Fibroblast growth factor 21 protects the heart from oxidative stress

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Aims Oxidative stress mediated by reactive oxygen species (ROS) plays a striking role in the pathogenesis of heart failure, and antioxidants have been shown to attenuate cardiac remodelling in experimental models of cardiac damage. We recently showed that fibroblast growth factor 21 (Fgf21) is produced by the heart and exerts protective effects, preventing cardiac hypertrophy development. The aim of the study was to determine the effects of Fgf21 during oxidative stress signalling in the heart.

Methods and results Fgf21 treatment in cardiomyocytes in culture induced the expression of genes encoding proteins involved in antioxidative pathways, including mitochondrial uncoupling proteins (Ucp2 and Ucp3) and superoxide dismutase-2 (Sod2) and reduced ROS production. In keeping with this, expression of antioxidant genes in response to lipopolysaccharide (LPS)-induced stimulation of pro-inflammatory pathways or isoproterenol-induced cardiac hypertrophy in the heart was reduced in Fgf21-null mice. Moreover, we found that Fgf21 is expressed in and released by cardiomyocytes in response to LPS, and its expression is under the control of the Sirt1 (sirtuin-1) pathway. This Fgf21 released by cardiomyocytes acts in an autocrine manner to protect cells against oxidative stress. Finally, failing human hearts showed up-regulation of Fgf21, Ucp3, and Sod2, confirming the association between Fgf21 induction and the control of cardiac oxidative stress pathways.

Conclusion Our data indicate that Fgf21 regulates genes involved in antioxidative pathways in an autocrine manner, thus preventing ROS production in cardiac cells. Therefore, Fgf21 acts as an antioxidant factor in the heart, preventing induction of pro-oxidative pathways by inflammatory or hypertrophic conditions.

Keywords Fgf21 • Oxidative stress • ROS • Cardiac disease

1. Introduction Fibroblast growth factor 21 (Fgf21) is a secreted protein involved in the control of glucose homeostasis, insulin sensitivity, and ketogenesis.1,2 The liver is considered the main site of production and release of Fgf21 into the blood,3,4 but extra-hepatic tissues, such as white and brown adipose tissues and skeletal muscle, also express Fgf21.3–6 Endocrine actions of Fgf21 include the promotion of glucose uptake by white adipocytes through induction of the glucose transporter Glut47 and activation of brown fat thermogenic activity.6 Action of Fgf21 on target cells requires FGF receptors (mainly Fgfr1 and Fgfr4 in adipose tissue and liver, respectively) and β-Klotho, a single-pass transmembrane protein that functions as an obligate cofactor for Fgf21 signalling.9,10 Recently, we showed that the heart is a target as well as a source of Fgf21. Fgf21 protects the heart against cardiac hypertrophy and in turn expresses and releases Fgf21.11 This study also demonstrated that Fgf21 expression in the heart is under the control of the protein deacetylase Sirt1 (sirtuin 1). Recent reports have identified Sirt1 as an important actor in cardiac protection, showing that Sirt1 protects against hypertrophy, ischaemia-reperfusion injury, and oxidative stress in the heart.12–15 However, the molecular basis of the cardioprotective action of Fgf21 remains incompletely understood.

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Oxidative stress mediated by reactive oxygen species (ROS) causes contractile failure and structural damage, and thus plays an important role in the pathogenesis of heart failure, especially after myocardial infarction. Increased ROS production may contribute to the development of cardiac damage through different mechanisms, including cardiomyocyte loss via apoptosis or other cell death mechanisms. Mitochondrial ROS production can be neutralized by uncoupling proteins (Ucps), which regulate proton leak across the inner mitochondrial membrane. In fact, recent studies have shown the importance of uncoupling protein-3 (Ucp3) in the heart in controlling ROS production and thereby mediating cardioprotection.

In the present study, we analysed the role of Fgf21 in cardiac tissue in relation to pathological situations associated with oxidative stress. We found that Fgf21 induced expression of antioxidative genes such as Ucp3, Ucp2, and Sod2, in the heart, and showed that pro-oxidant signals induced Ucp3 expression in an Fgf21-dependent manner. Activation of the Sirt1 pathway induced cardiac secretion of Fgf21, which acted in an autocrine manner to prevent oxidative stress in cardiomyocytes.

