Activation of the Signal Transducer and Activator of Transcription Signaling Pathway in Renal Proximal Tubular Cells by Albumin

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Abstract. Renal proximal tubular cells activated by reabsorption of protein are thought to play significant roles in the progression of kidney diseases. It was hypothesized that the signal transducer and activator of transcription (STAT) proteins may be activated by proteinuria in proximal tubular cells. To test this hypothesis, murine proximal tubular cells were treated with albumin (30 mg/ml medium) for various lengths of time. The results showed that albumin could activate Stat1 and Stat5 within 15 min in proximal tubular cells. The activation of STATs was mediated mostly by Jak2 and required no protein synthesis. In addition, activation of Stat1 occurred even after neutralization of IFN-γ. The activation of STATs was inhibited by *N*-acetyl-L-cysteine, a precursor of glutathione and a reactive oxygen species (ROS) scavenger, and fluorescence-activated cell sorter analysis showed upregulation of intracellular ROS after albumin overloading, suggesting that albumin *per se* could generate ROS in proximal tubular cells. The activation of STATs occurred by way of the ROS generating system, and especially through the membrane-bound NADPH oxidase system. Reduced activities of glutathione peroxidase and catalase could also be responsible for the accumulation of intracellular ROS. Hence, not only the ROS generating system, but also the ROS scavenging system may contribute to the induction of ROS by albumin. These findings support the hypothesis that proximal tubular cells are activated and generate ROS by reabsorption of abundant urinary proteins filtered through the glomerular capillaries, and as a consequence, various IFN-γ–inducible proteins are synthesized through IFN-γ–independent activation of STAT signaling.

Janus kinase (JAK) and the signal transducer and activator of transcription (STAT) proteins were originally defined largely in the context of interferon (IFN) signaling (1–3). The JAK/STAT signaling pathway was also first defined in the same context. A total of seven different STAT family members (Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b, and Stat6) have now been identified in mammalian cells (4), and a large number of cytokines, growth factors, reactive oxygen species (ROS), and others are now known to trigger STAT activation (5–7). The fundamental roles of STATs in highly diverse biologic processes have been identified by using STAT knockout mice and/or by tissue-specific deletions (8,9). These processes include innate and adaptive immune function, embryonic development, cell differentiation, cell proliferation, survival, and apoptosis (10–14), and the STAT family has become a therapeutic target in human cancer (15).

Renal proximal tubular cells play central roles in various kidney diseases by producing chemokines such as regulated upon activation, normal T cell expressed and secreted (RANTES) (16), and this is more pronounced in the presence of IFN-γ than IL-1 or TNF-α (17). Moreover, the level of proteinuria, which is independent of mean arterial BP, is reportedly one of the best predictors for disease progression toward end-stage renal failure (18,19). Microalbuminuria is known as an important early sign of diabetic nephropathy (20,21) and of progressive loss of renal function in the nondiabetic population (22). Recent studies have shown that proximal tubular cells are activated by reabsorption of abundant urinary proteins filtered through the glomerular capillaries, producing various chemokines that lead to kidney disease progression (23). Identification of the signal transduction pathway in activated proximal tubular cells is, however, still incomplete.

To study changes in *in vivo* gene expression in proximal tubular cells caused by proteinuria, we constructed an expression profile of proximal tubular cells isolated from an albumin-overloaded proteinuria mouse model by use of the body map procedure (24–29). These data showed that the expression patterns in proximal tubular cells were changed dramatically

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by proteinuria. In view of the renal damage caused by proteinuria, it is of considerable interest that several immunity-related genes, including interferon regulatory factor–1, major histocompatibility complex (MHC) class I, MHC class II, and monocyte chemoattractant protein–1 (MCP-1), were found to be upregulated (26). These proteinuria-induced genes are likely to participate in kidney disease progression, and it is noteworthy that almost all of them are also induced by IFN-γ (30).

Although IFN-γ is essentially a cytokine with direct antiviral activity, its properties also include regulation of the immune response, antigen presentation of phagocytes through the MHC class I and II pathways, and orchestration of leukocyte-endothelium interactions through an intermediary in the JAK/STAT signaling pathway (30). Hence, we were interested in the relationship between the STAT family and proteinuria-initiated gene expression in proximal tubular cells.

In previous *in vitro* experiments, protein-overloaded proximal tubular cells were found to activate transcription of a number of genes encoding inflammatory molecules (31,32). However, the signaling pathways involved have not been analyzed in detail. Because it is likely that many inflammatory cytokines have various effects on the later stages of these pathways, we have focused mainly on the early stage to avoid such complications.

In this report, we present our results showing that albumin *per se* can generate ROS in proximal tubular cells, resulting in the activation of the STAT signaling pathway. We suggest that the membrane-bound NADPH oxidase system is important as an ROS generating system. Albumin-induced activation of Stat1 and Stat5 in murine proximal tubular cells (mProx24) cells was found to be mediated mostly by Jak2, without the need for protein synthesis.

Materials and Methods

Antibodies

The Stat1, Stat3, Stat5, phospho-Stat1 (Y701), phospho-Stat3 (Y705) and phospho-Stat5 (Y694) antibodies were purchased from Cell Signaling (Beverly, MA). The Jak1, Jak2, and phospho-Jak2 (Y1007/Y1008) antibodies were obtained from Upstate Biotechnology (Lake Placid, NY), and the phospho-Jak1 (Y1022/Y1023) antibody from Affinity BioReagents (Golden, CO). The Tyk2 and phospho-Tyk2 (Y1054/Y1055) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-murine IFN-γ antibody from Peprotech (Princeton, NJ).

Chemicals

Cycloheximide (CHX) and AG490 were purchased from Calbiochem (La Jolla, CA). Protein A agarose beads, nylon membranes (hybond-P) for Western blot test and, Hyperfilm ECL films were obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK) and nylon membranes (Biodyne B) for electrophoretic mobility shift assays from Pall (East Hills, NY). The Pierce Supersignal substrate chemiluminescence detection kit and the BCA Protein Assay Reagent kit were purchased from Pierce Biotechnology (Rockford, IL). 5- (and 6-) Chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) was purchased from Molecular Probes (Eugene, OR), FCS and L-glutamine from InVitrogen (Carlsbad, CA), and Complete Mini from Boehringer Mannheim (Mannheim, Germany). Complete Mini tablets inhibit a broad spectrum of serine, cysteine, and metalloproteases as well as calpains. BSA (Fraction V, IgG free, low endotoxin), apo-transferrin (apoTf), *N*-acetyl-L-cysteine (NAC), diphenylene iodonium chloride (DPI), and all other chemicals were purchased from Sigma Chemical (St. Louis, MO).

