ATF3 Protects against Renal Ischemia-Reperfusion Injury

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ABSTRACT
Oxidative stress-induced cell death plays a major role in the progression of ischemic acute renal failure. Using microarrays, we sought to identify a stress-induced gene that may be a therapeutic candidate. Human proximal tubule (HK2) cells were treated with hydrogen peroxide (H₂O₂) and RNA was applied to an Affymetrix gene chip. Five genes were markedly induced in a parallel time-dependent manner by cluster analysis, including activating transcription factor 3 (ATF3), p21WAF1/CIP1 (p21), CHOP/GADD153, dual-specificity protein phosphatase, and heme oxygenase-1. H₂O₂ rapidly induced ATF3 approximately 12-fold in HK2 cells and approximately 6.5-fold in a mouse model of renal ischemia-reperfusion injury. Adenovirus-mediated expression of ATF3 protected HK2 cells against H₂O₂-induced cell death, and this was associated with a decrease of p53 mRNA and an increase of p21 mRNA. Moreover, when ATF3 was overexpressed in mice via adenovirus-mediated gene transfer, ischemia-reperfusion injury was reduced. In conclusion, ATF3 plays a protective role in renal ischemia-reperfusion injury and the mechanism of the protection may involve suppression of p53 and induction of p21.


Acute renal failure (ARF) is associated with high morbidity and mortality rates, although it was recently reported that in-hospital mortality has declined.¹,² Ischemia reperfusion injury (IRI), a common cause of ARF, occurs during kidney transplantation,³ cardiopulmonary bypass,⁴ aortic bypass surgery, and sepsis.⁵,⁶ There are a number of potential mediators of renal IRI, which are characterized as abnormal vascular reactivity, tubular obstruction, epithelial cell dysfunction, necrosis, and apoptosis. It is generally believed that reactive oxygen species are the key mediators of the renal IRI.

Many researchers are keenly interested in identifying key genes involved in renal IRI. To achieve this goal, we used DNA microarrays to elucidate candidate IRI genes that could have therapeutic potential. DNA microarray technology is a powerful tool for therapeutic discovery, optimization, and clinical validation.⁶ Several studies have exploited the high-throughput capability of DNA microarrays to obtain a molecular profile of oxidative stress in a variety of organs. We previously reported that the renal ischemia reperfusion animal model induces many sets of genes including activating transcription factor 3 (ATF3),⁷,⁸ which could represent a therapeutic target.

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ATF3 is a member of the mammalian activation transcription factor/cAMP responsive element-binding protein family of transcription factors. ATF3 represses rather than activates transcription from promoters with ATF binding elements. In animal models, ATF3 is induced by myocardial, renal, and liver ischemia reperfusion, by chemicals, and in the skin by wounding. In cultured cells, ATF3 is induced by a variety of signals including cytokines, genotoxic agents, and cytotoxic agents. It has been reported that the induction of ATF3 was almost completely inhibited by N-acetyl-L-cysteine, an oxidant scavenger, suggesting that ATF3 is induced by oxidative stress. However the role of ATF3 in renal ischemia reperfusion injury remains unclear.

Here we report that ATF3 is rapidly induced in human kidney cells in response to oxidative stress and may protect the cells. Overexpression of ATF3 in mice via adenovirus-mediated gene transfer ameliorated IRI, significantly decreasing creatinine and improving the pathologic score for acute tubular injury.

RESULTS

Microarray Analysis

Human kidney-2 cells (HK2 cells: human renal proximal tubular epithelial cells) were stimulated with 100 μM H2O2 for 15 min, 1, 4, and 16 h. After the indicated time, mRNAs were extracted and DNA microarray analysis was performed. Differential gene expression profiles after H2O2 stimulation were clustered into groups of coregulated genes as shown in Figure 1A. The changes were analyzed using the manufacturer’s software and fold changes were indicated by “affy-fold”. Among the groups clustered by expression, one group contained seven highly induced genes (Figure 2B) that included p21WAF1/CIP1 (p21), ATF3, CHOP/GADD153 (CHOP), dual-specificity protein phosphatase (DSP), heme oxygenase-1 (HO1), ID1, and Period. Using real-time reverse transcription (RT)-PCR we confirmed that five of these genes, namely p21, ATF3, CHOP, DSP, and HO1, were significantly upregulated in a time-dependent (Figure 2A) and dose-dependent (Figure 2B) manner. The time courses and dose-dependence for induction of these five genes were comparable (Figure 2B). Other gene clusters of up- and downregulated genes, including expressed sequence tags (ESTs) (AA149307, AA522537, etc.) or orphan genes (AB020713, etc.), were unremarkable in terms of either fold changes or known roles in cell injury and repair, and thus were not pursued further.

