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Differential gene expression in the recovery from ischemic renal injury

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Differential gene expression in the recovery from ischemic renal injury. Recovery from renal ischemia requires regeneration of damaged tubular epithelium. Previous studies have examined the expression of proto-oncogenes and growth factors after ischemia, but the response of genes coding for structural and functional genes has not been scrutinized. Rats were subjected to 40 minutes of renal artery occlusion and 60 minutes to 96 hours of reperfusion. Total RNA was isolated and mRNA for the structural protein actin, the enzymes superoxide dismutase and renin, the proto-oncogene c-*fos*, the nuclear protein histone H2b, and the putative marker for cell injury TRPM-2 was quantitated by Northern hybridization. Expression of the proto-oncogene c-*fos* was seen early but for only short duration. Histone gene expression was not markedly increased until 24 hours after ischemia, but remained increased for several days. Renin mRNA was undetectable one hour after ischemia, but was present in normal amounts at 24 and 48 hours. In contrast, superoxide dismutase mRNA was present in decreased amounts 24, 48, and 96 hours after ischemia. TRPM-2 gene expression was greatly increased 24 to 72 hours after ischemia and began decreasing at 96 hours. This selective sequence of gene expression or repression after renal ischemia might maximize the proliferative repair process. This information will be useful for designing therapies to further enhance recovery from acute renal injury.

The magnitude of renal dysfunction after a toxic or ischemic insult depends on the extent of the initial damage as well the pace of the repair process. Most investigations into the pathophysiology of acute renal failure have focussed on the "injury" phase. Thus, oxygen free radicals, ATP depletion, increased intracellular Ca^{++}, phospholipase activation, and mitochondrial dysfunction have all been implicated in contributing to acute renal injury. Far less attention has been paid to the "repair" phase of acute renal failure.

Nearly 20 years ago, Cuppage, Tate, and colleagues reported a series of studies using mercuric chloride-induced renal injury in the rat as a model for acute renal failure [1, 2]. They observed that even if approximately 90% of proximal tubule epithelial cells were lost due to necrosis or sloughing, the remaining cells would repopulate the denuded tubule along the tubular basement membrane [2]. In these studies, the cell doubling time was approximately 14 hours so that cells lost from the proximal nephron could be totally replaced within about five days. These investigators further observed that increased DNA synthesis could be observed within 24 hours of the initial insult with a peak in new DNA synthesis occurring at three days [1, 2].

More recently several groups have studied the importance of specific growth factors and their ability to influence the repair process. Epidermal growth factor (EGF) and transforming growth factor-alpha (TGF_α) have been found to favorably influence the course of experimental acute renal failure by increasing cellular proliferation [3, 4]. Whether EGF plays a regulatory role in the repair response, however, is unclear. After acute renal injury, although there is up-regulation of renal EGF receptors, there is decreased renal pre-pro-EGF message and decreased urinary EGF excretion [5–7].

Another approach to uncovering the secrets of the renal proliferative response to acute injury has been to catalogue the changes in gene expression following injury which precede and may, therefore, control epithelial cell growth. Norman et al observed a marked increase in the expression of proto-oncogenes as well as genes coding for structural and transport proteins for up to 48 hours after folic acid renal injury [8]. Ouellette et al noted a prompt, transient, and marked increase in expression of two "immediate early" genes, *Egr*-1 and c-*fos*, after ischemia in the mouse kidney [9]. Safirstein et al also reported similar findings in the rat after 50 minutes of renal ischemia [7].

The purpose of the present study was to examine the expression of a wider variety of genes involved in cellular proliferation as well as mature renal epithelial cell functions for a longer time period after renal ischemia in order to discern any sequential nature of gene expression after acute renal injury. In addition to studying genes controlling DNA synthesis and cellular proliferation, we were particularly interested in several specific genes. Because angiotensin II has been suggested to be involved in renal hypertrophy and hyperplasia, we evaluated renal renin gene expression after ischemia [10–12]. Since renal ischemic injury is mediated, in part, by oxygen free radicals we were interested in determining whether genes coding for antioxidant protective mechanisms, such as superoxide dismutase, would be selectively induced by this oxidant injury [13, 14]. We were also interested in studying TRPM-2 gene expression, a putative marker for renal injury, for an extended observation period [15–17].

