



Heterozygous disruption of *ALAS1* in mice causes an accelerated age-dependent reduction in free heme, but not total heme, in skeletal muscle and liver

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ABSTRACT

5-Aminolevulinic acid (ALA) is the rate-limiting intermediate in heme biosynthesis in vertebrate species; a reaction catalyzed by the mitochondrial ALA synthase 1 (ALAS1) enzyme. Previously we reported that knockdown of the ubiquitously expressed *ALAS1* gene in mice disrupts normal glucose metabolism, attenuates mitochondrial function and results in a prediabetic like phenotype when animals pass 20-weeks of age (Saitoh et al., 2018). Contrary to our expectations, the cytosolic and mitochondrial heme content of *ALAS1* heterozygous (*Al*^{+/−}) mice were similar to WT animals. Therefore, we speculated that regulatory “free heme” may be reduced in an age dependent manner in *Al*^{+/−} mice, but not total heme.

Here, we examine free and total heme from the skeletal muscle and liver of WT and *Al*^{+/−} mice using a modified acetone extraction method and examine the effects of aging on free heme by comparing the amounts at 8–12 weeks and 30–36 weeks of age, in addition to the mRNA abundance of *ALAS1*. We found an age-dependent reduction in free heme in the skeletal muscle and liver of *Al*^{+/−} mice, while WT mice showed only a slight decrease in the liver. Total heme levels showed no significant difference between young and aged WT and *Al*^{+/−} mice. *ALAS1* mRNA levels showed an age-dependent reduction similar to that of free heme levels, indicating that *ALAS1* mRNA expression levels are a major determinant for free heme levels. The free heme pools in skeletal muscle tissue were almost 2-fold larger than that of liver tissue, suggesting that the heme pool varies across different tissue types. The expression of *heme oxygenase 1 (HO-1)* mRNA, which is expressed proportionally to the amount of free heme, were similar to those of free heme levels. Taken together, this study demonstrates that the free heme pool differs across tissues, and that an age-dependent reduction in free heme levels is accelerated in mice heterozygous for *ALAS1*, which could account for the prediabetic phenotype and mitochondrial abnormality observed in these animals.

1. Introduction

Heme serves as an important regulatory component for hemoproteins such as those involved in translation and transcription [1], and is an essential component of complex II, III, and IV of the mitochondrial electron transport chain (ETC). Expectedly, deficiencies in heme can

dramatically impact cellular homeostasis and metabolic processes [2].

Regulatory heme, also known as “free heme”, is a form of heme that has yet to be incorporated into protein or that is nonspecifically associated with protein or the membrane. This mobile form of heme can act as a signal molecule [3] with the ability to influence transcription, translation and enzyme activity; *vide infra*.

The biosynthesis of heme begins with glycine and succinyl-CoA

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Abbreviations

ALAS1	5-Aminolevulinate synthase 1
A1 +/-	ALAS1 heterozygous
ALA	5-Aminolevulinic acid
BSA	Bovine serum albumin
WT	Wild-type

which are combined in the mitochondria to form 5-aminolevulinic acid (ALA) by the enzyme 5-aminolevulinate synthase (ALAS). This is the rate-limiting step in heme synthesis. Two isozymes of ALAS exist whose genes are encoded on separate chromosomes. Our previous studies on ALAS gene knockout mice showed that the ubiquitously expressed isozyme ALAS1 has an indispensable function for early embryogenesis [4], whereas the erythroid specific isozyme ALAS2 is crucial for heme production and iron metabolism in erythroid cells [5,6].

Although the original *ALAS1* knockout studies [4] identified no apparent abnormalities in heterozygous animals (*A1*+/- mice), we recently observed that *A1*+/- mice after 20-weeks of age (hereafter designated as Aged *A1*+/- mice), showed a prediabetic phenotype under normally fed conditions and also present glucose intolerance and insulin resistance in an age-dependent manner (as opposed to an overt diabetic phenotype) as well as abnormalities in the mitochondria of skeletal muscle [7]. Strikingly, dietary administration of ALA was found to reverse insulin resistance and glucose intolerance in aged *A1*+/- mice [7]. Somewhat surprisingly however, no significant reduction in total heme levels was observed in the cytosolic or mitochondrial fractions of skeletal muscle from aged *A1*+/- mice compared to aged wild-type (WT) mice. Therefore, by way of explanation for the aged phenotypes, we speculated that there may be a reduction in the regulatory “free heme” pool of *A1*+/- mice [7].

