STAT3 attenuates EGFR-mediated ERK activation and cell survival during oxidant stress in mouse proximal tubular cells

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We have shown that renal epithelial cell survival depends on the sustained activation of the extracellular signal-regulated protein kinase (ERK) and lack of this activation was associated with death during oxidative stress. ERK is activated via the canonical epidermal growth factor receptor (EGFR)–Ras–MEK pathway, which could be attenuated by oxidants. We now show that the failure to activate ERK in a sustained manner during severe oxidative stress is owing to the activation of the signal transducer and activator of transcription-3 (STAT3) rather than the failure to activate the EGFR. Tyrosine phosphorylation of the EGFR and STAT3 was studied in hydrogen peroxide (H_2O_2)-treated mouse proximal tubule (TKPTS) cells or in mouse kidney after ischemia/reperfusion (I/R) injury by Western blotting. STAT3 activation was inhibited by either pharmacologically (AG490) through its upstream janus kinase (JAK2) or by a dominant-negative STAT3 adenovirus. EGFR was inhibited by AG1478. Survival was determined by fluorescence-activated cell sorter analysis and trypan blue exclusion. We found that the EGFR was phosphorylated on its major autophosphorylation site (Tyr1173) regardless of the H_2O_2 dose. On the other hand, both I/R and severe oxidative stress – but not moderate stress – increased tyrosine phosphorylation of STAT3 in an EGFR and JAK2-dependent manner. Inhibition of JAK2 or STAT3 lead to increased ERK activation and survival of TKPTS cells during severe oxidative stress. Our data suggest a role of tyrosine-phosphorylated STAT3 in the suppression of ERK activation. These data suggest that the STAT3 pathway might represent a new target for improved survival of proximal tubule cells exposed to severe oxidant injury.

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During ischemia/reperfusion (I/R) injury, the excessive formation of reactive oxygen species and their intermediates, such as hydrogen peroxide (H_2O_2), contributes to the death of proximal tubules of the kidney.[1] In cultured mouse proximal tubule cells (TKPTS), we found that a moderate amount of H_2O_2 (0.5 mmol/l) resulted in sustained activation of extracellular signal-regulated kinase (ERK) and cell survival. By contrast, treatment with excessive H_2O_2 (1.0 mmol/l) led to a transient activation of ERK and cell death.[2] We also demonstrated that survival after H_2O_2 treatment requires the activation of the transcription factor cAMP-responsive element-binding protein, a downstream effector of the epidermal growth factor receptor (EGFR)–ERK pathway,[3] and this signaling is interrupted by severe oxidant stress.

The activation of ERK has been shown to be executed through the canonical EGFR–Ras–MEK pathway.[4,5] Epidermal growth factor (EGF) and EGF-like ligands bind and activate the intrinsic tyrosine kinase activity of the EGF receptor and initiate autophosphorylation of various tyrosine residues. H_2O_2 itself can also induce phosphorylation of the EGFR and initiate signaling through the EGFR–Ras–MEK pathway.[6–10] *In vivo* studies suggest that H_2O_2 generated during reperfusion could act as an activator of the EGFR.[11] Thus, moderate levels of H_2O_2 might serve as a second messenger in survival signaling including the EGFR/ERK pathway.[12–14] Proximal tubules of the kidney, which undergo necrotic cell death, express high levels of EGFR[15] and activate the EGFR during I/R injury;[11,16] yet, we and others have failed to demonstrate activation of ERK or other downstream elements of the canonical pathway in proximal tubule segments following I/R injury.[17,18]

In seeking an explanation for this observation we considered at least two possibilities. Severe stress might fail to activate the EGFR, usually accomplished by phosphorylation at a key activation site on the receptor,[6–9,19] or by a post-receptor mechanism involving the activation of transduction signaling arising from an alternate pathway that represses EGFR downstream signaling. Such an EGFR-repressing mechanism has been observed during reactive oxygen

species-activated janus kinase (JAK)/STAT signaling both *in vitro* as well as during I/R-induced injury,[20,21] whereby the activation of the signal transducer and activator of transcription-3 (STAT3) has been observed to downregulate the ERK1/2 pathway. It would appear that these alternate signaling pathways induced by severe stress result in the inhibition of the EGFR-mediated survival pathway.

