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Animal and human tissue Na,K-ATPase in normal and insulin-resistant states: regulation, behaviour and interpretative hypothesis on NEFA effects

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Summary

The sodium(Na)- and potassium(K)-activated adenosine-triphosphatase (Na,K-ATPase) is a membrane enzyme that energizes the Na-pump by hydrolysing adenosine triphosphate and wasting energy as heat, so playing a role in thermogenesis and energy balance. Na,K-ATPase regulation by insulin is controversial; in tissue of hyperglycemic-hyperinsulinemic ob/ob mice, we reported a reduction, whereas in streptozotocin-treated hypoinsulinemic-diabetic Swiss and ob/ob mice we found an increased activity, which is against a genetic defect and suggests a regulation by hyperinsulinemia. In human adipose tissue from obese patients, Na,K-ATPase activity was reduced and negatively correlated with body mass index, oral glucose tolerance test-insulinemic area and blood pressure. We hypothesized that obesity is associated with tissue Na,K-ATPase reduction, apparently linked to hyperinsulinemia, which may repress or inactivate the enzyme, thus opposing thyroid hormones and influencing thermogenesis and obesity development. Insulin action on Na,K-ATPase, *in vivo*, might be mediated by the high level of non-esterified fatty acids, which are circulating enzyme inhibitors and increase in obesity, diabetes and hypertension. In this paper, we analyse animal and human tissue Na,K-ATPase, its level, and its regulation and behaviour in some hyperinsulinemic and insulin-resistant states; moreover, we discuss the link of the enzyme with non-esterified fatty acids and attempt to interpret and organize in a coherent view the whole body of the exhaustive literature on this complicated topic.

Keywords: Diabetes, hypertension, obesity, tissue Na, K-ATPase.

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Introduction

The sodium(Na)- and potassium(K)-activated adenosine-triphosphatase (Na,K-ATPase; EC 3.6.1.37) is a complex membrane enzyme, which converts chemical energy from the hydrolysis of adenosine triphosphate (ATP) into active translocation of cations against an electrochemical gradient. Na,K-ATPase energizes the Na-pump responsible for maintaining the high K and low Na intracellular concentrations (1–4). This pump, by maintaining the Na gradient between intracellular and extracellular compartments, affects many cellular functions, influencing cell volume, absorption processes in kidney or intestine and excitability in nerve or muscle (3). In some specialized tissues (such as kidney, myocardium, brain or lung), there is a '*secondary active transport*'; for example, the active transport of sugars and amino acids in many animal tissues is also dependent on proper distribution of Na and K ions, mediated by Na,K-ATPase activity (2). ATP is the energy source for the transport through the pump mechanism energized by Na,K-ATPase (2). This process can be regarded as a special '*futile cycle*' that consists of cyclic translocation of the Na ions (5,6), which, by wasting energy (that is released as heat) and occurring in all cells of the organism, may play

a significant role in thermogenesis and energy balance regulation (Fig. 1).

The rate of energy wasted by this mechanism has been differently estimated; kidneys (one of the richest sources of Na,K-ATPase) invest 80% or more of the energy utilized in this active transport (7).

Na,K-ATPase enzyme is also associated with the control of calcium (Ca) fluxes (4).

In the last decades, increasing information concerning amino acid sequence, structure and specific isoforms of the Na,K-ATPase has been gained (8). A tetrameric ensemble of peptides conforms the system known as α- and β-subunits, each subunit having a particular membrane location (at cytoplasmic or extracellular membrane surface); the α-subunit (subdivided according to different location and properties in α1, α2 and α3) has a proper catalytic functional role whereas β-subunit probably acts as a receptor for soluble α-subunit (9).

The Na,K-ATPase is, moreover, the only known specific binding site for the cardiac glycosides (2,10), and this site exists on the catalytic α-subunit of the Na,K-ATPase; as stressed by Schwartz and co-workers (10), it is interesting that the Na,K-ATPase, one of the oldest proteins in terms of evolution, contains a specific receptor domain for the oldest plant origin cardiotonic drug, being involved in myocardial contractility.

In the human red cell, the Na,K-pump resides within the membrane as one component in a multi-enzyme complex (11). Glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase are membrane bound and could function to produce ATP, which is compartmentalized within the membrane, forming a pool of ATP that could be preferentially used by the Na,K-pump (11–13).

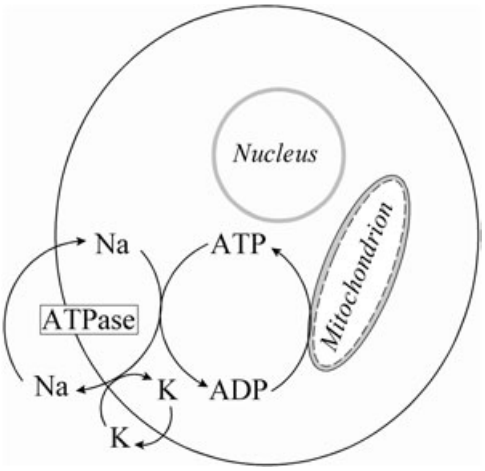

Figure 1 Mechanism of action of the Na,K-ATPase enzyme. Na entry in the cell is a passive diffusion whereas the extrusion is effected by an active mechanism of pump: Na,K-ATPase effects the coupled counter-transport of Na and K against their electrochemical gradients, so wasting energy (ATP degradation). ADP, adenosine diphosphate; ATP, adenosine triphosphate.

In the kidney, Na,K-ATPase activity is irregularly distributed: highest in the outer medulla, intermediate in the cortex and lowest in the inner medulla papilla (7). Recent data in rat kidney suggest that regulation of the degree of Na,K-ATPase α1-subunit phosphorylation at Ser-23 and enzyme activity has different mechanisms in medullary thick ascending limb of Henle than in proximal tubules, and may help us to understand the physiological heterogeneity of these segments (14).

Thyroid hormone stimulates oxygen consumption and active Na and K transport in many mammalian tissues, regulates the Na,K-ATPase rate in some tissues and exerts most of its calorigenic effects by stimulating Na,K-ATPase (15–17). Na,K-ATPase would also be involved in the non-shivering thermogenesis of cold acclimation (18). On these grounds, some authors studied the Na,K-ATPase activity in several tissues of genetically obese rodents, reporting a remarkable reduction of this enzyme in the liver, kidney and muscle (but not in brain) of the genetic ob/ob mouse (19–22) as well as in the liver of the genetically db/db mouse (19), but not in the livers of the spontaneously fatty rats (19) or in the erythrocyte membrane of the Zucker obese rats (23). These classical evidences led to the hypothesis that genetic obesity in mice may be due to the deficiency of Na,K-ATPase and to the consequent failure of thyroid hormone to stimulate the enzyme and therefore to elevate thermogenesis; this genetic enzyme deficiency (involving thermogenic capacity of the animals) would reduce wasting of energy, favouring a positive caloric balance and the occurrence of obesity. In a recent work (22), we reported a reduced Na,K-ATPase in some tissues (liver and kidney) of hyperglycemic-hyperinsulinemic ob/ob mice whereas in Swiss mice, made hypoinsulinemic and diabetic with streptozotocin (STZ), we found a marked increase of the enzyme activity in the same tissues; moreover, in the kidney of the ob/ob mice made hypoinsulinemic-diabetic with STZ, we also observed an enhanced enzyme level. These observations were against the occurrence of a genetic enzymatic defect and suggested, instead, a regulation by the hyperinsulinemia, present in ob/ob mice. In obese subjects, moreover, we observed a reduced activity of human adipose tissue Na,K-ATPase (22,24, Iannello *et al*., unpublished), in agreement with data obtained by others in red cells (25,26). We also observed that this diminished enzyme activity was negatively correlated with body mass index (BMI), insulinemic area during oral glucose tolerance test (OGTT) and mean arterial blood pressure (BP) (22,24, Iannello *et al*., unpublished). On these grounds, we hypothesized that animal and human obesity is associated with reduction of tissue Na,K-ATPase, apparently linked to hyperinsulinemia, which may repress or inactivate the enzyme, thus opposing to thyroid hormone and influencing thermogenesis and energy balance (22, Iannello *et al*., unpublished).

In the present paper, we analyse the Na,K-ATPase tissue level and regulation, and enzyme behaviour in some insulin-resistant states (such as animal and human obesity or diabetes or insulin resistance hypertension), focusing also on the possible correlation of this enzyme activity with hyperinsulinemia (and insulin resistance) and with non-esterified fatty acids (NEFA) – or free fatty acids, which act as circulating inhibitors of Na,K-ATPase (27,28) and which are reported to be increased in metabolic disease and hypertension (29,30). During the last decades, an enormous amount of information on Na,K-ATPase has been accumulated. Thus, we discuss our data focusing on the link of the enzyme with NEFA, and attempt to interpret and organize in a coherent view the whole body of the exhaustive literature on this complicated topic.

