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Comparative Proteomic Analysis of Extracellular Proteins Reveals Secretion of T-Kininogen from Vascular Smooth Muscle Cells in Response to Incubation with *S*-Enantiomer of Propranolol

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Abstract: Propranolol, a nonselective β blocker, exerts blocking activity both on β1 adrenoceptors and β2 ones, with the *S*-enantiomer being more active than the *R*-enantiomer. The aim of the study was to investigate the secreted proteins with differential protein expression levels in culture medium of vascular smooth muscle cells (A7r5) incubated separately with individual enantiomers of propranolol using isobaric tags for relative and absolute quantitation (iTRAQ)-coupled two-dimensional LC−MS/MS approach. Our results indicated that secretion of T-kininogen by *S*-enantiomer of propranolol incubated cells was greatly enhanced as compared with that of *R*-enantiomer incubated cells or control cells. It can be inferred that the *S*-enantiomer of propranolol will induce more Ile-Ser-bradykinin (BK) (T-kinin), the vasoactive peptides. This therefore provides molecular evidence and possible link of T-kininogen with treatment of cardiovascular disease associated with propranolol treatment.

Keywords: Propranolol; enantiomer; iTRAQ; T-kininogen; bradykinin

Introduction

The β adrenoceptors belong to the family of G-protein-coupled receptors[1] (GPRs) characterized by seven transmembrane spanning domains forming a pocket in which the agonists and competitive antagonists find their binding sites.[2] There are three known types of β adrenoceptors, designated as β1, β2 and β3; particularly β1 and β2 adrenoceptors are well-known pharmacologically. β1 adrenoceptors are located mainly in the heart and in the kidneys while β2 adrenoceptors are located mainly in the lungs, gastrointestinal tract, vasculature, and uterus; however, both receptors appear to be present in these organs.[3,4] β-Blockers are a class of drugs which compete with endogenous and exogenous β-adrenergic agonists. They inhibit these normal epinephrine-mediated sympathetic actions and reduce the effect of excitement/physical exertion on heart rate and force of contraction, dilation of blood vessels and opening of bronchi. Their specific effects depend on their selectivity for β1 adrenoceptors or β2 adrenoceptors. Propranolol is a nonselective β blocker, that is, it blocks the action of epinephrine on both β_1 and β_2 adrenoceptors. Despite the well-documented blocking action of catecholamines on β adrenoceptors, concerns regarding β-blockers' potential adverse effects such as β-blockers at usual doses carrying an unacceptable risk

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(1) Johnson, M. Molecular mechanisms of β2-adrenergic receptor function, response, and regulation. *J. Allergy Clin. Immunol.* **2006**, *117* (1), 18–24.

(2) Strader, C. D.; Candelore, M. R.; Hill, W. S.; Sigal, I. S.; Dixon, R. A. Identification of two serine residues involved in agonist activation of the beta-adrenergic receptor. *J. Biol. Chem.* **1989**, *264* (23), 13572–13578.

(3) Minneman, K. P.; Hedberg, A.; Molinoff, P. B. Comparison of betaadrenergic receptor subtypes in mammalian tissues. *J. Pharmacol. Exp. Ther.* **1979**, *211* (3), 502–508.

(4) Stiles, G. L.; Taylor, S.; Letkowitz, R. J. Human cardiac beta-adrenergic receptor subtype heterogeneity delineated by direct radioligand binding. *Life Sci.* **1983**, *33* (5), 476–483.

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of provoking type 2 diabetes[5] and particularly the underlying molecular mechanism remain to be addressed. To date most β-blockers are administered as the racemic mixture even though only the *S*-enantiomer is active at the receptor.