Cells and Cell Culture

The murine proximal tubular cells (mProx24 cells derivative; patent WO9927363, JP, US, EU) (33,34) were cultured in DMEM/F-12 medium containing 10% FCS. The mProx24 cells were examined beforehand to determine whether they could produce a valid activation of STATs in response to IFN-γ and IL-6. We used FITC-labeled BSA to confirm their ability to generate albumin endocytosis (data not shown). In addition, we verified the upregulation of some genes induced by IFN-γ when albumin was added to the medium (data not shown).

Confluent mProx24 cells were rinsed three times with serum-free medium and starved in the serum-free medium for 24 h to eliminate the effect of FCS. mProx24 cells were then treated with albumin (30 mg/ml medium). Albumin mediums were filtered with a 0.22-μm filter unit (Millipore, Carrigtwohill, County Cork, Ireland) and polymyxin B was added suppress the influence of lipopolysaccharide. When drugs or antibodies were added, mProx24 cells were pretreated with the drugs or antibodies for 1 h before exposure to albumin. The concentration (0.05 ng/ml) of anti–IFN-γ antibody was determined according to the manufacturer's instructions. This concentration resulted in a valid inhibition of Stat1-activation by IFN-γ (data not shown).

Trypan Blue Uptake Test

To evaluate cell viability by trypan blue dye exclusion, mProx24 cells were treated with the antioxidants, NAC (20 mM), rotenone (10 μM), or DPI (10 μM) 1 h before and during the 30-min incubation with albumin (30 mg/ml). At the end of the stimulation, the cells were rinsed three times with ice-cold PBS, detached with trypsin, and resuspended in medium diluted 1:20 with trypan blue solution. Live cells and stained dead cells were then counted by a hemocytometer.

Immunoprecipitation, SDS-PAGE, and Western Blot Analysis

At the end of the treatment with albumin, the cells were rinsed three times with ice-cold PBS with 1 mM Na_3VO_4, solubilized in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP40, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF, Complete Mini), and rotated for 1 h at 4°C. The total cell lysates were centrifuged at 14,000 × *g* for 10 min at 4°C. They were then incubated with the relevant antibody on ice for 2 h, and the antibody complexes were collected on protein A agarose beads during a 1-h incubation at 4°C. The beads were washed three times with the lysis buffer and boiled for 20 min in SDS-PAGE sample buffer. A total of 50 μg of each sample was separated by 7% SDS-PAGE and transferred to a nylon membrane (Hybond-P), where it was blocked by a 30-min incubation at room temperature in TPBS (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.05% Tween-20) plus 5% BSA and incubated overnight at 4°C with the relevant primary antibody. Subsequently, the membranes were washed three times for 5 min each with TPBS and incubated for 30 min with HRP-conjugated goat anti-rabbit IgG. After extensive washing, the bound antibody was visualized on Hyperfilm ECL film. Membranes were then incubated at 55°C for 30 min in stripping buffer (100 mM 2-ME, 2% SDS, 62.5

mM Tris-HCl, pH 6.8) to prepare them for a second round of immunoblotting.

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA)

After the indicated treatment, the cells were rinsed three times with ice-cold PBS containing 1 mM Na_3VO_4, scraped from the dish and pelleted at 14,000 × *g* for 1 min at 4°C. Cells were then resuspended in the same buffer and pelleted as described above. The resultant pellets were resuspended in 400 μl of ice-cold buffer A (10 mM HEPES-KOH pH 7.8, 10 mM KCl, 0.1 mM EDTA pH 8.0, 0.1% NP-40, 1 mM DTT, 0.5 mM PMSF, 1 mM Na_3VO_4, Complete Mini). The lysate was vortexed vigorously for 20 s, and the nuclei were pelleted at 14,000 × *g* for 1 min at 4°C, followed by resuspension of the nuclear pellets in 100 μl of buffer C (50 mM HEPES-KOH pH 7.8, 420 mM KCl, 0.1 mM EDTA pH 8.0, 5 mM $MgCl_2$, 20% glycerol, 1 mM DTT, 0.5 mM PMSF, 1 mM Na_3VO_4, Complete Mini) and rotation for 1 h at 4°C. The extracted proteins were separated from the residual nuclei at 14,000 × *g* for 15 min at 4°C, and the supernatant fractions were used as nuclear extracts. EMSA was performed with the Panomics' EMSA Kit (Panomics, Redwood City, CA) according to the manufacturer's instructions.

Assessment of Intracellular ROS

Intracellular ROS generation was assessed in mProx24 cells by means of an oxidant-sensitive dye, CM-H_2DCFDA. Suspensions of the cells (10^6 cells) were incubated with 5 μM CM-H_2DCFDA for 15 min at 37°C in the serum-free medium. After centrifugation and washing to remove unincorporated probe, cells were treated with several concentrations of albumin medium for 15 min at 37°C and placed on ice. Mean fluorescence intensity of DCF in the cells was measured by a flow cytometer (FACSCalibur; Becton Dickinson Biosciences, Franklin Lakes, NJ).

Measurement of SOD, Glutathione Peroxidase, and Catalase Activities, and the Total Reduced Form of Glutathione

The SOD activity was measured with a Superoxide Dismutase Assay Kit (Trevigen, Gaithersburg, MD). The cellular glutathione peroxidase (GPx) activity was determined with a Bioxytech GPx-340 (OxisResearch, Portland, OR). The catalase activity was measured with an Amplex Red Catalase Assay Kit (Molecular Probes, Eugene, OR). The total reduced form of glutathione (GSH) was determined with a Bioxytech GSH-400 (OxisResearch). Each of the kits was used according to the manufacturer's instructions.

Results

Activation of STATs in mProx24 Cells by Albumin

We first investigated whether members of the STAT family were activated by albumin. mProx24 cells were treated with albumin (30 mg/ml medium) for various lengths of time. Western blot analyses showed that Stat1 and Stat5 were rapidly activated within 15 min of exposure to albumin, with Stat5 in particular showing early and obvious activation. Stat3, however, demonstrated no detectable activation after 15 min (Figure 1A). To confirm Stat1 and Stat5 activation, nuclear extracts were prepared from the albumin-treated cells and analyzed by EMSA. This approach also showed rapid activation of Stat1 and Stat5 within 15 min of exposure to albumin. These results were finally confirmed by competition and supershift analyses (Figure 1B). To verify the later activation of Stat3 after albumin overloading, mProx24 cells were treated with albumin for longer lengths of time. Western blot analyses showed that Stat3 activation occurred only after a 4-h exposure to albumin (Figure 1C).

