INFLUENCE OF DIETARY-INDUCED OBESITY ON OPIATERGIC REGULATION OF APPETITE AND BLOOD PRESSURE IN SHEEP

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Dedicated to my father

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Synthesis and Secretion of Opioids

Discovery of Opioids

The name "Opioids" refers to a group of chemicals that produce a morphine-like effect. The discovery of specific receptors for opioides in animal tissue by Goldstein (1971) and Pert and Snyder (1973) led these authors to suggest that opioid-like chemicals were produced by humans and animals. Further evidence for the presence of endogenous opioid-like peptides (EOP) came from the discovery that electrical stimulation of the brain-stem produces analgesia (Reynolds 1969; Mayer and Liebekind 1974; Pasternak 1981), and that analgesia could be blocked by administering naloxone, a specific opioid antagonist (Pfiffer and Herz 1984, Akil et al 1984).

The search for identification and purification of EOP lead to the discovery of specific brain opioids identified as enkephalins, endorphins, and dynorphins (Hughes 1975; Li and chung 1976). The name endorphin refers to ß-endorphins and related peptides (Guillemin, 1978). Recently, "opiopeptins" has been proposed as a generic term to cover the three families of endogenous opioid peptides classified as enkephalinergic, dynorphinergic or endorphinergic (Morley et al 1982).

It is now clear that there are at least three genes responsible for producing large precursor peptides or prohormones, fragments of which have opioid activity.

These precursors are proopiomelanocorticotropin (POMC), proenkephalin and prodynorphin (Hollt 1983).

Opioid Synthesis Pathway

The prohormone, POMC, is a glycoprotein of approximately 285 amino acid with sugar residues attached near the N-terminal end of the molecule (Fenger 1990). POMC is synthesized by the anterior and intermediate lobes of the pituitary and a number of other tissues including hypothalamus, lung, placenta, and gastrointestinal tract (Drouin et al 1990; Fenger 1990). POMC is hydrolyzed to ß-lipotrophic hormone (ß-LPH), adrenocorticotropic hormone (ACTH) and γ-melanocyte-stimulating hormone (γ-MSH). ß-LPH, a large peptide with 91 amino acid (POMC 42-134) is cleaved to γ-lipotrophin (42-101) and ß-endorphin (104-134). The cleavage products may differ according to site of synthesis. The 31 amino acid ß-endorphin from different species varies only by one or two amino acids in the C-terminal region (Bloom 1983).

The precursor proenkephalin is hydrolysed to form pentapeptide methionine-enkephalin (Tyr-Gly-Gly-Phe-Met) and leucine-enkephalin (Tyr-Gly-Gly-Phe-Leu). Dynorphin (8 amino acid) and α-neo-endorphin (10 amino acid) are derived from prodynorphin (Lewis and Stern 1983). These peptides are distributed in brain tissues of a wide variety of animal species (Simantov et al 1976).

Sites of Opioid Production

Immunohistochemical techniques have been used to identify specific sites of opioid synthesis in the brain. The cellular distribution of enkephalins and ß-endorphins is not the same in different regions of brain. ß-Endorphin is found mostly in the hypothalamus, midbrain, amygdala, pituitary and brain stem. Although hypothalamus may contain high levels of ß-endorphin and enkephalins, other areas such as the

caudate hypothalamus and the globus pallidus contain much more enkephalin than ß-endorphin (Bloom 1978). Immunohistochemical staining of serial sections from rat hypothalamus indicates that enkephalins and ß-endorphins represent two separate neuronal systems (Watson 1978; Baile et al 1986; Ravindra and Grosvenor 1990).

ß-Endorphin is present at higher concentrations in plasma than cerebrospinal fluid (CSF) in humans (Atkinson 1987). Some ß-endorphin immunoreactivity has been detected in the stomach, duodenum, jejunum, pancreas, adrenal gland and follicular cells of the ovary (Baile et al 1986; Drouin et al 1990).

Opioid Receptors

The discovery of EOP indicated that perhaps receptors could be categorized as either enkephalin or endorphin receptors (Pert and Snyder 1973). It was Martin et al (1976) who hypothesized that multiple opioid receptors exist in brain. Subsequently, opiate receptors have been classified as mu (μ) receptors which have a high affinity for morphine and ß-endorphin, delta (δ) receptors which have high affinity for enkephalins, kappa (κ) receptors which have high affinity for dynorphin, and epsilon (ε) receptors which have highest affinity for ß-endorphin (Chang et al 1981; Goldstein et al 1989). In human brain, μ, δ and κ type of opiate receptors have been identified (Takemori and Portoghese 1985).

Mapping the location of opiate receptors in human brain showed that μ and k types predominated and that δ receptors were rare. The κ-receptors are most abundant in hypothalamus, amygdala and hippocampus, which are areas involved with the neuroendocrine effects of opioids. μ-Receptors also were present in these areas and other parts of the brain (Pfeiffer et al 1982). κ-receptors in human brain were further subdivided into 2 distinct types distinguishable by their low and very high affinities for the endogenous opioid peptides ß-endorphin and dynorphin, respectively.

The κ-receptor affinity for ß-endorphin is about 300-fold lower than that for dynorphin and thus ß-endorphin at physiological concentrations may not interact with κ-receptors. ß-endorphin and enkephalins may represent the physiological ligands for μ and δ opiate receptors (Miller, 1982).

Opioid Function

There is accumulating evidence that opioid peptides represent an unique class of regulatory chemicals in the endocrine system and in the central and somatic divisions of the nervous system. The fact that three types of opioid chemicals (endorphins, enkephalins and dynorphins) are found in the hypothalamus, pituitary and the adrenal gland, indicates that opioids play a powerful role in regulating the organism's response to physiological and environmental demands, including physical and psychological stress (Amir et al 1979; Zalman and Galina 1986). Like catecholamines, the opioids may have a basic, multisystem function essential to the maintenance of homeostasis and to the survival of the organism. Some of these basic functions relate to stress, appetite, and cardiovascular systems. It is often difficult to determine whether the effects of the endorphins on complex behaviors are primary or whether they are secondary to their ability to modify sensory or autonomic variables.

Opioid release during stressful conditions has been studied extensively. Stress appears to activate the hypothalamus-pituitary-adrenal axis of the peripheral sympathetic system, leading to release of endogenous opioid peptides from both the anterior pituitary and the adrenal medulla (Guillemin 1977, Holaday 1979). These stress-related opioid peptides may serve some analgesic function (Zalman and Galina 1986). The opioid mu-receptor, and even the delta type, may mediate analgesia when stimulated by opioid alkaloids (opium) or opioid peptides (Pasternak 1981). In addition, mu receptors may mediate local analgesia in peripheral tissues (Akil et al 1984).

There is extensive literature on the effect of opioids on feeding (Morely and Levine 1985). An overwhelming body of evidence favors a role for the endogenous opioid peptides in the regulation of ingestive behavior (Morley et al 1983; Gosnell 1987). More detailed involvement of opioid involvement in feed intake regulation will be discussed later in this chapter.

Opioids influence the cardiovascular system by their interaction with specific opioid receptors found in the central and peripheral nervous systems, the vascular tree and the heart (Holaday 1985). Exogenous administration of opioid peptides affects cardiovascular function by decreasing systemic arterial pressure and heart rate, and by suppressing baroreceptor reflex function (Holaday 1985). Furthermore, synthetic opioid agonists affect the urinary excretion of water and sodium (Akil et al 1984).

Opioids produce their biological effect by binding to cell-surface receptors (Childers 1991). Early studies indicated that opioid receptors (mu, kappa, delta and epsilon) were coupled to G-proteins that function as transducers in allowing the opioid receptor to inhibit adenylyl cyclase, thereby decreasing intracellular concentrations of cAMP (Giugliano et al 1989). However, some of the effects of mu and delta receptors may also be mediated by direct receptor interaction with G-proteins coupled to ion channels (Childers 1991). In a recent report, Giugliano et al (1989) suggested that opioids may inhibit insulin release by suppressing cytosolic accumulation of cAMP.

Regulation of Feed Intake

The regulation of feed intake and energy balance are influenced by a number of factors (Harris 1990). Central regulation of body composition is interfaced with central regulation of feed intake. This central control system balances energy input with energy output in regulating body weight (Harris 1990). Dysfunction in the central regulatory system can result in excessive weight gain or loss (Bray 1991).

Feeding behavior can be influenced by several external factors such as environmental conditions, sensory cues and nutrient type in the diet (Morley 1980; Kissileff and Van Itallie 1982). The internal regulatory systems, including gastrointestinal, hormonal and metabolic factors, also play a role in feeding behavior (Kissileff and Van Itallie 1982). The primary site responsible for the integrated control of feed intake and energy balance is the central nervous system (CNS), with the hypothalamus serving as the primary neuroendocrine transducer in regulation of feeding behavior. Peptides found in the CNS have been shown to have a direct effect on the control of metabolism, feed intake, and reproductive behaviors.

Hypothalamus and Feed Intake

The regulation of feed intake requires the integration of many signals including environmental factors and immediate and long term energy needs. Feed intake is regulated mainly by the central nervous system. Hypothalamus is an area of the brain classically associated with feed intake. It receives input from metabolic, hormonal, neurogenic, thermal and cortical factors, which describe the nutritional status of the organism (Figure 1). Electrical stimulation of the lateral hypothalamic area (LH) initiates feeding, but electric or chemical destruction of the LH results in aphagia. Electrical stimulation of the ventromedial hypothalamus (VMH) inhibits eating in hungry animals,

but ablation of this area produces hyperphagia and obesity. The interaction between the two areas is suggested to control feed intake. Other areas added to the list of regulatory centers in the central nervous system are the paraventricular nucleus and rostral areas such as the nucleus solitarius (Morley 1980; Sullivan and Gruen 1985; Harris 1990).

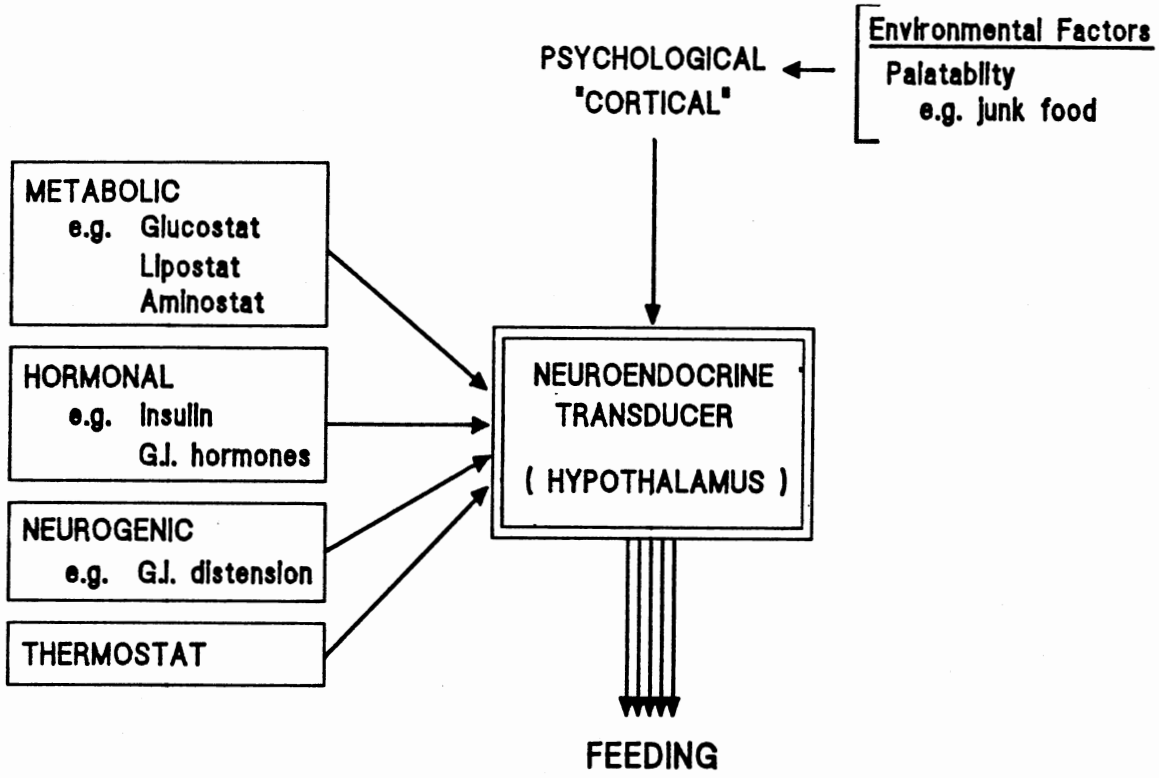

Figure 1. Diagram representing the role of hypothalamus as the central neuro-endocrine transducer in appetite regulation. (From Morley 1980)

The role of neurotransmitters in initiating feeding was shown by central administration of adrenergic, cholinergic, and serotonergic agonists as well as gamma aminobutyric acid (GABA) agonists (Blundell 1991). In sheep and cattle, feeding has

been stimulated by ventricular injection of α- and ß-adrenergic agonists and 5-hydroxytryptamine (Simpson 1975; Baile et al 1979). The GABA agonists, when injected in the CNS, have stimulated feeding in sheep (Seoane et al 1984).

Opioid Peptides and Feed Intake

Accumulating evidence suggests that opioids in the CNS are actively involved in the control of feed intake (Baile and McLaughlin 1987; Levine and Atkinson 1987). Central administration of ß-endorphin, met-enkephalin or dynorphin increases feed intake. Although most opioid peptide work has been done in rats (Morely and Levine 1891; Lowy 1981), some work has been done with humans and sheep. Injection of several met-enkephalin analogues and ß-endorphin into the central ventricles has stimulated feeding in satiated sheep and rats (Grandison and Guidotti 1977; Baile et al 1981; McKay et al 1981). Feeding in sheep was blocked by prior administration of naloxone, a pure opioid antagonist (Baile 1981; Alavi et al 1991). Intravenous administration of naloxone suppressed feed intake (Brown and Holtzman 1971, King et al 1979) whilst the quaternary analogue of naloxone, which is incapable of crossing the blood brain barrier, had no effect on feed intake (Carr and Simon 1982). Such results indicate that opioid regulation of feed intake occurs in the central nervous system.

Another determinant of the possible significance of opioid peptides in the control of feed intake is the concentration of opioid peptides in various areas of the brain of hungry or satiated animals. Measurements of peptides in sheep have shown that met-enkephalin concentration in the basomedial hypothalamus increased with increasing lengths of fasting from 0 to 2 h (Scallet et al 1985). In separate experiments, met-enkephalin concentrations in the VMH, paraventricular nucleus and anterior, dorsomedial and posterior hypothalamus were higher in fed than 4-h fasted

sheep (Scallet et al 1985), whereas concentrations of ß-endorphin were lower in the LH, and anterior and posterior hypothalamus of fed than in fasted sheep (Baile et al 1985). The differential response to hunger by opioids indicates that hypothalamic concentrations of opioids differ depending on the states of hunger and satiety (Margules et al 1978, Kaye et al 1987).

Animal experiments support the hypothesis that the endorphin system plays an important role in the biological response to stress and constitutes an "endorphin stress system" (Rossier et al 1979). ß-Endorphin and ACTH are concomitantly released by the hypophysis in response to acute stress (Guillemin et al 197, Holtzman 1974, Lowy et al 1981, Khawaja et al 1989). Clinical and laboratory studies have shown that "stress" may either increase or decrease food consumption depending on type and duration of the stress procedure. For example, mild tail pinching produces overeating and obesity in rats while immobilization stress may produce anorexia (Morely and Levine 1980). It is well known that acute exposure of animals to stressors of various kinds, for example heat, cold, immobilization , food deprivation, glucodeprivation or foot shock, provoke profound behavioral effects by activating either the sympatho-adrenal or the hypothalamo-hypophyseal-adrenal system.

Non-opioid Chemicals and Feed Intake

Serotonergic, alpha-adrenergic, beta-adrenergic and dopaminergic pathways are some of the well studied monoaminergic systems involved in feed intake regulation (Morely 1980; Sullivan and Gruen 1985; Blundell 1991). The satiety center in the VMH is thought to be under positive serotonergic control in that direct application of a serotoninergic agonist to this area causes anorexia (Shor-Posner et al 1986).

Alpha-adrenergic. An α-adrenergic system is thought to excite feeding by

inhibiting the VMH satiety center. Norepinephrine injections into the medial hypothalamus produce feeding through a strictly α-adrenergic effect. Long-lasting activation of the α-adrenergic receptors with clonidine produces overeating (Mayer and McCaleb 1980; Leibowitz and Hor 1982).

Beta-adrenergic. In contrast the to α-adrenergic system, the ß-adrenergic system is thought to cause satiety by inhibiting part of the classical feeding center in the VLH. Inhibition of feed intake in hungry rats occured when isoproterenol , a ß-adrenergic agonist, was injected into the lateral hypothalamus (Martin 1977). Inhibition of feed intake in rats after intraperitoneal injection of salbutamol, a ß-adrenergic stimulant, was reversed with intracerebroventricular injection of propranalol, a ß-adrenergic blocker (Borsini et al 1982).

Dopaminergic. Dopamine depletion in the nigrostriatal tract produces anorexia while dopamine agonists help restore food ingestion. Administration a of dopamine antagonist suppresses feeding elicited either by injection of 2-deoxy-D-glucose or fasting (Muller et al 1981).

Cholecystokinin. Cholecystokinin (CCK) initially found in the gastrointestinal (GI) tract, later in the brain, is one of several peptides involved in feed intake regulation (Baile 1986). In contrast to opioid peptides, CNS administration of CCK reduces feeding. Furthermore, it's likely that opioid and CCK-derived peptides may interact in regulating feed intake. Injections of physiological concentrations of CCK into the lateral ventricles of sheep decreased their feed intake (Della-fera and Baile 1979). Concentrations of CCK in specific hypothalamic areas of hungry and satiated sheep have been measured. In the anterior hypothalamus, the CCK concentrations were higher in fed than fasted sheep (Scallet et al 1985).

Obesity and Feed Intake

Obesity is characterized by excess adipose tissue. Development of obesity is a consequence of caloric intake that exceedes the maintenance energy requirements of the body. Increasing prevelance of obesity has directed attention of many researchers to study the mechanism by which obesity develops and the patho-physiological consequense of such condition. Opiate receptors and endogenous opioids play an important role in the central regulation of feed intake under both physiological and pathological conditions including obesity (Levine et al 1982; Morley et al 1983; Reid 1985).

In 1978, Margules and co-workers demonstrated for the first time elevated plasma and pituitary levels of ß-endorphin in genetically obese (ob/ob) mice and Zucker fatty rats (fa/fa). Margules et al (1978) found genetically obese (ob/ob) mice to be ten times more sensitive than their lean littermate controls to the anorectic effect of naloxone. In a similar study, Morley et al (1983) demonstrated an elevated pituitary concentrations of ß-endorphin in genetically obese rats (fa/fa) and mice (ob/ob). It is worth mentioning that obese Zucker fatty rats are also hyperinsulinemic and hyperglycemic (Margulese et al 1978). Diabetic animals are extremely sensitive to naloxone, as demostrated by Levine et al (1982). These investigators found spontaneously diabetic mice to be 80 times more sensitive to the feed inhibitory effect of naloxone than nondiabetic control and streptozocine-induced diabetic mice to be 1000 time more sensitive than their controls. Therefore, it is reasonable to suggest that greater sensitivity of obese animals to naloxone may be related to their hyperglycemia and glucose-induced increase in the activity of opiatergic recepotors.

Blood Pressure Regulation

Components of Blood Pressure Regulation

The circulatory, endocrine and nervous systems constitute the major integrating systems of the body. The circulatory system transports and distributes essential substances, removes by-products of metabolism, and maintains hemostatic balance during different physiological states. Blood serves as the vehicle for this homeostatic function, which depends on velocity of flow, blood pressure, and vessel diameters.

Velocity of flow is inversely related to the cross-sectional area of the vessel (Laplace law) and directly proportional to the difference between inflow and outflow pressure. Thus, changes in the diameter of the vessel (resistance) affect blood pressure and flow. Just as in electrical circuitry, vessels added in series to a circulatory pathway increase resistance and blood pressure for a given flow (for example in renal, splanchnic and hepatic capillaries), whereas addition of blood vessels in parallel reduces resistance and blood pressure (for example colateral blood vessles in skeletal muscle of atheletes).

Blood flow is regulated by complex regulatory systems. Superimposed on the intrinsic local tissue regulation (autoregulation) of blood flow are regulations imposed by the hormonal and autonomic nervous systems. The major hormonal regulatory system is the renin-angiotensin-aldosterone system. Autonomic regulation of blood pressure is in the realm of the sympathetic nervous system (SNS), which controls the blood pressure through sympathetic nerve fibers distributed over small arteries and arterioles. The vasomotor center in the brain stem (medulla) transmits impulses through the sympathetic fibers to regulate vascular smooth muscle tone. The upper and lower parts of the vasomotor center in the medulla serve as pressor and depressor

centers, respectively. The opposing actions of two parts regulate the vascular tone and thereby the vascular resistance (Figure 2).

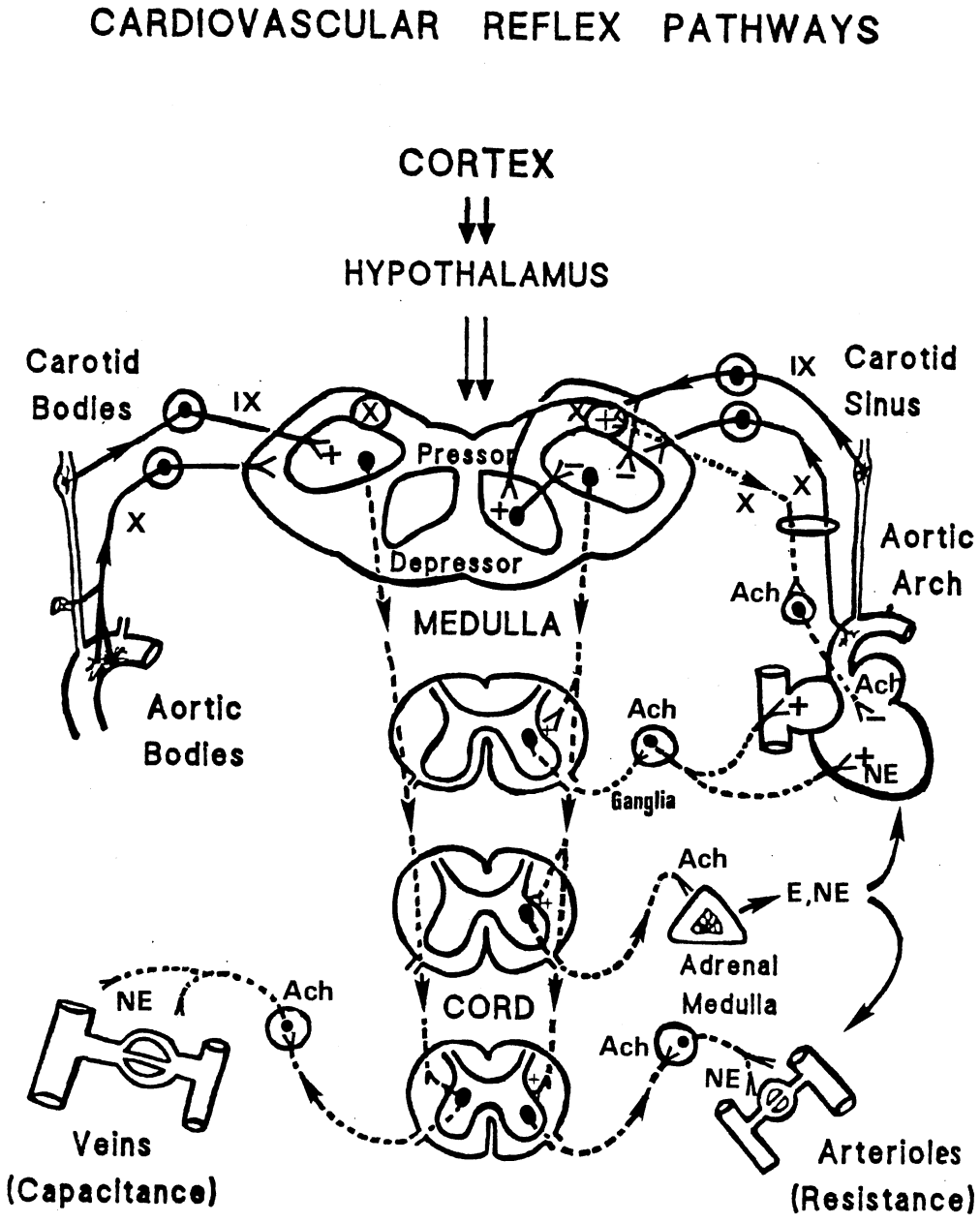

Figure 2. Autonomic control of blood pressure regulation

Blood pressure is a function of cardiac output and total vascular resistance. Any condition that increases either cardiac output or total peripheral resistance will cause an increase in mean arterial blood pressure. An increase in vascular resistance rather than cardiac output, is thought to be the principal factor contributing to essential hypertension in humans (Folkow et al 1958; Dustan et al 1981; Korner et al 1989; Shepherd 1990).

Adrenergic Regulation of Blood Pressure

Norepinephrine (NE) and epinephrine (EPI) are catecholamines involved in adrenergic regulation of blood pressure. Adrenal medulla is the major site of EPI production. Sympathetic nerve endings produce NE, which is the neurotransmitter at most sympathetic postganglionic nerve endings and at some synapses in the central nervous system. EPI secreted by the adrenal medulla functions as a neuroendocrine chemical. Both NE and EPI exert their effects by activating adrenoceptors located on the cell surface of target cells (Table 1).

Two major classes of adrenoceptors exist and these are termed alpha (α) and beta (ß) adrenoceptors (Raper 1987). Distribution of adrenoceptors varies from organ to organ (Table 1). The balance between the α- and ß-adrenoceptor and their subtypes known as α_1, α_2, $ß_1$, and $ß_2$ determines the characteristic cardiovacular responses to NE and EPI release.

TABLE 1

DISTRIBUTION OF ADRENOCEPTOR SUBTYPE AND ADRENERGIC RESPONSES OF SELECTED ORGANS OR TISSUES

Organ/Tissue	Receptor	Effect	
Heart	β_1	↑ force of contraction	↑ heart rate
Blood vessels	α_1, α_2, β_2	α_1, α_2 = vasoconstriction	β_2= vasodilation
Kidney	α_1, β_1	α_1= ↓ renin release	β_1= ↑ renin release
Pancreas	α_1, α_2, β_2	α_1, α_2 = ↓ secretion	β_2= ↑ secretion
Fat cells	α_2, β_1	α_2= ↓ lipolysis,	β_1= ↑ lipolysis
Liver	α_1, β_2	Glycogenolysis and gluconeogenesis	
Symp.Terminals	α_2, β_2	α_2= ↓ NE release,	β_2= ↑ NE release
Bronchioles	β_2	Dialation	

[1] Adapted from Maki et al 1990.

<u>α-Adrenoceptor.</u> The two subtypes of α-adrenoceptors are α_1 and α_2. Both types are located on the postsynaptic plasma membrane of target cells. The α_2-adrenoceptors also are located in the presynaptic membrane, where they function in the regulation of neurotransmitter release. Release of NE by exocytosis into the myoneuronal junction occurs when a nerve impulse is propagated along the postganglionic adrenergic neuron to the varicosities of the axon terminal. NE then activates α-adrenoceptors on the peripheral vascular smooth muscle causing vasoconstriction (Timmermans and Van Zwieten 1980; Bylund 1988). α_2-Adrenoceptors at the presynaptic sites mediate feedback inhibition of NE release (Brummelen 1987). The characteristic order of potency for agonists at both α_1 and α_2-adrenoceptors is epinephrine > norepinephrine > dopamine > isoproterenol.

α_1-Adrenoceptors produce their effect via phopholipase hydrolysis of

phosphotidyl biphosphate and attendent intracellular increase in calcium and protein kinase C. α_2-Adrenoceptors produce their effect by inhibiting adenyl cyclase, thereby decreasing production of intracellular cAMP from ATP (Maki et al 1990).

Plasma NE is almost entirely derived from sympathetic nerve terminals, and as such reflects sympathetic nerve activity (Cryer 1980). Previous studies in normotensive human subjects showed that changes in plasma NE levels mirrored the changes in blood pressure during barroreflex activation by vasoactive drugs (Grossman et al 1982).

ß-adrenoceptors. The ß-adrenoceptors are divided into two subtypes, generally referred to as $ß_1$- and $ß_2$-adrenoceptors. The $ß_1$-adrenoceptor is considered unique to the heart, kidney and intestine, with $ß_2$-adrenoceptors present in other tissues and heart (Brodde 1984). The characteristic order of potency for agonists at both $ß_1$ and $ß_2$-adrenoceptors is isoproterenol > epinephrine > norepinephrine > dopamine (Kalant and Roschlau 1989). Stimulation of the peripheral $ß_2$-adrenoceptor causes vasodilation, whereas stimulation of α-adrenergic receptors in blood vessels causes vasocons-triction. Most, if not all, of the actions of catecholamines at ß-adrenoceptor sites are mediated by the stimulation of the plasma membrane-bound adenyl cyclase enzyme, which stimulates the formation of intracelluar cAMP from ATP (Maki et al 1990).

Opioid Regulation of Blood Pressure

Neuroendocrine contributions to the CNS regulation of blood pressure and heart rate are well established (Ganong et al 1979). Endogenous opioids are among the neuropeptides identified in this regulatory role (Rubin 1984). Opiatergic peptides have been demonstrated in important cardiovascular control centers of the brain (Holaday

1983). Stimulation of central opiatergic receptors results in change in cardiovascular function. For example Haddard et al (1986) and Naranjo et al (1985) observed a decrease in blood pressure following central administration of ß-endorphin.

Central administration of opioids such as ß-endorphin or enkephalin causes a significant decrease in blood pressure in normotensive animals(Schatz et al 1980; Kunos et al 1981 Cuthbert et al 1989), whereas the opiate antagonist naloxone increases blood pressure in hypovolemic-shock-associated hypotension in non-human primates (McIntosh 1986). Proopiomelanocorticotrophin derived peptides are distributed throughout the brain and brain stem; opioid-containing axonal projections originate in the hypothalamus and extend to various brainstem areas, including the locus cerulus, nucleus tractus solitarius and area postrema. It is in these areas that opiate receptors exist and opiate containing neurons synapse with α-adrenergic neurons, suggesting a functional relationship between the two systems (Zamir et al 1984). Randich and co-workers (1991) found that intrathecal (spinal) administration of naloxone antagonized the vagal-mediated bradycardia produced by morphine.

There is experimental evidence to support the hypothesis that opioid peptides may function as neurotransmitters or as modulators in various peripheral effector organs innervated by sympathetic and parasympathetic neurons. Lang et al (1983) and Xiang et al (1984) reported that a considerable amount of enkephalins are stored in the heart, possibly in sympathetic nerve endings. Koyanagawa et al (1989) suggest that opioid receptors may mediate vagal bradycardia in dogs. Recently, Rogers and Henderson (1990) demonstrated that activation of presynaptic mu- and delta-opioid receptors depressed acetylcholine release in the hypogastric ganglion, thus indicating that opioids and opioid receptors may regulate neurotransmitter release in parasympathetic and sympathetic systems by a presynaptic mechanism.

Evidence for the interaction between opiatergic and sympathetic nervous systems in the regulation of blood pressure was demonstraed by Kunos et al (1981), who showed that the hypotensive effect of clonidine, an α_2-adrenergic agonist, was reversed by naloxone. Further, Laskey et al (1989) showed that the effects of ß-agonists on blood pressure were mediated via an opioid pathway.

Opioid receptors and peptides have been found in high concentration in the nucleus tractus solitarius (NTS) in the rat, cat and monkey brain, with the greatest concentration of each occurring in the area of NTS that receives afferent input from heart (Lesli 1985). It is therefore possible that stimulation of these afferents causes the release of an opioid peptide in the NTS, which in turn leads to reduced sympathetic outflow to renal and other vascular beds (Weinstock et al 1989).

Hypertension in Health

According to guidelines set by The World Health Organization (WHO), hypertension is defined as systolic pressure greater than 160 mmHg and diastolic pressure greater than 90 mmHg, although most US authors consider 140/90 mmHg as an adequate criterion (Trevisan et al 1988; Stoner and Parker 1991). The etiology of hypertension is complex and not always well understood. In situations where the etiology is known, such as in renal hypertension, the hypertension can be effectively treated . However, hypertension of unknown cause (essential hypertension) is difficult to treat (Folkow 1982).

The prevalence of hypertension is sufficiently high in western societies to warrant considering it as a serious public health problem. Epidemiological data reveal that premature cardiovascular morbidity and mortality are directly related to the level of diastolic and systolic blood pressure (Kannel, 1974, 1979; Hansen et al 1990).

Hypertension can produce two primary damaging effects on the cardiovascular system: (1) increased work load on the heart and (2) damage to blood vessles caused by excessive pressure (Messerli 1990; Morgan and Baker 1991).

Risk Factors for Hypertension

Certain risk factors increase the probability of developing hypertension. An individual with one or a combination of specific risk factors has a greater probability of becoming hypertensive than an individual without such risk factors. The most common risk factors for hypertension are obesity, diabetes, excess sodium ingestion and excess alcohol ingestion (Van Itallie 1986; Horan and Lenfant 1990; Reaven 1990; Mahler 1990; McKeigue et al 1991).

Obesity. An association between obesity and hypertension has been demonstrated clearly in several epidemiological studies (Stamler 1978; Van Itallie 1985; Dryer et al 1990; Falkner et al 1990; Xavier Pi-Sunyer 1990, Horswill and Zipf 1991). Conclusions from these studies are supported by experiments demonstrating hypertension in obese dogs (Rocchini et al 1989) and Zucker fatty rats (Kurtz et al 1989). Patients are considered obese when their body weight exceeds by 25 % their ideal body weight which is calculated according to the actuarial tables of the Metropolitan Life Insurance Company (Stamler et al 1978).

Although a high proportion of obese individuals develop hypertension, not all obese individuals are hypertensive. In a study of 1 million people, Stamler and co-workers (1978) reported that the prevalence rate for hypertension was 50% greater in overweight than normal weight people. The prevalence of hypertension increases with increasing age and body weight (Stamler 1978; Leitschuh et al 1991). The incidence of hypertension was higher in obese blacks as compared with obese caucasians.

Regardless of race, obese males have a higher prevalence of hypertension than female individuals (Stamler 1978, Ramirez et al 1991). Several adaptations in the cardiovascular system occur during the development of obesity that may lead to development of hypertension. For example, as a consequence of the expanding adipose tissue a greater demand is placed on the heart and blood supply, and this is associated with elevated cardiac output and eventually, left ventricular hypertrophy.

Diabetes. Distribution of adipose tissue may have a significant effect on the association observed between obesity, diabetes and hypertension (Krieger and Landsberg 1988; Evers et al 1989; Lundgren et al 1989; Mahler 1990; Despres et al 1991). Upper body (abdominal) obesity, assessed by increased waist-to-hip ratio, may contribute significantly more to high blood pressure and diabetes than lower body (gluteal) obesity (Bray 1990; Gerber et al 1990; Skarfors et al 1991). Studies show that hypertension also is strongly associated with diabetes in obese and nonobese individuals (Young and Landsberg 1982; Memeh 1990; Xavier Pi-Sunyer 1990, Muscelli et al 1990; Gans and Donker 1991). Diabetes and hyperinsulinemia play a significant role in the pathogenesis of hypertension and coronary heart disease (Ronnemaa et al 1991; Reaven 1990). This relationship between diabetes and hypertension has been demonstrated recently in humans (Ronnemaa et al 1991), and in spontaneously hypertensive rats (Hulman et al 1991). The interactions between diabetes and obesity and hypertension are not completely understood, but hyperinsulinemia and insulin resistance may serve as causative links (Swislocki 1990).

Hyperinsulinemia. Some of the suggested mechanisms (Fig. 3) linking hyperinsulinemia to hypertension include: (1) insulin's stimulatory effect on SNS activity (Rowe et al 1981); (2) insulin's stimulation of sodium absorption in the kidney

(DeFronzo 1981; Skott et al 1989); (3) insulin stimulation of locally active growth factors in the vascular wall (Bar et al 1988); and (4) insulin stimulation of endothelial-derived vasoactive hormones (Ferrari and Weidmann 1990; Ferrannini and Natali 1991).

Insulin, SNS activity and Hypertension. Insulin may affect hypertension by increasing sympathetic nervous system activity (SNS). Intravenous infusion of insulin increased plasma catecholamine concentrations in normal individuals independently of changes in glucose concentrations (Rowe et al 1981). Siani et al (1989) showed that iv administration of insulin increased heart rate and that this was reversible by propranolol, a ß-adrenergic blocker. Whether these results were caused directly by insulin or indirectly by insulin effect on glucose is still not clear, since some studies have shown that dietary glucose influences the sympathetic activity in rats (Kaufman et al 1991).

Insulin, Sodium Balance and Hypertension. Insulin increases sodium reabsorption in kidney distal tubules (DeFronzo et al 1981; Skott et al 1989; Gesek et al 1991). The increase in sodium reabsorption leads to water retention and, consequently, increases in blood volume and pressure. Insulin may also enhance sodium reabsorption indirectly by its effect on renal sympathetic activity (Gans and Donker 1991). Brands and co-workers (1991) showed that sustained iv infusion of insulin and glucose significantly increased mean arterial pressure and heart rate in rats.

