We recently proposed a comparative proteomic method utilizing proteolytic $^{18}$O labeling of peptides catalyzed by peptidyl-Lys metalloendopeptidase (Lys-N) (Rao, K. C. S., Carruth, R. T., and Miyagi, M. (2005) Proteolytic $^{18}$O labeling by peptidyl-Lys metalloendopeptidase for comparative proteomics. J. Proteome Res. 4, 507–514). Unlike trypsin, which generates a mixture of isotopic isoforms resulting from the incorporation of one or two $^{18}$O atoms into each peptide species, Lys-N incorporates only a single $^{18}$O atom into the carboxyl terminus of each proteolytically generated peptide in $H_2^{18}$O solvent. This study reports the first biological application of the Lys-N-based proteolytic $^{18}$O labeling method, characterizing the proteome changes of cytokine/lipopolysaccharide-treated versus untreated human retinal pigment epithelium (ARPE-19) cells. The study resulted not only in the identification of 584 proteins but also the determination of the relative abundances of 562 proteins in the two proteomes. The results demonstrate the usefulness of the Lys-N-based proteolytic $^{18}$O labeling method in comparative proteomic studies. The results also provide the most comprehensive description of the retinal pigment epithelium proteome to date. Molecular & Cellular Proteomics 4:1550–1557, 2005.

Large scale quantitative measurements of protein expressions in different sets of samples (comparative proteomics) are expected to be critical to the advance of our understanding of physiological processes and disease mechanisms. Although 2D$^1$ PAGE-based methods have been the primary choice in comparative proteomics, 2D gels are cumbersome to run, have a poor dynamic range, and are biased toward abundant and soluble proteins (1). Therefore alternative methods have been actively sought. One such method is mass spectrometry-based in vitro stable isotope labeling in which proteins from the control and experimental samples are proteolytically digested. The generated peptides from the control sample are then labeled with naturally abundant (light) isotope(s), whereas the peptides from the experimental sample are labeled with its heavier isotope(s) or vice versa. The samples are then mixed together in equal proportions, and the relative abundance of each particular peptide from the two samples is determined by mass spectrometry. By comparing the peak areas or intensities of the light and heavy peptides, the relative abundance of each particular peptide can be determined; this equals the relative abundance in the original samples of the parent protein from which the peptide was generated.

Among the mass spectrometry-based in vitro stable isotope labeling methods, the proteolytic $^{18}$O labeling method (2) is particularly attractive because it has the least technical variations. In this method, the stable isotopic ($^{18}$O atom) labeling of peptides is achieved concurrently with the proteolytic digestion of proteins. Therefore the yield of each isotopically labeled peptide depends only on the effectiveness of proteolytic digestion in both samples compared. In contrast, the other in vitro stable isotope labeling methods (such as the isotope-coded affinity tag method (3)) have the isotopic labeling and proteolytic digestion of proteins occurring at different steps, meaning that the yield of each isotopically labeled peptide has greater variability because it depends on both the yield of the isotope labeling and the effectiveness of the proteolytic digestion.

Trypsin has been the protease most utilized in proteolytic $^{18}$O labeling methods. However, it is known that trypsin generates a mixture of isotopic isoforms resulting from the variable incorporation of either one or two $^{18}$O atoms ($^{18}$O$_1$/$^{18}$O$_2$) into each peptide (4). This not only makes quantification of the peptides complicated but also increases the error in the experimentally determined $^{16}$O- and $^{18}$O-labeled peptides ratios. We recently reported a Lys-N-based technique, which is advantageous over trypptic digestion because it incorporates only a single $^{18}$O atom into the carboxyl terminus of each proteolytically generated peptide (5). Lys-N is a metalloendopeptidase (peptidyl-Lys metalloendopeptidase, EC 3.4.24.20) that cleaves specifically peptidyl-lysine bonds (-X-Lys-) in proteins and peptides (6). In this initial study with several model proteins the unique single $^{18}$O atom incorporation property of Lys-N was shown to provide accurate quan-
Classification results. Other advantages of the Lys-N-based technique shown in this initial study include no enzyme-catalyzed $^{18}$O back exchange and production of a less complex protein digest.

