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G. Jayakumar Amirtharaj
Christian Medical College, Vellore, India

Sathish Kumar Natarajan
University of Nebraska - Lincoln, snatarajan2@unl.edu

Anna Pulimood
Christian Medical College, Vellore, India

K. A. Balasubramanian
Christian Medical College, Vellore, India

Aparna Venkatraman
Christian Medical College, Vellore, India

See next page for additional authors

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Authors

G. Jayakumar Amirtharaj, Sathish Kumar Natarajan, Anna Pulimood, K. A. Balasubramanian, Aparna Venkatraman, and Anup Ramachandran

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Role of Oxygen Free Radicals, Nitric Oxide and Mitochondria in Mediating Cardiac Alterations During Liver Cirrhosis Induced by Thioacetamide

G. Jayakumar Amirtharaj,¹ Sathish Kumar Natarajan,^{1,3}
Anna Pulimood,¹ K. A. Balasubramanian,¹
Aparna Venkatraman,² and Anup Ramachandran¹

1 The Wellcome Trust Research Laboratory, Division of Gastrointestinal Sciences,
Christian Medical College, Ida Scudder Road, Vellore 632004, India

2 Center for Stem Cell Research, Christian Medical College, Ida Scudder Road,
Vellore 632004, India

3 IANR, Nutrition & Health Sciences, University of Nebraska–Lincoln, Lincoln, NE
68583-0806, USA

Corresponding author — Anup Ramachandran, wellcome@cmcvellore.ac.in

Abstract

Thioacetamide (TAA) administration is widely used for induction of liver cirrhosis in rats, where reactive oxygen radicals (ROS) and nitric oxide (NO) participate in development of liver damage. Cardiac dysfunction is an important complication of liver cirrhosis, but the role of ROS or NO in cardiac abnormalities during liver cirrhosis is not well understood. This was investigated in animals after TAA-induced liver cirrhosis and temporal changes in oxidative stress, NO and mitochondrial function in the heart evaluated. TAA induced elevation in cardiac levels of nitrate *before* development of frank liver cirrhosis, without gross histological alterations. This was accompanied by an early induction of P38 MAP kinase, which is influenced by ROS and plays an important signaling role for induction of iNOS. Increased nitrotyrosine, protein oxidation and lipid peroxidation in the heart and cardiac mitochondria, suggestive of oxidative stress, also preceded frank liver cirrhosis. However, compromised cardiac mitochondrial function with a decrease in respiratory control ratio and increased mitochondrial swelling was seen later, when cirrhosis was evident. In conclusion, TAA induces elevations in ROS and NO in the heart in parallel to early liver damage. This leads to later development of functional deficits in cardiac mitochondria after development of liver cirrhosis.

Keywords: Heart, Oxidative stress, Liver, p38 MAP kinase, Thioacetamide

Introduction

Liver cirrhosis is a chronic disease characterized by hepatic fibrosis induced by progressive hepatocyte injury and stellate cell activation. Thioacetamide (TAA) administration is a widely used model for development of liver cirrhosis in animals which reproduces the human disease induced by a number of different etiologies. TAA hepatotoxicity requires its biotransformation to TAA-S-oxide and subsequently to the highly reactive metabolite TAA-S-dioxide by hepatic microsomal CYP2E1 [1]. Early studies from the late 1940s suggest that the liver is the main organ affected by administration of TAA [2–4], and chronic feeding of TAA to rats affected only the liver with no changes to other organs like the lung, heart, spleen [3]. However, only gross abnormalities were generally examined in those studies, and the role of reactive oxygen species (ROS) and nitric oxide (NO) in cardiac changes after TAA is not well understood. This is relevant since cardiac alterations during later stages of cirrhosis in humans could be the underlying cause for development of life-threatening complications such as the hepatorenal syndrome, ascites, spontaneous bacterial peritonitis (SBP) and the hepatopulmonary syndrome [5]. Cirrhotic children have cardiac dysfunction mainly in the form of diastolic dysfunction [6], and functional deficits in the heart in patients with liver disease may only become evident during pharmacological or physiological stress, when compensatory mechanisms of the heart become insufficient to maintain adequate cardiac output [7]. Though mechanisms involved in cardiac dysfunction in hepatic disease are not fully understood, it has been suggested that early detection of this condition is important [8].