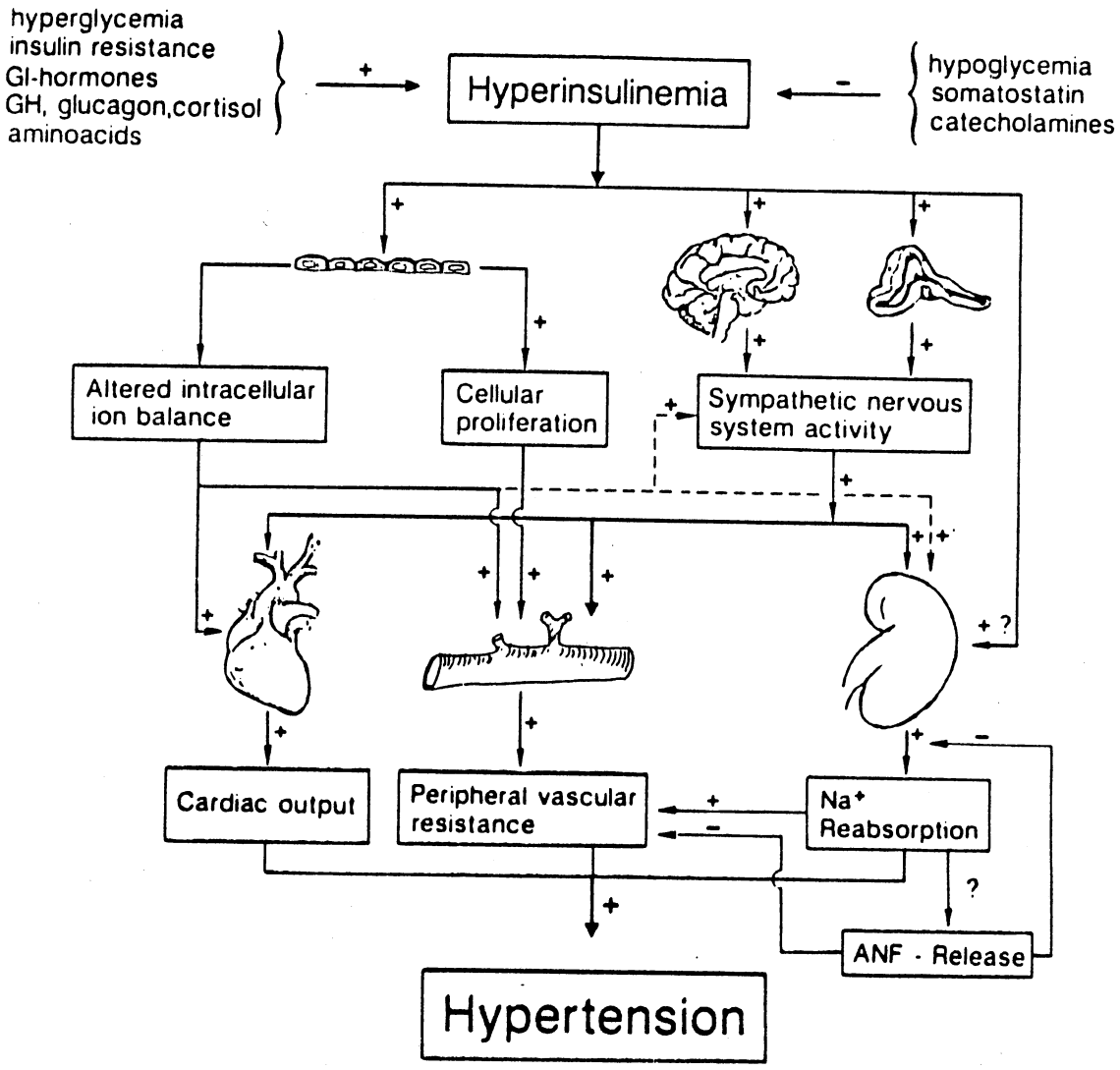

Figure 3. Schematic of proposed insulin-mediated mechanisms of blood pressure regulation. (GI, gastrointestinal; GH, growth hormone; ANF, atrial natriuretic factor). Adapted from Ferrari and Weidmann 1990.

Insulin, Vascular Growth and Hypertension. Abnormal cellular calcium homeostasis may link insulin resistance and high blood pressure in non-insulin dependent diabetic individuals (Levy at al 1989; Resnick et al 1991). These authors found that plasma concentrations of Ca^{2+} and Mg^{2+} were positively and negatively correlte with incidence of diabetes in humans, repectively. It has been suggested that

insulin may contribute to vascular hypertrophy leading to increased vascular tone, and subsequent increase in blood pressure (Raij 1991). Insulin inhibition of plasma membrane Ca^{++}/Mg^{++}-ATPase activity, increases intracellular calcium concentrations (Pershadsingh and McDonald 1979), which could potentially modulate cardiovascular function (Ferrari and Weidmann 1990).

Insulin, Endothelial Function and Hypertension. Vascular endothelium represents an extraordinary complex network of cells with multiple metabolic and immunological properties. These cells are continuously bathed by chemicals in blood. The presence of insulin receptors in endothelial cells (Frank and Partridge 1981) indicates that these cells respond to insulin regulation of glucose and amino acid transport and glucose metabolism (Bar et al 1988). Endothelial cells produce a number of factors that cause relaxation (nitric oxide, prostacyclin) and contraction (endothelin) of vascular smooth muscle (Miller 1991). Endothelial cells synthesize clotting factors and this can be affected by insulin. In both type I and type II diabetes, the levels of prostacyclin are depressed but may improve with normalization of hyperglycemia suggesting that high plasma glucose may compromise endothelial cell function (Stolar 1988). Therefore, one may suggest that a dysregulation of endothelial cell's function may lead to alterations in the regulation of the blood hemodynamics in the blood vessels of obese diabetic and nondiabetic individuals.

Objectives and Hypothesis

Opioids have an important role in regulation of feeding behavior and cardiovascular function (Morely 1982, Holaday 1983). Dysfunction in opioid biology has been associated with development of obesity in obese Zucker rats and also with development of hypertension in animals (Guillemin et al 1977, Holaday 1983).

The schematic in Fig. 4 displays a working model that illustrates the relationship between opiates and obesity and their interactive effect on blood pressure regulation. The model indicates that enhanced activity in the opiatergic system is essential for the overeating that leads to dietary obesity. Opiatergic regulation of intake during the induction or dynamic phase of obesity will differ from that during the maintenance or static phase of obesity because the obese state *per se* modifies opiatergic regulation of appetite. Obesity is closely linked with the development of hypertension.

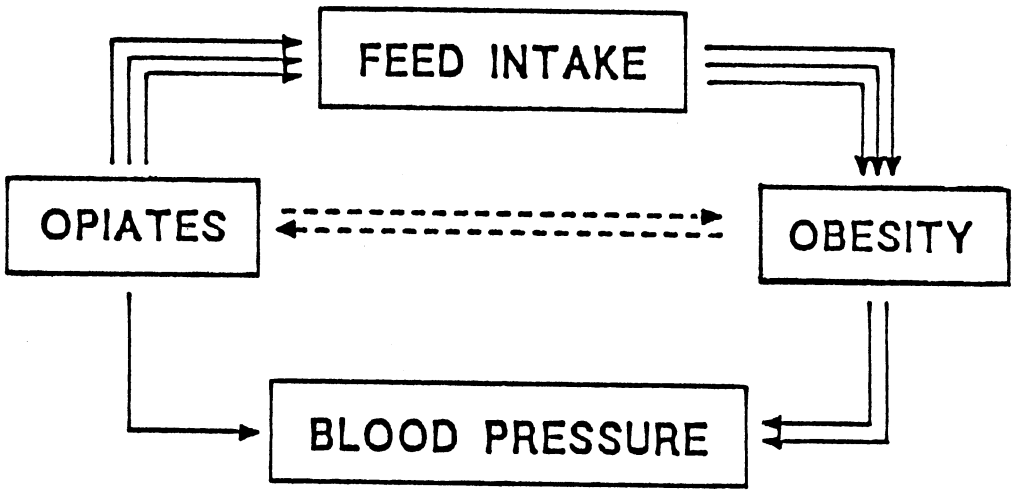

Figure 4. This model illustrates the possible relationship between obesity and opiates and their interactive role in feed intake and pressure regulation. The model is explained in the text.

The model further shows that abnormal cardiovascular function in obese individuals is partly the result of an obesity-induced defect in the opiatergic regulation of blood pressure and cardiovascular function. The model does not distinguish if the defects in opiatergic regulation of appetite and blood pressure are one-and-the-same or whether the defective site(s) of opiate dysfunction is exclusively central or is located at central and peripheral sites. The model subserves the thesis that static obesity changes opiatergic regulation of appetite and blood pressure. Experiments were designed to address this thesis and to further determine the cellular mechanisms explaining opiate involvement in obesity-associated hypertension.

Objectives in experiment 1 were to determine the dose-response curve for naloxone inhibition of feed intake in sheep and to establish doses that were without side effects.

Experiment 2 was designed to test the hypothesis that obesity alters the opiatergic regulation of feed intake. The inhibitory effect of naloxone on feed intake was evaluated in lean sheep and sheep in the static phase of obesity. Sheep became obese by overeating for 40 - 50 wk (see Chapter 2).

Experiments 3, 4 and 5 tested the hypothesis that obesity in sheep is associated with hypertension and that cardiovascular responses to opioid blockade differ in lean and obese sheep. Cardiovascular responses to clonidine were determined in the presence and absence of naloxone to examine the interactive effects of opioids and adrenergic receptors on cardiovascular function in lean compared with obese sheep.

Finally, the effect of obesity on the pharmacokinetics of iv injected naloxone was investigated. Results obtained in lean and obese sheep treated with naloxone can be readily compared only if body condition has no effect on plasma levels or degradation of the injected naloxone.

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CHAPTER II

DEVELOPMENT OF OBESE SHEEP MODEL

Introduction

Obesity, diabetes and hypertension are clinical diseases with high prevalence in western society. Epidemiological statistics reveal that diabetes and obesity are major predisposing factors for premature cardiovascular morbidity. It has been estimated that approximately 26 % of the US population (age 20-74 yr) currently are considered obese (Van Itallie 1985).

Obesity is an important risk factor for development of hypertension. Indeed, in the United States, national data for 1979-1980 suggest that 44% of the hypertensive population are obese, whereas only 16% of the normotensive population are obese. Conversely, 53% of all obese subjects are hypertensive, but only 22% of non-obese individuals are hypertensive (Horan and Lenfant 1990). There seems to be a clear association between obesity and high blood pressure.

Understanding and treating human diseases has depended to a great extent on the use of animal research models. Obese animals may represent models of human obesity in certain respects but not others. Studies in animal models may be aimed at elucidating etiological factors in obesity or the metabolic abnormalities and clinical diseases associated with obesity. Animals may be used to study neurotransmitters involved in the regulation of food intake, pharmacokinetic properties of therapeutic drugs in obese patients, and finally the pathophysiology of obesity-associated

hypertension and diabetes (Fuller and Yen 1987; Bohr and Dominiczak 1991).

Obesity can have different origins and etiologies in animals just as in humans. In animals, obesity can be genetic in origin or induced by chemical, surgical or dietary means (Bray 1979). Genetically obese animal models, e.g. ob/ob mouse and fa/fa fatty rats, might be good models for genetic obesity but not for nongenetic obesity in humans (Bray 1977; Fuller and Yen 1987).

Excessive caloric intake produces obesity in humans and animals. Animals can be induced to overeat either by force feeding, enhancing the palatability of food, manipulations such as hypothalamic lesions, or by tail-pressure stress to enhance feeding (Bray 1979; Penicaud et al 1989). Special effort is necessary to produce dietary-induced obesity in rodents. Rats become dietary obese by consuming a diet comprised of cheese, ham, salami, bread, and crackers; the so-called "cafeteria diet" (Sclafani and Springer 1976; Rolls et al 1980; Bubag et al 1990). The lack of homogeneity of diet introduces difficulty in estimating the type of nutrients and amount of calories that are ingested in dietary-obese rats as compared with control rats fed standard rat chow. Alternative procedures involve the feeding of high glucose, high fructose or high fat diets to induce dietary obesity in rats while control animals are fed standard rat chow (Levin et al 1983; Kaufman et al 1991).

Genetic and nongenetic obese animal models have been used to study the patho-physiology of hypertension in humans. The most studied animal models are genetically hypertensive such as the Dahl salt sensitive rat (DS) and the Japanese spontaneously hypertensive rat (SHR). Dahl and co-workers established a genetic stain of rats that became hypertensive when fed a high salt diet and a second salt-resistant strain (SR) that did not develop hypertension (Dahl 1962). The SHR rats were originally developed by Okamoto and Aoki (1963) by selectively breeding of the Wistar

Kyoto (WKY) rats for higher blood pressure. The SHR rats have many similarities to human essential hypertension and currently SHR rats are the most widely used animal model of essential hypertension. SHR rats have enhanced our understanding of some of the mechanisms associated with hypertension, but unfortunately this genetic animal model does not show the risk factors appropriate for obesity-associated hypertension. An animal model of renal hypertension, originally described by Goldblatt and associates in 1937, is produced by clamping one or both renal arteries (Goldblatt et al 1937). Variation of Goldblatt's original technique has been used by other investigators (Hall et al 1990; Xie et al 1990; Imig and Anderson 1991), to advance significantly our understanding of renal hypertension.

Dietary obese hypertensive dogs were first described by Wood and Cash (1939). Recently, Rocchini and associates reported that high fat feeding produces obesity and hypertension in dogs (Rocchini et al 1987). The obesity was produced by supplementing the regular diet of dogs with 2 lb of freshly cooked beef fat per day. After 5 wk on this diet, mean arterial pressure and heart rate were increased 24 and 21 %, respectively, in high-fat supplemented dogs compared with control animals fed only regular diet (Rocchini et al 1987).

Development of hypertension in genetically obese rats has been inconsistent. Kurtz et al (1989) reported an increase of approximately 20 mmHg in the mean arterial blood pressure of Zucker fatty rat compared with lean Zucker rats. However, others have failed to see any significant difference in the blood pressure of lean or obese Zucker rats (Ernsberger and Nelson 1988; Kasiske et al 1991). Furthermore, development of hypertension in the Zucker rat may be genetic in origin, and distinct from the obese state because caloric restriction did not attenuate the hypertension (Kurtz et al 1989).

In some models of dietary-induced obesity, rats and dogs are fed excess fat in their normal ration to develop obesity-associated hypertension (Levin et al 1983; Rocchini et al 1987; Bunag et al 1990). In these studies, obese animals are compared to lean ones which are fed normal diet without the excess amount of fat, and thus comparisons may not be appropriate since animals belong to different dietary treatments. We have developed an obese sheep model (Fig. 1) by dietary means, in which the composition of the diet is the same for both lean and obese sheep. This sheep model of obesity develops some of the risk factors associated with diabetes and hypertension in humans, such as hyperinsulinemia, hyperglycemia, and insulin resistance (McCann and Bergman 1988; McCann et al 1991). In this chapter we will present results pertaining to the development of dietary obesity in Rambouillet ewes that were used in this thesis.

Materials and Methods

Animals and Diet

Lean adult Rambouillet ewes aged 3-4 years were purchased from USDA-ARS, Forage and Livestock Research Laboratoy, Fort Reno, OK. The diet fed throughout the experiment was a pelleted hay-grain mixture (Stillwater Ag and Mill, Stillwater, OK) with a small amount of prairie hay to provide long-stem roughage for maintenance of rumen motility. Fresh water was always available to each sheep. Chemical composition of the pelleted feed and hay was relatively constant throughout the experiment (Table 1).

Dietary-Induction of Obesity

Ewes were assigned randomly to the lean (n=5) and obese (n=5) groups and housed individually in pens (1.1x1.8 m) bedded with sawdust in a room with constant.

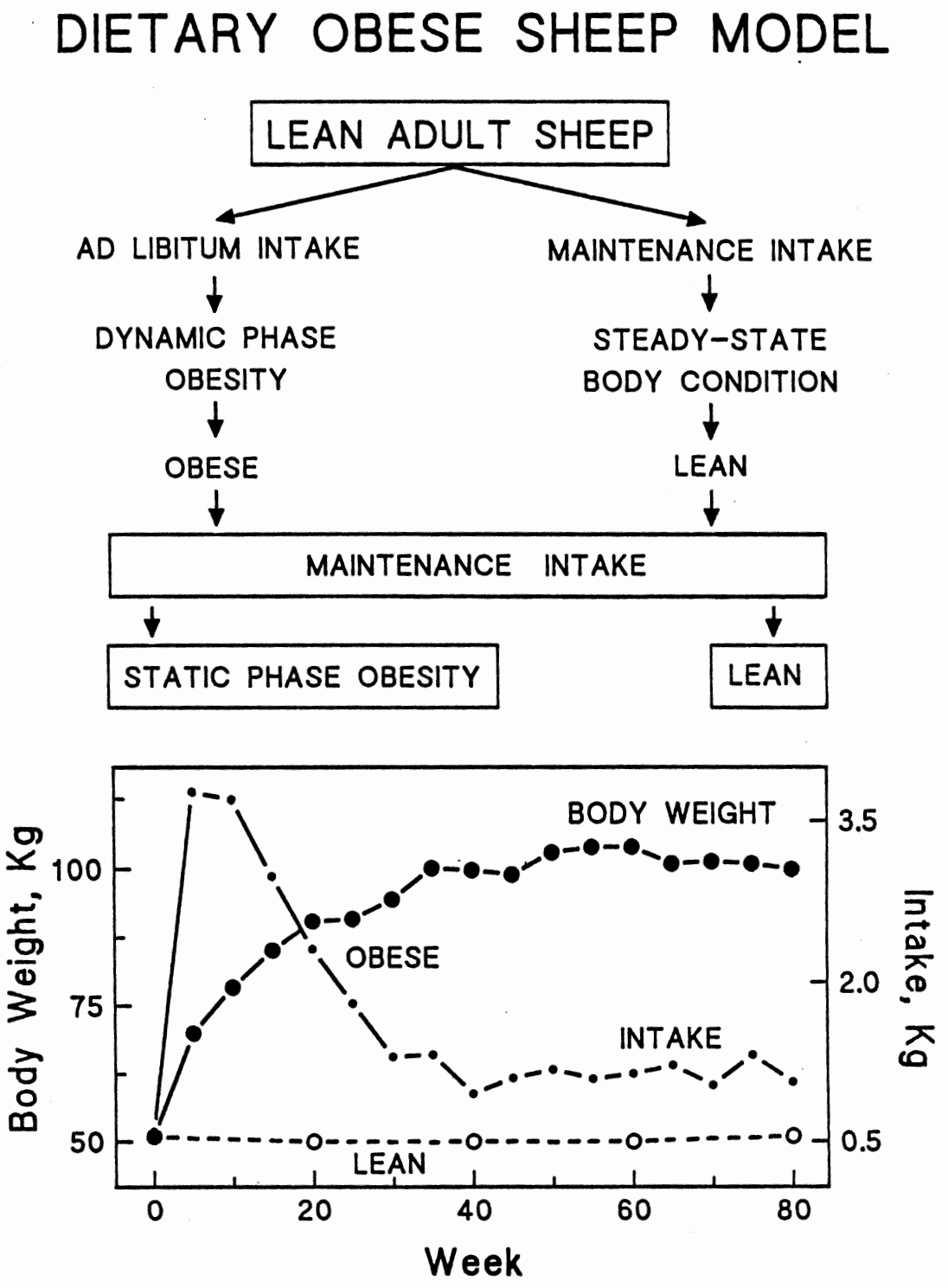

Figure 1. Schematic diagram of development of dietary obese sheep model. The graph depicts the changes in body weight and feed intake in sheep during dynamic and static phases of obesity. Dynamic obesity persists until nearly steady-state body weight and feed consumption occurs at which time animals enter static-phase obesity. Diagram provided by Dr. McCann, Oklahoma State University.

TABLE 1

NUTRIENT COMPOSITION OF DIET AS FED[1]

Nutrient	Pellet	Hay
	Values as g/kg	
Dry Matter (DM)	934.6 ± 2.0	942.0 ± 6.2
Total Digestible Nutrient (TDN)	671.4 ± 12.3	523.2 ± 7.9
Total Protein	152.4 ± 4.7	47.3 ± 3.3
Crude Fat	27.9 ± 0.4	12.8 ± 0.9
Nitrogen Free Extract	545.9 ± 9.1	480.1 ± 17.2
Crude Fiber	149.1 ± 12.6	373.8 ± 18.0
Ash	98.3 ± 3.4	58.9 ± 3.5
Calcium	13.2 ± 0.3	3.2 ± 0.3
Phosphorus	5.2 ± 0.3	0.89 ± 0.20
Magnesium	9.1 ± 0.4	1.03 ± 0.16
Potassium	15.8 ± 0.3	5.23 ± 1.59
Sodium	3.0 ± 0.2	0.23 ± 0.02
Sulfur	1.6 ± 0.2	1.07 ± 0.29
	Values as mg/kg	
Manganese	60.5 ± 6.5	48.7 ± 8.8
Copper	5.82 ± 0.79	1.44 ± 0.36
Cobalt	1.49 ± 0.08	0.212 ± 0.054
Zinc	67.4 ± 15.5	5.96 ± 1.80
Iron	994.6 ± 45.6	43.6 ± 6.6
Molybdenum	2.5 ± 0.22	0.50 ± 0.11

[1] Values are means (± SE) of means of 5 analyses done at intervals of approximately 4 months on 5 different batches of pellets and hay. Analyses performed by Triple "S" Labs, Loveland, CO, USA.

light, temperature (20 ± 1 ∘C) and music (radio). Sheep were acclimated to environment, jugular venipuncture and personel for 4 wk before experimental data were coolected.were collected. At this time, all sheep were fed maintenance levels of the pelleted feed (12.8 g/kg) and hay (1 g/kg). The target body weight associated with lean body condition was calculated using wither heights and a weight-to-wither height ratio of 0.65 kg/cm (McCann and Bergman 1988, McCann et al 1991). Target body weights were used in calculating maintenance intakes in all sheep during the acclimation period and in lean sheep throught the experiment.

Amount of pellets fed to obese sheep increased from maintenance to 1.5 kg per sheep per day in week (wk) 1, to 2.0 kg in wk 2 and to 2.5 kg per sheep per day in wk 3 of the experiment. Feed refusal was determined daily and amounts fed adjusted to provide weighbacks of approximately 10% of the amount fed. Pellets were fed in two equal amounts at 0900 and 1630 h. Hay (1 g/kg) was fed daily at 0900 h throughout the experiment.

Indices of Obesity and Chemical Composition of Carcass

Sheep were necropsied over a 4 month period at wk 110 to 130 of the experiment. A lean and obese sheep were necropsied as a pair after an overnight fast. All sheep were in equilibrium body weight at necropsy.

Sheep were electrically stunned, exsanguinated and eviscerated. The omental, perirenal and pericardial fat depots were removed and weighed to the nearest 1 g. Weights of heart, kidney and liver also were recorded. The carcass was split longitudinally into right and left halves and chilled overnight at 4 ∘C. The half-carcass was ground seven times through a 9 mm plate before subsampling for proximate analysis. Proximate composition was done by standard AOAC method (Association of Official Analytical Chemists 1970). Protein was determined by the macro-Kjehldal method. Lipid content was

determined using Soxhlet extraction procedure with anhydrous petroleum ether. Moisture content was the difference in weight of tissue before and after freeze-drying of subsamples (500 g) of comminuted carcass.

Results and Discussion

Body Weight and Feed Intakes

Body weights in the obese group increased in a curvilinear fashion until attaining a plateau value by wk 40 approximately (Fig. 2). Overeating for 40 wk increased body weight from 45 ± 3 kg to 85 ± 3 kg in obese sheep. Intakes of pellets and hay in obese sheep peaked at 1840 ± 105 g x $sheep^{-1}$ x day^{-1} at wk 3-5 and declined gradually thereafter until plateauing at nearly constant levels of 915 ± 32 g x $sheep^{-1}$ x day^{-1} at wk 30-60 of ad libitum feeding. Intakes of digestible energy mirrored feed intakes (Fig. 2), and clearly were decreasing from peak values (5490 ± 315 Kcal DE.$sheep^{-1}$. day^{-1}) at wk 3 while body weights continued to increase in obese sheep. Body weights and intakes in lean sheep were nearly constant throughout the experiment (Fig. 2).

Obese sheep were in static-phase obesity after wk 40 based on stable body weight and relatively constant consumption that was sufficient for maintenance of steady-state body weight and presumably steady-state body composition. Obese sheep were fed a specified maintenance intake starting wk 72 of the experiment. During static phase obesity (wk 40-120), average intakes of dry matter and digestible energy per kg body weight were practically equivalent in lean sheep fed maintenance and obese sheep fed maintenance (wk 72-120) or consuming maintenance when fed ad libitum (wk 40-71; Table 2).

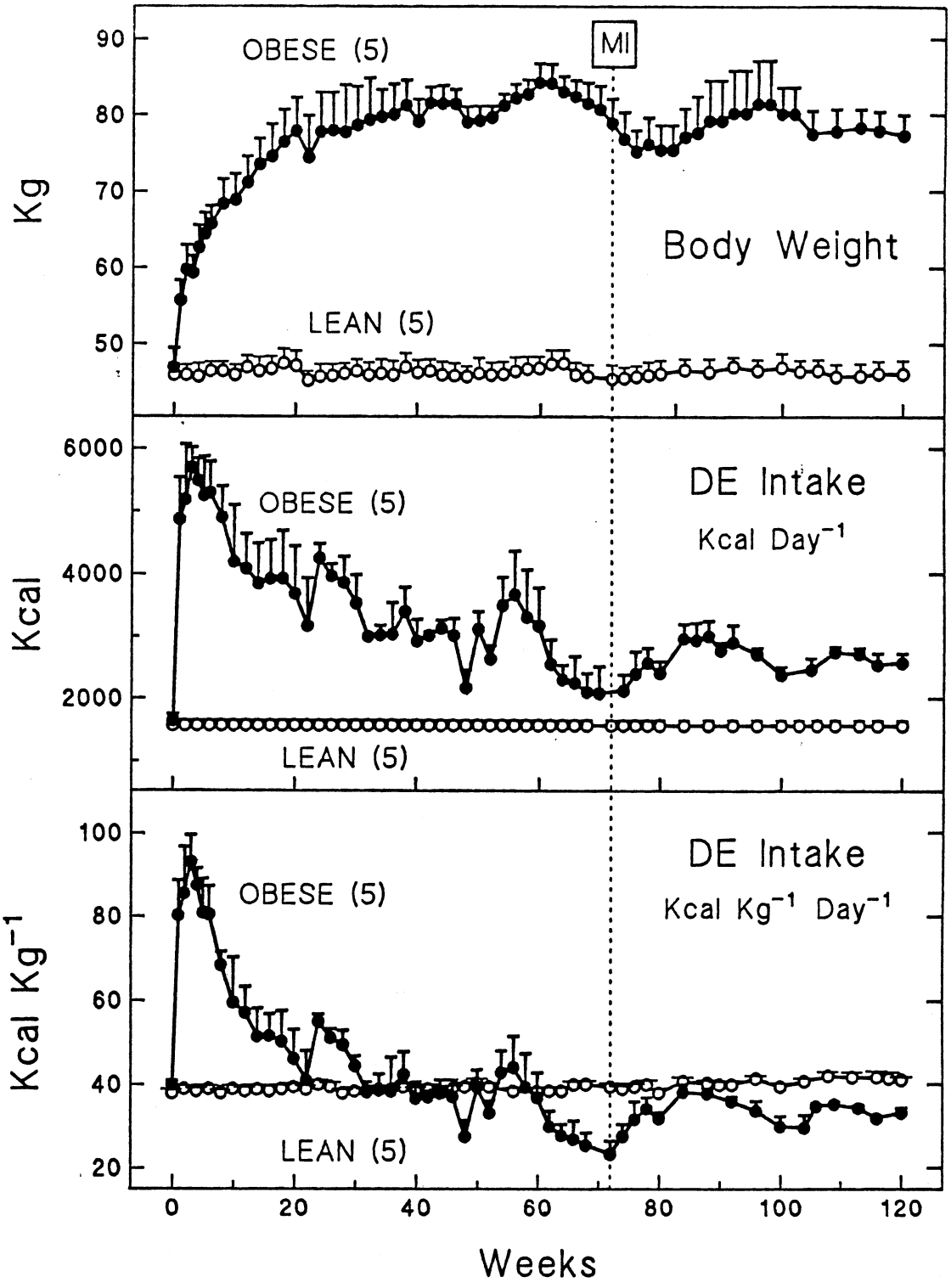

Figure 2. Body weight and digestible energy (DE) intakes in maintenance-fed lean sheep and obese sheep during the dynamic and static phases of dietary obesity. The bottom panel represents kcal DE consumed per kg body weight in lean and obese sheep. Average daily DE intake was obtained for 7 day (wk) intervals throughout the experiment.

Indices of Obesity and Carcass Composition

Body weights in obese sheep (79 ± 3 kg) were about twice those in lean sheep (46 ± 2 kg) at necropsy (Table 3). Weights of the omental, perirenal fat and pericardial fatdepots were markedly greater ($P<0.01$) in obese than lean sheep. Weights of some of the vital organs, (e.g., heart, liver and kidneys) were greater ($P<0.01$) in obese than lean sheep. The percentage of lipid was greater ($P<0.01$) and the percentage of protein less ($P<0.01$) in the heavier ($P<0.01$) carcass of obese compared with lean sheep.

TABLE 2

AVERAGE DAILY TOTAL INTAKES OF DRY MATTER (DM) AND DIGESTIBLE ENERGY (DE) DURING THE STATIC PHASE OF DIETARY OBESITY IN SHEEP[1]

		Lean (5)	Obese (5)	
Item		WK 50-120	WK 50-66	WK 70-120
Dry Matter	$g/kg^{1.0}$	12.5 ± 0.2	11.3 ± 0.3	10.3 ± 0.2
(DM)	$g/kg^{0.75}$	32.5 ± 0.6	34.5 ± 1.0	30.7 ± 0.5
Digestible Energy	$Kcal/kg^{1.0}$	38.7 ± 0.8	35.5 ± 0.9	32.0 ± 0.5
(DE)	$Kcal/kg^{0.75}$	101.1 ± 2.9	107.8 ± 3.3	95.3 ± 1.4

[1] Obese sheep were fed ad libitum but consuming maintenance during week (WK) 50-66 and were fed a specified maintenance during WK 72-120. Daily intakes of DM and DE were averaged for each sheep during each period and the average value used to calculate the mean ± SE values shown. DE intakes were calculated on the basis that 1 g of total digestible nutrient (TDN) provides 4.45 Kcal DE.

TABLE 3

WEIGHTS OF LIVE BODY, CARCASS, INTERNAL ORGANS, AND FAT DEPOTS AND CHEMICAL COMPOSITION OF CARCASS DURING STATIC PHASE OBESITY IN SHEEP

Item	Lean (5)	Obese (5)[1]
	Body and organ weights	
Body weight (kg)	46 ± 2	79 ± 3
Carcass weight (kg)	20 ± 0.5	40 ± 1
Omental fat (kg)	0.94 ± 0.14	4.42 ± 0.2
Perirenal fat (kg)	0.48 ± 0.09	2.17 ± 0.23
Pericardial fat (g)	91 ± 12	274 ± 26
Heart (g)	248 ± 17	287 ± 16
Liver (g)	530 ± 20	714 ± 49
Right kidney (g)	58 ± 2	76 ± 6
Left kidney (g)	54 ± 2	82 ± 7
	Carcass Percent Composition	
Lipid	23 ± 2	46 ± 1
Protein	14.9 ± 1.2	10.7 ± 0.5
Moisture	58 ± 1.0	24 ± 0.7
Ash	4.2 ± 0.4	3.1 ± 0.2

[1] Except for heart weights, values for lean and obese sheep within an item differ ($P<0.05$)

Dietary obese sheep portray some of the risk factors associated with obesity in humans such as hyperinsulinemia, hyperglycemia, insulin resistance. Our understanding of the etiology and pathophysiology of obesity relies heavily on the availability of suitable animal models. Genetically obese rodents are well suited to study pathophysiology of obesity with genetic origin. Dietary obese rodents are difficult

to produce. One of the limitations of genetically obese or dietary obese rodents is their small body size which precludes repetitive sampling. Obese sheep provide an ideal model of non-genetic origin for studies including diabetes, insulin resistance and blood pressure. Sheep are equivalent in size to humans, are docile, do not molest the experimenter, and are readily instrumented for short term or chronic physiological and pharmacological studies. As with any model, sheep have their limitations. Sheep are herbivorous ruminants whereas humans are omnivorous and nonruminants. However, sheep are established animal models for humans in areas of neurophysiology, cardiovascular physiology and reproductive biology.

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CHAPTER III

EFFECTS OF NALOXONE ON *AD LIBITUM* INTAKE AND PLASMA INSULIN, GLUCOSE, AND FREE FATTY ACIDS IN MAINTENANCE-FED SHEEP

Introduction

Changes in plasma levels of insulin, glucose and free fatty acids (FFA) are thought to play a role in systemic regulation of feed intake in animals (Bray 1986; Friedman et al 1986; Morley 1987). Endogenous opiates and their receptors also play important roles in the regulation of feed intake in physiological and pathological conditions (Morley et al 1983; Yim and Lowy 1984; Reid 1985). Opiate antagonists such as naloxone and naltrexone decrease feed intake in fasted or fed animals and humans (Morley 1987; Baile and McLaughlin 1987). In addition to their effects on appetite, endorphins also are capable of affecting the plasma concentrations of insulin, FFA and glucose directly via peripheral opiate receptors and(or) indirectly via opiate receptors in the central nervous system (Morley 1987; Appel et al 1987; Rudman and Kutner 1986; Curry et al 1987). Naloxone, therefore, may suppress feed intake by direct effects within the CNS alone or in combination with naloxone-induced changes in the concentrations of plasma chemicals such as insulin, glucose and FFA that serve as peripheral signals in appetite regulation. Conversely, plasma glucose itself may influence appetite by regulating the activity of central opiate receptors in rats (Morley and Levine 1982).

Endorphinergic regulation of appetite may differ among species and between animals of the same species that are in differing physiological states. For example, doses of naloxone that decreased feed intake in satiated chickens were ineffective in 12-h fasted chickens (Sangiah et al 1988). In these and other similar studies in domestic animals (Baile et al 1981), the endorphinergic link between feed intake and changes in plasma insulin, glucose and FFA were not examined. Therefore, the objectives of this study were to determine (1) dose-dependent inhibitory effects of naloxone on *ad libitum* intake in lean sheep fasted overnight, and (2) whether effects of naloxone on feed intake were dissociated from concomitant effects of naloxone on plasma concentrations of insulin, glucose and FFA.

Materials and Methods

Dorset ewes (n = 5), 3 to 4 years of age and weighing 36 ± 2 kg, were housed individually in a room with constant light and temperature (23-25 °C). Except on experimental days, sheep were fed a maintenance intake (540 g) of a pelleted hay-grain feed (14% CP; 12% fibre, 63% TDN; 93% DM) in two equal amounts at 0900 h and 1700 h and 90 g of hay at 0900 h. All feed routinely was consumed within 30 min of feeding. Previous work showed that sheep fed this ration were in zero-energy balance (McCann and Bergman 1988; McCann et al 1990) as indicated in this study by steady-state body weights over prolonged time intervals of at least several months before and during data collection. The weight-to-height ratio in these Dorset ewes indicated a lean body condition that has been associated with a stripped carcass lipid content of about 23% and a live body lipid content of 16% to 20% (McCann and Bergman 1988; McCann et al 1990).

Five of twenty four sequences possible for treatments of 0 (saline), 0.3, 1.0 and

3.0 mg/kg naloxone were assigned to sheep in a generalized block design with sheep considered as blocks. At least 7 d elapsed between successive treatments. Naloxone hydrochloride (DuPont, Wilmington, Delaware) was prepared fresh in sterile saline (5 ml) as needed on each experimental day, and was injected iv by jugular vein catheter 5 min before 16-h fasted sheep were allowed *ad libitum* intake of pellets for the ensuing 24 h. A sham trial consisting of blood sampling and 24 h of *ad libitum* feeding was done in all sheep to acclimate them to experimental procedures. Feed intakes were determined and blood samples obtained at 10, 20, 40 and 60 min and 2, 4, 8, 12, 16 and 24 h of *ad libitum* feeding; additional blood samples were taken 30, 20 and 5 min before *ad libitum* feeding. Blood samples were collected via jugular catheter that was inserted at least 12 h before sample collections. Plasma samples were stored (-22 C) after centrifugation (4 C) of whole blood for 20 min at 1000 x g. Glucose and FFA concentrations in plasma were determined using enzymatic colorimetric assays (McCann et al 1990; McCann et al 1986). Plasma concentrations of insulin were determined in duplicate by a validated solid-phase radioimmunoassay (McCann et al 1986).

Treatment effects of animal, naloxone dose, sample time and their interactions were tested by repeated measures analysis of variance using the General Linear Model (GLM) procedure in SAS (Freund 1986). An analysis of covariance model of SAS for split-unit and repeated measures (Meredith et al 1988) was done to adjust plasma concentrations of insulin, glucose and FFA for differences in feed intakes among treatment groups; feed intake was considered as the covariate changing with the subunit levels (time). Data are presented as mean ± SE.

Results

Saline-treated sheep, that previously had been meal fed a maintenance intake, consumed 1.79 kg of a high-energy diet during 24 h of *ad libitum* feeding (Table 1). Approximately 50% and 80% of their total 24-h intake was consumed in the first 60 min and 4 h of *ad libitum* intake, respectively. Relative to control intakes in saline-treated sheep, naloxone at doses of 0.3 and 1.0 mg/kg initially had no significant effect on intakes in the first 60 min of *ad libitum* intake, but both doses decreased ($P < 0.01$) the cumulative 2-h and 4-h intakes by approximately 35% (Table 1; Fig. 1). In contrast, the highest dose of naloxone (3 mg/kg) had immediate (10-min) and protracted (4 h)

TABLE 1

MEAN (± SE) CUMULATIVE FEED INTAKE (g) IN LEAN SHEEP (n=5) TREATED WITH NALOXONE AND FED *AD LIBITUM* [A]

	Dose of Naloxone (mg/kg)			
Time	Saline	0.3	1.0	3.0
10 min	630 ± 29^{b}	649 ± 39^{b}	690 ± 31^{b}	344 ± 79^{c}
20 min	885 ± 82^{b}	718 ± 64^{b}	718 ± 40^{b}	392 ± 115^{c}
40 min	908 ± 84^{b}	730 ± 58^{b}	718 ± 40^{b}	392 ± 114^{c}
60 min	947 ± 104^{b}	765 ± 64^{b}	720 ± 40^{bc}	475 ± 114^{c}
2 h	1,216 ± 149^{b}	822 ± 69^{c}	769 ± 61^{c}	482 ± 117^{d}
4 h	1,397 ± 216^{b}	987 ± 104^{c}	836 ± 74^{c}	560 ± 111^{d}
8 h	1,515 ± 220^{b}	1,225 ± 123^{c}	1,066 ± 114^{cd}	849 ± 171^{d}
12 h	1,615 ± 194^{b}	1,501 ± 95^{bc}	1,329 ± 129^{cd}	1,151 ± 157^{d}
16 h	1,699 ± 166^{b}	1,684 ± 69^{b}	1,489 ± 155^{b}	1,443 ± 152^{b}
24 h	1,788 ± 137^{b}	1,826 ± 56^{b}	1,638 ± 154^{b}	1,890 ± 85^{b}

[a]Sheep were allowed 24-h *ad libitum* intake after a 16-h fast.

