Biochimica et Biophysica Acta 1797 (2010) 1138–1148

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Diminished NADPH transhydrogenase activity and mitochondrial redox regulation in human failing myocardium

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ARTICLE INFO

Article history:
Received 15 November 2009
Received in revised form 30 March 2010
Accepted 5 April 2010
Available online 11 April 2010

Keywords:
NADPH transhydrogenase
Mitochondria
Glutathione
Heart failure
Oxidative stress
Myocardium
Human

ABSTRACT

Although the functional role of nicotinamide nucleotide transhydrogenase (Nnt) remains to be fully elucidated, there is strong evidence that Nnt plays a critical part in mitochondrial metabolism by maintaining a high NADPH-dependant GSH/GSSG ratio, and thus the control of cellular oxidative stress. Using real-time PCR, spectrophotometric and western blotting techniques, we sought to determine the presence, abundance and activity level of Nnt in human heart tissues and to discern whether these are altered in chronic severe heart failure. Left ventricular levels of the NNT gene and protein expression did not differ significantly between the non-failing donor (NF) and heart failure (HF) group. Notably, compared to NF, Nnt activity rates in the HF group were 18% lower, which coincided with significantly higher levels of oxidized glutathione, lower glutathione reductase activity, lower NADPH and a lower GSH/GSSG ratio. In the failing human heart a partial loss of Nnt activity adversely impacts NADPH-dependent enzymes and the capacity to maintain membrane potential, thus contributing to a decline in bioenergetic capacity, redox regulation and antioxidant defense, exacerbating oxidative damage to cellular proteins.

1. Introduction

NADPH transhydrogenase (Nnt, E.C.1.6.1.2), located within the inner mitochondrial membrane of animal cells, has a major function in the formation of NADPH and NAD^+ from $NADP^+$ and NADH by translocating protons across the inner membrane from the cytosol to the mitochondrial matrix [1]. Nnt activity is driven by the electrochemical proton gradient which promotes a conformational change in Nnt from an inactive to active state with catalytic sites for both NAD(H) and NADP(H) exposed on the matrix side of the inner membrane (see [2–5]). Based on flux studies, it has been estimated that Nnt is a major source of NADPH, accounting for up to 45% of the total NADPH supply, with the remainder arising from the pentose phosphate pathway, NADP-isocitrate dehydrogenase ($NADP^+$-ICDH) and the mitochondrial NAD(P)-malic enzyme (ME2) [6,7].

One proposed functional role for Nnt is the redox regulation of NADPH-dependent thiols, particularly mitochondrial glutathione, thioredoxin, peroxiredoxins and glutaredoxin [8,9]. As the conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH) requires NADPH, Nnt activity serves a critical task in maintaining a high GSH:GSSG ratio, thus protecting against reactive oxygen species-related damage and potentially limiting opening/induction of IMAC (inner mitochondrial anion channel) [10] and potentially also the MPT (mitochondrial permeability transition) pore [11]. Strong support for this role of Nnt was derived from work with Nnt deletion mutants of the nematode *Caenorhabditis elegans* which became highly sensitive to oxidative stress when superoxide formation was augmented with methylviologen, thus markedly decreasing surviving larvae that could successfully progress to the adult stage [12]. This effect was reversed by reintroduction of the NNT gene.

Although much of the previous work on Nnt has been performed in non-mammals, gene quantification using RT-PCR and northern blotting in the mouse has confirmed expression of the NNT gene in all major organs, with the highest expression being in the heart [13]. Nnt activity has been measured in numerous animal species, all demonstrating the highest rates in cardiac mitochondrial fractions [14]. However, to date Nnt activity and its gene or protein expression have not been previously reported for the human heart. There is now mounting evidence that the pathogenesis and progression of human heart failure involves augmented generation of reactive oxygen species and related oxidative stress [15–19]. Thus, the aim of the present study was to determine the expression and activity levels of Nnt in left ventricular myocardium from end-stage heart failure patients and non-failing donors.

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doi:10.1016/j.bbabio.2010.04.002

2. Methods

2.1. Patient sample preparation

Explanted heart tissue biopsies were obtained from patients with end-stage chronic heart failure (HF) undergoing cardiac transplantation (NYHA Class IV, mean age of 50 ± 3 years, left ventricular ejection fraction of 24 ± 2%, $n = 31$), with approval by the Alfred Hospital Human Ethics Committee for Discarded Tissue Research. Prior to transplantation all HF patients had a recent history of treatment with statins, diuretics, ACE inhibitors, β-adrenoreceptor antagonists and Ca^{2+}-antagonists. Non-failing donor hearts (NF, mean age of 55 ± 3 years, left ventricular ejection fraction of 64 ± 3%, $n = 20$) were obtained from multi-organ donors following subarachnoid hemorrhage-related brain death, and although they were free of overt cardiovascular disease, their hearts were unsuitable for transplantation for technical reasons. The use of these NF myocardial biopsies was approved for research by donor family consent and the Victorian Organ Donation Service, Australian Red Cross. Upon dissection human left ventricular myocardium was immediately frozen in liquid N_2 and stored at −80 °C. Tissue extracts were prepared by homogenizing frozen ground tissues in 10× volume of ice-cold phosphate buffer, pH 7.4, followed by centrifugation at 6000*g* for 10 min at 4 °C. For purified mitochondrial protein, mitochondria were isolated from 100 mg of frozen left ventricular tissues using the Qiagen Mitochondrial Isolation kit according to the manufacturer's protocol. Extracts were stored at −80 °C until use. Protein content was estimated using the bicinchoninic acid (BCA) assay (Sigma-Aldrich).

2.2. Human NADPH transhydrogenase antibody production and Nnt protein expression

Custom preparation of monoclonal antibodies against human Nnt and epitope mapping was as follows. Human NNT domain III cDNA was amplified by RT-PCR from human placenta total RNA and cloned into pQE30 vector (Qiagen, USA) at *Bam*HI/*Sal*I sites. Protein expression and purification by metal chelate affinity chromatography was performed under denaturing conditions using standard protocols. The protein was dialyzed against phosphate buffered saline and used for immunization of BalB/c mice. Cell fusion, cloning and selection were accomplished using standard hybridoma technology procedures and enzyme-linked immunoassays. One clone, 4F6, was chosen for further work due to its excellent ability to recognize Nnt in western blotting. The epitope has been mapped by analyzing its reactivity towards truncation mutants. 4F6 reacts with overlapping fragments 835–983 and 968–1043 but does not bind fragment 835–979. Therefore, the epitope for 4F6 lies within the fragment 968-TVNSAAQEDPNSIIAG-983 of the human enzyme. This fragment represents "loop D" of the domain III. Also, this antibody reacts with Nnt from other mammals (rat, mouse, pig, bovine), chicken and xenopus, but does not react with *C. elegans* or *Escherichia coli* proteins [12]. The sequence of the fragments is well conserved between the species and the only two non-conserved residues, E-975 and/or I-982, may be critically important for interaction with the antibody.

Nnt protein expression in isolated human heart mitochondria was determined using standard western blotting conditions. 5 μg protein per lane was run on a 10% SDS-PAGE gel and transferred to a PDVF membrane at 100 V for 1 h. Membranes were blocked for 1 h at room temperature with TBS-Tween (TBST) + 5% skim milk (TBST/skim) and incubated overnight with primary antibody at 4 °C. The primary antibody mixture contained custom-made mouse monoclonal α-Nnt (clone 4F6; 1:1000 dilution) and monoclonal α-porin (VDAC1) as a mitochondrial reference standard (Molecular Probes, 1:2000 dilution), and was prepared in TBST/skim. Membranes were washed with TBST and incubated with secondary Ab (goat α-mouse/HRPO conjugate; 1:2000 dilution in TBST/skim; Biorad) for 1 h at room temperature. Band detection was via ECL chemiluminescent reagent (Packard Biosciences). Membranes were then exposed to film using a Kodak X-Omat processor and bands analyzed using Visionworks analysis software (UVP).

