Role of β-adrenergic receptors in regulation of hepatic fat accumulation during aging

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Abstract

Excessive fat accumulation in liver (hepatic steatosis) predisposes to hepatic functional and structural impairment and overall metabolic risk. Previous studies noted an association between hepatic steatosis and age in humans and rodents. However, the mechanisms leading to age-associated hepatic fat accumulation remain unknown. Earlier work from our group showed that β-adrenergic receptor (β-AR) levels and β-AR-stimulated adenylyl cyclase activity increase in rat liver during aging. Here we investigated whether age-associated increases in β-AR signaling play a role in augmenting hepatic lipid accumulation. We demonstrate an increase in hepatic lipid content during senescence and a significant correlation between hepatic fat content and stimulation of adenylyl cyclase activity by the β-AR agonist isoproterenol in rat liver. Isoproterenol administration to young and old rodents in vivo increased hepatic lipid accumulation. Furthermore, in vitro overexpression of β1- and β2-AR subtypes in hepatocytes from young rodents increased cellular lipid content, whereas inhibition of β-ARs by receptor subtype-specific inhibitors reduced lipid levels in hepatocytes from senescent animals. Isoproterenol-induced hepatic lipid accumulation in vivo was prevented by the β-AR nonselective blocker propranolol, suggesting a novel therapeutic effect of this class of drugs in hepatic steatosis. Acipimox, which inhibits adipose tissue lipolysis, did not alter isoproterenol-mediated hepatic fat accumulation; thus β-AR responsive hepatic lipid accumulation does not appear to be related primarily to altered lipolysis. These findings suggest that augmented hepatic β-AR signaling during aging may increase lipid accumulation in liver and advocate a possible role for β-adrenergic blockers in preventing or retarding the development of hepatic steatosis.

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Introduction

The metabolic syndrome comprises a number of related disorders including obesity, insulin resistance, type 2 diabetes, and cardiovascular disease (Ford et al. 2002, Dowman et al. 2011). Aging is considered to be a risk factor for the metabolic syndrome, with a prevalence of more than 40% among Americans over the age of 60 (Ford et al. 2002). Nonalcoholic fatty liver disease (NAFLD), a common manifestation of patients with the metabolic syndrome (Dowman et al. 2011), includes a spectrum of pathological conditions ranging from hepatic steatosis, or augmented fat accumulation in the liver, through steatohepatitis, cirrhosis, and hepatocellular carcinoma (Clark 2006, Kotronen et al. 2007, Stefan et al. 2008, Cohen et al. 2011). Accumulating clinical evidence indicates a higher prevalence of cardiovascular disease in patients with NAFLD than in control subjects without steatosis (Targher et al. 2010). The mechanisms leading to hepatic fat accumulation are not well understood. Given the associations of NAFLD with liver dysfunction and cardiovascular disease, identification of therapeutic targets to reduce or prevent hepatic lipid content is of considerable clinical importance.

Recent epidemiological studies have revealed age as a risk factor for increased hepatic lipid accumulation (Park et al. 2006, Slawik & Vidal-Puig 2006). The prevalence of NAFLD and progression to more advanced stages are associated with older age (Clark 2006, Farrell & Larter 2006, Park et al. 2006, Stefan et al. 2008, Frith et al. 2009). Animal models of aging provide additional evidence of a relationship between age and increased hepatic lipid accumulation. For example, a study of dietary influences on age-related pathology in Fischer 344 rats noted, among other observations, an increase in hepatic lipid content in older compared to younger animals regardless of diet (Maeda et al. 1985). More recently, increased lipid accumulation in liver was observed with aging in senescence
accelerated P10 mice (Honma et al. 2011). Significantly, the cause of augmented lipid accumulation in the liver with age is yet to be clearly defined. The goal of the present study was to delineate the molecular basis for increased hepatic fat accumulation in rodents with aging.

Catecholamines acting via β-adrenergic receptors (β1-, β2-, or β3-AR subtypes) coupled to adenylyl cyclase and other effectors modulate important biological responses including lipid and glucose metabolisms in a wide variety of tissues (Katz et al. 1993, Nonogaki 2000, Xiao et al. 2006). Studies have documented the presence of β1- and β2-ARs in rodent and human liver tissues (Dax et al. 1987, Arner et al. 1990, Van Ermen et al. 1992, Krief et al. 1993, Cardani & Zavanella 2001). Our group and others have previously demonstrated that β-AR levels in rat liver increase during senescent aging, in association with increased β-AR-mediated stimulation of adenylyl cyclase and glucose output (Katz et al. 1987, 1993). Similar increases in β-AR coupling to adenylyl cyclase stimulation were also observed in aging mice (Katz et al. 1993). β3-ARs, which play an important role in increasing thermogenesis and lipolysis in brown and white adipose tissues, have generally not been identified in liver tissues from rats and humans (Krief et al. 1993, Sanghani & Scarpone 1994, Nonogaki 2000, Jin 2010). Notably, in marked contrast to increased β-AR responsiveness in rat liver during aging, the lipolytic response of fat cells to catecholamines has been found to decline with senescent aging in rats and humans (Lonnqvist et al. 1990, Gregerman 1994). Therefore, we have investigated whether age-related increases in hepatic β1- and β2-AR signaling contribute to augmented lipid accumulation in liver during aging.

