Dietary iron deficiency induces ventricular dilation, mitochondrial ultrastructural aberrations and cytochrome c release: involvement of nitric oxide synthase and protein tyrosine nitration

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ABSTRACT

Iron deficiency is associated with multiple health problems, including the cardiovascular system. However, the mechanism of action of iron-deficiency-induced cardiovascular damage is unclear. The aim of the present study was to examine the effect of dietary iron deficiency on cardiac ultrastructure, mitochondrial cytochrome c release, NOS (nitric oxide synthase) and several stress-related protein molecules, including protein nitrotyrosine, the p47phox subunit of NADPH oxidase, caveolin-1 and RhoA. Male weanling rats were fed with either control or iron-deficient diets for 12 weeks. Cardiac ultrastructure was examined by transmission electron microscopy. Western blot analysis was used to evaluate cytochrome c, endothelial and inducible NOS, NADPH oxidase, caveolin-1 and RhoA. Protein nitrotyrosine formation was measured by ELISA. Rats fed an iron-deficient diet exhibited increased heart weight and size compared with the control group. Heart width, length and ventricular free wall thickness were similar between the two groups. However, the left ventricular dimension and chamber volume were significantly enhanced in the iron-deficient group compared with controls. Ultrastructural examination revealed mitochondrial swelling and abnormal sarcomere structure in iron-deficient ventricular tissues. Cytochrome c release was significantly enhanced in iron-deficient rats. Protein expression of eNOS (endothelial NOS) and iNOS (inducible NOS), and protein nitrotyrosine formation were significantly elevated in cardiac tissue or mitochondrial extraction from the iron-deficient group. Significantly up-regulated NADPH oxidase, caveolin-1 and RhoA expression were also detected in ventricular tissue of the iron-deficient group. Taken together, these results suggest that dietary iron deficiency may have induced cardiac hypertrophy characterized by aberrant mitochondrial and irregular sarcomere organization, which was accompanied by increased reactive nitrogen species and RhoA expression.

Key words: caveolin-1, cytochrome c, heart, iron deficiency, NADPH oxidase, nitric oxide synthase (NOS).

Abbreviations: BW, body weight; HRP, horseradish peroxidase; LV, left ventricular; MLC, myosin light chain; NO, nitric oxide; NOS, NO synthase; eNOS, endothelial NOS; iNOS, inducible NOS; NOx, nitrite/nitrate; O2−, superoxide anion; ONOO−, peroxynitrite; RNS, reactive nitrogen species; TBS, Tris-buffered saline.

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INTRODUCTION

Deficiency in dietary iron intake has been demonstrated to be one of the most prevalent nutritional problems in the world with approximately 5 billion people afflicted. Nutritional iron deficiency is believed to trigger multiple cardiovascular diseases, including cardiac hypertrophy and chronic heart failure [1–3]. Although the precise mechanism of action responsible for dietary iron-deficiency-induced cardiac dysfunction and morphological aberration remains poorly understood, several adaptive or compensatory mechanisms have been postulated for altered cardiac function and morphology as a result of dietary iron deficiency. For example, erythropoietin production was elevated under iron deficiency and associated with increased intra-erythrocytic levels of 2,3-diphosphoglycerate [4]. In addition, structural remodelling and compensatory vascular dilatation may contribute to decreased systemic peripheral resistance and reduced afterload, which in turn increases stroke volume under iron deficiency [5,6]. Blood viscosity and sympathetic tone were reduced and elevated respectively, under iron-deficiency-induced anaemia, leading to enhanced venous return, preload and heart rate [7]. These haemodynamic and non-haemodynamic changes (e.g. preload, afterload, heart rate, stroke volume and erythropoietin) have validated the significance of iron content in the regulation of cardiovascular function and morphology.