Biochemical responses to disease or drug action are likely to be reflected in the patterns of protein expression and turnover in affected cells, tissues and, presumably, biological fluids such as blood, cerebrospinal fluid, saliva. Mass spectrometry (MS)-based proteomics has been widely used to identify potentially clinically useful molecular patterns in cancer,[6] heart disease,[7] and other common ailments. These approaches also allow the establishment of cellular signaling pathways in response to various external stimuli including comparing normal and diseased conditions,[8–11] and offer a nontargeted way to identify protein profiles or drug-activity markers since there is often no a priori knowledge of the particular proteins that are likely to change.[12] For example, it was well documented that the thermostable fractions of serum samples from patients with ovarian, uterus, and breast cancers, as well as samples from benign ovarian tumor, were analyzed by mass spectrometry system. Of them, the expression of α-1 acid glycoprotein and clusterin has been found to be downregulated in breast cancer, whereas that of transthyretin decreased specifically in ovarian cancer.[13] For quantitation of the protein expression levels some methods such as isotope-coded affinity tag,[14] chemical and enzymatic modifications[15] were established. Recently, an MS/MS-based quantitation method (iTRAQ) has been developed.[16] The system enables up to four samples to be analyzed within one experiment. They are differentially isotopically labeled such that all derivatized peptides will have an identical mass and LC retention time after tagging. Following collision-induced dissociation (CID) MS/MS analysis of the precursor ion, the four reporter groups appear as distinct ions (*m*/*z* 114−117). The relative concentration of the peptides is derived from the relative intensities of the reporter ions.

Our previous work focused on the comparative analysis of protein profile within the vascular smooth muscle cells incubated with *S*- and *R*-enantiomers of propranolol.[17] For a better understanding of the blocking effect exerted by propranolol it will be of great interest to detect changes in secretory proteins and to infer biological function combining with the observed intracellular patterns. In this study, we use a 4-plex multiplex strategy to simultaneously detect and quantitate differences in secretory proteins in untreated vascular smooth muscle cells and those incubated with *S*- and *R*-enantiomers of propranolol, respectively, which reflect pharmacologic action of eantiomers. To identify proteins from a complex mixture, the two-dimensional (2D) application is used. In this approach, a strong cation exchange (SCX) column is used for the first dimension, a reversed-phase (RP) column is used for the second, and two identical enrichment columns are used for trapping the peptides. The sample peptides bound on the SCX columns are then eluted by injected salt solution plugs of increasing concentration, trapped on a short enrichment column, and subsequently analyzed on a nano RP column interfaced with electrospray ionization (ESI)-MS/MS.

Experimental Section

Cell Culture Conditions. The vascular smooth muscle cell, A7r5 cells obtained from the American Type Culture Collection, were cultured in Dulbecco's modified Eagle's medium (DMEM, supplemented with 10% FBS, penicillin