[bcd]Means within a time period with similar superscripts are not different ($P > 0.05$).

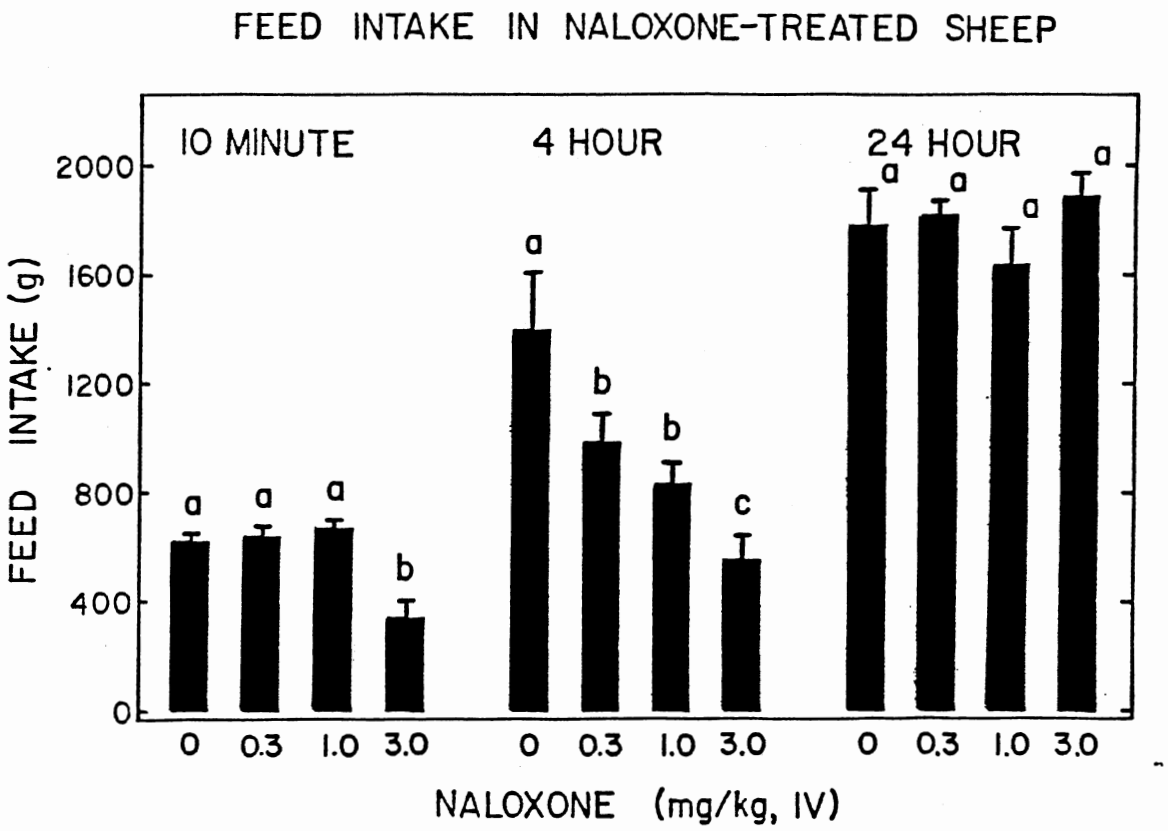

Figure 1. Cumulative feed intakes (mean ± SE) after 10 min, 4h, and 24 h of *ad libitum* intake in sheep (n=5) pretreated with 0, 0.3, 1.0, or 3.0 mg/kg of naloxone iv.

inhibitory effects on feed intake. Doses of naloxone used had no observable effects onthe gross motor behaviour or well-being of the sheep. Preliminary experiments by us in 2 sheep showed that 10 mg/kg naloxone clearly affected motor behaviour adversely.

Compared with saline-treated sheep, 0.3 and 1.0 mg/kg naloxone decreased ($P<0.01$) cumulative intakes during the first 8 to 12 h of *ad libitum* intake, whereas 3 mg/kg naloxone decreased ($P< 0.01$) cumulative intake during the first 12 to 16 h of *ad libitum* intake. Although 16-h cumulative intakes in naloxone-treated sheep generally were less than those in saline-treated sheep, the mean rate of intake

between 4 and 16 h in sheep treated with 0.3 mg/kg (2.9 g/min), 1.0 mg/kg (2.7 g/min) or 3.0 mg/kg (3.7 g/min) of naloxone exceeded ($P < 0.05$) that during saline treatment (1.2 g/min). These results suggest that pretreatment with naloxone effectively suppressed intake only in the first 2 to 4 h of *ad libitum* intake; thereafter the effects of naloxone waned and a rebound response was observed as evidenced by greater consumption rate in naloxone-treated than in control sheep. Consequently, total intakes at 24 h were similar in all groups of sheep (see Table 1 and Fig. 1).

Plasma concentrations of insulin (38 ± 7 vs 95 ± 22 fmol/ml) and glucose (2.7 ± 0.2 vs 3.6 ± 0.2 μmol/ml) first increased ($P < 0.01$) above fasting levels at 40 min and 4 h, respectively, of *ad libitum* intake in saline-treated sheep (Fig. 2).

Plasma FFA levels decreased ($P < 0.01$) from 291 ± 48 nmol/ml to a sustained low level of 92 ± 14 nmol/ml after 60 min of *ad libitum* intake in saline-treated sheep. Concentrations of insulin, glucose and FFA in plasma of *ad libitum*-fed sheep pretreated with three doses of naloxone were statistically similar to those in control sheep despite the fact that feed intakes were affected by naloxone. To determine naloxone effects *per se* on plasma variables, the plasma concentrations of insulin, glucose and FFA were adjusted statistically for naloxone-induced differences in feed intake (18,19). This analysis showed significant covariate relationship between plasma variables and feed intake across sheep in all treatment groups. The adjusted treatment means for glucose and FFA remained unaffected by naloxone treatment, but the highest dose of naloxone tended ($P=0.09$) to increase plasma insulin concentrations independent of feed intake. Collectively, results suggest that the inhibitory effects of naloxone on appetite in sheep likely were independent of peripheral feedback signals in plasma involving insulin, glucose, or FFA.

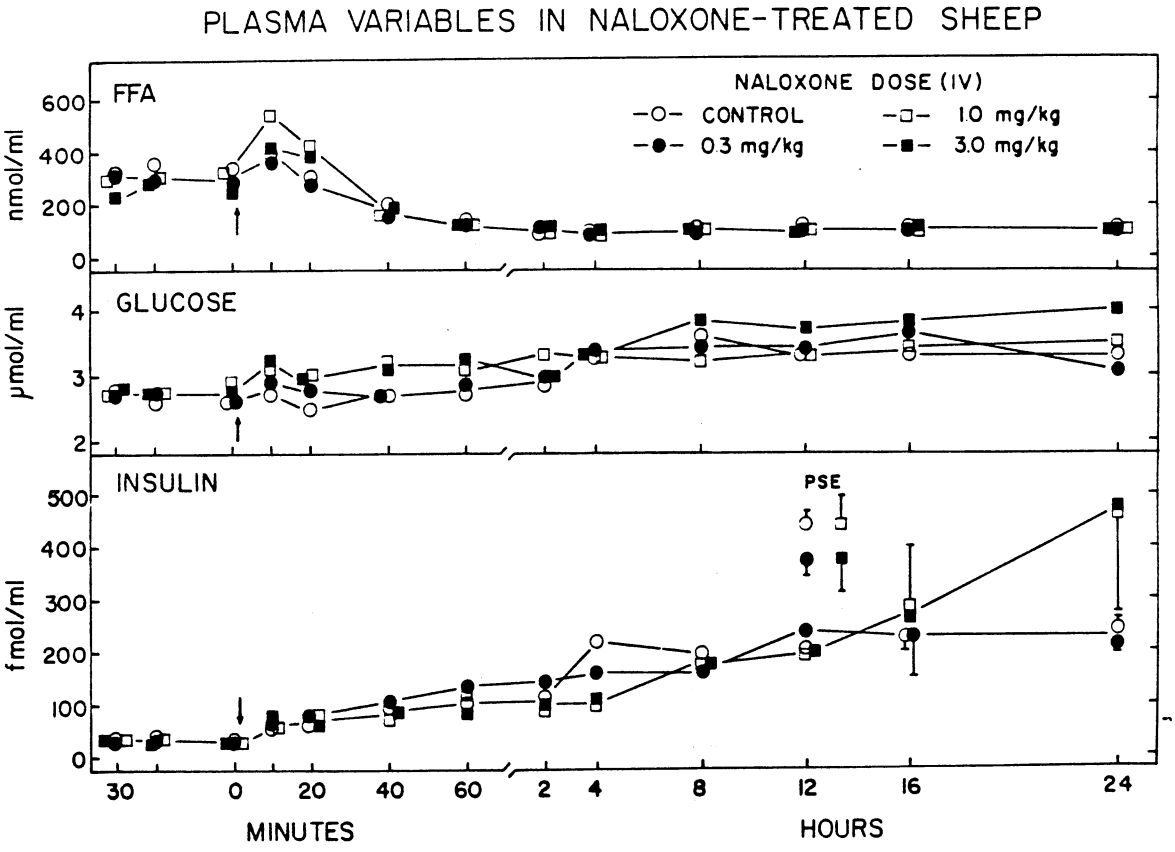

Figure 2. Plasma concentrations (mean ± SE) of free fatty acids, glucose and insulin before (fasted) and after (fed) naloxone treatment in lean sheep (n=5). Arrows indicate naloxone injection 5 min before start of 24 h of *ad libitum* feeding. The pooled standard error (PSE) for insulin concentrations are shown for each treatment group; standard errors (bar) at 16 and 24 h are shown because mean concentrations differed the most at these times. Standard error bars for glucose and FFA were numerically contained within symbols for mean concentration.

Discussion

This study demonstrated dose-dependent inhibitory effects of naloxone on *ad libitum* intake in 16-h fasted lean sheep that routinely had been meal fed a maintenance intake. However, only the highest dose of naloxone (3 mg/kg) had

immediate inhibitory effects on feed intake. Lower doses of naloxone did not reduce intake significantly until sheep had consumed feed in the initial h of *ad libitum* intake. The behavioral response of feed intake to low doses of naloxone in this study apparently was modified with time as animals ate. One interpretation is that the strong endorphinergic drive for hunger in our sheep could be antagonized immediately only by high dose naloxone, and that low dose naloxone became effective with time because endorphinergic drive for eating diminished as the animals consumed feed. This postulate is strengthened by comparing our results with those of Baile et al (Baile et al 1981) who determined dose-dependent effects of naloxone in 4-h fasted sheep that previously were maintained on *ad libitum* intake. Baile et al (1981) reported that as little as 0.03 mg/kg naloxone suppressed 2-h intake in their sheep, whereas much higher doses of 0.3 and 1.0 mg/kg naloxone were ineffective in our study. Fasting an overfed animal for 4 h (Baile et al 1981) should result in less hunger drive than fasting maintenance-fed animals for 16 h, as was done in our study. The degree of negative energy balance, therefore, would seem to be directly related to the endorphinergic drive for hunger in sheep, as illustrated by responses to naloxone in this study and that of Baile et al (1981). A further consideration is that our sheep purposefully were lean, whilst those of Baile et al (1981) should have been fatter, relatively, because their sheep chronically were overfed. Data in lean and obese Rambouillet ewes suggest that body condition *per se* alters opiate regulation of appetite in sheep (Chapter IV). Additionally, it should be noted that the supraphysiological dose of 3 mg/kg of naloxone was incapable of completely suppressing intake in these sheep. In broiler chicks, doses of naloxone as high as 10 mg/kg had no effect whatsoever on intake in 12-h fasted birds (Sangiah et al 1988). That intake was not suppressed completely by high dose naloxone in fasted sheep or chicks suggests that opiate and nonopiate

systems (e.g., monaminergic and gaba-receptors) in the CNS are involved to different degrees in initiating feeding in hunger-driven sheep and chicks. Girard and co-workers (1985; 1986) reported that initiation of feeding in sheep may involve neurons in the medial hypothalamus that are responsive to gamma-aminobutyric acid.

This study also sought to determine if the inhibitory effects of naloxone on feed intake were mediated in part by changes in the plasma levels of insulin, glucose, or FFA. Increases in the concentrations of insulin or glucose are purportedly feedback signals for satiety, whereas increases in plasma FFA may have a role in hunger drive (Rezek 1976; Morley 1987). Our results showed that dose-dependent effects of naloxone on appetite in sheep were manifested without concomitant naloxone-induced changes in plasma insulin, glucose, or FFA. However, the highest dose of naloxone tended to increase plasma insulin levels, independent of changes in feed intake, towards the latter half of *ad libitum* intake. This supports other studies in rats where naloxone treatment significantly increased the plasma insulin level (Fontela et al 1986; Knudtzon 1986). A direct effect of naloxone on pancreatic B-cell function in sheep should be considered because B-cells of rodents respond to opiate stimulation *in vitro* (Rudman and Kunter 1986; Curry et al 1987).

The biological half-life of naloxone in rats approximates 40 min (Tepperman et al 1983). It is assumed that the ability of naloxone to effectively block opiate action in sheep in this study waned within 2 to 4 h of iv injection of naloxone consistant with the naloxone short half life of 43 min sheep (Chapter VIII). We suggest that the recovery in feed intake in our naloxone-treated sheep after 4 h of *ad libitum* intake likely reflected loss of naloxone bioactivity because of its metabolism and elimination. Morley (1987) has addressed the teleological practicality of opiates suppressing reproductive function while stimulating feeding drive in hungry animals. It is well

established that naloxone treatment increases LH secretion in postpartum cows that are in negative energy balance and lactating (Whisnant et al 1986). Results of this study and those of Baile et al (1981) would suggest that the greater the degree of negative energy balance then the greater the opiate drive for hunger that directly or indirectly could curtail reproductive function in domestic ruminants. Indeed, a dose of naloxone that increased plasma LH in beef cows at d 42 postpartum was ineffective at d 14 or 28 postpartum when negative energy balance may have been more severe (Whisnant et al 1986a). Central opiate systems may be part of an intricate neural regulatory mechanism that links nutritional state and reproduction in domestic ruminants (McCann and Hansel 1986), and such a system would be influenced by the suckling calf (Whisnant et al 1986b).

We conclude that the ability of naloxone to suppress appetite in sheep is negatively associated with duration of fasting or severity of negative energy balance, and that the appetite inhibitory effects of naloxone in sheep do not involve peripheral changes in plasma insulin, glucose, or FFA.

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CHAPTER IV

OPIATERGIC REGULATION OF FEED INTAKE IN LEAN AND OBESE SHEEP

Introduction

Considerable evidence supports the involvement of the opiatergic system in regulation of appetite in mammalian and nonmammalian species (Sanger 1981; Morley et al 1983; Reid 1985; Sangiah et al 1988; Alavi et al 1991). Although opiatergic control of appetite requires opioid receptors located centrally in the brain and peripherally in the intestine, centrally located receptors in the hypothalamus and surrounding area are believed to play the dominant role in appetite regulation (Morley et al 1983; Atkinson 1987). Stimulation of opiate receptors in the central nervous system (CNS) is associated with hunger drive and eating. Opiate antagonists such as naloxone reduce intake in hungry animals, whereas opiate agonists can increase intake in satiated animals (Gosnell 1987; Baile et al 1981, 1987; Alavi et al 1991).

Dysfunction in opiatergic regulation of appetite may be involved in the development and maintenance of the obese state in humans (Baranowska et al 1987; Giugliano et al 1987) and animals (Margules et al 1978; Shimomura et al 1982). Concentrations of ß-endorphin were higher in pituitary and plasma of genetically obese than lean mice or rats during the development of the obese state (Margules et al 1978; Givens et al 1980). Plasma levels of ß-endorphin generally are greater in humans with established obesity than in humans of lean body condition (Ritter et al 1991). Further

support for opiatergic dysfunction in obesity comes from results consistently showing greater appetite inhibitory effects of naloxone in obese humans (Atkinson 1987) and genetically obese rodents (Margules et al 1978) than in their lean controls. However, the exact roles played by central and peripheral opioid receptors in appetite regulation during dynamic obesity and static phase obesity are not clear (Morley et al 1983; Yim and Lowy 1984)

Dysfunction in opioid regulation of appetite could be an initiating factor in the development of obesity in genetically obese rodents or it could be a prerequisite for allowing hyperphagia during the dynamic or induction phase of obesity. Most, if not all, studies comparing opioid regulation of appetite between genetically lean and obese rodents have examined opiatergic control of appetite in obese animals during the dynamic phase of obesity. In contrast, similar studies in humans compare responses to naloxone between lean individuals and individuals in the static or established phase of obesity. Unlike dynamic phase obesity, hyperphagia is not evident during the steady state conditions of static phase obesity if hyperphagia means that animals consume more than their maintenance requirements (McCann and Bergman 1988).

McCann et al (1991) has developed an animal model of dietary obesity in sheep. Dietary obese sheep are hyperinsulinemic, hyperglycemic and insulin resistant (McCann and Bergman 1988; McCann et al 1991). Sheep consume 3 to 6 times maintenance during dynamic obesity but consume only maintenance upon attainment of static phase obesity (McCann et al 1991). The impact of the obese state *per se* on opiatergic regulation of appetite in sheep therefore can be examined readily because diet type, level of intake, and energy balance are equivalent in both lean sheep and sheep in the static phase of obesity (McCann et al 1991). The objectives in this study , therefore, were to determine if intake differed between lean and obese sheep allowed

free access to a common diet, and whether obesity affected opiatergic regulation of appetite in sheep.

Materials and Methods

Animals

Rambouillet ewes aged 3 to 4 years were purchased from USDA-ARS, Forage and Livestock Research Laboratory, Fort Reno, Oklahoma. Detailed description of development of obesity is discussed in Chapter II. Briefly, sheep were fed a pelleted hay-grain diet maintain them in lean body condition (lean, n=5) or were fed the same diet ad libitum to induce dietary obesity (obese, n=5). Obese sheep were considered to be in static phase of obesity after 50 wk of overfeeding as indicated by nearly constant body weight and feed intake.

Lean (46 ± 2 kg) and obese (79 ± 3 kg) sheep were fed the pelleted hay-grain mixture (12.5 g/kg) in two equal amounts at 0900 and 1500 h; small amounts of hay (1 g/kg) were fed daily at 0900 h to maintain rumen tone. Uneaten feed was removed at 1700 h. This ration maintained zero-energy balance in lean and obese sheep. Chemical composition of the pellets and hay was presented in Chapter II (see Table 1). Sheep were housed and fed individually in a room with constant light and temperature (21-23 C).

Treatments

Lean and obese sheep were paired and assigned randomly by latin square to 5 treatments of 0 (saline), 0.01, 0.1, 1 and 3 mg/kg naloxone. Naloxone hydrochloride generously provided by DuPont Pharmaceutical (Wilminigton, Delaware, USA) was prepared fresh in sterile saline and filtered through a disposable 0.2 μ nylon filter

before injection on each experimental day. Saline (10 ml) and naloxone solutions (10 ml) were injected via a cannulated jugular vein 5 min before 16-h fasted sheep were allowed ad libitum intake of pellets for the ensuing 32 h. Cannula were flushed quickly with 2 ml of saline after each dose of naloxone. At least 7 d elapsed between successive treatments. Blood samples were collected and feed intakes were determined at 20, 40 and 60 min and 2, 4, 8, 12, 16, 24 and 32 h of ad libitum feeding. The amount of feed consumed was measured by replacing the uneaten feed with a fresh batch of pellets (1000 g) at each time interval. Blood samples (5 ml) were collected into glass tubes containing 50 µl mixture of benzamidine (200 mg/ml) and heparin (5000 U/ml) that were held in an ice-water bath. Chilled blood samples were centrifuged (4 C) for 10 min at 1000 x g and plasma stored (-30 C) for determination of plasma naloxone. Additional blood samples (5 ml) were collected into chilled glass tubes containing EDTA (7.2 mg) at 60, 30 and 1 min before each naloxone injection and the recovered plasma used for quantification of ß-endorphin concentrations. A sham trial was conducted to familiarize animals to all experimental procedures including ad libitum feeding for 32 h.

Chemical Analysis

Naloxone Assay. Plasma concentrations of naloxone immediately before and at 25, 45 and 65 min after the 4 highest doses of naloxone were quantified by Waters HPLC system equipped with a reverse-phase radial pak C18 column cartridge (Waters, Milford, MA, USA). Preliminary experiments indicated that a mobile phase (pH 4.5) comprising 0.1 M ammonium dihydrogen phosphate, 0.9 mM octasulfonic acid, 5.4 mM disodium EDTA, and 4.5 % n-propanol at a flow rate of 0.7 ml/min provided optimal conditions with respect to the separation of naloxone, naltrexone (internal standard)

and benzamidine added to blood tubes to prevent proteolytic degradation of peptide hormones. The intraassay and interassay coefficients of variation were 4.1 and 3.5%, respectively.

ß-Endorphin Assay. Plasma concentrations of immunoreactive ß-endorphin were measured by radioimmunoassay (IncStar, Stillwater, MN). Sepharose particles in suspension were added (0.5 ml) to columns provided by the manufacturer. The supernatant in the column was forced through using a rubber bulb, and then the bottom of the column was tightly capped. Columns received 1 ml of either human ß-endorphin (17-272 pg/ml), ovine ß-endorphin standard (25-200 pg/ml), sheep plasma samples, or control sheep plasma for determination of non specific binding in RIA. The columns were then capped and rotated end over end for 4 h (± 15 min) at 2-8 C before plasma was allowed to drain through the column. Any remaining fluid in the column was forced out gently with the rubber bulb. The remaining sepharose particles in the column were washed with 3 X 1 ml aliquots of saline (0.9% NaCl) until all the particles settled at the bottom of the column. Saline was forced to drain as before. To elute the sepharose bound ß-endorphin, two sequential additions of 250 µl of 0.025 N HCl were added and allowed to sit for 1 min and then the HCl was forced through the column and collected into a clean glass test tube. The extract collected was mixed and placed on crushed ice for immediate radioimmunoassay.

All samples were assayed in duplicate according to manufacturer's instructions. Briefly, 200 µl of extract were transferred to duplicate 12 X 75 mm glass tubes held in crushed ice. Neutralizing buffer (100 µl) was added to all tubes before 100 µl of rabbit anti-ß-endorphin serum were added to all tubes except total count (TC) and nonspecific binding tubes. Tubes were gently mixed and incubated for 16-24 h at 2-8 C, before 100 µl [^{125}I]ß-endorphin were added. Vials were mixed gently and incubated

for 16-24 h at 2-8 C. Goat anti-rabbit serum (0.5 ml) was added, vials were mixed and then incubated for 15-25 min at 2-8 C before centrifugation (20 C) at 1000 x g for 20 min. The supernatant was discarded and tubes allowed to drain for at least 2 min. The excess supernatant around the edge of the tubes was blotted before turning tubes upright. Using a Micromedic MEplus gamma-scintillation counter (Horsham, PA, USA), the activity of the precipitate of each tube was counted for 5 min to achieve statistical accuracy and results were calculated automatically from linear standard curves generated as log-logit plots by weighted least-squares regression.

The ß-endorphin RIA was validated for use in ovine samples as follows. Serial dilutions of plasma or serum from lean (n = 2) or obese (n= 2) sheep inhibited binding of [^{125}I]ß-endorphin to antibody in a manner that paralleled inhibition by either human ß-endorphin (IncStar, Stillwater, MN) or ovine ß-endorphin (Peninsula Laboratories, Belmont, CA). The RIA had reasonable accuracy for measuring ß-endorphin in sheep plasma because mean (± SE) recovery of ovine ß-endorphin was 82.5 ± 8.0% and 95.9 ± 19.9 % when sheep plasma samples (n=4) were spiked with 50 and 200 pg/ml of ovine ß-endorphin, respectively. Intraassay and interassay coefficients of variation were 6.6% and 13.5%, respectively. Reported values of immunoreactive ß-endorphin have not been corrected for recovery losses.

Calculations and Statistical Analyses

Differences in cumulative feed intake between lean and obese sheep in the first 32 h after 0 mg/kg naloxone were tested by univariate correlated t test for repeated measurements (Gill 1979). Effects of dose, time and their interaction on cumulative feed intake at each level of body condition were tested using repeated measures analysis of variance in the general linear model procedure of SAS (Freund et al 1986); Fisher's protected ($P<0.05$) LSD test was used for multiple comparisons among means

at a given sample time in each body condition. Effects of body condition, naloxone dose and their interaction on feed intake were examined using cumulative intakes after naloxone injections calculated as percentage of the corresponding control intake in each sheep. Percent cumulative feed intakes at +20 min, +2 h and +4 h of ad libitum feeding then were each analyzed by 2 x 4 split-plot analysis of variance; multiple comparisons among means were done using Duncan's new multiple range test if a significant F-value ($P<0.1$) was found.

Dose-response curves for naloxone effects on intake were constructed as follows. Area under the curve (AUC) for cumulative intakes in the first 2 h after each dose of naloxone were calculated by trapezoid method. For each sheep, the AUC after 0.01, 0.1, 1 and 3 mg/kg naloxone were calculated as percent of AUC after the control dose (0 mg/kg) of naloxone to provide data on relative feed intakes in naloxone-treated lean and obese sheep. Best fit of the relationship between relative feed intake and naloxone dose was determined using least squares polynomial regression analysis of observations (n=20) in each body condition group (SAS, Cary, NC, USA). Comparisons of regression residual sum-of-squares and coefficient of determination determined that a first degree polynomial best described the dose-response effect of naloxone on intake in lean and obese sheep. Dose of naloxone suppressing relative intake by 25% (D_{25}) was calculated using an equation derived from first degree (linear) polynomial regression analysis of mean data in each body condition group. Significance of difference in the mean D_{25} dose between lean and obese sheep was determined by comparing the 95% confidence interval range for the mean D_{25} dose in lean sheep with the mean D_{25} dose in obese sheep. Level of significance was 0.05. Values are presented as mean ± SE.

Results

Control Feed Intake.

Intakes after 0 mg/kg naloxone were greater ($P< 0.05$) in lean than obese sheep through the first 8 h of ad libitum feeding (Fig 1). In the first 20 min of ad libitum feeding, saline-treated lean and obese sheep had consumed 42% and 26%, respectively, of their 32-h total intake.

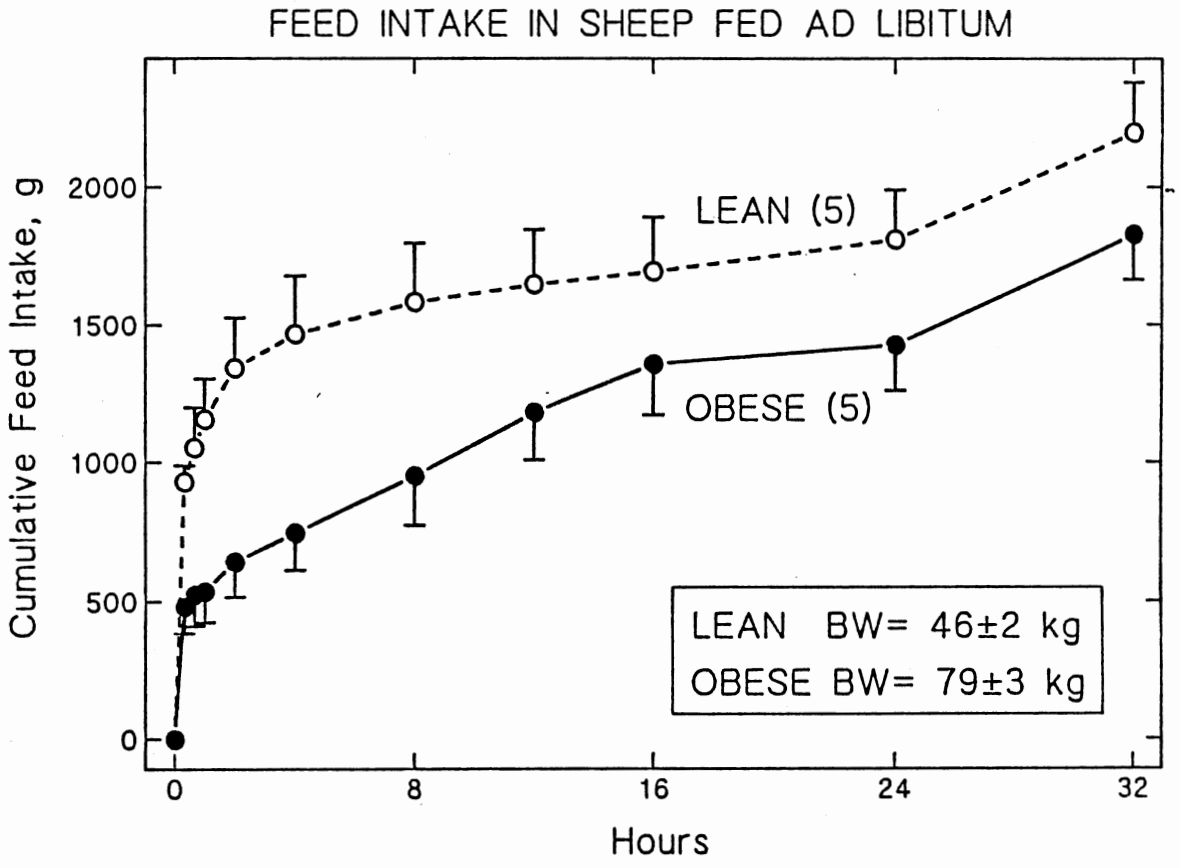

Figure 1. Cumulative feed intakes (mean ± se) in 16-h fasted lean and obese sheep allowed ad libitum intake of a pelleted hay-grain diet for 32 h. Sheep were in steady state and chronically fed maintenance of the hay-grain diet to keep them in zero-energy balance. Sheep were presented with fresh batches (1000 g) of pellets at time 0 and at each time point indicated when intakes were determined.

After 2 h of ad libitum feeding, lean sheep had consumed approximately twice as much as obese sheep, whether measured as absolute intake (1343 vs 645 g) or as percent of 32-h total intakes (61 vs 35 %). Cumulative intakes became equivalent statistically in both groups at +12 h, and thereafter, of ad libitum intake. Differences in shape of the control intake curves (see Fig. 1) may reflect differences in the chemical regulation of early (+20 min to +16 h), but not late, consummatory behavior in lean and obese sheep fed under the conditions of this experiment.

Naloxone Treatment Effect

Dose-dependent and time-dependent inhibitory effects of naloxone on intake were observed in lean and obese sheep (Fig. 2, 3 and Table I). Cumulative intakes were most affected by naloxone in the first 2 to 4 h of ad libitum feeding in both groups of sheep and these results are highlighted in Fig 4.

To allow comparison of the effects of naloxone in lean versus obese sheep, cumulative intakes during the first 4 h of ad libitum intake were recalculated as percent of corresponding control intakes in each sheep. The lowest dose of naloxone (0.01 mg/kg) increased cumulative intake nonsignificantly by 15 to 20 % in lean sheep in the first 4 h of ad libitum feeding (Fig. 5). This dose also increased cumulative intake nonsignificantly by 20 % in obese sheep only at +1 h of ad libitum intake. Of the ten sheep used in this experiment , four lean and two obese sheep displayed a stimulatory response to low dose naloxone in that their 2-h cumulative intake was 37 ± 9 % greater ($P<0.01$) after 0.01 mg/kg naloxone than after 0 mg/kg naloxone.

Maximum suppression of intakes occurred during the first 2 h after injection of naloxone (0.1, 1 and 3 mg/kg). Injection of 0.1 mg/kg naloxone had no effect on intake in lean sheep but the 2-h intakes in obese sheep were suppressed ($P<0.05$) 30 ±13% by this dose. Naloxone doses of 1 and 3 mg/kg suppressed ($P<0.0$) 2-h intakes in lean

sheep by approximately 29% and 50%, respectively. In contrast, intakes in obese sheep at +2 h were suppressed ($P<0.025$) 48% and 71% by 1 and 3 mg/kg naloxone, respectively. Interactive effects of body condition and naloxone on feed intake were determined using cumulative intakes calculated as percent of corresponding control intake at +20 min, +2 h and +4 h of ad libitum feeding. Intakes at these times were affected by body condition (+20 min only) and naloxone dose but not by their interaction (Fig. 6).

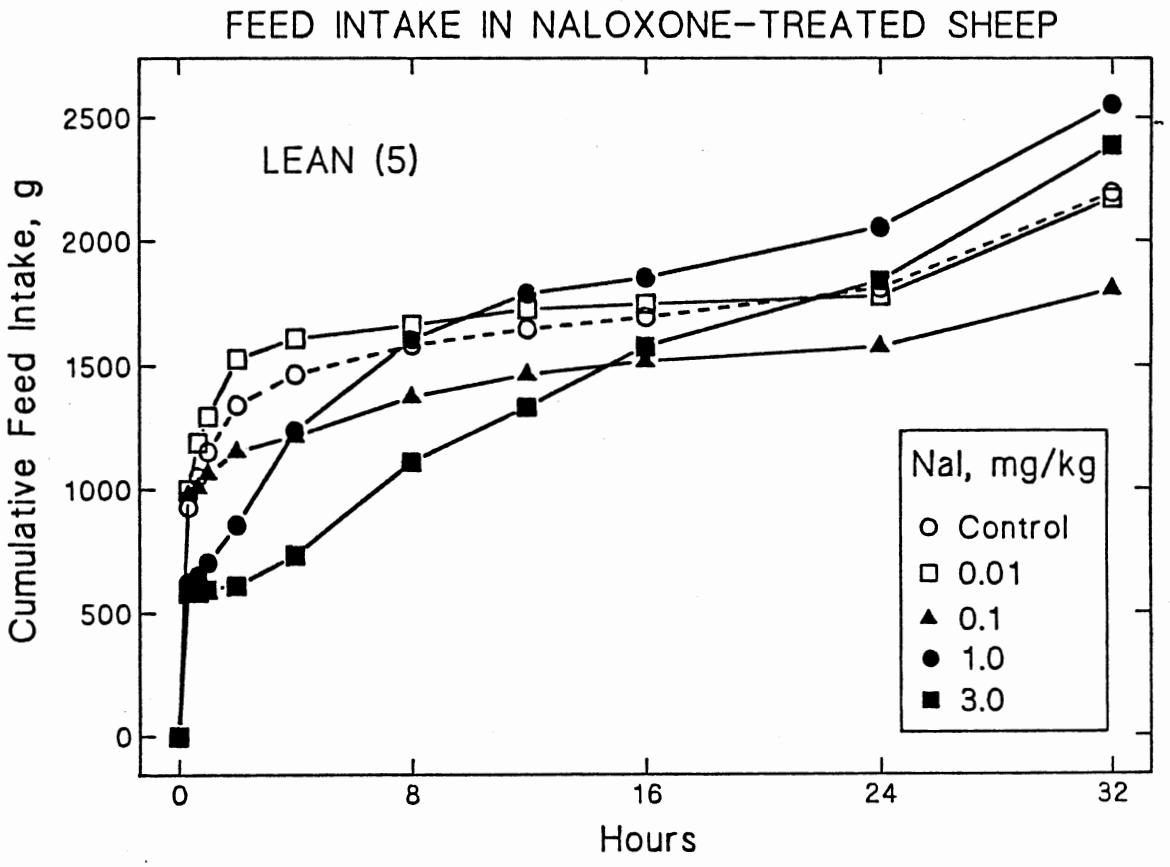

Figure 2. Cumulative feed intakes (mean ± SE) during 32-h of ad libitum intake in lean sheep pretreated with 0, 0.01, 0.1, 1 or 3 mg/kg of naloxone. Naloxone was injected iv 5 min before feeding began. Additional notes as in legend to Fig. 1.