2.3. Partial purification of Nnt by immunocapture

Nnt was partially purified from isolated human heart mitochondria using an immunocapture bead matrix (Mitosciences; Eugene, OR). 200 μl (1 mg) of mitochondrial protein was initially solubilized with 20 μl of 10% dodecyl-β-D-maltoside (Anatrace) for 30 min on ice, followed by a 10-min centrifugation at 16,000*g* (4 °C). The solubilized protein (200 μl supernatant) was then incubated with 10 μl of bead matrix (cross-linked to 25 μg anti-Nnt monoclonal antibody, in 160 μl PBS, pH 7.2) for 3 h at room temperature with continuous rotation. Excess sample was washed three times from the beads with 100 volumes of 50 mM Tris–HCl, pH 7.4, followed by gentle centrifugation at 1000*g* for 1 min. Nnt protein was eluted from the beads by the addition of 30 μl 20 mM glycine, pH 2.5, incubation at room temperature for 10 min, followed by 1 min of gentle centrifugation at 1000*g*. The supernatant containing enriched Nnt was taken from above the beads and the elution procedure repeated twice to maximize sample recovery. 10 μl of 1.5 mM Tris, pH 8.8 was added to the combined eluents to neutralize the samples, which were then stored at −80 °C for further analyses.

2.4. NNT gene expression

NNT gene expression was measured using real-time PCR techniques. RNA was freshly extracted from frozen left ventricular tissues using phenol/chloroform extraction (RNAwiz, Ambion) according to the manufacturer's instructions. Total RNA was quantified using the Nanodrop ND-1000 spectrophotometer and reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems; ABI). Real-time PCR reactions were prepared by the addition of 10 μl Taqman® Universal PCR master mix, 1 μl FAM-labelled pre-designed primer/probe for human NNT (Taqman Gene Expression Assay Id: 00202583_m1; ABI) and 9 μl cDNA (diluted 1/10 in RNAse-free H_2O) in triplicate to a 384-well reaction plate (ABI). Plates were run under standard thermal cycling conditions in an ABI Prism 7900HT PCR machine. Ct values for Nnt were normalized to those of human GAPDH (Assay Id; 99999905_m1), which were measured using the identical cDNA samples in a singleplex assay on the same plate. Primer/probe mixes were designed by Applied Biosystems to be highly specific to the gene of interest, with primers crossing at least two exon boundaries, preventing the reaction from detecting genomic DNA contamination.

2.5. Nnt activity

Nnt activity was measured in isolated human heart mitochondria using a Cary 300 UV-VIS double beam spectrophotometer (Varian Inc) in a 1-ml reaction volume, essentially as described [20]. The reaction mixture was composed of 50 mM Tris (pH 8.0), 0.5% Brij-35, 1 mg/ml lysolecithin, 300 μM APAD (3-acetylpyridine adenine dinucleotide; Sigma-Aldrich), an analogue of NAD^+, and 300 μM β-NADPH. APAD was used instead of NAD^+ because of the different absorption spectrum of APADH as compared to those of NADPH and NADH. The reaction was initiated by the addition of 50 μg mitochondrial protein (20 μl volume) and the change in absorbance monitored at 375 nm for 3 min. Rates were converted to nmol APAD reduced/min using the mmolar extinction coefficient of 5.1 mM^{-1}, cm^{-1} for APAD and normalized to protein, which was determined using the BCA assay.

2.6. Protein carbonylation

Protein carbonylation refers to side chain proteins oxidised by reactive oxygen species to form reactive aldehydes and ketones. These can be reacted further with 2,4-dinitrophenylhydrazine (DNP) to form hydrazones in which carbonyl species can be detected using immunological blotting techniques. Direct oxidation was therefore measured using the Oxyblot Protein Oxidation Detection Kit (Chemicon, USA) according to the manufacturer's protocol. Briefly, carbonyl groups were derivatized by reaction with DNP for 15 min and DNP-derivatized proteins were then resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. Membranes were incubated overnight with primary antibody to the DNP moiety and then with secondary goat anti-rabbit/horseradish peroxidase antibodies. Signals were visualized by chemiluminescence detection using a Kodak X-Omat film processor and band desitometry was determined using Visionworks analysis software (UVP, USA).

2.7. Determination of GSH and GSSG

GSH and GSSG, and total glutathione (GSH+GSSG) were quantified using the GSH/GSSG-412 assay kit (CAT: 21040; OxisResearch, USA). Tissue extracts for GSH + GSSG were prepared by homogenizing frozen ground tissues in 10 volumes of ice cold phosphate buffer, followed by centrifugation at 2000*g* for 10 min at 4 °C. Homogenates were stored at −80 °C until required. For quantification of GSSG, tissues were processed as for total glutathione, with the addition of 3 mM of the thiol scavenging agent, 1-methyl-2-vinylpyridinium trifluoromethanesulfonate (M2VP) to the homogenization buffer, which provides complete derivatization of GSH within 1 min, preventing its participation in the GSH cycling assay. Samples were then assayed spectrophotometrically according to the kit protocol, based on the method of Tietze [21].

2.8. Glutathione reductase activity

Glutathione reductase (GR) activity was measured in tissue extracts based on the rate of reduction of GSSG to GSH by NADPH. Activity was measured by the addition of 20 µl tissue extract to an assay medium (100 mM phosphate buffer, pH 7.5; 1 mM EDTA) containing 1 mM GSSG, 0.1 mM NADPH and 0.75 mM DTNB (1 ml final volume). Activity was measured by the rate of absorbance increase at 412 nm and compared to that of known standards (Sigma Aldrich).

2.9. Determination of NADPH/NADP

Myocardial NADPH/NADP was determined via high pressure liquid chromatography (HPLC) using the method of Bernocchi et al. [22], briefly detailed as follows. Frozen tissues were ground in liquid N_2 with 0.4 N $HClO_4$ and centrifuged at 4000 g (4 ºC, 10 min). The pH of the supernatant was adjusted between 6 and 6.5 with KOH, and these samples were assayed for NAD and NADP. For NADH and NADPH, tissues were ground in phenol buffer (0.64 M phenol, 0.07 M phosphate buffer, pH 7.8) and added to an equal volume of chloroform and thawed to 4 ºC in the dark. These homogenates were centrifuged and the aqueous phase was washed five times with 5 ml diethyl ether before extracts were used for HPLC. Samples for NADPH and NADP assay were run on a C_{18} 3 µm RP column (0.46 × 15 cm, Supelchem) via a mobile phase gradient of buffer A (2.5% v/v acetonitrile, 5 mM tetrabutylammonium and 0.1 M KH_2PO_4, pH 6.0) and buffer B (25% v/v acetonitrile, 5 mM tetrabutylammonium and 0.1 M KH_2PO_4, pH 5.5). Extract peaks were detected at 260 nm and identified by co-elution of known standards.

3. Results

3.1. Nnt protein levels

Fig. 1A shows the detection of the human myocardial Nnt protein at approximately 110 kDa on a 10% SDS-PAGE gel, as predicted following cleavage of transit peptide and precursor [12]. The Nnt bands were revealed maximally within 2-min film exposure using ECL detection reagent with a 5 µg/lane mitochondrial protein loading. Expression of VDAC1 was evident at 30 kDa and was highly abundant (Fig. 1B).