(5) Taylor, E. N.; Hu, F. B.; Curhan, G. C. Antihypertensive medications and the risk of incident type 2 diabetes. *Diabetes Care* **2006**, *29* (5), 1065–1070.
(6) Hanash, S. Integrated global profiling of cancer. *Nat. Rev. Cancer* **2004**, *4* (8), 638–644.
(7) Stanley, B. A.; Gundry, R. L.; Cotter, R. J.; Van Eyk, J. E. Heart disease, clinical proteomics and mass spectrometry. *Dis. Markers* **2004**, *20* (3), 167–178.
(8) Shiio, Y.; Donohoe, S.; Yi, E. C.; Goodlett, D. R.; Aebersold, R.; Eisenman, R. N. Quantitative proteomic analysis of Myc oncoprotein function. *EMBO J.* **2002**, *21* (19), 5088–5096.
(9) Han, D. K.; Eng, J.; Zhou, H.; Aebersold, R. Quantitative profiling of differentiation-induced microsomal proteins using isotope-coded affinity tags and mass spectrometry. *Nat. Biotechnol.* **2001**, *19* (10), 946–951.
(10) Griffin, T. J.; Gygi, S. P.; Ideker, T., B.; Rist, B.; Eng, J.; Hood, L.; Aebersold, R. Complementary profiling of gene expression at the transcriptome and proteome levels in Saccharomyces cerevisiae. *Mol. Cell. Proteomics* **2002**, *1* (4), 323–333.
(11) Tan, T. L.; Chen, W. A proteomics analysis of cellular proteins associated with HBV genotype-specific HBX: potential in identification of early diagnostic markers for HCC. *J. Clin. Virol.* **2005**, *33* (4), 293–298.
(12) Tyers, M.; Mann, M. From genomics to proteomics. *Nature* **2003**, *422* (6928), 193–197.
(13) Goufman, E. I.; Moshkovskii, S. A.; Tikhonova, O. V.; Lokhov, P. G.; Zgoda, V. G.; Serebryakova, M. V.; Toropygin, I. Y.; Vlasova, M. A.; Safarova, M. R.; Makarov, O. V.; Archakov, A. I. Two-dimensional electrophoretic proteome study of serum thermostable fraction from patients with various tumor conditions. *Biochemistry (Moscow)* **2006**, *71* (4), 354–360.
(14) Hansen, K. C.; Schmitt-Ulms, G.; Chalkley, R. J.; Hirsch, J.; Baldwin, M. A.; Burlingame, A. L. Mass spectrometric analysis of protein mixtures at low levels using cleavable C-13-isotope-coded affinity tag and multidimensional chromatography. *Mol. Cell. Proteomics* **2003**, *2* (5), 299–314.
(15) Zappacosta, F.; Annan, R. N-terminal isotope tagging strategy for quantitative proteomics: Results-driven analysis of protein abundance changes. *Anal. Chem.* **2004**, *76* (22), 6618–6627.
(16) Ross, P.; Huang, Y.; Marchese, J.; Williamson, B.; Parker, K.; Hatten, S.; Khainovski, N.; Pillai, S.; Dey, S.; Daniels, S.; Purkayastha, S.; Juhasz, P.; Martin, S.; Bartlet-Jones, M.; He, F.; Jacobson, A.; Pappin, D. J. Multiplexed Protein Quantitation in Saccharomyces cerevisiae Using Amine-reactive Isobaric Tagging Reagents. *Mol. Cell. Proteomics* **2004**, *3* (12), 1154–1169.
(17) Sui, J. J.; Tan, T. L.; Zhang, J. H.; Ching, C. B.; Chen, W. N. ITRAQ-coupled 2D LC-MS/MS analysis on protein profile in vascular smooth muscle cells incubated with S- and R-enantiomers of propranolol: Possible role of metabolic enzymes involved in cellular anabolism and antioxidant activity. *J. Proteome Res.* **2007**, *6* (5), 1643–1651.

(100 U/mL), and streptomycin (100 U/mL). Cells were maintained at 37 °C in an atmosphere of 5% CO_2. All culture media and media supplements were purchased from Life Technologies. After reaching 80% confluence the cells were incubated with the *S*-enantiomer and the *R*-enantiomer of propranolol at a concentration of 20 μM, respectively, in the absence of serum. The concentration of 20 μM was chosen by close analysis of results of MTT assay since no significant effect on the cell viability was observed for those concentrations below 20 μM.[6] After incubation for 24 h, the conditioned medium was carefully removed, and then filtered using a 0.20 μM syringe filter (Sartorius) to remove suspended cells. The filtrate was concentrated in Amicon ultra-4 centrifugal filter unit with ultracel-3 membrane (Millipore) at 4000*g* with swinging bucket rotor for 20 min and kept for further analysis.

Protein Quantification, Protein Digestion, and Labeling with iTRAQ Reagents. The protein was quantified using the 2-D Quant Kit (GE Healthcare). A standard curve was made using BSA as a control. A total of 40 μg of each sample was precipitated by the addition of four times the sample volume of cold (−20 °C) acetone to the tube for 2 h, and the supernatant was carefully decanted. The protein pellets were then dissolved in the solution buffer and denatured, and cysteines were blocked as described in the iTRAQ protocol (Applied Biosystems). Each sample was then digested with 20 μL of 0.25 μg/ μL sequence grade modified trypsin (Promega) solution at 37 °C overnight and labeled with the iTRAQ tags as follows: control A7r5 = iTRAQ 114; A7r5 incubated with the *S*-enantiomer = iTRAQ 115; A7r5 incubated with the *R*-enantiomer = iTRAQ 117. The labeled samples were then pooled before analysis.