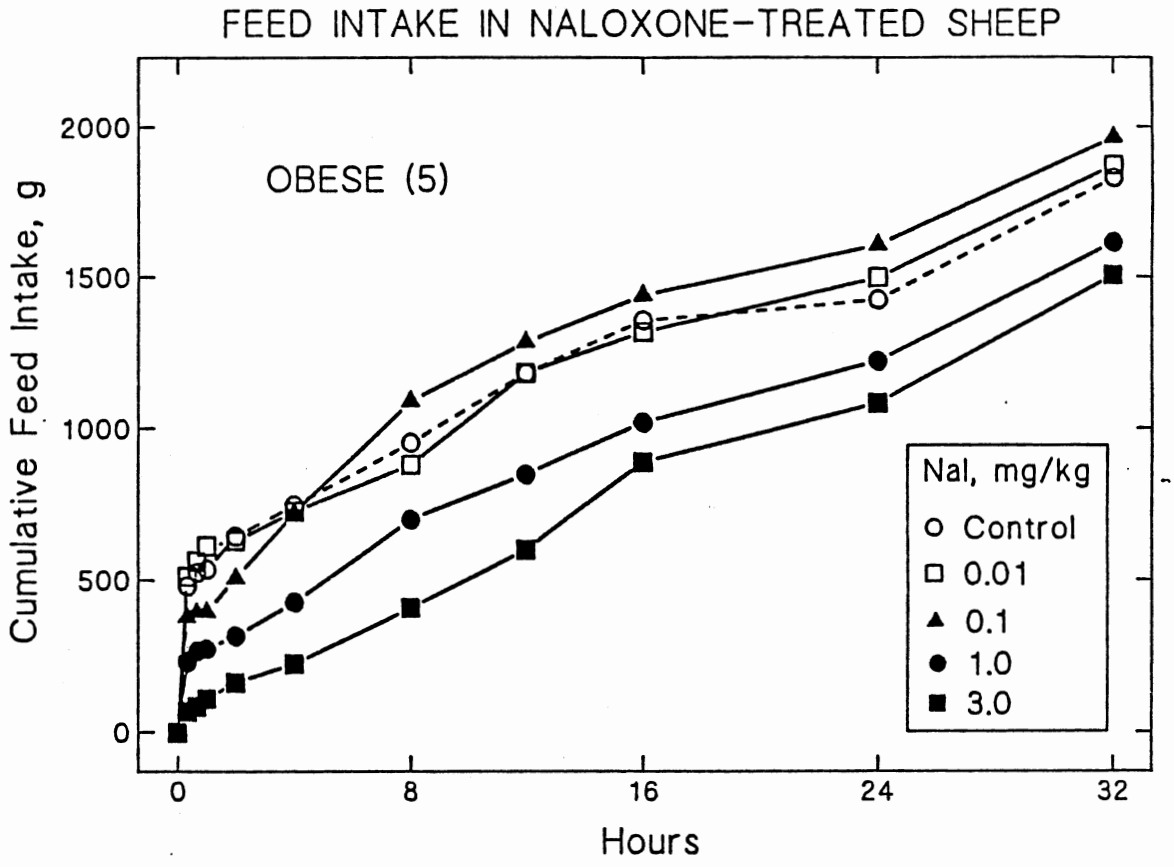

Figure 3. Cumulative feed intakes (mean ± SE) during 32-h of ad libitum intake in obese sheep pretreated with 0, 0.01, 0.1, 1 and 3 mg/kg naloxone iv. Additional notes as in legend to Fig. 2.

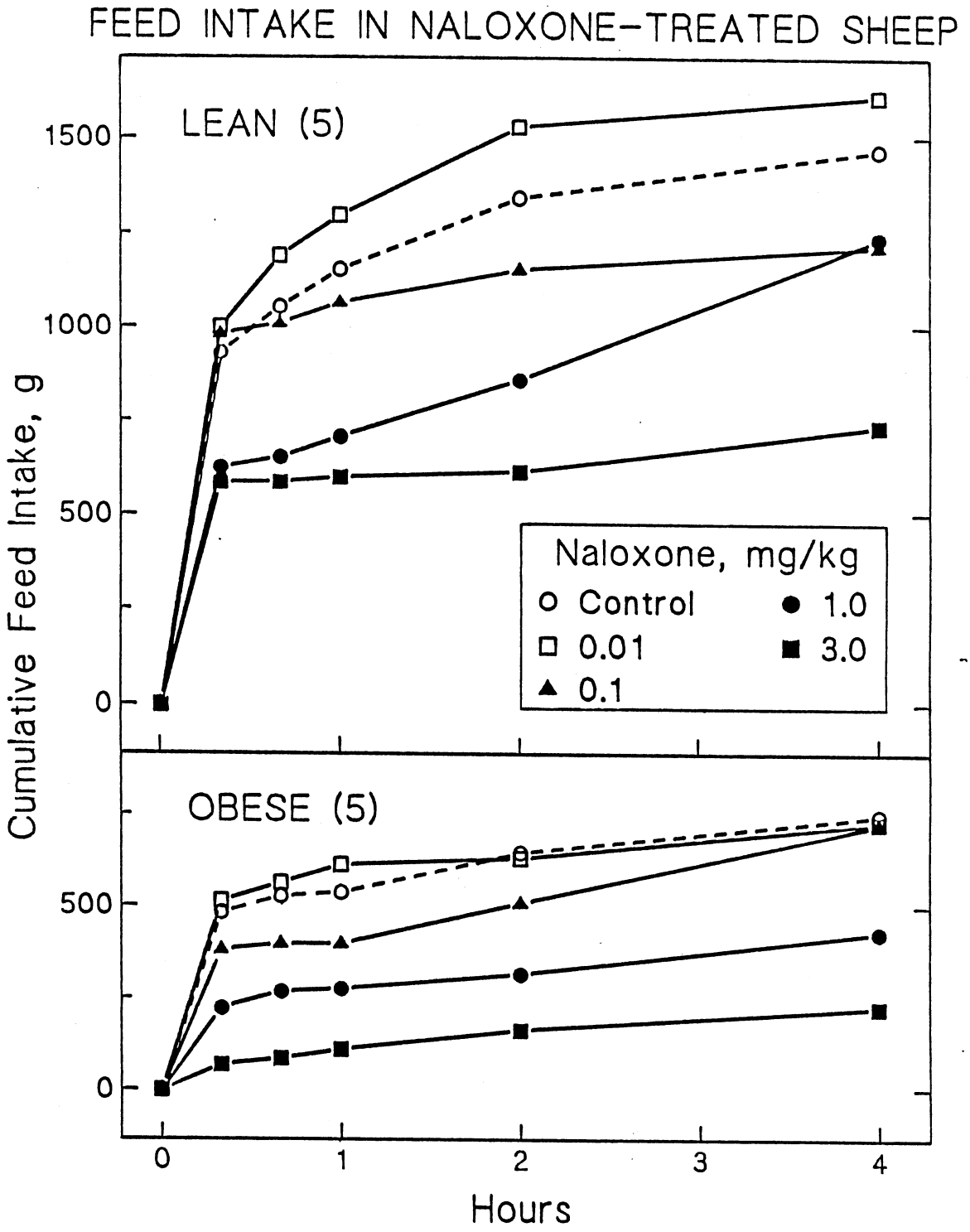

Figure 4. Cumulative feed intakes (mean ± SE) during the first 4 h of ad libitum intake in lean and obese sheep pretreated with 0, 0.01, 0.1, 1 and 3 mg/kg naloxone iv. For clarity of presentation, SE bars are not shown but the pooled SE values were 167, 142, 128, 112 and 90 in lean sheep and 131, 90, 170, 90 and 50 in obese sheep treated with 0.01, 0.1, 1 or 3 mg/kg naloxone, respectively. Additional notes in legend to Fig. 2.

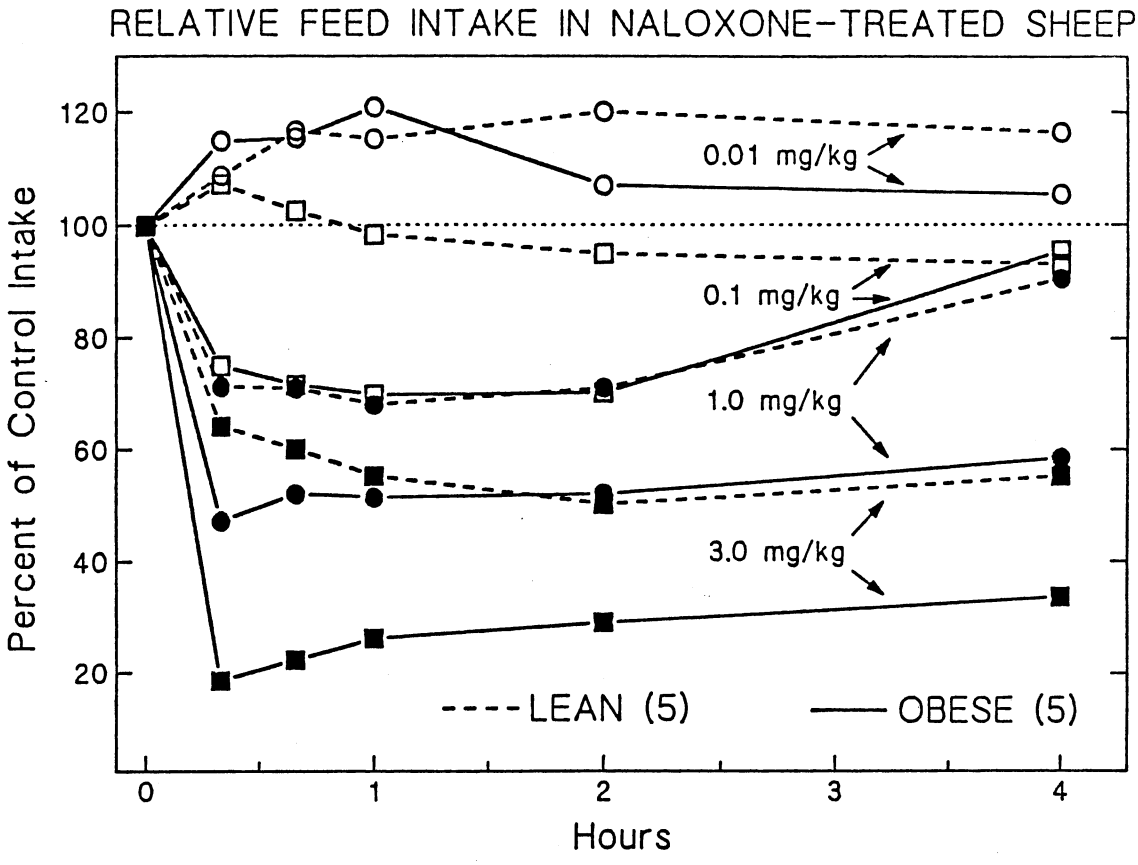

Figure 5. Cumulative feed intakes as percent of control intakes (mean ± SE) in lean and obese sheep pretreated with 0.01, 0.1, 1 or 3 mg/kg naloxone. Cumulative intakes during the first 4 h of ad libitum intake were expressed as percent of corresponding intake after 0 mg/kg naloxone (control) for each sheep and mean values calculated at each time point for the different doses of naloxone are shown. For clarity of presentation, SE bars are not shown but the pooled SE values were 12, 16, 13 and 11 in lean sheep and 16, 15, 12 and 12 in obese sheep treated with 0.01, 0.1, 1 or 3 mg/kg naloxone, respectively. Additional notes in legend to Fig. 2.

TABLE 1

MEAN (± SE) CUMULATIVE FEED INTAKE (g) IN LEAN (n=5) AND OBESE (n=5) SHEEP TREATED WITH NALOXONE AND FED *AD LIBITUM*

Naloxone	Body	Time, hr									
(mg/kg)	Condition	0.33	0.66	1	2	4	8	12	16	24	32
Saline	Lean	931 ± 66^{ab}	1054 ± 163^{a}	1156 ± 165^{a}	1343 ± 206^{a}	1468 ± 239^{ab}	1586 ± 240^{a}	1650 ± 222	1697 ± 220	1812 ± 200	2195 ± 205
	Obese	483 ± 109^{a}	527 ± 130^{a}	538 ± 127^{ab}	645 ± 143^{a}	748 ± 149^{a}	953 ± 200^{ab}	1182 ± 194	1357 ± 208	1427 ± 188	1831 ± 186
0.01	Lean	1000 ± 86^{a}	1191 ± 170^{a}	1297 ± 165^{a}	1530 ± 151^{a}	1611 ± 140^{a}	1665 ± 142^{a}	1730 ± 154	1749 ± 162	1781 ± 146	2172 ± 227
	Obese	513 ± 80^{a}	563 ± 90^{a}	613 ± 101^{a}	630 ± 102^{a}	727 ± 120^{a}	880 ± 270^{a}	1183 ± 224	1319 ± 245	1450 ± 285	1873 ± 397
0.1	Lean	981 ± 114^{a}	1010 ± 121^{ab}	1067 ± 115^{ab}	1156 ± 153^{ab}	1217 ± 138^{ab}	1376 ± 78^{a}	1467 ± 68	1520 ± 84	1578 ± 80	1806 ± 154
	Obese	382 ± 130^{ab}	398 ± 145^{ab}	398 ± 145^{abc}	509 ± 224^{a}	723 ± 207^{a}	1090 ± 273^{a}	1284 ± 280	1440 ± 295	1607 ± 263	1966 ± 256
1.0	Lean	625 ± 127^{ab}	654 ± 108^{bc}	707 ± 97^{bc}	859 ± 92^{bc}	1240 ± 136^{b}	1609 ± 214^{a}	1792 ± 262	1855 ± 306	2057 ± 318	2554 ± 367
	Obese	224 ± 55^{ab}	269 ± 76^{ab}	277 ± 83^{bc}	318 ± 101^{ac}	429 ± 138^{ab}	700 ± 168^{ab}	849 ± 144	1021 ± 154	1224 ± 175	1615 ± 198
3.0	Lean	587 ± 104^{b}	588 ± 103^{c}	600 ± 96^{c}	615 ± 90^{c}	736 ± 55^{c}	1113 ± 33^{a}	1337 ± 60	1580 ± 138	1842 ± 147	2389 ± 172
	Obese	69 ± 35^{b}	87 ± 43^{b}	112 ± 47^{c}	165 ± 52^{c}	227 ± 75^{b}	410 ± 125^{b}	601 ± 190	890 ± 257	1085 ± 279	1508 ± 376

[1] Sheep fasted 16 h were injected iv with saline or naloxone 5 min before they were allowed ad libitum feeding for 32 h. Means with the same superscript letters are not different ($P<0.05$) for comparison at each time interval within each level of body condition.

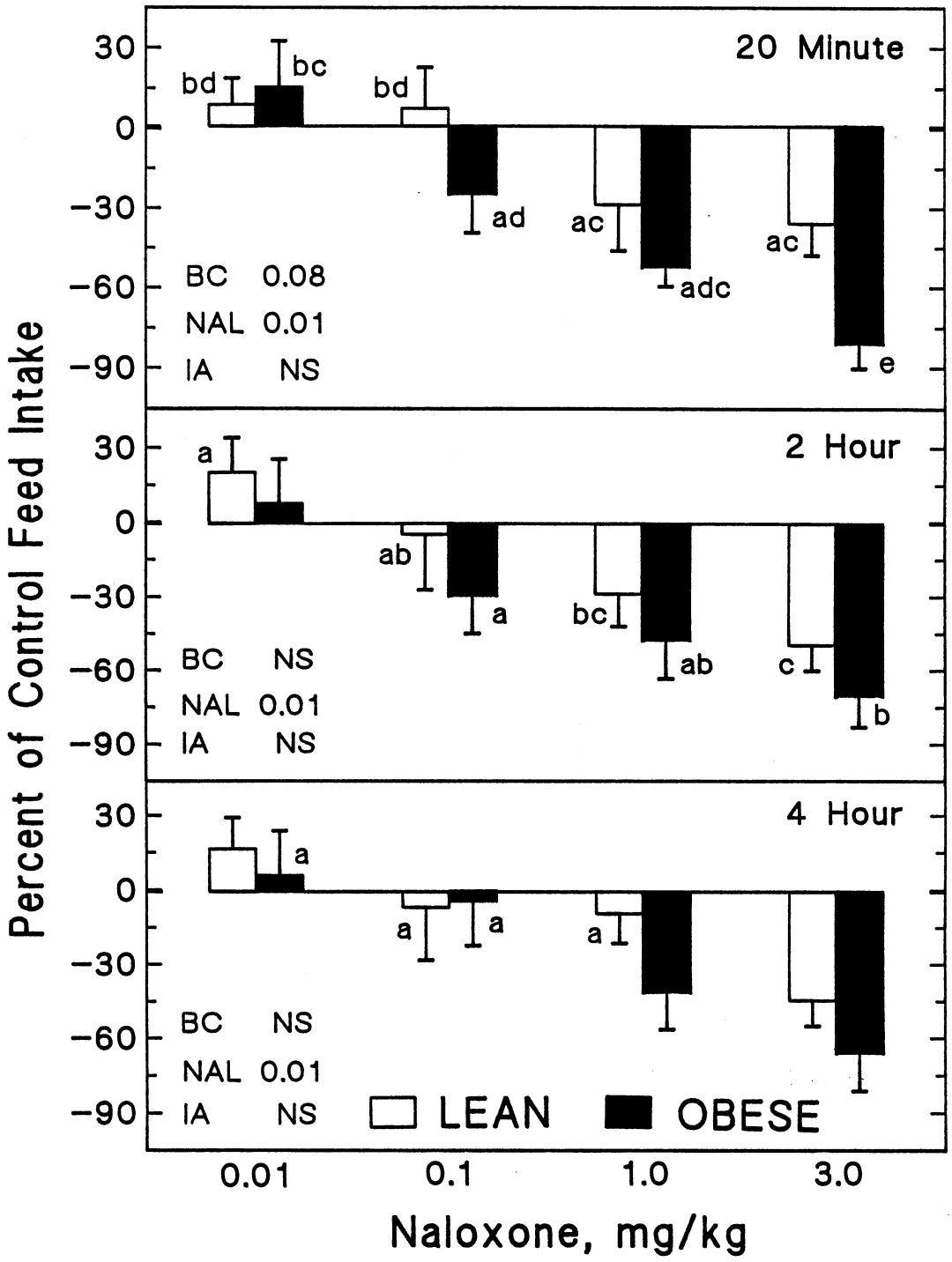

Figure 6. Feed intakes as percent of control intake at 20 min, 2 h and 4 h of ad libitum in lean and obese sheep injected with 0.01, 0.1, 1 and 3 mg/kg naloxone. Probability values shown indicate significance of treatment effects of body condition (BC), naloxone dose (NAL) and their interaction (IA) are derived from split-plot analysis of variance; means within each time period with similar superscript letter are not different ($P>0.05$); NS indicates no significant effect ($P>0.1$).

The dose-response curve for naloxone's effect on intake was shifted leftward in obese compared with lean sheep (Fig. 7). The significant ($P<0.05$) best fit regression line in lean sheep was Y = -25.04(log dose) + (-31.04) (r= 0.990) and that in obese sheep was Y = -33.87(log dose) + (-55.1) (r= 0.980). Regression analysis of variance showed that the linear regression fits were affected by body condition ($P<0.02$) and naloxone dose ($P<0.001$), but not by their interaction. Regression coefficients (slopes; b) of each equation were equivalent but the position of the lines differed ($P<0.02$) in lean and obese sheep. Dose of naloxone suppressing intake by 25% (D_{25}) was calculated from these equations and was significantly less in obese (0.129 mg/kg) than lean (0.574 mg/kg) sheep; endpoints for a 95 percent confidence interval for D_{25} dose of naloxone were 0.0654 and 0.257 mg/kg and 0.218 and 1.51 mg/kg in obese and lean sheep, respectively. Results show that obese sheep were about 4 times more sensitive to naloxone than lean sheep.

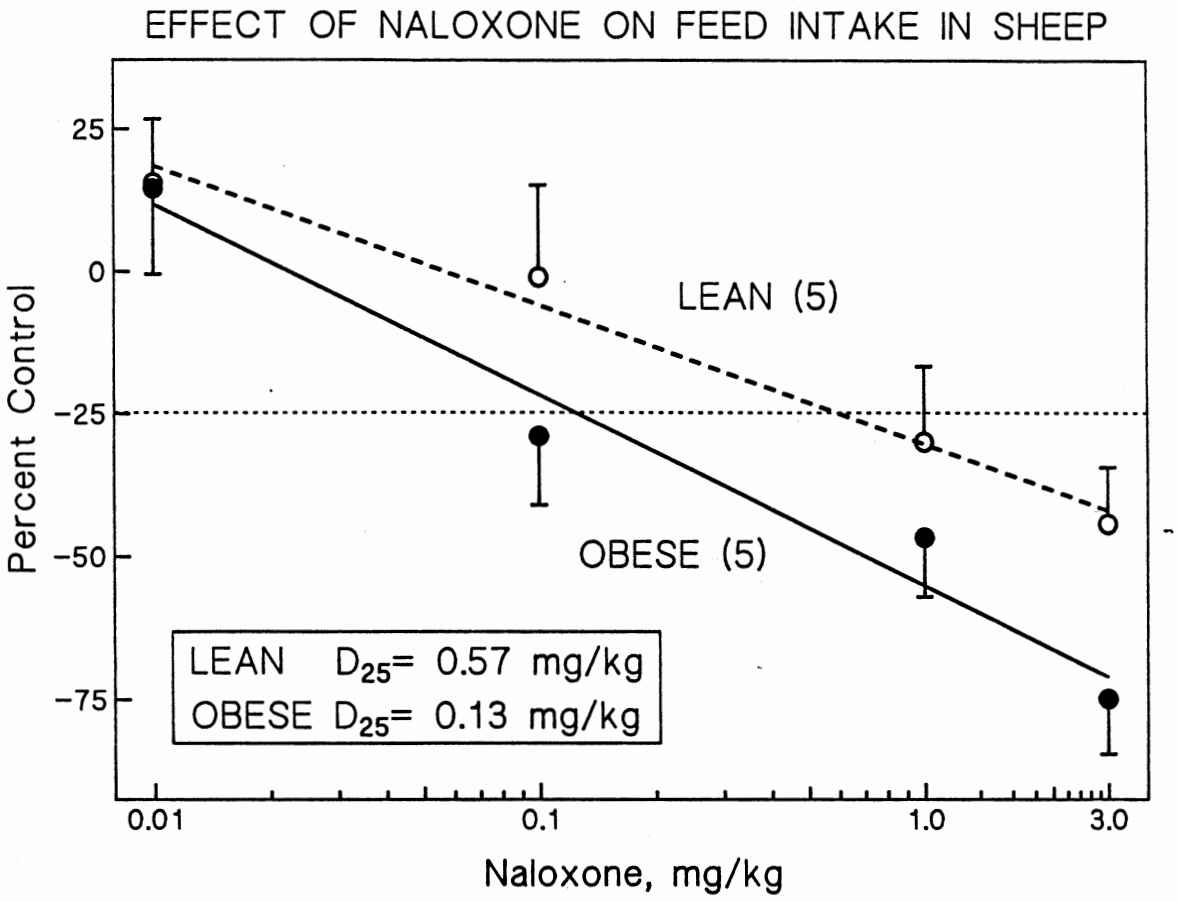

Figure 7. Dose-response effect of naloxone on acute feed intake in lean and obese sheep fed ad libitum. Area under the curve (AUC) for cumulative feed intake in the first 2 h of ad libitum intake was calculated in each sheep after each dose of naloxone. Within each body condition, the 2-h AUC after 0.01, 0.1, 1 and 3 mg/kg naloxone was expressed as percentage of 2-h AUC after 0 mg/kg (control) naloxone in each sheep and the mean (± SE) values shown were calculated for each dose. Regression equations for the mean data (lines shown) were Y= -33.88(log dose) + (-55.07) in lean and Y= -25.04(log dose) + (-31.0) in obese sheep. Dose of naloxone inhibiting intake by 25 percent (D_{25}) was calculated from these equations and was 0.13 mg/kg in obese sheep and 0.57 mg/kg in lean sheep as discussed in results. Additional notes in legend to Fig 1.

Plasma Chemicals

The quantitatively greater inhibitory effects of naloxone in obese than lean sheep were associated with similar plasma concentrations of naloxone in both groups after either 1 or 3 mg/kg naloxone (Fig. 7). Levels of naloxone in plasma after injection of 0.1 and 0.01 mg/kg naloxone were below the sensitivity (10 ng/ml) of the assay system.

Concentrations of ß-endorphin in plasma obtained from 16-h fasted sheep were similar ($P > 0.05$) in lean (33.3 ± 4.2 pg/ml) and obese (48.4 ± 8.6 pg/ml) sheep.

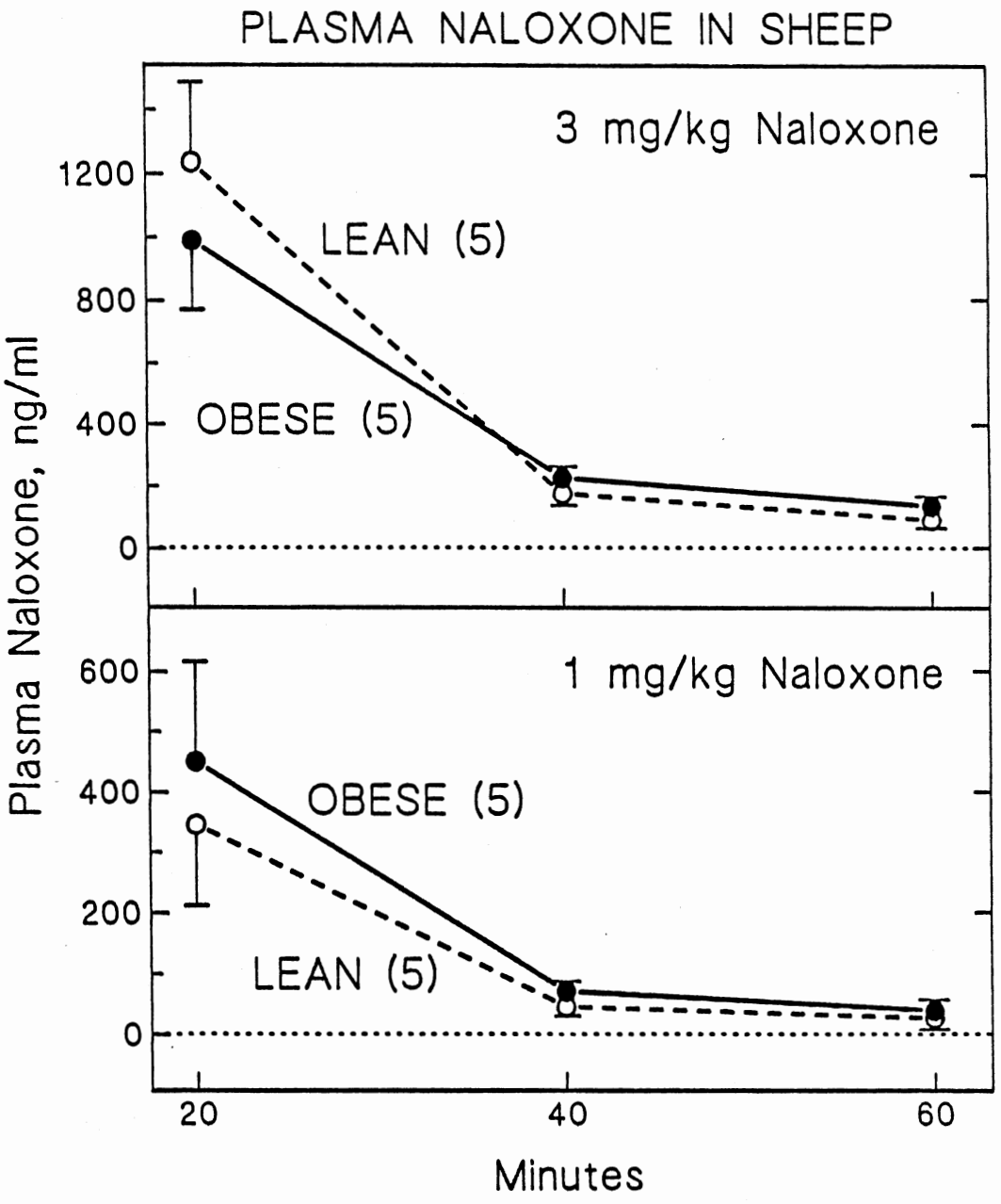

Figure 8. Plasma concentrations of naloxone (mean ± SE) in lean and obese sheep injected with 1 and 3 mg/kg naloxone iv. Concentrations of naloxone after iv injection of 0.01 and 0.1 mg/kg naloxone were below the sensitivity (10 ng/ml) of the assay.

Discussion

We have developed a nongenetic animal model of obesity in which the obesity is caused by excessive voluntary consumption of a normal diet fed at maintenance to lean control sheep. Our results show clearly that early consummatory behavior differs in lean and obese sheep. Hunger drive was greater in lean than obese sheep in the first 8 h of ad libitum feeding. The initial rapid consumption rate in lean sheep slowed to a nearly constant rate of consumption which was comparable with that in obese sheep. Cumulative intake consistently was less in obese than lean sheep despite an approximate doubling of body mass in obese compared with lean sheep.

The stronger appetite-inhibitory effect of naloxone in obese than lean sheep suggests that opiatergic regulation of appetite differs between lean and obese sheep in the static phase of obesity. Response to the appetite-inhibitory effect of naloxone also was greater in genetically obese rats (fa/fa) and mice (ob/ob) than in lean ones (Margules et al 1978). The D_{25} dose of naloxone was approximately three times lower in obese (ob/ob) mice (approximately 0.39 vs 1.12 mg/kg) and obese (fa/fa) rats (approximately 0.17 vs 0.57 mg/kg) than in lean controls when intakes were measured 1 to 2 h after feeding began in naloxone-treated animals (Margules et al 1978). Feed-inhibitory response to naloxone also was greater in genetically obese Zucker rats than in lean ones (McLaughlin and Baile 1984 a, b). Greater responsiveness to the feed-inhibitory effects of naloxone also has been reported in obese compared with lean humans (Atkinson 1987). In contrast, sensitivity to naloxone appears similar in control rats and rats with nongenetic forms of obesity due to ventromedial hypothalamic lesions, ovariectomy or drug administration (Gunion and Peters 1981). Reasons for the increased sensitivity to naloxone in dietary obese sheep, obese humans and genetically obese rodents are unknown but may involve down-regulation of central

opioid receptors, and(or) effects of hyperglycemia on opioid receptor activity (Morley et al 1983).

There is a good positive relationship between increased plasma ß-endorphin levels and overeating in normal and genetically obese rats (Margules et al 1978; Davis et al 1982). Plasma levels of ß-endorphin are higher in obese than lean humans, particularly when comparisons are restricted to female subjects (Ritter et al 1991). Margules et al (1978) reported higher plasma and pituitary, but unchanged hypothalamic, concentrations of ß-endorphin in obese mice (ob/ob) and Zucker rats (fa/fa) than in lean controls during the dynamic phase of obesity in these young animals aged 2 to 5 months. Others have reported markedly elevated ß-endorphin and met-enkephalin concentrations in the pituitary and hypothalamus of genetically obese (ob/ob) than lean mice aged 4 to 5 months (Khawaja et al 1989). Concentration of kappa and delta binding sites were greater and that of mu binding sites less in membranes isolated from brain of obese (ob/ob) than lean mice (Khawaja et al 1989). However, it remains controversial whether increased pituitary synthesis and release of opioids play an important role in the central control of appetite during development of the obese state (Morley et al 1982; Yim and Lowy 1984). Results of several studies support the claim that plasma ß-endorphin enters the CSF but not the interstitial fluid of brain (Banks and Kastin 1990). Pituitary-derived increases in the plasma concentrations of ß-endorphin do not always result in parallel increase in CSF levels of ß-endorphin in sheep, which are results suggesting that hypothalamic opioid receptors may respond only to centrally produced opioids in sheep (Smith et al 1986).

It is still not clear whether brain opioid peptides increase in hungry animals to initiate feeding, and thus would be involved with hunger drive, or whether opioid peptides increase after feeding commences so as to act as a positive reinforcement

and increase the "joy" of eating (Morley et al 1982; Kirkham and Blundell 1984; Carr and Simon 1984; Stein et al 1990). In our sheep model, obese sheep were continually exposed to feed during the dynamic phase of obesity and consequently were unlikely to experience significant increases in hunger drive despite a change in feed intake amounts as sheep progressed from the dynamic to static phase obesity. Nevertheless, increased opioid activity during dynamic phase obesity could have resulted in down-regulation of central opioid receptors, thus explaining the greater sensitivity to naloxone in obese than lean sheep during static phase obesity. Greater response to antagonist is an expected response in systems that have undergone receptor down-regulation.

Although plasma concentrations of immunoreactive ß-endorphin were similar in 16-h fasted lean and obese sheep in this study, it is possible that central opioid receptor activity and plasma ß-endorphin concentrations differed between lean and obese sheep during dynamic obesity and in the fed state during static phase obesity. Although there is no clear relationship between plasma levels of ß-endorphin and activity of central opioid control of appetite in animals (Morley et al 1982; Yim and Lowy 1984), plasma ß-endorphin levels routinely are used as a crude index of opioid biology in animals.

Plasma glucose concentration can affect opioid receptor activity and animal response to naloxone treatment. Levine et al (1982) reported that genetically obese diabetic mice (db/db) and streptozotocin-induced diabetic mice were 80 to 1000 times more sensitive, respectively, than control animals to naloxone's inhibitory effect on feed intake. On the other hand, streptozotocin-induced diabetes decreased the effectiveness of some opioid agonists in stimulating feed intake in rats (Gosnell et al 1989) and reduced the contractile response of guinea-pig ileum to stimulation by the opioid agonist, morphine (Shook et al 1986). Further, glucose added to incubation media

reportedly increased the amount and affinity of binding of naloxone to membranes isolated from rat brain (Morley et al 1981). Morley et al (1983) postulated that increasing concentrations of glucose alter conformation and binding properties of an opioid receptor such that this receptor in the presence of glucose preferentially binds naloxone with high affinity while failing to bind opioid agonists.

Because obese mice and rats are hyperglycemic, the greater effectiveness of naloxone in obese than lean rodents may be related to the increase in plasma glucose levels. Dietary obese sheep are hyperglycemic and hyperinsulinemic and the approximate increases in plasma glucose of 5 to 10 mg/dl and plasma insulin of approximately 10 to 30 μU/ml in obese sheep are very similar to the increases in plasma insulin and glucose observed in obese compared with lean humans. The increase in plasma glucose in genetically obese compared with lean rodents usually exceeds 10 mg/dl. It is possible that modest hyperglycemia in dietary obese sheep and obese humans sufficiently affects opioid receptor activity, as postulated by Morley et al (1983), resulting in greater inhibitory effects of naloxone in obese than lean subjects.

An interesting finding in this study was the response of four lean and two obese sheep to the lowest dose of naloxone (0.01 mg/kg) which stimulated their feed intake by approximately 37%. Relative to saline-treated controls, low doses of naloxone of 0.06 mg/kg (Lowy et al 1980) and 0.01 to 0.1 mg/kg (Brown and Holtzman 1979) increased food intake in rats by 20 to 35%. The ability of low dose naloxone to increase intake in rats may depend on duration of fasting before treatment and whether naloxone is administered during the light or dark cycle (Brown and Holtzman 1979). One possible explanation for stimulation of intake by low dose naloxone could be that of different affinities of naloxone for the opioid receptors involved in appetite regulation. Naloxone at very low concentration may bind preferentially to high affinity and few

opioid receptors that stimulate appetite, whereas higher concentration of naloxone bind to many opioid receptors coupled to appetite inhibitory pathways. In a recent study, Kelly et al (1990) demonstrated that autoregulation of ß-endorphin release by hypothalamic arcuate neurons may be controlled by presynaptic opioid receptors, a mechanism similar to the autoregulation of adrenergic neurons by α_2-adrenoceptors. In support of the finding of Kelly et al (1991), Rogers and Henderson (1990) previously had reported that activation of presynaptic mu and delta receptors by opioid agonists resulted in reduction of transmitter release from the same preganglionic nerve terminals. Possibly, naloxone at low concentration could block presynaptic opioid receptors leading to increased release of ß-endorphin and stimulation of feed intake, whereas high concentrations of naloxone could block both presynaptic and postsynaptic opioid receptors thereby resulting in naloxone suppression of feed intake.

In conclusion, early feeding behavior differs substantially in lean and dietary obese sheep. Sensitivity to the appetite-inhibitory effects of naloxone was at least four times greater in dietary obese than lean sheep, and this change in sensitivity was associated with comparable levels of ß-endorphin and naloxone in the plasma of lean and obese sheep.

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CHAPTER V

CARDIOVASCULAR RESPONSES TO ADRENERGIC AGONISTS IN LEAN NORMOTENSIVE AND OBESE HYPERTENSIVE SHEEP

Introduction

Hypertension or high blood pressure is a serious public health problem in western societies. Risk factors commonly associated with the development of hypertension are obesity, glucose intolerance, and dyslipidemia (Van Itallie 1986; Cambien et al 1987; Ferrari and Weidmann 1990; Reaven 1990; Crandall and DiGirolamo 1990). Origin and nature of irregularities in glucose and lipid metabolism associated with hypertension are still unknown (Ferrannini and Natali 1991; Ferrari and Weidmann 1990). Obesity-associated hypertension is very prevalent in the United States where obesity exists in 44% of the hypertensive population as compared with an incidence of obesity of only 16% in the normotensive population. Conversely, 53% of obese subjects are hypertensive as compared with an incidence of hypertension of 22% in nonobese subjects (Hoarn and Lenfant 1990). There seems to be a fairly clear association between sedentary lifestyle, overweight and high blood pressure (Stamler et al 1978; Van Itallie 1985).

Obesity frequently coexists with hyperinsulinemia, hyperglycemia and hypertension in humans and other animal models (Cambien et al 1987; Krieger and Landsberg 1988). Defects in glucose and insulin metabolism may play a role in both

the etiology and clinical course of hypertension because abnormalities of glucose and insulin metabolism occur more frequently in hypertensive patients than in normotensive ones (Krieger and Landsberg 1988; Haffer et al 1989; Falkner et al 1990). Patients with high blood pressure have been shown to be relatively glucose intolerant (Ferrannini et al 1987). In addition, spontaneously hypertensive rats tend to be hyperinsulinemic and insulin resistant when compared to normotensive Wistar-Kyoto rats (Hulman 1991).

Elevated plasma levels of very low-density lipoproteins (VLDL) and decreased levels of high-density lipoproteins (HDL-cholesterol) are common abnormalities of lipoprotein metabolism in hypertensive humans (Williams et al 1990; Frohlich and Pritchard 1989; Bjorntorp 1990; Despres et al 1990). The plasma HDL-cholesterol concentration is inversely related to the plasma insulin level in obese subjects (McKeigue et al 1991; Ronnemaa et al 1991). Hyperinsulinemia together with decreased plasma levels of HDL-cholesterol are frequent findings in obese hypertensive compared with obese normotensive subjects (Cambien et al 1987; McKeigue et al 1991).

