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# Difference in Mass Analysis Using Labeled Lysines (DIMAL-K)

A NEW, EFFICIENT PROTEOMIC QUANTIFICATION METHOD APPLIED TO THE ANALYSIS OF ASTROCYTIC SECRETOMES\*

Nicolas Delcourt, Patrick Jouin, Joël Poncet, Emmanuelle Demey, Eric Mauger, Joël Bockeaert, Philippe Marin, and Nathalie Galéotti‡

**Here we describe an original strategy for unbiased quantification of protein expression called difference in mass analysis using labeled lysine (K) (DIMAL-K). DIMAL-K is based on the differential predigestion labeling of lysine residues in complex protein mixtures. The method is relevant for proteomic analysis by two-dimensional electrophoresis and MALDI-TOF mass spectrometry. Protein labeling on lysine residues uses two closely related chemical reagents, S-methyl thioacetimidate and S-methyl thiopropionimidate. Using protein standards, we demonstrated that 1) the chemical labeling was quantitative, specific, and rapid; 2) the differentially labeled proteins co-migrated on two-dimensional gels; and 3) the identification by mass fingerprinting and the relative quantification of the proteins were possible from a single MALDI-TOF mass spectrum. The power of the method was tested by comparing and quantifying the secretion of proteins in normal and proinflammatory astrocytic secretomes (20  $\mu$ g). We showed that DIMAL-K was more sensitive and accurate than densitometric image analysis and allowed the detection and quantification of novel proteins. *Molecular & Cellular Proteomics* 4:1085–1094, 2005.**

Since the last decade, MS has become one of the most powerful techniques in proteomics. Two-dimensional (2-D)<sup>1</sup> PAGE in combination with MALDI-TOF analysis provides a sensitive method for protein identification. An important challenge in proteomics deals with quantification of relative expression levels of individual proteins in two different biological

samples. In this context, quantification by mass spectrometry has recently become a major alternative strategy to analysis based on densitometric or fluorescence technologies (1–4). Despite extensive work accomplished in the field of quantification, only few strategies have been associated with the widely used 2-D PAGE/MALDI.

Quantification by mass spectrometry requires the production of differentially labeled peptides from two sets of proteins. The difference in expression levels is obtained by measuring the relative intensities of MS signals of the mixed labeled peptides. Different strategies have already been described (for reviews, see Refs. 2 and 5–8). These strategies differ in 1) the pre- or postdigestion labeling, 2) the choice of the labeled function (thiol, primary amine, or carboxylic acid), 3) the use of stable isotopes or alternative chemicals, and 4) the type of MS analysis (LC-MS/MS or MALDI-TOF MS). Predigestion labeling allows for greater quantification accuracy than postdigestion labeling. Indeed introducing the chemical modification early in the experimental procedure reduces differential loss of proteins during further biochemical steps and guarantees accuracy of the quantification. Predigestion labeling often involves stable isotope incorporation through either metabolic labeling or chemical labeling on a specific residue of proteins. Metabolic labeling, such as the stable isotope labeling by amino acids in cell culture (SILAC) method, is introduced *in vivo* early in the process (9, 10) allowing for greater quantification accuracy than postdigestion labeling. However, metabolic labeling can only be used for cultured cells grown in controlled media. Chemical labeling with stable isotopes was mainly introduced by selective alkylation of cysteine residues with either a light or a heavy reagent. The most widely used methodology is based on a class of reagents referred to as ICATs (11, 12). In the same manner, cysteine residues of a mixture of proteins can be alkylated with  $d_0$ - or  $d_3$ -acrylamide (13–15) or by using the HysTag strategy (16). Very recently, isotope-coded protein labeling of free amino groups in intact proteins using succinimide activation was described (17). This contrasts with previously reported approaches based on lysine derivatization such as the global internal standard strategy (GIST) in which labeling is performed after enzymatic cleavage of protein samples (18–21). Beardsley and Reilly (22) have also proposed a method called QUEST (quantification

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<sup>1</sup> The abbreviations used are: 2-D, two-dimensional; DIMAL-K, difference in mass analysis using labeled lysine (K); LPS, lipopolysaccharide; VIME, vimentin; SPARC, secreted protein acid and rich in cysteine; VCA1, vascular cell adhesion protein 1; C3L1, chitinase-3-like protein-1, chain 1; CO3A, complement C3  $\alpha$  chain; CO3B, complement C3  $\beta$  chain; B2MG,  $\beta_2$ -microglobulin; NGAL, neutrophil gelatinase-associated lipocalin precursor; Me, acetamidine moiety or N<sup>ε</sup>-lysine; Et, propionamide moiety on N<sup>ε</sup>-lysine; AOP2, antioxidant protein 2; CYPHA, cyclophilin A.

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using enhanced signal tags) in which efficient amidination of N-terminal and  $\epsilon$ -amino groups is performed on peptides using S-methyl thioimide reagents. We propose here a highly efficient strategy for differential quantification of proteins from different physiological states that we call difference in mass analysis using labeled lysine (K) (DIMAL-K). DIMAL-K is based on the differential amidination of lysine residues in complex mixtures of intact proteins (before the digestion step) using the two closely chemically related reagents S-methyl thioacetimidate and S-methyl thiopropionimidate (22). The mixture of differentially labeled proteins was separated on 2-D gels, and identification and relative quantification of individual proteins were performed in a single MALDI-TOF mass analysis. The relative expression of individual proteins in each state was deduced from the difference in signal intensities of differentially lysine-labeled tryptic peptides separated by multiples of 14 Da. We applied DIMAL-K to quantify the variation of protein secretion in astrocytes secretomes (20  $\mu$ g) following exposure to a proinflammatory treatment.

#### EXPERIMENTAL PROCEDURES

**Materials**—S-Methyl thioacetimidate and S-methyl thiopropionimidate were synthesized according to Beardsley and Reilly (22). Ovalbumin (chicken egg), myoglobin (horse skeletal muscle), cytochrome *c* (horse heart), lysozyme (chicken egg), and lipopolysaccharide (type 055:B5) were from Sigma. Swiss mice were obtained from Elevage Janvier (Le Genest-Saint-Isle, France).