Activation of Albumin-Induced STATs through Jak2

The rapid phosphorylation of Stat1 and Stat5 indicated that albumin is, like cytokines, a direct stimulant of the JAK family. We examined the time courses of the phosphorylation of Jak1, Jak2, and Tyk2 in albumin-overloaded mouse mProx24 cells. Western blot analyses showed that rapid activation of Jak2 took place within 15 min of exposure to albumin, compared with no obvious activation of Jak1 or Tyk2 (Figure 2A). To confirm that the activation of Stat1 and Stat5 was under the control of Jak2, we tested the effect of AG490 (20 μM), a specific inhibitor of Jak2. The results in Figure 2B indicate that AG490 inhibited the activation of Jak2 and prevented the activation of Stat1 and Stat5, compared with the control. Given the strong evidence that JAK kinases are *in vivo* STAT kinases (35), our results suggest that the albumin-induced activation of Stat1 and Stat5 in mProx24 cells was mediated mostly by Jak2.

Moreover, to investigate whether protein synthesis was involved in the activation of Stat1 and Stat5, we tested the effect of CHX (2 μg/ml), a protein synthesis inhibitor. The results in Figure 3A indicate that CHX did not affect the albumin-induced Tyr-phosphorylation of STATs within 15 min, thus demonstrating that the albumin-induced activation of Stat1 and Stat5 occurred without protein synthesis.

Although the JAK/STAT signaling pathway appeared to be activated by albumin *per se*, some cytokines and/or growth factors may have been involved in this phenomenon. Hence, the effect of an inhibitory antibody on the JAK/STAT signaling pathway was examined. It is well known that Jak2 fulfills an essential function in response to IFN-γ (36). We therefore used an anti–IFN-γ antibody (0.05 ng/ml) as a representative cytokine-neutralization antibody to check whether albumin could induce IFN-γ secretion from mProx24 cells. mProx24 cells were pretreated with anti-IFN-γ antibody for 1 h before exposure to albumin. The results indicated that anti–IFN-γ antibody did not prevent the albumin-induced Tyr-phosphorylation of Stat1, suggesting that the albumin-induced activation of Stat1 was not a direct effect of IFN-γ (Figure 3B).

Activation of STATs in mProx24 Cells Was Not Specific to Albumin

To investigate whether the activation of the STATs was specific to albumin, mProx24 cells were treated with apoTf (30 mg/ml medium) for 15 min. ApoTf is one of the major components of plasma proteins and consists of transferrin that is not saturated with iron. Western blot analyses showed that there was also rapid activation of Stat1 and Stat5 within 15 min of exposure to apoTf (Figure 4), indicating that the activation of the STATs was not albumin specific.

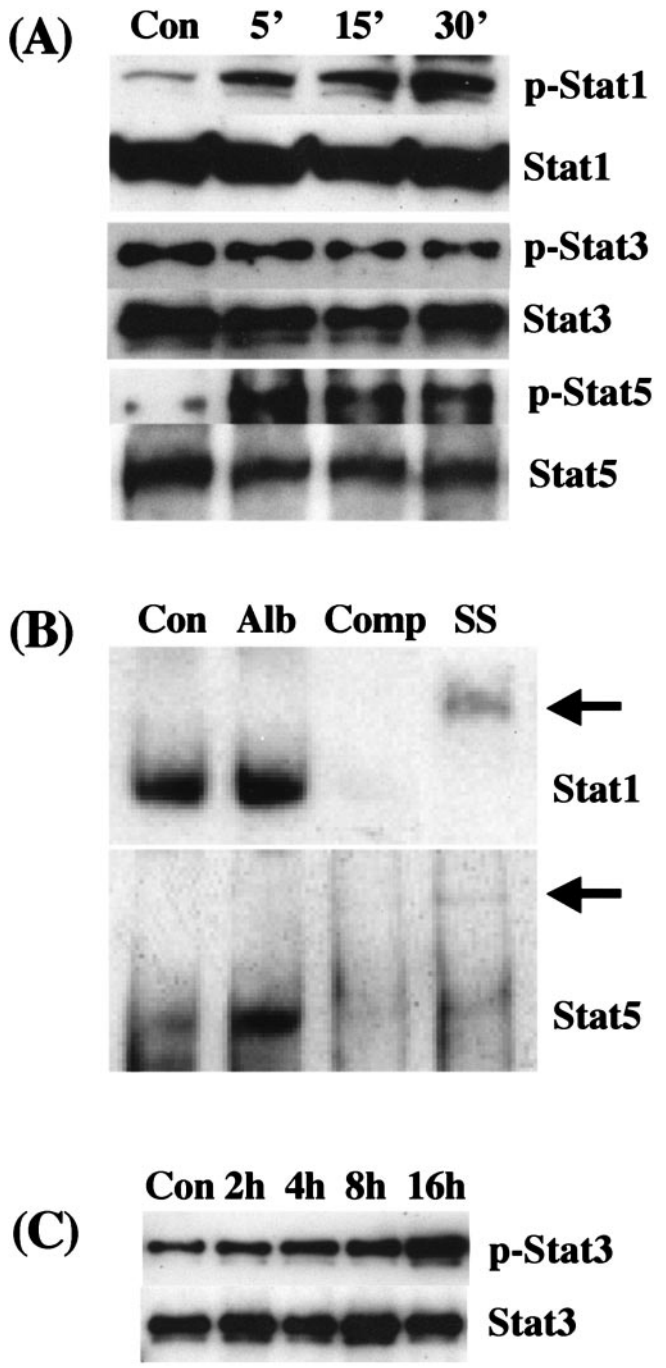

Figure 1. Western blot analysis and electrophoretic mobility shift assays (EMSA) of activated Stat1 and Stat5 in mouse renal proximal tubular cells (mProx24 cells) treated with albumin. mProx24 cells were treated with albumin for various time periods. (A) Western blot analyses detected phosphorylated Stat1 and Stat5 15 min after addition of albumin, indicating that albumin could induce signal transducer and activator of transcription (STAT) signaling pathways. Stat3 showed no significant changes. Western blot analyses of Stat1, Stat3, and Stat5 are shown as controls. (B) EMSA confirmed rapid activation of Stat1 and Stat5 within 15 min of exposure to albumin. These results were confirmed by competition (Comp) and supershift (SS) analyses by using control samples. Band shifts are indicated by arrows. (C) Western blot detection of phosphorylated Stat3 4 h after addition of albumin. Western blot analysis of Stat3 are shown as controls. Three independent experiments were performed, and results are shown for one representative experiment.