Mitochondria are crucial players for various physiological processes, including embryonic development, fat metabolism and aging. More importantly, mitochondria are essential organelles for the pro- and anti-oxidant balance responsible for the pathogenesis of a number of cardiovascular disorders [8]. Mitochondria dominate oxygen consumption and energy expenditure [9]. On the other hand, iron is a major cofactor for a number of respiratory chain enzymes [10], many of which reside in mitochondria [10]. It is thus natural to speculate that mitochondria may be a target for anaemia/hypoxia induced by dietary iron deficiency. As a potent vasodilator, NO (nitric oxide) regulates tissue blood flow, oxygen supply and respiratory substrates to mitochondria. Recent studies indicated that NO generated by eNOS [endothelial NOS (NO synthase)] and iNOS (inducible NOS) may be the ‘missing link’ in mitochondrial oxidative damage via reaction with O$_2^−$ (superoxide anion) and ONOO$^−$ (peroxynitrite) formation [9,11]. It is also suggested that the biogenesis of mitochondria is tightly regulated by the levels of eNOS, NO or its second messenger cGMP [9,11]. It could be suggested that the diverse interactions between NO and mitochondria may be linked to oxidative stress and cardiac damage, eNOS and iNOS are key enzymes determining the levels of NO en route to RNS (reactive nitrogen species) injury if overproduced [12]. Activation of NADPH oxidase generates O$_2^−$, which rapidly reacts with NO to form ONOO$^−$, one of the devastating RNS. Therefore the aim of the present study was to examine the impact of dietary iron deficiency on cardiac mitochondrial microscopic morphology and cytochrome c release, NOS expression in both whole-cell extraction and mitochondria, and protein nitrotyrosine formation as an indicator of RNS. Several other related cardiac stress markers such as NADPH oxidase, caveolin-1 and RhoA, an important G-protein, which is closely associated with sarcomere rearrangement during cardiac remodelling [13], were determined in ventricular tissues from adult rats.

MATERIALS AND METHODS

Animals and dietary feeding regime

The experimental procedures used in the present study were approved by the University of Wyoming Animal Use and Care Committee. In brief, 1-month-old male Sprague–Dawley rats were obtained from Charles River Laboratories and randomly divided into two groups fed either an iron-deficient diet (Diet #115109; Dyets Inc.) or a control diet (AIN-93G; Dyets Inc.) for 12 weeks. The iron-deficient diet was AIN-93G with reagent-grade salts (for low-iron purity) and no added iron. Rats were fed and given water (purified by reverse osmosis) ad libitum, kept on a 12 h light/dark cycle, weighed weekly, and maintained in accordance with the National Academy of Science’s Guide for the Care and Use of Laboratory Animals. BW (body weight) and organ weight were measured with a laboratory scales at the completion of the feeding period [6].

Morphometric measurement of hearts

The experimental animals were killed under anaesthesia and the hearts were cut open along the sagittal axis. Ventricular walls and chambers were examined ventrally. Morphometric measurements were performed using metric calipers and an 8× Agfa magnifying loupe. Cardiac length and width, right and LV (left ventricular) free wall thickness, and LV major and lesser diameter were measured to the nearest 0.2 mm. The LV major length was the distance perpendicular from a line drawn joining cranial aspects of mitral and tricuspid valves to the distal aspect of the LV lumen. LV lesser diameter was the width of the LV lumen. LV chamber volume was calculated as $(4/3)\pi \times (LV \ major \ length/2) \times (LV \ lesser \ diameter/2)^2$ [2].

Transmission electron microscopy

Tissue sections of the left ventricles of two rats selected randomly from each group were fixed with 2.5 % glutaraldehyde/1.2 % acrolein in fixative buffer [0.1 M cacodylate and 0.1 M sucrose (pH 7.4)], post-fixed with 1 % osmium tetroxide, followed by 1 % uranyl acetate,
dehydrated through a graded series of ethanol concentrations, and embedded in 1X112 resin (LADD Research Industries). Sections (1 μm thick) were cut on an ultramicrotome (RMC-MTXL), stained with 1 % Toluidine Blue in 1 % sodium borate, and viewed on an Axioskop light microscope (Carl Zeiss). Ultra-thin sections (approx. 50 nm) were cut on the ultramicrotome, stained with uranyl acetate, followed by lead citrate, and viewed on a 1200EX transmission Electron Microscope (Hitachi-7000) equipped with a 4K digital camera at 80 kV [14,15].

