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J Immunol 2003; 170:931-940; doi: 10.4049/jimmunol.170.2.931
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Up-Regulation of Early Growth Response Gene-1 Via the CXCR3 Receptor Induces Reactive Oxygen Species and Inhibits Na⁺/K⁺-ATPase Activity in an Immortalized Human Proximal Tubule Cell Line

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The CXCR3 chemokine receptor, a member of the CXCR family, has been linked to a pathological role in autoimmune disease, inflammatory disease, allograft rejection, and ischemia. In the kidney, expression of the CXCR3 receptor and its ligands is up-regulated in states of glomerulonephritis and in allograft rejection, but little is known about the expression and functional role the CXCR3 receptor might play. Here, we study the function of the CXCR3 chemokine receptor in an immortalized human proximal tubular cell line (IHKE-1). Stimulation of the CXCR3 receptor by its selective agonist monokine induced by IFN-γ leads via a Ca²⁺-dependent mechanism to an up-regulation of early growth response gene (EGR)-1. Overexpression of EGR-1 induces down-regulation of copper-zinc superoxide dismutase and manganese superoxide dismutase and stimulates the generation of reactive oxygen species (ROS) via the NADH/NADPH-oxidase system. EGR-1 overexpression or treatment with monokine induced by IFN-γ resulted in a ROS-dependent inhibition of basolateral Na⁺/K⁺-ATPase activity, compromising sodium transport in these cells. Thus, activation of the CXCR3 receptor in proximal tubular cells might disturb natriuresis during inflammatory and ischemic kidney disease via EGR-1-mediated imbalance of ROS. The Journal of Immunology, 2003, 170: 931–940.

Proximal tubular cells play a crucial role in several forms of renal injury such as acute kidney failure following ischemia, chronic allograft rejection, and chronic renal failure (13). Especially, tubulointerstitial disease is an important participant in the progression of chronic renal failure (14). After injury of proximal tubular cells various mediator systems can be activated leading to the enhanced local production of complement, chemokines, and matrix components and to the amplification of injury by the invasion of proinflammatory cells (13). As a result, tubular transport is disturbed (15) and overproduction of matrix leading to fibrosis occurs (14). There is a good body of evidence that chemokines and their ligands are involved in tubular injury during inflammation. For example, the CXCR3 ligand IFN-γ-inducible protein-10 (IP-10), is up-regulated in animal models of interstitial nephritis (16). In adriamycin nephropathy, high levels of glomerular IP-10 mRNA expression and glomerular and tubulointerstitial IP-10 protein expression are associated with proteinuria and interstitial cellular infiltrates. IP-10 mRNA expression has been detected in renal interstitial fibroblasts and tubular epithelial cells (17). Not only up-regulation of IP-10, but also increased expression of the CXCR3 receptor itself has been reported in a variety of inflammatory diseases (7) and in clinical states associated with ischemia (8, 9). The intracellular signal transduction of the CXCR3 receptor is widely unknown, but angiostatic properties (18, 19), antitumor effects (20, 21), induction of tissue necrosis (22), and chemotactic properties (23) have all been linked to the CXCR3 receptor. Because CXCR3 receptor and its ligands seem to play an important role in tubulointerstitial inflammation, we investigated the...

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Received for publication December 17, 2001. Accepted for publication November 12, 2002.

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1 This work was supported in part by Deutsche Forschungsgemeinschaft Grants Fi691/1-2, Pa483/5-1 and by the Else-Kröner-Fresenius Stiftung.

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Abbreviations used in this paper: IP-10, IFN-γ-inducible protein-10; MIG, monokine induced by IFN-γ, SOD, superoxide dismutase; EGR, early growth response gene; Cu/ZnSOD, copper-zinc SOD; MnSOD, manganese SOD; [Ca²⁺], free cytosolic calcium concentration; ROS, reactive oxygen species; HO, hemoxygenase; DMTU, 1,3-dimethyl-2-thiourea; PDTC, pyrroldinedithiocarbamate; PCK, protein kinase C.

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0022-1767/03/$02.00

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expression and function of the CXCR3 receptor in an immortalized proximal tubular cell line.

Materials and Methods

Materials

Recombinant human monokine induced by IFN-γ (Mig) was obtained from R&D Systems (Wiesbaden, Germany); osazin, 1,3-dimethyl-2-thio-urea of early cDNA 2 min at 50°C. Master mix (8 μl), catalse, superoxide dismutase (SOD), p-nitrophenyl phosphate, pyridinedithiocarbamate (PDTC), xanthine, and xanthine oxidase were all obtained from Sigma-Aldrich (Deisenhofen, Germany).

Cell culture

Immortalized human proximal tubule cells (IHKE-1) were cultured as recently reported (24). Briefly, cells were maintained in Ham’s F12/DMEM medium containing 1-glutamine (Life Technologies, Eggenstein, Germany) supplemented with 1% RBS (Roche Diagnostics System, Basel, Switzerland), 50 μg/ml ciprofloxin (Bayer, Leverkusen, Germany), 10 μg/L epidermal growth factor (Calbiochem, La Jolla, CA), 36 μg/L hydrocortisone (Sigma-Aldrich), 1.5 g/L NaHCO3 (Biochrom, Berlin, Germany), 100 mM sodium-pyruvate (Biochrom), 250 mg/L insulin-transferrin-sodium selenite supplement (Roche Diagnostics). The medium used for transfected cell lines contained 300 μg/ml genetin for selection purposes. Cells were switched to fresh media 24 h before the experiments and then exposed to various treatments.

