Hepatic Gluconeogenic Capacity From Various Precursors in Young Versus Old Rats

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Hepatic gluconeogenic capacity was studied in young (4 months of age) and old (24 months of age) male Fischer 344 rats fasted for 24 hours using the isolated hepatocyte technique. Following the isolation of liver cells, the following precursors were added to the cell suspensions and incubated for 30 minutes: lactate (5 mmol/L), pyruvate (5 mmol/L), alanine (5 mmol/L), glutamine (5 mmol/L), oxaloacetate (5 mmol/L), glycerol (5 mmol/L), dihydroxyacetone (10 mmol/L), fructose (10 mmol/L), or saline (no precursor addition). To confirm that glucose production reflects gluconeogenic capacity, there was significant depletion of hepatic glycogen after the 24-hour fast and minimal alterations in glycogen content once substrates were added. Adjusting the gluconeogenic rates to reflect 100% cell viability resulted in no difference between young and old animals for any substrate used with the sole exception of fructose. The hepatic glucose production from fructose was 34% greater for young versus old animals. The results suggest that following a period of starvation the basal glucose production rates from hepatocytes, incubated with precursors entering the gluconeogenic pathway prior to fructose-6-phosphate, are equivalent in young and old rats.

ENVIRONMENTAL (eg, social or occupational) and physiologic (eg, exercise or disease) stress can elevate the metabolic demands placed upon an individual. A distinguishing characteristic of senescence is the attenuated ability of the organism to metabolically respond to a given stress.[1] The reduced capacity to respond to stressful stimuli has been attributed to a decline in the function of various organs. The loss of function could be the result of specific alterations within the cells comprising the organ, changes in neurohormonal regulation, or impairments in the response to neurohormonal stimulation.[2] In this regard, there is mounting evidence that suggests a decline in glucose metabolism attributable to senescence. Specifically, glucose intolerance,[3] impaired insulin-mediated skeletal muscle glucose uptake,[3,4] and a diminished capacity to mobilize hepatic glycogen[5,6] are some of the age-related declines associated with glucose metabolism.

More recent reports in rats[7-10] have implicated an age-related decline in the gluconeogenic capacity of the liver that can further affect glucose metabolism. Podolin et al[8-10] observed glucose production rates that were more than 2-fold lower from liver slices of old versus young animals. This decline in hepatic function could hinder the maintenance of blood glucose levels that would subsequently limit the function of the brain and peripheral nerves. In combination with the other decrements in glucose metabolism associated with senescence, the capacity of an elderly individual to metabolically respond to and handle a given stress would be severely compromised. However, the decline in hepatic gluconeogenic capacity attributable to age is not a consistent observation. Kmiec and Mysliwski[11] reported no decline in gluconeogenic capacity in isolated hepatocytes from young and old animals when incubated with several precursors. In contrast, Satrustegui et al[12] measured in vivo basal gluconeogenesis using a radioactive tracer injected intraperitoneally and observed an increase in the aged rat. Finally, we failed to observe a decline in glucose production capacity from isolated hepatocytes of old versus young animals incubated with lactate.[13]

It is conceivable that the disparate observations pertaining to age and hepatic gluconeogenic capacity could be attributable to the different preparations used (ie, liver slices *v* isolated hepatocytes). In addition, most studies that examine the impact of age on hepatic glucose production use only lactate, the primary in vivo gluconeogenic precursor. However, it remains a possibility that glucose production capacity can be altered with age at gluconeogenic sites distinct from lactate. We are aware of only 1 study that specifically examined the impact of age on hepatic gluconeogenic capacity with use of various precursors.[11] However, the precursors examined were the prominent substrates found in vivo, which critically limits the number of entry sites into the gluconeogenic pathway. Thus, the purpose of the present investigation was to examine the impact of age on hepatic gluconeogenic capacity using isolated hepatocytes and a variety of precursors that enter the glucose synthesis pathway at numerous sites. The use of various precursors would help to delineate the specific location for any decline attributable to age. We chose the isolated hepatocyte technique to maximize our ability to examine numerous precursors and to minimize the damage to cells (this technique evokes less injury to cells compared with liver slices, in addition, cell viability can be quantified). Based upon our initial observation with lactate,[13] it was our hypothesis that the gluconeogenic capacity of the liver would not be different between these 2 age groups, a supposition in direct contrast to recent reports.