2. Methods

2.1 Animals
Sirt1-null mice were kindly supplied by Frederick W. Alt. Fgf21-null mice (B6N;129S5-Fgf21<sup>1tm1Lest</sup>/Mmcdd) were obtained from the Mutant Mouse Regional Resource Center, an NCRR-NIH funded strain repository; these mice were donated to the MMRRC by Genentech, Inc. Neonatal cardiac hypertrophy was induced by intraperitoneal (i.p.) injection of 6-day-old neonates with 15 mg/kg/day isoproterenol (ISO; Sigma, St. Louis, MO, USA) for 7 days, as previously reported. Effects of the inflammatory process were assessed in 6-day-old neonates from wild-type and Fgf21-null mice with 15 mg/kg/day isoproterenol (ISO; Sigma, St. Louis, MO, USA) for 7 days, as previously reported. Four-month-old male mice from wild-type and Fgf21-null mice were anaesthetized with 1.5% isofluorane, and subcutaneous Alzet osmotic minipumps containing PBS or ISO, the latter calibrated to release the drug at a rate of 15 mg/kg/day for 7 days, were surgically implanted subcutaneously in the interscapular region of the mouse to induce cardiac hypertrophy in adult mice, as previously reported. Effects of the inflammatory process were assessed in 6-day-old neonates from wild-type and Fgf21-null mice 4 h after i.p. injection of 5 mg/kg lipopolysaccharide (LPS; Sigma) as previously reported.

2.2 Cell culture
Neonatal (1–2 days old) Sprague Dawley rats were decapitated, and their hearts were removed. Hearts were digested with a collagenase solution to induce intraperitoneal (i.p.) injection of 6-day-old neonates from wild-type and Fgf21-null mice with 15 mg/kg/day isoproterenol (ISO; Sigma, St. Louis, MO, USA) for 7 days, as previously reported. Four-month-old male mice from wild-type and Fgf21-null mice were anaesthetized with 1.5% isofluorane, and subcutaneous Alzet osmotic minipumps containing PBS or ISO, the latter calibrated to release the drug at a rate of 15 mg/kg/day for 7 days, were surgically implanted subcutaneously in the interscapular region of the mouse to induce cardiac hypertrophy in adult mice, as previously reported. Effects of the inflammatory process were assessed in 6-day-old neonates from wild-type and Fgf21-null mice 4 h after i.p. injection of 5 mg/kg lipopolysaccharide (LPS; Sigma) as previously reported.

2.3 RNA isolation and real-time quantitative reverse transcription–PCR
Total RNA was extracted using TriPure (Roche, Indianapolis, IN, USA). cDNA was synthesized from total RNA (0.5 μg) by reverse transcription using random hexamer primers (Applied Biosystems, Foster City, CA, USA) in a total reaction volume of 20 μL. TaqMan Gene Expression Assays were used for PCR, with each 25 μL reaction mixture containing 1 μL cDNA, 12.5 μL TaqMan Universal PCR Master Mix (Applied Biosystems), 250 nM primers, and 900 nM primers from Assays-on-Demand Gene Expression Assay Mix or Assays-by-Design Gene Expression Assay Mix (Applied Biosystems). Each sample was run in duplicate for improved accuracy, and the mean value was used to calculate the mRNA expression of the gene of interest and the housekeeping reference gene cyclophilin A (PPIA). The mRNA level of the gene of interest in each sample was normalized to that of the reference control using the comparative (2^-ΔΔCT) method following the manufacturer’s instructions.

2.4 Adenoviral-mediated gene transfer
A recombinant adenovirus expressing murine Sirt1 cDNA was constructed (Ad5-CMV-Sirt1, CEBATEG, Barcelona, Spain). NCMs were transduced with the Sirt1 adenoviral vector (AdSirt1) or an AdCMV-GFP control vector (AdGFP) at 10 infectious units (IFU)/cell for 24 h in serum-free medium. An assessment of GFP fluorescence showed that this treatment led to an infection efficiency of ~90%. Adenoviral transduction at 10 IFU induced no significant cell death.

