Quantitation of NAD+ biosynthesis from the salvage pathway in *Saccharomyces cerevisiae*

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Abstract

Nicotinamide adenine dinucleotide (NAD+) is synthesized via two major pathways in prokaryotic and eukaryotic systems: the *de novo* biosynthesis pathway from tryptophan precursors, or by the salvage biosynthesis pathway from either extracellular nicotinic acid or various intracellular NAD+ decomposition products. NAD+ biosynthesis via the salvage pathway has been linked to an increase in yeast replicative lifespan under calorie restriction (CR). However, the relative contribution of each pathway to NAD+ biosynthesis under both normal and CR conditions is not known. Here, we have performed lifespan, NAD+ and NADH (the reduced form of NAD+) analyses on BY4742 wild type, NAD+ salvage pathway knockout (*npt1*Δ), and NAD+ *de novo* pathway knockout (*qpt1*Δ) yeast strains cultured in media containing either 2% glucose (normal growth) or 0.5 % glucose (CR). We have utilized ^{14}C labeled nicotinic acid in the culture media combined with HPLC speciation and both UV and ^{14}C detection to quantitate the total amounts of NAD+ and NADH and the amounts derived from the salvage pathway. We observe that wild type and *qpt1*Δ yeast exclusively utilize extracellular nicotinic acid for NAD+ and NADH biosynthesis under both the 2% and 0.5% glucose growth conditions suggesting that the *de novo* pathway plays little role if a functional salvage pathway is present. We also observe that NAD+ concentrations decrease in all three strains under CR. However, unlike the wild type strain, NADH concentrations do not decrease and NAD+:NADH ratios do not increase under CR for either knockout strain. Lifespan analyses reveal that CR results in a lifespan increase of approximately 25% for the wild type and *qpt1*Δ strains, while no increase in lifespan is observed for the *npt1*Δ strain. In combination these data suggest that having a functional salvage pathway is more important than the absolute levels of NAD+ or NADH for lifespan extension under CR.

Keywords

Calorie restriction; NAD; NADH; yeast; NPT1; QPT1; salvage; *de novo*; AMS

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Introduction

Nicotinamide adenine dinucleotide (NAD$^+$) is synthesized via two major pathways in both prokaryotic and eukaryotic systems (Anderson *et al.,* [2002]; Grubmeyer *et al.,* [1999]; Panozzo *et al.,* [2002]; Sandmeier *et al.,* [2002]; Smith and Boeke, [1997]) (Figure 1). In one pathway, NAD$^+$ is synthesized from tryptophan (the *de novo* pathway). In the other, NAD$^+$ is generated by recycling NAD$^+$ degradation products such as nicotinamide (the salvage pathway). In the salvage pathway nicotinic acid can be taken up by the yeast from the extracellular environment via the nicotinic acid permease transporter (Llorente and Dujon, [2000]). Other points of entry into the salvage pathway are by *de novo* synthesis of NAD$^+$ from tryptophan (Kucharcyzk *et al.,* [1998]).

