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Superoxide Anions Are Involved in Mediating the Effect of Low K Intake on c-Src Expression and Renal K Secretion in the Cortical Collecting Duct*

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Abstract

We previously demonstrated that low K intake stimulated the expression of c-Src and that stimulation of protein tyrosine kinase inhibited ROMK channel activity (Wei, Y., Bloom, P., Lin, D. H., Gu, R. M., and Wang, W. H. (2001) *Am. J. Physiol.* 281, F206–F212). Decreases in dietary K content significantly increased O_2^- levels and the phosphorylation of c-Jun, a transcription factor, in renal cortex and outer medulla. The role of O_2^- and related products such as H_2O_2 in stimulating the expression of protein tyrosine kinase is suggested by the observation that addition of 50–200 μM H_2O_2 increased the phosphorylation of c-Jun and the expression of c-Src in M1 cells, a mouse collecting duct principal cell line. The effect of H_2O_2 on c-Src expression was completely abolished with cyclohexamide or actinomycin D. The treatment of animals on a K-deficient (KD) diet with tempol for 7 days significantly decreased the production of O_2^-, c-Jun phosphorylation, and c-Src expression. Moreover, low K intake decreased the activity of ROMK-like small conductance channels from 1.37 (control K diet) to 0.5 in the cortical collecting duct and increased the tyrosine phosphorylation of ROMK in the renal cortex and outer medulla. In contrast, the tempol treatment not only increased channel activity to 1.1 in the cortical collecting duct but also decreased the tyrosine phosphorylation of ROMK from rats on a KD diet. Finally, suppressing O_2^- production with tempol significantly increased renal K excretion measured with metabolic cage and lowered the plasma K concentration in comparison with those on a KD diet alone without tempol. We conclude that O_2^- and related products play a role in mediating the effect of low K intake on c-Src expression and in suppressing ROMK channel activity and renal K secretion.

It is well known that K restriction suppresses renal K excretion (2). This is achieved, at least in part, by decreasing the apical K conductance in the cortical collecting duct (CCD)[1] and by stimulating K absorption in the outer medullary collecting duct (3,4). However, the mechanism by which low K intake suppresses the apical K channels is not completely understood. We previously demonstrated that low K intake increased the expression of Src family protein

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[1]The abbreviations used are: CCD, cortical collecting duct; PTK, protein tyrosine kinase; KD, K-deficient; MOPS, 4-morpholinepropanesulfonic acid; SK, small conductance.

tyrosine kinase (PTK) such as c-Src and c-Yes (1) and that inhibition of PTK increased the apical ROMK-like small conductance (SK) channels (1). This suggests that PTK is involved in mediating the effect of low K intake on the apical K channels and that increases in PTK activity and expression are important for suppression of renal K secretion during K depletion.

Low K intake has been reported to increase the production of O_2^- anion in rabbit carotid arteries (5). Moreover, it has been shown that H_2O_2 stimulates the phosphorylation of c-Jun in endothelial cells, an indication of activation of transcription factor (6). Therefore, it is possible that increases in O_2^- or related products induced by low K intake may be an upstream signal responsible for mediating the effect of low K intake on PTK expression and K secretion in the kidney. This hypothesis was tested in the present study by examining whether O_2^- and related products such as H_2O_2 can mimic the effect of low K intake and stimulate the expression of PTK in the CCD. We also examined whether decreases in O_2^- and related products with tempol could attenuate the effect of low K intake on c-Src expression, ROMK channel activity, and renal K excretion.

EXPERIMENTAL PROCEDURES

Animals

Sprague-Dawley rats (6–8 weeks, either sex) were purchased from Taconic Farms (Germantown, NY). Rats were housed in metabolic cages for 7 days to study urinary K excretion. After 3 days of training in the cage, rats were divided into three groups: 1) control group in which animals were kept on a normal K (1.1%) diet and had a daily intraperitoneal injection of saline for 1 week; 2) the low K group in which rats were maintained on a K-deficient (KD) diet and received a daily intraperitoneal injection of saline for 7 days; and 3) the tempol-treated group in which rats were also fed with KD diet and had a daily intraperitoneal injection of tempol (15 mg/kg) for 1 week. Data regarding the 24-h food intake, body weight, and urine output were recorded. Urinary Na and K concentrations were measured by a flame photometer, and daily Na and K excretion were calculated as mEq/24 h.

Animals were anesthetized with pentobarbital (60 mg/kg), and blood samples were drawn from the heart to measure the plasma K and Na concentrations. Rats were then killed, and the abdomens were opened to remove the kidneys.

Tissue Preparation

The renal cortex and the outer medulla were separated under a dissecting microscope and suspended in radioimmune precipitation assay buffer solution (1:8 ratio, w/v) containing 1× phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS. 10 μl of phenylmethylsulfonyl fluoride (10 mg/ml stock solution in isopropanol). 10 μl of a mixture of protease inhibitors (Sigma) were added per ml of buffer at the time of lysis. The samples were homogenized on ice for 15 min with a mortar and pestle. The suspension was incubated at 4 ° C for 1 h in the presence of DNase (5 μg/ml) followed by centrifugation at 1800 rpm for 10 min. The resultant supernatant was collected. Protein concentrations were measured in duplicate using a Bio-Rad DC protein assay kit.

Preparation of M1 Cells

M1 cells, a mouse CCD line, were purchased from the American Type Culture Collection (Mannasas, VA) and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. Before H_2O_2 treatment, the cells were cultured in medium containing 1% fetal bovine serum for 16 h, followed by incubation for an additional 30 min in a solution containing 22 mM HEPES (pH 7.4), 124 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 0.16 mM HPO_4, 0.4

m_M H_2PO_4, 5 m_M $NaHCO_3$. 5.6 m_M H_2O_2 (50–200 μM final concentration) was directly added to the cells in HEPES buffer for 30–120 min. The viability of M1 cells treated with H_2O_2 as determined by the Trypan Blue dye exclusion method was ~90% of the corresponding control cells. After treatment with H_2O_2, the cells were washed with ice-cold phosphate-buffered saline twice and incubated for 30 min in radioimmune precipitation assay lysis buffer.

Immunoprecipitation and Western Blot

The corresponding antibody was added to the protein samples (500 μg) harvested from kidneys at a ratio of 5 μl/ml solution. The mixture was gently rotated at 4 °C overnight, followed by incubation with 25 μl of protein A/G-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) for an additional 2 h at 4 °C. The tube containing the mixture was centrifuged at 3000 rpm and washed twice with phosphate-buffered saline containing 10 μl/ml phenylmethylsulfonyl fluoride and 10 μl of protease inhibitor mixture/ml. The agarose pellet was resuspended in 25 μl of 2× SDS sample buffer containing 4% SDS, 100 m_M Tris-HCl (pH 6.8), 20% glycerol, 200 m_M dithiothreitol, 0.2% bromphenol blue. After boiling the sample for 5 min, proteins were separated by electrophoresis on 10% SDS-polyacrylamide gels and transferred to Immuno-Blot polyvinylidene difluoride membrane (Bio-Rad). The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) and incubated overnight with the primary antibody at 4 °C. The membrane was washed 3× 15 min with TBS containing 0.05% Tween 20 followed by incubation for 30 min with respective secondary antibody horseradish peroxidase conjugate. ECL Plus (Amersham Biosciences) was used to detect the protein bands; the intensity of the bands of interest was determined using Alpha DigiDoc 1000 (Alpha Innotech, San Leandro, CA).

Measurement of Superoxide Anion

The method for measurement of O_2^- has been previously described (7). Briefly, renal cortex and outer medulla were isolated from rats on a normal or KD diet for 7–10 days. The tissue (100 mg) was cut into small pieces with a sharp blade and suspended in air-equilibrated MOPS-sucrose buffer (pH 7.4) containing 5 μM lucigenin. The chemiluminescence elicited in the presence of lucigenin was measured in a liquid scintillation counter with a single active photomultiplier tube positioned in out-of-coincidence mode. Blanks were substracted from the average level of chemiluminescence signal.

