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Regulation of Expression of the Na^+/H^+ Exchanger by Thyroid Hormone

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The Na^+/H^+ exchanger is a pH regulatory protein with a ubiquitous distribution in eukaryotic cells. Several isoforms of the Na^+/H^+ exchanger are known. The first isoform to be characterized and cloned, NHE1, is present on the plasma membrane of cells and functions to remove one intracellular proton in exchange for one extracellular sodium ion. It is involved in pH regulation, cell growth, differentiation, and cell migration. NHE1 is also involved in the cycle of damage that occurs in the heart with ischemic heart disease. Recent studies have shown that the Na^+/H^+ exchanger is regulated in response to thyroid hormone. Reduction in circulating thyroid hormone levels reduces the amount of both protein and mRNA of NHE1. Conversely, an elevation of thyroid hormone levels has the opposite effects. Transcriptional regulation of NHE1 expression has been demonstrated. The NHE1 promoter contains a $TR\alpha_1$ binding site located between −841 to −800 bp. This element responds positively to $TR\alpha_1$. This regulation of the NHE1 promoter by thyroid hormone is proposed to be responsible for postnatal changes in expression of the Na^+/H^+ exchanger.

I. INTRODUCTION

The Na^+/H^+ exchanger is a ubiquitous protein present in mammalian cells. In higher eukaryotes this integral membrane protein removes one intracellular H^+ for one extracellular Na^+, serving to protect cells from intracellular acidification. In addition, the Na^+/H^+ exchanger participates in regulating sodium fluxes and cell volume. The Na^+/H^+ exchanger is essential in the myocardium, where it prevents intracellular acidosis that inhibits contractility (Dibrov and Fliegel, 1998; Putney *et al.*, 2002; Slepkov and Fliegel, 2002; Wiebe *et al.*, 2001). It also plays a key role in the damage that occurs to the mammalian myocardium during ischemia and reperfusion (Avkiran and Marber, 2002), and it is involved in hypertrophy of the myocardium (Ennis *et al.*, 2003; Karmazyn, 2001). The Na^+/H^+ exchanger is regulated via several mechanisms, including hormonal activation. The exchanger is composed of two domains: a membrane domain that transports cations and a hydrophilic cytoplasmic domain that modifies the activity of the membrane domain (Orlowski and Grinstein, 1997; Schmitt *et al.*, 1996; Wakabayashi *et al.*, 1994) (Fig. 1). Though several isoforms of the Na^+/H^+ exchanger exist (Orlowski and Grinstein, 2003), we (Fliegel, 1999; Fliegel *et al.*, 1991, 1993a,b) and others (Karmazyn *et al.*, 1999; Orlowski *et al.*, 1992; Takaichi *et al.*, 1992) have shown that the Va^+/H^+ exchanger isoform 1 (NHE1) is the only significant plasma membrane isoform in the myocardium. The purpose of this chapter is to discuss the recent finding that thyroid hormone regulates the expression of the NHE1 isoform of the Na^+/H^+ exchanger. We emphasize the regulation of the Na^+/H^+

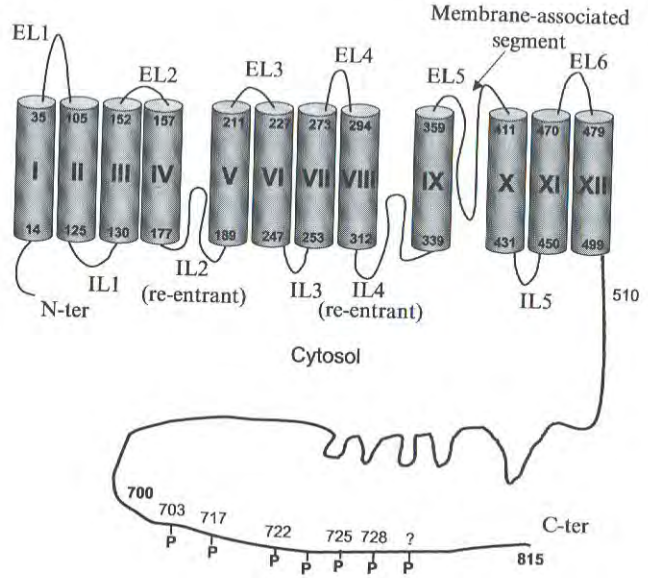

FIGURE 1. Hypothetical topology of the Na^+/H^+ exchanger. I–XII indicate transmembrane segments. IL1–5 indicate intracellular loops. EL1–6 indicate extracellular loops. P, Putative phosphorylation sites by regulatory protein kinases. The model is modified from Wakabayashi *et al.*, 2000

exchanger in the myocardium, where it plays an important physiological role and where thyroid hormone has been shown to play an important role in regulation of NHE1 expression. We discuss the importance of this protein, the physiological significance of regulation of NHE1, and the new information that shows how thyroid hormone regulates expression of NHE1 through the promoter. We also discuss avenues that future research can explore.