MATERIALS AND METHODS

The experimental protocol for this study was preapproved by the Chapman University Institutional Review Board and conformed with the Public Health Service policy on the use of experimental animals for research. Male Fischer 344 rats were obtained from the National Institute on Aging and were either 4 months (n = 7) or 24 months (n = 7) of age upon arrival. Animals were individually housed, had free

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access to food and water, and were kept on a 12-hour light/dark cycle. All animals were acclimated to the living conditions for 1 week prior to the initiation of any experiments, and all experiments were performed during the same time of day. Finally, an equivalent number of young and old animals were used on given day. As such, all solutions were prepared on the day of the experiment and used on both young and old hepatocytes.

Twenty-four hours before the experiment, all food was withdrawn, whereas water continued to be provided ad libitum. We have previously demonstrated significant depletion of liver glycogen stores after a 24-hour fast, thereby minimizing the glucose production derived from glycogenolysis.[13-15] Following the 24-hour fast, the liver was surgically isolated as described by Mortimore[16] and as previously reported by our laboratory.[14,15] After the surgical isolation of the liver, the animal was placed in a humidified and temperature-controlled (37°C) Plexiglas perfusion chamber, identical to the perfusion chamber described in detail by Exton and Park.[17] Before entering the liver, the perfusate was sequentially filtered through a nylon mesh, oxygenated (95%:5%, O_2:CO_2), and then passed through a bubble trap.

Hepatocytes were isolated using a minor modification to the method described by Chan.[18,19] Briefly, calcium-free Krebs-Hensleit bicarbonate buffer (pH 7.35 to 7.45) and fresh thoroughly washed bovine red blood cells (hematocrit of 13% to 15%) perfused the liver (single-pass) for 10 minutes at a rate of 35 mL/minute. Collagenase (30 mg) was then added to the perfusate, the flow rate was adjusted to approximately 2 mL/min × g liver, and the system was switched to a recirculation mode for 20 to 30 minutes while being visually monitored for digestion. The liver was carefully removed from the animal and placed in a petri dish containing an incubation buffer similar to the perfusate, but with the addition of calcium (2.4 mmol/L) and the absence of collagenase. Scissors were used to open the liver capsule until there was a concentrated homogenate of cells. The cells were then funneled into a 250-mL Nalgene flask (Rochester, NY), aerated, and shaken in a 37°C water bath for 10 minutes. The cells were filtered through 2 layers of a nylon mesh and centrifuged. The supernatant was aspirated, and the cells were washed 3 more times using an incubation buffer now containing 1% (wt/vol) gelatin (DIFCO, Detroit, MI). Prior to the wash and every time after each wash, a small aliquot was tested for viability using a trypan blue exclusion test. Following the final wash, the cell volume was measured, and the cells were reconstituted (14 mL buffer/1 mL cells) in an incubation buffer now containing 1.5% (wt/vol) gelatin (DIFCO). The cell suspensions from a single liver were then separated into 50-mL plastic flasks and attached to a 10-lane manifold that allowed for continual gassing with O_2:CO_2 (95%:5%) while being gently shaken in a 37°C water bath.

The following precursors were added to the cell suspensions to bring the final concentration indicated in parentheses: lactate (5 mmol/L), pyruvate (5 mmol/L), alanine (5 mmol/L), glutamine (5 mmol/L), oxaloacetate (5 mmol/L), glycerol (5 mmol/L), dihydroxyacetone (10 mmol/L), fructose (10 mmol/L), or saline (no precursor addition). Precursor solutions were neutralized (where applicable) prior to their addition to the cell suspensions. All cell suspensions were incubated for 30 minutes. Aliquots of the cell suspension from each precursor were assayed for glucose,[20] glycogen,[21] and when appropriate, protein[22] using bovine albumin as the standard. The pH of the cell suspension was checked after the incubation to ensure a range between 7.35 to 7.45.