**Astrocyte Cultures**—Primary cultures of mouse striatal astrocytes were prepared as described previously (23). Briefly striata from 18-day-old Swiss mouse embryos were mechanically dissociated in PBS supplemented with 33 mM glucose (PBS-glucose). Cells were seeded ( $0.5 \times 10^6$  cells/ml) in 100-mm (15 ml/dish) culture dishes previously coated with poly-L-ornithine (1.5  $\mu$ g/ml,  $M_r$  40,000). The culture medium consisted of a 1:1 mixture of Dulbecco's modified Eagle's medium and F-12 nutrient supplemented with 30 mM glucose, 2 mM glutamine, 13 mM  $\text{NaHCO}_3$ , 5 mM HEPES buffer (pH 7.4), penicillin-streptomycin (100 IU/ml and 100  $\mu$ g/ml, respectively), and 10% N-serum (BD Biosciences). The culture medium was changed every 3 days, and cells were maintained for 21 days at 37 °C in a humidified atmosphere containing 5%  $\text{CO}_2$ . At this stage, cultures were shown to be highly enriched in astrocytes (more than 98% of glial fibrillary acidic protein-positive cells) that had formed a confluent monolayer and devoid of neuronal and microglial or endothelial cells (assessed by the lack of microtubule-associated protein 2 and isolectin B4 immunostaining, respectively) (24).

**Preparation of Astrocyte-conditioned Media**—Cells were washed six times with the serum-free culture medium. This washing procedure efficiently eliminated all serum proteins because we did not detect any bovine albumin, the major serum protein, in the astrocyte-conditioned medium. Cells were then covered with a minimal volume of the serum-free medium (6 ml/dish) for 18 h at 37 °C and 5%  $\text{CO}_2$  in the presence or absence of lipopolysaccharide (LPS) (1  $\mu$ g/ml). Astrocyte-conditioned media were harvested and centrifuged successively at  $200 \times g$  (5 min),  $1,000 \times g$  (10 min), and  $20,000 \times g$  (25 min) to remove non-adherent cells and debris. Samples (about 20  $\mu$ g of protein/dish) were precipitated for 2 h with 10% ice-cold TCA. Precipitates were then washed three times with diethyl ether, dried, and solubilized in 6 M urea. Protein concentration in conditioned media and cell extracts was determined by the bicinchoninic acid method (25).

**Labeling of Standard and Astrocyte-secreted Proteins**—150  $\mu$ g of labeling reagent (S-methyl thioacetimidate or S-methyl thiopropionimidate synthesized according to Beardsley and Reilly (22)) dissolved in 10  $\mu$ l of 200 mM  $\text{NaHCO}_3$  (pH 8.7) was added to each standard protein solution (30  $\mu$ g in 10  $\mu$ l of either 6 M urea, 6 M urea plus 4% CHAPS, or 6 M urea plus 1% SDS (or a solution of proteins secreted by the astrocytes (about 20  $\mu$ g of protein in 10  $\mu$ l of 6 M urea)). The mixture was stirred for 90 min at room temperature and precipitated with 10% ice-cold TCA during 2 h. Centrifugation ( $38,000 \times g$  for 30 min) was then performed. Precipitates were washed three times with diethyl ether and dried.

**Two-dimensional Gel Electrophoresis, Image Acquisition, and Spot Pattern Analysis**—Proteins were resuspended in 350  $\mu$ l of isoelectrofocusing medium containing 7 M urea, 2 M thiourea, 4% CHAPS, ampholines (preblended, pl 3.5–9.5, 8 mg/ml, Amersham Biosciences), 100 mM DTT, 0.2% Tergitol NP7 (Sigma), and traces of bromophenol blue (26). Proteins were first separated according to their isoelectric point along non-linear IPG strips (pH 3–10, 18 cm long) using the IPGphor apparatus (Amersham Biosciences). Sample loading for the first dimension was performed by passive in-gel reswelling. After the first dimension, the IPG strips were equilibrated for 10 min in a buffer containing 6 M urea, 50 mM Tris-HCl (pH 6.8), 30% glycerol, 2% SDS, and 10 mg/ml DTT and then for 15 min in the same buffer containing 15 mg/ml iodoacetamide instead of DTT. For the second dimension, the strips were loaded onto vertical 10–15% gradient SDS-polyacrylamide gels. The gels were silver-stained according to the procedure of Shevchenko and Wim (27). Gels to be compared were always processed and stained in parallel. Gels were scanned using a computer-assisted densitometer. Spot detection, gel alignment, and spot quantification were performed using Melanie 5 software (Amersham Biosciences). Quantification of proteins was expressed as volumes of spots. To correct for variability resulting from silver staining, results were expressed as relative volumes of all spots in each gel. Data are the means of values from four gels originating from experiments performed on different sets of cultured astrocytes.

**Q-TOF Mass Spectrometry Analysis**—10  $\mu$ g of standard protein labeled with either S-methyl thioacetimidate or S-methyl thiopropionimidate were solubilized in 20  $\mu$ l of 1% formic acid, and 2  $\mu$ l were transferred in a tip emitter (Protana, Odense, Denmark). Nano-electrospray mass spectrometry was performed off line on a Q-TOF mass spectrometer (QSTAR Pulsar-*i*, Applied Biosystems, Foster City, CA) fitted with a Protana nanospray inlet system. Spectra were recorded using Analyst QS software (Applied Biosystems). Parameters were adjusted as follows: ion spray voltage, 900 V; curtain gas, 25; declustering potential, 45–75 V; focusing potential, 265 V; declustering potential 2, 15 V.

**MALDI-TOF Mass Spectrometry and Protein Identification**—Proteins of interest were excised and digested in-gel using trypsin (sequencing grade, Promega, Charbonnières, France) as described previously (27). Digested proteins were dehydrated in a vacuum centrifuge, solubilized in 10  $\mu$ l of formic acid (2%), desalted using  $\text{C}_{18}$  ZipTips (Millipore, Bedford, MA; elution with 10  $\mu$ l of 0.1% TFA in 60% acetonitrile), and concentrated to a 2- $\mu$ l volume. 0.3  $\mu$ l was mixed with the same volume of  $\alpha$ -cyano-4-hydroxy-*trans*-cinnamic acid (10 mg/ml in 0.1% TFA in 50% acetonitrile), deposited on a 384-well MALDI target using the dry droplet procedure (28), and air-dried at room temperature. Analyses were performed using an UltraFlex MALDI-TOF/TOF mass spectrometer (Bruker-Franzen Analytik, Bremen, Germany) operating in the reflectron mode with a 20-kV accelerating voltage and a 70-ns delayed extraction. Mass spectra were acquired in the automatic mode using the AutoXecute™ module of Flexcontrol™ (Bruker-Franzen Analytik) (laser power ranged from 30 to 70%, 500 shots). Spectra were analyzed using FlexAnalysis™ software (Bruker-Franzen Analytik) and calibrated internally with the

