Biogerontology (2008) 9:169–176
DOI 10.1007/s10522-008-9126-x

RESEARCH ARTICLE

Caloric restriction counteracts age-dependent changes in prolyl-4-hydroxylase domain (PHD) 3 expression

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Received: 9 November 2007 / Accepted: 17 January 2008 / Published online: 31 January 2008

Abstract Caloric restriction remains the most reproducible measure known to extend life span or diminish age-associated changes. Previously, we have described an elevated expression of the prolyl-4-hydroxylase domain (PHD) 3 with increasing age in mouse and human heart. PHDs modulate the cellular response towards hypoxia by regulating the stability of the α-subunit of the transcriptional activator hypoxia inducible factor (HIF). In the present study we demonstrate that elevated PHD3, but not PHD1 or PHD2, expression is not restricted to the heart but does also occur in rat skeletal muscle and liver. Elevated expression of PHD3 is counteracted by a decrease in caloric intake (40% caloric restriction applied for 6 months) in all three tissues. Age-associated changes in PHD3 expression inversely correlated with the expression of the HIF-target gene macrophage migration inhibitory factor (MIF), which has been previously described to be involved in cellular HIF-mediated anti-ageing effects. These data give insight into the molecular consequences of caloric restriction, which influences hypoxia-mediated gene expression via PHD3.

Keywords Ageing · HIF-1α · Hypoxia · Prolyl-4-hydroxylase domain

Abbreviations

ARNT	Aryl hydrocarbon receptor
HIF-1	Hypoxia-inducible factor-1
MIF	Macrophage migration inhibitory factor
PHD	Prolyl-4-hydroxylase domain

Introduction

One of the hallmarks of ageing is the decline of physiological functions. This is accompanied with progressive organ failure and an increased incidence of age-related diseases. Caloric restriction remains the most reproducible measure known to extend the maximum lifespan and to retard a variety of age-associated changes in several species (Speakman and Hambly 2007). Recent evidence suggests that caloric restriction, besides preventing accumulation of energy-related metabolites like reactive oxygen species or advanced glycation end products, triggers a regulatory response (Koubova and Guarente 2003).

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As a result of the limited energy intake, a modulated expression of genes involved in various regulatory integrative functions has been discovered. In a recent study we described age-related changes in the expression of the prolyl-4-hydroxylase domain (PHD) 3 and as a consequence in the expression of the hypoxia-inducible factor (HIF)-1α transcription factor (Katschinski 2006; Rohrbach et al. 2005).

PHD3 and HIF belong to the cellular oxygen sensing system and regulate therefore the adaptation of cells towards a decreased oxygen supply via hypoxia-inducible gene expression. This involves genes associated with angiogenesis, pH control, glucose metabolism, oxygen transport etc. (Wenger et al. 2005). In this regard, it should be noted that some ageing-associated diseases such as ischemic diseases, atherosclerosis and cancer are associated with an altered oxygen supply. Moreover, the tolerance to ischemia and hypoxia is reduced in elderly (Abete et al. 1999; Bosch-Marce et al. 2007; Mariani et al. 2000; Paolucci et al. 2003).

HIF is a heterodimer comprising the oxygen labile α-subunit and the oxygen-independently expressed β-subunit, which is also called ARNT. The expression of HIF-1α is regulated by three described PHDs, i.e. PHD1, PHD2 and PHD3, at the protein level (Ivan et al. 2001; Jaakkola et al. 2001). PHD-dependent hydroxylation is directly depending on the oxygen availability (Bruick and McKnight 2001; Epstein et al. 2001). Hydroxylation of HIF-1α allows binding of the von Hippel Lindau tumor suppressor protein, which triggers rapid ubiquitination and degradation (Maxwell et al. 1999). An age-dependent decline of HIF-1α protein levels and HIF-target gene expression has been described in different organs like rat cerebral cortex, mouse heart, carotid body and smooth muscle cells isolated from rabbit aorta (Di Giulio et al. 2005; Rivard et al. 2000; Rohrbach et al. 2005). The age-related expression pattern of PHDs in other organs besides heart is not known.

