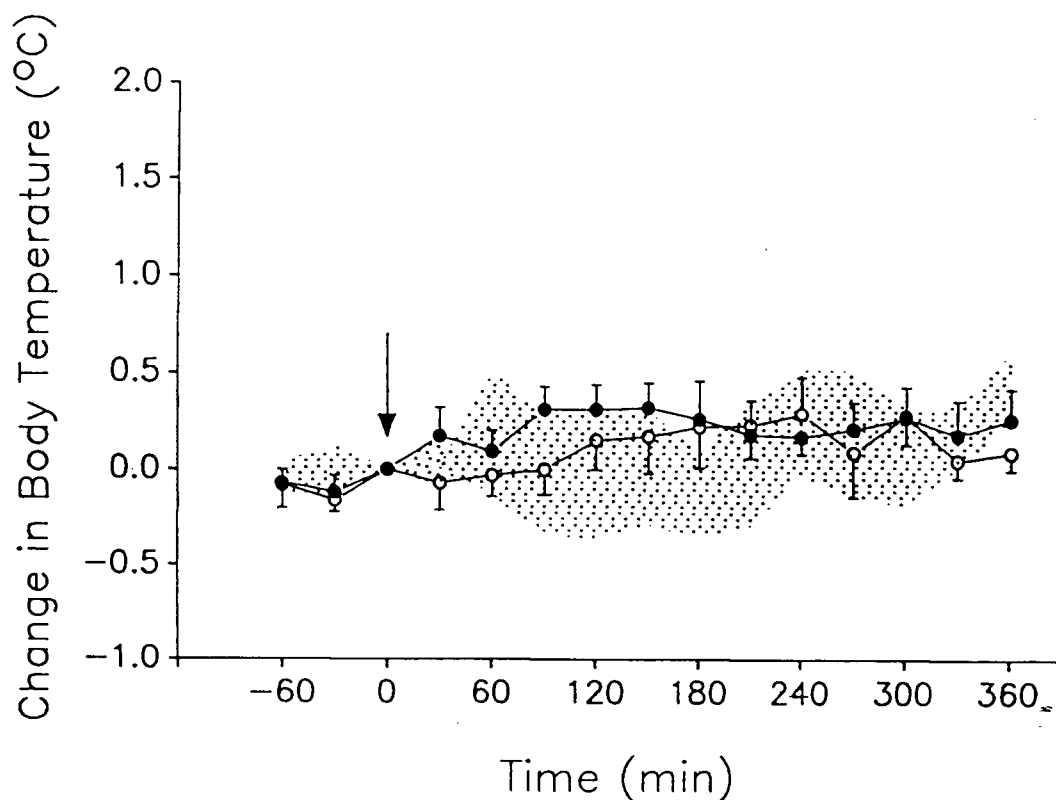


Figure 32. Mean change  $\pm$  SEM in body temperature ( $^{\circ}\text{C}$ ) in response to  $0.5\ \mu\text{L}$  saline (open circles),  $0.43\ \text{nmol}$  (closed circles) or  $4.3\ \text{nmol}$  (shaded area) of the  $\text{V}_1$  antagonist,  $\text{d}(\text{CH}_2)_5\text{Tyr}(\text{Me})\text{AVP}$  bilaterally injected into the VSA (arrow) of endotoxin tolerant rats. Baseline body temperature: saline (VSA),  $37.60 \pm 0.12\ ^{\circ}\text{C}$  ( $n=9$ );  $0.43\ \text{nmol}$   $\text{V}_1$  antagonist (VSA),  $37.25 \pm 0.14\ ^{\circ}\text{C}$  ( $n=9$ ),  $4.3\ \text{nmol}$   $\text{V}_1$  antagonist (VSA),  $37.15 \pm 0.12\ ^{\circ}\text{C}$  ( $n=6$ ).



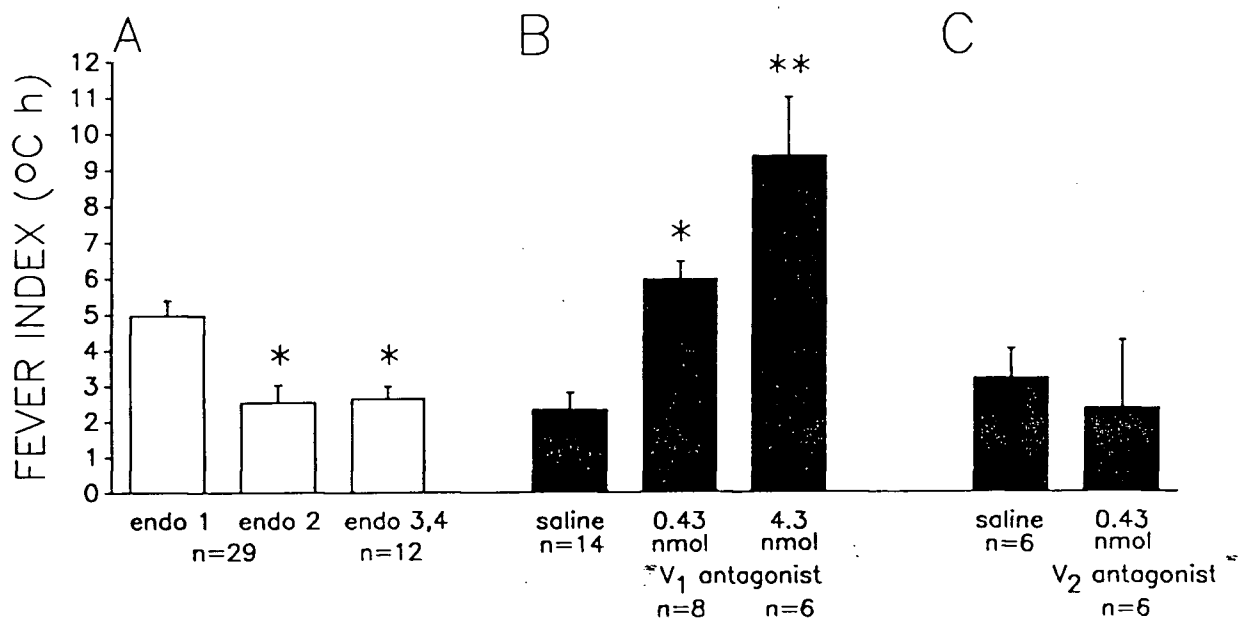


Figure 33. Cumulative 6h thermoregulatory responses (Fever Index) to endotoxin alone and endotoxin preceded by VSA microinjections. Panel A: Fever indices depicting tolerance development to intravenous endotoxin from 1st injection (endo 1) to 3rd and 4th injection (endo 3,4). \*  $p < 0.05$ , ANOVA, Newman-Keuls. Panel B: Fever indices of tolerant rats after VSA pretreatment with saline or V<sub>1</sub> antagonist followed by intravenous endotoxin (3rd or 4th injection). \*  $p < 0.05$ , \*\*  $p < 0.01$ , ANOVA, Newman-Keuls. Panel C: Fever indices of tolerant rats after VSA pretreatment with saline or V<sub>2</sub> antagonist followed by intravenous endotoxin (3rd or 4th injection).