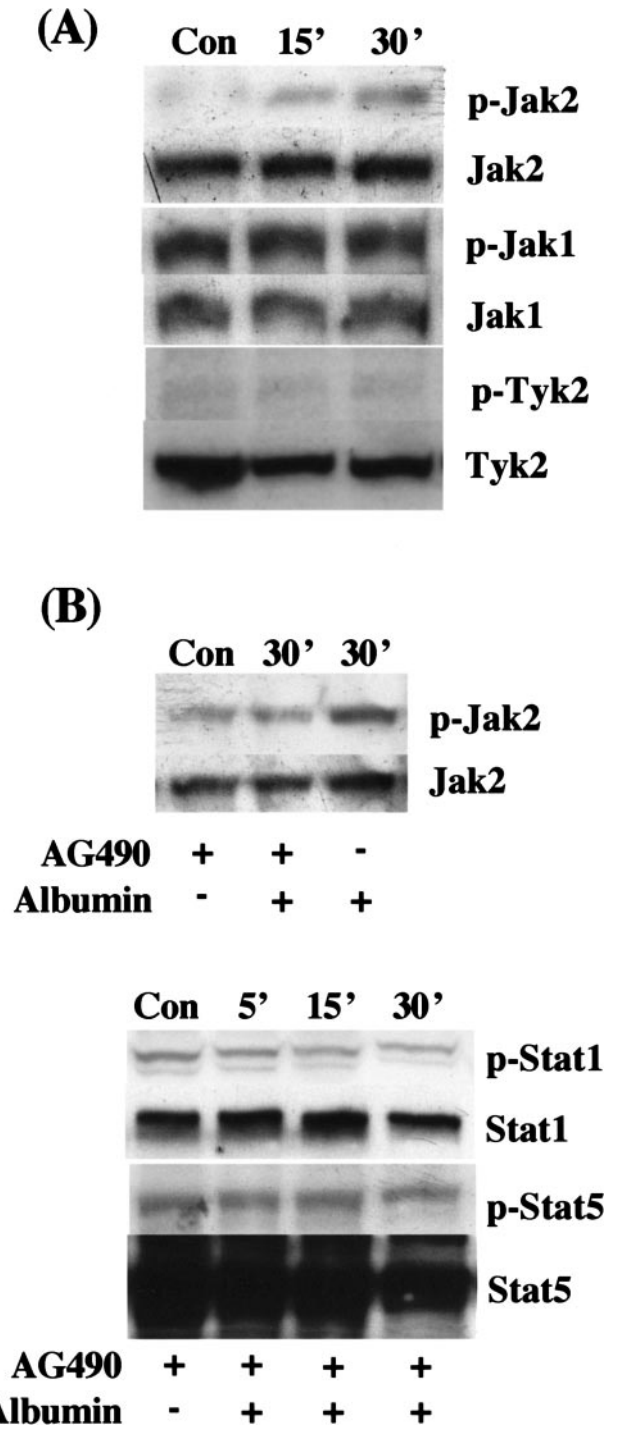

Figure 2. Activation of Jak2 in mouse renal proximal tubular (mProx24) cells treated with albumin mediates the activation of Stat1 and Stat5. (A) Phosphorylated Jak2 was detected 15 min after addition of albumin, demonstrating that albumin induced JAK signaling pathways. Jak1 and Tyk2 showed no significant changes. Western blot results for Jak2, Jak1, and Tyk2 are shown as controls. (B) mProx24 cells were transferred to a medium containing albumin (30 mg/ml medium) in the presence of AG490 (20 μM). AG490 inhibited the activation of Jak2 (only control and 30-min data shown) and prevented the activation of Stat1 and Stat5 compared with the control, indicating that albumin-induced activation of Stat1 and Stat5 was mediated mostly by Jak2. Western blot analyses for Stat1 and Stat5 are shown as controls. Three independent experiments were performed, and results are shown for one representative experiment.

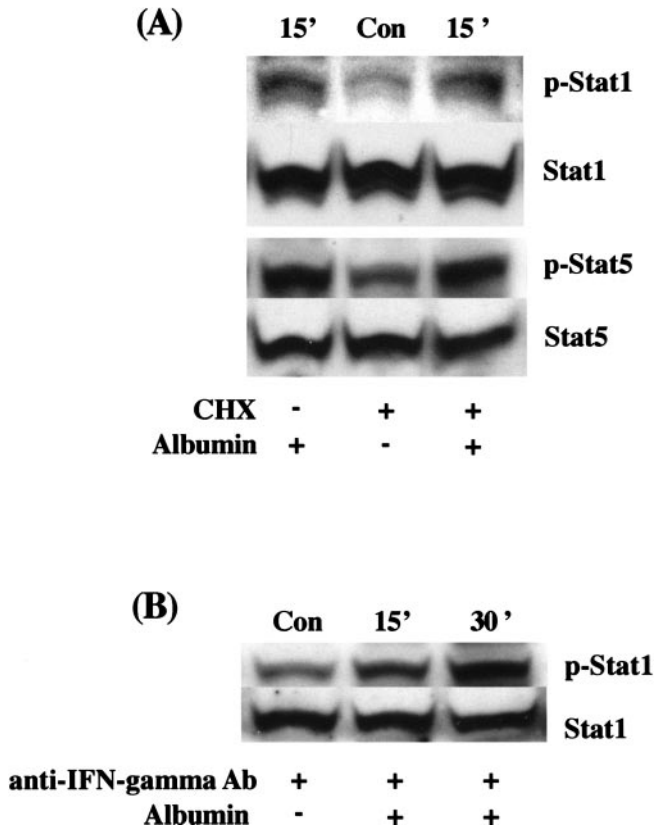

Figure 3. Effects of cycloheximide or anti–IFN-γ antibody on albumin-induced Stat1 and Stat5 activation. Mouse renal proximal tubular (mProx24) cells were transferred to a medium containing albumin (30 mg/ml medium) in the presence of cycloheximide (2 μg/ml) or anti–IFN-γ antibody (0.05 ng/ml). (A) Cycloheximide could not inhibit albumin-induced activation of Stat1 and Stat5. (B) Anti–IFN-γ antibody could not inhibit albumin-induced activation of Stat1. Three independent experiments were performed, and results are shown for one representative experiment.

