Atenolol is a β<sub>1</sub>-selective drug, which exerts greater blocking activity on β<sub>1</sub>-adrenoceptors than on β<sub>2</sub>-adrenoceptors, with the S-enantiomer being more active than R-enantiomer. The aim of this study was to investigate the proteins with differential protein expression levels in the proteome of vascular smooth muscle cells (A7r5) incubated separately with individual enantiomers of atenolol using an iTRAQ-coupled two-dimensional LC-MS/MS approach. Our results indicated that some calcium-binding proteins such as calmodulin, protein S100-A11, protein S100-A4, and annexin A6 were down-regulated and showed relatively lower protein levels in cells incubated with the S-enantiomer of atenolol than those incubated with the R-enantiomer, whereas metabolic enzymes such as aspartate aminotransferase, glutathione S-transferase P, NADH-cytochrome b<sub>5</sub> reductase, and α-N-acetylgalactosaminidase precursor were up-regulated and displayed higher protein levels in cells incubated with the S-enantiomer relative to those incubated with the R-enantiomer. The involvement of NADH-cytochrome b<sub>5</sub> reductase in the intracellular anabolic activity was validated by NAD<sup>+</sup>/NADH assay with a higher ratio of NAD<sup>+</sup>/NADH correlating with a higher proportion of NAD<sup>+</sup>. The down-regulation of the calcium-binding proteins was possibly involved in the lower intracellular Ca<sup>2+</sup> concentration in A7r5 cells incubated with the S-enantiomer of atenolol. Ca<sup>2+</sup> signals transduced by calcium-binding proteins acted on cytoskeletal proteins such as nestin and β-tropomyosin, which can play a complex role in phenotypic modulation and regulation of the cytoskeletal modeling. Our preliminary results thus provide molecular evidence on the metabolic effect and possible link of calcium-binding proteins with treatment of hypertension associated with atenolol. Molecular & Cellular Proteomics 7:1007–1018, 2008.

The β-adrenoceptors belong to the family of G protein-coupled receptors (GPRs) (1) characterized by seven transmembrane–spanning domains forming a pocket in which the agonists and competitive antagonists find their binding sites (2). The signal transduction process triggered by agonist binding to β-adrenoceptors consists of discrete steps, and one of the earliest is the coupling of this complex to heterotrimeric G protein that dissociates in Gα and Gβγ subunits (3). Gα and Gβγ subunits activate or inhibit a number of effector enzymes such as adenylyl cyclase, phospholipase C, ion channels, or mitogen-activated protein kinases (MAPKs), resulting in a variety of cellular functions. One of the well documented signal transduction steps is that the coupling of this complex to the heterotrimeric G protein Gα subunits will stimulate adenylyl cyclase activity, which produces the second messenger cAMP. Protein kinase A activated by cAMP phosphorylates L-type calcium channels and myosin light chain kinase (4, 5), facilitating Ca<sup>2+</sup> entry and producing the positive inotropic effect in atria and ventricles. In addition to mechanisms that indirectly lead to alterations in ion transport, β-adrenoceptor activation is more directly linked to ion channels (6). After agonist stimulation, most GPRs rapidly lose their ability to respond to agonist. For many GPRs, this process, commonly referred to as desensitization, appears to be primarily mediated by two protein families: G protein-coupled receptor kinases (GRKs) and arrestins. GRKs specifically bind to the agonist-occupied receptor, thereby promoting receptor phosphorylation, which in turn leads to arrestin binding. Arrestin binding precludes receptor/G protein interaction leading to functional desensitization (7, 8). The activated Gα can in turn be inhibited by regulator of G protein signaling proteins, which are GTPase-activating proteins that reduce the signal transmitted by the receptor-activated (GTP-bound) Gα subunit by rapidly returning it to the inactive state (GDP-bound) (9).