Calorie restriction (CR) extends life span in a wide spectrum of organisms although the mechanism by which this regimen slows aging is unknown. CR can be imposed in yeast by reducing the glucose concentration in the media from 2% to 0.5% (Lin *et al.,* [2000]). As cells continue to feed on other culture materials, which are rich in amino acids, nucleotides, and vitamins, the growth rate remains strong with the lower glucose levels, but imposes a state of partial energy (ATP) limitation. Under these CR conditions, mother cells divide ~30% more. This increase in replicative life span does not occur in strains in which NAD$^+$ synthesis is reduced by removal of salvage pathway function linking an increase in yeast replicative lifespan with the salvage pathway for NAD$^+$ biosynthesis (Lin *et al.,* [2000]). Deletion of *NPT1*, the gene responsible for the conversion of nicotinic acid into nicotinic acid mononucleotide, a precursor to NAD$^+$ in the salvage pathway, prevented an increase in yeast life extension under calorie-restricted conditions. Deletion of *QPT1*, the gene responsible for conversion of tryptophan products into NAD$^+$ through the *de novo* biosynthesis pathway, failed to affect lifespan increases under CR. Over-expression of Npt1, a salvage pathway protein responsible for the conversion of nicotinic acid into nicotinic acid mononucleotide, has also been shown to extend lifespan in a manner similar to calorie restriction (Anderson *et al.,* [2002]). Increases in life span have also been observed when Pnc1, Nma1, and Nma2, all proteins involved in the NAD$^+$ salvage pathway, were over-expressed, further supporting a role for salvage pathway activity in life span extension (Anderson *et al.,* [2002]). However, the relative contribution of each pathway to NAD$^+$ biosynthesis under both normal and CR conditions is not known. Here we report on the relative contribution of the salvage pathway to NAD$^+$ biosynthesis under both normal and CR conditions.

Materials and Methods

Yeast culture

Yeast *Saccharomyces cerevisiae* BY4742 strain *MATα his3 1 leu2 1 lys2 0 ura3 0* was acquired from Open Biosystems. The salvage and *de novo* knockout strains, *npt1* and *qpt1* , respectively, were generated by replacing the WT genes with the *Kan^r* marker as described (Lin *et al.,* [2004]). Both wild type and knockout strains were cultured in synthetic complete media with 20 g/L glucose (to simulate normal growth conditions) or 5g/L glucose (to simulate CR conditions) (Sigma-Aldrich, St Louis, Mo) as previously described (Sporty *et al.,* [2008]). The nicotinic acid content of the synthetic complete media was determined

using the protocol of Hengen *et al.* [1978] to be 570 μg/L. The unlabeled media was supplemented with (54 mCi/mmol) [carboxy-^{14}C]-nicotinic acid (Moravek Biochemicals, Brea, CA) so that the final activity of the media was 45 pCi per 25-mL culture corresponding to a total additional nicotinic acid supplement of 103 pg. and 6.34 μmol of ^{14}C labeled nicotinic acid per mol of unlabelled nicotinic acid in the growth media. Cultures were maintained until they contained ~ 7 x 10^6 cells/mL corresponding to mid-log phase growth.

NAD$^+$ and NADH Extraction and Quantitation

NAD$^+$ and NADH were extracted using a single sample extraction and HPLC processing procedure that enables the isolation and quantitation of total cellular NAD$^+$ and NADH from pools of yeast (Sporty *et al.,* [2008]). UV absorbance was monitored at 260 nm for NAD$^+$ and NADH and at 340 nm for NADH only and pertinent peak areas were integrated using area under the curve algorithms. Peak identification and calibration of NAD$^+$ and NADH areas for quantitation were determined using standard solutions of authentic NAD$^+$ and NADH (Sigma-Aldrich, St Louis, MO).

^{14}C Quantitation

HPLC fractions were collected, dried and converted to graphite as previously described (Buchholz *et al.,* [2000]; Getachew *et al.,* [2006]; Miyashita *et al.,* [2001]). ^{14}C contents in the graphite samples were quantified by AMS (Ognibene *et al.,* [2002]). ^{14}C contents (units of attomol ^{14}C) for fractions that contributed to either the NAD$^+$ or NADH peaks were integrated, corrected for recovery efficiency (Sporty *et al.,* [2008]) and converted to salvage pathway NAD$^+$ or NADH contents assuming that ^{14}C-labeled NAD$^+$ and NADH were derived from the ^{14}C-labeled nicotinic acid and using the ratio of mol of ^{14}C labeled nicotinic acid: mol of unlabeled nicotinic acid in the growth media of 6.34 x 10^{-6}:1.