We therefore postulated that the ERK survival signaling pathway is interrupted by severe oxidant stress in TKPTS. Accordingly, we tested the hypotheses that severe oxidant stress fails to activate ERK either by failure to activate the EGFR or by activation of the JAK2/STAT3 pathway. Furthermore, we sought to re-establish ERK signaling by manipulation of these pathways in order to restore cell survival during severe oxidant stress.

RESULTS

EGFR is phosphorylated independent of the dose of oxidative stress in mouse renal proximal tubular cells

TKPTS cells were treated with 0.5 or 1 mmol/l H_2O_2 or 10 ng/ml EGF for 30 min. Phosphorylation of the EGFR at one of the major autophosphorylation sites (tyrosine(Tyr) 1173) and the unphosphorylated EGFR was determined by Western blotting (Figure 1a). The results show that the EGFR was significantly phosphorylated at Tyr 1173 both at 0.5 and 1.0 mmol/l H_2O_2 similar to treatment by its cognate ligand EGF (Figure 1b). Pretreatment of TKPTS cells with 50 μM/l AG1478 (an EGFR inhibitor) 1 h before treatment with 0.5 mmol/l H_2O_2 resulted in extensive necrotic cell death similar to that seen after treatment with 1 mmol/l H_2O_2 (Figure 1c), suggesting that EGFR activation is critical for cell survival. For comparison control (untreated), 0.5 mmol/l- and 1 mmol/l H_2O_2-treated cells are also included. Note the G_2/M arrest induced by 0.5 mmol/l H_2O_2, which we have previously shown to accompany cell survival under these conditions.[2] These results suggest that attenuation of ERK phosphorylation by 1 mmol/l H_2O_2[2] may be downstream from the activated EGFR.

Severe oxidant stress tyrosine phosphorylates STAT3 both *in vitro* and *in vivo*

We next explored the role of STAT3 in the interruption of EGFR-induced ERK1/2 activation.[22] TKPTS cells were treated with 0.5 or 1 mmol/l H_2O_2 for 30 min and Tyr-705 phosphorylated STAT3 was determined by Western blotting (Figure 2a). The results demonstrate that only 1 mmol/l H_2O_2 phosphorylates STAT3 significantly at Tyr-705 (Figure 2b). Tyrosine phosphorylation of STAT3 is also detectable in lysates from mouse kidneys undergoing I/R injury (Figure

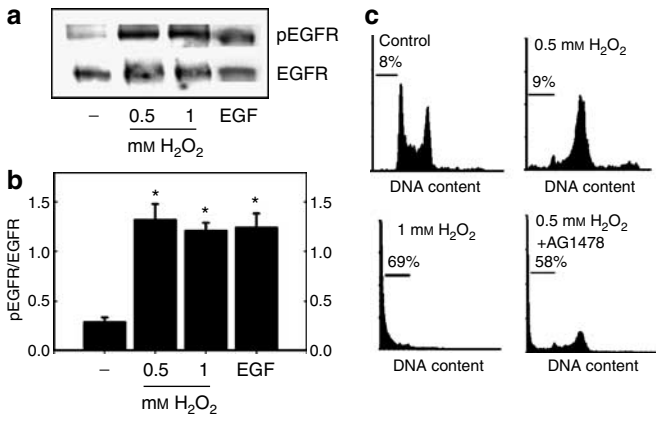

Figure 1 | EGFR activation after various levels of oxidant stress and its role in survival. (**a**) TKPTS cells were treated with either 0.5 or 1 mmol/l H_2O_2 or 10 ng/ml EGF for 30 min. Cell lysates were prepared and phosphorylation of EGFR determined by Western blotting using a phospho-EGFR (Tyr 1173) antibody that recognizes one of the major autophosphorylation site on EGFR. Status of the unphosphorylated EGFR was also determined. Data shown are representative of three independent experiments. (**b**) Densitometric analysis of Western blots as shown in (**a**). The extent of EGFR tyrosine phosphorylation was determined by normalizing phospho-EGFR levels to the total EGFR. Values are given as mean $\pm$ s.d. ($n = 3$). *$P < 0.001$ compared to the untreated control. (**c**) TKPTS cells were pretreated with 50 μmol/l AG1478 1 h before treatment with 0.5 mmol/l H_2O_2. Twenty-four hour after H_2O_2 treatment FACS analysis was carried out to determine viability of cells. Cells reside in the sub-G1 fraction are considered dead. For comparison, control (untreated), 0.5 mmol/l and 1 mmol/l H_2O_2-treated cells are also included. Note the G_2/M arrest induced by 0.5 mmol/l H_2O_2. Data shown are representative of three independent experiments.