Nitric oxide (NO) is an important vasodilator in the splanchnic and systemic circulation at low concentrations, but at higher levels, the molecule can have a number of damaging effects. Patients with cirrhosis exhibit elevated NO levels in the systemic and splanchnic circulation [9], and inhibition of iNOS in cirrhotic rats abrogates the effects of NO and improves cardiac contractility, suggesting a role for NO in cardiac pathophysiology [10]. In addition, NO can react with free radicals such as superoxide resulting in the generation of peroxynitrite, which can enhance cellular toxicity [11].

Oxygen free radicals have been implicated in the pathogenesis of toxic liver injury [12], and oxidative stress during liver cirrhosis is responsible for intestinal and renal damage [13–15]. Oxidative stress has been suggested to be involved in cardiac dysfunction in the cirrhotic heart [16], and an increase in lipid peroxidation markers has been demonstrated in the kidney, brain and heart of rats with cholestatic liver disease suggesting oxidative damage to different organs [17]. Mitochondria are an important cellular source as well as target of oxygen free radicals, and intestinal and renal mitochondria have been shown to be significantly affected in liver cirrhosis [13, 15]. However, the temporal relationship between TAA-induced development of liver cirrhosis and changes in ROS and NO in the heart as well as its effect on cardiac mitochondrial function are not well understood and were investigated in this study. We hypothesized that

generation of oxygen free radicals and NO during TAA-induced cirrhosis results in oxidative and nitrosative damage in the heart, which affects mitochondrial function. This hypothesis was tested by examining alterations in the rat heart and liver over time after administration of TAA.

Methods

Animals and Induction of Liver Cirrhosis

The study was approved by the Institutional Animal Ethics Committee (IAEC) at the Christian Medical College, Vellore, and experimentation, transportation and care of the animals were performed in compliance with relevant laws and institutional guidelines. Adult Wistar rats of both sexes (125–200 g) were administered intraperitoneal injections of TAA (200 mg/kg, ip) in saline twice a week for 2, 3, 4, 5 and 6 months. Some experiments had a group treated for 1 month. Control rats received vehicle alone [18]. After each treatment period, TAA was stopped and the animals were killed after 1 week.

Light and Electron Microscopy (EM)

For light microscopy, tissues were fixed in 10 % buffered formalin and processed. Four-micron sections were cut and stained with hematoxylin and eosin. For EM, heart tissue was fixed in 2.5 % glutaraldehyde, post-fixed in osmium tetroxide and embedded in araldite (epoxy resin). One-micron-thick sections were cut and stained with toluidine blue. Ultrathin sections were cut on an LKB UM4 ultramicrotome, sections mounted on copper grids, and stained with uranyl acetate and lead citrate. The grids were examined under a Philips TECHNAI T12 SPIRIT transmission electron microscope.

Measurement of Nitrate and Oxidative Stress Parameters

Nitrate in the samples was measured by the Griess reaction as described [19]. Malondialdehyde (MDA), conjugated dienes and protein carbonyl content was measured as described [20–22]. Protein was estimated by Lowry's method [23].

Western Blot for p38 MAPK

Twenty-microgram samples of heart protein were separated by SDS-PAGE, blotted onto PVDF membrane and probed with anti-p38 MAPK (rabbit polyclonal, Cell Signaling Technology) and anti-phospho-p38 MAPK (mouse monoclonal, Cell Signaling Technology). Following treatment with HRP-linked secondary antibodies, membranes were developed by enhanced chemiluminescence and bands quantitated with Image J software.