Calculations and Statistics

Gluconeogenic rates are expressed as the difference between exogenous glucose production (with substrate) and endogenous glucose synthesis (no substrate). The final gluconeogenic rate and glycogen content are divided by the cell viability and expressed as the concentration (nmol) per milligram of protein. For glutamine and alanine, the determination of protein content could not be distinguished between the precursor used and the content within the hepatocytes. As such, an average of the protein content of the hepatocytes from a given animal incubated with the other substrates (that varied less than 15%) was used as the protein content for glutamine and alanine. A Student's *t* test was used to compare rates, levels (eg, pH), and concentrations (eg, hepatic glucose production) between groups with the level of significance set at $P < .05$. For the examination of alterations in glycogen content following the addition of a substrate, an analysis of variance (ANOVA) was used ($P < .05$) and when appropriate, a Tukey post hoc test was used to identify significant differences. All values are expressed as the mean ± SE.

RESULTS

The body weights for the young animals, 262.8 ± 1.4 g, were significantly lower than the old animals, 370.4 ± 10.1 g, after the 24-hour fast. Following the hepatocyte incubation, the pH of the cell suspensions did not significantly differ between young, 7.40 ± 0.02, and old animals, 7.41 ± 0.01. There was no decrement in cell viability attributable to the washing procedure within a group (for brevity, only the results from the prewash and final wash are reported), young animals 87.2% ± 0.5% (prewash) versus 87.4% ± 0.4% (final wash) and old animals, 71.3% ± 2.5% (prewash) versus 70.8 ± 1.1 (final wash), respectively. However, the hepatocyte viability was significantly greater for young compared with old animals.

The protein content from the cell suspensions was not significantly different between groups, 3.62 ± 1.08 mg protein/mL versus 3.57 ± 0.98 mg protein/mL, young versus old, respectively. The initial glycogen content in the liver cells prior to the addition of any substrate was not significant between young, 8.81 ± 0.89 nmol/mg protein and old animals, 8.17 ± 1.05 nmol/mg protein. The glycogen content 30 minutes after the addition of a given substrate was not significantly different from the initial glycogen content in either group, indicative of minimal glycogen deposition or synthesis. In addition, the hepatic glycogen content following the addition of a substrate was not significantly different between groups (Fig 1).

Adjusting the rate to reflect 100% cell viability resulted in no difference between young, 44.15 ± 4.91 nmol/mg protein and old animals, 42.76 ± 8.35 nmol/mg protein for endogenous (no substrate) hepatic glucose production. In addition, expressing the gluconeogenic rate as the difference between exogenous and endogenous glucose production, as well as adjusting it to reflect 100% cell viability resulted in no significant difference between young and old animals for any substrate used with the sole exception of fructose (Fig 2). The hepatic glucose production from fructose was significantly greater for young versus old animals.

DISCUSSION

In the current study, there was a decline in liver cell viability attributable to age. It is unknown whether the lower cell viability from the old animals is an inherent loss in organ function (ie, at any given time, there are less viable cells) or another aspect of the isolation procedure that compromises the integrity of apparently fragile senescent cells. In support, the same solutions were used on both young and old hepatocytes eliminating the possibility of contaminants. In addition, the loss of

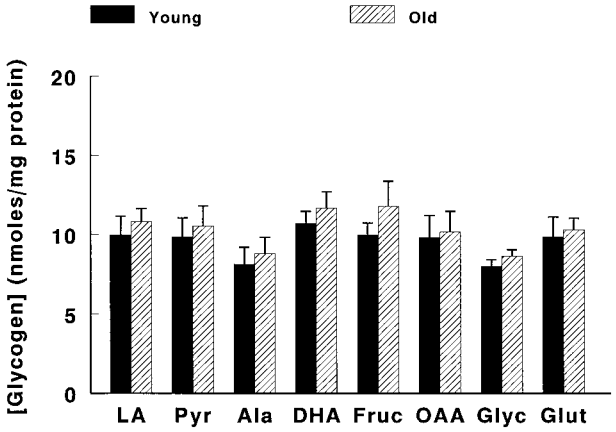

Fig 1. Effects of precursor incubation on glycogen content in isolated hepatocytes from young (n = 7) and old (n = 7) animals. The precursors added to the cell suspensions were: lactate (LA), pyruvate (Pyr), alanine (Ala), dihydroxyacetone (DHA), fructose (Fruc), oxaloacetate (OAA), glycerol (Glyc), and glutamine (Glut). Values are means ± SE. Differences between groups for each precursor were not significant.

cell integrity from old animals was not due to the washing procedure (the protocol where the greatest damage to cells can occur). Despite the difference in cell viability, the fact that the hepatocytes synthesized glucose at rates similar to that reported by others who used distinct substrates[11,17] suggests that the remaining cells maintained their integrity. As such, when cell viability was equalized, there was no decline in gluconeogenic capacity from isolated hepatocytes of young and old animals using various precursors. However, the gluconeogenic capacity from fructose was 34% lower for old animals compared with young animals. That the glucose production reflects gluconeogenic capacity is supported by the depletion of hepatic glycogen after the 24-hour fast, as measured prior to the incubation with precursors, and the nonsignificant alterations in glycogen content once substrates were added.

