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Stimulation of Na,K-ATPase by Low Potassium Is Dependent on Transferrin

W. Yin[1], G. Jiang[2], K. Takeyasu[3], X. Zhou[1]
Departments of Medicine[1] and Anatomy[2], Uniformed Services University of the Health Sciences, 4301, Jones Bridge Rd, Bethesda, MD 20814, USA
[3]Department of Natural Environment Sciences, Kyoto University, Kyoto, Japan

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Abstract. We took advantage of the fact that confluent MDCK cells can survive in a serum-free medium for several days to examine whether the upregulation of Na,K-ATPase by low K^+ required serum. We found that serum was essential for low K^+ to induce an increase in the cell surface Na,K-ATPase molecular number as quantified by ouabain binding assays. Further analyses identified that transferrin, not EGF or IGF-1, could simulate the effect of serum. Moreover, transferrin was also required for low-K^+-induced increases in α1-subunit promoter activity, α1- and β1-subunit protein abundance of the Na,K-ATPase. In the presence of transferrin, low K^+ enhanced cellular uptake of iron. Inhibition of intracellular iron activity by deferoxamine (40 μM) abrogated the effect of low K^+ on the Na,K-ATPase. Like deferoxamine, catalase (100 U/ml) also ablated the effect of low K^+. We conclude that stimulation of the Na,K-ATPase by low K^+ is dependent on transferrin. The effect of transferrin is mediated by increased iron transport and reactive oxygen species activity.

Key words: Iron — Reactive oxygen species — MDCK cells

Introduction

Na,K-ATPase, or sodium pump, is a heterodimer membrane protein that is composed of α and β subunits. Under physiological condition, the Na,K-ATPase expels three molecules of Na^+ out of cells in exchange for two molecules of K^+ at the expense of 1 molecule of ATP. In most animal cells, the Na,K-ATPase represents the only pathway to extrude Na^+ and to uptake K^+, and this process is absolutely required to form and maintain inward Na^+ and outward K^+ gradients across the plasma membrane. The Na^+ and K^+ gradients are essential for nutrient uptake, apoptosis, regulation of cellular Ca^{2+} concentration, osmolarity and pH, and electrical excitability as well. Therefore, it is not surprising that the Na,K-ATPase is delicately regulated according to cell demands for Na^+ and K^+ gradients. Decreases in these ion gradients usually result in upregulation of the pump. For example, a decrease in Na^+ gradient by activation of voltage-sensitive Na^+ channels in cultured chick skeletal muscles or by hypertonic medium in Madin Darby canine kidney cells (MDCK) cells stimulates the enzyme (Wolitzky & Fambrough, 1986; Bowen, 1992). Similarly, decreases in both Na^+ and K^+ ion gradients by partial inhibition of Na,K-ATPase activity with ouabain or with a decrease in extracellular K^+ concentration increase the capacity of the sodium pump both in cultured cells and in kidneys (Pressley et al., 1986, 1988; Bowen & McDonough, 1987; Hayashi & Katz, 1987; Rayson, 1989; McDonough, Magyar & Komatsu, 1994; Buffin-Meyer et al., 1998). Among the conditions that lead to upregulation of the Na,K-ATPase, the effect of low extracellular K^+ has been most extensively studied. Studies have demonstrated that low K^+ stimulates the Na,K-ATPase activity, which is preceded by increased mRNA level and protein synthesis in most cells (Bowen & McDonough, 1987; Pressley et al., 1988; Lescale-Matys et al., 1999) including the medullary collecting duct (Buffin-Meyer et al., 1998), except in a human cervical cancer cell line (Hela cells) in which low K^+-induced Na,K-ATPase activity is attributed to increased stability of the enzyme (Pollack, Tate & Cook, 1981).

As in many other biological events, a cascade of biochemical reactions must take place before low K^+ increases the transcription and translation of the

Correspondence to: X. Zhou; email: xiazhou@usuhs.mil

Na,K-ATPase. Aydemir-Koksoy and Allen (2001) have demonstrated that LY 294002, a specific inhibitor of PI3 kinase, blocks the upregulation of ouabain binding sites in the vascular smooth muscle cells. The direct measurement of PI3 kinase activity in p85 subunit immunoprecipitates reveals that low K^+ increases the activity of the kinase (Zhou et al., 2001). In many respects, ouabain elicits similar reactions as low K^+. In rat cardiac myocytes, ouabain induces gene expressions that are related to cardiac hypertrophy, and this process is mediated by reactive oxygen species (ROS) (Xie et al., 1999). However, most of the components that transduce the effect of low K^+ remain to be identified.

Transferrin is a single-chain glycoprotein with molecular weight of near 80,000, bearing two structurally similar but functionally distinct iron-binding sites. Although many tissues can synthesize transferrin, livers are the primary source of plasma transferrin in adults. The transferrin receptor is a disulfide-linked homodimer present in the plasma membrane with two subtypes, binding one transferrin molecule per monomer. In kidneys transferrin receptors show uneven distribution, the highest level of expression being in the convoluted parts of the distal tubules of the cortex and in the collecting ducts of the medulla (Ponka, 1999). The transferrin/transferrin receptor system is mainly responsible for cellular uptake of iron and is also the best-characterized iron transport in mammals. The complex of diferric transferrin and transferrin receptors is internalized by receptor-mediated endocytosis, ultimately entering the endosomal compartment of the cytoplasm. Endosomal acidification, to a pH of about 5.5, is needed for release of iron from transferrin. After releasing iron, the apo-transferrin-transferrin receptor complex is recycled back to the cell surface to mediate further rounds of endocytosis (Aisen, Enns & Wessling-Resnick, 2001). Iron is involved in a broad spectrum of crucial biologic functions, including oxygen binding and metabolism, electron transfer and energy metabolism, and DNA synthesis (Ponka, 1999).

