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Maternal perinatal calorie restriction temporally regulates the hepatic autophagy and redox status in male rat

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Abstract

Intrauterine growth restriction (IUGR) leads to adult obesity, cardiovascular disease, and non-alcoholic fatty liver disease/steatohepatitis. Animal models have shown that combined intrauterine and early postnatal calorie restriction (IPCR) ameliorates these sequelae in adult life. The mechanism by which IPCR protects against adult onset disease is not understood. Autophagy, a lysosomal degradative process, recycles cellular constituents and eliminates damaged organelles, proteins, and oxidants. In this study, we hypothesized that IPCR could regulate autophagy in the liver of male rat offspring. At birth (d1) of male IUGR rat offspring and on day 21 (p21) of life, IPCR male rat offspring had a profound decrease in hepatic autophagy in all three stages of development: initiation, elongation, and maturation. However, upon receiving a normal diet ad-lib throughout adulthood, aged IPCR rats (day 450 of life (p450)), had increased hepatic autophagy, in direct contrast to what was seen in early life. The decreased autophagy at d21 led to the accumulation of ubiquitinated proteins and lipid oxidative products, whereas the increased autophagy in late life had the opposite effect. Oxidized lipids were unchanged at d1 by IUGR treatment indicating that decreased autophagy precedes oxidative stress in early life. When cellular signaling pathways regulating autophagy were examined, the 5′ adenosine monophosphate-activated protein kinase pathway (AMPK), and not endoplasmic stress pathways, was found to be

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Competing interests

The author declares that there are no competing interests associated with this manuscript

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.freeradbiomed.2018.09.029.

altered, suggesting that autophagy is regulated through AMPK signaling pathway in IPCR rats. Taken together, this study reveals that the perinatal nutritional status establishes a nutritionally sensitive memory that enhances hepatic autophagy in late life, a process that perhaps acts as a protective mechanism to limited nutrition.

Keywords

Intrauterine growth restriction; Autophagy; Perinatal calorie restriction; Intrauterine and postnatal calorie restriction; Autophagy; Oxidative stress; AMP activated protein kinase; Glutathione; Glucose-6-phosphate dehydrogenase

1. Introduction

The fetal origin of adult disease hypothesis posits that the nascence of adult chronic disease occurs during *in utero* growth. A large body of long-term observational human studies, pioneered by the late David Barker established that a simple biometric assay of birth weight could largely predict the development of adult onset diseases such as obesity and cardiovascular diseases [1]. Guided animal studies performed by different laboratories have confirmed the observations made in humans and have provided a mechanistic association of fetal growth conditions to long term animal health [2]. Recently we have published a discovery driven study examining hepatic transcriptome changes in the early (p21, at the weaning stage-p21) and late (day 450-p450) life of male rats subjected to perinatal calorie restriction [3]. In these studies, there was an interesting group of animals treated with combined intrauterine and postnatal calorie restriction (IPCR) which were protected from the long-term adult sequelae found in the fetal only calorie restriction group. In addition to being protected from the maladaptive fetal programming which leads to adult onset disease, these animals were diminutive in final adult size [3,4].

Autophagy is an intracellular recycling pathway that is active at basal levels in most cell types where it is claimed to play a housekeeping role in maintaining the integrity of intracellular organelles by removing damaged organelles and oxidative products [5]. In addition, autophagy is known to be strongly induced by starvation/calorie restriction and is thought to be an adaptive response which promotes cell survival until sufficient nutrients become available again [5]. Many signaling cascades are known to be involved in autophagy regulation: AMPK- activated protein kinase, endoplasmic stress, mammalian target of rapamycin, hypoxia induced factor 1α and reactive oxygen species (ROS) [5-10]. Further, the relationship of autophagy to ROS induced oxidative stress is not straightforward, as there are biological examples where, autophagy is increased in acute oxidative stress conditions (*In vitro*) in order to eliminate the oxidative modified proteins/lipids. In alternative examples, chronic increased oxidative stress leads to decreased autophagy. Understanding that fetal growth restriction is largely a result of limited nutritional resources, we hypothesized that autophagy, would be increased in the animals subjected to IPCR. Contrary to our intuitive conclusion, as well as other published data describing increased autophagy in calorie restricted adult animals, the discovery driven transcriptome studies found that p21 IPCR male rats exhibited decreased hepatic expression of autophagy related proteins.

Furthermore, our current studies have revealed that IPCR results in decreased autophagy function such as clearance of ubiquitinated and oxidized proteins/lipids.To complete a comprehensive life analysis of autophagy, further samples collected at birth, revealed decreased autophagy without an increase in oxidative stress, while aged liver samples collected at p450, were characterized by an increase in autophagy that was associated with decreased oxidative stress. In addition, we observed that the modulation of autophagy is associated with AMPK signaling. Presented herein are conclusive studies which demonstrate that perinatal calorie restriction temporally regulates hepatic autophagy in IPCR male rats. The nutritionally sensitive signals established in the perinatal period, augments hepatic autophagy in late life and may be a defensive mechanism against metabolic syndrome associated disease.

2. Methods

2.1. Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Research Committee of the University of California, Los Angeles (Permit Numbers: 2014-127-03 (WAF) and 1999-104-42 (SUD)) and all efforts were made to minimize suffering.

2.2. Animals

Pregnant Sprague-Dawley rats (8–10 weeks old, 225–250 g; Charles River Laboratories, Hollister, CA) were housed in individual cages with ad libitum access to water, exposed to 12-h light/dark cycles at 21–23 °C, and were fed standard rat chow (NIH-31 Modified Open Formula Mouse/Rat Sterilizable Diet composed of 63.9% carbohydrate, 6.25% fat, and 18.6% protein; product number 7013, Harlan Industries, Indianapolis, IN). Upon birth, gender was identified and litters were culled to 6 male newborn pups with litter birth weights closest to the median weight. Maternal cross-fostering was employed in all experimental groups.