Methods

Renal ischemia model

Male Sprague-Dawley rats weighing 225 to 275 g were allowed unlimited access to food and water until the time of

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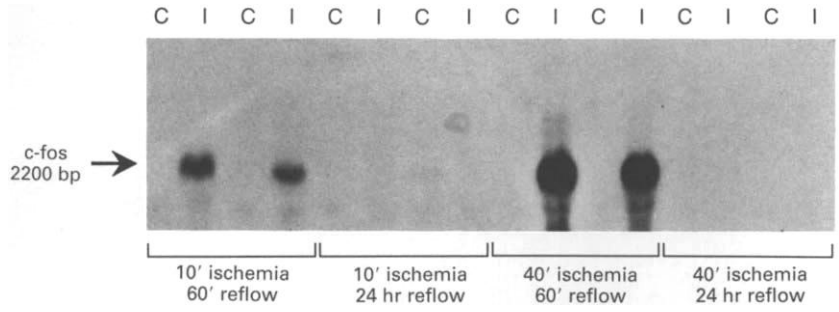

Fig. 1. *Northern blot analysis of c-fos mRNA in paired control (C) and ischemic (I) kidneys.*

study. Rats were anesthetized with sodium pentobarbital (60 mg/kg i.p.). Bilateral flank incisions were made and the right kidney was removed. The left kidney perirenal fat was removed and the left renal artery was exposed. A non-traumatic vascular clamp was then placed across the renal artery for 40 minutes. After removal of the clamp the animal was sutured and allowed to recover, except when the kidney was removed after only 60 minutes of reperfusion in which case the animal remained on a temperature-controlled heated table. For some studies of c-*fos* gene regulation the renal artery was occluded for only 10 minutes or a sham occlusion of the renal artery was performed. After the desired period of reperfusion (60 min to 96 hr), the animal was anesthetized and a midline abdominal incision was made to provide rapid access to the kidney. The kidney was excised, decapsulated, wrapped in aluminum foil, and plunged in liquid nitrogen. Frozen kidneys were stored at −70°C until needed. For these studies, the nonischemic right kidney removed at the time of initial surgery and processed and frozen in an identical manner served as a paired control. Because of inter-animal variation in mRNA content for specific genes, we studied two to five rats for each time period.

The model of renal ischemia was specifically chosen because it is well-studied, less traumatic than a two-kidney occlusion model, and provides the nonischemic, nephrectomized kidney as a control specimen. A potential disadvantage of this model is that a stimulus for renal hypertrophy might be superimposed upon that for hyperplastic repair. Since all renal tissue was made ischemic, the stimulus for independent hypertrophy was probably minimized.

RNA isolation and Northern hybridization

Total renal RNA was extracted by the guanidinium isothiocyanate/cesium chloride procedure [18]. The RNA was dissolved in sterile water and RNA concentrations determined by absorbance readings at 260 nm. Aliquots (20 μg) of total kidney RNA were electrophoresed in a 1% agarose gel containing 20 mM MOPS, 1 mM EDTA, 5 mM sodium acetate pH 7.0, and 2.2 M formaldehyde and transferred to nylon membranes (Duralon UV™, Stratgene, La Jolla, California, USA). In each gel equivalent loading of RNA, absence of degradation, and the positions of the 28 S and 18 S ribosomal RNA were determined by ethidium bromide staining. RNA was fixed to the nylon membranes by UV light (Stratalinker™, Stratgene). The membranes were prehybridized at 60°C for four hours in a buffer containing 5X SSC, 5X Denhardt's reagent, 50 mM Tris-hydrochloride, pH 7.5, 0.1% sodium pyrophosphate, 0.2% SDS, 200 μg/ml sonicated, denatured salmon testes DNA, and 100 μg/ml yeast tRNA. The membranes were then hybridized at 42°C with random oligomer primer labelled cDNA probes (see below) for 16 to 18 hours in a buffer containing 50% formamide (deionized), 5X SSC, 1X Denhardt's reagent, 50 mM Tris-hydrochloride, pH 7.5, 0.1% sodium pyrophosphate, 1% SDS, 100 μg/ml salmon testes DNA, and 100 μg/ml yeast plus RNA. The membranes were washed for 45 minutes in 2X SSC, 0.1% SDS twice at room temperature and once at 60°C, and were then washed in 0.2X SSC and 0.1% SDS at 60°C for 45 minutes. Autoradiographs (Kodak XAR-5 film) were obtained and quantitated by computer-assisted videodensitometry [19]. Exposure times were varied to make sure densitometric readings were in the linear range of the films. For each RNA sample at least two gels were made and the resulting membranes probed to confirm the initial findings.