Serum is often included in a cell culture medium because it is important to cell growth. However, the presence of serum in a culture medium has complicated studies concerning regulation of cellular functions in vitro. Specifically, it is impossible to identify whether changed cellular functions are directly caused by a manipulation itself or mediated by a component in serum. Bowen and McDonough (1987) demonstrated that low K^+ induced upregulation of the Na,K-ATPase in MDCK cells. Confluent MDCK cells can survive in a serum-free medium for several days. We took advantage of this to examine whether the effect of low K^+ on the Na,K-ATPase required serum. We found that withdrawal of serum abolished the effect of low K^+. In an attempt to identify which component was critical, we screened a number of factors and discovered that transferrin was the most important factor that mimicked about 70% of the effect of serum.

Methods and Materials

Cell Culture

MDCK cells were purchased from the American Type Culture Collection (Manassas, VA). The cells were kept in Dulbecco's Modified Essential Medium (DMEM) with 10% fetal bovine serum at 37°C in an atmosphere containing 5% CO_2. The cells were placed down at confluence with the complete growth medium overnight, deprived of serum for 20 to 24 hours, washed with 0.9% NaCl, and then incubated with either the control or low K^+ medium for appropriate periods of time. The control medium was regular DMEM (US Biological, Swampscott, MA). The low K^+ medium was identical to the control medium except some of K^+ was substituted by Na^+. In experiments that required serum, the media were supplemented with 7.5% horse serum and 2.5% fetal bovine serum, which had been dialyzed against Ca^{2+}, K^+-free Hanks' solution (in mM: 150 NaCl, 0.5 $MgCl_2$, 0.2 Na_2HPO_4, 0.4 NaH_2PO_4). The antibiotic gentamycin (25 μg/ml, GIBCO BRL, Grand Island, NY) was included in all culture media.

Ouabain binding Assay

The cells were plated at 6×10^4 well in a 96-well plate. After treatments the confluent cells were washed twice with Ca^{2+}, K^+-free Hanks' solution and incubated in the same solution plus 2 mM EGTA at 37°C for 15 minutes to disrupt tight junctions of the cells. Then, the cells were incubated in ouabain-binding medium at 37°C for additional 15 minutes, washed four times with ice-cold Ca^{2+}, K^+-free Hanks' solution and solubilized in 0.4 N NaOH. Radioactivity retained in the cells was quantified by scintillation counting. The total binding medium contained 4×10^{-7} M [^{3}H]ouabain in Ca^{2+}, K^+-free Hanks' solution. This concentration of ouabain approached the saturation of the specific binding sites (*data not shown*), and there is no evidence that argues for low K^+ altering the affinity of the Na,K-ATPase to ouabain (Pollack et al., 1981). Nonspecific binding was measured in the presence of 10^{-4} M unlabeled ouabain. Nonspecific binding was less than 2% of total binding. Specific ouabain binding was defined as the difference between total binding and nonspecific binding.

Western Analysis

The cells were placed down at 8×10^5 cells/well in a 12-well plate. After treatments, the confluent cells were rinsed with ice-cold phosphate buffered saline (PBS), scraped with a rubber policeman in a loading buffer supplemented with 5% β-mercaptoethanol and 0.1 mg/ml phenylmethysulfonyl fluoride (PMSF) and 0.04 μg/ml aprotinin. After they were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the samples were electrophoretically blotted onto PVDF membranes. The membranes were first hybridized with primary antibodies and then with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG secondary antibody (Sigma, St. Louis, MO). Antibody binding was visualized by the enhanced chemiluminescence method (Amersham, UK). The antibody against the α1-subunit of the Na^+,K^+-ATPase was a generous gift from Dr. Thomas A. Pressley (1992). Antibodies against the β1-subunit and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Upstate Biotechnology (Lake

Placid, NY) and RDI Research Diagnostics (Flanders, NJ), respectively.

Transfection and Chloramphenicol Acetyl Transferase (CAT) Assay

The cells were transfected with the expression construct mixed with plasmid pCB6 that has the G418-resistant cassette with Lipofectamine (GIBCO BRL, Grand Island, NY) according to the manufacturer's instructions. The cells were selected under 600 μg/ml G418. The mixture of G418-resistant cells was used without subcloning. After treatments, the cells were transferred into an Eppendorff tube with the lysis buffer included in the assay kit. Freeze-thawing was used to rupture the cells. CAT assays were performed according to the manufacturer's procedures (Promega, Madison, WI).

Iron uptake Assay

Transferrin receptors are restricted to the basolateral membrane of MDCK cells (Odorizzi & Trowbridge, 1997). The confluent MDCK cells have tight apical membrane junctions that prevent large molecules like transferrin from access to the basolateral side. It takes about 15 min to disrupt the tight junctions of the cells, which is not suitable for iron uptake assays. Therefore, the cells were grown in transwells with polyester membranes (Corning, NY), which allowed uptake medium to access the basolateral side without the need for disrupting tight junctions. After serum deprivation, the membranes were detached from wells, washed with 0.9% NaCl and treated with the control or low K^+ medium at 37°C for 10 min, and then incubated with the uptake medium at 22°C for 10 min, washed with ice-cold balanced Hanks' solution four times, solubilized in 0.4 N NaOH, and counted for radioactivity with a β-counter. The uptake medium contained 3.3 μM ^{59}Fe, 10 μM nitrilotriacetic acid, and 4 μg/ml transferrin in balanced Hanks' solution (Van Renswoude et al., 1982). Nitrilotriacetic acid enhances iron binding to transferrin by acting as a bidentate ligand (Zak et al., 2002).