2.5 Measurement of ROS generation
Intracellular ROS generation was assessed using the intracellular ROS assay kit from Cell Biolabs (San Diego, CA, USA), following the manufacturer’s instructions. Briefly, cells were incubated with the peroxide-specific probe 2’7’-dichlorofluorescein diacetate (DCF-DA) for 1 h followed by a 1-h stimulation with LPS. Fluorescence signals were measured using a fluorescent reader.

2.6 Sod2 and Fgf21 protein levels
Sod2 protein levels were determined using specific Sod2 ELISA kit (Uscn Life Science Inc., Wuhan, China). Fgf21 protein levels were measured using an Fgf21 ELISA kit (Biovendor) following the manufacturer’s instructions.

2.7 Western blotting and caspase 3/7 activity
Preparation of whole-cell extracts and immunoblotting procedures have been described previously. Whole-cell extracts (40 μg protein) were immunoblotted with antibodies against Ucp3 (Chemicon, EMD Millipore Corporation, Billerica, MA, USA) and α-tubulin (Sigma). Coomassie blue staining was used as an internal loading control in human samples. Caspase 3/7 activity was measured using the fluorimetric CaspACE Assay System (Promega), which is based on the fluorimetric detection of amino-4-trifluoromethyl coumarin after proteolytic cleavage of the synthetic substrate DEVD-amino-4-trifluoromethyl coumarin.

2.8 Aconitase activity and protein carbonyl content determination
Protein extracts from adult heart mice from wt and Fgf21-null mice subjected to ISO infusion were used for these assays. Aconitase activity was measured...
FGF21 and cardiac oxidative stress

using a colorimetric assay kit (Cayman Chemical Company, Ann Arbor, MI, USA) following the manufacturer’s instructions. Oxidized proteins were determined using a protein carbonyl colorimetric assay kit (Cayman) following the manufacturer’s instructions.

2.9 Human samples

Human hearts were obtained from patients with dilated cardiomyopathy and final-stage heart failure who underwent cardiac transplantation (six patients). Controls were obtained from heart samples from organ donors without clinical history of heart failure and whose heart could not be used for any reason (10 controls). Written informed consent was obtained from all participants in the human population study which conformed to the principles outlined in the Declaration of Helsinki. The study was approved by the institutional ethical committee of the Hospital Clnic of Barcelona (Barcelona, Spain) following standard procedures.

2.10 Statistics

Cell culture experiments were conducted on at least three independent cell culture isolates obtained on different days. For in vivo experiments, four to five mice were used per group. Results are presented as means ± SEM. Data were analysed by one- or two-way analysis of variance (ANOVA), followed by Tukey post hoc test, using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). A P-value of < 0.05 was considered to be statistically significant.

3. Results

3.1 ISO and LPS induce antioxidant gene expression in an Fgf21-dependent manner in vivo

We recently showed that Fgf21-null mice develop enhanced signs of cardiac hypertrophy compared with wild-type mice in response to 7-day ISO infusion, a model of experimentally induced cardiac hypertrophy in neonates previously described. In these conditions (see Supplementary material online, Table S1 for heart morphology parameters), considering that cardiac hypertrophy is known to induce cardiac oxidative stress, we analysed cardiac genes encoding proteins involved in the antioxidant pathways, including uncoupling protein (Ucp)-2, Ucp3, superoxide dismutase-2 (Sod2), glutathione peroxidase-1 (Gpx1), peroxiredoxin (Prdx)-2, Prdx5, catalase (Cat), and sequestosome-1 (Sqstm1), under basal conditions and in response to ISO, in wild-type and Fgf21-null mice (Figure 1A). We found that aconitase activity was significantly decreased in the Fgf21-null mice compared with wild-type mice under both CT and ISO-infused conditions. This decrease in the aconitase activity was accompanied by significant increases in the protein carbonyl content (Figure 2B). We found that aconitase activity was significantly decreased in the Fgf21-null mice compared with wild-type mice under both CT and ISO-infused conditions. This decrease in the aconitase activity was accompanied by significant increases in the protein carbonyl content of Fgf21-null mice, especially following ISO infusion.

Collectively, these data indicate that a lack of Fgf21 induces cardiac oxidative stress in adult mice developing cardiac hypertrophy.