II. PHYSIOLOGICAL SIGNIFICANCE OF NHE1

A. IMPORTANCE OF THE Na^+/H^+ EXCHANGER AND pH REGULATION IN THE MYOCARDIUM

The Na^+/H^+ exchanger is important in intracellular pH homeostasis in the myocardium. With the proton generation of intermediary metabolism and the effect of negative membrane potential, protons tend to accumulate within the cytosol and inhibit contractility. Thus, pH regulation is necessary. With its steep relationship between intracellular pH and activity, the Na^+/H^+ exchanger is maximally active at low intracellular pH ($pH \leq 6.5$), removing

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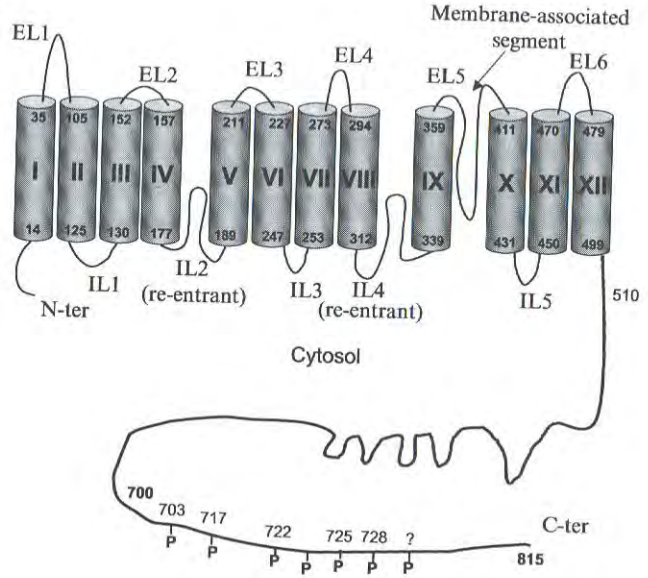

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excess acid of intermediary metabolism. This pH dependence can be shifted to a more alkaline range by α_1-adrenergic stimulation and by hormones such as endothelin (Haworth and Avkiran, 2003; Karmazyn and Moffat, 1993). Mitogen-activated protein kinase (MAPK) dependent phosphorylation is important in this response (Moor and Fliegel, 1999; Moor *et al.*, 2001).

Although HCO_3^--based transporters contribute to recovery from intracellular acidosis, these antiporters generally contribute less than 50% of total acid efflux in the myocardium. In addition, HCO_3^--based transporters are mostly active at more alkaline pHs (Dart and Vaughan-Jones, 1992; Lagadic-Gossmann *et al.*, 1992a,b; Liu *et al.*, 1990). Therefore, at acidic pHs the Na^+/H^+ exchanger is the major mechanism for removal of protons from the myocardium (Dart and Vaughan-Jones, 1992; Grace *et al.*, 1993; Lagadic-Gossmann *et al.*, 1992a,b; Liu *et al.*, 1990; Vandenberg *et al.*, 1993, 1994).

The Na^+/H^+ exchanger plays a key role in the damage that occurs during ischemia and reperfusion. When intracellular acidosis occurs during ischemia, the Na^+/H^+ exchanger removes excess protons from the cell. This results in a buildup of intracellular Na^+ that either inhibits the Na^+/Ca^{2+} exchanger's ability to extrude Ca^{2+} or reverses the Na^+/Ca^{2+} exchanger such that it accumulates Ca^{2+} within the cell. This increased intracellular Ca^{2+} is detrimental to the heart. Inhibition of Na^+/H^+ exchanger activity has beneficial effects on the myocardium in a vast number of studies. Drugs that inhibit the Na^+/H^+ exchanger include amiloride and its derivatives and a variety of guanidine derivative compounds, such as cariporide, that have been developed for clinical treatment of heart disease (Avkiran, 2001; Avkiran and Marber, 2002; Chakrabarti *et al.*, 1997; Karmazyn, 1996, 2001, 2003a,b; Karmazyn *et al.*, 1999, 2001; Scholz and Beier, 2003; Scholz *et al.*, 1993, 1995, 1999; Yasutake *et al.*, 1994).