In this study we demonstrate increased fat content in liver of aging rodents, and observed a significant positive correlation between hepatic fat content and liver adenylyl cyclase activity stimulated by the β-AR agonist isoproterenol. We also found that in vivo administration of isoproterenol to young and old rodents, and in vitro overexpression of β1- and β2-ARs in hepatocytes from young animals, increased fat accumulation, whereas fat content of hepatocytes from old rodents was reduced by β1- and β2-AR selective antagonists. Moreover, isoproterenol-induced hepatic fat accumulation in vivo appeared to reflect mechanisms intrinsic to liver since acipimox, an inhibitor of adipose tissue lipolysis, did not alter hepatic lipid levels. Taken together, these studies suggest an important role of hepatic β-AR signaling in the induction of liver steatosis during aging.

Materials and Methods

Materials

All tissue culture reagents were obtained from Gibco-BRL. BioCoat collagen–coated plates were purchased from Becton Dickinson (Franklin Lakes, NJ, USA). Lipofectamine 2000 and dithiothreitol were from Invitrogen. DNase I and Complete Mini tablets were obtained from Roche Diagnostics. CGP 20712, ICI 118, 551, and acipimox were from Tocris Bioscience (Ellisville, MO, USA). Bradford protein assay reagents were purchased from Bio-Rad Laboratories. ECL Advance kit was purchased from Amersham Biosciences and Cell Staining Solution was from SA Biosciences (Frederick, MD, USA). All other chemicals were obtained from Sigma–Aldrich.

Animals

Young adult (6 months old) and old (24 months old) male Fischer 344 rats and young (6 months old) male C57BL/6 mice were obtained from the National Institute on Aging, Bethesda, MD, USA. Upon receipt, the animals were housed within the Veterinary Medical Unit of the Audie L. Murphy Veterans Hospital (AMVH), San Antonio, TX, USA; rodents were maintained for at least 1 week prior to use. For in vivo studies, rodents were injected i.p. with saline, isoproterenol (20 µg/g), propranolol (50 µg/g), or acipimox (50 µg/g) as specified in the descriptions of individual experiments. We did not observe any evident morbidity or mortality in animals injected with these agents individually or in combination. Animals were treated in accordance with the guidelines approved by the Institutional Animal Care and Use Committee at the AMVH.

Preparation of liver samples

Rats were killed by exsanguination after anesthesia as previously described (Kamat et al. 2008). The livers were rapidly removed and cut into pieces, which were quick-frozen in liquid nitrogen and stored at −80 °C until use.

Lipid quantitation in liver tissues

Frozen liver tissue sections were stained with Oil Red O (ORO). In some studies hematoxylin was used as a counterstain. Photographic images were taken of three random fields of each stained specimen using an Olympus AX70 research microscope equipped with a 20× objective and 1:25× multiplier connected to a DP70 digital camera (Olympus America, Inc., Center Valley, PA, USA). Red staining (representing fat) was digitally extracted using the segmentation tool of Image-Pro Plus 4.5 Software (Media Cybernetics, Bethesda, MD, USA). The software was calibrated to a stage micrometer and the area of ORO staining was quantified and expressed as a percentage of total area in each field.

Grading of fatty change in liver

Hepatic fatty change was graded in liver tissues stained with hematoxylin and eosin. Slides were scored in a blinded fashion by a pathologist (Y I) as grades 0, 1, 2, or 3, by a previously described method (Maeda et al. 1985). Briefly, tissues with no appreciable fat droplets received grade 0; tissues with few
small fat droplets in hepatocytes near the portal region were graded as 1; many moderately sized fat droplets in hepatocytes near midzonal and portal regions were scored as grade 2; and many large fat droplets in hepatocytes distributed throughout the liver tissue received grade 3.

Isolation of hepatocytes

Hepatocytes were isolated from rats and mice, as described previously (Kamat et al. 2008). Briefly, the animals were anesthetized using pentobarbital sodium (65 mg i.p. injection per kg body weight); and the livers were perfused in situ with calcium-free Earle’s Balanced Salt Solution (EBSS), pH 7.4, followed by calcium-free EBSS containing 0.05% collagenase (type I), pH 7.4. Hepatocytes from collagenase-perfused livers were suspended in calcium-containing EBSS, filtered through a nylon mesh, and washed twice by low-speed centrifugation (52 g at 4 °C for 2 min; Sorvall RT7 centrifuge). Freshly isolated hepatocytes were then suspended in Williams’ medium E. Cell viability (~85–90%) and yield were determined by trypan blue dye exclusion.