Online 2D Nano LC−MS/MS Analysis. The analysis was performed on an Agilent 1200 nanoflow LC system (Agilent Technologies) interfaced with a QSTAR XL mass spectrometer (Applied Biosystems/MDS Sciex). In the first dimension 3 μL of the combined peptide mixture was loaded onto the PolySulfethyl A SCX column (0.32 × 50 mm, 5 μm) and was eluted stepwise by injecting salt plugs of 10 different molar concentrations of 10, 20, 30, 40, 50, 60, 80, 100, 300, 500 mM of KCl solution. In the second dimension, with the 10-port valve being in position 1, the sequentially eluted peptides from SCX column were trapped onto the ZORBAX 300SB-C18 enrichment column 1 (0.3 × 5 mm, 5 μm) and washed isocratically with buffer A (5% acetonitrile, 0.1% formic acid) at 0.005 mL/min for 100 min to remove any excess reagent. Meanwhile, the peptides bound on the ZORBAX 300SB-C18 enrichment column 2 during the previous run were eluted with buffer B (0.1% formic acid) and buffer C (a nanoflow gradient of 5−80% acetonitrile + 0.1% formic acid) at a flow rate of 500 nL/min. Further separation was achieved onto the analytical Zorbax 300SB C-18 reversed-phase column (75 μm × 50 mm, 3.5 μm). In the next run with the 10-port valve having been switched to position 2, column 1 was switched into the solvent path of the nanopump and column 2 was used to trap the newly eluted peptides from SCX. Altogether 11 runs were performed to finish one experiment. For MS/MS analysis, survey scans were acquired from *m/z* 300−1500 with up to two precursors selected for MS/MS from *m/z* 100−2000 using dynamic exclusion, and the rolling collision energy was used to promote fragmentation. To gain statistical evidence for differential expression of proteins another two separate experiments were performed as described above.

Data Analysis and Interpretation. Relative abundance quantitation and peptide and protein identification were performed using ProteinPilot Software 2.0 (Applied Biosystems, Software Revision 50861). Each MS/MS spectrum was searched for species of *Rattus norvegicus* against the uniprot_sprot_20070123 database. Parameters such as tryptic cleavage specificity, precursor ion mass accuracy and fragment ion mass accuracy are built-in functions of ProteinPilot software. The database allowed for iTRAQ reagent labels at N-terminal residues, internal K and Y residues, and the methylmethanethiosulfate-labeled cysteine as fixed modification plus one missed cleavage, and biological modification "ID focus" was set. The following criteria were required to consider a protein significant: 2 or more high confidence (>95%) unique peptides had to be identified, the *P* value had to be $P < 0.05$ and the fold difference had to be greater than 1.2. To account for small differences in protein loading, all protein ratios have been normalized using the overall ratios for all proteins in the sample, as recommended by Applied Biosystems. ProteinPilot Software 2.0 pooled data from all the 11 runs in the complete experiment. Relative quantification of proteins in the case of iTRAQ was performed on the MS/MS scans and was the ratio of the areas under the peaks at 114, 115, and 117 Da which were the masses of the tags that correspond to the iTRAQ reagents. The relative amount of a peptide in each sample was calculated by dividing the peak areas observed at 115.1 and 117.1 *m/z* by that observed at 114.1 *m/z*. The calculated peak area ratios were corrected for overlapping isotopic contributions, and were used to estimate the relative abundances of a particular peptide. The unused protein score is ProteinPilot's measurement of protein identification confidence taking into account all peptide evidence for a protein, excluding any evidence that is better explained by a higher ranking protein. Sequence coverage is calculated by dividing the number of amino acids observed by the protein amino acid length. Error factor (EF) is a statistic that has been created for reporting errors in ratios and expresses the 95% confidence interval for an average ratio ($EF = 10^{95\%\text{confidenceinterval}}$, where 95% confidence interval = (ratio × ER) − (ratio/ER)).