2.3. Prenatal and postnatal nutrition management

Control-fed rats (Con) were given ad libitum access to feeding throughout gestation, and when appropriate for age at tissue collection, through lactation, and adulthood. Control offspring were cross fostered by mothers who received ad libitum feeding throughout gestation and lactation, and upon weaning at parturition p21 (p21), received ad libitum feeding as juveniles and adults. The rats exposed to intrauterine calorie restriction and postnatal calorie restrictions were born from mothers who had 50% calorie restriction (11 g/day embryonic period from embryonic day 11 (e11) through birth; 20 g/day lactation period-birth through weaning at the 21st day of life (p21)). For day 450, the IPCR group received ad libitum feeding as juveniles and adults beginning after weaning.

2.4. Gene expressions study by Quantitative reverse-transcription polymerase chain (QPCR)

Hepatic Gene expression was determined by reverse transcription and Taqman based quantitative polymerase chain reaction (RT-qPCR). Sequences of the primers and probes assay id are found in Supplementary Table S1. One μg of RNA was used as a template for reverse transcription using Super Script III in conjunction with an oligo-dT primer according to manufacturer's protocol (Invitrogen). This cDNA was used as a template for RT-qPCR using the Step One Plus Real Time PCR System thermocycler (Applied Biosystems, Foster City, CA) using the following parameters: 50 °C for 2 min, 95 °C for 20 s, then 40 cycles of 95 °C for 15 s and 60 °C for 20 s. Data were normalized to the amplification of Rpl13a and relative quantification to the control sample was made using the ΔΔCt. Data is displayed as relative quotient (RQ) with error bars representing the standard error of the mean calculated from the power base 2 transformed variance of the ΔΔCt.

2.5. Western blot analysis

Hepatic tissues were homogenized with lysis buffer containing 0.1 M NaCl, 5 mM EDTA, 50 μM sodium orthovanadate, 1% Triton X-100, and protease inhibitor tablet (Roche Diagnostics, Indianapolis, IN) in 50 mM Tris buffer, pH 7.5. 50 μg of proteins per sample were resolved by 4–20% SDS-PAGE, transferred onto nitrocellulose membranes for 1 h and blocked in TBST containing 3% milk protein for 1 h. Atg12, Atg7, Beclin, Bcl2, Atg3, LC3I/II, AMPK, p-AMPK antibodies were used at 1:1000 (Cell signaling technology, Danvers, MA), and mouse vinculin at 1:5000 (Sigma Aldrich, St. Louis, MO, USA). Primary antibodies were diluted in TBSTcontaining 3% milk protein, incubated at 4 °C overnight. The membranes were probed with their respective secondary antibodies (1:5000) for 1 h and proteins illuminated using an ECL Plus Western blotting kit (GE Healthcare, UK).

2.6. Enzymes linked immunosorbent assays

4-Hydroxynonenal (4HNE) (Abcam, Cambridge, MA, USA) was measured by ELISA. Briefly 50 μg liver tissue homogenate were loaded onto wells and were incubated at 4 °C for overnight. Wells were blocked with 5% milk at 37 °C for 1hrs. HNE antibody was diluted in TBS containing 5% milk protein and was incubated at 4 °C for overnight. After washing three times with washing buffer, HRP-peroxidase was added to each well and incubated for 1 h at RT with gentle shaking. Following this, wells were washed three times with TMB solution (100 μL) was added, and was incubated for 30 min at RT in the dark room. Color density was measured at 450 nm after adding stop solution. For ubiquitinated proteins, mono and polyubiquitinylated conjugated monoclonal antibody HRP conjugate was used at 1:5000 and remaining steps were carried out as described above.

2.7. Biochemical assays

Catalase, and G6PD activity and GSH levels were measured as described previously [11-13].

2.8. Electron microscope study

Pieces of liver were dissected, fixed with 5% glutaraldehyde, 2% paraformaldehyde in 0.1 M phosphate buffer, 0.9% sodium chloride (PBS) for 2 h at room temperature in the same fixative and stored at 4 °C until processing. Then tissues were washed with PBS, post fixed in 1% OsO4 in PB for 1 h, dehydrated in a graded series of ethanol, treated with propylene oxide and infiltrated with eponate 12 (Ted Pella) overnight. Tissues were embedded in fresh eponate, and polymerized at 60 °C for 48 h. Approximately 60–70 nm thick sections were cut on a RMC Powertome ultramicrotome and picked up on formvar coated copper grids. The sections were stained with uranyl acetate and reynolds lead citrate and examined on a JEOL 100CX electron microscope at 60 kV. Images were collected on type 4489 EM film and the negatives scanned to create digital files.

2.9. Estimation of Protein in the liver of IPCR animal

Liver tissue was homogenized with triton lysate buffer and protein was estimated using Bradford method as described in the manufacture protocol.

2.10. Statistical analysis

Data was expressed as mean ± SEM where indicated. Statistical differences were analyzed using the Student's *t*-test using Prism software. *p* values less than 0.05 were considered statistically significant.

3. Results

3.1. Decreased autophagy markers in the liver of p21 IPCR male rat offspring

Discovery-driven microarray analysis of male offspring subjected to IPCR reveals hepatic down-regulation of many of the genes that are involved in the autophagy process. (Supplementary Fig. 1). As a survey of autophagy related genes, QPCR was performed. As seen in Fig. 1A hepatic gene expression of the autophagy initiation protein Beclin [14] (Fig. 1A) and the autophagy elongation proteins Atg 7, Atg3, Atg12 and Atg 5, (Fig. 1A) are significantly down-regulated by perinatal calorie restriction, confirming the results found in the microarray screen. Surprisingly the expression of Map1lc3a, a known autophagy elongation protein, was increased [14,15] (Fig. 1A). The autophagy termination phase gene p62 was significantly up-regulated [15] (Fig. 1A).

To verify that the identified gene expression changes resulted in a change at the protein level, immunoblotting was performed. Consistent with the gene expression results beclin 1, Atg7, Atg3, Atg12and Atg5 levels were decreased while P62 level was increased in the IPCR animal (Fig. IB-C). IPCR treatment resulted in an increase in the level of LC 3A/B-I (LC3-I) protein. However, LC 3A/B-II (LC3-II), was decreased. (Fig. 1B-C). These decreased autophagy proteins translate into significantly less autophagosomes as seen in the transmission electron microscope studies (Fig. 1D).