Preparation of probes

The cDNA probes were labeled with ^{32}P-dCTP (3000 Ci/mmol; ICN Biomedicals, Irvin, California, USA) by random oligomer priming [20]. The following probes were used for the hybridization studies: mouse c-*fos* [21], rat renin (pRen44.ceb) [22], rat histone H2b [23], mouse α-actin (pAM91) [24], rat TRPM-2 [25], and rat SOD [26].

Results

The c-*fos* gene was rapidly induced by renal ischemia, but expression of c-*fos* was observed for only short duration. Northern blots for c-*fos* mRNA are shown in Figure 1. C-*fos* was expressed at very low levels in non-ischemic control kidneys. After just 10 minutes of ischemia and 60 minutes of reflow there was a marked increase in c-*fos* message (second and fourth lane). By 24 hours after ischemia c-*fos* mRNA was no longer detectable. Expression of the c-*fos* gene was far greater after 40 minutes of ischemia (lanes 10 and 12). However, 24 hours later c-*fos* message was no longer detectable despite such prominent expression only 23 hours earlier.

When kidneys were subjected to surgical manipulation and isolation of the renal artery without occlusion, c-*fos* message was increased fourfold ($N = 2$). This increased c-*fos* expression was small in magnitude when compared to the 39-fold induction after just 10 minutes of ischemia or the 289-fold induction after 40 minutes of ischemia. Thus, the induction of c-*fos* was not, for the most part, secondary to nonspecific renal trauma.

Expression of the histone H2b gene which codes for a histone protein required for new DNA synthesis lagged behind that observed for c-*fos*. After 60 minutes of reperfusion there were decreased levels of histone H2b message compared with non-

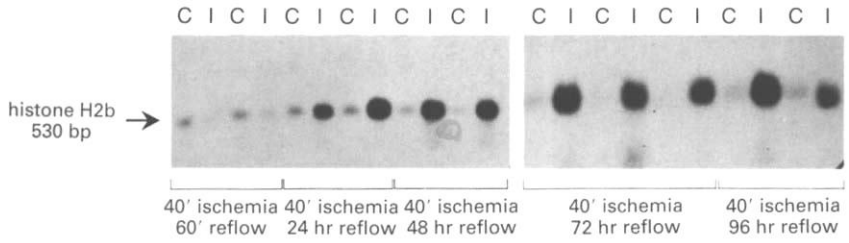

Fig. 2. *Northern blot analysis of histone H2b mRNA in paired control (C) and ischemic (I) kidneys.*

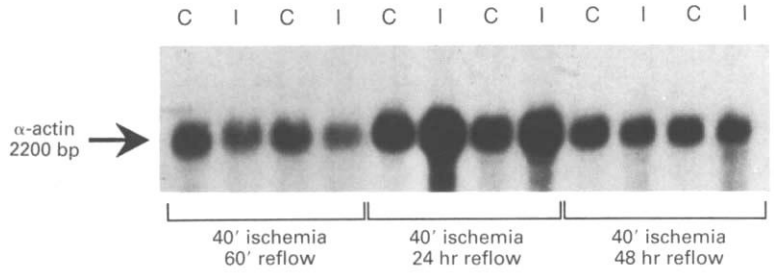

Fig. 3. *Northern blot analysis of α-actin mRNA in paired control (C) and ischemic (I) kidneys.*

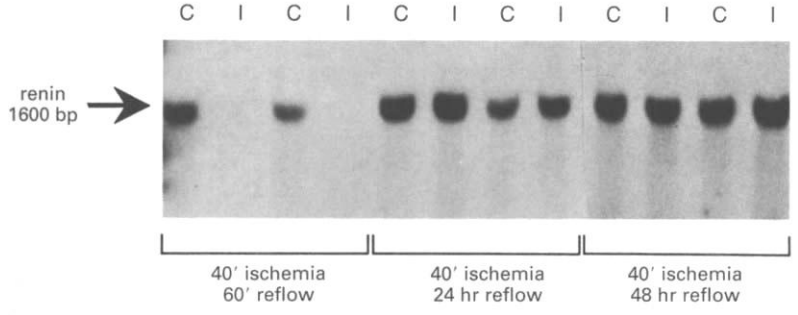

Fig. 4. *Northern blot analysis of renin mRNA in paired control (C) and ischemic (I) kidneys.*