Cell culture

Freshly isolated hepatocytes were resuspended in Williams' medium E containing 1% glutamine and 1% penicillin/streptomycin, and plated on collagen-coated dishes in the presence of 5% fetal bovine serum (FBS). The cells were plated at a density of 3 × 10⁶ cells/100 mm dish or 150 000 cells/well in a 24 well plate at 37 °C in a humidified 5% CO₂ atmosphere. Two hours after plating, the cells were washed and fresh Williams’ medium E containing glutamine and antibiotics was added to the plates. Cells were cultured for an additional 24–72 h in the absence or presence of appropriate ligands.

Transfection of hepatocytes

After overnight culture in Williams’ medium E containing 5% FBS, hepatocytes plated in 24 well dishes were transfected with β₁- or β₂-AR pcDNA3 expression plasmids (generous gifts of Dr R J Letkowitz, Duke University Medical Center, Durham, NC, USA) or empty vector (500 ng) using Lipofectamine Plus and OPTI-MEM medium. Three hours after transfection fresh medium containing 5% FBS was added to the cells. Forty-eight hours after transfection hepatocytes were fixed in 10% (v/v) formaldehyde for at least 2 h at room temperature for cellular lipid accumulation measurements as described below.

Adenoviral infection of hepatocytes

Two hours after culturing hepatocytes in medium containing 5% FBS, cells in 24 well plates were uninfected or infected with adenovirus vectors expressing β₁- or β₂-AR (generously provided by Dr Walter Koch, Thomas Jefferson University, Pennsylvania, PA, USA) or the green fluorescent protein (control) at a multiplicity of infection (moi) of 100 for 1 h at room temperature. The plates were then shifted to 37 °C in a humidified 5% CO₂ atmosphere. After 24 h fresh medium containing isoproterenol (10⁻⁵ M) was added to the cells and changed daily for the next 48 h. Seventy-two hours after infection hepatocytes were fixed in 10% (v/v) formaldehyde for at least 2 h at room temperature for cellular lipid accumulation measurements as described below.

Cellular lipid accumulation measurements

For lipid quantitation, hepatocytes were stained with ORO, and the accumulated cellular lipid measured as described previously (Sanchez-Hidalgo et al. 2007). Briefly, fixed cells in 24 well plates were rinsed in distilled water and then stained with 150 µl/well ORO solution for 2 h. After washing the stained wells with distilled water, accumulated lipids in the cells were extracted with isopropanol and the extracted ORO absorbance was read spectrophotometrically at 510 nm using the Spectra Max M2 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). Cellular lipid accumulation was then calculated by dividing ORO absorbance per well by relative cell number determined spectrophotometrically at 595 nm upon addition of Cell Staining Solution (SABiosciences; Sanchez-Hidalgo et al. 2007).

Adenyl cyclase activity

Adenyl cyclase activity was measured as the conversion of [α-³²P]ATP to [³²P]cAMP in liver homogenates of rats in the absence or presence of the β-AR agonist isoproterenol. cAMP product was isolated by a two-column chromatographic method (Katz et al. 1987).

Statistical analysis

Data from multiple experiments are expressed as means ± S.E.M. For in vitro studies statistical significance of single comparisons was determined by Student’s t-test, and correlation analyses were performed using Spearman rank correlation coefficients. For in vivo studies single comparisons between control and isoproterenol treated groups were performed by the nonparametric Kruskal–Wallis (χ²) test, while ANOVA followed by Dunnett’s post hoc test was used for multiple comparisons.

Results

Fat accumulation increases with age in rat liver

Hepatic fat accumulation, as assessed by ORO staining, was measured in liver sections from young (6 months, n = 10) and old (24 months, n = 7) Fischer 344 male rats. Representative liver sections from a young and an old rat are shown in Fig. 1A (left panel). Lipid droplet staining by ORO was greater in...
the liver section from the old rat than in the preparation from the young animal. Quantitation of the accumulated fat in liver tissue, as described in Materials and methods section, demonstrated a 3.7-fold increase \((P<0.0001)\) with age (Fig. 1A, right panel). We also compared the grade of fatty change in liver tissues from the same group of young and old animals. The results demonstrated that liver samples from old rats all exhibited fat accumulation with a grade from 1 to 3, while the liver samples from young rats uniformly revealed a grade of 0 (Fig. 1B). These results demonstrate an age-related increase in hepatic lipid accumulation.

