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# Comparison of Isotope-labeled Amino Acid Incorporation Rates (CILAIR) Provides a Quantitative Method to Study Tissue Secretomes\*<sup>§</sup>

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Adipose tissue is an endocrine organ involved in regulation of whole-body energy metabolism via storage of lipids and secretion of various peptide hormones (adipokines). We previously characterized the adipose tissue secretome and showed that [<sup>13</sup>C]lysine incorporation into secreted proteins can be used to determine the origin of identified proteins. In the present study we determined the effect of insulin on the secretome by comparing incorporation rates of <sup>13</sup>C-labeled lysine in the presence and absence of insulin. Human visceral adipose tissue from one patient was divided over six dishes. After subsequent washes to remove serum proteins, [<sup>13</sup>C]lysine-containing medium was added. Three dishes also received 60 nM insulin. The other three were controls. After 72 h of culture, media were collected and processed separately, involving concentration by ultrafiltration and fractionation by SDS-PAGE followed by in-gel digestion of excised bands and LC-MS/MS analyses. The obtained spectra were used for database searching and calculation of heavy/light ratios. The three control data sets shared 342 proteins of which 156 were potentially secreted and contained label. The three insulin-derived data sets shared 361 proteins of which 141 were potentially secreted and contained label. After discarding secreted proteins with very low label incorporation, 121 and 113 proteins remained for control and insulin data sets, respectively. The average coefficient of variation for control triplicates was 10.0% and for insulin triplicates was 18.3%. By comparing heavy/light ratios in the absence and presence of insulin we found 24 up-regulated proteins and four down-regulated proteins, and 58 proteins showed no change. Proteins involved in the endoplasmic reticulum stress response and in extracellular matrix remodeling were up-regulated by insulin. In conclusion, comparison of isotope-labeled amino acid incorporation rates (CILAIR) allows quantitative assessment of changes in protein secretion without the need for 100% label in-

corporation, which cannot be reached in differentiated tissues or cells. *Molecular & Cellular Proteomics* 8: 316–324, 2009.

Adipose tissue plays an important role in the development of energy metabolism-related diseases such as metabolic syndrome and type 2 diabetes. Especially visceral adipose tissue has been implicated in the development of these diseases (1–3). Adipose tissue is an endocrine organ involved in the regulation of whole-body energy metabolism via storage of lipids and secretion of peptide hormones (adipokines) such as leptin, adiponectin, visfatin, and retinol-binding protein-4 (1, 2, 4). Besides adipocytes, adipose tissue contains endothelial cells, macrophages, and fibroblasts that may modulate the overall peptide secretion pattern via cross-talk between the various cell types. Factors secreted by macrophages have been shown to induce changes in the secretion of adipokines, free fatty acids, and glucose uptake by murine 3T3-L1 adipocytes (5). On the other hand, the adipocyte-derived hormone leptin has been shown to activate macrophages to produce tumor necrosis factor- $\alpha$ , which can be inhibited by adiponectin (6). These interactions between cells from the stromal fraction and adipocytes are important for physiological functions of adipose tissue, and deregulation of this cross-talk is regarded as an important mechanism leading to insulin resistance and type 2 diabetes (7–10). By studying the tissue as a whole rather than adipocytes alone, this cross-talk is preserved. To study hormonal regulation of the human adipose tissue secretome, a quantitative proteomics approach that is compatible with tissue culture is needed. Here we describe a newly developed quantitative method, comparison of isotope-labeled amino acid incorporation rates (CILAIR),<sup>1</sup> that is es-

<sup>1</sup> The abbreviations used are: CILAIR, comparison of isotope-labeled amino acid incorporation rates; SILAC, stable isotope labeling with amino acids in cell culture; FA, formic acid; CV, coefficient of variation; ER, endoplasmic reticulum; GRP, glucose-regulated protein; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; STRING, Search Tool for the Retrieval of Interacting Genes/Proteins; CXCL5, CXC motif chemokine 5; CSF1, macrophage colony-stimulating factor 1; CHI3L1, chitinase-3-like protein 1.

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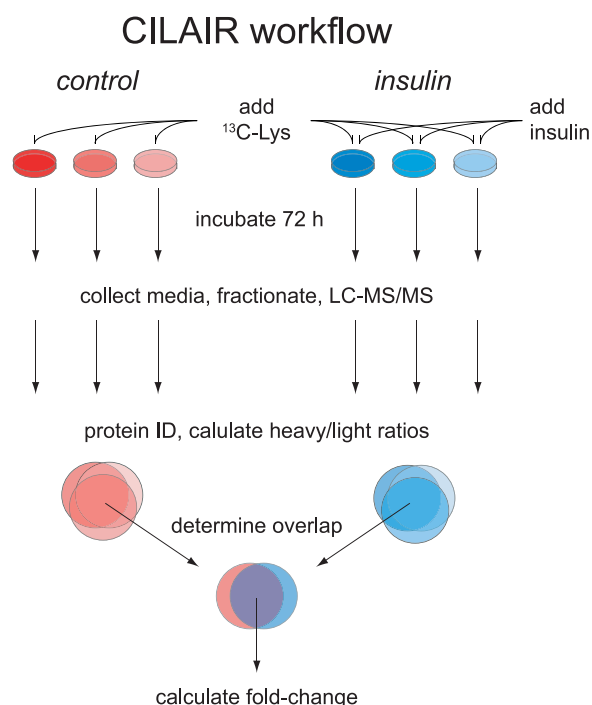
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pecially suitable for use in combination with tissue culture. To obtain relevant proteomics data when using tissue culture as a model, several difficulties have to be overcome. The presence of serum proteins inside tissue pieces that slowly diffuse into the culture medium and the presence of intracellular proteins that are released from damaged cells can dramatically influence the dynamic range of the sample. This will affect detection of low abundance secreted proteins. Also the relevance of identified proteins may be difficult to assess if the source (secreted, serum, or intracellular) is not clear. We have shown previously that incorporation of  $^{13}\text{C}$ -labeled lysine in secreted proteins can be used for validation of the origin (tissue or serum) of detected proteins (11). Proteins that contain label are synthesized by the tissue and are not derived from serum. In the present study we assessed whether comparing  $^{13}\text{C}$ -labeled lysine incorporation into newly synthesized secreted proteins at a fixed culture period could serve as a reliable method to assess insulin-induced changes in protein expression. Insulin is well known for its regulatory role in energy metabolism and has profound effects on protein synthesis (12). We show that the CILAIR method allows quantitative assessment of changes in protein secretion without the need for 100% label incorporation. This makes CILAIR a good alternative for stable isotope labeling with amino acids in cell culture (SILAC) (13, 14) for which complete labeling of cells is essential. However, this cannot be reached in differentiated tissues or cells. In addition to relative quantification of changes in protein expression, CILAIR provides information on synthesis rates and on the source of secreted proteins.