Staining for Nitrotyrosine

Paraffin sections were blocked with universal blocking buffer (Biogenex) after antigen retrieval, followed by incubation with an anti-nitrotyrosine antibody (Clone-39B6) (1:100 dilution, Santa Cruz) overnight at 4 °C. Slides were then incubated with secondary antibody, and immunostained sections visualized using a confocal microscope (Olympus FluoView™ FV1000).

Isolation of Heart Mitochondria and Mitochondrial Functional Assays

Heart mitochondria were isolated by differential centrifugation as described [13] after homogenization in buffer containing 225 mM Mannitol, 75 mM Sucrose, 10 mM Tris, 1 mM EDTA pH 7.4. Mitochondrial oxygen uptake and swelling were then measured [13, 24]. MTT assay was carried out as described [25].

Statistical Analysis

Data are expressed as mean $\pm$ SEM. Statistical analysis was performed using Student's *t* test. A probability value of <0.05 was taken to indicate statistical significance.

Results

To confirm development of liver cirrhosis in rats after TAA treatment, liver sections from rats treated with either saline or TAA for 3 or 6 months were evaluated by light microscopy. Control livers showed normal liver architecture (Fig. 1a), with no significant changes in hepatocytes and portal tracts. These features were not altered after 2 months of TAA treatment (data not shown). However, by 3 months, rats treated with TAA showed characteristics of cirrhosis, with the formation of nodules separated by septae, infiltration by moderate numbers of inflammatory cells and fibroblasts with proliferating bile ductules (Fig. 1b). By 6 months of TAA treatment, histological changes in the liver were similar to those seen at 3 months, but with more uniformly thick septae (Fig. 1c).

Histological changes in the heart were then examined, and the heart from control rats showed normal cardiac muscle fibers with elongated nuclei exhibiting fine chromatin and eosinophilic cytoplasm. There was no evidence of vacuolization, fibrosis or inflammation (Fig. 1d). The heart from cirrhotic rats at both 3 and 6 months of TAA treatment did not show any gross cellular abnormality compared to hearts from control rats, though congestion of capillaries between cardiac muscle fibers was visible (Fig. 1e, f).

Nitric oxide has been shown to be involved in cardiac damage after liver cirrhosis [11], and further experiments examined the temporal profile of nitrate, an NO metabolite, in the heart during TAA-induced cirrhosis. As seen in Fig. 2a, nitrate levels increased significantly by 2 months. These levels do not change considerably by 3 months, but begin to progressively increase further from 4 months, increasing up to 5 months after TAA.

Probable signaling events controlling this elevation in NO in the heart during liver cirrhosis were then examined. Cardiac levels of iNOS mRNA and protein have been shown to be elevated in cirrhotic rats [10], and p38 MAPK has been suggested to be essential for the expression of iNOS in cardiac tissue [26]. Western blot analysis showed an increase in levels of p38 protein in the heart within a month of treatment with TAA, which did not alter significantly subsequently. Activation of p38, as measured by phosphorylation at Thr180/Tyr182, was significantly elevated by 1 month and increased further at 3 and 5 months after TAA treatment (Fig. 2b). Since p38 MAPK can be induced by reactive oxygen species (ROS) [27], the presence of ROS was next evaluated in the cirrhotic heart.

Markers of lipid peroxidation such as malondialdehyde and conjugated diene began rising in heart tissue by 2 months of TAA treatment (before establishment of gross cirrhotic changes by histology) and peaked by 3 months when cirrhotic changes in the liver were evident by histology (Fig. 3a, b). Oxidative changes to cardiac proteins were also evident, as seen by an elevation in protein carbonyl content, which was also initiated at 2 months, but peaked at 4 months, in contrast to lipid peroxidation data (Fig. 3c). These elevations in malondialdehyde, conjugated diene and protein carbonyl content suggest significant oxidant stress in the heart.

Concurrent elevations in free radicals such as superoxide and NO can result in the formation of peroxynitrite, which modifies proteins to generate nitrotyrosine. Since both NO and oxidant stress were elevated in the heart at early time points, the presence of nitrotyrosine was evaluated in the heart as an index of peroxynitrite formation. As seen in Fig. 4, a significant increase in staining for nitrotyrosine was evident in the cardiomyocytes of the heart by 1 month, concurrent with elevation in NO as well as oxidant stress markers.