The Na^+/H^+ exchanger and its inhibition are important to a number of other pathological and physiological cardiovascular events. Perhaps the most important is its role in mediating heart hypertrophy. Congestive heart failure is a major clinical problem in North America, and hypertrophy is an early maladaptive response (Cohn, 1996). Blockade of the Na^+/H^+ exchanger can prevent hypertrophy (Camilion de Hurtado *et al.*, 2002; Chen *et al.*, 2001; Cingolani *et al.*, 2003; Ennis *et al.*, 2003; Karmazyn, 2001, 2003b). Thus, NHE1 blockade has potential as a new therapeutic approach for the treatment of heart failure (Karmazyn, 2001).

The Na^+/H^+ exchanger is also involved in hypertension. The activity of the Na^+/H^+ exchanger is elevated in essential hypertension in humans and in animal models of hypertension (reviewed in Alvarez *et al.*, 2002). This involvement may be critical because it affects sodium reabsorption in the kidney and ultimately blood pressure (Ng *et al.*, 2000). The elevation in

activity of the Na^+/H^+ exchanger in hypertension is due to regulation by phosphorylation (Ng *et al.*, 2000; Phan *et al.*, 1997).

Other studies have demonstrated that NHE1 inhibition, in conjunction with cardioplegic solutions, improves recovery of cardiac output (Cox *et al.*, 2002; Myers, 2003; Shipolini *et al.*, 1997; Tritto *et al.*, 1998). In addition, exciting recent results have shown that NHE1 inhibition can enhance the efficacy and outcome of closed-chest resuscitation from cardiac arrest. In the US alone, over 350,000 persons suffer episodes of cardiac arrest per year and less than 5% of these return to productive lives (Gazmuri and Ayoub, 2003; Gazmuri *et al.*, 2001a,b).

Clinical trials on the use of Na^+/H^+ exchanger inhibitors are underway. The Guardian trial showed that the guanidine derivative inhibitors are safe for patient use. Patients undergoing coronary artery bypass grafting showed significant improvements when they were treated with the Na^+/H^+ exchanger inhibitor cariporide. The Expedition trial further elaborates these results (Avkiran and Marber, 2002; Scholz and Beier, 2003).

B. PHYSIOLOGICAL SIGNIFICANCE IN OTHER TISSUES

The Na^+/H^+ exchanger has a number of important physiological roles in other tissues. Briefly (for extensive reviews, see Fliegel, 1996; Karmazyn *et al.*, 2003; Putney *et al.*, 2002), the Na^+/H^+ exchanger is involved in cell growth, proliferation, and differentiation (Alvarez *et al.*, 1989; Grinstein *et al.*, 1989; Hazav *et al.*, 1989; Pouyssegur *et al.*, 1984), including neuronal differentiation of P19 cells (Wang *et al.*, 1997b). We have found that Na^+/H^+ exchanger expression varies during myocardial development, suggesting that it plays an important role in embryogenesis (Fliegel, 2003; Rieder and Fliegel, 2002, 2003). The Na^+/H^+ exchanger is also important in programmed cell death of a number of cell types because it suppresses apoptosis (Perez-Sala *et al.*, 1995; Rajotte *et al.*, 1992). In other cells, such as pro-β-cell lines, we found that trophic factor withdrawal activates the Na^+/H^+ exchanger by p38-mediated phosphorylation. This elevates intracellular pH, causing alkalinization that activates the death protein Bax and disrupts mitochondrial metabolism (Khaled *et al.*, 2001).

Recent results have shown that the Na^+/H^+ exchanger acts as a plasma membrane anchor for actin filaments through an association with actin binding proteins, and that NHE1-deficient cells have impaired cytoskeletal organization (Denker *et al.*, 2000). NHE1 also regulates cell migration (Denker and Barber, 2002).

The Na^+/H^+ exchanger regulates cell volume in a number of cell types (Grinstein and Rothstein, 1986; Grinstein *et al.*, 1985). We have demonstrated this function in the myocardium (Moor *et al.*, 2000). It is also critical in intracellular pH regulation in the preimplantation embryo (Lane *et al.*, 1998; Phillips *et al.*, 2000), and Na^+/H^+ exchanger-dependent intracellular

alkalinization is essential in malignant transformation of some cells (Reshkin *et al.*, 2000). The Na^+/H^+ exchanger is also a target for tumor therapy, especially for solid tumors (Maidorn *et al.*, 1993; Rotin *et al.*, 1989; Yamagata and Tannock, 1996). Overall it is clear that the Na^+/H^+ exchanger is an extremely important protein both within and outside of the cardiovascular system.