Analysis of protein band density corresponding to Nnt indicated that although the Nnt levels in the HF group tended to be lower, these did not differ significantly from NF and HF groups ($p = 0.248$), even when each sample was normalized to the VDAC1 level ($p = 0.275$; Fig. 2A). There was no significant difference in VDAC1 expression for these samples (Fig. 2B; $p = 0.829$ NF vs. HF), indicating that under identical protein loading conditions, mitochondrial content was similar between the two groups. This was further confirmed by the presence of similar citrate synthase levels in both groups (data not

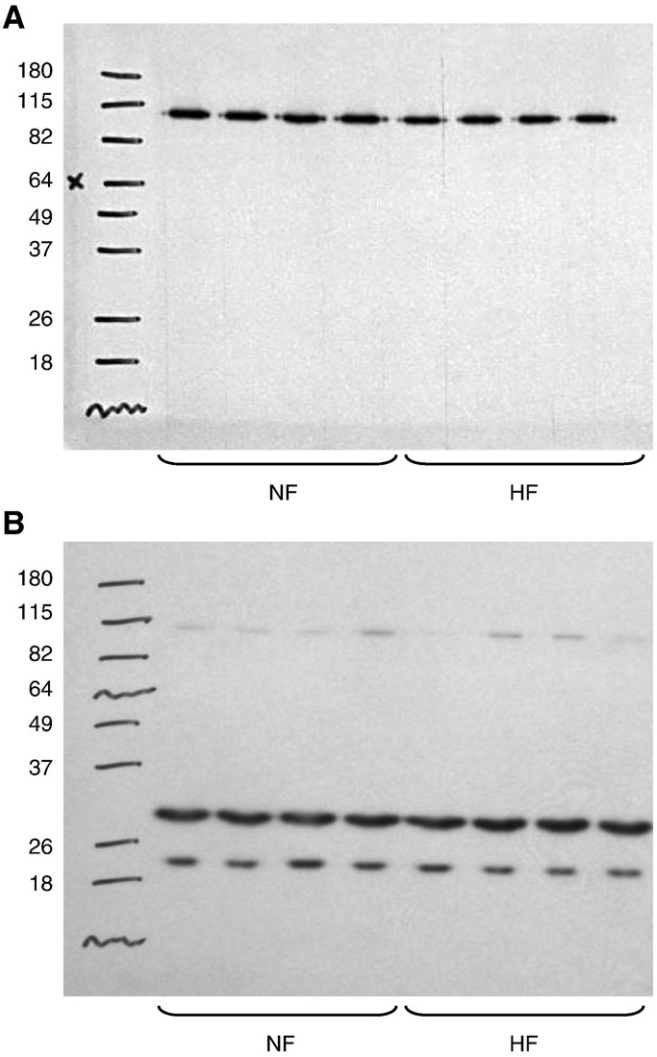

Fig. 1. Protein expression of Nnt and Porin (VDAC1) in isolated human heart mitochondria. 5 µl mitochondrial protein from NF ($n = 20$) and HF ($n = 31$) hearts (left ventricle) was run on a 10% SDS-PAGE gel, transferred to a PVDF membrane and blotted with monoclonal antibodies to Nnt and VDAC1. (A) Nnt expression following 1 min film exposure; (B) VDAC1 expression following 20 sec film exposure.

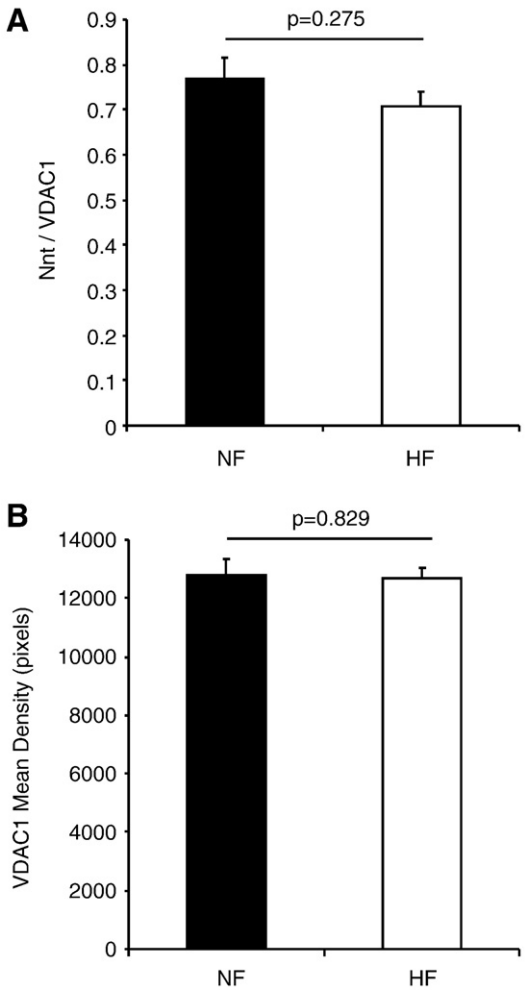

Fig. 2. Nnt protein and porin (VDAC1) in non-failing and failing human left ventricle. (A) Nnt was detected in isolated human heart mitochondria using western blotting techniques with monoclonal Nnt antibody. Loading: 5 μg mitochondrial protein/lane. Values represent mean density of Nnt bands normalized to VDAC1 expression. (B) VDAC1 was detected in isolated human heart mitochondria with monoclonal VDAC1 antibody. Loading: 5 μg mitochondrial protein/lane. Values represent mean density (pixels) of VDAC1 expression. All values are expressed as mean ± SEM. ($n = 20$) vs. HF ($n = 31$).

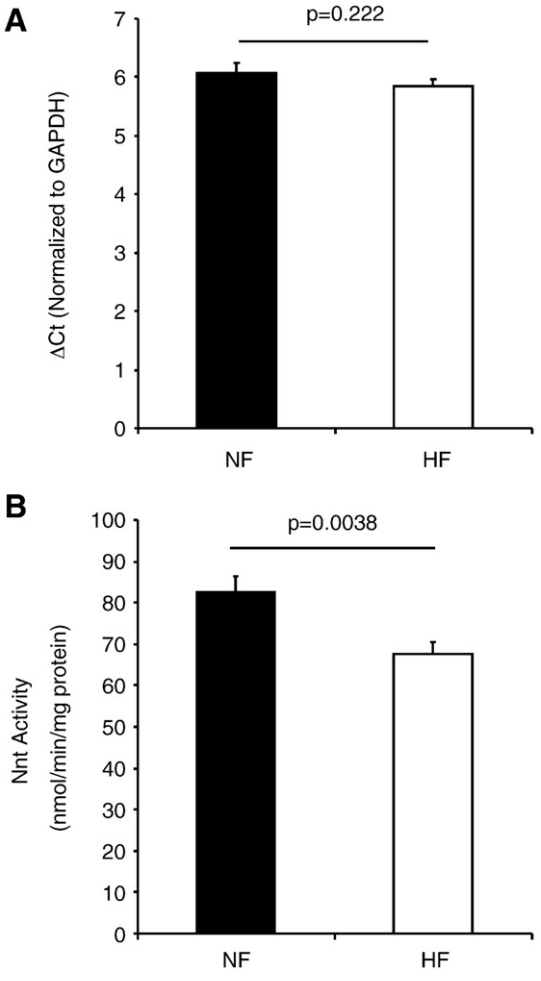

Fig. 3. (A) NNT gene expression in non-failing and failing left ventricle. Values represent mean ΔCt ± SEM (normalized to GAPDH). As each cycle represents a doubling in gene product, this difference equates to a 1.2-fold decrease in expression in the heart failure group, $p = 0.222$, NF ($n = 20$) vs. HF ($n = 31$). (B) Nnt Activity in Non-failing and Failing human heart. Nnt activity was measured spectrophotometrically using 50 μg (20 μl) isolated human heart mitochondria in a 1-ml reaction volume. Values represent mean ± SEM, $p = 0.0038$, NF ($n = 20$) vs. HF ($n = 31$).

shown). Thus, Nnt protein levels are abundant in the human left ventricle, even in end-stage chronic heart failure.