Literature review and discussion

Na,K-ATPase, by affecting the distribution of ions between the intracellular and extracellular space, is responsible for total-body Na homeostasis. As expected, Na,K-ATPase activity is regulated by the concentration of its substrates, Na, K and ATP; and, *in vivo*, tissue ATP level may be a rate-limiting factor (2). Some data suggested that the changes in intracellular Na content and the amount of ATP provided by glycolysis are closely related (31). The activity of the ion-pump is hormonally regulated, and especially catecholamines and peptide hormones have a great impact on salt balance and, hence, on vascular contractility (32). Renal Na,K-ATPase activity is bidirectionally regulated by natriuretic and anti-natriuretic hormones, and a shift in the balance between these forces may lead to salt retention (32). Dopamine (DA) certainly plays a key role in this interactive regulation (32). An excess of the circulating ligand of Na,K-ATPase, ouabain, by inhibiting vascular Na,K-ATPase activity, may increase Ca concentration in vascular cells and lead to increased vascular contractility (32). A direct interaction of Na,K-ATPase with cytoskeleton proteins might also regulate ion-pump, and mutations in cytoskeleton proteins may stimulate renal Na,K-ATPase activity by way of protein/protein interaction and lead to salt retention (32).

Various other crucial factors, such as age, racial extraction, gender, national origin and region of birth, may affect Na,K-ATPase and energy expenditure (33).

In this section, we will review in detail and discuss the most important regulators of Na,K-ATPase, as well as the enzyme behaviour in health status and in some metabolic or vascular diseases.

Hormonal regulators of Na,K-ATPase (Table 1)

Trachtenberg and co-workers (34), in the past, classified as '*intrinsic*' the immediate modulation (from seconds to minutes) of Na,K-ATPase activity by its direct interaction with different substrates (intracellular ATP, Na and Mg or extracellurar K) and by modifiers or inhibitors, and as '*extrinsic*' the long-term regulation (from hours to days) induced by some endocrine or development events. Thus, the hormonal regulators fall into two classes, those that act with minimal latency (vasopressin, catecholamines and insulin) and those that act with a latent period of 1–12 h (steroids and thyroid hormone) (35). The former group would affect Na- and K-ion permeability, causing rapid changes in the substrate concentrations (local transport), or would act with direct activation of Na,K-ATPase; the latter group would carry out biogenesis of new pumps or related processes that effect pump abundance (35). Moreover, Na would be a primary modulator of Na,K-ATPase activity and the intracellular '*messenger*' of the effect of certain hormones and factors on expression of the pump (36). Unfortunately, the data on hormonal regulation of Na,K-ATPase are rather discordant, being the effects often variable or of little magnitude.

Table 1 Hormonal regulators of K,Na-ATPase

1. Thyroid hormone (15–17,35,37–44)
2. Adrenal steroid hormones
   - Cortisol (45,48,80)
   - Dexamethasone (46)
   - Glucocorticoids (35,47,48)
   - Mineralocorticoids (35,48,49,51,55)
   - Deoxycorticosterone acetate (50)
   - Aldosterone (7,52–56,80)
3. Catecholamines (35,57,62,63,116)
   - Epinephrine (58–62)
   - Norepinephrine (62,63,135)
   - In brown adipose tissue (64–70)
   - 86Rb-uptake method (71)
4. Dopamine (32,57,72–78,80,106,123,145,146)
5. Progesterone (79)
6. Serotonin (9,80)
7. Growth hormone (81)
8. Glucagon (22,58,82,83)
9. C-peptide (84–88)
10. Insulin (29,30,35,89–114)
    - Skeletal muscle tissue (92–98)
    - Liver tissue (82,99)
    - Brain (100)
    - Adipose tissue (101–105)
    - Kidney tissue (106,107)
    - Rat uterus (108)
    - Rat small intestine (109)
    - Human erythrocyte plasma membrane (110)
    - Human lymphocyte plasma membrane (111)
    - Vascular smooth muscle (112)
    - Rabbit corneal endotelium (113)
    - Vascular smooth muscle of rabbit aorta (114)
11. Vasopressin (35,115,116)
12. Leptin (117,118)

Thyroid hormone

T4 (thyroxine) and T3 (triiodothyronine) stimulate oxygen consumption and active Na- and K-ion transport in various mammalian tissues (15–17,35,37), the skeletal muscle being the major target organ for the thermogenic action of thyroid hormone (38). Treatment of either euthyroid or hypothyroid animals with T3 increased Na,K-ATPase of liver, skeletal muscle, kidney, intestine and cardiac muscle, whereas brain Na,K-ATPase enzyme of adult rats was unaffected by thyroid status (although, in the developing rat, brain enzyme activity was thyroid dependent) (35). Moreover, administration of T3 to thyroidectomized rats increased Vmax (ATP) of skeletal muscle, renal cortex and heart (35). The high degree of correlation between nuclear receptor occupancy by T3 and renal or hepatic oxygen consumption (and Na,K-ATPase activity) supported a nuclear T3-mediated mechanism (35). Researches of Gick and co-workers (35) demonstrated that, in the responsive tissues, T3 clearly increased Na,K-ATPase gene expression. In its study, Clausen and co-workers (38) provided the evidence that, in human skeletal muscle, the active Na,K transport is correlated to energy expenditure and thyroid status. In the past, they also reported an enhanced number of Na-pumps in skeletal muscle of patients with hyperthyroidism (39), while others observed an increased Na-pump activity of erythrocytes and leucocytes in thyroid disease (40). In contrast, other authors reported, in red cells of hyperthyroid patients, a reduction of Na,K-ATPase activity and number of Na-pump sites, with a subsequent enhanced intracellular Na concentration, perhaps via increased protein breakdown, including the enzymatic proteins (41,42).

The neurotransmitter dopamine (see also below), precursor of noradrenaline, induces a variety of cardiovascular and renal physiological responses, including an increase in myocardial contractility and cardiac output, diuresis and natriuresis. Renal dopamine synthesis is, to some extent, dependent on thyroid hormone levels, and the response of dopamine receptors is altered by thyroid hormone deficiency (43).

A recent study of Luo and MacLean (44) reported that, in rats, increased food intake in response to T3 may be secondary to decreased hypothalamic ATP content, perhaps resulting from both increased Na,K-ATPase activity in the hypothalamus and metabolic signalling induced by whole-body caloric deficit.

Adrenal steroid hormones

Cortisol was reported to exert a stimulating effect *in vivo* of Na,K-ATPase activity in kidney tissue (45) and a similar effect was observed for *dexamethasone* (46). In rat kidney, specific and distinct glucocorticoid and mineralocorticoid receptors would exist, and the administration of *glucocorticoids* in adrenalectomized rats restored Na,K-ATPase activity, the increase of enzyme being associated with a parallel increase of 3H-ouabain binding (35). A direct *in vitro* effect of glucocorticoids (mediated by Na-ion content) was also reported on Na,K-ATPase of kidney tubules (47). It is probable that glucocorticoids act directly (inducing an increase in the number of Na,K-pumps) rather than via effects on Na content, even if a permissive role of intracellular Na has been suggested (35). Some evidence suggested that corticosteroids might modulate ATPase activity also at extrarenal sites, such as myocardium, which contains glucocorticoid receptors to which mineralocorticoids can also bind (48). For the effect of *mineralocorticoids* on Na,K-ATPase, many works exist and a simple model cannot yet be constructed to account for the regulation of Na,K-ATPase activity (35). Gick and co-workers (35) reviewed numerous studies that have convincingly demonstrated a significant reduction in renal Na reabsorption and Na,K-ATPase activity after adrenalectomy, whereas mineralocorticoid administration to adrenalectomized animals resulted in restoration of enzyme activity. Under physiologic conditions, Na,K-ATPase in the cortical collecting tubule (a probable target nephron-segment of hormones) is under mineralocorticoid rather than glucocorticoid control (49). Ouabain or digitalis-like factor (see also below), although itself devoid of hypertensive action, amplified the hypertensive action of small doses of *deoxycorticosterone acetate (DOCA)* and caused a hypertensive state similar to that induced by larger doses of DOCA (50). Human distal nephron and distal colon both exhibited mineralocorticoid-sensitive electrogenic Na absorption and made significant contributions to Na homeostasis (51). *Aldosterone* may exert an actual action in the renal tubule by activating two Na,K-pumps located on the luminal and serosal membranes (52). At variance, in the study of Diez (53), aldosterone *in vitro* did not influence ouabain-sensitive Na efflux from fresh human erythrocytes and did not alter Na-transport activity of stimulated Na,K-ATPase of Na-loaded erythrocytes. Moreover, Na efflux from Na-loaded human erythrocytes was not changed by preincubation of the cells with aldosterone (53). On the other hand, Seok and co-workers, in the renal cortex of rats with aldosterone-induced hypertension, confirmed that aldosterone stimulated Na,K-ATPase activity in basolateral renal membrane (54). Another work demonstrated that aldosterone directly stimulated Na,K-ATPase α1-subunit mRNA synthesis and protein accumulation in cardiac cells (55). A recent study reported that, in mouse kidney cells, quantitative basolateral localization of the Na,K-ATPase and its responsiveness to aldosterone required α1-subunit-specific sequences that differentiate this isoform from the α2- and α3-subunit isoforms (56).