#### EXPERIMENTAL PROCEDURES

**Adipose Tissue Culture**—A human omental adipose tissue explant was obtained from a woman (age, 39 years; body mass index, 21.7) undergoing laparotomy to remove an intramural myoma. The study had the approval of the local ethics committee.

The adipose tissue culture protocol is described in Alvarez-Llamas *et al.* (11). Briefly adipose tissue explants were transported from the operating room to the laboratory in transport buffer (PBS, 5.5 mM glucose, 50  $\mu\text{g}/\text{ml}$  gentamicin) at room temperature. The following procedures were carried out under a laminar flow hood using sterile equipment. Immediately upon arrival, the tissue was transferred to a Petri dish containing 20 ml of PBS and was finely minced in 20–80-mg pieces using scissors. The tissue pieces were extensively washed with 400 ml of PBS over a filter containing sterile cotton bandage fabric. Thereafter the tissue pieces were transferred to a 75-cm<sup>2</sup> culture flask containing 200 ml of PBS and were gently shaken for a short period. Next the contents of the flask was poured over the filter, and the tissue pieces were washed with 300 ml of warm PBS (37 °C). The tissue pieces were transferred to a tube containing 50 ml of PBS and centrifuged for 1 min at  $277 \times g$  at room temperature to remove red blood cells and debris. The tissue was then removed from the tube, and the weight was determined. To six dishes with 1.2 g of tissue each, 12 ml of lysine-free M199 medium (reference number 22340 Lys-free, Invitrogen) supplemented with 50  $\mu\text{g}/\text{ml}$  gentamicin was added to deplete lysine from other sources (blood in the tissue). The dishes were incubated at 37 °C at 5% CO<sub>2</sub>. The medium was renewed after 1, 18.5, 22.5, and 26.5 h. After the last wash (time point, 26.5 h), all six dishes received fresh M199 medium



**FIG. 1. The CILAIR work flow.** To six culture dishes with human adipose tissue pieces [ $^{13}\text{C}$ ]lysine was added. Three dishes also received 60 nM insulin. The tissues were cultured for 72 h. After the incubation was completed, media were collected and prepared separately for LC-MS/MS analyses. Samples were concentrated by ultrafiltration and subsequently fractionated by SDS-PAGE. After LC-MS/MS analyses, proteins were identified, and heavy/light ratios were determined. In this way three control data sets and three data sets from insulin-treated tissue were obtained. The control and insulin-treated data sets were compared to obtain one data set for each condition with proteins that contain label and were identified in all three dishes. Subsequently the overlap between these data sets was determined, and the -fold change values were calculated for each protein by dividing their heavy/light ratios in the insulin and control data set.

containing 70 mg/liter  $^{13}\text{C}$ -labeled lysine ( $[\text{L-}^{13}\text{C}_6,^{14}\text{N}_2]$ lysine (Invitrogen)). To three of the six dishes also 60 nM human insulin was added. Tissues were maintained in culture for an additional 72 h to allow incorporation of the label into newly synthesized proteins. Thereafter media were collected and stored at  $-80^\circ\text{C}$  until analysis.

**Protein Identification by LC-MS/MS**—Eleven milliliters of adipose tissue culture medium was concentrated by ultrafiltration (Vivaspin 15R, 3-kDa cutoff). This concentrated sample was further concentrated by ultrafiltration (Microcon, 3 kDa, Millipore) to a final volume of approximately 40  $\mu\text{l}$ . Proteins present in the concentrated adipose tissue medium sample were fractionated by SDS-PAGE on a 4–12% bis-Tris gel with a MOPS buffer system according to the manufacturer's protocol (NuPAGE<sup>®</sup>-Novex, Invitrogen). Protein separation occurred for 50 min at 200 V, and visualization of bands was performed overnight by Coomassie Brilliant Blue G-250-based staining (Page-Blue Staining Solution, Fermentas). The whole lane was excised into 25 bands that were processed for tryptic digestion. Each band was cut into small pieces and stored at  $-20^\circ\text{C}$  until analysis. Then they were washed in ultrapure water and dehydrated in ACN. In-gel reduction with dithiothreitol (for 1 h at room temperature) and carbamidomethylation with iodoacetamide (for 45 min at room temperature in the dark) were performed. Gel pieces were subsequently washed with