Confirmation of Upregulation of ATF3 Expression

All five upregulated genes are known to have roles in oxidative stress. However, of these genes, ATF3 was most markedly changed by H2O2 treatment and is a known transcriptional regulator. For these reasons ATF3 was selected as a key regulatory gene that might mediate injury events associated with oxidative stress. To confirm that H2O2 induced ATF3 expression at the protein level, Western blot analysis was performed. Induction was clearly observed at 1, 4, and 16 h (Figure 3A). Immunohistological examination showed that, under control, nonstress conditions, ATF3 is present in the cytosolic regions of HK2 cells (Figure 3B). The staining of ATF3 shifted to the nucleus after 4 h of H2O2 treatment (100 μM) (Figure 3C). These observations are consistent with ATF3 functioning as an activated transcription factor in response to oxidative stress.
Interaction between ATF3 and Other Upregulated Genes

To analyze the interaction between ATF3 and other upregulated genes, ATF3 was silenced using small RNA interference (siRNA) methodology. H$_2$O$_2$-Induced ATF3 expression was suppressed by ATF3-siRNA transfection (Figure 4) of HK2 cells. Interestingly, suppression of ATF3 with ATF3-siRNA blocked induction of p21 but did not influence the increased expression of CHOP, DSP, or HO1. Thus, p21 appears to be a downstream target of ATF3.

ATF3 Prevented H$_2$O$_2$-Induced Cell Death in HK2 Cells

Next, we examined whether overexpression of ATF3 could protect cells from oxidant stress. For this purpose, HK2 cells were infected with AdATF3 or AdLacZ, treated with H$_2$O$_2$, and cell viability was examined with an assay using acetomethoxy ester of calcein (calcein-AM; Molecular Probes, Eugene, OR). As shown Figure 5A, AdATF3-treated HK2 cells were significantly protected from cell death. Thus our in vitro experiment indicated that ATF3 could diminish H$_2$O$_2$-induced cell death. To examine a whether this protective effect of ATF3 could involve a decreased expression of p53 as previously reported, we investigated the p53 mRNA expression in the HK2 cells with or without AdATF3. Real-time RT-PCR revealed that p53 mRNA was significantly downregulated in the AdATF3-treated HK2 cells by approximately 0.7-fold compared with AdLacZ-overexpressed HK2 cells (Figure 5B). Notably, p21 mRNA was induced by AdATF3 by approximately 1.8-fold.

ATF3 Overexpression by Adenovirus Gene Transfer Protected against Renal IRI In Vivo

In an in vivo study we next examined whether ATF3 plays a role in IRI. First we characterized the changes in ATF3 levels in the kidney of a mouse model of IRI. In the kidney, at 12 and 24 h after ischemia there was a significant increase in ATF3 mRNA levels (Figure 6A) and corresponding increases in ATF3 protein levels (Figure 6B). Immunofluorescent staining of kidney (Figure 6C) showed that, with IRI, ATF3 protein was localized principally in the nuclei of the distal tubules that were not stained for NHE3.

Because ATF3 overexpression inhibited oxidative stress induced cell death in HK2 cells, we hypothesized that ATF3 might protect the kidney from IRI. Therefore, we examined the protective effect of overexpression of ATF3 in our model of IRI in the mouse kidney. First, ATF3 gene transfer was performed by hydrodynamic injection of AdATF3 into the mouse tail vein. Overexpression of ATF3 mRNA and protein in the kidney was confirmed by RT-PCR and Western blot analysis (Figure 7, A and B). Immunofluorescence revealed that ATF3 was prominently overexpressed in all nephron segments of mice injected with AdATF3 (Figure 7D) but not with AdLacZ (Fig-
In the kidneys of mice injected with AdLacZ, marked damage (i.e., exfoliation of epithelial cells, tubular cast formation, and tubular dilation) was observed (Figure 7E). In contrast, the injury observed in the kidneys of mice injected with AdATF3 was attenuated compared with control mice injected with AdLacZ (Figure 7, E and F). An associated improvement in renal function was observed in AdATF3-treated mice as serum creatinine levels were significantly ameliorated compared with control mice injected with AdLacZ (Figure 8A). An improvement in the acute tubular necrosis (ATN) score was also observed in the kidneys of IRI mice with ATF3 overexpression (Figure 8B).

