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RESEARCH ARTICLE | *G Protein-Coupled Receptor Signaling in Metabolic Disease*

Altered expression of hepatic β-adrenergic receptors in aging rats: implications for age-related metabolic dysfunction in liver

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[1]*Geriatric Research, Education and Clinical Center, Audie L. Murphy Division, South Texas Veterans Health Care System, San Antonio, Texas;* [2]*Department of Medicine, University of Texas Health Science Center at San Antonio, San Antonio, Texas;* [3]*Department of Comprehensive Dentistry, University of Texas Health Science Center at San Antonio, San Antonio, Texas;* [4]*Barshop Institute for Longevity and Aging Studies, University of Texas Health Science Center at San Antonio, San Antonio, Texas;* [5]*Departments of Urology and Biochemistry, University of California Davis, Sacramento, California; and* [6]*Research Service, Veterans Affairs Northern California Health Care System, Mather, California*

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Shi Y, Shu Z-J, Wang H, Barnes JL, Yeh C-K, Ghosh PM, Katz MS, Kamat A. Altered expression of hepatic β-adrenergic receptors in aging rats: implications for age-related metabolic dysfunction in liver. *Am J Physiol Regul Integr Comp Physiol* 314: R574–R583, 2018. First published December 6, 2017; doi:10.1152/ajpregu.00372.2017.—Increased β-adrenergic receptor (β-AR)-mediated activation of adenylyl cyclase (AC) in rat liver during aging has been linked to age-related increases in hepatic glucose output and hepatosteatosis. In this study, we investigated the expression of β-ARs, individual receptor subtypes, and G protein-coupled receptor (GPCR) regulatory proteins in livers from aging rats. Radioligand-binding studies demonstrated that β-AR density increased by greater than threefold in hepatocyte membranes from senescent (24-mo-old) compared with young adult (7-mo-old) rats and that this phenomenon was blocked by food restriction, which is known to retard aging processes in rodents. Competition-binding studies revealed a mixed population of β_1- and β_2-AR subtypes in liver membranes over the adult life span, with a trend for greater β_2-AR density with age. Expression of both β-AR subtype mRNAs in rat liver increased with age, whereas β_2- but not β_1-AR protein levels declined in livers of old animals. Immunoreactive β_2- but not β_1-ARs were preferentially distributed in pericentral hepatic regions. Levels of GRK2/3 and β-arrestin 2 proteins, which are involved in downregulation of agonist-activated GPCRs, including β-ARs, increased during aging. Insofar as sympathetic tone increases with age, our findings suggest that, despite enhanced agonist-mediated downregulation of hepatic β-ARs preferentially affecting the β_2-AR subtype, increased generation of both receptor subtypes during aging augments the pool of plasma membrane-bound β-ARs coupled to AC in hepatocytes. This study thus identifies one or both β-AR subtypes as possible therapeutic targets involved in aberrant hepatic processes of glucose and lipid metabolism during aging.

β-arrestin; food restriction; G protein-coupled receptor; G protein-coupled receptor serine/threonine kinase; hepatocytes

INTRODUCTION

Elevations of hepatic glucose output and lipid accumulation are hallmarks of type 2 diabetes mellitus and nonalcoholic fatty liver disease, two widely occurring metabolic diseases with increasing prevalence during aging (7, 18). Although disordered hepatic glucose and lipid metabolism in these age-related diseases is strongly associated with reduced sensitivity of liver to the actions of insulin (39), altered hepatic responsiveness to counterregulatory factors such as catecholamines might also play a role in the development of liver dysfunction during aging. Sympathetic nervous system activity, as reflected by basal plasma levels of catecholamines noradrenaline (norepinephrine) and adrenaline (epinephrine), increases during aging (9, 17). The responses of many target tissues to adrenergic stimuli are mediated by the classical β-adrenergic receptor (β-AR) coupled adenylyl cyclase (AC)/cAMP cascade (26, 33, 42). Likely in adaptation to increased sympathetic tone with age, β-adrenergic responsiveness of a number of target organs declines during aging, together with decreases in β-AR numbers and/or receptor-linked signaling function(s) (43, 44, 51, 55). We and others, however, established some years ago that membrane content of β-ARs in liver increases during postmaturational or senescent aging in Fischer 344 male rats in association with progressive increases in β-adrenergic sensitive AC stimulation and hepatic glucose output (11, 14, 24, 26, 27). More recent work in our laboratory further suggested that increased hepatic β-AR signaling may also contribute to lipid accumulation in liver during aging (13, 49). Age-related increases in membrane content of β-ARs coupled to AC-mediated functions, with potentially deleterious metabolic consequences, appear to be unique to liver and may reflect a tissue-specific defect in adaptive mechanisms modulating adrenergic responses to increased circulating levels of catecholamines at advanced age. Although ample evidence points to increased β-AR density in whole liver of aging rats, it has not been clarified whether this change with age reflects an increase in receptor content in hepatocytes that may be linked to cellular dysfunction during aging.

β-Adrenergic receptors, which are prototypical members of the guanine nucleotide-binding G protein-coupled receptor

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(GPCR) superfamily, include β_1-, β_2-, and β_3-AR subtypes (33). β-ARs detected in rat liver by radioligand binding were initially reported to be predominantly, or even exclusively, of the β_2-subtype (11, 36). However, subsequent work also documented the presence of β_1-ARs in rat liver by competition-binding assays using β-AR subtype selective antagonists and by immunohistochemistry (6, 35, 54). β_3-ARs, which play an important role in adipose tissue lipolysis, are undetected or expressed at very low levels in rodent liver (20, 40). β_1- and β_2- but not β_3-AR expression has also been demonstrated in human liver samples by receptor binding, immunoblot, PCR, and/or Southern hybridization experiments (1, 19, 30, 41). Little is known about changes in density or expression of β_1- and β_2-AR subtypes with age in liver; of the few relevant data published in this area, earlier studies utilizing competition binding described a mixed population of β_1/β_2-ARs in rat liver over the lifespan (35, 54), whereas preliminary semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) performed in our laboratory suggested an increase in hepatic β_1-AR expression with senescent aging in rats (20).

β-Adrenergic receptors, like most GPCRs, undergo a complex process of desensitization and internalization in response to agonist stimulation. Agonist-bound receptor assumes a conformation allowing phosphorylation by one or more GPCR serine/threonine kinases (GRKs) (50). GRK-induced phosphorylation of the receptor promotes binding of the arrestin family of proteins to the receptor, which in turn interrupts receptor coupling to G proteins (hence, "desensitizing" classical GPCR signaling) and targets the receptor for sequestration/internalization via clathrin-coated pits. Of the seven members of the GRK family, the GRK2 subfamily members (GRK2 and GRK3, also known as β-ARK1 and β-ARK2) are the most widely expressed and phosphorylate a range of GPCRs. Among the four family members of arrestin molecules, two (visual arrestin and cone arrestin) are expressed exclusively in the retina and two [β-arrestin-1 (βarr1) and β-arrestin-2 (βarr2)] are expressed ubiquitously in other tissues (50). The two β-arrestins do not appear to be functionally redundant, since βarr2 has been shown to be much more effective than βarr1 in supporting internalization of the β_2-AR (29). Internalized GPCRs have long been known to undergo degradation or recycling to the plasma membrane, albeit by sorting mechanisms that remain obscure. Altered expression of GRKs and/or β-arrestins in livers of old rats could play a modulatory role in age-related changes of plasma membrane content of β_1- and/or β_2-AR subtypes and their internalization, but to our knowledge this has not been evaluated.