C. Na^+/H^+ EXCHANGER PRESENCE IN THE MYOCARDIUM

The ubiquitously expressed NHE1 was the first Na^+/H^+ exchanger isoform cloned (Sardet *et al.*, 1988); subsequently, NHE2–NHE8 have been discovered and they have a more restricted tissue distribution. NHE1–NHE5 are predominantly plasmalemmal, with NHE6 found in mitochondria or endosomes (Nass and Rao, 1998; Numata *et al.*, 1998). The NHE3 isoform is predominantly present in the kidney (Fliegel, 1999; Fliegel *et al.*, 1991; Orlowski and Grinstein, 2003), whereas NHE7 is in the golgi and endosomes, and the distribution of NHE8 is not yet clear (Orlowski and Grinstein, 2003). Homology between the isoforms varies from 20 to 75% (Orlowski and Grinstein, 2003). Our laboratory first identified NHE1 in the myocardium and in isolated myocytes (Fliegel *et al.*, 1991, 1993a). There is little or no expression of other isoforms in the heart (Brant *et al.*, 1995; Collins *et al.*, 1993; Klanke *et al.*, 1995; Orlowski *et al.*, 1992; Takaichi *et al.*, 1992; Tse *et al.*, 1993; Wang *et al.*, 1993). In the myocardium, we (Dyck *et al.*, 1995a; Fliegel *et al.*, 1993a) and others (Gan *et al.*, 1999) have shown that the levels and activity of the Na^+/H^+ exchanger increase with ischemia and reperfusion. We also showed that the Na^+/H^+ exchanger is transcriptionally regulated in cardiomyocytes (Wang *et al.*, 1997b; Yang *et al.*, 1996b).

III. Na^+/H^+ EXCHANGER BASIC STRUCTURE

The Na^+/H^+ exchanger is composed of two major domains: a transmembrane domain of 500 amino acids and a large intracellular cytoplasmic "tail" of 315 amino acids (see Fig. 1). The N-terminal membrane domain is responsible for the movement of cations across the membrane (Wakabayashi *et al.*, 1992). The large C-terminal cytoplasmic tail regulates the membrane domain (Counillon and Pouyssegur, 2000; Orlowski and Grinstein, 1997; Wakabayashi *et al.*, 1992). The membrane domain has 12 integral membrane segments (Wakabayashi *et al.*, 2000) (see Fig. 1). In addition, intracellular loops 2 and 4 may invaginate into the lipid bilayer and extracellular loop 6 may be a re-entrant loop. The membrane domain has both N- and O-linked carbohydrate groups (Counillon *et al.*, 1994; Haworth *et al.*, 1993). The cytoplasmic domain is the site of phosphorylation by kinases, and it binds regulatory proteins and cofactors (see later). Although the detailed physical structure of NHE1 is not yet known, we have demonstrated that it is a dimer (Fliegel *et al.*, 1993b). Our laboratory also has shown that the cytoplasmic tail is primarily monomeric and undergoes pH dependent changes in conformation (Li *et al.*, 2003; Rieder and Fliegel, 2003). Finally, our laboratory demonstrated that the Na^+/H^+ exchanger exists in both detergent-resistant rafts and in higher density membrane fractions, and that NHE1 exhibits strong hydrophobic interactions that cause temperature-dependent aggregation (Bullis *et al.*, 2002).

IV. GENERAL ASPECTS OF REGULATION OF EXPRESSION OF NHE1

It has long been known that regulation of expression of the NHE1 isoform of the Na^+/H^+ exchanger varies in response to the environment. In the kidney, a number of early studies showed that chronic acidosis treatment of either intact animals or kidney cells in culture results in an increase in NHE1 expression (Horie *et al.*, 1990; Moe *et al.*, 1991; Presig and Alpern, 1988). This increase is linked to increased rates of transcription of the NHE1 and an increase in mRNA content of NHE1 (Alpern *et al.*, 1993).