The data were expressed as mean ± SD. One-sample *t* tests were performed if the quantitative information met the criteria for further statistical analysis as described above and was in a minimum of two independent experiments. Correction for multiple comparisons was done according to the

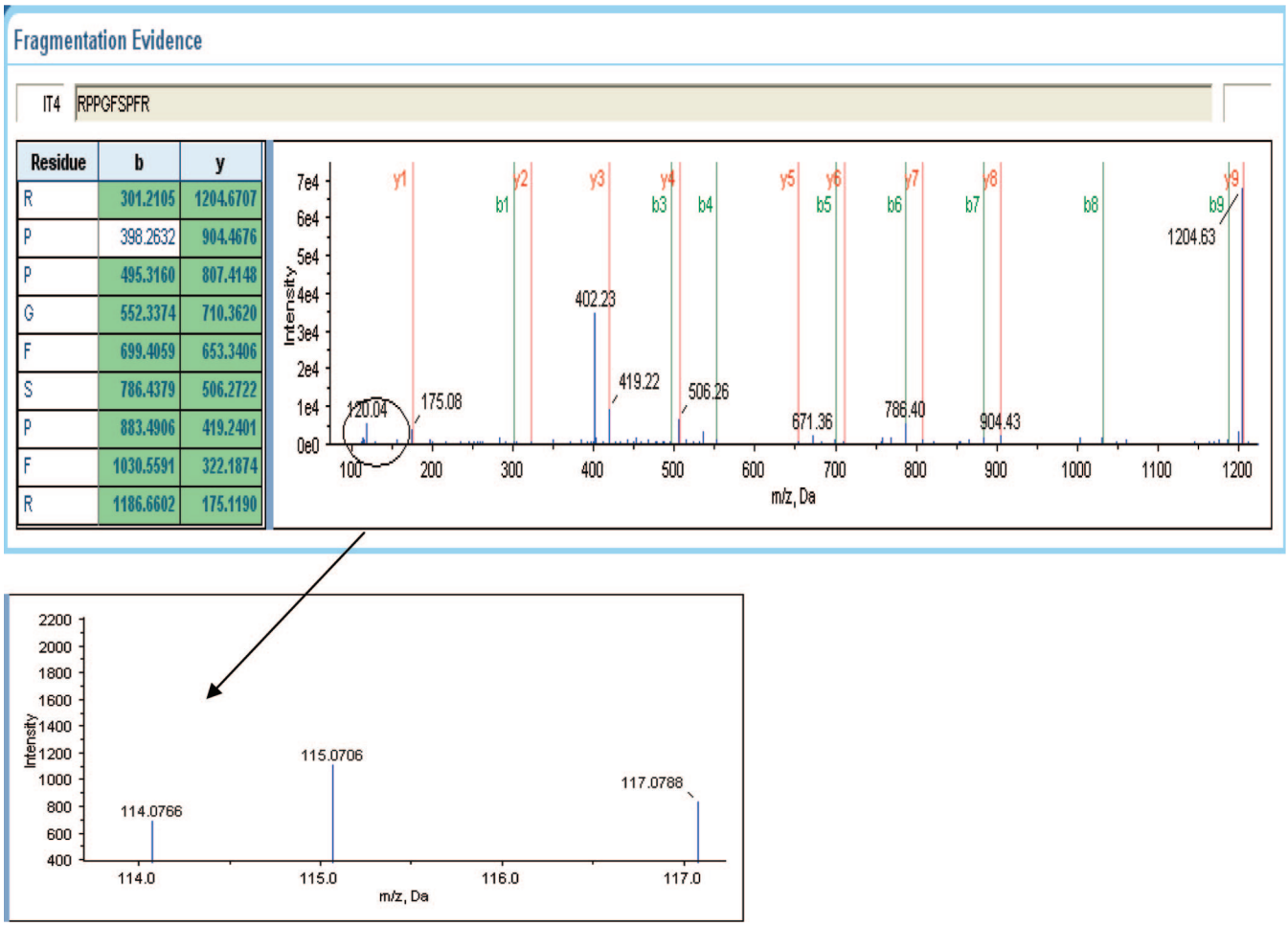

Figure 1. A representative MS/MS spectrum showing a peptide, RPPGFSPFR, from T-kininogen 1. The ion assignments are as follows: control A7r5 = iTRAQ 114; A7r5 incubated with the *S*-enantiomer of propranolol = iTRAQ 115; A7r5 incubated with the *R*-enantiomer of propranolol = iTRAQ 117.

false discovery rate (FDR) method described by Benjamini[18] using the FDR software available to the public at www-.math.tau.ac.il/∼ybenja/ with a significance value of 0.05. Calculated p values by using this software larger than the FDR α value were considered statistically nonsignificant.

Western Blot Analysis. Total protein obtained from the experiments as described above for the LC/MS/MS analysis was used for the Western blot analysis. Specifically, 60 μg of total proteins from each concentrated medium of control, the *R*-enantiomer-, and the *S*-enantiomer-incubated cells was separated on an 8% SDS−PAGE. Proteins were then transferred onto nitrocellulose membrane (GE Hybond), and the detection of the respective proteins was performed using specific antibodies. Enhanced chemiluminescence (ECL) was performed using SuperSignal West Pico Chemiluminescent reagent, and CL-XPosure film was used for detection (GE-Amersham). Developed X-ray film was then scanned using Bio-Rad's densitometer and analyzed using Bio-Rad's QualityOne software. The antibody used in this study was goat-anti kininogen (Santa Cruz SC-25889).

(18) Benjamini, Y.; Drai, D.; Elmer, G.; Kafkafi, N.; Golani, I. Controlling the false discovery rate in behavior genetics research. *Behav. Brain Res.* **2001**, *125* (1−2), 279–284.

Results