3.2. NNT gene expression

For NNT gene expression the human NNT gene sequence was accessed from the Entrez Gene (NCBI) Database (Gene ID: 23530) and complementary FAM-labelled primer/probe reaction mixes were manufactured to this sequence by Applied Biosystems (ABI). Primers crossed at least two exon boundaries and therefore would not pick up any genomic DNA contamination. RNA was isolated from frozen ground human heart tissues and analyzed for NNT gene expression using real-time PCR techniques. NNT gene expression was normalized to that of GAPDH run for identical cDNA samples on the same reaction plate.

Although NNT gene expression values in the HF group were marginally lower than those in NF these were not significantly different (Fig. 3A; $p = 0.222$). Gene expression was relatively abundant, with fluorescence intensity reaching the cycle threshold at an average of 24.3 cycles for NF and 24.1 for HF tissues, compared to 17–18 cycles for GAPDH. Expression was also highly conserved between individuals with small variation between individual values. ΔCt values normalized to GAPDH were 6.1 ± 0.2 for NF vs. 5.8 ± 0.1 in HF tissues, ($p = 0.222$). Neither GAPDH nor 18srRNA expression differed between NF and HF ($p = 0.859$, $p = 0.167$, respectively). However, heart failure severity, based on measurement of molecular markers of heart failure, was confirmed via real-time PCR. Atrial natriuretic peptide and β-myosin heavy chain gene expression were increased in left ventricular samples by 4-fold ($p = 0.009$) and 1.8-fold ($p = 0.003$), respectively, in the heart failure group (data not shown). These results indicate that both Nnt protein levels and gene expression do not significantly differ between NF and HF human heart tissues.

3.3. Cardiac Nnt activity

Although *in vivo* the formation of NADPH by Nnt is highly favored, the reaction has been proposed to operate in reverse under certain physiological conditions, e.g. ischemic stress [9]. For the assay of Nnt activity, the reaction was performed in reverse using the analogue of NAD^+, APAD, which has a higher oxidation potential than NAD^+ and a different absorption spectrum especially for the reduced form, and is thus useful for the measurement of activity in dehydrogenase or transhydrogenase enzymes [23,24]. Nnt activities were estimated at 60–100 nmol/min/mg protein, which is in line with that reported for other solubilised mitochondrial transhydrogenases [25]. Comparing

NF and HF samples, Nnt activity was reduced by 18% in the HF, with activity rates of 82.6 ± 2.0 in the NF and 67.8 ± 2.1 nmol/min/mg protein in the HF group ($p = 0.0038$; Fig. 3B).

To confirm that this activity was specific for Nnt, isolated mitochondria were incubated for 5 min with increasing concentrations of 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD chloride) an inhibitor of Nnt [26]. Rates following exposure to 10 μM, 100 μM and 1 mM NBD chloride were 89%, 67% and 34% of control values respectively ($n = 6$ per group; Fig. 4). Inhibitory effects were also examined via incubation with palmitoyl CoA, which specifically interferes with Nnt activity by competition with NADPH-binding sites [27] (Fig. 5A, B).

We also sought to determine whether Nnt activity was influenced by oxidative stress and antioxidant status. While post-oxidative products peroxynitrite and 4-hydroxynonenal had no effect on Nnt activity even at millimolar concentrations (data not shown), there was a moderate increase in Nnt activity following exposure to hydrogen peroxide (Fig. 6). Incubation with 10 μM, 100 μM and 500 μM H_2O_2 resulted in a 113%, 127% and 136% respective increase in Nnt activity compared to controls ($n = 4$). Thus, Nnt activity may be augmented as an important mechanism of response to increased hydrogen peroxide, since mitochondria rely mainly on the production of reduced glutathione for the removal of peroxides rather than catalase (but see Section 4). However, extremely high, supra-pathophysiological concentrations of peroxide above 1 mM caused a decline in Nnt activity (not shown).

To confirm that antibody binding was not affected by oxidative agents, peroxide-treated samples were run on a 10% SDS-PAGE gel and blotted for Nnt. As shown by western blotting, antibody binding was not prevented by NBD chloride nor H_2O_2 exposure, (Insets, Figs. 4 and 6). Thus, activity measured can be considered to be specific, while modification of the enzyme by oxidizing agents or thiol modification appeared to have no effect on the binding capacity of the antibody.

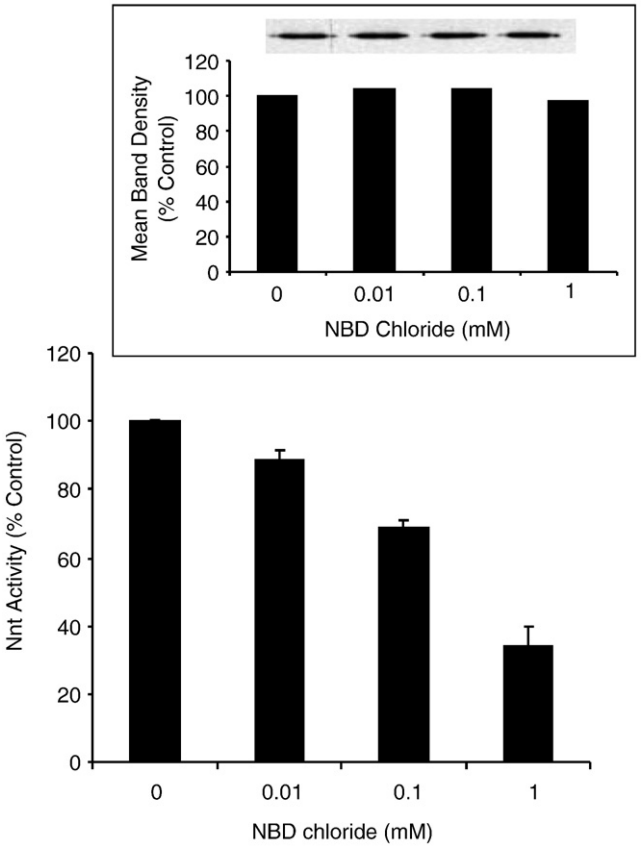

Fig. 4. Effect of NBD chloride on Nnt activity. 50 μg (20 μl) isolated non-failing human heart mitochondria was incubated for 5 min with NBD chloride and Nnt activity measured. $n = 6$ per group. Results expressed as mean ± SEM. (Inset above: Nnt protein expression in identical mitochondrial samples treated with NBD chloride).

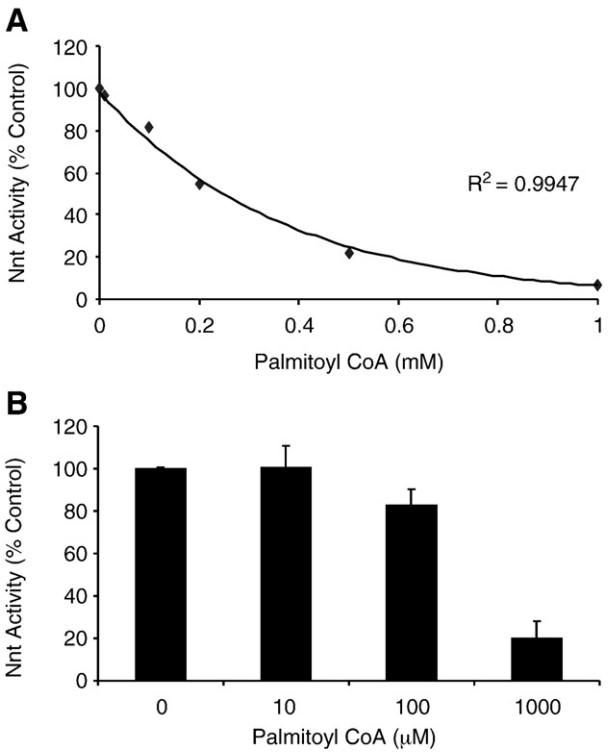

Fig. 5. Effect of palmitoyl CoA on Nnt activity. (A) 50 μg (20 μl) isolated non-failing human heart mitochondria were incubated for 5 min prior to assay with increasing doses of palmitoyl CoA. (B) Group average effect of palmitoyl CoA at 10, 100 and 1000 μM, $n = 4$ per group. Results expressed as mean ± SEM.