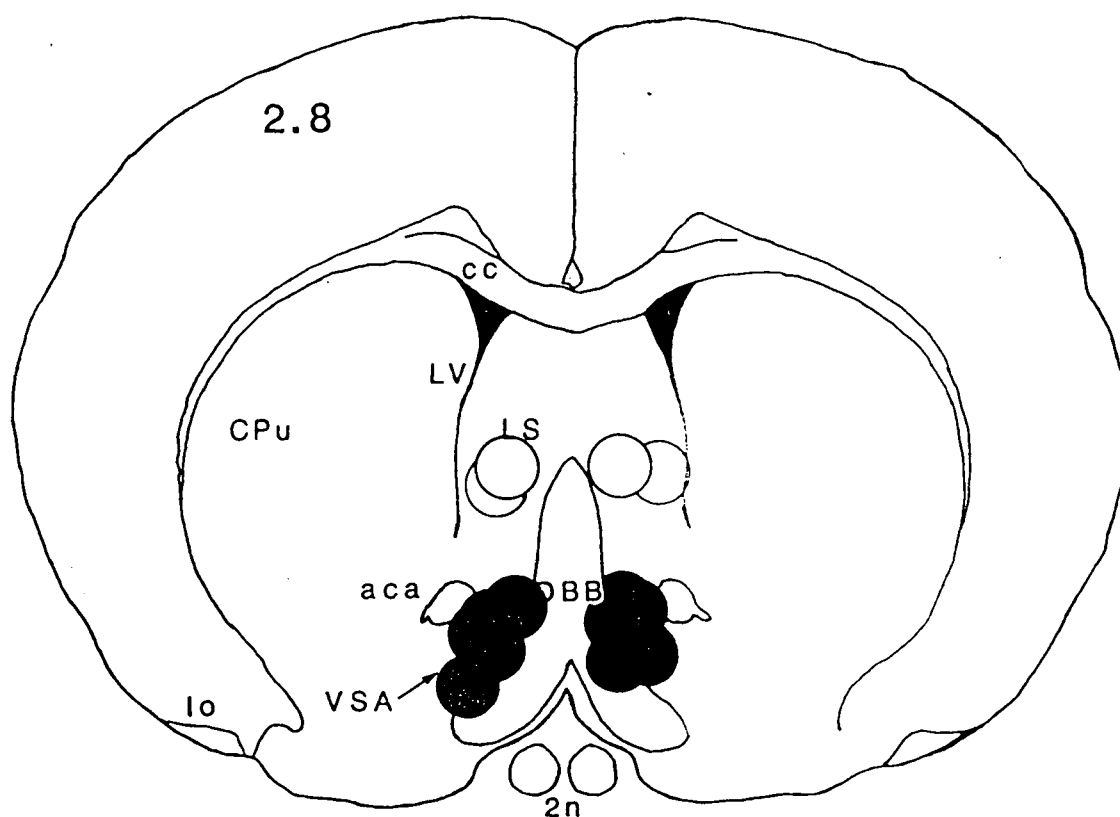


Figure 34. Representative histological section depicting injection sites where the  $V_1$  antagonist,  $d(CH_2)_5Tyr(Me)AVP$ , enhanced (closed circles) or had no effect (open circles) on the fever evoked by intravenous endotoxin in endotoxin tolerant rats. aca, anterior commissure; cc, corpus callosum; CPu, caudate putamen; DBB, diagonal band of Broca; lo, lateral olfactory tract; LS, lateral septum; LV, lateral ventricle; VSA, ventral septal area; 2n, optic tracts. Number in top left corner refers to approximate distance from bregma.

for immunoreactive AVP within the septal region of the guinea pig brain during pyrogen tolerance. Since there is strong evidence that AVP acts as an endogenous antipyretic, it is possible that this peptide may also constitute a CNS component contributing to the induction or maintenance of endotoxin tolerance.

The present data indicate that blockade of VSA vasopressin V<sub>1</sub> receptors using d(CH<sub>2</sub>)<sub>5</sub>Tyr(Me)AVP, markedly enhances the febrile response to *E. coli* endotoxin despite demonstrable tolerance to this pyrogen. This effect was not observed if saline was administered into the VSA prior to endotoxin. The AVP antagonist, d(CH<sub>2</sub>)<sub>5</sub>Tyr(Me)AVP, is a peripheral V<sub>1</sub> vasopressor receptor antagonist (Kruzynski et al, 1980) which binds competitively to VSA V<sub>1</sub> receptors *in vitro* (Lawrence et al, 1988; Poulin et al, 1988). The effects of d(CH<sub>2</sub>)<sub>5</sub>Tyr(Me)AVP on endotoxin fever in the tolerant rat cannot be attributed to a thermogenic effect of the analogue since it had no dramatic thermoregulatory effects when injected into the VSA without subsequent intravenous endotoxin. Although there was a small rise in core temperature (peak rise,  $0.32 \pm 0.13$  °C) in V<sub>1</sub> antagonist treated rats (0.43 nmol), this was not statistically different over time, nor was the time course of this response sufficient to explain the results observed in Figure 30A. The observed effect of d(CH<sub>2</sub>)<sub>5</sub>Tyr(Me)AVP on normal core temperature in this study are in close agreement with previous work (Cooper et al, 1987; Naylor et al, 1988; see also chapter III and IV).

Blockade of AVP V<sub>1</sub> receptors within the VSA prior to a pyrogenic challenge results in an enhanced febrile response in non-tolerant animals (Cooper et al, 1987; Naylor et al, 1988). In addition, kainic acid lesions (Martin et al, 1988) or depletion of VSA AVP by castration (Pittman et al, 1988) also augments fever evoked by PGE<sub>1</sub>. Specifically, and perhaps more important to this study, is the trend in these experiments for the fevers to be enhanced and prolonged in the later stages of the febrile episode (Cooper et al, 1987; Martin et al, 1988; Naylor et al, 1988; Pittman et al, 1988). In the

present study VSA  $V_1$  receptor antagonism enhanced the febrile reaction to endotoxin in the tolerant rat. However, there is an essential difference between the augmentation of fever reported here and those previously cited. From the results described here, it is evident that VSA administration of the small dose (0.43 nmol) of  $d(CH_2)_5Tyr(Me)AVP$  transiently augmented the tolerant reaction to endotoxin. However, from 150 min post endotoxin, the body temperature in these animals declined in a manner similar to controls. Previously, this same dose was sufficient to significantly enhance and prolong the fever evoked by either icv IL-1 (Cooper et al, 1987) or  $PGE_1$  (Naylor et al, 1988). In order to obtain a sustained pyrexia in endotoxin tolerant animals, a 10x greater concentration of the  $V_1$  antagonist was required. Under these conditions, the febrile episode, despite tolerance to endotoxin, was elevated and prolonged in a manner similar to previous studies utilizing  $d(CH_2)_5(Me)AVP$  or AVP antisera prior to a pyrogenic stimulus (Cooper et al, 1987; Malkinson et al, 1987). The present observation that 0.43 nmol of  $d(CH_2)_5Tyr(Me)AVP$  was unable to sustain the fever in the tolerant animals indicates that the tolerance process somehow modifies the AVP antipyretic system normally operating within the VSA during fever.