Gene expression array

Differential gene expression was tested using the Atlas cDNA Expression Array kit (Clontech Laboratories, Heidelberg, Germany): 5 μg of RNA was mixed with 1 μl of 10× primer mix and incubated at 70°C for 2 min followed by another 2 min at 50°C. Master mix (8 μl), containing 2 μl of 5× reaction buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl2), 1 μl of 10× dNTP mix for dATP labeling (5 mM each dCTP, dGTP, dITP), 3.5 μl of [32P]dATP (10 μCi/μl; Amersham Pharmacia Biotech, Freiburg, Germany), 0.5 μl of 100 mM DTT, and 1 μl of Moloney murine leukemia virus reverse transcriptase (250 U/μl; Promega). The reaction was stopped with 1 μl of 10× termination mix (0.1 M EDTA, pH 8.0; 1 mg/ml glycerol). Labeled cDNA was purified on Chroma Spin-200 DEPC-H2O columns (all obtained from Clontech Laboratories). Hybridization was performed according to the manufacturer’s manual. In brief, microarray membranes were placed into roller bottles, washed with deionized H2O, and prehybridized with 500 μg of sheared salmon testes DNA in ExpressHyb hybridization solution (Stratagene, Heidelberg, Germany) at 68°C for 30 min. Labeled cDNA probes (2–10 × 106 cpm) were mixed with 10× denaturing solution (1 M NaOH; 10 mM EDTA) and incubated at 68°C for 20 min. C1–1 DNA and 2× neutralizing solution (1 M NaH2PO4, pH 7.0) was added to the cDNA probes, incubated at 68°C for 30 min, and transferred together with hybridization solution to the membranes. Membranes were hybridized overnight at 68°C, washed with prewarmed washing solutions, and wrapped in plastic wrap. Membranes were exposed to x-ray film (Biomax MS; Kodak, München, Germany) at ~80°C using intensifying screens. For best evaluation, films were exposed for varying lengths of time (from 12 h to 7 days).

Polymerase chain reaction

Isolation of human nephron segments was performed from unaltered cortices of patients (with their consent) undergoing tumor nephrectomy (25, 26). Proximal tubules of a total length of 200 μm or IHKE-1 cells were lysed in a 4 M guanidinium chloride buffer and total RNA was isolated and incubated with 10 U DNase I (Promega, Heidelberg, Germany) at 68°C for 30 min to digest traces of genomic DNA. RNA and DNase I were then sheared in 4 M guanidinium chloride buffer and total RNA was isolated and purified on Chroma Spin-200 DEPC-H2O columns (all obtained from Clontech Laboratories). Reaction conditions were as follows: CXCR3 primer, 5′-CCACCCACTGCAATACAA-3′ and 5′-CCGA ACTTGGACCTCTACAA-3′; size: 379 bp, denaturing 94°C/30 s, annealing 55°C/2 min, elongation 55°C/2 min for 32 cycles; GAPDH, 5′-GGT GAAGGTTCCGATCAG-3′ and 5′-CAAAGTTGATCAGTATGACA-3′; size: 496 bp, annealing 60°C/2 min, elongation 72°C/1 min for 24 cycles. PCR products were analyzed by agarose gel electrophoresis. Positive signals obtained from PCR products were either directly sequenced or subjected to restriction enzyme digestion. GAPDH expression was used as a positive control for nonquantitative PCR.

RNA isolation and Northern analysis

To confirm results of gene-expression arrays with Mig-stimulated and control cells, 10 μg of total RNA was isolated and subjected to Northern analysis. Amplification products from the PCR were labeled with [α-32P]dATP and used as a probe for Northern analysis as described recently (27). Hybridization with a probe for the housekeeping gene GAPDH was used as an internal control. Autoradiography signals were analyzed by scanning and volume integration.

Cloning of human early growth response gene (EGR-1)

Human EGR-1 was cloned using a cDNA template generously provided by Dr. T. McCaffrey (Cornell University, New York, NY). In brief, 5′ primers containing a Xhol site and a Kozak sequence and 3′ primers containing an EcoRI site were used to amplify the complete coding sequence of human EGR-1. The PCR product was fully sequenced and cloned into the expression vector pRES2-EGFP (Clontech) at the Xho/EcoRI site. After transfection of IHKE-1 cells (Superfect; Qiagen) with vector and vector containing the cDNA for EGR-1, selection was performed with medium containing 300 μg/ml genetin (Calbiochem). Clones were screened for green fluorescent protein expression using fluorescence microscopy. Fluorescent cells stably expressing EGR-1 were selected and expression levels for EGR-1 were analyzed using Western blot techniques. Two clones for each cell type (EGR-1 overexpressing and control cells) were tested in additional experiments.