Statistical Analysis

Data are expressed as means ± standard errors. Statistical analyses were performed using analysis of variance (ANOVA) or Student's *t*-test, as appropriate. Post hoc comparisons were made by the Dunnett test. The null hypothesis was rejected at the 0.05 level of significance.

Abbreviations

CAT, chloramphenicol acetyl transferase; DFO, deferoxamine; EGF, epidermal growth factor; IGF, insulin-like growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGE1, prostaglandin E1; PMA, phorbol-1,2-myristate -1,3-acetate; ROS, reactive oxygen species.

Results

A subclone of MDCK cells, A4, was used throughout the study. Our previous analyses demonstrated a maximum response of the Na,K-ATPase at 0.1 mM K^+ (Zhou et al., 2003). Therefore, this concentration was used. We found that withdrawal of serum abolished the effect of low K^+ on the cell surface Na,K-ATPase molecular sites, as quantified by ouabain binding assays (Fig. 1*A*). The absolute ouabain binding sites were approximately 20 pmoles/mg protein under control condition, and deprivation of serum decreased the binding sites by 10% to 20%. In an attempt to identify which component was indispensable, we screened a number of factors. Although both prostaglandin E1 (PGEl) and transferrin potentiated the effect of low K^+, the latter received a better response (Fig. 1*B*). It appears that the duration of serum deprivation is critical. Low K^+ still had some degree of stimulation if serum was withheld for less than 16 hours. On the other hand, transferrin could not potentiate the effect of low K^+ if serum was withheld for more than 30 hours (Fig. 1*C*). Transferrin also raised the basal Na,K-ATPase binding sites by 10% to 20%.

To further examine the role of transferrin in the effect of low K^+, we performed concentration-response and time-course studies. Our concentration-response analyses revealed a maximal response between 2 μg/ml to 6 μg/ml (Fig. 2*A*). The time course of the effect of low K^+ in the presence of transferrin was similar to that in the presence of serum (Zhou et al., 2003). Low K^+ increased the ouabain binding sites after a lag of more than 12 hours and reached a plateau by 20 hours. During this interval the number of binding sites increased by approximately 40% (Fig. 2*B*). The basal ouabain binding sites in the presence of transferrin did not significantly change over the entire incubation period (*data not shown*). The increased activity of the Na,K-ATPase induced by low K^+ is associated with enhanced mRNA and protein abundance (Pressley et al., 1986, 1988; Bowen & McDonough, 1987). We previously demonstrated that in the presence of serum the 96 bp located immediately upstream from the coding region of the α1-subunit gene of the avian Na,K-ATPase was sufficient to possess basal transcription activity and to respond to the stimulation by low K^+ (Zhou et al., 2003). We found that in the absence of serum, transferrin was required for low K^+ to increase the activity of this promoter, as measured by heterologous expression of the CAT reporter gene (Fig. 3*A*). Transferrin was also necessary for the increased α1- and β1-subunit abundance induced by low K^+, as detected by immunoblotting analyses (Fig. 3*B*). Similar to what was observed with serum, the β1-subunit displayed a better response to low K^+ than its counterpart.

One of the major functions of transferrin is to facilitate cellular uptake of iron via transferrin receptors. We found that low K^+ markedly increased ^{59}Fe transport activity in the presence of transferrin (Fig. 4). The iron uptake assays were performed after the cells were deprived of serum for 24 hours and then treated with low K^+ with or without serum for