**Fat content in liver tissues correlates with isoproterenol stimulation of adenylyl cyclase activity**

Previous studies from our group and others have shown that β-AR-stimulated adenylyl cyclase activity in rodent liver increases with age (Katz *et al.* 1987, 1993). In the present study we determined whether increased fat accumulation in liver was associated with an increase in β-AR-mediated stimulation of adenylyl cyclase activity. Stimulation of adenylyl cyclase activity by the β-AR agonist isoproterenol \(\left(10^{-5} \text{ M}\right)\) was measured in homogenates prepared from the same liver tissues used for determination of fat accumulation (Fig. 1). In support of previous results (Katz *et al.* 1987, 1993), activation of adenylyl cyclase activity by isoproterenol increased more than twofold \(\left(P<0.002\right)\) in liver homogenates of rats between 6 and 24 months of age (Fig. 2A). The isoproterenol-induced increase in adenylyl cyclase activity (above basal) in liver homogenates from 24-month-old rats was about fourfold greater \(\left(P<0.001\right)\) than in preparations from 6-month-old animals (Fig. 2B). Next, we correlated the levels of isoproterenol-induced increase in adenylyl cyclase activity with liver fat content and grade of fatty liver change. The increase in adenylyl cyclase activity upon isoproterenol stimulation was found to correlate positively with both liver fat content (Spearman correlation coefficient \(=-0.66, P=0.004\); Fig. 2C) and grade of fatty liver change (Spearman correlation coefficient \(=0.83, P<0.0001\); Fig. 2D). Collectively, these results implicate a role for increased β-AR-mediated adenylyl cyclase activation in augmented hepatic lipid accumulation during aging.

**Isoproterenol treatment in vivo increases hepatic lipid accumulation**

We hypothesized that if increased β-AR-mediated stimulation of adenylyl cyclase activity during aging augments lipid accumulation in the liver, then raising catecholamine levels in young rats would increase hepatic lipid content. To test this

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**Figure 1** Fat accumulation in rat hepatocytes increases with age. (A) Liver sections from ten young (6 months old) and seven old (24 months old) Fischer 344 male rats were stained with ORO and hematoxylin. (Left panel) Representative liver sections from one young and one old rat are shown. (Right panel) Quantitation of fat content in the liver sections, as described in Materials and methods, indicates a 3.7-fold increase in tissues from old rats. Data are presented as mean values ± S.E.M. \(*P<0.0001\) vs liver fat content in young animals. (B) Liver sections from the same group of young and old animals used to measure fat content in (A) were stained with hematoxylin and eosin and scored as grades 0, 1, 2, or 3 based on fatty change as previously described (Maeda *et al.* 1985). (Upper panel) Representative liver sections for each grade are shown. (Lower panel) Table depicting the percentage of young and old rat liver tissues graded as 0, 1, 2, and 3. Full colour version of this figure available via http://dx.doi.org/10.1530/JOE-11-0406.
presented as mean values of lipid droplet staining showed that hepatic fat content in saline-treated animals (Fig. 3A, upper panels). Quantitation in preparations from isoproterenol-injected rats compared to ORO staining of frozen liver sections, was markedly greater removed and frozen. Lipid droplet accumulation, assessed by over the course of a 24-h period; after 24 h livers were increase

Iso stimulation in young animals) (B) Each bar represents mean increase±S.E.M. in AC activity (above basal levels) upon Iso stimulation. †P<0.001 vs activity in young animals. A positive correlation is observed between the increase in AC activity upon Iso treatment and both (C) hepatic fat content (P=0.004; Spearman correlation coefficient=0.66) and (D) grade of fatty liver (P<0.0001; Spearman correlation coefficient=0.83).

Figure 2 β-AR-mediated stimulation of adenylyl cyclase (AC) in rat liver is positively correlated with both hepatic fat content and grade of fatty liver. (A) Liver homogenates from the ten young and seven old rats used in Fig. 1 were used to measure AC activity, as described in Materials and methods, in the absence (basal) and presence of the β-AR agonist isoproterenol (Iso, 10⁻⁵ M). Data are presented as mean values±S.E.M. (⁎P<0.002 vs AC activity upon Iso stimulation in young animals) (B) Each bar represents mean increase±S.E.M. in AC activity (above basal levels) upon Iso stimulation. †P<0.001 vs activity in young animals. A positive correlation is observed between the increase in AC activity upon Iso treatment and both (C) hepatic fat content (P=0.004; Spearman correlation coefficient=0.66) and (D) grade of fatty liver (P<0.0001; Spearman correlation coefficient=0.83).

hypothesis, we injected young rats i.p. with isoproterenol (20 μg/g) or saline (control; n=3–5 in each group) two times over the course of a 24-h period; after 24 h livers were removed and frozen. Lipid droplet accumulation, assessed by ORO staining of frozen liver sections, was markedly greater in preparations from isoproterenol-injected rats compared to saline-treated animals (Fig. 3A, upper panels). Quantitation of lipid droplet staining showed that hepatic fat content in isoproterenol-injected young rats was 3.5-fold times that measured in control animals (χ²=5.000; P=0.025; Fig. 3A, lower panel). Parallel experiments performed in old rats (n=3 in each group) also demonstrated an increase in lipid droplet accumulation (Fig. 3B, upper panels) and a 1.8-fold increase in hepatic fat content after isoproterenol injection (χ²=3.857; P=0.049; Fig. 3B, lower panel). These studies confirm that isoproterenol treatment in vivo augments hepatic lipid content.