Caloric restriction has been consistently described to challenge biological ageing (Rohrbach et al. 2006b). Regarding the HIF system, it is interesting to note that caloric restriction counteracts age-related changes in the angiogenic response and can protect cardiomyocytes from hypoxic death in rodent models of ischemic diseases (Ahmet et al. 2005; Facchetti et al. 2007). To gain more insight into the impact of nutrient availability on the adaptive transcriptional response towards hypoxia, we quantified the expression of the HIF-1α regulating PHD1–3 in heart, liver and skeletal muscle in young and old rats undergoing a well-defined protocol of caloric restriction.

Materials and methods

Animals and diet protocol, 40% caloric restriction for 6 months

Male young (4 months) and senescent (22 months) Sprague-Dawley rats were obtained from Charles River (Germany), caged individually with a light/dark cycle of 12 hrs and with tap water ad libitum. Food (Altromin^{R} 1244; 2550 cal/g) was offered ad libitum. Prior to the application of the specific diet protocols, daily food intake of the normal standard diet was monitored for 14 days and averaged for each rat individually. Thereafter, rats at the age of 6 months (young rats) or at the age of 24 months (old animals) were randomly assigned to one of the following diets for the next six months: rats on "control diet" received their individual prediet average of Altromin^{R} 1244 (2,550 cal/g), but not more, in order to avoid any degree of diet-induced obesity. Rats subjected to caloric restriction received also their prediet average, but of a calorically reduced, fibre-rich diet (Altromin^{R} 1344/1500; 1,550 cal/g). Thus, in young rats on control diet ($n = 6$), the daily energy intake during six months was 64.8 ± 2.2 kcal and in young rats on -40% caloric restriction ($n = 6$) the daily intake was 37.8 ± 1.9 kcal. In old rats the daily energy intake amounted to 57.9 ± 1.8 kcal with control diet ($n = 3$) and to 36.6 ± 0.1 kcal with 40% caloric restriction ($n = 3$). All animals were fasted for 12 hours before killing.

RNA extraction, RT-reaction and quantification by PCR

The RNA was isolated from left ventricle, liver and skeletal muscle (M. gastrocnemicus) tissue as described before (Rohrbach et al. 2007). Integrity and quality of the RNA was confirmed by agarose gel electrophoresis and the concentration determined by measuring UV-absorption.

Real time PCR

Reverse transcription (RT) of RNA samples was carried out for 30 min at 42°C. Real-time PCR and data analysis were performed using the Mx3000P Multiplex Quantitative PCR System (Stratagene). DNA amplification was performed as follows: initial denaturation at 95°C for 10 min, 40 cycles of amplification (denaturation at 95°C for 30 s, annealing at 60°C for 60 s, and extension at 72°C for 60 s), followed by a denaturation at 95°C for 60 s and a melting curve over the range from 55°C up to 95°C. Fluorescence data were collected at the end of the annealing stage of amplification. We performed Real-time PCR of PHD1, PHD2, PHD3, MIF-1 and 18S rRNA (18S rRNA Control kit, Yakima Yellow®-Eclipse® Dark Quencher, Eurogentec) in samples derived from rat left ventricles, skeletal muscle and liver. The following primers were used: PHD1 sense CGTGAGGCATGTTGACAATC, PHD1 antisense AACACCTTTCTGTCCCGATG; PHD2 sense TACA GGATAAACGGCCGAAC, PHD2 antisense GGCTT GAGTTCAACCCTCAC; PHD3 sense GGCCGCT GTATCACCTGTAT, PHD3 antisense TTCTGCCC TTTCTTCAGCAT; MIF sense CAGAACCGCAA CTACAGCAA, MIF antisense GAACAGCGGTGCA GGTAAGT. Each assay was performed in duplicate and validation of PCR-runs was assessed by evaluation of the melting curve. All data of mRNA are given as relative units of 18S rRNA concentrations.

Western blot

Liver tissue was rapidly homogenized in a buffer containing 50 mmol/l Tris·HCl, 1% SDS, 1 mmol/l sodium-orthovanadate, 5 mmol/l EGTA, 1 mmol/l PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin. Proteins were quantified using the bicinchoninic acid protein assay (Pierce). Protein (50 μg) in 2× Laemmli SDS sample buffer were boiled for 5 min and after centrifugation loaded onto a SDS-PAGE gel. After electrophoresis, proteins were transferred to a nitrocellulose membrane at 100 V for 90 min. The filters were blocked with 0.01% Tween, 2% nonfat milk, and then incubated with antibodies directed against MIF (Abcam) and GAPDH (Abcam).