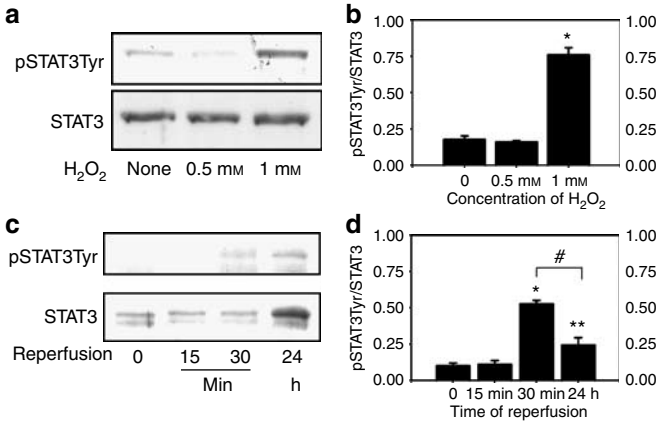

Figure 2 | Tyrosine 705 phosphorylation of STAT3 in TKPTS cells and in the kidney. (**a**) TKPTS cells were treated with 0.5 or 1 mmol/l H_2O_2 for 30 min. Tyr 705 phosphorylation of STAT3 (pSTAT3Tyr) was determined by Western blotting together with the total STAT3. Data shown are representatives of three independent experiments. (**b**) Densitometric analysis of Western blots as shown in (**a**). The extent of STAT3 tyrosine phosphorylation was determined by normalizing phospho-STAT3 levels to the total STAT3. Values are given as mean $\pm$ s.d. ($n = 3$). *$P < 0.001$ compared to the untreated control. (**c**) Protein lysates were obtained from kidneys 15 and 30 min as well as 24 h after re-establishing the blood flow after 50 min ischemia and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Western blotting. Blots were hybridized with antibodies that recognize either the phospho-(Tyr705)-STAT3 or STAT3, respectively. Data shown are representatives of three independent experiments. (**d**) Densitometric analysis of Western blots as shown in (**c**). The extent of STAT3 tyrosine phosphorylation was determined by normalizing phospho-STAT3 levels to the total STAT3. Values are given as mean $\pm$ s.d. ($n = 3$). *$P < 0.001$, **$P < 0.05$ compared to the untreated control. #$P < 0.05$ compared to the 30 min reperfusion.

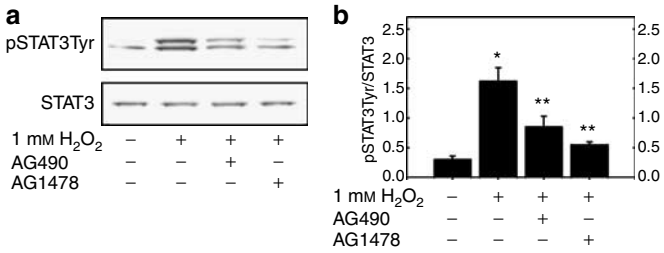

Figure 3 | Tyr 705 phosphorylation of STAT3 by 1 mmol/l H_2O_2 is both EGFR- and JAK2-dependent in TKPTS cells. (**a**) TKPTS cells were pretreated with either the JAK2 inhibitor AG490 (50 μmol/l) or the EGFR inhibitor AG1478 (50 μmol/l) for 1 h before treatment with 1 mmol/l H_2O_2 for 30 min. Levels of the tyrosine-phosphorylated and total STAT3 were determined by Western blotting. Results shown are representatives of three independent experiments. (**b**) Densitometric analysis of Western blots as shown in (**a**). The extent of STAT3 tyrosine phosphorylation was determined by normalizing phospho-STAT3 levels to the total STAT3. Values are given as mean $\pm$ s.d. ($n = 3$). *$P < 0.005$, **$P < 0.05$ compared to the untreated control.

