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Immunoglobulin Light Chains Activate Tubular Epithelial Cells through Redox Signaling

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ABSTRACT

The renal proximal tubule metabolizes circulating low-molecular-weight proteins such as Ig free light chains. In the setting of plasma cell dyscrasias, the burden of filtered protein can be very high. Endocytosis of certain nephrotoxic light chains induces H_2O_2 production and monocyte chemoattractant protein-1 (MCP-1) release, leading to recruitment of inflammatory cells and interstitial fibrosis, but how these processes are linked mechanistically is not well understood. This study investigated the relationship between H_2O_2 generated after light chain endocytosis by human proximal tubular (HK-2) cells and activation of c-Src, a redox-sensitive tyrosine kinase. HK-2 cells exposed to two different light chains upregulated c-Src activity, which increased the production of MCP-1. In parallel, we observed a time-dependent oxidation of c-Src. Inhibition of c-Src activity and silencing c-Src expression abrogated the light chain–induced MCP-1 response, but had no effect on H_2O_2, indicating that production of H_2O_2 is upstream of c-Src in the signaling cascade. Silencing megalin and cubilin expression inhibited the MCP-1 response, whereas extracellular catalase did not, indicating that endocytosis is required and that intracellular generation of reactive oxygen species activates c-Src. These data show that intracellular H_2O_2 induced by endocytosis of monoclonal free light chains oxidizes and activates c-Src, which promotes release of MCP-1.

J Am Soc Nephrol 21: 1165–1173, 2010. doi: 10.1681/ASN.2009101089

During immunoglobulin (Ig) synthesis, a surplus of κ and λ light chains are produced, resulting in free light chains being released into the circulation.[1] These are low-molecular-weight proteins that are primarily cleared from the circulation by the kidneys.[2] They are freely filtered at the glomerulus and are presented to the proximal tubule; thus, the rate of clearance is linked to GFR.[1,3–5] Light chains in the filtrate are actively endocytosed into proximal tubule epithelial cells (PTECs) by means of megalin-cubilin receptor complexes on their luminal surfaces.[6–9] Following endocytosis into cytoplasmic vesicles, the receptor is recycled back to the cell surface, whereas the vesicular contents are acidified and subsequently hydrolyzed by the action of lysosomal enzymes before being returned to the circulation.[10–12]

In health, approximately 500 mg of free light chain is produced per day, nearly all of which is removed by the kidneys, with only 1 to 10 mg/d appearing in the urine.[3,13] In multiple myeloma, where an aberrant B cell clone can produce prodigious quantities of free light chain, serum concentrations can rise considerably, sometimes approaching 100,000 mg/L.[14] This leads to a greatly

Received October 26, 2009. Accepted March 29, 2010.

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increased burden of light chain on PTECs and saturation of the megalin-cubilin pathway,[6,8,9] allowing light chain to travel to the distal nephron where they may interact with Tamm-Horsfall protein and appear in the urine.[15–17]

There is a mounting body of evidence pointing to exposure of PTECs to excess filtered proteins, resulting in cytokine release, recruitment of inflammatory cells, and the acceleration of interstitial fibrosis.[18] Light chains have been shown to cause nuclear translocation of Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB), resulting in the release of interleukin-6 (IL-6), IL-8, monocyte chemoattractant protein-1 (MCP-1), and transforming growth factor-β (TGF-β), and are much more potent inducers of these cytokines than other proteins, such as albumin, which may be filtered and enter the proximal tubule in significant amounts, especially in glomerular disease states.[19,20] Exposure to light chains has also been shown to activate mitogen-activated protein kinases.[21,22] The single initiating event for signal transduction, however, has remained elusive.

A series of studies had recently discovered that intact Ig and antigen-binding (Fab) fragments could generate hydrogen peroxide (H_2O_2).[23–25] Following on from these results, our laboratory was able to show that light chains are also capable of producing H_2O_2 and induce oxidative stress in immortalized human PTECs (HK-2 cells)[26] and that MCP-1 production and cytotoxicity induced by the same light chains was H_2O_2-dependent.[27] These data pointed to a key role played by H_2O_2 in the signal transduction cascades that are set in motion after internalization of excess light chain.

Reactive oxygen species (ROS) are known to function as second messengers for postreceptor signal transduction in many cell types.[28–32] c-Src, the 60-kDa product of *c-src*, is a member of the Src tyrosine kinase family, which plays a role in signal transduction in response to many external stimuli[33] and its activity is under tight redox control. When reduced by phosphorylation at Y527, it is inactive.[34] However, in the oxidized state, it is dephosphorylated at Y527, undergoes conformational change, is autophosphorylated at Y416, and becomes active.[35,36] This process of activation has been shown to be dependent on ROS.[28,34] Studies from this laboratory have shown c-Src also to be a participant in production of TGF-β by endothelial cells.[37]

Here, we studied the effects of light chain–generated H_2O_2 on c-Src in HK-2 cells. We report that intracellular generation of ROS after endocytosis of light chains by proximal tubular cells promotes the oxidation and activation of c-Src. MCP-1 release was dependent on c-Src activation; however, generation of H_2O_2 was independent of c-Src activity.