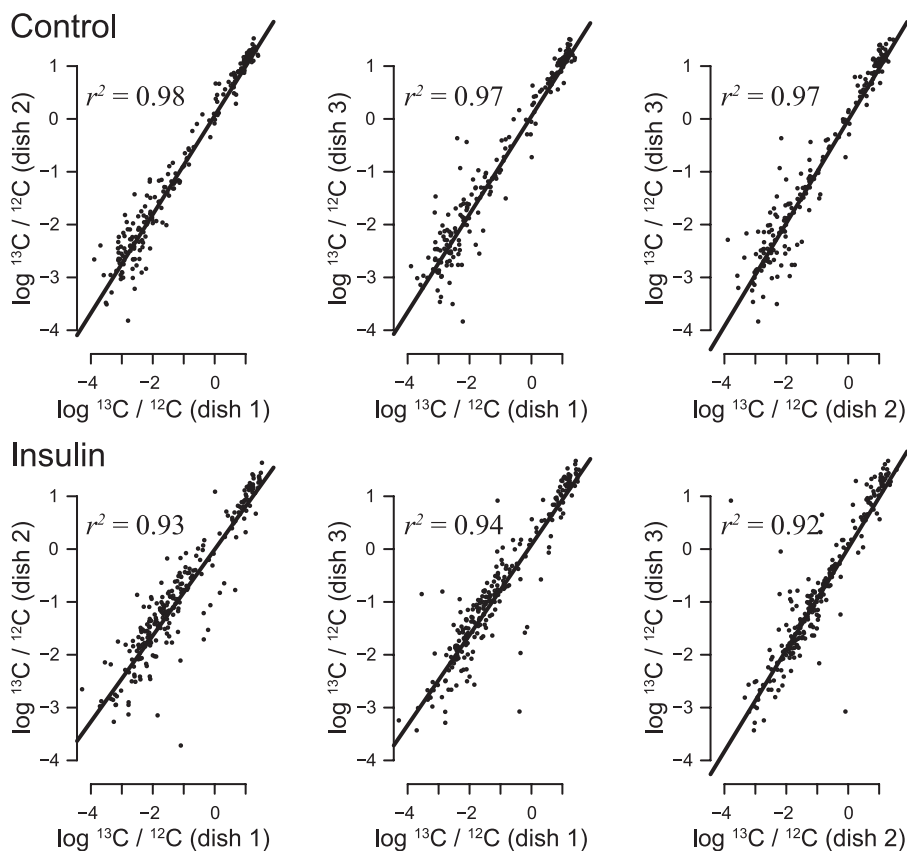


FIG. 2. **Correlation between dishes for label incorporation.** For each condition the  $\log^{13}\text{C}/^{12}\text{C}$  ratios of the proteins detected in the dishes were plotted against each other. The correlation coefficient of the trend lines is indicated.

ultrapure water, 50% ACN, and pure ACN. Next 0.4  $\mu\text{g}$  of modified trypsin (Promega) in 20 mM ammonium bicarbonate was added, and gel pieces were allowed to rehydrate on ice for 20 min. Digestion was carried out overnight at 37  $^{\circ}\text{C}$ .

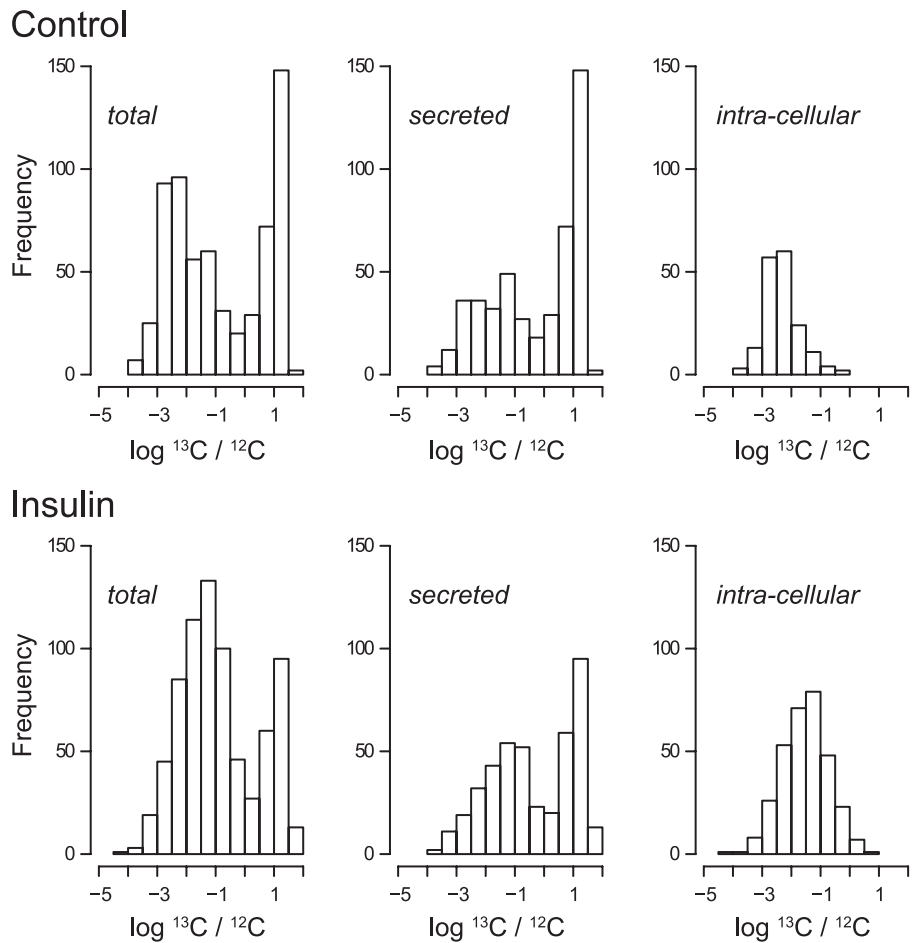
Separation of the resulting tryptic peptide mixtures was performed by nanoscale reversed-phase LC-MS/MS. The Agilent 1100 nano-flow/capillary LC system was equipped with a trapping column ( $5 \times 0.3$  mm,  $\text{C}_{18}\text{RP}$ ) (Dionex/LC Packings, Amsterdam, The Netherlands) and a nanocolumn ( $150 \times 0.075$  mm,  $\text{C}_{18}\text{PepMap}$ ) (Dionex/LC Packings). Peptides mixtures were injected into the trapping column at a flow rate of 20  $\mu\text{l}/\text{min}$  (0.1% formic acid (FA)). After 5 min the trapping column was switched into the nanoflow system, and the trapped peptides were separated using the nanocolumn at a flow rate of 0.3  $\mu\text{l}/\text{min}$  in a linear gradient elution from 95% A (0.1% FA) to 50% B (90% ACN, 0.1% FA) in 53 min followed by an increase up to 80% B in 3 min. The eluting peptides were on-line electrosprayed into the QStar XL hybrid ESI quadrupole time-of-flight tandem mass spectrometer (ESI-qTOF-MS/MS, Applied Biosystems, Framingham, MA; MDS Sciex, Concord, Ontario, Canada) provided with a nano-spray source equipped with a Proxeon stainless steel needle (25- $\mu\text{m}$  diameter). Typical values for emitter voltage were 2.25 kV in positive ion mode. Analyst QS 1.1 software (Applied Biosystems) was used for data acquisition in the positive ion mode typically with a selected mass range of 300–1500  $m/z$ . Peptides with +2 to +4 charge states were selected for tandem mass spectrometry, and the time of summation of MS/MS events was set to be 2 s. The three most abundant charged peptides above a 30-count threshold were selected for MS/MS and dynamically excluded for 30 s with 30-ppm mass tolerance.

