Investigation of High Salt Intake and Adipogenesis in Rats

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by

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Abstract

Sodium chloride has been scrutinized of late, not only for its liberal use as a food preservative but for its contribution to disease states. The causal role of habitual high salt intake in increasing blood pressure and exacerbating hypertension has been established. A growing body of evidence strongly suggests that hypertension contributes to other conditions such as cardiovascular disease, obesity, stroke, and type II diabetes. Rates of hypertension are significantly higher in obese individuals. These comorbidities have been positively associated with the modern Western diet. Worldwide, many government and health organizations have called for public policy measures to mandate sodium reductions in processed foods. The objective of this study is to assess the possible direct association between high salt intake and adipocyte hypertrophy and hyperplasia as it relates to obesity.
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Introduction

Dietary Sodium

Sodium chloride holds a unique position in the annals of human history and in health and disease research. For millions of years of evolution, hominids consumed no more salt than other mammals; our diet contained less than 0.25 g of salt per day (He and MacGregor, 2009). Within the past five thousand years, human societies spent enormous amounts of energy procuring salt for food preservation as it allowed for the storage and stockpiling of food to provision them over the barren winter months. This principal use catapulted salt’s economic importance and it became the most taxed and traded commodity in the world (He and MacGregor, 2009). Many a war was fought over its control and salt has infamously been used as a means of compensation – we earn our salary or salt. For over a century, the medical profession has recognized that there exists a relationship between dietary sodium consumed in excess and high blood pressure. Today, we are at the cusp of worldwide governmental actions to reduce salt intake on a population-wide scale.

Dietary sodium is usually consumed in the form of sodium chloride, which contains 40% Na⁺ by weight. In addition to sodium chloride, Na⁺ in foods can be found in the form of sodium alginate, sodium sulfite, monosodium glutamate, sodium caseinate, disodium phosphate, sodium citrate, sodium benzoate and a whole host of other food additives commonly found in processed foods. He and MacGregor (2009) have traced the patterns of human salt consumption and describe how historically, sodium intake peaked in the late 1800s, just before the advent of refrigeration and at the
height of pre 20th century global trade. Since then, sodium intake had been declining until recently with the increase in consumption of highly salted processed and convenience foods.

Americans currently consume on average 9-12 grams of salt per day or 3,600-4,800 mg of sodium (Palar and Sturm, 2009). This is almost twice the amount recommended by the current 2005 U.S. Dietary Guidelines for Americans. Many recent epidemiological analyses and reviews have been published in anticipation of the 2010 Dietary Guidelines for Americans, due for release this Fall. These guidelines are the cornerstone of U.S. nutrition policy and nutrition education and influence the funding of various governmental nutrition programs – from the K – 12 school lunch program to nutritional guidelines for those individuals in incarceration. Issued every five years since 1980, the publication of this 7th Edition is expected to take into consideration the recent epidemiological research on processed food consumption and other worldwide salt reduction programs, and further reduce their current 2,300 mg/day recommendation. The Institute of Medicine (IOM), the World Action on Salt and Health (WASH), and the World Health Organization (WHO) recommend that adults consume no more than 2,300 mg of sodium per day, approximately one teaspoon of table salt, with even lower recommended levels of consumption for those over 40 years of age, African-Americans, salt-sensitive, and hypertensive individuals (Palar and Sturm, 2009). No more than 1500 mg/day is recommended for the above subgroup that encompasses 70% of the U.S. population. Palar and Sturm (2009) suggest that fewer than 30% of Americans actually consume less than 2,400 mg of sodium per day. Clearly, there exists a mismatch between stated public health targets and dietary habits of Americans.
Many in the medical and public health communities propose that the only way to bring about population-wide reductions in sodium intake is to mandate salt reduction in foods made by the manufacturers of processed foods (Mohan et al., 2009, Havas et al., 2003, Palar and Sturm, 2009, He and MacGregor, 2009). The consensus among public health scientists is that disease-based approaches to caring for individuals at high risk for sodium-related health problems are resource-intensive and ultimately benefit few people (Mohan et al., 2009). In contrast, population-wide reductions in sodium intake are cost effective and could prevent large numbers of cardiovascular and stroke events in normotensive, hypertensive, and susceptible populations alike. Public health interventions to reduce the burdens of paying for health costs incurred by caring for those with hypertension and related conditions have been implemented in countries such as Finland, since the 1970s, and more recently the U.K., Canada, France, and Australia. Proponents of sodium reduction policies cite numerous studies that analyze and predict the potential societal savings of taking public policy action and which project savings in the billions, of both dollars and quality-adjusted life years. Reducing dietary salt by 3 g/day is projected to save 194,000 – 392,000 quality-adjusted life years and $10 - 24 billion in health care costs in the U.S. annually (Bibbins-Domingo et al., 2010). Bibbins-Domingo et al. (2010) suggest that such interventions could be cost saving - even if a modest population-wide reduction of 1 g/day were achieved between 2010 and 2019, the public policy changes would be more cost effective than using medications to lower blood pressure in all persons with hypertension in that same time frame. Most recently, in 2009, the New York City Department of Health has coordinated a nationwide effort to mandate sodium reductions in packaged and restaurant foods, the goal of
which is to reduce Americans’ salt intake by 25% over the next 5 years (NYC.gov, 2010). This is seen by many as necessary action to avert a critical health care crisis. To date, the FDA and the U.S. Department of Agriculture, unlike the Food Standards Agency in the U.K, have not required any reductions in the sodium content in processed foods in spite of the preponderance of medical research. Some scientists, a minority, have argued that humans have a natural salt appetite (innate behaviors that drive us to consume salt) and that sodium intake is tightly controlled by the central nervous system by way of renin, angiotensin II, and aldosterone hormonal signals - with excess sodium excreted by the kidneys. McCarron et. al (2009) suggest that efforts to reduce sodium consumption beyond physiological set points would be futile.

Reducing dietary sodium can only be effective if the sources of sodium are known. The innate sodium in foods account for 5% of dietary intake, another 15% is added at the table or in the cooking, and 80% is consumed in the form of processed foods (He and McGregor, 2009). High dietary salt intake is then a result of a large portion of daily calories consisting of processed food. Some estimates claim that on average, 1000 calories of processed food contributes 1,600 mg of sodium (FSA, 2009). Processed foods that do not taste salty often contain hidden sodium. A breakfast cereal, Multigrain Cheerios™ contains 200 mg of sodium per 28 g serving; the same serving size of potato chips averages 160 mg of sodium. Notably, processed food consumption has increased the most in children and it is possible that children from the age of three to four onwards consume similar amounts sodium as adults (St-Onga, 2003). Changing the population’s exposure to sodium in the food supply, it would
seem, represents the challenge most amenable to a public health solution (Havas et al., 2003).

