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Cell Injury, Repair, Aging and Apoptosis

Renal Ischemia-Induced Cholesterol Loading

Transcription Factor Recruitment and Chromatin Remodeling along the HMG CoA Reductase Gene

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Acute kidney injury evokes renal tubular cholesterol synthesis. However, the factors during acute kidney injury that regulate HMG CoA reductase (HMGCR) activity, the rate-limiting step in cholesterol synthesis, have not been defined. To investigate these factors, mice were subjected to 30 minutes of either unilateral renal ischemia or sham surgery. After 3 days, bilateral nephrectomy was performed and cortical tissue extracts were prepared. The recruitment of RNA polymerase II (Pol II), transcription factors (SREBP-1, SREBP-2, NF-κB, c-Fos, and c-Jun), and heat shock proteins (HSP-70 and heme oxygenase-1) to the *HMGCR* promoter and transcription region (start/end exons) were assessed by Matrix ChIP assay. HMGCR mRNA, protein, and cholesterol levels were determined. Finally, histone modifications at *HMGCR* were assessed. Ischemia/reperfusion (I/R) induced marked cholesterol loading, which corresponded with elevated Pol II recruitment to *HMGCR* and increased expression levels of both HMGCR protein and mRNA. I/R also induced the binding of multiple transcription factors (SREBP-1, SREBP-2, c-Fos, c-Jun, NF-κB) and heat shock proteins to the *HMGCR* promoter and transcription regions. Significant histone modifications (increased H3K4m3, H3K19Ac, and H2A.Z variant) at these loci were also observed but were not identified at either the 5′ and 3′ HMGCR flanking regions (±5000 bps) or at negative control genes (*β-actin* and *β-globin*). In conclusion, I/R activates the HMGCR gene via multiple stress-activated transcriptional and epigenetic pathways, contributing to renal cholesterol loading. *(Am J Pathol 2009, 174:54–62; DOI: 10.2353/ajpath.2009.080602)*

Diverse forms of tissue injury evoke cellular responses that confer protection against subsequent ischemic or toxic attack. This adaptation has been denoted by heterogeneous terms that include: acquired cytoresistance, the stunning phenomenon, the heat shock response, and ischemic preconditioning. It has been recognized for a century that the kidney can undergo this same injury adaptation.[1] This is based on observations that exposing the kidney to one nephrotoxin elicits protection against the same, or a different (cross resistance), nephrotoxic agent.[1] In 1984, this laboratory demonstrated that this phenomenon is not restricted to nephrotoxic injury: when mild ischemic renal injury was induced in rats, dramatic protection against subsequent, and more severe, renal ischemia resulted.[2] Indeed, this was probably the first demonstration of the so-called ischemic preconditioning phenomenon, subsequently confirmed in many extra-renal tissues (eg, heart, liver, intestine, brain).[3–7] It should be recognized that ischemic preconditioning is not a specific postischemic state. As noted above, this same preconditioning develops after toxic renal damage. Furthermore, nonischemic/nontoxic insults can also induce the renal cytoresistant state. Examples include heat shock,[8,9] oxidant stress,[10–12] endotoxemia,[13–15] partial ablation of renal mass,[16] urinary tract obstruction,[17,18] and acute glomerulonephritis.[19] Therefore, the phenomenon of ischemic preconditioning is best considered to be one of any number of stressors that can elicit subsequent protection against acute renal failure.

There are undoubtedly multiple pathways by which injured renal tubular cells acquire resistance to further damage. The one that has been most widely recognized is the induction of cytoprotective stress proteins, such as the heat shock protein-70 (HSP-70), HSP-32 (ie, heme

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oxygenase-1; HO-1), and ferritin.[8,9,20,21] Alterations in lipid homeostasis may also be involved. For example, arachidonic acid release from phospholipids,[22–24] as well as sphingomyelin-generated sphingolipid products,[25–27] can evoke a cytoprotective response. Probably the most consistent, and stable, renal injury-induced lipid alteration is renal tubular cholesterol accumulation.[15,28–34] For example, in each of the above-mentioned models of renal cytoresistance (IR; myoglobinuric acute renal failure, glomerulonephritis, nephrotoxins, heat shock, ureteral obstruction, endotoxemia), an ~25 to 50% sustained increase in renal tubular cholesterol concentrations result.[28–34] The precise mechanism(s) by which excess cholesterol exerts its protective action remains to be defined. However, the available data suggest that cholesterol increases plasma membrane and mitochondrial membrane rigidity, and this serves to maintain mitochondrial energetics and plasma membrane integrity during superimposed ischemic or toxic attack.[28,30,32] Of note, after injury cholesterol accumulation and cytoresistance are not renal-specific phenomena. For example, we have observed that when acute myelogenous leukemia cells are exposed to cancer chemotherapeutic agents, cholesterol accumulates, conferring resistance to further chemotherapy.[35–37] Therefore, the mechanisms that are responsible for injury-induced cholesterol accumulation likely have broad-based biological relevance.

Evidence gathered to date indicates that increased tubular cell cholesterol synthesis can contribute to the cholesterol loading state after injury. This assertion is based on observations that statin-mediated inhibition of HMG CoA reductase, the rate-limiting enzyme in cholesterol synthesis, abrogates cholesterol accumulation after injury.[31,35–39] However, whether increased cholesterol synthesis reflects increased *HMGCR* gene transcription, and which transcription factors might stimulate this response, have not been assessed. Hence, the present study was undertaken to explore the following issues: i) Does renal ischemic preconditioning activate the *HMGCR* gene (as assessed by RNA polymerase II recruitment to its transcription sites)? ii) If so, which transcription factor(s) might be involved? iii) Do epigenetic modifications exist at the *HMGCR* gene, potentially facilitating an increased transcriptional state? Investigations into each of these issues form the basis of this study.