**Data Analyses**—ProteinPilot 2.0 software (Applied Biosystems) was used to generate peak lists and as search engine to identify proteins from the mass spectrometric data sets using the UniprotKB/Swiss-

Prot database (release 54, January 2008, 276,256 entries). The search engine makes use of the Paragon<sup>TM</sup> algorithm (15). Search parameters such as modifications, substitutions, cleavage events, and mass tolerance are modeled with probabilities and do not need discrete user-controlled settings. Options that were chosen within the program were: label, Lys +6; Cys alkylation with iodoacetamide; digestion with trypsin; gel-based identification; species, *Homo sapiens*; identification focus for biological modifications; thorough search. The software automatically detects the heavy/light peak pairs and calculates the heavy/light ratios based on the peak areas. The automatic correction for mixing errors (normalization to median) when performing SILAC was turned off. Identifications with a probability score higher than 95% were included in the results list. When the heavy/light ratio of a protein differed more than two standard deviations from the average ratio of the triplicate, spectra were manually inspected for correct label detection and, if necessary, corrected. This was the case for eight proteins in the control data set and one in the insulin data set. The statistical analysis and visualization was performed in R, a language for statistical computing and graphics. We analyzed ratios on a logarithmic scale.

Classification of identified proteins as potentially secreted was performed by SecretomeP 2.0 (16). Those proteins with a predicted signal peptide were considered as secreted via the classical pathway (endoplasmic reticulum/Golgi-dependent pathway). If no signal peptide was predicted but the NN score exceeded the value of 0.5, proteins were classified as secreted via the non-classical pathway. Proteins that did not match these criteria were considered as being intracellular.

Functional interactions between up- or down-regulated proteins were determined using STRING with the high confidence setting. STRING is a database of known and predicted protein-protein interactions derived from genomic context, high throughput experiments, (conserved) co-expression, and published knowledge (17).



**FIG. 3. Frequency distribution of heavy/light ratios.** The figure depicts the frequency distribution of the heavy/light ratios observed for proteins detected in control and insulin conditions. Also the contribution of heavy/light ratios for potentially secreted and intracellular proteins to the overall distribution is shown.