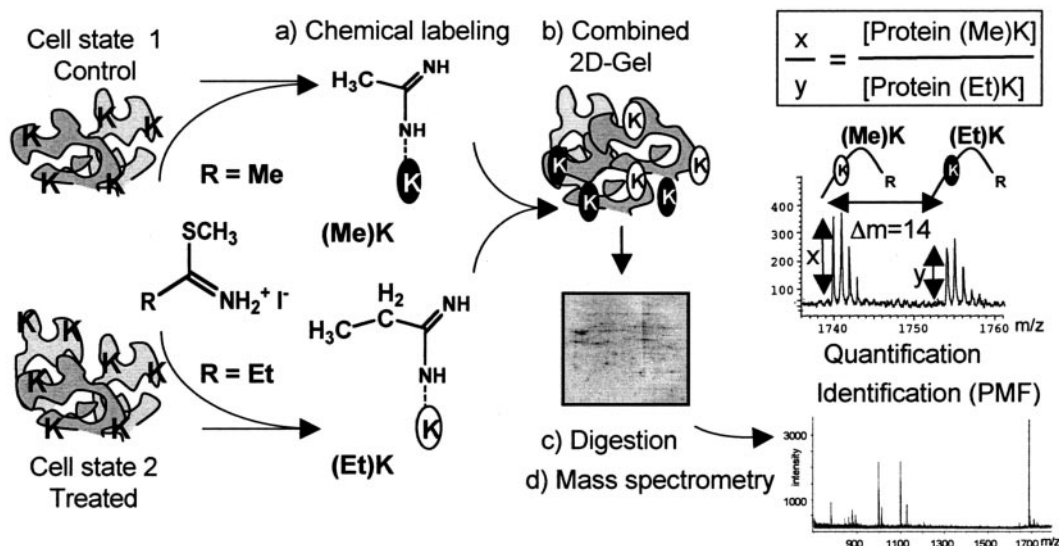


FIG. 1. **Principle of the DIMAL-K method.** a, the proteins from cell states 1 and 2 are first labeled on lysine residues with *S*-methyl thioacetimidate and *S*-methyl thiopropionimidate, respectively. b, the two sets of labeled proteins are then combined and separated by two-dimensional electrophoresis in the same gel. c, individual spots are excised, and proteins are digested with trypsin. d, the tryptic peptides are analyzed by MALDI-TOF MS to identify and quantify the protein. The relative amount of protein in each state is determined by the ratio of the peak intensity of the peptide signals corresponding to the differentially labeled peptides Me and Et. PMF, peptide mass fingerprint.

autoproteolysis peptides of trypsin ( $m/z$  842.51, 1045.56, and 2211.10). Peptides were selected in the mass range of 900–4,000 Da. Peptide mass fingerprint identification of labeled proteins was performed by searching against the Swiss-Prot Databases using an in-house Mascot software package (Matrix Science) with Arg-C enzyme specificity and no trypsin missed cleavages. Acetimidoyl and propionimidoyl were set as fixed lysine modifications for searches on single labeled samples or as variable lysine modifications for searches on mixed labeled samples. A mass tolerance of 50 ppm was allowed for identification.

**MALDI-TOF MS Quantification**—Quantification was performed on pairs of identified labeled peptides by measuring the intensity of the  $^{12}\text{C}$  isotopic peaks. Only good quality signals (resolution > 8,000, signal to noise ratio > 15, and no overlap) were selected. Two inverse labeling experiments were performed for each sample: either LPS-treated samples were labeled with *S*-methyl thioacetimidate, and the untreated ones were labeled with *S*-methyl thiopropionimidate, or inversely LPS-treated samples were labeled with *S*-methyl-thiopropionimidate, and the untreated ones were labeled with *S*-methyl-thioacetimidate.

**Protein Data Base Statistics**—Statistics given in Table II were calculated from a Fasta-formatted Swiss-Prot Database (November 23, 2004 update) by using home-made lua scripts ([www.lua.org](http://www.lua.org)). These scripts were written to run on various platforms (Win, MacOSX, Linux, and UNIX) and can be obtained freely on request ([eric.mauger@ccipe.cnrs.fr](mailto:eric.mauger@ccipe.cnrs.fr)).

## RESULTS

The DIMAL-K method described in Fig. 1 was designed to measure changes in expression levels of individual proteins between two biological samples. The efficiency and accuracy of the DIMAL-K method was first investigated using standard proteins: myoglobin from horse skeletal muscle, albumin from chicken egg white, cytochrome *c* from horse heart, and lysozyme from chicken egg.

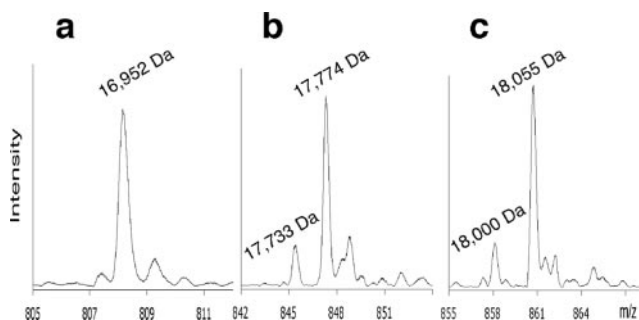
**Efficiency of Chemical Labeling**—30  $\mu\text{g}$  of each standard

protein was dissolved in either 6 M urea, 6 M urea plus 1% SDS, or 6 M urea plus 4% CHAPS. The reaction was performed under mild pH conditions (pH 8.7) with either *S*-methyl thioacetimidate or *S*-methyl thiopropionimidate (30-fold molar excess of reagent over the total concentration of the amine groups). The gain in molecular weight between unlabeled and labeled standard proteins was assessed by ESI-Q-TOF mass analysis indicating that all  $\epsilon$ -amino lysine groups were derivatized under these experimental conditions. For example, the labeling of myoglobin with *S*-methyl thioacetimidate resulted in a mass shift from 16,952 Da (unlabeled protein) to 17,774 Da (labeled protein), which corresponds to the expected  $20 \times 41$  Da for the 19 lysine residues and the N-terminal amine (Fig. 2). Similar results were obtained from *S*-methyl thiopropionimidate. We detected an additional mass peak at 17,733 Da (of about 20% intensity compared with the mass peak of myoglobin with 20 labeled amine functions) corresponding to myoglobin bearing 19 labeled lysine residues. The pool of partially labeled myoglobin includes the unlabeled N-terminal residue because N-terminal amine shows lower reactivity with the reagent than the  $\epsilon$ -amine of lysines. Similar results were obtained for cytochrome *c* and lysozyme (data not shown).