Pathologies Related to High Dietary Sodium

During the past century, dietary sodium has been the subject of intense research and scientific debate. Papers on the pathophysiological roles of salt and risks imposed on human health by excess consumption number in the tens of thousands. The causal role of habitual high dietary salt intake or chronic salt loading in increasing blood pressure and exacerbating hypertension has been established through experimental, epidemiological, longitudinal, migration, meta-analysis, and intervention studies (Strazzullo et al., 2009). High salt intake, or salt loading, has also been positively associated with cardiovascular disease, left ventricular hypertrophy, stroke, obesity, stomach cancer, diabetes, metabolic syndrome, end-stage renal disease, oxidative stress in various tissues, and even preventable causes of death in animals and humans. Heart disease and stroke are, respectively, the number one and three ranked causes of death in the United States, with cancer ranking at number two (Census, 2005). Salt’s role in blood pressure increases is indisputable (Appel, 2009) and high blood pressure is the single largest risk factor for both stroke and cardiovascular mortality in the U.S., responsible for an estimated 45% of all cardiovascular deaths (Goodarz et al, 2009). In studies with borderline hypertensive rats, psychological stress due to physical crowding was shown to increase salt appetite
and the resultant increase in salt intake was shown to modify pressor responses to stress, setting up a vicious feedback loop (Ely, 2007).

Worldwide, raised blood pressure also accounts for 62% of strokes (Appel, 2009). High blood pressure levels – systolic levels above 120 mmHg and diastolic levels above 80 mmHg – and the risk of developing cardiovascular disease is strong, continuous, graded, consistent, independent, and etiologically significant (Havas et al., 2003). The risks of heart attack, congestive heart failure, stroke, and end-stage renal disease, also increase as blood pressure rises. Hypertension, defined as systolic pressure of 140 mmHg or higher and a diastolic blood pressure of 90 mmHg or higher, affects 50 million U.S. adults and 1 billion worldwide, with 17 - 30% of those cases of hypertension attributable to excess dietary sodium (Kearney et al., 2005). Most hypertension is uncontrolled and nearly half of patients with hypertension do not receive treatment (Kearney et al., 2005). Recent data from the Framingham Heart Study (Havas et al., 2003), one of the longest running longitudinal studies on cardiovascular health in a large specific population, show that hypertensive individuals have a 1.2 to 2.5 times greater risk of experiencing a heart attack, a stroke, or heart failure within 10 years than those whose blood pressure level is below 120/80 mmHg. Based on the effects of high salt intake on blood pressure and hypertension, and their prominent role in promoting cardiovascular disease, it has been suggested that a population-wide reduction in salt intake could substantially reduce the incidence of cardiovascular disease (WHO, 2007).

It has been recognized that acquired factors such as increased weight, obesity, excessive salt intake, alcohol consumption, physical inactivity, and environmental stresses are involved in the pathogenesis of hypertension (Agarwal et al., 2005). Even
modest reductions in salt intake have brought about significant effects on blood pressure in both hypertensive and normotensive individuals. The landmark Dietary Approaches to Stop Hypertension (DASH)-Sodium trial was a randomized trial that compared the effects of three levels of sodium intake (1150, 2300 and 3450 mg/day) and the consumption of more fresh fruits and vegetables than the typical American diet on blood pressure. A clear dose-response relationship was found. Sodium reduction alone from a high level to a low level reduced mean blood pressure by 8.3/4.4 mmHg among individuals with hypertension and by 5.6/2.8 mmHg among normotensives; the greatest reductions in blood pressure were seen in the two groups most at risk for developing hypertension, African-Americans and older persons (Sacks et al., 2001). The combination of salt reduction from 3450 mg to 1150 mg and the DASH diet lowered mean blood pressure by 11.5/5.7 mmHg and 7.1/3.7 mmHg, respectively, among those with and without hypertension (Sacks, et al., 2001). Similar meta-analyses, most notably, He et al. (2002) and Strazzullo et al. (2009) also suggested a dose-response relationship with salt reduction; they assert that higher salt intake is unequivocally associated with high blood pressure, greater incidence of stroke, and greater incidence of cardiovascular events. The National Health and Nutrition Examination Survey Epidemiologic Follow-up Study showed that among participants aged 25-74 years who were overweight, a 100 mmol/day or 2,300 mg/day increase in intake of sodium was associated with an increased relative risk of coronary disease mortality of 61%, an increase in stroke mortality of 89%, and an increase in all-cause mortality of 39% (Havas et al., 2003). These assessments were reached after adjustment for blood pressure, age, and BMI. Given that more than 2/3 of Americans are now overweight or
obese (NIH, 2009), this study has major implications with regard to the importance of reducing sodium intake above and beyond concerns over blood pressure.

In addition to hypertension, two of the most intensely studied pathologies related to high dietary sodium are insulin resistance (as it relates to Type II diabetes) and obesity. Both of these comorbidities coalesce in insulin resistance syndrome or syndrome X, also known as metabolic syndrome. Metabolic syndrome is a constellation of abnormalities associated with an increased risk of the development of obesity, Type II diabetes, and atherosclerotic vascular disease – leading to heart disease and stroke (Lago et al., 2009). Excessive salt intake has been found to induce the development of insulin resistance - the condition where normal amounts of insulin are inadequate to produce normal insulin responses from cells (Usukura et al., 2009). Donovan et al. (1993) showed that normotensive subjects given a high salt diet were more insulin resistant than those on a low salt diet. A common condition of insulin resistance is hyperinsulinemia or excess levels of circulating insulin in the blood (Rocchini, 1992). This is commonly referred to as pre-diabetes and may develop into diabetes if left unmonitored. Insulin resistance and enhanced sensitivity of blood pressure to sodium are thought to be underlying mechanisms for the development of metabolic syndrome (Hoffman et al., 2009). Metabolic syndrome is characterized as having at least 3 of the following: (1) abdominal obesity with large waist circumference, (2) serum triglycerides ≥ 150 mg/dL or 1.7 mmol, (3) serum HDL ≤ 40 mg/dL or 1 mmol, (4) blood pressure ≥ 130/85 mmHg, and (5) fasting plasma glucose (FPG) ≥ 110 mg/dL (NHLBI, 2009).