Given the link between Nnt, NADPH and glutathione, we further explored the possibility of feedback regulation of Nnt by GSH and GSSG. However, GSH (up to 1 mM) or GSSG (up to 5 mM) had no or little effect (data not shown). In contrast, S-nitrosoglutathione (NO-GSH) above 1 mM inhibited Nnt significantly with 100% inhibition at 3 mM or higher concentrations (Fig. 7). Inhibition by NO-GSH is likely mediated via release of nitric oxide.

3.4. Oxidative modification of Nnt

Nnt partially purified from isolated human heart mitochondria by immunocapture was used to determine whether it represented a significant target of oxidative damage under both normal conditions and the diseased state. Nnt from both NF and HF mitochondria displayed evidence of protein oxidation (DNP-related carbonylation) (data not shown). These same samples were confirmed to be Nnt-specific by immuno-blots. There was a higher state of DNP-related carbonylation in the HF group (DNP:Nnt density ratio 0.54 NF vs. 0.70 HF). Thus, the high generation of DNP indicates that Nnt is a significant target of oxidative damage, to some extent observed even in the NF group, which is subject to the brief, brain death-related acute oxidative stress that is induced by the Cushing reflex effect.

3.5. GSH/GSSG

Samples from the heart failure group showed a significant decrease in total GSH (Fig. 8A), i.e. GSH+GSSG, measuring an average of 60.9 ± 2.7 nmol/mg protein in the NF and 54.0 ± 1.6 nmol GSH/mg protein in HF tissues ($n = 20$ NF, $n = 29$ HF; $p = 0.022$), which corresponded to a decrease in reduced GSH levels in the HF group ($p = 0.027$). Concurrently, there was a three-fold increase in GSSG in HF tissues (34.98 ±

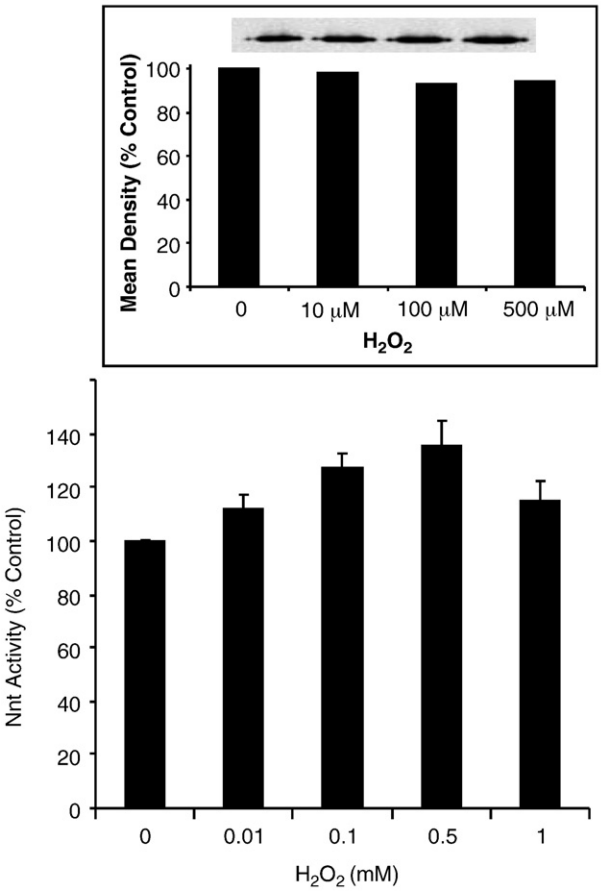

Fig. 6. Effect of hydrogen peroxide on Nnt activity. 50 μg (20 μl) isolated non-failing human heart mitochondria was incubated for 5 min prior to assay with increasing doses of hydrogen peroxide. $n=4$ per group. Results expressed as mean ± SEM. (Inset above: Nnt protein expression in identical mitochondrial samples treated with H_2O_2).

7.7 pmol/mg protein NF, $n=17$; vs. 109.73 ± 24.6 pmol GSSG/mg protein HF, $n=27$; $p=0.0068$).

In contrast to our findings, Biagioli et al., [28] have reported GSH and GSSG values for the donor human heart during transplantation that are markedly lower compared to our present study [GSH (7.8 ± 2.47 pmol/

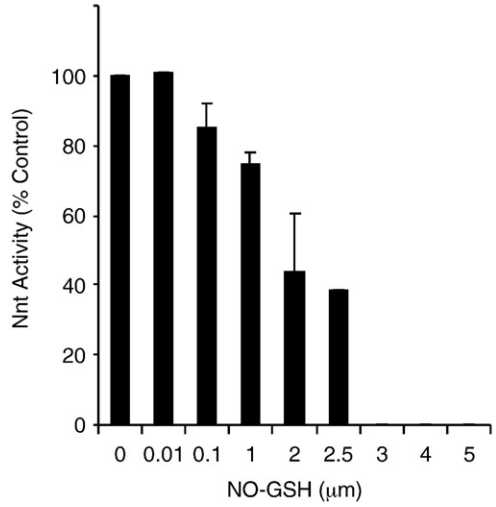

Fig. 7. Effect of S-nitrosoglutathione (NO-GSH) on Nnt activity. Isolated human heart (left ventricle) mitochondria (50 μg protein in 20 μl) were incubated for 5 min with NO-GSH and Nnt activity was measured spectrophotometrically. Results are expressed as mean ± SEM, $n=6$ per group.

mg protein and GSSG (1.58 ± 0.55 pmol/mg protein). Janssen et al [29] in a study of failing human hearts, that were subsequently subjected to additional experimental ischemia-reperfusion stress, reported GSSG levels (0.61 ± 0.12 μmol/g protein) that are generally comparable with those of our present study however GSH values are higher (25.3 ± 4.5 μmol/g protein). Studies in blood from healthy individuals have estimated GSH levels at 1.01 mmol/L [30], while in control human skeletal muscle biopsies, values of 1.6 nmol GSH+GSSG/mg wet weight have been reported [31].

GSSG levels were comparable to other reported values, with the present study values of 35–110 pmol GSSG/mg protein comparable to 200 pmol/mg protein reported in *C. elegans* [12]. In patients undergoing coronary artery bypass graft (CABG) surgery, coronary sinus blood GSSG levels have been reported as negligible prior to aortic cross clamping, increasing to 100–500 pmol/ml within minutes of cross-clamp removal and returning to near zero levels within 10 min following coronary bypass [32]. Similar results have been reported in more recent studies of elective CABG patients [33,34]. In human skeletal muscle biopsies, values of 60 pmol GSSG/mg wet weight have been reported [31].

The GSH/GSSG ratio was lower in the failing left ventricular tissues, with a ratio of 2148 ± 591 in NF ($n=20$) and 892 ± 169 in HF ($n=29$), $p=0.049$, Fig. 8B. Our GSH/GSSG ratios were markedly higher than the ratio of 60 reported for the nematode *C. elegans* [12] and 150 reported for the transgenic glutaredoxin-2-overexpressing mouse heart [35] although a ratio of 441 was reported in *Rhodobacter sphaeroides* [36]. The high GSH/GSSG ratio is mainly attributed to both the high GSH level as well as the low GSSG values in the human myocardial tissues, being in the pmol/mg protein range. A predicted GSH/GSSG range of 30 to 300 has been quoted by Aon et al. [37] in their study of isolated guinea pig cardiomyocytes. They found that reversible membrane depolarization occurred when the GSH/GSSG ratio was clamped at ratios between 150 and 100, however when the ratio was clamped below 50 the myocytes progressed to irreversible depolarization, maximal production of reactive oxygen species (ROS) and loss of matrix contents [37]. These results suggest that a higher (>150), rather than lower (<100) GSH/GSSG ratio is preferable in these cardiomyocytes.