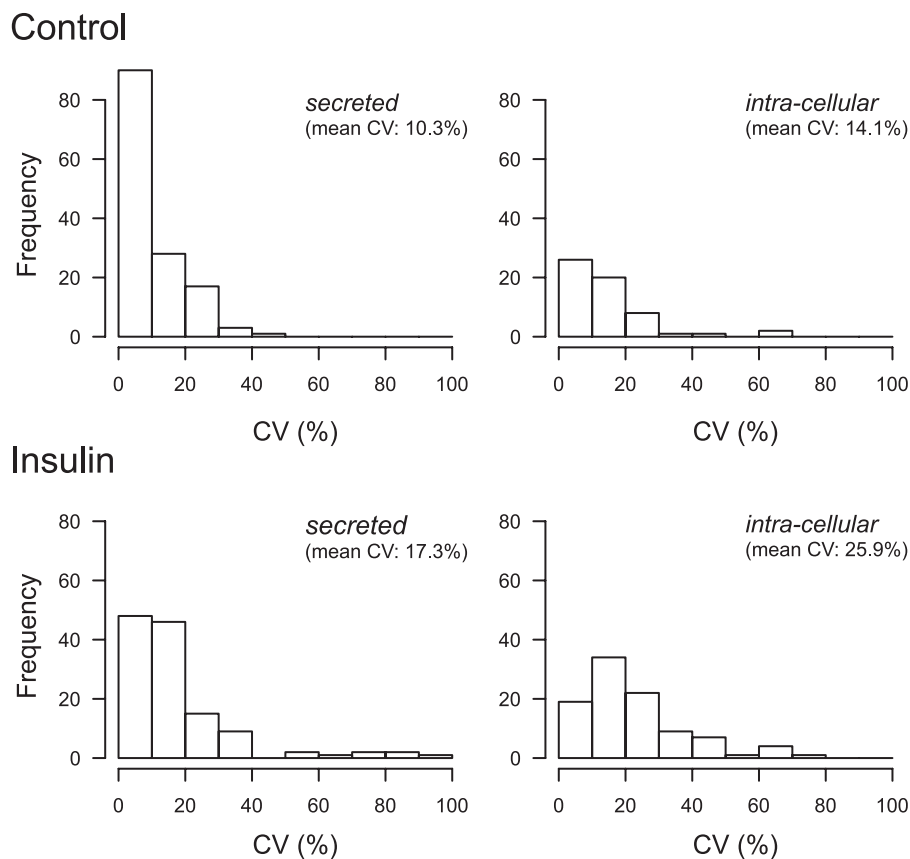
RESULTS

A human visceral adipose tissue explant, obtained from a lean person to assure good insulin sensitivity, was prepared for tissue culture as described under “Experimental Procedures.” The experiment was performed in triplicate to allow assessment of variation in the outcome of the method. The complete CILAIR work flow is depicted in Fig. 1. Tissue pieces were equally divided over six culture dishes and were cultured in lysine-free medium for 26.5 h with intermittent washing steps to remove serum enclosed in the tissue pieces and to reduce the [<sup>12</sup>C]lysine pool in the tissue. Thereafter [<sup>13</sup>C]lysine was added to all six dishes. At this point, three dishes also received 60 nM insulin. The tissues were cultured for an additional 72 h. In previous experiments, this period showed sufficient label incorporation in secreted proteins (11). After the incubation was completed, media were collected and prepared separately for LC-MS/MS analyses. Samples were concentrated by ultrafiltration and subsequently fractionated by SDS-PAGE. Twenty-five bands per lane were cut, and in-gel digestion was performed. After LC-MS/MS analyses, proteins were identified, and heavy/light ratios were determined. In this way three control data sets and three data sets from insulin-treated tissue were obtained. For the three con-

trol dishes 440, 390, and 378 proteins were identified. For the insulin-treated dishes 447, 496, and 425 proteins were identified. The control data sets were compared and showed an overlap of 342 proteins. The data sets from the insulin-treated dishes showed an overlap of 361 proteins. After removal of unlabeled proteins, 213 proteins remained in the control data set that contained label and were identified in all three dishes. For the insulin-treated data set, 247 proteins contained label and were detected in all three dishes (see Supplements 1 and 2 for control and insulin data sets, respectively). Unlabeled proteins may be identified only by arginine-containing peptides or may be derived from serum which, despite the vigorous washing protocol, is enclosed in the tissue pieces and is slowly released during culture. High correlation for label incorporation between dishes within triplicates was obtained (Fig. 2) with correlation coefficients ranging from 0.97 to 0.98 for controls and from 0.92 to 0.94 for insulin-treated dishes. On a log scale, the frequency distribution of the ratios observed for both control and insulin data appears as a mixture of two normal distributions (Fig. 3): one distribution for proteins with heavy/light ratios lower than 1 (log 0) and the other distribution for proteins with heavy/light ratios higher than 1 (log 0). An explanation for this mixture of distributions may be

**FIG. 4. Variation in the measurement of secreted and intracellular proteins.**

The frequency of the observed variations in label incorporation within the triplicate (CV) was plotted for proteins that are potentially secreted and proteins that are potentially intracellular for control and insulin conditions as indicated. Also the mean CV for these groups is given.



provided by the presence of intracellular proteins in the culture medium. Intracellular proteins can make up more than 50% of the proteins in the medium (11). Therefore, the contribution of secreted and intracellular proteins to this distribution was established using SecretomeP. The control data set contained 155 potentially secreted proteins and 58 potentially intracellular proteins. Of the 247 proteins in the insulin data set, 141 were potentially secreted. For both control and insulin data sets, intracellular proteins show low label incorporation and mainly contribute to the left part of the distribution (see Fig. 3). Secreted proteins can be found in both distributions. However, the right part of the distribution (high label incorporation) consists completely of secreted proteins. Therefore, the mixture of two normal distributions can at least partly be explained by the presence of intracellular proteins in the culture media. Furthermore it is remarkable that insulin shifts the maxima of both distributions to the right, indicating that insulin has a stimulating effect on label incorporation for both secreted and intracellular proteins.

To assess the variation in the measurement, the coefficient of variation (CV) was calculated for all the proteins in the two data sets. Because of the use of log ratios for calculating the mean and standard deviation of the triplicates, calculation of CVs for proteins with a mean log ratio close to 0 can return very high CVs. Because this is not due to the measurement but due to the way of calculation, we did not include the few

proteins with a mean log ratio between  $-0.5$  and  $+0.5$ . Fig. 4 shows the frequency distributions of the CVs for secreted and intracellular proteins in the control and insulin data sets. The average CV for secreted and intracellular proteins in the control was 10.3 and 14.1%, respectively. For the insulin-treated data sets average CV for secreted and intracellular proteins was 17.3 and 25.9%, respectively. This indicates that intracellular proteins present in the medium have on average lower heavy/light ratios and show higher variation than secreted proteins in both control and insulin data sets. This can be explained by assuming that intracellular proteins are released randomly by the tissue within the 72-h labeling period, leading to lower label incorporation and higher variation compared with secreted proteins. Therefore, intracellular proteins were removed from both data sets.