Statistical analysis

For statistical comparison of age groups or the influence of caloric restriction, a non-paired t-test was used. A P-value < 0.05 was considered to represent a significant difference.

Results

Prevention of age-dependent increase of PHD3 in heart, liver and skeletal muscle

Age-dependent changes of PHD1-3 expression were investigated in three different organs obtained from young (12 months old) and old (30 months old) rats. PHD1 and PHD2 mRNA expression did not differ significantly between the two age groups neither in heart, liver nor in skeletal muscle (Figs. 1 and 2). However, in all three different organs investigated, there was a significant increase in PHD3 mRNA expression in the tissues obtained from the old rats compared to the young animals (Fig. 3).

Young and old rats were subsequently challenged by caloric restriction. Daily caloric intake was reduced to 40% in young and old animals for 6 months. Control animals received their prediet average caloric intake. Using this regimen, we previously have observed that thioredoxin reductase 2 is significantly reduced in ageing skeletal and cardiac muscle and renormalized after caloric restriction (Rohrbach et al. 2006a). Whereas no significant effect of caloric restriction was observed on the expression of PHD1 or PHD2 (Figs. 1 and 2), the reduced food intake significantly counteracted the age-dependent increase in PHD3 expression in heart, liver and skeletal muscle (Fig. 3). PHD3 levels in old rats after caloric restriction was comparable to the PHD3 expression levels in young animals.

Caloric restriction affects the expression of the macrophage migration inhibitory factor

Numerous HIF-target genes, including erythropoietin, vascular endothelial growth factor, carbonic anhydrase IX etc., have been identified (Wenger et al. 2005). Recently, the expression of the macrophage migration inhibitory factor (MIF) has been

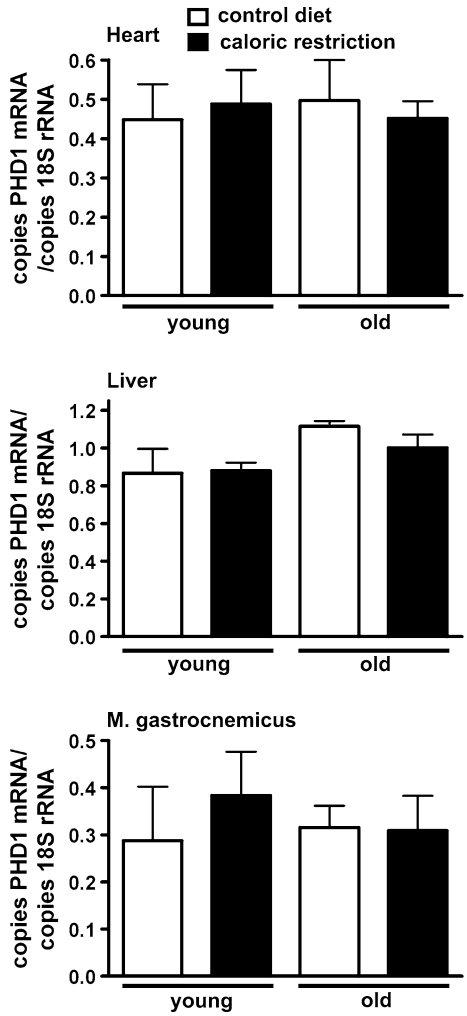

Fig. 1 Effect of age and caloric restriction on PHD1 mRNA expression. Tissue samples (heart, liver and skeletal muscle (M. gastrocnemicus)) were obtained from young (6 months old) and old (24 months old) rats with or without a 40% caloric restriction applied for 6 months. Subsequently, RNA was extracted and PHD1 mRNA quantitated by real time PCR