3.2 Lack of Fgf21 induces cardiac oxidative stress in adult mice

Next, we analysed cardiac gene expression in adult wt and Fgf21-null mice in which cardiac hypertrophy had been induced by a 7-day ISO infusion. We first examined cardiac genes encoding proteins involved in the antioxidant pathways, such as Ucp2, Ucp3, Cat and Sqstm1, under basal conditions and in response to ISO, in wild-type and Fgf21-null mice (Figure 2A). We did not observe any difference in the expression levels of the studied genes between wild-type and Fgf21-null mice under basal conditions. After ISO treatment, the cardiac expression levels of the Ucp2, Cat, and Sqstm1 genes were markedly induced in wild-type mice, but no such change was observed in ISO-infused Fgf21-null mice. No change in Ucp3 gene expression was observed under the tested conditions.

To assess the occurrence of oxidative stress in these hearts, we next analysed aconitase activity, which is decreased under oxidative stress conditions, and oxidized protein levels, which were assessed as the protein carbonyl content (Figure 2B). We found that aconitase activity was significantly decreased in the Fgf21-null mice compared with wild-type mice under both CT and ISO-infused conditions. This decrease in the aconitase activity was accompanied by significant increases in the protein carbonyl contents of Fgf21-null mice, especially following ISO infusion.

Collectively, these data indicate that a lack of Fgf21 induces cardiac oxidative stress in adult mice developing cardiac hypertrophy.

3.3 Fgf21 induces expression of antioxidant genes and prevents ROS formation in cardiac cells

Because Fgf21 was shown to modulate antioxidant genes in the heart in response to oxidative stress signals, we determined whether Fgf21 directly influences the expression of these genes by treating cultured NCMs in vitro with Fgf21. As shown in Figure 3A, Fgf21 significantly induced expression of Glut1, a known gene target of Fgf21, but not Glut4. Fgf21 also significantly increased Ucp2, Sod2, and Ucp3 mRNA levels in cardiac cells. Western blot analyses further showed that Fgf21 treatment significantly induced the Sod2 protein levels as well as the Ucp3 protein in accordance with the mRNA expression levels (Figure 3B). These data indicate that Fgf21 directly up-regulates the expression of antioxidant proteins in the heart.

Considering that Fgf21 induces the expression of Ucp2, Ucp3, and Sod2, which protect against oxidative stress, we studied the effects of Fgf21 in NCMs in response to ROS, using LPS as a tool for stimulating ROS production. We found that LPS treatment induced ROS in...
Figure 1  Antioxidant signals in hearts from wild-type and Fgf21-null neonatal mice: effects of ISO and LPS treatment. (A–C) Neonatal wt (black bars) and Fgf21−/− mice (white bars) were treated with ISO for 7 days to induce oxidative stress in the heart. (A) mRNA expression levels of the antioxidant genes, Ucp2, Sod2, Ucp3, Gpx1, Prdx2, Prdx5, Cat, and Sqstm1. (B) Western blot analysis of Ucp3 protein levels. (C) Caspase 3/7 activity. (D) mRNA expression levels of the antioxidant genes, Ucp2, Sod2, Ucp3, Gpx1, Prdx2, Prdx5, Cat, and Sqstm1, in 6-day-old neonates from wild-type and Fgf21-null mice 6 h after injection (i.p.) with LPS (5 mg/kg). Results are expressed as means ± SEM (n = 4–5 mice/group). Data were analysed by two-way ANOVA followed by Tuckey post hoc test (*P < 0.05 compared with corresponding control (CT) mice; #P < 0.05 compared with corresponding wt mice; &P < 0.05 compared with wild-type ISO- or LPS-treated mice).
NCMs, and pre-treatment of cells with Fgf21 significantly reduced the extent of ROS formation, both under basal conditions and in response to LPS (Figure 3C).