Experiments utilizing 4.3 nmol of the  $V_1$  antagonist suggest that during tolerance the endogenous AVP activity within the VSA is amplified compared to non-tolerant animals. This process could be accomplished by either AVP receptor upregulation, increased synaptic release of AVP or a combination of these two events. While it is tempting to suggest that vasopressin receptor blockade results in reversal of the tolerance to endotoxin, a cautious interpretation is forwarded primarily due to the observation that the endotoxin-induced fever profile in tolerant rats pre-treated with 4.3 nmol of  $V_1$  antagonist is different from naive animals receiving endotoxin alone. These differences may underscore the hepatic contribution to the tolerance phenomenon.

However, our incomplete understanding of the mechanisms involved in tolerance and the febrile process in general does not allow adequate resolution of this issue.

Although tolerance is easily induced by intravenous endotoxin the present data raise the question as to why tolerance does not develop to other pyrogenic agents, particularly if the AVP antipyretic system is generally activated during fever. The simplest explanation may be that peripherally administered endotoxin stimulates a cascade of events, including the release of various cytokines, endocrines and perhaps, as yet unidentified substances. These components may have synergistic effects that contribute to the tolerance process, possibly resulting in enhanced vasopressin release within the VSA neuropil. Indeed, a recent report has shown that recombinant IL-1 or TNF alone cannot induce endotoxin tolerance. However, together these cytokines act synergistically to induce tolerance to endotoxin (Vogel et al, 1988). Therefore, the strength of the AVP antipyretic response, and subsequent participation in tolerance, may directly depend upon the combined action of several endogenous mediators.

In contrast to the effects of the  $V_1$  antagonist, administration of an equimolar dose of the  $V_2$  antagonist,  $d(CH_2)_5[D-Ile^2, Abu^4]AVP$ , prior to intravenous endotoxin, did not affect the febrile response in the tolerant rat. This indicates that fever enhancement during tolerance is specific to the  $V_1$  receptor and is unlikely to involve  $V_2$ -like receptors. Consistent with this are the observations that a  $V_2$  agonist (desamino-8-D-arginine vasopressin) is not antipyretic (Naylor et al, 1987) and the  $V_2$  antagonist,  $d(CH_2)_5-D-ValVAVP$ , does not enhance fever (Cooper et al, 1987; Naylor et al, 1988). Furthermore, the same  $V_2$  antagonist utilized in the present study was ineffective, within the VSA, in attenuating indomethacin-induced antipyresis, contrasting that seen during  $V_1$ -blockade (see chapter III).

The present data provide further support for an endogenous antipyretic function for centrally acting vasopressin. Furthermore, the results demonstrate that V<sub>1</sub> blockade within the VSA reverses at least some portion of endotoxin tolerance and therefore endogenous vasopressin may contribute, along with hepatic mechanisms, to the state of pyrogenic tolerance. The nature of the vasopressinergic mechanism contributing to endotoxin tolerance is not clear from these data and thus the tolerance-induced changes within the VSA remain speculative. It is clear, however, that additional work will be required to further investigate this new hypothesis.

## **IX. THE THERMOREGULATORY EFFECTS OF CENTRALLY ADMINISTERED PYROGENS DURING ACTIVE PYROGENIC TOLERANCE TO INTRAVENOUS ENDOTOXIN**

### **Introduction**

The phenomenon of pyrogenic tolerance has thus far been demonstrated only following intravenously administered endotoxin and in some cases for TNF $\alpha$  (Nakamura et al, 1988; Kawasaki et al, 1989). The previous chapter presented data which showed pyrogenic tolerance to intravenous endotoxin in the rat and the association of this tolerance to a central antipyretic system involving AVP. Pyrogenic tolerance is not observed, however, when pyrogens are introduced into the cerebral ventricles. Yet, activation of the AVP endogenous antipyretic pathway has been demonstrated following both peripherally (Cooper et al, 1979; Malkinson et al, 1987) and centrally administered pyrogens (Cooper et al, 1987; Naylor et al, 1988; Landgraf et al, 1990b). It is unclear, then, why stimulation of this antipyretic pathway does not confer tolerance to centrally administered pyrogenic agents.

There is little doubt that the physiological mechanisms which prompt the febrile process differ following peripheral or central administration of pyrogens (Morimoto et al, 1987b; Morimoto et al, 1988d; Stitt et al, 1985). It is precisely these differences which may contribute to the mechanisms, central and peripheral, that ultimately lead to the development of pyrogenic tolerance. If, however, endotoxin tolerance indeed activates the AVP endogenous antipyretic pathway, then it should be possible to demonstrate this activated antipyretic state by observing a diminished thermal response to centrally delivered pyrogens. The present study was conducted to test this hypothesis

by comparing the febrile response to icv PGE<sub>2</sub> or endotoxin during active pyrogenic tolerance to intravenous endotoxin. In, addition, the endogenous release of AVP within the VSA was determined, using push-pull perfusion, following intravenous endotoxin in naive and endotoxin tolerant rats.

## Methods

Male Sprague-Dawley rats were surgically prepared with an intravenous catheter for the administration of endotoxin (50 µg/kg) and a guide cannula directed towards a lateral cerebral ventricle for icv injections of PGE<sub>2</sub> (50 ng in 5 µL saline), endotoxin (10 ng in 5 µL saline) or saline alone. Body temperature was measured via biotelemetry. Animals were rendered tolerant to intravenous endotoxin by 2 daily injections of endotoxin, as established in the preceding chapter. Experiments were conducted on the third and fourth day of endotoxin challenge. On these days endotoxin was injected iv and after 180 min, saline, PGE<sub>2</sub> or endotoxin were introduced into the lateral cerebral ventricles, by gravity flow, and the core temperature monitored for 3h. The 180 min point was chosen for the icv injections because body temperatures were usually close to baseline levels by this time and thus any diminished febrile responses to icv treatments could not be construed as due to the differences in pre-injection temperatures. A tolerant animal received only two icv treatments with one trial being a saline control. The order of saline or pyrogen administration was random. A separate naive (non-tolerant) group of rats served as controls to allow comparisons between the thermoregulatory responses to icv injections in tolerant and non-tolerant rats. A fever index was calculated for the 3h period following the icv injections.

A separate group of animals were prepared with intravenous catheters and an unilateral withdrawal cannula for push-pull perfusion of the VSA. The perfusion system was identical to that described in chapter II. Eight consecutive 30 min VSA



push-pull perfusions were conducted in the conscious and unrestrained rat. Following a control perfusion, endotoxin (50  $\mu\text{g/kg}$ ) was injected intravenously and the VSA perfusions were continued for 3h. The following day the rats received a second endotoxin challenge but were not perfused. One day later the same animals were perfused again for 30 min before and for 3h subsequent to a third endotoxin injection. The 30 min perfusion samples were collected on ice and later assayed for AVP. Perfusion loci were verified histologically.