Catecholamines

These hormones play certainly a central role in the regulation of Na homeostasis and Na,K-pump activity (and BP)

(35,57). In the past, an *in vitro* inhibition of Na,K-ATPase by *epinephrine* was reported in rat liver plasma membrane (58) whereas an *in vivo* acute activating effect has been demonstrated in mouse and rat brain (59,60). Clausen and Hansen (61) reported that, in isolated rat soleus muscle, addition of adrenaline stimulated Na efflux and K influx, these effects being blocked by β-adrenergic antagonists (propranolol) and unaffected by α-adrenergic blockers. The mechanisms suggested for epinephrine and *norepinephrine (NE)* activation of the enzyme would involve specific receptors or non-specific chelating action by divalent cations; another possibility is that NE might remove an endogenous inhibitory factor present in the cytoplasm (62). On the other hand, it was reported that stimulation of Na,K-ATPase in bovine myocardial tissue was not mediated via a direct adrenergic mechanism (63).

Na,K-ATPase is involved in the NE-mediated stimulation of respiration in *brown adipose tissue* (BAT) (64–69). Under normal aerobic conditions, it is likely that in BAT there is a separate, more active transport system for Na than the Na,K-ATPase, perhaps a Na/Na-exchange activity (68). Rothwell and co-workers (65) reported that Na,K-ATPase of rat BAT may be involved in the thermogenic responses to cafeteria diet, catecholamines and thyroid hormones. Knehans and Romsos (70) demonstrated that BAT of ob/ob mice was responsive to thyroxine treatment, whereas this treatment decreased functional rates of NE turnover in BAT of lean mice, but had no effect on BAT turnover of ob/ob mice.

It is noteworthy that there is unsuitability of the *86Rb-uptake method* for the estimation of Na,K-ATPase activity in innervated tissues (71). Indeed, at high concentrations of cardenolide acetylstrophanthidin, NE is released from the nerve endings and causes 86Rb efflux from the smooth muscle cells consequent upon α-adrenoreceptor activation (71). Since this efflux reduces the extent of Rb accumulation, the measurement of the latter does not adequately reflect uptake mediated by the activity of Na,K-ATPase (71). This is significant because the estimate of Rb accumulation (made in the presence of high concentration of cardenolide) forms the basis of all subsequent calculations of Na,K-ATPase activity.

Dopamine

While NE stimulates Na,K-ATPase activity and decreases urinary Na excretion, DA (an intrarenal natriuretic hormone involved in Na homeostasis) inhibits tubular Na,K-ATPase activity (as well as many other important Na-influx pathways in the nephron, such as Na,H-exchanger) and increases Na excretion. In the past, Aperia and co-workers (72) demonstrated that, in proximal convolute tubule segments, locally generated DA inhibited Na,K-ATPase activity via an intracellular signal system, a GTP-dependent regulatory G protein. DA coordinates the effects of antinatriuretic and natriuretic factors, and that renal DA system is very important for the maintenance of Na homeostasis (and normal BP) (32,73). Nowadays, it is well known that DA inhibits Na,K-ATPase via activation of renal D1-receptors, DA responses resulting from DA interaction with the DA receptors, D1-like (D1 and D5) and D2-like (D2, D3 and D4) (74,75). All 5-receptor subtypes are expressed in the kidney, albeit in low copy (73). Ibarra and co-workers (76) demonstrated that phosphorylation of renal Na,K-ATPase activity was modulated by the level of intracellular Na, the effect involving protein kinase C (PKC) and Ca-signalling pathways. Lokhandwala and co-workers (77) observed that DA and a D1-like receptor agonist (SKF 38393) caused inhibition of Na,K-ATPase activity in the proximal tubules of adult (6 months) but not of old (24 months) Fischer 344 rats, due, at least in part, to the higher basal PKC activity and to the Na,K-ATPase hyperphosphorylation. The results of another their recent study reported that DA (via the D1-like receptor adenylyl-cyclase pathway) recruited D1A-receptors to the plasma membrane in proximal tubules of Sprague-Dawley rats; these newly recruited receptors, coupled to G proteins, increased cAMP and participated in DA-mediated inhibition of Na,K-ATPase (78).

Progesterone

In a study, progesterone *in vitro* induced a dose-dependent inhibition of the canine kidney Na,K-ATPase activity (79).

Serotonin

Na,K-ATPase is activated by serotonin (9). Serotonin agonists did activate the brain enzyme whereas serotonin antagonists neutralized this activation (9). A study demonstrated that stimulation of hormonal receptors of proximal tubule cells with a serotonin agonist (8-OH-DPAT) induced an increased activity of Na,K-ATPase, which resulted from the recruitment of the enzyme molecules to the plasmalemma (80). Besides the antagonistic effects of DA and 8-OH-DPAT, intracellular Na modulated whether an activation or an inhibition of Na,K-ATPase was produced (80).

Growth hormone

In rat liver plasma membrane, an *in vivo* stimulating effect by this hormone was reported (81).

Glucagon

This pancreatic hormone produced *in vitro* Na,K-ATPase inhibition in rat liver plasma membrane (58), whereas in rat isolated hepatocytes and in rat perfused liver a stimulating effect on Na,K-ATPase has been reported (82,83). Recently, we observed that *in vitro* glucagon (3 microg/mL) exerted a statistically significant inhibitory effect in human liver Na,K-ATPase (22).

C-peptide

The proinsulin C-peptide has been held to be merely a by-product in insulin biosynthesis whereas recent reports showed that it is a hormonally active peptide that elicits both molecular and physiological effects (84). In rat medullary thick ascending limb, C-peptide via PKC-α stimulates Na,K-ATPase activity (within a physiological concentration range) (85). Specific binding of C-peptide to the plasma membranes of intact human erythrocyte cells (because of the presence of a cell-surface receptor) has been demonstrated (84,86). Some researches demonstrated an effect of human C-peptide on microvascular function (compatible with the C-peptide effects in type-1 diabetics), which might be mediated by increased nitric oxide (NO) production and by activation of the erythrocyte Na,K-ATPase (84,87). This effect was due to an increase in enzyme turnover rate, which is most likely mediated by PKC-α phosphorylation of the Na,K-ATPase α-subunit (88). Many of these C-peptide effects were inhibited by pertussis toxin, supporting the interaction of C-peptide with a G-protein-coupled receptor (84).

Insulin

This hormone is, certainly, the most interesting hormonal factor to investigate in various metabolic disorders (such as obesity or non-insulin-dependent diabetes mellitus) (29,30) and cardiovascular disorders (such as essential hypertension or atherosclerosis) (89), having these two groups of diseases some features in common, such as resistance to insulin-mediated glucose uptake and vascular endothelial dysfunction. It is well known that intracellular Na regulates Na,K-ATPase in most mammalian cells; then, insulin stimulation of the process that increases influx of Na ions may stimulate Na,K-ATPase (90). DeFronzo and co-workers (91), reviewing clinical disorders of hyperkalemia, stressed that insulin stimulates the Na,K-ATPase pump, which could regulate mainly intracellular K ions. Tissue-specific differences existed in the mechanism of insulin stimulation of the Na,K-pump, which appears to be a receptor-mediated process (35). Most reports on the effect of insulin on Na,K-ATPase have been obtained primarily with *in vitro* experiments of short duration, suitable to detect the rapid effects of activation or inhibition. Concerning the mechanism through which insulin regulates Na,K-ATPase, in the *in vitro* experiments, the insulin effect appeared within a few minutes, because of a rapid inhibitory action of insulin on pre-existent enzyme molecules (35).

The available data about insulin effects on Na,K-ATPase are contrasting.

Concerning *skeletal muscle tissue*, a lack of insulin effect on Na,K-ATPase in rat skeletal muscle was reported, either *in vivo* or *in vitro* (92). Afterwards, in rat muscle sarcolemma (93), an *in vitro* enzyme activating insulin action was observed. It was reported that insulin lowered K-ion concentration, stimulating net cellular K uptake and activating Na-ion transport in skeletal muscle (94). An insulin recruitment of latent Na,K-pumps in skeletal muscle cells of rat, mouse and guinea pig has been suggested (95), and it is possible that the hormone might unmask cryptic pumps that were accessible to ouabain but not involved in *trans*-membrane Na-ion transport, prior to the exposure to insulin (35). In rat skeletal muscle cells, Flatman and Clausen (96) demonstrated that the activating action of insulin on active electrogenic Na,K transport was unlikely to be evoked by a lowering of the intracellular concentration of cAMP, which had been proposed as an early signal in the action of insulin. In isolated rat skeletal muscle, Weil and co-workers (97) suggested the following model of insulin regulation: insulin augments Na,K-ATPase activity by a sequence of events comprising activation of Na,H-antiporter, increased intracellular Na-ion concentration and stimulation of Na,K-pump. Yet, an equal stimulation of Na,K-ATPase by insulin was found when intracellular Na was elevated (under monensin) or lowered (under amiloride), suggesting that the Na,K-ATPase activation in rat soleus muscle by insulin was not secondary to a stimulation of Na,H-antiporter (97). In mammalian skeletal muscle, insulin induces isoform-specific translocation of Na-pump subunits from different intracellular sources to the plasma membranes, the hormone-responsive enzyme in this tissue being an α2:β1 dimer (98).