3.6. Glutathione reductase activity

The activity of glutathione reductase, which reduces GSH from GSSG, was significantly higher in the NF, (9.6 ± 0.26 mU/min/mg protein) compared to HF (8.7 ± 0.16 mU/min/mg protein, $p=0.0028$, Fig. 9A).

3.7. NADPH/NADP

Compared to NF, NADPH (NF: 795 ± 81 vs. HF: 298 ± 29 nmol/mg protein, $p=0.00013$) and NADP (NF: 318 ± 34 vs. HF: 226 ± 26 nmol/mg protein, $p=0.0475$) levels were markedly lower in the HF group with a corresponding lower NADPH/NADP ratio (Fig. 9B).

4. Discussion

In this study it was demonstrated for the first time that, despite relatively normal expression of Nnt and comparable Nnt protein levels between the NF and HF groups, there is a significant decrease in Nnt activity in the failing human heart. As we have detected DNP-related carbonylation of Nnt isolated by monoclonal Nnt-immunocapture technique it is likely that Nnt activity may be impeded by post-oxidative protein modification. However, it is important to note that our determination of decreased Nnt activity is the maximal activity of the reverse reaction, thus it is possible that the forward-reaction driven Nnt activity may be even lower. As the rate of these Nnt reactions are dependent on mitochondrial membrane potential and this is decreased in HF [15,38], then the Δp-driven forward reaction

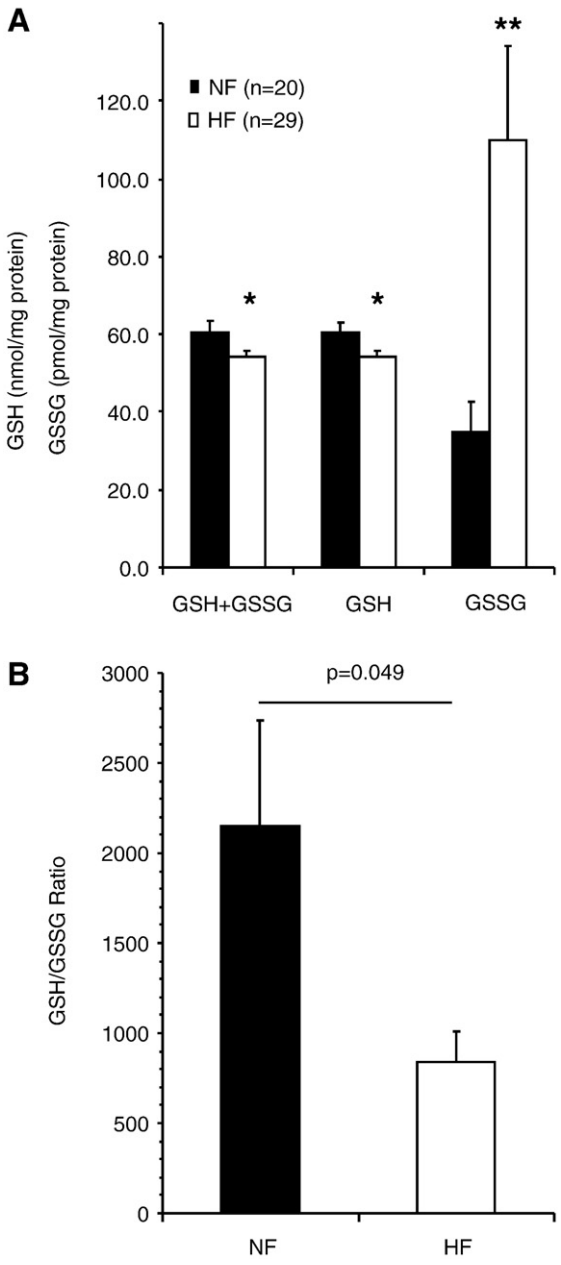

Fig. 8. (A) Total, reduced and oxidized glutathione in non-failing and failing human heart. Left ventricular tissue extracts were analyzed spectrophotometrically using the GSH-GSSG-412 assay kit. Values represent mean ± SEM. *p*-Values NF vs. HF: Total GSH (GSH+GSSG): $p = 0.029$, GSH: $p = 0.027$, GSSG: $p = 0.0068$. (B) GSH/GSSG ratio in human non-failing and failing heart. The GSH/GSSG ratio was determined by dividing GSH (nmol/mg protein) by 2GSSG (nmol/mg protein) for each LV sample. Values represent mean ± SEM, ($n = 20$) vs. HF ($n = 31$).

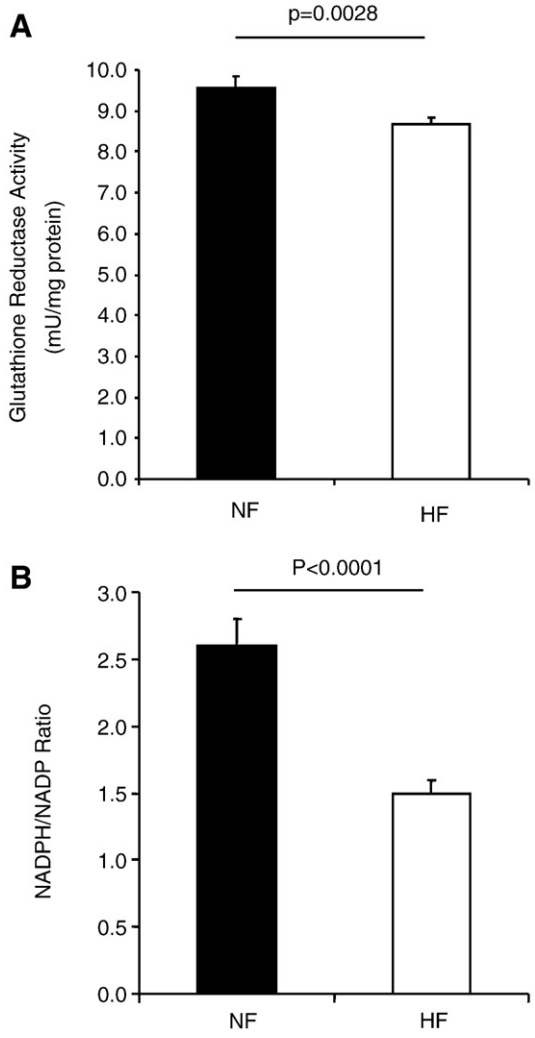

Fig. 9. (A) Glutathione reductase activity and (B) NADPH/$NADP^+$ ratio in human non-failing and failing left ventricle. Values represent mean ± SEM. NF ($n = 20$) vs. HF ($n = 31$).

catalyzed by Nnt is expectedly diminished in HF. Thus, diminished Nnt activity has significant implications relating to the reduced tolerance of the failing heart to minimize oxidative damage to proteins and lipids.

Although the role of Nnt and its contribution to the overall bioenergetics of the mitochondrion is yet to be fully defined, there is substantial evidence for a number of roles of Nnt, which include:

1) Control of ROS and cellular redox state by replenishment of antioxidant systems, namely GSH and mitochondrial repair enzymes (thioredoxin, glutaredoxin, peroxiredoxins and phospholipid hydroperoxidase), directly or indirectly using NADPH as an electron donor [7,12,36].
2) Maintenance of mitochondrial membrane potential through generation of a proton gradient by reverse reaction under conditions of transient ischemia [9].
3) Fine regulation of Krebs cycle flux by interaction with the $NADP^+$-ICDH [39]. There is also increasing evidence that Nnt, by regulating the level of NADPH, is also involved in calcium homeostasis and apoptosis, possibly through the bcl-2 and mitochondrial pore proteins [40,41].