Overexpression of β₁- and β₂-ARs increases fat content in isolated hepatocytes

We next determined whether increased hepatocellular β-AR complement, as occurs during aging, augments lipid content. Of the three well-characterized β-AR subtypes, only β₁- and β₂-ARs are demonstrable in rodent liver at all ages (Sanghani & Scarpace 1994). Hence, hepatocytes from young rats were transfected with β₁- or β₂-AR expression plasmids or empty vector (control). Representative photomicrographs (Fig. 4A, upper panels) show greater ORO staining in cells transfected with individual β-AR subtypes than control cells. A significant increase (P=0.048) in measured lipid accumulation was observed in cells transfected with β₂-AR expression plasmid compared to cells transfected with empty vector, while a lesser effect was apparent upon overexpression of β₁-AR in hepatocytes (Fig. 4A, lower panel).

To investigate whether the effect of β-AR overexpression on hepatocellular lipid accumulation is species specific, we conducted analogous experiments in mouse hepatocytes. Hepatocytes isolated from young mice were uninfected or infected with recombinant adenoviruses expressing β₁- or β₂-AR subtypes or green fluorescent protein (negative control) and treated with isoproterenol (10⁻⁵ M) for 72 h. As with rat hepatocytes, mouse hepatocytes overexpressing β₂-AR subtypes exhibited greater ORO staining than did uninfected cells (Fig. 4B, upper panels). Measurement of

Figure 3 Administration of the β-AR agonist isoproterenol increases hepatic lipid accumulation in rats. Young (6 months old; n=3–5 in each group; Panel A) and old (24 months old; n=3 in each group, Panel B) rats were injected i.p. with isoproterenol (Iso; 20 μg/g) or saline twice over a 24-h period. After 24 h the animals were killed and livers were removed and frozen. Frozen liver sections were stained with ORO and hepatic fat content measured as described in Materials and methods. (A and B; upper panels) Representative ORO stained liver sections from individual saline and Iso-treated rats are shown. (A; lower panel) Quantitation of lipid levels in liver from young rats determined by ORO staining. A 3.5-fold increase (χ²=5.006; *P=0.025) in hepatic lipid content upon Iso treatment is observed. (B; lower panel) Quantitation of lipid levels determined by ORO staining in liver from old rats liver tissues. A 1.8-fold increase (χ²=3.857; **P=0.049) in hepatic lipid content upon Iso treatment is observed. Data are presented as mean values±S.E.M. Full colour version of this figure available via http://dx.doi.org/10.1530/JEO-11-0406.
cellular lipid accumulation demonstrated a 152% increase ($P < 0.0023$) in lipid content in $\beta_2$-AR-overexpressing hepatocytes compared to uninfected cells; a lesser (47%) increase in lipid accumulation was observed in $\beta_1$-AR overexpressing mouse hepatocytes compared to uninfected controls (Fig. 4B, lower panel). Lipid accumulation measurements were equivalent in uninfected cells and cells infected with adenovirus expressing green fluorescent protein (data not shown). Taken together, these results indicate that increased expression of $\beta$-ARs, as occurs in liver during aging, augments fat accumulation in hepatocytes from both rats and mice.

**$\beta$-AR subtype selective antagonists suppress fat accumulation in hepatocytes from old rats**

Inasmuch as $\beta$-AR responsive signaling and fat accumulation both increase in rat hepatocytes with age, we determined whether treatment of hepatocytes from old rats with $\beta$-AR subtype selective antagonists can reduce cellular fat content. Figure 5 shows that the $\beta_1$- and $\beta_2$-AR selective antagonists CGP 20712A (CGP) and ICI 118 551 (ICI) inhibited cellular lipid accumulation in hepatocytes from old rats by 40% ($P < 0.003$) and 30% ($P = 0.06$) respectively compared to untreated cells. These data suggest that lipid accumulation in hepatocytes from old animals can be blocked by inhibitors of $\beta_1$- and $\beta_2$-AR subtypes. Comparable experiments were performed with hepatocytes from young rats; in these cells, with low levels of $\beta$-AR signaling, we were unable to detect any changes in cellular lipid levels upon treatment with $\beta$-AR subtype selective antagonists (Fig. 5).