Concerning *liver tissue*, in rat cell-culture systems (isolated hepatocytes or hepatoma cells), insulin binding to high-affinity receptors was associated with a stimulatory effect, without apparent change in the number of Na,K-pump (82,99), perhaps occurring through the Na ions provided to internal, unsaturated, transport sites of the Na,K-pump.

In *brain*, a depressing effect on enzyme activity was reported in which a role of insulin as a neuromodulator was suggested, the insulin inhibitory effect being regulated in a dose-dependent manner by a cytoplasmic factor (Mg^2) (100).

In *adipose tissue*, an activating effect of insulin has been postulated on the grounds of evidence obtained *in vitro* (101). In rat adipocytes, Resh and co-workers (102) reported that insulin rapidly stimulated ouabain-inhibitable uptake of 86Rb with contemporary increase in intracellular concentration of K ions. In the same cells, they identified by immunoassay the high ouabain-affinity insulin-stimulated subset (α^+-isoform) and the low ouabain-affinity subset (α-isoform), and proposed that insulin stimulates Na,K-ATPase by shifting the Na affinity of the α^+-isoform without a concomitant increase of intracellular Na (103). In gosts isolated from rat adypocytes, Na,K-ATPase was reported to be low and insulin insensitive (presumably for its already maximal activity) (104). The

same group of authors reported that in rat adipocytes, insulin stimulated Na,K-ATPase by decreasing Km for intracellular Na of both α1- and α2-isoenzymes and by increasing Vmax of the α2-isoenzyme, which is very sensitive to insulin action (105).

Concerning *kidney tissue*, data obtained by Feraille and co-workers (106) showed that physiological concentrations of insulin inhibited by 44% the initial rate of ouabain-sensitive 86Rb uptake in the medullary and cortical thick ascending limb, whereas increased it by 40% in proximal tubules and by 60% in both cortical and medullary collecting tubules. Banday and co-workers (107) suggested that chronic exposure of cells to insulin causes both reduction in the abundance of DA D1-receptors and their uncoupling from G proteins; these phenomena might account for the diminished inhibitory effect of DA on Na,K-ATPase activity in primary proximal tubule epithelial cells obtained from kidneys of hyperinsulinemic-hypertensive Sprague-Dawley rats.

In *rat uterus*, an activating effect of insulin has been obtained (108).

In brush border membrane of the mucosal cells of the *rat small intestine*, a lowering action of insulin on Na,K-ATPase was observed (deduced from the increased activity in alloxan-diabetic rats) (109).

In *human erythrocyte plasma membrane*, insulin significantly inhibited *in vitro* Na,K-ATPase activity and, at the same time, decreased membrane fluidity (110) whereas, in *human lymphocyte plasma membrane*, an *in vitro* enzyme activating insulin action was observed (111).

Insulin action predisposes to increased vascular smooth muscle tone (the hallmark of hypertension associated with diabetes) and the *vascular smooth muscle* is an insulin-sensitive tissue (like skeletal muscle and adipocytes), because insulin stimulated glucose uptake in this tissue (112). In vascular smooth muscle, insulin regulates the intracellular cation metabolism by attenuating the effects on inward Ca-ion currents and by direct effects on cell Na,K-ATPase-pump expression (112). In the past, in *rabbit corneal endotelium*, a biphasic *in vitro* insulin effect was demonstrated: i.e. transient activation at low insulin concentration and inhibition at high concentration (113). In *vascular smooth muscle of rabbit aorta*, on the other hand, insulin did not seem to regulate Na,K-ATPase (114).

Vasopressin

This neurohypophyseal peptide regulates water reabsorption and *trans*-epithelial active Na transport by activation of adenylate cyclase system (115). This was a short-term action and whether hormone increased the abundance of Na,K-ATPase molecules in responsive tissues over a longer period of time remains to be clarified (35). Lynch and co-workers (116) proposed that vasopressin (and also catecholamines) stimulates rat hepatic Na,K-ATPase via increased synthesis of diacylglycerol and PKC activation.

Leptin

This hormone is the product of the obesity ob-gene that controls energy intake and expenditure, by acting on the central nervous system and peripheral tissues; it was found to inhibit Na,K-pump activity in 3T3-L1 fibroblasts (likely via PI 3-kinase), thus explaining its physiological natriuretic effect and cardiovascular or metabolic implications of the leptin (117). Beltowski and co-workers (118) demonstrated that human leptin, administered intraperitoneally in the rat, stimulated natriuresis primarily by inhibiting tubular Na reabsorption; this effect was mediated, at least partially, by decreased Na,K-ATPase activity in the renal medulla and is impaired in rats with dietary-induced obesity.

Non-hormonal regulators

Nitric oxide

Data of Beltowski and co-workers (119) suggested that NO decreases Na,K-ATPase activity in the renal medulla through a mechanism involving the cGMP (i.e. through a protein kinase G dependent mechanism) and the cytochrome P450-dependent arachidonate metabolites (see below). In contrast, NO had no effect on Na,K-ATPase in the renal cortex (119). Recently, the same authors (120) demonstrated that chronic hyperleptinemia caused up-regulation of renal Na,K-ATPase and decreased urinary Na excretion, by inducing oxidative stress-dependent NO deficiency. Antioxidant treatment was effective in animal leptin-induced hypertension (120) and should be considered in controlling BP in hyperleptinemic obese individuals.

Cytochrome P450-dependent metabolites of arachidonic acid

Considerable evidence has been accumulated, over the last decade, implicating a role of cytochrome P450-dependent metabolites of arachidonic acid (CYP-AA) in the pathogenesis of hypertension (121,122). The CYP-AA metabolites have different biological properties based on the sites of production, and can be stored in tissue lipids and released in response to hormonal stimuli (121). Inhibition of the formation of NO by CYP-AA metabolites mediated most of the cGMP-independent component of the vasodilator response to NO (121). In the kidney, CYP-AA metabolites (produced by vascular smooth muscle) are potent vasoconstrictors and inhibit Na transport in the proximal tubule by blocking Na,K-ATPase activity (122).

Adducin

This membrane-bound skeletal protein regulates Na,K-ATPase activity: for example, the interaction between adducin and Na,K-pump is the most likely biochemical

mechanism responsible for the increased tubular Na reabsorption and hypertension in Milan hypertensive strain rats (123). α-Adducin 460Trp allele was reported to be correlated with erythrocyte Na-transport rate in North Sardinian primary hypertensives (124). α-Adducin polymorphism in humans was associated with abnormal renal Na handling and high BP (124).

Endogenous ouabain-like factor(s)

These digitalis-like Na-pump ligands may play a role in the pathogenesis of volume-dependent hypertension, by affecting natriuresis via inhibition of renal tubular Na,K-ATPase and by raising intracellular free Ca ions in vascular smooth muscle cells as a consequence of the inhibition of the Na-pump (125). Genes of these factors involved in Na,K-ATPase regulation should be related to the development of hypertension (125,126). The ouabain-like factor has been proposed as a modulator of the renal Na,K-pump and considered as a new pharmacological target for hypertension therapy (126,127). It is noteworthy that the digitalis-like immunoreactivity of the above-mentioned inhibitors might create cross-reactivity and is of prevalence and magnitude sufficient to distort the interpretation of the *digoxin assay* in clinical situations (128).

Dietary factors and Na,K-ATPase (Table 2)

Some data have been reported in animals and humans about the influence of dietary factors on tissue Na,K-ATPase activity.

Table 2 Dietary factors and Na,K-ATPase

1. Fasting and meal (129–131)
2. Sucrose supplement
   - ob/ob obese mice (132)
3. Fructose
   - FF-Sprague-Dawley rats (133,134)
4. High-energy diet
   - Psammomys obesus (135)
   - Spiny mice (Acomys cahirinus) (136)
5. Cafeteria diet (137)
   - BAT rat (138)
6. Overfeeding
   - Rats (139)
   - Obese men (140)
   - Human obesity, after a meal (141)
7. High-salt load (142–144)
   - Wistar fatty rats (145)
   - Obese Zucker rats (146)
8. Dietary K
   - K adaptation (7,147)
   - K tolerance (7)
   - Increased K load (7,147)
   - Low K diet (7)
9. Oral magnesium supplementation (148)

BAT, brown adipose tissue.