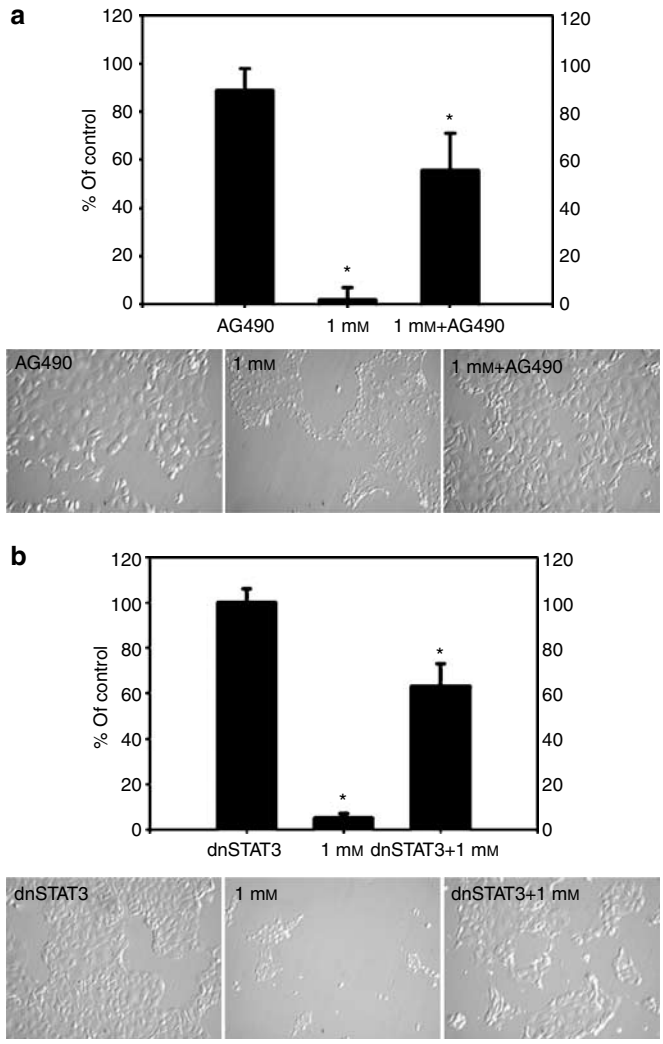

Figure 4 | Inhibition of JAK2 or STAT3 ameliorates survival of TKPTS cells after treatment with 1 mmol/l H_2O_2. (**a**) TKPTS cells were pretreated with 50 μmol/l AG490 for 1 h before treatment with 1 mmol/l H_2O_2. After 24 h, cell counts were determined by trypan blue exclusion. Data are expressed as the percentage of pretreatment (control) values ($N = 3$; mean $\pm$ s.d.). Cell morphology was assessed by light microscopy. AG490: cells treated with 50 μmol/l AG490 for 24 h; and 1 mM: cells treated with 1 mmol/l H_2O_2 for 24 h; 1 mM + AG490: cells pretreated with 50 μmol/l AG490 for 1 h before treatment with 1 mmol/l H_2O_2 for 24 h. *$P < 0.001$. (**b**) TKPTS cells were infected with 25 multiplicity of infection/ml dominant-negative STAT3 adenovirus 24 h before treatment with 1 mmol/l H_2O_2. After 24 h, cell counts were determined. Data are expressed as the percentage of pretreatment (control) values ($N = 3$; mean $\pm$ s.d.). dnSTAT3: cells infected with dnSTAT3 for 24 h; 1 mM: cells treated with 1 mmol/l H_2O_2 for 24 h; and dnSTAT3 + 1 mM: cells infected with dnSTAT3 before treatment with 1 mmol/l H_2O_2 for 24 h. *$P < 0.001$

2c). As is shown in Figure 2, 30 min as well as 24 h reperfusion significantly increased tyrosine phosphorylation of STAT3 in the mouse kidney. The relative decrease in STAT3 tyrosine phosphorylation 24 h after reperfusion is owing to the fact that ischemic injury also increased total STAT3 protein at that time point (Figure 2d). Regardless, the levels of tyrosine-phosphorylated STAT3 are still significantly higher compared to the normal kidney. These results suggest that tyrosine phosphorylation of STAT3 might be a key negative regulator of ERK activation. Furthermore, tyrosine-phosphorylated STAT3 might be an important element in death signaling.