Collectively, these data indicate that Fgf21 has direct effects on cardiac cells, inducing the expression levels of antioxidant genes such as Ucp2, Ucp3, and Sod2.
Fgf21 targets the heart and protects it from oxidative stress. (A) Expression levels of the glucose transporter genes, Glut1 and Glut4, and the antioxidant genes, Ucp2, Sod2, Ucp3, Gpx1, Cat, Sqstm1, and Prdx5, in NCMs stimulated with Fgf21 (5 and 50 nM) for 24 h. (B) Sod2 protein levels determined by ELISA, and Ucp3 protein levels analysed by western blotting in NCMs treated with Fgf21 (50 nM) for 24 h. (C) ROS production in NCMs exposed to LPS (10 ng/mL) alone or following pre-treatment with Fgf21 (50 nM). Data are presented as means ± SEM. Cell culture experiments were conducted on three independent cell culture isolates obtained on different days. Data were analysed by one (A and B) or two-way ANOVA followed by Tukey post hoc test (C) [*P < 0.05 compared with control (CT) cells; #P < 0.05 compared with LPS-treated cells].
3.4 Cardiac Fgf21 is expressed and secreted after LPS treatment and acts in an autocrine manner to induce antioxidant genes

To this point, we have shown that Fgf21 protects the heart from pro-oxidative stress by inducing antioxidant genes. Next, we sought to determine the effects of oxidative stress on the expression levels of Fgf21 in the heart. First, we analysed the expression levels of Fgf21 in the hearts of mice injected (i.p.) with the pro-inflammatory agent LPS. These studies showed that LPS alone significantly increased Fgf21 expression levels in the heart (Figure 4A). However, the plasmatic levels of Fgf21 were unchanged after LPS injection. In parallel studies performed in NCMs, we observed that Fgf21 mRNA levels were also significantly induced by a 24-h exposure to LPS (Figure 4A). Finally, to determine whether Fgf21 was also secreted by cardiomyocytes, we analysed Fgf21 protein levels in NCM culture media. Low levels of secreted Fgf21 were detected in media under basal conditions, whereas treatment with LPS significantly increased Fgf21 protein levels in cardiac cell culture media.

Considering the duality of Fgf21—protecting the heart against LPS-induced oxidative stress (inducing antioxidant genes such as Ucp3) and being produced by the heart after LPS stimulation—we sought to establish a potential autocrine role of FGF21 by exploring the consequences of LPS-dependent induction of Fgf21 in cardiac cells from wild-type and Fgf21-null mice (Figure 4C). As expected from data obtained in rat NCMs, shown above, LPS treatment of mouse NCMs from wild-type mice significantly induced expression of the antioxidant genes Ucp2, Sod2, Ucp3, Gpx1, and Sqstm1. However, LPS treatment only induced the mRNA levels of Ucp2 in cardiac cells from Fgf21-null mice; the expression levels of Sod2, Ucp3, Gpx1, and Sqstm1 were unchanged. Moreover, LPS treatment reduced the expression levels of Cat and Prdx5 in cultured cells from Fgf21-null mice.

Taken together, these results indicate that LPS induces Fgf21 expression and release from cardiac cells, and further shows that induction of the antioxidant genes in cardiac cells in response to LPS is mediated by secreted Fgf21 acting in an autocrine manner.

3.5 Sirt1 controls antioxidant gene expression in the heart

It has recently been shown that the Sirt1 pathway protects the heart from pro-oxidative stimuli,1,3 and that Sirt1 induces Fgf21 gene expression in the heart.2 Accordingly, we studied F21 and antioxidant gene expression in hearts from Sirt1-deficient mice (Figure 5A). Fgf21 mRNA levels in the heart were significantly decreased in Sirt1-deficient mice compared with those in wild-type mice. Moreover, the expression levels of Ucp2, Sod2, Ucp3, Cat, Sqstm1, and Prdx5 were also significantly reduced in Sirt1-deficient mice. Next, we further confirmed the involvement of Sirt1 in antioxidant gene expression in vitro in cardiomyocytes by determining the effects of Sirt1 overexpression. As previously reported,10 overexpression of Sirt1 significantly increased the expression levels of Fgf21 (Figure 5B). Moreover, the expression levels of the antioxidant genes, Ucp2, Sod2, and Ucp3, were significantly up-regulated by Sirt1 overexpression, consistent with data obtained in Sirt1-deficient mice.

Thus, Sirt1 controls the expression levels of Fgf21 and antioxidant genes both in vivo and in vitro.