Fasting and meal

Cellular Na transport via the Na,K-ATPase contributes significantly to daily energy expenditure (129). Ng and Hockaday (129), studying the leucocyte Na-pump response to the need for thermogenesis, demonstrated that, in fasting lean normal subjects (investigated at room temperature of 23 and 33 degrees), the ouabain-sensitive efflux rate (which reflects active Na-transport) and the intracellular electrolytes were similar. At 2 h after a meal of 1000 kcal, the ouabain-sensitive Na-efflux rate rose when the room temperature was 23 degrees but not 33 degrees (129). They concluded for an effect of environmental temperature on prandial changes of human leucocyte transport and for a blunted postprandial activation of active Na efflux when the need for thermogenesis was reduced (129). The influence of environmental temperature, as well as energy intake, on 3H-ouabain binding sites was reported in porcine skeletal muscle by Dauncey and Burton (130). Moreover, Lupien and co-workers reported an increased GDP binding to BAT mitochondria after a single meal (131).

Sucrose supplement

A high energy intake, in the form of sucrose supplement, increased Na,K-ATPase-mediated ion transport in liver and muscle of lean ob/ob control mice whereas *ob/ob obese mice* seemed to have an impairment in such dietary control (132).

Fructose

In the *fructose-fed (FF) Sprague-Dawley rats*, results obtained suggested that in these animals the influence of insulin on the vascular Na,K-pump is increased and might modulate changes in the vascular responsiveness (133). FF rats develop hyperinsulinemia and hypertension, and provide a good animal model. In a study, there were no differences, along the nephron, in basal 86Rb uptakes and Na,K-ATPase activities between control rats and FF-hypertensive rats; in FF-hypertensive rats, 86Rb influx remained unresponsive to insulin in contrast to control rats in which insulin stimulated 86Rb uptake in the proximal convoluted tubule and cortical collecting duct but exerted an inhibitory action in the medullary thick ascending limb (134).

High-energy diet

Psammomys obesus (a desert rodent that develops diabetes when displaced from its natural environment and fed a high-energy diet) was examined about variations in renal changes of Na,K-ATPase activity, in relation to the diabetes (135). Animals fed a high-energy diet showed increased Na,K-ATPase activity in the kidney cortex and medulla and became hyperglycemic-hyperinsulinemic or diabetic-hypoinsulinemic with elevated glomerular filtration rate (GFR), exhibiting metabolic similarity to type-2 and type-

1 diabetes (135). On the other hand, in insulin-treated diabetic animals with normalized GFR, Na,K-ATPase activity was similar to control (135). There was a linear and significant correlation between GFR and Na,K-ATPase activity both in the cortex and in the medulla, whereas variations in glucose and insulin did not correlate with Na,K-ATPase activity (135). The enzyme activity in this animal model seemed to be determined by GFR and to be independent of plasma glucose or insulin levels (135).

Recent interesting data on *Spiny mice (Acomys cahirinus)* were also reported. This animal model (which lives in semidesert regions of the eastern Mediterranean countries), transferred to Geneva in the 1950s and maintained on a fat-rich diet, became obese with pancreatic islet hyperplasia or hypertrophy and low insulin secretion response (136). In Jerusalem, Shafrir placed on different diets this brand of Spiny mice: one contained 50% sucrose and the other was rich in fat seed. The animals on sucrose-rich diet developed hepatomegaly, increased lipogenic enzyme activity and elevation in very low density lipoprotein (but did not develop overt diabetes), whereas the mice on fat-rich seed diet exhibited obesity with low hepatic lipogenesis, mild hyperglycemia and hyperinsulinemia (136). The sucrose diet induced elevation of thyroid hormone and of both T3-inducible hepatic mitochondrial FAD-glycerophosphate oxidase and Na,K-ATPase (as well as of body temperature), indicating that sucrose diet was associated with enhanced thermogenesis and energy-wasting metabolic cycling and with protection against excessive obesity and pancreatic islet disintegration (136).

Cafeteria diet

Romsos (137), in a comprehensive review, reported that genetically obese rodents and animals with dietary-induced thermogenesis represent two extremes in efficiciency of energy retention: the former deposit dietary energy with high efficiency, whereas the latter deposit dietary energy with low efficiency. These differences in efficiency of energy retention, at the cellular level, must be associated with changes in efficiency and/or rate of formation and/or utilization of ATP; metabolic reactions that alter the rate of ATP utilization include Na,K-ATPase (especially in BAT, which posesses a unique proton-conductance pathway that reduces the efficiency of ATP synthesis) (137). Rothwell and co-workers reported that cafeteria-fed rats showed large increases in metabolizable energy intake, energy expenditure and BAT mass, Na,K-ATPase activity and mitocondrial GDP binding (138). The involvement of BAT in diet-induced thermogenesis is potentiated by hyperthyroidism (138).

Overfeeding

In rats, diet-induced obesity alters BAT and Na,K-pump activities (139). An increased erythrocyte Na efflux during overfeeding was reported in obese men, without measurable changes in thyroid hormone or catecholamine levels (140). On the other hand, an increase in erythrocyte Na-pump (60 min after a meal) was reported in human obesity, although a great interindividual variability was noted (141).

High-salt load

High NaCl feeding increased the plasma level of the circulating inhibitor of Na,K-ATPase (142–144). In *Wistar fatty rats*, the salt load tended to develop a salt-sensitive hypertension, which could be caused by the excessive Na retention occurring as the results of a defective DA system in the kidney tissue that failed to inhibit Na,K-ATPase activity (145). Lucas-Teixeira and co-workers (146) observed that in *obese Zucker rats*, the inhibition of jejunal Na,K-ATPase activity through DA D1-receptors was dependent on salt intake whereas, in lean Zucker rats, the enzyme failed to respond to the receptor activation irrespective of their salt intake.

Dietary K

Animals or humans, chronically subjected to increased K in the diet, become conditioned to it and excrete all the ingested K ('*K adaptation*'), and are able to display an enhanced capacity to excrete an acute K load ('*K tolerance*') (7). Silva and co-workers (147) demonstrated increased Na,K-ATPase in kidney homogenates of K-loaded rats and suggested a role of the enzyme in renal K adaptation. Katz (7), analysing the literature concerning the complex problem of K adaptation, found that the enzyme increase involves the cortical collecting tubules and that the various components of the renal response to the *increased K load* are facilitated by increased availability of aldosterone, which plays an important permissive role in K adaptation, even though it is not essential for its occurrence. By examining the effect of a *low K diet* on Na,K-ATPase from various nephron segments, in K-depleted animals, Katz (7) reported a striking, time-dependent, selective increase of the enzyme activity in the medullary collecting tubules.

Oral magnesium supplementation

In a Japanese study, MgO administered orally three times a day for a period of 2 weeks (at a daily dose of 1.0 g) showed hormonal and antihypertensive effects in 17 inpatients with mild-to-moderate essential hypertension (148). Mg suppressed a circulating Na,K-ATPase-inhibitor activity, thus attenuating vascular tone and thereby reducing BP (148).

Non-esterified fatty acids as inhibitors of NA,K-ATPase

Non-esterified fatty acids might be endogenous factors that can modulate the Na,K-ATPase or mediate the regulator

effects on it. In fact, NEFA have been reported to inhibit Na,K-ATPase (27,28,128,149–151), and a similar effect would be exerted also by lysophospholipids (128). Huang and co-workers (151) demonstrated that low levels of CoA-esters of fatty acids (palmitoyl-CoA), at low concentrations of substrates (especially of ATP), had activating effects on the enzyme. On this basis, CoA-esters of long-chain fatty acids may be intracellular regulators, which protect the pump in the face of substrate depletion (151). An inhibitory effect of NEFA in rat brain Na,K-ATPase was, firstly, signaled by Ahmed and Thomas (149), and in the last decades some researches suggested that unsaturated fatty acids might be the elusive endogenous digitalis-like substances (27,151). In another study, the common fatty acids, linoleic and linolenic acid, were reported as effective Na,K-ATPase inhibitors in hogs, exhibiting a rather high inhibitory constant, well beyond their physiological range of concentrations (150). A circulating inhibitor of the enzyme activity (having NEFA-like properties and dose-dependent inhibiting effects) has been observed in relation to the pathophysiology of essential hypertension (27,151).

Obesity and Na,K-ATPase (Table 3)

In literature, there are several conflicting data on Na,K-ATPase activity in obesity, but the obesity of both man and laboratory rodents seems to be associated with a reduction of the enzyme activity in some tissues. The cause of this diminished Na,K-ATPase activity in obesity is not definitely established, as it will appear from the discussion that follows.