RESULTS

Human Light Chains, But Not Delipidated Human Albumin, Induce the Release of MCP-1 and IL-6 by Human Proximal Tubular Epithelial Cells

Incubation of HK-2 cells with the κ2 and λ2 light chains, 1 mg/ml, increased ($P < 0.05$) MCP-1 production, compared with cells incubated in medium alone. For κ2, MCP-1 production increased ($P < 0.05$) from 367.5 ± 41.1 to 980.3 ± 15.7 pg/d, and for λ2, production increased ($P < 0.05$) from 434.7 ± 56.5 to 956.5 ± 78.4 pg/d. Incubation of HK-2 cells with delipidated albumin, 15 mg/ml, produced no change (470.2 ± 89.2 *versus* 597.1 ± 126.3 pg/d) in MCP-1 production. IL-6 production was also examined. Although albumin produced no change in production (data not shown), incubation of HK-2 cells with both light chains increased ($P < 0.05$) IL-6 production from a mean baseline of 23.2 ± 1.7 pg/h to 192.7 ± 7.9 pg/h when the cells were exposed to κ2 and 225.5 ± 8.9 pg/h when the cells were incubated with λ2.

Similar experiments were repeated using HEK293 cells. Although albumin had no effect on production of either MCP-1 or IL-6 (data not shown), both κ2 and λ2 light chains increased ($P < 0.05$) production of both MCP-1 (390.4 ± 23.9 pg/d for medium alone, 1190.6 ± 59.1 pg/d for κ2, and 1352.5 ± 78.7 pg/d for λ2) and IL-6 (0.9 ± 0.2 for medium alone, 3.6 ± 0.4 pg/d for κ2, and 16.2 ± 0.6 pg/d for λ2). Subsequent experiments used primarily HK-2 cells.

Ig Light Chains Activate c-Src

c-Src activation by phosphorylation (phospho-c-Src) was detected by Western blot analysis of cell lysates using a primary antibody that specifically detects phosphorylation at Y416. The amount of active c-Src in cells exposed to light chain (1 mg/ml) relative to the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the lysate were determined by densitometry (Figure 1). After exposure to both κ2 and λ2 light chains, phospho-c-Src levels increased rapidly, in a time-dependent manner, with a peak being observed at 12 hours. At this point they were up to eight- to ninefold higher than the amounts seen in cells treated with vehicle alone. No time-dependent change in phospho-c-Src levels were seen in cells exposed to vehicle alone. After 12 hours, phospho-c-Src concentrations declined, reducing to <50% of the peak levels by 24 hours. There were no significant differences in the relative increase of phospho-c-Src between the two species of light chains at each time point. Co-incubation of HK-2 cells with either light chain and 1,3-dimethyl-2-thiourea (DMTU), 30 mM, a cell permeable chemical trap for H_2O_2, prevented activation of c-Src (Figure 2).

Inhibition of c-Src Suppresses MCP-1 Production But Not H_2O_2 Production

Overnight incubation of HK-2 cells with both κ2 and λ2 light chains (1 mg/ml) increased production of MCP-1 and H_2O_2 in the cell culture supernatant, when compared with medium alone, as measured by sandwich ELISA and Amplex Red, respectively (Figure 3). When 10 μM 4-amino-5-(4-chlorophenyl)-7-(*tert*-butyl)pyrazolo[3,4-*d*]pyramidine (PP2), an inhibitor of c-Src activity, was added to the culture medium, MCP-1 concentrations in the supernatant remained at baseline levels. However, PP2 did not have such an effect on H_2O_2 in the supernatant. PP2 also inhibited light chain–induced MCP-1 production by HEK293 cells (data not shown).