Currently, 47 million adults in the U.S., ≈25%, have metabolic syndrome. Normally, the postprandial state is characterized by a temporal rise in blood glucose and a
concomitant rise in plasma insulin levels. Insulin has anabolic effects on skeletal muscle and adipose tissue and regulates glucose uptake by those tissues. Insulin-mediated glucose uptake in adipose cells (adipocytes) in particular account for the “disposal” of approximately 20% of this rise in blood glucose, with the rest being taken up by skeletal muscle, nervous tissue, erythrocytes and other tissues (Whitney and Rolfes, 2008). Insulin resistance by muscle and adipose tissue, associated with high dietary sodium, therefore should have lipolytic effects. Both processes of lipogenesis and lipolysis are metabolically in flux in white adipose tissue and contribute to the size of adipose deposits (Fonseca-Alaniz et al., 2007). More will be said about the metabolic activity of adipose tissue in the next section.

Dietary sodium’s role in hypertension has been established. The prevalence of hypertension in the U.S. has been mirrored by the rise in obesity and obesity-related health problems and has led many researchers (and research dollars) down the road of trying to find the connections between the two – obesity-hypertension is a robust axis of study. The causes of obesity are extremely complex and varied and the urgency seen in finding solutions to this epidemic reflect the magnitude of the problem. In the past two decades, obesity has increased in every state, in both genders, and across all ages, races, and educational levels (Whitney and Rolfes, 2008). In 1990, no state in the nation had prevalence rates of obesity above 15%, in 2005 only four states had prevalence rates below 20% (CDC, 2010). An estimated 66% of adults in the U.S. are now overweight or obese, as defined by a BMI ≥ 25 and BMI ≥ 30, respectively. According to the World Health Organization, this epidemic of obesity has spread worldwide, affecting over 300 million adults, in both industrialized nations and
developing countries (Ogden et al., 2006). Genetics plays a causative role in few modern cases of obesity, as in the case of Prader-Willi syndrome – a genetic disorder characterized by excessive appetite, massive obesity, short stature, and often cognitive disabilities (Whitney and Rolfes, 2008). Even with the discovery of the hormone leptin in 1994 and the consequences of the ob/ob recessive gene in mice, environmental influences such as diet and lifestyle are the more probable causes of the dramatic change we have seen within the past twenty years. Increasing consumption of soft drinks, fats and oils, and sodium appear to be the major dietary factors that are positively associated with childhood obesity (Boumtje et al., 2005). A genetic component, however, does play a central role in individual variations in energy balance, metabolism, susceptibility to obesity-related comorbidities, chronic disease, and higher risk distribution of body fat. Individual hormonal variation, learned behaviors, and neural influences involved in appetite regulation add an additional layer of complexity to the etiology of obesity. These topics are beyond the scope of this discussion and the integration of all of these pathophysiological factors remains to be clarified.

Although there is no clear establishment of cause-and-effect in obesity and hypertension, the rate of occurrence between the two disorders (often coupled within the same individual) cannot be explained by mere coincidence (Davey and Hall, 2004). Roughly 29% of the U.S. population over the age of 18 and 65% of the population over the age of 60 is hypertensive (Hajar and Kotchen, 2003). Hypertension occurs in 50% of obese individuals, which is significantly higher than the prevalence in the general population (Bravo et al., 2006). One simple explanation is that blood volume and thus cardiac output increases in proportion to body mass, however, Zhang and Reisin (2000)
point out that obesity is associated with systemic and renal oxidative stress and sodium retention, which in turn lead to increases in extracellular volume. The resultant lymphatic transport of this excess fluid increases cardiac output leading to activation of the renin-aldosterone-angiotensin II (RAAS) system and sympathetic nervous system stimulation; the combination of which, contributes to the development of hypertension and left ventricular hypertrophy. Current evidence suggest that sodium retention due to either kidney compression and/or abnormal renal sodium handling, associated with obesity, is the major influence behind the mean increase in arterial pressure that often accompanies weight gain and obesity.

Obesity in the population could be considered a measure of how well adapted we are to efficiently storing excess energy during positive energy balance states. The mammalian ability to ensure continuous availability of energy despite the unpredictable supplies in the environment is a major determinant of survival (Frühbeck et al., 2001). The capacity to efficiently store excess energy as triglycerides in adipose tissue, and quickly release those stores when required has contributed to our evolutionary success. Although this capacity if often maladaptive and detrimental in our modern environment, adipose tissue and adipocytes still function as principal regulators of energy homeostasis. We now turn our attention to this tissue in particular.