**DISCUSSION**

We have three major findings. First, five markedly upregulated genes, including ATF3, were identified in response to H$_2$O$_2$ treatment in HK2 cells. Second, ATF3 plays a protective role in the H$_2$O$_2$-induced cell death in the HK2 cells. Third, ATF3 also plays a protective role in mouse renal IRI.

A cluster analysis was used to search for key genes, because genes with similar patterns of expression can represent genes that are coregulated at the transcription level. Genes involved in a specific biochemical pathway or developmental process have been shown to have coordinated transcriptional regulation, and functionally unknown genes showing the same regulation can be consequently implicated in the same pathway.

We found five H$_2$O$_2$-induced genes in HK2 cells using DNA microarrays. These genes included ATF3, p21, CHOP, DSP, and HO1. All of these genes have been reported to be involved in oxidative stress. Yuen et al. reported about microarray-identified genes, including ATF3, p21, and HO1. HO1 is known to be upregulated in response to an ischemic insult and to play an important role in protection against oxidative stress including renal IRI, which was supported by our result. DSP was reported to be induced by H$_2$O$_2$ stimulation and is implicated in ERK and JNK inactivation in the nucleus. Therefore this gene could also be related to the mechanism of IRI. Id1 was observed as increased in the microarray analysis, but this was not confirmed by real-time PCR for reasons that are unclear. Nonetheless, further work with Id1 may be warranted because Kang et al. suggested that ATF3 can mediate Id1 repression as a common target of TGF-$\beta$/Smad signals and stress signals via p38 kinase.

In this article we demonstrated that ATF3 was dramatically induced by H$_2$O$_2$ in HK2 cells and IRI in the mouse kidney.
ATF3 is a member of the ATF/cAMP-responsive element-binding protein family and is known to be activated under a variety of extracellular stresses. The gene products of this family have a basic region followed by the leucine-zipper motif and bind to the consensus DNA sequences TGACGT(C/A)(G/A) as transcription factors. Several reports have noted that ATF3 is rapidly and preferentially induced by various stimuli. It has been reported that the ATF3 gene is induced when organs such as the liver, heart, and kidney are subjected to oxidative stresses caused by ischemia reperfusion. Our result is consistent with these previous reports. Our previous report did not report the upregulation of ATF3 gene in the animal model of IRI because the probe of ATF3 was not on the microarray. A report by Supavekin et al. also did not show the induction of ATF3 for reasons that are not apparent.

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Herein we described the protective effect afforded by ATF3 against H2O2-induced cell death in a cultured cell line and in renal IRI in the mouse. The mechanism, however, is not clear. As shown in our results, the increase in p21 was suppressed by silencing the ATF3 induction by H2O2 stimulation. AdATF3 induced p21. The cyclin-dependent kinase inhibitor p21 plays a pivotal role in apoptosis through regulation of the cell cycle. Therefore, the mechanism could be that the oxidative stress-mediated increase in p21 associated with overexpression of ATF3 contributed to the protection against oxidative stress through cell-cycle arrest (G1 arrest) and thus inhibited apoptosis. The experiment for further detail about apoptosis and proliferation should be achieved in the next project. Another possibility is that overexpression of ATF3 suppressed the downregulation of p53, which can cause apoptosis. Nobori and colleagues reported that overexpression of ATF3 inhibited doxorubicin-induced apoptotic cell death in cardiac myocytes. They suggested that the downregulation of p53 may function as a cytoprotective transcription factor in doxorubicin-treated cardiac myocytes. Kawauchi et al. also reported that ATF3 protects human umbilical-vein endothelial cells from TNF through downregulation of p53. These reports are compatible with our results. Therefore, the downregulation of p53 also may play a protective role in renal tubules against IRI.