3.2. Decreased autophagosomes result in increased polyubiquitinated proteins and oxidative stress in the liver of p21 IPCR animals

Autophagosomes are known to degrade and recycle proteins. This process is initiated by ubiquitin attachment followed by sequestration to the autophagosome by the transport protein p62 [16]. It is generally thought that the rate-limiting step of ubiquitinated protein clearance correlates to the level of autophagy present. Therefore, polyubiquitinated protein content was measured by ELISA and p62 were assayed using Western blotting. Polyubiquitinated protein and p62 protein levels were significantly increased in the liver of IPCR treated rats. (Fig. 2A and IB). Impaired autophagy leads to increased polyubiquitinated proteins via decreased clearance, which collectively leads to oxidative stress. Therefore, we measured both 4-HNE, a pro-oxidant created by lipid peroxidation, as well as glutathione, an important intracellular reducing agent which acts as an antioxidant. As seen in Fig. 2B and C, 4HNE was significantly increased and glutathione was significantly decreased in the liver of the IPCR animals as compared to control rats.

3.3. AMPK down-regulation is associated with decreased autophagy in the liver of p21 IPCR animals

Previously it has been shown that autophagy is regulated by endoplasmic reticulum stress and by AMP activated protein kinase (AMPK) [17,18]. Therefore we examined the ER stress markers, ATF4, XBP, and ATF6. The levels of phospho-AMPK were significantly decreased by IPCR treatment, while the amount of total AMPK remained the same in both groups (Fig. 3 A-C). However, there was no difference in the ER stress markers between control and IPCR treated animals (Fig. 3D).

3.4. Antioxidant enzymes levels and activities in the liver of p21 IPCR rats

The cell responds to oxidative stress by inducing or repressing a group of antioxidant enzymes which catalytically reduce reactive oxygen species. These enzymes include catalase, copper-containing superoxide dismutase (CuSOD), glutathione peroxidase, glutathione reductase and glucose-6-phosphate dehydrogenase (G6PD) [19]. We examined the expression of these antioxidant genes through QPCR and found that catalase and G6PD are the only examined antioxidants decreased by IPCR treatment (Fig. 4A) at p21. To verify that the identified gene expression changes resulted in a change at the protein level, immunoblotting and enzyme activity were performed for catalase and G6PD. Both catalase and G6PD were decreased at both the protein and activity level (Fig. 4B-F). Other antioxidants enzymes including PON1, PON2, and PON3 were unchanged by IPCR treatment (data not shown).

3.5. Hepatic Autophagy related proteins, AMPK, p-AMPK ubiquitinated protein and oxidative stress marker in the liver of d450 IPCR animal

To understand the effects of aging on the IPCR liver, IPCR treated animals were given ad lib access to a normal diet from weaning to old age (d450). Liver samples were then interrogated by Western blotting for the autophagy genes Beclin, Atg7, Atg3, Atg12, Atg5, LC3-I/LC3-II, and p62. All autophagy genes were increased compared to control (Fig. 5A-B) with decreased polyubiquitinated protein and 4HNE (Fig. 5C-D). The level of p-AMPK

(Fig. 5E-G) was significantly increased in the liver of IPCR animals compared to control. There was no change in total AMPK levels (Fig. 5E-G). There were no differences in G6PD and catalase levels between control and IPCR (data not shown).

3.6. Hepatic autophagy related proteins, ubiquitinated protein, oxidative stress marker in the liver of day 1 IUGR rats

To understand the baseline attributes of liver autophagy, male offspring subjected to intrauterine growth restrictions were examined at birth. Liver samples were interrogated by Western blotting for the autophagy genes namely, Beclin, Atg7, Atg3, Atg12, Atg5, LC3-I/LC3-II, and p62. All the autophagy genes were decreased (Fig. 6A-B) in response to intrauterine calorie restriction, except p62, which was increased. Consistent with decreased autophagy, ubiquitinated protein levels were increased in the liver of IPCR animal (Fig. 6C), however there was no change in oxidative stress marker 4HNE (Fig. 6D). p-AMPK levels were decreased in the liver of IUGR animal as compared to that of control (Fig. 6E-G), however there was no change in total AMPK levels (Fig. 6E-G). There were no differences in G6PD and catalase levels between control and IUGR (data not shown).

Table 1 describes the effect of IPCR on the different stages of autophagy and oxidative stress

4. Discussion

4.1. Late life hepatic autophagy is altered by nutritional cues set during late fetal and early postnatal life

Autophagy plays a crucial role in maintaining homeostasis in nutrient limiting conditions by degrading and recycling redundant or aged cytoplasmic components. Experimental evidence suggests that calorie restriction increases autophagy, reduces the oxidative stress and ameliorates many of the metabolic associated diseases. However, autophagy regulation by maternal perinatal calorie restriction has not been investigated. Here we demonstrate that exposure to limited nutrition during a critical 31 day of the embryonic fetal and early neonatal period (E11 throughout p21) decreases hepatic autophagy in early life, however, increase hepatic autophagy in late life (at d450) despite exposure to an *ad lib* normal diet for 429 days. There are evidences suggesting that phosphorylation of AMPK regulates the autophagy and it is quite possible that in the present study AMPK signaling governs this process. The altered autophagy is positively associated with clearance of ubiquitinated and oxidative products. These studies reveal that late life hepatic autophagy is altered by nutritional signals established during late fetal and early postnatal life.

4.2. Every step of autophagy- initiation, elongation, and maturation- are coordinately altered by IPCR animals

Interestingly, there is coordinate regulation of autophagy markers at all three stages of autophagy development- initiation, elongation, and maturation at p21 and remarkable coordinated responses remain to old age. Table 1 summarizes the relative expression (versus controls) of autophagy initiation, elongation, and maturation proteins at birth, d1, p21, and p450. The autophagy initiation protein Beclin 1 has been shown to link the PI3 kinase signaling cascade to the pre-autophagsosmal structure, the site of initiation of autophagy

formation [14,15]. In addition, beclin 1 can interact with other proteins, most importantly Bcl2, which links the autophagic process to the cell survival pathways [14,15]. At birth and at p21, active calorie restriction leads to a decrease in beclin expression; however late in life, after the animals have received access to a normal diet throughout adulthood, beclin expression increases.

The elongation phase requires proteolytic cleavage of the ubiquitous cytoplasmic protein microtubule associated protein 1 light chain 3 alpha (Map1lc3a) to the LC3-I form, which subsequently becomes localized to the inner layer of the autophagosome membrane, thereby creating the LC3-II form [14,15].