The Physiology and Organization of White Adipose Tissue

The predominant type of adipose tissue, commonly referred to as “fat” in mammals is white adipose tissue (WAT) (Ahima, 2006). White adipose tissue is the
body’s largest energy reservoir and the typical adult with 15 kg of body fat has over
110,000 kcal of lipid fuel stores – potentially provisioning 2000 kcal/day for 2 months
(Ramsay, 1996). The primary role of this organ is to store free fatty acids (FFA) as
triglycerides (triacylglycerol) during periods of caloric excess (lipogenesis) and to
mobilize this reserve and release free fatty acids for ATP synthesis via the Kreb’s Cycle
during periods when expenditure of calories exceeds intake (lipolysis). This central
function distinguishes WAT from brown adipose tissue (BAT) in mammals. Brown
adipose tissue’s contribution to energy balance involves its thermogenic capabilities, the
transfer of food calories to heat production. This organ is essential for classical
norepinephrine and SNS-induced non-shivering thermogenesis that distinguishes
mammals from other vertebrates (Cannon and Nedergaard, 2004). Heat production
from BAT is especially necessary for cold-acclimation, post-natal survival, entry into the
febrile state to fight off infection, and during arousal from hibernation. Research points
to the unique qualities of uncoupling protein-1 (UCP-1), found in high concentrations in
internal membranes of BAT mitochondria, to bring about this function necessary to life
in cold surroundings (Cannon and Nedergaard, 2004). UCP-1 or thermogenin acts as a
proton channel, diverting H⁺ from ATP-synthase and allowing the stored electrochemical
energy to dissipate as heat. BAT in mammals and to a lesser extent in adult humans
makes up a small percentage of total fat stores, is centrally located with a limited
distribution; it’s function and/or dysfunction is generally understood to not be
responsible for obesity and obesity-related health problems. The distribution,
composition, histology, endocrine function, and dysfunction of WAT have been the
focus of obesity research.
In mammals, WAT is the only tissue in the body that can dramatically change in mass after adult size is reached and appears to have an unlimited ability for caloric storage (Hausman et al., 2001). Normal values for fat mass in humans range from 13 – 21% in males and 22 – 31% in females with mean values shifting up or down depending upon physical condition (Whitney and Rolfes, 2008). In obesity, fat mass exceeds 22% in males and 32% in females, and the genetically and hormonally influenced distribution of this fat is associated with the most deleterious effects of weight gain. WAT is located in three major anatomical areas – subcutaneous, dermal, and intraperitoneal (Hausman et al., 2001). In addition, region-specific adipose tissue localized to one site does not always respond in the same manner as adipose tissue in another. The basic organization of WAT reveals the presence of a heterogeneous amalgam of mature adipocytes, adipocyte precursors, monocytes, macrophages, fibroblasts, connective tissue, and stromal cells with mature adipocytes normally making up 30% tissue (Vásquez-Vela et al, 2008). The exact contribution of each cell type varies with location, vascularization, innervation, hormonal influence, and particular disease state. Adipocytes can vary enormously in size, from 20 µm – 200 µm in diameter and after a critical size is reached, growth is followed by division and proliferation (Frühbeck et al., 2001). Research has revealed that when the population of adipocytes increases in both size and number (hypertrophy and hyperplasia), as in the case of obesity, the tendency is for this normal heterogeneity to diminish. In states of obesity, 50% of cellular content of adipose tissue may actually be inflammatory macrophages compared to only 10% in lean individuals (Weisberg et al., 2003). Increasingly, researchers are discovering that obese adipose tissue differs structurally and functionally from lean adipose tissue. WAT
adipocytes are unilocular, ≈ 90% of the cell volume is a single large lipid droplet surrounded by a phospholipid monolayer and associated coat proteins of the perilipin family (Ducharme and Bickel, 2008). The cytoplasm forms a thin layer around this central triglyceride droplet and the nucleus is flattened and pushed to the periphery of the cell. BAT adipocytes, by contrast, are multilocular cells of a more polygonal shape, containing considerable cytoplasm and dispersed lipid droplets. The high relative concentration of mitochondria and cytochrome oxidase contributes to their brown color.

The cellular development of adipocytes involves both hypertrophy and hyperplasia of the stem cell population and subsequent differentiation into either adipocytes or vascular cells (Figure 1). Both neural and hormonal activities govern proliferation and differentiation of adipocytes, and although preadipocytes begin their transformation into adipocytes in the late embryonic stage, this ability is not lost later in life (Vásquez-Vela et al, 2008). Although difficult to culture, the majority of laboratory studies involving development of adipose cells in vitro have taken advantage of two distinct cell lines. A pluripotential preadipocyte culture derived from the stromal-vascular fraction of adipose tissue from various species and a unipotential murine cell line (3T3-L1) that can only give rise to adipocytes have provided researchers with a suitable system for exploring adipocyte differentiation and a number of regulatory mechanisms (Vásquez-Vela et al, 2008). Advances in understanding of adipocyte gene expression, membrane transport, differentiation, and cellular metabolism stem from in vitro manipulations of these cells lines. However, in vivo studies demonstrate that adipocyte proliferation and differentiation and their interactions with systemic neural, endocrine, paracrine, and autocrine factors are very complex. Notable progress has
been made since the 1990s to improve in vivo studies in both rats and humans. *In situ* microdialysis techniques and open-flow microperfusion allow stable sampling of macromolecules from the interstitial space of adipose tissue (Lafontan, 2008). From these and other advances in gene expression profiling, a clearer picture of how WAT serves as both endocrine and immunologic organ is emerging.
Figure 1. Schematic representation of adipose tissue proliferation and differentiation into either adipocytes or vascular cells. (Reprinted with permission from Hausman et al., 2001).
Adipose Tissue as Endocrine and Immune Organ

Some of the most exciting research within the past ten years has dramatically changed how adipose tissue has been perceived. Classically referred to as a storage depot for excess dietary triglycerides, the image of WAT is now one of a dynamic organ that serves both endocrine and immunological purposes. Some would argue that it also has a tendency to be “self-serving” – an insidious secretory powerhouse of endocrine, paracrine, and autocrine hormones - or adipokines. WAT has the ability to regulate its metabolism, cell size, number, and composition using these signals.

The identification of leptin, the adipocyte-derived hormone, and the leptin gene (Lep) in 1994, followed by its cognate receptor Ob-R located within the ventral medial hypothalamus (VMH) in 1995 paved the way for this area of research (Kershaw and Flier, 2004). Leptin is a 167 amino acid residue hormone almost exclusively derived from adipose tissue that shares structural similarities with cytokines. Adipocytes have been shown to secrete leptin at rates proportional to adipose tissue mass and leptin is considered to be a homeostatic hormone that functions to reduce appetite and increase energy expenditure through sympathetic nervous system stimulation (Bravo et al., 2006). Excess caloric intake increases the size of adipocytes, which in turn influences leptin synthesis, with larger cells containing and secreting higher levels than smaller cells (Ahima and Flier, 2000). To date, six leptin receptor isoforms are known and have been found both within the CNS and the periphery (blood vessels, skeletal muscle, adipocytes, immune cells, etc). The primary effects of leptin involve the mobilization of lipolytic pathways of energy expenditure as it signals to the hypothalamus that energy
sufficiency has been met (Kershaw and Flier, 2004). Specifically, leptin is known to inhibit the strongly anabolic effects of insulin-adipocyte binding and subsequent lipoprotein lipase (LPL) activity, increase fatty acid oxidation in skeletal muscle, and inhibit the synthesis of ACC, an enzyme essential in the conversion of carbohydrates into long chain fatty acids (Lago et al., 2009). Both expression and secretion of leptin is reduced with calorie restriction and weight loss and therefore the manipulation of the anorexigenic effects of leptin were once thought to offer a solution for obesity. As it turned out, the exogenous introduction of leptin to obese patients with already elevated leptin levels did not result in decreased food intake, or increased energy expenditure and weight reduction. The theory of selective leptin-resistance was proposed to explain this contradiction. Support for this theory was demonstrated in studies with obese and lean mice, whereby lean littermates exhibited the appropriate leptin-mediated SNS responses while the obese mice remained in a positive energy balance state (Bravo et al., 2006). The exact mechanism for leptin resistance remains to be clarified, however, Kershaw and Flier (2004) point out that the most sensitive portion of the leptin dose-response curve resides within the food restriction and re-feeding ranges in murine models – not the supraphysiological range associated with obesity.