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There are three known types of β-adrenoreceptors, designated as β₁, β₂, and β₃. Particularly β₁- and β₂-adrenoreceptors are well known pharmacologically. β₁-Adrenoreceptors are located mainly in the heart and in the kidneys, whereas β₂-adrenoreceptors are located mainly in the lungs, gastrointestinal tract, vasculature, and uterus; however, both receptors appear to be present in these organs (10, 11). β-Blockers are a class of drugs that compete with endogenous and exogenous β-adrenergic agonists. They inhibit the 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-adrenoreceptors or β₂-adrenoreceptors. Atenolol is a so-called β₁-selective drug, which means that it exerts greater blocking activity on β₁-adrenoreceptors than on β₂-adrenoreceptors. It has been shown that in humans a plasma level of 1 μg/ml atenolol is associated with a 30% reduction of exercise-accelerated heart rate and that a clear linear relationship could be obtained between log plasma concentration and percentage of reduction in heart rate (12). Despite the well documented blocking action of catecholamines on β-adrenoceptors, concerns regarding their potential adverse effects such as β-blockers at usual doses carrying an unacceptable risk of provoking type 2 diabetes (13) and particularly the underlying molecular mechanism remain to be addressed. To date most β-blockers are administered as the racemic mixture, although only the S-enantiomer is active at the receptor. The mechanism of action of β-blockers has been studied at the gene expression level (14). However, little is known about their action at the protein level. It will be of great interest to detect changes in quantitative protein profiles and to infer biological function from the observed patterns. Proteomics analysis has been widely used to establish cellular signaling pathways in response to various external stimuli, including comparing normal and diseased conditions (15–18). Established methods for relative quantitation of proteins involve isotope-coded affinity tag (19) and chemical and enzymatic modifications (20). Recently an MS/MS-based quantitation method (iTRAQ) has been developed (21). 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 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. In this study, we used a fourplex multiplex strategy to simultaneously detect and quantify differences in expression levels of proteins in untreated vascular smooth muscle cells and those incubated with the S- and R-enantiomers of atenolol, respectively, that reflect the pharmacologic action of enantiomers. To identify proteins from a complex mixture, a two-dimensional (2D) application was used. In this approach, a strong cation exchange (SCX) column was used for the first dimension, a reversed-phase column was used for the second, and two identical enrichment columns were used for trapping the peptides.

Our results indicated that some calcium-binding proteins such as calmodulin, protein S100-A11, protein S100-A4, and annexin A6 were down-regulated and showed relatively lower protein levels in cells incubated with the S-enantiomer of atenolol than those incubated with the R-enantiomer, whereas some metabolic enzymes such as aspartate aminotransferase, glutathione S-transferase P, NADH-cytochrome b₅ reductase, and α-N-acetylgalactosaminidase precursor were up-regulated and displayed higher protein levels in cells incubated with S-enantiomer than those incubated with the R-enantiomer. The findings of our investigation of the detected proteins with differential expression levels provide molecular evidence on the anabolic activity and blocking effect induced by atenolol incubation.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures**—Vascular smooth muscle cells, A7r5 cells obtained from the American Type Culture Collection, were cultured in Dulbecco’s modified Eagle’s medium (supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin). Cells were maintained at 37 °C in an atmosphere of 5% CO₂. All culture media and media supplements were purchased from Invitrogen. After reaching 90% confluence the cells were incubated with the S-enantiomer and the R-enantiomer of atenolol at a concentration of 20 μM, respectively, for 24 h in the absence of serum.

**MTT Assay**—Cytotoxic concentrations were determined by the MTT reduction test. After chemical exposure, the medium was removed, and cells were incubated for 3 h with 5 mg/ml MTT dissolved in PBS. MTT was cleared out, and the formazan salts were solubilized in 100 μl of DMSO. Plates were read at 570 nm against a 660-nm reference wavelength on a microplate reader (Benchmark Plus). The cell viability was expressed as a percentage of the corresponding control value.

**Cell Lysis, Protein Digestion, and Labeling with iTRAQ Reagents**—Cells were harvested and lysed in 150 μl of 8 μM urea, 4% (w/v) CHAPS, and 0.05% SDS (w/v) on ice for 20 min with regular vortexing. Protein was centrifuged at 15,000 × g for 60 min at 4 °C, supernatant was removed, and protein was quantified using the 2-D Quant kit (GE Healthcare). A standard curve was made using BSA as a control. A total of 100 μg of each sample was precipitated by the addition of 4 times the sample volume of cold (−20 °C) acetone to the tube for 2 h and carefully decanting the supernatant. 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 sequencing 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 of atenolol, iTRAQ 115; and A7r5 incubated with the R-enantiomer of atenolol, iTRAQ 117. The labeled samples were then pooled before analysis.