The localization mechanism by which the LC3-I becomes tethered to the phosphatidylethanolamine, contained within the inner membrane of the autophagy vesicle, employs a three step enzymatic step process similar to the ubiquitin localization enzymes E1, E2 and E3. Atg7, Atg3 and the Atg5/Atg12 complex, homologous to the E1, E2 and E3 enzymes respectively, were all coordinately regulated by IPCR in both early and late life (Table 1). At p21 despite increased expression of Map1lc3a, active LC3-II form was reduced due to decreased expression of Atg3, Atg5, Atg7 and Atg 12. Furthermore, this striking display of coordinate regulation of autophagy related proteins indicate that there are likely conserved cis acting elements contained within the 5′ regulatory regions of these genes which are controlled by nutrient sensor that are yet to be identified.

Final maturation of an autophagosome requires association of LC3-II, which is bound to the inner autophagosome membrane, and to the sequestosome 1 protein (Sqst1, p62); this binding allows for ubiquitin degradation of the LC3-II/p62 complex [14,16]. Knockout mouse models of hepatic Atg7 demonstrate that autophagy deficiency is associated with increased p62 levels [20,21]. It is thought that when autophagy is rampant there is active ubiquitin-mediated destruction of the LC3-II/p62 complex. Interestingly, p62 levels are induced at the transcriptional level which is translated to protein level by IPCR treatment at all examined time points- birth, weaning, and late in life. It is known that p62 is a multifunction protein involved in the signaling pathways of NFKB and NRF2, as well as ubiquitin proteolysis [20,22,23] and autophagy [24]. Further directed studies will need to clarify the non-autophagy functions of p62 in IPCR treatment.

4.3. Early perinatal and adult postnatal nutrient limitation affects hepatic autophagy differently

Studies in adult rats have shown that postnatal calorie restriction leads to an increase in the autophagic recycling processes, ameliorates the age-dependent disease and promotes life extension [25,26]. Although these observations indicate that there is a fundamentally different hepatic response to adult postnatal versus early perinatal calorie restriction, neither the reasons for this difference are clear nor the point at which a juvenile liver response transitions to an adult response is known. It could be hypothesized that postnatal adult animals have adequate nutrient stores prior to the initiation of the calorie restriction, a luxury likely not afforded to chronically IPCR rats, and this may be a factor in determining the hepatic response. This further implies that impaired autophagy in early IPCR might be an adaptive mechanism, as the extremely starved status would result in a decreased need for

ATG related protein expression. Therefore, understanding and quantifying the antecedent hepatic stores of glycogen and lipids prior to the initiation of calorie restriction would be an important component for future examination.

4.4. Temporal regulation of autophagy is positively associated with clearance of ubiquitinated and oxidative products in IPCR offspring

Free radicals are formed from enzymatic and nonenzymatic reactions, and formed ROS oxidize various lipids or proteins under normal physiological conditions [27]. It is well documented that ROS are formed during phagocytosis [28], mitochondrial electron transports [29], prostaglandin synthesis [30], microsomal fatty acid oxidation [31], uric acid biosynthesis and cytochrome P-450 system [31], and once formed these ROS oxidize lipids and proteins under physiological conditions [27]. The constantly formed oxidized lipids/ 4HNE/ oxidized proteins are removed by the autophagy process [32,33]. IPCR leads to a significant hepatic oxidative stress as seen in the elevated levels of the oxidized protein 4HNE at p21. An important observation from these studies is that at birth 4HNE levels are comparable to control tissues indicating that the oxidative stress seen at weaning on p21 is likely an effect of the decreased autophagy. Note that the coordinated transcriptional response decelerating autophagy is already present at birth; however there is no evidence of oxidative stress. Furthermore, in day 1 IPCR, decreased autophagy is sufficient to remove the oxidative molecules similar to control group in contrast to ubiquitinated proteins. We therefore conclude that during active calorie restriction in the fetal and neonatal periods there is first a decrease in autophagy, which in turn leads to an increasein oxidative stress. In support of our findings, animal models employing knock out Atg7 mice [34] or Atg5 deficiency [35] which have shown that the loss of autophagy is associated with increased oxidized lipid levels and enhanced oxidative stress in advanced macrophage lesions, respectively. In our current study, it is also possible that chronic accumulation of ubiquitinated proteins disturb the cellular redox homeostasis thereby elevating ROS levels, which leads to increased 4HNE in p21 rats in addition to its decreased clearance. It is well documented that cells have a defensive pathway with GSH redox cycle and several enzymatic systems to protect oxidative stress [27]. However, on p21, there was no induction of antioxidant genes; rather expression of catalase and G6PD were decreased. It has been reported that 4-HNE pro-oxidant activates the survival signaling by inducing antioxidant enzymes in acute stress condition, whereas in chronic stress condition it inhibits the antioxidant gene expressions, further inducing tissue injury and inflammation [36]. In contrast, late in life this increased autophagy is associated with decreased 4HNE oxidative stress markers without altering the antioxidant gene expression suggesting that the increased autophagy results in the increased clearance of oxidized proteins.

4.5. Further directed mechanistic studies are needed to discern the actual mechanism by which IPCR regulates autophagy: epigenetic analysis and genetically modified animal models

It has been well documented that nutrient limitation during pregnancy results in changes in promoter methylation which directly alters gene expression in disparate physiologic processes [37]. The striking display of coordinated transcriptional regulation of autophagy genes found in early and late life suggests that these genes may share conserved 5′ upstream

regulatory regions. With this in mind, the presence of CpG islands in autophagy genes were examined using Methprimer software (http://www.urogene.org/methprimer). Remarkably, all of the autophagy genes studied in this study except Atg7 contains CpG islands. Future studies examining the epigenetic architecture of autophagy genes may yield insight into the mechanism by which nutrition controls gene transcription. Additionally, oxidative stress is known to affect the gene expression epigenetically [38] so understanding the role of oxidant and antioxidant factors will be of importance. Our studies suggest that nutritionally sensitive memory established in the late fetal and perinatal period, which enhances the hepatic autophagy in late life, might be a protective mechanism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

