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Extracellular Signal-Regulated Kinase Mediates Renal Regeneration in Rats with Myoglobinuric Acute Renal Injury

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***In vitro* data support that extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK), members of mitogen-activated protein (MAP) kinases, mediate the signal transduction pathways responsible for the cell proliferation. However, *in vivo* role of these MAP kinases is poorly understood. Intramuscular injection of 50% glycerol solution induces acute renal failure in rats. This injury is known as a model of rhabdomyolysis in human. To investigate the molecular mechanism of the signaling pathway in this injury, we examined the role of ERK and JNK. After the glycerol injection JNK was rapidly and transiently activated at about 4 h, while the activation of ERK was gradually increased and the levels were sustained at least to 24 h. Next, we examined the expression of cell-cycle related proteins after the glycerol injection using Western blot analysis. The levels of proliferating cell nuclear antigen (PCNA) protein as a marker for cell proliferation were induced at 2 h and significantly increased to 24 h after the injection. In addition, cyclins D1, D2, and D3 as markers for G_1 phase also increased with similar time courses. To examine whether activation of ERK and/or JNK are involved in the renal regeneration after the glycerol injection, we examined the effect of genistein, which is an inhibitor of tyrosine kinase, on the activation of ERK and JNK. Administration of genistein to rats with this injury decreased the activation of ERK, but not JNK. The induction of PCNA and cyclin D1 was also prevented by this treatment. In this condition, renal function was further worsened as compared to control rats. These results provide the first evidence that ERK may be involved in the repair process of renal tubules damaged by this injury.**

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Glycerol-induced acute renal failure (glycerol-ARF) in rats is induced by intramuscular injection of hypertonic glycerol solution and causes rapid myoglobinuria, oliguria, and reduction in glomerular filtration rate. The morphology of glycerol-ARF is acute tubular necrosis, particularly in the proximal tubules. Glycerol-ARF is known as a model of rhabdomyolysis in human. Recently, a role of reactive oxygen metabolites in glycerol-ARF has been suggested based on the protective effect of hydroxyl radical scavengers (1). Oxidative stress seems to be one of the causes in glycerol-ARF (2-4). However, little is known about the molecular mechanism of this injury.

Mammalian cells express multiple mitogen-activated protein (MAP) kinases that mediate the effects of extracellular signals on a wide array of biological process. Three distinct MAP kinase cascades have been described, which appear to be linked to separate signal transduction pathways resulting in the final activation of either extracellular signal-regulated kinase (ERK) (5), c-Jun N-terminal kinase (JNK) (6-8) and recently identified p38 MAP kinase (p38) (9-11). The two ERKs (ERK1 and ERK2) are known to be involved in many mitogenic pathways (12-14). As like ERK, JNK is strongly activated by growth factors and proinflammatory cytokines including EGF, TNF, and IL-1 (8). It has been also known that the direction of cell survival and death (apoptosis) are settled by the balance of activation of ERK and JNK/p38 kinase pathways (15). The function of these MAP kinases in glycerol-ARF also remains unclear.

A number of cell-cycle regulatory proteins have been identified and classified as cyclins, cyclin-dependent kinases, and cyclin-dependent kinase inhibitors. Induction of D-type cyclins is growth factor dependent and D1, D2, and D3 are expressed in G1 phase (16).

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D-type cyclins and their cyclin-dependent kinase partners can also form complexes with proliferating cell nuclear antigen (PCNA), a DNA polymerase δ associated protein that is required for cell proliferation (17).

Our present study focuses our attention on the signaling cascade(s) responsible for the renal regeneration in rats with glycerol-induced acute injury. After the glycerol injection, cell-cycle related proteins are elevated at several hours later. Although ERK and JNK are both activated in this condition, ERK signaling cascade may be involved in renal cell regeneration induced by glycerol-induced acute injury. To our knowledge, our findings are the first report in vivo that ERK signaling pathway involves in the renal regeneration after acute renal injury.

MATERIALS AND METHODS

Materials. Antibody against JNK was purchased from Santa Cruz. Phospho-specific antibodies against JNK (Thr 183/Tyr 185) (pJNK) and ERK (Tyr 204) (pERK), and antibody against ERK were from New England Biolabs. Alkaline phosphatase-conjugated goat anti-rabbit IgG and alkaline phosphatase-conjugated goat anti-mouse IgG were from Vector Laboratories. Mouse monoclonal antibody against proliferating cell nuclear antigen (PCNA) (Clone PC-10) was from Progen. Mouse monoclonal antibodies against cyclin D1, D2, and D3 were from Calbiochem. Genistein was from Wako Chemicals (Osaka, Japan). Hybond-C extra nitrocellulose membrane was from Amersham. Protein assay kit was from Bio-Rad.