Lately, many studies have highlighted the importance of free heme and its regulatory functions. For example, progesterone receptor membrane component 2 (PGRMC2) is a heme chaperone that carries free heme into the nucleus. Knockout of this gene in mice, decreases free heme levels in the nucleus and disrupts normal mitochondrial homeostasis. The reduction in free heme levels experienced by these mice results in glucose intolerance and insulin resistance [8], similar to the phenotype displayed in our mice [7]. BTB Domain and CNC Homolog 1 (BACH1) is a nuclear transcription factor, with a proven role in the metastasis of several types of cancer [9–11], and is affected directly through free heme levels inside the nucleus [11]. When nuclear free heme levels are low, BACH1 functions as a transcriptional repressor for heme oxygenase 1, a key enzyme in heme degradation [12]. When exposed to high free heme, BACH1 is exported from the nucleus and degraded [13,14], removing its repression, and the subsequent recruitment of the Nrf2 transcription factor to the *HO-1* promoter which positively activates transcription [14]. Aside from mouse model studies, yeast studies have uncovered an important role for free heme in the oxidative stress response, mitochondrial biogenesis and in its trafficking by chaperones [15–17].

Currently, studies on free heme in mice employ either an HRP-based assay or a peroxidase assay to determine free heme levels [8]. Here we employed a more facile method, that was first described by Espinas et al. [18] to examine heme fractions in plants. Espinas et al. validated this method by using authentic hemoproteins such as hemoglobin and myoglobin. They observed that acidic acetone could quantitatively extract protein-bound heme, whereas no heme was extracted by neutral acetone. Specifically, when a 3.7 M ratio of heme was mixed with BSA—which has two heme binding sites—neutral acetone was only capable of extracting unbound heme. Therefore, the fraction of acidic acetone-extracted heme corresponds to total heme non-covalently bound to protein, whereas neutral acetone-extracted heme

corresponds to free unbound heme. Through the use of neutral and acidic acetone separation, we were able to extract free and total heme from tissue homogenates of skeletal muscle and liver, from both *A1*+/- and WT mice.

Presently, the principal means by which ALAS activity is regulated has not been defined, whether at the level of transcription [19], mRNA stability [20] translation [21] or post-translation [22,23], or if the age-dependent decrease in ALAS activity occurs in other tissues apart from the liver. However, an age-dependent decrease in hepatic ALAS activity in rats had been highlighted as early as 1983 [24]. Moreover, an age-dependent decline in heme levels, especially free heme, remains unexplained to this day. Consequently, in this study, we examine the effect of aging on *ALAS1* mRNA levels in addition to the free and total levels of heme.

In the present study, we demonstrate that free heme levels decrease with age, but differ between liver and skeletal muscle, and obtain evidence that a reduction in free heme may be a causative factor in the phenotype of *A1*+/- mice. We also observe that the patterns between *ALAS1* mRNA expression and free heme levels overlap, confirming an age-dependent decrease in these tissue types.

2. Materials and methods

2.1. Mice

ALAS1 heterozygous mice (*A1*+/-) were generated on a mixed background, 129Sv/C57BL/6, as previously described [4]. *A1*+/- animals were maintained by crossbreeding to BDF1 (F1 hybrid of C57BL/6 and DBA2) with male *A1*+/- and wild-type littermates (WT) used for experiments. Mice 8–12 weeks-old and 30–36 weeks-old were assigned as young *A1*+/-/WT and aged *A1*+/-/WT, respectively. Mice were housed in a 14 h–10 h light-dark cycle and allowed access to regular chow diet and water *ad libitum*. All animal studies were conducted in accordance with The Regulation of Animal Experiments in Yamagata University and approved by The Institutional Animal Care and Use Committee of Yamagata University (Approved Number R2–131).

2.2. ALA administration

ALA was orally administered for 6 weeks *ad libitum* by adding it to drinking water (2 mg/ml). Mice typically drank 6 ml of water per day, amounting to a daily intake of 12 mg of ALA, 400 mg/kg in a 30 g mouse.

2.3. Fluorometric assay of heme in skeletal muscle and liver tissue

Heme extraction was performed based on the method described by Espinas et al. [18]. In case of liver, mice were sacrificed and perfused with 48 mg/L heparin in PBS to remove red blood cells. Homogenate for skeletal muscle and liver was made by crushing tissue and briefly sonicating in ice cold PBS containing protease inhibitors (cOmplete™ Protease Inhibitor Cocktail, Roche) before being spun at 600 g for 10 min at 4 °C. Supernatants was transferred to a new tube and 50 µl was pipetted to a different tube containing 950 µl of either acidic acetone (80% (v/v) acetone (WAKO) containing 20% (v/v) 1.6 M HCl) for total heme or neutral acetone (80% (v/v) acetone containing 20% (v/v) MilliQ ultrapure water) for free heme. Samples was incubated on a tube rotator for 5–10 min and subsequently spun down at 600 g for 10 min at 4 °C. Heme content was quantified as previously described [7]. The total heme (acidic acetone) and free heme (neutral acetone) fractions of tissue homogenates were each added in separate tubes to 10 volume of 1 M oxalic acid and immediately heated for 2 h at 100 °C. After cooling, fluorescence was determined in a microplate reader (Excitation 400 nm/Emission 622 nm; Thermo scientific Varioskan Flash).