We next asked if the activation of STAT3 is mediated by the EGFR or JAK2.[23–26] Pretreatment of TKPTS cells with either the JAK2 inhibitor AG490 or the EGFR inhibitor AG1478 partially but significantly attenuated 1 mmol/l H_2O_2-induced STAT3 tyrosine phosphorylation (Figure 3b), suggesting that both JAK2 and EGFR participate in STAT3 phosphorylation.

Inhibition of JAK2 or STAT3 ameliorates survival through restoring ERK activation

To determine whether ERK activation and survival could be restored by inhibition of the JAK2/STAT3 pathway, TKPTS cells were pretreated with either the JAK2 inhibitor AG490 or infected with a dominant-negative STAT3 adenovirus (Ad-dnSTAT3) before treatment with 1 mmol/l H_2O_2. After 24 h, cell numbers were counted. As shown in Figure 4a, pretreatment with AG490 significantly increased survival of cells after treatment with 1 mmol/l H_2O_2. Similarly, inhibition of STAT3 also reduced 1 mmol/l H_2O_2-induced cell death (Figure 4b).

We next explored whether JAK2/STAT3 inhibition restores ERK activation. Accordingly, TKPTS cells were either pretreated with AG490 (Figure 5) or infected with an adeno-dnSTAT3 (Figure 6) before treatment with 1 mmol/l H_2O_2. The phosphorylation status of ERK was determined 2 and 4 h after treatment with H_2O_2 by Western blotting. Figures 5 and 6 show that 1 mmol/l H_2O_2 significantly inhibited the phosphorylation of ERK at those time points. However, pretreatment with AG490 (Figure 5) or by direct inhibition of STAT3 via a dominant-negative mutant (Figure 6) restored ERK activation (phosphorylation).

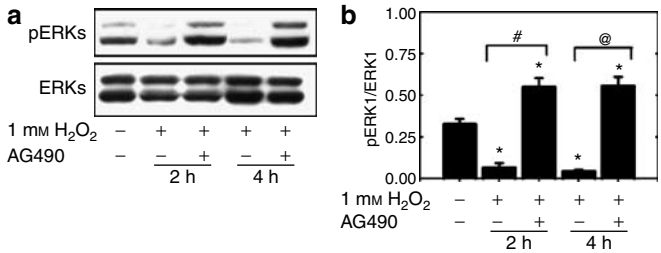

Figure 5 | Effects of JAK2 inhibition on ERK phosphorylation in TKPTS cells treated with 1 mmol/l H_2O_2. (**a**) TKPTS cells were treated with 1 mmol/l H_2O_2 for 2 and 4 h in the presence or absence of 50 μmol/l AG490. ERK phosphorylation was determined by Western blotting. Data shown are representatives of three independent experiments. (**b**) Densitometric analysis of Western blots as shown in (**a**). The extent of ERK phosphorylation was determined by normalizing phospho-ERK1 levels to the total ERK1. Values are given as mean $\pm$ s.d. ($n = 3$). $^{*}P < 0.05$ compared to the untreated control. $^{\#}P < 0.05$ compared to the 1 mmol/l H_2O_2-treated cells, $^{@}P < 0.05$ compared to the 1 mmol/l H_2O_2-treated cells.