Preparation of CCDs for Patch Clamping

Single CCDs were isolated, placed on a 5 × 5-m_M coverglass coated with polylysin and transferred to a chamber (1000 μl) mounted on an inverted Nikon microscope. The CCDs were superfused with HEPES-buffered NaCl solution containing (in m_M) 140 NaCl, 5 KCl, 1.8 $CaCl_2$, 1.8 $MgCl_2$, and 5 HEPES (pH 7.4). The pipette solution was composed of (in m_M) 140 KCl, 1.8 $MgCl_2$, and 5 HEPES (pH 7.4). The temperature of the chamber was maintained at 37 ± 1 °C by circulating warm water around the chamber. The CCD was cut open with a sharpened micropipette to expose the apical membrane.

Patch Clamp Technique

We followed the methods described previously to dissect the CCD from rats on a normal K and KD diet and on tempol-treated rats on a KD diet (1). An Axon200A patch clamp amplifier was used to record channel current. The current was low-pass filtered at 1 kHz by an 8-pole Bessel filter (902LPF; Frequency Devices, Haverhill, MA) and digitized with Axon interface (Digidata 1200). Data were analyzed using the pClamp software system 6.04 (Axon Instruments, Burlingame, CA). Channel activity was defined as NPo, which was calculated from data samples of 60-s duration in the steady state as follows, $NPo = \Sigma(t_1 + t_2 + \ldots\ldots t_i)$, where t_i is the fractional open time spent at each of the observed current levels. The slope

conductance of the channel was calculated by measurement of K^+ current at several cell membrane potentials.

Experimental Materials and Statistics

Antibodies to c-Src and β-actin were purchased from Santa Cruz Biotechnology; anti-tyrosine-phosphorylation antibody (4G10) and ROMK antibody were obtained from Upstate USA (Charlottesville, VA) and Alomone (Jerusalem, Israel), respectively. Antibodies to phospho-c-Jun (serine 73) and c-Jun were obtained from Cell Signaling Technology (Beverly, MA). Actinomycin D and cyclohexamide were purchased from Sigma.

Data are shown as mean ± S.E., and paired or unpaired Student's *t* test was used to determine the significance between the two groups. Statistical significance was taken as $p < 0.05$.

RESULTS

Low K intake has been shown to increase the generation of O_2^- anions in rabbit arteries (5). Thus, we investigated whether low K intake also increased O_2^- production in the kidney. We used the lucigenin enhanced chemiluminescence method described elsewhere (7,8) to determine O_2^- production in the renal cortex and outer medulla from rats on a KD diet and on a normal K diet. Fig. 1 summarizes results from seven measurements demonstrating that concentrations of O_2^- in the renal cortex and outer medulla increased by ~110 ± 5% in the rats on a KD diet in comparison with those on a normal chow.

Because O_2^- anion and related products such as H_2O_2 have been demonstrated to activate c-Jun, a transcription factor, in endothelial cells (6), we speculated that high O_2^- concentrations induced by low K intake may be responsible for increased expression of c-Src in the kidney through activation of c-Jun. Therefore, we used the antibody recognizing the phosphorylated c-Jun on serine residue 73, an indication of c-Jun activation (9), to determine the effect of low K intake on c-Jun phosphorylation. Fig. 2 is a Western blot showing that phosphorylation of c-Jun on serine residue 73 is 95 ± 5 and 110 ± 5% ($n = 7$ rats) higher in the renal cortex and outer medulla from rats on a KD diet than those from animals on a normal K diet, respectively.

To further determine the role of O_2^- in stimulating c-Jun phosphorylation, we investigated the effect of H_2O_2 on c-Jun phosphorylation in M1 cells, a mouse CCD cell line (10–12). M1 cells were incubated in 200 μM H_2O_2-containing medium for 30, 60, and 120 min, and c-Jun phosphorylation was examined with Western blot. Fig. 3 is a Western blot demonstrating that incubation of M1 cells in 200 μM H_2O_2-containing medium for 60 and 120 min significantly increased phospho-c-Jun by 45 ± 5 and 110 ± 20% ($n = 5$) in comparison to the control value, respectively. We also studied the dose response curve of c-Jun phosphorylation to the stimulation of H_2O_2. Fig. 4*A* is a Western blot showing the effect of H_2O_2 on c-Jun phosphorylation on serine residue 73. Data summarized in Fig. 4*B* demonstrate that 100 and 200 μM H_2O_2 significantly stimulated the phosphorylation of c-Jun by 90 ± 10 ($n = 4$) and 120 ± 10%, respectively. Thus, we used 200 μM H_2O_2 in the following experiments.

We examined the effect of H_2O_2 on c-Src expression in M1 cells to determine whether H_2O_2 increases the expression of c-Src, a representative member of Src family PTK (13). M1 cells were incubated in 200 μM H_2O_2-containing medium for 30, 60, and 120 min, followed by incubation in the control medium for an additional 3 h. Fig. 5*A* is a Western blot demonstrating that incubation of M1 cells in H_2O_2-containing medium for 120 min significantly increased c-Src by 190 ± 20% ($n = 7$) in comparison to the control value. Moreover, the effect of H_2O_2 on c-Src was completely blocked by actinomycin D (5 ng/ml) and cyclohexamide (20 ng/ml) (Fig. 5*B*). In addition, in the presence of actinomycin D, the c-

Src expression level decreased progressively. The decrease was most likely because of the degradation of c-Src.

To further examine the role of O_2^- and related products in mediating the effect of low K intake on PTK expression, we employed tempol, an agent that has been used to decrease O_2^- formation (14). We measured O_2^- levels in rats on a normal K (1.1%) diet, KD diet, and KD diet plus tempol treatment. Data summarized in Fig. 6 demonstrate that tempol treatment significantly decreased O_2^- levels by 70 ± 10% (n = 5) in the renal cortex and outer medulla in comparison with that without tempol treatment, whereas low K intake significantly increased O_2^- production by 150 ± 20% (n = 5). We also examined the effect of low K intake on c-Jun phosphorylation in the tempol-treated and untreated rats. Fig. 7 is a Western blot showing that low K intake increased c-Jun phosphorylation by 90 ± 10% (n = 4). In contrast, the c-Jun phosphorylation in rats on KD diet plus tempol was not significantly different from the control value. Thus, suppression of O_2^- production abolished the effect of low K intake on c-Jun activation.

We have previously shown that low K intake increased the expression of Src family PTK (1). If O_2^- is responsible for mediating the effect of low K on PTK expression, decreases in O_2^- production are expected to suppress the low K intake-induced increases in Src family PTK. Thus, we examined whether tempol treatment inhibits the effect of low K intake on the expression of c-Src as a representative member of Src family PTK. Fig. 8 is a Western blot showing that low K intake significantly increased the expression of c-Src by 220 ± 15% (n = 4) in comparison with the control. In contrast, tempol treatment abolished the stimulatory effect of low K intake on c-Src expression because c-Src expression in rats on KD diet plus tempol was not significantly different from those on the control K diet.

After showing that tempol treatment suppressed the effect of low K intake on O_2^- levels, c-Jun phosphorylation, and c-Src expression, we examined the effect of tempol on ROMK tyrosine phosphorylation. We confirmed the previous finding that low K intake stimulates the tyrosine phosphorylation of ROMK (15). However, the stimulatory effect of low K intake on tyrosine phosphorylation of ROMK was almost absent in tempol-treated rats (Fig. 9*A*). Data summarized in Fig. 9*B* show that low K intake stimulates the tyrosine phosphorylation of ROMK by 150 ± 10% (n = 6), whereas the tyrosine phosphorylation of ROMK in rats on KD diet plus tempol was not significantly different from the control value.