The efficiency of derivatization of proteins was further confirmed by MALDI-TOF MS analysis of tryptic peptides. In MALDI-TOF MS analysis of the tryptic digest of unlabeled ovalbumin, among the four major signals detected, three of them came from cleavage after lysine: (K)HIATNAVLFFGR, (K)AFKDEDTQAMPFR, and (K)ISQAVHAAHAEINEAGR. In contrast, the MS analysis of the tryptic digestion of the la-

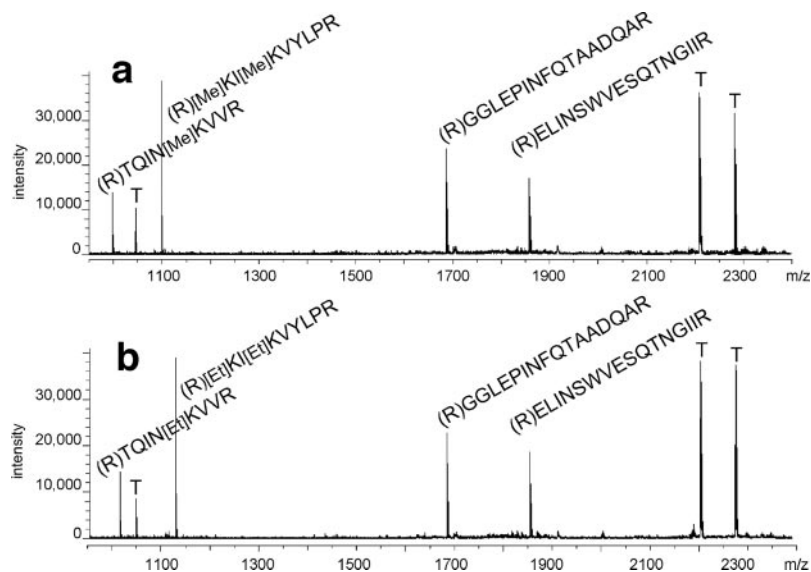
beled ovalbumin showed a different pattern in which only peptides resulting from cleavage after arginine were detected, including some peptides with labeled lysines in their sequence (Fig. 3). Moreover no peptide with unlabeled lysine was detected. These observations in addition to the Q-TOF mass analyses of labeled proteins prove that the reaction on the  $\epsilon$ -position of lysine was total, leading to an Arg-C protease-like behavior of trypsin toward the derivatized proteins. In addition, we did not observe any ion signal corresponding to a potential side reaction. The same results were obtained for labeled myoglobin, cytochrome *c*, and lysozyme (data not shown).

**Protein Identification**—Ovalbumin labeled with either *S*-methyl thioacetimidate or *S*-methyl thiopropionimidate was identified by peptide mass fingerprint analysis with a high Mascot score of 170 and 24% sequence coverage (the statistical significance threshold was 62), indicating that the labeling reaction does not preclude identification. The specific parameters include Arg-C as the proteolytic enzyme and acetimidoyl or propionimidoyl as fixed lysine modifications.



**FIG. 2. Q-TOF mass analysis of unlabeled myoglobin (a), myoglobin labeled with *S*-methyl thioacetimidate (b), and myoglobin labeled with *S*-methyl thiopropionimidate (c).** Average masses were calculated from the +21 charge state. Peaks at 17,774 and 18,055 correspond to labeling of 20 amine functions. Peaks at 17,733 and 18,000 correspond to labeling of 19 lysine residues.

**FIG. 3. MALDI-TOF analysis of tryptic peptides from ovalbumin labeled with *S*-methyl thioacetimidate reagent (a) and ovalbumin labeled with *S*-methyl thiopropionimidate reagent (b).** *T* represents ion signals of trypsin autolysis peptides.



We detected two peptides with high intensities containing one (TQINKVVR) and two amidinated lysines (KIKVYLPR) in the peptide mass fingerprint of each labeled ovalbumin digest (Fig. 3). Identification could also be performed on mixtures of differentially labeled ovalbumin with a Mascot score of 136 and 24% sequence coverage. In this case, acetimidoyl and propionimidoyl labeling was set as variable lysine modifications leading to a scoring decrease.

**Quantitative Analysis of Standard Proteins Using DIMAL-K Strategy**—The accuracy of quantification by the DIMAL-K strategy is conditioned by the co-migration of the differentially labeled proteins on 2-D gels. Two ovalbumin samples were labeled with either *S*-methyl thioacetimidate or *S*-methyl thiopropionimidate and then mixed at various ratios ranging from 0.4 to 2 (Me/Et) to evaluate the sensitivity of the method for small differences in expression. 1  $\mu$ g of unlabeled protein and each protein mixture were resolved by 2-D gel electrophoresis. Image analysis showed that protein labeling did not alter the profile of ovalbumin isoform separation by 2-D gel electrophoresis and preserved a good resolution (Fig. 4). Labeling only induced a slight increase in the apparent protein molecular weight. In contrast, 2-D analysis of mixtures of differentially labeled proteins showed no detectable  $M_r$  or  $pI$  shifts for proteins labeled with either of the two reagents, indicating that the proteins co-migrated, and thus that quantification was not biased by spot picking. This is consistent with the low difference in mass between differentially labeled proteins (14 mass units per lysine residue), which is below the resolution of SDS-PAGE.

After excision, digestion, and MALDI-TOF mass analysis of 2-D spots of each isoform, quantitative analysis of differentially labeled ovalbumin mixtures was carried out from all spots detected on 2-D gels by measuring the ion signal intensities of two couples of tryptic peptides, which contain one (TQINKVVR,  $M_r = 998.6$  and 1012.6) or two amidinated ly-

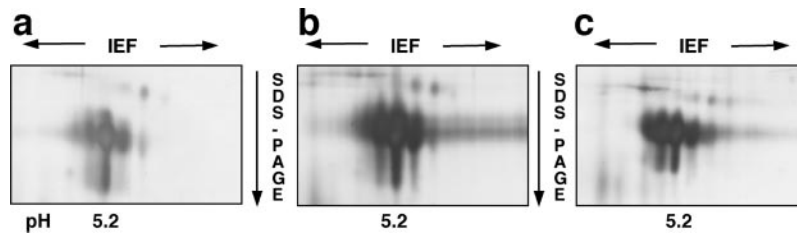


FIG. 4. Two-dimensional gel analysis of unlabeled ovalbumin (a), a mixture of 1:1 unlabeled ovalbumin and ovalbumin labeled with S-methyl thioacetimidate reagent (b), and a mixture of 1:1 ovalbumin labeled with S-methyl thioacetimidate reagent and S-methyl thiopropionimidate reagent (c).