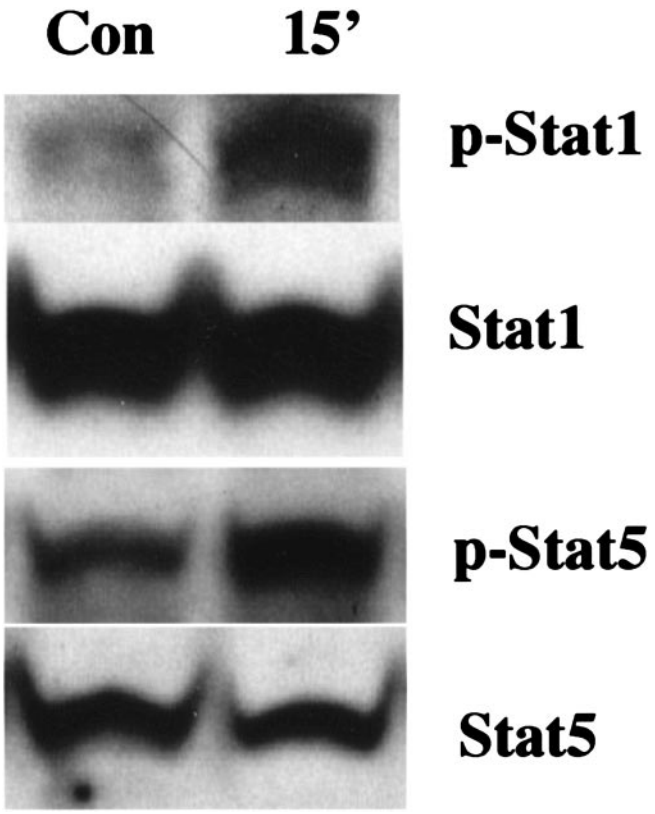

Figure 4. Western blot analyses of activated Stat1 and Stat5 in mouse renal proximal tubular (mProx24) cells treated with addition of apoTf. Phosphorylated Stat1 and Stat5 were detected 15 min after addition of apoTf, indicating that apoTf induced signal transducer and activator of transcription (STAT) signaling pathways. Western blot analyses for Stat1 and Stat5 are shown as controls. Three independent experiments were performed, and results are shown for one representative experiment.

Assessment of ROS Generation by Albumin and apoTf

Recently, it was reported that albumin upregulated ROS generation in proximal tubular cells (37,38). Therefore, to directly assess whether albumin could induce oxidative stress in mProx24 cells, we used the CM-H_2DCFDA method and fluorescence-activated cell sorter (FACS) analysis. After CM-H_2DCFDA incubation, mProx24 cells were treated with albumin for 15 min. Accumulation of DCF in mProx24 cells was measured with a flow cytometer by monitoring the fluorescence at 526 nm (39). ROS generation was found to be upregulated compared with the control within 15 min after either albumin or apoTf had been added to the medium (Figure 5). Dose-dependent upregulation of intracellular ROS generation was observed, as previously reported (37). The result was not changed by addition of DMSO, which was used as the solvent for AG490, rotenone, and DPI (data not shown).

To inhibit ROS generation by albumin, we checked the effect of NAC (20 mM), a precursor of GSH and a ROS scavenger, rotenone (10 μM), an inhibitor of complex I of the mitochondrial respiratory chain, and DPI (10 μM), an inhibitor of membrane NADPH oxidase. All three were able to block ROS generation by albumin (Figure 5). These results supported our finding that albumin could induce oxidative stress in mProx24 cells. ROS generation was observed even in the control mProx24 cells after starvation, compared with the findings with antioxidants.

Activation of Albumin-Induced STATs through ROS

FACS analyses showed that albumin-induced ROS generation could be blocked by the three antioxidants. To determine whether the activation of Stat1 and Stat5 occurred by way of oxidative stress, we first tested the effect of NAC (20 mM). The results presented in Figure 6 indicate that NAC prevented albumin-induced Tyr-phosphorylation of Stat1 and Stat5 within 15 min (Figure 6, A and B). Moreover, even the activation of Stat1 and Stat5 in the control cells was suppressed by pretreatment with NAC (Figure 6A). This suggests that the activation of Stat1 and Stat5 could take place through the upregulation of ROS generation.

The mitochondrial respiratory chain is one of the major intracellular sources of ROS and indispensable to cell function.

