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

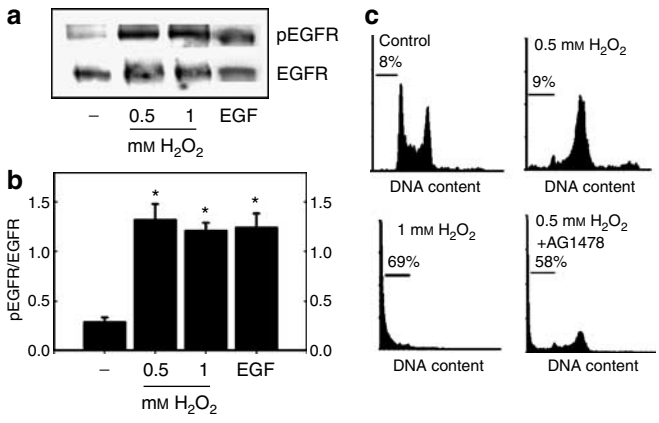

Figure 1 | EGFR activation after various levels of oxidant stress and its role in survival. (**a**) TKPTS cells were treated with either 0.5 or 1 mmol/l H_2O_2 or 10 ng/ml EGF for 30 min. Cell lysates were prepared and phosphorylation of EGFR determined by Western blotting using a phospho-EGFR (Tyr 1173) 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.

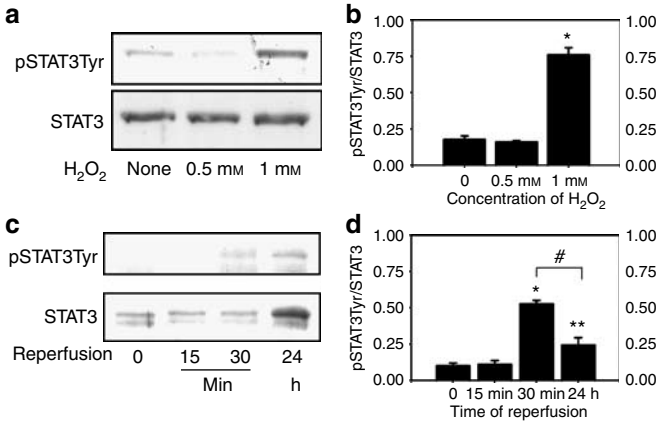

Figure 2 | Tyrosine 705 phosphorylation of STAT3 in TKPTS cells and in the kidney. (**a**) TKPTS cells were treated with 0.5 or 1 mmol/l H_2O_2 for 30 min. Tyr 705 phosphorylation of STAT3 (pSTAT3Tyr) was determined by Western blotting together with the total STAT3. Data shown are representatives of 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.