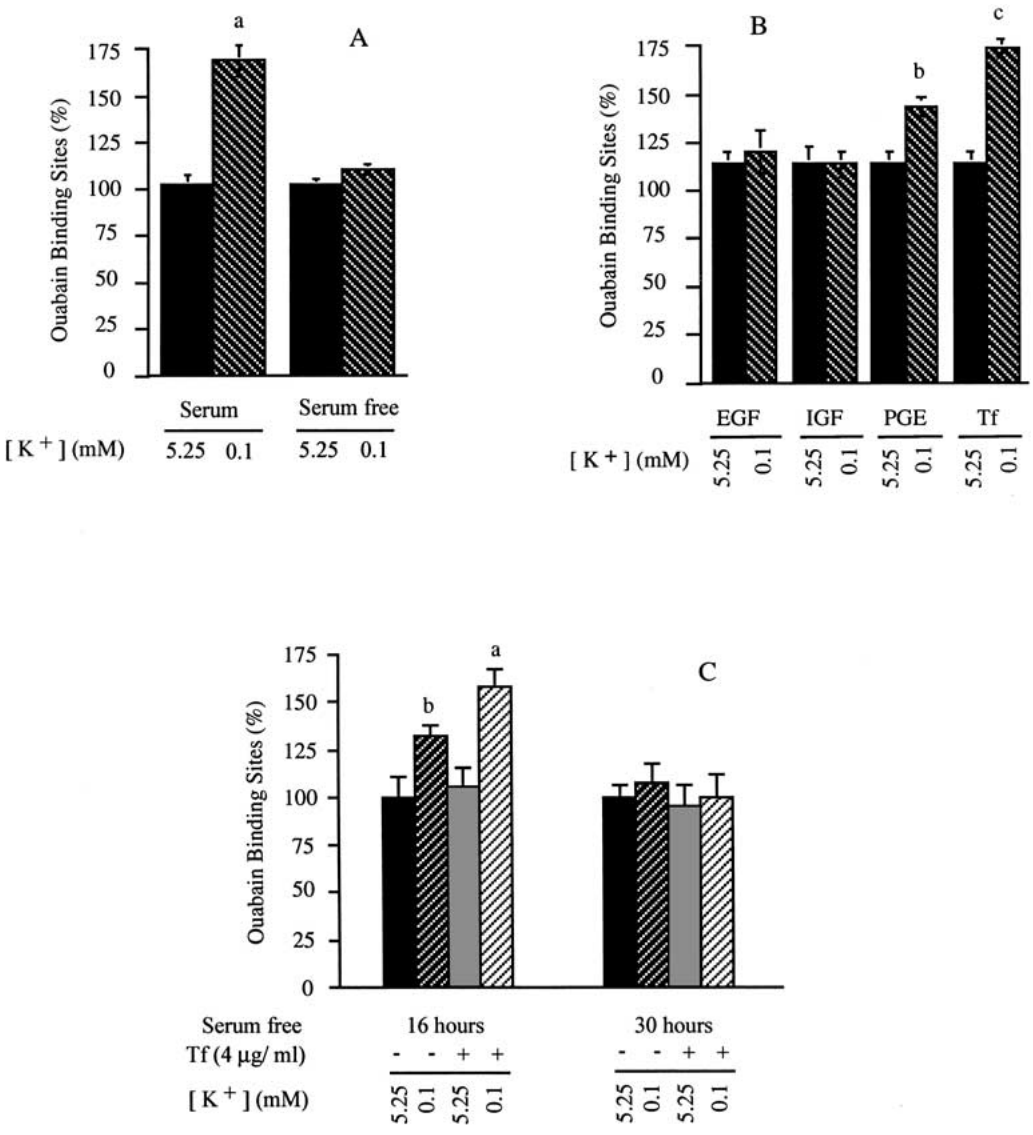

Fig. 1. The increase in the cell surface Na,K-ATPase molecular number induced by low K^+ is dependent on serum (*A*) or transferrin (Tf, *B*), and the duration of serum deprivation affects the potentiation effect of Tf (*C*) in MDCK/A4 cells. The cell surface Na,K-ATPase molecular number was quantified by ouabain-binding assays. Prior to ouabain-binding assays, the cells were treated with low-K^+ medium for 24 hours after deprivation of serum for 20 to 24 hours unless indicated otherwise. Each experiment was performed in duplicate or triplicate. The first ouabain-binding data from control was arbitrated as 100%. The rest of the data were normalized to this value. EGF: 10^{-8} M; IGF-1: 500 ng/ml; PGE1: 25 ng/ml; and Tf: 5 μg/ml. $^aP < 0.005$, $^bP < 0.05$ and $^cP < 0.01$ (Student's *t*-test, $n = 3$).

10 min. It appears that serum repletion rapidly suppressed the iron transport under both control and low K^+ conditions. Deferoxamine, a chelator of iron, ablated the effect of low K^+ on the ouabain binding sites (Fig. 5*A* and 5*B*). A higher concentration of deferoxamine was needed to inhibit the effect of low K^+ in the presence of serum than in the absence of serum. This may be due to factors in serum that counteract the effect of deferoxamine. Deferoxamine also inhibited low K^+-induced α1-promoter activity, α1- and β1-subunit protein abundance (Fig. 5). Iron participates in the generation of reactive oxygen species (ROS). Catalase abrogated the effect of low K^+ on the ouabain binding sites, α1-promoter activity, α1- and β1-subunit protein abundance (Fig. 5), Taken together, these data suggest that transferrin potentiates the effect of low K^+ on the Na,K-ATPase via increased iron transport and ROS activity.

Discussion

Use of a serum-free and chemically-defined medium not only allows dissection of the mechanism under-

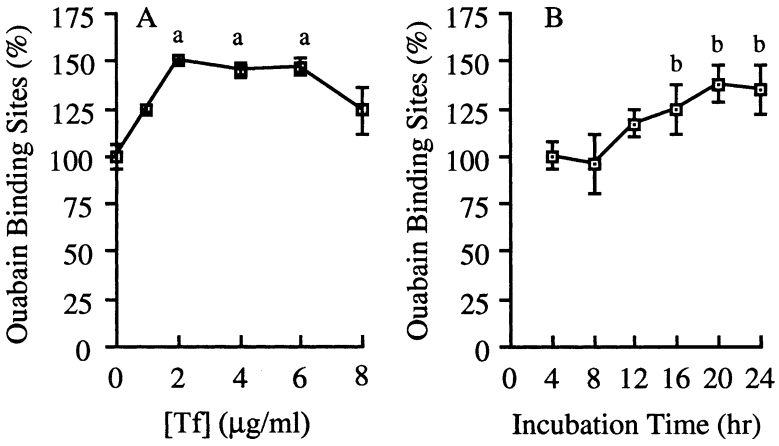

Fig. 2. The concentration response and time course of the effect of transferrin. (*A*) Concentration response. (*B*) Time course. The cells were deprived with serum for 20 to 24 hours prior to introduction of low K^+. In the dose-response study, the cells were treated with low K^+ and transferrin for 24 hours. In the time-course study, the concentration of transferrin was 4 μg/ml. $^aP < 0.01$, $^bP < 0.05$ as compared with respective control; ANOVA.