Antisense experiments

Antisense experiments were performed using morpholino antisense oligonucleotides. The following oligonucleotides were used: EGR-1 antisense oligonucleotide, 5′-ACGGACGACGTGAGCTGTTGCT-3′; control oligonucleotide (inverted antisense sequence), 5′-CTGGTGTCGAGGTGCGAGGCAGCA-3′ (Gene Tools, Philomath, OR). IHKE-1 cells were grown in six-well plates to 100% confluency. Antisense/control oligonucleotide (16.8 μl, 300 nmol) 566.4 μl of sterile water, and 16.8 μl of ethoxylated-polyethyleneimine (200 μM) were mixed and incubated for 20 min at room temperature. After addition of 5.6 ml of serum-free cell medium to the antisense/ethoxylated-polyethyleneimine mixture, cells were incubated for 3 h with the antisense oligonucleotide complex. The antisense oligonucleotide complex was replaced by fresh serum-containing medium and the cells were incubated for 16 h under standard incubator conditions. Thereafter, cells were harvested for Western blot experiments and measurement of Na+/K+ -ATPase activity.

Western blots

Western blotting was performed using standard techniques (28). In brief, cells were washed once with PBS, scraped with lysis buffer containing 2 mM EDTA, 2 mM EGTA, 100 mM NaCl, 20 mM Tris, 0.1% SDS, 1% Nonidet P-40, 2 mM PMSF, and a protease inhibitor mixture (Roche Diagnostics) and sonicated. The samples were resuspended in Laemmli sample buffer, boiled (5 min), and subjected to SDS-PAGE and transfer electrophoresis. The transblots were stained with Ponceau solution to prove equal amounts of protein were loaded on the membrane and probed with the following primary Abs: rabbit-anti-EGR-1, goat-anti-p47phox, goat-anti-p67phox, goat-anti-gp91phox (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-oxidase-DNA complex (Oxidaso, Hamburg, Germany), The rabbit anti-p22phox Ab was a generous gift from Dr. M. Quinn (Department of Veterinary Molecular Biology, Montana State University, Bozeman, MT); the rabbit anti-human Nox4 Ab was a generous gift from Dr. D. Lambeth (Department of Pathology and Laboratory Medicine, Emory University Medical School, Atlanta, GA), followed by peroxidase-labeled secondary Abs (donkey anti-rabbit, rabbit anti-goat; Amersham Pharmacia, Piscataway, NJ), and detected by chemiluminescence detection reagents (ECL, Amersham Pharmacia).

Measurement of the free cytosolic calcium concentration ([Ca2+]i)

Measurement of [Ca2+]i was performed in single IHKE-1 cells with an inverted fluorescence microscope as recently described (29). In short, IHKE-1 cells were incubated with the Ca2+-sensitive dye fura-2/AM (5 mmol/L; Sigma-Aldrich) for 30 min at 37°C and mounted in a bath chamber on the stage of an inverted microscope. Perfusion was performed with
a ringer-like solution containing (in millimoles per liter) NaCl 145, K\textsubscript{2}HPO\textsubscript{4} 1.6, KH\textsubscript{2}PO\textsubscript{4} 0.4, Ca\textsubscript{2}Cl\textsubscript{2} 1.3, MgCl\textsubscript{2} 1.03, glucose 5, pH 7.4. Transmission maxima at 340, 360, and 380 nm were measured with a photomultiplier, digitized with 12-bit resolution, and recorded continuously on the hard disc of an AT computer. 

**Measurement of superoxide anion production** was performed as described recently (31). In brief, cells were washed once with cold PBS and resuspended at a density of 10\textsuperscript{6} cells/ml in 300 \mu l of Krebs solution (pH 7.35) containing 99 mM NaCl, 4.7 mM KCl, 1.8 mM CaCl\textsubscript{2}, 1.2 mM MgCl\textsubscript{2}, 25 mM NaHCO\textsubscript{3}, 1.03 mM K\textsubscript{2}HPO\textsubscript{4}, 20 mM Na-HEPES, and 11.1 mM glucose, and centrifuged (200 \times g, 4°C, 5 min). The supernatant was discarded and the pellet was resuspended in fresh Krebs buffer. Cell suspension (100 \mu l) was added to Krebs solution containing 5 \mu M lucigenin and stimulated with either 100 \mu M NADH or NADPH. Bioluminescence was measured with Lumat LB9501 (Berthold, Wildbad, Germany). To calculate the amount of superoxide produced, total counts were analyzed by integrating the area under the signal curve. These values were compared with a standard curve generated using xanthine/xanthine oxidase as described (32).

**Statistical analysis**

Data were expressed as mean ± SEM and were analyzed by ANOVA for repeated measures when comparing within groups and one-way ANOVA when comparing among groups; Student’s t test was used for a two-group comparison. \( p < 0.05 \) was considered statistically significant.

**Results**

**Human proximal tubules express the CXCR3 chemokine receptor**

Previous analysis of renal CXCR3 receptor expression showed high expression of the chemokine receptor CXCR3 only in states of inflammatory renal disease (10) or during human renal transplant rejection (12) but recent experiments have shown that the CXCR3 receptor can likewise be expressed in the unaltered kidney in vivo (34). To further evaluate the expression of the CXCR3 receptor in the healthy human kidney, RT-PCR experiments were performed using microdissected human proximal tubules from otherwise healthy subjects undergoing tumor nephrectomy and an immortalized human proximal tubule cell line (IHKE-1) known to express many characteristics of proximal tubules (35). Using primers specific for the human CXCR3 receptor, a 379-bp fragment of the receptor could be amplified both in microdissected human proximal tubules and in IHKE-1 cells (Fig. 1A). The presence of the CXCR3 receptor was confirmed through direct sequencing of the PCR products. To further characterize the localization of the CXCR3 receptor in the kidney, immunohistochemical stains were performed. The CXCR3 receptor was found on the apical side of proximal tubules (Fig. 1B).