The current results support our hypothesis indicating that hepatic gluconeogenic capacity was not different between young and old animals. This is predicated upon the use of various precursors entering the gluconeogenic pathway at distinct locations. Specifically, lactate, pyruvate, and alanine converge at a common site, pyruvate, prior to its entry into the mitochondria. Glutamine enters the mitochondria at a different site, while oxaloacetate enters the gluconeogenic pathway outside the mitochondria prior to its conversion to phosphoenolpyruvate. Glycerol and dihydroxyacetone also enter the gluconeogenic pathway in the cytosol, but at the level of the triose phosphates. Glucose production was similar between groups from each of these precursors. As such, to the extent that these in vitro observations can be extrapolated to in vivo conditions, the results suggest that any decline in glucose metabolism in response to stress are not due to a loss in hepatocyte function. In contrast, that glucose production from fructose was different between groups suggests an alteration in the enzymatic conversion(s) above the level of fructose-6-phosphate or a possible difference in transport mechanisms for glucose out of the cell. As observed by others,[11,17] fructose yields the greatest hepatic gluconeogenic rates since it is beyond the influence of phosphoenolpyruvate carboxykinase (PEPCK), the rate-limiting enzyme for hepatic gluconeogenesis[7,23] from the principal 3 carbon precursors found in vivo. While the technique utilized does not mimic in vivo conditions, it does attempt to stress the gluconeogenic pathway with use of distinct precursors at specific sites. Thus, the difference in glucose production from fructose suggests that with age, glucose-6-phosphatase or glucose transport capacity could not be sustained at the rates observed for the young animals.

Our results are consistent with Kmiec and Mysliwski,[11] who similarly reported no alterations in gluconeogenic capacity attributable to age in isolated rat hepatocytes when incubated with lactate/pyruvate, alanine, and glycerol. We have now extended the observation to include: glutamine, oxaloacetate, dihydroxyacetone, and separate observations for both lactate and pyruvate (rather than a lactate/pyruvate combination). However, in contrast to Kmiec and Mysliwksi,[11] we observed greater glucose synthesis from fructose in young versus old animals. Our observation is consistent with Bois-Joyeux et al,[23] who reported enhanced glucose-6-phosphatase activity in young rats following a period of starvation. Albeit changes in maximal enzyme activity do not represent the actual flux through the gluconeogenic pathway nor does it address potential changes in transport capacity. As glucose-6-phosphatase is the limiting enzyme for glucose production from fructose, this could help to explain the elevated gluconeogenic rates in the hepatocytes from young animals. In addition, Podolin et al[10] reported greater [^{14}C]fructose incorporation into glycogen in liver slices from young compared with old animals in the absence of an increase in net glycogen content. Similar to Podolin et al,[10] we did not observe any increase in net glycogen deposition from young versus old animals from fructose or any substrate used. Alternatively, if the conversion of fructose to glucose from old liver cells was compromised due to glucose-

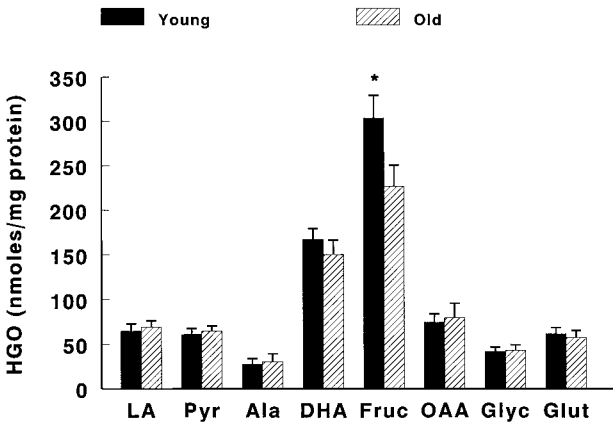

Fig 2. Effects of age on hepatic glucose production (HGO) in isolated hepatocytes from young (n = 7) and old (n = 7) animals. The precursors added to the cell suspensions were: lactate (LA), pyruvate (Pyr), alanine (Ala), dihydroxyacetone (DHA), fructose (Fruc), oxaloacetate (OAA), glycerol (Glyc), and glutamine (Glut). Values are means ± SE. *Significant difference between young and old animals, $P < .05$.