3.6 Fgf21 acts in an autocrine manner to control antioxidant pathways in response to Sirt1

Considering that Sirt1 has a strong impact on the expression levels of Fgf21 and antioxidant genes, we sought to establish a potential autocrine role of Fgf21 in the antioxidant response to Sirt1. To assess the involvement of Fgf21 in the actions of Sirt1, we treated NCMs overexpressing Sirt1 with Fgf21-neutralizing or control antibodies and then assessed the expression of antioxidant genes by RT–qPCR (Figure 6A). Incubating NCMs with the anti-Fgf21 antibody abolished Sirt1 overexpression-induced up-regulation of Ucp2, Sod2, and Ucp3, suggesting that blocking the activity of endogenously released Fgf21 impairs antioxidant gene expression in cardiac cells. Furthermore, the presence of the anti-Fgf21 antibody in Sirt1-overexpressing cells reduced the expression levels of Gpx1, Sqstm1, and Prdx5. To further investigate the cardiac consequences of the lack of Fgf21 on antioxidant genes in the context of Sirt1 overexpression, we used NCMs from wild-type and Fgf21-null mice as a second independent approach (Figure 6B). Using this model, we found that Sirt1 overexpression induced the mRNA expression of Ucp2, Sod2, Ucp3, and Sqstm1 in NCMs from wild-type mice. However, these changes in mRNA expression were abrogated in Fgf21-null mice, confirming the involvement of Fgf21 in the expression of antioxidant genes in cardiac cells in response to Sirt1 overexpression.

Next, we analysed Sod2 and Ucp3 protein levels in Sirt1-overexpressing NCMs in the presence or absence of Fgf21-neutralizing antibodies (Figure 6C). An analysis of intracellular Sod2 protein levels revealed that Sirt1 overexpression significantly increased Sod2 protein levels in NCMs. This Sirt1-induced increase in Sod2 protein levels was prevented by Fgf21-neutralizing antibodies. Furthermore, Fgf21-neutralizing antibodies reduced Ucp3 protein levels in Sirt1-overexpressing NCMs.

Finally, we examined ROS production in Sirt1-overexpressing NCMs treated with Fgf21-neutralizing or control antibodies (Figure 6D). We found that Sirt1 overexpression significantly decreased ROS production, whereas blockade of Fgf21 significantly reversed the protective effects of Sirt1. Collectively, these data indicate that the antioxidant effects of Sirt1 in cardiac cells are at least partially mediated by secreted Fgf21 acting in an autocrine manner.

3.7 Fgf21 expression levels are up-regulated in the failing human heart

Previous studies have shown that ROS production is enhanced in the failing heart.16,30 To determine whether the expression of Fgf21 and antioxidant genes are regulated in failing hearts, we studied the mRNA expression levels of Fgf21, Ucp2, Ucp3, and Sod2 in samples from healthy control and failing human hearts (Figure 7A). Fgf21 mRNA expression levels were significantly up-regulated in failing hearts compared with control hearts. Furthermore, we found that Ucp3 and Sod2 transcript levels were significantly increased, and Ucp2 showed a tendency to increase, in heart failure patients. In contrast, we did not observe any change in the studied glucose transporters (Glut1 and Glut4), Sirt1, or other fibroblast growth factors known to be relevant for cardiac remodelling (e.g. Fgf2 and Fgf23). Moreover, our analysis revealed that the protein levels of Ucp3 were also increased in failing hearts compared with control hearts (Figure 7B). Thus, we herein report that Fgf21, Ucp3, and Sod2 are up-regulated in samples from failing human hearts compared with controls, providing additional evidence for the association between Fgf21 induction and control of the...
Figure 4  Fgf21 is up-regulated in cardiac cells in response to LPS. (A) Fgf21 expression levels (right) and plasmatic levels of Fgf21 (left) in mice 6 h after injection with LPS (n = 5 mice/group). (B) Fgf21 mRNA levels in NCMs and protein levels in culture media 24 h after stimulation with LPS. (C) mRNA expression levels of the antioxidant genes, Ucp2, Sod2, Ucp3, Gpx1, Cat, Sqstm1, and Prdx3, in NCMs from wild-type and Fgf21−/− mice exposed to LPS for 24 h. Results are expressed as means ± SEM. Cell culture experiments were conducted on four independent cell culture isolates obtained on different days. Data were analysed by one (A and B) or two-way ANOVA followed by Tukey post hoc test (C) [*p < 0.05 compared with corresponding control (CT) mice or cells; #p < 0.05 compared with wild-type LPS-treated cardiomyocytes].
cardiac oxidative stress pathways. Furthermore, other cardiac remodelling-associated members of the fibroblast growth factor family were not altered in failing human hearts, indicating that Fgf21 is specifically up-regulated during heart failure.