In addition, a threshold heavy/light ratio was applied to correct for false positives that are generated when small  $^{13}\text{C}$  peaks have to be distinguished from noise peaks by the software. For example, serum albumin, which is derived from blood and not synthesized by adipose tissue, was mistakenly detected as containing label ( $^{13}\text{C}/^{12}\text{C}$  ratio of 0.05 and 0.06 in control and insulin, respectively). To avoid this problem an arbitrarily chosen cutoff ratio of 0.15 was applied to both data sets to obtain more relevant data. This reduces the control and insulin data sets to 121 and 115 proteins and the average CV to 10.0 and 18.3%, respectively.

TABLE I

Secreted proteins with the highest label incorporation in the control triplicate

AVG H/L, average heavy/light ratio; SPARC, secreted protein acidic and rich in cysteine.

Accession no.	Name	AVG H/L	CV
			%
P42830	CXCL5	4.28	10.12
P09603	CSF1	4.09	10.72
P19021	Peptidyl-glycine $\alpha$ -amidating monooxygenase	3.74	9.12
Q06481	Amyloid-like protein 2	3.70	9.57
O95633	Follistatin-related protein 3	3.67	3.84
P02452	Collagen $\alpha$ -1(I) chain	3.64	1.99
P02649	Apolipoprotein E	3.61	18.10
P03956	Interstitial collagenase	3.61	15.15
P16870	Carboxypeptidase E	3.60	15.40
P02461	Collagen $\alpha$ -1(III) chain	3.59	5.55
P05156	Complement factor I	3.58	18.51
P00751	Complement factor B	3.53	8.49
P12109	Collagen $\alpha$ -1(VI) chain	3.53	4.04
P08571	Monocyte differentiation antigen CD14	3.51	15.77
P24592	Insulin-like growth factor-binding protein 6	3.40	4.82
Q15113	Procollagen C-endopeptidase enhancer 1	3.40	3.28
P16035	Metalloproteinase inhibitor 2 (TIMP-2)	3.39	6.19
P09486	SPARC	3.34	8.83
P08253	72-kDa type IV collagenase (MMP-2)	3.34	3.80
P34096	Ribonuclease 4	3.33	5.3

TABLE II

Secreted proteins with the highest label incorporation in the insulin-treated triplicate

AVG H/L, average heavy/light ratio.

Accession no.	Name	AVG H/L	CV
			%
P13500	CC motif chemokine 2 (CCL2)	4.85	6.70
P24592	Insulin-like growth factor-binding protein 6	4.57	15.46
P09603	CSF1	4.30	17.14
P12109	Collagen $\alpha$ -1(VI) chain	4.29	10.37
P18428	Lipopolysaccharide-binding protein	4.22	6.46
P16035	Metalloproteinase inhibitor 2 (TIMP-2)	4.20	14.21
P02452	Collagen $\alpha$ -1(I) chain	4.11	11.28
P42830	CXCL5	4.03	15.34
P36222	Chitinase-3-like protein 1	3.87	3.37
P03956	Interstitial collagenase (MMP-1)	3.79	18.59
P01034	Cystatin-C	3.74	7.54
P15018	Leukemia inhibitory factor	3.71	10.24
P09871	Complement C1s subcomponent	3.62	17.95
Q08380	Galectin-3-binding protein	3.61	9.10
P02461	Collagen $\alpha$ -1(III) chain	3.59	14.65
P05155	Plasma protease C1 inhibitor	3.58	4.38
P00751	Complement factor B	3.49	7.30
P09919	Granulocyte colony-stimulating factor	3.48	9.38
P05121	Plasminogen activator inhibitor 1 (PAI-1)	3.46	8.70
O00391	Sulfhydryl oxidase 1	3.44	8.57

In Table I a list is presented showing the top 20 proteins with highest label incorporation in the control situation. Table II shows this list for the insulin-treated tissue. The CXC motif chemokine 5 (CXCL5) had the highest synthesis rate of the identified proteins in the control situation with a heavy/light ratio within the 72-h culture period of 4.28, which is equal to a label incorporation of ~80%. Other proteins with high synthesis rates were macrophage colony-stimulating factor 1 (CSF1) with a ratio of 4.09 and peptidyl-glycine  $\alpha$ -amidating monooxygenase with a ratio of 3.74. When insulin was present the highest synthesis rate was found for CC motif chemokine 2 with a heavy/light ratio of 4.85, which equals 83% label incorporation. Second was insulin-like growth factor-binding protein 6 with a ratio of 4.57, and third was CSF1 with a ratio of 4.30. The latter protein had a similar ratio (4.09) in the control situation, indicating there was no change in synthesis rate due to insulin. Also for CXCL5 there was no change with ratios of 4.28 and 4.03 for control and insulin, respectively. However, insulin-like growth factor-binding protein 6 appeared up-regulated 1.34-fold by insulin because its ratio in the control is 3.40, whereas in the insulin-stimulated tissue it is 4.57. -Fold change values were determined for all proteins present in both data sets. The overlap between control and

insulin data sets was 86 proteins. -Fold change factors were calculated by dividing heavy/light ratios for the insulin condition by the corresponding values in the control data set. In Fig. 5, the -fold change values for the 86 proteins are plotted, ranked to decreasing -fold change value. Assuming a normal distribution significance of observed change was calculated by Student's *t* test ( $p < 0.05$ ) (Table III). Fifty-eight proteins were unchanged. Twenty-four proteins were up-regulated with the highest difference being 2.7-fold for calreticulin. Four proteins were down-regulated. CD14 was reduced 1.6-fold.