The other risk factor associated with hypertension is excess sodium ingestion (Beard 1990). It appears that approximately one fourth of normotensive subjects and one half of hypertensive patients are characterized as sodium sensitive in that excess dietary sodium increases their blood pressure. The relationship between excess dietary sodium and blood pressure is complicated. It seems that obesity associated-hyperinsulinemia may significantly interact with sodium metabolism in the development of hypertension in humans (Rocchini 1990; Horan and Lenfant 1990; Folkow 1990).

Blood pressure is a function of cardiac output (CO) and total peripheral resistance (TPR). Established human hypertension is associated with increased TPR

and normal rates of CO (Folkow et al 1958; Lund-Johansen 1983). Several mechanisms may explain the increased TPR. Increased vascular wall diameter in hypertensive humans correlated positively with increased vascular response to adrenergic agonist stimulation (Aalkjaer et al 1987). Sympathetic outflow from the central nervous system (CNS) modulates activity of the resistance vessels and thus systemic blood pressure. Hyperinsulinemia in obesity may affect sympathetic nervous system (SNS) activity and its regulation of resistance vessels, and thus hyper-insulinemia could increase TPR (Krieger et al 1988).

Attempts have been made to develop animal models to further understanding of the mechanisms responsible for hypertension in obesity. Spontaneously hypertensive rats (SHR) have been used extensively in hypertension research, but unfortunately this genetically developed model is not obese and lacks other risk factors associated with essential hypertension in humans (Wexler 1981). Although genetically obese rats are hyperinsulinemic, development of hypertension in these animals has not been a consistent finding. Kurtz et al (1989) reported that obese Zucker rats become hypertensive, whereas others found no significant difference in blood pressure between lean and obese Zucker rats (Levin et al 1984; Contreras and Williams 1989).

Dietary obese hypertensive models were first demonstrated by Wood and Cash (1939). Recently, Rocchini and associates (1987) produced hypertension in dogs fed a high fat diet as compared with control dogs fed a different diet devoid of high fat. Differences in diet, levels of energy balance, and the probability that high-fat feeding incites renal damage (Grone et al 1989; Kasiske et al 1991) confounds any conclusions that hypertension in dogs fed a high diet is strictly obesity associated. Furthermore, high-fat feeding prevented development of hypertension in spontaneously hypertensive rats (Wexler 1981). Feeding rats a high fructose diet results in

hyperinsulinemia and hypertension (Hwang et al 1987). Such results also are confounded by differences in diet, energy balance and unknown effects due to abnormally high intakes of fructose.

We have developed a dietary obese sheep model that is hyperinsulinemic, hyperglycemic, and insulin resistant (McCann et al 1991). This animal model also portrays most of the above mentioned risk factors associated with essential hypertension in humans. Our objectives were therefore to determine if dietary obese sheep are hypertensive, whether obesity affects cardiovascular responses to the adrenergic agonists norepinephrine and phenylephrine, and whether sympathetic activity, indirectly assessed by arterial plasma catecholamines, differs in lean and obese sheep.

Materials and Methods

Animals

Lean and obese Rambouillet ewes (n=5/group) aged 3-4 years were housed individually in a room with constant light and temperature (21 ± 1 C). Sheep were fed a pelleted hay-grain mixture (12.5 g/kg) and hay (1g/kg) to provide approximately 38 Kcal DE/kg, an amount sufficient to maintain sheep in zero-energy balance. Nutrient composition of the diet and detailed results on events during the induction and maintenance phase of dietary obesity are reported elsewhere (Chapter 2). Briefly, lean adult sheep were fed a maintenance intake of pelleted hay-grain diet to keep them in lean body condition, or were fed the same diet ad libitum to induce obesity. Sheep were in static phase obesity after 50 wk of ad libitum intake at which time body weight, feed intakes and body composition were in relatively steady state. At wk 70 of ad libitum feeding, obese sheep were a maintenance intake of pellets in two equal

amounts at 0900 and 1700 h, with small amounts of hay (1 g/kg) fed at 0900 h.

Animal Preparation and Experimental Protocol

Surgery was conducted with aseptic technique for survival surgery. Anesthesia was induced with pentobarbital sodium (Nembutal®, Abbott, IL; 50 mg/kg) and maintained with mixture of 1-2% halothane (Fluthane®, Ayerst Lab, NY) and oxygen. The gas mixture was delivered to the sheep through an endotracheal tube using a surgical anesthesia machine (Ohio Chemical & Surgical Equipment, Madison, WI).

Catheters with diameters of 12.7 mm (ID) were implanted in the caudal aorta and the caudal vena cava through the femoral artery and vein, respectively. The distal end of the catheters were exteriorized from the right hind leg and brought to the mid back of the animal. The catheters were connected to Tuohy-Borst adapters (Perfektum, New Hyde Park, NY, USA) and wrapped in surgical adhesive tape which was glued to the animal. Sheep were treated with penicillin (300,000 U/kg) daily for 5 days after surgery. Catheters were flushed thrice weekly with sterile heparinized saline (10 U/ml) and filled with 1.5 ml of heparin (1000 U/ml). At least 2 wk elapsed between surgery and experiments in any sheep. Inspection at necropsy confirmed that both catheter tips were positioned about 3 to 4 cm cranial to the ileac bifurcations.

Basal Blood Pressure Measurements. One lean and one obese sheep constituted a replicate and replicates were assigned randomly by latin square design to sequence of norepinephrine (0.25, 0.5, 1.0, 2.0, and 4.0 μg/kg) and phenylephrine (1, 3, 7, 10 and 30 μg/kg) dose. The doses of norepinephrine (NE; Sigma, St. Louis, MO) and phenylephrine (PE; Sigma, St. Louis, MO) were paired by dosage and coded for assignment as experimental days within a replicate. All experiments were done between 0900 and 1200 h in 16-h fasted sheep with at least 4 d between PE and NE

experiment.

NE and PE were given as a bolus injection via the vena cava catheter to conscious standing sheep. Order of administration of the paired doses of NE and PE in each experiment day was alternated across replicates. Cardiovascular variables returned to basal values for at least 20 min before the second adrenergic agonist was given on any experimental day. The venous catheter was flushed immediately with 2 ml saline after each agonist injection. Doses of NE and PE used were established in preliminary experiments with lean and obese sheep.

Blood pressure transducers and physiograph were calibrated using a water column with 1.36 cm H_2O height equal to 1 mmHg (West 1985); the physiograph pen deflection set at 5 cm for 100 mmHg. Relationship between water pressure developed and physiograph pressure measured was determined by least square linear regression for each transducer assigned for use in lean or obese sheep. The regression equation in lean sheep was $Y= 0.8305(X) + 0.471$ ($r=0.999$) and that in obese sheep was $Y=0.8342(X) + 0.2449$ ($r=0.999$), where Y equals physiograph (transducer) pressure and X equals absolute water pressure converted to mmHg. All blood pressure readings consequently were divided by the correction factor of 0.831 (lean sheep) or 0.834 (obese sheep) to obtain absolute pressure. The aortic catheter was connected to a calibrated pressure transducer (Narco Bio-System, International Biomedics, Houston USA) which was located at the level of the heart. Changes in blood pressure were recorded continuously by 4-channel physiograph (Narcotrace Model 40 MKIV, Narco Bio-Systems). Heart rates were determined simultaneously using a Narco biotachometer model 7302 (Narco Bio-Systems) coupled to the pressure transducer. On each experimental day, recordings of blood pressure and heart rate were made for 45 min before agonist injection. Three observations on blood pressure and heart rate

were done during this stabilization period at approximately 15 min intervals for each sheep. These basal recordings were done once a wk for 5 wk to provide a mean basal pressure and heart rate measurement in each sheep which was comprised of 5 separate observations of three values each. Mean arterial pressure (MAP) was calculated 2/3 diastolic plus 1/3 of systolic pressure (West 1985)

Arterial Plasma Catecholamines. Arterial blood (5 ml) was collected into heparinized syringes during the stabilization period on three separate experimental days. Blood was immediately transferred to a prechilled (4 C) glass tube containing 100 µl of mixture of EGTA (ethyleneglycol-bis-[ß-aminoethylether] N,N,N',N'-tetraacetic acid; 90 mg/ml) and GSH (reduced glutathione; 75 mg/ml). The chilled blood was centrifuged at 1000 x g for 10 min at 4 C and the recovered plasma stored at -20 C for later HPLC analysis. NE and epinephrine (EPI) were determined by HPLC and electrochemical detection system comprised of Waters Model 510 pump, C18 reverse phase column (3.9 X 150 mm) with 5µ particles, and Model 460 electrochemical detector (Waters, Division of Millipore, Milford, MA, USA). The concentrations of NE and EPI were determined by the method described by Water's catecholamine analysis kit (Waters, Milford, MA USA). Briefly, to 1.5 ml of plasma, 50 µl of internal standard (3,4-dihydroxybenzylamine; 10 pg/µl) and 10 mg of aluminum oxide were added. The mixture was alkalinized with 400 µl of 2 M Tris buffer (pH 8.7), and shaken for 15 min. The solution was centrifuged for 1 min at 1000 x g. The supernatant was eluted and the precipitated alumina washed and the supernatent discarded 3 successive times with 1 ml of 0.0165 M Tris buffer (pH 8.1). The alumina-bound catecholamines were desorbed from the alumina by 100 µl of a mixture (10 ml) containing 100 µl of glacial acetic acid, 50 µl sodium disulfite (10%, w/v), 50 µl EDTA (5%, w/v) and 9.8 ml of distilled water. The suspension was centrifuged and 50 µl of the supernatant containing the catecholamines was gently separated from the precipitated alumina and injected

for analysis. The mobile phase contained 5% methanol, 0.1 M anhydrous sodium acetate, 0.1 M citric acid anhydrous, 0.5 mM 1-octane sulfonic acid and 0.15 mM EDTA. The flow rate was 1.0 ml/min. The voltage of the glassy carbon electrode was maintained at +0.60 mV against the Ag/AgCl reference electrode throughout the experiment.

Plasma Hormone and Metabolites. Arterial blood (5 ml) was collected into heparinized syringes during the 45 min stabilization period in wk 1 and 5 of the experiments. Blood was immediately dispensed into glass tubes containing 50 µl solution of heparin (5,000 U/ml) and benzamidine (200 mg/ml) that were held in an ice-water bath (4 C). Plasma was recovered by centrifugation (1000 x g, 15 min, 4 C) and stored frozen (-22 C). Plasma concentrations of insulin and glucagon were quantified by validated radioimmuno-assay (McCann et al 1991; Blackett et al 1991), and those of glucose, free fatty acid (FFA), total cholesterol, HDL-cholesterol and total triglyceride were quantified by enzymatic assay (McCann et al 1991). A single serum sample was obtained from each sheep at wk 5 of the experiment and used to quantify serum electrolyte concentrations of Na^+, K^+, Cl^-, Ca^{2+}, Mg^{2+} and PO_4^-, and serum clinical chemistry levels of creatine, creatine phosphate kinase (CPK), albumin, alkaline phosphatase (ALP), lactate dehydrogenase (LDH), gamma glutamyl transferase (SGGT), and blood urea nitrogen (BUN); analyses were done by automated enzymatic procedures (Cobas Mira, Roach Diagnostic System , Nutley, NJ, USA) in the Department of Clinical Pathology, College of Veterinary Medicine , Oklahoma State University.

Statistical Analysis

Differences in plasma and serum variables, basal blood pressures and heart

rates between lean and obese sheep were evaluated by Students unpaired two-tailed t test (Steel and Torrie 1980). Main and interactive effects of body condition and agonist dose on maximum increments in MAP and maximum decrement in heart rate observed after each agonist dose were determined using 2 x 5 split-plot analysis of variance; multiple comparisons among means were done using Duncan's new multiple range test if a significant f-value ($P<0.1$) was found (Steel and Torrie 1980). Dose-response curves of blood pressure response to adrenergic agonist were constructed as follows. The maximum increment in MAP after each dose of agonist was determined for all sheep. Best fit of the relationship between blood pressure increase and agonist dose was determined using noniterative least squares polynomial regression analysis (*Fig P*; Biosoft, Ferguson, MO) of observations (n=25) in each body condition group. Comparison of residual sum-of-squares and coefficient of determinations determined that a first polynomial adequately described the data as compared with higher degree polynomial, exponential, logarithmic, or power fits. Best fit of group data dictated fit used in individual sheep to determine dose of agonist increasing MAP by 25 (D_{25}) or 50 (D_{50}) mmHg; differences in mean agonist dose for D_{25} and D_{50} between lean and obese sheep were evaluated by Student's two-tailed t test (Steel and Torrie 1980).

Results

Plasma Chemical Analyses

Compared with lean sheep, obese sheep had higher ($P< 0.05$) plasma concentrations of insulin, glucagon and glucose but lower ($P< 0.05$) concentrations of HDL-cholesterol (Table 1). Plasma levels of total cholesterol and triglyceride were unaffected by obesity, but levels of total cholesterol tended to be higher ($P<0.1$) in lean than obese sheep.

TABLE 1

PLASMA CONCENTRATIONS OF SELECTED HORMONES AND METABOLITES IN LEAN AND OBESE SHEEP FASTED 16 h

Item	Lean (5)	Obese (5)
Insulin (μU/ml)	7 ± 1	19 ± 2*
Glucose (mg/dl)	47 ± 1	53 ± 2*
Glucagon (pg/ml)	68 ± 7	113 ± 8*
HDL Cholesterol (mg/dl)	52 ± 4	31 ± 3*
Total Cholesterol (mg/dl)	74 ± 4	60 ± 5
Triglycerides (mg/dl)	13 ± 1	15 ± 3

* Lean and obese differ ($P < 0.05$).

Serum ion concentrations were not remarkable and equivalent concentrations were measured in serum of lean and obese sheep (Table 2). The serum magnesium concentration, however, apparently was increased ($P < 0.05$) in obese compared with lean sheep. Serum levels of clinically relevant chemicals were similar in lean and obese sheep (Table 3).

Arterial plasma catecholamine levels were unaffected by obesity in sheep (Table 4). Large variation was observed in the data even though the average of three observations was used to calculate the final value in each sheep.

TABLE 2

SERUM ION CONCENTRATIONS IN LEAN AND OBESE SHEEP FASTED 16 h

Item	Lean (5)	Obese (5)
Sodium (meq/l)	139.8 ± 0.5	140.8 ± 0.9
Potassium (meq/l)	4.5 ± 0.1	4.4 ± 0.2
Chloride (meq/l)	105.0 ± 5.3	105.6 ± 4.3
Calcium (mg/dl)	8.1 ± 0.4	8.5 ± 0.4
Magnesium (mg/dl)	2.0 ± 0.04	2.5 ± 0.15*
Phosphorus (mg/dl)	7.1 ± 0.8	7.3 ± 0.6

* Lean and obese differ ($P < 0.05$)

TABLE 3

SERUM CLINICAL CHEMISTRY IN LEAN AND OBESE SHEEP FASTED 16 h

Item	Lean (5)	Obese (5)
BUN (mg/dl)	15.8 ± 1.5	14.8 ± 1.7
Creatine (mg/dl)	0.9 ± 0.1	1.0 ± 0.1
Albumin (mg/dl)	2.7 ± 0.2	3.0 ± 0.1
Total Protein (mg/dl)	6.7 ± 0.2	7.2 ± 0.3
Alkaline Phosphatase (U/L)	99.2 ± 14.5	64.2 ± 15.4
LDH (U/L)	198.0 ± 19.4	203.6 ± 27.7
CPK (U/L)	48.0 ± 12.7	38.8 ± 8.2
SGGT (U/L)	68.4 ± 5.1	72.4 ± 2.5

TABLE 4

ARTERIAL PLASMA CONCENTRATION OF NOREPINEPHRINE AND EPINEPHRINE IN LEAN AND OBESE SHEEP FASTED 16 h

Item	Lean (5)	Obese (5)
Norepinephrine (pg/ml)	167 ± 34	106 ± 27
Epinephrine (pg/ml)	110 ± 27	48 ± 7

Basal Blood Pressure and Heart Rate

Systolic, diastolic and mean arterial blood pressure were approximately 25% greater ($P < 0.01$) in obese than lean sheep (Table 5). The obesity-associated hypertension coexisted with a marked 50 percent increase in the heart rate of obese compared with lean sheep. Blood pressure variability in obese sheep appeared similar to that in lean sheep because the mean (± SE) coefficient of variation in blood pressure was similar in lean (7.0 ± 0.3%) and obese (6.4 ± 0.2%) sheep.

TABLE 5

BASAL CARDIOVASCULAR VARIABLES IN LEAN AND OBESE SHEEP FASTED 16 h

Item	LEAN (5)	OBESE (5)
Systolic Blood Pressure, mmHg	96.6 ± 2.9	121.6 ± 3.0*
Diastolic Blood Pressure, mmHg	65.2 ± 2.2	81.1 ± 2.7*
Mean Arterial Blood Pressure, mmHg	75.7 ± 2.2	94.6 ± 2.8*
Heart Rate, beats/min	49.6 ± 1.4	74.8 ± 5.6*

* Lean and obese differ ($P < 0.01$).

Cardiovascular Responses to Adrenergic Agonists

Overall mean arterial pressure before NE or PE injections was similar ($P > 0.05$) in lean sheep (77.0 ± 2.3 vs 78.0 ± 2.8 mmHg) as also was the case in obese sheep (95.8 ± 3.2 vs 95.7 ± 2.2 mmHg).

NE and PE increased ($P<0.01$) MAP in a dose-dependent manner in lean and obese sheep (Fig. 1). Body condition did not affect peak blood pressure response to NE or PE dose, although mean response in obese sheep always exceeded that in lean sheep. Peak blood pressure response and nadir heart rates after each dose of agonist are summerized in Table 6.

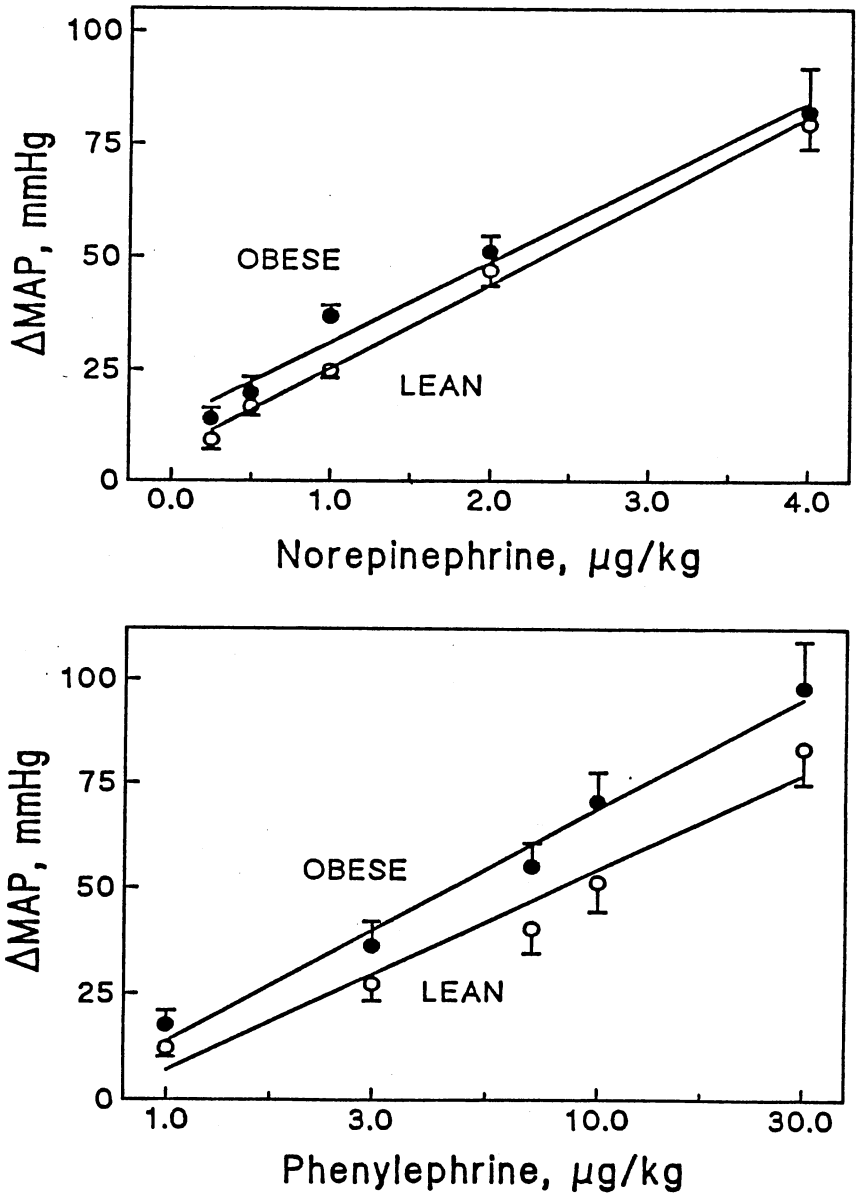

Figure 1. Mean arterial blood pressure (MAP) response to dose of norepinephrine (upper panel) or phenylephrine (lower panel). Values are mean ± SE. The pressure increase above basal (Δ MAP) is plotted as a function of each agonist dose. Best fit of the relationship between mean increment in MAP and dose of agonist was determined by noniterative least squares regression (see Methods). First degree polynomial was best ($P<0.01$) fit of mean data shown here and all observations ($n=25$) in each group (see results and Table 7). Equations describing the relationship shown for NE were $Y= 18.5(X) + 6.8$ ($r= 0.96$) in lean sheep and $Y= 17.7(X) + 13.4$ ($r= 0.89$) in obese sheep; equations for PE were $Y=46.2(\log X) + 7.1$ ($r= 0.84$) in lean sheep and $Y=54.3(\log X) + 14.0$ ($r= 0.88$) in obese sheep. Neither regression coefficients (b; slope) nor intercepts for each agonist differed ($P>0.05$) between lean and obese sheep as determined by regression analysis of variance and Student's t test.

TABLE 6

ABSOLUTE SYSTOLIC (SBP), DIASTOLIC (DBP) AND MEAN (MAP) ARTERIAL PRESSURE AND HEART RATE (HR) RESPONSE TO INTRAVENOUS NOREPINEPHRINE (NE) AND PHENYLEPHRINE (PE) IN LEAN AND OBESE SHEEP[1]

	SBP (mmHg)		DBP (mmHg)		MAP (mmHg)		HR (beats/min)	
NE (μg/kg)	Lean	Obese	Lean	Obese	Lean	Obese	Lean	Obese
Control	99.2 ± 3.5	122.9 ± 4.1	65.7 ± 2.4	82.3 ± 3.4	77.0 ± 2.3	95.8 ± 3.2	51.7 ± 2.9	77.2 ± 6.0
0.25	109.3 ± 3.0	143.3 ± 2.8	73.8 ± 4.0	93.7 ± 1.7	71.0 ± 3.1	110.2 ± 2.0	41.6 ± 2.4	56.8 ± 3.6
0.5	118.5 ± 3.2	148.1 ± 5.9	81.4 ± 4.0	100.0 ± 2.8	79.2 ± 3.2	116.0 ± 3.7	38.6 ± 2.7	59.8 ± 1.9
1.0	129.3 ± 3.7	175.5 ± 5.3	87.7 ± 4.3	112.2 ± 3.0	86.9 ± 3.5	133.3 ± 3.7	39.6 ± 3.2	55.8 ± 3.0
2.0	158.4 ± 8.2	192.3 ± 8.1	106.9 ± 4.8	123.1 ± 4.6	109.5 ± 5.3	146.2 ± 5.7	37.2 ± 3.4	49.2 ± 1.2
4.0	199.2 ± 9.2	234.6 ± 11	135.8 ± 6.2	149.5 ± 10	142.3 ± 6.2	177.9 ± 10	36.8 ± 3.5	48.0 ± 2.3
PE (μg/kg)								
Control	100.1 ± 3.8	123.2 ± 2.6	66.9 ± 2.6	81.9 ± 2.1	78.0 ± 2.8	95.7 ± 2.2	46.9 ± 1.5	70.8 ± 5.1
1.0	114.1 ± 4.8	144.7 ± 4.6	77.6 ± 4.3	96.6 ± 3.0	89.8 ± 4.9	112.6 ± 3.9	40.4 ± 1.7	58.0 ± 4.0
3.0	129.1 ± 6.1	170.7 ± 6.6	91.5 ± 5.5	113.9 ± 6.4	104.0 ± 6.0	132.8 ± 7.1	33.6 ± 1.5	47.2 ± 3.5
7.0	152.6 ± 10	194.7 ± 6.2	101.6 ± 7.1	129.8 ± 6.1	118.6 ± 8.9	151.4 ± 6.7	34.0 ± 2.3	46.4 ± 5.5
10.0	166.5 ± 12	211.5 ± 6.2	111.7 ± 7.1	143.3 ± 7.1	130.0 ± 9.5	166.0 ± 7.4	30.8 ± 0.7	40.8 ± 4.5
30.0	194.9 ± 15	246.5 ± 7.4	140.1 ± 10	167.1 ± 14	158.4 ± 13	193.6 ± 12	27.8 ± 1.8	37.3 ± 5.8

[1] Peak blood pressure and nadir heart rates were measured in each sheep 0 to 3 min after each agonist injection and mean (±SE) values shown were calculated.

Noniterative least squares regression indicated that a first degree polynomial was the best fit of relationship between peak increment in MAP and dose of NE and log dose of PE in lean and obese sheep. Regression equations for observations in lean (n=25) sheep after NE and PE were Y= 18.5(X) + 6.8 (r= 0.96) and Y= 46.2(log X) + 7.1 (r= 0.84), and those for observations (n=25) in obese sheep were Y= 17.7(X) + 13.4 (r= 0.89) and Y= 54.3(log X) + 14.0 (r= 0.88), respectively. Equations derived from linear regression of data in individual sheep were used to estimate dose of NE and PE that increased their MAP by 25 (D_{25}) or 50 (D_{50}) mmHg. The mean D_{25} and D_{50} doses of NE and PE were similar (P>0.05) in lean and obese sheep, although D_{25} for NE was lower (P<0.08) in obese than lean sheep (Table 7). The D_{25} dose of NE and PE on average was 34% and 42% lower, respectively, in obese than lean sheep.

TABLE 7

EFFECTIVE ADRENERGIC AGONIST DOSE IN LEAN AND OBESE SHEEP

		Dose, µg/kg		Probability Values		
Agonist Type	BC	D_{25}	D_{50}	BC	Dose	IA
Norepinephrine	Lean	0.97 ± 0.11	2.37 ± 0.15	0.1	0.05	NS
	Obese	0.64 ± 0.11	2.47 ± 0.56			
Phenylephrine	Lean	2.95 ± 0.6	4.84 ± 5.6	0.1	0.05	NS
	Obese	1.71 ± 0.281	5.49 ± 1.25			

Agonist dose for D_{25} and D_{50} were determined in each sheep using equations derived from linear regression analysis (see text) and mean (± SE) values shown were calculated for lean (n=5) and obese (n=5) sheep. Probability values for significance of treatment effects from split plot analysis of variance; treatments were body condition (BC), dose of agonist (Dose) and their interaction (IA). Means within each agonist type with similar superscript letter are not different (P>0.05) as determined by Duncan's new multiple-range test.

Regardless of dose, heart rate (HR) was decreased ($P<0.01$) approximately 27% in lean sheep treated with NE (Fig. 2). In contrast, heart rate in obese sheep was decreased ($P<0.01$) approximately 25% by 0.25, 0.5 and 1 mg/kg NE and approximately 37% by 2 and 4 mg/kg NE. Overall decrease in heart rate was affected ($P<0.05$) by body condition with greater decrement in obese than lean sheep.

Body condition markedly affected ($P<0.01$) the heart rate response to PE (Fig. 2). The decrease in heart rate (beats/min) was greater ($P<0.05$) in obese than lean sheep after 1 (6.5 vs 12.8), 3 (13.3 vs 23.6), 7 (13.7 vs 24.4), 10 (16.1 vs 30) and 30 (19.1 vs 33.5) μg/kg PE. Duration of blood pressure response increased with increasing dose of NE or PE equally in lean and obese sheep (Fig. 3). Equivalent duration of response suggests similar blood levels of the injected NE and PE in lean and obese sheep.

Baroreceptor reflex function was assessed by plotting absolute HR as function of absolute MAP after NE or PE treatment (Fig. 4). Although the regulation of baroreceptor control as function of increasing blood pressure appears similar in both groups of sheep, it is clear that for any given blood pressure the heart rate was greater in obese than lean sheep. The shift in the position of the curve indicates that the set point about which this regulation operates is different in lean and obese sheep. That regulation of the baroreceptor reflex was similar in lean and obese sheep was attested to by calculating similar baroreceptor sensitivity ratios in lean and obese sheep (Fig. 5).

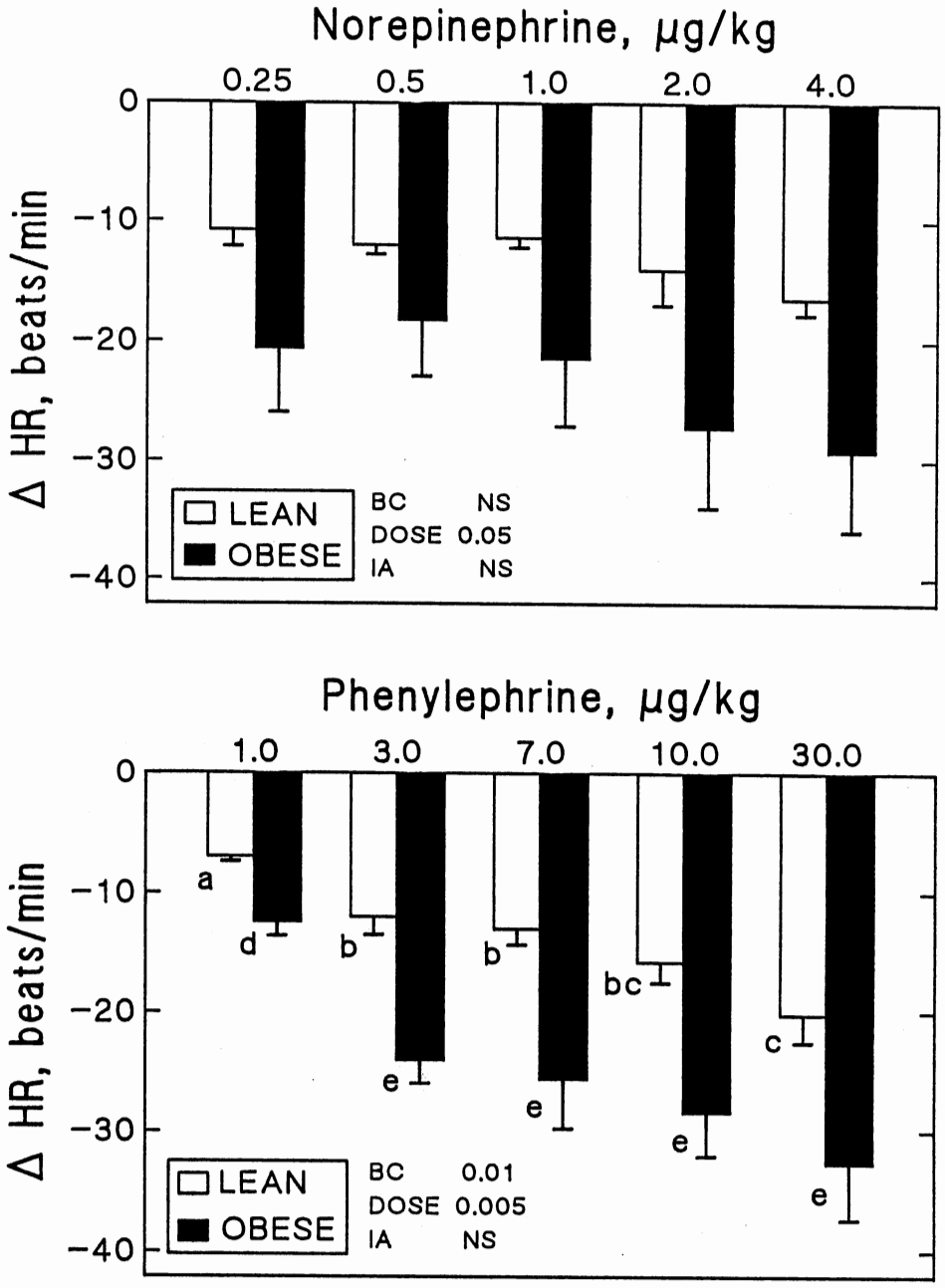

Figure 2. Decrements in heart rate (ΔHR) in lean and obese sheep (n=5/group) injected with different doses of norepinephrine (upper panel) or phenylephrine (lower panel). The maximum decrement in heart rate (beats/min) after each agonist dose was used to calculate the mean (± SE) values shown. Probability values shown for significances if treatment effects of body condition (BC), agonist dose (NE; PE) and their interaction (IA) were derived from 2 x 5 split-plot analysis of variance; means within each agonist type with similar lower case letter are not different ($P<0.05$) as determined by Duncan's new multiple range test.

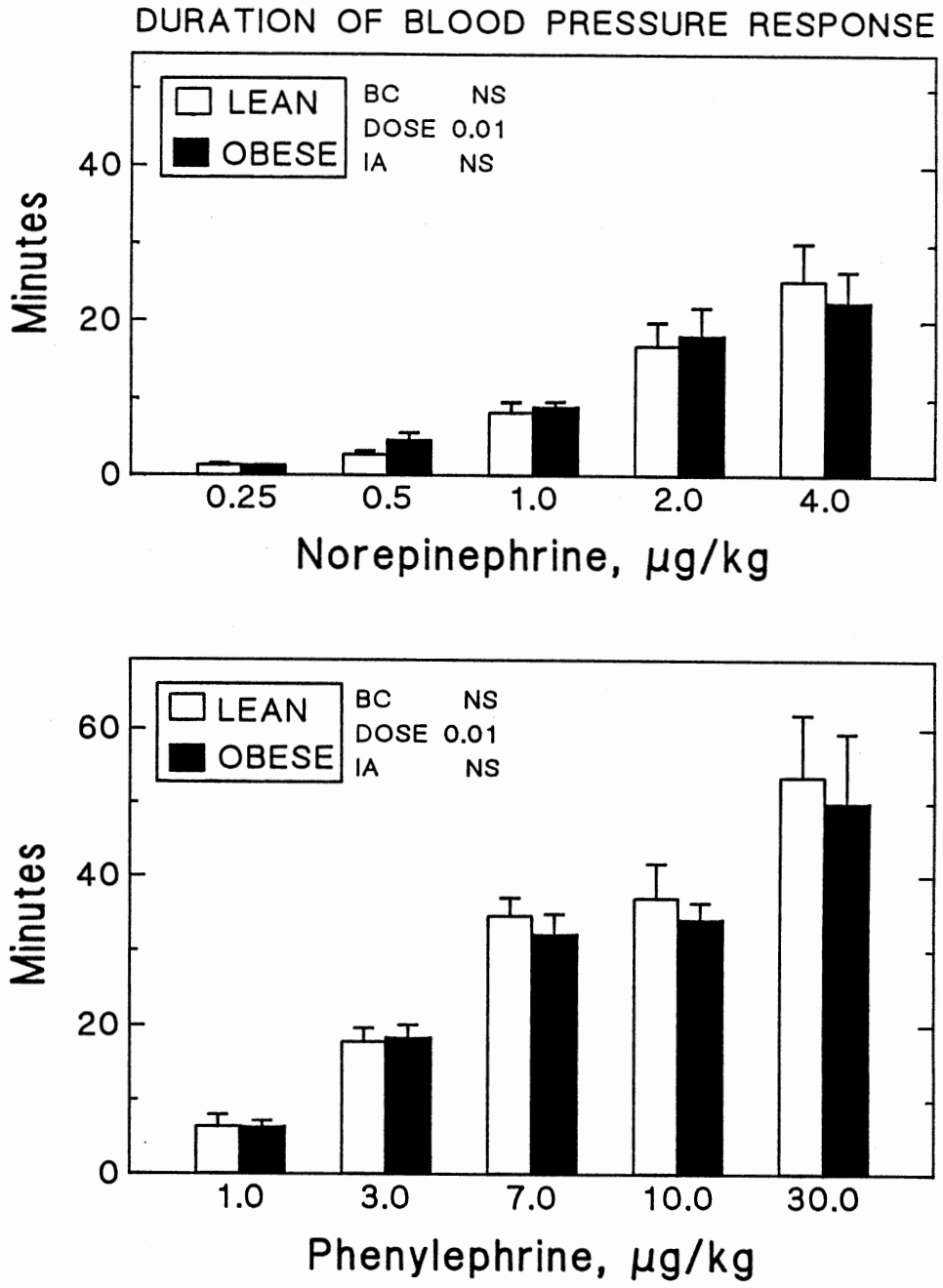

Figure 3. Duration of pressor response in lean and obese sheep (n=5/group) injected with different doses of norepinephrine (upper panel) or phenylephrine (lower panel). Duration was time (min) for the increased MAP to return to basal values after iv injection of agonist dose. Probability values shown for significances if treatment effects of body condition (BC), agonist dose (NE; PE) and their interaction (IA) were derived from 2 x 5 split-plot analysis of variance.