**Stimulation of the CXCR3 receptor increases [Ca\textsuperscript{2+}]\textsubscript{i}, in IHKE-1 cells**

With the results shown in Fig. 1, we proceeded to evaluate the functional role of the CXCR3 receptor in IHKE-1 cells. Stimulation of IHKE-1 cells with MIG, a selective agonist for the CXCR3 receptor, induced a reversible increase of [Ca\textsuperscript{2+}]\textsubscript{i} in IHKE-1 cells. The MIG-induced increase of [Ca\textsuperscript{2+}]\textsubscript{i} consisted of an initial peak followed by a plateau (MIG 10\textsuperscript{-11} M; peak \( \Delta \text{[Ca}\textsuperscript{2+}]\textsubscript{i}; 172 ± 15 nmol/L; plateau: \( \Delta \text{[Ca}\textsuperscript{2+}]\textsubscript{i}; 145 ± 17 nmol/L, n = 12)). Reduction of the extracellular Ca\textsuperscript{2+} from 2 × 10\textsuperscript{-3} M to 10\textsuperscript{-6} M did not change the MIG-induced peak (peak \( \Delta \text{[Ca}\textsuperscript{2+}]\textsubscript{i}; 170 ± 11 nmol/L, 933The Journal of Immunology
The CXCR3 receptor activates EGR-1

Little is known about genes regulated by the CXCR3 receptor. To obtain more information about MIG-induced gene expression, we performed cDNA gene expression arrays with IHKE-1 cells stimulated with MIG (0.5 nmol/L) or vehicle. Fig. 3A shows part of a cDNA expression array with a strong up-regulation of EGR-1 in MIG-stimulated IHKE-1 cells. To confirm these results, we performed Northern blot and Western blot analyses of IHKE-1 cells stimulated with MIG (0.5 nmol/L). Fig. 3 shows the time-dependent increase in EGR-1 mRNA expression (Fig. 3B) (relative density; 1 h: control = 0.18 ± 0.03, MIG = 1.63 ± 0.38, n = 3, *, p < 0.05, Student’s t test). This increase was comparable to the increase in [Ca^{2+}]_{i} in IHKE-1 cells stimulated with MIG. An 9.5-fold increase in EGR-1 protein expression was found at 1 h with a smaller, but still significant, increase at 4 h.

Increase of [Ca^{2+}]_{i} induces EGR-1 expression

Next we investigated whether an increase in [Ca^{2+}]_{i} can lead to the up-regulation of EGR-1. Stimulation with ionomycin (10^{-8} M) induced an ~50-fold reversible increase of [Ca^{2+}]_{i} (baseline: 9 ± 5 nmol/L peak: 454 ± 57 nmol/L, baseline post stimulation: 15 ± 4 nmol/L, n = 4, p < 0.05 peak vs baselines, paired Student’s t test). This increase was comparable to the increase in [Ca^{2+}]_{i}, induced by MIG (Figs. 2B and 4B). Western blot experiments showed the time-dependent up-regulation of EGR-1 in IHKE-1 cells at 1 and 2 h, but not at 4 h after stimulation with ionomycin (Fig. 4A). Because previous experiments have shown that the CXCR3 receptor can induce chemotaxis in eosinophils via a
cAMP-dependent protein kinase A signaling pathway (36), additional experiments with forskolin were performed. In IHKE-1 cells, forskolin (10−3 M), a substance known to activate adenyllyl cyclase activity, did not up-regulate EGR-1 (Fig. 4A).

EGR-1 down-regulates Cu/ZnSOD and MnSOD protein expression and activates NADH/NADPH-oxidase activity