2.4. Gene expression analysis

Total RNA was isolated from tissue and cells using ISOGENE (Nippon Gene). Isolated RNA was reverse-transcribed into cDNA using ReverTra Ace™ qPCR RT Master Mix with gDNA Remover (Toyobo). Quantitative RT-PCR analyses were performed using THUNDERBIRD SYBR qPCR Mix (Toyobo) with a CFX96 Real-Time PCR Detection System (Bio-Rad). Expression levels were calculated using the comparative critical threshold (Ct) method. Each reaction was run in duplicate using specific primer sets. Primer melting temperature (T_m) is 59 °C for all primers. *ALAS1*: Forward Primer (Exon 8): 5'ACTGGAAGAGCTGTGTGACG'3, Reverse Primer (Exon 9): 5'GGAGGTGGTGAAGATGAAGC'3. Amplicon: 243bp *HO-1*: Forward Primer (Exon 3): 5'AAGACCGCCTTCCTGCTCAAC'3, Reverse Primer (Exon 4): 5'CGAAGTGACGCCATCTGTGAGG'3. Amplicon: 101bp *HPRT*: Forward Primer (Exon 7): 5'GGACCTCTCGAAGTGTGGATACAGG'3, Reverse Primer (Exon 9): 5'CTGGCAACATCAACAGGACTCCTCGT'3 Amplicon: 210bp.

2.5. Western blot

Whole protein extracts were prepared from quadriceps muscles and liver tissue by homogenization in RIPA buffer containing protease inhibitor (cOmplete™ Protease Inhibitor Cocktail, Roche). Homogenates were centrifuged and the supernatant was collected. Protein extracts were quantified and an equal amount of protein was loaded onto an SDS-polyacrylamide gel. The gel was blotted on a polyvinylidene Fluoride (PVDF) microporous membrane (Millipore). Membranes were blocked in Tris Buffered Saline with 0.1% Tween 20 (TBST) containing 5% skim milk. Primary anti-body reactions were performed using antibodies against the following proteins: α -Tubulin (Cell Signaling Technology), HO-1 (Enzo Lifesciences). Secondary antibodies used were anti-rabbit antibodies (Cell signaling Technology). Immunocomplexes were detected by ImmunoStar Zeta (WAKO) and optical densities were measured using Light-Capture and CS Analyzer software (ATTO).

3. Results

3.1. Age-dependent decrease in *ALAS1* mRNA expression in skeletal muscle and liver of wild-type and *ALAS1* heterozygous mice

An age-dependent reduction in ALAS enzyme activity has been reported previously by Beattie et al. (1983) [24]. However, to our knowledge, the relative changes in *ALAS1* mRNA transcript levels as a

result of aging has not been reported. To gain insight on the effects of aging, *ALAS1* mRNA expression was measured by qPCR in skeletal muscle and liver of WT and *A1+/-* mice at 8–12 and 30–36 weeks of age (Fig. 1). It was observed that *ALAS1* mRNA expression in skeletal muscle was significantly decreased in both genotypes (Fig. 1A). Moreover, the levels of *ALAS1* mRNA of skeletal muscle at 30–36 weeks of age for *A1+/-* mice (hereinafter referred to as aged *A1+/-* mice) were significantly reduced compared to aged WT mice. By contrast, in mice that were 8–12 weeks of age (hereafter referred to as young mice) the reduction in *ALAS1* mRNA expression did not differ significantly between *A1+/-* mice and WT mice. This suggests that the levels of *ALAS1* mRNA in skeletal muscle of *A1+/-* mice decreased with age, beyond a minimum threshold level which could possibly be a causative factor for the aberrant glucose metabolism and mitochondrial function observed in aged *A1+/-* mice.

Unlike the observations in skeletal muscle, an age-dependent reduction in hepatic *ALAS1* mRNA levels was seen only in *A1+/-* mice, and hepatic *ALAS1* mRNA levels in aged *A1+/-* mice were comparatively smaller than those in aged WT mice (Fig. 1B). WT mice showed no age-dependent reduction in hepatic *ALAS1* mRNA levels, differing from previous findings on hepatic ALAS activity in rats by Beattie, S. et al. [24].

In summary, *ALAS1* mRNA levels decreased with age in skeletal muscle of both WT and *A1+/-* mice while only decreasing in an age-dependent manner in the liver of *A1+/-* mice and not WT mice.

3.2. Age-dependent decrease in free heme, but not total heme, is accelerated in *ALAS1* heterozygous mice compared to wild-type mice

With aging, a reduction in the amount of free heme in the skeletal muscle of both WT and *A1+/-* mice was observed (Fig. 2A). However only *A1+/-* mice showed a significant reduction in free heme, with WT mice only exhibiting a minor reduction in free heme with age. On the contrary, total heme levels in skeletal muscle showed no significant decrease across genotypes nor across age groups (Fig. 2B), consistent with the findings of our previous study [7]. Next, we investigated how administration of ALA to aged *A1+/-* mice may affect free heme levels. Aged *A1+/-* mice orally administered ALA for 6 weeks showed more than a 2-fold increase in the levels of both free and total heme in skeletal muscle when compared to aged *A1+/-* mice that had not been administered ALA (Fig. 2A–B).