**On-line 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 PolySulfoethyl 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, and 500 mM KCl solution. In the second dimension, while the...
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Each MS/MS spectrum was searched for species of *Rattus norvegicus* against the UniProt_sprot_20070123 database. The searches were run using the following parameters: fixed modification of methylnethanethiosulfate-labeled cysteine, fixed iTRAQ modification of free amine in the amino terminus and lysine, variable iTRAQ modifications of tyrosine, and allowing serine and threonine residues undergoing side reaction with the iTRAQ reagent. Other parameters such as tryptic cleavage specificity, precursor ion mass accuracy, and fragment ion mass accuracy are built-in functions of ProteinPilot software. 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 was 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 = \(10^{95\%\text{ confidence interval}}\)). The following criteria were required to consider a protein for further statistical analysis: two or more high confidence (>95%) unique peptides had to be identified, the p value in the Protein Quant had to be p < 0.05, and the -fold difference had to be greater than 1.2. The candidate proteins were carefully examined in the Protein ID of the ProteinPilot software. The peptides without any modification of free amine in the amino terminus or without iTRAQ modification of free amine in the lysine were excluded from calculation of the protein ratios. To account for small differences in protein loading, all protein ratios were normalized using the overall ratios for all proteins in the sample as recommended by Applied Biosystems.

**NAD*/NADH Assay**—Each of the three types of A7r5 cells (control, the cells incubated with S-enantiomer of atenolol at a concentration of 20 μM, and the cells incubated with R-enantiomer of atenolol at a concentration of 20 μM) were cultured for 24 h to reach nearly 80% confluence in the absence of serum for the NAD*/NADH quantification assay (BioVision). Three independent experiments were performed for each type of cells. A total of 4 × 10^5 cells were used in each assay, and intracellular NAD*/NADH was extracted with 400 μl of NAD*/NADH extraction buffer by subjecting the cells to two cycles of freeze/thaw (20 min on dry ice followed by 10 min at room temperature). To detect total NAD⁺ and NADH (NADt), 50 μl of each extracted sample was transferred into a 96-well plate in duplicates. The plate was incubated at room temperature for 5 min in the presence of 100 μl of NAD Cycling Mix to allow the conversion of NAD⁺ to NADH. To detect NADH, 200 μl of extracted solution was taken from each sample and heated at 60 °C for 30 min in a heating block. Under these conditions, all NAD⁺ was decomposed, whereas NADH remained intact. Then 50 μl of each NADH sample was taken into a 96-well plate in duplicates. Subsequently 10 μl of NADH developing solution was added into each well. After 40 min in the dark, the plates were read at 450-nm wavelength on a microplate reader (BenchMark Plus). The ratio of NAD⁺/NADH was calculated as follows: (NAD⁺ − NADH)/NADH.

**Real Time PCR**—Each of the three types of A7r5 cells were cultured for RNA isolation using an RNeasy minikit (Qiagen). Three independent experiments were performed for each type of cells.
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TABLE I

| Protein name | Primers |
|--------------|---------|
| Calmodulin   | 5'-CCGAAAGAACAGATTGCAGAGT-3' |
|              | 5'-ATTGCCATACGATCCACCTC-3' |
| Protein S100-A11 | 5'-CAGTTGGAAGGTGAAGATG-3' |
|              | 5'-AGCCACCAATAAGGTGAGAA-3' |
| Protein S100-A4  | 5'-GCCCTGGATGTAATAGTGTGTC-3' |
|              | 5'-TTCAATTGTCCCTGCTTGCTGTC-3' |
| Annexin A6   | 5'-CAGGAGCTCGACAGAGTTATG-3' |
|              | 5'-GATGAGGTCCTTGCCCATAGAG-3' |

DNase was used to treat RNA to remove genomic DNA contamination. The iSCRIPT One-step RT-PCR kit (Bio-Rad) was used for the precise real time quantification of RNA targets. SYBR Green was used in real time PCR as a dye to emit the fluorescence signal. Primers that were specific to the following cDNAs that displayed changes in the respective protein level (Table I) were chosen: calmodulin, protein S100-A11, protein S100-A4, annexin A6, and nestin, are shown in Table I. These primers were designed to yield amplification products within 300 bp in size to reduce nonspecific binding of SYBR Green. Quantification was performed by calculating the fluorescence density in each lane through the amplification cycle. The real time PCR was carried out in an iQ5 multicolor real time PCR detection system (Bio-Rad). The cycling program was as follows: the initial cycle with 10 min at 95 °C and 5 min at 95 °C followed by 40 cycles with 10 s at 95 °C and 30 s at 60 °C. The dissociation analysis was routinely carried out by acquiring a fluorescence reading for each 1 °C increase from 55 to 95 °C. Microsoft Excel-formatted data including amplification analysis, experimental report, melting curve analysis, and threshold cycle number were produced automatically by DNA IQ5 optical system software version 2.0 (Bio-Rad). The -fold changes were calculated as shown in the following formula: \( \Delta \Delta Ct = Ct_{sample} - Ct_{actin} \), where \( \Delta Ct = Ct_{sample} - Ct_{control} \); Fold of sample versus control = \( 2^{\Delta \Delta Ct} \).