## Results

The febrile response following a first and second intravenous endotoxin challenge is depicted in Figure 35. The monophasic response characteristic of tolerance is evident on day 2 of intravenous endotoxin. Figure 36 summarizes the results of experiments employing icv administration of saline,  $\text{PGE}_2$  or endotoxin in naive (panel A) and endotoxin tolerant rats (panel B). As in naive animals, pyrogens injected into the brain of tolerant rats elicited a febrile reaction. In naive animals the maximum change in body temperature following icv  $\text{PGE}_2$  and endotoxin was  $1.42 \pm 0.03$  °C (after 30 min) and  $1.54 \pm 0.18$  °C (after 180 min) respectively. Whereas in animals experiencing the latter stages of a tolerant endotoxin reaction the maximal thermogenic responses were  $1.29 \pm 0.11$  °C (after 30 min) and  $0.90 \pm 0.27$  °C (after 90 min) for icv  $\text{PGE}_2$  and endotoxin, respectively. The maximal hyperthermic responses following icv  $\text{PGE}_2$  were not significantly different (unpaired t-test) in naive or tolerant animals, nor were the body temperatures just prior to injection of the pyrogen (naive:  $37.06 \pm 0.15$  °C, versus tolerant:  $37.52 \pm 0.23$  °C). In animals receiving icv endotoxin, there was a significant difference ( $p < 0.05$ , unpaired t-test) in fever height between naive and tolerant rats at 180 min post-injection ( $1.54 \pm 0.18$  °C versus  $0.68 \pm 0.31$  °C, respectively). This occurred despite the fact that the naive animals started from a

significantly higher mean baseline temperature than their tolerant counterparts (naive:  $38.09 \pm 0.17$  °C, versus, tolerant:  $37.68 \pm 0.15$  °C;  $p < 0.05$ , unpaired t-test).

Examination of the 3h fever indices calculated from the naive and tolerant animals revealed that the thermoregulatory responses to centrally administered pyrogens elicited significant increases in core temperature compared to saline controls in both groups (Figure 37). Comparison of the fever indices between icv endotoxin or PGE<sub>2</sub> in tolerant or non-tolerant rats did not reflect significant differences.

Figure 38 shows the AVP levels in the VSA push-pull perfusates from rats experiencing an initial (naive) and third (tolerant) intravenous endotoxin challenge. Time 0 represents the basal AVP release prior to endotoxin treatment. There were no changes in AVP release during the fever evoked by the first intravenous endotoxin injection compared to basal (pre-endotoxin) levels. In contrast, there was a significant ( $p < 0.05$ , ANOVA, Newman-Keuls) pulse of AVP released into the perfusates in the first 30 min following a third endotoxin challenge. Thereafter, AVP concentrations were not different compared to time 0.

## Discussion

The present experiments were conducted to examine whether the tolerant response to intravenous endotoxin is transferable to the fevers evoked by centrally administered pyrogens. The idea for these experiments was born out of the observations that endogenous AVP has an apparent role in the endotoxin tolerance process (see previous chapter). Because there is evidence that endogenous AVP is active during centrally initiated fevers it was hoped that activation of this system during endotoxin tolerance would render tolerant animals hyporesponsive to centrally administered pyrogens as well.

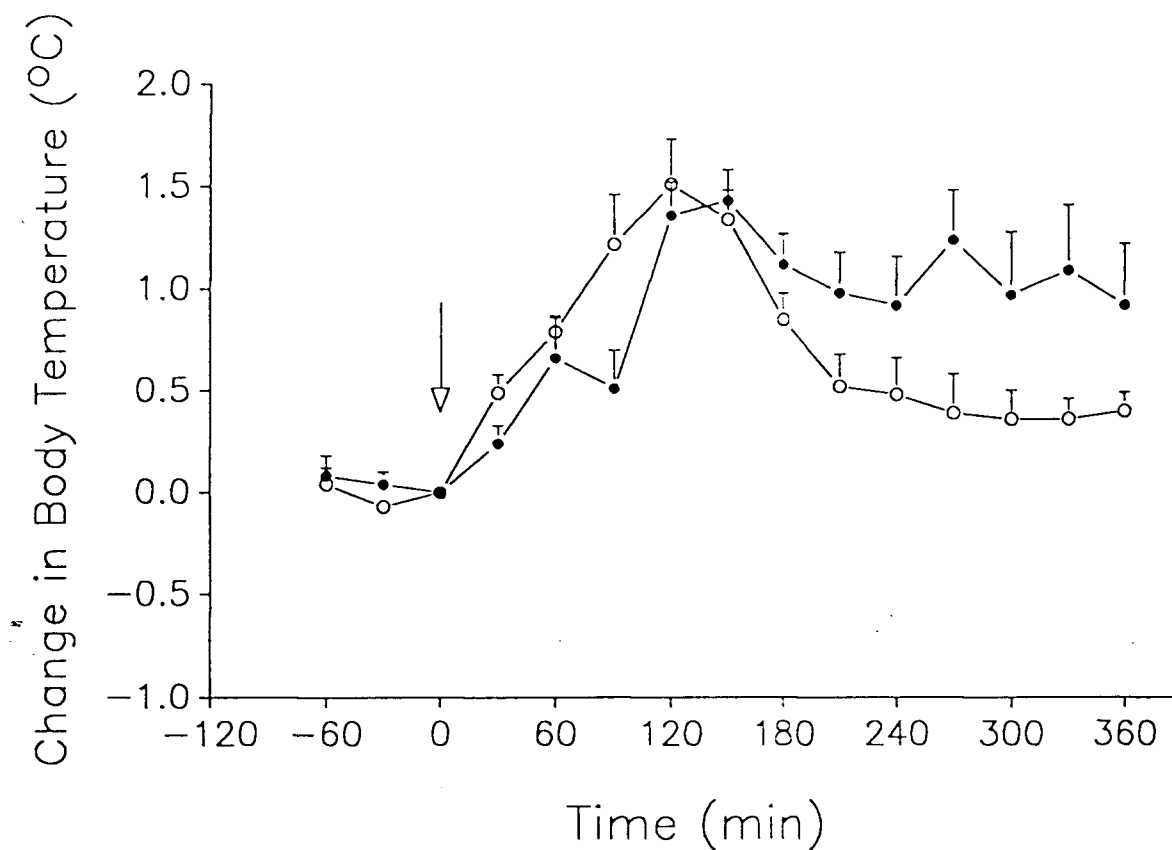


Figure 35. Mean change ( $\pm$  S.E.M.) in body temperature ( $^{\circ}$ C) in response to a first (solid circles) and second (open circles) daily intravenous injection of *E. coli* endotoxin at time 0 (arrow). Mean baseline body temperatures were  $37.29 \pm 0.10$   $^{\circ}$ C and  $37.19 \pm 0.09$   $^{\circ}$ C for naive and tolerant animals respectively ( $n=8$ ).