To measure free and total heme levels in the liver (an organ of high blood-content) we perfused the animals with heparin-PBS thereby

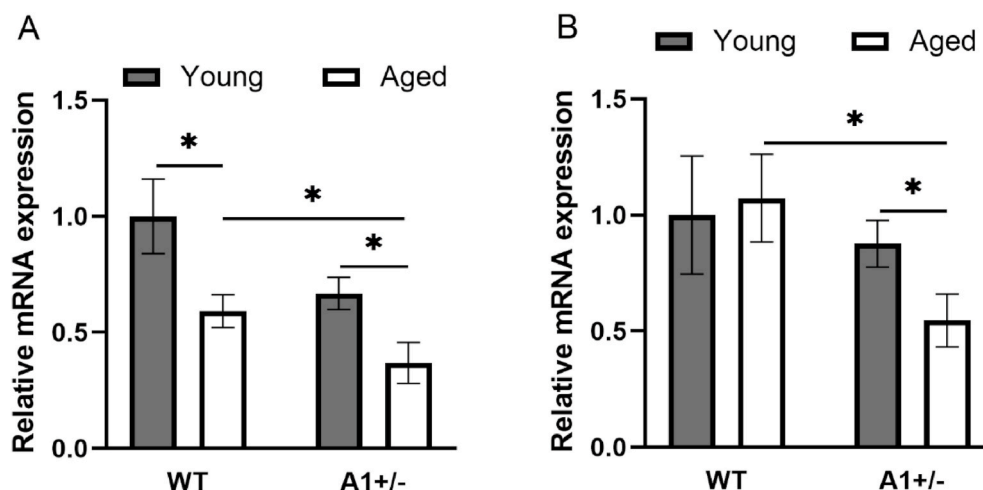


Fig. 1. *ALAS1* mRNA expression in skeletal muscle and liver in young and aged mice.

(A) Skeletal muscle, (B) Liver tissue. Black bars, Young mice, aged 8–12 weeks. White bars, Aged mice, 30–35-weeks old mice. N = 4 for each genotype and age group. Values are means $\pm$ s.e.m. for the indicated number of measurements. Statistical significance was determined by 2-tailed unpaired Student's t-test, *P < 0.05.