In the current study, we have extended previous observations of β-ARs in liver during aging by *1*) investigating β-AR density, assessed by radioligand binding, in hepatocytes isolated from aging rats; *2*) identifying by several methods the β-AR subtype(s) that may be involved in augmenting β-AR-mediated AC activity with age in rat liver; and *3*) determining whether hepatic expression levels of GRK2/3 and βarr2 are age dependent. The intent of this study is to begin to identify specific therapeutic targets involved in aberrant β-AR-mediated processes of glucose and lipid metabolism occurring in liver during aging.

MATERIALS AND METHODS

Materials. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. TRI reagent was obtained from Molecular Research Center (Cincinnati, OH). Complete Mini tablets and DNAse I were obtained from Roche Diagnostics (Indianapolis, IN), whereas DTT was purchased from Invitrogen (Carlsbad, CA). TaqMan Gene Expression Assays [β_2-AR (Rn00560650), β_1-AR (Rn00824536), and 18S (Hs99999901)], TaqMan universal master mix, high-capacity cDNA reverse transcription kit, magnesium chloride, and plasticware required for performing quantitative real-time PCR were obtained from Applied Biosystems (ABI; Foster City, CA). Bicinchoninic acid (BCA) assay and enhanced chemiluminescence (ECL) kit were from Pierce (Rockford, IL). Polyvinylidene fluoride (PVDF) membranes were obtained from GE Osmonics (Minnetonka, MN), whereas collagenase (type II) was purchased from Worthington Biochemical (Lakewood, NJ). Bradford protein assay reagents were purchased from Bio-Rad Laboratories (Hercules, CA). (−)-[^{125}I]iodopindolol (2,200 Ci/mmol) was obtained from Perkin-Elmer (Waltham, MA), whereas Whatman glass fiber filters (GF/C) were purchased from Brandel (Biomedical Research and Development Laboratories, Gaithersburg, MD). In Western blot analyses, antibodies specific for β_2-AR (1:500 dilution, no. ab36956) and β_1-AR (1:1,000 dilution, no. ab3442) were ordered from Abcam (Cambridge, MA); β-actin (1:5,000 dilution, no. sc-47778), GAPDH (1:5,000 dilution, no. sc-47724), GRK2/3 (1:250 dilution, no. sc-8329), and β-arrestin2 (1:250 dilution, no. sc-13140) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody used in immunohistochemical analysis of β_2-AR (1:100 dilution, no. ab36956) was also from Abcam, whereas the β_1-AR (1:20 dilution, no. sc-568) antibody was from Santa Cruz Biotechnology. Antibodies utilized in this study were previously validated according to manufacturers' data sheets and product citations therein [Abcam (www.abcam.com) and Santa Cruz Biotechnology (www.scbt.com)]. Appropriate peroxidase-labeled secondary antibodies and ImmPRESS polymer detection kit were purchased from Vector Laboratories (Burlingame, CA), whereas ICI-118,551 and ICI-89,406 were obtained from Tocris (Bristol, UK). Williams' E Medium was obtained from GIBCO-BRL (Gaithersburg, MD). Krebs-Henseleit Buffer was from Sigma.

Animals. For saturation-binding experiments assessing total β-AR content in hepatocyte membranes (Fig. 1), specific pathogen-free (SPF) Fischer 344 male rats were obtained as weanlings from Charles River Laboratories (Wilmington, MA) and were maintained singly in a SPF barrier facility on standard 12-h light-dark cycles at the University of Texas Health Science Center at San Antonio (UTHSCSA). Rats were fed ad libitum a diet of previously specified composition until 6 wk of age and then continued on the same diet ad libitum or were restricted to 60% of the mean ad libitum intake (i.e., food restricted, FR) until the animals were used at 7, 14, 20, or 24 mo of age (24, 27, 59). For all other experiments young adult (3–6 mo old) and senescent (22–24 mo old) SPF Fischer 344 male rats were obtained from the National Institute on Aging, National Institutes of Health (Bethesda, MD), and housed on standard 12-h light-dark cycles for ≥1 wk in a SPF barrier facility within the Veterinary Medical Unit, Audie L. Murphy Division (ALMD)-South Texas Veterans Health Care System, before use; during the equilibration period, animals were fed ad libitum a diet approximating that used in the experiments of Fig. 1 (59). All animals were treated in accordance with the guidelines approved by the joint Institutional Animal Care and Use Committee at the UTHSCSA/ALMD.

Preparation of liver samples. Rats were euthanized by exsanguination after anesthesia, as previously described (23). Livers were rapidly removed, cut into pieces, and quick-frozen in liquid nitrogen for storage at −80°C until further use. For competition-binding studies (Fig. 2 and Table 1), liver pieces were homogenized, followed by filtration and centrifugation of the homogenate to obtain the

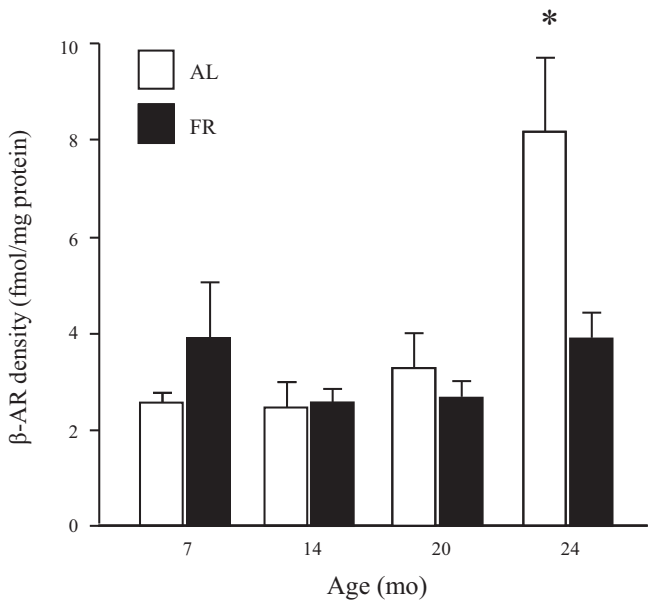

Fig. 1. Density of β-adrenergic receptors (β-ARs) in hepatocyte membranes from aging rats: modulation by food restriction. Hepatocytes were isolated from 7-, 14-, 20-, and 24-mo-old Fischer 344 male rats either fed ad libitum (AL) or food restricted (FR) to 60% of the mean ad libitum intake. β-AR density (B_{max}) in particulate membrane preparations from freshly isolated hepatocytes was determined by Scatchard analysis of saturation-binding experiments using the β-AR antagonist (−)-[^{125}I]iodopindolol as the radioligand. Values represent means ± SE from 5 to 11 rats in each age/diet group. *P = 0.026 vs. B_{max} in 20-mo-old AL-fed rats. β-AR-binding affinity [dissociation constant (K_d)] varied from 50 to 80 pM, with no statistically significant differences among groups.