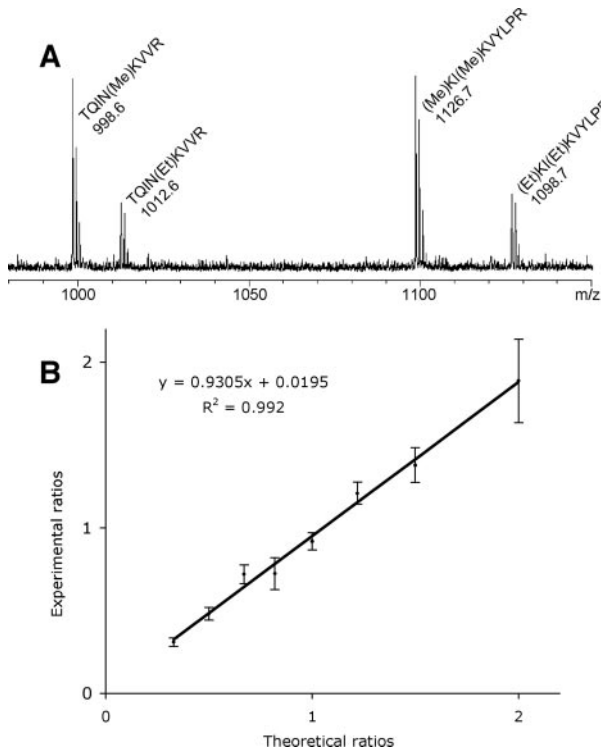


FIG. 5. A, MALDI-TOF mass spectrum of a 7:3 mixture of Me- and Et-labeled ovalbumin. B, observed versus expected ratios obtained from MALDI-TOF analyses of mixtures at various ratios ranging from 0.4 to 2 of labeled Me-ovalbumin and Et-ovalbumin for four experiments.

sines (KIKVVYLPR,  $M_r = 1,098.7$  and  $1,126.7$ ) (Fig. 5a). Comparing the experimental ratio of the measured signal intensities of the monoisotopic peaks with the theoretical ratio indicated that the signal intensities were neither biased by differences in the reactivity of each reagent nor by differences in desorption-ionization potency between peptides derivatized by both reagents (Fig. 5b) because the average deviation from expected ratios was only 15%, yielding a linear dynamic range with a slope close to 0.93 and an  $R^2$  value of 0.992. To prove that the DIMAL-K method provides accurate and sensitive quantification, we also evaluated the differential quantification using a broader dynamic range up to a 120-fold quantitative difference over a protein amount range varying from 5 to 300 fmol. The observed ratios were linear across a

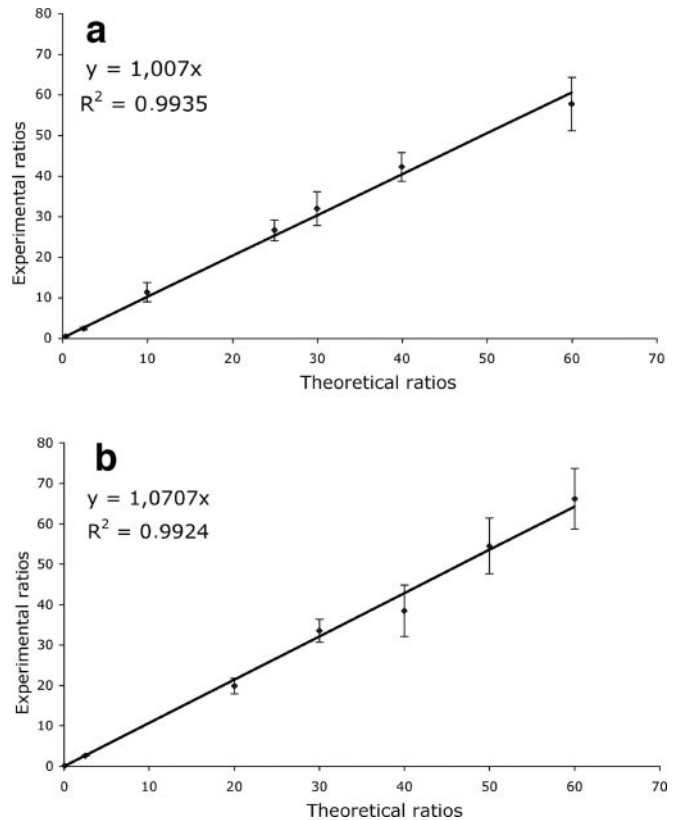


FIG. 6. Observed versus expected ratios obtained from MALDI-TOF analyses of concentration ratios ranging from 0.5 to 60 over amounts ranging from 5 to 300 fmol of mixture of labeled Me-ovalbumin and Et-ovalbumin (a) and Me-myoglobin and Et-myoglobin (b). For each mixture of proteins, three experiments were performed for the entire range of relative concentrations.

120-fold range of concentration ratios from 0.5 to 60 with  $R^2$  values of 0.993 for ovalbumin and 0.992 for myoglobin and with an error of less than 15% (Fig. 6).

*Quantification of Variations in Astrocytic Protein Secretion Induced by a Proinflammatory Treatment by Using the DIMAL-K Strategy*—Previously we have identified the major proteins secreted by astrocytes, the major cell population in the mammalian central nervous system, using a proteomic approach combining the separation of proteins recovered from astrocyte culture-conditioned medium by 2-D electrophoresis and their identification by MALDI-TOF MS (24). We also

showed that exposing cultured astrocytes to LPS or proinflammatory cytokines (interleukin 1 $\beta$  or tumor necrosis factor  $\alpha$ ) modified the profile of astrocytic protein secretion (24).