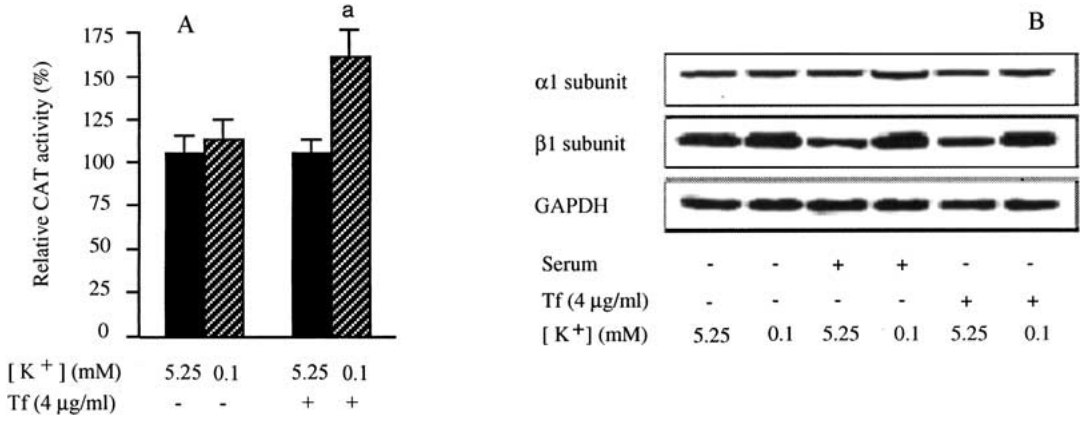

Fig. 3. Transferrin (Tf) is required for low K^+ to induce upregulation of the α1-subunit promoter activity and the α1- and β1-subunit protein abundance of the Na,K-ATPase. (*A*) Promoter activity was measured by chloramphenicol acetyl transferase (CAT) assays. The first CAT activity data from control was arbitrated as 100%. The rest of the data were normalized to this value ($^aP < 0.05$, Student's *t*-test; $n = 4$). (*B*) The α1- and β1-subunit protein abundance was estimated by Western analyses, a representative of four independent experiments. The cells were treated with low K^+ medium for 24 hours after deprivation of serum for 20 to 24 hours.

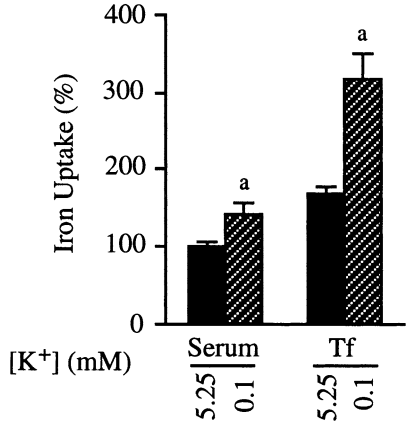

Fig. 4. Low K^+ increases iron uptake in the presence of serum or transferrin (Tf, 4 μg/ml). The cells were treated with low K^+ medium for 10 min after deprivation of serum for 24 hours. $P < 0.05$ (Student's *t*-test).

lying a specific hormone action, but also the role of this hormone in altered cellular functions induced by another manipulation. By using a defined medium, Sekine et al. demonstrated that glucose-induced insulin secretion in cells of the rat insulinoma cell line INS-1 was dependent on factors present in fetal calf serum (Sekine et al., 1997). We took advantage of the fact that confluent MDCK cells can survive in a serum-free medium for several days and examined whether the effect of low K^+ on the Na,K-ATPase required serum, which led us to the discovery of the critical role of transferrin in the effect of low K^+. Although EGF and IGF-1 can replace serum in culturing MDCK cells (Taub et al., 1979), EGF or IGF-1 did not potentiate the effect of low K^+ (Fig. 1*B*). Therefore, the effect of transferrin cannot be explained solely based on maintaining cell health.

The main function of transferrin is to transport nonheme iron and to facilitate cellular uptake of iron. In the presence of transferrin, low K^+ increased iron