Cell growth and differentiation also cause large changes in regulation of expression of the Na^+/H^+ exchanger. Several different models have demonstrated this effect, including differentiation of human leukemic cells (HL-60), (Ladoux *et al.*, 1988; Rao *et al.*, 1992), differentiation of L6 cells from myoblasts to myotubes (Yang *et al.*, 1996a), and differentiation of mouse P19 cells to neuronal like cells (Dyck and Fliegel, 1995). The level of induction of expression of NHE1 varies up to 18-fold in some tissues (Ladoux *et al.*, 1988; Rao *et al.*, 1992). We examined the developmental regulation of NHE1 during embryonic and postnatal development in mice. We made transgenic mice with the NHE1 promoter-driving expression of green fluorescent protein and β-galactosidase. The level of expression varied greatly with the stage of development and the tissue examined. The greatest level of NHE1 transcription was in the liver and hearts of 12-day-old fetal mice. This expression declined until it reached a low basal level at birth (Rieder and Fliegel, 2002, 2003). There also were changes in postnatal expression of the NHE1 protein. After birth, the levels of protein expression increase in the heart, lung, liver, kidney, and brain up to the age of 14 days and then decline. The heart had over 6-fold changes in the level of expression when comparing the peak at 2 weeks with the neonatal myocardium. Detailed analysis of the expression of the NHE1 promoter-driven green fluorescent protein confirmed that the NHE1 promoter was active in cardiomyocyte cells of the heart (Rieder and Fliegel, 2002, 2003).

Changes in expression of NHE1 suggest that the protein plays an important role in development. This was demonstrated in a study on P19 cells that showed that differentiation of these cells is blocked by either inhibiting NHE1 protein activity or knocking out the NHE1 gene in these cells (Wang *et al.*, 1997b).

It is also significant physiologically that the level of expression of NHE1 protein and mRNA vary with cardiac hypertrophy. Na^+/H^+ exchanger message levels are greatly increased in both the spontaneously hypertensive rat and in monocrotaline-induced hypertrophy (Chen *et al.*, 2001; Takewaki *et al.*, 1995). In experimental models in culture, stretching of myocytes on deformable plates mimics cardiac hypertrophy and increases NHE1 message levels (Takewaki *et al.*, 1995). Because of the role that NHE1 plays in cardiac hypertrophy (see earlier), changes in the level of expression may have important clinical significance.

V. INITIAL STUDIES ON CLONING AND CHARACTERIZATION OF THE MOUSE NHE1 PROMOTER

We cloned the mouse NHE1 promoter in our laboratory in 1995 (Dyck *et al.*, 1995b). We examined a 1.2-kb fragment of the promoter that contains the transcriptional start sites and a number of putative transcription factor binding sites. Our studies demonstrated that the regulation of the NHE1 promoter varies from one tissue to another. An AP-2 binding site was found at −95 to −111 bp (base pairs) from the start site. In fibroblasts and P19 embryonal carcinoma cells, deletion of the regions distal to this site reduced basal activity of the promoter by about 40% and 25%, respectively (Dyck and Fliegel, 1995; Dyck *et al.*, 1995b); However, in isolated cardiac myocytes (Yang *et al.*, 1996b), the same mutation reduced basal activity by about 75%. Deletion of the AP-2 site, along with the removal of the more distal regions of the promoter, completely eliminated activity in all these tissues. However, it should be noted that although these effects were readily apparent in cells in culture, in intact animals the AP-2α transcription factor is not as important. When we examined transgenic mice with a knockout of this transcription factor, expression of the NHE1 protein was not reduced (Rieder and Fliegel, 2003). It is possible that, *in vivo*, other transcription factors compensate for the lack of AP-2α or that other members of the AP-2 transcription factors such as AP-2β and AP-2γ (Talbot *et al.*, 1999) are the critical elements regulating the NHE1 promoter at this site.

Several other regions of the mouse NHE1 promoter are important in regulating expression in varying cell types and under different circumstances. At −155 to −169 bp there is a highly conserved (dA:dT) region that acts as an enhancer for the NHE1 promoter and other promoters and is protected by nuclear extracts in DNase I footprinting experiments (Yang *et al.*, 1996c). High-mobility group nuclear proteins bind to this sequence (Wang *et al.*, 1997a), and they may be important in regulation in cell growth and differentiation (Patel *et al.*, 1994).

In 1998, we showed that distal regions of the NHE1 promoter are necessary for activation of the gene in response to various mitogenic stimuli (Besson *et al.*, 1998). Mitogenic stimuli such as serum, insulin, thrombin, and epidermal growth factor increased the activity of a 1.1-kb fragment of the promoter, and the increase in activity required the distal region of the gene. These experiments were followed by a detailed analysis of the transcription factors that bound to the distal region of the gene. Two of these were the chicken ovalbumin upstream promoter transcription factors (COUP-TF) type I and II (Fernandez-Rachubinski and Fliegel, 2001). Nucleotides at −841 to −800 bp upstream of the start site bound COUP-TFs and were protected in footprint analysis of the mouse promoter. Expression of the COUP-TF in NIH 3T3 cells in culture increased NHE1 message levels. In addition, expression of the proteins in cells in culture activated transcription of the NHE1 gene. This activation was dependent on the presence of an intact COUP-TF binding site.