**Analysis of mitochondrial cytochrome c release**
The ventricles were minced and homogenized using a Polytron homogenizer in ice-cold MSE buffer (220 mM mannitol, 70 mM sucrose, 2 mM EGTA, 5 mM Mops (pH 7.4), 0.2 % BSA and a protease inhibitor cocktail containing AEBSF [4-(2-aminoethyl)benzenesulphonyl fluoride], E-64, bestatin, leupeptin, aprotinin and EDTA (Sigma)). The homogenates were centrifuged for 10 min at 600 g to remove unbroken tissue and nuclei, and the supernatants were centrifuged for 10 min at 3000 g to pellet mitochondria. The supernatants were centrifuged further for 30 min at 100 000 g to obtain the cytosolic fraction. The mitochondrial pellet was dissolved in lysis buffer [20 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 % Triton, 0.1 % SDS and 1 % protease inhibitor cocktail] and centrifuged at 10 000 g for 30 min at 4 °C to produce a soluble protein fraction. A portion (50 μg) of the mitochondrial protein or cytosolic protein fraction was separated by SDS/PAGE [15 % (w/v) acrylamide] and analysed by Western blot using anti-cytochrome c antibody (Upstate) [16].

**Western blot analysis of eNOS, iNOS, p47phox subunit of NADPH oxidase, caveolin, RhoA and β-actin**
Total protein (whole-cell extraction) was prepared as described previously [17]. In brief, tissue samples from heart ventricles were removed and homogenized in lysis buffer. Samples were then sonicated for 15 s and centrifuged at 12 000 g for 20 min at 4 °C. The protein concentration of the supernatant was evaluated using Protein Assay Reagent (Bio-Rad Laboratories). Equal amounts (50 μg of protein/lane) of the protein from the whole-cell extraction, or mitochondria and prestained molecular mass markers (Gibco-BRL), were separated on 7 % (iNOS and eNOS), 10 % (p47phox and RhoA) or 15 % (caveolin-1 and β-actin) (w/v) polyacrylamide gels in a minigel apparatus (Mini-PROTEAN II; Bio-Rad Laboratories). Separated proteins were then transferred electrophoretically on to nitrocellulose membranes (0.2 μm pore size; Bio-Rad Laboratories). Membranes were incubated for 1 h in a blocking solution [TBS (Tris-buffered saline; 20 mM NaCl and 2mM Tris/HCl, pH 7.4) containing 5 % (w/v) non-fat milk], washed briefly in TBS, and then incubated overnight at 4 °C with the appropriate dilution of antibodies: anti-p47phox (1:1000 dilution); monoclonal antibody kindly provided by Dr Mark T. Quinn, Montana State University, Bozeman, MT, U.S.A., anti-eNOS (1:2000 dilution; BD Pharmigen), anti-iNOS (1:1000 dilution; Santa Cruz Biotechnology), anti-RhoA (1:1000 dilution; Upstate), anti-caveolin-1 (1:1000 dilution; Sigma) and β-actin (1:5000 dilution; Cell Signaling Technology) antibodies. After washing the blots to remove excess primary antibody binding, blots were incubated for 1 h with HRP (horseradish peroxidase)-conjugated secondary antibody (1:5000 dilution). Antibody binding was detected using enhanced chemiluminescence (Amersham Biosciences), and the resulting film was scanned and the intensity of immunoblot bands was detected with a calibrated densitometer (Model GS-800; Bio-Rad Laboratories).

**Myocardial nitrotyrosine content**
Nitrotyrosine content, a footprint of in vivo ONOO− formation, was determined using an ELISA method. Myocardial tissue was homogenized in ice-cold PBS (1/10, w/v) using a PRO 200 homogenizer, followed by sonication with a dismembrator (medium intensity for 30 s; Fisher Scientific). Homogenates were centrifuged for 10 min at 12 500 g at 4 °C. Supernatants were collected and protein concentrations were determined. Tissue samples from hearts (50 μg of protein) were applied to disposable sterile ELISA plates (Corning Glassworks) and incubated overnight at 4 °C. The plate was then washed with 200 μl of PBS/0.1 % Tween buffer, followed by incubation with heat-inactivated 10 % (v/v) goat serum in PBS for 1 h at 37 °C to block non-specific binding. The primary antibody (mouse monoclonal antibody against nitrotyrosine; 1:2000 dilution; incubation for 2 h at 37 °C; Alexis Biochemicals) and secondary antibody (HRP-conjugated anti-mouse IgG; 1:2000 dilution; incubation for 1.5 h at 37 °C) were added, and the peroxidase reaction product was then generated using 2.2 mM O-phenylenediamine dihydrochloride solution (Abbott Diagnostics). Plates were incubated for 20 min in the dark at room temperature, and the reaction was stopped by addition of 50 μl of 1 M HCl to each well. Absorbance was measured at 450 nm with a Spectra Max 190 Microplate Spectrophotometer (Molecular Devices) [18].