Previously, it was shown that expression of both the CXCR3 receptor and its agonist MIG is increased in brain tissue after ischemia (8). Generation of reactive oxygen species (ROS) during and after induction of ischemia is a well-known mechanism for tissue damage (37). To evaluate whether EGR-1 is involved in the generation of ROS, we stably overexpressed human EGR-1 in IHKE-1 cells. Expression of EGR-1 was significantly (5.5-fold) increased in EGR-1-transfected compared with vector-only transfected cells (n = 3, *, p < 0.05 vs control, Student’s t test) (Fig. 5A). We then studied protein expression of Cu/ZnSOD, MnSOD, and HO-1 in these cells. In addition, we addressed the question of whether overexpression of EGR-1 could modify the generation of ROS by the NADH/NADPH-oxidase system. The results show that overexpression of EGR-1 inhibits total cellular protein expression of Cu/ZnSOD (expression percentage of control: 36 ± 4) and MnSOD (expression percentage of control: 41 ± 3), but does not change expression of HO-1 (expression percentage of control: 81 ± 4.2) (Fig. 5B). In addition, cells overexpressing EGR-1 show an increase in NADH/NADPH-oxidase activity after stimulation with either NADH (Fig. 6A) or NADPH as a substrate (Fig. 6B). Determination of superoxide anion generation was performed using the xanthine/xanthine-oxidase reaction as described recently (32). There was an excellent correlation between chemiluminescence and superoxide anion generation (Fig. 6C). To quantify the total amount of superoxide anions generated during our experimental period (15 min), we integrated the areas under the curves and compared control cells and EGR-1-overexpressing cells incubated either with vehicle, DMTU or SOD. Addition of the ROS scavenger DMTU (10 mM) and SOD (1000 U/ml) to control cells and cells overexpressing EGR-1 significantly reduced the measurable amount of superoxide anions, demonstrating the specificity of this assay for superoxide. Because of one outlier, the data for DMTU in EGR-1-overexpressing cells stimulated with NADH were not significant (Fig. 6D). To evaluate whether the increase in the NADH/NADPH-oxidase activity was caused by a change in enzymatic activity or a change in protein expression, we determined the protein expression of the p22phox, p47phox, p67phox, and gp91phox subunits of the NADH/NADPH-oxidase complex. The p47phox and gp91phox subunits of the NADH/NADPH-oxidase complex could not be found, neither in PCR experiments nor in Western blot experiments (data not shown). The p22phox and p67phox expression did not differ between control cells and EGR-1-overexpressing cells (Fig. 7, A and B). Recently, a new NADH/NADPH-oxidase isoform (Nox4) with high expression in the kidney has been identified. To test the expression of this NADH/NADPH-oxidase in our cell line, Western blot experiments with control cells and EGR-1-overexpressing cells were performed (Fig. 7C). Nox4 expression was significantly increased in cells

FIGURE 4. Increase of [Ca2+]i induces EGR-1. A. Western blot analysis for EGR-1 in IHKE-1 cells stimulated with either vehicle, ionomycin (10−8 M), or forskolin (10−3 M). Stimulation with ionomycin (Ion) but not forskolin (For) induced a time-dependent increase in EGR-1 expression with a maximum at 1 h. Experiment showing the effect of ionomycin (10−8 M) on [Ca2+]i in IHKE-1 cells. Ionomycin induced a long-lasting, reversible increase of [Ca2+]i. Functional viability of cells was confirmed by stimulation with ATP (10−4 M) at the end of the experiment.

B. Western blot analysis for EGR-1 in IHKE-1 cells stimulated with either 5M ionomycin, 8M forskolin (Ion and For, respectively). Densitometric analysis shows an ~5.5-fold increased expression of EGR-1 in EGR-1-transfected compared with vector-only transfected cells. Summary of experiments (graph) shows an ~5.5-fold increased expression of EGR-1 in EGR-1-transfected compared with vector-only transfected IHKE-1 cells (*, p < 0.05 vs control, Student’s t test; the number in parentheses indicates the number of experiments).

FIGURE 5. EGR-1 down-regulates SOD. A. Overexpression of EGR-1 in stably transfected IHKE-1 cells. Summary of three experiments (graph) shows an ~5.5-fold overexpression of EGR-1 in EGR-1-transfected compared with vector-only transfected IHKE-1 cells ( *, p < 0.05 vs control, Student’s t test; the number in parentheses indicates the number of experiments). B. Experiment showing the protein expression for Cu/ZnSOD, MnSOD, and HO-1 in cells overexpressing EGR-1. Summary of four experiments (graph) shows a strong down-regulation of Cu/ZnSOD (~65%) and MnSOD (~59%), but no change in the expression of HO-1 ( *, p < 0.05 vs control, Student’s t test; the number in parentheses indicates the number of experiments).
overexpressing EGR-1. In addition, protein expression of Rac1, a member of the Rho family of small GTPases known to assemble with the cytosolic p47^phox and p67^phox and the membrane-associated flavocytochrome b558 to form the multicomponent respiratory burst oxidase, was investigated (Fig. 7D). Rac1 protein expression was markedly up-regulated in cells overexpressing EGR-1.

**MIG activates NADH/NADPH-oxidase activity**

To demonstrate that the effects of EGR-1 on cellular ROS generation could be replicated by MIG, we stimulated untransfected IHKE-1 cells with either vehicle or MIG (0.5 nmol/L) for 4 h and measured NADH/NADPH-oxidase activity thereafter (Fig. 8A). To quantify the total amount of superoxide anions generated during the experimental period (15 min), we again integrated the areas under the curves and compared control cells and MIG-stimulated cells. These results indicate that stimulation with MIG likewise induces an increase in superoxide anion generation with either NADH or NADPH as substrate (Fig. 8B).

**MIG via EGR-1-induced ROS generation inhibits cellular Na^+/K^-ATPase activity**

Several studies have demonstrated the effects of ROS on cellular ion transport mechanisms (38). In the proximal tubule, most of the secondary active ion transporter like sodium-glucose and sodium-amino acid cotransporter and sodium-hydrogen antiporter depend at least in part on the sodium gradient generated by the basolateral Na^+/K^-ATPase activity (39). To investigate the role of MIG on the cellular Na^+/K^-ATPase activity of human proximal tubules, we first measured the activity of this enzyme in EGR-1-overexpressing and control cells. In EGR-1-overexpressing cells, basal Na^+/K^-ATPase activity is reduced by ~60% (basal activity: 12.7 ± 2.5 nmol Pi/mg protein/minute) compared with vector-only transfected cells (basal activity: 29 ± 4.2 nmol Pi/mg protein/minute) (Fig. 9).