Materials and Methods

Ischemia-Reperfusion Injury

Male CD 1 mice (30 to 35 g; Charles River Laboratories, Wilmington, MA), maintained under routine vivarium conditions and subjected to Institutional Animal Care and Use Committee approved protocols were used for all experiments. They were subjected to 30 minutes of left renal pedicle occlusion performed through an abdominal incision under pentobarbital anesthesia (40 to 50 mg/kg; IP) and followed by two-layer abdominal wall suturing. Approximately 72 hours after surgery, they were re-anesthetized, and bilateral nephrectomy was performed.

Analyses

The renal cortices were dissected (4°C) and subjected to either lipid, protein, or RNA extraction or chromatin cross linking (formalin).[40] Lipid extracts were analyzed for free and esterified cholesterol levels by gas chromatography (expressed as nmol/μmol phospholipid phosphate).[29,40] Protein samples were probed for HMGCR protein by Western blotting/chemiluminescence.[31] HMGCR mRNA was quantified by competitive polymerase chain reaction (PCR) (expressed as a ratio to simultaneously measured GAPDH product).[34] The validity of using the contralateral kidneys as controls was ascertained by identifying a lack of cholesterol/cholesterol ester accumulation in them, compared with kidneys obtained from six mice undergoing sham unilateral ischemia surgery.

Microplate-Based Matrix Chromatin Immunoprecipitation (ChIP)

Supplies and Equipment

ChIP assays were done using the Matrix ChIP platform in 96-well polystyrene high-binding capacity microplates (no. 9018; Corning, Corning, NY).[41] The following reagents and equipment were used: protein A (no. P7837; Sigma, St. Louis, MO); proteinase K (no. 25530hyphen]015; Invitrogen, Carlsbad, CA); formaldehyde (no. 2106-02; J.T. Baker, Phillipsburg, NJ); bovine serum albumin (no. A9647, Sigma); phenylmethyl sulfonyl fluoride (no. P-7626, Sigma); leupeptin (no. L-2884, Sigma); SYBR Green PCR master mix (2×SensiMix, no. QT6T3; Quantace, Norwood, MA); salmon sperm DNA (no. D1626, Sigma); Misonix Sonicator 3000 with micro tip (no. S3000; Misonix, Farmingdale, NY); ultrasonic bath (no. B3510-MT CPN-952-316; Branson, Danbury, CT); heat blocks (analog heat block, no. 13259032: VWR Scientific, West Chester, PA; Isotemp 125: Fisher Scientific, Pittsburgh, PA); quantitative PCR (ABI 7900HT system; ABI Biotechnology, Foster City, CA); and MixMate (Eppendorf, Westbury, NY).

Buffers

The following buffers were used: phosphate-buffered saline (PBS): 137 mmol/L NaCl, 10 mmol/L Na phosphate, 2.7 mmol/L KCl, pH 7.4; TE buffer: 10 mmol/L Tris, 1 mmol/L ethylenediaminetetraacetic acid, pH 7.0; immunoprecipitation (IP) buffer: 150 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.5, 5 mmol/L ethylenediaminetetraacetic acid, NP-40 (0.5% v/v), Triton X-100 (1.0% v/v); blocking buffer: 5% bovine serum albumin, 100 μg/ml sheared salmon sperm DNA in IP buffer; elution buffer: 25 mmol/L Tris base, 1 mmol/L ethylenediaminetetraacetic acid, pH 9.8, 200 μg/ml proteinase K (20 mg/ml stock, stored at −20°C).

Sonication of Renal Cortex Chromatin

Approximately 25 mg of minced renal cortex was fixed with formaldehyde (final concentration 1.42% in PBS for

Table 1. List of Antibodies Used in Matrix ChIP Assay

Antibody	Type	Source	Catalog	Amount/ChIP
Pol II CTD (4h8)	Monoclonal	Gene Tex	GTX25408	0.25 μg
p65/Rel NF-κB	Rabbit anti-serum	See Reference 70	na	0.5 μl
c-Jun	Rabbit polyclonal	Santa Cruz	sc-044	0.5 μg
c-Fos	Rabbit polyclonal	Santa Cruz	sc-652	0.5 μg
SREBP-1	Monoclonal	PharMingen	67351A	1.0 μg
SREBP-2	Monoclonal	BD Pharmingen	557037	1.0 mg
HO-1	Rabbit polyclonal	Oncogene Research	PC340	1.0 μg
HSP-72/73	Monoclonal	Oncogene Research	HSP01	1.0 μg
H2A.Z	Rabbit polyclonal	Abcam	ab4174	0.5 μg
H3K4m3	Rabbit polyclonal	Abcam	Ab8580	0.5 μg
H3K9Ac	Rabbit polyclonal	Cell Signaling	No. 9671	0.5 μg

These antibodies were used in the ChIP assay, as described in the text.