Isoproterenol-induced hepatic fat accumulation in vivo is prevented by the $\beta$-AR blocker propranolol but not by the antilipolytic drug acipimox

Since the above studies with $\beta$-AR antagonists were conducted using isolated hepatocytes, we performed additional experiments to determine whether the effect of $\beta$-AR inhibition on fat accumulation in liver is also demonstrable in vivo. Fat accumulation in livers of young mice treated with isoproterenol was measured in the absence or presence of the nonselective $\beta$-AR antagonist propranolol. As shown earlier in rats (Fig. 3), isoproterenol treatment of mice induced a marked (7.2-fold) increase in hepatic fat content ($P = 0.014$ vs saline). In the presence of propranolol,
however, the stimulatory effect of isoproterenol on hepatic fat accumulation was largely eliminated (Fig. 6). Studies were also performed to distinguish whether the observed hepatic lipid accumulation in response to isoproterenol in vivo reflects intrinsic β-adrenergic sensitive processes in liver or augmented levels of fatty acids presented to the liver via catecholamine-responsive adipose tissue lipolysis. Figure 6 shows that the addition of the antilipolytic drug acipimox (50 μg/g; Al-Shurbaji et al. 1990, Ahrén 2001) had no effect on isoproterenol-induced hepatic fat accumulation (P=0.005 vs saline). Administration of propranolol or acipimox alone did not alter basal hepatic fat content. These data suggest that hepatic fat accumulation responsive to isoproterenol treatment is not primarily related to alterations in flux of fatty acids from adipose tissue, and hence may reflect β-AR-mediated mechanisms intrinsic to the liver.

Discussion

Aging is associated with changes in lipid metabolism and redistribution of body fat to nonadipose tissues such as liver, skeletal muscle, heart, and pancreatic β-cells (Slawik & Vidal-Puig 2006, Sepe et al. 2011). Excessive fat accumulation in the liver (hepatic steatosis) is the earliest manifestation of NAFLD, which occurs with other aspects of the metabolic syndrome including obesity, insulin resistance, type 2 diabetes, dyslipidemias, and atherosclerotic cardiovascular diseases (Kotronen et al. 2007, Stefan et al. 2008). Age appears to be a risk factor for both NAFLD and the metabolic syndrome (Ford et al. 2002, Stefan et al. 2008, Frith et al. 2009). However, the pathogenetic mechanisms leading to an increase in hepatic lipid accumulation during aging are not well understood.

In this study we present data demonstrating that increased hepatic β-AR signaling may play an important role in augmenting fat accumulation in liver during aging. Our findings extend previous evidence linking increased hepatic β-AR levels and age-related metabolic dysfunction of liver. Earlier work from our laboratory and others showed that β-AR content and β-adrenergic responsive adenylyl cyclase activity increase in rat liver during senescent aging (Dax et al. 1987, Katz et al. 1993). Progressive increases in β-AR-mediated stimulation of adenylyl cyclase in hepatocytes from aging rats are in turn associated with increasing β-adrenergic responsive glucose output (Graham et al. 1987, Katz et al. 1993). Recently, we have also reported that isoproterenol treatment in vivo acutely induces insulin resistance in liver and increases hepatic glucose production in young and old fasted rats (Muscogiuri et al. 2011); our findings further supported the development of hepatic insulin resistance in older rats. These observations have led us to hypothesize that the emergence of β-adrenergic-mediated insulin resistance in liver during aging, with attendant increases in fasting hepatic glucose output and plasma glucose levels, could contribute to glucose dysregulation and diabetes developing with advancing age (Muscogiuri et al. 2011). Hepatic steatosis is also closely associated with – and may be caused by – insulin resistance (Cohen et al. 2011). Hence, taken together with earlier findings our current results demonstrating a significant correlation between increasing steatosis and isoproterenol-stimulated adenylyl cyclase activity in liver of aging rats (Figs 1 and 2) further suggest that increased hepatic β-adrenergic signaling may contribute broadly to the development of multiple insulin resistance-related metabolic disorders during aging. It is important to note in this regard that, irrespective of mechanism, insulin resistance during aging is likely to play a major role in the development of increased hepatic lipid content in old animals. Whether our findings in rodent models are applicable to aging humans remains to be determined, although catecholamine-induced insulin resistance in human liver is known to be exerted predominantly by β-adrenergic-mediated mechanisms (Diebert & DeFronzo 1980, Rizza et al. 1980). It should also be emphasized that increased β-adrenergic signaling with age is a phenomenon apparently unique to liver; and β-AR levels and/or function in multiple other tissues decline or remain unchanged with age (Scarpace et al. 1991, Katz et al. 1993, Gettys et al. 1995, Xiao et al. 1998). Since circulating catecholamine levels are thought to increase during aging, metabolic changes associated with an age-related increase in hepatic β-AR levels could reflect the consequences of a tissue-specific defect in adaptive mechanisms otherwise modulating end-organ responses to increased sympathetic tone (Muscogiuri et al. 2011).

Figure 5 β-AR antagonists decrease lipid accumulation in hepatocytes from old rats. Hepatocytes from young (6 months old) and old (24 months old) rats (n=3 in each group) were treated with the selective β1-AR antagonist CGP 20712 (CGP, 1 μM) or the β2-AR antagonist ICI 118, 551 (ICI, 100 nM) for 72 h. The cells were then fixed and stained with ORO to measure cellular lipid accumulation, as described in Materials and methods. Results indicate a reduction in hepatocellular lipid accumulation by 40 and 30% in cells isolated from old rats and treated with CGP and ICI, respectively, compared to vehicle-treated hepatocytes (control, C). No reduction in hepatocellular lipid accumulation was observed in cells isolated from young rats and treated with β-AR selective antagonists. Each experiment was performed in triplicate. Data are presented as mean values ± S.E.M. and expressed relative to control (100%); *P<0.003 vs C; †P<0.06 vs C.