Here we report the first biological application of the Lys-N-based proteolytic $^{18}$O labeling method, demonstrating the technique on cytokine/lipopolysaccharide (LPS)-treated versus untreated (control) human retinal pigment epithelium (ARPE-19) cells. Retinal pigment epithelium (RPE) performs many functions required for the neural retina to work properly, including transport of nutrients and removal of waste products into/from the photoreceptor cells, regeneration of bleached visual pigment, and phagocytosis of the outer segments of the rods and cones. ARPE-19 was developed as a spontaneous derived cell line using cells from a 19-year-old male donor and is an in vitro model system of RPE that is ideal for physiological and biochemical studies (7). Effects of cytokine treatment, including tumor necrosis factor-γ, on cultured RPE and ARPE-19 cells have been studied extensively, and the induction of several proteins have been reported (8–11). In addition, several proteomic studies on RPE or RPE-derived cell lines have also been reported (12–15). Therefore, ARPE-19 cells (+ cytokine treatment) will serve as a good model system to evaluate the Lys-N-based proteolytic $^{18}$O labeling method.

The goal of this study was to evaluate the practicability of Lys-N-based proteolytic $^{18}$O labeling method with biological samples. The study resulted in identification of about 600 proteins along with semiquantitative information on their expressions in the cytokine/LPS-treated and untreated cells with a minimum detection accuracy of a 2-fold change. This method can thus be used as a general comparative proteomic technique applied to other systems. The results also provide a more complete description of the proteome in ARPE-19 cells and proteome changes resulting from cytokine/LPS treatment.

**EXPERIMENTAL PROCEDURES**

**Materials**

Lys-N from *Grifola frondosa* was obtained from Seikagaku Corp. (Tokyo, Japan). Oxygen-18-enriched water (>95%) and HPLC grade water were obtained from Spectra Stable Isotopes (Columbia, MD) and Fisher Scientific, respectively. All other chemicals used were of analytical grade or the highest quality that was commercially available.

**Cell Culture, Cytokine/LPS Treatment, and Cell Harvest**

Human retinal pigment epithelium (ARPE-19) cells were obtained from the American Tissue Culture Collection (Manassas, VA). Cells were cultured to ~80% confluency in T-175 flasks as described previously (16) at 37 °C under 95% air and 5% CO₂ in Dulbecco’s modified Eagle’s medium; nutrient mixture F-12 (Ham’s) 1:1 (DMEM-F12) with 10% fetal calf serum, 2% l-glutamine, and 0.5% antibiotic/antimycotic. The cells, before harvesting, were either 1) treated in growth medium for 24 h with a combination of cytokines/LPS consisting of human tumor necrosis factor α (3.25 ng/ml, Upstate Biotechnology, Lake Placid, NY), human interferon-γ (50 ng/ml, Upstate Biotechnology), and *Escherichia coli* LPS (10 μg/ml, Sigma-Aldrich, St. Louis, MO) or 2) untreated (control), keeping them in medium for 24 h. After 24 h, the medium was removed from the flask, and the cells were washed with PBS twice and DMEM-F12 once and harvested in DMEM-F12 by scraping the cells from the flask. The harvested cell suspension was centrifuged at 150 × g for 10 min at 4 °C, the supernatant was removed, and the cell pellet was stored at −80 °C until use.

**Protein Extraction and S-Carbamidomethylation**

The stored cell pellet was resuspended in 2.5 ml of 2% SDS in 50 mM Tris-HCl, pH 7.5, buffer and sonicated for 30 s. The resulting homogenate was centrifuged at 8,000 × g for 30 min at 4 °C, and the supernatant was recovered. The extracted proteins were reduced by adjusting the solution to 1 mM DTT and incubating for 2 h at 50 °C. An S-alkylation treatment was then done by adjusting the solution to 2.5 mM iodoacetamide and incubating for 1 h at 25 °C in the dark. After the S-carbamidomethylation, 4 times the volume of cold acetone (10 ml) was added to the protein solution and allowed to stand at 4 °C for 60 min in the dark to precipitate the proteins before centrifugation at 8,000 × g for 30 min at 4 °C. The resulting pellet was dried in a SpeedVac concentrator, redissolved in 25 μl of 100 mM pH 10.0 buffer consisting of glycine-NaOH and 8 M urea, and then diluted 8 times with 100 mM glycine-NaOH buffer (pH 10.0). The protein concentration was determined by the modified Bradford method (17). After protein quantification, protein concentrations of the untreated control and cytokine/LPS-treated sample were adjusted to be exactly equal in concentration (4 μg/μl) with 100 mM pH 10.0 buffer consisting of glycine-NaOH and 1 M urea and used for the experiments described below.