Animal experimentation. Male Wistar rats weighing 200-250 g were used. All animal experiments were performed in strict accordance with the institution's criteria for the care and use of laboratory animals as approved by an institutional governing committee. Rats were first deprived of water for 24 h, then anesthetized with ether and administered intramuscularly with 10 ml/kg body weight of 50% (vol/vol) glycerol solution distributed equally in both hind limbs. Five to six rats were used for each group. Genistein (10 mg/kg body weight, 20 mg/ml dimethyl sulfoxide) was intraperitoneally administered 1 h before the glycerol injection. Control rats was treated with vehicle only before the glycerol injection.

Western blot analysis. At the indicated times rats were anesthetized with ether and sacrificed by the collection of blood from the hearts. For the immunoblot analysis of cyclin D1, D2, D3 and PCNA, kidneys were homogenized with a glass-Teflon homogenizer in 10 vol. of 20 mM Hepes (pH 7.5), 2 mM EDTA, 25 mM β-glycerophosphate, 1 mM dithiothreitol, 1 mM orthovanadate, 1% Tween 20, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin and then the homogenate samples were used as tissue extracts. For the immunoblot analysis of activated ERK and JNK, nuclei fraction was prepared as follows. Kidneys were homogenized in 0.25 M sucrose and 3 mM $MgCl_2$ solution containing 25 mM β-glycerophosphate, 1 mM dithiothreitol, 1 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and then centrifuged at 600×g for 10 min. The pellet was suspended in the same solution. Each tissue extract (100 μg protein/lane) was separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose filters. The filters were blocked with 6% bovine serum albumin (BSA) in TBST (50 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0.05% Tween 20) overnight at 4°C, then each filter was incubated with appropriate primary antibody in 6% BSA-TBST for 1 h at room temperature. The blots were washed with TBST three times each for 10 min to remove unbound antibodies and then incubated with alkaline phosphatase-conjugated second antibody in 6% BSA-TBST for 2 h at room temperature. After washing with TBST (3 times 10 min), the immunoreactive bands were visualized using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium chloride as recommended by supplier. Protein concentration was determined using protein assay kit.

Renal function. For the renal function, serum creatinine contents were determined using an analyzer (AU510; Olympus, Tokyo, Japan).

Statistical analysis. Significance of experimental results between animals was evaluated using the unpaired Student's *t*-test. Results were expressed as means ± SE. The differences were considered statistically significant when P value was less than 0.01.

RESULTS

Activation of ERK and JNK

To investigate whether MAP kinases mediate the biological effects in the kidney with glycerol-ARF, we examined the time courses of ERK and JNK activation levels after 50% glycerol solution injection (Fig. 1). Because ERK and JNK were both activated by phosphorylation, we determined the levels of phosphorylated forms of ERK (pERK) and JNK (pJNK) using each phospho-specific antibody against pERK and pJNK, respectively. Fig. 1a shows that the antibody against pERK used in this experiment detects two pERK bands, pERK1(44 kDa) and pERK2 (42 kDa), respectively. Both pERK1 and pERK2 bands were faintly detected in normal and 24-h dehydrated rat kidneys. At 2 h after the glycerol injection, the activation of ERK1/ERK2 was both elevated and reached maximum levels at about 8 h and the levels were sustained at least to 24 h. Then the levels of pERK1 and pERK2 were returned to the normal level at 72 h after the treatment (data not shown). On the other hand, the levels of both ERK1 and ERK2 proteins were not changed during these experimental periods (data not shown). As shown in Fig. 1c, the phospho-specific antibody against JNK also detected two bands, pJNK1 (46 kDa) and pJNK2 (54 kDa), respectively. In contrast to pERK, the activation of pJNK1 and pJNK2 was both transiently induced at about 4 h after the glycerol injection (Fig. 1c and 1d). In normal and 24-h dehydrated rat kidneys both pJNK1 and pJNK2 were scarcely detected. During experimental periods, the levels of JNK1 and JNK2 proteins were unchanged (data not shown). Because ERK and JNK signaling pathways were known to be related to cell proliferation (10), we speculated that renal cell regeneration might be occurred in this injury.