Discussion

Aging is associated with changes in lipid metabolism and redistribution of body fat to nonadipose tissues such as liver, skeletal muscle, heart, and pancreatic β-cells (Slawik & Vidal-Puig 2006, Sepe et al. 2011). Excessive fat accumulation in the liver (hepatic steatosis) is the earliest manifestation of NAFLD, which occurs with other aspects of the metabolic syndrome including obesity, insulin resistance, type 2 diabetes, dyslipidemias, and atherosclerotic cardiovascular diseases (Kotronen et al. 2007, Stefan et al. 2008). Age appears to be a risk factor for both NAFLD and the metabolic syndrome (Ford et al. 2002, Stefan et al. 2008, Frith et al. 2009). However, the pathogenetic mechanisms leading to an increase in hepatic lipid accumulation during aging are not well understood.

In this study we present data demonstrating that increased hepatic β-AR signaling may play an important role in augmenting fat accumulation in liver during aging. Our findings extend previous evidence linking increased hepatic β-AR levels and age-related metabolic dysfunction of liver. Earlier work from our laboratory and others showed that β-AR content and β-adrenergic responsive adenylyl cyclase activity increase in rat liver during senescent aging (Dax et al. 1987, Katz et al. 1993). Progressive increases in β-AR-mediated stimulation of adenylyl cyclase in hepatocytes from aging rats are in turn associated with increasing β-adrenergic responsive glucose output (Graham et al. 1987, Katz et al. 1993). Recently, we have also reported that isoproterenol treatment in vivo acutely induces insulin resistance in liver and increases hepatic glucose production in young and old fasted rats (Muscogiuri et al. 2011); our findings further supported the development of hepatic insulin resistance in older rats. These observations have led us to hypothesize that the emergence of β-adrenergic-mediated insulin resistance in liver during aging, with attendant increases in fasting hepatic glucose output and plasma glucose levels, could contribute to glucose dysregulation and diabetes developing with advancing age (Muscogiuri et al. 2011). Hepatic steatosis is also closely associated with – and may be caused by – insulin resistance (Cohen et al. 2011). Hence, taken together with earlier findings our current results demonstrating a significant correlation between increasing steatosis and isoproterenol-stimulated adenylyl cyclase activity in liver of aging rats (Figs 1 and 2) further suggest that increased hepatic β-adrenergic signaling may contribute broadly to the development of multiple insulin resistance-related metabolic disorders during aging. It is important to note in this regard that, irrespective of mechanism, insulin resistance during aging is likely to play a major role in the development of increased hepatic lipid content in old animals. Whether our findings in rodent models are applicable to aging humans remains to be determined, although catecholamine-induced insulin resistance in human liver is known to be exerted predominantly by β-adrenergic-mediated mechanisms (Diebert & DeFronzo 1980, Rizza et al. 1980). It should also be emphasized that increased β-adrenergic signaling with age is a phenomenon apparently unique to liver; and β-AR levels and/or function in multiple other tissues decline or remain unchanged with age (Scarpace et al. 1991, Katz et al. 1993, Gettys et al. 1995, Xiao et al. 1998). Since circulating catecholamine levels are thought to increase during aging, metabolic changes associated with an age-related increase in hepatic β-AR levels could reflect the consequences of a tissue-specific defect in adaptive mechanisms otherwise modulating end-organ responses to increased sympathetic tone (Muscogiuri et al. 2011).

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Data from both in vitro and in vivo experiments implicate a causal role for hepatic β-AR signaling in age-related liver steatosis. In hepatocytes from young rats and mice overexpression of β2-AR and, to a lesser extent, β1-AR subtypes were found to increase cellular lipid accumulation (Fig. 4). The conditions for receptor overexpression in these experiments were designed to simulate those occurring with aging, insofar as preliminary data from our laboratory have suggested increased expression of both β-AR subtypes in rat liver during aging (Kamat et al. 2004, Jin 2010). After 24 h livers were removed and frozen. Frozen liver sections were stained with ORO and hepatic fat content measured as described in Materials and methods. (Upper panels) Representative photomicrographs demonstrate accumulation of lipid droplets in liver sections from mice treated with Iso and Iso + Acip, but not with Iso + Prop. (Lower panel) Quantitation of lipid droplet accumulation from livers of animals treated as described. Data are presented as mean values ± S.E.M. *P = 0.014 vs Sal; ‡P = 0.005 vs Sal. Full colour version of this figure available via http://dx.doi.org/10.1530/JOE-11-0406.