4,500-g particulate fraction (25). Particulates were stored at −80°C before use in the binding assay. Unless otherwise specified, protein concentration of liver samples and also hepatocyte membranes (see below) was determined by the method of Bradford.

Isolation of hepatocytes and preparation of hepatocyte membranes. Hepatocytes were isolated as previously described (27). Briefly, rats were anesthetized using pentobarbital sodium (65 mg ip injection/kg body wt), and livers were perfused in situ with 0.03% collagenase (type II)-containing Krebs-Henseleit bicarbonate buffer (pH 7.4). For preparation of hepatocyte membranes, filtered and washed hepatocytes were resuspended to a density of 10^7 cells/ml in ice-cold Krebs-Henseleit buffer and subjected to sequential freeze-thawing and homogenization with a Polytron homogenizer (two 10-s bursts at *setting 6*). Cell lysates were resuspended (1.8 ml:10 ml) in tissue buffer [0.154 M NaCl and 20 mM HEPES (pH 7.4) with 1 mM $MgCl_2$] and centrifuged at 27,000 *g* for 15 min at 4°C. Membrane pellets were resuspended in 1.125 ml of tissue buffer, pooled, and homogenized on ice in a 7-ml Dounce homogenizer with 10 strokes using a loose pestle; pellet fractions were then placed in 1-ml aliquots in cryotubes, snap-frozen in liquid nitrogen, and stored at −80° until use in the binding assay.

Receptor-binding assay. Receptor binding in membrane preparations from freshly isolated hepatocytes or liver homogenates was measured by an equilibrium-binding assay using (−)-[^{125}I]iodopindolol, as described previously (25, 57, 60). About 100 μg of membrane protein was incubated with (−)-[^{125}I]iodopindolol in 125–250 μl of reaction buffer [12.5 mM HEPES (pH 7.5), 115 mM NaCl, and 0.66 mM L-ascorbic acid] for 30 min at 30°C. Reactions were then terminated by adding 4 ml of wash buffer [10 mM Tris (pH 7.5) and 154 mM NaCl] at room temperature, and membrane-bound radioligand was collected on Whatman glass fiber filters (GF/C) with a Brandel Cell Harvester (Gaithersburg, MD). Nonspecific binding of ^{125}I-labeled receptor ligand was defined as the amount of radioligand bound in the presence of an excess (10^{-4} M) of the unlabeled ligand. Saturation-binding curves were constructed by measuring specific binding of the ^{125}I-labeled receptor ligand at eight different concentrations in the range of 0.01–5 nM. Competition-binding studies were performed by measuring binding of (−)-[^{125}I]iodopindolol (at a concentration approximating the dissociation constant, K_d) in the presence of increasing concentrations of nonlabeled β_2-AR antagonist ICI-118,551 or β_1-AR antagonist ICI-89,406 in the range of 10^{-11} to 10^{-4} M.

Quantitative real-time PCR. Total RNA was isolated from frozen liver pieces using TRI Reagent according to the manufacturer's instructions. The RNA samples were treated with DNAse I (RNAse-free) and then reverse transcribed. The complementary DNA (cDNA) synthesis was carried out in a thermocycler at 25°C/10 min, 42°C/50 min, 72°C/10 min, and 4°C. Real-time RT-PCR assay was then performed by the $\Delta\Delta C_T$ method with TaqMan Gene Expression Assays for rat β_1-AR and β_2-AR using an ABI 7900 Sequence Detection System. In each experiment, mRNA levels were normalized to 18S RNA, which did not vary with animal age (data not shown).

Western blotting. Frozen liver pieces were homogenized in lysis buffer [50 mM NaCl, 1% NP-40, and 50 mM Tris·HCl (pH 7.4)] containing protease and phosphatase inhibitors. Homogenates were rocked at 4°C for 60 min, followed by centrifugation at 10,000 *g* for 15 min at 4°C. The supernatant proteins were estimated by BCA assay. Protein samples (40–70 μg) were added to 10 μl of 4× sample buffer [150 mM Tris·HCl (pH 8.8), 1% SDS, and 40% glycerol] and β-mercaptoethanol (355 mM) and then diluted with lysis buffer to a

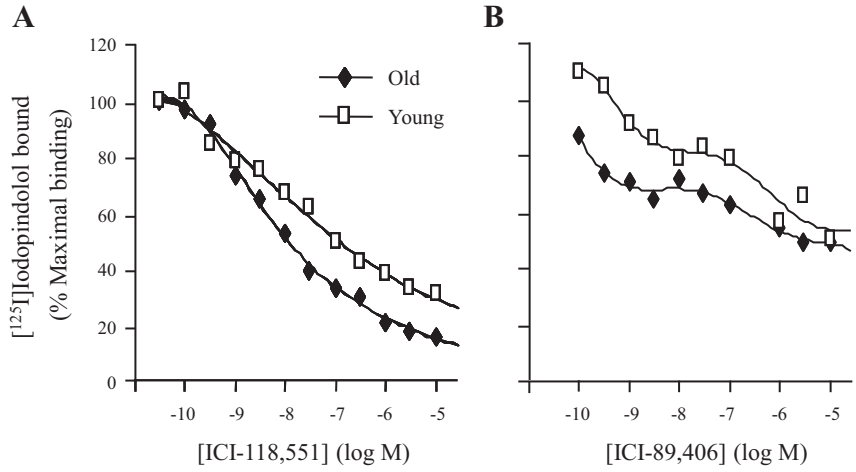

Fig. 2. Competition for (−)-[^{125}I]iodopindolol-binding sites in rat liver by ICI-118,551 and ICI-89,406: effects of age. Binding of (−)-[^{125}I]iodopindolol to liver particulates from individual young (6 mo old) vs. old (24 mo old) rats was assayed in the presence of increasing concentrations of unlabeled ICI-118,551 (*A*) or ICI-89,406 (*B*), as described previously (57). Results from single representative animals are shown; curve-fitting analysis of competition data from 4 young (3–6 mo old) and 5 old (24 mo old) rats is presented in Table 1.