To find some evidence toward biological difference between propranolol incubated A7r5 cells and control A7r5 cells, the secreted protein profile in A7r5 cells incubated with individual enantiomers of propranolol was established. iTRAQ tags shown in Figure 1 were as follows: control A7r5 cells = iTRAQ 114; A7r5 cells incubated with the *S*-enantiomer of propranolol= iTRAQ 115; A7r5 incubated with the *R*-enantiomer of propranolol = iTRAQ 117. In three experiments we were able to identify about 10 proteins with unused protein score more than 2 (99% confidence) per experiment on the basis of one or more peptide hits per protein at >95% confidence per peptide. A total of 13 unique proteins were identified in the three independent experiments and were displayed in Table 1. For each experiment, we examined the original protein list generated by ProteinPilot software and filtered it according to the criteria for considering a protein significant mentioned in the experimental procedures. Further statistical comparisons by FDR from quantitative information obtained from the three experiments led to 1 protein, T-kininogen 1 precursor, which was considered significant for further analysis and displayed in

Table 1. List of Secreted Proteins in Conditioned Culture Medium[a]

accession	name	av S:C (±SD)	av R:C (±SD)	no. of experiments
P02770	serum albumin precursor	1.00 ± 0.03	0.98 ± 0.02	3
P12346	serotransferrin precursor	0.89 ± 0.06	0.96 ± 0.05	3
P02767	transthyretin precursor	1.01 ± 0.11	1.03 ± 0.09	3
P24090	alpha-2-HS-glycoprotein precursor	0.91 ± 0.16	0.95 ± 0.13	3
P01026	complement C3 precursor	0.98 ± 0.08	0.98 ± 0.14	3
P11762	galectin-1	1.15 ± 0.17	1.18 ± 0.20	3
P01048	T-kininogen 1 precursor	1.39 ± 0.11	1.07 ± 0.06	3
Q64268	heparin cofactor 2 precursor	0.97 ± 0.24	0.99 ± 0.15	2
P49745	thrombopoietin precursor	0.81 ± 0.21	0.89 ± 0.12	2
P12843	insulin-like growth factor-binding protein 2 precursor	1.02 ± 0.17	1.03 ± 0.11	2
O88959	inhibin beta E chain precursor	0.97 ± 0.23	0.86 ± 0.19	1
5XI67	F-box only protein 30	1.06 ± 0.19	1.03 ± 0.15	1
Q07936	annexin A2	1.05 ± 0.07	1.27 ± 0.30	1