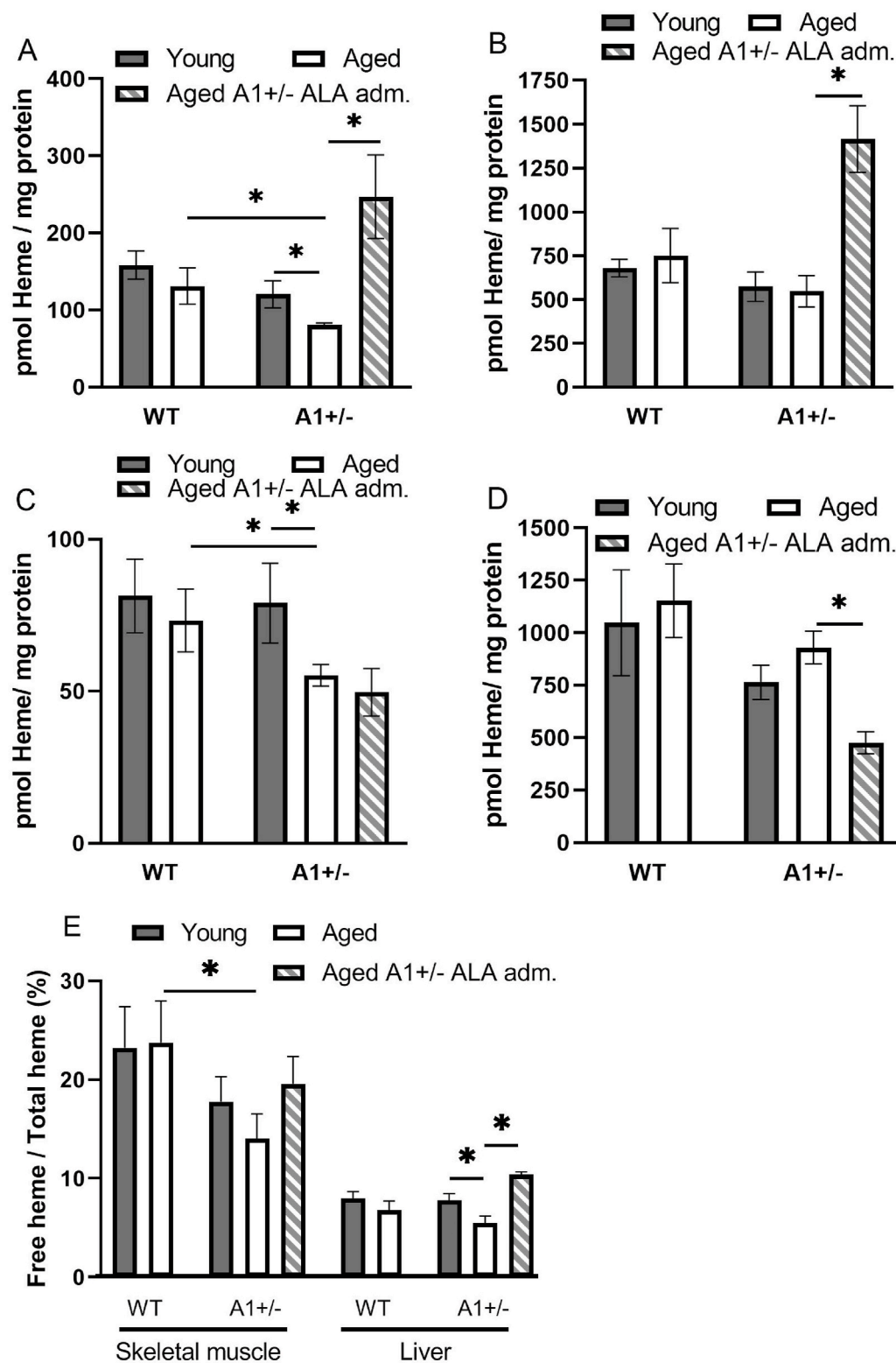


Fig. 2. Free and total heme levels and the ratio of free heme/total heme in skeletal muscle and liver in young and aged mice.

(A) Free heme, (B) total heme in skeletal muscle. Black bars, Young mice, aged 8–12 weeks. White bars, Aged mice, 30–35-weeks old mice. Striped bars, ALA administered aged A1+/- mice. N = 5 for each genotype, age group and ALA administered aged A1+/- mice. (C) Free heme, (D) total heme in the liver. Black bars, Young mice, aged 8–12 weeks. White bars, Aged mice, 30–35-weeks old mice. Striped bars, ALA-treated mice. N = 6 for young mice, N = 9 for aged mice, N = 3 for ALA administered aged A1+/- mice. (E) The ratio of free/total heme in percentages. Data used from Fig. 2 (A–D). Values are means $\pm$ s.e.m. for the indicated number of measurements. Statistical significance was determined by 2-tailed unpaired Student's t-test, $P < 0.05$.

removing red blood cells and minimizing contamination of the tissue extract with hemoglobin. Only A1+/- mice showed a significant age-dependent decrease in hepatic free heme levels, with no changes observed in WT mice (Fig. 2C). The hepatic levels of total heme were decreased slightly but not significantly in A1+/- mice compared to WT mice, and no age-dependent decrease was observed in either genotype (Fig. 2D). ALA-treated aged A1+/- mice showed a reduction in hepatic total heme levels compared to non-treated A1+/- mice (Fig. 2D). A

possible explanation as to why ALA has the opposite effect to what was observed in skeletal muscle is that HO-1 is induced to a greater level in liver: this is a point of discussion which we will address hereafter. The profile of free heme levels, rather than total heme, mirrored that of ALAS1 mRNA expression (Fig. 1A–B, 2A and C).

An overview of these results are provided in Table 1. Although the total heme levels in skeletal muscle were smaller than those in liver (Fig. 2B and D, and Table 1), the free heme levels in skeletal muscle were

Table 1

A summary of free, total heme levels and free/total heme ratio.

tissue type	genotype	age	free heme pmol/mg protein	total heme pmol/mg protein	free/total heme ratio (%)
Skeletal muscle	WT	young (10w. o.)	158 ± 18	679 ± 49	23% ± 4
		aged (30 w. o.)	115 ± 22	750 ± 154	24% ± 4
	A1+/-	young (10w. o.)	120 ± 18	573 ± 84	18% ± 3
		aged (30 w. o.)	76 ± 5	547 ± 89	14% ± 2
	ALA-admin. A1+/-	aged (30 w. o.)	247 ± 24	1328 ± 154	20% ± 3
Liver	WT	young (10w. o.)	81 ± 12	1047 ± 251	7.9 ± 0.7
		aged (30 w. o.)	73 ± 10	935 ± 72	7.4 ± 1.1
	A1+/-	young (10w. o.)	79 ± 13	763 ± 81	7.8 ± 0.7
		aged (30 w. o.)	55 ± 3	893 ± 30	5.9 ± 0.7
	ALA-admin. A1+/-	aged (30 w. o.)	50 ± 4	476 ± 30	10 ± 0.3

almost two-fold higher than those in the liver regardless of age or genotype (Fig. 2A and C, and Table 1), suggesting that the free heme pool in skeletal muscle is much larger than that in the liver.

When calculating the overall ratio of free heme to total heme in each mouse (Fig. 2E), the ratios of free/total heme in skeletal muscle were higher than those in the liver similar to what was observed with the free heme levels (Fig. 2A and C, and Table 1). A significant difference in the ratio of free/total heme in skeletal muscle was only observed between aged WT and A1+/- mice. A1+/- mice had a tendency of age-dependent decrease in the ratio of free/total heme in both tissues, while those of WT mice did not change in both tissues with aging. ALA administration

to aged A1+/- mice increased the ratio of free/total heme similar to the effect on free heme levels.