Since mitochondria are an important target as well as source of free radicals within the cell, we next evaluated cardiac mitochondrial function during development of liver cirrhosis. Cardiac mitochondria showed a decreased respiratory control ratio (Fig. 5a) which was evident by 3 months of TAA treatment and continued until 6 months. This was accompanied by an increase in mitochondrial swelling as seen by the decrease in absorbance at 540 nm (Fig. 5b) indicative of activation of the mitochondrial permeability transition (MPT). This was initiated by 3 months and progressively increased until 6 months of TAA treatment. These changes were confirmed by electron microscopy, which demonstrated patchy cardiac mitochondrial dilation with loss of cristae in animals after 3 (Fig. 6b, e) or 6 months (Fig. 6c, f) of TAA treatment, while normal control rat hearts showed cardiomyocytes with normal mitochondria and myofibrils (Fig. 6a, d). Thus, measurements of cardiac mitochondrial functional

parameters and electron microscopy indicate that significant functional impairment of cardiac mitochondria occur in rats after development of cirrhosis. While the oxidative stress in heart tissue was found to start increasing at 2 months and peak at 3–4 months followed by a decrease, mitochondrial functional alterations were initiated later and were progressive (Fig. 5c).

The role of oxidative stress in mediating these mitochondrial alterations was then investigated by measuring the rate of radical production within mitochondria using MTT reduction, which has been shown to be elevated in kidney and intestinal mitochondria during cirrhosis [13, 15]. As seen in Fig. 7a, an increased rate of MTT reduction was evident in cirrhotic rats beginning by 3 months, which stayed elevated subsequently. The consequence of this elevated mitochondrial radical production was next examined, and an increase in malondialdehyde levels was evident in heart mitochondria by 2 months of TAA treatment, which peaked by 3 months, similar to changes seen in the heart (Fig. 7b). Conjugated diene levels also increased by 2 months of TAA treatment and reached a maximum by 3 months and stayed elevated till 6 months of TAA treatment (Fig. 7c). Protein carbonyl content in mitochondria, however, started to increase significantly only by 3 months and then peaked at 4 months after TAA treatment (Fig. 7d). Thus, lipid peroxidation in the mitochondria seems to precede changes in protein oxidation as well as functional defects in cardiac mitochondria, implicating it in the subsequent derangement of cardiac mitochondrial function during TAA-induced liver cirrhosis.

Discussion

Thioacetamide Induces Early Elevations in Cardiac Nitric Oxide Levels Prior to Liver Cirrhosis

Nitric oxide (NO) has been implicated in renal and cardiac complications during liver cirrhosis [11]. Early studies examining cardiac function in biliary cirrhosis in the rat suggested a role for NO in pathophysiology of cirrhotic cardiomyopathy [28]. It was subsequently shown that cardiac levels of TNF- α and iNOS mRNA and protein, as well as serum nitrite/nitrate levels, were significantly higher in cirrhotic rats than sham controls [10]. However, most of the earlier studies examined cardiac alterations *after* development of liver cirrhosis, and our data now show that elevations of NO in the heart occur *prior* to development of frank cirrhosis in the liver after administration of TAA. While this is unlikely to be a direct effect of TAA since the compound requires hepatic metabolism to produce toxicity [29], the role of circulating TAA metabolites in the process cannot be ruled out. However, it has been shown that TAA administration does not directly produce alterations in the heart [30], and cardiac modifications occurring in ascitic rats with biliary cirrhosis are related to NO [28]. The fact that bile duct ligation per se, without cirrhosis, resulted in elevation of cardiac

mRNA, and protein for iNOS [16] lends further credence to this possibility that early liver damage itself can upregulate NO production in the heart irrespective of the trigger.