[a] S:C is the ratio of the level of each protein secreted by *S*-enantiomer of propranolol incubated cells relative to that by the control cells; R:C is the ratio of the level of each protein secreted by *R*-enantiomer of propranolol incubated cells relative to that by control cells. "no. of experiments" indicates the number of times the protein was detected in the three independent experiments.

Table 2. Differentially Expressed Protein, T-Kininogen 1 in Conditioned Culture Medium[a]

accession	name	sequence coverage	no. of peptides	av S:C (±SD)	av R:C (±SD)	*p* (S:C)	*p* (R:C)	no. of experiments
P01048	T-kininogen 1 precursor	64.9%	2	1.39 ± 0.11	1.07 ± 0.06	0.0175	0.0084	3

[a] S:C is the ratio of the level of each protein secreted by *S*-enantiomer of propranolol incubated cells relative to that by the control cells; R:C is the ratio of the level of each protein secreted by *R*-enantiomer of propranolol incubated cells relative to that by control cells. "no. of experiments" indicates the times the protein was detected in the three independent experiments. "no. of peptides" indicates the total number of detected peptides (with 99% confidence) for the individual protein in the three independent experiments. The *p* values indicated statistical significance of the observed differences.

Protein	S:C	R:C
T-kininogen 1	1.79±0.12	1.23±0.06

Figure 2. Western blot analysis of T-kininogen 1 in the conditioned medium of A7r5 cells incubated with individual enantiomers of propranolol. Lane C, control, cells in the absence of propranolol. Lane S, cells incubated with the *S*-enantiomer of propranolol. Lane R, cells incubated with the *R*-enantiomer of propranolol. The quantification of the protein level based on Western blot analysis was displayed in the table below the Western blot results. Ratio between *S*-enantiomer incubated cells and control cells (S:C), as well as between *R*-enantiomer incubated cells and control cells (R:C) was shown.

Table 2. T-kininogen 1 in culture medium of *S*-enantiomer incubated A7r5 cells showed higher protein levels from those in culture medium of *R*-enantiomer incubated cells or control cells (absence of propranolol). To verify the differential protein levels quantitated by iTRAQ coupled 2D-LC/MS/MS Western blot analysis was performed. The results in Figure 2 showed a similar trend as the iTRAQ analysis, which indicated that the level of the T-kininogen 1 was higher in culture medium of *S*-enantiomer of propranolol incubated cells compared with the level in those two types of culture medium ($p < 0.05$). However by close observation of the quantification of the protein level based on Western blot analysis in Figure 2B, the changes in the protein were more dramatic than those in iTRAQ analysis. The cause for this phenomenon is most likely a bias due to the normalization of total protein content, which is much more suitable for high abundant proteins, leading to relatively lower values for less abundant proteins in the iTRAQ quantitation. The experimental molecular weight was 66 kDa determined by Western blot analysis, higher than the theoretical molecular weight of 47 kDa. It was most possibly due to the glycosylation of T-kininogen 1.

Discussion

In rats, T-kininogen is a major positive acute phase protein,[19] a cysteine proteinase inhibitor[20] with an Ile-Ser-bradykinin (BK) (T-kinin) sequence in its structure.[21] The responses to vasoactive kinin peptides are mediated by the activation of bradykinin receptor B1 (B1R) and B2 (B2R) according to the relative potencies and affinities to their agonists.[22] B2R is constitutively expressed in most tissues