Time-dependent incubation of EGR-1-overexpressing cells with either catalase (5000 U/ml), an enzyme that metabolizes hydroperoxide (1 h: activity: 11.5 ± 1.8 nmol Pi/mg protein/minute; 24 h: activity: 12.2 ± 2 nmol Pi/mg protein/minute) or PDTC (50 μM), an inhibitor of inducible NO synthase induction (1 h: activity: 16.9 ± 2 nmol Pi/mg protein/minute, 24 h: activity: 16.4 ± 2.1 nmol Pi/mg protein/minute), did not change basal Na^+/K^-ATPase activity. In contrast, incubation of EGR-1-overexpressing cells with either DMTU (10 mM) (1 h: activity: 32.6 ± 3.6 nmol Pi/mg protein/minute) or SOD (1000 U/ml) (1 h: activity: 25.7 ± 3 nmol Pi/mg protein/minute), both superoxide anion scavengers, reversed the EGR-1 induced inhibition of basal Na^+/K^-ATPase activity to control values (Fig. 9). To demonstrate that the effects of EGR-1 on basal Na^+/K^-ATPase activity could be replicated by MIG, we stimulated untransfected IHKE-1 cells with either vehicle (activity: 28.6 ± 2.1 nmol Pi/mg protein/minute, n = 21) or MIG (0.5 nmol/L) (activity: 17.1 ± 2.6 nmol/minute, n = 21).
Pi/mg protein/minute, n = 11) for 4 h. Stimulation with MIG likewise induced an inhibition of the Na⁺/K⁺-ATPase activity in untransfected IHKE-1 cells (Fig. 11). To prove that EGR-1 via generation of superoxide anions is responsible for the effects of MIG on Na⁺/K⁺-ATPase activity, antisense experiments with EGR-1 antisense oligonucleotides were performed. First, the specificity of anti-EGR-1 antisense oligonucleotides on EGR-1 protein expression was tested (Fig. 10). Stimulation of untransfected IHKE-1 cells with MIG (0.5 nmol/L) for 1 h induced the known up-regulation of EGR-1 as compared with vehicle-stimulated control cells. Pretreatment of IHKE-1 cells with control antisense oligonucleotides slightly reduced total MIG-induced EGR-1 expression. Pretreatment of IHKE-1 cells with anti-EGR-1 antisense oligonucleotides significantly reduced MIG-induced EGR-1 expression (46.6 ± 6.2%) compared with experiments with control antisense oligonucleotides. To show linkage between the CXCR3 receptor and its effect on Na⁺/K⁺-ATPase activity via EGR-1, untransfected IHKE-1 cells were preincubated with control antisense and antisense. Preincubation with control antisense did not influence MIG-induced inhibition of Na⁺/K⁺-ATPase activity (activity: 15.1 ± 2.6 nmol Pi/mg protein/minute, n = 12). In contrast, preincubation with antisense partially reversed MIG-induced inhibition of Na⁺/K⁺-ATPase activity (activity: 26.5 ± 3.1 nmol Pi/mg protein/minute, n = 12) (Fig. 11).

**Discussion**

The CC and the CXC chemokines are important chemotactic molecules that control leukocyte trafficking and function. The CXCR3 receptor is expressed in a cell type-specific manner in subsets of leukocytes (3), but also in some nonhemopoietic cells, such as neurons (40), and cells of the mammary gland (41). A variety of functions of the CXCR3 receptor have been described (18, 19, 20, 21, 22, 23), but the molecular mechanisms leading to these diverse biological functions are widely unknown. A recent study has shown that the CXCR3 receptor via activation of the p21^{ras} oncprotein/extracellular signal-regulated kinase cascade leads to increased chemotaxis (42). In the present study, we investigated the
expression and function of the CXCR3 receptor in an immortalized proximal tubular cell line. We demonstrate expression of the CXCR3 receptor in microdissected human proximal tubule cells, an immortalized human proximal tubule cell line (IHKE-1) and in rat kidney sections. Furthermore, MIG reversibly and concentration-dependently increased [Ca\(^{2+}\)], in IHKE-1 cells, indicating that this receptor is functionally active. The half maximal concentration of MIG was 20 pM, which fits well to that described in other cell types such as lymphocytes (43). Experiments with reduction of the extracellular calcium concentration show that increase of [Ca\(^{2+}\)], depends on both release of Ca\(^{2+}\) from intracellular stores and transmembranous influx of Ca\(^{2+}\). In addition, MIG induces a up-regulation of EGR-1 mRNA expression in IHKE-1 cells at 1 h and up-regulation of EGR-1 protein expression at 1 and 4 h. The regulation of EGR-1, a zinc-finger transcription factor that has been linked to ischemia, has been studied in detail and several mechanisms are thought to be involved in its up-regulation. In our experiments, up-regulation of EGR-1 was Ca\(^{2+}\)-dependent because stimulation with ionomycin, a calcium ionophore, but not forskolin, an activator of adenylyl cyclase activity, increased EGR-1 expression in a time-dependent manner similar to the up-regulation of EGR-1 induced by MIG. These data are in agreement with experiments described in the literature. Stimulation of islet β cells with glucose increased EGR-1 mRNA expression via a calcium/calmodulin-dependent pathway (44). Furthermore, experiments in the murine erythroleukemia cell line ELM-1 show that exposure of these cells to the Ca\(^{2+}\)-ionophore A23187 leads to a rapid transient rise in EGR-1 and c-fos mRNA expression followed by an increase in EGR-1 and c-fos protein levels as well as an increase in EGR-1 and AP-1 DNA-binding activity (45). A recent study has shown the essential role of c-fos for the activation of the transcription factor complex AP-1 and the subsequent stimulation of downstream genes such as tyrosine hydroxylase. The results of this study show that hypoxia causes Ca\(^{2+}\) influx through L-type voltage-gated Ca\(^{2+}\) channels and that hypoxia-induced c-fos gene expression is Ca\(^{2+}\)/calmodulin-dependent (46). But conflicting data exist. Experiments performed in mouse cortical cultures show that although zinc, N-methyl-d-aspartate, or ionomycin induced