Because stimulation of tyrosine phosphorylation of ROMK1 has been shown to decrease ROMK channel activity in the CCD (16), tempol treatment-induced inhibition of ROMK tyrosine phosphorylation is expected to increase ROMK channel activity in the CCD. Thus, we used the patch clamp technique to examine the ROMK-like SK channels in the CCD from rats on a control (1.1%) or a KD (<0.001%) diet and in rats on KD diet plus tempol. Data summarized in Fig. 10*A* show that SK channel activity in the CCD from rats on KD diet (NPo = 0.5 ± 0.1, n = 11) was significantly lower than that (NPo = 1.37 ± 0.2, n = 10) in the CCD from rats on a control K diet. However, SK channel activity in the CCD from rats on a KD diet plus tempol was significantly higher than those (NPo = 1.1 ± 0.1, n = 10) in rats on KD diet. This value is not significantly different from the control value.

Because ROMK-like SK channels are responsible for K secretion, increases in channel activity were expected to stimulate K secretion. We measured renal K excretion in rats on KD diet plus tempol for 7 days with metabolic cage. Results summarized in Fig. 10*B* show that renal K excretion decreased from 3.5 ± 0.3 mEq/day in rats on a control K diet (n = 7) to 0.03 ± 0.01 mEq in rats on a KD diet (n = 7). Tempol treatment significantly increased renal K excretion to 0.5 ± 0.1 mEq (n = 7). This increase in K excretion leads to a severe hypokalemia in rats on KD diet plus tempol treatment. Plasma K concentration decreased from 3.8 ± 0.3 m_M in animals

on the control K diet to 2.8 ± 0.2 mM in rats on a KD diet. However, tempol treatment further lowered plasma K (to 2.2 ± 0.1 mM) in rats on KD diet.

DISCUSSION

The main findings of the present study are that low K intake increases O_2^- levels in the kidney and that O_2^- and the related products mediate the inhibitory effect of low K intake on ROMK channel activity in the CCD. The notion that O_2^- may play a role in suppressing renal K excretion induced by low K intake is supported by several lines of evidence. First, low K intake significantly increased O_2^- production in the kidney. Second, application of H_2O_2 mimicked the effect of low K intake on c-Jun phosphorylation and the expression of c-Src in M1 cells. Third, decreasing O_2^- levels induced by tempol significantly suppressed c-Jun phosphorylation, expression of c-Src, and tyrosine phosphorylation of ROMK. Finally, tempol treatment abolished the inhibitory effect of low K intake on the ROMK-like SK channel activity and increased renal K excretion. Therefore, increases in renal O_2^- production from K-restricted rats play an important role in mediating the effect of low K intake on ROMK channel activity and renal K excretion.

It is well established that a low K intake suppresses renal K excretion (2). This is achieved by both inhibition of renal K secretion in the connecting tubule and the CCD and stimulation of K absorption through K-H-ATPase in the outer medullary collecting duct (3). The inhibition of K secretion in the CCD induced by low K intake is at least in part the result of decreases in the apical K conductance. Two types of K channels are present in the apical membrane of the CCD (17): Ca^{2+}-dependent maxi K and ROMK-like SK channel. Although maxi K channels may be involved in K secretion in the CCD when tubule flow rate is high (18,19), ROMK-like SK channels play an important role in mediating K secretion during normal tubule flow. Thus, alterations in ROMK channel activity in the CCD could affect renal K secretion in the CCD.

We previously demonstrated that low K intake significantly increased the expression of Src family PTK such as c-Src and c-Yes (1). Furthermore, we have shown that ROMK1 was a substrate of PTK and that tyrosine phosphorylation of ROMK increased during K depletion and decreased by high K intake (15). The role of PTK in regulating ROMK channel activity in the CCD is further established by the observation that inhibition of PTK increases (1), whereas inhibition of protein tyrosine phosphatase decreases (20), ROMK channel activity. The inhibitory effect of PTK on ROMK1 is the result of stimulation of the ROMK internalization. In the present study, we have demonstrated that the ROMK channel activity was significantly lower in the CCD from rats on KD diet than those on a control K diet. However, we observed previously that the SK channel activity was not significantly different in the CCD of rats on a normal K diet (0.7%) from that on KD diet (1). The discrepancy may be because a 0.7% K-containing rat chow was used as a control K diet in previous experiments and the content of K was 1.1% in the present experiment. Thus, it is conceivable that a physiological variation of dietary K intake could have an effect on ROMK channel activity in the CCD.

Although tempol treatment increased ROMK channel activity in the CCD from rats on a KD diet to a similar extent observed in the CCD from the control animal, renal K excretion in tempol-treated rats was still significantly lower than that of control animals. It is possible that stimulation of K absorption in the outer medullary collecting duct during K restriction plays an important role in preserving K (3). However, the observation that suppressing O_2^- production with tempol significantly increased renal K excretion in comparison with rats on a KD diet alone strongly indicates the role of O_2^- in mediating the effect of low K on renal K secretion.

We hypothesized that low K intake stimulates O_2^- production, which stimulates transcription factors such as c-Jun and increases PTK expression. As a consequence, tyrosine phosphorylation of ROMK increased and channel activity decreased. The notion that O_2^- and the related products are involved in mediating the effect of low K intake on tyrosine phosphorylation of ROMK is supported by the observation that suppressing O_2^- production by tempol significantly attenuated the tyrosine phosphorylation of ROMK in rats on a KD diet in comparison with those without tempol treatment. There are at least two possibilities by which O_2^- and related products can stimulate the tyrosine phosphorylation of ROMK: 1) O_2^- stimulates the expression of Src family PTK, which in turn increases the tyrosine phosphorylation of ROMK; 2) O_2^- and the related products facilitate the tyrosine phosphorylation of ROMK by directly modulating the activity of PTK and protein tyrosine phosphatase. $O_2^-$and H_2O_2 have been demonstrated to modulate the activity of a variety of protein kinases and phosphatases (21–26). H_2O_2 has been shown to inhibit protein tyrosine phosphatase (24,27) and activate several members of Src family PTK, such as Lck and Fyn (25,26). Thus, it is possible that O_2^- can stimulate the tyrosine phosphorylation of ROMK channels by increasing PTK expression and activity.

O_2^- generation increases by activation of enzymes such as NAD(P)H oxidases and xanthine oxidase (28). Superoxide dismutases convert O_2^- to H_2O_2, which is then metabolized to water by catalase. Thus, increases in O_2^- are expected to raise H_2O_2 concentrations. O_2^- and H_2O_2 have initially been identified to be involved in the regulation of immuno response and programmed cell death. However, a large body of evidence has supported the notion that O_2^- and H_2O_2 may play an important role in mediating a variety of cell functions (28). O_2^- has been shown to mediate the effect of nerve growth factor (NGF) in neuronal cells (29) and epidermal growth factor (EGF) in human epidermoid carcinoma cells (30). Stimulation of NGF and EGF receptors results in transient increases in O_2^- and H_2O_2 concentrations. Moreover, elimination of H_2O_2 by catalase has been demonstrated to inhibit EGF and NGF receptors. Stimulation of insulin receptors has been shown to augment the formation of O_2^- (31), and low concentrations of H_2O_2 can potentiate the insulin effect in insulin-responsive tissues (32). Moreover, high concentrations of H_2O_2 can induce insulin-like effects in the absence of insulin via stimulation of the insulin-independent tyrosine phosphorylation of the insulin receptor (33). H_2O_2 mediates the stimulatory effect of angiotensin II on nitric oxide production in endothelial cells (34). In addition, H_2O_2 stimulates cGMP generation and causes the transient relaxation of calf coronary arteries (35). Thus, it is possible that O_2^- and the related products play a physiological role in the regulation of renal K secretion.

The mechanism by which low K intake increases O_2^- production is not known. Several hormones such as growth factor and angiotensin II have been shown to stimulate NAD(P)H and increase O_2^- production (29,30,34). K depletion has been shown to increase the concentration of growth factors such as insulinlike growth factor (36,37). Thus, it is possible that growth factors and angiotensin II may be involved in O_2^- generation during low K intake. Further experiments are required to test these speculations. We conclude that low K intake increases O_2^- and the related products, which in turn stimulate the expression of PTK, and that increases in PTK activity stimulate tyrosine phosphorylation of ROMK channels and inhibit ROMK channel activity.