**Statistical analysis**
Due to specific requirements of the measurement technique, it was not possible to use the same sample for all experimental indices. In this case, different rats were used for different parameters using randomized sample selection criteria. Data are expressed as means ± S.E.M. Differences between variables were analysed by the non-parametric Mann–Whitney rank sum test (SPSS). The
pattern of data distribution (normality) was assessed using Kolmogorov–Smirnov test (SAS software). All data in Table 2, with the exception of LV lesser diameter and LV chamber volume, were non-normally distributed. The data in Figures 3–6 that were non-normally distributed included control mitochondrial cytochrome c release, control whole-cell homogenate eNOS, mitochondrial eNOS and iNOS (except for control iNOS), p47phox subunit of NADPH oxidase, RhoA and caveolin-1 expression (except for p47phox in the iron-deficient group), and all data used for protein nitrotyrosine formation (except for the whole-cell formation in iron-deficient groups). P < 0.05 was considered statistically significant.

**RESULTS**

**General features of experimental animals fed the control or iron-deficient diets**

As shown in Table 1, rats consuming an iron-deficient diet were anaemic (as assessed by significantly reduced haematocrit values) compared with the control rats. Feeding rats the iron-deficient diet for 12 weeks did not significantly affect BW and liver weight or size of liver (organ/BW ratio) compared with the control diet. However, kidney and brain organ weights as well as brain size were significantly smaller in rats fed the iron-deficient diet compared with the control group. Kidney size (kidney weight/BW ratio) was comparable between the two dietary groups. As expected, absolute heart weight and heart size (heart/BW ratio) were both significantly larger in the iron-deficient group compared with the control group. As shown in Table 2, the heart length, heart width, and LV and right ventricular free wall thickness were all similar between the control and iron-deficient groups. However, following 12 weeks of feeding with the iron-deficient diet, LV major length, LV lesser diameter and LV chamber volume were all significantly increased compared with the control group, suggesting the presence of dilated cardiomyopathy.

**Electron microscopic characteristics of hearts from rats fed control or iron-deficient diets**

No ultrastructural abnormalities were observed in cardiac samples from ventricular samples of rats fed the control diet (Figures 1A and 1C). However, feeding rats an iron-deficient diet induced extensive focal damage in myocardial tissue sections (Figures 1B and 1D). This was indicated by cyto-architectural damage, including mitochondrial swelling, changes in shape and loss of integrity, significant disruption of sarcomeres and the arrangement of cardiac contractile filaments. However, the number of mitochondria did not differ in myocardial sections between the two groups (52.833 ± 4.875 in controls compared with 49.714 ± 4.144 in iron-deficient rats). Ultrastructural microscopy also revealed changes in sarcomeres in ventricular myocytes from rats fed the iron-deficient diet compared with those on the control diet. Figure 2 shows that LV myocytes from rats fed the iron-deficient diet displayed severely disrupted sarcomere and interfibrillar mitochondrial structure in comparison with the typically organized sarcomere and interfibrillar mitochondria in LV myocytes from rats fed the control diet.

**Effects of dietary iron-deficiency on cytochrome c release, protein expression of eNOS, iNOS, NADPH oxidase, RhoA, caveolin-1 and β-actin, and protein nitrotyrosine formation**

To elucidate the possible contributing factors responsible for dietary iron-deficiency-induced cardiac enlargement,
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Figure 1  Transmission electron microscopic micrographs exhibiting ultrastructural changes and number of mitochondria in LV myocytes from rats fed with control or iron-deficient diet

Mitochondria from LV myocytes from rats fed the control diet (A) and iron-deficient diet (B). In (B), note that mitochondria are swollen with altered morphology. Magnification, × 15 000. Higher-power magnification (× 30 000) of mitochondria from LV myocytes from rats fed the control (C) and iron-deficient (D) diets. In (D), note the degenerated mitochondria with membrane loss.