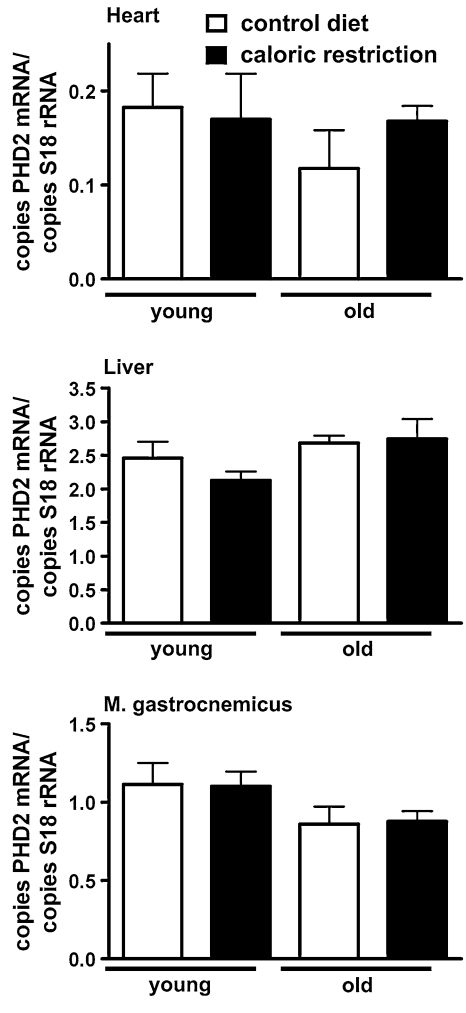

Fig. 2 Effect of age and caloric restriction on PHD2 mRNA expression. Tissue samples (heart, liver and skeletal muscle (M. gastrocnemicus)) were obtained from young (6 months old) and old (24 months old) rats with or without a 40% caloric restriction applied for 6 months. Subsequently, RNA was extracted and PHD2 mRNA quantitated by real time PCR

demonstrated to be inducible by hypoxia. In addition, functional HIF binding sites in the promoter have been identified. In a recent study evidence was provided that HIF-1 plays a critical role in delaying the onset of senescence in rodent cells via transcriptional activation of MIF and thereby inhibition of the p53-mediated pathway (Welford et al. 2006). To gain insight into the functional consequences of age and caloric restriction-mediated changes on the HIF-induced signal transduction pathways, we analyzed the mRNA expression of MIF as a function of age and caloric restriction. In line with the suggestion that HIF-dependent gene expression is hampered with increasing age, MIF RNA and protein expression was significantly lower in the heart, liver and skeletal muscle obtained from the old rats compared to the expression found in the young rats (Figs. 4, 5). Restriction of food intake reversed the age-dependent decline in MIF expression (Fig. 4). MIF levels after caloric restriction were comparable to the expression levels in young rats in all three tissues investigated.

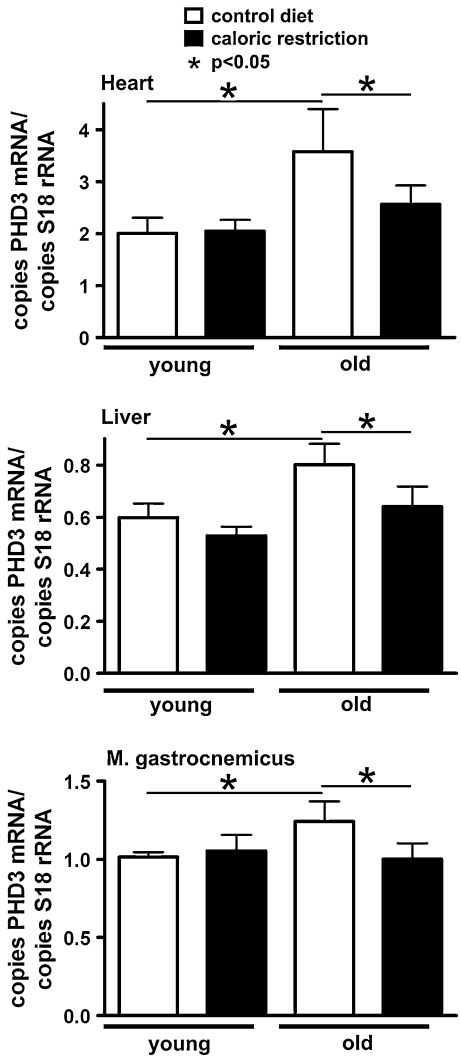

Fig. 3 Effect of age and caloric restriction on PHD3 mRNA expression. Tissue samples (heart, liver and skeletal muscle (M. gastrocnemicus)) were obtained from young (6 months old) and old (24 months old) rats with or without a 40% caloric restriction applied for 6 months. Subsequently, RNA was extracted and PHD3 mRNA quantitated by real time PCR