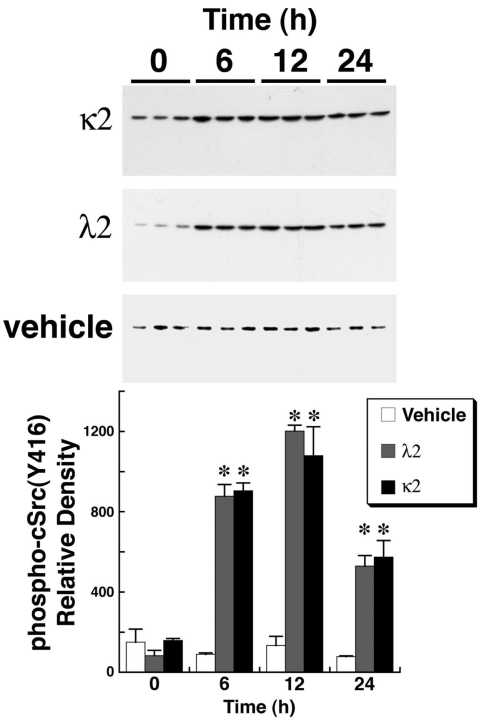

Figure 1. Time dependent activation of c-Src by light chains. Exposure to both κ2 and λ2 light chain resulted in an increase in phosphorylation at Y416, representing activation of this enzyme, when compared with lysates of cells exposed to vehicle alone. Densitometric analysis of bands shows the increase was similar for both light chains and occurred in a time-dependent manner, reaching a peak at 12 hours, and declining thereafter. $n = 3$ experiments in each group.

Removal of Extracellular H_2O_2 by Catalase Has No Effect on MCP-1 Production

To investigate whether H_2O_2 involved in signaling was produced intracellularly or extracellularly, we added catalase to the medium. As a powerful extracellular scavenger of H_2O_2, exogenously applied catalase would quickly destroy any H_2O_2 in the supernatant. Cells exposed to medium containing catalase produced MCP-1 at a rate of 302.8 ± 10.3 pg/d. Addition of catalase to the medium along with κ2 and λ2 light chains did not prevent the increase in MCP-1 (672.5 ± 37.3 and 1018.3 ± 28.7 pg/d, respectively; $P < 0.05$ compared with control).

Silencing of c-Src Expression Suppresses MCP-1 Production in Response to Light Chain Exposure

c-Src expression was silenced by transfecting HK-2 cells with siRNA specifically targeted to c-Src. Western blot analysis of cell lysates confirmed successful silencing of total c-Src production (Figure 4A). Densitometry relative to GAPDH expression showed an approximate 80% reduction in c-Src expression when compared with lysates from cells exposed to the nontargeting siRNA. When HK-2 cells were incubated with κ2 and λ2 light chains, cells in which c-Src expression was reduced did not release MCP-1 into the supernatant above baseline levels (Figure 4B).

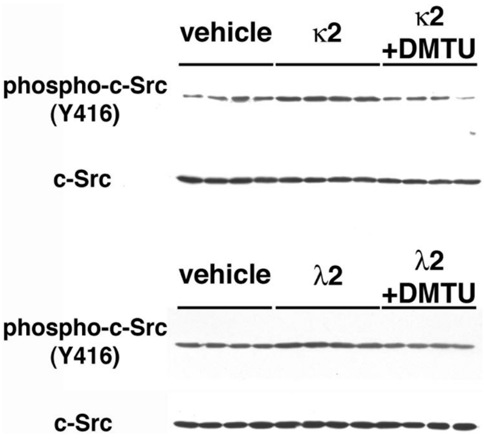

Figure 2. Addition of DMTU inhibits light chain-induced activation of c-Src in HK-2 cells, as determined by the ratio of phospho-c-Src to total c-Src in the lysate obtained following overnight incubation. Compared with κ2 alone, the increase in the relative amount of phospho-c-Src induced by the κ2 light chain was inhibited ($P < 0.05$) by DMTU (0.28 ± 0.04 for vehicle alone; 0.96 ± 0.07 for light chain alone; 0.28 ± 0.04 for light chain plus DMTU). Compared with λ2 light chain alone, the increase in phospho-c-Src induced by the λ2 light chain was inhibited ($P <$ 0.05) by DMTU (0.46 ± 0.04 for vehicle alone; 0.80 ± 0.12 for light chain alone; 0.41 ± 0.02 for light chain plus DMTU). $n = 4$ experiments in each group.

c-Src Is Oxidized after Light Chain Treatment

To determine whether oxidation of c-Src occurs in response to exposure to light chain in HK-2 cells, *N*-(biotinoyl)-*N*′-(iodoacetyl)ethylenediamide (BIAM), a thiol-reactive biotinylating reagent for proteins, was used. BIAM specifically identifies the thiolate form of cysteine residues when they are in the reduced state, making it a very useful tool to detect redox-regulation of proteins.[38,39] Using this method, we measured reduced c-Src levels in HK-2 cells after exposure to κ2 and λ2 light chains (Figure 5). There was a time-dependent reduction in reduced c-Src levels, when compared with those treated with vehicle alone. Data in the bottom panels show that total c-Src levels in the samples did not differ among the groups. These data confirm that c-Src is directly oxidized when cells are treated with light chains.