Life-span analyses

Life span analyses for wild type yeast, *qpt1* and *npt1* mutants grown on 2% and 0.5 % glucose were carried out as previously described (Lin *et al.,* [2000]). All life-span analyses in this study were carried out independently at least twice with >45 cells per strain per experiment.

Conversions and statistics

Metabolite contents in units of attomol/cell were calculated from both the measured UV absorbance or measured ^{14}C content, using the number of cells in the extraction. Metabolite contents (attomol/cell) were converted to concentrations (mM) using a derived cell volume for each yeast strain. Mean yeast diameters were determined for all three strains by measuring diameters of at least thirty individual yeast cells using a high magnification optical Zeiss microscope (Carl Zeiss Maple Grove, MN) and assuming yeast were spherical. Differences in metabolite contents were assessed by unpaired two-tailed Student's *t*-tests. A significance level of less than 0.05 was considered meaningful. A significance level between 0.05 and 0.10 was considered evidence of a possible trend, while a significance level of greater than 0.10 was considered to indicate no significant difference.

Results and Discussion

Total yeast cellular NAD$^+$ and NADH contents (attomol/cell) measured by UV detection, and ^{14}C-NAD$^+$ and ^{14}C-NADH yeast contents measured by AMS, under normal and calorie-restricted conditions are presented in Table 1. Optical microscopy yielded a mean diameter of 5.1 +/− 0.3 μm for wild type yeast. Assuming yeast to be spherical with a volume of 4/3*π*R^3, where R is the radius of 2.5 μm yields a mean volume of approximately 70 μm^3. Both knockout strains were slightly smaller than the wild type strain. Optical microscopy of the *de novo* pathway knockout (*qpt1*) and salvage pathway knockout (*npt1*) yeast strains respectively revealed mean diameters of 4.3 +/− 0.3 μm^3 and 4.2 +/− 0.4 μm^3 and a mean volume of 40 μm^3 per cell was derived for each strain. Thus the attomol/cell data in Table 1 were converted to mM concentrations shown in Table 2 using a volume of 70 μm^3 for the wild type yeast and 40 μm^3 for the two knockout strains. Total NAD$^+$ and NADH concentrations (mM) and NAD$^+$:NADH ratios for the BY4742 wild type, NAD$^+$ salvage pathway knockout, and NAD$^+$ *de novo* pathway knockout yeast strains are shown in Table 2 together with previously published data on NAD$^+$ and NADH concentrations in wild type yeast. Life span analyses for wild type yeast, *qpt1* and *npt1* mutants grown on 2% and 0.5 % glucose are shown in Figure 2.

Here, we have used ^{14}C-nicotinic acid to specifically label NAD$^+$ and NADH through the salvage pathway. The sensitivity provided by AMS allows for low levels of nicotinic acid, about 103 pg, to be added to 25-mL of media that has an endogenous content of 14.25 μg yielding a molar ratio of ^{14}C labeled nicotinic acid: to total nicotinic acid in the growth media of 6.34x 10^{-6}:1. Consequently, addition of the labeled nicotinic acid does not significantly perturb media conditions and the growth media remains physiologically relevant. UV quantitation provides total NAD$^+$ and NADH concentrations in cells while AMS quantitates ^{14}C-labeled NAD$^+$ and NADH contents. Only NAD$^+$ and NADH derived from the importation of ^{14}C-nicotonic acid into the salvage pathway should contain a ^{14}C label. The AMS:UV ratios for NAD$^+$ and NADH in Table 1 indicate the fraction of total NAD$^+$ or NADH that is synthesized from the salvage pathway.

Table 1 reveals that UV-quantified NAD$^+$ and NADH levels were statistically similar to AMS-quantified ^{14}C-NAD$^+$ and ^{14}C-NADH levels for both normal and CR conditions in the *qpt1* knockout strain. Furthermore, the AMS:UV ratios in Table 1 for the *qpt1* knockout strain indicates all cellular NAD$^+$ and NADH is derived from extracellular nicotinic acid via the salvage pathway, under both normal growth and CR conditions. This is not surprising since deletion of *QPT1*, should prevent conversion of tryptophan products into NAD$^+$ through the *de novo* biosynthesis pathway. Under such a scenario all cellular NAD$^+$ and NADH must come from the salvage pathway.