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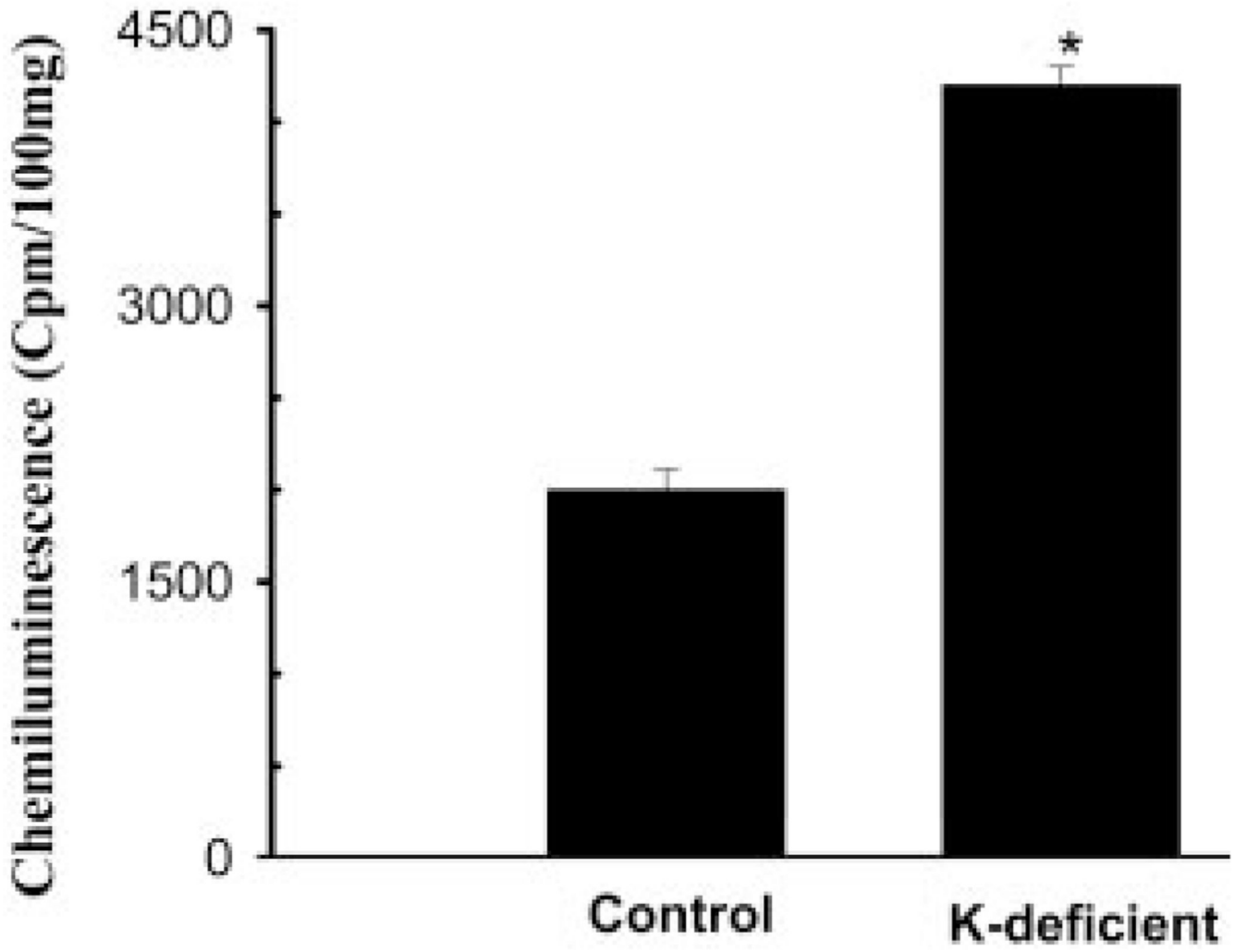

FIG. 1. Superoxide concentration in the renal cortex and outer medulla from rats on control (1.1%) and K-deficient diets

Relative superoxide concentration from 100 mg of wet tissue was determined by lucigenin chemiluminescence. The fluorescence intensity is proportional to the superoxide concentration. Asterisk indicates that the difference is significant ($p < 0.01$).

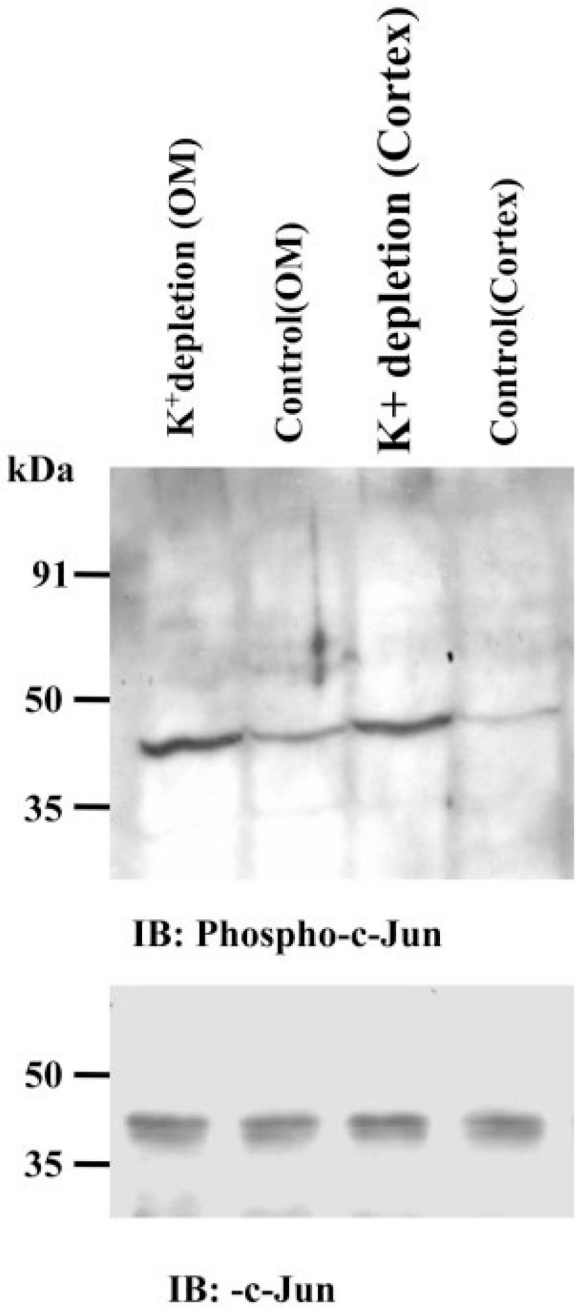

FIG. 2. Effect of low K intake on c-Jun phosphorylation in the renal cortex and outer medulla (*OM*)

The *top panel* shows the phosphorylated c-Jun on serine residue 73 (*phospho-c-Jun*), whereas the *bottom panel* demonstrates the total c-Jun.