Table 1. *Effects of age on high- and low-affinity binding sites for ICI-118,551 and ICI-89,406 in rat liver particulates*

	Ligand Competing for (−)-[^{125}I]Iodopindolol Binding					
	ICI-118,551			ICI-89,406		
	Young	Old	*P* value	Young	Old	*P* value
R_H, fmol/mg	2.34 ± 0.82	7.17 ± 2.85	0.165	2.30 ± 0.30	3.24 ± 1.12	0.450
R_L, fmol/mg	1.90 ± 0.33	2.83 ± 0.83	0.346	2.48 ± 0.83	3.73 ± 1.11	0.399
$\%R_H$	51.7 ± 5.8	68.1 ± 2.9	0.064	53.8 ± 11.5	44.3 ± 3.0	0.478
$\%R_L$	48.3 ± 5.8	31.9 ± 2.9	0.064	46.2 ± 11.5	55.7 ± 3.0	0.478
K_H, nM	2.13 ± 1.31	9.05 ± 5.80	0.309	0.50 ± 0.09	0.86 ± 0.49	0.528
K_L, μM	1.14 ± 0.76	12.97 ± 9.13	0.266	7.55 ± 6.19	3.72 ± 0.46	0.581

Values are means ± SE from 4 young and 5 old rats. R_H and R_L, nos. of sites binding ICI-118,551 or ICI-89,406 with high and low affinity; K_H and K_L, dissociation constants of high- and low-affinity binding sites. Competition binding curves using liver particulate preparations from young (3–6 mo old) and old (24 mo old) rats were constructed as described in Fig. 2, and the data were analyzed with a weighted, nonlinear, least-squares curve-fitting program (38). ICI-118,551 and ICI-89,406 competition curves were best fit by a 2-site model describing high- and low-affinity binding sites.

total volume of 40 μl. Samples were size-fractionated on SDS-PAGE gels and electroblotted onto 0.2-μm PVDF membranes. Membranes were immunoblotted overnight at 4°C with a primary antibody, followed by a secondary horseradish peroxidase-conjugated antibody for 1 h at room temperature. Specific proteins were visualized using an enhanced chemiluminescence kit, and immunoblots were quantified with ImageJ software (45) and normalized against β-actin or GAPDH. Molecular weights of target proteins were determined using standard molecular weight markers (Bio-Rad Precision Plus Dual Marker, cat. no. 161-0374).

Immunohistochemistry. Six-micrometer frozen sections cut from liver pieces were blocked with 0.1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) and then stained with rabbit antibody directed against β_1-AR (1:20 dilution, no. sc-568) or β_2-AR (1:200 dilution, no. ab36956), followed by incubation with an ImmPRESS polymer detection kit according to the manufacturer's instructions. The stained tissue was mounted on coverslips, viewed, and photographed using an Olympus AX70 research microscope equipped with a DP-70 digital camera (Olympus America, Melville, NY). For image analysis, photographic images were taken of three to five random fields using a ×4 objective magnification for a total of ≥10.8 sq. mm of each tissue. The area of diaminobenzidine (DAB) reaction product in each image was measured using the segmentation tool of Image-Pro Plus 4.5 imaging software (Media Cybernetics, Silver Spring, MD) calibrated to a stage micrometer. DAB staining in the image was extracted by selecting a lower and upper range of grayscale within the limits of background and the highest intensity of staining (12). The image data were then masked and pseudo-colored for measurement of staining area relative to the total field.

Data analysis. Data from multiple experiments are expressed as means ± SE. Statistical significance of single comparisons was determined by Student's *t*-test. Scatchard analysis of (−)-[^{125}I]iodopindolol saturation-binding data was used to determine β-AR receptor density (B_{max}) and K_d. Competition-binding curves describing (−)-[^{125}I]iodopindolol binding in the presence of increasing concentrations of ICI-118,551 or ICI-89,406 were analyzed with a weighted, least-squares curve-fitting program (38).

RESULTS

β-Adrenergic receptor density in rat hepatocyte membranes increases with senescent aging: effect of food restriction. Earlier radioligand-binding studies in our laboratory and others demonstrated age-related increases in numbers of β-AR-binding sites measured in membrane preparations from whole liver (11, 14, 20, 24, 54). In the present study, we have extended these previous findings in experiments measuring β-AR binding in hepatocytes isolated from rats of increasing age. As shown in Fig. 1, β-AR density in hepatocyte membranes increases more than threefold between 7 and 24 mo of age; interestingly, the increase in receptor number in old rats occurs almost entirely during senescent aging, i.e., after 20 mo of age. In the same experiments, we demonstrated that food restriction, which has long been known to retard aging processes in rodents and other species, blocks the senescent increase of β-AR density in rat hepatocyte membranes (Fig. 1).

β-Adrenergic receptor-binding sites in liver comprise a mixed population of β_1- and β_2-AR subtypes over the adult lifespan of the rat. To evaluate whether increased density of β-AR-binding sites in livers of old rats represents changes in the levels of β_1- and/or β_2-AR subpopulations, the relative proportions of the two receptor subtypes in whole liver particulates from young (3–6 mo old) and old (24 mo old) rats were analyzed in competition-binding experiments using antagonists selective for β_2- and β_1-ARs (ICI-118,551 and ICI-89,406, respectively). Our results (Fig. 2 and Table 1) reveal high- and low-affinity binding sites for the two antagonists in liver preparations from both young and old animals. The high-affinity binding sites for ICI-118,551 and ICI-89,406 presumably represent authentic β_2- and β_1-ARs, respectively; in contrast, low-affinity binding by ICI-118,551 and ICI-89,406 is generally believed to occur at β_1- and β_2-ARs, respectively (41, 54, 57). Inspection of representative binding curves suggests that liver preparations from 24-mo-old rats exhibit a greater degree of binding by both antagonists than do preparations from 6-mo-old animals (Fig. 2). Curve-fitting analysis of competition-binding data may implicate an age-related increase in high-affinity binding for ICI-118,551 but not ICI-89,406, although no apparent age difference reaches the level of statistical significance (Table 1). Overall, these results are consistent with the expression of both β_1- and β_2-ARs in rat liver over the adult lifespan, with a trend for an age-related increase in the density of β_2-ARs.

β_1- and β_2-AR subtypes in rat liver demonstrate differential changes in gene and/or protein expression levels during aging. To begin an investigation into the processes by which β-AR expression is regulated in livers of aging rats, we measured the levels of β_1- and β_2-AR mRNAs and also receptor subtype protein levels in whole liver from young and old rats. Figure 3 shows that hepatic expression of both β-AR subtype mRNAs, determined by quantitative real-time PCR (qRT-PCR), increased ~2.5- to threefold between 6 and 24 mo of age. In contrast to these results, immunoblot analysis revealed no change in hepatic β_1-AR levels with age and decreased β_2-AR

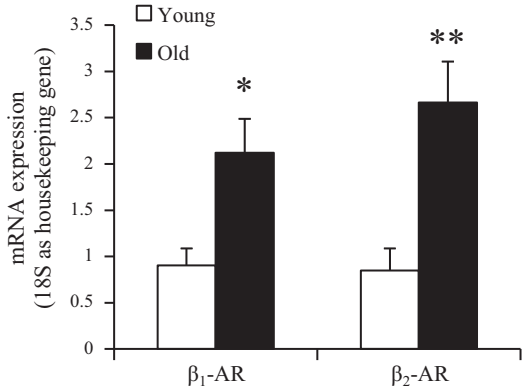

Fig. 3. Effects of age on β_1- and β_2-AR mRNA levels in rat liver. β_1- and β_2-AR mRNA levels in liver specimens from young (6 mo old) and old (24 mo old) rats were determined by quantitative RT-PCR, as described in materials and methods. mRNA levels were normalized to 18S RNA and then expressed as fold change relative to the value from a single animal (calibrator). Values shown represent means ± SE from 7 young and 7 old rats. *$P = 0.026$ vs. β_1-AR mRNA level at 6 mo; **$P = 0.018$ vs. β_2-AR mRNA level at 6 mo.

protein expression in livers of senescent rats compared with young animals (Fig. 4). Levels of β-AR subtypes and their distribution within hepatic parenchyma during aging were further evaluated by immunohistochemical staining. Commensurate with the results from immunoblot analysis, β_1-AR staining was equivalent in livers of young and old rats, whereas β_2-AR staining declined significantly with age (Fig. 5, *A* and *B*). Notably, the distribution of staining at both ages appeared to differ between the two isoforms; whereas β_1-AR stained diffusely, β_2-AR staining tended to concentrate in hepatocytes surrounding the central vein (pericentral region, or zone 3) (Fig. 5*C*).