3.3. The HO-1 mRNA expression profile mirrors that of free heme in skeletal muscle and the liver

As mentioned in the introduction, the dynamics of HO-1 production and BACH1 function can be seen as indicators of changes in the amount of free heme. With HO-1 being expressed in a proportional manner to the amount of free heme. Thus, to further support our data on the reduction of free heme, HO-1 mRNA levels were analyzed (Fig. 3A–B). Profiles of HO-1 mRNA expression in skeletal muscle (Fig. 3A) and the liver (Fig. 3B) of young and aged WT and A1+/- mice were similar to those of free heme levels (Fig. 2A and C). Taken together, these data conform to the theory that the amount of free heme dictates a proportional expression of HO-1 mRNA transcript, and also validates our free heme quantification data.

3.4. Protein expression profiles of HO-1 gives insight as to how induction through ALA administration differs in skeletal muscle compared to liver tissue

As demonstrated above, the amount of total heme was reduced upon treatment with ALA in the liver (Fig. 2D) which is in contrast to what was seen in skeletal muscle (Fig. 2B). This may be attributed to the difference in the degree of induction of HO-1 protein in these two tissue types, causing liver to catabolize heme faster than skeletal muscle. Therefore, the expression of HO-1 protein was analyzed in skeletal muscle and liver of aged WT, aged A1+/- and aged ALA-treated A1+/- mice (Fig. 4). The same amount of chemiluminescent protein marker was loaded in the lanes indicated by “M” (Fig. 4A and B).

Under normally fed conditions HO-1 protein expression of aged WT and A1+/- mice was scarcely detectable in both skeletal muscle and liver (Fig. 4A and B). However, by comparative estimation using the protein marker bands (Fig. 4C), we determined that the relative hepatic HO-1 protein expression was about 20–80 times greater than skeletal muscle.

Treatment with ALA increased the expression significantly, especially so in the liver. Hepatic HO-1 protein expression in aged ALA-administered A1+/- mice was about 150 times greater in intensity than that of skeletal muscle, underlining the fact that HO-1 protein expression levels are vastly below that of liver tissue (Fig. 4C).

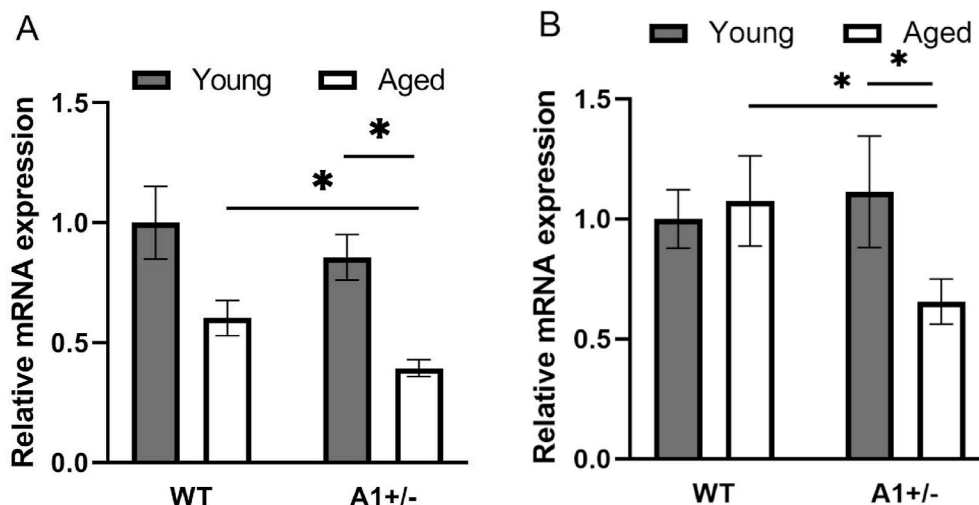


Fig. 3. HO-1 mRNA expression in skeletal muscle and liver in young and aged mice. (A) Skeletal muscle, (B) the liver. Black bars, Young mice, 8–12 weeks-old. White bars, aged mice, 30–35-weeks old. N = 4 for each genotype and age group. Values are means ± s.e.m. for the indicated number of measurements. Statistical significance was determined by 2-tailed unpaired Student's t-test, *P < 0.05.