VI. REGULATION OF NHE1 BY THYROID HORMONE

A. THYROID HORMONE REGULATION OF EXPRESSION

Thyroid hormones have profound effects on the heart and other tissues (Klein and Ojamaa, 2001). Cardiac functions such as heart rate, cardiac output, and systemic vascular resistance are linked to thyroid status. Triiodothyronine (T3) decreases systemic vascular resistance, increases tissue thermogenesis, increases cardiac inotropy and chronotropy, and increases blood volume (Klein and Ojamaa, 2001). T3 is the biologically active form of thyroid hormone, and, once inside cardiomyocytes, it binds to nuclear receptors that target it to affect gene expression. The actions of thyroid hormone are largely viewed as cell nucleus mediated, through nuclear or genomic actions. They are generally thought to require gene transcription and changes in protein synthesis, although significant acute actions of thyroid hormones also occur (Davis and Davis, 2002; Lin *et al.*, 1999). Long-term regulation of protein levels by thyroid hormone has been demonstrated for many genes. The α-myosin heavy chain, the sarcoplasmic reticulum Ca^{2+}-ATPase, β_1-adrenergic receptors, Na^+/K^+ ATPase, and some voltage-gated potassium channels respond positively to thyroid hormone in the myocardium. Some other genes such as phospholamban and the Na^+/Ca^{2+} exchanger are down-regulated by thyroid hormone (Klein and Ojamaa, 2001). Because transcriptional regulation of membrane proteins such as the Ca^{2+}-ATPase,

β_1-adrenergic receptors, and Na^+/K^+ ATPase has been demonstrated, it is clear that regulation of membrane proteins in the myocardium by thyroid hormone is not an unusual phenomenon.

Early studies examined the effect of thyroid status on the mRNA levels of several isoforms of the Na^+/H^+ exchanger. Azuma *et al.* (1996) found that in the renal cortex, neither NHE1, NHE2, nor NHE3 mRNA levels were altered in the change from euthyroid to hypothyroid states. Also, only NHE2 and NHE3 mRNA levels were increased when comparing hypothyroidism with hyperthyroidism. However, the levels of protein were not altered in response to thyroid hormone. In contrast to these results, later reports showed that NHE3 protein levels are elevated in the kidney brush border membranes after T3 treatment (Baum *et al.*, 1998; Cano *et al.*, 1999). The NHE3 gene does contain putative binding sites for thyroid hormone receptors (Kandasamy and Orlowski, 1996), and the effect on NHE3 has been demonstrated to be due to transcriptional activation of the NHE3 gene (Cano *et al.*, 1999).

Short-term nongenomic effects of thyroid hormones on the Na^+/H^+ exchanger also have been demonstrated. Both T3 and T4 (tetraiodothyronine) activate Na^+/H^+ exchanger activity in L6 cells from rat skeletal muscle. Treatment with T3 for as little as 2 min elevated intracellular pH. In addition, both intracellular pH recovery from an acid load and the set-point of the Na^+/H^+ exchanger were elevated after T3 and T4 treatments. The effects of T3 were much more rapid than those of T4 (Incerpi *et al.*, 1999). Chick embryo hepatocytes show similar effects that mediate through a pathway involving protein kinase C, MAPK, and phosphatidylinositol 3-kinase (Incerpi *et al.*, 2002).

In the myocardium, Doohan *et al.* (1997) examined the effect of treatment of hypothyroid rats on intracellular sodium levels and on Na^+/H^+ exchanger activity. This study showed that thyroid hormone enhances both Na^+/H^+ exchange activity and sodium influx rates (Doohan *et al.*, 1997). Wolska *et al.* (1997) gave a more systematic study of the effect of thyroid state on intracellular pH and Na^+ homeostasis in the rat heart. Myocytes from hypothyroid hearts demonstrated increased intracellular sodium and a decrease in intracellular pH. NHE1 mRNA levels varied with thyroid hormone status, increasing in the hyperthyroid hearts. These results confirmed that the NHE1 message is regulated in the heart in response to thyroid hormone. Our study (Li *et al.*, 2002) demonstrated that thyroid hormone also affects NHE1 protein levels in the myocardium. We showed that hypothyroid rat hearts had approximately 3-fold decreased levels of NHE1 protein compared with euthyroid rat hearts. Conversely, hyperthyroid rat hearts had the NHE1 protein levels increased almost 3-fold compared with euthyroid rat hearts (Fig. 2). Similar results were obtained with T3 treatment of isolated cardiomyocytes (Li *et al.*, 2002).