Figure 2  Ultrastructural microscopic changes of sarcomere in ventricular myocytes from rats fed the control or iron-deficient diets

(A) LV myocytes from rats fed the control diet showing typically organized sarcomere structure and interfibrillar mitochondria. (B) LV myocytes from rats fed the iron-deficient diet exhibiting disrupted sarcomeres. Magnification, × 8000.

mitochondrial damage and sarcomere disruption, we examined mitochondrial cytochrome c release, a marker of mitochondrial damage, and several stress signalling molecules involved in RNS-mediated mitochondrial injury. Immunoblot analysis revealed that the mitochondrial cytochrome c release was significantly elevated (by approx. 70%) in the iron-deficient group compared with that from the control group (Figure 3). Expression of the housekeeping protein β-actin was similar between the two groups (Figure 3). As shown in Figure 4, the
Figure 3 Effect of dietary iron deficiency on cardiac cytochrome c release

Ventricular tissues from rats fed the control or iron-deficient diets were homogenized and separated by differential density centrifugation to yield membrane (mitochondria) and soluble (cytosol) fractions. Membrane and soluble proteins (50 µg) were separated by SDS-PAGE and analysed by Western blotting using an anti-cytochrome c antibody (Cyt C) or anti-β-actin antibody, a housekeeping protein used as an internal control. Quantification of protein expression (means ± S.E.M.) is also shown; n = 12. * P < 0.05 compared with control group.

Figure 4 Effect of dietary iron deficiency on cardiac protein expression of eNOS (A) and iNOS (B)

Representative blots probed with anti-eNOS or -iNOS antibodies in heart mitochondria and whole-cell extracts are shown. Quantification of protein expression (means ± S.E.M.) is also shown; n = 8–9 per group. * P < 0.05 compared with control.

DISCUSSION

The results of the present study show that dietary iron deficiency is associated with cardiac hypertrophy, ultrastructural changes in mitochondria and sarcomeres, and increased release of cytochrome c from mitochondria into cytosol in hearts. Enhanced NOS expression and elevated protein nitrotyrosine formation were demonstrated for the first time in hearts from iron-deficient rats. In addition, increased expression of the p47phox subunit of NADPH oxidase (Figure 5B), the membrane structural protein caveolin-1 (Figure 6A) and the small G-protein RhoA (Figure 6B) were all significantly increased in ventricular tissue from the iron-deficient group compared with the control group. Finally, as shown in Figure 5(A), protein nitrotyrosine formation, a key marker for RNS-induced protein injury, as detected by ELISA, was significantly increased in the mitochondrial, but not whole-cell, fraction from the iron-deficient group compared with the control group.

Protein expression of eNOS and iNOS were significantly higher in whole-cell homogenates from the iron-deficient group. Protein expression of iNOS, but not eNOS, was significantly increased in the mitochondrial fractions from the iron-deficient group. Protein expression of the p47phox subunit of NADPH oxidase (Figure 5B), the membrane structural protein caveolin-1 (Figure 6A) and the small G-protein RhoA (Figure 6B) were all significantly increased in ventricular tissue from the iron-deficient group compared with the control group. Finally, as shown in Figure 5(A), protein nitrotyrosine formation, a key marker for RNS-induced protein injury, as detected by ELISA, was significantly increased in the mitochondrial, but not whole-cell, fraction from the iron-deficient group compared with the control group.

tyrosine formation through O2•−-mediated production of ONOO•. Caveolin-1, a constituent protein of caveolae, regulates NO signalling by inhibiting eNOS [19]. Its expression was up-regulated in hearts from rats fed an iron-deficient diet, indicating multi-regulation of NO signalling in cardiac damage. Significantly enhanced levels of RhoA protein might suggest a contribution of small G-protein signalling to sarcomere disruption in hearts from iron-deficient rats.