(19) Esnard, F.; Gauthier, F. Rat al-cysteine proteinase inhibitor: An acute phase reactant identical with al-acute phase globulin. *J. Biol. Chem.* **1983**, *258* (20), 12443–12447.
(20) Miiller-Esterl, W.; Iwanaga, S.; Nakanishi, S. Kininogens revisited. *Trends Biol. Sci.* **1986**, *11* (8), 336–339.
(21) Okamoto, H.; Greenbaum, L. M. Isolation and structure of T-kinin. *Biochem. Biophys. Res. Commun.* **1983**, *112* (2), 701–708.
(22) Park, S. S.; Zhao, H.; Mueller, R. A.; Xu, Z. L. Bradykinin prevents reperfusion injury by targeting mitochondrial permeability transition pore through glycogen synthase kinase 3beta. *J. Mol. Cell Cardiol.* **2006**, *40* (5), 708–716.

and responsive to intact BK and kallidin (Lys-BK).In contrast, B1R which has a higher affinity for des-Arg^9-BK (DBK) and des-Arg^{10}-kallidin, is weakly expressed under physiological conditions but induced by pathological stimuli such as inflammation and tissue injury.[23] Hang Yin and co-workers reported that the B2 receptor, but not the B1 receptor, protects against ischemia/reperfusion (I/R) induced cardiac dysfunction by inhibiting apoptosis and limiting ventricular remodeling.[24] Propranolol, a nonselective β blocker, is mostly used in the treatment of hypertension,[25] angina,[26] and for the prevention of reinfarction in patients who have suffered from myocardial infarction.[27] It was well documented that the *S*-enantiomer of propranolol is more potent than the *R*-enantiomer. In this study, we demonstrated for the first time that A7r5 cells can secrete T-kininogen by using the iTRAQ-coupled 2D LC−MS/MS and Western blot analysis. However, secretion of T-kininogen by *S*-enantiomer incubated cells was greatly enhanced as compared with that of *R*-enantiomer incubated cells or control cells. Since T-kininogen is the precursor of active peptide BK, *S*-enantiomer of propranolol incubated A7r5 cells will produce much more BK or active metabolites of BK as compared with other two types of cells, indicative of more pharmacologically.

In conclusion, the application of quantitative proteomics provides us an effective approach to understand the mechanism of action of drug and build a better understanding of protein targets and their roles in disease. Our preliminary results indicated that the *S*-enantiomer of propranolol was able to trigger the secretion of T-kininogen by A7r5 cells much more than control cells or *R*-enantiomer of propranolol incubated cells. This provides molecular evidence and a possible link of T-kininogen with treatment of cardiovascular disease associated with propranolol treatment.

Abbreviations Used

iTRAQ, isobaric tags for relative and absolute quantification; SCX, strong cation exchange; RP, reversed-phase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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(23) Regoli, D.; Allogho, S. N.; Rizzi, A.; Gobei, F. J. Bradykinin receptors and their antagonists. *Eur. J. Pharmacol.* **1998**, *348* (1), 1–10.

(24) Yin, H.; Chao, J.; Bader, M.; Chao, L. Differential role of kinin B1 and B2 receptors in ischemia-induced apoptosis and ventricular remodeling. *Peptides* **2007**, *28* (7), 1383–1389.

(25) Witte, K.; Engelhardt, S.; Janssen, B.; Lohse, M.; Lemmer, B. Circadian and short- term regulation of blood pressure and heart rate in transgenic mice with cardiac overexpression of the beta(1)-adrenoceptor. *Chronobiol. Int.* **2004**, *21* (2), 205–216.

(26) Detry, J.; Sellier, P.; Pennaforte, S.; Cokkinos, D.; Marthes, P. Dargie, H. A new concept in the treatment of angina-comparison with propranolol in patients with stable angina. *Br. J. Clin. Pharmacol.* **1994**, *37* (3), 279–288.

(27) Shivkumar, K.; Schultz, L.; Goldstein, S.; Gheorghiade, M. Effects of propranolol in patients entered in the beta-blocker heart attack trial with their first myocardial infarction and persistent electrocardiographic ST-segment depression. *Am. Heart J.* **1998**, *135* (2), 261–267.