15 minutes; 22°C) and then quenched with 125 mmol/L glycine (5 minutes, 22°C). The cross-linked tissues were then extensively washed with PBS (4°C). To shear the chromatin, the washed cross-linked tissue pellets were resuspended in 1 ml of IP buffer (containing the following inhibitors: 0.5 mmol/L dithiothreitol, 10 μg/ml leupeptin, 0.5 mmol/L phenylmethyl sulfonyl fluoride, 30 mmol/L *p*-nitrophenyl phosphate, 10 mmol/L NaF, 0.1 mmol/L Na_3VO_4, 0.1 mmol/L Na_2MoO_4, and 10 mmol/L β-glycerophosphate) and sheared using six rounds of sonication (power 5, 15 seconds, on ice). The suspension was cleared by centrifugation at 12,000 × *g* (10 minutes at 4°C), and the supernatant, representing sheared chromatin, was aliquoted and stored at −80°C.

Immunoprecipitation and DNA Isolation

Ninety-six-well plates were washed once with 200 μl of PBS per well and were incubated overnight with 0.2 μg of protein A in 100 μl of PBS per well. After washing (200 μl of PBS per well), well walls were blocked with 200 μl of blocking buffer (15 to 60 minutes, 22°C). The wells were cleared and the used antibodies (Table 1) were added with 100 μl of blocking buffer per well (60 minutes, 22°C). Chromatin samples (5.0-μl chromatin preparations/100 μl of blocking buffer) were added (100 μl/well) and plates were floated in an ultrasonic water bath (60 minutes, 4°C) to accelerate protein-antibody binding.[41] The wells were washed three times with 200 μl of IP buffer and 1 times with 200 μl of TE buffer. Wells were incubated with 100 μl of elution buffer (15 minutes at 55°C, followed by 15 minutes at 95°C). Total DNA (input) was isolated using the same plate and concurrently with immunoprecipitated DNA by suspending 5.0 μl of chromatin in 100 μl of elution buffer (15 minutes at 55°C, followed by 15 minutes at 95°C). DNA samples were stored (−20°C) in the same Matrix ChIP plates for repeated use.

Real-Time PCR (qPCR)

ChIP DNA samples were assayed by qPCR. The reaction mixture contained 2.5 μl of 2× SYBR Green PCR master mix (SensiMix, Quantace), 2.3 μl of DNA template, and 0.2 μl of primers (10 μmol/L) in 5-μl final volume in a 384-well optical reaction plate (Applied Biosystems, Foster City, CA). Amplification (three step, 40 cycles), data acquisition, and analyses were done using the 7900HT real-time PCR system and SDS Enterprise Database (Applied Biosystems). All PCR reactions were run in triplicate. At least four samples were run for each determination. The used qPCR primers are presented in Table 2. PCR calibration curves were generated for each primer pair from a dilution series of total mouse sheared genomic DNA. The PCR primer efficiency curve was fit to cycle threshold (Ct) versus $\log_e$ (genomic DNA dilutions) using an *r*-squared best fit. DNA concentration values for each ChIP and input DNA samples were calculated from their respective average Ct values. Final results were expressed as percent input DNA.[41]

Table 2. List of Primers Used for qPCR Analyses in ChIP Analyses

Hmgcr (ID15357)	Right	5′-CAGAACCAGAAGAGGCCTTG-3′
−5′ (−5000 bp to TSS)	Left	5′-GACCTCTGGCGGAGACATAC-3′
Hmgcr	Right	5′-GGAAGGACTGCGCTTACG-3′
Promoter (−100 bp to TSS)	Left	5′-GTTGTTAGGGAGACCGTTCG-3′
Hmgcr	Right	5′-AGGATCCAAGGACTGTGAGG-3′
Exon1	Left	5′-AGGGCACTCATAATTCCAGC-3′
Hmgcr	Right	5′-TGTTCAAGGAGCATGCAAAG-3′
Exon19	Left	5′-CTTACCTGTTGTGAACCATGTG-3′
Hmgcr	Right	5′-CAGCTCTCCTATCCAATGCC-3′
+5′ flanking (+5000 bp to end of gene)	Left	5′-TTGAAGGACATGCTGCTCAC-3′
β-Actin (ID 11461)	Right	5′-AGGAAGGAAGGCTGGAAGAG-3′
Exon 1	Left	5′-GCTGAGAGGGAAATTGTTCG-3′
β-Globin (ID 2440)	Right	5′-ACCCATGATAGCAGAGGCAG-3′
Int 1-Exon 2	Left	5′-GGTGCTTGGAGACAGAGGTC-3′

Primers used in qPCR analyses after chromatin immunoprecipitation.

Calculations and Statistics

All values are presented as means ± 1 SEM. Paired Student's *t*-test was used to compare results obtained from left versus right kidney samples. Comparisons between different mice were performed by unpaired Student's *t*-test. An *n* of four to eight tissue samples were used for all comparisons. Significance was judged by a *P* value of <0.05.

Results

We previously demonstrated that by 18 to 24 hours after ischemia-reperfusion (I/R) injury, an ~25 to 50% increase in renal cortical cholesterol levels result.[28] The present study demonstrates the durability of this response: at 3 days after unilateral renal ischemia, a time that corresponds to persistent renal tubular histological injury (Figure 1), cholesterol levels were elevated by ~35%, compared to the values observed in contralateral (nonischemic) control kidneys (Figure 2, left). Of note, the contralateral controls retained normal cholesterol levels, compared to sham-operated animals (data not shown). This indicates that the post-ischemic kidney elevations reflected the impact of I/R, rather than a nonspecific adaptation to surgical stress. The free cholesterol levels were accompanied by an approximate sixfold increase in cholesterol ester content (Figure 2, middle). Because acyl transferase-mediated esterification represents a shunting of excess free cholesterol into the cholesterol ester storage pool,[40] cholesterol ester serves as a biomarker for degrees of cell cholesterol/sterol excess.[29,42]