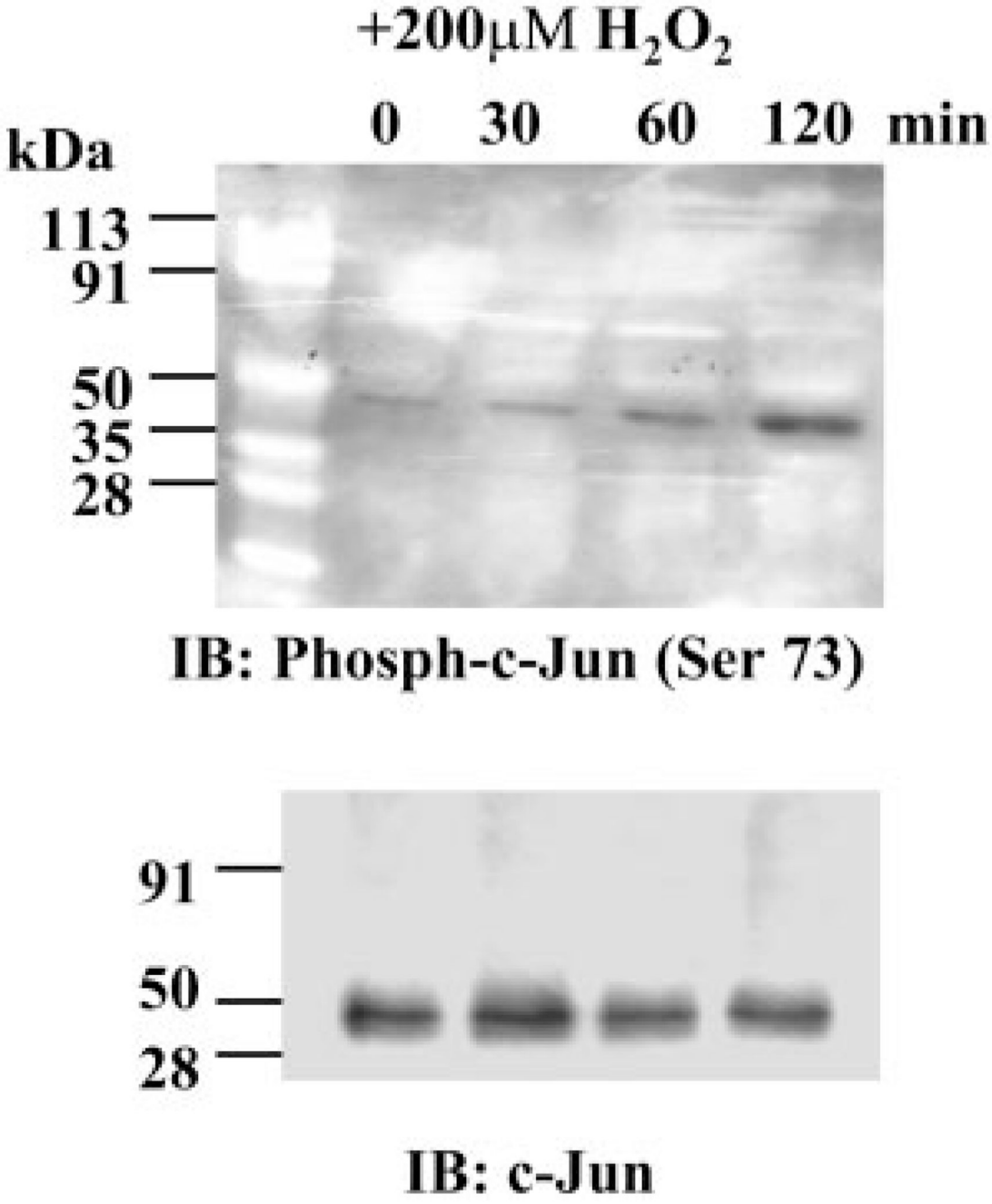

FIG. 3. H_2O_2 stimulates the phosphorylation of c-Jun (*phospho-c-Jun*) on serine residue 73 in M1 cells

M1 cells were treated with 200 μM H_2O_2 for 30, 60, and 120 min. The *top panel* shows the effect of H_2O_2 on phospho-c-Jun, whereas the *bottom panel* demonstrates the total c-Jun.

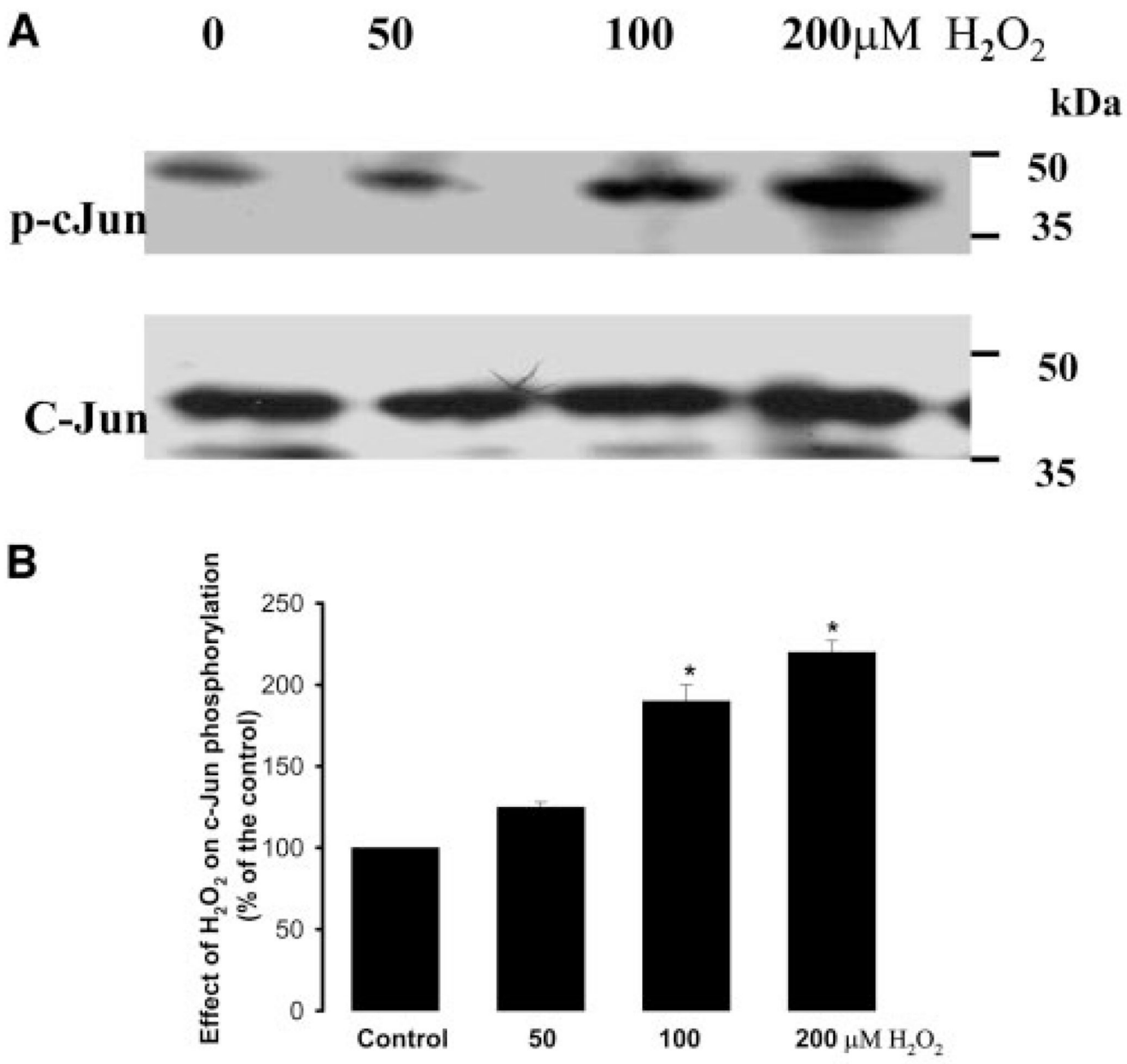

FIG. 4.

A, dose-dependent response of c-Jun phosphorylation to H_2O_2. The *top panel* is phosphorylated c-Jun (*p-c-Jun*); total c-Jun is shown on the *bottom panel*. *B*, *bar graph* summarizes the effect of H_2O_2 at different concentrations on c-Jun phosphorylation. The results are normalized with the control value. Asterisk indicates that the difference is significant (p <0.01) from control value.