Expression of Cell-Cycle Related Proteins

To investigate whether the renal regeneration is induced or not in the kidneys with glycerol-ARF, we examined the levels of PCNA and cell-cycle related proteins as markers for cell proliferation using immunoblot analysis. Fig. 2 shows that monoclonal antibody against PCNA detects a band of 36 kDa which is corresponding to PCNA protein. At 2 h after the injection, PCNA protein was slightly detected and then gradually increased. The levels reached a peak at about 24 h. This result suggested

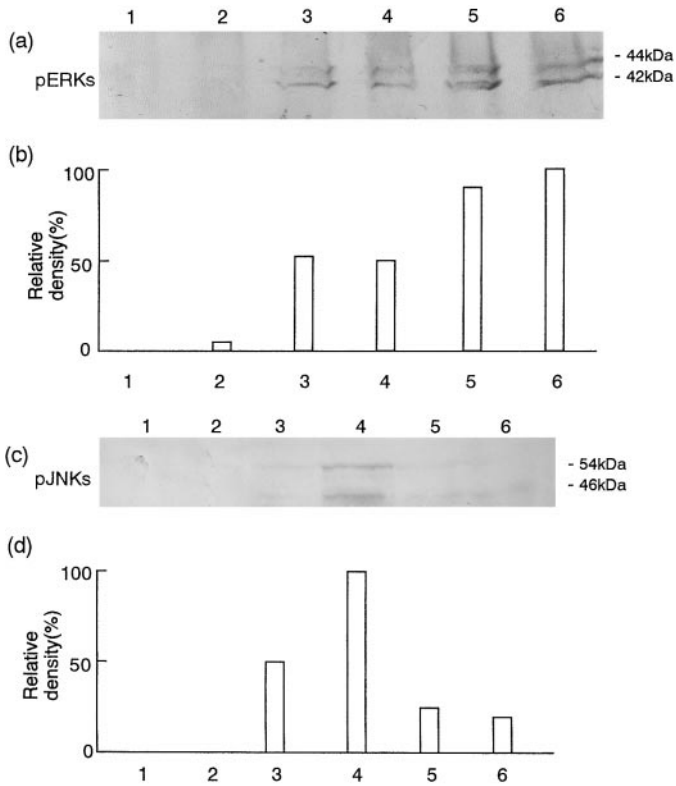

FIG. 1. Time courses of the levels of phosphorylated ERKs (pERKs) and JNKs (pJNKs) proteins in rat kidneys after intramuscular glycerol injection. (a) Immunoblot analysis of pERKs (44 kDa ERK1/42 kDa ERK2) proteins in rat kidneys at the indicated times after the glycerol injection. Lane 1, normal rat kidneys; lane 2, 0 h (24 h dehydration only); lane 3, 2 h; lane 4, 4 h; lane 5, 8 h; lane 6, 24 h. (b) Relative amounts of pERKs proteins. Densitometric scanning of both bands was performed using immunoblot analysis filter as shown in (a). A relative amount was expressed as compared with a maximum level. (c) Immunoblot analysis of pJNKs (46 kDa JNK1/54 kDa JNK2) proteins in rat kidneys at indicated times after the glycerol injection. Lane 1, normal rat kidneys; lane 2, 0 h (24 h dehydration only); lane 3, 2 h; lane 4, 4 h; lane 5, 8 h; lane 6, 24 h. (d) Relative amounts of pJNKs proteins. Densitometric scanning of both two bands was performed using immunoblot analysis filter as shown in (c) and a relative amount was expressed as (b).

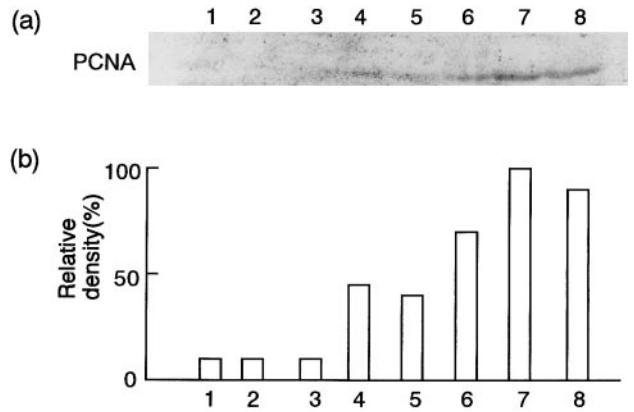

FIG. 2. Time course of the levels of PCNA protein in rat kidneys after intramuscular glycerol injection. (a) Immunoblot analysis of PCNA protein levels in rat kidneys after the glycerol injection. Lane 1, normal rat kidney; lane 2, 0 h (24 h dehydration only); lane 3, 1 h; lane 4, 2 h; lane 5, 4 h; lane 6, 8 h; lane 7, 24 h; lane 8, 36 h. (b) Relative amounts of PCNA protein levels. Densitometric scanning was performed using immunoblot filter as shown in (a). A relative amount was expressed as compared with a maximum level.