**FIGURE 9.** Superoxide anions inhibit Na\(^+/\)K\(^{-}\)-ATPase activity. Na\(^+/\)K\(^{-}\)-ATPase activity in EGR-1-overexpressing cells and control cells. Basal Na\(^+/\)K\(^{-}\)-ATPase activity was reduced by ~50% in EGR-1-overexpressing cells compared with control cells. The addition of catalase (5000 U/ml) or PHTC (50 μM) for 1 or 24 h did not significantly change basal Na\(^+/\)K\(^{-}\)-ATPase activity in EGR-1-overexpressing cells. The addition of either DMTU (10 mM) or SOD (1000 U/ml) reversed the EGR-1 induced reduction in basal Na\(^+/\)K\(^{-}\)-ATPase activity (*, p < 0.05 vs control; +, p < 0.05 vs DMTU-treated; ●, p < 0.05 vs SOD treated; ANOVA, Scheffe’s test; the number in parentheses indicates the number of experiments).

**FIGURE 10.** Antisense oligonucleotides inhibit MIG-induced EGR-1 expression. A, EGR-1 protein expression in untransfected IHKE-1 cells after stimulation with vehicle (Co) and MIG (0.5 nmol/L) for 1 h. Again, there is a significant increase in EGR-1 expression in MIG-stimulated cells. Stimulation of IHKE-1 cells with MIG (0.5 nmol/L) after preincubation with control antisense oligonucleotides (CoAS) slightly reduced overall EGR-1 expression. Stimulation of IHKE-1 cells with MIG (0.5 nmol/L) after preincubation with anti-EGR-1 antisense oligonucleotides (AS) significantly reduced EGR-1 expression compared with experiments with CoAS. B, Summary of three experiments showing the percent change of EGR-1 expression (*, p < 0.05 vs control; Student’s t test; the number in parentheses indicates the number of experiments).

**FIGURE 11.** MIG inhibits Na\(^+/\)K\(^{-}\)-ATPase activity. Left two bars, Na\(^+/\)K\(^{-}\)-ATPase activity in untransfected IHKE-1 cells after stimulation with MIG (0.5 nmol/L, 4 h) or vehicle. There is a significant decrease in Na\(^+/\)K\(^{-}\)-ATPase activity in MIG-stimulated cells compared with control cells (*, p < 0.05 vs control; Student’s t test; the number in parentheses indicates the number of experiments). Right two bars, Na\(^+/\)K\(^{-}\)-ATPase activity in untransfected IHKE-1 cells after preincubation with either control antisense oligonucleotides (CoAS) or anti-EGR-1 antisense oligonucleotides (AS) and stimulation with MIG (0.5 nmol/L) for 4 h. CoAS did not influence MIG-induced inhibition of Na\(^+/\)K\(^{-}\)-ATPase activity. In contrast, preincubation of IHKE-1 cells with AS significantly decreased the MIG-induced inhibition of Na\(^+/\)K\(^{-}\)-ATPase activity (*, p < 0.05 vs control; Student’s t test; the number in parentheses indicates the number of experiments).
comparable neuronal death, only zinc increased EGR-1 expression, which was attenuated by blocking zinc influx (47). Therefore, it might be possible that induction of EGR-1 via Ca^2+ depends on cell type-specific mechanisms. It has been suggested that a Ca^2+-dependent protein kinase C (PKC) isoform might be involved in the up-regulation of EGR-1. Recent data support this mechanism. Using homozygous null mice for the PKC β-isozyme gene, Yan et al. (48) could show that PKC-β, a Ca^2+-dependent PKC isoform, is essential for the hypoxia-induced expression of EGR-1. In addition, rapid ischemia-mediated activation of EGR-1 has been shown to up-regulate chemokine receptors, adhesion receptors, and procoagulant- and permeability-related genes. Deletion of the gene encoding EGR-1 strikingly diminished the expression of these mediators of vascular injury in a murine model of lung ischemia/reperfusion, and enhanced animal survival and organ function (49). These data indicate that EGR-1 activation plays a central role in the pathogenesis of ischemic tissue damage.