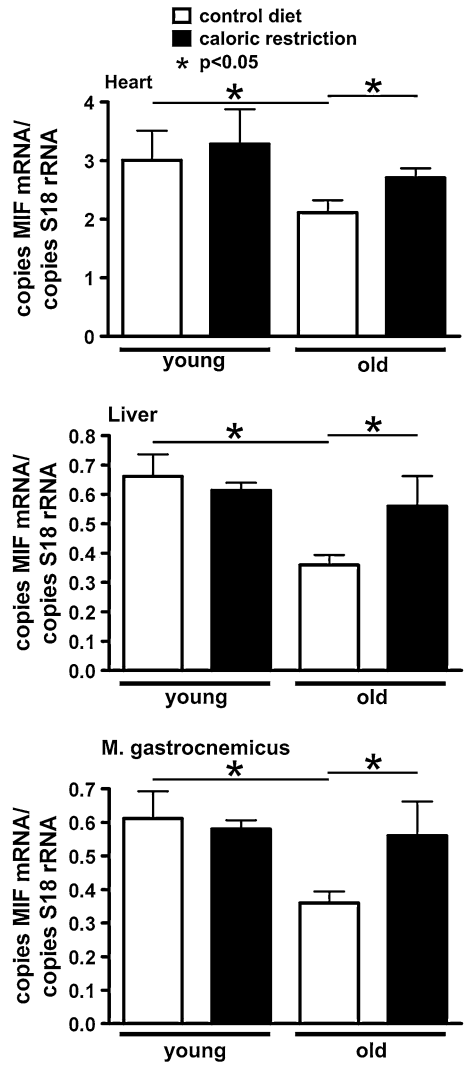

Fig. 4 Effect of age and caloric restriction on MIF mRNA expression. Tissue samples (heart, liver and skeletal muscle (M. gastrocnemicus)) were obtained from young (6 months old) and old (24 months old) rats with or without a 40% caloric restriction applied for 6 months. Subsequently, RNA was extracted and MIF mRNA quantitated by real time PCR

Discussion

Caloric restriction has been described to extend life in a variety of different mammalian and non-mammalian species (Masoro 2000). In addition, restriction of food intake delays the progression of several age-associated diseases. Identification of age-dependently expressed genes is one of the most direct approaches in ageing research. In a previous study, we identified PHD3 as one age-dependently expressed gene in human and mouse heart (Rohrbach et al. 2005). The data presented here demonstrate that PHD3 expression is additionally increased in rat heart, liver and skeletal muscle with increasing age. These findings indicate that age-dependent changes in PHD3 expression are tissue- and species-independent. Furthermore our data presented here demonstrate that PHD3 expression is decreased by caloric restriction in old rats, whereas no change in the expression of PHD1 or PHD2 was observed. Previous findings indicate that

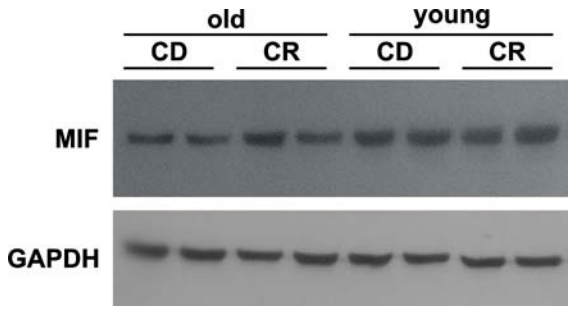

Fig. 5 Effect of age and caloric restriction on MIF protein levels. Liver tissue samples were obtained from young (6 months old) and old (24 months old) rats with or without a 40% caloric restriction applied for 6 months. Subsequently protein was extracted and MIF and GAPDH protein levels were investigated by immunoblot analysis

each PHD-isoform displays its own tissue and cell-line-specific expression pattern (Appelhoff et al. 2004). In addition, the susceptibility of the PHD isoform expression by change in the oxygen availability differs greatly. Whereas PHD3 is highly inducible by hypoxia, there is just a slight hypoxia-mediated induction described in case of PHD2. In case of PHD1 no hypoxia-inducible expression has been observed (Appelhoff et al. 2004; Marxsen et al. 2004). Including the age-dependent and caloric restriction-induced changes in PHD3 expression, PHD3 seems to be the most flexible PHD isoform regarding stimuli-induced change in expression.