Western Blot Analysis—Total protein lysates extracted from the experiments as described above for the LC-MS/MS analysis were used for the Western blot analysis. Specifically 30 μg of total proteins from each of the three types of cells was separated by 12% SDS-PAGE. Proteins were then transferred onto nitrocellulose membrane (GE Hybond), and the detection of the respective proteins was performed using specific antibodies (see below). ECL was performed using SuperSignal West Pico Chemiluminescent reagent, and CL-XPose film was used for detection (Amersham Biosciences). Developed x-ray film was then scanned using a Bio-Rad densitometer and analyzed using Bio-Rad QuantityOne software. The antibodies used in this study were as follows: 1) goat anti-annexin VI (Santa Cruz Biosciences), 2) mouse anti-calmodulin (GeneTex GTX22860), and 3) mouse anti-β-actin (Sigma A5441).

Intracellular Ca\(^{2+}\) Concentration Measurements—A Flu-4 NW Calcium Assay kit (Invitrogen) was used to measure intracellular Ca\(^{2+}\) concentration on a fluorometer (Tecan) following the manufacturer’s protocols. Briefly each of the three types of A7r5 cells was cultured separately in a 96-well plate. The growth medium was replaced with 100 μl/well Flu-4 dye solution containing probenecid to prevent extrusion of the dye out of cells. The plate was incubated at 37 °C for 30 min and then at room temperature for an additional 30 min. The assay was done at 494 nm for excitation and 516 nm for emission.

Immunostaining and Fluorescence Microscopy—Each of the three types of A7r5 cells were seeded separately on glass coverslips for 24 h, washed with prewarmed PBS, and fixed with 1 ml of 3% paraformaldehyde, PBS followed by another wash with PBS. Cells were then permeabilized with 0.2% Triton X-100, PBS and blocked with 10% FBS, 0.1% Triton-X100, PBS followed by a wash with PBS. To probe the structure of cytoskeleton, the fixed and permeabilized A7r5 cells were incubated with FITC-phalloidin for 1 h. The coverslip was mounted with mounting solution, and the fluorescence imaging of cells stained with actin was performed with a Pascal 5 confocal microscope (Pascal 5, Carl Zeiss). The sample was excited by an argon-ion laser with a wavelength of 488 nm, and the emitted light was detected with a band pass filter of 520 nm.

RESULTS

MTT Assay and Concentration of Atenolol—Drugs have been shown to have an impact on cell growth in vitro as cell death has been observed depending on the concentration of drugs and the time of incubation (24). Disturbances in metabolism and cell signaling as a result of cell death, however, may be different in nature to normal cellular responses to exposure to drugs. In the case of drug treatment, such cell death-related signaling would not be relevant for drugs that were designed and synthesized for non-anticancer applications. As the purpose of this study was to establish the cellular protein profile in response to the non-anticancer drug, it was critical to minimize the cell death associated with the incubation of drugs. To determine the appropriate concentration of atenolol used in our study, A7r5 cells were incubated with an increasing concentration of the respective enantiomer of atenolol, and the viability was examined by the MTT assay. Results shown in Fig. 2 indicated that either of the two enantiomers of atenolol was toxic to the cells with a drastic decrease in cell viability.
viability for any concentration higher than 50 \text{\mu M}. In addition, the overall comparison of the MTT values obtained showed that the R-enantiomer of atenolol was somewhat less toxic than the S-enantiomer of atenolol. Our results also indicated that no significant effect on the cell viability was observed for those concentrations below 25 \text{\mu M}. For this study the concentration of 20 \text{\mu M} was chosen in all experiments.