**Protein Digestion by Lys-N**

**Validation Study—**Two equal aliquots of the above prepared untreated control cell protein samples (200 μg of protein/50 μl for each) were dried in a SpeedVac concentrator. In one tube, 25 μl of $[^{18}$O]$\mathrm{H_2O}$ was added and mixed with 4 μg of Lys-N in 25 μl of $[^{15}$O]$\mathrm{H_2O}$. In another tube, 25 μl of $[^{15}$O]$\mathrm{H_2O}$ was added and mixed with 4 μg of Lys-N in 25 μl of $[^{18}$O]$\mathrm{H_2O}$. The final concentration of glycine-NaOH buffer (pH 10.0) and urea should thus be 100 mM and 1 M, respectively, in both the reaction tubes. The resulting two reaction tubes were then incubated at 25 °C for 18 h to digest the proteins. After incubation, the two digests were mixed in a 1:1 ratio and analyzed by liquid chromatography-tandem mass spectrometry.

**Comparative Proteomic Study—**A 50-μl aliquot from each protein extract sample (treated and untreated, 200 μg of protein/50 μl for each) was dried in a SpeedVac concentrator. The protein pellet from the untreated control sample was redissolved in 25 μl of $[^{18}$O]$\mathrm{H_2O}$ and mixed with 4 μg of Lys-N in 25 μl of $[^{15}$O]$\mathrm{H_2O}$. The protein pellet from the cytokine/LPS-treated sample was redissolved in 25 μl of $[^{15}$O]$\mathrm{H_2O}$ and mixed with 4 μg of Lys-N in 25 μl of $[^{18}$O]$\mathrm{H_2O}$. The resulting two reaction mixtures were then incubated at 25 °C for 18 h to digest the proteins.

**Cation Exchange Chromatography**

The two digests (treated sample and untreated control) were then mixed in a 1:1 ratio and separated by strong cation exchange chromatography as follows. The cation exchange chromatography was performed using a strong cation exchange MicroBulge cartridge (12 μ, 300 Å, 2.0-mm inner diameter × 25 mm, Michrom BioResources, Inc., Auburn, CA) using a syringe pump (NP 70-2213 pump, Harvard Apparatus, Holliston, MA) as a solvent delivery system.
strong cation exchange column was first equilibrated with 1 ml of 5 mM ammonium formate adjusted to pH 3.2. An amount of 150 μg of protein of the mixed digest (37.5 μl) was diluted to 500 μl with 5 mM ammonium formate (adjusted to pH 3.2) and loaded onto the strong cation exchange column using a flow rate of 100 μl/min. The bound peptides from the strong cation exchange column were eluted stepwise with 200 μl each of the following eluents at a flow rate of 200 μl/min: 10, 40, 50, 80, 100, 150, and 500 mM ammonium formate (adjusted to pH 3.2). The unbound fraction and each of the stepwise eluents were collected and dried in a SpeedVac concentrator. Each dried fraction was then reconstituted in 20 μl of 0.1% TFA in [16O]water and analyzed by reversed-phase LC-MS/MS. Adjustment of ammonium formate solutions to pH 3.2 was done with formic acid.