Immunoreactive levels of GPCR kinase 2/3 and β-arrestin-2 increase with age in rat liver. In response to agonist stimulation, β-ARs and other GPCRs undergo rapid uncoupling from plasma membrane effectors and internalization mediated by the sequential actions of GPCR kinase (GRK) and arrestin proteins (29, 50). We performed experiments comparing immunoreactive GRK2/3 and βarr2 protein levels in livers of 6- and 24-mo-old rats. In these experiments, livers from old rats exhibited an approximately twofold increase in GRK2/3 protein levels relative to levels in livers from young adult animals (Fig. 6). A significant increase in βarr2 protein levels was also observed with age in rat liver (Fig. 7). These data suggest that increased expression of GRK2/3 and βarr2 may play a role in modifying β-AR internalization during aging.

DISCUSSION

The results of this study implicate age-related changes in expression of hepatic β-ARs, GRK2/3, and β-arrestin-2 likely underlying previously reported increases in β-AR-responsive signaling, glucose output, and lipid accumulation in livers of aging rats (11, 13, 14, 24, 26, 27, 49). We have demonstrated that β-AR density in membrane preparations from isolated rat hepatocytes increases with senescent aging and that the increase in receptor content in cells from old animals is attenuated by food restriction (Fig. 1). We have further shown by competition-binding studies a mixed population of β_1- and β_2-AR subtypes in rat livers over the adult lifespan, with a trend for an age-related increase in the density of β_2-ARs (Fig. 2 and Table 1). Expression of both β-AR subtype mRNAs in rat liver increases with age (Fig. 3); in apparent contrast to these results, comparison of β-AR subtype protein levels in livers from young and old rats reveals no change with age in

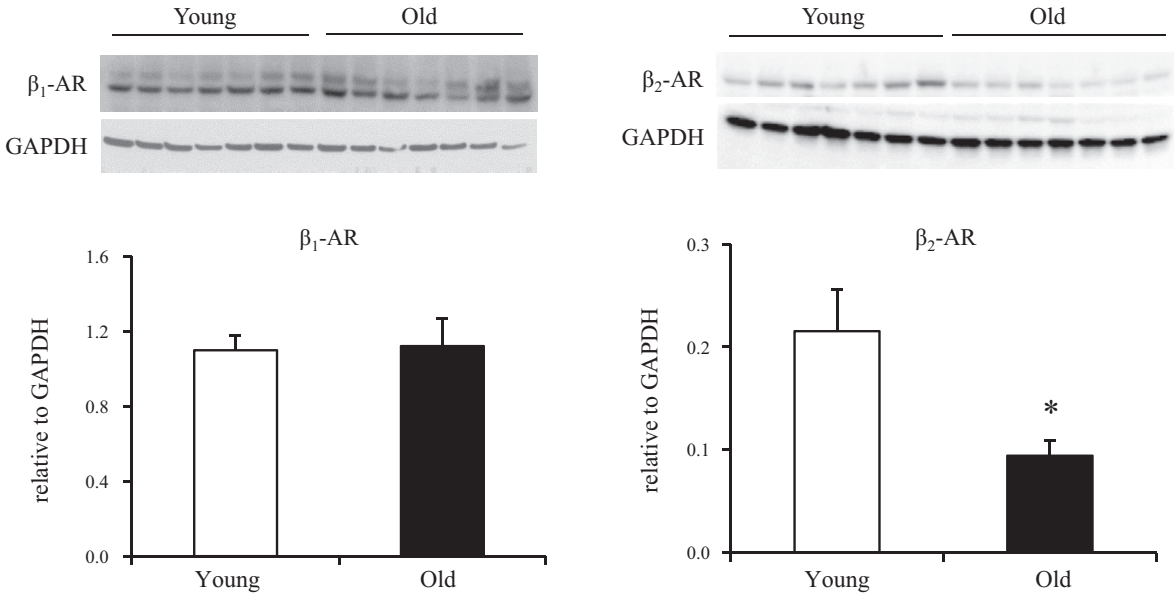

Fig. 4. β_1- and β_2-AR protein levels in rat liver with age. Protein levels of the two β-AR subtypes were measured by Western blotting of lysates prepared from the same liver specimens used for mRNA determinations. All lysates were prepared at the same time and processed in parallel. *Top*: representative immunoblots depicting β_1- and β_2-AR protein levels in livers from young (6 mo old) and old (24 mo old) animals. GAPDH was used as loading control. Protein levels were quantified as integrated intensity and then normalized to the loading control. *Bottom*: bar values represent means ± SE from 7 young and 7 old rats. *$P = 0.03$ vs. β_2-AR protein level at 6 mo. An age-related decline in β_2-AR levels was confirmed in additional experiments (not shown) utilizing a different antibody to β_2-AR (no. 182136; Abcam) from that employed in the immunoblot shown (no. ab36956; Abcam).