iTRAQ Analysis and Differentially Expressed Proteins—To establish the biological difference between atenolol-incubated A7r5 cells and control A7r5 cells, the protein profile in A7r5 cells incubated with individual enantiomers of atenolol was analyzed by 2D LC-MS/MS. Cells incubated with individual enantiomers of atenolol as well as the control cells were collected, lysed, and labeled prior to 2D LC-MS/MS. In this study, iTRAQ 114 represented control A7r5 cells, iTRAQ 115 represented A7r5 cells incubated with the S-enantiomer of atenolol, and iTRAQ 117 represented A7r5 cells incubated with the R-enantiomer of atenolol (Figs. 3 and 4). More than 200 proteins were identified from each of the three independent experiments conducted (268, 249, and 257 proteins, respectively) by the following criteria: unused protein score was more than 2 (99% confidence) per experiment, and one or more peptide hits were found per protein at >95% confidence per peptide. Combining these three experiments, a total of 407 unique proteins were identified in the three independent experiments and were classified into distinct categories (Fig. 5) according to their molecular functions and represented by percentage of proteins found for each category. It is apparent that metabolism and structural proteins account for a large proportion of the detected proteins. For each experiment, we examined the original protein list generated by ProteinPilot software and filtered it according to the criteria considering a candidate protein for further statistical analysis as described under “Experimental Procedures.” Among the three experiments, the range of proteins in the filtered list was 19–27. Testing for multiple comparisons from quantitative information obtained from the three experiments eventually led to the 13 proteins listed in Table II. All 13 proteins showed statistically significant changes ($p < 0.05$) in cells incubated with S-enantiomer of atenolol compared with control cells. In contrast, in R-enantiomer of atenolol-incubated cells the changes in most of the proteins were not significant ($p > 0.05$).
These proteins were subsequently categorized into four groups according to their cellular functions (Table II). These included four calcium-binding proteins, four proteins involved in cellular metabolism, three cytoskeletal proteins, and two proteins involved in cellular signal transduction. In general, proteins in A7r5 cells incubated with the S-enantiomer of atenolol showed differential levels from those in cells incubated with the R-enantiomer of atenolol. These results were consistent with the fact that the S-enantiomer of atenolol is more biologically active than the R-enantiomer of atenolol.

The metabolic enzymes listed in Table II were significantly up-regulated in A7r5 cells incubated with the S-enantiomer of atenolol compared with those incubated with the R-enantiomer of atenolol. In addition, the four calcium-binding proteins were significantly down-regulated in A7r5 cells incubated with the S-enantiomer of atenolol compared with those incubated with the R-enantiomer of atenolol. In contrast to the metabolic enzymes and calcium-binding proteins, cytoskeletal proteins and signal proteins in Table II showed random changes with some down-regulated and others up-regulated regardless of the enantiomer of atenolol used in the experiment.

**Up-regulation of Metabolic Enzymes**—Anabolism is the set of metabolic pathways that construct molecules from smaller units, which is the opposite of catabolism. One common feature of anabolic metabolic pathways is the oxidation of...
would correlate with a higher proportion of NADH to NAD+. Anabolic enzymes with such a feature in our study included aspartate aminotransferase (mitochondrial), which facilitates the conversion of aspartate and α-ketoglutaric acid to oxaloacetate and glutamate (25); glutathione S-transferase P, which catalyzes the conjugation of reduced glutathione via the sulfhydryl group to electrophilic centers on a wide variety of substrates (26) and is considered to contribute to the phase II biotransformation of xenobiotics (27); and NADH-cytochrome b5 reductase, which functions in the desaturation and elongation of fatty acids (28, 29), cholesterol biosynthesis (30), and drug metabolism (31). NADH-cytochrome b5 reductase is involved in the oxidation of NADH to NAD+ in the following catalytic reaction: NADH + 2 ferrocytochrome b5 = NAD+ + H+ + 2 ferrocytochrome b5.

To strengthen the involvement of NADH-cytochrome b5 reductase in cellular response to the individual enantiomers of atenolol, the level of intracellular NADH to NAD+ was measured. Results shown in Table III indicated that the ratio of NAD+/NADH was significantly higher in cells incubated with the S-enantiomer of atenolol (with an average of 1.248) compared with the level in either control cells (with an average of 0.773) or cells incubated with the R-enantiomer of atenolol (with an average of 0.863). The higher ratio of NAD+/NADH would correlate with a higher proportion of NAD+, indicative of higher anabolic activity.

**Down-regulation of Calcium-binding Proteins**—A closer analysis of the proteins in Table II revealed that the calcium-binding proteins such as calmodulin, protein S100-A11, protein S100-A4, and annexin A6 were all down-regulated with iTRAQ ratios ranging from 0.68 to 0.81-fold for S-enantiomer-incubated cells. Further evaluation by Student’s t tests indicated that the down-regulation of these calcium-binding proteins was statistically significant (p < 0.05) in cells incubated with the S-enantiomer of atenolol compared with the control cells (Table II). Although a down-regulation of the same calcium-binding proteins was also observed in cells incubated with the R-enantiomer of atenolol, evaluation by Student’s t tests suggested that the changes were not significantly different (p > 0.05) compared with the control cells (Table II).