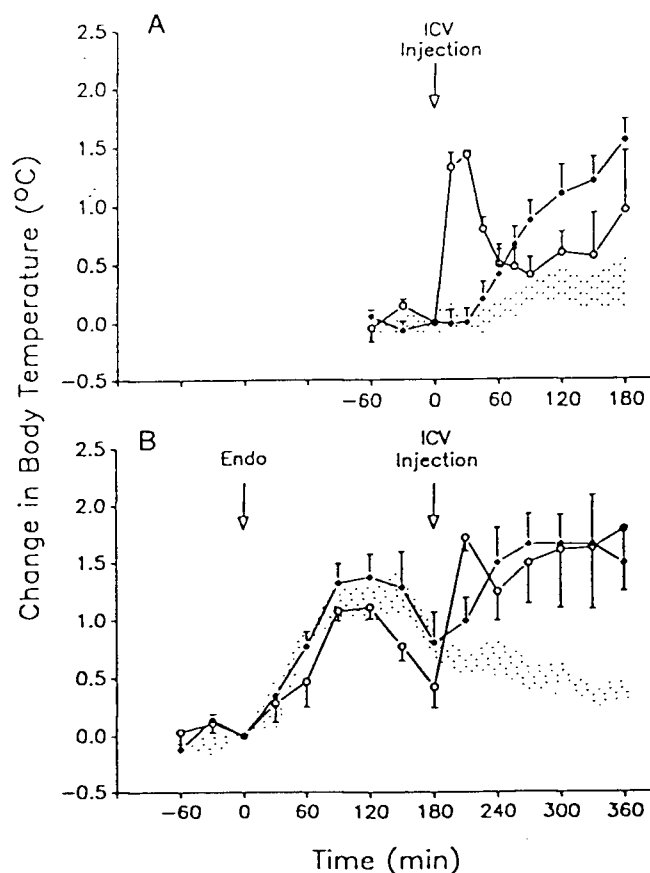


Figure 36. Mean change ( $\pm$  S.E.M.) in body temperature ( $^{\circ}$ C) in response to centrally administered saline (shaded area), PGE<sub>2</sub> (open circles) or endotoxin (closed circles) in naive (no previous iv endotoxin; panel A) or during active tolerance to intravenous endotoxin (two previous iv endotoxin injections; panel B). Panel A: Naive animals received icv injections at time 0 (arrow). Mean baseline body temperatures were saline,  $37.28 \pm 0.16$   $^{\circ}$ C,  $n=7$ ; PGE<sub>2</sub>,  $37.06 \pm 0.15$   $^{\circ}$ C,  $n=7$ ; endotoxin,  $38.09 \pm 0.17$   $^{\circ}$ C,  $n=6$ . Panel B: Tolerant animals received a third or fourth intravenous injection of endotoxin at time 0 (first arrow) followed 180 min later (second arrow) by icv saline (baseline temperature,  $36.94 \pm 0.14$   $^{\circ}$ C,  $n=5$ ), PGE<sub>2</sub> (baseline temperature,  $37.10 \pm 0.17$   $^{\circ}$ C,  $n=6$ ) or endotoxin (baseline temperature,  $36.88 \pm 0.14$   $^{\circ}$ C,  $n=4$ ). See Figure 37 for statistical analysis.

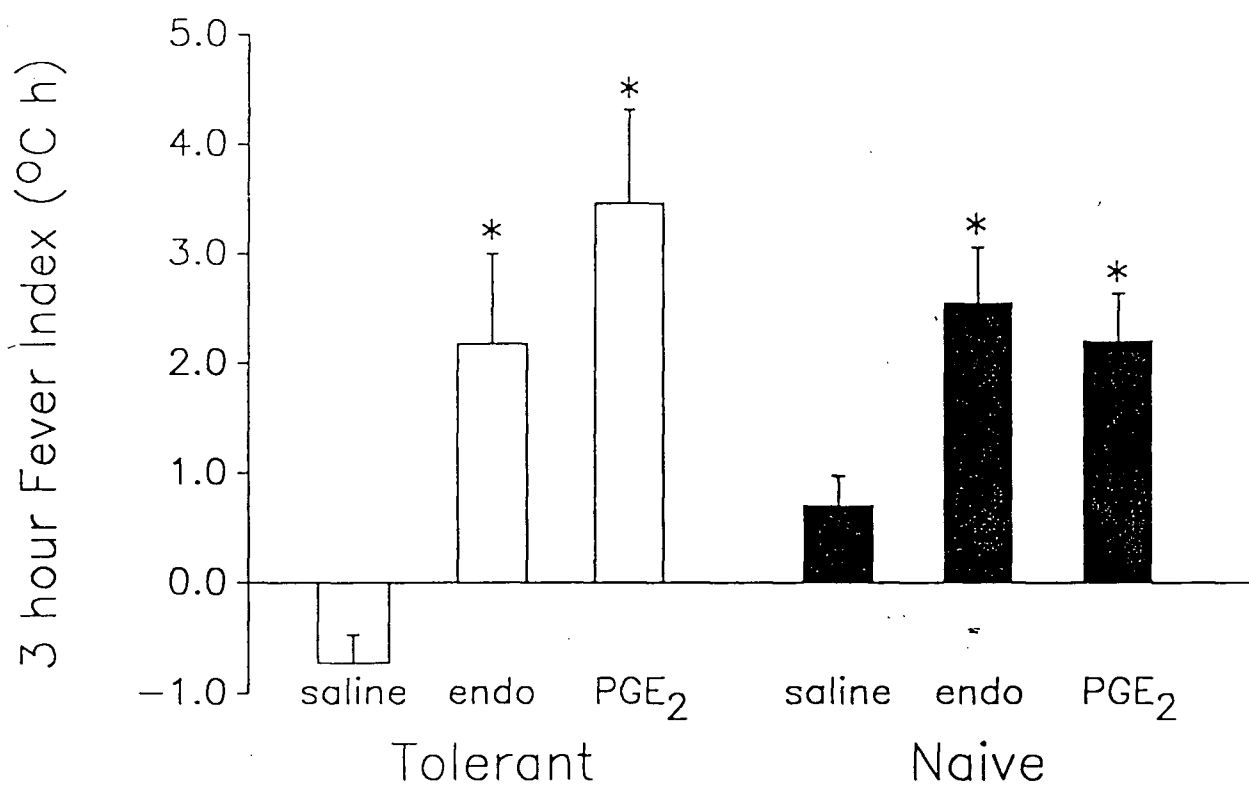


Figure 37. Cumulative 3h thermoregulatory response (fever index) to centrally administered pyrogens in naive (solid bars) and endotoxin tolerant rats (open bars). \*  $p < 0.05$ , one way ANOVA, Newman-Keuls.