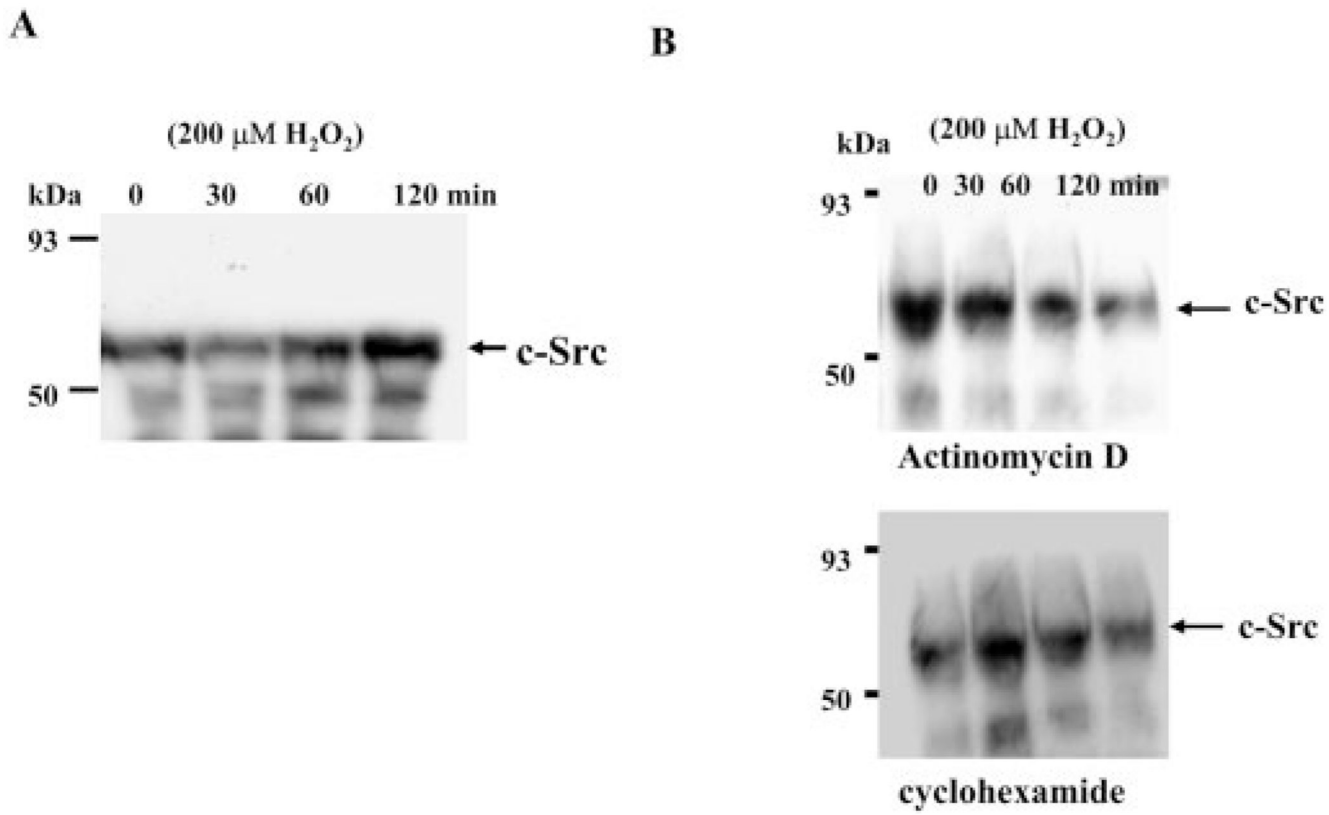

FIG. 5.

A, effect of H_2O_2 on c-Src expression in M1 cells. The cells were treated with 200 μM H_2O_2 for different times, followed by incubation in the control medium for an additional 3 h. *B*, effect of H_2O_2 on c-Src expression in M1 cells in the presence of actinomycin D (*top panel*) or cyclohexamide (*bottom panel*).

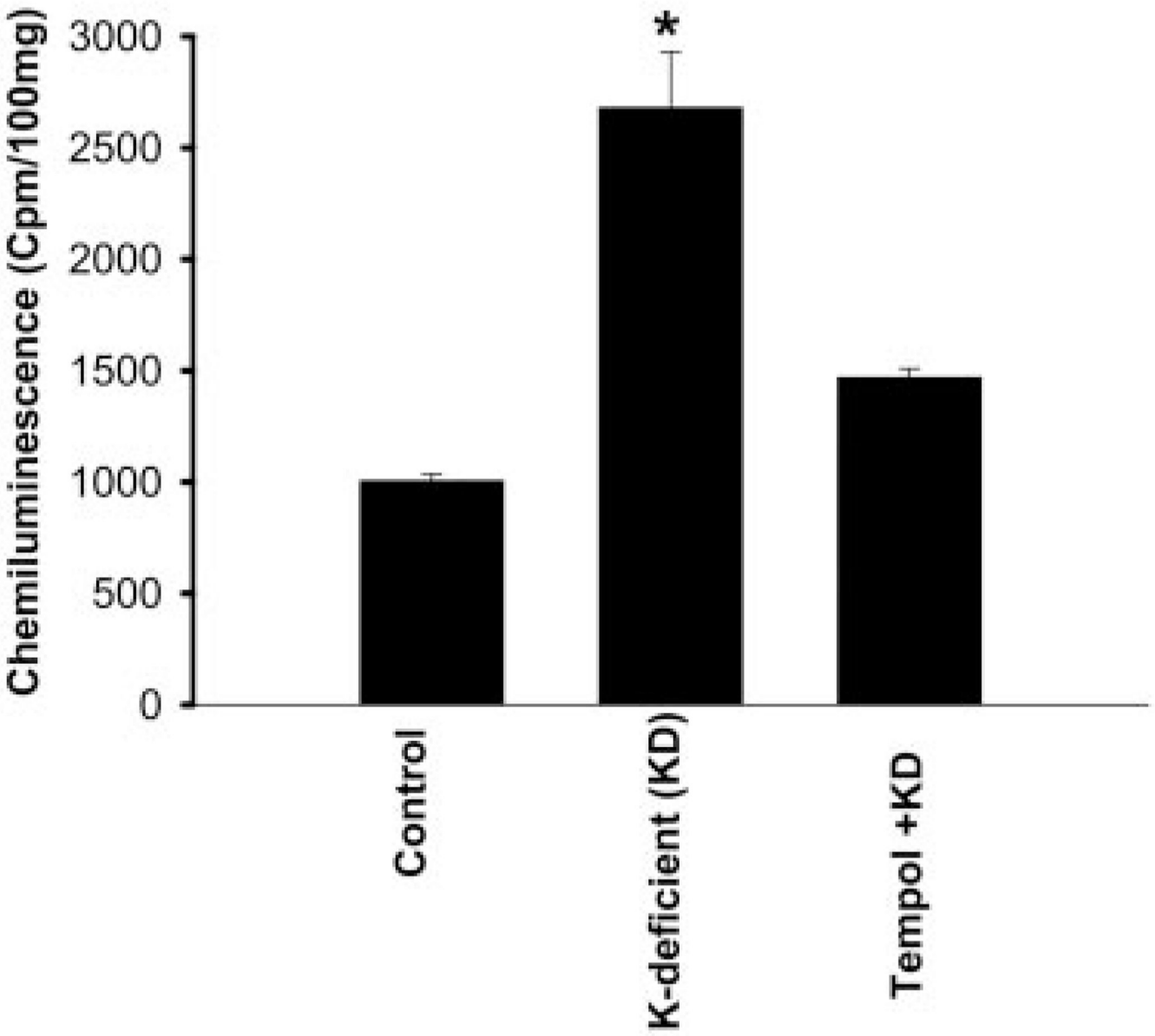

FIG. 6. Superoxide concentration in the renal cortex and outer medulla from rats on a control (1.1%) diet, K-deficient (*KD*) diet, and KD diet plus tempol treatment

Relative superoxide concentration from 100 mg of wet tissue was determined by lucigenin chemiluminescence. Asterisk indicates a significant difference ($p < 0.01$) between control and KD group