To substantiate the down-regulation as quantitated by iTRAQ-coupled 2D LC-MS/MS, the level of two proteins (calmodulin and annexin A6) was examined by Western blot...
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(a)

Annexin A6 (66 KDa)
Calmodulin (17KDa)
β- Actin (42 KDa)

(b)

| Protein      | S:C p | R:C p | p(S:C) p(R:C) |
|--------------|-------|-------|---------------|
| Annexin A6   | 0.65±0.05 | 1.04±0.05 | 0.0000 | 0.0510 |
| Calmodulin   | 0.35±0.07 | 0.75±0.05 | 0.0067 | 0.0110 |
| β-Actin      | 1.02±0.06 | 0.97±0.04 | 0.05    | 0.05    |

Fig. 6. a, Western blot analysis of protein levels of calmodulin and annexin A6 in S-enantiomer- and R-enantiomer-incubated cells. Lane Control, cells in the absence of atenolol; Lane S, cells incubated with the S-enantiomer of atenolol; Lane R, cells incubated with the R-enantiomer of atenolol. b, quantification of the protein level based on Western blot analysis. The ratio between S-enantiomer-incubated cells and control cells (S:C) as well as between R-enantiomer-incubated cells and control cells (R:C) is shown. The p values indicated statistical significance of the observed differences with p < 0.05 considered as statistically significant and p > 0.05 considered as not statistically significant.

Analysis. Similarly to the iTRAQ analysis, results shown in Fig. 6 indicated that the level of the calmodulin and annexin A6 was lower in cells incubated with the S-enantiomer of atenolol compared with the level in the control cells. In addition, the level of annexin A6 showed no statistically significant changes in R-enantiomer of atenolol-incubated cells compared with control cells (p > 0.05), whereas the level of calmodulin in cells incubated with the R-enantiomer of atenolol was found to be significantly different compared with the control cells (p < 0.05). Quantification of the protein level based on Western blot analysis indicated that the reduction in these two proteins was more dramatic than that in iTRAQ analysis (Fig. 6b). One possible explanation for the observed discrepancy between iTRAQ and Western blot analyses could be that it is due to the normalization of total protein content leading to less dramatic values for lower abundance proteins in iTRAQ quantitation.

To confirm the expression of the genes encoding the four identified calcium-binding proteins, real time PCR was carried out for calmodulin, protein S100-A11, protein S100-A4, and annexin A6. Results shown in Fig. 7 indicated that the changes in gene expression for these four proteins in cells incubated with the S-enantiomer of atenolol were statistically significantly lower than those in control cells (p < 0.05). On the other hand, only the gene coding for calmodulin in cells incubated with the R-enantiomer of atenolol showed statistically significant changes in its expression (p < 0.05) compared with that in the control cells. The expression of the other three genes (coding for protein S100-A11, protein S100-A4, and annexin A6, respectively) in cells incubated with the R-enantiomer of atenolol was found to be not significantly different (p > 0.05) compared with the control cells. Our real time PCR results were consistent with the Western blot analysis at two levels. First, all calcium-binding proteins showed statistically significant down-regulation in cells incubated with the S-enantiomer of atenolol (at both gene and protein levels). Second, calmodulin was the only protein showing down-regulation in cells incubated with the R-enantiomer of atenolol (at both gene and protein levels).

To investigate the Ca^{2+} entry into the cytoplasm intracellular Ca^{2+} concentration was measured. The results in Table IV showed that intracellular Ca^{2+} concentration in A7r5 cells incubated with S-enantiomer of atenolol was about 8% lower than that in control cells and about 3% lower in the R-enantiomer of atenolol-incubated cells compared with the control cells. Statistical analysis based on three independent experiments indicated statistically significant changes (p < 0.05). Combining our results from real time PCR, Western blot, and the measurement of intracellular Ca^{2+} concentration, it is plausible that the reduction of intracellular Ca^{2+} concentration in cells incubated with the R-enantiomer of atenolol was due to the reduced level of calmodulin. The immunofluorescence images in Fig. 8 showed that the A7r5 cells incubated with the S-enantiomer of atenolol spread more than the control cells and those cells incubated with the R-enantiomer of atenolol.