A previous investigation [20] has suggested that altered myocardial function develops in iron-deficient rats with changes in ventricular contractility, maximal rates of contraction and relaxation, action potentials and L-type Ca2+ currents. In addition, the twitch duration was prolonged similar to that observed in diabetes and heart failure [20]. These electrophysiological changes may contribute to depressed cardiac function with iron deficiency. On the other hand, dietary iron deficiency has been shown to induce cardiac eccentric hypertrophy.
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Figure 5 Effect of dietary iron deficiency on cardiac protein nitrotyrosine formation (A) and expression of NADPH oxidase p47phox subunit (B)

(A) Nitrotyrosine formation was determined by ELISA. (B) Representative blot showing immunostaining with an anti-p47phox antibody in ventricular tissue. Quantification of protein expression (means ± S.E.M.) is also shown; n = 4 per group. *P < 0.05 compared with control group.

Figure 6 Effect of dietary iron deficiency on cardiac expression of caveolin-1 (A) and RhoA (B)

Representative blots showing immunostaining with anti-caveolin-1 and -RhoA antibodies. Cardiac proteins (20 and 50 µg respectively) were separated by SDS/PAGE [20% or 10% (w/v) acrylamide respectively]. Quantification of protein expression (means ± S.E.M.) is also shown; n = 8 (caveolin-1) or 12 (RhoA) per group. *P < 0.05 compared with control group.

[2], further indicating the aetiology of heart dysfunction in anaemic (iron-deficient) patients. The morphological findings from our present study revealed that the major/minor ventricular diameters and ventricular volume were significantly enhanced in the iron-deficient group, consistent with cardiac hypertrophy (absolute heart weight and heart weight/BW ratio) observed in hearts from iron-deficient rats.

Mitochondria generate energy and heat by metabolizing oxygen and nutrients in addition to regulation of cellular metabolism and apoptosis [21]. Our results revealed an enhanced cytosolic fraction of cytochrome c associated with reduced mitochondrial cytochrome c levels in hearts from iron-deficient rats, indicating a 'translocation' of cytochrome c from mitochondria, which is a hallmark of mitochondrial injury and a key pre-apoptotic mitochondrial event. Our data also showed elevated mitochondrial nitrotyrosine formation in iron-deficient groups, indicating NO-related cardiac damage. NO controls oxygen supply and respiratory substrates to mitochondria and redistribution of heat generated by mitochondria [22]. However, NO itself could be cytotoxic, since it may inactivate mitochondrial respiratory chain enzymes and directly stimulate the mitochondrial pathway of apoptosis. Recent evidence suggests that nitrogen-derived stress is a unique form of cell injury under a wide variety of pathophysiological conditions [12]. Enhanced nitrotyrosine formation, a footprint of ONOO- formation, has been demonstrated in a number of pathological conditions such as ischaemia/reperfusion injury [23]. Excessive NO produced from eNOS, or more frequently iNOS, may react with O2- to form ONOO-, resulting in protein nitrotyrosine formation. Both eNOS and iNOS were found in mitochondria in cardiac myocytes [24,25]. Meanwhile, iNOS was induced in mitochondria providing a possible role of RNS in mitochondrial damage in iron deficiency. Although we did not measure local NOx (nitrite/nitrate), enhanced eNOS and iNOS expression observed in our present study supports the observation of enhanced protein nitrotyrosine formation due to elevated NOx levels [25a].
Our present study also revealed that iron deficiency may trigger the up-regulation of stress signalling molecules, including NADPH oxidase and RhoA. NADPH oxidase activation may directly promote generation of free radicals such as O$_2^-$, leading to inactivation of NO and production of ONOO$^-$ [26]. The up-regulation of NADPH oxidase under iron deficiency coincides with elevated eNOS, iNOS and protein nitrotyrosine formation in our present study. On the other hand, RhoA regulates cell morphology and contraction. Cardiac-specific overexpression of RhoA leads to prolongation of action potential duration and diminished ventricular contractility [27,28], suggesting a role of RhoA in impaired myocardial function in iron deficiency. RhoA is essential for the progression of cardiac hypertrophy through the regulation of the actin/myosin cytoskeleton. The effects of RhoA on cellular architecture may be mediated through Rho-dependent Ser/Thr protein kinases. RhoA activation of Rho kinase promotes phosphorylation of MLC (myosin light chain), although this increase in MLC phosphorylation regulates cytoskeletal organization [13]. Thus the data also suggest that up-regulated RhoA might contribute to aberrant sarcomere organization. One interesting finding from the present study is the elevated caveolin-1 expression in iron deficiency. Caveolin-1 acts through packing proteins in caveolae by protein–protein interactions, allowing finely tuned regulation of physiological responses [29]. Caveolin-1 usually suppresses growth and cell proliferation and is closely associated with apoptosis [30–32]. Although the mechanism of action of elevated caveolin-1 expression is not clear at present, one would speculate that elevated expression of caveolin-1 during iron deficiency may sensitize myocardial cells to apoptotic stimuli via activation of apoptotic enzymes (such as caspase 3) [33]. Caveolin-1 has been suggested to participate in the regulation of eNOS protein function and may thus contribute to iron-deficiency-induced cardiac damage through an eNOS-dependent mechanism [34].