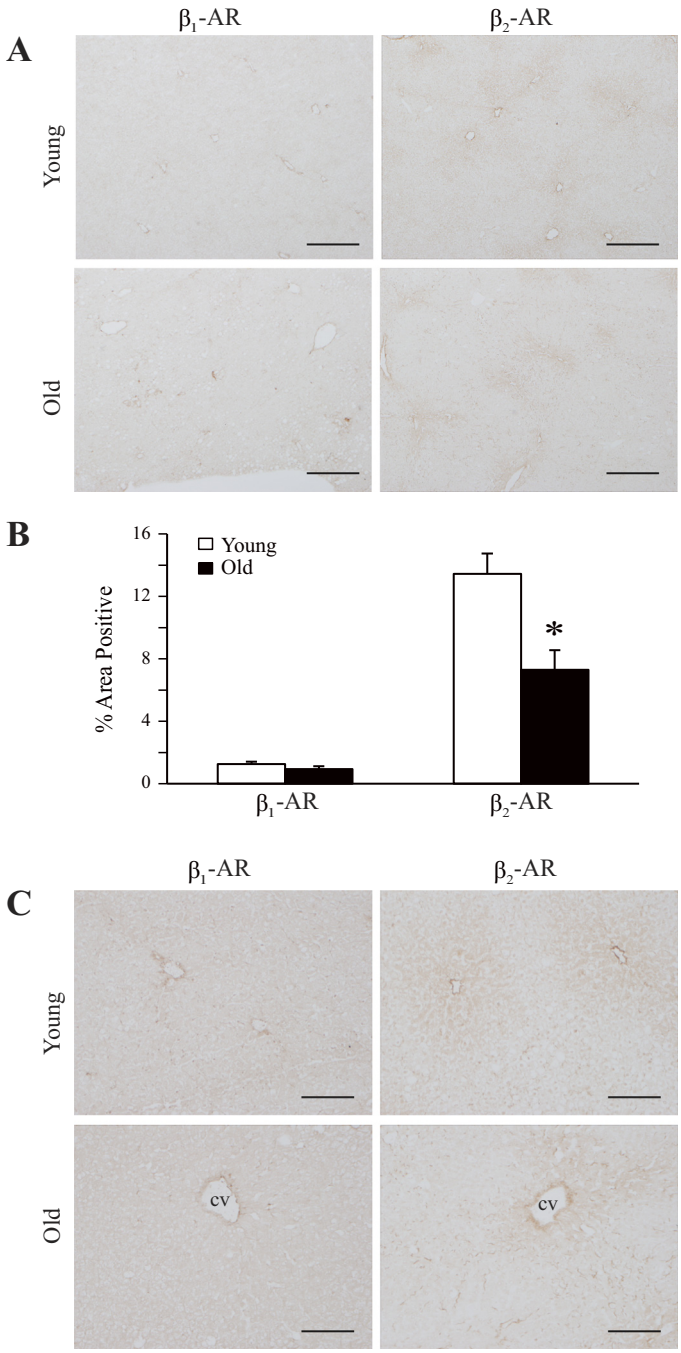

Fig. 5. β_1- and β_2-AR immunoreactivity in liver sections from young and old rats. Frozen liver sections were immunostained for β_1-AR and β_2-AR. *A*: representative diaminobenzidine (DAB)-stained liver sections from individual young (6 mo old) and old (24 mo old) rats are shown (×4 objective magnification). Scale bar, 300 μm. *B*: areas of staining for β_1- and β_2-AR proteins in young vs. old rat liver sections, as in *A*, determined by quantitation of DAB staining, are represented as bar graphs. Antibody to β_1-AR yielded relatively low signal. Data are expressed as means ± SE from 8 to 9 young and 6 to 8 old rats. *$P = 0.002$. *C*: representative liver sections exhibiting greater detail are shown at higher magnification (×12.5 objective). Scale bar, 100 μm. cv, Central vein.

β_1-AR content yet an age-related decrease in β_2-AR levels (Figs. 4 and 5). Finally, we have observed increased expression of GRK2/3 and βarr2 proteins in livers of old rats compared with young animals (Figs. 6 and 7).

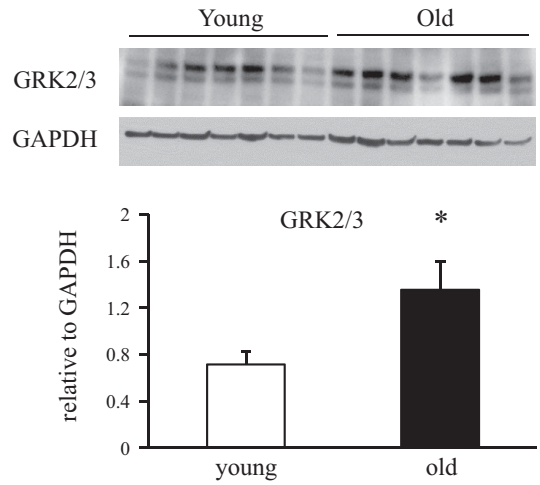

Fig. 6. Effects of age on G protein-coupled receptor kinase (GRK) protein expression levels in rat liver. Protein levels of GRK2/3 in whole liver lysates from 6- and 24-mo-old rats were determined by Western blotting. All lysates for Western blotting were prepared at the same time and processed in parallel. *Top*: immunoblots of GRK2/3 (both bands) in livers from young and old animals. GAPDH was used as loading control. *Bottom*: proteins levels were quantified as integrated intensity and then normalized to the values of loading controls. Bar values are means ± SE from 7 young and 7 old animals. *$P = 0.03$ vs. value at 6 mo.

To our knowledge, the present investigation is the first to show that the age-related increase in β-AR density previously observed in whole liver preparations from rats (11, 14, 20, 24) reflects a senescent change in the receptor content of parenchymal liver cells, i.e., at the level of hepatocytes. That the increased number of β-AR binding sites observed in hepato-

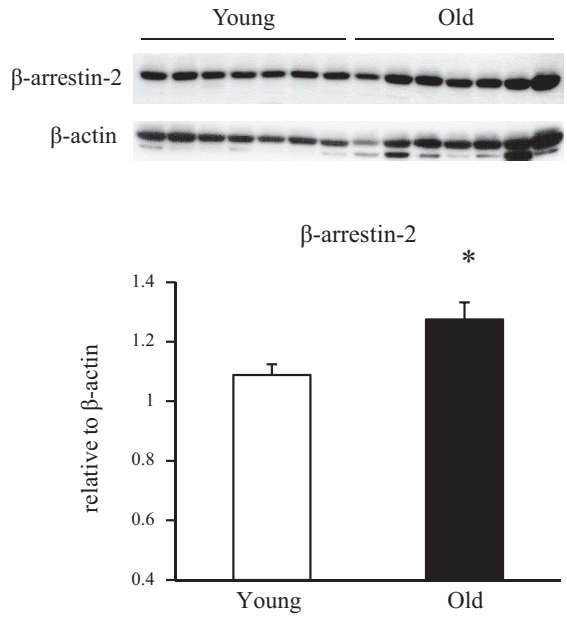

Fig. 7. β-Arrestin 2 (βarr2) protein levels in livers from young and old rats. Protein levels of βarr2 in whole liver lysates from 6- and 24-mo-old rats were determined by Western blotting. All lysates were prepared at the same time and processed in parallel. *Top*: representative immunoblots of βarr2 in livers from young and old animals ($n = 7$ in each group). *Bottom*: protein levels were quantified as integrated intensity and then normalized to the values of respective loading controls. Bar values are means ± SE from 7 young and 7 old animals. *$P = 0.018$ vs value at 6 mo.

cytes from 24-mo-old rats does in fact represent a phenomenon of cellular aging is substantiated by the finding that food restriction, long known to extend the lifespan of rats and delay or prevent age-related changes in hormone action and other physiological processes (24), blocks the increase in β-AR binding occurring at advanced age. Our observations further extend earlier work in the Fischer 344 male rat model of aging by demonstrating that the postmaturational increase in hepatic β-AR binding apparent in previous comparisons of adult (4–6 or 12 mo old) and senescent (24 mo old) animals (14, 20, 24) more precisely corresponds to increased hepatocyte β-AR density occurring only late in the lifespan, with receptor density remaining stable between 7 and 18 mo of age and increasing thereafter by two- to threefold at 24 mo. Moreover, the increase in hepatocyte β-AR binding occurring late in life appears to be closely linked to β-adrenergic-sensitive adenylate cyclase (AC) activation and glycogenolytic functional responses, both of which exhibited equivalent two- to threefold increases between 20 and 24 mo of age in earlier studies of hepatocytes from aging Fischer 344 male rats (10, 27). In these same studies, the late-life increases in AC and glucose output responses to adrenergic stimulations were, like the accompanying increase in β-AR number, also attenuated by food restriction (10).