Silencing of Megalin and Cubilin Suppresses MCP-1 Production

To confirm that MCP-1 production was dependent on cellular uptake of light chain through interaction with megalin and cubilin, expression of these two proteins was silenced by transfecting HK-2 cells with specific siRNAs. Successful knockdown was confirmed by Western blot analysis for both proteins (Figure 6A). Silencing of megalin and cubilin significantly reduced MCP-1 release by HK-2 cells in response to exposure to κ2 and λ2 light chains (Figure 6B). MCP-1 production between controls, where cells were transfected with nontargeting sequence siRNA or exposed to vehicle alone, did not differ. MCP-1 production increased when cells were exposed to light

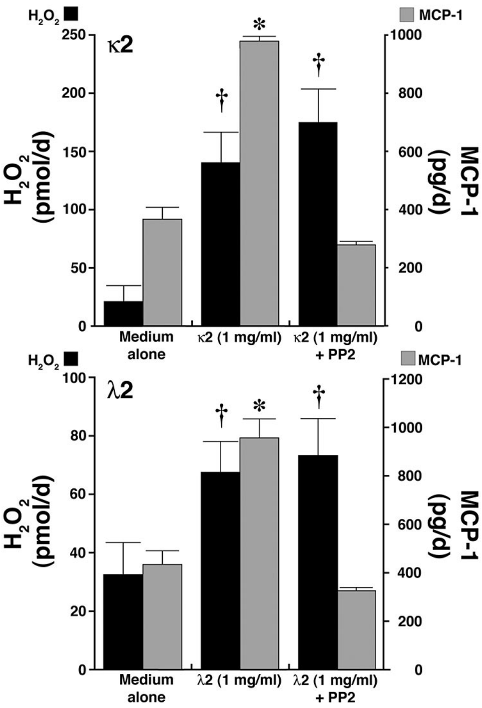

Figure 3. Inhibition of c-Src prevents light chain-induced production of MCP-1, but not H_2O_2. There is a rise in H_2O_2 after exposure of HK-2 cells to both κ2 (top) and λ2 (bottom) light chains. This is unaffected by the addition of 10 μM PP2, an inhibitor of c-Src activity, indicating that the production of H_2O_2 takes place upstream of c-Src. There is also an increase in MCP-1 release into the supernatant. Inhibition of c-Src activity by PP2 returns MCP-1 levels to baseline, indicating that c-Src activation is necessary for the release of MCP-1. *n* = 6 experiments in each group.

chains, the response being stronger with λ2 compared with κ2. After megalin and cubilin knockdown, this response was markedly reduced, by approximately 60% with κ2 and 63% with λ2, but remained slightly above production levels seen in the control samples.

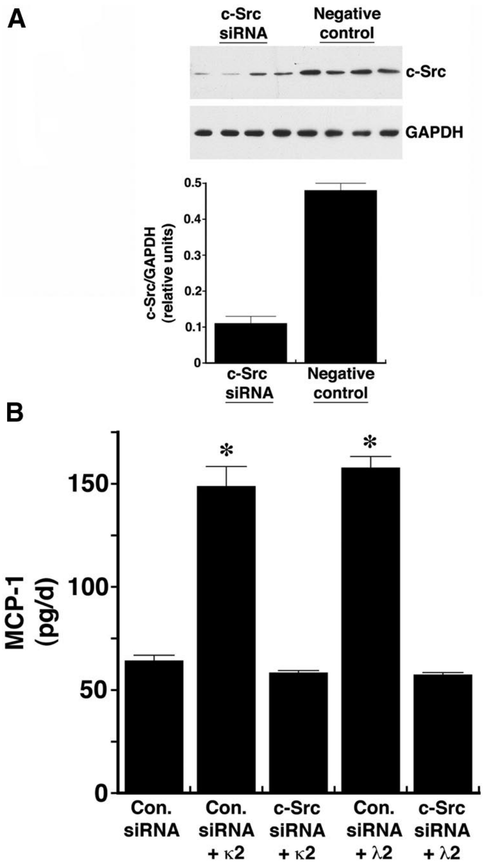

Figure 4. Silencing c-Src expression in HK-2 cells abrogates the MCP-1 response that follows exposure to either κ2 or λ2 light chains. Cells were transfected with siRNA specific for c-Src. (A) Successful knockdown of c-Src expression was confirmed by Western blot analysis, normalized to a GAPDH loading control. Densitometric analysis shows an approximate 80% reduction compared with negative control. Bars represent means of four individual experiments. (B) MCP-1 release from transfected cells after light chain challenge. Knockdown of c-Src expression abrogates the MCP-1 response, indicating that the presence of c-Src is necessary for signal transduction, leading to MCP-1 release. *n* = 8 experiments in each group.