4.1. Implications of reduced Nnt activity on tissue glutathione levels

Glutathione is synthesized in the cytoplasm but can be exchanged with other cellular compartments. While mitochondria are not able to synthesize GSH, they are able to accumulate it up to a percentage of 10–25% of total cellular GSH [42,43]. Cytosolic and mitochondrial GSH pools are distinct, although there may be an "overflow" or exchange mechanism between the two compartments. The cell possesses a number of powerful antioxidant defenses capable of counteracting ROS and repair of oxidized molecules. These mechanisms work to maintain cellular components in a predominantly reduced redox state and are maintained by two major factors: (1) the reduction potential of the electron carriers (NADH/NAD^+ and $FADH_2$/FAD) and (2) the reducing capacity of linked redox couples present in the cytoplasm or in the intra-organellar compartments (e.g. mitochondrial matrix) of

the cell [37]. Those included in the second are reduced/oxidized glutathione (GSH/GSSG), thioredoxin ($Trx(SH)_2$/TrxSS), glutaredoxin, peroxiredoxins (Prx3 and Prx5) and NADPH/$NADP^+$, of which the latter provides the driving force for the glutathione and thioredoxin systems. The GSH/GSSG pool is the largest of the cell and is considered to be a major indicator of cellular redox status [44,45].

Superoxide formed by the mitochondrial respiratory chain is removed by the action of superoxide dismutases (SOD), resulting in the formation of H_2O_2. In the cytoplasm, H_2O_2 is further converted to water and oxygen by the catalase enzyme. Mitochondria, however, instead rely mainly on GSH for the removal of H_2O_2, with the reduction of GSH fuelled by the NADPH-producing enzymes, even though mitochondrial catalase has been proposed to play a role [46]. Notably, incubation with pathophysiological concentrations of H_2O_2 increased Nnt activity, rather than decreased it, possibly due to an increased demand for GSH (Fig. 6). Being a relatively unreactive ROS, H_2O_2 may work as a signaling substance. GSH can also act as a potent antioxidant in its own right, providing defense against mitochondrial-produced ROS. GSSG is then reduced back by the action of GR whose activity is regulated by cellular GSSG levels and which requires NADPH as an electron donor [47]. A significant contribution of mitochondrial NADPH required for this reaction is supplied by Nnt, with contributions also from the $NADP^+$-ICDH and ME2 [7,48,49]. Thus, mitochondrial Nnt, which is interlinked with the reduction of GSH by production of NADPH, is of fundamental importance in the control of ROS.

Nnt knockout mutants of *C. elegans* displayed a significant decline in the GSH/GSSG ratio and increased sensitivity to oxidative insult (Paraquat), determined by a dramatic decline in those progressing to the adult stage [12]. The implications of GSH deficiency on mitochondrial function have been demonstrated in rats injected with buthionine sulfoximine, a selective inhibitor of γ-glutamylcysteine synthetase, which results in a significant loss of glutathione, in which those treated with the drug displayed grossly swollen brain mitochondria which lacked inner membrane structural features [50]. Based on the findings in the Nnt knockout nematodes, it would be predicted that an 18% reduction in Nnt enzyme activity in the failing human heart would have an impact on tissue GSH levels, which was indeed seen as a moderate (11%) decrease in total glutathione (Fig. 8) in the failing heart tissue. Given that GSSG levels in the myocardial tissues were very low (pmol/mg protein amounts vs. nmol GSH+GSSG/mg protein) it can be assumed that almost all of the total GSH pool was in the reduced form, translating to lower reduced GSH levels in the failing heart tissues. Assuming that the 11% fall in total GSH levels was consistent across all cellular compartments, and given that mitochondrial GSH accounts for 10–25% of the cellular total [43], this would translate to an effective loss of a significant part of the total mitochondrial GSH pool.

There are relatively few in depth studies examining myocardial tissue levels of GSSG and GSH in humans that are generally free of disease or intervention-dependent ischemia-reperfusion. However it is likely that the differences in myocardial GSH and GSSG levels reported in the present study, compared to other human studies which are also variable, may relate to a combination of differences in handling of tissue, assay methodology and disease state (varying severity of heart failure and donor hearts with subtle but variable ischemic injury). Most studies referencing values for glutathione (such as [29,32,51,52]), have utilized the enzymatic method of Tietze [21], as we have. These studies though have used the older generation agent, N-ethylmaleimide, to derivatise GSH so that it does not participate in the GSH cycling assay. However this agent has limitations in terms of inhibitory action on glutathione reductase, time required for derivatisation and capacity to permit complete conversion of GSH. In contrast, as first introduced by Griffith [53], we used the thiol scavenger 1-methyl-2-vinylpyridine (M2VP) which provides rapid and complete removal of GSH (within a minute vs. >1 h) but without inhibition of glutathione reductase. In a study of mitochondria isolated from pulmonary artery epithelial cells (and not myocardium) GSH/GSSG ratios up to 250 were measured using M2VP and were in keeping with higher ratio values also reported by Aon et al. [37]. It is therefore possible that in older studies using N-ethylmaleimide in the cycling assay, the level of GSSG may have been overestimated. This methodology with M2VP may possibly result in consistently lower human myocardial GSSG values thus influencing a higher GSH/GSSG ratio, however whether this permits a more accurate measure of GSSG requires a validation study with HPLC assay methodology.

Other factors found in our study which would also affect the decline in the reduced GSH pool in the failing tissues include lower NADH and the NADH/NAD ratio, as well as reduced activities of $NADP^+$-ICDH and ME2 (not shown). Most notably, we measured NADPH levels and the NADPH/NADP to be markedly lower in HF compared to the NF group, which supports our findings of the concomitantly lower level of Nnt activity, GR activity and GSH. Aon and colleagues [37] have also shown a direct relationship between mitochondrial redox potential (mV) and GSH and NADPH levels. Given that state III respiration and the activities of several OXPHOS proteins are markedly impaired in the failing heart [54–57], it is likely that mitochondrial membrane potential is also affected, influencing the rate of GSH and NADPH reduction. This, together with an increased demand on NADPH supply due to augmented oxidative stress and NADPH oxidase activity in the failing human heart [58], means that NADPH availability for GSH reduction is markedly impaired.

There are a number of implications of decreased NADPH and GSH availability in terms of antioxidant defense in the failing heart tissues. Reduced NADPH formation by the Nnt and other NADPH-linked enzymes would also have a direct impact on repair of oxidatively damaged proteins and lipids, namely through the action of repair enzymes such as glutathione peroxidase, mitochondrial thioredoxin, glutaredoxin, peroxiredoxins and phospholipid hydroperoxidases. Given the increased levels of mitochondrial-released superoxide [59] and myocardial release of oxidative intermediates in the failing heart [60–62], it is therefore likely that NADPH-linked repair mechanisms supported by Nnt are diminished, leading to increased oxidative damage to proteins and lipids in the failing human heart. In isolated rat cardiomyocytes, inhibition of glucose-6-phosphate dehydrogenase by dehydroepiandrosterone (DHEA) led to a marked reduction in the GSH/GSSG ratio, resulting in contractile dysfunction due to disrupted calcium handling [63].

GSH, in particular, is a major indicator of cellular redox status, with the GSH/GSSG ratio influencing a number of critical mitochondrial ion channels including the IMAC and the MPT pore. The MPT pore is a major player in initiating both apoptotic and necrotic cell death, with oxidation of GSH and other critical thiols favouring pore opening [64–66]. It has been previously found in isolated rat cardiomyocytes that substrate deprivation or localized ROS generation triggered collapse of mitochondrial membrane potential ($\Delta\psi_m$) in parallel with cyclical GSH oxidation [67,68]. The same authors went on to further demonstrate (see also Section 4.2 below) that decreasing the GSH/GSSG ratio to below 50 resulted in irreversible depolarization of the mitochondrial membrane, maximal rates of oxygen consumption, together with sequential opening of IMAC and the MPT pore leading to release of mitochondrial matrix contents and initiation of the apoptotic cascade [37].