**LC-MS/MS Analysis**

All LC-MS/MS analyses were done using an UltiMate nano-HPLC system (Dionex, San Francisco, CA) consisting of an isocratic pump, an autosampler, a gradient pump module, and a column switching module interfaced to a QStar quadrupole/time-of-flight mass spectrometer (Applied Biosystems-HPLC Sciex, Foster City, CA) via a nano-electrospray ion source (Applied Biosystems-MDS Sciex) and a metal sprayer (New Objective Inc., Woburn, MA). Protein digests (5 μl) were injected into a reversed-phase C4 trapping column (300-μm inner diameter × 1 mm, Dionex) equilibrated with 0.1% formic acid, 2% acetonitrile (v/v) and washed for 5 min with the equilibration solvent at a flow rate of 10 μl/min using an isocratic pump that pumped the solvent through an autosampler. After the washing, the trapping column was switched in-line with the reversed-phase analytical column (0.075 × 50 mm, New Objective Inc.) packed in-house with Jupiter C18 media (10 μm, 300 Å, Phenomenex, Torrance, CA). A gradient pump module was used to produce a linear gradient of acetonitrile from 2 to 35% in aqueous 0.1% formic acid over a period of 100 min at a flow rate of 200 nl/min. After the gradient elution, the acetonitrile concentration was increased by a step change to 80% and maintained for 20 min at a flow rate of 200 nl/min. The column effluent was passed directly into the nano-electrospray ion source attached to the metal sprayer on which 2,050 V of electrospray voltage was applied. The mass spectrometer was operated in a data-dependent MS to MS/MS switching mode with the three most intense ions in each MS scan subjected to MS/MS analysis. Survey MS spectra (from m/z 400 to 2,000) were acquired in the TOF analyzer with 1-s accumulation time. The three most intense ions in each survey TOF-MS analysis were sequentially selected in the first quadrupole mass analyzer and fragmented in the collision cell by collision-induced dissociation with nitrogen gas, and then the generated fragment ions were analyzed in the TOF analyzer using a 2-s accumulation time per precursor ion. The collision energy used for each precursor ion was dynamically selected based on its m/z value and charge state. Previously selected precursor ions were excluded for 60 s. AnalystQS software (version 1.0, Applied Biosystems-MDS Sciex) was used for instrument control, data acquisition, and data processing.

**Protein Identification**

Proteins were identified by comparing all of the experimental product ion spectra of the peptides to the Swiss-Prot database using the Mascot database search software (Matrix Science, London, UK). Only human proteins were searched. S-Carbamidomethylation of cysteine was set as a fixed modification. 18O labeling of the carboxyl terminus of the peptide and the oxidation of methionine (methionine sulfoxide) were set as variable modifications in the database search. Mass tolerances for protein identification on precursor and product ions were both set to 0.2 Da. Strict Lys-N specificity was applied while allowing for one missed cleavage. A minimum Mascot search score of 15 was used as the cutoff for a positive identification.

**Calculation of Corrected 16O/18O-Peptide Ratios**

Precursor peptide ions identified by the Mascot database search were manually extracted from the LC-MS/MS raw data on AnalystQS software, and the peak intensities of 16O- and 18O-labeled peptides were obtained from the extracted signal. The peak intensities for the 16O- and 18O-labeled peptides were corrected for 1) the effect of 5% H218O in the [18O]water and 2) the contribution of the M + 2 isotope of the 18O-labeled peptide to 16O-labeled peptide peak. A corrected 16O/18O peptide ratio was calculated as described previously (5).