DISCUSSION

Renal prognosis in multiple myeloma is poor and is associated with significant morbidity, with 10% of new cases requiring dialysis and of these, 80% not recovering independent renal function.[40,41] Renal fibrosis can progress rapidly despite treatment in this condition.[42] Understanding pathways to inflammation driven by cytokine release from the proximal tubule becomes important from a translational point of view, if ways of preventing the resulting irreversible renal fibrosis are to be found.[22] The purpose of the present studies was to investigate the links between light chains, oxidative stress, c-Src activation, and production of MCP-1, a key chemokine in inflammation. Our data from this series of experiments show that two unique light chains, in concentrations relevant to levels exposed to proximal tubule cells *in vivo*, induce activation of c-Src, a tyrosine kinase known to be involved in several signal transduction pathways. Sengul *et al.* demonstrated that NF-κB is activated in HK-2 cells when they are exposed to and internalize light chains, resulting in the release of MCP-1, and also IL-6 and IL-8.[19] Findings in the present study agree with these data and complement our previous findings that production of MCP-1 was also dependent on H_2O_2 and NF-κB because inhibition of ROS with DMTU and inhibition of NF-κB with pyrrolidine dithiocarbamate suppressed MCP-1 release.[27] The present data further demonstrate that c-Src is integrally involved in production of MCP-1 by proximal tubule cells after exposure to light chains.

Because of the observed capability of light chain to generate H_2O_2,[27] this study therefore focused on activation of c-Src as

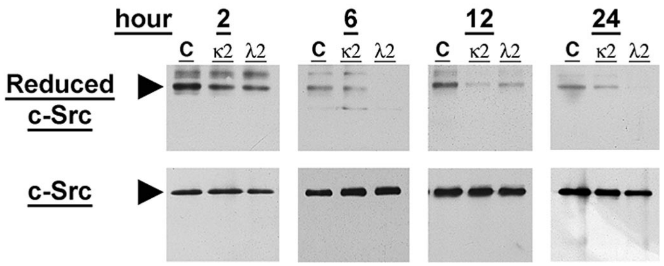

Figure 5. Incubation of HK-2 cells with the two light chains oxidizes c-Src. Cells were exposed to either κ2 or λ2 light chain, and c-Src oxidation was assessed at 2-, 6-, 12-, and 24-hour time points. Cells were lysed and reduced c-Src was labeled with BIAM and immunoprecipitated before detection by immunoblotting. Half of each sample was probed with an anti-total-c-Src antibody for the purpose of normalization. Reduced-c-Src levels declined during the time course of the experiment, indicating that direct oxidation and activation of c-Src was taking place.

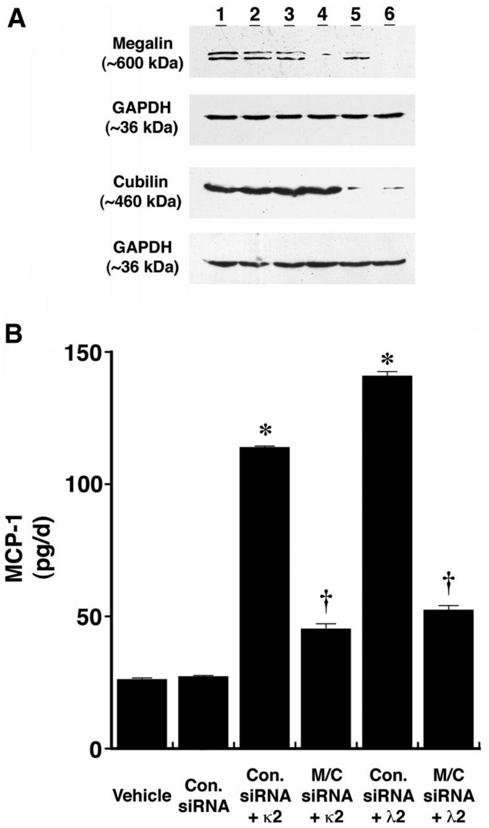

Figure 6. Silencing megalin and cubilin expression inhibits light chain-induced MCP-1 production by HK-2 cells. Megalin and cubilin expression was silenced by specific siRNAs. (A) Successful knockdown was confirmed by Western blot analysis, normalized to a GAPDH loading control. Lane 1, control; lane 2, vehicle; lane 3, addition of nontargeting siRNA; lane 4, addition of siRNA targeted for megalin; lane 5, addition of siRNA targeted for cubilin; and lane 6, addition of siRNA targeted for both megalin and cubilin. Densitometric analyses showed a greater than 85% reduction in megalin and an approximate 95% reduction in cubilin. (B) MCP-1 release is significantly reduced after knockdown, compared with nontargeting scramble sequence siRNA. However, the response was not completely abrogated, indicating ongoing signal transduction likely due to incomplete knockdown of the machinery involved in transport of the light chains into the cell. *n* = 6 experiments in each group.