The downstream mechanisms involved in cellular injury mediated by EGR-1 are poorly understood. In this study, we show that in cells overexpressing EGR-1, expression of Cu/ZnSOD and MnSOD, two enzymes essential for the detoxification of superoxide anions, is down-regulated. This mechanism most likely depends on the transcriptional regulatory domain of EGR-1. Interestingly, forced overexpression of EGR-1 in NIH3T3 cells leads to increased MnSOD transcription in a dose-dependent manner (50), but an EGR-1-mediated negative role on the basal promoter activity of MnSOD has also been shown (51). In addition, the 3′-untranslated region of the MnSOD RNA has influence on the expression of this enzyme (52, 53). The same is true for the Cu/ZnSOD (54, 55). Experiments with an EGR-1 protein, where a serine/threonine/proline-rich region between aa 174 and 270 was eliminated, show that the activation domain of EGR-1 is critical for the activity of the protein (56). Our data show that EGR-1 in addition to the down-regulation of SODs stimulates NADH/NADPH-oxidase activity both in response to NADH and to NADPH. This effect could, at least in part, be explained by an increase in protein expression of the NADH/NADPH-oxidase Nox4. Interestingly, Rac1 expression was increased in cells overexpressing EGR-1. An increased binding of Rac1 to the p67^{phox} subunit with activation of the cytochrome b558 complex could explain an increase in NADH/NADPH-oxidase activity but despite the presence of the p22^{phox} subunit in our cell line, no mRNA or protein for the gp91^{phox} subunit of the cytochrome b558 complex could be found. Addition of the superoxide anion scavenger anagonized the effect of EGR-1 on NADH/NADPH-oxidase activity indicating that superoxide anions were involved. Additional experiments with untransfected cells showed that stimulation of untransfected IHKE-1 cells with MIG for 4 h has increased NADH/NADPH-oxidase activity Nox4. Interestingly, Rac1 expression was increased in cells over-expressing EGR-1. An increased binding of Rac1 to the p67^{phox} subunit with activation of the cytochrome b558 complex could explain an increase in NADH/NADPH-oxidase activity but despite the presence of the p22^{phox} subunit in our cell line, no mRNA or protein for the gp91^{phox} subunit of the cytochrome b558 complex could be found. Addition of the superoxide anion scavenger anagonized the effect of EGR-1 on NADH/NADPH-oxidase activity indicating that superoxide anions were involved. Additional experiments with untransfected cells showed that stimulation of untransfected IHKE-1 cells with MIG for 4 h can indeed induce NADH/NADPH-oxidase activity via an EGR-1-dependent mechanism. Our data corroborate present data of ischemia-induced tissue damage because ischemia increases superoxide anion (ROS) generation in hypoxic tissues. Experiments with a cytochrome c-coated platinized carbon electrode have for instance shown that hypoxia, focal ischemia, and reperfusion all induce superoxide anion generation in the brain (37). Therefore, our data might explain part of the cellular mechanisms involved in ROS generation induced by ischemia.

What might be the function of increased superoxide anion production in proximal tubule cells? Several earlier studies have shown that ROS can alter ion transport mechanisms (38). Experiments with hypoxic alveolar epithelial cells have shown that ion transport is significantly affected by decreasing or increasing cellular ROS levels (57). Interestingly, our data indicate that EGR-1 via generation of superoxide anions inhibits the basolateral Na^+/K^+-ATPase activity of proximal tubules. The reduction of basolateral Na^+/K^+-ATPase activity in EGR-1-overexpressing cells could be normalized by treatment of those cells with either SOD or DMTU, both potent scavengers for superoxide anions, but not by treatment with PTDC, an inhibitor of inducible NO synthase induction, or catalase, an enzyme that detoxifies hydrogen peroxide. These data indicate that the superoxide anion, but not hydrogen peroxide or ROS downstream of hydrogen peroxide, is responsible for the inhibition of Na^+/K^+-ATPase activity in EGR-1-overexpressing cells. This mechanism could be reproduced by stimulating untransfected IHKE-1 cells with MIG for 4 h at a time point, where a significant difference in NADH/NADPH-oxidase activity between MIG stimulated and unstimulated cells has been found. Basolateral Na^+/K^+-ATPase activity is essential for secondary active Na^+-dependent transport mechanisms present in the luminal membrane (39) and depends on the intracellular availability of ATP, a product of an intact oxygen-dependent cell metabolism (58). The ROS-dependent reduction of basolateral Na^+/K^+-ATPase activity in states of oxygen deprivation might be a mechanism to reduce the oxygen consumption of cells already in jeopardy. Moreover, mild ischemia-induced acute renal failure is known to be associated with significant impairment of tubular Na^+ reabsorption and an increased fractional urinary Na excretion (59). In addition, inflammatory kidney diseases like granulomatous interstitial nephritis (60), membranoproliferative glomerulonephritis, lupus nephritis (61), and biopsy-verified chronic glomerulonephritis (62) all have been shown to be associated with an increased fractional sodium excretion. Therefore, the CXCR3 receptor via EGR-1 stimulation might participate in the pathogenesis of renal injury and natriuresis during inflammatory and ischemic kidney disease.

Acknowledgments
We thank Dr. Timothy McCaffrey (Cornell University, New York, NY) for generously providing us with a cDNA template of human EGR-1. We further thank Dr. Mark Quinn (Department of Veterinary Molecular Biology, Montana State University, Bozeman, MT) for a sample of his anti-p22^{phox} Ab, and Dr. David Lambeth (Department of Pathology and Laboratory Medicine, Emory University Medical School, Atlanta, GA) for the generous gift of rabbit anti-human Nox4 Ab. We thank Temel Kilic and Petra Daemisch for their excellent technical assistance.

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