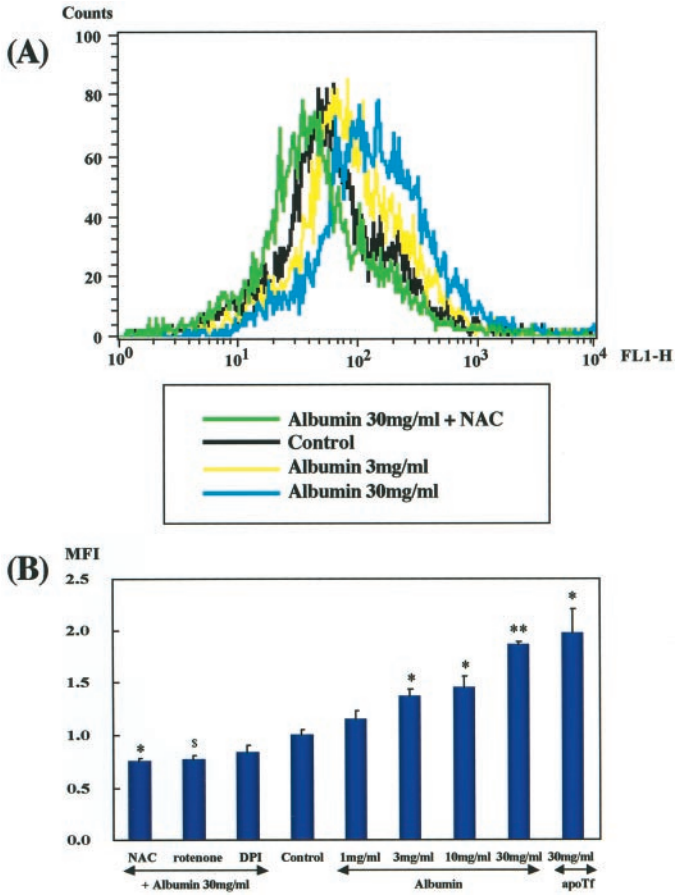

Figure 5. Assessment of reactive oxygen species (ROS) by fluorescence-activated cell sorter analysis. After CM-H_2DCFDA incubation, mouse renal proximal tubular (mProx24) cells were treated with albumin for 15 min. Accumulation of DCF was measured with a flow cytometer by monitoring fluorescence at 526 nm. The increase was assumed to be proportional to the concentration of superoxide anions and hydrogen peroxide in the mProx24 cells. (A) Results are shown for one representative experiment. ROS generation was observed even in the control mProx24 cells (black line). The ROS generation was upregulated within 15 min after albumin (yellow line; 3 mg/ml, blue line; 30 mg/ml) overloading, but NAC (green line), a precursor of glutathione (GSH) and a ROS scavenger, blocked ROS generation. (B) Intracellular ROS formation was expressed as a ratio of the mean fluorescence intensity of control cells incubated in an albumin-free medium. Results are the means ± SD of triplicate experiments. $\$P <$ 0.01 *versus* control cells. $*P < 0.005$ *versus* control cells. $**P <$ 0.001 *versus* control cells.

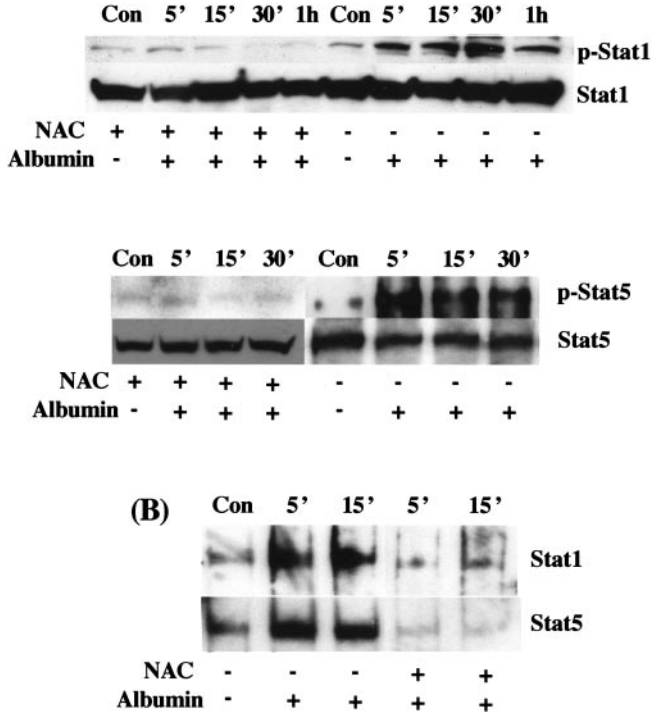

Figure 6. Effects of *N*-acetyl-L-cysteine (NAC) on albumin-induced Stat1 and Stat5 activation. Mouse renal proximal tubular (mProx24) cells were transferred to a medium containing albumin (30 mg/ml medium) in the presence of NAC (20 mM). (A) Western blot analyses showed that activation of Stat1 and Stat5 was inhibited by NAC, indicating that the activation of Stat1 and Stat5 is mediated by reactive oxygen species (ROS). Western blot analyses for Stat1 and Stat5 are shown as controls. (B) Electrophoretic mobility shift assay (EMSA) also confirmed the inhibition of Stat1 and Stat5 by NAC. Three independent experiments were performed, and results are shown for one representative experiment.

We investigated the involvement of mitochondria in the albumin-induced ROS with an inhibitor of the mitochondrial respiratory chain, rotenone (10 μM). The results showed that rotenone prevented the albumin-induced Tyr-phosphorylation of Stat1 and Stat5 within 15 min, compared with the positive control (Figure 7, A and B). Pretreatment with rotenone also suppressed the activation of Stat1 and Stat5 in the control cells (Figure 7B). This indicated that inhibition of the mitochondrial respiratory chain could suppress ROS generation by albumin or by starvation.

Intracellular generation of ROS in response to ligands is often mediated by the activity of membrane-bound NADPH oxidase (40,41). DPI (10 μM) also prevented the albumin-induced Tyr-phosphorylation of Stat1 and Stat5 (Figure 7, A and C), but did not seem to suppress the activation of Stat1 and Stat5 in the control cells (Figure 7C). This suggests that ROS generated by albumin are mainly derived from the membrane-bound NADPH oxidase system.

Because pretreatment with NAC and rotenone even suppressed the activation of Stat1 and Stat5 in the control cells, we evaluated the cell viability in the presence of NAC, rotenone and DPI by trypan blue dye exclusion. The antioxidants did not have much effect on cell viability, at least during the 30-min incubation with albumin (30 mg/ml) (Table 1).

Measurement of SOD, GPx, and Catalase Activities

The membrane-bound NADPH oxidase system produces superoxide anions in cells, and intracellular SOD is the main enzyme that metabolizes these anions. Because interference with the SOD activity could induce accumulation of superoxide anions, we measured the SOD activity after mProx24 cells had been treated with albumin for 10 min. No marked change