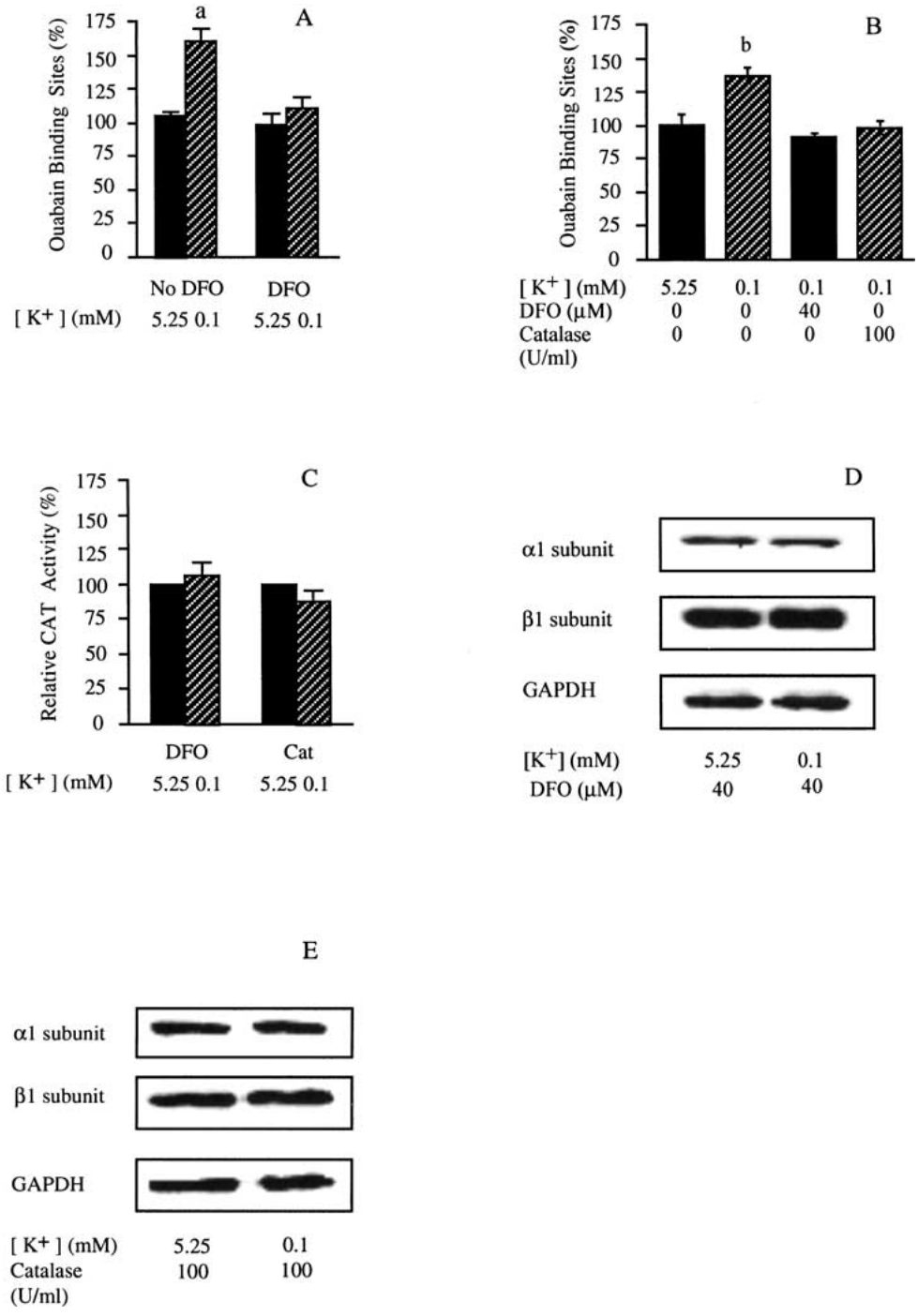

Fig. 5. Deferoxamine (DFO) or catalase (Cat) inhibits the effect of low K^+. (*A*) Ouabain binding assays in the presence of serum, DFO: 400 μM. (*B*) Ouabain binding assays in the absence of serum. (*C*). The α1-subunit promoter activity assays in the absence of serum but supplemented with 4 μg/ml transferrin ($n = 4$). (*D* and *E*) Western analyses of α1- and β1-subunit protein expression in the absence of serum but supplemented with 4 μg/ml transferrin. A representative of four independent experiments. $^aP < 0.005$, $^bP < 0.01$ (Student's *t*-test, $n = 3$).

uptake (Fig. 4). Inhibition of iron activity by deferoxamine abrogated the effect of low K^+ on the Na,K-ATPase (Fig. 5). These data suggest that the permissive effect of transferrin is mediated by increased iron uptake. Iron is needed for the function of NADH/NADPH oxidase that generates ROS (Minotti & Gennaro, 1990; Kaufmann & Lovley, 2001). Moreover, iron also catalyzes conversion of hydrogen peroxide to hydroxyl radical through the Fenton reaction. Hydroxyl radical is the most powerful oxidant

in biological systems. During the past decade, ROS have been identified as important second messengers in signaling cascades originating from growth factors receptors, receptor serine/threonine kinases, and G protein-coupled receptors (Finkel, 2000). Besides interacting with other signaling pathways, ROS may control gene expression by directly regulating the activity of transcription factors like NF-κB, AP-1 and SP-1 (Wendt et al., 2000; Donepudi et al., 2001). ROS have been shown to mediate ouabain-induced gene expressions that are related to cardiac hypertrophy (Xie et al., 1999). Our previous study has suggested the involvement of ROS in the effect of low K^+ on the Na,K-ATPase (Zhou et al., 2003). In the present study, we found that catalase completely inhibited the up-regulation of the α1-promoter activity, α1- and β1-subunit protein abundance and ouabain binding sites induced by low K^+ (Fig. 5). Because of the critical role of iron in producing ROS and because ROS are involved in the effect of low K^+, it is plausible to speculate that in the presence of transferrin low K^+ increases iron transport, thereby increasing generation of ROS signals, which in turn mediate the effect of low K^+ on the biosynthesis of the Na,K-ATPase. EGF increases production of ROS that mediate the mitogenic effect of the hormone (Bae et al., 1997). EGF also stimulates exocytosis of transferrin receptors (Davis et al., 1987). The possibility that EGF increases generation of ROS via increasing transferrin-dependent iron uptake cannot be ruled out.

Iron is a double-edged sword. An excessive amount of iron may result in over-formation of free radicals that damage cellular constituents. Therefore, the amount of iron within cells is carefully regulated. A major mechanism for the regulation of iron homeostasis relies on the post-transcriptional control of ferritin and transferrin receptor mRNAs, which are recognized by two cytoplasmic iron-regulatory proteins (IRP-1 and IRP-2) that modulate their translation and stability, respectively. Ferritin is an intracellular iron storage protein. Transferrin receptors control access of transferrin to cells, thereby controlling transferrin-dependent iron uptake. Increased intracellular iron activity stimulates synthesis of ferritin and destabilizes transferrin receptor mRNA. Decreased intracellular iron activity has an opposite effect (Aisen, Enns & Wessling-Resnick, 2001). However, it is unlikely that low K^+ regulates the transferrin receptors at the post-transcriptional level, because low K^+ increased iron uptake within 10 min (Fig. 4).