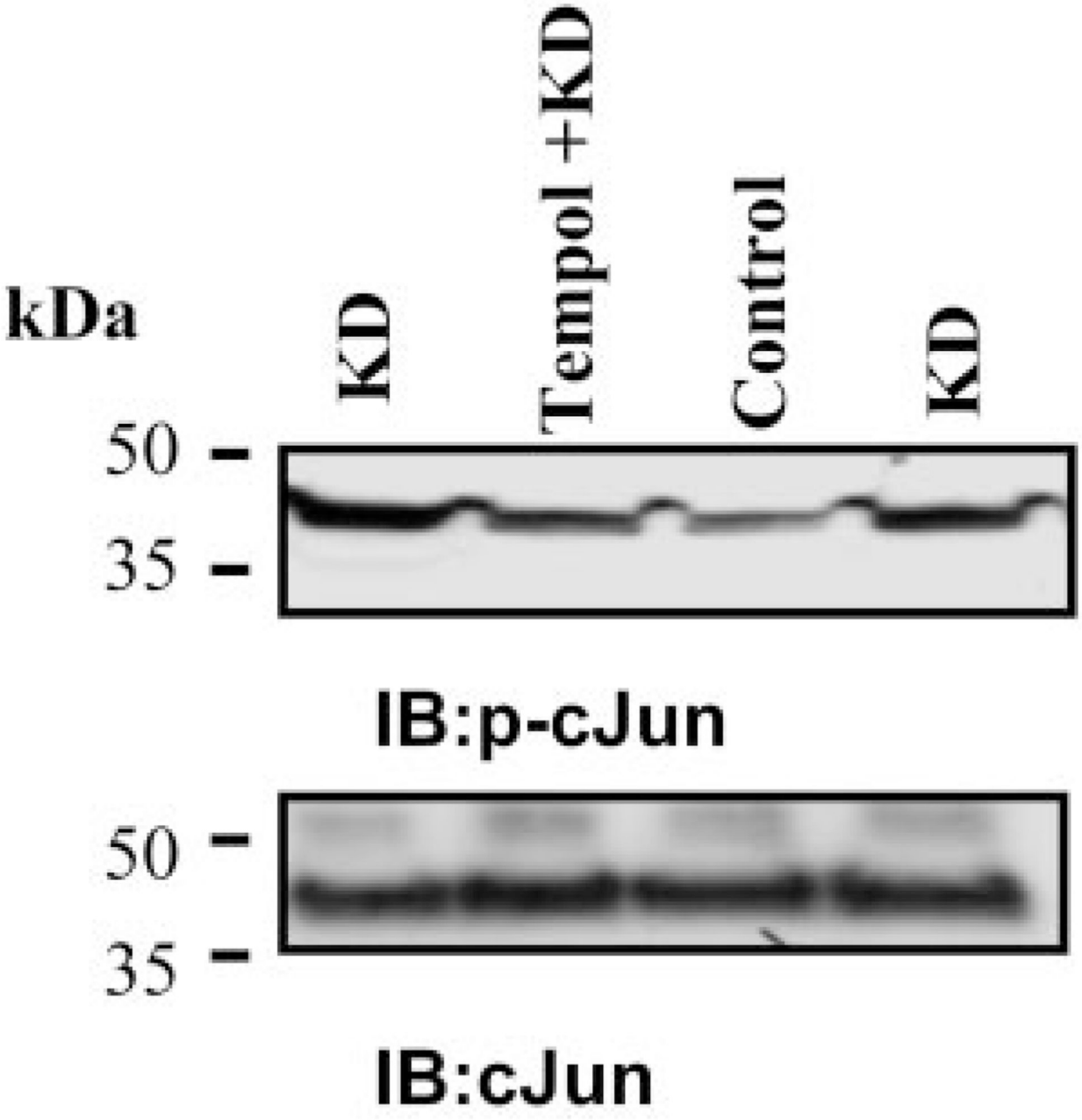

FIG. 7. Effect of low K intake on c-Jun phosphorylation in the renal cortex and outer medulla in rats with or without tempol treatment

The *top panel* shows the phosphorylated c-Jun on serine residue 73 (*p-c-Jun*), whereas the *bottom panel* demonstrates the total c-Jun.

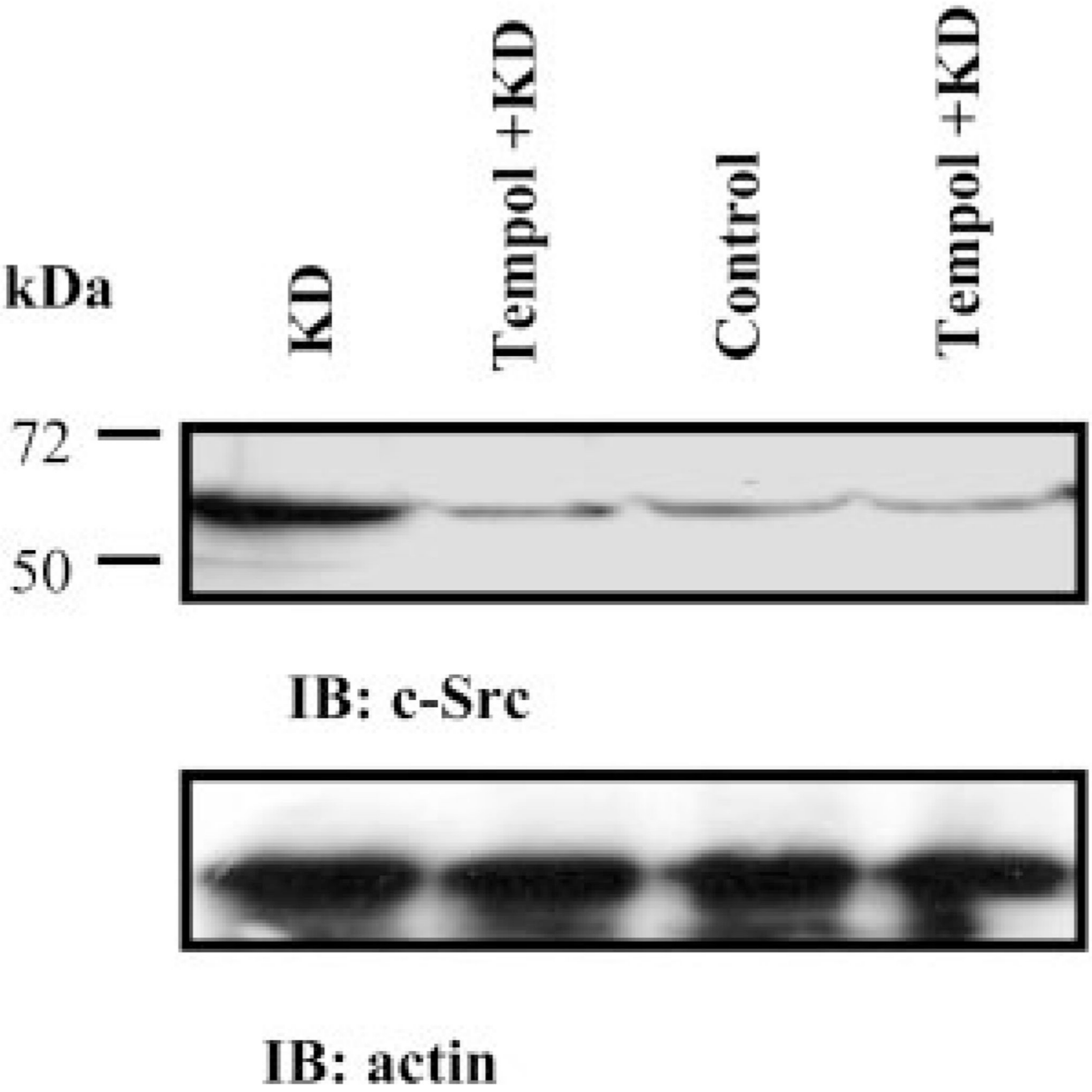

FIG. 8. Effect of low K intake on the expression of c-Src in the renal cortex and outer medulla of rats with or without tempol treatment

The *top panel* demonstrates the expression of c-Src; the *bottom panel* shows the expression of β-actin.