CHOP is a key gene in renal IRI that physically interacts with ATF3. Our results indicated that CHOP was elevated by H2O2 stimulation but unaffected by siRNA silencing of ATF3, which suggests that its induction is not mediated by ATF3. CHOP is a member of the CCAAT/enhancer binding protein (C/EBP), can function as a complex with ATF3, and is known to be an apoptosis-induced gene. ATF3 and CHOP transcripts were regulated in parallel, and ATF3/CHOP heterodimers may be formed with and related to necrotic cell death. Therefore, CHOP would play an important role with ATF3 in the caspase signaling pathway in oxidative stress–induced apoptosis in the human proximal tubule. Moreover, we would suggest that during oxidant stress the mechanism responsible for ATF3 induction may also be also responsible for CHOP induction as well.

Other mechanisms for ATF3 in the protection against cell death have also been reported. Nakagomi et al. hypothesized...
another mechanism in which ATF3 binds with c-Jun and the heterodimer of ATF3/c-Jun induces heat shock protein 27 (Hsp27), which contains a heat shock element in the promot-
er. Hsp27 is known to inhibit cell death in neuronal cells. These authors further suggested that Hsp27 activated the Akt pathway and inhibited MEKK1-c-Jun-JNK, thereby inhibiting apoptosis in neuronal cells. Therefore, Hsp27 may play an
important role with ATF3 in IRI. In another study, Alam et al. reported that ATF3 as well as the transcription factors Nrf2 and MaIG bind to stress-response elements and contribute to the cytoprotective function of HO1 activity during heme-mediated cellular injury.

In conclusion, in this study we have shown that there is a marked induction of ATF3 by oxidative stress and renal isch-
emic injury. Furthermore, ATF3 promotes a protective effect against renal IRI, potentially through regulation of p53 and p21. Thus, overexpression of ATF3, either by gene therapy or by infusion of recombinant ATF3 protein, may provide a new therapeutic intervention in ischemia-mediated acute renal fail-
ure. However, further study may be needed to clarify the role of ATF3 because alternative mechanisms for the protective effect of ATF3 have been reported. In addition, an appropriate ATF3 gene delivery system needs to be developed to put this ap-
proach into clinical practice.

**CONCISE METHODS**

**Cell Culture**

HK2 cells were cultured in RPMI1640 (Life Technology, Tokyo, Ja-
p) supplemented with 10% FBS (JRH Biosciences, Lexena, KS), 100
units/ml penicillin, and 100 µg/ml streptomycin (Life Technology).
Cells were grown at 37°C in 5% CO₂ and 95% air.

**Adenovirus and Gene Transfer**

An adenovirus vector encoding the human ATF3 gene (AdATF3) and
β-galactosidase (AdLacZ) using an Adenovirus Expression Vector kit was a kind gift from Dr. Nobori of Tokyo Medical and Dental Uni-
versity (Tokyo, Japan). For adenovirus-mediated gene transfer, HK2
cells were exposed to the adenoviral vectors and cultured for the indi-
cated time on the basis of the DNA-terminal protein complex
method as described elsewhere.

Purified recombinant adenoviruses were added to HK2 cells at a dose of 3.3 × 10⁶ plaque-forming units. For hydrodynamic injection, AdATF3 or AdLacZ in 1 ml of PBS was
injected within 10 s into the mouse tail vein.

**Animals and Sample Preparation**

C57BL/6j mice (20 to 25 g) were housed in a 12-h light/dark cycle and
allowed free access to food and water. AdATF3, human recombinant
ATF3, or AdLacZ was injected 24 h before treatment to mice. Nobori reported that human recombinant ATF3 functioned in mice.
IRI was carried out as described previously. Briefly, animals were anes-
ethetized intraperitoneally with pentobarbital sodium (65 mg/kg).
Both renal arteries were exposed with a midline incision, then
clamped for 30 min and followed by reperfusion on the heat pad.

Sham animals had an incision plus 30 min of waiting time without
clamping. Animals were then followed for 12 h, 1 d, or 2 d (n = 6 per
group per time point). The animals were killed humanely at the end of
the experiment. The kidneys were quartered, then two of the kidney
specimens were snap-frozen in liquid nitrogen, and the remaining
specimens were fixed with either 15% neutral buffered formalin or
4% paraformaldehyde in PBS. Total RNA was isolated from the kid-
neys with Trizol (Invitrogen, Tokyo, Japan) extraction.