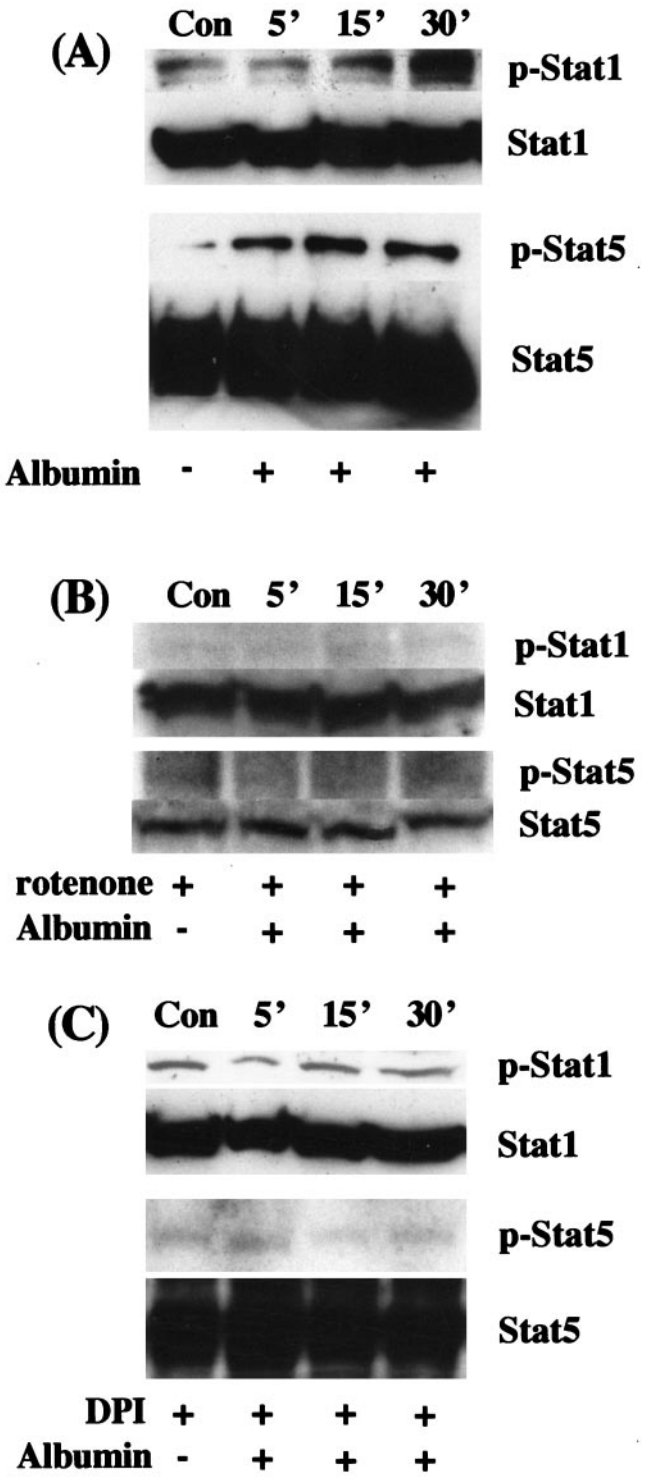

Figure 7. Effects of rotenone or diphenylene iodonium chloride (DPI) on albumin-induced Stat1 and Stat5 activation. Mouse renal proximal tubular (mProx24) cells were transferred to a medium containing albumin (30 mg/ml medium) in the presence of rotenone (10 μM) or DPI (10 μM). Western blot analyses showed that activation of Stat1 and Stat5 was inhibited by either rotenone (B) or DPI (C), compared with the positive control (A), indicating that the activation of Stat1 and Stat5 was mediated by reactive oxygen species (ROS). Western blot analyses for Stat1 and Stat5 are shown as controls. Three independent experiments were performed, and results are shown for one representative experiment.

Table 1. Viable cell count by trypan blue dye exclusion

	% of Control
Control	100.00
Albumin	103.84 ± 3.18
Albumin + NAC	102.29 ± 2.96
Albumin + rotenone	102.67 ± 4.42
Albumin + DPI	99.66 ± 2.12

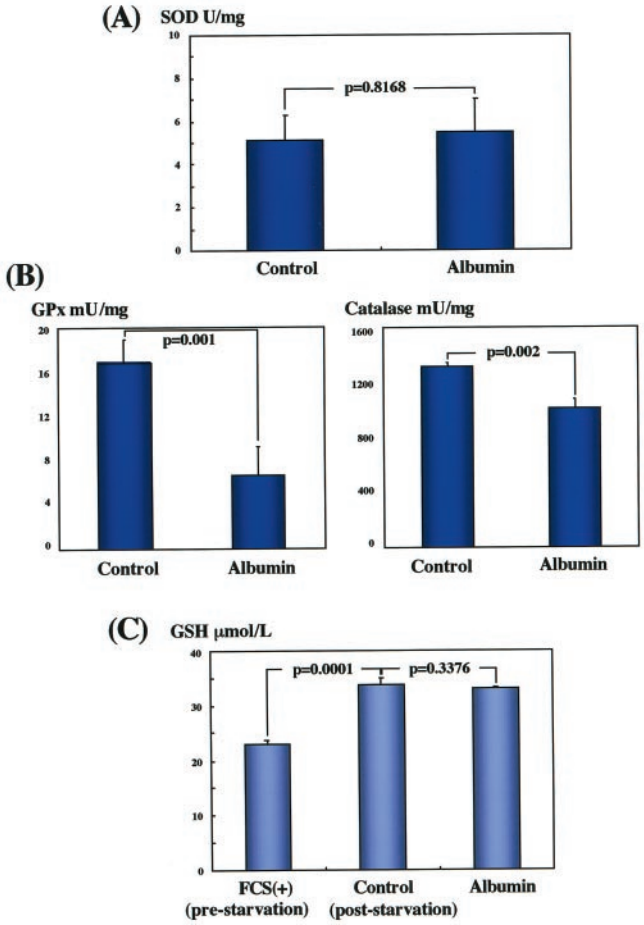

Figure 8. Measurement of SOD, glutathione peroxidase (GPx), and catalase activity, and the total reduced form of Glutathione. (A) SOD activity was measured after 10-min albumin treatment of mouse renal proximal tubular (mProx24) cells, but showed no significant change. (B) GPx and catalase activities were measured after 10-min albumin treatment of mProx24 cells. Both GPx and catalase activities were reduced after albumin overloading. (C) Quantity of total glutathione (GSH) was determined after 10-min albumin treatment of mProx24 cells. The quantity increased after starvation but showed no significant change after treatment with albumin compared with control. The histograms represent the means of three independent experiments (means ± SD).

was observed (Figure 8A). These results indicate that altered SOD activity was not responsible for the accumulation of intracellular ROS after exposure of mProx24 cells to albumin.

SOD produces hydrogen peroxide in cells, and intracellular GPx and catalase are the main enzymes that convert hydrogen

peroxide into water to prevent production of hydroxyl radicals. Because reduction of GPx and catalase activities could induce accumulation of hydrogen peroxide, we measured the GPx and catalase activities after mProx24 cells had been treated with albumin for 10 min. Figure 8B showed that both enzymatic activities were reduced by albumin. These results indicate that changes in GPx and catalase activities could be responsible for the accumulation of intracellular ROS after exposure of mProx24 cells to albumin.

Measurement of the Total Reduced Form of Glutathione

Generally, intracellular proteins do not contain disulfide bonds because the high cytosolic concentration of free sulfhydryl (-SH) reducing agents breaks such bonds (42). We hypothesized that a large amount of proteins with disulfide bonds, such as albumin and apoTf, might lead to oxidative stress when ingested into cells. To address this, we measured the shift in intracellular GSH levels. GSH is a naturally occurring tripeptide whose nucleophilic and reducing properties play a central role in the antioxidant system of most aerobic cells. GSH is crucial to the maintenance of the -SH level of proteins in cells (43). Hence, we determined the quantity of the total reduced form of GSH under various conditions of albumin loading. The quantity of GSH increased after albumin starvation, but did not show any marked change compared with control cells after treatment with albumin for 10 min (Figure 8C). Our findings suggest that the increase in GSH was induced by long-term upregulation of intracellular ROS, and that GSH was not consumed for at least 10 min after the cells were exposed to albumin.