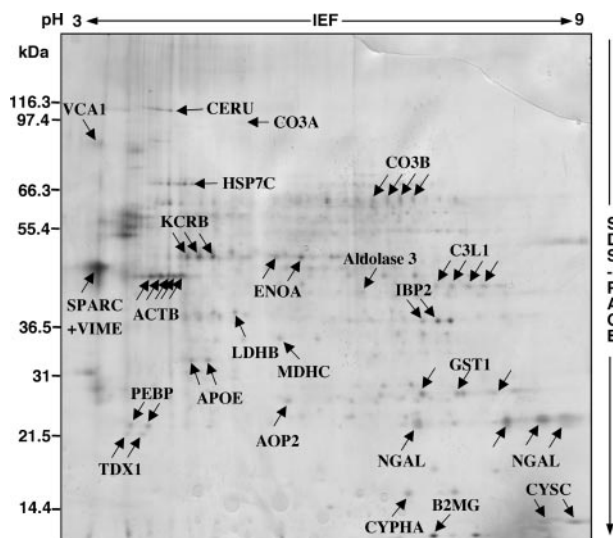


FIG. 7. Two-dimensional gel analysis of 20  $\mu$ g of proteins secreted by LPS-treated astrocytes and labeled with S-methyl thioacetimidate. Proteins were first separated according to their isoelectric point along non-linear IPG strips (pH 3–10, 18 cm long). For the second dimension, the strips were loaded onto vertical 10–15% gradient SDS-polyacrylamide gels. The gels were silver-stained according to the procedure of Shevchenko and Wim (27). *GST1*, glutathione S-transferase, class Mu; *CYSC*, cystatin C; *PEBP*, phosphatidylethanolamine-binding protein; *APOE*, apolipoprotein E; *LDHB*, lactate dehydrogenase B; *MDHC*, methyl 2,5-dihydroxycinnamate; *ENOA*,  $\alpha$  enolase; *ACTB*, actin  $\beta$ ; *CERU*, ceruloplasmin chain 1; *KCRB*, creatine kinase B chain; *HSP7C*, heat shock protein; *IBP2*, insulin-like growth factor-binding protein 2; *TDX1*, thioredoxin peroxidase 1.

Here we used the DIMAL-K strategy to quantify the variations in astrocytic protein secretion induced by treating cells with LPS (1  $\mu$ g/ml, type 055:B5). We first evaluated the efficacy of the labeling reaction on astrocyte-conditioned medium containing 20  $\mu$ g of total proteins using either S-methyl thioacetimidate or S-methyl thiopropionimidate. 2-D gel analysis of the derivatized proteins showed a profile (Fig. 7) similar to that found for unlabeled secreted proteins (24). Unambiguous identifications consistent with those previously obtained in unlabeled samples (24) were obtained from their peptide mass fingerprint with significant protein scores based on Arg-C type digestion (Table I). For proteins appearing on gels as trains of spots, identification was performed independently for each spot indicated by an arrow on the gel image (Fig. 7). As expected, the peptide mass fingerprints included only peptides terminated with an Arg residue and/or containing uncleavable labeled lysines (Table I).

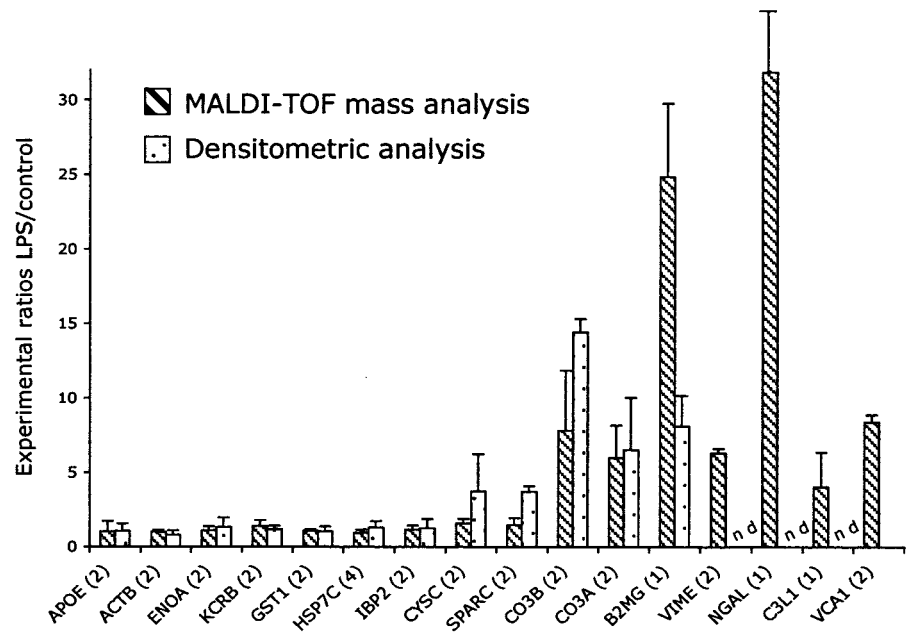
To get an unambiguous quantification of proteins released by astrocytes exposed to sham treatment or LPS, reverse labeling experiments were performed in parallel for each sample. Either the LPS-treated sample was labeled with S-methyl thioacetimidate and mixed with the S-methyl thiopropionimidate-labeled control sample, or inversely the secretion product of LPS-treated cells was labeled with S-methyl thiopropionimidate and mixed with the S-methyl thioacetimidate-labeled untreated secretion. The differential expression of 16 protein species was quantified by MALDI-TOF MS and compared with the densitometric quantification of the corresponding unlabeled protein spots using Image Master software (Fig. 8).

These experiments indicated that quantification by mass

TABLE I  
MALDI-TOF MS identification of astrocyte-secreted proteins labeled with S-methyl thioacetimidate

Protein	Swiss-Prot accession number	Mascot score (significant score = 53, $p < 0.05$ )	Sequence coverage of labeled protein (%)	Number of peptides (number of labeled peptides detected)
$\alpha$ enolase (ENOA)	P17182	67	24	4 (3)
Actin $\beta$ (ACTB)	P02570	91	34	7 (2)
Apolipoprotein E (APOE)	P08226	172	37	9 (3)
Ceruloplasmin chain 1 (CERU)	Q61147	55	16	3 (1)
C3L1	Q61362	94	15	3 (1)
CO3A	P01027	67	20	6 (2)
CO3B	P01027	71	15	8 (3)
Cystatin (CYSC)	P21460	86	54	4 (2)
Creatine kinase B chain (KCRB)	Q04447	60	24	7 (4)
Glutathione S-transferase, class Mu (GST1)	P10649	112	50	7 (4)
Heat shock protein (HSP7C)	P08109	120	26	9 (4)
Insulin-like growth factor-binding protein 2 (IBP2)	P47877	83	25	5 (3)
B2MG	P01887	62	25	2 (2)
NGAL	P11672	105	26	5 (2)
SPARC	P07214	101	22	4 (2)
Thioredoxin peroxidase 1 (TDX1)	O88376	70	19	3 (1)
VIME	P20152	99	24	9 (6)
VCA1	P29533	83	24	5 (4)