**Measurement of Biochemical Parameters**

Plasma concentrations of creatinine were measured with an autoana-
lyzer (SRL, Tokyo, Japan) as an indicator of impaired glomerular
function.

**RNA Isolation, Reverse Transcription, and Real-Time
RT-PCR**

Total RNA was extracted from the kidney using Trizol (Invitrogen), cDNA was prepared with the Super Script kit (Invitrogen) from 5 µg
total RNA with Oligo (dT) for analysis with real-time PCR using
Prism 7700 (Applied Biosystems, Tokyo, Japan) according to the
manufacturer’s instructions. Commercially available mouse glyceral-
dehyde-3-phosphate dehydrogenase (GAPDH) primers (Rodent, To-
kyo, Japan) were used; the PCR primers were as follows:

- **Mouse ATF3 (product size 195)**
  - 5’ AACTGGCTTCCTGTGCACTT’ (forward)
  - 5’ TGAGGCCAGCTAGGTCTCT’ (reverse)
- **Human ATF3 (product size 115)**
  - 5’ CCTCCGGAAG TGAGTGCTTCT’ (forward)
  - 5’ ATGGCAAACTCAGCTTCTC’ (reverse)
- **Human p53 (product size 173)**
  - 5’ AGCCACCTGGA TCCAAAAA3’ (forward)
  - 5’ CAAGGG TTCAAAGCCAAA 3’ (reverse)
- **Human GAPDH primers (product size 127)**
  - 5’ CCACCCAGAAGACTGTGGAT’ (forward)
  - 5’ TTCAGCTGAGGATGACCTT’ (reverse)

**Western Blot Analysis**

For whole-cell extract preparation, HK2 cells treated as indicated were
washed in PBS, resuspended in 50 µl of lysis buffer (50 mM Hepes-
KOH (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1.5 mM MgCl₂, 1
mM EGTA, 0.1 mM PMSF, 10 µg/ml each leupeptin and aprotinin,
200 µM Na vanadate, and 100 mM NaF, and 10% glycerol), and
incubated on ice for 10 min. The cells were then centrifuged at 10,000
rpm for 10 min, and the supernatant was taken as the whole-cell
extract. The amounts of protein were measured with the Lowry
method using BSA as a standard. Whole-cell extracts (20 µg of protein)
were separated on an SDS-PAGE, transferred onto a nitrocellu-
lose membrane, and subjected to a Western blot antibody against
mouse ATF3 at a 1:4000 dilution. Bands were visualized and their intensity was calculated with Image J (an image pro-
cessing and analysis program; National Institutes of Health, Bethesda,
MD).
Cell Viability Assay
To measure cell viability, the cell culture medium containing H$_2$O$_2$ was removed and replaced with D-PBS containing 10 μM calcein AM (Molecular, Tokyo, Japan) and cells were then incubated at 25°C for 30 min. Fluorescence was measured with a multilabel fluorescence plate reader (PerkinElmer Life Sciences, Tokyo, Japan).