Leptin secretion is mediated by a variety of other hormones and macromolecules - most importantly, insulin (reciprocal effects), glucocorticoids, TNF-α, steroid hormones, free fatty acids, growth hormone, thyroid hormone T3, and peroxisome proliferator-activated receptor gamma agonists (Kershaw and Flier, 2004). Additional endocrine and paracrine effects of leptin include regulation of hematopoiesis, angiogenesis, and bone development. With the expansion of adipose tissue comes the requirement of
increased vascular tissue support and leptin may be the essential messenger. The implication of leptin in immune responses in addition to its well-documented endocrine role has been supported by studies showing direct effects on macrophage cytokine production and proliferation of T cells (Frühbeck et al., 2001).

Adiponectin is a 244-residue protein that is produced exclusively by mature adipocytes and primarily acts upon receptors found in skeletal muscle and the liver (Lago et al., 2009). It is unique in the fact that unlike most adipokines, levels of adiponectin decrease with increased adiposity. Adiponectin displays protective anti-inflammatory and antiatherosclerotic effects (Stehno-Bittel, 2008). An inverse relationship also exists between adiponectin levels and insulin resistance, when insulin sensitivity improves (as occurs after weight loss) it is followed by an increase in adiponectin levels. The metabolic effects of adiponectin include increased hepatic insulin sensitivity and decreased non-esterified fatty acid (NEFAs) influx, increased skeletal muscle glucose use (insulin receptor phosphorylation) and fatty acid oxidation, and decreased expression of adhesion molecules in vascular epithelium reducing macrophage accumulation (Kershsaw and Flier, 2004). Attempts at administering exogenous adiponectin to reverse insulin resistance, has only been effective in rodent models of diabetes so far (Stehno-Bittel, 2008).

Two of the most studied pro-inflammatory adipokines include tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6). Although these cytokines are not unique to adipose tissue, evidence suggests that when adipose tissue enlarges in the obese state – it acts dysfunctionally and is involved in the hyper-secretion of pro-inflammatory and pro-diabetic adipokines accompanied by the decreased production of protective
cytokines like adiponectin (Hajer et al., 2008). It cannot be overstated that all fat is not alike and obesity leads to an immunologically pathological state where chronic inflammation ensues from secretory dysfunction of obese adipocytes. Additionally, most of these harmful adipokines are released as paracrine agents which recruit macrophages and T helper cells to the tissue, changing the composition of the WAT and the normal metabolic cycles of lipogenesis and lipolysis found in non-obese fat. Hajer et al. (2008) point out that “obesity leads to adipose tissue dysfunction and dysfunction of adipose tissue leads to obesity”.

The expression and secretion of TNF-α are increased in the obese state and there exists a positive correlation between TNF-α levels and insulin resistance, especially in women (Fonseca-Alaniz et al., 2007). The underlying mechanism is understood as a stress response by the adipocytes in a state of chronic hypoxia characterized by increased levels of secreted FFAs (Stehno-Bittel, 2008). The majority of TNF-α is secreted by macrophages (specifically M1-macrophages) which take up a disproportionate share of space in obese WAT. This local paracrine loop involves large adipocytes and their tendency to release more saturated FFAs into the interstitial space between the cells (Hajer et al., 2008). These FFAs then bind to toll like receptors (specifically TLR-4) on the macrophages resulting in signal transduction pathways that lead to NF-κB activation and increased TNF-α production. The TNF-α then activates the adipocytes producing further lipolysis and expression of various genes and translation of gene products, among them IL-6 (Figure 2). IL-6 is an inflammatory mediator that plays a role in impairing insulin signaling, reducing LPL activity, and
increasing lipolysis. While this reduction in LPL activity may limit weight gain in the short term, chronically

Figure 2. Adipocyte-macrophage interaction leading to dysfunction. As adipocytes enlarge, increasing levels of adipocyte-derived FFAs are released which stimulate already present macrophages to produce TNF-α. Saturated FFAs bind to the toll-like receptor-4 (TLR-4) which is expressed in macrophages resulting in NF-κB activation and increased TNF-α production. TNF-α activates human adipocytes in vitro, thereby enhancing expression of various genes (ICAM-1, IL-6, MCP-1). (Reprinted with permission by Hajer et al., 2008).
elevated IL-6 increases hepatic synthesis of pro-coagulant molecules (Frühbeck et al., 2001). While secreted by adipose and other tissues during basal conditions, this stress induced adipokine has the potential to increase 60-fold (Frühbeck et al., 2001).

Clearly, the emerging picture of WAT as an endocrine and immunological organ incorporates cross-talk between and co-stimulation of a multitude of hormonal signals. Localized hormonal positive and negative feedback loops interact with the tissue itself. Obesity research has centered on uncovering the distinguishing characteristics of both normal/basal activity and abnormal/elevated activity of WAT. If triglycerides were sequestered into adipocytes without further metabolic influence, we would not observe the collection obesity-related health problems that we do. Obviously, the scenario is more complex. To date, more than one hundred different adipokines have been isolated from fat cells, including fatty acids, prostaglandins, steroids, and those similar to the aforementioned hormones that regulate energy balance (Stehno-Bittel, 2008). While it is not within the scope of this paper to describe all of them, Table 1 summarizes a few of the most well-studied protein and non-protein substances produced and secreted by WAT. Regional differences in WAT metabolism also differ in their innervation density, further frustrating obesity researchers in their attempts to transpose \textit{in vitro} processes to \textit{in situ} explanations. Our focus now is to shed some light on a few key factors that control adipogenesis and then return full circle to obesity-hypertension and high salt diets.