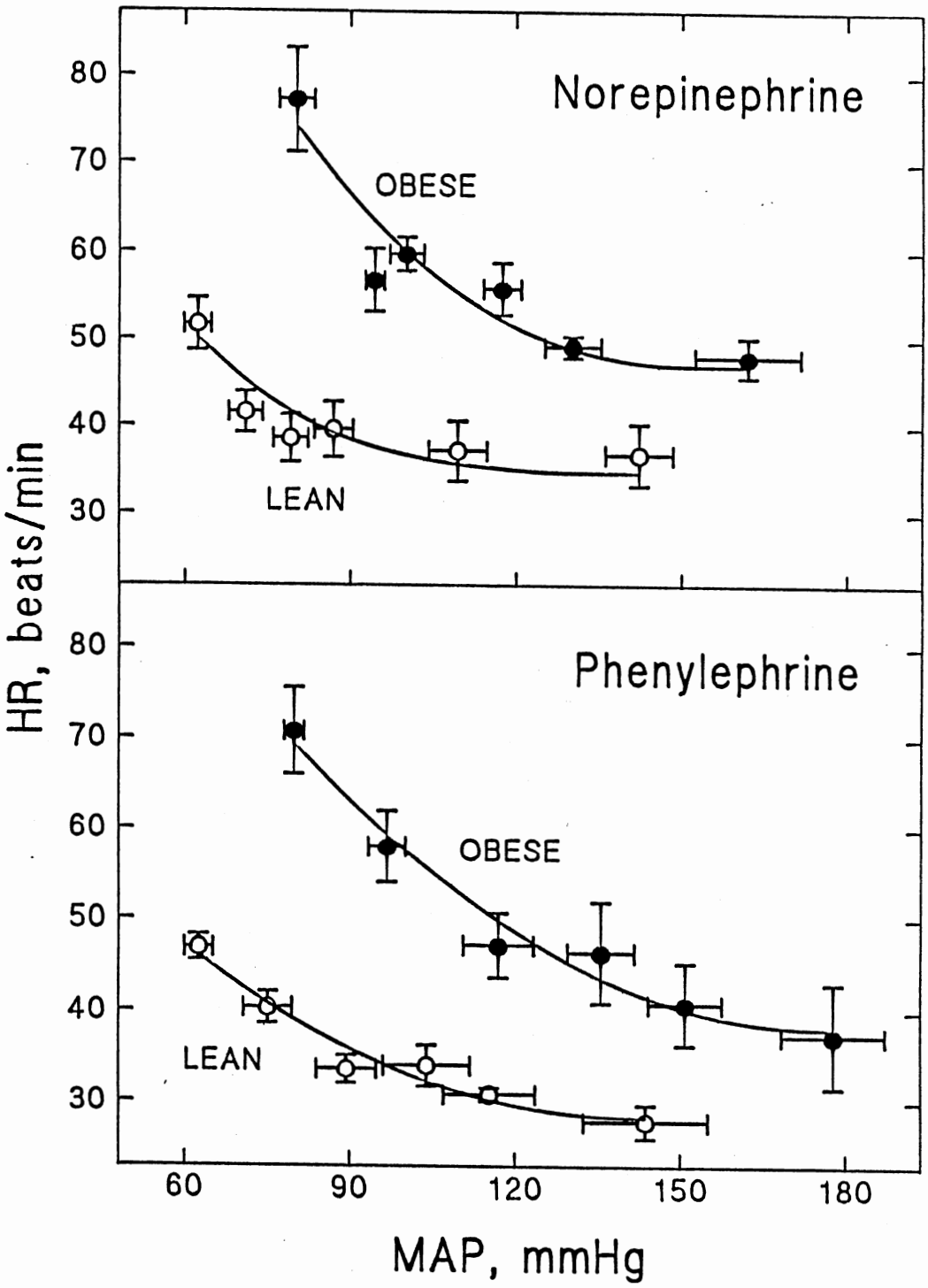

Figure 4. Relationship between heart rate and MAP in lean and obese sheep (n=5/group) under basal conditions and after iv injection of adrenergic agonists. Reading left-to-right, data points (mean ± SE) correspond to basal conditions and then lowest-to-highest dose of norepinephrine (upper panel) or phenylephrine (lower panel). Peak pressure response and the attendant heart rate for dose of adrenergic agonist are shown.

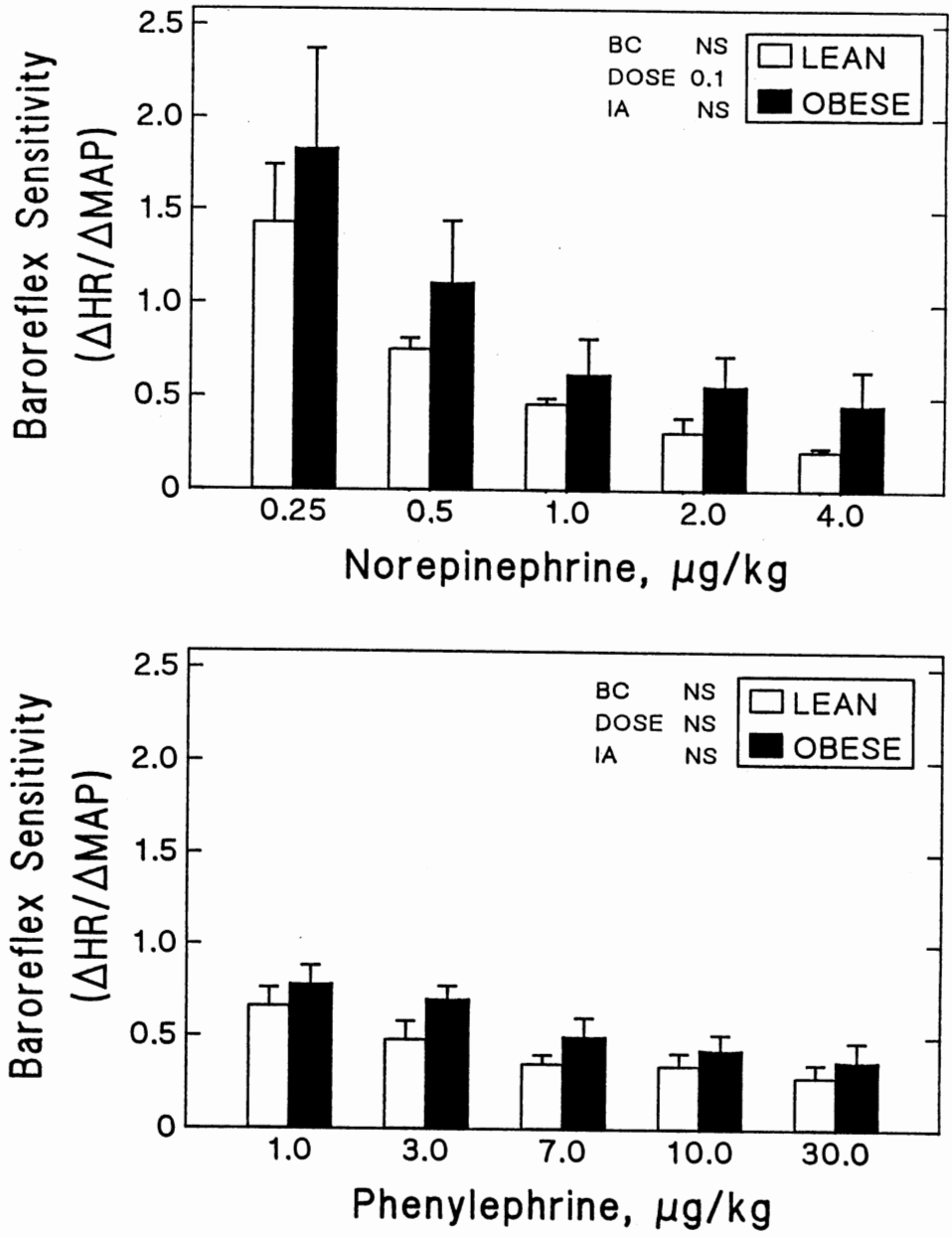

Figure 5. Baroreflex sensitivity ratios (mean ± SE) in lean and obese sheep (n=5/group) calculated at peak pressor response to dose of norepinephrine (upper panel) or phenylephrine (lower panel). Ratios calculated as decrement in heart rate (beats/min) below basal divided by peak increment in MAP (mmHg) above basal in response to agonist dose. Values for decrement in heart rate were those coincident with peak pressor response and usually this coincided with the nadir heart rate observed after the iv injection of adrenergic agonist. Probability values shown for significance of treatment effects of body condition (BC), agonist dose (NE; PE) and their interaction (IA) were derived from 2 x 5 split-plot analysis of variance.

Discussion

Many epidemiological studies have demonstrated a strong relationship between obesity and essential hypertension (Stamler 1978; Van Itallie 1985; Krieger et al 1988). The pathophysiological mechanisms involved in obesity-associated hypertension are unknown, but suggested mechanisms include insulin's effects on vascular structure and reactivity and insulin's effects on sympathetic nervous system activity (Krieger and Landsberg 1988; Swislocki 1990; Ferrari and Weidmann 1990).

We have established the dietary obese sheep as an alternative animal model applicable to obese humans with or without type II diabetes mellitus (McCann and Bergman 1988; McCann et al 1991). We report here that dietary obese sheep also are hypertensive. MAP and HR were 25% and 50% greater, respectively, in obese than lean sheep under basal, defined conditions. Dietary means have been used to induce obesity-associated hypertension in dogs (Rocchini 1989; Wehberg et al 1990) and rats (Kaufmann et al 1991) fed a high fat diet and in rats fed a high fructose diet (Hwang et al 1987); MAP was increased approximately 13 to 22% in these models of obesity-associated hypertension.

Ernberger and Nelson (1988) reported obesity-associated hypertension in rats made dietary obese by recurrent bouts of fasting/refeeding but not in rats made obese by chronic ad libitum feeding. Results in obese sheep, however, clearly show that hypertension was associated with obesity per se and not obesity interacting with diet type, level of intake, or non-steady state conditions of the dynamic phase of obesity. Our sheep became obese over 40 to 50 wk by consuming ad libitum the same normal diet fed at maintenance to lean control sheep. Blood pressure recordings were done in our study when lean and obese animals were fed equivalent levels of a common diet such that each was in zero-energy balance. Further, we purposefully measured basal

cardiovascular variables over 5 wk when cardiovascular variables and animal metabolism were in pseudo-steady state conditions and this is seldom the case in other animal models of obesity-associated hypertension.

Obesity induced by feeding a diet different from that fed to control animals may have ontoward effects other than those associated with the obese state. For example, high fat feeding has produced renal damage in rodents (Grone et al 1989; Kasiske et al 1991). Moreover, surgical manipulation of the animal (e.g., catheterization) requires suitable recovery time before reliable measurement of cardiovascular variables can be made. Simple catheterization of leg blood vessels in sheep required at least 7 d recovery before the surgery-induced increase in MAP abated and MAP returned to basal values; this effect was more pronounced in obese than lean sheep.

Obesity-associated hypertension in sheep coexisted with hyperinsulinemia, hyperglycemia and decreased plasma concentrations of HDL-cholesterol, which are results consistent with obesity-associated hypertension in humans. However, unlike humans, plasma triglyceride concentration was similar in lean and obese sheep. These results suggest that hypertriglyceridemia is not a prerequisite finding in obese mammals that are hypertensive. Plasma levels of Ca^{2+}, Na^{+} and K^{+} were similar in lean and obese sheep indicating that the obesity-associated hypertension likely was unrelated to abnormal metabolism of sodium, potassium or calcium.

It has been argued that the hyperinsulinemia of obesity increases sympathetic nervous system activity leading to increased vascular constriction and hypertension (Krieger and Landsberg 1988). Fasting hyperinsulinemia was evident in obese sheep since approximately wk 2 to 7 of ad libitum feeding (J. P. McCann, unpublished data), but plasma catecholamines at wk 100 to 120 of obesity in this study were similar in lean and obese sheep. In fact, arterial plasma catecholamines tend to be greater in

lean than obese sheep. O'Hare et al (1989) also reported that obese men had greater plasma insulin, glucose and MAP than lean controls and that plasma NE concentrations did not differ. The SHR rat and some hypertensive humans reportedly have elevated plasma venous levels of catecholamines relative to values in normotensive controls (Vlachkis and Alexander 1981; Tuck 1986; Ludwig et al 1991). Dogs fed a high fat diet for 6 wk developed hypertension, but the elevated levels of plasma catecholamines noted at wk 1 remained steady throughout the next 5 wk of high-fat diet feeding (Rocchini et al 1989). Other workers, however, reported a decrease in plasma catecholamine levels in dietary-obese compared with lean rats (Levin et al 1983). It appears that hyperinsulinemia and hypertension can coexist in obesity without concomitant significant elevation in the plasma catecholamine levels.

Hyperinsulinemia definitely plays some role in the development and sustenance of hypertension, coronary heart disease and atherosclerosis in obese and nonobese humans with or without diabetes (Swislocki 1990; Gwinup and Elias 1991; Reaven 1990; Ronnemaa et al 1991). The exact mechanism linking hyperinsulinemia to cardiovascular disease are unknown. Experimental efforts to induce hypertension in animals infused with insulin have been largely unsuccessful. Five-fold elevation in plasma insulin in euglycemic dogs for 7 d did not affect MAP (Hall et al 1990) and high-sucrose feeding together with 5-d iv infusion of insulin in rats barely increased MAP above values in chow-fed control rats (Brands et al 1991). High fructose (Hwang et al 1987) feeding alone has produced obesity, hyperinsulinemia, insulin resistance and a modest rise in systolic blood pressure.

Increased TPR is believed responsible for the increased blood pressure in obesity-associated hypertension (Folkow 1982). Enhanced vascular reactivity to adrenergic stimulation will increase vascular resistance and this could explain the

increased blood pressure in obese subjects (Landsberg and Kreiger 1989). Blood pressure increases in response to NE or PE injections were similar in lean and obese sheep in this study, although dose of NE increasing MAP by 25 mmHg was greater ($P<0.08$) somewhat in obese than lean sheep. However, increases in MAP after iv injections of PE were significantly greater in obese, high-fat fed dogs than in lean dogs fed a low-fat diet (Wehberg et al 1990). Pressure response to iv infusion of PE was greater marginally in obese rats chronically fed high fat than in lean rats fed regular chow (Bunag et al 1990). The relative contribution of high-fat feeding and the obese state to adrenergic agonist-induced increase in blood pressure in such syudies are not clear. Hypertensive dogs and rats made obese by high-fat feeding become normotensive if fed a normal levels of fat intake (Rocchini et al 1987; Kaufman et al 1991). Results comparing pressor response to alpha-adrenergic agonists in lean versus obese humans apparently are not available, but dose of NE increasing blood pressure by 20 mmHg was less in nonobese humans with essential hypertension than in normotensive humans (Weidmann 1989).

Several studies suggest that baroreflex function may be altered in hypertensive animals and humans (Aars 1968; Dustan 1983; Corner 1989; Xie et al 1991). Alteration in baroreflex control of heart rate can involve resetting of the threshold pressure for inhibition of heart rate and(or) change in sensitivity of the baroreflex pressure-activity curve (Korner 1989). Baroreflex sensitivity was normal but baroreflex response was reset to function at higher pressure levels in rats (Jones and Floras 1980) and rabbits (Guo et al 1983; Xie et al 1991) with renal hypertension and in nonobese humans with essential hypertension (Bristow et al 1969; Eckberg 1979). That the curve relating change in heart rate to change in MAP was shifted rightward in obese compared with lean sheep in this study, suggests that baroreflex control of heart rate was impaired in

the dietary obese sheep and that this impairment was characterized by resetting of the reflex without change in sensitivity of the reflex. Similar results on baroreflex control of heart rate were found in dogs that developed hypertension and obesity after 6 wk of high-fat feeding (Wehberg et al 1990) and in Wistar rats chronically fed high fat to develop obesity and border-line hypertension (Bunag et al 1990).

In summary, we report a new model of obesity-associated hypertension in sheep in which the obesity is induced by overconsumption of a normal diet. Hypertension in our dietary obese sheep model coexists with hyperinsulinemia, hyperglycemia, insulin resistance, hyperglucagonemia and lower plasma levels of HDL-cholesterol. Moderately greater hypertensive responses to adrenergic agonists in obese than lean sheep suggest the obesity-associated hypertension may be related to enhanced reactivity of adrenoceptor mechanisms in vascular smooth muscle of the obese sheep; this interpretation is valid only if the hypertensive response was caused directly via agonist-induced constriction of vascular smooth muscle and not indirectly by adrenergic agonist modification of central sympathetic outflow to resistance vessels. Chronic hyperinsulinemia in obese sheep may enhance vascular reactivity to vasoconstrictive agents and it also may play a role in resetting of the baroreflex control of heart rate, possibly by inducing structural changes in thickness and collagen content of vascular smooth muscle.

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CHAPTER VI

EFFECT OF CLONIDINE ON BLOOD PRESSURE AND HEART RATE IN LEAN AND OBESE SHEEP

Introduction

α-Adrenergic receptors mediate vasoconstriction and elevation of blood pressure. Modulation of synaptic norepinephrine release by presynaptic adrenergic receptors and the subclassification of α-adrenoceptors into α_1- and α_2- subtypes have been well documented (Kobinger 1978). Postsynaptic α_1-adrenoceptors located in the plasma membrane of vascular smooth muscle are the α-adrenoceptor type most responsible for vasoconstriction and blood pressure elevation (Timmermans and Van Zwieten 1980; Van Brummelen et al 1984). Adrenergic α_2-adrenoceptors are widely distributed in the body and are usually pre-synaptic, but they appear to be also located postjunctionally on vascular smooth muscle cells where they mediate vasoconstriction (Docherty and McGrath 1980; Timmermans and Van Zwieten 1980; Van Brummelen et al 1984; Goldberg and Robertson 1984). Activation of presynaptic α_2-adrenoceptors results in a decrease in release of endogenous transmitter (Schmitt 1967; Kobinger 1978).

Many drugs used in therapy of hypertension decrease blood pressure either by reducing the sympathetic drive to the cardiovascular system or by blocking the transduction of this signal to the effector organ. Clonidine hydrochloride is a hypotensive drug with agonistic properties for α_2-adrenoceptors (Bentley and Li 1968;

Langer et al 1980). Because of its pKa of 8.05, about 85% of administered clonidine exists in the protonated form in extracellular fluid (pH 7.36). Further, the lipid-solubility of clonidine enables it to cross the blood brain barrier and reach the pressure regulatory centers in the brain (Kobinger 1978).

Clonidine activation of α_2-adrenoceptors in the cardiovascular control center in the brain reduces sympathetic outflow to heart and vasculature and enhances parasympathetic tone to the heart, processes that promote lowering of blood pressure and heart rate (Schmitt et al 1967; Kobinger and Walland 1971; Pettinger 1980). Clonidine-induced decrease in heart rate and blood pressure is accompanied by decreased sympathetic activity as measured by electrical activity in peripheral sympathetic nerves of animals and by decreased plasma levels of catecholamines in animals and humans (Isaac 1980; Pettinger 1980).

As discussed above, activation of central α_2-adrenoceptors produces peripheral hypotension in humans and rodents. However, clonidine has an initial transient hypertensive effect when given iv to humans, rats, and dogs (Hoefke and Kobinger 1966; Shaw et al 1971), and this hypertensive response is effected by clonidine activation of postjunctional vascular smooth muscle α_2-adrenoceptors (Timmermans and Van Zwieten 1980). Thus, the hemodynamic effects of clonidine depend on the balance between activation of peripheral post-junctional and cental pre-synaptic α_2-adrenoceptors (Timmermans and Zwieten 1980).

Mechanisms responsible for obesity-associated hypertension in humans and animals are unknown but likely include dysfunctions in the neural and humoral control of cardiovascular function (Krieger and Landsberg 1988; Ferrari and Weidmann 1990; Rocchini 1990). The role of α_2-adrenoceptors in obesity-associated hypertension is not clear but available evidence suggests that down-regulation of central α_2-adrenoceptors

may play a role in obesity-associated hypertension (Pettinger 1980; Levin 1990; Gulati 1991). The objectives of this experiment were to determine if cardiovascular α_2-adrenoceptor-dependent responses differed in lean normotensive sheep and obese hypertensive sheep. The hypertensive sheep model is a new and alternative animal model that displays hyperinsulinemia, hyperglycemia and insulin resistance (McCann and Bergman 1988; McCann et al 1991).

Materials and Methods

Animal Preparation

Surgery was conducted with aseptic technique for survival surgery. Anesthesia was induced with 50 mg/kg pentobarbital sodium (Nembutal®, Abbott Lab., IL) and maintained with 1-2% halothane (Fluthane®, Ayerst Lab, NY) in oxygen delivered to the sheep through an endotracheal tube by anesthesia machine (Ohio Chemical & Surgical Equipment, Madison, WI).

Catheters with diameters of 12.7 mm (ID) were implanted in the abdominal aorta and the abdominal vena cava through the femoral artery and vein, respectively. The distal end of the catheters were exteriorized from the right hind leg and brought to mid back of the animal. The catheters were connected to Tuohy-Borst adapters, and wrapped in surgical adhesive tape which was glued to the animal. Sheep were treated with penicillin (300,000 U/kg) daily for 5 days after surgery. Catheters were flushed thrice weekly with sterile heparinized saline (10 U/ml) and filled with heparin (1,000 U/ml). At least 2 wk elapsed between surgery and experiments in any sheep. Inspection at necropsy confirmed that both catheter tips were positioned about 3 to 4 cm cranial to the ileac bifurcations.

Experimental Protocol

All experiments were done between 0900 and 1200 h in 16-h fasted lean (n=5) and obese (n=5) Rambouillet ewes that were, loosely held by halter in their own pen. The aortic catheter was connected to a calibrated blood pressure transducer (Narco Bio-System, Int. Biomedics, Houston, TX) located at the level of heart. Changes in blood pressure were recorded with a 4-channel Narcotrace model 40 MKIV physiograph (Narco Bio-System). Heart rates were determined using Narco biotachometer model 7302 (Narco Bio-System) linked to the blood pressure transducer, and occasionally from direct counting of the systolic waves for 1 min at slow (0.5 cm/sec) chart speed. Before each clonidine injection, blood pressure and heart rate were recorded for 45 min (stabilization period), during which time three measurements were made at 15 min intervals to establish base line values of blood pressure and heart rate. After the stabilization period, 5 or 10 μg/kg clonidine (10 to 15 ml volume) were infused in 2 min via the venous catheter which was immediately flushed with 5 to 10 ml of saline. Clonidine hydrochloride (Sigma, St. Louis, MO) solutions were freshly prepared for each experiment in saline, filtered through a 0.2 μm filter, and kept on ice until injected. Blood pressure and heart rate were recorded throughout each experiment, but blood pressure and heart rate measurements were done at 2, 5, 10, 15, 20, 30, 45, 60, 75, 90, 105 and 120 min post injection. Experiments were done simultaneously in a lean and obese sheep pair, with sequence of clonidine dose randomized among pairs; at least 5 days elapsed between successive doses of clonidine in each pair of sheep.

Calculations and Statistical Analysis

The mean arterial pressure (MAP) was determined as 2/3 of diastolic blood

pressure plus 1/3 of systolic blood pressure (West 1985). Differences between lean and obese sheep in blood pressure and heart rate after clonidine injections were compared using univariate correlated t test for repeated measurements (Gill 1979). The interactive effects of body condition and clonidine dose on blood pressure and heart rate variables were tested by 2 x 2 split-plot analysis of variance and Duncan's new multiple range test if a significant F-value ($P<0.1$) was found (Steel and Torrie 1980); for these analyses, clonidine-induced change in blood pressure for each sheep was quantified by area-under-the-curve (AUC) and peak heart rate and peak MAP also were used. AUC in each sheep was calculated by trapezoid method. Level of significance was 0.05. Values are mean ± SE.

Results

Basal MAP (91.5 ± 3.8 vs 73.8 ± 2.2 mmHg) and HR (72.7 ± 5.3 vs 50.6 ± 2.5 beats/min) were greater ($P< 0.01$) in obese than lean sheep. Values of blood pressure variables and heart rates for each clonidine dose are summarized for lean and obese sheep in Table 1 and 2. Clonidine produced a dose-dependent increase in MAP in both lean and obese sheep (Fig. 1). MAP was greater ($P< 0.05$) after 10 μg/kg than after 5 μg/kg clonidine in lean sheep and in obese sheep. Maximum increment in MAP developed in response to clonidine was unaffected by body condition but was affected significantly by clonidine dose (Table 3). However, the overall MAP response to 5 μg/kg clonidine was less ($P< 0.05$) in obese than lean sheep (Fig. 2). The response to low dose clonidine in obese sheep was only 46 % of that of lean sheep. Lesser MAP response to low dose clonidine together with similar MAP response to high dose clonidine are results suggesting fewer vasoactive α_2-adrenoceptors in obese compared with lean sheep.

TABLE 1

BLOOD PRESSURE VARIABLES AND HEART RATE IN SHEEP AFTER ADMINISTRATION OF 5 μg/kg CLONIDINE

	Lean (n=5)				Obese (n=5)			
Time (min)	SBP (mmHg)	DBP (mmHg)	MAP (mmHg)	HR (beat/min)	SBP (mmHg)	DBP (mmHg)	MAP (mmHg)	HR (beat/min)
-30	94.7 ± 2.4	64.1 ± 2.1	73.3 ± 2.0	52.0 ± 2.6	119.2 ± 3.2	81.2 ± 3.2	93.9 ± 3.1	71.6 ± 3.8
-15	93.7 ± 2.9	62.6 ± 2.4	73.1 ± 2.4	51.2 ± 2.9	118.7 ± 4.2	79.3 ± 3.4	92.4 ± 3.7	70.8 ± 3.3
0	94.2 ± 3.4	63.1 ± 2.4	73.5 ± 2.5	51.6 ± 2.4	120.1 ± 3.0	79.5 ± 2.9	93.1 ± 2.9	67.2 ± 2.9
2	119.6 ± 8.9	86.7 ± 6.8	97.7 ± 7.5	40.0 ± 2.2	135.5 ± 2.2	93.7 ± 1.4	107.3 ± 1.5	44.4 ± 2.6
5	124.4 ± 7.0	83.8 ± 3.6	97.4 ± 4.7	52.6 ± 5.3	145.6 ± 2.5	95.1 ± 2.9	112.5 ± 2.5	65.6 ± 7.5
10	121.6 ± 7.3	82.8 ± 4.2	95.8 ± 5.2	55.2 ± 6.5	134.1 ± 2.8	90.8 ± 3.3	105.2 ± 3.1	65.8 ± 5.5
15	118.7 ± 6.8	79.5 ± 3.8	92.6 ± 4.5	54.0 ± 3.6	127.8 ± 3.2	85.0 ± 2.1	99.3 ± 2.2	68.0 ± 4.0
20	111.9 ± 7.3	76.1 ± 4.4	88.1 ± 5.1	60.4 ± 6.0	121.1 ± 3.9	78.8 ± 3.5	92.9 ± 3.6	74.8 ± 6.8
30	107.1 ± 5.5	70.8 ± 3.1	83.0 ± 3.5	55.2 ± 1.0	121.1 ± 3.7	77.3 ± 3.0	91.9 ± 3.2	72.4 ± 4.9
45	101.4 ± 5.6	67.5 ± 3.4	78.8 ± 4.0	56.4 ± 2.4	124.5 ± 7.5	80.7 ± 5.3	95.3 ± 6.0	70.0 ± 2.4
60	98.0 ± 5.0	65.5 ± 3.0	76.4 ± 3.4	54.4 ± 1.7	124.5 ± 11.2	83.6 ± 8.3	97.2 ± 9.3	67.2 ± 3.3
75	96.6 ± 3.8	65.1 ± 3.1	75.6 ± 2.9	52.8 ± 1.9	125.9 ± 10.1	84.1 ± 7.6	98.0 ± 8.6	66.4 ± 3.8
90	96.1 ± 3.5	64.1 ± 2.8	74.8 ± 2.7	52.2 ± 2.4	125.9 ± 9.5	85.0 ± 7.4	98.7 ± 8.1	66.4 ± 2.9
105	94.2 ± 3.5	63.1 ± 3.1	73.5 ± 3.0	52.4 ± 1.7	122.5 ± 9.9	82.6 ± 7.8	95.9 ± 8.5	68.4 ± 2.5
120	93.7 ± 2.6	62.6 ± 2.5	73.1 ± 2.2	52.8 ± 1.7	121.1 ± 9.9	81.2 ± 6.0	94.5 ± 7.5	67.6 ± 2.9

SBP= Systolic Blood Pressure; DBP= Diastolic Blood Pressure; MAP= Mean Arteial Pressur; HR= Heart Rate

TABLE 2

BLOOD PRESSURE VARIABLES AND HEART RATE IN SHEEP AFTER ADMINISTRATION OF 10 μg/kg CLONIDINE

	Lean (n=5)				Obese (n=5)			
Time (min)	SBP (mmHg)	DBP (mmHg)	MAP (mmHg)	HR (beat/min)	SBP (mmHg)	DBP (mmHg)	MAP (mmHg)	HR (beat/min)
-30	92.5 ± 1.9	64.1 ± 2.2	73.6 ± 1.9	49.6 ± 2.4	115.3 ± 4.4	76.4 ± 4.2	89.4 ± 4.2	76.4 ± 7.0
-15	93.9 ± 1.9	64.6 ± 2.1	74.4 ± 1.9	49.6 ± 2.2	116.8 ± 4.1	76.4 ± 4.6	89.9 ± 4.4	75.2 ± 7.3
0	92.7 ± 2.3	64.6 ± 2.9	74.0 ± 2.6	49.6 ± 2.4	115.8 ± 4.3	77.4 ± 5.1	90.2 ± 4.8	75.2 ± 7.4
2	118.4 ± 8.9	84.8 ± 6.6	96.0 ± 7.3	35.6 ± 0.7	133.1 ± 4.4	91.8 ± 3.5	105.6 ± 3.7	44.0 ± 2.8
5	137.2 ± 4.7	93.9 ± 2.8	108.3 ± 2.9	37.6 ± 1.6	174.0 ± 16.3	110.0 ± 7.1	131.4 ± 10.1	57.4 ± 7.9
10	136.2 ± 3.8	95.9 ± 2.9	109.3 ± 1.9	43.6 ± 4.1	167.7 ± 15.3	106.2 ± 4.7	126.7 ± 8.1	63.2 ± 8.5
15	131.4 ± 4.0	90.1 ± 3.2	103.8 ± 3.0	44.8 ± 3.8	161.0 ± 12.5	104.8 ± 4.3	123.5 ± 6.6	67.6 ± 9.3
20	127.1 ± 4.6	84.8 ± 4.4	98.9 ± 4.3	47.8 ± 3.3	141.3 ± 4.3	95.7 ± 3.0	110.8 ± 3.3	74.4 ± 12.0
30	118.4 ± 6.0	78.1 ± 5.1	91.5 ± 5.3	52.0 ± 2.6	126.4 ± 6.9	85.6 ± 4.5	99.2 ± 5.2	74.8 ± 9.2
45	104.0 ± 6.4	74.2 ± 4.4	84.2 ± 4.9	56.0 ± 5.0	120.6 ± 5.1	81.2 ± 3.5	94.4 ± 4.0	75.0 ± 9.0
60	98.8 ± 8.1	69.9 ± 4.4	79.5 ± 5.5	63.6 ± 9.6	116.8 ± 4.5	79.3 ± 3.8	91.8 ± 3.9	75.2 ± 7.0
75	98.3 ± 6.6	67.0 ± 4.2	77.4 ± 4.9	54.4 ± 2.4	114.8 ± 4.0	77.9 ± 4.4	90.2 ± 4.1	74.0 ± 5.4
90	96.8 ± 5.7	65.6 ± 4.3	76.0 ± 4.6	52.4 ± 1.6	115.8 ± 4.0	80.3 ± 4.2	92.1 ± 4.0	43.2 ± 4.7
105	94.9 ± 5.3	66.1 ± 3.7	75.7 ± 4.2	54.2 ± 1.0	112.4 ± 3.2	76.0 ± 4.2	88.1 ± 3.8	72.0 ± 4.6
120	95.9 ± 5.7	66.1 ± 3.8	76.0 ± 4.2	52.8 ± 2.0	113.9 ± 4.5	76.9 ± 4.6	89.2 ± 4.6	71.2 ± 4.4

SBP= Systolic Blood Pressure; DBP= Diastolic Blood Pressure; MAP= Mean Arteial Pressur; HR= Heart Rate

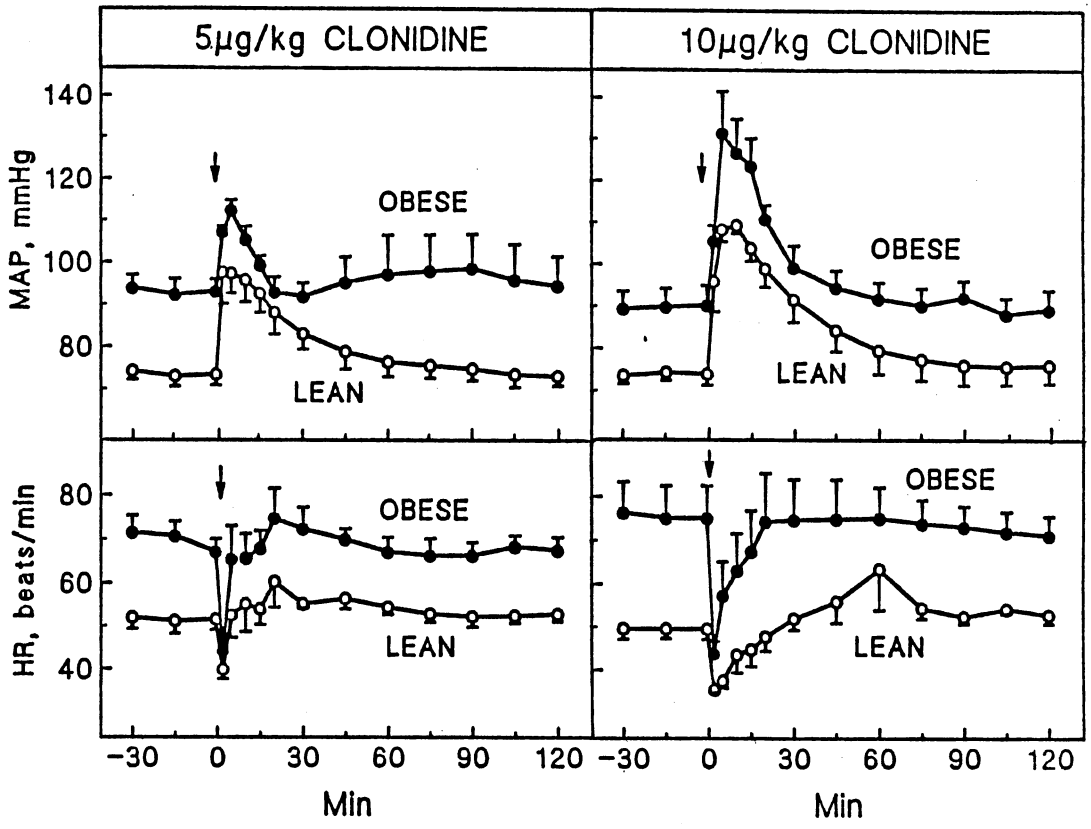

Figure 1. Hemodynamic effects of 5 and 10 μg/kg clonidine in lean (n=5) and obese (n=5) sheep. Arrows indicate iv injection of clonidine.

TABLE 3

MAXIMUM CHANGE IN MAP AND HR AFTER CLONIDINE INJECTION IN LEAN AND OBESE SHEEP[1]

		Clonidine Dose		Probability Value		
Item	Body Condition	5 μg/kg	10 μg/kg	BC	Dose	IA
MAP, mmHg	Lean	24.0 ± 5.3^{ab}	35.4 ± 1.6^{c}	NS	0.025	NS
	Obese	18.8 ± 3.7^{a}	41.6 ± 12.0^{bc}			
HR, beats/min	Lean	11.1 ± 1.0^{a}	13.0 ± 3.0^{a}	0.01	NS	NS
	Obese	25.3 ± 3.0^{b}	31.0 ± 5.0^{b}			

[1] Values are mean ± SE and represent the difference between the peak MAP and nadir HR developed and corresponding basal values in each sheep. Peak MAP and nadir HR values were recorded 1 to 5 min after clonidine injection. Probability values for significance of treatment effects from split-plot analysis of variance; means within each item with similar superscript letter are not different (P>0.05) as determined by Duncan's new multiple range test. (BC=body condition, IA= interaction).

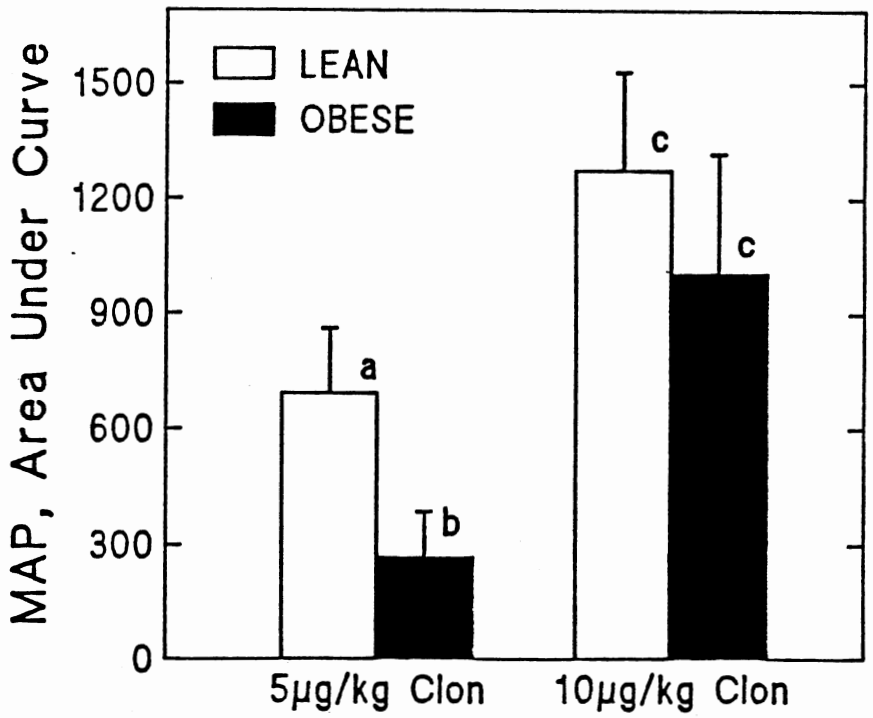

Figure 2. MAP response to 5 and 10 µg/kg clonidine in lean and obese sheep. Response calculated as area (AUC) above basal MAP plus one standard deviation of basal. Probability values from split-plot analysis of variance for treatment effects were body condition 0.1; clonidine dose 0.005; interaction 0.1. Means with similar superscript letters are not different ($P>0.05$) as determined by Duncan's new multiple range test.