Conversely, the UV and AMS data and AMS:UV ratios in Table 1 for *npt1* knockout cells indicate that ^{14}C-labeled NAD$^+$ and NADH are completely absent under both normal growth and CR conditions. ^{14}C-labeled NAD$^+$ and NADH contents quantified by AMS were not statistically significant from zero for all *npt1* knockout samples grown under either normal or CR conditions. Again, such a finding is not surprising since deletion of *NPT1* should disrupt the salvage pathway and prevent conversion of recycled and imported

nicotinic acid into NAD$^+$ through the *salvage* biosynthesis pathway. All cellular NAD$^+$ and NADH must come from the *de novo* pathway in that situation. The AMS data and AMS:UV ratios for NAD$^+$ and NADH in Table 1 from the *qpt1* and *npt1* knockout strains indicate that both our experimental design and extraction/purification/labeling strategies appear robust.

The AMS:UV ratio data in Table 1 indicate that UV-quantified NAD$^+$ and NADH levels were statistically similar to AMS-quantified ^{14}C-NAD$^+$ and ^{14}C-NADH levels in wild type yeast. The AMS:UV ratios in Table 1 for the wild type yeast indicate that, like the *qpt1* knockout strain, all cellular NAD$^+$ and NADH is derived from extracellular nicotinic acid under both normal growth and CR conditions. Because disruption of the salvage pathway in the *npt1* knockout strain prevents incorporation of ^{14}C from extracellular labeled nicotinic acid into NAD$^+$ and NADH, it is clear that NAD$^+$ and NADH in the wild type strain are derived solely from the salvage pathway. These findings are consistent with the absence of salvage pathway activity in *npt1* yeast grown under CR (Anderson *et al.,* [2002]). Furthermore, the salvage pathway is exclusively used under both normal and CR conditions. Under normal growth conditions in which extracellular nicotinic acid concentrations are not growth-limiting, yeast preferentially use the salvage pathway over the *de novo* synthesis pathway, likely conserving tryptophan, the NAD$^+$ precursor for *de novo* biosynthesis, for protein building ultimately resulting in exponential cell growth.

Table 1 also reveals that both knockout strains have lower NAD$^+$ and NADH contents (units of attomol/cell) than the wild type strain. Such a finding is not surprising since the knockout strains had smaller sizes and smaller volumes (40 μm^3) than the wild type strain (70 μm^3). The calculated volume of 70 μm^3 per cell for the wild type yeast strain is identical to that assumed previously (based on the work of Sherman, [2002]) in calculating yeast metabolite concentrations (Lin *et al.,* [2004]; Sporty *et al.,* [2008]) and indicates that our methodology for calculating yeast cell volumes is appropriate. Other investigators have also noted that yeast knockout strains can have significantly smaller volumes than wild type yeast (Jorgensen *et al.,* [2002]). Converting NAD$^+$ and NADH contents (attomol/cell) from Table 1 to NAD$^+$ and NADH concentrations (mM) shown in Table 2 allows us to make more meaningful comparisons between the three strains and also to compare our data with that from previously reported studies.

Table 2 reveals that NAD$^+$ concentrations from wild type yeast grown under normal or CR conditions are comparable to those found in previously published reports (Anderson *et al.,* [2002]; Lin *et al.,* [2004]; Sporty *et al.,* [2008]). Table 2 also reveals that NAD$^+$ concentrations from the *qpt1* knockout strain are statistically similar to NAD$^+$ concentrations from wild type yeast for both normal and CR growth conditions. However, the *npt1* knockout strain possesses a NAD$^+$ concentration that is only three-quarters of that found in the wild type strain during normal growth conditions and possesses an NAD$^+$ concentration that is only two thirds of that found in the wild type strain during CR. These differences in NAD$^+$ concentration are both significant ($p < 0.5$). This finding is consistent with previous studies that found that deleting the *NPT1* gene decreases the NAD$^+$ level, whereas deleting the *QPT1* gene had no effect on NAD$^+$ level (Sandmeier *et al.,* [2002]; Smith and Boeke, [1997]).