6-phosphatase, it is conceivable that the radioactive fructose taken up would subsequently remain within the cell. The radioactive fructose within the cell could be misinterpreted as glycogen when radioactive counts are used as an indicator for glycogenesis. This could account for the elevation in [^{14}C]fructose incorporation into glycogen in the absence of any net increase in glycogen deposition.

The preservation of hepatic gluconeogenic capacity with increasing age is in contrast to the recent studies of Podolin et al,[8-10] who reported age-related declines in 15- and 25-month-old rats. However, if a decrease in cell viability, as observed in the current study, represents an overall loss in organ function and/or the fragile integrity of older hepatocytes, then this may partially explain the discrepancy. In addition, Podolin et al used liver slices to assess hepatic gluconeogenic rates, a technique that can result in further damage to cells. Nevertheless, the decline in cell viability cannot account for their observation of a substantial decrease in PEPCK activity in whole liver homogenates from 25-month-old animals[8-10] that was observed to occur as early as 15 months in rats.[8] However, the age-related decline in PEPCK activity is not a consistent observation in animals.[6,23] Victorica et al[24] failed to observe a decline in hepatic PEPCK activity in rats attributable to senescence (17 months of age). Similarly, Van Remmen and Ward[25] reported no difference in PEPCK activity prior to and in response to fasting in young (3 months) versus older (18 months) rats. The specific discrepancy between these observations compared with Podolin et al[8-10] is unknown, but taken as a whole, these varying reports in PEPCK could be indicative of an advanced age when liver function declines and is not reversed. In support, Wimonwatwatee et al[26] observed an age-related decline in both PEPCK activity and PEPCK mRNA from 26-month-old compared with 6-month-old fasted rats. In addition, it is also possible that hepatic gluconeogenic capacity can fluctuate during the 4- and 24-month period examined in this study. As such, our results are confined to these 2 time periods with recognition that positive and negative alterations could occur during the age range of these animals.

In stark contrast to the maintenance or decline in hepatic gluconeogenic capacity, Satrustegui et al[12] reported a 3-fold increase in basal gluconeogenic capacity in old (21 months) rats as assessed by in vivo rates of lactate incorporation into blood glucose. However, they also reported higher glucagon levels and lower plasma glucose concentrations from the old animals at the time of sacrifice. As such, this may reflect differences in diurnal variation between young and old animals rather than a persistent increase in basal gluconeogenic capacity. Alternatively, this could confirm the fluctuation in hepatic gluconeogenic capacity prior to a critical age when cell function irreversibly declines. Finally, Satrustegui et al[12] reported greater body weights for old compared with young animals. In like manner, we also observed greater body weights for the older animals. Thus, it remains a possibility that obesity could contribute to an elevation in basal hepatic gluconeogenesis. This potential factor is minimized by the similar rates we observed between young and old animals for endogenous hepatic glucose production.

In summary, the results suggest that following a period of starvation the basal glucose production rate from hepatocytes, incubated with precursors entering the gluconeogenic pathway prior to fructose-6-phosphate, are not different between young and old animals. However, it remains to be determined if hepatic gluconeogenic capacity fluctuates between the time period examined and if it declines irreversibly with more advanced age (ie, greater than 24 months in rats). Moreover, whether the absence of any difference in basal hepatic gluconeogenic capacity at 24 months of age translates into the maintenance of glucose homeostasis under various in vivo conditions of stress and in response to neurohormonal stimulation remains to be elucidated. Several investigators have implicated an age-related decline in hepatic gluconeogenic response to glucagon[9,11] and norepinephrine[8] in rats. This would lend support to the contention that age-related declines in hepatic gluconeogenic capacity would be due to alterations in neurohormonal regulation and/or impairments in neurohormonal response, but not a loss in basal hepatocyte function.

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