#### DISCUSSION

Metabolic labeling is a very useful proteomics tool when using tissue culture. As we have shown previously (11), it facilitates validation of the source of the identified secreted proteins. When proteins have incorporated the label they should be synthesized by the tissue and not be derived from blood present within the tissue. In the present study we show that insulin-induced changes in the rate of label incorporation into secreted proteins can be used as a relative measure to quantify changes in expression. The CILAIR method proved to be robust and reproducible with CVs ranging from 10% for the control and 18% for insulin-treated tissue. The latter CV is

FIG. 5. **Insulin-induced changes in secreted proteins.** Proteins are ranked according to decreasing -fold change on a log scale. Twenty-four proteins were significantly up-regulated, four proteins were down-regulated, and 58 showed no change. Up- and down-regulated proteins are listed in Table III. Significance of changes was determined by Student's *t* test ( $p < 0.05$ ).

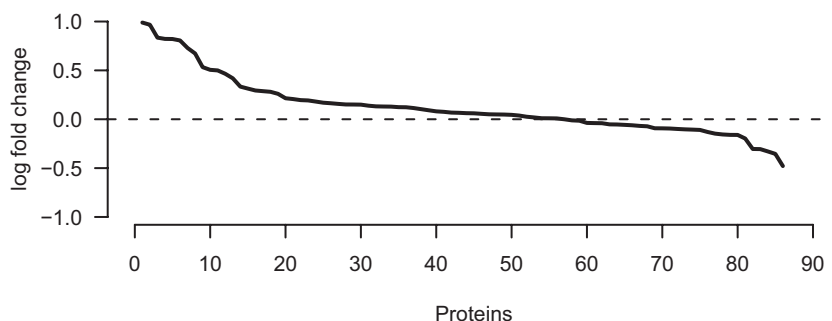


TABLE III  
Secreted proteins of which expression is affected by insulin

Significance of changes was determined by Student's *t* test ( $p < 0.05$ ). H/L, heavy/light ratio; TGF, transforming growth factor.

Accession no.	Name	H/L		-Fold change
		Control	Insulin	
P27797	Calreticulin (CALR, GRP60)	0.28	0.75	2.69
P30101	Protein-disulfide isomerase A3 (GRP58)	0.23	0.60	2.63
Q969H8	Interleukin-25 (IL25)	0.29	0.66	2.31
P37802	Transgelin-2 (TAGLN2)	0.17	0.38	2.28
P14314	Glucosidase 2 subunit $\beta$ (PRKCSH)	0.16	0.36	2.27
P07237	Protein-disulfide isomerase A1 (P4HB)	0.27	0.61	2.24
P14625	Endoplasmic (GRP94, HSP90B1)	0.45	0.94	2.07
P07602	Proactivator polypeptide (ENSP00000350063)	1.09	2.13	1.96
P06733	$\alpha$ -Enolase (ENO1)	0.25	0.43	1.70
P11021	78-kDa glucose-regulated protein (GRP78, HSPA5)	1.13	1.88	1.66
P23284	Peptidyl-prolyl cis-trans isomerase B (PPIB)	0.29	0.48	1.65
P24821	Tenascin (TNC)	0.98	1.49	1.52
P24592	Insulin-like growth factor-binding protein 6 (IGFBP6)	3.40	4.57	1.34
P18428	Lipopolysaccharide-binding protein (LBP)	3.17	4.22	1.33
P23141	Liver carboxylesterase 1 (SES1)	0.33	0.43	1.32
P09919	Granulocyte colony-stimulating factor (CSF3)	2.68	3.48	1.30
P22692	Insulin-like growth factor-binding protein 4 (IGFBP4)	2.86	3.66	1.28
Q08380	Galectin-3-binding protein (LGALS3BP)	2.94	3.61	1.23
P12109	Collagen $\alpha$ -1(VI) chain (COL6A1)	3.53	4.29	1.22
P36222	CHI3L1	3.23	3.87	1.20
P00746	Complement factor D (DF)	2.72	3.18	1.17
P01033	Metalloproteinase inhibitor 1 (TIMP-1)	2.97	3.38	1.14
P05155	Plasma protease C1 inhibitor (SERPING1)	3.15	3.58	1.14
P35442	Thrombospondin-2 (THBS2)	3.05	3.29	1.08
P08123	Collagen $\alpha$ -2(I) chain (COL1A2)	3.15	2.69	0.85
P10145	Interleukin-8 (IL8)	2.79	2.38	0.85
Q15582	TGF $\beta$ -induced protein ig-h3 (TGFB1)	0.57	0.47	0.82
P08571	Monocyte differentiation antigen CD14	3.51	2.17	0.62

probably somewhat higher because of the extra variation that can be attributed to differences in the response to insulin of the tissues in the separate dishes. To reduce variation, intracellular proteins were removed from the data sets. Intracellular proteins present in the medium showed on average lower heavy/light ratios and higher variation than secreted proteins in both control and insulin data sets. This can be explained by assuming that intracellular proteins are released randomly by damaged cells in the tissue and thus do not use the full period of 72 h for label incorporation. Furthermore intracellular proteins probably have lower synthesis rates and are regulated more by post-translational modification rather than synthesis and degradation, whereas secreted proteins are synthesized on demand. This implies lower heavy/light ratios for intracellular proteins compared with secreted proteins.