Thioacetamide-Induced Liver Injury Results in Early Activation of the p38 MAPK

Cholestasis in the liver results in elevated plasma TNF- α in mice, accompanied by increased cardiac mRNA and protein expression of p38 MAPK and iNOS [16]. Activation of p38 MAPK can contribute to loss of cardiac contractility [31] and also regulate NO production [32]. Our data now show that activation of molecular mediators such as p38 MAPK in the heart occurs early in the disease process, in parallel to development of liver injury. The initial elevation could be due to increased protein synthesis, but subsequently there is a robust phosphorylation of the protein resulting in its sustained activation. Activation of p38 MAPK in cholestatic mice was accompanied by changes in glutathione and antioxidant enzymes suggestive of oxidant stress [16], and the damaging effects of NO can be amplified several fold by its reaction with free radical intermediates such as superoxide [33], implicating oxidative stress in cardiac damage during TAA-induced cirrhosis.

Oxidative and Nitrosative Stress in the Heart Precedes Development of Frank Cirrhosis in the Liver

Liver cirrhosis can induce functional alterations in the intestine [13, 14] and kidney [15] due to oxidant stress, which has also been demonstrated in mice cardiac cells after bile duct ligation [34]. Our data now show that elevations in protein carbonyl and malondialdehyde are evident in the heart before development of frank cirrhosis in the liver after TAA administration. This indicates that oxidant stress in the heart is not merely a consequence of liver cirrhosis, but is initiated earlier, before frank cirrhosis is evident in the liver. Simultaneous elevations in NO and superoxide can result in formation of peroxynitrite, which compromises protein function by formation of nitrotyrosine [35]. It has been demonstrated that nitration of cardiac proteins is associated with abnormal cardiac chronotropic responses in rats with biliary cirrhosis [36]. Our data now show elevated nitrotyrosine levels in the heart of TAA-treated animals within a month after treatment indicating that formation of peroxynitrite and nitrosative stress in the heart is also an early event in conjunction with oxidant stress.

Liver Cirrhosis Induces Significant Functional Alterations in Cardiac Mitochondria

Mitochondria are critical organelles for cellular function, especially in energy intensive organs such as the heart, and mitochondrial oxidative

stress plays a role in dysfunction of the organelle in heart failure [37]. Impaired mitochondrial function and dynamics have been shown to induce myocardial contractile dysfunction in patients with type 2 diabetes [38], and our data now show that this organelle is a sensitive target in the heart, albeit at later stages after development of liver cirrhosis. The uncoupling of mitochondrial respiration and oxidative phosphorylation, producing the decrease in respiratory control ratio, was evident by 3 months, at which time cirrhosis is evident histologically in the liver. A similar decrease in respiratory control ratio has been observed in failed human myocardial tissue [39], and diabetic hearts were also shown to have lower ATP levels and compromised mitochondrial respiration [40]. An important feature of cellular injury is activation of the mitochondrial permeability transition (MPT) pore, which can be induced by oxidative stress [41]. It is interesting that while uncoupling of respiration was evident by 3 months and subsequently did not change, the induction of the mitochondrial permeability transition was significant only at 4 months and then continued to worsen each month until the sixth month. This indicates that the MPT induction in cardiac mitochondria was a later event subsequent to mitochondrial uncoupling.

Lipid Peroxidation Could Initiate Cardiac Mitochondrial Dysfunction in TAA-Induced Cirrhosis

The mitochondrion is a source as well as a target for reactive oxygen species, and production of ROS within mitochondria has been demonstrated in heart failure [42]. The early elevation in lipid peroxidation within cardiac mitochondria by 2 months of TAA, prior to development of frank liver cirrhosis, may result in downstream effects since lipid peroxidation has been shown to induce uncoupling of mitochondrial respiration [43], probably by activation of uncoupling proteins in mitochondria [44]. Lipid peroxidation has also been shown in mouse heart mitochondria after myocardial infarction when mitochondrial dysfunction was evident [45]. Cardiac mitochondrial uncoupling then results in elevated free radical production, as seen by MTT reduction which was significantly elevated by 3 months, and sustained subsequently. This seems to be similar to that seen in both the intestine and the kidney in cirrhotic rats [13, 15]. This then suggests that early induction of lipid peroxidation in the heart could be responsible for the subsequent mitochondrial dysfunction, which exacerbates as the liver injury intensifies.