β-Adrenergic responsive liver steatosis was confirmed in vivo in experiments demonstrating that isoproterenol treatment of rats and mice increases hepatic lipid content, and that the stimulatory effect of isoproterenol on liver fat was blocked by the β-AR antagonist propranolol (Figs 3 and 6). Previous studies have demonstrated hepatic lipid accumulation after treatment in vivo with isoproterenol, albeit at high doses (Wexler & Greenberg 1978, Wexler & McMurtry 1983). We also found that isoproterenol-induced hepatic fat accumulation in vivo was unaffected by acipimox, an inhibitor of hepatic lipase.
of adipose tissue lipolysis (Fig. 6; Al-Shurbaji et al. 1990, Ahren 2001). Interestingly, acipimox administration to both rats and humans has been shown to have no effect on liver fat content, despite marked suppression of circulating free fatty acids (al-Shurbaji et al. 1990, Rigazio et al. 2008). In the context of these previous findings, the lack of inhibition of isoproterenol-induced liver fat by acipimox in our experiments further suggests that β-adrenergic responsive increases in liver fat reflect processes intrinsic to liver and do not appear to be related primarily to altered flux of fatty acids from adipose tissue. The increase in hepatic fat content observed in senescent rats (Fig. 1) is also unlikely to be caused by changes in lipolysis related to increased sympathetic tone during aging, since the lipolytic response to catecholamines declines with age in rats (Gregerman 1994).

The cellular and molecular mechanisms by which increased hepatic β-AR signaling contributes to liver steatosis during aging are currently unknown. Fat may accumulate in the liver as a consequence of multiple abnormalities of hepatic lipid metabolism, including increased de novo lipogenesis, decreased β-oxidation of fatty acids, and/or decreased synthesis or secretion of very-low-density lipoproteins (VLDL; Donnelly et al. 2005, Stefan et al. 2008). Previous studies indicated that high concentrations of cAMP in rat hepatocytes increase the activity of phosphatidate phosphohydrolase, a rate-limiting enzyme of triglyceride synthesis, and thereby provide the cells with an increased capacity to synthesize triglycerides (Pittner et al. 1985). Additionally, investigations using knockout mice carrying a mutated form of cAMP-dependent protein kinase A (PKA) demonstrated that disruption of PKA reduced the development of diet-induced fatty liver, and suggested a potential role for altered mechanisms of intrinsic hepatic lipogenesis (Enns et al. 2009). Other studies revealed that treatment of rat hepatocytes with cAMP and isoproterenol increased lipid content of cells by suppressing secretion of triglycerides, cholesterol, and VLDL (Bjornsson et al. 1994, Rasouli & Zahraie 2006). Decreased β-oxidation of fatty acids via reduced hepatic expression of the nuclear receptor peroxisome proliferator-activated receptor alpha has also been implicated as a cause of/ contributor to hepatic triglyceride accretion and hypertriglyceridemia associated with old age (Sanguino et al. 2004). As also indicated previously, multiple studies point to a causal relationship between hepatic insulin resistance and liver steatosis (Cohen et al. 2011); and our own recent work may implicate a role for hepatic β-adrenergic signaling in promoting age-related insulin resistance (Muscogiuri et al. 2011). Detailed investigations relating the β-AR/adenyl cyclase/cAMP/PKA pathway to insulin responsive lipid metabolism in liver will be required to define the specific mechanisms underlying β-adrenergic-mediated fat accumulation in liver during aging.

In summary, our studies using rodent models demonstrate accumulation of lipid in the liver with aging, and suggest a role for increased β-AR-mediated signaling intrinsic to the liver as a mediator of age-related steatosis. Our findings are likely to have important clinical implications, since fatty liver may be involved in the pathogenesis of multiple metabolic risk factors and atherosclerotic cardiovascular disease (Stefan et al. 2008, Targher et al. 2010). Interventions to prevent or treat hepatic steatosis (and more advanced stages of NAFLD) are limited. In this regard, although β-AR antagonists are in wide clinical use for the treatment of cardiovascular disorders (Aronow et al. 2007, Wisler et al. 2007), the effects of these agents on lipid metabolism in the liver during aging are not currently known. Notably, fatty liver also occurs in severely burned patients subject to increased circulating catecholamine levels; and treatment of these patients with propranolol can reduce hepatic fat storage not only by decreasing lipolysis and delivery of free fatty acids to the liver but also by increasing the efficiency of the liver in secreting re-esterified fatty acids as VLDL triglycerides (Aarsland et al. 1996, Morio et al. 2002). Our current results showing inhibition of hepato-cellular lipid accumulation by β-AR subtype selective and nonselective antagonists suggest the possible utility of hepatic β-AR blockade as a therapeutic modality in older individuals with, or at risk of developing, fatty liver. Ongoing studies in our laboratory to define the pathogenetic mechanisms involved in β-AR-mediated hepatic steatosis during aging are expected to provide additional rationale for new therapeutic targets in the treatment or prevention of metabolic disorders common in the elderly.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References


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