an intermediate in the signal transduction process that produced MCP-1 by light chain. The addition of PP2 abrogated the MCP-1 response quite effectively, indicating that activation of c-Src plays a key role in MCP-1 production. To reaffirm that c-Src was necessary for the production of MCP-1 after light chain exposure, we silenced c-Src synthesis with the use of siRNA. After successful knockdown, the release of MCP-1 into the supernatant in response to light chains was abolished. This was further evidence that as well as H_2O_2 and NF-κB, c-Src served as a vital link in the chain of events leading to MCP-1 release. Experiments then investigated whether the H_2O_2 generated after light chain challenge led to oxidation of c-Src. The data show that c-Src in the reduced state (as detected by BIAM labeling) is depleted in a time-dependent fashion temporally associated with c-Src activation and furthermore that addition of DMTU, a scavenger of H_2O_2, prevented light chain–induced activation of c-Src. These observations are consistent with our previous results demonstrating the presence of intracellular oxidative stress within HK-2 cells.[27] The data are also supported by the results published by Giannoni *et al.*[28] showing that intracellular oxidative stress causes direct oxidation of c-Src at Cys245 and Cys487, thereby facilitating c-Src activation.

Inhibition of c-Src by PP2 however had no effect on H_2O_2 levels in the supernatant. This would suggest that H_2O_2 generation occurs independently of c-Src activation. Although the major source of H_2O_2 is likely from the light chain itself,[27] the precise intracellular location where H_2O_2 was produced in these experiments remains unclear. In a fashion similar to immunoglobulins,[23–25] light chains alone in solution are capable of catalyzing the production of H_2O_2.[27] Although all proteins have the intrinsic ability to do this, the effect is usually quickly saturable, resulting in low levels of production of ROS. In contrast, light chains are much more efficient and have a much higher capacity for catalyzing this reaction when compared with non–Ig-derived proteins. Although the ability of immunoglobulins to generate H_2O_2 may improve the ability of the antibody to destroy pathogens, the present series of experiments show that the generation of H_2O_2 by light chains may have deleterious effects on the kidney proximal tubule by initiating inflammatory signaling pathways.

There are other potential sites of ROS production. Morigi *et al.* have reported that NF-κB activation in HK-2 cells after protein overload requires the upstream generation of H_2O_2.[31] In the pathway they describe, activation of protein kinase C was required for the generation of H_2O_2. Both membrane nicotinamide adenine dinucleotide phosphate-oxidase and the mitochondrial respiratory chain served as sources of H_2O_2 after protein (other than light chains) challenge to HK-2 cells.[31]

A recent study by Li *et al.* from the laboratory of Batuman has shown that by silencing the expression of megalin and cubilin with siRNAs, endocytosis of light chains could be blocked, resulting in the amelioration of toxic effects, cytokine release such as that of MCP-1, and epithelial-to-mesenchymal transition.[43] Our own findings from the current study were in agreement with the role of megalin and cubilin in these processes. Along with the lack of efficacy of extracellular catalase, these data indicate that endocytosis of light chains is an important step in signal transduction leading to cytokine release. However, an elegant series of experiments by DeYulia *et al.* has revealed that the interaction of receptor and its ligand can generate H_2O_2, independent of subsequent signal transduction.[44] This ability was conserved even when cells were fixed, or purified receptor and ligand interacted in the absence of cells. Although this phenomenon has not been shown when megalin-cubilin receptors interact with proteins, it is possible that this could be yet another source of H_2O_2. However this possibility is much less likely because albumin, which is endocytosed into PTECs through the same megalin-cubilin mechanism, did not reproduce the findings observed with the light chains. It has been repeatedly observed that the ability of light chains to induce cytokine production in PTECs far exceeds that of other filtered proteins such as albumin.[19,27] One possibility is that free fatty acids that are normally bound to albumin promote the observed effects;[45] in our study, delipidated albumin was used. In the context of inflammation in the proximal tubule, the intrinsic ability of light chains to generate H_2O_2 and the highly efficient manner in which they do this puts them in a class of proteins quite separate from non–Ig-derived proteins. In this series of experiments, we have obtained data that show that in order for PTECs to reach an inflammatory state in response to light chains, the activation of the tyrosine kinase c-Src is necessary. The generation of H_2O_2 occurs upstream of c-Src activation, which in turn is dependent on H_2O_2. Indeed, this event appears to be mediated by H_2O_2 directly oxidizing this enzyme. Finally, although the data in this study, which examined the effect of human light chains on human proximal tubular cells, have potential clinical relevance, the findings are limited by the absence of an *in vivo* model.