4.2. Oxidative modification of Nnt and mitochondrial proteins

Increased superoxide production and markers of lipid peroxidation have been reported in the failing human heart [60,61,69–71]. ROS released from the mitochondrial respiratory chain may oxidatively modify membrane-bound proteins and lipids, resulting in enzyme dysfunction, thereby transiently contributing to the mitochondrial membrane potential. Nnt has been shown to support protection against singlet oxygen [72]. Previous *in vitro* studies using bovine heart submitochondrial particles have demonstrated that Nnt is susceptible to oxidative damage by Fe-ascorbate and peroxynitrate through two separate mechanisms, with increases in endogenous ubiquinone

preventing Fe-ascorbate damage only [73]. There was a significant oxidation of Nnt even under basal conditions, and a slight increase in the proportion of oxidatively-modified Nnt in the heart failure group, with a ratio of 0.54 in the non-failing and 0.70 in the failing group ($n=3$ per group), although this was not significant due to the low sample numbers. However, it can be concluded that Nnt represents a significant target of ROS-induced damage, which may lead to a decline in enzyme function and further exacerbate a loss of GSH and the capacity to handle oxidative stress.

4.3. Interaction between Nnt and mitochondrial membrane potential ($\Delta\psi_m$).

Of the NADPH-producing enzymes, Nnt is unique in that it is the only enzyme which couples production of NADPH to the rate of aerobic respiration, and hence the production of ROS. Early studies of the structure and function of Nnt revealed that the reaction is energy-dependent, with Nnt activity being driven either directly by respiration or ATP hydrolysis through a reversal of the reaction sequences involved in the respiratory chain-linked synthesis of ATP [1,74]. In fact, unlike $NADP^+$-ICDH and ME2, Nnt is the only NADPH-generating enzyme that is linked to mitochondrial membrane potential. The electrochemical proton gradient (Δp) generated by respiration stimulates the Nnt forward reaction and thus, the rate of reduction of $NADP^+$ ($\approx$10-fold) is also linked to an inhibition of the reverse reaction [75,76]. This linkage to Δp makes Nnt a more efficient generator of NADPH than other NADP-linked enzymes. These findings appear to relate to a conformational change in the enzyme from the inactive to active state under conditions where a proton gradient exists [75–78]. As the catalytic site for both substrates (one each for NAD(H) and NADP(H)) are exposed on the matrix side of the inner membrane, the enzyme can, in principle, either consume Δp generated by the respiratory chain in the forward reaction or generate Δp in the reverse reaction in the absence of other sources of Δp [9,39]. This led to the proposal by Hoek and Rydström [9] that under conditions of transient ischemia, Nnt may act in the reverse direction, thereby maintaining the mitochondrial membrane potential.

The relationship between the GSH/GSSG ratio, the NAD(P)(H)/NAD(P) redox state and mitochondrial membrane potential ($\Delta\psi_m$) was comprehensively studied in a series of experiments by Aon et al [37] on isolated guinea pig cardiomyocytes. In these experiments, the (external) GSH/GSSG ratio was clamped at ratios ranging from 300:1 to 20:1. At ratios of 150:1 to 100:1, reversible membrane depolarization occurred in saponin-permeabilized TMRM-loaded cardiomyocytes, with matrix components retained. At ratios of 50:1 however, irreversible membrane depolarization occurred resulting from opening of the mitochondrial ion channel IMAC, and later, by an unknown mechanism, a more dramatic and irreversible opening of the MPT pore, which also induced maximal rates of ROS production, NAD(P)H oxidation and loss of matrix constituents. Further probing of the relationship between GSH redox status and membrane polarization revealed that it was not the GSH/GSSG ratio *per se* which influenced membrane depolarization, but rather the concentration of GSSG. Maintaining GSSG at very low levels (10 μM) and altering the GSH/GSSG ratio by increasing the GSH concentration, mitochondria were able to resist membrane depolarization by a GSH/GSSG ratio as low as 20:1. Higher GSSG levels in failing myocardium, as shown in Fig. 8, may therefore lower the threshold for initiation of IMAC and membrane depolarization leading to cardiomyocyte death. Notably, inhibition of Nnt with NBD chloride resulted in irreversible membrane depolarization at ratios of 150:1 (1 μM NBD chloride) and 200:1 (10 μM NBD chloride), compared to the control value of 50:1, highlighting the critical role Nnt plays in maintaining the NADPH pool and a high GSH/GSSG ratio [37]. Given that Nnt is estimated to account for at least 45% of NADPH supply [6,7], a reduction in activity could have considerable consequences on the cellular GSH pool, increasing susceptibility to ROS-induced cell death.

In this context it should be stressed that it has been known for a long time that Nnt is rapidly saturated with Δp, and that the final extent of reduction of NADPH represents a steady-state rather than an equilibrium [75]. This represents only one aspect of the full complexity of the cellular systems for mitochondrial NADPH generation and utilization and their regulation. It may therefore be concluded that the overall regulation of the redox state of mitochondrial glutathione by Δp is complex and indirect, but where Nnt plays a key role.

4.4. Relationship between Nnt and NADP-linked Krebs cycle enzymes

While the precise interaction between Nnt and other NADPH-producing enzymes remains to be fully defined, several links between Nnt and $NADP^+$-ICDH have been identified. The interaction between these two was initially proposed by Sazanov and Jackson [39], who hypothesized that when excess NADPH by Nnt was present, the $NADP^+$-ICDH reaction could operate in reverse, effectively providing a fine regulation of Krebs cycle flux in response to changes in energy demand. Notably, this control also limits cellular oxidative damage [79]. In mutants of *E. coli* lacking the membrane-bound Nnt, growth on glucose medium was at a significantly reduced rate compared to wild-types but still possible [6], indicating that absence of Nnt was not critically lethal for growth if NADPH could be supplied by other enzymes, namely ICDH. Indeed in *E. coli* transhydrogenase mutants grown on acetate, growth rate was significantly impaired in the absence of $NADP^+$-ICDH [80]. In isolated mitochondria from rat forebrain, uncoupling by CCCP (carbonyl cyanide m-chlorophenylhydrazone) inhibited Nnt activity through the dissipation of the mitochondrial membrane potential. However, in these mitochondria, GSSG reduction was still evident when isocitrate or malate were used as substrates [37], thus NADPH production may have continued via $NADP^+$-ICDH or ME2. Detailed studies of $NADP^+$-ICDH and ME2 activities in chronic human heart failure are currently in progress in our laboratory.

4.5. Conclusions

Recent studies have identified a profound effect of Nnt dysfunction on the perturbation of glucose and insulin homeostasis in β-cells [81]. We hypothesize that Nnt would have a major role in myocardium which is dense with mitochondria, has a high oxygen and bioenergetic demand and a critical requirement to manage high rates of oxidative metabolism. This is the first study to report NNT gene expression and protein levels in human non-failing donor hearts and chronic end-stage failing human hearts. Of particular note is the finding of a marked reduction in Nnt activity in severe heart failure. Nnt represents a significant target of oxidative damage, which may contribute to a decline in activity and subsequent glutathione depletion, further exacerbating oxidative damage to mitochondrial proteins and diminishing the capacity of the myocardium to defend itself against oxidative insult.

Acknowledgments

The present work was supported in part by NHMRC Australia project grants (S. Pepe) and a Dora Lush NHMRC Postgraduate Scholarship (F. Sheeran). The design and preparation of monoclonal Nnt antibodies was supported in part by the Russian Foundation for Basic Research (grant 07-04-01497-a), and we thank Maria V. Ivanova and Maxim V. Egorov for their technical assistance in this production.

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