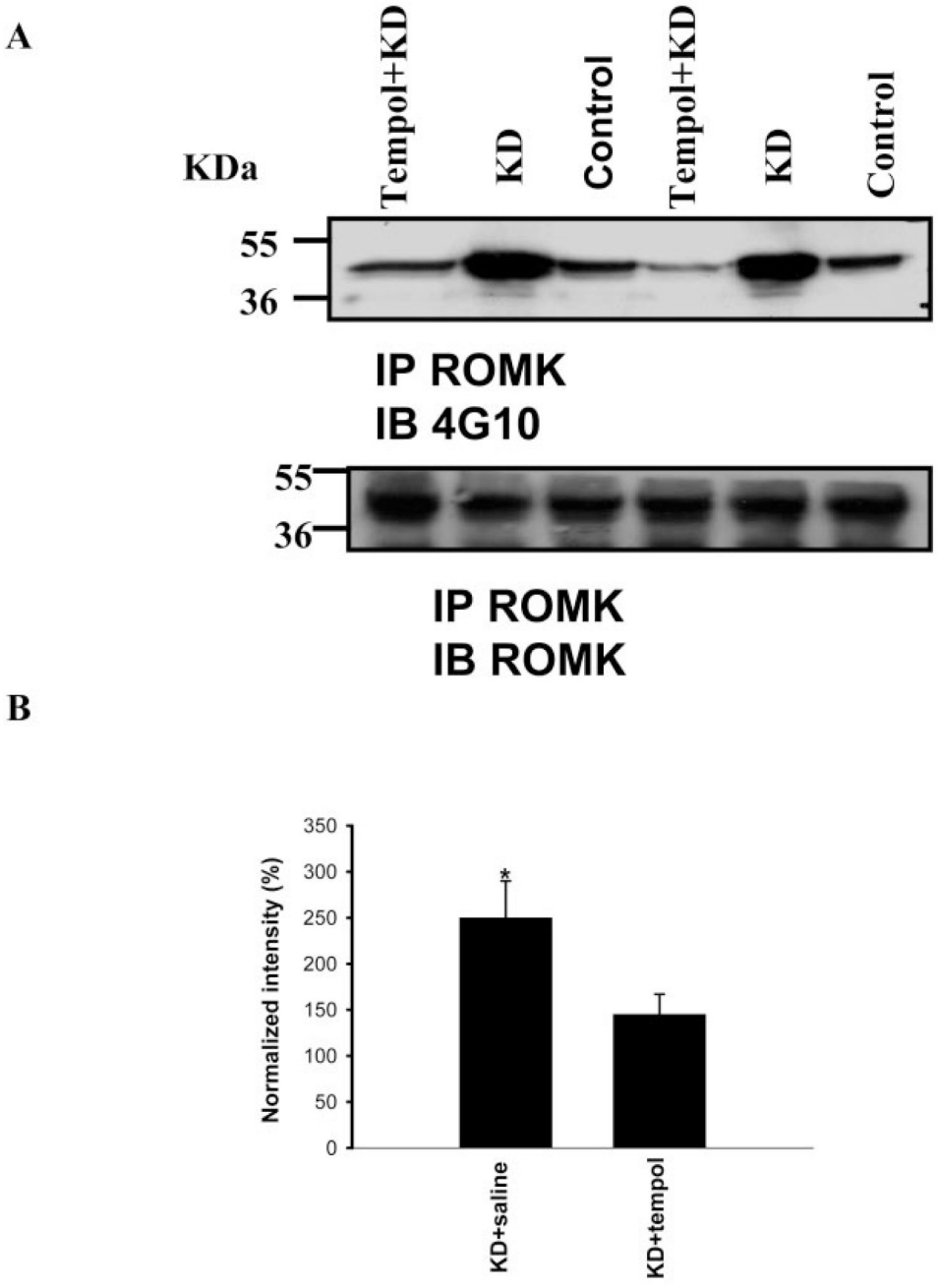

FIG. 9.

A, Western blot showing the effect of low K intake on the tyrosine phosphorylation of ROMK harvested from rats with tempol or without tempol treatment. *B*, *bar graph* summarizes the changes of tyrosine phosphorylation of ROMK in comparison with that from the control rats. Asterisk indicates that the difference between two groups is significant ($p < 0.01$).

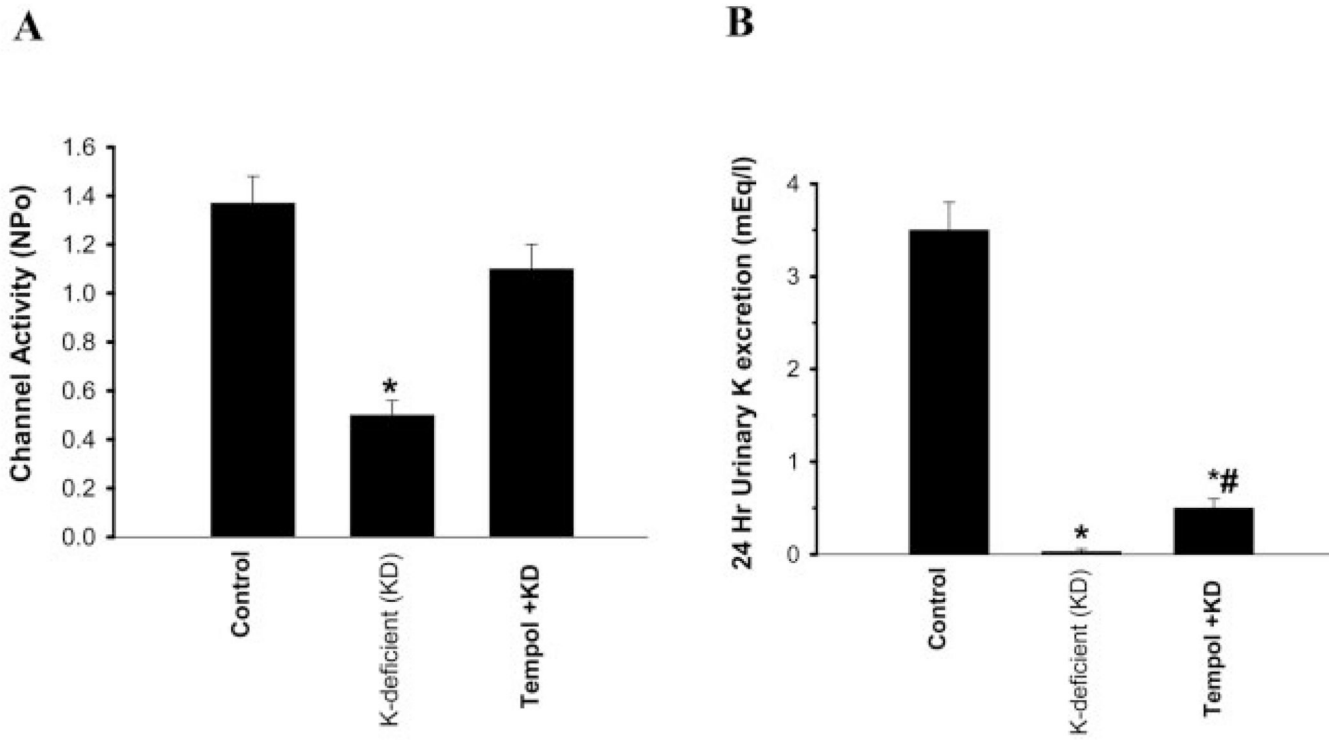

FIG. 10.

A, activity of ROMK-like SK channels in the CCD from rats on a control K diet (1.1%), KD diet, and KD diet plus tempol for 7 days. *B*, 24 h of urinary K excretion in rats on a normal K diet, KD diet, and KD diet plus tempol for 7 days. *Asterisk* indicates the significant difference between the control group and experimental group. *# indicates that the renal K excretion in rats on a KD diet with tempol is significantly different from those without tempol treatment.