Animal obesity

Researches on genetic susceptibility suggested that altered energy expenditure (Na,K-ATPase and uncoupling protein) and/or preferential substrate utilization (fatty-acid-binding protein) were likely to be involved in the aetiology of obesity (33). Concerning experimental obesity, Bray and co-workers reported a remarkable reduction of the Na,K-ATPase activity in tissues of the genetic *ob/ob mice* (19,21), as well as in liver of the genetically db/db mouse (19). On the basis of these observations, Bray and co-workers hypothesized that the reduction of the enzyme activity in the ob/ob mice was due to a genetic enzyme defect. We reported similar data in ob/ob mice and suggested a regulation of the enzyme by the high level of insulinemia (22). Guernsey and Morishige (152), on the grounds of data obtained with liver and lung tissues, suggested that the low enzyme activity in ob/ob mice may be due to a reduction in nuclear receptors for triiodothyronine, a defect that inactivated the stimulating action of thyroid hormone on Na,K-ATPase activity. In this mice strain, Lin and co-workers (20) observed reduction of enzyme activity in skeletal muscle, probably because of the diminution in the number of enzyme units, which, of course, cannot be due to an inhibitory effect but could be the result of a repressing effect. More recently, Elmi (153) postulated an increased (and not reduced) number of Na,K-ATPase enzyme units in ob/ob-mouse pancreatic islets.

Zemel and co-workers (23) did not evidence significant difference in enzyme activity of liver or erythrocyte membrane between spontaneously *hypertensive-obese Zucker rats* and their lean controls. On the other hand, other authors reported in the hypertrophic liver of this strain of obese rats a coordinate induction of several Na-dependent transport systems and of Na,K-ATPase, and an enhanced expression of the Na-pump in tissues that are directly involved in nutrient uptake and processing (154). Bickel and co-workers (155) reported that the abundances of the α1-subunit of Na,K-ATPase, the thiazide-sensitive Na,Cl-cotransporter and the β-subunit of the epithelial Na channel were all significantly increased in the kidneys of the obese hypertensive-hyperinsulinemic Zucker rats. These changes might possibly enhance Na retention by the kidney and therefore could play a role in obesity-related hypertension. In obese-hypertensive Zucker rats, the natriuretic and diuretic response to exogenously administered and endogenously produced DA was reduced; this altered response resulting from diminished DA-induced inhibition of the Na,K-ATPase (156). This defective D1-receptor function in obese Zucker rats is not inherited, but is provoked by hyperinsulinemia and/or other circulating factors associated with obesity (156).

Data of Tsuchida and co-workers (145) indicated that *Wistar fatty rats* tend to develop salt-sensitive hypertension, which could be caused by the excessive Na retention occurring as the results of a defective kidney DA system that failed to inhibit Na,K-ATPase activity.

Table 3 Obesity and Na,K-ATPase

1. Animal obesity (33)
   - ob/ob mice (19–22,152,153)
   - Hypertensive-obese Zucker rats (29,154–156)
   - Wistar fatty rats (145)
2. Human obesity (22,24,25,33,158–162)
   - Obese Pima Indians (26,157)
3. Hyperinsulinemia and insulin resistance (22–24,29,30,163,164)
   - Degree of obesity or BMI (22,24,26,159,165–168)
4. NEFA effects (22,27,28,128,149–151,169–175)

BMI, body mass index; NEFA, non-esterified fatty acids.

Human obesity

Twin and family studies seem to confirm that a strongly heritable component affects resting energy expenditure, substrate utilization and thermogenic response to feeding (33). Recent data in young sib pairs reported also a moderate heritability of non-resting energy expenditure (33).

In diverse studies, no evidence has been observed for intrinsic cellular changes in basal Na,K-pump activity, related to obesity. In cultured fibroblasts from *obese Pima Indians*, no abnormalities in insulin-regulation of Na,K-pump activity were found (157). Mir and co-workers (141) failed to detect a diminished erythrocyte Na,K-ATPase activity in obese people. Similar observation was reported by Simat and co-workers (158), who (measuring ouabain binding, electrolyte concentrations in erythrocytes and red cell ghost Na,K-ATPase) found no correlations between these variables and the percentage of ideal body weight, concluding that Na,K-ATPase does not directly influence human obesity. On the contrary, reduction of erythrocyte Na,K-ATPase activity in obese subjects was observed by DeLuise and co-workers (25), and similar data were obtained by ourselves in the adipose tissue from obese patients (22,24) as well as by Mott and co-workers (159). Ouabain-sensitive and ouabain-insensitive ATPase activities were also reported to be decreased in the polymorphonuclear leucocytes of obese patients, with considerable restoration in the cells obtained from obese subjects after treatment (160). It is noteworthy that Bray and co-workers (161) observed that in liver of obese subjects, the enzyme activity was not reduced but increased (although the number of the studied patients was very little). DeLuise and co-workers (162) noted that two populations of adolescent obese subjects could be biochemically distinguished into a group with primary obesity (and with low erythrocyte membrane Na,K-ATPase sites) and another group with secondary obesity (and normal or slightly elevated numbers of erythrocyte Na,K-ATPase sites). In this study, however, the actual ability of these sites to hydrolyze ATP was not estimated.

Hyperinsulinemia and insulin resistance

Hyperinsulinemia is the major alteration of obesity, which is an insulin-resistant condition (29,30,163). Insulin is a potent stimulus for Na,K-ATPase activity *in vitro*, and a defect in such activity has been postulated in insulin-resistant states (such as obesity or overweight type-2 diabetes or hypertension) (22,23,29,30,163) and in the related complications (30,163). It has been suggested that, in human skeletal muscle, hyperinsulinemia induces activation of Na,K-ATPase (located in endothelial or vascular smooth cells) and contributes to vasodilation that favours insulin-stimulated glucose uptake; this effect would be reduced in various insulin-resistant states (164). In obese-hyperinsulinemic subjects, we reported that adipose Na,K-ATPase activity reduced and negatively correlated with OGTT-insulinemic area (22, Iannello *et al*., unpublished).

Hyperinsulinemia and *degree of obesity* (measured as BMI) are certainly strongly correlated. Available data concerning the effects of BMI on the Na,K-ATPase activity are conflicting. In a research on Na,K-ATPase levels in erythrocytes of normal children and infants of diabetic mothers, the enzyme resulted in no correlation with body weight, appearing primarily regulated by genetic factors (165). A diminished insulin-stimulated Na,K-ATPase activity was demonstrated in adipocytes from obese subjects, with basal adipocyte enzyme per cell positively correlated with BMI (159). However, percent insulin stimulation above basal adipocyte Na,K-ATPase activity was negatively correlated with BMI (159). In our recent studies (22), we observed that in obese-hyperinsulinemic subjects the reduced adipose tissue Na,K-ATPase activity was negatively correlated with BMI. In lymphocytes of insulin-resistant obese patients, a negative correlation of Na,K-ATPase (measured by 86Rb uptake) with BMI and a positive one with insulin sensitive index (Conard's K) were also reported by others (166). In non-diabetic euthyroid male Pima Indians with varying degrees of obesity (BMI from 22 to 60 kg m^{-2}), Na,K-ATPase was decreased in intact red blood cells and inversely correlated with BMI (26). In obese subjects, a reduction of erythrocyte Na,K-ATPase was confirmed by others, with a negative correlation between the degree of obesity and the increase of enzyme activity after 12 weeks of weight loss (167). Finally, in normal healthy subjects, also, an inverse relationship between erythrocyte ouabain sites and BMI was reported (168).

Non-esterified fatty acids effects

Concerning NEFA in obesity, there are no data in literature. According to our data (22, Iannello *et al*., unpublished), human obesity (like the obesity of laboratory rodents) in adipose tissue is associated with a reduction of Na,K-ATPase activity, which is inversely correlated with the high insulin level. Moreover, we have reported evidences that the inhibitory action of insulin on Na,K-ATPase was more evident *in vivo* than *in vitro* (22, Ianello *et al*., unpublished). On the other hand, we have also observed (169) that, in obese-hyperinsulinemic patients, the insulin and NEFA response to glucose load increased in parallel manner, OGTT-NEFA areas being strongly and positively correlated with OGTT-insulinemic areas (because of the NEFA unresponsiveness to the suppression by insulin). Based on such findings and on the available data of literature on the role of NEFA as circulating inhibitors of Na,K-ATPase (27,28,128,149–151), we formulated the working hypothesis that hyperinsulinemia with insulin resistance (often present in obesity and in overweight type-2 diabetes) may repress the Na,K-ATPase activity through their association with increased circulating NEFA, whose elevation have been reported in animal (170) or human (169,171,172) obesity and in type-2 diabetes (169,173). It is noteworthy that, in patients affected by genetic forms of obesity (syndromes of Bardet-Biedl and Alstrom), who were highly insulin-resistant and refractory to dietary treatment, we observed a very high value of fasting serum NEFA (which

were non-responsive to suppression by insulin during OGTT) (174,175). This high NEFA level might act to suppress Na,K-ATPase, thus affecting energy balance and favouring the development of severe obesity.

Diabetes and Na,K-ATPase (Table 4)

Na,K-ATPase activity would be decreased in many tissues of animals or humans with diabetes, and this impairment could be, at least in part, responsible for the development of diabetic complications (176,177). However, contrasting data have been reported in literature for Na,K-ATPase activity in diabetes, as shown by the following discussion.