that the renal cell proliferation was occurred in the kidneys with glycerol-ARF. To investigate the renal cell proliferation in more detail, we examined the expression of cyclin D1, D2 and D3 in the kidneys as markers for G_1 phase in this model (Fig. 3). These G1-phase related proteins examined in this experiment were all detected at about 4 h and increased at least to 24 h after the glycerol injection. These results obviously showed that the renal regeneration was occurred during glycerol-induced acute renal injury.

Relationship between ERK Activation and Renal Regeneration

To investigate the relationship among ERK, JNK, and cell-cycle related proteins in glycerol-ARF, we examined the effect of genistein, an inhibitor of tyrosine kinase, on these proteins after the glycerol injection. Genistein, which was administered intraperitoneally 1 h before the glycerol injection, clearly reduced the levels of PCNA protein (Fig. 4a). In addition, the induction of cyclin D1 was prevented by genistein treatment (Fig. 4c). Cyclins D2 and D3 were also prevented (data not shown). It was of particular interest that genistein treatment obviously prevented the activation of ERK (Fig. 4c), but not JNK (data not shown). In these conditions, renal function in genistein-treated rats with glycerol-ARF was further worsened as compared to that of genistein-untreated and glycerol-injected rats (genistein + glycerol, 4.2 ± 0.05 mg/dl vs.

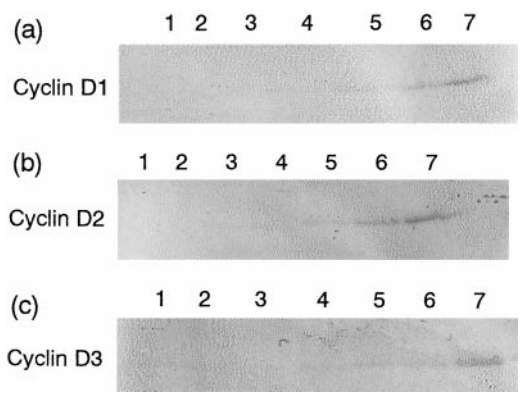

FIG. 3. Time courses of the levels of cyclins D1, D2 and D3 proteins in rat kidneys after intramuscular glycerol injection. Immunoblot analysis of cyclins D1, D2 and D3 proteins in rat kidneys at the indicated times after the glycerol injection. Lane 1, normal rat kidney; lane 2, 0 h (24 h dehydration only); lane 3, 1 h; lane 4, 2 h; lane 5, 4 h; lane 6, 8 h; lane 7, 24 h.