HMGCR-mediated conversion of HMG CoA to mevalonate is the rate limiting step in cholesterol synthesis.[43,45] Under physiological conditions, increased cellular cholesterol suppresses *HMGCR* expression via inhibition of the sterol regulatory element binding protein (SREBP) transcription pathway.[43–47] Furthermore, cholesterol loading accelerates HMGCR protein catabolism.[48,49] Therefore, if I/R were to induce cholesterol loading via a nonsynthetic pathway (eg, by low-density lipoprotein receptor-mediated uptake of plasma cholesterol), decreased HMGCR protein levels should result (decreased transcription/increased proteolysis). Conversely, an increase in HMGCR protein would be consistent with increased *HMGCR* transcription, translation, and hence cholesterol synthesis. To help differentiate between these two possibilities, HMGCR protein levels were assessed by Western blotting. As shown in Figure 2, right, at 72 hours after ischemia, an ~35% increase in HMGCR protein levels was observed, paralleling the total cholesterol gain. Thus, these findings are consistent with an activated cholesterol synthesis pathway.

To gain additional support for the concept that I/R activates the *HMGCR* gene, HMGCR mRNA levels were assessed. As shown in Figure 3A, at both 4 and 72 hours after ischemia, HMGCR mRNA levels were elevated by ~30 to 35%, quantitatively paralleling the HMGCR protein and cholesterol increases. The 4-hour mRNA elevations are noteworthy in that they preceded any increase in renal cholesterol content (data not shown), suggesting mechanistic relevance. HMGCR mRNA levels were still increased at the 72-hour time point, a time of marked cholesterol excess. Because suppressed HMGCR mRNA would be expected with cholesterol overload, a failure of

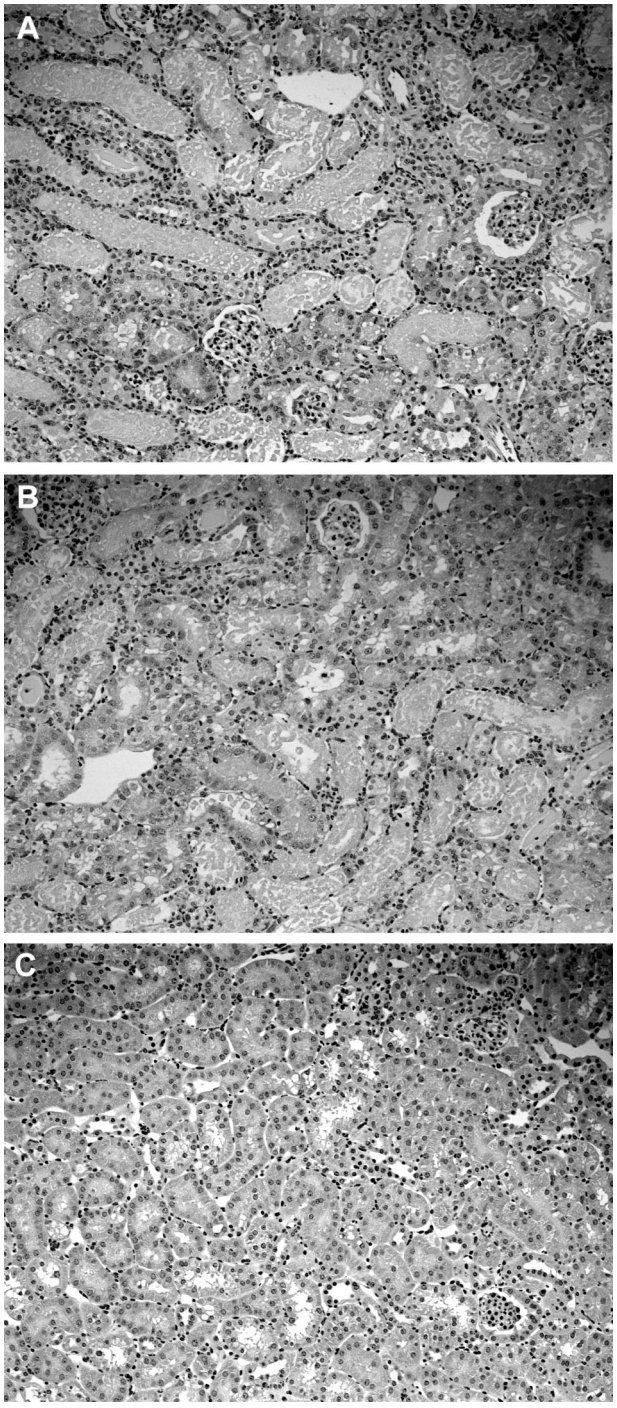

Figure 1. Renal histological injury, as assessed at 3 days after unilateral ischemic injury. As a frame of reference for interpreting the biochemical data, 4-μm kidney sections were cut from 10% formalin-fixed tissues and stained with H&E. Extensive proximal tubular necrosis and cast formation was observed in the renal cortex (**A**) and in the outer medullary stripe (**B**). **C:** The contralateral kidney manifested normal histology. Thus, extensive renal injury was present in the kidney samples that were used to study the HMG CoA reductase pathway.