**RESULTS**

**Validation of Lys-N-based 18O Labeling Method**—Previously we have shown that the Lys-N-catalyzed 18O labeling method provides accurate quantification results using several model proteins (5). What remains to be done is validation of the applicability of the method for biological samples. This was done by taking equal amounts of reduced and S-carbamidomethylated proteins extracted from the control (untreated ARPE-19 cells) and then digesting in separate experiments using Lys-N in either H216O or in H218O. After the digestion, the two digests were mixed in a 1:1 ratio and analyzed directly by LC-MS/MS. As described under “Experimental Procedures,” identities of the peptides were determined through database searching, and the corrected ratios of 18O- and 18O-labeled peptides were calculated. Theoretically the 16O- and 18O-labeled peptide ratios of all the generated peptides should be 1:1. Corrected ratios of
more of the following amino acid sequences: 1) -Lys-
discrepant peptides were products of cleavage on one or
shown in Table I. The analysis revealed that 11 of the 21
after the cleavage sites of these peptides. The results are
crepant peptides as well as amino acid sequences before and
ations specified.
biological samples analyzed by this method under the condi-
or above 2.0, implying false results for 17% of the peptides for
ever, 21 peptides (17% of 121 peptides) gave ratios below 0.5
0.5–2.0 range compared with the theoretical ratio of 1. How-
results to 8% (10 peptides of 121) in this experiment. We
produced from these sequences, reducing the 17% false
peptides the corrected16O/18O peptide ratios were within the
from 40 different proteins are given in Fig. 1. For most of the
ments of the b-type ions (20). As can be seen in the product
ion spectra arising, at least in part, from internal rearrange-
are generally more predominant than b-type ions (19). This
product ion spectra of tryptic peptides in which y-type ions
resentative Lys-N peptides are shown in Fig. 2. The first
sociation used in this work. Product ion spectra of two rep-
peptides produced by the low energy collision-induced dis-
acy in quantification.
It is instructive to examine product ion spectra of Lys-N
peptides produced by the low energy collision-induced dis-
siation in this work. Product ion spectra of two re-
representative Lys-N peptides are shown in Fig. 2. The first
peptide is KNQLTSNPENTVFDA. This peptide shows a typical
product ion spectrum obtained for a Lys-N peptide. As can be seen in Fig.
2a, the predominant fragment ions observed in the product
spectrum were b-type ions. This is in contrast to the
product ion spectra of tryptic peptides in which y-type ions
are generally more predominant than b-type ions (19). This
may be attributed to the preferential localization of positive
charges on α- and/or ε-amino group(s) of the amino-terminal
lysine residue for Lys-N-generated peptides. The second
peptide is KYQNULLRIEEELGS (Fig. 2b). This peptide has a
basic amino acid residue (arginine) in the middle of the pep-
tide. Such peptides are known to produce complex product
ion spectra arising, at least in part, from internal rearrange-
ments of the b-type ions (20). As can be seen in the product
ion spectrum (Fig. 2b), predominant ions were still b-type with y-type ions containing the arginine residue also present. The spectra presented and the fact that 1,046 peptides from 584 proteins were identified in the comparative proteomic study (see below) confirm the effectiveness of the Mascot database search software in identifying proteins from Lys-N-generated peptides. This is seen even though the fragmentation patterns of Lys-N peptides are different from those of tryptic peptides and for peptides that have a basic amino acid residue in the middle of the sequence.

Comparative Proteomic Study on Cytokine/LPS-treated ARPE-19 Cells—Proteins from cytokine/LPS-treated and untreated (control) ARPE-19 cells were digested in H$_2$O and H$_2$O$_{16}$ solvent, respectively, mixed in a 1:1 ratio, and then separated into eight fractions by strong cation exchange chromatography. The eight fractions were analyzed one-by-one by reversed-phase LC-MS/MS. The obtained LC/MS/MS data were subjected to a Mascot database search for peptide sequencing and subsequent protein identification. Peak intensities of each identified 16O- and 18O-labeled peptide were obtained and corrected as described under “Experimental Procedures.” These analyses resulted in the identification of 584 proteins in the cells, listed in Supplemental Table I along with peptide sequences identified. Relative abundances for most of these proteins were determined except for 22 proteins that were identified solely by a single carboxyl-terminal peptide because the carboxyl-terminal peptide of a protein does not incorporate 18O atom by this method. A total of 1,046 peptides were quantified through manual data analysis to determine the ratio of each peptide in the two samples. Note that any corrected 16O/18O peptide ratios greater than 10 or smaller than 0.1 were recorded as ratios >10 or <0.1 because this method may not be accurate for these large-fold changes (5). Proteins were identified from various cellular compartments. The highest number came from the cytoplasm (~19%) followed by 17% from the membrane, 13% from the nucleus, 7% from the mitochondrial, 2% from the endoplasmic reticulum, and 1% from lysosomes. Approximately 32% of proteins were from unknown locations. As expected, this method has the capability of identifying membrane proteins, which are generally difficult to identify by the commonly used 2D PAGE method. As far as we know this is the most comprehensive description of a proteome in RPE that has been reported.
The expression levels of 11 proteins were found to be greater than 2-fold in cytokine/LPS-treated cells compared with untreated control cells (16O/18O peptide ratio \( > 0.5 \) \( \) (Table II). Among the 11 proteins, five proteins (indoleamine 2,3-dioxygenase \( \) (KGTGTDLMNFL), interferon-induced guanylate-binding protein \( \) (KPEVSEGTEVT) (KGIQAEILQTYL), Methyltetrahydrofolate dehydrogenase \( \) (KMPILGLGTW), colonic and hepatic tumor expressed protein \( \) (KITSELVS), plasminogen activator inhibitor-1 \( \) (KELMGPWN)), and tryptophanyl-tRNA synthetase \( \) (KGTGGTDLMNFL) were previously known to be induced by cytokines, providing further evidence supporting the quantitative reliability of the technique. The other six proteins are not previously known to be induced by cytokines. Further work will need to be done to substantiate that these identified proteins are in fact induced by cytokines.