Animal diabetes

Concerning data in experimental diabetes, Cohen and co-workers (178), in *STZ-diabetic rats*, reported a decreased *kidney* glomerular Na,K-ATPase in animals with acute (<18 days) diabetes, but a significantly increased enzyme activity in rats with more chronic (>32 days) disease, which suggested that the time of the examination after the induction of diabetes is critical for the influence on Na,K-ATPase activity. Other investigators, however, found an increased kidney Na,K-ATPase in animal diabetes (179–183). In a recent study (22, Iannello *et al*., unpublished), we observed an increased enzyme activity in the kidney of *STZ-diabetic Swiss mice and ob/ob mice*. Some of the above reported studies were performed with renal tubules or whole-tissue homogenates (which contain tubular components), thus the stimulation of tubular ATPase activity may be the result of increased Na-ion transport and/or renal hypertrophy in diabetes.

In *myocardium*, diabetes or experimental galactosemia of a 2-month duration in rats significantly increased oxidative stress while the activities of Na,K-ATPase and Ca-ATPase were subnormal (184). Administration of supplemental antioxidants prevented both the diabetes- and galactosemia-induced elevation of oxidative stress and inhibited the decreases of myocardial ATPases; these metabolic disturbances appeared as secondary to elevated blood hexose levels and were contrasted by supplemental antioxidants (184). By examining with immunoblotting the levels of Na,K-ATPase subunit isoforms of *heart* and *skeletal muscle* in diabetic rats, a decrease of α2- and β1-subunit levels was found in diabetic cardiac muscle with decreased Na,K-ATPase activity, whereas a significant increase was noted for α1- and α2-subunit levels in skeletal muscle with no changed Na,K-ATPase activity (as well as increased α1- and β1-levels with enhanced Na,K-ATPase in kidney) (185). Seven days of subcutaneous insulin treatment partially reversed some of these alterations, without significant effect on α1-subunit level in diabetic cardiac muscle and on β1-subunit level in skeletal muscle (185). Thus, STZ-diabetes exerted isoform- and tissue-specific regulation of the Na,K-ATPase.

In *erythrocytes* from STZ-diabetic rats, a defect of Na,K-ATPase was found, accompanied by an increase in cell volume, which could play a role in the filterability and in the evolution of the microvascular changes of diabetes and which was reversed or prevented *in vivo* by insulin (186). Reddi and co-workers (187), in long-term STZ-diabetic rats, observed that erythrocyte Na,K-ATPase was not altered and was not affected by insulin. Agarwal and co-workers (188), in *alloxan-diabetic rats*, reported that erythrocyte-membrane Na,K-ATPase activity was significantly decreased; preincubation of normal ghosts with insulin resulted in marked reduction, whereas a similar treatment of diabetic erythrocyte membrane resulted in significant increase of enzyme activity (the effect depending on the hormone concentration and on the duration of preincubation).

Concernig *liver* Na,K-ATPase, STZ-diabetes induced increased hepatic Na,K-ATPase activity and enhanced expression of the β1-subunit, whereas there were no changes in the amount of the α1- and β3-isoenzymes, and no α2- and α3-isoenzymes could be detected (189). Biphasic ouabain-inhibition curves were obtained for diabetic groups, indicating the presence of low- and high-affinity sites (189). In SZT-diabetic Swiss mice, we reported an increased activity of liver Na,K-ATPase activity (22, Iannello *et al*., unpublished).

In the *aorta* of STZ-induced diabetic rats, a research of Michea and co-workers (190) demonstrated that Na,K-pump was reduced and Na,K,2Cl-cotransporter was

Table 4 Diabetes and Na,K-ATPase

1. Animal diabetes (176,177,182–184)
   - STZ-diabetic Swiss mice (22)
   - STZ-diabetric ob/ob mice (22)
   - STZ-diabetic rats (178–181,185–187,189,190)
   - STZ-diabetic Sprague Dawley rats (191)
   - Alloxan-diabetic rats (188)
   - Diabetic db/db rat (192)
   - Kidney (22,178–183)
   - Myocardium (184,185)
   - Skeletal muscle (185)
   - Erythrocytes (186–188)
   - Liver (22,189)
   - Aorta (190)
   - NEFA effect (192)
2. Human diabetes (176,177,196,200,201)
   - Genetic component (165,193)
   - Type-1 diabetes (86,88,194,197–199,202)
   - Type-2 diabetes (88,193–195,197)
   - C-peptide (86,88,194)
   - Diabetic nephropathy (195)
   - Conformational modifications of the protein (199)
   - Platelet membrane (201,202)

NEFA, non-esterified fatty acids; STZ, streptozotocin.

increased, and that endothelium was responsible for both Na,K-ATPase and Na,K,2Cl-dependent 86Rb/K uptake.

In *STZ-diabetic Sprague-Dawley rats*, the natriuretic response to D1-receptor activation is reduced as result of a decrease in D1-receptor expression and defective receptor G protein coupling (191). These abnormalities may contribute to the Na retention associated with type-1 diabetes.

With reference to the *NEFA effect* on tissue Na,K-ATPase activity in animal diabetes, only the data of Smith and co-workers (192) are available: in the *diabetic db/db rat* vascular smooth muscle, these authors demonstrated that, despite the diminished glycolysis and glucose oxidation, Na,K-ATPase activity was comparable in the control and diabetic tissue, in the absence or presence of exogenous long-chain fatty acids. In the same tissue, the accelerated oxidation of palmitate had no additional inhibitory effect on glycolysis or Na,K-ATPase activity (192). These data suggested that Na,K-ATPase activity in vascular smooth muscle was not impaired by the altered pattern of substrate utilization, which occurred in insulin-deficient db/db rats.

Human diabetes

Na,K-ATPase activity was reported to be decreased in the red blood cell membranes of subjects affected by *type-1 diabetes* (irrespective of the degree of diabetic control) and less impaired or even normal in those with *type-2 diabetes* (86,88).

Concerning the *genetic component*, a study, performed in relatives of type-2 diabetic patients and in control subjects, before and after glucose infusion and before and after treatment with dexamethasone (which decreases insulin sensitivity), showed that muscle Na, K and Mg content and 3H-ouabain binding capacity did not differ between the two groups (193). Infusion of glucose increased muscle Na content and decreased muscle K and Mg content, whereas muscle K/Mg ratio decreased only in relatives (193). Thus, subjects who were predisposed to the development of type-2 diabetes exhibited increased interdependency between glucose-, Na- and K-handling in skeletal muscle. On the other hand, theoretically, disturbances in Na and K homeostasis and Mg deficit could be possible factors in the development of obesity, type-2 diabetes and hypertension. In another study, performed in erythrocyte, Na,K-ATPase activity of normal children and infants of diabetic mothers representative of three ethnic groups (black, Caucasian and Hispanic), significant differences were found among the three ethnic groups (Caucasian having the highest values), suggestive of a genetic component influencing the enzyme activity (165).

Vague and co-workers (88) reported that, in the red blood cells of type-2 diabetic patients, Na,K-ATPase activity was strongly related to blood *C-peptide* levels in non-insulin-treated patients (in whom C-peptide concentration reflects that of insulin) as well as in insulin-treated patients. Furthermore, a gene–environment relationship has been found. The α1-isoform of the enzyme (predominant in erythrocytes and nerve tissue) is encoded by the ATP1A1 gene; a polymorphism in the intron 1 of this gene was associated with lower enzyme activity in patients with C-peptide deficiency either with type-1 or type-2 diabetes, but not in normal individuals (88,194). Short-term C-peptide infusion to type-1 diabetic patients restored normal Na,K-ATPase activity (88). Islet transplantation (which restores endogenous C-peptide secretion) enhanced Na,K-ATPase activity proportionally to the rise in C-peptide level (88). The effect of C-peptide was not indirect; indeed, incubation of diabetic red blood cells with C-peptide at physiological concentrations induced the increase of Na,K-ATPase activity. The impairment of Na,K-ATPase activity, mainly secondary to the lack of C-peptide, plays probably a role in the development of diabetic complications (88). The diabetes-induced decrease in enzyme activity would compromise microvascular blood flow by affecting microvascular regulation and decreasing red blood cell deformability, which lead to increased blood viscosity (88). C-peptide infusion restored red blood cell deformability and microvascular blood flow concomitantly with Na,K-ATPase activity (88). Vague and co-workers (88) suggested that physiological C-peptide infusion could be beneficial for the prevention of diabetic complications. The same authors reported that insulin and C-peptide directly act on erythrocyte Na,K-ATPase, restoring the decreased tissue Na,K-ATPase activity observed in type-1 diabetic patients (86).