Besides post-transcriptional regulation of transferrin receptors, the number of transferrin receptors on the cell surface can be also regulated by exocytosis and endocytosis. In some types of cells only 20% of the transferrin receptors is on the cell surface with 80% residing in cytosol (Ajioka & Kaplan, 1986). PMA, EGF and IGF-1 increase the surface receptor number by stimulating exocytosis of the receptors (Davis et al., 1987; McGraw, Dunn & Maxfield, 1988), whereas ROS and iron decrease the surface receptor number by stimulating endocytosis of the receptors (Malorni et al., 1998; Van Gelder, 1998). It is likely that low K^+ in the presence of serum or transferrin stimulates membrane insertion of transferrin receptors, thus increasing iron uptake. In the A431 epidermoid carcinoma cells, EGF or IGF-1 increases the rate of transferrin receptor exocytosis (Davis et al., 1987). Whether a similar effect is present in MDCK cells is unknown. However, even though EGF or IGF-1 stimulated externalization of the receptors in MDCK cells, EGF or IGF-1 could not increase transferrin-mediated iron transport, because there was no transferrin in the culture medium. Therefore, it is not surprising that EGF or IGF-1 failed to potentiate the effect of low K^+ on the Na,K-ATPase binding sites (Fig. 1*B*).

Transferrin receptors are localized in the basolateral membrane of MDCK cells (Odorizzi & Trowbridge, 1997). Gentamicin-induced proximal tubular injury downregulates transferrin receptor expression in the distal nephron (Nouwen, 1994). Transferrin receptor expression is upregulated on cultured mesangial cells as well as on glomerular mesangial cells in patients with IgA nephropathy (Moura, 1994). However, knowledge of the regulatory mechanism underlying the expression of transferrin receptors in kidneys is scant. The distal tubule has the highest expression of transferrin receptors in kidneys (Ponka, 1999) and is also the final tuning site for Na^+ and K^+ excretion. The present study suggests that transferrin receptors may be subject to regulation by the Na^+ or K^+ balance.

In summary, the upregulation of the Na,K-ATPase by low K^+ is dependent on transferrin. The effect of transferrin is mediated by increased iron transport and ROS activity. Hypokalemia is one of the most common electrolyte disorders. K^+ depletion decreases Na^+ excretion (Buffin-Meyer et al., 1998; Eiam-Ong & Sabatini, 1999). The present study contributes to our understanding of how kidneys increase the Na,K-ATPase activity and Na^+ retention under K^+ depletion.

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References

Aisen, P., Enns, C., Wessling-Resnick, M. 2001. Chemistry and biology of eukaryotic iron metabolism. *Int. J. Biochem. Cell Biol.* **33:**940–959

Ajioka, R.S., Kaplan, J. 1986. Intracellular pools of transferrin receptors result from constitutive internalization of unoccupied receptors. *Proc. Natl. Acad. Sci. USA* **83:**6445–6449

Aydemir-Koksoy, A., Allen, J.C. 2001. Regulation of Na^+ pump expression by vascular smooth muscle cells. *Am. J. Physiol.* **280:**1869–1874

Bae, Y.S., Kang, S.W., Seo, M.S., Baines, I.C., Tekle, E., Chock, P.B., Rhee, S.G. 1997. Epidermal growth factor (EGF)-induced generation of hydrogen peroxide. Role in EGF receptor-mediated tyrosine phosphorylation. *J. Biol. Chem.* **272:**217–221

Bowen J.W. 1992. Regulation of Na^+-K^+-ATPase expression in cultured renal cells by incubation in hypertonic medium. *Am. J. Physiol.* **262:**C845–C853

Bowen, J.W., McDonough, A. 1987. Pretranslational regulation of Na,K-ATPase in cultured canine kidney cells by low K^+. *Am. J. Physiol.* **252:**C179–C189

Buffin-Meyer, B., Verbavatz, J.M., Cheval, L., Marsy, S., Younes-Ibrahim, M., Le Moal, C., Doucet, A. 1998. Regulation of Na^+, K^+-ATPase in the rat outer medullary collecting duct during potassium depletion. *J. Am. Soc. Nephrol.* **9:**538–550

Davis, R.J., Faucher, M., Racaniello, L.K., Carruthers, A., Czech, M.P. 1987. Insulin-like growth factor I and epidermal growth factor regulate the expression of transferrin receptors at the cell surface by distinct mechanisms. *J. Biol Chem.* **262:**13126–13134

Donepudi, M., Raychaudhuri, P., Bluestone, J.A., Mokyr, M.B. 2001. Mechanism of melphalan-induced B7-1 gene expression in P815 tumor cells. *J. Immunol.* **166:**6491–6499

Eiam-Ong, S., Sabatini, S. 1999. Age-related changes in renal function, membrane protein metabolism, and Na,K-ATPase activity and abundance in hypokalemic F344 × BNF(1) rats. *Gerontology* **45:**254–264

Finkel, T. 2000. Redox-dependent signal transduction. *FEBS Lett.* **476:**25–52

Hayashi, M., and Katz, A.I. 1987. The kidney in potassium depletion. I. Na^+-K^+-ATPase activity and [^{3}H]ouabain binding in MCT. *Am J. Physiol.* **252:**F437–F446

Kaufmann, F., Lovley, D.R. 2001. Isolation and characterization of a soluble NADPH-dependent Fe (III) reductase from Geobacter sulfurreducens. *J. Bacteriol.* **183:**4468–4476

Lescale-Matys, L., Hensley, C.B., Crnkovic-Markovic, R., Putnam, D.S., McDonough, A.A. 1999. Low K^+ increases Na,K-ATPase abundance in LLC-PK1/C14 cells by differentially increasing beta, and not alpha, subunit mRNA. *J. Biol. Chem.* **265:**17935–17940

Malorni, W., Testa, U., Rainaldi, G., Tritarelli, E., Peschle, C. 1998. Oxidative stress leads to a rapid alteration of transferrin receptor intravesicular trafficking. *Exp. Cell Res.* **241:**102–116