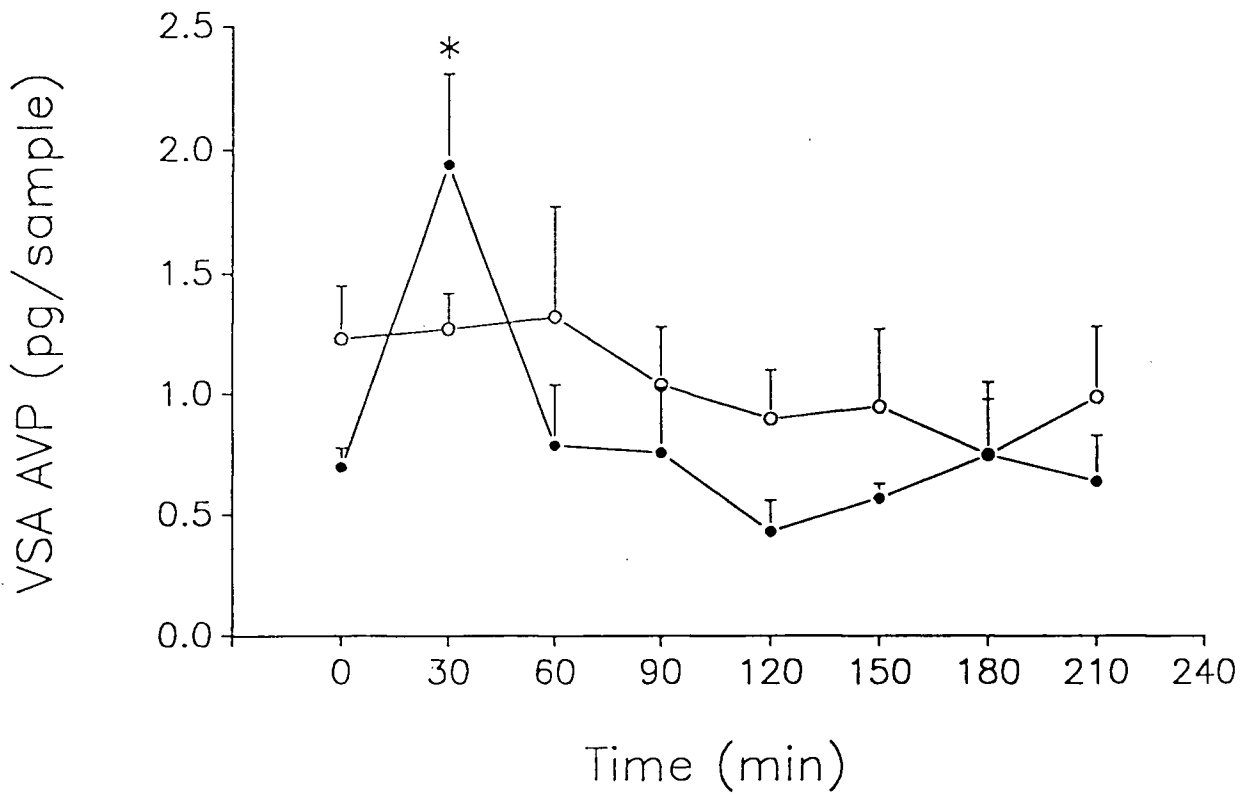


Figure 38. AVP levels in 30 min VSA push-pull perfusates in naive (open circles,  $n=6$ ) and endotoxin tolerant rats (closed circles,  $n=4$ ) before (time 0) and after intravenous endotoxin. \*  $p < 0.05$ , one way ANOVA, Newman-Keuls.

The lack of development of pyrogenic tolerance in response to centrally delivered pyrogens in endotoxin naive animals has long been recognized (Sheth and Borison, 1960). While, the physiological relevance of centrally administered pyrogens has been questioned (Stitt, 1986), it is nonetheless apparent, that the negative feedback mechanism(s) which limit fever height and duration remain functional regardless of the route of pyrogen administration (for e.g. Cooper et al, 1987; chapter VIII). Collectively, the present results do not indicate that tolerance to intravenous endotoxin renders these animals hyporesponsive to either PGE<sub>2</sub> or endotoxin when these pyrogens are introduced into the cerebral ventricles. Indeed, if one examines either the maximal hyperthermic response or the 3h fever index in animals treated with icv PGE<sub>2</sub> there is no major difference between tolerant or naive groups. Interestingly, even the maximum change in temperature following PGE<sub>2</sub> were similar (approximately 1.5 °C). The same was not the case following icv endotoxin. Although the maximal febrile response to icv endotoxin appeared attenuated the data was obtained from a small number of animals (n=4) and examination of the responses from the individual animals indicated that only 1 out of the 4 rats lacked an appreciable febrile response following icv endotoxin. It is provoking, however, that the response to icv endotoxin in naive animals was greater even though they started from a higher body temperature. This is important because the starting, or pre-pyrogen body temperature greatly influences the magnitude of a febrile response (Kasting, 1986a; Malkinson et al, 1988; Bibby and Grimble, 1989). In light of this, the differences in maximal fever height following centrally administered endotoxin may be important. However, analysis of the fever index data revealed a lack of significant difference between tolerant or naive animals with respect to their response to icv endotoxin. These experiments may be more revealing if repeated using larger sample sizes.

The present experiments confirm that the intravenous route of endotoxin administration is an essential requirement for induction of tolerance. The data would also suggest that either the AVP antipyretic system is not generally activated during tolerance or that the AVP system undergoes a refractory period which enables the animal to respond to the centrally administered pyrogen. It would be of interest to this hypothesis by observing the thermoregulatory responses to other *intravenously* injected pyrogens (e.g. PGE<sub>2</sub> or TNF) following a tolerant reaction to endotoxin.

Another way to assess the activity of endogenously acting vasopressin is to directly measure release of the peptide during a tolerant response to endotoxin. In this way an estimate of the antipyretic activity can be made and this can be related to the thermal responses to centrally administered PGE<sub>2</sub> or endotoxin. These experiments were conducted using the technique of push-pull perfusion of the VSA in animals undergoing an initial and tolerant febrile reaction to intravenous endotoxin. Interestingly, intravenous endotoxin in the naive rat did not affect the release of AVP into VSA perfusates. This is in contrast to similar push-pull perfusion studies carried out in the sheep (Cooper et al, 1979) and rabbit (Malkinson et al, 1987). In these studies AVP release decreased during the rising phase of a peripherally induced endotoxin fever. While the present results did show a trend towards decreasing AVP levels at no point were these significantly different from pre-pyrogen levels. Unfortunately, the difficulty of long term push-pull perfusions prevented the perfusion experiments from continuing into the defervescent phase of fever where AVP release was initially reported to be correlated with the decline in febrile body temperature (Cooper et al, 1979).

Perfusion of the VSA during the tolerant endotoxin response revealed a significant release of AVP in the 30 min period following intravenous endotoxin. If the release of the endogenous antipyretic occurs at this time it would indicate that the



endogenously acting peptide requires at least 60 min before the body temperature begins to respond. From the previous experiments employing the AVP antagonists (chapter VIII) it was suggested that the endogenous activity of the AVP system is altered during endotoxin tolerance. The present data confirm this assumption, albeit in an unexpected manner. In addition to the apparent lack of effect an initial injection of endotoxin had on VSA AVP release, the AVP response during tolerance was only evident immediately following the intravenous injection of the pyrogen. Thus other components are likely to be involved in this phenomenon.