Another issue is the time window for label incubation in which the method can be applied. A minimal amount of label incorporation is necessary to be able to detect the label. On the other hand, label incorporation should not reach a 100% before the end of the incubation period because this would lead to an underestimation of incorporation rates. Based on previous experiments (11), we chose for a period of 72 h. Highest label incorporation in this period was ~83% (CC motif chemokine 2 with a heavy/light ratio of 4.85 in the presence of insulin). Shorter incubation times may lead to fewer proteins that incorporate label. Longer periods may lead to more proteins with complete label incorporation. The optimal label incubation time should be determined for each tissue/cell type and condition. In addition, a threshold ratio was applied to correct for false positives. Low label incorpo-



ration in some proteins leads to small  $^{13}\text{C}$  peaks that are difficult to distinguish from noise in the spectra or noise peaks are mistaken for  $^{13}\text{C}$  peaks by the software. This is e.g. the case with hemoglobin and serum albumin, which are clearly serum-derived but were detected as containing low levels of label. By applying a threshold ratio of 0.15 (7.5% label incorporation) these proteins were removed. Of course this threshold is arbitrary and may vary from experiment to experiment. One may also choose not to apply a threshold because the -fold change values for these proteins will most likely not reach significance. However, with this correction a more reliable list of proteins that incorporated label at different rates within the 72-h incubation period is obtained. Tables I and II show proteins ranked according to decreasing heavy/light ratios for control and insulin, respectively. This ranking is equivalent with decreasing protein synthesis rates because label incorporation rate is solely determined by the protein synthesis rate. However, this ranking does not necessarily refer to the relative abundance of the proteins in the medium because CILAIR does not take into account possible breakdown. For example, the chemokine CXCL5, which had the highest label incorporation of the proteins listed for the control situation (Table I), may not necessarily have had the highest abundance in the media of the proteins listed in Table I. This depends on the turnover of this protein. However, protein breakdown should not affect the relative quantitation of expression changes induced by insulin, assuming that breakdown of a particular protein is not influenced by insulin. Therefore, the -fold change value, as calculated for proteins listed in Table III, reflects the actual situation in the media under the two conditions. In total we found 24 proteins that were significantly up-regulated, four proteins that were down-regulated, and 58 proteins that showed no change. When analyzing the functional relationship of up-regulated proteins with the protein network program STRING (17), two major functional clusters can be identified (see Fig. 6). One cluster, containing calreticulin (GRP60), 78-kDa glucose-regulated protein (GRP78), protein-disulfide isomerase A3 (GRP58), endoplasmic reticulum (ER) stress response (18). Glucose-regulated proteins (GRPs) normally reside in the ER and have roles as chaperones for protein folding and as calcium-binding proteins. However, under conditions that perturb normal ER function leading to accumulation of misfolded proteins in the ER, these proteins are secreted and may function outside the cell to aid in cell survival by regulating the immune response towards stressed cells (19, 20). In our model, chronic incubation (72 h) with a relatively high concentration of insulin may lead to a high demand on the protein synthesis and folding machinery causing ER stress. The other functional cluster that was up-regulated, although less pronounced, contains tenascin, chitinase-3-like protein 1 (CHI3L1), and TIMP-1. These proteins are involved in extra-

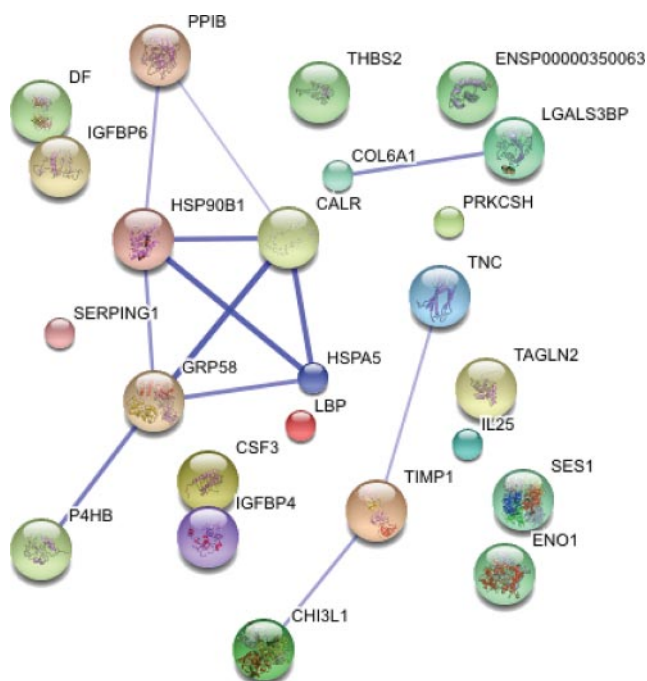


Fig. 6. **Functional relationships between up-regulated proteins.** Accession numbers of proteins that were increased in expression due to the insulin treatment (Table III) were submitted to STRING to determine their functional relationship. Two major clusters were identified that are related to the ER stress response (PP1B, HSP90B1, calreticulin (CALR), GRP58, HSPA5, and P4HB) and to extracellular matrix remodeling (tenascin (TNC), TIMP1, and CHI3L1). For protein abbreviations see Table III.

cellular matrix formation. Tenascin and CHI3L1 are part of the extracellular matrix as well as type I and VI collagens that may also be included in this group. TIMP-1 is an inhibitor of matrix metalloproteases. Matrix metalloproteases are involved in remodeling of extracellular matrix (21). As an anabolic hormone, insulin stimulates the storage of glucose and lipids in fat tissue in the form of triglycerides. This implies a higher demand for storage capacity that involves modulation of the extracellular matrix to facilitate the associated increase in adipocyte size (22, 23).

In conclusion, CILAIR allows quantitative assessment of changes in protein secretion. Because CILAIR is based on the rate of label incorporation, it does not require 100% label incorporation, which is needed for SILAC (12, 13). Complete labeling of cells is only feasible with proliferating cell lines. Therefore, CILAIR is especially suited for application with differentiated cells and tissues. In addition, it provides information on protein synthesis rates and facilitates validation of the origin of proteins. Use of CILAIR is not limited to adipose tissue but should also be applicable to other tissues and differentiated cell types in culture.

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