After clonidine injection, HR decreased acutely in lean and obese sheep before returning to basal levels immediately, except in lean sheep injected with 10 µg/kg clonidine (Fig. 1). The maximum decrements in HR after clonidine in each group of sheep were unaffected by dose , but the decrement was 2 to 3 times greater ($P<0.05$) in obese than lean sheep (Table 3). The bradycardiac response to clonidine appeared unrelated to the dose-dependent hypertensive effects of clonidine. Results in Table 3 show that for the same time interval (+2 to +10 min) after each dose of clonidine, the

decrement in HR was considerably greater in obese than lean sheep despite less or similar increment in peak MAP being developed in obese than lean sheep.

Additional qualitative observations on behavior of sheep injected with clonidine are noteworthy. Salivation was noticeably increased in all sheep by either dose of clonidine. However, distinct respiratory distress was evident in obese but not lean sheep and this response was exacerbated by increasing dose of clonidine. Respiration rate increased along with marked reduction in volume of air breathed in the first +5 to +15 min after clonidine injection.

Discussion

In this experiment 5 and 10 μg/kg clonidine produced marked hypertension in lean and obese sheep. Blood pressure did not decrease significantly below basal values in these clonidine-injected sheep when observations were made up to 4 h after clonidine treatment. The blood pressure response to clonidine is biphasic in many species including man (Kobinger 1978; Pettinger 1980). After iv injection, clonidine activation of vascular smooth muscle α_2-adrenoceptors produces an initial short-lived hypertension response which precedes a chronic hypotensive response mediated by clonidine activation of central α_2-adrenoceptors (Timmermans and Van Zwieten 1980, Jie et al 1987). The hypertensive response in man is evident after high but not low doses of clonidine (Pettinger 1980). Neither acute nor chronic hypotensive responses were observed in our sheep injected with doses of clonidine which were comparable to those used in studies reporting biphasic pressure responses to iv clonidine.

Hypertension without any hypotensive response was reported previously for normal sheep injected iv with clonidine (Eisenach 1988; Castro and Eisenach 1989), but the degree of hypertension noted by these workers was less than that in our sheep

despite employment of similar doses of clonidine. Blood pressure response to iv injection of clonidine in sheep apparently differs from the biphasic hyper-hypotensive response observed in dogs (Schmitt and Schmitt 1969), goats (Eriksson and Tuomisto 1982) and rats (Grichois et al 1990) treated with clonidine doses similar to those used in this study. The biphasic blood pressure response to iv clonidine has been explained by clonidine activation of α_2-adrenoceptors in the peripheral vasculature and in the CNS that mediate clonidine's hypertensive and hypotensive responses, respectively (Kobinger and Walland 1967; Pettinger 1980; Head and DeJong 1986). Intravenously injected clonidine can interact with both central and peripheral adrenoceptors. Thus the blood pressure change after iv clonidine reflects the balance between the central hypotensive and peripheral vasoconstrictive effects of clonidine.

Because ruminants are more sensitive to α-agonists than nonruminants (Hopkins 1972), it would be difficult to inject a dose of clonidine iv that would, on basis of dose, preferentially affect central but not peripheral adrenoceptors. Accordingly, the typical blood pressure response to iv clonidine in sheep is singularly hypertensive reflecting the dominant role of adrenoceptors in the peripheral vasculature, relative to those in the CNS, when both are exposed to iv clonidine. This is substantiated by the results of Eisenach (1988) and Eisenach and Tong (1991) in sheep where clonidine produced hypertension when administered iv but produced hypotension when similar dosage was administered intrathecally into spinal fluid. A few reports in ruminants, however, have noted mild hypotension after iv injection of 7 μg/kg clonidine in goats (Eriksson and Tuomisto 1982) and after epidural injection of approximately 2-5 μg/kg clonidine (Eisenach and Tong 1991). Reasons for the discrepancy in results between sheep and goats are not clear, but it is reasonable to assume that clonidine-induced hypotension in sheep could be masked by clonidine's dominant effect of peripheral vasoconstriction. Although central and peripheral adrenoceptors likely were stimulated

by iv clonidine, hypertension without development of hypotension occurred in the lean and obese sheep injected with clonidine in this experiment.

The maximum increment in MAP after each dose of clonidine and the overall hypertensive response to high dose clonidine were comparable in lean and obese sheep. However, the hypertensive response to low dose clonidine was significantly less in obese than lean sheep. That the difference in response to clonidine between lean and obese sheep was observed in the presence of submaximal concentrations of clonidine, but not in the presence of saturating concentrations of clonidine, suggests that obese sheep had fewer α_2-adrenoceptors than lean sheep. Relative to control conditions, fewer cell-surface receptors are associated with a rightward shift in the dose-response curve for agonist action (Kahn 1985). This would be applicable if our results could be explained entirely by the peripheral vasoconstrictive actions of clonidine in vascular smooth muscle. If clonidine indeed has central and peripheral effects in sheep that are concurrent but antagonistic, then the lesser hypertensive response to low dose clonidine in obese than lean sheep could be explained by a greater central vasodilatory effect of clonidine in obese sheep than lean sheep without difference in response to the peripheral vasoconstrictive effects of clonidine.

Reflex bradycardia was two to three times more prominent in obese than lean sheep a despite similar, or lesser, hypertensive response in obese than lean sheep. These results suggest that clonidine affected baroreceptor function by affecting baroreceptor sensing of increased MAP, central cardiovascular processing of baroreceptor information, and(or) efferent signalling to cardiac muscle in obese compared with lean sheep. Centrally acting clonidine inhibits sympathetic and enhances parasympathetic nervous activity (Pettinger 1980). The greater bradycardia in obese than lean sheep might indicate greater parasympathetic vagal response to

clonidine in the obese sheep. Others have suggested that clonidine increases the sensitivity of the baroreflex response in normal rats (Grichois et al 1990).
The observed bradycardia in sheep in this study dissipated within 10 min even though MAP was increased for up to 30 to 60 min after clonidine injection. In contrast, bradycardia persisted throughout the hypertensive response in sheep injected with phenylephrine, an α_1-agonist (see Chapter V).

Clonidine can affect respiration in sheep by central and peripheral mechanism (Eisenach 1988; Eisenach et al 1988; Bolme and Fuxe 1973). Bolme and Fuxe (1973) hypothesized that clonidine interacts with a central inhibitory noradrenergic mechanism to control respiration. Clonidine had slight inhibitory effect on respiration in goats (Eriksson and Tuomisto 1982) but significantly reduced incidence and duration of breathing in foetal lambs (Bamford et al 1990; Bamford and Howkins 1990). In this experiment, respiratory effects of clonidine were distinct in obese sheep but barely detectable in lean sheep. However, lean and obese sheep exhibited similar parietal gland response as illustrated by noticeable salivation in each sheep in the first 10 min approximately after each dose of clonidine. Furthermore, preliminary experiments to determine appropriate dose of clonidine revealed that 20 μg/kg clonidine produced severe dyspnea in obese (n=2) but not lean (n=2) sheep, and consequently only doses of 5 and 10 μg/kg clonidine were used in the experiment. The marked difference in respiratory response to clonidine in obese compared with lean sheep suggests that central α_2-adrenoceptor activity was greater in obese hypertensive than in lean normotensive sheep. It would appear that obesity in sheep affects central and peripheral α_2-adrenoceptor regulation of vasoconstriction and central α_2-adrenoceptor regulation of respiration. Levin (1990) reported that density of binding sites for α_2-agonists was greater in brain membranes of obese than lean rats. Putative differences in α_2-adrenoceptor activity in obese compared with lean sheep may be the

consequence of hypertension and not the obese state *per se* because the central response to α_2-agonist also was greater in spontaneously hypertensive rats than in their normotensive littermates (Yarbrough et al 1983; Tibirica et al 1988).

In summary, the cardiovascular response to low and high doses of iv clonidine differed in lean and dietary-obese sheep. Hypertensive response to low dose clonidine was greater in lean than obese sheep, whilst bradycardic response to clonidine was greater in obese than lean sheep regardless of clonidine dose. Hypertension without hypotension suggested that iv clonidine predominantly affected peripheral vasoconstrictive α_2-adrenoceptors in sheep. However, clonidine-induced apnea and dyspnea were markedly more noticeable in obese compared with lean sheep. Dietary obesity may differentially affect function of central and peripheral α_2-adrenoceptors in sheep.

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CHAPTER VII

HEMODYNAMIC EFFECTS OF CLONIDINE AND NALOXONE IN NORMOTENSIVE LEAN AND HYPERTENSIVE OBESE EWES

Introduction

Clonidine a is potent antihypertensive drug in humans and animals (Kobinger 1978; Pettinger 1980; Isaac 1980; Jarrott et al 1987). The hypotensive action of clonidine is due both to a centrally-mediated decrease in sympathetic tone to the heart and vascular together with an enhanced vagal baroreceptor reflex (Kobinger 1978; Isaac 1980; Jarrot et al 1987; Maze and Tranquilli 1991). Despite the well documented hypotensive effect of clonidine in various species, the blood pressure response to iv clonidine is biphasic in humans, dogs, cats and certain strains of rats (Boissier et al 1968; Kobinger 1978; Frisk-Holmberg et al 1984). Clonidine causes an initial short-lived hypertension that precedes chronic hypotension and both responses are attended by bradycardia (Rhee and De Lapp 1988; Grichois et al 1990).

The blood pressure response to clonidine reflects the balance of clonidine 's activation of centrally located α_2-adrenoceptors, which mediate hypotension, and peripheral α_2-adrenoceptors in vascular smooth muscle which mediate hypertension (Schmitt et al 1969; Kobinger 1978; Isaac 1980; Langer 1985). The biphasic blood pressure response to clonidine depends on the dose of clonidine and accessibility of clonidine into brain tissue after its systemic administration by intravenous or oral routes

(Frisk-Holmberg et al 1984; Rhee and De Lapp 1988). Low levels of clonidine are associated with a hypotensive response, where increasing concentrations of plasma clonidine are associated with hypertensive response (Wing et al 1977; Pettinger 1980).

The central effects of clonidine on blood pressure seem to involve opioid receptors active within the hypothalamus, nucleus tractus solitarius and (or) nucleus reticularis lateralis (Schmitt 1967; Jarrot et al 1987; Tibirica et al 1989; Bousquet et al 1989). Concomitant administration of the opioid antagonist, naloxone, has inhibited or abolished the hypotensive response to iv clonidine in anaesthetized normotensive and hypertensive rats (Farsang and Kunos 1979; Shropshire and Wendt 1983; Mastrianni and Ingenito 1987; Mosquede-Garcia and Kunos 1988; Chang et al 1989) and in normotensive dogs (Pinto et al 1989). However, naloxone co-treatment had no effect on the hypotensive response to clonidine in normotensive and hypertensive humans (Watkins et al 1980; Bramnert and Hokfelt 1983) and normotensive Wistar-Kyoto rats(Mastrianni and Ingenito 1987). The ability of naloxone to affect the blood pressure response to clonidine may be species specific.

Obese humans are frequently hypertensive (Stamler 1978; Van Itallie 1985). It has been suggested that dysfunction in central α_2-adrenoceptors could lead to enhanced sympathetic outflow and development of hypertension in obese subjects (Bramnert and Hokfelt 1983; Veith et al 1984). We reported that the blood pressure response to iv clonidine differed between normotensive lean and dietary obese hypertensive sheep (Chapter VI). The pressor response was less and the bradycardic response was greater in obese than lean sheep injected iv with 5 μg/kg clonidine, whereas the hypertensive and bradycardic responses were comparable in both group after 10 μg/kg clonidine. The objectives of this experiment were to determine if naloxone affected the hypertensive and bradycardiac response to high dose clonidine (10 μg/kg) in sheep and whether these responses differed in normotensive lean sheep

and hypertensive obese sheep. The dietary obese sheep is hyperinsulinemic, hyperglycemic, insulin resistant, and hypertensive (see Chapter V).

Materials and Methods

Animal Preparation

Five normotensive lean (46 ± 2 kg) and 5 hypertensive obese (79 ± 3 kg) Rambouillet ewes were housed individually in a room with constant light and temperature (20-21 C). Procedures for induction of dietary obesity in sheep were described in full in Chapter II. Briefly, lean adult Rambouillet ewes weighing 47 ± 3 kg were fed a pelleted hay-grain diet at maintenance (lean; control) or fed the same diet ad libitum (obese) until the maximum voluntary capacity for adipose accretion was attained at which point animals were in static-phase obesity characterized by steady-state body weights and steady-state levels of maintenance intake. Consequently, all sheep were fed a specified maintenance intake of approximately 38 Kcal DE/kg in two equal amounts at 0900 and 1700 h such that each was in zero-energy balance before and during the experiments.

Surgery was conducted with aseptic technique for survival surgery. Anesthesia was induced with 50 mg/kg pentobarbital sodium (Nembutal®, Abbott Lab., IL) and maintained with 1-2% halothane (Fluthane®, Ayerst Lab. NY). The gas mixture was delivered from a surgical anesthesia machine (Ohio Chemical & Surgical Equipment, Madison, WS, USA) to the sheep through an endotracheal tube.

Catheters with diameters of 12.7 mm (ID) were implanted in the abdominal aorta and the abdominal vena cava through the femoral artery and vein, respectively. The distal end of the catheters were exteriorized from the right hind leg and brought to mid back of the animal. The catheters were connected to Tuohy-Borst adapters, and

wrapped in surgical adhesive tape which was glued to the animal. Sheep were treated with penicillin (300,000 U/kg) daily for 5 days after surgery. Catheters were flushed thrice weekly with sterile heparinized saline (10 U/ml) and filled with heparin (1,000 U/ml). At least 2 wk elapsed between surgery and experiments in any sheep. Inspection at necropsy confirmed that both catheter tips were positioned about 3 to 4 cm cranial to the ileac bifurcations.

Experimental Protocol

One lean and one obese sheep constituted a replicate and replicates were assigned randomly to one of the six sequences possible for treatment of clonidine (10 µg/kg), naloxone (3 mg/kg) and combined clonidine and naloxone (see below). On each experimental day, clonidine hydrochloride (Sigma, St. Louis, MO) and naloxone (Sigma) were prepared fresh in saline, filtered through a 0.2 µm filter and kept on ice until injected in via the venous catheter. At least 5 d separated successive treatments.

Experiments were done between 0900 and 1200 h in conscious 16-h fasted sheep that were free-standing in their own pen. The aortic catheter was connected to a calibrated blood pressure transducer (Narco Bio-System, Int. Biomedics Houston TX USA) located at the level of heart. Changes in blood pressure were recorded with a 4-channel Narcotrace model 40 MKIV physiograph (Narco Bio-System). Heart rates were determined using Narco biotachometer model 7302 (Narco Bio-System) linked to the blood pressure transducer. On each experimental day, recordings of blood pressure and heart rate were made for 45 min before iv injection of drugs. Three observations on blood pressure and heart rate were done during this stabilization period at approximately 15 min interval for each sheep. After the stabilization period, drugs in 5 to 10 ml of saline were infused in 2 min and the venous infusion catheter flushed with similar volume of saline. Blood pressure and heart rate measurements were done at 2,

5, 10, 15, 20, 30, 45, 60, 75, 90, 105, and 120 min after injection of naloxone or clonidine. In the combined treatment, naloxone was injected iv 5 min before clonidine and blood pressure and heart rate measurements done at times indicated relative to clonidine injection and also at 2 min after naloxone.

Statistical analysis

The mean arterial pressure (MAP) was calculated as 2/3 of diastolic plus 1/3 systolic blood pressure (West 1985). Effects of drug treatment on absolute values of MAP and heart rate within each body condition level were tested by repeated measures method of Gill (1979). Interactive effects of body condition and drug treatment on blood pressure and heart rate variables were determined using 2 x 3 split-plot analysis of variance with separation of means by Duncans' new multiple range test if a significant F-value ($P<0.1$) was found (Steel and Torrie 1980). For this analysis the maximum change in MAP and heart rate measured after drug injection were used; additionally, the net effect of drug-induced change in MAP was quantified by area-under-the-curve (AUC) measured from time 0 until MAP returned to the mean plus one SD of that mean for basal MAP values in each sheep. Level of significance was 0.05 and reported values are means ± SE.

Results

Blood pressure variables and heart rates in lean and obese sheep before and after injection of drug are summarized in Table 1, 2 and 3. Overall basal MAP (92.6 ± 3.7 vs 74.6 ± 2.4 mmHg) and basal HR (69.8 ± 3.3 vs 51.6 ± 2.6 bpm) were greater ($P<0.01$) in obese than lean sheep (Table 1, 2, 3). Clonidine injection caused pronounced hypertension in lean and obese sheep (Fig. 1). The hypertensive response

to clonidine was equivalent in lean and obese sheep whether measured as maximum change in MAP (Fig. 3) or net change in MAP response (Fig. 4). Clonidine-induced bradycardia was greater in obese than lean sheep (Fig. 2; 5).

Naloxone by itself induced noticeable hypertension in all sheep. In contrast to clonidine, hypertensive response to naloxone was greater in obese than lean sheep (Fig. 1, 3, 4). Interestingly, the hypertensive response to naloxone was accompanied by tachycardia, not bradycardia as was observed after clonidine injection (Fig. 1, 2). The degree of tachycardia in naloxone-treated sheep was two-to-three times greater in obese than lean sheep (Fig. 5). The clonidine-induced hypertension and bradycardia in both group of sheep were completely unaffected by antecedent treatment with naloxone (Fig. 4, 5).

Other gross behavioral effects of clonidine were observed but not quantified. Clonidine induced noticible salivation in both group of sheep, but clonidine caused distinct respiratory stress (apnea) in obese but not lean sheep. Naloxone by itself had no observable effect on respiration or salivation, but naloxone appeared to attenuate the respiratory-inhibiting effects of clonidine in the obese sheep without affecting in a major way the salivation response to clonidine.

TABLE 1

BLOOD PRESSURE VARIABLES AND HEART RATE IN SHEEP AFTER IV ADMINISTRATION OF 10 μg/kg CLONIDINE

	Lean (n=5)				Obese (n=5)			
Time (min)	SBP (mmHg)	DBP (mmHg)	MAP (mmHg)	HR (beat/min)	SBP (mmHg)	DBP (mmHg)	MAP (mmHg)	HR (beat/min)
-30	92.5 ± 1.9	64.1 ± 2.2	73.6 ± 1.9	49.6 ± 2.4	115.3 ± 4.4	76.4 ± 4.2	89.4 ± 4.2	76.4 ± 7.0
-15	93.9 ± 1.9	64.6 ± 2.1	74.4 ± 1.9	49.6 ± 2.2	116.8 ± 4.1	76.4 ± 4.6	89.9 ± 4.4	75.2 ± 7.3
0	92.7 ± 2.3	64.6 ± 2.9	74.0 ± 2.6	49.6 ± 2.4	115.8 ± 4.3	77.4 ± 5.1	90.2 ± 4.8	75.2 ± 7.4
2	118.4 ± 8.9	84.8 ± 6.6	96.0 ± 7.3	35.6 ± 0.7	133.1 ± 4.4	91.8 ± 3.5	105.6 ± 3.7	44.0 ± 2.8
5	137.2 ± 4.7	93.9 ± 2.8	108.3 ± 2.9	37.6 ± 1.6	174.0 ± 16.3	110.0 ± 7.1	131.4 ± 10.1	57.4 ± 7.9
10	136.2 ± 3.8	95.9 ± 2.9	109.3 ± 1.9	43.6 ± 4.1	167.7 ± 15.3	106.2 ± 4.7	126.7 ± 8.1	63.2 ± 8.5
15	131.4 ± 4.0	90.1 ± 3.2	103.8 ± 3.0	44.8 ± 3.8	161.0 ± 12.5	104.8 ± 4.3	123.5 ± 6.6	67.6 ± 9.3
20	127.1 ± 4.6	84.8 ± 4.4	98.9 ± 4.3	47.8 ± 3.3	141.3 ± 4.3	95.7 ± 3.0	110.8 ± 3.3	74.4 ± 12.0
30	118.4 ± 6.0	78.1 ± 5.1	91.5 ± 5.3	52.0 ± 2.6	126.4 ± 6.9	85.6 ± 4.5	99.2 ± 5.2	74.8 ± 9.2
45	104.0 ± 6.4	74.2 ± 4.4	84.2 ± 4.9	56.0 ± 5.0	120.6 ± 5.1	81.2 ± 3.5	94.4 ± 4.0	75.0 ± 9.0
60	98.8 ± 8.1	69.9 ± 4.4	79.5 ± 5.5	63.6 ± 9.6	116.8 ± 4.5	79.3 ± 3.8	91.8 ± 3.9	75.2 ± 7.0
75	98.3 ± 6.6	67.0 ± 4.2	77.4 ± 4.9	54.4 ± 2.4	114.8 ± 4.0	77.9 ± 4.4	90.2 ± 4.1	74.0 ± 5.4
90	96.8 ± 5.7	65.6 ± 4.3	76.0 ± 4.6	52.4 ± 1.6	115.8 ± 4.0	80.3 ± 4.2	92.1 ± 4.0	43.2 ± 4.7
105	94.9 ± 5.3	66.1 ± 3.7	75.7 ± 4.2	54.2 ± 1.0	112.4 ± 3.2	76.0 ± 4.2	88.1 ± 3.8	72.0 ± 4.6
120	95.9 ± 5.7	66.1 ± 3.8	76.0 ± 4.2	52.8 ± 2.0	113.9 ± 4.5	76.9 ± 4.6	89.2 ± 4.6	71.2 ± 4.4

SBP= Systolic Blood Pressure; DBP= Diastolic Blood Pressure; MAP= Mean Arterial Pressur; HR= Heart Rate

TABLE 2

BLOOD PRESSURE VARIABLES AND HEART RATE IN SHEEP AFTER IV ADMINISTRATION OF 3 mg/kg NALOXONE

	Lean (n=5)				Obese (n=5)			
Time (min)	SBP (mmHg)	DBP (mmHg)	MAP (mmHg)	HR (beat/min)	SBP (mmHg)	DBP (mmHg)	MAP (mmHg)	HR (beat/min)
-30	96.8 ± 4.5	65.1 ± 1.8	75.7 ± 2.5	54.0 ± 2.5	120.1 ± 4.4	81.7 ± 3.3	94.5 ± 3.6	71.0 ± 5.2
-15	96.4 ± 3.1	64.1 ± 2.0	74.9 ± 2.1	54.8 ± 3.4	119.7 ± 4.2	80.2 ± 3.4	93.4 ± 3.7	70.6 ± 4.9
0	96.8 ± 3.0	65.1 ± 1.4	75.7 ± 1.7	54.8 ± 3.8	120.6 ± 4.8	81.2 ± 4.3	94.4 ± 4.4	71.6 ± 5.1
2	119.9 ± 7.7	80.4 ± 4.5	93.6 ± 5.6	62.8 ± 2.9	161.5 ± 6.3	112.2 ± 6.2	129.1 ± 6.3	105.4 ± 10.4
5	115.1 ± 4.8	74.7 ± 2.9	88.2 ± 3.4	61.6 ± 4.3	151.4 ± 6.1	101.1 ± 7.0	118.1 ± 6.6	104.2 ± 12.2
10	110.2 ± 3.8	69.4 ± 2.5	83.0 ± 2.8	58.4 ± 3.5	143.2 ± 5.2	92.7 ± 6.2	109.6 ± 5.9	95.4 ± 9.4
15	107.8 ± 3.4	68.4 ± 2.1	81.6 ± 2.2	58.0 ± 3.3	138.4 ± 4.6	91.8 ± 5.9	107.3 ± 5.4	93.6 ± 10.1
20	105.4 ± 3.0	67.9 ± 2.0	80.5 ± 2.1	58.4 ± 3.5	134.6 ± 5.3	91.3 ± 6.3	105.7 ± 6.1	96.2 ± 9.5
30	103.0 ± 3.2	67.0 ± 1.9	79.0 ± 2.1	57.8 ± 3.4	132.2 ± 4.8	88.9 ± 5.9	103.3 ± 5.6	93.6 ± 9.4
45	103.0 ± 4.1	66.5 ± 2.2	78.7 ± 2.7	59.8 ± 2.9	126.4 ± 3.5	86.0 ± 5.0	99.5 ± 4.6	87.6 ± 6.7
60	100.6 ± 4.0	66.5 ± 2.0	77.9 ± 2.6	60.0 ± 3.0	122.5 ± 4.3	82.2 ± 4.6	95.7 ± 4.6	88.0 ± 7.0
75	98.3 ± 4.4	65.1 ± 2.5	76.2 ± 3.0	58.8 ± 4.1	121.1 ± 4.4	83.1 ± 4.8	95.8 ± 4.7	87.2 ± 6.5
90	96.8 ± 4.3	63.1 ± 2.9	74.4 ± 3.2	56.4 ± 4.0	119.7 ± 4.2	82.6 ± 4.7	95.0 ± 4.6	88.0 ± 5.2
105	95.4 ± 4.5	62.6 ± 2.8	73.6 ± 3.2	56.6 ± 4.3	120.1 ± 4.5	83.1 ± 4.2	95.5 ± 4.4	85.2 ± 6.2
120	93.5 ± 4.1	62.2 ± 2.9	72.6 ± 3.2	56.0 ± 4.2	122.1 ± 4.0	83.6 ± 3.8	96.5 ± 3.8	86.0 ± 5.4

SBP= Systolic Blood Pressure; DBP= Diastolic Blood Pressure; MAP= Mean Arterial Pressur; HR= Heart Rate

TABLE 3

BLOOD PRESSURE VARIABLES AND HEART RATE IN SHEEP INJECTED IV WITH NALOXONE (3 mg/kg) 5 MIN BEFORE CLONIDNE (10 mg/kg)

	Lean (n=5)				Obese (n=5)			
Time (min)	SBP (mmHg)	DBP (mmHg)	MAP (mmHg)	HR (beat/min)	SBP (mmHg)	DBP (mmHg)	MAP (mmHg)	HR (beat/min)
-30	93.9 ± 4.0	64.6 ± 3.1	74.4 ± 3.2	49.2 ± 2.3	118.7 ± 3.6	79.3 ± 4.0	92.5 ± 3.9	70.8 ± 3.7
-15	93.9 ± 3.5	64.6 ± 3.3	74.4 ± 3.2	47.6 ± 2.1	121.1 ± 3.9	82.2 ± 3.4	95.2 ± 3.5	70.0 ± 3.3
-5	93.5 ± 3.4	64.6 ± 2.6	74.2 ± 2.6	48.6 ± 2.0	121.1 ± 3.9	81.2 ± 3.2	94.5 ± 3.4	69.6 ± 4.4
-3	119.9 ± 7.4	80.9 ± 3.0	93.9 ± 4.1	58.8 ± 4.5	166.3 ± 5.9	111.6 ± 6.1	129.8 ± 6.0	95.6 ± 4.8
0	114.1 ± 5.1	74.7 ± 2.8	87.8 ± 3.1	59.2 ± 4.5	151.9 ± 5.6	99.5 ± 5.6	116.9 ± 5.5	96.0 ± 7.6
2	136.7 ± 4.0	97.7 ± 3.8	110.7 ± 3.5	39.4 ± 2.6	168.2 ± 5.2	109.2 ± 7.2	128.8 ± 6.0	49.2 ± 4.9
5	141.0 ± 6.1	91.5 ± 5.7	108.0 ± 5.5	44.8 ± 5.2	169.7 ± 3.7	111.1 ± 3.5	130.6 ± 3.4	55.2 ± 6.9
10	141.5 ± 5.7	92.9 ± 4.7	109.1 ± 4.8	42.8 ± 3.6	168.7 ± 5.8	111.6 ± 4.1	130.6 ± 4.6	56.0 ± 6.3
15	134.3 ± 4.7	86.2 ± 3.7	102.2 ± 3.9	48.6 ± 3.0	158.6 ± 2.5	104.7 ± 2.1	122.7 ± 2.2	61.2 ± 6.1
20	127.6 ± 4.5	82.8 ± 3.6	97.8 ± 3.8	51.6 ± 3.0	152.8 ± 4.0	102.5 ± 2.8	119.2 ± 3.1	66.4 ± 7.4
30	118.9 ± 4.9	77.6 ± 5.7	91.4 ± 5.4	55.4 ± 3.9	141.3 ± 4.8	93.2 ± 4.1	109.2 ± 4.3	68.4 ± 8.2
45	107.8 ± 5.8	73.2 ± 4.8	84.8 ± 4.8	62.4 ± 12	132.2 ± 2.8	85.5 ± 2.5	101.1 ± 2.5	71.2 ± 5.7
60	98.8 ± 5.5	67.9 ± 3.8	78.2 ± 3.9	67.4 ± 15	127.8 ± 3.2	85.0 ± 3.0	99.4 ± 3.1	73.6 ± 4.1
75	93.5 ± 5.5	67.0 ± 3.5	75.8 ± 3.4	62.8 ± 9.4	124.0 ± 4.9	82.6 ± 3.4	96.5 ± 3.8	73.6 ± 3.0
90	95.4 ± 3.3	65.5 ± 3.1	75.5 ± 2.9	55.2 ± 3.7	122.5 ± 4.6	81.7 ± 3.5	95.3 ± 3.8	71.6 ± 3.3
120	94.9 ± 3.4	64.6 ± 2.1	74.7 ± 2.6	64.0 ± 2.1	120.6 ± 6.8	79.8 ± 5.0	93.4 ± 5.6	71.2 ± 2.8

SBP= Systolic Blood Pressure; DBP= Diastolic Blood Pressure; MAP= Mean Arterial Pressur; HR= Heart Rate

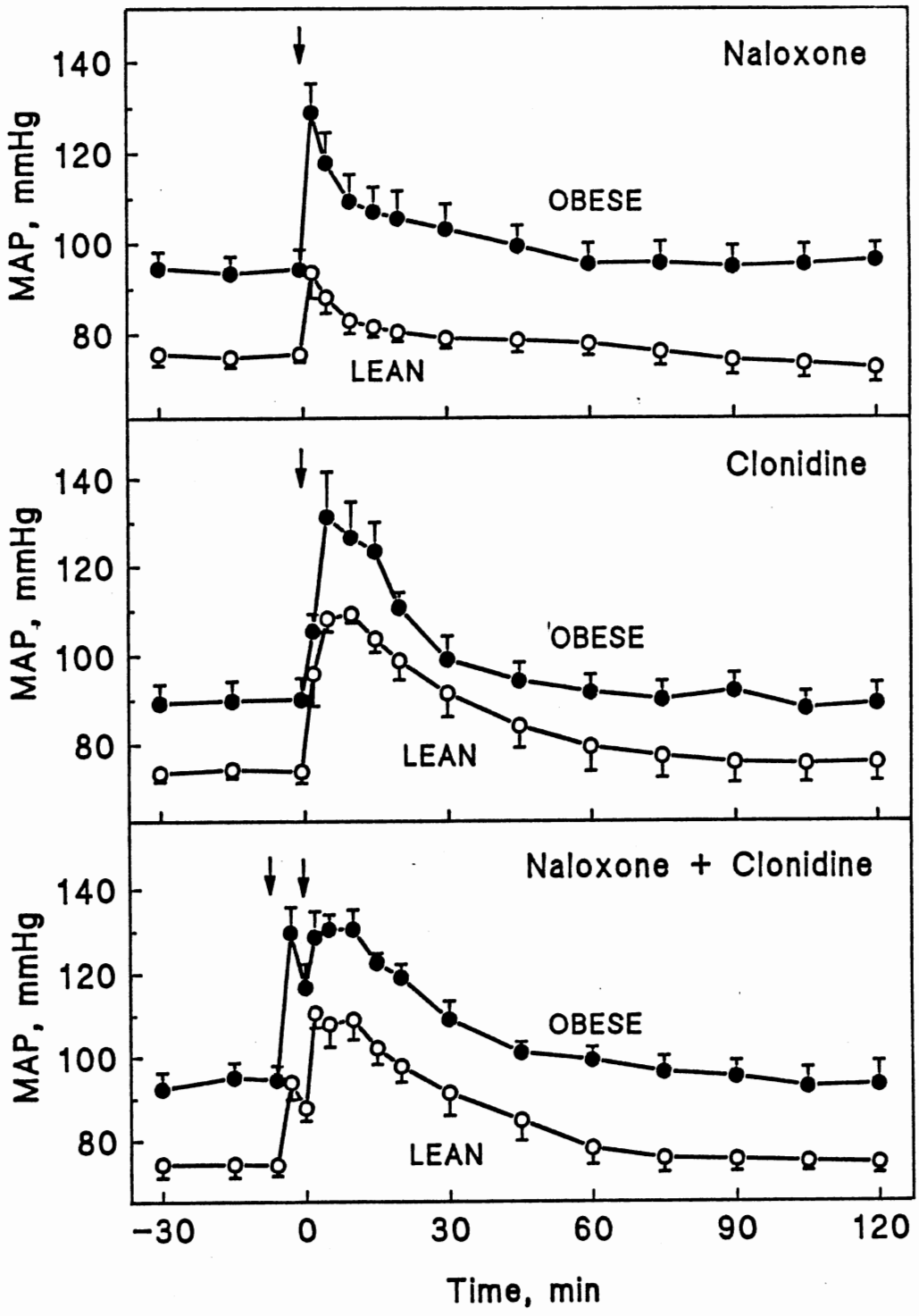

Figure 1. Changes in absolute mean arterial pressure (MAP) in lean (n=5) and obese (n=5) sheep injected with clonidine (10μg/kg) alone, naloxone (3 mg/kg) alone, or naloxone 5 min before clonidine (bottom panel). Arrows indicate time of injections. Values are means ± SE.

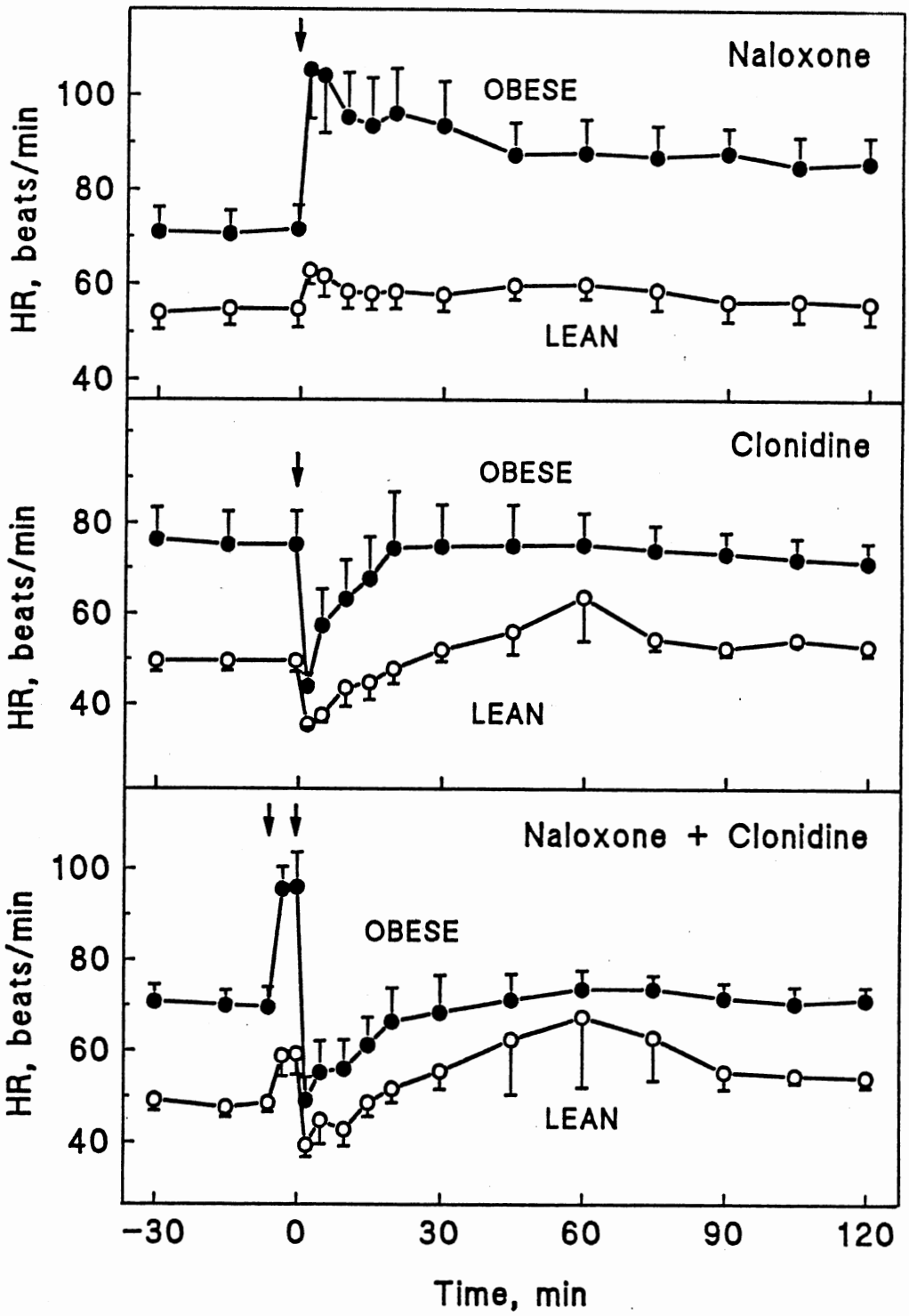

Figure 2. Changes in absolute heart rate (HR) in lean (n=5) and obese (n=5) sheep injected with clonidine (10μg/kg) alone, naloxone (3 mg/kg) alone, or naloxone 5 min before clonidine (bottom panel). Arrows indicate time of injections. Values are means ± SE.