CONCISE METHODS

Human Ig Light Chains and Albumin

Two unique monoclonal Ig light chains, one κ and one λ, labeled $\kappa 2$ and $\lambda 2$, were purified from the urine of patients who had multiple myeloma and light chain proteinuria using standard methods.[46] Both patients had clinical evidence of significant renal damage that was presumed to be cast nephropathy, although renal biopsy was not routinely performed. Both of these light chains have previously been shown to generate H_2O_2 from water when stimulated with near-UV light radiation, and also increase H_2O_2 concentrations in HK-2 cell culture media.[27] Endotoxin assay showed levels in each light chain preparation to be below the detection limit (Limulus Amebocyte Lysate, QCL-1000; Lonza, Walkersville, MD). Delipidated human serum albumin was obtained from a commercial vendor (Sigma-Aldrich Corporation, St. Louis, MO).

Cell Culture

HK-2 cells were obtained from the American Type Culture Collection and have been characterized previously.[26] Cells were grown on plates that were coated with 5 μg/cm^2 type 1 collagen (Rat Tail Collagen type 1; BD Biosciences, San Diego, CA), and incubated at 37°C with 5% CO_2/95% air in keratinocyte serum-free medium (Life Technologies, Invitrogen, Carlsbad, CA) supplemented with recombinant human epidermal growth factor (5 ng/ml) and bovine pituitary extract (50 μg/ml). Medium was exchanged at 48-hour intervals and cells were not passaged beyond 25 to 30 times.

For experiments, medium was exchanged for keratinocyte serum-free medium containing light chains (1 mg/ml) and incubated for up to 24 hours. Light chain concentration, 1 mg/ml, used in these studies was within the expected concentration range to which proximal tubule cells are exposed, on the basis of the serum levels found in patients with multiple myeloma[1,14] and the estimated glomerular sieving coefficients for these low-molecular-weight proteins.[2,47] Human serum albumin was used in a higher concentration (15 mg/ml).

Harvested Supernatants Were Clarified by Centrifugation and Promptly Assayed

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer containing a protease inhibitor cocktail (Complete; Roche, Indianapolis, IN), clarified by centrifugation, and lysates were stored at −70°C until they were assayed. Total soluble protein in lysates were measured using a kit (BCA Protein Assay Kit; Pierce, Rockford, IL).

Western Blot Analysis

Protein extracts (20 to 60 μg) were boiled for 3 minutes in Laemmli buffer and separated by 7 to 12% SDS-PAGE (BioRad, Hercules, CA), before being electrophoretically transferred onto polyvinylidene difluoride membranes. These were blocked in 5% skim milk and then incubated at 4°C overnight with one of the following primary antibodies: rabbit-anti-human phospho-c-Src Y416 (1:1000 dilution), rabbit-anti-human total c-Src (1:1000 dilution; Cell Signaling Technology, Danvers, MA), and goat-anti-human megalin (C19) and cubilin (Y20) (1:250 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). GAPDH served as a loading normalization control and was determined using mouse-anti-human GAPDH (1:10,000 dilution; Abcam Inc., Cambridge, MA). Blots were incubated for 1 hour at room temperature with horseradish peroxidase (HrP)-conjugated goat-anti-mouse (Pierce, Rockford, IL; 1:2000 dilution), goat-anti-rabbit (1:2000 dilution; Pierce), or anti-goat (1:10,000 dilution; BioRad) secondary antibodies. For detection of BIAM-labeled c-Src, blots were incubated with HrP-conjugated streptavidin (Biolegend, San Diego, CA). Visualization was by enhanced chemiluminescence (SuperSignal West Dura; Pierce) on film (BioMax MR; Carestream Health, Rochester, NY). Films were scanned (Microtek Int., Taiwan) and densitometry was performed using Quantity One software (Bio-Rad, Hercules, CA).

Light Chain–Induced H_2O_2 Production in HK-2 Cells

H_2O_2 concentrations in cell culture supernatants were measured using a kit (Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit; Invitrogen, Carlsbad, CA). Standards and samples were mixed with Amplex Red working solution and incubated at room temperature for 30 minutes, protected from light. Fluorescence was detected at an excitation frequency of 535 nm and emission at 560 nm (Packard Fusion Universal Microplate Reader; Packard Instrument Co., Meriden, CT). All assays were performed in duplicate and averages taken.