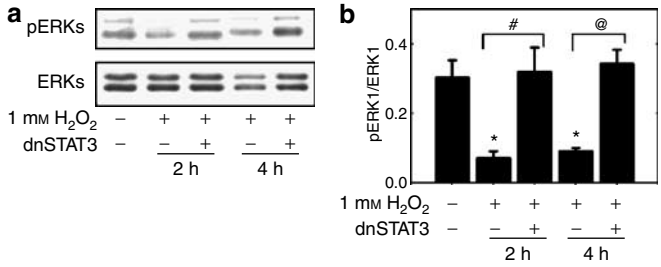

Figure 6 | Effects of STAT3 inhibition on ERK phosphorylation in TKPTS cells treated with 1 mmol/l H_2O_2. (**a**) TKPTS cells were treated with 1 mmol/l H_2O_2 for 2 and 4 h in the presence or absence of a dnSTAT3 adenovirus. ERK phosphorylation was determined by Western blotting. Data shown are representatives of three independent experiments. (**b**) Densitometric analysis of Western blots as shown in (**a**). The extent of ERK phosphorylation was determined by normalizing phospho-ERK1 levels to the total ERK1. Values are given as mean $\pm$ s.d. ($n = 3$). $^{*}P < 0.05$ compared to the untreated control. $^{\#}P < 0.05$ compared to the 1 mmol/l H_2O_2-treated cells, $^{@}P < 0.05$ compared to the 1 mmol/l H_2O_2-treated cells.

DISCUSSION

We have shown that survival of renal epithelial cells during oxidant injury depends on the activation of the ERK–cAMP-responsive element-binding protein pathway.[2,3] We now show that an activated EGFR is indispensable for survival as AG1478, an EGFR inhibitor, attenuates survival of TKPTS cells during moderate oxidant stress (Figure 1c). This is the first demonstration of EGFR-dependent survival signaling in renal proximal tubules.

Although proximal tubules activate the EGFR after renal I/R injury,[27] they fail to activate ERK or other downstream elements of the proximal tubule survival pathway[17,18] and undergo necrotic death. We considered at least two possibilities to explain this observation: failure to phosphorylate the EGFR or activation of alternative pathways that interrupt EGFR–ERK signaling. We found that EGFR is phosphorylated on one of its major autophosphorylation site (Tyr 1173) regardless of the dose of oxidant in proximal tubule cells *in vitro* (Figure 1a). We then focused our attention on activation of alternate pathways that may interrupt EGFR-mediated survival signaling.

We next explored the effects of severe oxidant stress signaling at post-receptor levels. Aware that reactive oxygen species activates the JAK/STAT pathway,[24,28] we found that 1 mmol/l but not 0.5 mmol/l H_2O_2 phosphorylated STAT3 at Tyr 705 in TKPTS cells (Figure 2a, b). Similarly, I/R injury increased tyrosine phosphorylation of STAT3 in the kidney (Figure 2c, and d). We also found that tyrosine phosphorylation of STAT3 was mediated by both the EGFR and JAK2, as inhibition of either the EGFR or JAK2 diminished tyrosine phosphorylation of STAT3 *in vitro* (Figure 3). Thus, activation of STAT3 by 1 mmol/l H_2O_2 requires both the EGFR and JAK2. Inhibition of JAK2 by AG490 or inhibition of STAT3 function by Ad-dnSTAT3 reduced cell death caused by 1 mmol/l H_2O_2 treatment (Figure 4a, and b), thus revealing a pro-death role of this pathway in oxidant injury in these cells.

STAT3 is capable of suppressing the ERK pathway,[22,29] although the mechanism of this inhibition is not clear. A direct role of STAT3 in the inhibition of ERK activation was revealed in experiments in which inhibition of either JAK2 or STAT3 increased ERK phosphorylation after treatment with 1 mmol/l H_2O_2 (Figures 5 and 6). One possibility is that the tyrosine-phosphorylated STAT3 competes with growth factor receptor-bound protein 2 for the binding site on the activated EGFR[30] and thus terminates ERK activation. Oxidative stress can also trigger STAT3-mediated activation of SOCS3,[31] which can inhibit EGFR signaling.[32] To our knowledge, this is also the first determination of the pro-death role of the JAK/STAT pathway in the kidney. The precise mechanism by which the activated (tyrosine phosphorylated) STAT3 suppresses ERK activation remains to be determined.