We also found 49 proteins whose expression levels appeared to be decreased by a factor of 2 or more in the cytokine/LPS-treated cells compared with those in the control untreated cells (16O/18O peptide ratio \( > 2.0 \) \( \) ). A list of these proteins is provided in Supplemental Table II. Note that proteins that were identified exclusively by peptides that were the products of cleavage of one or more of the following amino acid sequences: 1) -Lys- \( \) X 0-3-Lys-, 2) -Glu-Lys-, and 3) -Pro-Lys-, were not included in Table II and Supplemental Table II due to the variability considerations described previously even though the experimentally obtained corrected 16O/18O peptide ratios for these proteins are given in Supplemental Table I.

### Table II

| Accession number\(^a\) | Protein name (peptides identified) | -Fold change\(^b\) | Total no. of MS/MS spectra interpreted |
|------------------------|-----------------------------------|--------------------|---------------------------------------|
| P15121\(^c\)          | Aldose reductase (KMPILGLGTW)     | 2.5                | 3                                     |
| Q14008                 | Colonic and hepatic tumor expressed protein (KITSELVS) | >10               | 1                                     |
| P14902                 | Indoleamine 2,3-dioxygenase (KGTGTDLMNFL) | >10               | 1                                     |
| P05362                 | Intercellular adhesion molecule-1 precursor (KPEVSEGTEVT) (KGIQAEILQTYL) | >10               | 4                                     |
| P32456                 | Interferon-induced guanylate-binding protein (KEDVADALLQTDQLSL) (KESMTDAIQLQTDQTLTE) (KGIQAEILQTYL) | >10               | 6                                     |
| P13995                 | Methyltetrahydrofolate dehydrogenase (KASISEEELNIN) | 2.0                | 1                                     |
| P51970                 | NADH-ubiquinone oxidoreductase (PGIVLPTLEEL) | >10               | 1                                     |
| P05121                 | Plasminogen activator inhibitor-1 (KELMGPWN) | >10               | 1                                     |
| P20648                 | Potassium-transporting ATPase (KNLEAVETSIVICSD) | >10               | 1                                     |
| P04179                 | Superoxide dismutase (Mn) (KGDVTAQIALOPAL) (KGEELIAI) | 5.0                | 3                                     |
| P23381                 | Tryptophanyl-tRNA synthetase (KDLTDGAVYA) (KMSASDPNSFNSTDA) (KGIQFGTDSICG) | >10               | 6                                     |

\(^a\) Swiss-Prot accession number.

\(^b\) Calculated from the observed peak area of 16O- and 18O-peptides. When multiple peptides were found for a particular protein, -fold changes are mean of the individual peptides -fold change. The -fold change of individual peptide can be seen in the supplemental data.

\(^c\) Mascot also identified this peptide to an identical sequence in alcohol dehydrogenase (accession number P14550).

The expression levels of 11 proteins were found to be greater than 2-fold in cytokine/LPS-treated cells compared with untreated control cells (16O/18O peptide ratio \( < 0.5 \) \( \) ) (Table II). Among the 11 proteins, five proteins (indoleamine 2,3-dioxygenase \( \) (KGTGTDLMNFL), interferon-induced guanylate-binding protein \( \) (KPEVSEGTEVT) (KGIQAEILQTYL), Methyltetrahydrofolate dehydrogenase \( \) (KMPILGLGTW), colonic and hepatic tumor expressed protein \( \) (KITSELVS), plasminogen activator inhibitor-1 \( \) (KELMGPWN)), and tryptophanyl-tRNA synthetase \( \) (KGTGGTDLMNFL) were previously known to be induced by cytokines, providing further evidence supporting the quantitative reliability of the technique. The other six proteins are not previously known to be induced by cytokines. Further work will need to be done to substantiate that these identified proteins are in fact induced by cytokines.