Lin *et al.,* [2004] have suggested that CR extends yeast life span by lowering the level of NADH resulting in increased NAD^+:NADH ratios. However, they have only reported NAD^+ and NADH measurements in wild type yeast. Our NAD^+ and NADH concentrations and NAD^+:NADH ratios (Table 2) for wild type yeast under normal and CR conditions are comparable to those of Lin *et al.,* [2004] and our previously data (Sporty *et al.,* [2008]). However, under normal growth conditions total NAD^+:NADH ratios for the knockout strains were significantly higher than those observed in wild type cells and NADH levels were significantly depressed. Low NADH levels for the knockout strains under normal growth could be associated with smaller cell sizes for the *npt1* and *qpt1* knockout strains. The citric acid cycle, which reduces NAD^+ to generate NADH, is coupled to oxidative phosphorylation by the oxidation of NADH yielding ATP that is required for cell growth, reproduction and survival. Such low levels of NADH may inhibit cellular anabolic processes and plausibly cell growth.

Our metabolite data for CR reveals that, similar to the work of Lin *et al.,* [2004] and Sporty *et al.,* [2008], NAD^+:NADH ratios are observed to increase for the wild type strain (Table 2). However, NAD^+:NADH ratios do not increase under CR conditions for either of the knockout strains. Furthermore, unlike wild type yeast where NADH concentrations decrease, NADH levels show evidence of a possible increase in the *qpt1* mutant and do not decrease in the *npt1* mutant during CR. In contrast to previous studies (Lin *et al.,* [2004]; Sporty *et al.,* [2008]) we also find that NAD^+ concentrations decrease in all three strains under CR.

Our life span data (Figure 2) for wild type yeast, *qpt1* and *npt1* mutants grown on 2% glucose and 0.5 % glucose are consistent with those previous reports (Lin *et al.,* [2000]). CR results in an average increase in lifespan of approximately 25% in the wild type yeast and *qpt1* mutant, while no increase in lifespan is observed for the *npt1* mutant.

Our data togther with that of others show that rDNA silencing and lifespan extension under CR are lost in the *npt1* mutant (Lin *et al.,* [2000]; Smith and Boeke, [1997]). For the *npt1* mutant, it is possible that although our data indicate that the NAD^+:NADH ratio is high, the NAD^+ level is too low to activate selected NAD^+-dependent enzymes. For the *qpt1* mutant, our data and that of others show deleting *QPT1* has no effect on total NAD^+ levels or rDNA silencing or life span (Lin *et al.,* [2000]; Sandmeier *et al.,* [2002]; Smith and Boeke, [1997]). Since lifespan extension for both wild type and *qpt1* yeast occurs under CR while the NAD^+:NADH ratio significantly decreases for the mutant but increases for the wild type strain our data suggest that a high NAD^+:NADH ratio under CR alone is not sufficient to extend life span. This suggests that perhaps a threshold level of NAD and/or NADH is required for CR-induced beneficial effects and increased metabolic activity may also play an important role in CR.