DISCUSSION

Atenolol is a cardioselective β_{1}-receptor blocking agent without intrinsic sympathomimetic activity. It mainly causes its hypotensive effect by decreasing heart rate and cardiac contractility in both humans and experimental animals. However, because β_{1}-receptors appear to be present in vascular blood vessels it is possible that atenolol will reduce blood pressure by decreasing the peripheral vascular resistance like...
the nonselective blockers such as propranolol (32). In addition, advances in industrial chemical processes have made it economical for pharmaceutical manufacturers to take drugs that were originally marketed in racemic form and market the individual enantiomers, each of which may have unique properties. Investigation of the protein profiles in A7r5 cells incubated with individual enantiomers of atenolol and thus identification of signaling effectors downstream of the β-block-receptor will provide a better understanding of the β-block effect and potential adverse effects.

One up-regulated metabolic enzyme in Table II is glutathione S-transferase P. The rat glutathione S-transferase P has been found to be dramatically up-regulated in its expression in preneoplastic and neoplastic cells (33, 34) and is widely used as a specific marker in the basic analysis of chemical carcinogenesis (35). Another metabolic enzyme associated with lipid and drug metabolism is NADH-cytochrome b5 reductase. It has been well documented that the membrane-bound cytochrome b5 is located in the endoplasmic reticulum where it can accept an electron from NADH-cytochrome b5 reductase. Reduced cytochrome b5 then provides reducing equivalents for the biosynthesis of selected lipids and drugs (36). We performed the NAD+/NADH assay comparing the cellular anabolism in S-enantiomer-incubated cells with that in R-enantiomer-incubated cells and control cells. Interestingly the NAD+/NADH ratio in cells incubated with the S-enantiomer of atenolol was about 61% higher than that for control cells, and the ratio in R-enantiomer-treated cells was 12% higher than that for control cells; these results were in line with the protein level for NADH-cytochrome b5 reductase in individual type of cells (about 65 and 16% higher in S-enantiomer-treated cells and R-enantiomer-treated cells, respectively). The increase in the anabolic activity preceded by higher levels of metabolic enzymes such as NADH-cytochrome b5 reductase therefore provided molecular evidence on the metabolic effect associated with atenolol treatment.

The Ca2+ ion is a highly versatile intracellular signal regulating many different cellular functions, including fertilization, cell cycle, apoptosis, muscle contraction, vision, and memory. In eukaryotic cells, cytoplasmic Ca2+ entry and outflow are governed by two sources: intracellular stores such as the endoplasmic reticulum and extracellular Ca2+ that enters the cell through various transporters on the plasma membrane (37). The Ca2+ flux machinery, consisting of ion channels, pumps, and exchangers, gives rise to highly localized and transient Ca2+ signals that are, in turn, transduced by calcium-binding proteins acting on various enzymes and downstream effector proteins. The ubiquitous calcium-binding protein calmodulin plays a crucial role in various cellular signaling cascades through regulation of numerous target proteins in a Ca2+-dependent manner. It can activate the Ca2+-pump of plasma membranes by interacting with a domain next to its carboxyl terminus (38) and can interact with GRK5, a member of the GRK family that is associated with homologous desensitization of G protein-coupled receptor, to reduce GRK5 binding to the membrane (39). Annexins are a family of proteins that bind in a calcium-dependent manner to phospholipid membranes. It was reported that annexin A6 increased the activity of the sarcoplasmic reticulum Ca2+-ATPase (40). S100-A11 is a member of the S100 family of calcium-binding proteins that is expressed in smooth muscle and other tissues. Ca2+ binding to S100-A11 induces a conformational change that exposes a hydrophobic surface for interaction with target proteins such as annexin A6. It was hypothesized that an increase in cytosolic free Ca2+ leads to formation of a complex of S100-A11 and annexin A6, which forms a physical connection between the plasma membrane and the cytoskeleton, or plays a role in the formation of signaling complexes at the level of the sarcolemma (41). S100-A4 is another member of the S100 family, which is associated with active stress fibers (probably regulating contraction) and the sarcoplasmic reticulum (regulating Ca2+ homeostasis) (42). The down-regulation of the calcium-binding proteins was therefore closely correlated with the decrease in the intracellular Ca2+ concen-

**Table IV**

| Ratio of intracellular Ca2+ concentration | S:C (±S.D.) | R:C (±S.D.) | p | S:C | R:C |
|-----------------------------------------|-------------|-------------|---|-----|-----|
| Ratio between S-enantiomer-incubated cells and control cells (S:C) as well as between R-enantiomer-incubated cells and control cells (R:C) is shown. | 0.919 ± 0.016 | 0.968 ± 0.018 | 0.0064 | 0.0456 |

Fig. 8. The immunofluorescence images of control A7r5 cell (A), S-enantiomer of atenolol incubated A7r5 cells (B), and R-enantiomer of atenolol incubated A7r5 cells (C). The scale bar represents 20 μm.
The lower intracellular Ca\(^{2+}\) concentration in A7r5 cells incubated with S-enantiomer of atenolol would induce less control of actin-myosin-based contraction in A7r5 cells that was mediated by the myosin light chain kinase-calsodulin complex (43). The cells incubated with S-enantiomer of atenolol spread much more (Fig. 8) compared with the other two types of cells, indicative of less contraction and more relaxation.