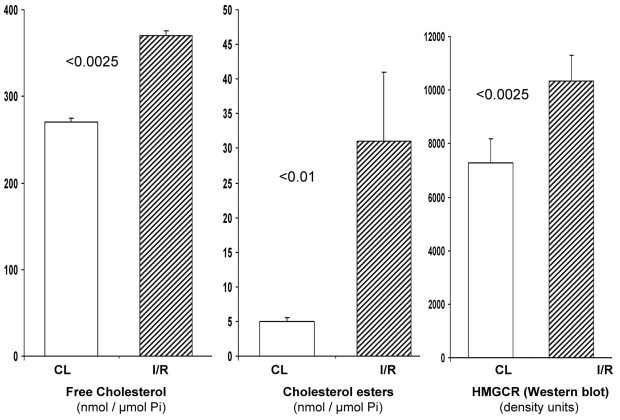

Figure 2. Free cholesterol (**left**), cholesterol esters (**middle**), and HMG CoA reductase (HMGCR) protein levels (**right**, by Western blot) were assessed 3 days after unilateral I/R and in contralateral (CL) control kidneys. An ~35% increase in free cholesterol levels was observed. This was accompanied by a sixfold increase in cholesterol esters, a cholesterol storage form. Increases in HMGCR protein were also observed, the magnitude of which paralleled the extent of the free cholesterol accumulation (each ~35%).

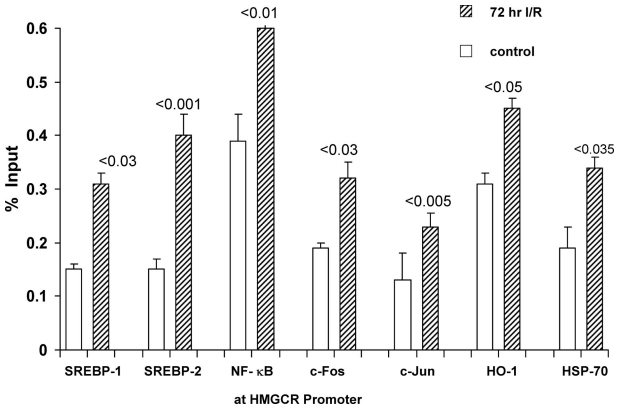

Figure 4. The *HMGCR* promoter region was probed for: i) sterol-responsive element binding proteins (SREBP 1, 2); ii) general transcription factors (NF-κB, c-FOS, c-Jun); and iii) two heat shock proteins (HSP-70 and HSP-32, ie, HO-1). I/R caused twofold to threefold increases in the densities of both SREBP 1 and 2 at the *HMGCR* promoter. Significantly greater amounts of NF-κB, c-Fos, c-Jun, HSP-70, and HO-1 were also observed in the I/R versus control kidneys. Thus, multiple factors appear to activate *HMGCR* transcription as part of the stress response.

feedback inhibition of mevalonate/cholesterol signaling pathway(s) clearly existed.[43–46]

It could be argued that the HMGCR mRNA increases reflected increased mRNA stability, rather than increased gene transcription. Therefore, to lend additional support for the latter hypothesis, degrees of RNA polymerase II (Pol II) recruitment along the *HMGCR* gene were assessed. In this regard, Pol II is the critical enzyme that drives mRNA synthesis from the DNA template. Hence, the relative degree of Pol II recruitment at a target gene serves as a semiquantitative index of transcription rates.[50–53] As shown in Figure 3B, I/R induced approximately threefold Pol II increases at both the start and end exons of the *HMGCR* gene. A smaller, although significant, increase was also observed at the *HMGCR* promoter. In sum, then, the evidence presented above indicates that I/R activated the entire cholesterol synthetic pathway (from increased Pol II binding to *HMGCR* → increased HMGCR mRNA → increased HMGCR protein → increased cholesterol → increased cholesterol esters).

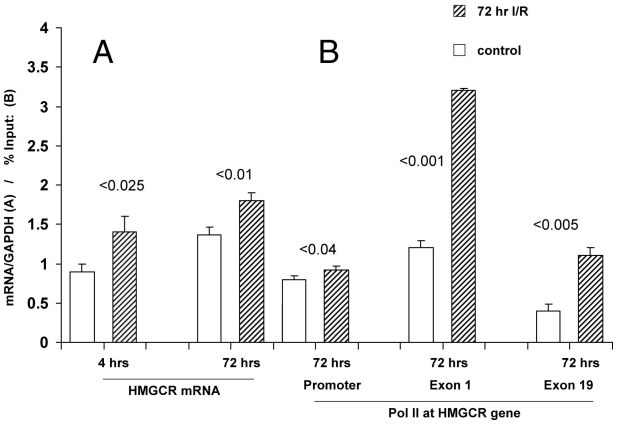

Figure 3. A: *HMGCR* mRNA levels in control and post-I/R cortical kidney samples obtained at 4 or 72 hours after surgery were quantified by PCR. In both instances, an ~30% increase in mRNA levels was observed (expressed as a ratio to GAPDH). **B:** RNA polymerase II (Pol II) densities were assessed at the *HMGCR* promoter, and at the start and end exons of the *HMGCR* gene (exons 1 and 19). I/R caused a small, albeit statistically significant, Pol II increase at the *HMGCR* promoter. Conversely, I/R induced an approximately threefold increase in Pol II at both exons 1 and 19. As expected, the absolute Pol II amounts in both the control and post-I/R kidneys were greater at the start versus the end exon.