In a manner similar to the thermoregulatory and central AVP response to icv PGE<sub>2</sub> (see chapter VI), the tolerant reaction to endotoxin was accompanied by a pulse of AVP release. Oxytocin is co-released with AVP in response to PGE<sub>1</sub> (Landgraf et al, 1990b) and sensitizes the antipyretic effects of AVP (Poulin and Pittman, 1989), therefore it may also be involved in the tolerance phenomenon; perhaps by sensitizing the AVP receptors such that only a short pulse of the antipyretic peptide is required to mediate its effects. This may also help to explain why a larger dose of the AVP antagonist was required to more fully reverse the tolerance to endotoxin (see previous chapter). The AVP antagonist utilized in the previous chapter has some anti-oxytocic activity (Manning and Sawyer, 1986) thus the use of higher concentrations may have been necessary to block both endogenous AVP and oxytocin activity and thus explain the observed results.

The present data demonstrate that tolerance to intravenous endotoxin does not render the tolerant animal hyporesponsive to centrally delivered PGE<sub>2</sub>. However, confident conclusions regarding the effects of tolerance on the pyrogenic response to centrally injected endotoxin must await further experimental results. The small number of animals used in these endotoxin-endotoxin experiments reflect the preliminary nature of these particular results. As to the endogenous release of vasopressin during

tolerance, there are indeed dramatic changes within the VSA which occur during endotoxin tolerance. The retention of a full pyrogenic response to icv PGE<sub>2</sub> and perhaps to endotoxin in tolerant animals may be explained by the idle AVP system at the time of the central injections. However, these release studies would suggest that VSA AVP activity alone cannot fully explain the results of the experiments utilizing AVP antagonists (see previous chapter).

## **X. SUMMARY AND GENERAL DISCUSSION**

The experiments described in this thesis were designed to investigate the role of AVP in the process of drug-induced antipyresis and the suppression of fever during pyrogenic tolerance to intravenous endotoxin. A variety of experimental techniques have been utilized to demonstrate that AVP plays a role in the control of body temperature during fever. It was the intent of this research to further delineate this antipyretic function particularly with regards to stimuli which lower or suppress the febrile response. The major findings of this research were:

- (1) The antipyretic mechanism of action for peripherally administered indomethacin and sodium salicylate involves a vasopressinergic component acting on V<sub>1</sub> but not V<sub>2</sub>-like receptors within the ventral septal area of the rat brain. However, the antipyretic action of acetaminophen does not seem to include this vasopressin-mediated neural pathway.
- (2) Endogenous AVP released in response to fever and drug-induced antipyresis was measured in the extracellular fluid surrounding VSA nerve terminals using the technique of push-pull perfusion. AVP release was found to increase in response to antipyretic intervention with indomethacin but not acetaminophen. This was consistent with experiments employing AVP receptor antagonists. This stimulatory effect on central AVP release was observed only during endotoxin fever.

(3) Endogenously released AVP in response to fever and drug-induced antipyresis was also measured in the CSF and blood plasma. The febrile response to intravenous endotoxin did not result in alterations to the release of AVP into the CSF. Indomethacin, however, significantly suppressed CSF AVP. Peripherally released AVP was markedly stimulated by intravenous endotoxin; this response was not affected by antipyretic treatment. In contrast, plasma AVP was stimulated by both indomethacin and to some extent, by acetaminophen in non-febrile animals.

(4) The effects of indomethacin on central AVP release were investigated utilizing an alternative pyrogenic agent, PGE<sub>2</sub>. The rising phase of PGE<sub>2</sub>-induced hyperthermia was accompanied by significant increases in AVP levels from VSA push-pull perfusates. Under these conditions, however, indomethacin inhibited rather than stimulated, AVP release in VSA perfusates.

(5) The mechanism of pyrogenic tolerance to intravenous endotoxin appears to include a central vasopressinergic component, that includes V<sub>1</sub> but not V<sub>2</sub> vasopressin receptors.

(6) The tolerant response to intravenously injected endotoxin does not appear to render an animal hyporesponsive to centrally injected pyrogens. This is likely due to the dynamics of AVP release within the VSA during active endotoxin tolerance.

The experiments reported in this thesis have utilized two basic approaches of investigation: pharmacological blockade of endogenous AVP receptors and the *in vivo* measurement of the endogenously released receptor ligand. These techniques yielded evidence which strongly suggests that the mechanism of action of antipyretic drugs can no longer be explained simply as an action on prostaglandin biosynthesis. The investigations with indomethacin and the effects of this drug on body temperature and the central release of AVP are the first to directly link the action of an antipyretic drug with an endogenously acting neuropeptide. Moreover, they provide an example of the multiple sites of action for indomethacin as well as the independent regulation of vasopressin release into various biological compartments. The indomethacin studies do not necessarily rule out an action on PG biosynthesis. However, the currently accepted notion as to how indomethacin exerts its varied biological effects must surely be modified. The studies utilizing sodium salicylate would indicate that the novel effects observed during the course of the indomethacin investigations can probably be extended to salicylate as well.

It remains a curiosity, however, that acetaminophen was an exception despite the drug's similar effects on body temperature. It may be that acetaminophen acts strictly as a PG synthesis inhibitor or perhaps activates alternate antipyretic pathways. Indeed, other endogenous neuropeptides, such as  $\alpha$ -melanotrophin stimulating hormone ( $\alpha$ -MSH) (Lipton et al, 1981), may be involved in the regulation of fever in general or in specific instances, perhaps acetaminophen-induced antipyresis. To date no research has attempted to address the interaction between  $\alpha$ -MSH or AVP during fever. Therefore it is not known whether the two peptidergic systems act redundantly under similar circumstances or are activated specifically in response to particular thermoregulatory stimuli. It is clear, though, that prevention of AVP (Cooper et al, 1987; Malkinson et al, 1987) or of  $\alpha$ -MSH (Shih et al, 1986) from acting on their

respective central receptors does not result in uncontrolled increases in body temperature during a febrile episode.

The experiments in which VSA AVP release was measured in response to PGE<sub>2</sub>-induced hyperthermia, with and without indomethacin treatment, support the many previous observations which demonstrated the lack of effect antipyretic drugs have on PG-mediated thermoregulatory perturbations (reviewed in Clark, 1979). What is unique about the present data is that the PG-mediated increase in VSA AVP levels was suppressed by indomethacin. Thus the antipyretic drug has different actions on AVP neurons of the antipyretic pathway, depending upon the pyrogenic stimulus. In addition, the suppression of the AVP response had no effect on the PG-induced hyperthermia. This inhibitory response on AVP release would suggest that AVP does not have an antipyretic function during PGE<sub>2</sub>-elicited hyperthermia. However, the majority of the published evidence is contrary to this (Ruwe et al, 1985b; Martin et al, 1988; Naylor et al, 1988; Pittman et al, 1988; Landgraf et al, 1990b), although the lack of effect of VSA V<sub>1</sub> blockade on PGE<sub>1</sub> hyperthermia has been published (Fyda et al, 1989). This disparity remains to be adequately explained.

It may be fair to say that the hyperthermia produced by the central administration of PGs is not a suitable model for the study of antipyretic mechanisms. Indeed it becomes very difficult to reconcile much of this thesis research if one assumes a dogmatic PG-mediated fever mechanism. Therefore the most conservative evaluation of the present results as well as in the field in general, should be viewed as the febrile process being the result of more than one febrogenic pathway and that the physiological antipyretic mechanisms (endogenous and exogenous) also involve more than one pathway (see Figure 39).