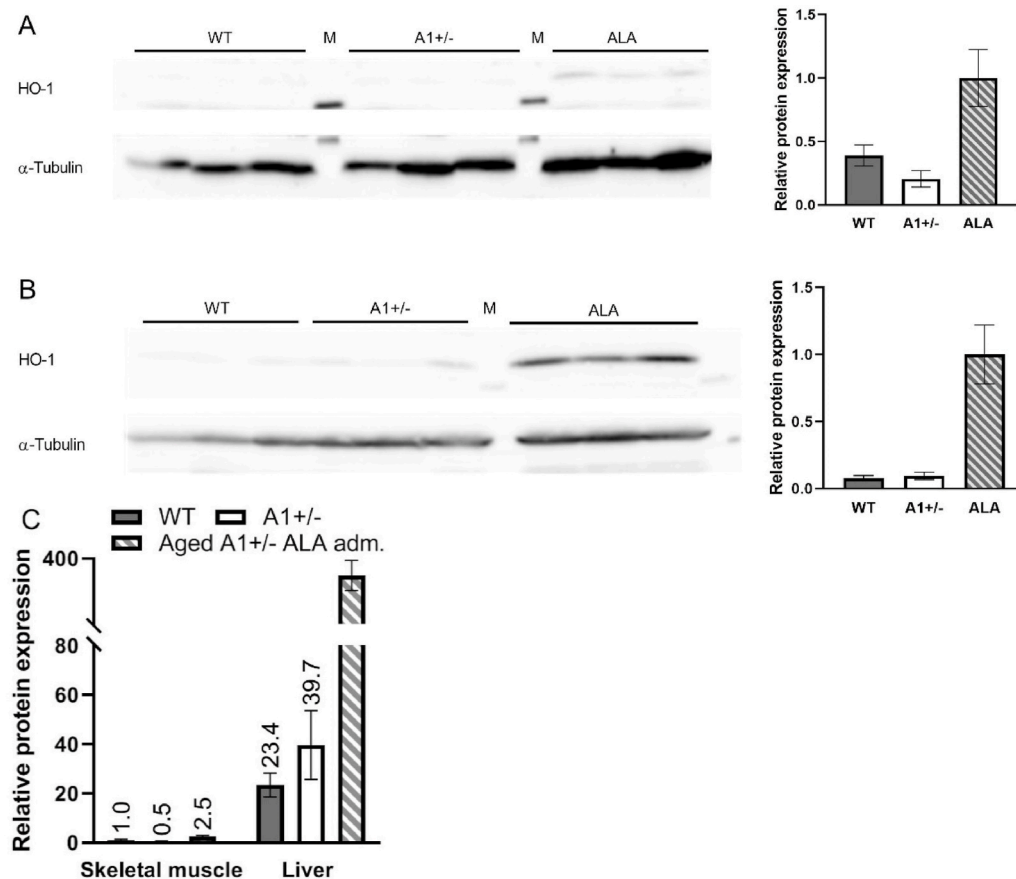


Fig. 4. Protein expression of HO-1 in skeletal muscle and liver tissue of aged mice. Protein expression levels of HO-1 in (A) skeletal muscle and (B) liver tissue of aged WT, aged A1+/- and aged A1+/- mice treated with ALA. Right panels show quantification of HO-1 protein bands on the blot. (C) Relative HO-1 protein expression across skeletal muscle and liver tissue.

Values are means $\pm$ s.e.m. for the indicated number of measurements. M = chemiluminescent marker, the same amount was loaded in both blots.

4. Discussion

Previously, through *in vivo* experiments, we established a number of links between *ALAS1* deficiency, insulin resistance, glucose intolerance and mitochondrial dysfunction [7]. We observed that those defects were reversed by ALA administration, however, no difference was observed in the amount of total heme when comparing A1+/- mice to WT mice [7]. Previously, we tested whether an insufficient amount of ALA or heme leads to impaired glucose homeostasis by treating C2C12 myocytes with succinylacetone (SA), an inhibitor of the enzyme delta-aminolevulinic acid dehydratase (ALAD). Upon treatment with SA the cells showed a significant reduction in glucose uptake, thus confirming that heme contributes to impaired glucose metabolism and not ALA [7].

In this study, by exploiting an acetone extraction method, we confirm our previous data [7] showing total heme levels do not change significantly between WT and A1+/- mice (Fig. 2B and D). By contrast, a profound age-dependent reduction was observed in the free heme levels in the skeletal muscle and liver of aged A1+/- mice, which greatly differs from the slight reductions seen in aged WT mice and the significant decrease in the free heme levels of aged A1+/- mice compared to aged WT mice (Fig. 2A and C). The results show that loss of one *ALAS1* allele exacerbates the age-dependent decrease in free heme *in vivo*. Interestingly, when aged A1+/- mice were administered ALA, an increase in free heme levels was observed only in skeletal muscle but not in the liver. Therefore, we can ascribe the insulin resistance and mitochondrial abnormality in skeletal muscle of aged A1+/- mice, reported previously [7], to such an accelerated age-dependent decrease in free heme rather than total heme. Galmozzi et al. have also reported that a reduction of nuclear free heme levels in adipocytes led to glucose intolerance and insulin resistance in *PGRMC2* -/- mice [8]. These results, similar to ours, suggest that a reduction of free heme levels leads to impaired glucose homeostasis.