The maintenance of cellular cholesterol homeostasis is generally believed to be under the transcriptional control of SREBP-2, and to a lesser extent, SREBP-1.[43–45] Under conditions of normal cholesterol supply, the SREBPs bind cholesterol and remain inactive, being anchored within the endoplasmic reticulum.[43–47] Under conditions of cholesterol depletion, the SREBPs are mobilized from the endoplasmic reticulum, and they are escorted to the Golgi apparatus by SCAP (SREBP-cleavage-activating protein). Two proteolytic steps then ensue (via S1P and S2P), generating active transcription factors. These gain nuclear access via importin β, and they then bind to multiple genes that regulate lipid synthesis (including *HMGCR*). Under conditions of cholesterol excess, these signaling pathways should be suppressed. Therefore, to assess whether SREBP signaling is activated by I/R, SREBP-1 and SREBP-2 densities at the *HMGCR* promoter were determined. As shown in Figure 4, approximately threefold elevations of both SREBPs were observed at the promoter region. This indicates both a failure of physiological suppression and that the SREBPs likely participate in mediating the cholesterol loading state. The reason for the inappropriate SREBP nuclear translocation in the setting of cholesterol excess remains unknown. However, one possibility is that I/R activates intracellular/endoplasmic reticulum proteases, causing nonphysiological SREBP-endoplasmic reticulum release.

Although I/R increased SREBP nuclear binding, it remains possible that this is not the only transcription factor that might participate in activating the *HMGCR* gene. Hence, we addressed the possibility that other stress-activated early response genes[54] might also participate. Toward this end, degrees of nuclear factor (NF)-κB (p65RelA), c-Fos, and c-Jun densities at the *HMGCR*

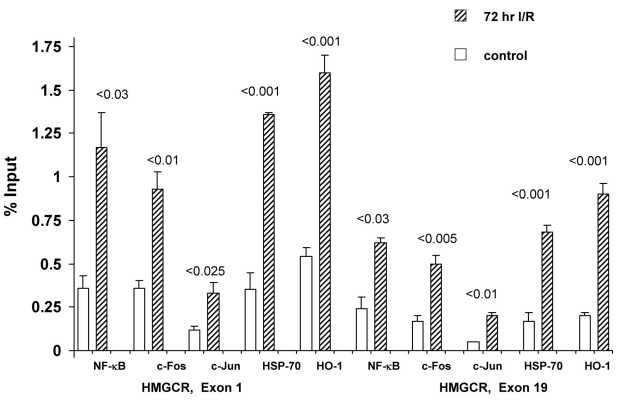

Figure 5. Localization of general transcription factors (NF-κB, c-Fos, c-Jun), HSP-70, and HO-1, at the start and end *HMGCR* exons. I/R induced significant increases of each protein at both *HMGCR* exons 1 and 19, compared to control kidneys. The absolute levels were consistently greater at the start versus the end exon (as would be expected with gene transcription).

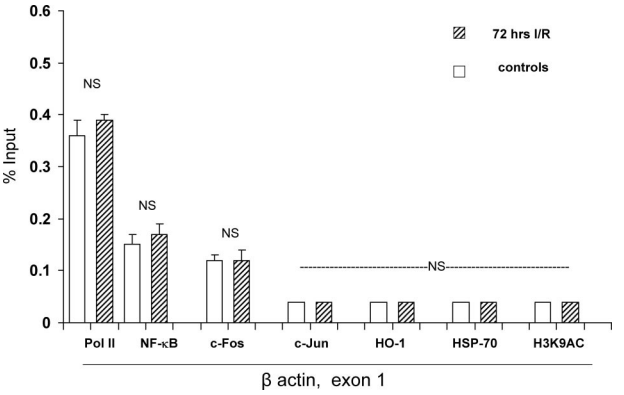

Figure 6. Assessments performed at a control gene (*β-actin*, exon 1). I/R did not significantly alter Pol II levels at *β-actin*, exon 1, serving as a negative control for the data shown at the right of Figure 2. Furthermore, I/R did not impact NF-κB, c-Fos, or c-Jun binding to *β-actin*, (negative control for the data in Figure 4). Finally, no difference in the extent of trimethylation of histone 3 lysine 4 (H3K4m3) was observed, indicating the relative specificity for data presented in Figure 8. [Note: H3K9AC and H2A.Z variants at *β-actin* were not assessed.]

promoter were assessed. In each instance, I/R caused dramatic increases (Figure 4). It has recently been suggested that heat shock proteins (HSPs) are also capable of translocation to the nucleus, potentially impacting gene transcription rates.[55–59] Hence, we assessed whether I/R facilitates heat shock protein—nuclear access with subsequent binding to *HMGCR*. As shown in Figure 4, right, this was indeed the case: I/R-injured kidneys had 50 to 100% greater HSP-70 and HO-1 densities at the *HMGCR* promoter, compared to control kidney samples. Thus, it appears that classic cholesterol transcription factors (SREBPS), inducible transcription factors (c-Fos c-Jun, NF-κB), and HSPs may each participate in I/R-induced *HMGCR* activation.

Recently, there have been suggestions that transcription factors can bind along the length of an activated gene, rather than simply being confined to the promoter region. For example, Martone and colleagues[60] documented NF-κB along the length of chromosome 22. This suggests that in addition to initiation of transcription, the classical transcription factor(s) may also participate in elongation and pre-mRNA processing. To test whether the above noted changes in NF-κB, c-Fos, c-Jun, HO-1, and HSP-70 might extend beyond the promoter, each was probed at both start and end exons of the *HMGCR* gene. As shown in Figure 5, the postischemic kidneys did, indeed, demonstrate increased amounts of each of these five proteins at these loci. Thus, these findings seemingly provide a new paradigm for stress-mediated cholesterol accumulation: the stress response, in this case induced by I/R, leads to binding of not only SREBPs, but also of inducible transcription factors, as well as HSPs, to the *HMGCR* promoter and transcribed regions.