In conclusion, we demonstrate early elevations in NO, activation of p38 MAP kinase, induction of lipid peroxidation and nitrosative stress in cardiac tissue prior to development of frank liver cirrhosis after TAA administration. Though the mitochondria are likely sources for these free radicals, organelle function in the heart is only compromised at later time points after development of liver cirrhosis. Thus, while functional cardiac alterations occur after development of liver cirrhosis and the resultant circulatory abnormalities, molecular alterations within the heart could be occurring earlier. While the cardiac changes are probably not responsible

for the changes in the liver, the data imply that early changes in the liver before development of frank cirrhosis probably induce oxidative and nitrosative stress in the heart, which primes the organ for functional deficits at later time points after frank cirrhosis has developed. Hence, signaling events and subcellular alterations are initiated very early in cardiac tissue during development of liver cirrhosis after TAA administration, and the cumulative effect of these alterations could play an important role in cardiac complications later in liver disease.

Fig. 1. Histology of liver and heart from rats treated with TAA: light microscopy of liver (**a–c**) and heart (**d–f**) from control and rats treated with TAA for either 3 or 6 months. **a, d** Control; **b, e** 3 months; **c, f** 6 months. Original magnification $\times 400$.

Fig. 2. Nitric oxide and p38 MAPK in the heart during development of liver cirrhosis: **a)** nitrate levels in heart from controls and TAA-treated rats. The data are expressed as mean $\pm$ SEM, $n = 4-6$. * $P < 0.05$ when compared to controls. # $P < 0.05$ when compared to 5 months. **b)** Phospho-p38 MAP kinase protein expression in heart from controls and TAA-treated rats. The data are expressed as mean $\pm$ SEM, $n = 4$. * $P < 0.05$ when compared to controls.

Fig. 3. Oxidative stress parameters in heart from controls and TAA-treated rats: malondialdehyde (**a**), conjugated diene (**b**) and protein carbonyls (**c**). The data are expressed as mean $\pm$ SEM, $n = 3-5$. * $P < 0.05$ when compared to controls.

Fig. 4. Nitrotyrosine staining in the heart after administration of TAA: immunofluorescence staining for nitrotyrosine in sections of cardiac tissue from controls and rats treated with TAA for 1 month. *NT* nitrotyrosine staining, *DIC* differential interference contrast image, *DAPI* nuclear staining).

Fig. 5. Cardiac mitochondrial function after administration of TAA: cardiac mitochondrial functional parameters such as respiratory control ratio (**a**) and mitochondrial swelling (**b**) from controls and TAA-treated rats. The data are expressed as mean $\pm$ SEM, $n = 3-5$. * $P < 0.05$ when compared to controls. (**c**) Overlay of temporal changes in oxidant stress in the heart and cardiac mitochondrial function in animals treated with TAA when compared to controls.

Fig. 6. Electron micrographs of hearts from rats treated with TAA: control animals showing normal mitochondria (**a** $\times 6000$, **b** $\times 43,000$) (arrows). Cardiac tissue after 3 (**c** $\times 6000$, **d** $\times 43,000$) and 6 (**e** $\times 6000$, **f** $\times 43,000$) months showing mitochondrial dilation with loss of cristae (asterisks).

Fig. 7. Superoxide production and oxidative stress parameters in cardiac mitochondria from cirrhotic rats: MTT reduction **(a)** and oxidative stress parameters such as malondialdehyde **(b)**, conjugated diene **(c)** and protein carbonyls **(d)** in cardiac mitochondria from controls and TAA-treated rats. The data are expressed as mean $\pm$ SEM, $n = 3-5$. * $P < 0.05$ when compared to controls.

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