\textbf{Adipocyte Lipolysis and Lipogenesis}
At a fundamental level, energy storage and release from adipocytes in the form

Table 1. Protein and non-protein factors produced and secreted by white adipose tissue (WAT)

<table>
<thead>
<tr>
<th>Substance</th>
<th>Main Biological Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptin</td>
<td>Signals to the CNS about the body's energy status, stimulation of lipolysis, autocrine regulation of leptin expression</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>Increases sensitivity to insulin, anti-inflammatory cytokine, attenuates the progression of atherosclerosis</td>
</tr>
<tr>
<td>Resistin (in rodents)</td>
<td>Increases insulin resistance, acts on glucagon receptors</td>
</tr>
<tr>
<td>Tumor necrosis factor-α (TNF-α)</td>
<td>Stimulation of lipolysis, reduces sensitivity to insulin, regulation of leptin secretion, inhibition of adipocyte differentiation</td>
</tr>
<tr>
<td>Interleukin-6 (IL-6)</td>
<td>Inhibition of LPL activity, induction of lipolysis, pro-inflammatory cytokine, reduces insulin sensitivity</td>
</tr>
<tr>
<td>Adipsin</td>
<td>Activates alternative complement pathway</td>
</tr>
<tr>
<td>Acylation stimulating protein (ASP)</td>
<td>Stimulates triglyceride synthesis in WAT</td>
</tr>
<tr>
<td>Angiotensinogen</td>
<td>Angiotensin II precursor, regulation of arterial vasoconstriction, increases lipogenesis</td>
</tr>
<tr>
<td>Plasminogen activation inhibitor-1 (PAI-1)</td>
<td>Blocks fibrinolysis</td>
</tr>
<tr>
<td>Tissue Factor</td>
<td>Initiates coagulation cascade, acts on TNF-α receptors</td>
</tr>
<tr>
<td>Vascular endothelial growth factor (VEGF)</td>
<td>Stimulates angiogenesis in WAT</td>
</tr>
<tr>
<td>Visfatin</td>
<td>Imitates insulin, predominantly produced by visceral fat, glucose lowering effect</td>
</tr>
<tr>
<td>Monobutyrin</td>
<td>Vasodilator, induces angiogenesis</td>
</tr>
<tr>
<td>Transforming growth factor-β (TGF-β)</td>
<td>Regulates differentiation and proliferation in pre-adipocytes, development and apoptosis of adipocytes</td>
</tr>
<tr>
<td>Insulin-like growth factor-1 (IGF-1)</td>
<td>Stimulates proliferation and differentiation in adipocytes, mediates growth hormone</td>
</tr>
<tr>
<td>Hepatocyte growth factor (HGF)</td>
<td>Stimulates development and differentiation of adipocytes</td>
</tr>
<tr>
<td>Macrophage migration inhibitory factor (MIF)</td>
<td>Paracrine action in WAT, pro-inflammatory processes and immunoregulation</td>
</tr>
<tr>
<td>Lipoprotein lipase (LPL)</td>
<td>Enzyme controlling hydrolysis of triglycerides in lipoproteins</td>
</tr>
<tr>
<td>Cholesterol ester transfer protein (CETP)</td>
<td>Transfers cholesterol esters between lipoproteins</td>
</tr>
<tr>
<td>Apo-E</td>
<td>Protein component of lipoproteins, especially VLDL</td>
</tr>
<tr>
<td>Prostaglandins</td>
<td>Regulation, active during inflammation, blood coagulation</td>
</tr>
<tr>
<td>Glucocorticoids</td>
<td>Generated by action of 11-hydroxysteroid dehydrogenase, transforms cortisone into cortisol in WAT</td>
</tr>
<tr>
<td>Estrogens</td>
<td>Produced by action of aromatase, control WAT distribution</td>
</tr>
<tr>
<td>Apelin</td>
<td>Energy metabolism</td>
</tr>
</tbody>
</table>
of triglycerides is the more readily quantifiable measure of metabolic activity. Elevated rates of lipolysis and lipogenesis reflect elevated rates of energy metabolism. As previously described, enlarged adipocytes release FFAs into the surrounding interstitial space and induce not only inflammatory processes but the proliferation and differentiation of pre-adipocytes, increasing the potential for lipid storage (Vásques-Vela et al., 2008).

Adipocytes have the ability to grow in size by *de novo* lipogenesis from non-lipid substrates such as amino acids, but more commonly by the uptake of FFAs from the plasma via chylomicrons and very low density lipoproteins (VLDL) (Lafontan, 2008). *De novo* lipogenesis in humans occurs more efficiently in the liver than in adipocytes. The primary route of triglyceride accumulation involves the processing of these large lipoproteins in the luminal space of capillaries adjacent to adipocytes. Lipoprotein lipase (LPL) is synthesized and secreted by adipocytes and is upregulated within the cell under the influence of insulin binding to its cognate receptor. Through mechanisms not fully delineated, LPL translocation occurs and this enzyme finds its way to the luminal surface of endothelial cells where it acts to hydrolyze triglycerides into its constituents of glycerol and non-esterified fatty acids (NEFAs) (Lafontan, 2008). After the NEFAs are absorbed, re-esterification is necessary for efficient storage as triglycerides – again, insulin plays a central role in esterification. Insulin-mediated glucose uptake by adipocytes via GLUT 4 receptors also contributes to the formation of fatty acids within the cell. Glucose excess is oxidized via glycolysis to acetyl-CoA and the subsequent formation of acyl-CoA is esterified in the endoplasmic reticulum to form triglycerides.
Several additional enzymes such as fatty acid synthase (FAS), acetyl CoA carboxylase (ACC) and malic enzyme (MA) are involved in this esterification process (Vásques-Vela et al., 2008). Both sources of NEFAs must then interact with a heterogeneous mixture of coat proteins to emerge from the ER in vesicle form and accumulate in a nascent lipid droplet within the cytoplasm of the adipocyte (Ducharme and Bickel, 2008). This migration and fusion of vesicles towards a central droplet is regulated by perilipin coat proteins of the monolayer surrounding the central lipid droplet. It has been suggested that these coat proteins form a hormonally regulated barrier between cytoplasmic lipases and the triglycerides within, helping to explain the surprising resistance of adipocytes to any toxic effects associated with such vast lipid accumulation (Ducharme and Bickel, 2008).