DNA Microarray Analysis
Affymetrix GeneChip technology was used as described previously. Briefly, cDNA was synthesized from total RNA using a Superscript Choice kit (Gibco BRL, Tokyo, Japan) and a T7 polymerase (Mega Script T7 kit; Ambion, Tokyo, Japan). Total RNA (8.0 μg) was annealed to T7-(dt)$_{24}$ primer (100 pmol/μL) at 70°C for 10 min. Reverse transcription was carried out at 42°C for 1 h in a mixture with final concentrations of 1 × First Strand Buffer, 10 mM dithiothreitol, 500 μM dNTP, and 20,000 U of Superscript II reverse transcriptase per ml. Second-strand synthesis was carried out in 150 μl, incorporating the entire 20-μl first-strand reaction mixture and a 130-μl second-strand reaction mixture containing final concentrations of 1 × Second Strand Buffer, 250 μM dNTP, 1.2 mM dithiothreitol, 65 U of DNA ligase per ml, 250 U of DNA polymerase I per ml, and 13 U of RNase H per ml. The mixture was incubated at 16°C for 2 h, whereupon 2 μl of T4 DNA polymerase at 5 U/μl were added and incubation at 16°C was prolonged for 5 min. After purification, the cDNA was precipitated with 5 M ammonium acetate and absolute ethanol at −20°C for 20 min. The pellet was resuspended in 1.5 μl of water. Synthesis of biotin-labeled cRNA was carried out by using the MEGA script T7 In Vitro Transcription Kit (Ambion). The amplified cRNA was purified with an affinity resin column (RNasy, Qiagen, Tokyo, Japan). The cRNA was fragmented by incubation at 94°C for 35 min in the presence of 40 mM Tris-acetate (pH 8.1), 100 mM potassium acetate, and 30 mM magnesium acetate. Twenty micrograms of the fragmented cRNA was hybridized to the GeneChip U74A Array Set (Affymetrix, Santa Clara, CA), which allowed us to monitor the abundance of about 12,000 mRNA transcripts. A 220-μl hybridization solution of 1 M NaCl, 10 mM Tris (pH 7.6), 0.005% Triton X-100, 50 mM control oligonucleotide B2, control cRNA, 0.1 mg of herring sperm DNA per ml, and 0.05 μg of the fragmented cRNA per μl was heated to 95°C and then cooled to 40°C. Hybridization occurred at 45°C in a rotisserie hybridization oven at 60 rpm for 16 h. Subsequent washing and staining of the arrays was carried out by using the GeneChip fluidics station protocol EukGE-WS2. After washing and staining, probe arrays were scanned twice at 3 μm resolution using the GeneChip System confocal scanner (Hewlett-Packard, Santa Clara, CA). Data analysis was performed using Affymetrix GeneChip 3.1 software (Affymetrix).

siRNA Design and Transfection
Control siRNA (scramble) and siRNA for ATF3 were designed as follows: Control siRNA, 5’-CTGAAGGCTCAGATTGAGGAGC-3’; ATF3 siRNA, 5’-CTGTGAGATAAGCGGGACTCAG-3’. One picomole of the siRNA dissolved in 500 μl of culture medium was transfected into HK2 cells using 20 μl Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol.

Histological Examinations
Kidneys were fixed with 15% neutral buffered formalin and then embedded in paraffin. For morphological evaluations, paraffin sections (3 μm thick) were routinely stained with periodic acid–Schiff stain. Histological examinations were performed by three renal pathologists in a blinded fashion. Histological changes caused by tubular necrosis were quantified by calculation of the percent of tubules that displayed cell necrosis, loss of the brush border, cast formation, and tubule dilation as follows: 0, none; 1, 10%; 2, 11% to 25%; 3, 26% to 45%; 4, 46% to 75%; and 5, >76%. At least 15 fields (×200) were reviewed for each slide in the HE stained specimens (ATN score).

Frozen Immunohistochemical Analysis for ATF3, NHE3, and Nuclear DNA
Kidneys were fixed with 4% paraformaldehyde in PBS for 16 h at 4°C, were immersed in 10%, 15%, and 20% sucrose in PBS for 12 h at 4°C, embedded in optimal cutting temperature compound, and immediately frozen in liquid nitrogen. Triple labeling for ATF3, NHE3, and 4,6-diamidine-β-phenylindole dihydrochloride (DAPI) were performed. For immunohistochemical analysis, a primary antibody against recombinant human ATF3 and a secondary detection antibody (Alexa Fluor 488 goat anti-mouse IgG (H+L), Molecular Probes, Tokyo, Japan) were used. To counterstain for NHE3, chicken anti-Na+/H+ exchanger-3 polyclonal antibody was used. Nonspecific binding sites were blocked in sections (3 μm thick) with goat serum (Life Technologies, Tokyo, Japan) for 1 h at room temperature, blocked with anti-mouse IgG (Vector Laboratories, Tokyo, Japan) for 3 h with TBS (20 mM Tris, 225 mM NaCl), and then incubated with the primary antibody that was used at a 1:300 dilution for 16 h at 4°C. After three 5-min washing steps with TBS, the sections were incubated with the secondary antibody that was used at a 1:200 dilution for 1 h at room temperature in the dark. After three 5-min washing steps with TBS, the sections were mounted using VectaShield (Vector Laboratories). Staining for nuclear DNA was performed using DAPI dye (1 μg/ml, Kirkegaard Perry Laboratories, Tokyo, Japan).

Statistical Analyses
All data are presented as means ± SEM. Differences between groups were analyzed with the t test. P < 0.05 was considered significant.

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DISCLOSURES
None.

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