In a model of physiological stress as multifaceted as iron deficiency, it is difficult to distinguish between changes that result from lack of dietary iron, from anaemia or tissue hypoxia, or from compensatory physiological responses to anaemia. For example, iron deficiency resulted in a dilated eccentric cardiac hypertrophy, as demonstrated in the present study and elsewhere [2]. This morphological alteration was found whether anaemia was severe [2] or moderate, as in our present study (Table 1). With dietary copper deficiency, a concentric hypertrophic pattern (increased ventricular wall thickness in the absence of increased chamber size) was found without or with anaemia [2,35]. This might suggest that the iron-deficiency-induced dilated hypertrophy pattern is secondary to the physiological stimuli related to anaemia (whether or not that anaemia is severe), whereas copper-deficiency-induced hypertrophy [35] is unrelated to anaemia. Increased venous return, decreased peripheral resistance, increased inotropic response to noradrenaline (norepinephrine) and vascular remodelling to a larger arterial diameter have all been demonstrated with iron-deficiency-induced anaemia [5–7]; a chronic hypersympathetic state coupled with the haemodynamic changes has been suggested to be essential for cardiac hypertrophy [6]. In contrast, copper-deficiency-induced hypertrophy has been attributed to mitochondrial proliferation [36].

Similarly, the mitochondrial changes reported in the present study appear to have significant differences compared with the copper-deficiency model as a direct result of disparate mineral deficiencies. For example, copper deficiency induces an increase in cardiac mitochondrial number, which may allow normal levels of oxidative phosphorylation [37]. We found no increase in mitochondrial number with iron deficiency; furthermore, it would appear unlikely that oxidative phosphorylation is unchanged with iron deficiency given our findings of ultrastructural damage to mitochondria, the translocation of cytochrome c from mitochondria to cytoplasm and an unchanged mitochondrial number. Copper deficiency has also been shown [38] to reduce the number of subunits of cytochrome c oxidase that are transcribed in the nucleus, but not mitochondria. However, this is not true with iron deficiency [38]. Thus, although electron transport Complex IV requires both iron and copper to function properly and both deficiencies result in cardiac hypertrophy [2,35,36], there appear to be significant differences in cardiac mitochondrial alterations that are best explained as direct responses to mineral deficiency.

Experimental limitations of the present study

One major limitation of the present investigation is that our conclusions from this ‘uncontrolled comparison’ study are essentially based upon observed differences between groups, rather than any cross-sectional associations between parameters within the same group. Therefore any causal relationship between reactive oxygen species and cardiac damage during iron deficiency cannot be drawn in a convincing manner at the present stage. In addition, since it was impossible to use the same sample for all experimental indices, use of different rats for different parameters reduces the potential usefulness of examining associations between various parameters. This also contributed to the relatively small sample sizes (i.e. different rats were, in some instances, used for different parameters due to requirement of the measuring technique). Nevertheless, samples were randomly chosen for any parameter tested (if not all rat tissue samples were available for that given parameter).

In summary, the findings of the present study have shown that dietary iron deficiency and/or anaemia...
may trigger alterations in cardiac morphology, including enlarged (dilated) ventricles, ultrastructural aberrations in mitochondria and sarcomeres, elevated release of cytochrome c from mitochondria, elevated protein nitrotyrosine formation, and higher levels of eNOS, iNOS, NADPH oxidase, caveolin-1 and RhoA. These data suggest the presence of RNS and cardiac damage during iron deficiency, and may enhance our understanding of the mechanism and possible therapeutic regime for dietary iron-deficiency-related cardiac dysfunction.

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