Given the dramatic results that are described above, we carefully considered the possibility that they might have represented nonspecific transcription factor/protein binding to chromatin, induced by the used tissue preparation technique. To exclude this possibility, extensive controls were undertaken. First, as shown in Figure 6, control and I/R kidney samples had virtually identical amounts of Pol II, NF-κB, c-Fos, c-Jun, HO-1, and HSP-70 at exon 1 of the *β-actin* gene (selected to serve as a negative control). Second, as shown in Figure 7 (and its legend), I/R did not increase Pol II, SREBP-1, SREBP-2, NF-κB, c-Fos, c-Jun, HO-1, or HSP-70 binding at the silent *β-globin* gene. Third, Pol II, NF-κB, c-Fos, c-Jun, HSP-70, and HO-1 densities were measured at the 5′ and 3′ flanking regions of *HMGCR* (−5 kb relative to the transcription start; +5 kb relative to the end of the gene). Again, no differences were seen between control and I/R kidney samples (Table 3); and fourth, it is notable that in each instance in which Pol II/transcription factor/or HSP binding was observed at the *HMGCR* gene, greater amounts were seen at exon 1, compared to exon 19. This is typical of Pol II recruitment, with the highest levels being observed at the transcription start sites, with progressive decreases along the gene.[61] Had the binding results simply been an artifact, rather than a physiological

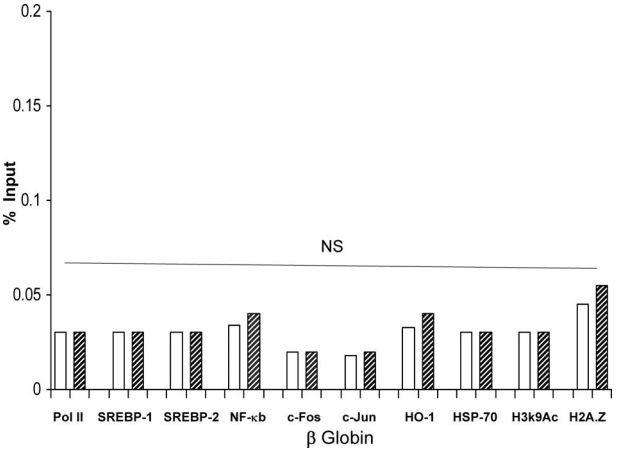

Figure 7. Assessments performed at the *β-globin* gene. As additional controls for the experiments depicted in Figures 1 to 5 and Figure 7, the SREBPs, general transcription factors, heat shock proteins, and histone modifications/variants were assessed at the normally silent *β-globin* gene (assessed between exon 1 and intron 1). In no instance was a statistically significant difference observed between control and the I/R kidney samples. Not shown (because of a different *y* axis scale), the amount of H3K4m3 was also not increased by I/R (2.2% versus 2.5% input for control and I/R samples; NS).

Table 3. Pol II, Transcription Factors, Heat Shock Proteins, and Histone Modifications/Variants in *HMGCR* Flanking Regions (±5 kb)

HMGCR flanking regions (±5 kb)	Contralateral kidney	72 hours I/R
Pol II		
−5 kb	0.29 ± 0.02	0.24 ± 0.03 (NS)
+5 kb	0.26 ± 0.06	0.31 ± 0.05 (NS)
SREBP-1		
−5 kb	0.067 ± 0.01	0.064 ± 0.006 (NS)
+5 kb	0.06 ± 0.01	0.052 ± 0.006 (NS)
SREBP-2		
−5 kb	0.05 ± 0.005	0.049 ± 0.01 (NS)
+5 kb	0.052 ± 0.007	0.055 ± 0.02 (NS)
NF-κB		
−5 kb	0.26 ± 0.05	0.26 ± 0.05 (NS)
+5 kb	0.20 ± 0.01	0.18 ± 0.01 (NS)
c-Fos		
−5 kb	0.12 ± 0.01	0.14 ± 0.01 (NS)
+5 kb	0.11 ± 0.01	0.14 ± 0.01 (NS)
c-Jun		
−5 kb	0.04 ± 0.005	0.04 ± 0.005 (NS)
+5 kb	0.03 ± 0.005	0.03 ± 0.005 (NS)
HSP-70		
−5 kb	0.08 ± 0.01	0.09 ± 0.01 (NS)
+5 kb	0.03 ± 0.005	0.03 ± 0.005 (NS)
HO-1		
−5 kb	0.06 ± 0.005	0.05 ± 0.005 (NS)
+5 kb	0.03 ± 0.05	0.03 ± 0.005 (NS)
H2A.Z		
−5 kb	0.14 ± 0.01	0.12 ± 0.01 (NS)
+5 kb	0.10 ± 0.01	0.11 ± 0.01 (NS)
H3K4m3		
−5 kb	6.5 ± 0.4	6.5 ± 0.3 (NS)
+5 kb	6.0 ± 0.02	6.3 ± 0.03 (NS)
H3K9Ac		
−5 kb	0.08 ± 0.01	0.09 ± 0.01 (NS)
+5 kb	0.07 ± 0.01	0.08 ± 0.01 (NS)

Values are percent of DNA input. No significant differences were observed between control (contralateral) and postischemic kidney samples.

process, one would have expected approximately equal binding at the start and end of *HMGCR,* as well as binding at its flanking regions, *β-actin*, and the *β-globin* genes. The relative importance of each of these proteins to gene activation, and ultimately mRNA synthesis (eg, elongation along the gene), remain to be defined.