FIG. 8. Experimental ratios ( $\pm$ S.D.) of astrocyte-secreted proteins (LPS/control) obtained by MS and densitometry. *nd*, ratio not determined due to the lack of detection of the proteins under the control conditions. Numbers in parentheses represent the number of pairs of differentially labeled peptides used to calculate the ratios. For densitometric ratios, data are the means of values from four independent gels performed on different sets of cultured astrocytes ( $n = 4$ ). MS ratios are the means of values from four MS analyses ( $n = 4 \times$  number in parentheses). *GST1*, glutathione S-transferase, class Mu; *CYSC*, cystatin C; *APOE*, apolipoprotein E; *ENOA*,  $\alpha$  enolase; *ACTB*, actin  $\beta$ ; *KCRB*, creatine kinase B chain; *IBP2*, insulin-like growth factor-binding protein 2; *HSP7C*, heat shock protein 71 kDa protein.



spectrometry provided a greater accuracy and sensitivity than those obtained by image analysis. Two sets of proteins emerged from this analysis: the first one included proteins whose secretion was not altered by LPS treatment (ratio between 0.99 and 1.5 in mass analysis). Among these proteins, seven were accurately quantified by image analysis (ratio between 0.8 and 1.3) and corresponded to well resolved spots on 2-D gels. In contrast, results obtained by image analysis diverged from those obtained by mass spectrometry for cystatin C (ratios measured by MS and densitometric analysis,  $1.4 \pm 0.3$ ,  $n = 12$  and  $3.7 \pm 0.39$ ,  $n = 4$ , respectively) and secreted protein acid and rich in cysteine (SPARC; ratios measured by MS and densitometric analysis,  $1.4 \pm 0.46$ ,  $n = 8$  and  $3.7 \pm 0.39$ ,  $n = 4$ , respectively), the two proteins exhibiting acidic and basic pI (4.7 and 9, respectively) and poorly resolved on 2-D gels. The uncertain densitometric value for SPARC was probably not only related to the unresolved spots but could be linked to spot overlapping caused by protein co-migration. In fact, peptide mass fingerprint revealed the presence of a mixture of two proteins with a Mascot score of 84 (the statistical significance threshold was 63), SPARC and vimentin (VIME), which was not previously detected (Fig. 9). LPS did not increase SPARC secretion (ratio, 1.4; assessed by MALDI-TOF MS), whereas this treatment increased the amount of vimentin released by astrocytes in the extracellular medium (ratio,  $6.3 \pm 0.31$ ;  $n = 8$ ).

The second protein set showed an enhanced secretion (4–32-fold increase, assessed by MALDI-TOF MS) following exposure of astrocytes to LPS, including proteins identified in our previous study (CO3A, CO3B, B2MG, NGAL, and C3L1) and those not previously identified, namely, vascular cell adhesion protein 1 (VCA1) and VIME. Again major discrepancies with the corresponding densitometric ratios were found. For

example, C3L1, VCA1, and NGAL exhibited increased factors of 4, 8, and 32, respectively, in mass analysis, whereas these proteins could not be measured by 2-D gel image analysis because they were not detectable on silver-stained 2-D gels obtained from untreated cells. This highlights the enhanced sensitivity of the DIMAL-K procedure, which allowed comparisons of peptides in the subfemtomole range using last generation MALDI-TOF mass spectrometers compared with 2-D gel image analysis (1). Finally the higher accuracy of the DIMAL-K procedure compared with 2-D gel image analysis was confirmed by the quantification of CO3A and CO3B secretion by cells treated or not with LPS. CO3A and CO3B are produced from the same secreted precursor protein by disulfide bond reduction during the analytical process and should exhibit similar increased secretion following LPS exposure. Consistently mass spectrometry quantification indicated a -fold increase of  $6.0 \pm 2.18$ ,  $n = 8$  and  $7.8 \pm 4.05$ ,  $n = 4$ , respectively, for CO3A and CO3B secretion from astrocytes exposed to LPS. In contrast, divergent ratio values were obtained by densitometric analysis of 2-D gels ( $6.5 \pm 3.5$ ,  $n = 4$  and  $14.4 \pm 0.9$ ,  $n = 4$ , respectively).

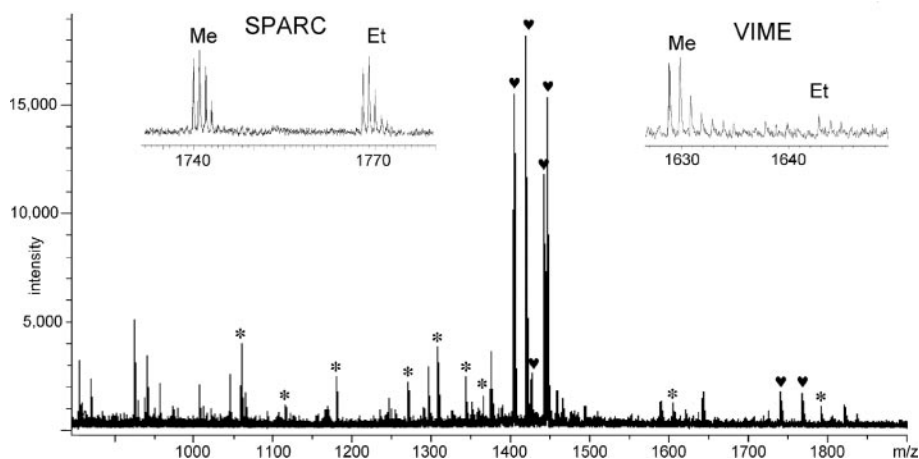
#### DISCUSSION

In this study, we described and validated a new quantification procedure to compare protein expression in two biological samples. This procedure, which we call DIMAL-K, is based on the differential labeling of lysine residues on entire proteins (before enzymatic cleavage) and is relevant for proteomic approaches based on separation of proteins by 2-D electrophoresis and their identification by MALDI-TOF mass spectrometry.