IPCR	Intrauterine and postnatal calorie restriction
IUGR	Intrauterine growth restriction
G6PD	glucose-6-phosphate dehydrogenase
4-HNE	4-Hydroxynonenal
AMPK	Adenosine monophosphate-activated protein kinase

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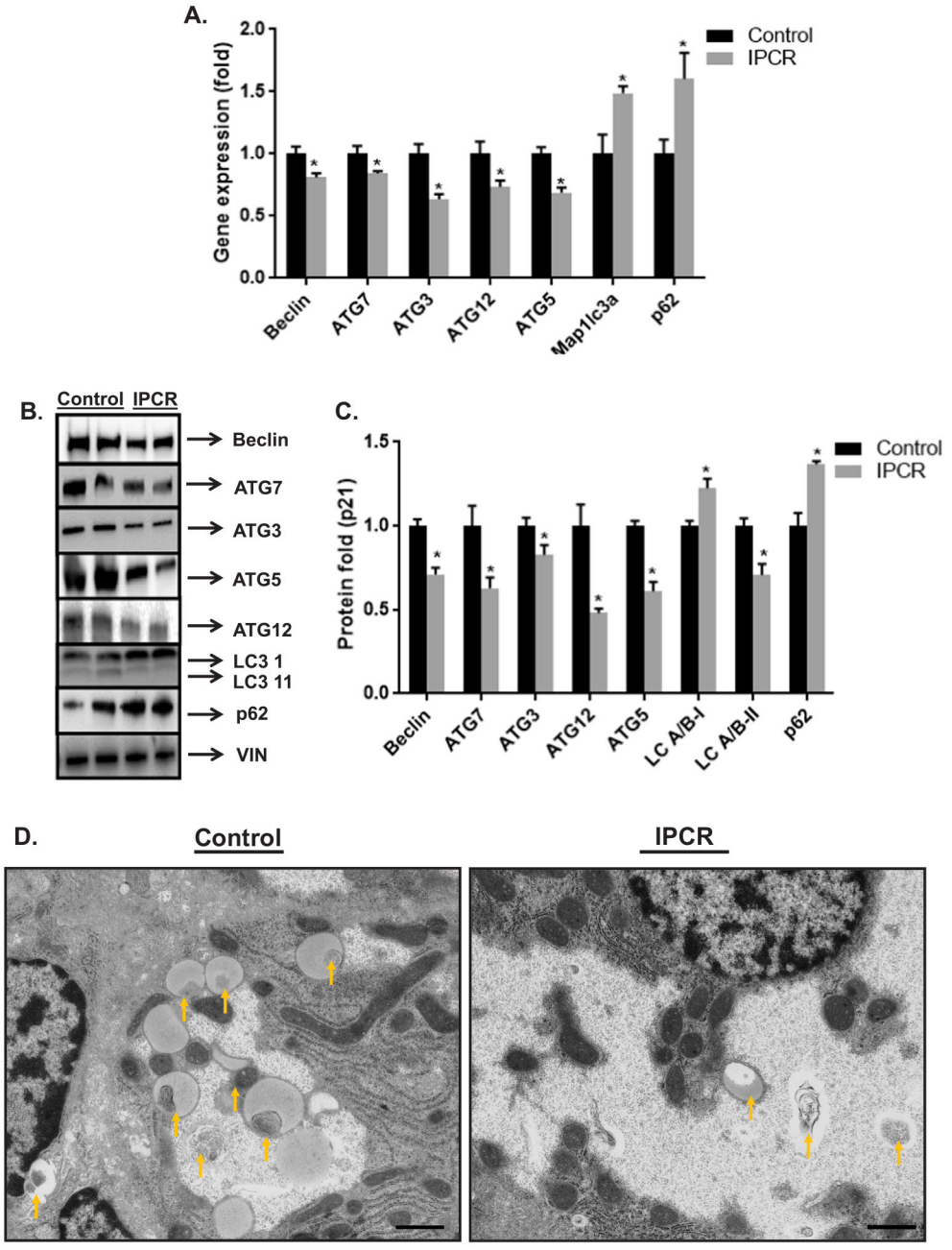

Fig. 1.

Decreased autophagy markers in the liver of p21 IPCR male rat offspring. At p21, IPCR animals sacrificed and livers were collected, RNA isolated, CDNA prepared, and genes expression performed by QPCR for beclin, Atg7, Atg3, Atg12, Atg5, Map11c3 and p62 and normalized to RLP13a (Fig. 1A). IPCR liver tissue was homogenized with protein lysate buffer, centrifuged, supernatant was taken and protein measured. 50 μg protein was loaded on a gradient (4–20%) gel and Western blotting was performed as described in the Methods section with the following antibodies beclin, Atg7 Atg3, Atg5, Atg12, LC3-I/ LC3-II and vinculin as an internal control. Representative Western blot images are shown in Fig. 1B. Protein bands were quantified (Fig. 1C) with Image J software. T test was performed with

Prisms software. Values were expressed as fold change ($n = 6$). *$p < 0.05$, as compared to control. Ultrastructural analyses using electron microscope confirmed a decreased in autophagic structures in the liver of IPCR rats. Arrows denote auto phagosome; Scale bars: 1 μm (Fig. 1D).

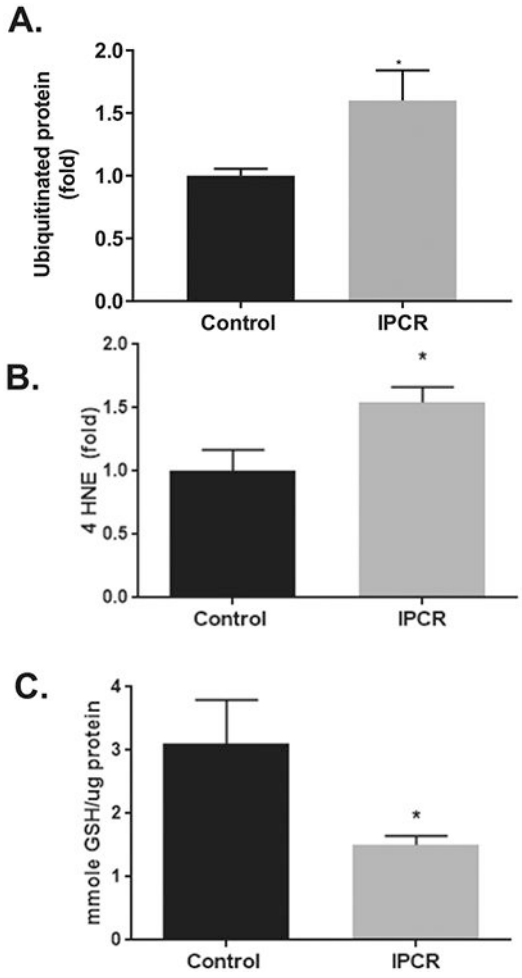

Fig. 2.