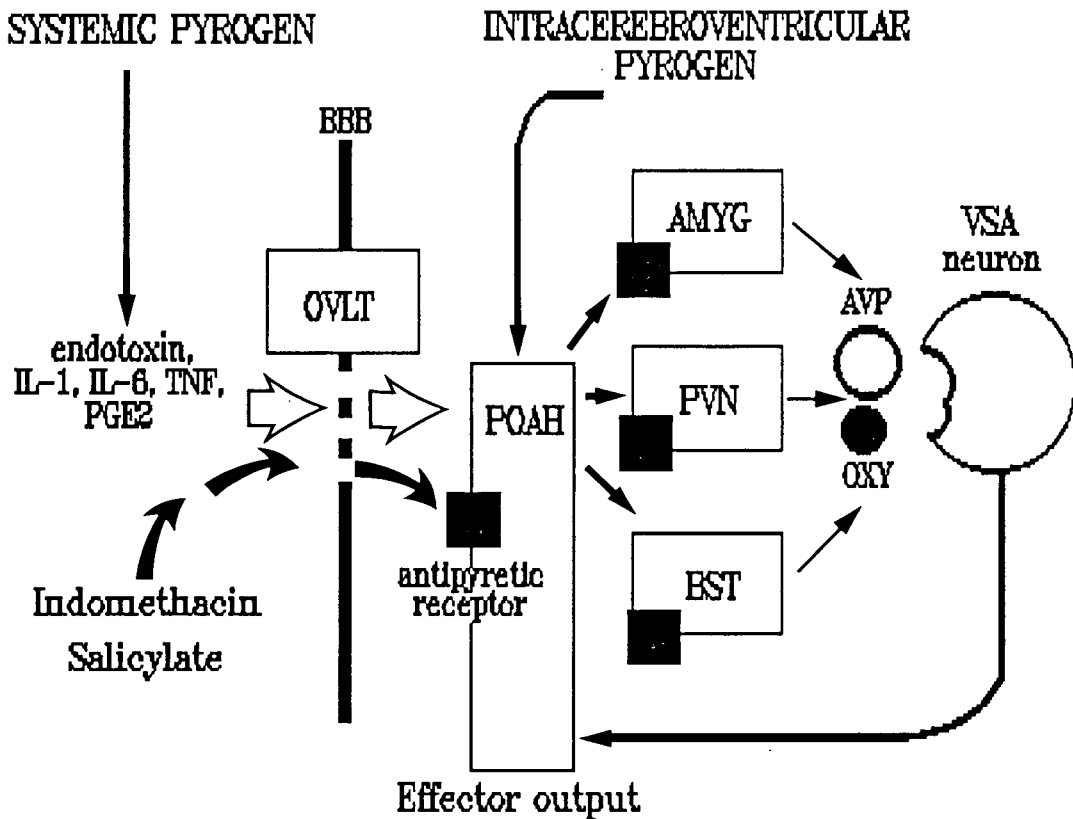


Figure 39. Schematic diagram outlining the pathways of activation for the endogenous antipyretic system within the VSA. The mechanisms by which peripherally or centrally administered pyrogens induce fever in all likelihood differ and are indicated as such by separating systemic and intracerebroventricular compartments. BBB, blood brain barrier; OVLT, organum vasculosum of the lamina terminalis; POAH, preoptic and anterior hypothalamus; AMYG, amygdala; PVN, paraventricular nucleus; BST, bed nucleus of the stria terminalis; VSA, ventral septal area; AVP, arginine vasopressin; OXY, oxytocin.

The existence of multiple febrogenic and antipyretic pathways receives support not only from the present experiments utilizing acetaminophen, but also from the data demonstrating, for the first time, a role for AVP in endotoxin tolerance. The specificity for the activation of the AVP system was underscored by the lack of effect the tolerant condition had on the hyperthermia evoked by icv PGE<sub>2</sub>. These particular results also serve to support the contention that PGE<sub>2</sub>-induced hyperthermias may be an inappropriate model for fever investigation. The results from central endotoxin administration during tolerance, while perhaps suggestive, remain too incomplete to allow firm conclusions. However, the results from push-pull perfusion of the VSA indicate that tolerance indeed modifies the release of AVP from VSA nerve terminals and does so in a manner which is consistent with both the effects of V<sub>1</sub> receptor blockade and the effects of centrally administered pyrogens during the pyrogenic tolerance to intravenous endotoxin.

New directions in this field should examine the potential interactions between AVP and oxytocin as well as  $\alpha$ -MSH in the regulation of fever and drug-induced antipyresis. Although oxytocin itself is not antipyretic its effects on AVP function suggest an important role. As to  $\alpha$ -MSH, the overwhelming majority of evidence supporting an antipyretic function for this peptide comes from studies in the rabbit. Clearly, to establish a general antipyretic function for this peptide its effects must be observable across species. Moreover it is most unlikely that either AVP or  $\alpha$ -MSH acts in isolation. Experiments to this end await to be done.

While there is compelling evidence for an antipyretic function for centrally acting AVP, the afferent and efferent neural circuitry conveying signals to and from the VSA remain virtually unknown. Further studies are needed to characterize the BST-VSA vasopressinergic pathway, both during fever, as well as during normal defervescence and drug-induced antipyresis. The only study to yet address these issues



utilized PGE<sub>1</sub> as the pyrogenic stimulus (Mathieson et al, 1989) and the majority of BST neurons were unaffected. It would be of interest to examine further the affects of other pyrogens, particularly those sensitive to antipyretic drug action, on BST as well as other VSA afferent neurons.

The existence of physiological binding sites for indomethacin has been reported on blood platelets (Magous et al, 1985). Do receptors for indomethacin exist in thermoregulatory active neural areas? If indomethacin receptors do exist centrally, are they on the vasopressinergic neuronal soma (e.g. BST) or the nerve terminals (e.g. VSA)? It is also possible that a receptor complex linking AVP, indomethacin and perhaps the cyclooxygenase enzyme is present within the central nervous system. Undoubtedly, investigations into the "antipyretic" receptor dynamics within central thermoregulatory structures will yield important new information to this field.

The physiological processes which lead to the development of febrile body temperatures encompass virtually every organ system and are directed through the actions of the central nervous system. It is sufficient to say that the process is complex and much is yet to be learned. The mechanisms which control and ultimately reverse febrile body temperature are undoubtedly as complex. The research conducted in this thesis has attempted to understand the mechanism by which febrile temperatures are negatively modulated either during naturally occurring antipyresis or that evoked by exogenously administered substances. The results have established a link between the AVP endogenous antipyretic system and the mechanism of drug-induced antipyresis and pyrogenic tolerance. The vasopressin V<sub>1</sub> receptor is an important connection in this antipyretic pathway. However, the precise stimuli which evokes AVP release as well as the identity of the post synaptic neuron expressing

the AVP receptor remain to be clarified. It is clear that this area of investigation will continue to provide a wide range of research questions that should be approached by an equally wide range of research disciplines.

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