Nestin is an intermediate filament protein expressed in dividing cells during the early stages of development in the central nervous system and myogenic and other tissues and can play a complex role in regulation of the assembly and disassembly of intermediate filaments together with vimentin or \(\alpha\)-internexin and in remodeling of the cell (44). Tropomyosin in muscle and non-muscle occurs in tight association with actin filaments, and in skeletal and cardiac muscle tropomyosin plays a central role in regulation of contraction through mediation of the calcium response of the troponin complex to actin filaments (45). However, the physiological function of tropomyosin in smooth muscle cells and non-muscle cells is not fully understood due in part to the absence of troponin. It has been suggested that tropomyosin is involved in stabilization of actin filaments, cytoskeletal modeling, and cell motility (46), and \(\beta\)-tropomyosin plays a significant role in the process of phenotypic modulation of smooth muscle cells (47). Unlike nestin and \(\beta\)-tropomyosin, which were down-regulated in A7r5 cells incubated with atenolol, tubulin \(\alpha-2\) chain was up-regulated. Cooper and co-workers (48–50) reported that the isolated myocytes showing contractile dysfunction were accompanied by increased cytoskeletal stiffness characterized by an augmentation of the amount of total tubulin and an elevated degree of polymerization. However, the role of tubulin in the development of hypertrophy and heart failure has been questioned by Collins et al. (51) and Bailey et al. (52).

Transforming protein RhoA is a small GTPase protein that directly stimulates actin polymerization through activation of diaphanous-related formins (DRF proteins). DRF proteins stimulate addition of actin monomers to the fast growing end of actin filaments. DRFs act together with Rho kinases (ROCKs) to mediate Rho-induced stress fiber formation (53). In addition, ROCKs induce actomyosin-based contractility and phosphorylate several proteins involved in regulating myosin and other actin-binding proteins (54). It was reported that inhibition of the RhoA/Rho kinase pathway is effective in reducing pulmonary hypertension (55). Bi et al. (56) reported that the expression level of RhoA plays a crucial role in regulating the contractility of cultured vascular smooth muscle cells. 14-3-3 protein \(\eta/\delta\) was up-regulated in S-enantiomer-incubated cells and was found to be significantly up-regulated in breast tumor cells (57).

Collectively, calmodulin plays a crucial role in activating the Ca\(^{2+}\) pump of plasma membranes, and the lower calmodulin expression level in A7r5 cells incubated with S-enantiomer of atenolol possibly led to the lower intracellular calcium concentration in those cells compared with the other two types of cells. It can be inferred from the above discussion that the lower calmodulin expression level can increase GRK5 binding to the membrane, resulting in overdesensitization of G protein-coupled receptor. In turn, a relative lower intracellular Ca\(^{2+}\) concentration in S-enantiomer-incubated cells would induce less interaction of S100-A11 with annexin A6, resulting in less physical connection between the plasma membrane and the cytoskeleton. The Ca\(^{2+}\) signals were transduced by calcium-binding proteins acting on cytoskeletal proteins such as nestin and \(\beta\)-tropomyosin, which can play a complex role in regulation of the cytoskeletal modeling and cell contraction in conjunction with S100-A4. In addition a lower RhoA expression level in S-enantiomer-incubated cells might result in less actin polymerization and thus induce less contractility with DRF proteins and ROCKs compared with control cells and \(R\)-enantiomer-incubated cells.

In summary, our results demonstrate that the application of quantitative proteomics based on iTRAQ is an effective approach to evaluate cellular changes with atenolol. We were able to identify and quantify some proteins with differential protein expression levels from A7r5 cells incubated with individual enantiomers of atenolol. Our preliminary results indicate significant alterations of proteins that were involved in the intracellular anabolism, Ca\(^{2+}\) signal transduction pathway. This provides molecular evidence on the metabolic effect and possible link of calcium-binding proteins with treatment of hypertension associated with atenolol treatment.

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