A primary aim of this study was to investigate the expression of hepatic β_1- and β_2-ARs in young versus old rats and thereby provide some insight into the relative contributions of the individual receptor subtypes to the age-related increases in β-AR binding linked to AC signaling and downstream cellular functions. The two β-AR subtypes exhibit considerable differences in G protein coupling, AC activation, and feedback regulation. In numerous studies in which the receptor subtypes have been expressed individually in cells lacking intrinsic β-ARs, the β_2-AR has exhibited greater high-affinity agonist binding (i.e., formation of the high-affinity agonist-receptor-G protein ternary complex), increased functional coupling to stimulatory G protein (G_s)-mediated AC activation, and a higher degree of agonist-induced regulation via GRK- and/or arrestin-mediated receptor desensitization, sequestration/internalization, and downregulation when compared with the properties displayed by the β_1-AR subtype (15, 16, 32, 52, 61). Moreover, although both receptor subtypes appear to be subject to agonist-induced internalization by clathrin-mediated endocytosis, internalized β_2- and β_1-AR receptors may be sorted into different intracellular compartments (32).

Our findings in competition-binding experiments using receptor subtype-selective antagonists (Table 1 and Fig. 2) confirm and extend earlier results by others (35, 54) showing a mixed population of β_1- and β_2-AR receptors in livers of young adult and senescent rats. In our experiments, individual competition-binding curves revealed a greater degree of binding by both β_1- and β_2-AR selective antagonists in liver preparations from old rats compared with young animals (Fig. 2). As in earlier studies, our results are in general consistent with about equal numbers of β_1- and β_2-AR-binding sites in liver over the adult lifespan of the rat, although we observed a trend toward an age-related increase in the density of β_2-ARs (Table 1). Previously, isoproterenol stimulation of hepatic AC activity in liver preparations from old rats was found to be inhibited to a greater extent by β_2-AR-selective antagonist than by β_1-AR antagonists (54). Overall then, our results and those of others suggest that increased content of both β_1- and β_2-AR subtypes may contribute to enhanced β-AR binding and signaling in liver of senescent rats but that β_2-AR signaling could predominate at advanced age. Definitive conclusions regarding the relative contributions of β_1- and β_2-AR subpopulations to increased total β-AR density in livers of aging rats would require additional experiments employing more detailed competition-binding curves, i.e., with larger numbers of data points, to improve precision of estimated numbers of the two receptor subtypes. It should also be noted that in early studies comparing coupling of β-ARs (irrespective of subtype) to G protein in liver preparations from young and old rats, we and others found no age-related differences in physical coupling, assessed by high-affinity agonist binding (i.e., K_H, or high-affinity dissociation constant for isoproterenol) and guanine nucleotide induced conversion of receptors to a low-affinity binding state (11, 14, 24), or in functional coupling to AC, measured as the concentration of β-adrenergic agonist producing half-maximal AC response (EC_{50}) (22). Moreover, an increase with age in the proportion of hepatic β-ARs in the high-affinity binding state was reported in one study (14) but not in others (11, 24). In general, the binding and functional properties of β-ARs measured in these earlier studies would appear to be of limited sensitivity in distinguishing age-related differences in a mixed population of hepatic β-AR subtypes.

In the present study, steady-state levels of β_1- and β_2-AR mRNAs in rat liver, measured by qRT-PCR, were found to increase up to threefold during postmaturational aging (Fig. 3), providing evidence that increased numbers of hepatic β-AR binding sites in livers of old animals may well reflect age-related changes in expression of the two receptor subtypes. The current findings extend those of an earlier preliminary report from our laboratory in which increased β_1-AR mRNA levels in livers of senescent compared with young adult rats were demonstrable by semiquantitative RT-PCR, whereas Northern analysis was not sufficiently sensitive to detect the age-related increase in hepatic β_2-AR mRNA levels revealed in the present work by qRT-PCR (20). The mechanism(s) by which β_1- and β_2-AR transcript levels increase in livers of aging rats has not been determined. Transcriptional and posttranscriptional processes are known to govern β-AR subtype expression in liver and other tissues. For example, β_1- and β_2-ARs in multiple species are transcriptionally activated or repressed by hormones and transcription factors acting at response elements in the 5′-flanking promoter regions of the two receptor subtype genes, whereas at the posttranscriptional level, stability, or turnover, of β_1- and β_2-AR mRNAs appears to be regulated via protein binding to A + U rich elements within the 3′-untranslated regions of the β-AR mRNAs (5, 37). Interestingly, both types of regulatory processes have been invoked to account for changes of β_2-AR mRNA levels in rat liver occurring during early postnatal development (2, 3); but whether related modifications of hepatic β-AR transcripts might also occur during postmaturational aging remains to be studied.

In apparent contrast to the observed increases with age in β-AR-binding sites and mRNA levels of both β-AR subtypes in rat liver, immunoblot and immunohistochemistry analyses demonstrated a decrease in hepatic β_2-AR protein levels with no change in β_1-AR protein levels during aging (Figs. 4 and 5). It should be noted here that in immunohistochemistry experiments, β_2- but not β_1-ARs were found to be distributed

preferentially in pericentral regions (zone 3) of liver parenchyma (Fig. 5*C*). Although of unclear significance, this finding could be related to the steep oxygen gradient existing from proximal (periportal) to distal (pericentral) regions of the liver acinus. Differences in oxygen tension are recognized to play a key role in regulation of "metabolic zonation," i.e., hepatic region-specific metabolic functions, under physiological conditions and also in modulation of liver disease (21). For example, low oxygen tension favors optimal glycogenolytic activity in pericentral hepatocytes, and hepatic steatosis in nonalcoholic fatty liver disease, which is most commonly localized to zone 3 (58), has been linked to pericentral hypoxia (21, 34). In separate studies, a pathway of oxygen-responsive β_2-AR regulation has been identified, in which hydroxylation and ubiquitination of β_2- (but not β_1-) ARs by the von Hippel-Lindau tumor suppressor protein (pVHL)-E3 ligase complex target receptors for downregulation by proteasomal degradation; under hypoxic conditions, the activity of this degradative pathway is markedly diminished, thereby increasing β_2-AR receptor abundance (56). Our own previous studies have related increased β-AR binding and signaling functions in liver of aging rats to enhanced β-adrenergic responsive glycogenolysis and lipid accumulation (13, 27), with a specific role for the β_2-AR subtype implicated in hepatic steatosis with age (49). Evidence of hepatic hypoxia at advanced age is variable; a preliminary immunohistochemical study assessing nitroimidazole-adduct formation under hypoxic conditions failed to detect differences in pericentral or periportal hypoxia between liver specimens from young adult vs. senescent rats, although the nitroimidazole pimonidazole marker utilized in this study was apparently sensitive only to dramatic decreases in intracellular oxygen tension (8). Whether more moderate decreases in oxygen tension of pericentral hepatocytes might play a role in zone-specific increases in β_2-AR mediated glucose output and/or fat accumulation during aging is in our view a provocative hypothesis deserving further investigation. Although in the current study pericentral β_2-AR staining appeared to decline with age (Fig. 5*C*), as did total hepatic β_2-AR protein levels (Figs. 4 and 5, *A* and *B*), the status of β-AR-binding sites coupled with downstream signaling in pericentral hepatocytes from aging animals remains to be determined (also see discussion in the following paragraph).