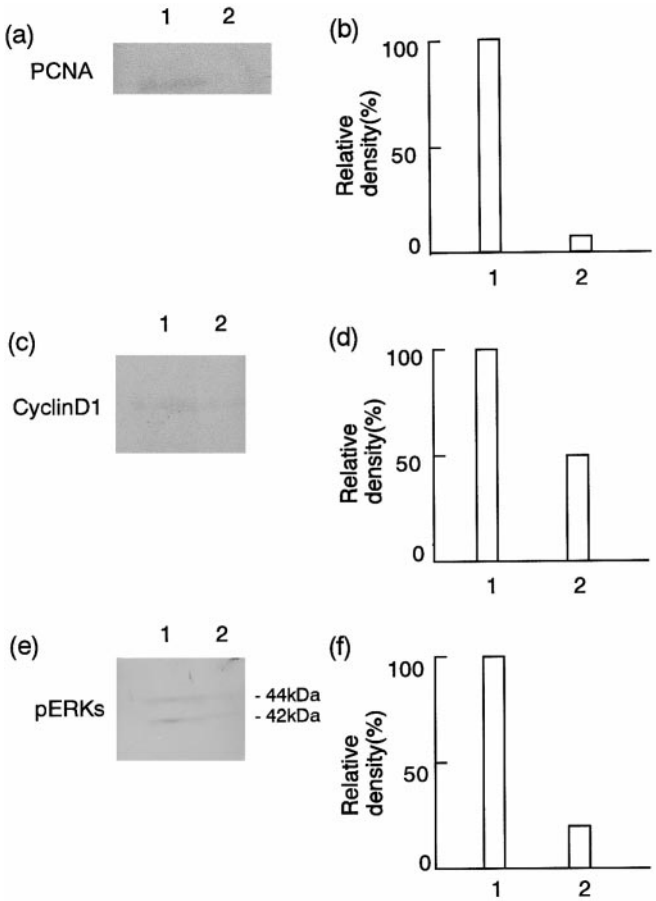

FIG. 4. Effect of genistein administration on the expression of PCNA, cyclin D1, and pERK proteins in rat kidneys with glycerol-ARF. Immunoblot analysis of PCNA (a), cyclin D1 (b), and pERK proteins in rat kidneys with glycerol-ARF treated with or without genistein. Lane 1, without genistein; lane 2, with genistein. Animal experiment and immunoblot analysis were performed as described in "Materials and Methods". Tissue extracts were prepared at 24 h after the glycerol injection.

glycerol alone, 3.1 ± 0.11, $p<0.01$) (Fig. 5). These results suggested that ERK might mediate the signaling pathway leading to renal cell proliferation induced by glycerol-induced acute renal injury.

DISCUSSION

Intramuscular injection of hypertonic glycerol solution induces acute renal tubular injury. Proliferation of renal tubules after acute injury is essential process for the recovery of the renal function. However, the mechanisms leading to the recovery of the kidney after glycerol-ARF are poorly understood. Our aim of this experiment is to understand the signal transduction pathway responsible for this process *in vivo*.

In our experiment cyclins D1, D2, D3 and PCNA were induced by the glycerol injection. Our findings clearly indicated that the renal cell proliferation process seemed to start at 24 h after the treatment when injury in the proximal tubular epithelium was most prominent (3). It was noteworthy to describe that the induction of PCNA was observed at 2 h after the glycerol injection and the expression was faster than those of cyclins D1, D2 and D3. Previous *in vitro* experiments suggested the involvement of PCNA in DNA repair after DNA damage (19,20). Therefore, the role of the early induction of PCNA may participate in DNA repair and the late expression of PCNA may function for DNA replication. In addition, immunohistochemical analysis showed that PCNA protein induced by the glycerol injection was exclusively restricted to the renal tubule cells (data not shown). The localization was coincident with the injured region. Our results clearly showed that the renal cell proliferation induced by glycerol-ARF was occurred in renal tubules. Using another acute renal failure induced by ischemia similar results have been recently reported that the renal cell proliferation is occurred in the proximal tubules (21). However, the mechanisms of signal transduction pathway leading to this process were not examined.

Members of MAP kinases, ERK and JNK are known to be regulated by several growth factors and cytokines and play important roles in cell proliferation (12-14,22). They are regulated by distinctive signal transduction pathways and show different function (1,5,23-25). However, these results were exclusively obtained *in vitro* experiments. Therefore, it is important to clarify the function of these MAP kinases in pathological conditions. In our *in vivo* experiment clearly indicated that ERK and JNK were both activated in the kidneys with glycerol-ARF. When genistein, an inhibitor of tyrosine kinase, was administered to rats with glycerol-ARF, ERK activation was only prevented. In this situation, expression of PCNA, cyclins D1, D2, and D3 were also prevented. These results suggest that ERK signaling pathway may involve in renal cell regeneration occurred in the kidneys with glycerol-ARF. In addition, genistein administration to rats with glycerol-ARF impaired the renal function further.

To our knowledge, these findings are the first report that ERK signaling pathway is strongly related to the

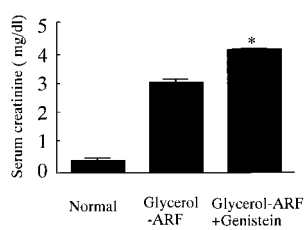

FIG. 5. Effect of genistein administration on renal function. Rats were sacrificed at 24 h after 50% glycerol injection. Genistein (10 mg/kg body weight) was intraperitoneally injected 1 h before the glycerol injection. Values were expressed as mean ± SE ($n = 6$) (*$p <$ 0.01, Glycerol-ARF vs. Glycerol-ARF plus genistein).

renal function and renal cell regeneration after the glycerol injection. Furthermore, it is of interest to examine whether ERK activation that appears to be required for the improvement of renal function is induced in the kidneys with other acute renal failures. Although it is well known that JNK is activated by oxidative stress and also plays an important role to induce apoptosis (15,26), the role of the early response of JNK which is activated by the glycerol-induced acute renal injury is still unclear. Further work will be required to understand the different roles of ERK and JNK signaling pathways induced by the glycerol-ARF in more detail.

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