In an unbiased screen using white adipose tissue it was found that energy restriction lowers the expression of genes linked to angiogenesis indicating that hypoxia-inducible gene expression is affected by caloric restriction (Higami et al. 2006). Similarly, the age-dependent and caloric restriction-induced expression of PHD3 described here seems to have consequences. Quantification of the HIF-target gene MIF demonstrates a decrease of MIF expression in heart, liver and skeletal muscle in the old animals, which was reversed by caloric restriction. In line with our observation, in previously published microarray experiments increased levels of MIF RNA were found in liver lysates from young adult mice treated with caloric restriction or with a methionine-deficient diet as well as in young mice of the long-lived Snell dwarf stock (Dozmorov et al. 2002; Miller et al. 2005; Miller et al. 2002). Since MIF affects cellular senescence (Welford et al. 2006), changes in PHD3 expression with increasing age seem to be one important feature of the molecular senescence phenotype. This conclusion is in line with a recent report demonstrating increased HIF-1α hydroxylation in aged versus young primary murine fibroblasts (Chang et al. 2007). Together with a previously published study demonstrating that under hypoxic conditions senescent cells failed to induce HIF-1α compared to presenescent cells our data indicate that the age-dependent changes in the oxygen sensing system may indeed have impact for the ageing process (Coppe et al. 2006).

Up to now a reduced protein expression of HIF-1α with increasing age has been demonstrated in smooth muscle cells isolated from rabbit aorta, rat cerebral cortex, mouse lung, liver, kidney, heart and in the carotid body (Di Giulio et al. 2005; Frenkel-Denkberg et al. 1999; Rivard et al. 2000; Rohrbach et al. 2005). A decreased HIF-1α expression was also demonstrated in tissue lysates from ischemic limbs comparing 20 months old mice with 2 months old mice (Bosch-Marce et al. 2007). Hwang et al described a diminished HIF-1α stabilization in old rats in response to hypoxia, although in nomoxic breathing animals there was an age-related increase in basal levels of HIF binding to DNA. Finally, mouse and human fibroblasts isolated from different age groups express less HIF-1α as a matter age (Chang et al. 2007). However, it also should be noted that Kang et al demonstrated an increased HIF activity and HIF-1α expression in old rats and conflicting to the presented results a decrease in HIF activity as a result of caloric restriction in liver lysates (Kang et al. 2005). Comparing the chow protocols, these rats were fed a soybean protein diet, which may have resulted in the described increase in redox metabolites and HIF-1α protein expression. The degree and management of caloric restriction is not described (Hwang et al. 2007). Taken collectively, several papers indicate a diminished HIF-response with increasing age with some exceptions, which may be tissue or diet-specific.

Whereas ageing thus is mostly associated with a decreased hypoxia-inducible gene expression, in endometrial cancer cells mutation of PHD2 has been associated with increased expression of HIF-1α and immortality (Kato et al. 2006). Downregulation of HIF-1α by overexpression of functional PHD2 altered the cancer cell phenotype and led to the occurrence of senescence markers (Kato et al. 2006). The impact of

the HIF-system for cell fate decisions additionally was described by Welford et al demonstrating in a well defined nontransformed/nonimmortalized knock out cell model that HIF-1 delays the onset of senescence via transcriptional activation of MIF and inhibition of p53 dependent pathways (Welford et al. 2006). Alltogether these data demonstrate the necessity of tight regulation of the HIF system to prevent unrestricted cell proliferation or early cellular senescence.

Acknowledgments This work was supported by grants to D.M.K. (DFG Ka 1269/8–1) and S.R (DFG RO 2328/2–1; Deutsche Stiftung für Herzforschung F/05/05). We appreciate the technical assistance of B. Heinze.

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