Notably, ALA administration dramatically increased the free heme pool in skeletal muscle. Therefore, the capacity to supply sufficient ALA might be the major determinant for free heme levels especially in skeletal muscle. In this regard, an age-dependent decrease in *ALAS1* mRNA expression could explain the onset of a prediabetic state and mitochondrial abnormality in A1+/- mice. Possibly, the mechanism leading to the age-dependent reduction in free heme could be a transcriptional repression of the *ALAS1* gene as a result of epigenetic changes; a question which future research efforts can hopefully clarify.

Previously, we have shown that aged A1+/- mice can be rescued from prediabetic conditions and mitochondrial abnormality by the oral administration of ALA [7] which the current work can ascribe to an elevation of the free heme pool in skeletal muscle. Given the phenotypic changes that occur in A1+/- mice, this work highlights the importance of maintaining a sustained level of free heme. It is possible that over time, aged WT mice will present a similar phenotype to aged A1+/- mice since the free heme pool in skeletal muscle was reduced with age in WT mice as well. If we were to attribute age-dependent reduction in free heme as part of the natural aging processes, A1+/- mice could be considered to be experiencing premature aging. Therefore, oral administration of ALA might prevent the onset of disease(s) related to age dependent free heme reduction through its ability to replenish the free heme pool.

Many previous studies have described feedback regulation of *ALAS1* in the liver [19–23], but not in skeletal muscle. In fact, young A1+/- mice were shown to have similar *ALAS1* mRNA expression levels in liver when compared to WT mice. While in skeletal muscle, *ALAS1* mRNA expression was halved for young A1+/- mice (Fig. 1). This suggests that *ALAS1* feedback regulation in skeletal muscle is different from that of the liver. Much like the *ALAS1* mRNA profile a similar pattern was observed for *HO-1* mRNA (Fig. 3). However, in the liver of aged A1+/- mice, *ALAS1* feedback regulation seems impaired because aged A1+/-

mice showed a significant decrease in *ALAS1* and *HO-1* mRNA compared to aged WT mice (Figs. 1B and 3B).

In this study, we found interesting differences between skeletal muscle and the liver with respect to *ALAS1* mRNA expression and the free heme levels. It is notable that the free heme pool and the ratio of free/total heme in skeletal muscle were much larger than those in the liver; albeit the total heme levels in skeletal muscle were less than those of liver (Fig. 2 and Table 1). Also, ALA administration was seen to not increase free heme in the liver and ALA-treated mice had reduced total heme levels compared to *A1*+/- mice. (Fig. 2C–D and Table 1).

HO-1 can degrade free heme to reduce free heme levels [25]. The larger free heme pool in skeletal muscle might be ascribed to less expression of the HO-1 protein. Interestingly, our estimation from protein marker standards (Fig. 4C), show the basal expression levels of HO-1 protein in the liver are about 20–80 times higher than in skeletal muscle (Fig. 4). From these data we speculate that lower HO-1 expression is responsible for a larger free heme pool and ratio of free/total heme in skeletal muscle. Additionally, negative feedback regulation is not or only weakly present in skeletal muscle.

Moreover, the liver showed about 150 times higher relative expression of HO-1 protein than skeletal muscle in ALA-administered *A1*+/- mice. ALA administration in aged *A1*+/- mice causes an enormous increase in HO-1 protein ultimately leading to reduced hepatic free and total heme levels, even lower than those of non-treated aged *A1*+/- mice. Evidently, higher HO-1 protein expression in liver can profoundly affect free heme levels, whereas the lower levels of HO-1 expression typical of skeletal muscle do not.

We speculate that *HO-1* promotor activity in skeletal muscle is much less responsive to ALA compared to liver, probably because of lower expression of HO-1 related transcriptional factors such as Nrf2 [14].

5. Conclusions

In this study, we found an age-dependent reduction in the levels of free rather than total heme, both in the skeletal muscle and the liver of *A1*+/- mice, while wildtype mice showed only a slight decrease (especially in the liver). The data suggest that the age-dependent reduction in free heme levels can be exacerbated by heterozygous levels of *ALAS1* that could account for the observed prediabetic phenotypes and mitochondrial abnormality seen in *A1*+/- mice. It was found that *ALAS1* mRNA levels also showed an age-dependent reduction mirroring the decreases in free heme levels, indicating that *ALAS1* mRNA expression level is a major determinant of free heme. The free heme pool in skeletal muscle is almost 2-fold larger than that of the liver, suggesting that heme pools can vary across different tissues. Also, skeletal muscle appears susceptible to an age-dependent reduction in free heme whereas the liver appears relatively robust against this change, albeit HO-1 can dominantly affect the hepatic free and total heme pools.

Why different organs should show variability in their susceptibility to reduced ALA production with age is a very interesting question which we are currently striving to address.

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Authors' contributions

Study conceptualization and design: M. Nakajima, O. Nakajima; Investigation: K. van Wijk, T. Akabane, T. Kimura, S. Saitoh, S. Okano, O. Nakajima; Data curation: K. van Wijk, V. P. Kelly, K. Kodama, K. Takahashi, T. Tanaka, M. Nakajima, O. Nakajima; Funding acquisition:

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Declaration of competing interest

The authors declare that they have no conflict of interest.

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