We also found 49 proteins whose expression levels appeared to be decreased by a factor of 2 or more in the cytokine/LPS-treated cells compared with those in the control untreated cells (16O/18O peptide ratio \( > 2.0 \) \( \) ). A list of these proteins is provided in Supplemental Table II. Note that proteins that were identified exclusively by peptides that were the products of cleavage of one or more of the following amino acid sequences: 1) -Lys- \( \) X 0-3-Lys-, 2) -Glu-Lys-, and 3) -Pro-Lys-, were not included in Table II and Supplemental Table II due to the variability considerations described previously even though the experimentally obtained corrected 16O/18O peptide ratios for these proteins are given in Supplemental Table I.

**DISCUSSION**

The advantages of Lys-N-based 18O labeling method compared with trypsin-based methods are as follows: 1) incorporation of only a single 18O atom, 2) no enzyme-catalyzed 18O back-exchange, and 3) production of a less complex protein digest (5). In this study, a total of 1,046 peptides were sequenced and quantified, leading to the identification and
quantification of 562 proteins (excluding 22 proteins that were identified solely by a single carboxyl-terminal peptide), comparing the relative abundances of proteins of the cytokine/LPS-treated and untreated (control) ARPE-19 cells. This is the most comprehensive description of the RPE proteome that has been reported. These results demonstrate the usefulness of Lys-N-based proteolytic 18O labeling method in proteomic studies.

Our validation study revealed that peptides generated by cleavages on one or more of the following amino acid sequences: 1) -Lys-Xaa-Lys-, 2) -Glu-Lys-, and 3) -Pro-Lys-, may not be accurately quantified due to presumed incomplete cleavage at these sites under the given digestion conditions. The recognition of amino acid sequences that have been shown to give inaccurate quantification results is significant because the inaccuracy of the technique can be reduced by excluding peptides generated from one or more of these sequences. The incomplete protein digestion for particular amino acid sequences is a drawback of this method. Efforts to achieve complete digestion of substrate proteins by Lys-N are in progress.

We must assume that our results contain false positive results (~8%) based on the validation experiment. One effective way to reduce false positive results would be to perform a reverse labeling experiment in which proteins from the control and experimental sample are digested in H2 16O and H2 18O, respectively, and compare the results with the results presented (control and experimental sample were digested in H2 16O and H2 18O, respectively). However, such an experiment would require an enormous amount of effort to complete unless software that can accelerate the data interpretation process becomes available as discussed below.

In the present study, quantification of each peptide was performed manually without the help of computational tools. It was an extremely time-consuming process. It took an experienced mass spectrometrist more than 3 months to complete the quantification of all 1,046 positively identified peptides (~4 h/day). The manual analysis included the following: 1) extraction of each 16O- and 18O-labeled peptide peak from the LC/MS/MS raw data based on Mascot database search results, 2) measurement of signal intensities of each 16O- and 18O-labeled peptide, and 3) calculation of corrected 16O- and 18O-labeled peptide ratio by incorporating the contribution of 18O-labeled peptide, and 3) calculation of corrected 16O- and 18O-labeled peptide ratio by incorporating the contribution of 18O-labeled peptide to the 16O-labeled peptide peak. Recently Halligan et al. (26) developed a stand-alone computational tool that quantifies the mass spectra of 16O-labeled peptides from an ion trap instrument. Development of such software that can handle data produced by any type of commercial instruments will greatly facilitate the use of the proteolytic 18O labeling method in comparative proteomics.

The identified proteins are assumed to be relatively high abundance proteins. In this study, we separated peptides by strong cation exchange chromatography followed by reversed-phase chromatography. We observed many peptides that were not subjected to MS/MS analysis in the survey MS spectra because their signal intensities were not within the three most intense signal peaks. Therefore there was no sequence information for these peptides, indicating that more peptides could be analyzed with the development of better peptide separation techniques. This confirms the importance of achieving better peptide separation before mass spectrometric analysis to identify low abundance proteins.

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