In summary we have shown that, both wild type BY4742 and *qpt1* yeast exclusively utilize extracellular nicotinic acid for NAD^+ biosynthesis during exponential growth under both normal and CR conditions, suggesting that the de novo pathway plays little role if a functional salvage pathway is present. Under CR conditions, cells do not alter biosynthesis pathway activity and continue to synthesize NAD^+ from extracellular nicotinic acid. We also

observe that NAD$^+$ concentrations decrease in all three strains under CR. However, unlike the wild type strain, NADH concentrations do not decrease and NAD$^+$:NADH ratios do not increase under CR for either knockout strain. Since lifespan analyses reveal that CR results in an average increase in lifespan of approximately 25% in the wild type yeast and *qpt1* mutant, while no increase in lifespan is observed for the *npt1* mutant, these data suggest that having a functional salvage pathway is more important than the absolute levels of NAD$^+$ or NADH for lifespan extension under CR.

Acknowledgments

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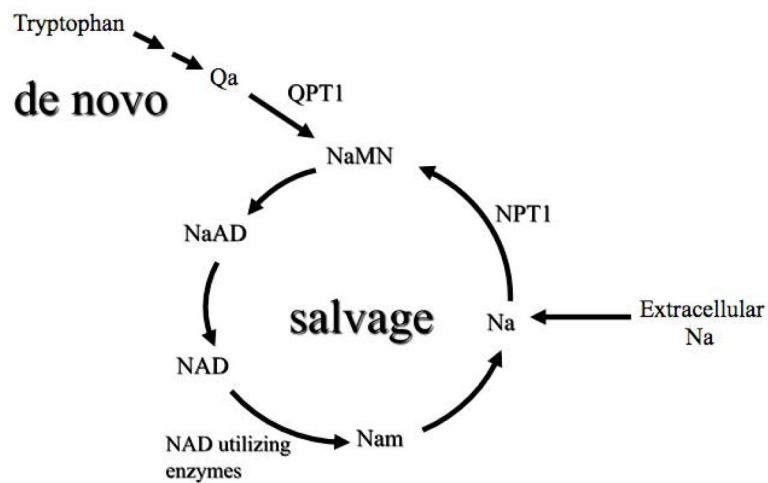
Figure 1.
Summary of the NAD$^+$ synthetic pathways in yeast. Key: **NaMN**: nicotinic acid mononucleotide; **NaAD**:deamido-NAD; **Nam**: nicotinamide; **Na**: nicotinic acid; **Qa**: quinolinic acid; *NPT1*:nicotinate phosphoribosyl transferase;; *QPT1*: quinolinate phosphoribosyl transferase.
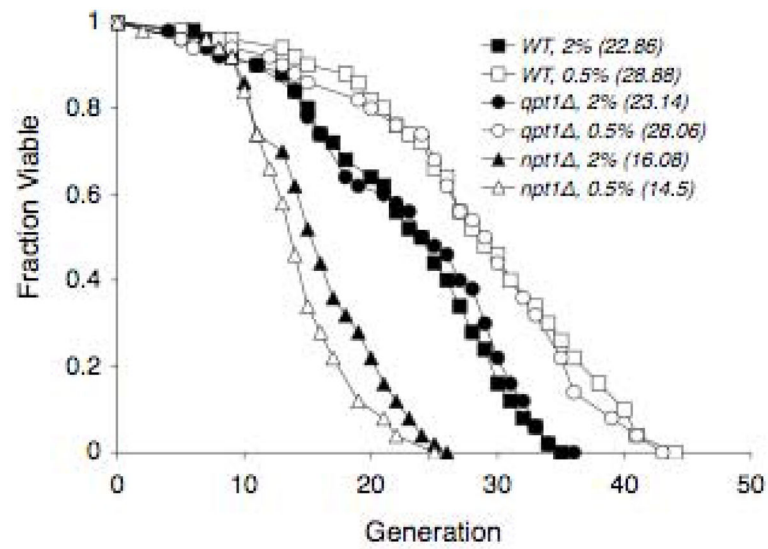
Figure 2.
Calorie restriction extends life span in a wild type yeast and *qpt1* but does not extend lifespan in *npt1*. Average life spans on 2% glucose: wild type 22.86, *qpt1 23.14, npt1 16.08*. Average life spans on 0.5% glucose: wild type 28.88, *qpt1* 28.06, *npt1* 14.5.