An approach to reconciling discordant findings from receptor-binding and mRNA experiments with receptor protein level measurements may be suggested in the context of additional experiments (Figs. 6 and 7) showing increases with age in hepatic protein levels of GRK2/3 and βarr2. Rapid signal termination of agonist-activated GPCRs occurs via GRK- and βarr-mediated receptor desensitization and sequestration/internalization, whereas in response to prolonged agonist exposure receptors undergo downregulation by complex processes involving proteolytic degradation within lysosomes (4, 53). In the case of β_2-ARs, downregulation appears to be initiated by typical GRK-mediated receptor phosphorylation and binding of the phosphorylated receptor to the βarr2 adaptor protein, followed by β_2-AR internalization and degradation requiring ubiquitination of βarr2 and β_2-AR, respectively; it should be noted that ubiquitin-dependent lysosomal degradation of β_2-ARs is of no clear relationship to the oxygen-dependent process of proteasomal degradation described above (48, 56). Downregulation of β_2-ARs might be expected to be amplified in tissues expressing greater levels of GRK2/3 and βarr2, such as in liver of senescent rats. Also in this regard, β-adrenergic responsiveness of a number of tissues (excluding liver) declines with age, likely as a reflection of heightened desensitization or downregulation of β-ARs exposed for prolonged periods to increased circulating concentrations of catecholamines in older animals (9, 17, 43, 44, 51, 55). Moreover, age-related increases in the expression of GRKs and βarr have been invoked as mediators of reduced β-AR-responsive vasorelaxation observed in rats during postmaturational aging (46, 47). Earlier studies by others have demonstrated that, unlike β_2-ARs, β_1-ARs are resistant to agonist-mediated downregulation via lysosomal degradation (31). In view of the above considerations then, reduced β_2-AR but not β_1-AR protein levels in livers of senescent rats could relate to an increase with age in receptor-degradative activity preferentially affecting the β_2-AR subtype. Similarly, insofar as blood catecholamine levels, like oxygen tension, exhibit a steep zonal concentration gradient in liver (21), preferential distribution of immunoreactive β_2-ARs (but not β_1-ARs) in pericentral areas of liver from both young and old animals (Fig. 5*C*) could reflect, at least in part, zone-specific differences in agonist-mediated downregulation differentially affecting the two β-AR subpopulations. It should be emphasized, however, that no prior studies have comprehensively investigated agonist-mediated trafficking and downregulation of β-AR subpopulations in liver during aging.

Notwithstanding an hypothesized increase in hepatic β_2-AR downregulation during aging, a relationship between age-related changes in hepatic β-AR-binding sites and immunoreactive receptor levels assessed in the current study remains elusive and has yet to be determined. Immunoblots and immunohistochemical analysis revealing a decline with age in β_2- but not β_1-AR levels were conducted using whole liver preparations and thus suggest a decrease in the total β-AR pool from livers of old animals. In contrast, increased β-AR binding in hepatocyte membranes prepared from old rats likely reflects an age-related change in a more limited subcellular fraction of β-ARs, i.e., plasma membrane receptors functionally linked to effector signaling. Importantly, it is the finding of increased numbers of β-AR-binding sites coupled with activation of AC-linked signaling during aging that is unique to liver, whereas decreased immunoreactive β_2-AR content consistent with augmented agonist-mediated receptor downregulation is an age-related characteristic shared by liver with other tissues demonstrating reduced β-adrenergic responsiveness with age (43, 44, 51, 55). In this context, the increases in steady-state mRNA levels of both β-AR subtypes observed in liver from old rats (Fig. 3) assume significance in possibly resolving the apparent discrepancies in the present data. Agonist-responsive downregulation of β_2-ARs is thought to occur not only via proteolytic degradation but also by β_2-AR mRNA destabilization or possibly modulated β_2-AR gene transcription, leading to reduced steady-state receptor mRNA levels (53). Future studies will be required to determine whether the increase in β-adrenergic responsiveness observed in liver of senescent animals might be related to disrupted downregulation pathways involving β_2-AR gene transcription and/or mRNA stability. Of note is that whereas β_1-ARs do appear to be resistant to agonist-mediated downregulation characteristic of β_2-ARs (31), stability of β_1-AR protein content despite increasing steady-state β_1-AR mRNA levels in liver during aging sug-

gests an age-related increase in β_1-AR turnover by an unclear mechanism.

Perspectives and Significance

The present study, together with earlier findings from our laboratory and by others, strengthens the contention that increased expression of β-ARs in rat liver during aging augments β-adrenergic signaling pathway activities with deleterious consequences on hepatic glucose and lipid metabolism. Our results may implicate a preferential role for age-related changes in expression of the β_2-AR subtype, which would appear to be subject to dynamic agonist-mediated regulation in the context of increased circulating concentrations of catecholamines in aging animals. Involvement of the β_2-AR in age-related changes in liver metabolism would likely be of relevance to equivalent processes in older humans insofar as β-ARs in human liver plasma membranes are predominantly of the β_2-AR subtype (28). Expression changes with age in the hepatic β_1-AR, however, are not excluded by the current data in rats. Additional studies are suggested, as discussed above, to determine the mechanisms underlying modulated expression of one or more β-AR subtypes in liver during aging and thereby to identify possible therapeutic targets relevant to metabolic diseases of human aging such as type 2 diabetes mellitus and nonalcoholic fatty liver disease.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Y.S., Z.-J.S., and A.K. performed experiments; Y.S., M.S.K., and A.K. analyzed data; Y.S., J.L.B., C.-K.Y., P.M.G., M.S.K., and A.K. interpreted results of experiments; Y.S., Z.-J.S., H.W., J.L.B., and A.K. prepared figures; Y.S. and A.K. drafted manuscript; Y.S., J.L.B., C.-K.Y., P.M.G., M.S.K., and A.K. edited and revised manuscript; M.S.K. and A.K. conceived and designed research; Y.S., Z.-J.S., J.L.B., C.-K.Y., P.M.G., M.S.K. and A.K. approved final version of manuscript.

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