Light Chain–Induced MCP-1 Production in HK-2 Cells

MCP-1 concentrations in cell culture supernatants were measured using a sandwich enzyme immunoassay (Human CCL2/MCP-1 Immunoassay; R&D Systems, Minneapolis, MN). Standards and samples were incubated in the assay microplate wells precoated with capture antibody, for 2 hours at room temperature. The wells were then washed and incubated with HrP-conjugated detection antibody for 1 hour at room temperature, washed again, and incubated with enzyme substrate for 20 minutes before the addition of a stop solution. Absorbance was measured at 450 nm (VersaMax Microplate Reader; Molecular Devices, Sunnyvale, CA). Assays were performed in duplicate, with averages taken. IL-6 concentrations in medium were also determined using a sandwich enzyme immunoassay (Human IL-6 ELISA Ready-SET-Go Kit; eBioscience, San Diego, CA).

Inhibition of c-Src Activity

4-Amino-5-(4-chlorophenyl)-7-(*tert*-butyl)pyrazolo[3,4-*d*]pyramidine (PP2; EMD Biosciences, Gibbstown, NJ) is a potent selective chemical inhibitor of c-Src activity.[48] To suppress c-Src activity in HK-2 cells during experiments, PP2 was added to the medium to a final concentration of 10 μM at the same time the light chain was added.

Removal of H_2O_2

For experiments where we desired the effect of extracellular H_2O_2 to be abolished, catalase from bovine liver (Sigma-Aldrich) was added to the medium to a final concentration of 500 U/ml before addition to wells containing HK-2 cells. The effect of inhibition of intracellular reactive oxygen species on light chain–induced MCP-1 production was examined by overnight co-incubation of HK-2 cells exposed to the κ2 and λ2 light chains with DMTU, 30 mM, a cell-permeable chemical trap for H_2O_2.[49]

Silencing of Gene Expression

All reagents for silencing of gene expression were obtained from Santa Cruz Biotechnology, unless otherwise stated. We employed siRNAs specifically targeting mRNA for human c-Src, megalin, and cubilin and a nontargeting scramble-sequence siRNA as a negative control. c-Src expression was silenced by transfecting HK-2 cells with a pool of four target-specific 20- to 25-nucleotide siRNAs (50 pmol) (sc-29228). Targeted knockdown of megalin and cubilin was achieved using pools of three target-specific 20- to 25-nucleotide siRNAs (50 pmol) (sc-40103 and sc-40099). HK-2 cells were transfected according to the vendor's protocol. Cells in log phase were plated onto six-well plates in antibiotic-free growth medium. At 60 to 80% confluence, cells were washed with transfection medium, then overlaid with siRNA-transfection reagent complexes, and returned to the incubator. After 6 hours, fresh medium was added to minimize toxicity. Cells were incubated for a further 48 hours, before protein expression was assessed by Western blotting.

Detection of c-Src Oxidation by Carboxymethylation

Cells were grown on 100-mm dishes and allowed to reach 80 to 90% confluence. Medium was then removed, the cells were rinsed briefly with PBS, and then medium containing either κ2 or λ2 light chain (1 mg/ml) was added. At 2, 6, 12, and 24 hours, medium was removed and cells were snap-frozen in liquid nitrogen. RIPA buffer containing 100 μM BIAM (Molecular Probes, Invitrogen, Carlsbad, CA) was rendered free of oxygen by bubbling with nitrogen gas at a low flow rate for 20 minutes. Frozen cells were then exposed to 0.5 ml of this RIPA buffer, followed by sonication for three periods of 1 minute each separated by 30-second intervals, and then incubated for 15 minutes at room temperature. Lysates were then clarified by centrifugation and immunoprecipitated with anti-human total c-Src antibody (Cell Signaling Technology) using Protein G PLUS-Agarose immunoprecipitation reagent (Santa Cruz Biotechnology). Total soluble protein concentration was determined by BCA assay, before separation by SDS-PAGE and transferred to polyvinylidene difluoride membranes as above. Each sample was divided into two equal parts: one half was used for detection of c-Src labeled with BIAM by HrP-conjugated streptavidin and the other half was probed for total c-Src for normalization, as above.

Statistical Analysis

Means were calculated ± SEM. Significant differences between groups were determined by ANOVA (Statview; SAS Institute, Cary, NC). A *P* value of <0.05 was assigned statistical significance.

ACKNOWLEDGMENTS

Portions of the data contained within this manuscript have been submitted in abstract form to the 42nd Annual Meeting and Scientific Exposition of the American Society of Nephrology; October 27 through November 1, 2009; San Diego, CA. National Institutes of Health grant (R01 DK46199) and P30 DK079337 (George M. O'Brien Kidney and Urological Research Centers Program) and the Office of Research and Development, Medical Research Service, Department of Veterans Affairs, supported this research. We thank the Binding Site Ltd. for their unrestricted support of Dr. Basnayake's clinical fellowship and their expertise in protein chemistry.

DISCLOSURES

None.

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See related editorial, "Receptor-Mediated Endocytosis Is a Trojan Horse in Light-Chain Nephrotoxicity," on pages 1065–1066.