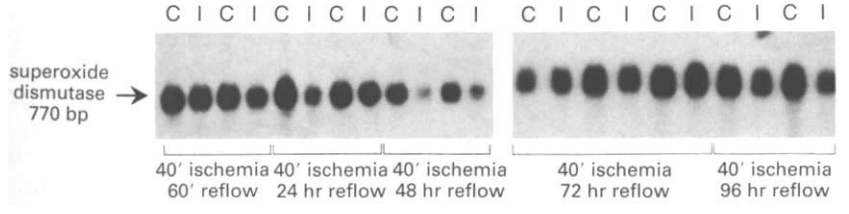

Fig. 5. *Northern blot analysis of SOD mRNA in paired control (C) and ischemic (I) kidneys.*

ischemic control kidneys (Fig. 2, first 4 lanes). However, by 24 hours of reperfusion there was a striking increase in histone H2b gene expression in postischemic kidneys (Fig. 2, lanes 5 to 8). High levels of histone H2b mRNA persisted at 48 hours after ischemia and remained elevated at 72 and 96 hours.

The level of mRNA for α-actin, a structural protein, did not show the marked changes seen for the proto-oncogene c-*fos* or the DNA synthesis-related histone H2b. Figure 3 shows that there was a slight decrease in α-actin expression 60 minutes after ischemia and a slight increase 24 hours after ischemia. However, at 48, 72, and 96 hours there were generally comparable levels of α-actin mRNA in control and postischemic kidneys (data not shown).

Genes coding for "functional" proteins showed yet a different pattern of expression. Renin mRNA was undetectable one hour following ischemia, but was present in normal amounts 24 and 48 hours after ischemia (Fig. 4). The decrease in renin mRNA at 72 and 96 hours may be related to volume expansion. In contrast, superoxide dismutase mRNA was present in decreased amounts 24 and 48 hours after renal ischemia, with complete or partial recovery by 72 hours followed by a decrease at 96 hours (Fig. 5).

Expression of the TRPM-2 gene was greatly increased 24 to 72 hours after ischemia (Fig. 6). By 96 hours after ischemia, although TRPM-2 mRNA was still found in increased levels, the amount was diminished compared with earlier time points.

To help synthesize the data the results from all of the experiments have been compiled in Table 1. This format emphasizes the several patterns of gene expression observed after renal ischemia.

Discussion

One of the most interesting findings in this study is the sequential nature of gene expression following ischemic renal injury. Expression of some genes occurred after the expression of others. However, we did not determine whether the expres-

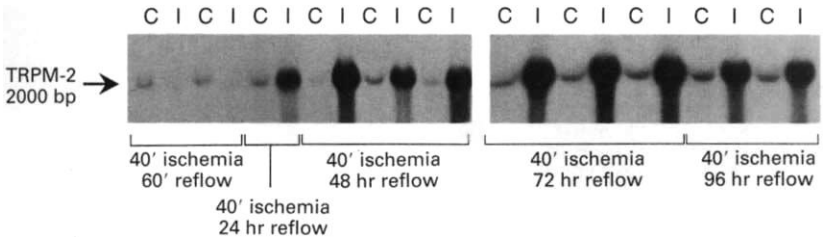

Fig. 6. *Northern blot analysis of TRPM-2 mRNA in paired control (C) and ischemic (I) kidneys.*

Table 1. Summary of results

Reperfusion:	60 minutes	24 hours	48 hours	72 hours	96 hours
c-*fos*	289.0	0	0	ND	ND
Histone H2b	0.1	11.0	18.0	20.0	26.0
TRPM-2	0.6	6.0	11.0	13.1	7.7
α-actin	0.6	1.7	0.9	1.6	0.8
Superoxide dismutase	0.7	0.5	0.3	1.0	0.4
Renin	0.1	1.2	0.9	0.3	0.7

Results are expressed as the ratio of mRNA (O.D. units) of the ischemic to paired control kidneys, and are the mean values of 2 to 3 animals at each time point. ND, not done.

sion of one gene was truly dependent upon that of the preceding one. For ultimate recovery, the repair process requires cellular proliferation followed by differentiation of the new cells into mature, functioning cells. After ischemic injury most of this activity involves proximal tubule epithelial cells in the S2 and S3 segment [27]. Some of our findings were predictable, whereas others were not.

In other cell types as well as in renal epithelial cells, mitosis is preceded by new DNA synthesis. This new DNA synthesis is induced by intracellular signals that allow a quiescent G_0 phase cell to re-enter the cell cycle. The proto-oncogene c-*fos* is one of several "immediate early" genes that are induced early in this process. Expression of c-*fos* does not require new protein synthesis and may be involved in transcriptional modulation [28]. Our findings are consistent with those of other investigators in demonstrating a prompt but transient expression of this gene [7–10]. Some time after the induction of c-*fos* other genes necessary for DNA synthesis, such as histone H2b, showed increased expression. The pattern of histone H2b expression is similar to that seen for ^{3}H-thymidine incorporation (a more integrated marker for new DNA synthesis) by postischemic kidneys which is maximal 48 to 72 hours after ischemia [3]. In pointing out this sequential nature of gene expression we presume that the same cells that make c-*fos* and histone H2b eventually proliferate.