Table 1

Quantitation of NAD^+ and NADH contents (amol/cell) via HPLC with UV detection (UV) and HPLC with ^{14}C detection (AMS) for wild type, *qpt1* and *npt1* knockout yeast under both normal and CR growth conditions. The ratio of AMS to UV-quantitated values under the two growth conditions is also shown (AMS:UV). Each value is the mean and standard deviation of at least three replicate experiments (n = 3–6).

| | Normal (2% Glucose) | | | | | | Calorie-Restricted (0.5% Glucose) | | | | | |
| | NAD^+ | | | NADH | | | NAD^+ | | | NADH | | |
	UV	AMS	AMS:UV	UV	AMS	AMS:UV	UV	AMS	AMS:UV	UV	AMS	AMS:UV
Wild Type	82 (10)	86 (12)	1.1 (0.2)	85 (8)	88 (15)	1.0 (0.1)	54 (4)	56 (5)	1.0 (0.1)	20 (7)	21 (8)	1.0 (0.1)
qpt1 knockout	53 (10)	58 (9)	1.1 (0.1)	9 (2)	9 (1)	1.1 (0.2)	31 (4)	35 (3)	1.1 (0.1)	15 (3)	15 (3)	1.0 (0.1)
npt1 knockout	37 (5)	0.1 (0.1)	0.00 (0.01)	5 (2)	0.04 (0.02)	0.01 (0.01)	22 (2)	0.00 (0.02)	0.00 (0.01)	3 (2)	0.01 (0.03)	0.00 (0.01)

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Table 2

Previously reported total cellular NAD$^+$ and NADH concentrations (mM) and ratios together with those from this study for wild type yeast, *npt1* and *qpt1* knockout strains under normal (2% glucose) and calorie-restricted (0.5% glucose) growth conditions.

	NAD$^+$ (mM)		NADH (mM)		NAD$^+$:NADH	
	Normal	Calorie-Restricted	Normal	Calorie-Restricted	Normal	Calorie-Restricted
Present study Wildtype	1.2 (0.1)	0.8 (0.1) [e]	1.2 (0.1)	0.3 (0.1) [f]	1.0 (0.2)	2.7 (1.3) [i]
Sporty et al. [2008]	1.4 (0.2)	1.7 (0.3)	1.4 (0.2)	0.9 (0.2)	1.0 (0.1)	1.9 (0.30)
Lin et al. [2004]	1.3 (0.1)	1.2 (0.1)	0.9 (0.1)	0.4 (0.1)	1.5	3.1
Anderson et al. [2002] [a]	2	-	0.8	-	2.6	-
Present study *qpt1* knockout	1.3 (0.3) [c]	0.8 (0.1) [c] [e]	0.2 (0.1) [b]	0.4 (0.1) [c] [g]	6.2 (2.4) [b]	2.1 (0.7) [c] [i]
Present study *npt1* knockout	0.9 (0.1) [b]	0.5 (0.1) [b] [e]	0.13 (0.05) [b]	0.08 (0.05) [b] [h]	8.1 (3.1) [b]	6.6 (3.3) [d] [j]

[a] Reported values were converted to concentrations (mM) as previously described (11),

[b] P < 0.05 compared to present study wild type,

[c] P > 0.1 compared to present study wild type,

[d] 0.05 < P < 0.10 compared to present study wild type,

[e] P < 0.05 compared to NAD$^+$ under normal growth,

[f] P < 0.05 compared to NADH under normal growth,

[g] 0.05 < P < 0.10 compared to *qpt1* NADH levels under normal growth,

[h] P > 0.10 compared to *npt1* NADH levels under normal growth,

[i] P < 0.05 compared to NAD$^+$:NADH ratio under normal growth conditions,

[j] P > 0.1 compared to NAD$^+$:NADH ratio under normal growth.