Discussion

We have previously constructed gene expression profiles by means of direct sequencing procedures (26,29), and identified several immunity-related genes, such as MHC class I and II, by comparing the profiles of control and disease model proximal tubular cells. Renal MHC proteins are expressed in response to inflammation and renal injury, and their expression is thought to be regulated solely by IFN-γ (44). Because the IFN-γ pathway usually requires the activation of Stat1 (45,46), we hypothesized that proteinuria could activate proximal tubular cells directly by activation of the STAT signaling pathway. Our results indicate that albumin activates proximal tubular cells in an IFN-γ–independent manner after ROS-mediated STAT activation.

Our data also demonstrate that Stat1/5 and Stat3 show different responses to albumin. The activation of Stat1 and Stat5 was clearly identified within 15 min. In contrast, no manifest activation of Stat3 could be identified after a 1-h exposure to albumin, and activation of Stat3 was observed only after a 4-h exposure. The mechanisms responsible for these different responses are still obscure, but the differences may depend on the cell type. In macrophages, Stat1 has been reported to induce sterilizing activity and the production of various complements, chemokines, and adhesion molecules (3). Stat5, which was originally identified as a mammary gland factor regulated by prolactin, is also activated by multiple cytokines, including IL-2, IL-3, and GM-CSF, which are macrophage-activating factors. In contrast, IL-10–induced Stat3 activation suppresses macrophage activity, and mice lacking Stat3 show abnormally enhanced macrophage activity and develop chronic inflammation (47). The different responses of Stat1/5 and Stat3 in the renal proximal tubular cell line seem to be similar to those in macrophages, suggesting that albumin overloading can induce proinflammatory processes. Stat3 is activated by various cytokines, such as IL-6, that are also induced by IFN-γ (30). Therefore, the activation of Stat3 at a late stage after albumin overloading might be due to a secondary response after Stat1 activation by albumin.

ROS generation was even observed in the control mProx24 cells after starvation, leading to a slight activation of Stat1 and Stat5. This is a previously reported result that supports our finding that serum deprivation can induce elevated ROS and an oxidative state in cells (48). Nuclear factor kappa B (NF-κB) is also induced by ROS (38) and is one of the key components that cause immune reactions in kidneys (49). A recent report of a new and interesting Stat5 signal transduction mechanism suggests that Stat5 could be a potential activator of NF-κB in some types of hematopoietic cells (50). In addition, many IFN-γ–inducible genes, such as RANTES and MCP-1, were found to be induced synergistically by IFN-γ and TNF-α (51). This synergism is reportedly involved in the interaction between activated Stat1 and NF-κB (11,30). Hence, the activation of Stat1 and Stat5 by albumin may be important in the induction of transcripts that cause kidney disease progression.

Recent reports have demonstrated rapid and significant increases in intracellular ROS after growth factor or cytokine stimulation. These types of ROS appear to be essential for a host of downstream signaling events, including cell proliferation, apoptosis, and inflammation, and thus contribute to the development of diseases (52). Simon *et al.* (7) reported that members of the STAT family of transcription factors were activated in fibroblasts and A-431 carcinoma cells in response to H_2O_2. This activation occurred within 5 min, could be inhibited by antioxidants, and did not require protein synthesis. These findings hence indicate that the JAK/STAT pathway responds to intracellular ROS.

Our results indicate that the membrane-bound NADPH oxidase system seems to be relevant in the induction of ROS by albumin. It has been found that the activity of a membrane-bound NADPH oxidase often mediates intracellular generation of ROS in response to ligands (40,41). The ROS in macrophages are also produced by NADPH oxidase after phagocytosis (53). Hence, it is particularly interesting that the endocytic uptake of albumin in renal proximal tubular cells seems also to be responsible for ROS generation. Further investigation is needed, however, to clarify the precise mechanisms of ROS generation by NADPH oxidase after albumin overloading. Our results also indicate that the GPx and catalase activities changed within 10 min after albumin loading, suggesting that albumin could interfere with GPx and catalase activities by way of glia maturation factor–beta (54). The low activities of GPx and catalase could be responsible for the accumulation of the intracellular ROS after albumin ingestion into mProx24 cells. Hence, it seems that not only the ROS generating system but also the ROS-scavenging system may contribute to the induction of ROS by albumin.

We next focus on the amino acid sequences of albumin and apoTf. The amino acid sequences of human, bovine, and rodent albumin are highly homologous. Albumin itself contains a high percentage of cysteine that is organized into a characteristic repeating disulfide pattern (55). Generally, intracellular proteins do not contain disulfide bonds because the high cytosolic concentration of free sulfhydryl (−SH) reducing agents breaks such bonds (42). We hypothesized that a large amount of proteins with disulfide bonds, such as albumin and apoTf, might cause oxidative stress when ingested into the cells. However, our data indicate that the amount of GSH was not changed for at least 10 min after albumin overloading, suggesting that disulfide bonds are not a primary cause of ROS induction.

Several mechanisms have been proposed by which ROS might activate intracellular kinases such as Jak2. One of these mechanisms is inactivation of protein tyrosine phosphatases (PTPs) that may be susceptible to oxidation (56). Tyrosine phosphorylation of proteins is dependent on the balance between kinases and PTPs within a cell. Therefore, it is feasible that inactivation of PTPs could induce tyrosine phosphorylation of Jak2. Another possible mechanism involves Fyn, a member of the *src* family, which can regulate the activation of Jak2 by H_2O_2 in fibroblasts (57). These proposed mechanisms need to be verified in future studies.

The fact that almost all cytokines activate the JAK/STAT pathway suggests that STATs play important roles in the inflammatory process. Our results suggest that albumin, a major component of proteinuria, exerts cytokine-like effects to activate proximal tubular cells after ROS-mediated STAT activation. The activated STAT signaling could then result in cell proliferation, production of several cytokines/growth factors, and induction of immune responses that are presumably involved in the progression of kidney diseases. The results reported here could thus provide an insight into the intrinsic toxicity of proteinuria and may provide further clues regarding therapeutic approaches to inhibition of the signal transduction pathways involved in proteinuria.

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