We provide evidence that the DIMAL-K strategy is highly efficient for differential quantification of relatively complex



FIG. 9. MALDI-TOF mass spectrum of the digest of the single spot corresponding to SPARC (♥) whose expression level did not change and VIME (\*), which underwent a 4-fold increase in secretion after LPS treatment versus the control sample. Insets show expanded views of labeled peptide pairs (Me- and Et-). For clarity, only major peptide signals are indicated.



proteomes containing small amounts of proteins (a few dozen micrograms). We demonstrated the following. 1) This procedure yields complete labeling of  $\epsilon$ -amino groups of lysines in entire proteins with identical reactivity for the two homologous imidate reagents and no side reactions such as deamidation, carbamylation, acetylation, or dehydration during amidination of the lysines. 2) The labeling reaction can be performed in commonly used protein solubilization buffers containing either chaotropic agents (urea), non-ionic (CHAPS), or ionic (SDS) detergents. 3) The labeling does not alter the net positive charge on the lysine residues, preserving the overall 2-D gel protein profile. 4) The differentially labeled proteins comigrate on 2-D gels. 5) No ionization bias occurs between differentially amidinated peptides. This allows accurate quantification and identification of individual protein species in a single mass analysis step. Introducing the chemical modification early in the experimental procedure reduces differential loss of proteins during further biochemical steps (*i.e.* extraction, separation, and digestion) and also guarantees accuracy of the quantification. Compared with 2-D gel image analysis, the DIMAL-K strategy circumvents several limitations inherent to 2-D electrophoresis: 1) streaking leading to poor resolution, 2) presence of several proteins in a single spot, 3) the small linear dynamic range of staining and saturation, and 4) gel-to-gel variability. DIMAL-K can also be compared with DIGE (4), technology in which quantification is carried out by differential labeling on the same gel in contrast with classical densitometric analysis in which 2-D protein patterns on different gels are compared (4). However, the DIMAL-K strategy has an advantage over DIGE in that the identification and quantification is carried out on a single protein sample, whereas proteins quantified with the DIGE technology still need to be identified, and therefore subsequent mass analysis is required. In this regard, it is important to note that metabolic labeling approaches, which proved to be effective for both quantification and identification, are not applicable to all protein samples such as tissue extracts and body fluids.

To date, DIMAL-K constitutes one of the first differential

quantification methods by mass spectrometry of lysine-modified proteins and that is compatible with 2-D electrophoresis. Alternative strategies are based on early cysteine labeling of protein mixtures with either ICATs (29) or acrylamide ( $d_0/d_3$ ) (14, 15). Labeling lysine residues provides several advantages over cysteine labeling. The first one is the higher abundance of lysine in protein compared with cysteine, which is absent in the sequence of a large set of proteins. The second relies on the Arg-C-like digestion profile for lysine-labeled proteins. Comparing the proportion of peptides containing at least one cysteine after trypsin digestion (used in cysteine labeling) with that of peptides containing at least one lysine after Arg-C digestion (Table II) by *in silico* trypsin and Arg-C digestion of 8,446 mouse proteins in the Swiss-Prot Database revealed that the latter condition provided the highest mean protein coverage (45.5 versus 15.4%). Furthermore taking into account peptide masses ranging from 900 to 4,000, which are mostly used in peptide mass fingerprint analysis, a strategy based on lysine labeling like DIMAL-K is still more powerful than cysteine tagging methods (27.4 versus 10.3% mean protein coverage).

Only a few examples of global lysine labeling on entire proteins before enzymatic cleavage have been described, and these procedures have not received widespread application in proteomics (17, 30, 31). They include the classical succinimide activation of amino groups, which unfortunately leads to nonspecific *O*-acylated side products that must be subsequently hydrolyzed under highly basic conditions (pH 11–12). In contrast, the thioimidate activation used here was performed at low basicity. Moreover it retained a net positive charge on lysine residue preserving the overall 2-D gel protein patterns. In the recently described isotope-coded protein labeling method, lysine modification induces an acidic shift of the pI of labeled proteins due to the loss of the basic amino groups, which precludes comparison between labeled and unlabeled proteins on 2-D gels (17).

DIMAL-K yielded efficient protein identification by peptide mass fingerprint. Consistent with an Arg-C digestion, data

TABLE II  
Number (percentage) of human and mouse proteins and tryptic and Arg-C peptides that contain cysteine or lysine residues annotated in the Swiss-Prot Database (164,201 entries November 23, 2004)

	Proteins		Tryptic peptides		Arg-C peptides	
	Human	Mouse	Human	Mouse	Human	Mouse
Total number	11,638	8,446	675,765	451,915	339,921	228,813
Cys-containing (%)	11,241 (96.6)	8,171 (96.7)	103,948 (15.4)	69,564 (15.4)		
900–4,000 Da (%)			69,488 (10.3)	47,072 (10.3)		
Lys-containing (%)	11,565 (99.4)	8,417 (99.7)			153,576 (45.2)	104,208 (45.5)
900–4,000 Da (%)					92,404 (27.2)	62,730 (27.4)

base searching by peptide mass fingerprint using the Mascot software required the selection of Arg-C as enzyme and setting acetimidoyl and propionimidoyl as new lysine modifications. A few additional points must be stressed. First less peptides were produced from labeled protein digests, leading to simplified mass spectra with reduced spectral suppression due to the desorption-ionization competition. About a 50% reduction in the number of digested peptides is expected with an Arg-C digestion (228,915 for 8,446 mouse proteins) compared with a trypsin digestion (451,765) (Table II). Comparing experimental mass data with a reduced data set of calculated peptide masses theoretically produced with the Arg-C digestion allowed a better discrimination in the data search. The discrimination was also improved by setting the number of allowed missed cleavage sites to zero (instead of one for the usual trypsin digestion to include possible identified missed cleavage sites such as basic doublets or the presence of acidic residues adjacent to the cleavage site). This contributed to correct scoring with in some cases no more than two or three matching mass values. In this regard, the DIMAL-K approach proved to be a successful method for identifying proteins already identified as secreted products of astrocytes (24).

Interestingly, using our approach, we identified for the first time vimentin in the medium conditioned by astrocytes. Vimentin was not detected in our previous study probably because this protein migrates at a position similar to that of SPARC on 2-D gels and is secreted in smaller amounts than SPARC. Vimentin identification using the DIMAL-K approach is also consistent with the power of Arg-C-based peptide mass fingerprint analysis, which is more discriminative than commonly used approaches based on trypsin peptide mass fingerprint. Vimentin is the most abundant intermediate filament protein. It was initially regarded as an exclusively intracellular protein. However, it has been shown recently that vimentin can be secreted by macrophages in a process that is positively regulated by proinflammatory cytokines (32). This is consistent with our finding indicating that LPS strongly stimulated vimentin secretion from cultured astrocytes. It has been suggested that extracellular vimentin contributes to the inflammatory response of macrophages by generating oxidative metabolites (32). Vimentin released by astrocytes in response to proinflammatory signals might participate in central nervous system inflammatory processes, consistent with the

pivotal role of astrocytes in central nervous system immune and inflammatory responses. Altogether these findings indicate that DIMAL-K provides not only accurate quantification of the relative amount of proteins in two biological samples but also extensive resolution and high sensitivity that are essential to obtain a deeper insight into complex proteomes containing low amounts of protein.

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