Overall, it is clear that a number of studies have demonstrated that thyroid hormone stimulates NHE1 message and protein levels in addition to

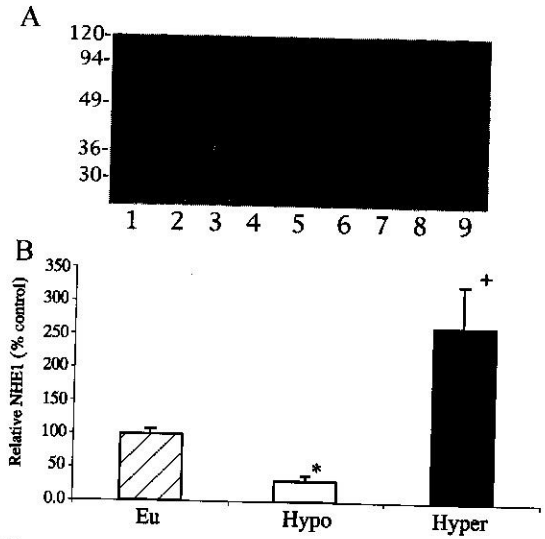

FIGURE 2. Western blot analysis of protein levels of the NHE1 isoform of the Na^+/H^+ exchanger in intact heart microsomes made from hypothyroid, euthyroid, and hyperthyroid rats. (A) Western blot of microsomes prepared from euthyroid (1–3), hypothyroid (4–6), and hyperthyroid (7–9) rat hearts. (B) Summary of experiments. $^+$ indicates significance at $p < 0.05$, and * indicates significance at $p < 0.01$. Eu, Euthyroid; (Hypo), hypothyroid; (Hyper), hyperthyroid. Republished with permission of L. Fliegel, Li *et al.* (2002). *J. Biol. Chem.* **277**, 28656–28662; permission conveyed through Copyright Clearance Center, Inc.

stimulating the activity of the protein. This has been demonstrated especially clearly in the myocardium.

B. THYROID HORMONE REGULATION OF THE NHE1 PROMOTER

We (Fernandez-Rachubinski and Fliegel, 2001; Li *et al.*, 2002) have recently investigated the mechanism by which thyroid hormone regulates expression of the NHE1 promoter. While determining the mechanism by which the distal region of the promoter mediates increases in NHE1 transcription, we surveyed a variety of synthetic oligonucleotides, testing for their ability to block nuclear extract binding to the native −841 to −800 element. Synthetic oligonucleotides coding for the thyroid response element (palindrome) were able to compete with nuclear extracts for binding to this region (Fernandez-Rachubinski and Fliegel, 2001). The −841 to −800 region was demonstrated to bind to COUP-TF and thyroid hormone receptor ($TR\alpha_1$) transcription factors. We found several critical mutations that eliminated binding of COUP-TFs to this region (Table I). The M1 mutation (Table I) reduced the binding of both COUP-TF1 and $TR\alpha_1$. The

TABLE I. Effect of Mutations on Binding to the −841 to −800 Element of the Mouse NHE1 Promoter

Type	DNA sequence −841 to −800	COUP-TF binding	$TR\alpha_1$ binding
Wild-type	GGGTCTCCCT ACTGACCTCA GCCTGGTCTA GAACTCACTT	++++	++++
M1	GGGCGATATA ACTGACCTCA GCCTGGTCTA GAACTCACTT	++	–
M2	GGGTCTCCCT ACCAAAACCA GCCTGGTCTA GAACTCACTT	–	++++
M3	GGGTCTCCCT ACTGACCTCA GCAAACCCTA GAACTCACTT	++++	++++

In vitro binding to the −841 to −800 element of the mouse NHE1 promoter was examined as described earlier (Li *et al.*, 2003). The mutations indicated (**shadow**) were introduced into the −841 to −800 element, and the effect on COUP-TF binding and $TR\alpha_1$ binding were examined.

M2 mutation did not bind COUP-TF1, but bound $TR\alpha_1$ both as a monomer and a dimer. Binding of $TR\alpha_1$ and COUP-TF1 was unaltered with the M3 mutation (Table I). The results showed that the −838 to −832 region is critical for $TR\alpha_1$ binding, but not as critical for COUP-TF binding. Conversely, the −829 to −824 region is critical for COUP-TF binding, but is not required for $TR\alpha_1$ binding. The binding of $TR\alpha_1$ could partially block the binding of COUP-TF to the −841 to −800 element. However, COUP-TF1 did not reduce the binding of $TR\alpha_1$. Overall these results suggest that the $TR\alpha_1$ binding site is different from that of COUP-TF, and that when bound, the $TR\alpha_1$ protein overlaps with the −829 to −824 region.