Decreased autophagosome result in increased ubiquitinated proteins and oxidative stress markers in the liver of p21 IPCR animals. Livers were collected from experimental animals and homogenized with protein lysate buffer, centrifuged, supernatant was taken and protein measured. Mono and polyubiquitinated protein and 4 HNE were measured by ELISA as described in the Method Section. (Fig. 2A-B). Glutathione level (Fig. 2C) was measured as described in the Method Section. Values were expressed as fold change ($n = 9$). *T*-test was performed with Prisms software. * $p < 0.05$, as compared to control.

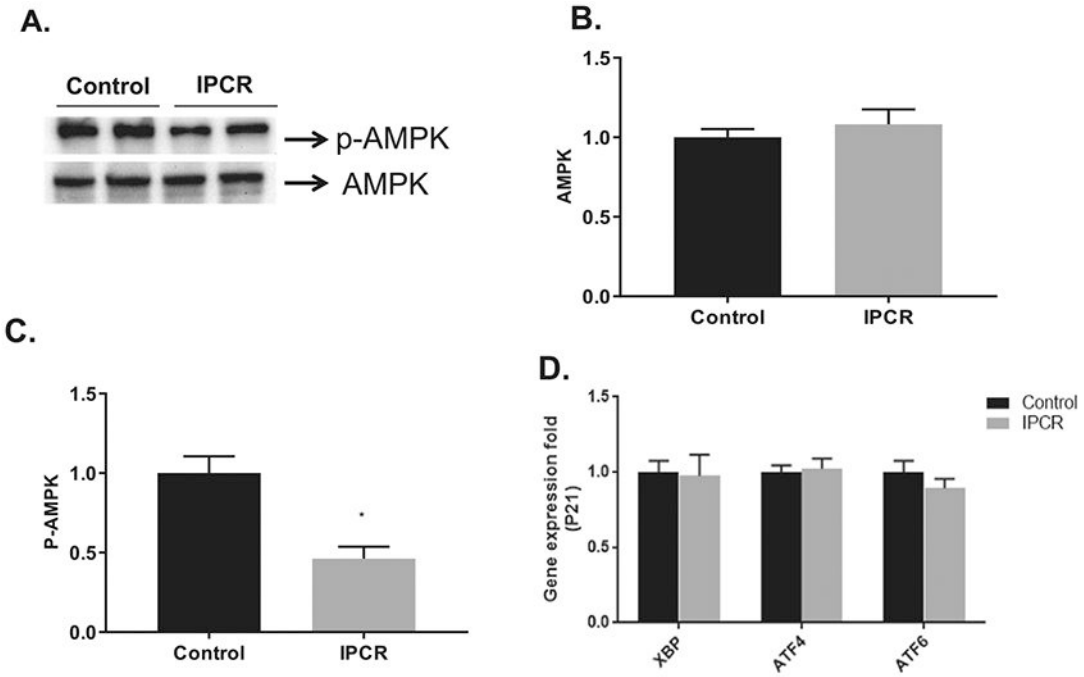

Fig. 3.

AMPK and ER stress markers in the liver of p21 IPCR animals. Livers were collected from IPCR animals on p21, homogenized with protein lysate buffer, centrifuged, supernatant isolated and protein quantified. 50 μg protein was loaded on a gradient (4–20%) gel and Western blotting performed for AMPK and p-AMPK antibodies as described in the Method Sections and quantified with Image J software (Fig. 3A-C). Values were expressed as fold change ($n = 6$). RNA was isolated from the liver sample, cDNA was prepared andgene expression for XBP, ATF4 and ATF6 were analyzed (Fig. 3D). * $p < 0.05$, as compared to control. T test was performed with Prisms software. Values were expressed as fold change ($n = 6$). * $p < 0.05$, as compared to control.