Once inside the adipocyte, the presence of dietary fatty acids has been shown to influence more than just vesicle membrane proteins. Genetic manipulations of mice revealed the presence of peroxisome proliferator-activated receptors (PPAR α, δ), members of the nuclear receptor superfamily, that act as transcription factors to coordinate energy metabolism (Evans et al., 2004). PPARδ knock-out mice failed to develop adipose tissue (or survive) and experiments demonstrated that PPARδ was both necessary and sufficient for adipogenesis (Vásques-Vela et al., 2008). Mounting evidence suggests that their transcriptional activity is controlled by the ligand binding of NEFAs and other small lipophilic substances within the cell - the proteins formed play an essential role in adipocyte differentiation and FFA uptake and storage (Heikkinen et al., 2007). Differentiation of pre-adipocytes, clonal expansion, and FFA uptake, the hallmarks of adipogenesis, are a result of continual activation of the PPARδ gene.
Some of the most potent anti-diabetic drugs, the thiazolidinediones (TZDs), are PPAR\(\gamma\)-agonists. These medications are widely prescribed and take into account the balance of both positive and negative effects; both increased insulin sensitivity and weight gain, respectively. In addition to insulin-sensitizing and increasing the capacity to store lipids, pleitropic effects of PPAR\(\gamma\) include stimulating the production of adipodenectin, increasing GLUT4 translocation in any number of tissues, osteoblast differentiation, and the promotion of metabolic syndrome (Heikkinen et al., 2007).

Lipolysis, or the hydrolysis of stored triglyceride, functions to provide other tissues with fatty acids and energy during fasting states or otherwise when glucose reserves are low. Several lipases are known to come into play. Triglycerides from within adipocytes are first hydrolyzed by the enzyme adipose triglyceride lipase (ATGL) to produce diacylglycerol and fatty acids (Vásques-Vela et al., 2008). Levels of ATGL are increased and decreased depending upon fasting or feasting state. Diacylglycerol is subsequently hydrolyzed by hormone-sensitive lipase (HSL) and in turn monoglyceride lipase (MGL) producing glycerol along with the liberated fatty acids (Vásques-Vela et al., 2008). As discussed earlier, coat proteins of the perilipin family act to buffer lipid droplets from the enzymatic action of these cytosolic enzymes. However, phosphorylation due to the \(\beta\)-adrenergic stimulation of protein kinase A results in a loss of membrane integrity and the translocation of HSL into the vesicle where it acts to mobilize this energy store. The importance of regional innervation of adipose deposits in lipolysis cannot be overstated. In their comparison of regional differences in metabolism, Hausman et al. (2001) illustrate how there exists an inverse relationship between the degree of sympathetic innervation and the propensity for adipocyte
proliferation. Highly innervated mesenteric adipocytes are smaller than those of other deposits and have rates of SNS-mediated glucose metabolism and lactate production that are higher than other fat deposits (Hausman et al., 2001). Women may have something to complain about when they say that fat located in thighs and buttocks are not affected by diet and exercise. Figure 3 provides a concise diagram of both processes in adipocyte energy metabolism.
Figure 3. Lipolysis and lipogenesis. Glucose excess is oxidized via glycolysis to acetyl-CoA in the adipocyte and then converted to acyl-CoA, which are then esterified in the ER to triglycerides for storage. Translocation into the lipid droplet of fatty acids via two pathways. Under fasting conditions, lipolysis is activated by G-protein coupled receptors resulting in an increase in cAMP that phosphorylates perilipin coat. cAMP also phosphorylates HSL and triggers translocation from the cytoplasm into the lipid droplet. Initial ATGL hydrolysis of triglycerides results in the liberation of glycerol and fatty acids. (Reprinted with permission by Vásques-Vela et al., 2008).
Objective and Statement of Problem

Chronic salt loading is a public health concern which many argue deserves a public health solution. Millions of health care dollars and quality-adjusted life years could be saved by implementing population-wide salt reduction policies – most effectively, sodium restrictions on processed and convenience foods. The comorbidities of hypertension, diabetes, and obesity are the focus of obesity-hypertension research and are greatly influenced by the modern Western diet. Combating the alarming rise in childhood and adult obesity in the U.S. demands the coordinated efforts of researchers, medical professionals, the food industry, and nutritional public policy. Current research into the etiology of obesity takes into account environmental, behavioral, and genetic influences. The causal role of habitual high salt intake in increasing blood pressure and exacerbating hypertension has been established. A growing body of evidence suggests that chronic salt loading may contribute to other conditions such as cardiovascular disease, obesity, stroke, end-stage renal disease, and GI cancers.

The objective of this study is to assess the possible direct association between chronic salt loading and adipocyte hypertrophy and hyperplasia as it relates to obesity. Chronic salt loading is defined as the habitual high dietary intake of sodium and is used to describe diets that consist mainly of processed foods in the form of pre-packaged, ready-to-eat, and convenience foods. Rates of hypertension are significantly higher in obese individuals. Hypertension has been positively correlated with the obese state and with obesity-related chronic illness in both human and animal subjects via indirect relationships (i.e. insulin-resistance, fluid retention). Few studies have shown any direct
and causal relationship between high salt intake and white adipose tissue (WAT) growth as measured by hypertrophy and hyperplasia. To my knowledge, this is the first systematic review of the current literature to identify such studies and report on the results.

**Review Method**

A systematic search for applicable original peer-reviewed publications was performed using key words on Medline (1985-present), Embase (1988-present), CINAHL (1985-present), and Google Scholar (1985-present). The Cochrane Library (The Cochrane Controlled Trails Register and Cochrane Database of Systematic Reviews) was also searched with the terms of “dietary sodium”, “dietary salt”, “high salt diet”, “adipose tissue”, “fat”, “lipids”, and “adipogenesis”. The reference lists of applicable original and review articles were also searched for more trials. Searches were restricted to English language publications. For inclusion, trials had to satisfy the following criteria:

1) Random allocation into low, high, or control salt intakes.

2) No concomitant interventions (i.e. pharmacological interventions).

3) Greater than or equal two months duration of randomized salt intake.

4) Studies conducted on rats maintained in an ambient environment with no restrictions on food or water intake.

5) Results had to measure quantifiable criteria for adipogenesis: fat weight, fat cell size, fat cell number, glucose uptake activity in adipocytes.
Appropriate studies were recorded for publication reference, sample size, length of study, level of salt intake, and outcome reported.