Another aspect of the sequential nature of gene expression, that of repression of other genes early in the recovery phase of ischemic injury, was a more surprising finding. Sixty minutes after ischemia expression of histone H2b was repressed despite its being expressed in much increased magnitude just 23 hours later. The level of renin mRNA was also markedly decreased just 60 minutes after ischemia. On the other hand, superoxide dismutase message was less dramatically decreased but the diminished levels persisted for several days. Yoshioka and coworkers similarly reported no change in SOD activity in the glomerulus three days after a mild ischemic insult (30 min ischemia), although they found a substantial increase in activity six days after ischemia [29]. The finding of initial repression of the SOD gene suggests that when the kidney is subjected to severe oxidant stress, as is ischemia-reperfusion injury, the subsequent response favors cellular proliferation to replace lost cells rather than enhancing mechanisms of protection against other oxidant insult. Although this appears to be a wise choice by the kidney, this is a different response from that seen in oxidant injury in other organs in which anti-oxidant protective mechanisms are subsequently enhanced. For example, when rats were exposed to 95% oxygen, SOD mRNA in the lung increased by approximately 50% [14].

The term "repression of genes" has been used loosely here. Only the steady state level of mRNA for a particular gene was measured. A decrease in this level could be due to differentially increased degradation of a specific message as well as gene inactivation. In addition, only changes in total kidney message can be detected with the methods we employed. Thus, neither inter- nor intra-nephron heterogeneity could be evaluated. In situ hybridization studies could better address those issues.

A potential explanation for the decreased levels of histone H2b, renin and SOD mRNA is the loss of a population of cells that constitutively express these genes with relative preservation of cells that express these genes at lower levels or not at all. We do not favor such an explanation since the time pattern for renin and histone H2b expression was different from that for SOD; this would require the existence of at least three major cell populations with differential sensitively to ischemic injury, two of which have equal ability to proliferate after injury. Instead, we favor the hypothesis that cells surviving critical injury can differentially regulate gene expression during regeneration to both induce expression of usually silent genes and to repress expression of constitutively expressed genes.

Recent evidence has implicated angiotensin II (Ang II) in the growth response of a number of cell types including renal proximal tubular and mesangial cells [10–12, 30–32]. Conversion of angiotensinogen to angiotensin I by renin is the rate limiting step in the generation of Ang II. Although not providing any definitive information about Ang II levels, the examination of renin message provides an opportunity to examine local renal renin synthesis. The lack of an increase in renin mRNA suggests that the renal renin-angiotensin system is not critical for epithelial cell regeneration following ischemia. Blockade of Ang II formation by captopril actually enhanced renal recovery following ischemia, a finding which could be related to altered renal hemodynamics [33, 34]. The late suppression of renin mRNA at 72 hours may have been related to volume expansion secondary to renal failure.

Testosterone-repressed prostate message-2 (TRPM-2) is an androgen-repressed mRNA cloned from regressing rat ventral

prostate [25]. This mRNA encodes for a protein identical to rat sulfated glycoprotein-2 (SGP-2 and also known as clusterin) produced by rat sertoli cells [35–38], and is highly homologous (82% amino acid homology in the coding region) to human complement cytolysis inhibitor (CLI; SP-40,40), a protein which inhibits the lysis of cells by the C5b-9 complex [39–41]. In the rat kidney increased expression of TRPM-2 was found at 24 and 48 hours, but not at one week, following ureteral obstruction [15]. In preliminary reports, increased expression of this gene followed ischemic and nephrotoxic injury [16, 17]. The prolonged expression of TRPM-2 after an acute ischemic insult raises the possibility that TRPM-2 is induced not only by tissue injury, but may also be in some manner related to tissue repair processes.

The selective and orderly sequence of gene expression or repression after renal ischemia, a process which presumably maximizes the proliferative repair response, might suggest approaches to therapeutic maneuvers to enhance recovery. Such interventions might be optimized by careful attention to timing or by administering agents that act at different stages of the repair process sequentially. For this to occur we must continue to unravel the intricacies of renal epithelial cell proliferation and identify those genes whose expression is critical to the recovery process.

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