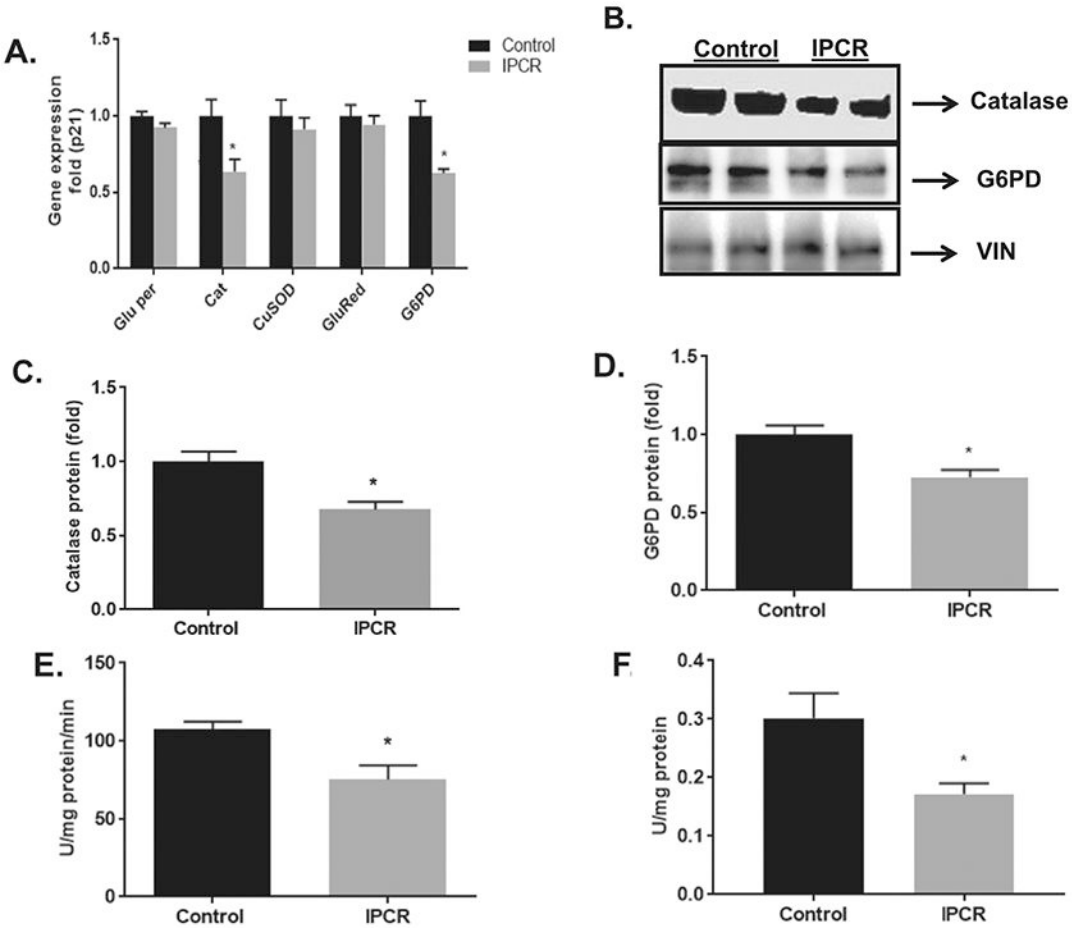

Fig. 4.

Antioxidant enzymes levels and activities in the liver of p21 IPCR rats. On p21, IPCR animal were sacrificed and livers were collected, RNA isolated, CDNA prepared and antioxidant genes expression for glutathione peroxidase, catalase, CuSOD, glutathione reductase and G6PD were analyzed with RLP13a is an internal control (Fig. 4A). Livers were homogenized and Western blotting was performed with (4–15%) a gradient gel using catalase and G6PD antibodies. Representative figures are displayed in Fig. 4B. Protein bands were quantified with Image J software (Fig. 4C-D) Values were expressed as fold change (n = 9–12). Using tissue extract, catalase (Fig. 4E) and G6PD (Fig. 4F) activity were measured. T test was performed with Prisms software. Values were expressed as fold (n = 9–12). * $p <$ 0.05, as compared to control.

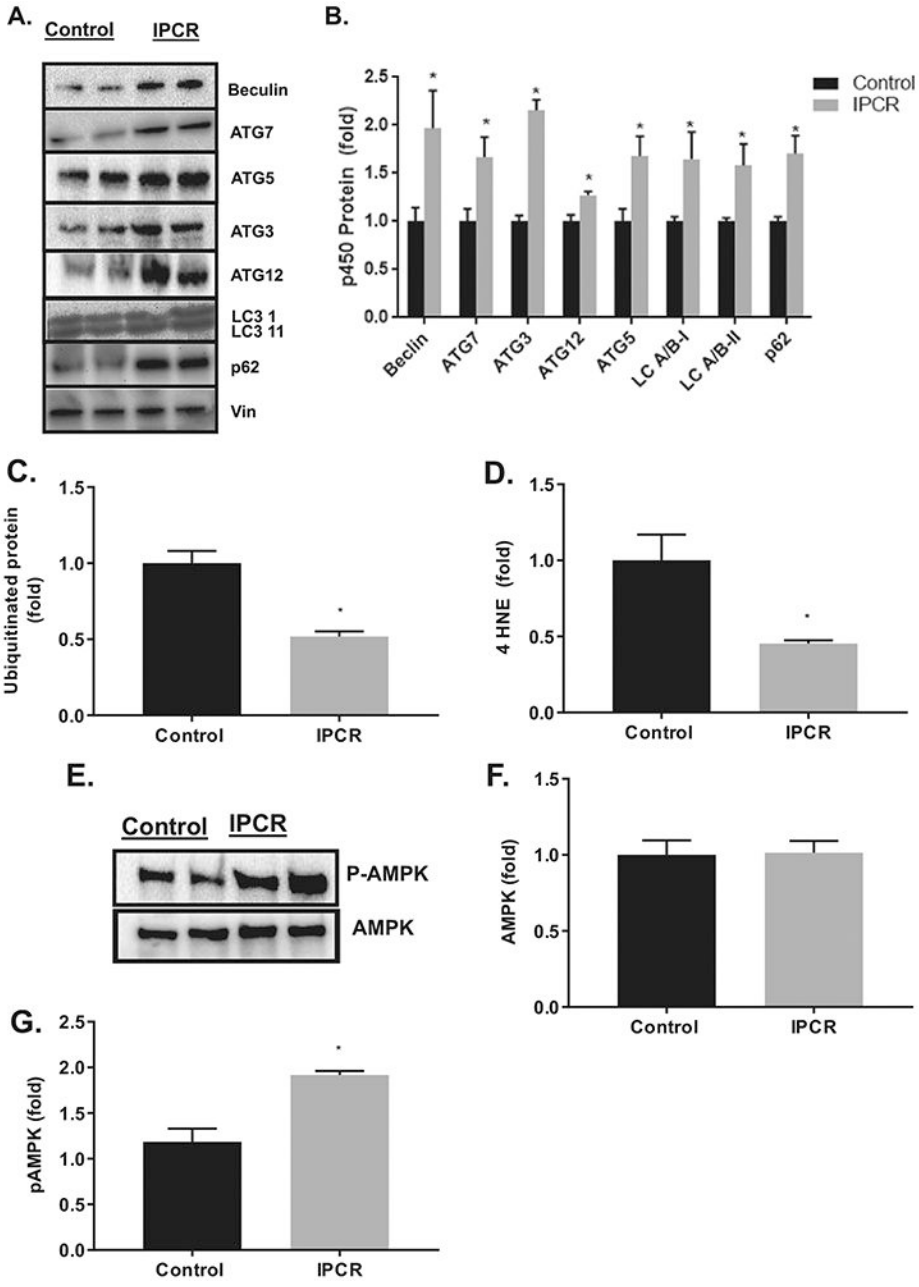

Fig. 5.