**Results**

A total of nine studies were found that met the search criteria. Dates of publication ran from 1988 to 2008. Sample sizes of rats ranged from 12 to 161 in the individual studies with a pooled total of 462 rats. The duration of studies on salt intakes lasted from 9 to 12 weeks after weaning (3 weeks of age). 8 out of the 9 studies used male Wistar rats, a multipurpose albino outbred strain of laboratory rat. One study used the Sprague-Dawley strain of lab rat. Table 2 summarizes the detailed outcomes of each study based upon low to high salt diets. One study compared the effects of low, normal, and high salt diets on both Wistar-Kyoto rats and spontaneously hypertensive rats (SHR). One study examined dose-response results among obesity prone (OP) and obesity resistant (OR) rats compared to controls. Sodium intakes were measured as a percent of rat chow and ranged from a low of 0.06% to a high of 7.94% Na⁺. 6 of the 9 studies used similar salt diets with a low salt diet (LSD) = 0.06% Na⁺, normal salt diet (NSD) = 0.50% Na⁺, and a high salt diet (HSD) = 3.12% Na⁺. One study coupled low, normal, and high sodium dosage with similar increases in calcium dosage. In 3 of the 9 studies, rats were culled at 3 weeks, 6 weeks, and 9 weeks of diet duration to analyze age-dependent responses to salt dosage. Mean body weights were taken during and at the end of the diets in 8 of the 9 studies. Mean blood pressure was reported in 6 of the 9 studies and surprisingly, only 4 of the 6 showed increases in blood pressure due to...
higher salt intakes. In 5 of the studies, researchers weighed fat pads (in grams) taken from typical locations found in rodents: periepidymal, subcutaneous, and retroperitoneal deposits. One study actually liquefied whole rodents and measured percent body fat of the homogenate. One study gravimetrically measured the lipid content of fat deposits per 100g of adipose tissue. Only 2 of the studies were able to report adipocyte cell volume. Some heterogeneity was found in between studies in collected morphometric data; however, 5 of the studies reported either none or an inverse relationship between salt intake and fat pad mass; 2 studies reported statistically significant increases in fat pad weights with increasing salt intake. 3 of the 9 studies were able to report the changes found in adipocyte size at the end of the study period. One study showed that adipocyte hypertrophy had occurred across all rodent groups fed 2% Na⁺ compared to 0.8% Na⁺. This trend was also found in the two most recent studies, with significant increases in periepideydimal adipocyte volume in the HSD group compared to both the NSD and LSD groups. Table 3 summarizes the trends found in each of the studies. Additional outcomes were reported in each of the studies in an attempt to measure other known indicators of adipocyte activity such as: WAT tissue GLUT 4 receptor density (AU/µg protein), plasma glucose levels before and after acute insulin treatment (mmol/L), plasma leptin levels (ng/mL), and glucose conversion to CO₂.
(See attached Excel file for table)
(See attached Excel file for table)
(See attached Excel file for table)
(See attached Excel file for table)
The use of animal models, historically rats, has provided practical research tools for examining mammalian physiological, histological, and genetic responses to various treatments. In an examination of chronic salt loading on adipocyte hypertrophy and hyperplasia, the whole organism provides valuable information that the 3T3-L1 cell line cannot. Because the conditions of hypertension and obesity have pleiotropic effects on the body, *in situ* examination is required to reveal a more complete picture of hormonal and neural interactions. We are lucky that this model has also been found to express both hypertension and obesity. Ethical considerations come into play when considering if humans should be subjected to chronic salt loading in trials – I propose that those controlled experiments are few and far between.

The identification of just nine research papers specific to the issue reveals several insights. The most obvious conclusion being that much more work is needed in this area. From 2000 on, the papers begin to share more similarities in methodology, with standardized dosages and research techniques. Although results are mixed as to the fundamental question, these initial steps show both focus and promise. The current data appears to be too thin to conduct any in depth statistical analysis. For example, pooling data on fat pad weights from four papers studying just 373 rats makes for underwhelming analysis. However, within the confines of the individual studies, researchers were able to generate meaningful data when comparing dose and response. Extrapolating those numbers to paint larger trends may be a bit premature.
The papers do point to the theory that measurement of adipokine levels is a useful tool for measuring the metabolic activity of WAT. The study on high salt dosage with obesity prone (OP), obesity resistant (OR), and control rats found across the board significant increases in leptin levels due to the HSD. Combining the implications of this study with what we know reveals that high salt diets may increase the abnormal secretory function and metabolic activity of WAT. Leptin secretion as measured by plasma leptin levels, may not lead to the mobilization of fatty acids – as we have learned, increases in adipocyte size brings about increased leptin production, the plasma concentration of leptin in this study may reflect growing dysfunction of adipocytes, regardless of weight, BP etc. Chronic salt loading may increase dysfunction of fat cells in lean and obese alike.

Some other useful data points come in the form of GLUT 4 receptor density and measures of glucose uptake (2GDU). Three of the studies found increases from 140 - 260% in adipocyte GLUT 4 receptor density in HSD versus LSD rats. The surprising thing is that the HSD rats in these studies all weighed significantly less than their LSD counterparts. If HSD is supposed to increase incidence of hypertension and fluid retention, these results are then contrary to expectations. The coupling of lower body weight with increased anabolic activity of adipocytes (recall GLUT 4 and lipogenesis) is puzzling. Furthermore, insulin-mediated glucose incorporation into adipocytes was shown to increase in HSD in 4 of the 5 studies which measured this parameter. This implies that some evidence exists for high salt diets leading to adipocyte hypertrophy via glucose uptake.
The collection and evaluation of these findings offers us a base from which we can build. Clearly, more data needs to be collected with regard to salt dosages and their direct effects on fat mass and composition. If in the future, such studies are conducted, then appropriate dose-response curves could be generated. Correlation and regression analyses could be performed on low to high salt doses versus mean results. ANOVA followed by any number of post-tests for multiple comparisons among groups would offer us more concrete information about trends which could then lead to the determination of cause-and-effect.

This investigation of this data reinforces the adage that original research is a laborious and painstaking process that may or may not lead to expected and significant outcomes. Finding so few papers suggests that the market has little to gain from the testing of this hypothesis. Sifting through the abundance of research on obesity-hypertension, I was struck by how many papers were analyzing drug/pharmaceutical effects on hypertension and/or obesity. If future studies do elucidate a direct connection between chronic salt loading and WAT growth, who stands to gain from this information? Could any group capitalize on this these findings and turn that into a competitive advantage? It seems that the simplest interventions with respect to chronic disease, like consuming less dietary sodium and increasing physical activity, may also prove to be the most cost-effective for patients and for society.
References