McDonough, A.A., Magyar, C.E., Komatsu, Y. 1994. Expression of Na^+-K^+-ATPase α- and β-subunits along rat nephron: isoform specificity and response to hypokalemia. *Am. J. Physiol.* **267:**C901–C908

McGraw, T.E., Dunn, K.W., Maxfield, F.R. 1988. Phorbol ester treatment increases the exocytic rate of the transferrin receptor recycling pathway independent of serine-24 phosphorylation. *J. Cell Biol.* **106:**1061–1066

Minotti, G., Gennaro, M. 1990. Microsomal iron-dependent NADPH oxidation: evidence for the involvement of membrane-bound nonheme iron in NADPH oxidation by rat heart microsomes. *Arch. Biochem. Biophys.* **282:**270–274

Moura, I.C., Centelles, M.N., Arcos-Fajardo, M., Malheiros, D.M., Collawn, J.F., Cooper, M.D., Nankivell, B.J., Chen, J., Boadle, R.A., Harris, D.C. 1994. The role of tubular iron accumulation in the remnant kidney. *J. Am. Soc. Nephrol.* **4:**1598–1607

Nouwen, E.J., Verstrepen, W.A., Buyssens, N., Zhu, M.Q., De Broe, M.E. 1994. Hyperplasia, hypertrophy, and phenotypic alterations in the distal nephron after acute proximal tubular injury in the rat. *Lab Invest.* **70:**479–493

Odorizzi, G., Trowbridge, I.S. 1997. Structural requirements for basolateral sorting of the human transferrin receptor in the biosynthetic and endocytic pathways of Madin-Darby canine kidney cells. *J. Cell Biol.* **137:**1255–1264

Ponka, P. 1999. Cellular iron metabolism. *Kidney Int. Suppl.* **69:**S2–S11

Pollack, L.R., Tate, E.H., and Cook, J.S. 1981. Na^+,K^+-ATPase in HeLa cells after prolonged growth in low K^+ or ouabain. *J. Cell Physiol.* **106:**85–97

Pressley, T.A. 1992. Phylogenetic conservation of isoform-specific regions within alpha-subunit of Na^+-K^+-ATPase. *Am. J. Physiol.* **262:**C743–C751

Pressley, T.A., Haber, R.S., Loeb, J.N., Edelman, L.S., Ismail-Beigi, F. 1986. Stimulation of Na,K-activated adenosine triphosphatase and active transport by low external K^+ in a rat liver cell line. *J. Gen. Physiol.* **87:**591–606

Pressley, T.A., Ismail-Beigi, F., Gick, G.G., Edelman, I.S. 1988. Increased abundance of Na,K-ATPase mRNA in response to low external K *Am. J. Physiol.* **24:**C252-C260

Rayson, B.M. 1989. Rates of synthesis and degradation of Na^+-K^+-ATPase during chronic ouabain treatment. *Am. J. Physiol.* **256:**C75–C80

Sekine, N., Fasolato, C., Pralong, W.F., Theler, J.M., Wollheim, C.B. 1997. Glucose-induced insulin secretion in INS-1 cells depends on factors present in fetal calf serum and rat islet-conditioned medium. *Diabetes* **46:**1424–1433

Taub, M., Chuman, L., Saier, M.H., Sato, G. 1979. Growth of Madin-Darby canine kidney epithelial cell (MDCK) line in hormone-supplemented, serum-free medium. *Proc. Natl. Acad. Sci. USA* **76:** 3338–3342

Van Gelder, W., Huijskes-Heins, M.I., Cleton-Soeteman, M.I., van Dijk, J.P., van Eijk, H.G. 1998. Iron uptake in blood-brain barrier endothelial cells cultured in iron-depleted and iron-enriched media. *J. Neurochem.* **71:**1134–1140

Van Renswoude, J., Bridges, K.R., Harford, J.B., Klausner, R.D. 1982. Receptor-mediated endocytosis of transferrin and the uptake of Fe in K562 cells: identification of a nonlysosomal acidic compartment. *Proc. Natl. Acad. Sci. USA* **79:**6186–6190

Wendt, C.H., Gick, G., Sharma, R., Zhuang, Y., Deng, W., Ingbar, D.H. 2000. Up-regulation of Na,K-ATPase beta 1 transcription by hyperoxia is mediated by SP1/SP3 binding. *J. Biol. Chem.* **275:**41396–41404

Wolitzky, B.A., Fambrough, D.M. 1986. Regulation of the (Na^+ K^+)-ATPase in cultured chick skeletal muscle. Modulation of expression by the demand for ion transport. *J. Biol. Chem.* **261:**9990–9999

Xie, Z., Kometiani, P., Liu, J., Li, J., Shapiro, J.I., Askari, A. 1999. Intracellular reactive oxygen species mediate the linkage of Na^+/K^+-ATPase to hypertrophy and its marker genes in cardiac myocytes. *J. Biol. Chem.* **274:**19323–19328

Zak, O., Ikuta, K., Aisen, P. 2002. The synergistic anion-binding sites of human transferrin: chemical and physiological effects of site-directed mutagenesis. *Biochemistry* **41:**7416–7423

Zhou, X., Yin, W., Doi, S.Q., Robinson, S.W., Takeyasu, K., Fan, X. 2003. Stimulation of Na, K-ATPase by low potassium requires reactive oxygen species. Am. J. Physiol. In press

Zhou, X., Jiang, G., Zhao, A., Bondeva, T., Hirszel, P., Balla, T. 2001. Inhibition of Na,K-ATPase activates PI3 kinase and inhibits apoptosis in LLC-PK1 cells. *Biochem. Biophys. Res. Commun.* **285:**46–51