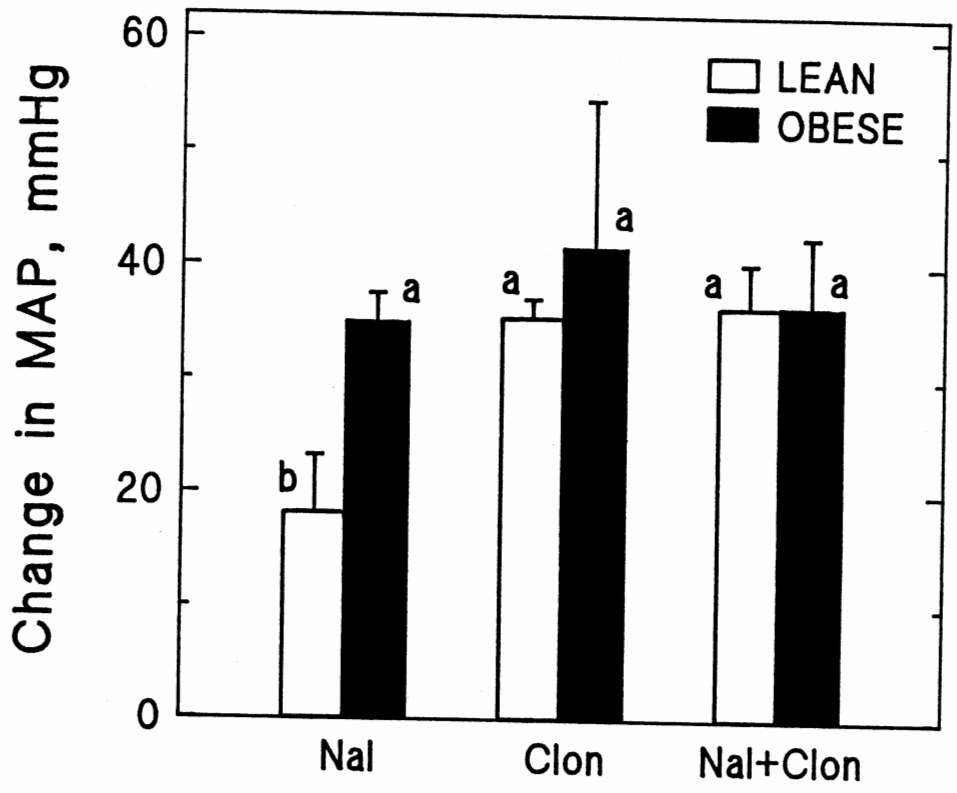

Figure 3. Maximum changes in mean arterial pressure (MAP) in lean (n=5) and obese (n=5) sheep injected iv with clonidine (10 μg/kg) alone, naloxone (3 mg/kg) alone, or naloxone 5 min before clonidine. Values are means ± SE and were calculated using the difference between the peak MAP developed and the corresponding basal values in each sheep. The greatest change in MAP from basal occurred in the first 5 min after time 0 (see Fig 2, 3). Probability values from split-plot analysis of variance for treatment on MAP response were body condition NS, treatment 0.005, and their interaction 0.1. Mean with similar lower case letter are not different (P>0.05) as determied by Duncan's new multiple range test.

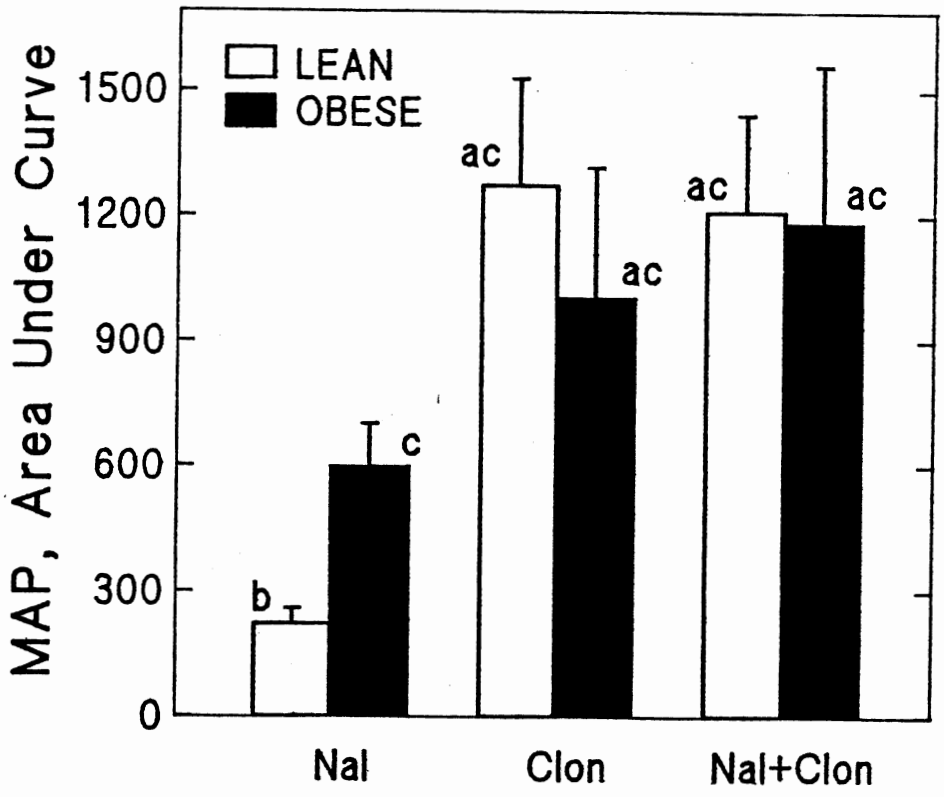

Figure 4. Mean arterial pressure (MAP) response in lean (n=5) and obese (n=5) sheep injected iv with clonidine (10μg/kg) alone, naloxone (3mg/kg) alone, or naloxone 5 min before clonidine. Response calculated as area above basal MAP plus one standard deviation of basal. Probability values from split-plot analysis of variance for treatment effects were body condition NS, drug treatment 0.005, and their interaction 0.1. Mean with similar lower case letter are not different (P>0.05) as determined by Duncan's new multiple range test.

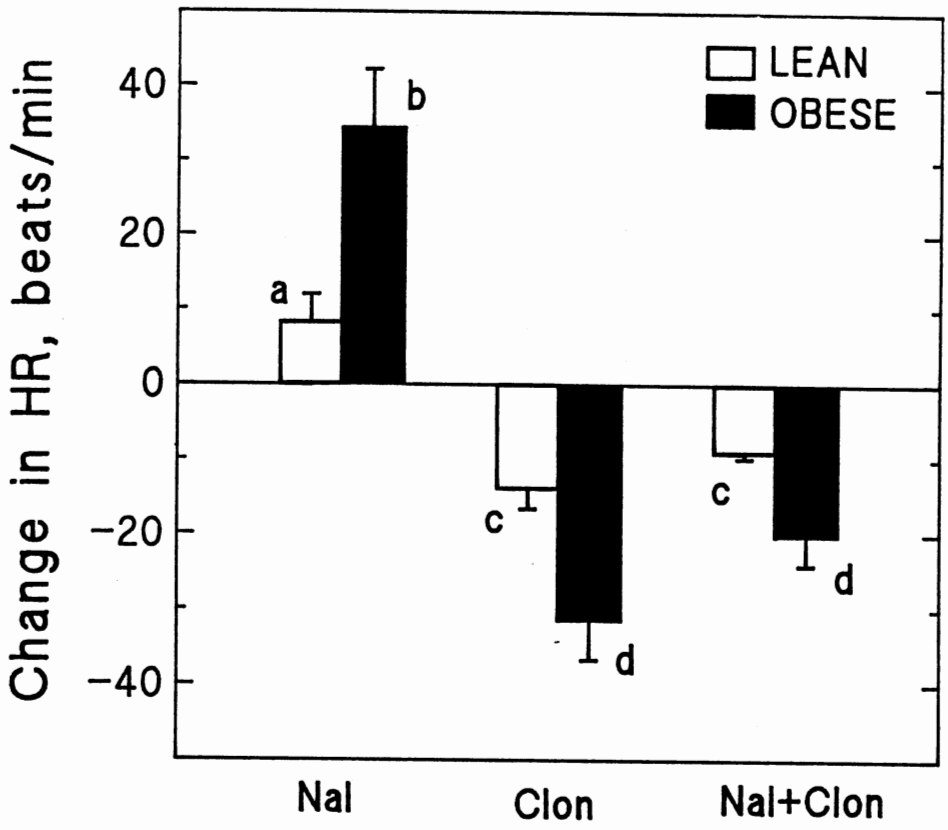

Figure 5. Maximum changes in heart rate (HR) in lean (n=5) and obese (n=5) sheep injected iv with clonidine (10 μg/kg) alone, naloxone (3 mg/kg) alone, or naloxone 5 min before clonidine. Values are means ± SE and were calculated using the difference between greatest or lowest HR developed and the corresponding basal values in each sheep. The greatest change in HR from basal occurred in the first 5 min after time 0 (see Fig 2). Probability values from split-plot analysis of variance for treatment on HR were body condition NS, drug treatment 0.001, and their interaction 0.05. Means with similar lower case letters in each panel are not different (P>0.05) as determined by Duncan's new multiple range test.

Discussion

The blood pressure response to iv clonidine in many species is biphasic in that a brief hypertensive response precedes the development of a chronic hypotensive response (Kobinger 1978; Isaac 1980). In humans, high doses of clonidine given per os or iv results in hypertensive then hypotensive relatively whereas relatively lower doses result in hypotension generally (Pettinger 1980). Clonidine induces hypotension and bradycardia via activation of central α_2-adrenoceptors that lead to suppressed sympathetic outflow to heart and vasculature and enhanced vagal drive to the heart (Kobinger 1978; Pettinger 1980). On the other hands, the hypertensive response to clonidine is elicited by clonidine activation of vasoconstrictive α_2-adrenoceptors in vascular smooth muscle. The blood pressure response to clonidine activation has been explained on the basis of the balance between clonidine activation of central hypotensive-related α_2-adrenoceptors vis-a-vis activation of peripheral vasoconstrictive-related α_2-adrenoceptors (Pettinger 1980; Isaac 1980).

In this study, clonidine had a singularly hypertensive response in both lean and obese sheep. Clonidine-induced hypertension without hypotension was reported previously in sheep by Eisenach and colleagues (1987). Explanation of divergence in blood pressure response to iv clonidine between humans and sheep is not clear. The same dose of clonidine that produced hypotension in humans may induce hypertension in sheep because ruminants in general are more responsive to α_2-agonists than nonruminants (Hopkins 1978). Additionally, less than 2% of iv injected clonidine enters the brain in nonruminants (Jarrott et al 1987) and differences in brain uptake of clonidine between sheep and humans may explain the divergence in demonstration of clonidine-induced hypotension between these species. The initial, transient hypertension seen in other species injected iv with clonidine (Pettinger 1980; Isaac

1980) was intensified in sheep possibly because of a greater reactivity of peripheral α_2-adrenoceptors and (or) reduced brain uptake of clonidine in sheep as compared with other species. The suggestion of lesser brain uptake of clonidine in sheep as compared with other species is tenuous because α_2-agonists clearly have potent centrally-mediated analgesic effects in ruminants (Eisenach et al 1987) and other species (Maze and Tranquilli 1991).

The hemodynamic responses to iv naloxone in sheep were unusual in that many previous studies reported no cardiovascular effects of naloxone in normotensive or hypertensive rats (Mastrianni and Ingenito 1987; Mosqueda-Garcia and Kunos 1988; Lasky et al 1989), normotensive dogs (Pinto et al 1989), or hypertensive humans (Bramnert and Hokfelt 1983). Naloxone caused hypertension and tachycardia in sheep and these responses were greater in obese than lean sheep. Dunlap et al (1989) increased blood pressure without affecting heart rate in foetal sheep injected iv with very high doses of 10 to 40 mg/kg naloxone. The same authors (Dunlap et al 1989) obtained hypertension and tachycardia when foetal sheep were exposed to an extraordinarily high dose of 80 mg/kg naloxone.

Sites and mechanisms of action for naloxone's effects on blood pressure and heart rate in sheep are not clear from this study. Ordinarily, increased blood pressure is associated with reflex bradycardia (West 1985), but naloxone-treated sheep displayed tachycardia in the face of increased blood pressure. This suggests that naloxone somehow masked the baroreceptor reflex in sheep. The hypertensive response to naloxone could be due to central and(or) peripheral effects of naloxone that resulted in vasoconstriction and increased blood pressure. Feria et al (1990) reported that naloxone potentiates cardiovascular responses to sympathetic amines possibly by enhancing catecholamine discharge in peripheral adrenergic nerve

endings. Central effects of naloxone on blood pressure and heart rate cannot be ignored. Petty and DeJong (1982) reported that direct injection of the opioid agonist, ß-endorphin, into the nucleus tractus solitarius (NTS) produced hypotension and bradycardia at low doses of ß-endorphin but a rise in blood pressure and heart rate at high doses of ß-endorphin. That direct injection of enkaphalins in the NTS of rats also caused an increase in blood pressure (Petty and DeJong 1983), suggests that different opioid receptors involved in cardiovascular regulation exist in the NTS. Naloxone blockade of one or more types of opioid receptors in the NTS of sheep might explain their naloxone-induced hypertension and tachycardia.

Pretreatment with naloxone had no effect on clonidine's ability to increase blood pressure and decrease heart rate in sheep in this study and naloxone pretreatment also did not reverse clonidine's antinocioceptive effect in sheep (Eisenach et al 1987). Pretreatment with naloxone by iv or intracerebroventricular routes also did not affect the hypotensive response to clonidine in normotensive cats (Shropshire and Wendt 1983), normo-tensive and hypertensive humans (Watkins et al 1980; Bramnert and Hokfelt 1983) and normotensive, anaesthetized Wistar-Kyoto rats (Mastrianni and Ingenito 1987). On the other, naloxone pretreatment abolished or inhibited significantly the hypotensive response to clonidine in normotensive conscious dogs (Pinto et al 1989), spontaneously hypertensive rats (SHR) that were anaesthetized (Farsang and Kunos 1979; Mastrianni and Ingenito 1987) and in normotensive rats that were anaesthetized (Mosqueda-Garcia and Kunos 1988) or conscious and unrestrained (Chang et al 1989). The ability of naloxone to block the cardiovascular effects of clonidine appears to be readily demonstratable in normotensive and hypertensive rodents but not so in normotensive or hypertensive sheep or humans.

Clonidine and naloxone injected alone had similar effects on blood pressure

increase but markedly divergent effect on heart rate in sheep. Response to combined clonidine and naloxone treatment in sheep equalled that of clonidine injected alone. Feria et al (1990) showed convincingly that naloxone enhances pressor response to epinephrine, norepinephrine and phenylephrine, all α-adrenoceptor agonist, mechanism might involve naloxone blockade of presynaptic delta-type opioid receptors in peripheral nerve endings. Naloxone pretreatment potentiated the initial pressor response to clonidine in conscious normotensive Sprague-Dawley rats (Dixon and Chandra 1985; Chang et al 1989), which are results somewhat consistent with the finding of Feria et al (1990). Lack of an antagonistic, additive or synergistic effect of naloxone on the pressor response to clonidine in sheep is not readily explainable. One might suggest that naloxone and clonidine affect blood pressure in sheep by different and independent routes, or that naloxone works upstream of and with diametrically opposite effects to that of clonidine in their regulation of the same central neural relay system that controls baroreceptor function, vasoconstriction, and heart rate. In species where naloxone blocks cardiovascular response to clonidine, the locus of naloxone effect is within the baroreceptor relay center of the medulla (Isaac 1980).

Naloxone-induced tachycardia was strikingly much greater in obese than lean sheep both in terms duration and magnitude. Naloxone-induced tachycardia was still prominent in obese sheep through the full 180-min period after naloxone injection; the greater naloxone-induced rise in blood pressure in obese than lean sheep was dissipated by 60 min after naloxone injection. These results suggest differences in opioid receptors between lean and obese sheep, particularly opioid receptor mechanisms concerned with increasing heart rate. Previous work showed that naloxone had approximately 4-fold greater inhibitory effects on feed intake in obese than lean sheep (see Chapter IV). Collectively, results of these studies suggest

down-regulation of opioid receptors, or specific receptor types, in anatomical sites concerned with appetite and cardiovascular regulation. The greater responses to naloxone in obese than lean sheep are expected findings to antagonist stimulation of a system that has undergone receptor down-regulation or desensitization.

In summary, the hypertensive and bradycardic responses to iv clonidine in lean and obese sheep were unaffected by prior exposure to naloxone. The naloxone-induced hypertension and tachycardia were greater in obese than lean sheep, which are results considerably different from the absent cardiovascular effects of naloxone in dogs and rodents.

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CHAPTER VIII

INFLUENCE OF OBESITY ON PHARMACOKINETICS OF NALOXONE IN RAMBOUILLET SHEEP

Introduction

Drug distribution, biotransformation and excretion may be changed due to alteration in body composition in obese compared with normal body weight humans and animals. Increased proportion and absolute amount of adipose tissue and increased lean body mass occur in human obesity (Baecke et al 1982; Forbes and Wells 1983). Changes in the size of these compartments in obese compared with lean individuals may alter distribution of lipophilic and lipophobic drugs (Abernethy and Greenblatt 1982, 1984). Additionally, adiposity may lead to altered protein binding of drug as well as altered processes of hepatic and renal biotransformation and excretion (Benedek et al 1984; Abernethy and Greenblatt 1986).

Volume of distribution of drugs and their distribution between intravascular and extravascular compartments may be affected by solubility properties of drug in fat, or by altered binding of drug to plasma proteins (Abernethy and Greenblatt 1986). Obesity had no effect on plasma protein binding of verapamil or cimetidine to albumin, whereas increased concentration of α_1-acid glycoprotein concentration explained the greater protein-bound fraction of propranolol in plasma of obese compared with lean humans (Benedek et al 1983; 1984). Biotransformation of drugs in obesity may be influenced by pathophysiological processes associated with obesity, such as fatty infiltration and

fibrosis of the liver (Adler and Schaffner 1979) and cardiovascular dysfunction, which may alter hepatic blood flow and peripheral organ perfusion (Rodighiero 1989; Crandall and DiGirolamo 1990). Finally, increased renal glomerular filtration in some obese subjects might be predicted to increase clearance of drugs that can be filtered and excreted by the kidney (Stokholm and Brochner-Mortensen 1980). These data support the importance of understanding parameters of drug disposition in obese individuals because drug therapy is commonplace in combating the pathophysiological problems of dyslipidemia, hypertension and diabetes that are associated with obesity (Abernethy et al 1986; O'Connor and Feely 1987).

Animal models can serve as valuable tools in studies to distinguish the potential differences in drug disposition between lean and obese individuals. Two animal models, the Zucker obese rat (fa/fa) and the cafeteria-fed male Sprague Dawley rat, have provided preliminary data on the effects of obesity on pharmacokinetics of prednisolone, phenobarbital, verapamil, and d-fenfluramine (Blouin et al 1987; Fracasso et al 1988; Nichols et al 1989; Rice et al 1989). However, the genetically obese Zucker rat may have limited application because of its impaired renal function and cytochrome P-450 activity that are unrelated to the obese state (Litterst 1979; Abernethy and Greenblatt 1986). Limitations of the cafeteria-fed Sprague Dawley rat model of obesity identified to date are that only male rats become obese and that the unusual cafeteria diet itself can affect drug pharmacokinetics; obese rats consume a cafeteria diet of chocolate chip cookies, orange juice, condensed milk and high fat while lean control rats are fed standard rat chow (Abernethy and Greenblatt 1986).

Naloxone is generally recognized as a short acting pure opiate antagonist. It is rapidly metabolized by the liver and eliminated by the kidney (Weinstein et al 1971; 1973). The primary metabolite of naloxone is naloxone-3-glucuronide, although the

N-dealkylation and reduction of the 6-ketone group of naloxone also takes place to some extent (Fujimoto, 1969; Weinstein et al 1971; 1973). Although naloxone has been used in numerous studies in sheep, the pharmacokinetics of naloxone in sheep and domestic ruminants is unknown. Where necessary, investigators have extrapolated available data on the pharmacokinetics of naloxone in rats to sheep. The objectives of this study were to determine the pharmacokinetics of naloxone in sheep and determine how dietary obesity affected naloxone kinetics in sheep.

Materials and Methods

Animals

Five lean (46 ± 2 kg) and 5 obese (77 ± 3 kg) ewes used in this experiment were housed individually in a room with constant light and temperature (21 ± 1 °C). Conditions for induction of dietary obesity and maintenance of lean sheep are described in detail elsewhere (Chapter II). Briefly, lean adult Rambouillet ewes were fed a pelleted hay-grain diet at maintenance to keep them in lean body condition or were fed the same diet at ad libitum (obese) until a static body weight was achieved. During static phase obesity, lean and obese sheep were fed the same diet at maintenance such that all sheep were in zero-energy balance. Body fat estimated as percent of body weight was approximated 27% in lean sheep and 37% in obese sheep (McCann et al 1991). Percent lipid in the eviscerated carcass was 23 ± 2% in lean sheep and 46 ± 1 % in obese sheep. Chronic catheters were placed in the caudal vena cava as described in Chapter 4.

Drug Administration and Sample Collection

Naloxone (Sigma, St.Louis, MO, USA) solution was prepared in sterile saline

and filtered through a 0.2 μm cellulose acetate filter (Micro Filtration System, USA) immediately before each experiment. Catheters were placed in the jugular vein of sheep at least 12 h before sample collection. Naloxone (3 mg/kg) in saline was injected (0900 h) in 16-h fasted sheep via the vena cava catheter which was flushed immediately with 10 ml of saline. Blood samples (5 ml) were collected via the jugular catheter at 30, 15, and 1 min before and 2, 5, 10, 20, 30, 45, 60, 90, 120, 240 and 480 min after naloxone injection. Samples were dispensed immediately into chilled glass tubes containing 50 μl of mixture of benzamidine (200 mg/ml) and heparin (5000 U/ml). Plasma obtained by centrifugation (1000 x g, 4 C) was stored frozen (-30 C). Experiments were conducted in a one lean and one obese sheep at a time.

Naloxone Assay

Instruments and Chemicals. Plasma naloxone concentrations were determined by the HPLC and electrochemical detection using a modification of method described by O'Connor et al (1989). The HPLC system (Waters, Millipore Co, Milford, MA, USA) comprised of a pump (model 510), autosampler with refrigerator unit (WISP, model 712), electrochemical detector (model 460), and chromatographic software (Maxima 820). A C-18 reverse phase radial pack cartridge column (10 x 0.5 cm) with 4 μ particles (Waters, Millipore Co. Milford, MA, USA) were used to resolve naloxone and naltrexone (internal standard). The voltage of the glassy carbon electrode was maintained at +8.5 mV against the Ag/AgCl reference electrode.

Chloroform, 2-propanol, ammonium dihydrogenphosphate, octanesulfonic acid and sodium bicarbonate were all analytical grade chemicals from Fisher Scientific Pittsburgh, PA. Disodium EDTA was obtained from Sigma, St. Louis, MO, and 1-propanol was purchased from Curtis Matheson, Houston, TX.

Sample Extraction and HPLC Analysis. Plasma (0.5 ml) was placed in a 5 ml vial and alkalinized with 0.5 ml of a mixture (pH 8.6) of 0.1 M ammonium dihydrogen-phosphate, 0.05 M sodium bicarbonate and 0.043 M sodium carbonate. Vials were capped and mixed for 10 sec before 3.0 ml chloroform-2-propanol (9:1, v/v) were added and mixed until the contents turned buttery. Vials were centrifuged at 3000 X g for 15 min at room temperature, vigorously tapped once and then centrifuged again. Approximately 2.7 ml of the lower organic phase containing naloxone and naltrexone were transferred into clean 12x75 mm borosilicate test tubes. Tubes were evaporated to dryness under nitrogen at room temperature (90-120 min) and their contents redissolved in 100 µl of mobile phase. Tubes were vortexed, centrifuged at 1000 x g for 5 min at 22 C and their contents were transferred to autoinjector vials, which were then loaded into refrigerated (8 C) autosampler. Standards for naloxone (10, 50, 100, 200, 400, 800 ng/ml) and naltrexone (400 ng/ml) were prepared in sheep plasma. Inter-assay precision was determined by analysis of pooled sheep plasma spiked with naloxone and naltrexone (400 ng/ml each). In a preliminary analysis, detector linearity was demonstrated for standards containing naloxone concentrations of 10 to 2400 ng/ml.

Mobile phase (pH 4.5) consisted of 0.1 M ammonium dihydrogenphosphate, 0.54 M disodium EDTA, 0.9 µM octanesulfonic acid and 3 to 10 % 1-propanol. All solvents were filtered (0.2 µm HA filter; Waters, Millipore Co. Milford, MA, USA) and degassed by helium sparging. An additional 0.2 µm filter (Puradisc 25 AS, Whatman Inc, Clifton, NJ) was connected to the pump inlet to filter and degas the mobile phase. The flow rate of the mobile phase was set at 0.7 ml/min for best separation of eluting peaks.

Pharmacokinetic Analysis

Data were analyzed using a microcomputer program for pharmacokinetic modelling (Bourne 1986). Initial estimates of the first-order rate constants were obtained by subjecting mean data for each body condition to analysis using several different models. Choice of the appropriate pharmacokinetic model was based on lowest weighted sum of squares, F test, and Akaike's information criterion (AIC) value for individual data (Akaike 1974; Boxenbaum et al 1974; Yamaoka et al 1978). Areas-under-the-curve (AUC) were calculated by trapezoidal approximation between the first (0 h) and last sampling times.

Pharmacokinetic values of lean and obese were compared using Students' unpaired t test and Mann-Whitney U test ($t_{½\beta}$, $t_{½\alpha}$, Vc, $Vd_{(area)}$, $Vd_{(ss)}$ and Cl_B). Significance was tested at $P<0.05$.

Results

Mobile phase containing 4.5 % 1-propanol provided adequate separation of naloxone, naltrexone (internal standard) and benzamidine (anticoagulant). At an electrode potential of +0.85 V , the lower limit of detection was 1 ng/ml. Intra- and inter-assay coefficients of variation were 4.1 and 4.3%, respectively. Recovery of naloxone and naltrexone averaged 78 ± 4 and 76 ± 4%, respectively. Deterioration of sensitivity after 50 to 60 injections was obviated by refurbishing the electrode (-0.6 V for 60 sec) after every 30 injections. After refurbishing, electrode was stabilized by pumping (0.1 ml/min) mobile phase for 12-16 h.

Disposition of naloxone in plasma after intravenous administration was best described by a two-compartmental open model using the equation:

$$C_p=Ae^{-\alpha t}+ Be^{-\beta t}$$

where C_p is the concentration of naloxone in plasma at time t, A and B are the zero-time blood concentration intercepts of the biphasic disposition curve, and α and ß are the hybrid rate constants related to slopes of the distribution (α) and elimination (ß) phases. Pharmacokinetic parameters were calculated from exponents of coefficients of disposition curves (Gibaldi and Perrier 1975; Baggot 1977).

Pharmacokinetic variables of naloxone in lean and obese sheep are presented in Table 1. Naloxone was rapidly eliminated (t½ ß) after intravenous administration in both lean 43.08 (range= 29.3-53.7 min) and obese 37.7 min (range= 24.4-82.8 min) sheep.

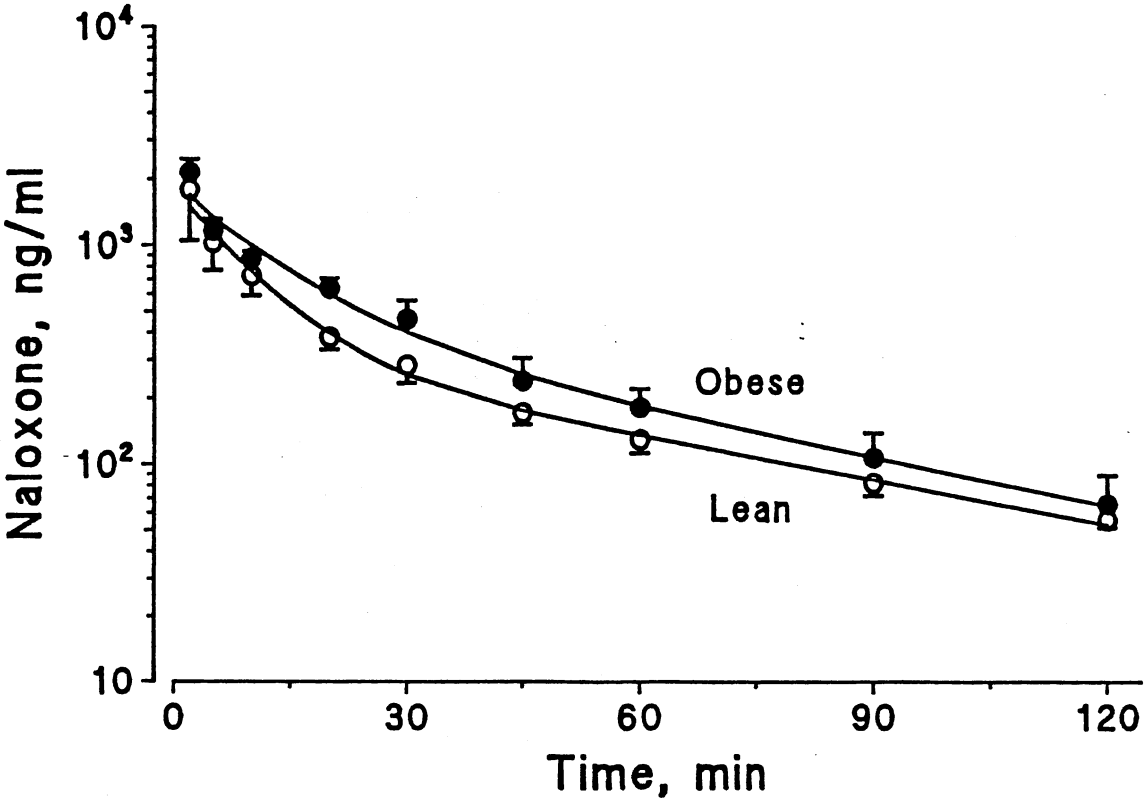

Figure 1. Disposition of naloxone (3 mg/kg) in plasma after intravenous administration to lean (n= 5) and obese (n=5) ewes. Data are mean ± SD. Components of equation describing curves are shown in Table 1.

Median volume of distribution ($Vd_{(area)}$) of naloxone was large in lean (4.51 L/kg) and obese (3.5 L.kg) sheep, indicating extensive distribution and possibly accumulation within extravascular tissue. Median clearance of naloxone was higher in lean (88.18 $ml.kg^{-1}.min^{-1}$) than obese (77.11 $ml.kg^{-1}.min^{-1}$) sheep.

TABLE 1

PHARMACOKINETIC PARAMETERS (mean ± SD) OF INTRAVENOUSLY INJECTED NALOXONE (3 mg/kg) IN LEAN AND OBESE SHEEP.

Parameters	Lean (n=5)	Obese (n=5)
A, ng/ml	1814 ± 968	2282 ± 772
B, ng/ml	413 ± 172	573 ± 301
α, min^{-1}	0.19048 ± 0.09222	0.28154 ± 0.23220
β, min^{-1}	0.01710 ± 0.00448	0.01851 ± 0.00743
K_{10}, min^{-1}	0.06296 ± 0.01814	0.06690 ± 0.02796
K_{12}, min^{-1}	0.08668 ± 0.04924	0.15643 ± 0.14971
K_{21}, min^{-1}	0.05794 ± 0.04577	0.07672 ± 0.06342
t½α, min [a]	3.55 (2.07-7.06)	2.41 (1.12-15.26)
t½β, min [a]	43.08 (29.3-53.7)	37.73 (24.36-82.83)
V_c, L kg^{-1} [a]	1.35 (0.81-2.94)	1.08 (0.73-1.71)
$V_{d(area)}$, L kg^{-1} [a]	4.51 (4.03-8.63)	3.54 (2.89-5.95)
$V_{d(ss)}$, L kg^{-1} [ab]	3.62 (2.80-6.43)	2.92 (2.55-3.23)
Cl_B, ml kg^{-1} min^{-1} [ab]	88.18 (71.08-111.44)	77.11 (49.77-82.31)
AUC, ng h ml^{-1} [b]	35900 ± 6440	47972 ± 9336

k_{10} = first-order elimination rate constant; k_{12}, k_{21} are the first-order rate constants describing distribution between central (plasma) and peripheral compartment (tissue); t½ α = distribution half-life after intravenous administration; t½ ß = elimination half-life after intravenous administration; V_c = volume of the central compartment; $Vd_{(area)}$ = apparent volume distribution calculated using AUC; $Vd_{(ss)}$ = apparent volume of distribution at steady state; Cl_B = body clearance of the drug.

[a] presented as median and range

[b] lean and obese differ ($P<0.05$)

Discussion

The objectives of this experiment were to describe naloxone pharmacokinetics in sheep and assess the impact of obesity on such kinetics. The elimination half-life of naloxone in sheep (≈ 40 min) was similar to that in rats (40 min) (Tepperman 1983). Fishman et al (1973) and Berkowitz (1975) reported an elimination half-life of 57 to 90 min for naloxone in man whereas Kleiman-Wexler et al (1989) reported a half-life of 16 to 31 min in rats. The relatively rapid elimination of naloxone is consistent with its short duration of action. Rapid distribution of naloxone throughout the body is important for its antagonistic effect on opiatergic receptors in CNS and peripheral tissues. Naloxone rapidly accesses the brain tissue of rats (< 5 min; Weinstein et al 1973) and can attain a two fold greater concentration in brain than serum of rats (Tepperman et al 1983).

The volume of distribution ($Vd_{(area)}$) in lean (4.5 L/kg) sheep was similar to that in obese (3.5 L/kg) sheep. A larger $Vd_{(SS)}$ of naloxone in lean than obese sheep may represent the significantly greater percent water composition in the body of lean compared with obese sheep. Obese sheep have more fat per unit body weight than lean sheep. Fat tissue has a low blood perfusion rate (Rowland and Toze 1980). The greater percent fat in obese sheep is associated with reduced percent body water composition. Therefore, the larger $Vd_{(SS)}$ of naloxone in lean than obese sheep could be explained by their greater. In addition, because fat has a relatively low blood perfusion rate, the distribution of naloxone among the compartments of unit weight of volume of distribution would differ between lean and obese sheep with relatively greater perfusable nonfat tissue compartment in the lean than obese sheep.

Vd of naloxone was similar to Vd (2.8 to 3.9 L/kg) of naloxone in man (Fishman et al 1973). Although the Vd of naloxone was large in sheep, a greater Vd (8 L/kg) was reported in rats (Tepperman et al 1983). Rapid metabolism of naloxone by

the liver in vitro (Fujimoto 1969; Weinstein et al 1971; Weinstein et al 1973) explains the very low bioavailability (5%) of oral naloxone in man (Kalant and Roschlau 1989). In fact, Weinstein and co-workers (1973) emphasized that the low potency of oral administration of naloxone in man is associated with a very effective first-pass hepatic extraction of naloxone. Cheymol et al (1987) point out that altered hepatic function and blood flow need to be considered when dealing with pharmacokinetic disposition of drugs in obese individuals. Rapid distribution and clearance of the drug into well perfused tissues such as liver and kidney, with an accompanying rapid elimination, may have reduced the impact of adiposity on the disposition of naloxone.

Generally, lipophilic drugs have larger volume of distribution than nonlipophilic drugs (Galletti et al 1989). A larger Vd of naloxone in lean than obese sheep is contrary to the characteristics of lipophilic drugs in that the lipophylic nature of naloxone should have increased its distribution in fat tissue of obese sheep. It seems clear that differences in drug lipophilicity are insufficient to explain complications of drug pharmacokinetics in obese individuals (Bickel 1984; Cheymol et al 1987). A recent pharmacokinetic study of propranolol, a lipophilic antihypertensive drug, showed an equal half-life but reduced Vd and clearance in obese compared with lean humans (Cheymol et al 1987), which are results agreeing with those reported here for naloxone in lean and obese sheep.

We conclude that obesity did not alter the elimination half life, but had reduced the clearance and volume of distribution of naloxone in sheep.

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CHAPTER VIII

SUMMARY

Obesity is a pathophysiological condition in humans that is associated with hyperinsulinemia, hyperglycemia and hypertension. How obesity may lead to dysregulation of appetite and blood pressure has been the topic of many investigations in humans and rodents. The opiatergic system clearly plays an important role in the regulation of appetite and blood pressure. Therefore, the primary objectives of this thesis were to study whether a dietary obesity alters opiatergic regulation of appetite and blood pressure in sheep, a new animal model of obesity.

Our experiments showed that dietary obesity altered appetite drive in sheep. Chronic obesity reduced appetite drive in sheep. The appetite-inhibitory effects of the opioid antagonist, naloxone, were 4 times greater in obese than lean sheep. This appetite effects of naloxone were considered to be central and independent of coincidental changes in plasma insulin, glucose and free fatty acids.

Obesity in sheep was associated with a 25 % increase in mean arterial pressure (MAP) and 50 % increase in heart rate (HR). The baroreflex sensitivity was found to be similar in lean normotensive and obese hypertensive sheep, but the baroreceptor system setting had shifted rightward in obese compared with lean sheep. The reflex bradycardiac response to α_1-agonist (phenylephrine) was greater in obese than lean sheep.

Greater hypertensive and tachycardiac responses to naloxone in obese than lean sheep indicated that obesity had altered opiatergic regulation of blood pressure.

The altered appetite and cardiovascular responses in obese sheep to naloxone cannot be attributed to differences in disposition of naloxone because the pharmacokinetic parameters of naloxone were similar in lean and obese sheep.

Clonidine, a hypotensive drug produced hypertension rather than hypotension in normotensive lean and hypertensive obese sheep. The degree of clonidine-induced hypertension was less in obese than lean sheep at low dose clonidine (5 μg/kg) but was similar in both groups at high dose clonidine (10 μg/kg). The clonidine-induced rise in mean arterial pressure and reflex bradycardia in lean and obese sheep were unaffected by antecedent treatment with iv naloxone.

We conclude that development of dietary obesity alters opiatergic regulation of appetite and blood pressure in sheep possibly by down regulation of opioid receptors, and that obesity alters adrenergic receptor control of blood pressure.

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