In order to elucidate the causal relationship between Na,K-ATPase and *diabetic nephropathy*, the erythrocyte enzyme activity was studied in type-2 diabetic patients (with microalbuminuria and without microalbuminuria) and in control subjects (195). Na,K-ATPase activity was significantly reduced in diabetic patients with hypertension and in microalbuminuric patients who had higher systolic BP and greater frequency of parental hypertension than those without microalbuminuria (195). Moreover, enzyme activity in diabetic patients with parental hypertension was significantly reduced (195). In another research, the Na-pump activity was found to be higher in the diabetic than in the normal group, but the number of Na-pumps was not significantly different (196). This increased activity of erythrocyte Na-pump in diabetes mellitus might suggest an increase of cation permeability associated with a possible disorder in the diabetic membrane. Conversely, the Na-pumping activity (estimated from both Na,K-ATPase and ouabain-binding) was observed by others to be significantly decreased in type-1 and type-2 diabetic patients and to retain the insulin sensitivity only in young type-1 diabetics (197). A significant reduction of Na,K-ATPase activity in erythrocyte membranes of type-1 diabetic patients, compared with matched controls, with similar contents of erythrocyte Na- and K-ion was also reported; when eryth-

rocyte membranes of diabetic patients were incubated with their own plasma (probably containing higher concentration of a specific activator of Na,K-ATPase enzyme), a significant increase was found in enzyme activity, this effect being not influenced by the metabolic control (198).

Rabini and co-workers (199) observed that the structure of erythrocyte membranes, obtained from type-1 diabetic patients, showed a uncompetitive inhibition of the Na,K-ATPase, linked to the presence of *conformational modifications of the protein*. Modifications of the interactions between the enzymatic subunits and the membrane lipid environment might be at the basis of the Na,K-ATPase alteration in diabetes.

With a microcalorimetric study, which allows a direct measurement of the Na,K-ATPase activity in living red blood cells, a reduced Na,K-pump activity in diabetic patients was reported with a slower velocity of response to ouabain (200).

Compared with control subjects, diabetic patients showed reduced activity of Na,K-ATPase in *platelet* membranes (201), even if this observation was not found by others in type-1 diabetic patients with neuropathy, in whom platelet Na,K-ATPase activity was observed significantly higher (202).

With reference to the *NEFA effect* on tissue Na,K-ATPase activity in human diabetes, despite its potential interest, no data have been reported in literature.

Na,K-ATPase and hypertension in obesity and diabetes

There are increasing evidences that the Na-pump plays a role in hypertension through intrinsic alterations in pump activity or through external modification of pump activity by circulating inhibitors or through an endothelial mediation of endogenous inhibitor effects on the vascular response (203). On the other hand, it is well known that patients with essential hypertension exhibit several red blood cell ion transport abnormalities associated with insulin resistance and have increased risk of developing type-2 diabetes (204–209).

With reference to the *hypertension in obesity*, numerous clinical, pathophysiological and epidemiological studies demonstrated that obesity and overweight type-2 non-insulin-dependent diabetes are associated with higher prevalence of hypertension (210–213). Thus, hyperinsulinemia and decreased insulin sensitivity can be the common factors that link hypertension, abdominal obesity, impaired glucose tolerance, diabetes mellitus, dyslipidemia and accelerated atherosclerosis, alterations that form the so-called 'syndrome X' or 'metabolic syndrome' (29,30), even if the link may be associative and not pathogenetic (213). Concerning the link between hyperinsulinemia (and insulin resistance), obesity, hypertension and Na,K-ATPase, our recent data (22, Iannello *et al*., unpublished) showed a statistically significant increase of mean BP in obese patients (compared with lean subjects) and a negative correlation between the adipose tissue enzyme activity and mean BP, BMI and OGTT-insulinemic areas. In another study (169), we observed that BP in obese subjects was increased and positively correlated with basal insulinemia and OGTT-insulinemic areas, as well as with OGTT-NEFA areas, which suggests a link between NEFA, Na,K-APTase and BP. The above-mentioned observations are in agreement with the older reports of reduced erythrocyte Na,K-ATPase activity, increased cellular Na-ion concentration and reduced Na-ion excretion in hypertensive-obese patients and in their normotensive parents, who are all hyperinsulinemic subjects (214,215). In women with normal (NGT) or impaired (IGT) glucose tolerance, peripheral glucose disposal (assessed with the euglycemic-hyperinsulinemic clamp technique) and Na,K-ATPase-mediated Na efflux (determined in leucocytes) were evaluated: diastolic BP was inversely correlated with glucose-disposal rate in the insulin-resistant IGT group but not in the NGT subjects; in IGT women, insulin resistance was correlated with high BP and increased androgenic activity, while in NGT women a low level of Na,K-ATPase-mediated Na efflux was associated with hypertension (216).

With regard to the *hypertension in diabetes*, a study of Jannot and co-workers (217) underlined the association between diabetic neuropathy and hypertension, on the one hand, and diabetic neuropathy and decreased Na,K-ATPase, on the other, and reported that hypertension in type-1 diabetic patients was not associated with decreased red blood cell Na,K-ATPase. Moreover, *ACE-inhibitor treatment* in these patients (whether hypertensive or not) was associated with higher levels of cellular Na,K-ATPase, which could account for its beneficial effect on diabetic neuropathy (217).

Concerning the role of *plasma digitalis-like Na,K-pump inhibitor*, Pamnani and co-workers (218), in the hypertension of STZ-induced insulin-dependent diabetes, observed that the increase in BP was associated with an increase of Na,K-pump inhibitor in extracellular fluid volume and plasma and with a decrease in myocardial Na,K-ATPase activity. Thus, in type-1 diabetes and hypertension, an increased plasma Na,K-pump inhibitor (which inhibits cardiovascular muscle cell Na,K-ATPase activity) may be involved in the mechanism of hypertension. Some works demonstrated elevated serum concentrations of digoxin-like immunoactivity in patients affected by type-2 diabetes and hypertension (219,220) and in subjects with essential hyperinsulinemic hypertension (220), with a good correlation between insulinemia and GFR (220). It is difficult, however, to speculate whether the elevation of this digoxin-like immunoactivity is a secondary result associated with hypertension and reduced GFR, or whether it has patho-

physiologic significance. In subjects with obesity and hypertension, the levels of serum digoxin-like immunoreactivity were increased, but control and obese subjects had similar 24-h digoxin-like immunoreactivity urinary excretion (221). A more recent study of Carroll and co-workers (222) suggested that a digitalis-like factor may be increased by the *hyperinsulinemia* accompanying the euglycemic-hyperinsulinemic clamp or the OGTT, thus providing an interesting mechanism by which insulin may increase BP.

Concerning the *NEFA level*, in obese-hypertensive patients, the increase of basal plasma NEFA (and the reduced suppression of plasma NEFA by insulin) is correlated with hypertension (28,169,223,224). Moreover, Grekin and co-workers (225) demonstrated a pressor effect of portal venous oleate infusion and proposed a mechanism for obesity hypertension.

Discordance on Na,K-ATPase behaviour

It is noteworthy that discordance exists in the literature on Na,K-ATPase, as it results from some of the data discussed in this review. Thus, for instance, in erythrocytes, adipocytes or liver cells of obese subjects and type-1 or type-2 diabetic patients, various authors reported contrasting data, which probably may depend on differences in the study populations, or in the recovery of the enzyme from tissues, or in other methodological approaches (226,227). Moreover, it should be underlined that the erythrocytes, which have been much studied, are non-nucleated cells whose metabolism is not representative of that of the other tissues; indeed, Fagerberg and co-workers (140) correlated the discrepancies between several studies on Na,K-ATPase enzyme to the question of whether erythrocytes are a representative model for *trans*-membrane ion fluxes in all tissues. In fact, in human obesity as well as in hyperthyroidism, comparison of studies made in liver tissue with those made in red blood cells showed conflicting results (140). In addition, it should be considered that the activity of Na,K-ATPase (like the activity of several other enzymes) markedly decreases during erythrocyte maturation and therefore a shifting of mean cell age may greatly influence erythrocyte enzyme activity (228). Moreover, in healthy men, an effect of age on red cell membrane Na,K-ATPase activity was reported (229) as well a reduced lymphocyte enzyme in aged people (230).

Conclusion

Na,K-ATPase is an enzyme that hydrolyzes ATP and waste energy as heat; it may have a significant role in cellular thermogenesis. Reduced thermogenesis and increased accumulation of unused calories in the form of fat could result from reduced Na,K-ATPase activity in obese insulin-resistant man (157). Enzyme regulation is complex, mediated by several factors, and may vary according to the various tissues. Human obesity, like the obesity of laboratory rodents, is associated with reduction of Na,K-ATPase in some tissues, so that it appears justified to formulate the working hypothesis that hyperinsulinemia with insulin resistance (often present in obesity but also in overweight type-2 diabetes and hypertension) may repress Na,K-ATPase enzyme activity in several tissues, probably by indirect way, through mediation of NEFA, which are reported to act as enzyme inhibitors and which are elevated in the above-mentioned insulin-resistant syndromes.

The decreased Na,K-ATPase enzyme activity in obesity and type-2 diabetes might entail reduced thermogenesis, which in turn might facilitate a positive caloric balance, thus favouring the development of obesity. The same mechanism, perhaps, would enhance Na-ion retention, influencing the increased BP often present in obese and obese-hyperglycemic subjects.

Conflict of Interest Statement

No conflict of interest was declared.

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