Hepatic Autophagy related proteins, p-AMPK, AMPK ubiquitinated protein and oxidative stress marker in the liver of d450 IPCR animals. Rats were subjected to perinatal calorie restriction for 31 days then food for given *ad libitum*. On d450, animals were sacrificed, livers collected, proteins extracted with lysate buffer and autophagy related proteins analyzed by Western blot Method. Representative Western blotting are displayed in Fig. 5 A and protein band quantified with Image J software (Fig. 5B). Ubiquitinated protein (Fig. 5C) and 4-HNE levels (Fig. 5D) were measured using ELISA method as described in the Methods Section. p-AMPK and AMPK were detected by Western blotting method. Representative Western blotting (Fig. 5E) and proteins bands quantified with Image J

software (Fig. 5E-G). T test was performed with Prisms software. Values were expressed as fold change (n = 9-12). *$p < 0.05$ as compared to control.

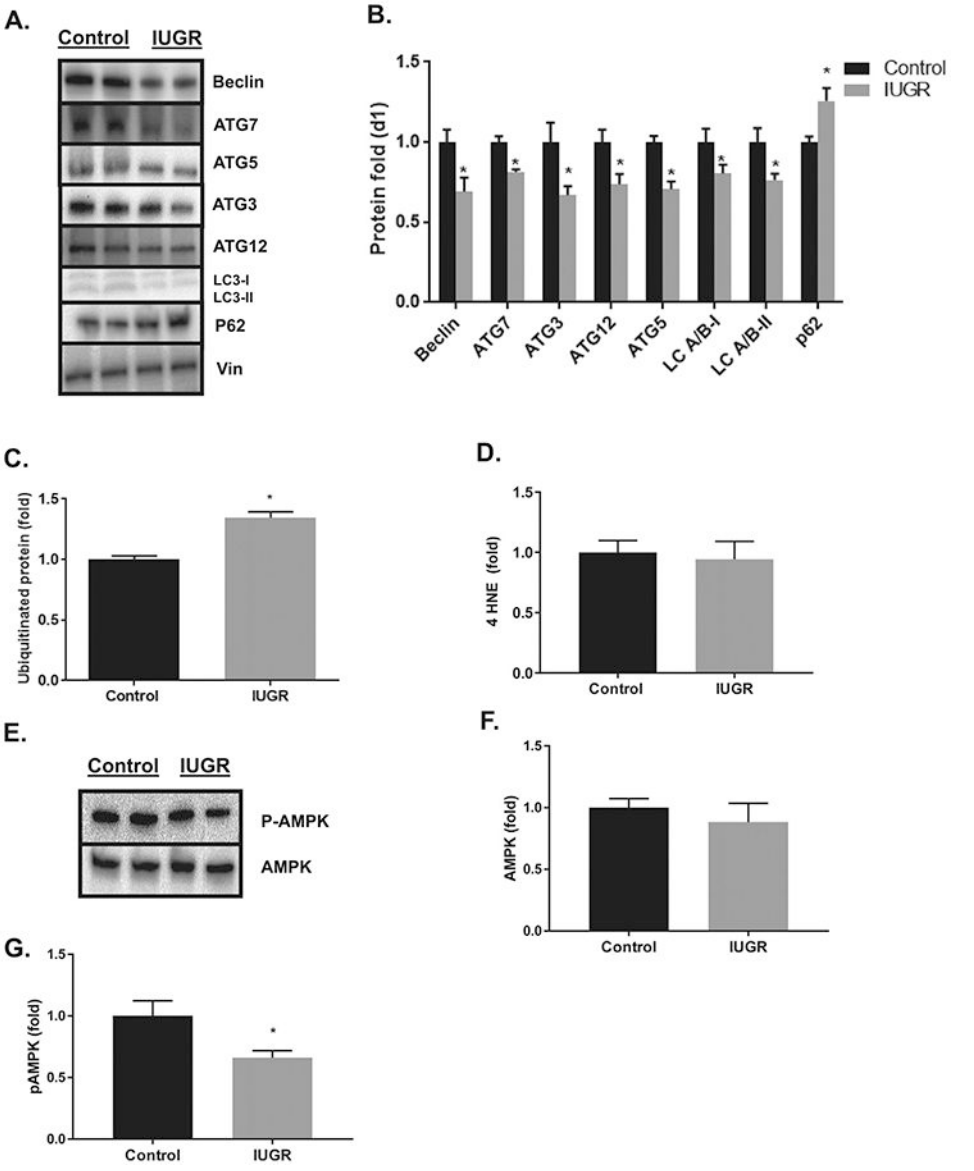

Fig. 6.

Hepatic autophagy related proteins, ubiquitinated protein, and oxidative stress marker in the liver of day 1 IUGR Rats: Rats were subjected to IUGR. On day 1, animals were sacrificed, livers collected and proteins extracted with lysate buffer and autophagy related proteins were analyzed by Western blot Methods. Representative Western blotting are shown in Fig. 6 A and quantified with Image J software (Fig. 6 B). Tissue lysate was prepared, and Ubiquitinated protein (Fig. 6 C) and 4HNE (Fig. 6 D) were measured using ELISA and p-AMPK and AMPK were detected by Western blotting method. Representative Western blotting (Fig. 6 E) and proteins band were quantified with Image J software (Fig. 6 F-G). T test was performed with Prisms software. Values were expressed as fold change (n = 9-12). *$p < 0.05$ as compared to control

Table 1

The effect of IPCR on the differnt stages of autophagy and oxidative stress tsin

	d1	p21	p450
Protein levels involved in autophagy initiation Processes *(Beclin)*	Beclin is decreased	Beclin is Decreased	Beclin is Increased
Protein levels involved in autophagy elongation processes *(Atg7, Atg3, Atg5)*	Atg7, Atg3 and Atg5 levels were decreased	Atg7, Atg3 and Atg5 levels were decreased	Atg7, Atg3 and Atg5 levels were increased
Protein levels involved in autophagy termination processes LC3-I and LC3-II	LC3-I and LC3-II were decreased	LC3-I increased LC3-II were decreased	LC3-I and LC3-II were increased
Ubiquitinated level	Increased	Increased	Decreased
4 HNE level	No change	Increased	Decreased
Total AMPK	No change	No change	No change
P-AMPK	Decreased	Decreased	Increased