Further analysis showed that transfection of cells with $TR\alpha_1$ activated the NHE1 promoter. Tandem copies of the −841 to −800 element activated a minimal fragment of the NHE1 mouse promoter, and could also activate a truncated region of the thymidine kinase promoter. This effect occurred in both NIH 3T3 cells and in CV1 cells (Li *et al.*, 2002). A single copy of the −1085 to −800 region of the NHE1 promoter could activate transcription from the NHE1 minimal promoter, whereas the −1085 to −842 region of the gene was unable to do so. We also found that there was some synergy between COUP-TF1 and $TR\alpha_1$. Cotransfection of COUP-TF1 and $TR\alpha_1$ resulted in greater activation of the NHE1 promoter than the sum of their individual effects on the promoter.

C. PHYSIOLOGICAL SIGNIFICANCE OF THYROID HORMONE REGULATION OF NHE1 EXPRESSION

The results discussed previously clearly show that thyroid hormone regulates transcription and protein expression of the NHE1 isoform of the Na^+/H^+ exchanger. The physiological significance of this regulation remains to be determined. Circulating thyroid hormone is responsible for regulation of expression of many genes. For example, it mediates a switch in the expression of myosin heavy chain isoforms. The β-myosin heavy chain is expressed in the fetus, whereas the α-myosin heavy chain is expressed after birth. Thyroid hormone mediates this change. In the mouse, this change is correlated with an increased level of circulating thyroid hormone that mediates these effects through thyroid response elements that are present in the proximal region of the α-myosin heavy chain promoter (Rindt *et al.*, 1995). Thyroid hormone levels vary after birth. In the rat, T3 levels increase progressively to a peak at 26 days after birth, and subsequently decline to adult levels by 40 days of age (Walker *et al.*, 1980). One possibility is that a similar type of regulation is responsible for postnatal changes in the level of Na^+/H^+ exchanger.

Supporting this hypothesis is the observation that in the kidney proximal straight tubule, the maturational increases in NaCl and Na^+/H^+ exchanger activity were prevented in hypothyroid animals. However, in this study it was not determined which isoform of the Na^+/H^+ exchanger was involved, and it is likely that the NHE3 isoform was responsible for this observation (Shah *et al.*, 2000). Baum *et al.* (1998) also have shown that thyroid hormone regulates NHE3 mRNA abundance in a manner that is consistent with the postnatal increase in NHE3 activity. We (Rieder and Fliegel, 2002) have recently demonstrated that expression of the NHE1 protein increases postnatally in several tissues of the mouse. The increase peaks at approximately 2 weeks of age. Notably the greatest increase in NHE1 protein expression occurred in the heart, where there was over a 6-fold increase in NHE1 protein levels. These results suggest that thyroid hormone could be responsible for this postnatal increase in NHE1 protein levels. The function of this change in protein levels has not yet been determined.

VII. SUMMARY AND FUTURE DIRECTIONS

Recent studies have demonstrated that thyroid hormone regulates expression of the NHE1 isoform of the Na^+/H^+ exchanger. Changes in transcription, protein levels, and activity of NHE1 have been noted. It is clear that these effects are mediated at least in part by transcriptional activation of the NHE1 promoter through $TR\alpha_1$ acting on the promoter. The site of action of $TR\alpha_1$ appears to be within the −841 to −800 element.

The heart appears to be the primary organ involved in this regulation. Thyroid hormone has a large number of physiological effects on the myocardium that affect both inotropy and developmental changes in protein expression. The NHE1 protein is clearly one of the many proteins whose expression levels are affected.

More studies on the physiological significance of this effect are warranted. It would be useful to demonstrate directly that thyroid hormone regulates developmental changes in NHE1 protein levels in the newborn mouse. This could be done by examining changes in NHE1 protein levels in these mice with varying thyroid hormone levels. In addition, it would be informative to use nuclear run on experiments to confirm that changes in NHE1 message levels induced by changes in levels of thyroid hormone are due to changes in transcription and not to changes in RNA stability. A comparison of the ability of "hypo," "hyper," and "euthyroid" cardiomyocytes to tolerate pH stress and "ischemic" stress would also provide useful information. This could be coupled with an examination of the compensatory role, if any, of other pH-regulatory proteins such as anion exchangers. Given that many cardiovascular abnormalities occur in both hyperthyroidism and hypothyroidism (Klein and Ojamaa, 2001), such experiments would be of great interest.

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