The final goal of this study was to ascertain whether the I/R-induced changes in transcription factor binding and Pol II expression at *HMGCR* were accompanied by epigenetic modifications at this gene. Two changes that are thought to enhance Pol II recruitment to target genes by opening chromatin structure are trimethylation of histone 3-lysine 4 (H3K4m3) and acetylation of H3K9 (H3K9Ac).[62–66] As shown in Figure 8, I/R injury dramatically increased the levels of each, as noted by their densities at the promoter as well as at exons 1 and 19. Another epigenetic change that may enhance, or reflect, gene activity is replacement of canonical H2A with histone 2A.Z variant.[67,68] I/R did, indeed, increase the deposition of H2A.Z at the promoter and transcribed regions (Figure 8). As negative controls, H3K4m3, H3K9Ac, and H2A.Z were probed at *HMGCR* flanking regions and at the *β-actin* or *β-globin* genes. In no instance did I/R increase any of these three histone marks (H3K4m3, H3K9Ac, H2A.Z) at these regions (Figures 6 and 7; Table 3). This strongly implies that these histone modifications at *HMGCR* loci were not simply nonspecific markers of cell stress.

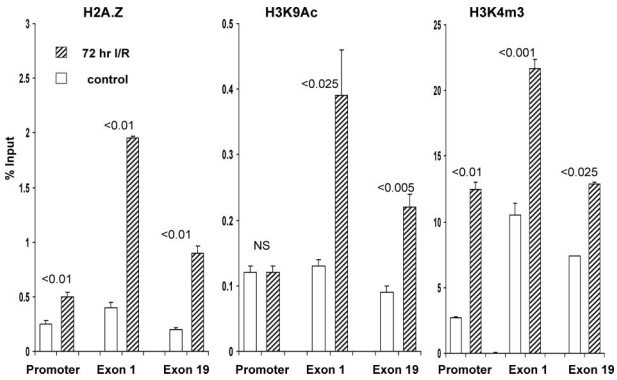

Figure 8. Histone variant H2A.Z levels and H3 histone modifications at the *HMGCR* promoter, and along the gene. I/R caused a twofold to fourfold increase in the amounts of H2A.Z variant and of H3K4m3 at all assessed sites. H3 lysine 19 acetylation was also increased at exons 1 and 19 (but not at the promoter).

Discussion

It has recently been suggested, based on data obtained in yeast, that *HMGCR* can function as a stress responsive element and that the resultant cholesterol increases can confer a survival advantage.[69] The results of the present study demonstrate quite clearly that this same response can be observed in mammals (or, at least, in the mouse). As discussed above, in response to a classic form of renal stress, ie, I/R, a 35% increase in free cholesterol concentrations resulted, culminating in a sixfold increase in the cholesterol ester storage pool. That injury-induced cholesterol increases can confer a survival advantage on mammalian cells has previously been demonstrated in a variety of studies from this laboratory that used freshly isolated mouse proximal tubules, cultured human proximal tubular (HK-2) cells, and acute myelogenous leukemia cells.[15,17,28–30,35–37] It needs to be underscored that these injury-induced cytoprotective cholesterol increases cannot be totally attributed to HMGCR-mediated cholesterol synthesis. Clearly, increased low-density lipoprotein receptor-mediated cholesterol uptake, as well as decreased cholesterol efflux (reverse cholesterol transport) may also be involved.[14,39] However, to separate out each of these different components *in vivo* is extremely difficult because any single experimentally induced change in one particular cholesterol accumulation pathway (eg, statin-mediated HMGCR inhibition) can cause compensatory alteration(s) in the others (eg, a secondary increase in low-density lipoprotein receptor-mediated uptake). Hence, these types of determinations are most easily resolved in cell culture experiments in which individual pathways can be controlled.[39]

The purpose of this study was to focus on the molecular determinant(s) of just one critical cholesterol homeostatic pathway: the *HMGCR* gene. The stimulus for this investigation was the present observation that I/R caused

increases in both HMGCR protein and its cognate mRNA. Hence, we sought to gain further support for increased *HMGCR* activity by attempting to document increased Pol II recruitment to multiple loci within the transcribed and promoter regions. Because this was observed, as discussed at length in the Results section, we sought to identify the transcription factors that might be involved. The results obtained indicated that classic sterol regulatory transcription factors (SREBP-1, SREBP-2), stress-activated transcription factors (NF-κB, c-Fos, C-Jun), and heat shock proteins (HSP-70, HO-1) may all play a role. That non-SREBP gene-activating elements may impact HMGCR regulation during cell stress represents, to the best of our knowledge, a novel insight.

Finally, we sought to test the hypothesis that I/R-induced *HMGCR* activation might either arise from, or conceivably induce, histone modifications at its gene loci. The results obtained support this possibility given that three potential gene activating epigenetic modifications at the *HMGCR* promoter and along the gene (increased H3K4m3, H3K9Ac, and H2A.Z) were observed. It remains to be proven that these modifications are mechanistically involved in activating the HMGCR gene, and hence, culminate in increased Pol II recruitment. An answer to this question remains a goal for future studies. These will undoubtedly require techniques to alter specific histone modifications at specific locations within the genome. Until such methodologies are available, the physiological relevance of the dramatic H3K4m3, H3K9AC, and H2A.Z changes noted in this study must be considered speculative as of this time.

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