Activation of the JAK/STAT pathway following I/R injury has broad implications. STAT3 can mediate inflammatory responses as well[33] by increasing the expression of tumor necrosis factor α[34] or interleukin-8,[35] both of which are induced during renal I/R[36,37] and may contribute to the pathogenesis of tubular injury. Tumor necrosis factor α is a known mediator of tubular cell death and plasma or urinary interleukin-8 levels predict high mortality in patients with acute renal failure.[38,39] Tumor necrosis factor α[34] and interleukin-8[35] possess a STAT3 binding site in their promoter proximal region. Thus, activation of STAT3 in addition to its demonstrated role in suppressing EGFR–ERK signaling might regulate transcription and secretion of inflammatory cytokines that could contribute to tubular epithelial cell death.

In summary, we found that severe oxidant stress leads to the tyrosine phosphorylation of STAT3. This event depends on the activation of the EGFR and JAK2 kinase in renal tubular epithelial cells. The pathway is directly linked to cell

death as inhibition of STAT3 function enables cells to survive severe oxidant stress. Part of the mechanism entails interruption of ERK activation via an activated EGFR. Thus, the STAT3 pathway might represent an important new target to protect proximal tubule cells from oxidant injury. These results also suggest a dual function for the EGFR (pro-survival or pro-death) and might explain the death of proximal tubules in the kidney in the presence of activated EGFR during I/R injury.

MATERIALS AND METHODS

Cell culture

The immortalized TKPTS cells are gift from Dr Elsa Bello-Reuss.[40] They were maintained in Dulbecco's modified Eagle's medium: Ham's F12 media supplemented with 8% fetal calf serum at 37°C and 5% CO_2 atmosphere. Experiments were performed on logarithmically growing cells (approximately 50–60% confluency). Cell viability was determined by trypan blue exclusion.

Animal preparation

Male 129Sv mice (6–8 weeks old) were anesthetized with 50 mg/kg body weight of sodium pentobarbital and a 50 min period of ischemia was induced by bilateral renal hilum clamping, as described earlier.[20] Sham operation was also performed without induction of ischemia. The clamps were removed and kidneys were harvested for Western blotting at various time points after re-establishing perfusion.

Protein lysates and Western blotting

Monolayers of TKPTS cells were lysed in a radioimmunoprecipitation assay buffer that contained 100 μg/ml phenylmethylsulfonyl fluoride (Sigma, St Louis, MO, USA), 100 mmol/l sodium orthovanadate (Sigma, St Louis, MO, USA), and 50 μl/ml of proteinase inhibitor cocktail (Sigma, St Louis, MO, USA) as described earlier.[2] Kidneys were homogenized in the same buffer as described earlier.[2,3,41] Protein content was determined by using a BioRad Protein Determination assay (BioRad Hercules, CA, USA) as described earlier.[2] Proteins (50–100 μg) from cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidine difluoride membrane (BioRad, Hercules, CA, USA). The filters were hybridized with the appropriate primary antibodies followed by a horseradish peroxidase-conjugated secondary antibody. The bands were visualized by an enhanced chemiluminescence method (Amersham) and quantified by densitometry (UnScan-It, Silk Scientific, Ore, UT, USA).

Adenoviral infection of TKPTS cells

TKPTS cells were grown in six-well-plates and incubated in the presence of 25 multiplicity of infection/ml adenovirus vector that contained a dominant-negative STAT3 construct[21] for 24 h at 37°C as described earlier.[2,3] Total cell lysates from these infected cells were prepared for later analysis. For survival studies the infected cells were treated with H_2O_2 for additional 24 h and cell counts were determined by trypan blue exclusion. Earlier we determined that the efficiency of infection was around 80% using a control (Ad-green fluorescent protein) adenovirus (unpublished data). We also determined that infection with a control adenovirus (Ad-green fluorescent protein) did not influence cell survival or activation of ERK.[2,3]

Fluorescence-activated cell sorter analysis

Cell cycle analysis was performed by propidium iodide staining as described earlier.[2,41] Briefly, TKPTS cells were collected after trypsinization and fixed in 70% ethanol overnight. After RNAse treatment, cells were incubated with 5 μg/ml propidium iodide and analyzed with a Becton Dickinson FACSCalibur analyzer. The cell cycle profile was analyzed using the CellQuest software.

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