4. Discussion

In the present study, we demonstrated that Fgf21 induces an antioxidant response in the heart by promoting the expression of certain antioxidant genes (e.g. Ucp2, Ucp3, and Sod2) and preventing ROS formation. Moreover, we found that Fgf21 gene expression is induced by pro-oxidative stimuli, establishing a feedback loop whereby the pro-oxidative stimuli themselves enhance the production of Fgf21, which confers local protection against ROS formation. Therefore, Fgf21 stimulates an endogenous antioxidant response in cardiac tissue by acting in an autocrine manner.

Recently, we showed that Fgf21 prevents the development of cardiac hypertrophy in both in vivo and in vitro murine models. The data presented here demonstrate that ISO-induced cardiac hypertrophy is also associated with up-regulation of the antioxidant protein Ucp3 and other antioxidants, including Prdx5, Cat, and Sqstm1, in an Fgf21-dependent manner. Consistent with a role for Fgf21 in the

Figure 5 SIRT1 controls the expression levels of Fgf21 and antioxidant genes. (A) Expression levels of Fgf21 and antioxidant genes in hearts from wild-type (wt; black bars) and Sirt1-null mice (Sirt1−/−; white bars). Results are expressed as means ± SEM (n = 4–5 mice/group). Data were analysed by one-way ANOVA (*P < 0.05 compared with wild-type mice). (B) Expression levels of glucose transporter (Glut1 and Glut4), Fgf21, and antioxidant genes in NCMs infected with AdSirt1 or AdGFP control vector. Results are expressed as means ± SEM. Cell culture experiments were conducted on three independent cell culture isolates obtained on different days. Data were analysed by one-way ANOVA followed by Tuckey post hoc test (*P < 0.05 compared with AdGFP-infected cells).
Figure 6 Role of endogenously produced Fgf21 in cardiac cells. (A) NCMs infected with AdSirt1 or AdGFP control vector alone or co-treated with anti-Fgf21 antibody or control antibody, added to the culture medium. (B) mNCMs from wild-type (wt; white bars) and Fgf21−/− mice (black bars) infected with AdSirt1 or AdGFP control vector. (C) Sod2 and Ucp3 protein levels in NCMs infected with AdSirt1 or AdGFP control vector alone or co-treated with anti-Fgf21 antibody, added to the culture medium. (D) Levels of ROS production, expressed as DCF-DA fluorescence (nM) in NCMs infected with AdSirt1 or AdGFP control vector alone or co-treated with anti-Fgf21 antibody, added to the culture medium. Results are expressed as means ± SEM. Cell culture experiments were conducted on three independent cell culture isolates obtained on different days. Data were analysed by one (C) or two-way ANOVA (A, B, D) followed by Tuckey post hoc test (*P < 0.05 compared with corresponding control cells; #P < 0.05 compared with AdSirt1-infected cells).
Figure 7 Fgf21 expression is up-regulated in the failing human heart. Samples from control (CT; n = 10) and failing (HF; n = 6) human hearts were analysed. (A) mRNA expression levels of Fgf21, the antioxidant genes, Ucp3, Ucp2, and Sod2, the glucose transporter genes, Glut1 and Glut4, the Sirtuin-1 (Sirt1), and the fibroblast growth factors 2 and 23 (Fgf2 and Fgf23). (B) Levels of Ucp3 protein in control and failing human hearts determined by western blotting (*C, Coomassie blue staining was used as an internal loading control). Results are expressed as means ± SEM. Data were analysed by one-way ANOVA followed by Tuckey post hoc test [*P < 0.05 compared with corresponding control (CT) hearts].
control of antioxidant activity, biological markers of oxidative stress were altered in hearts from Fgf21-null mice. It is known that increased oxidative stress activates a variety of hypertrophy signalling cascades and transcription factors [see recent review]. At the failing heart stage, oxidative stress further increases, facilitating cell dysfunction and induction of cardiac cell apoptosis. Accordingly, we found an increase in the apoptotic rate in Fgf21-null mice in association with a reduction in the levels of Ucp3 after ISO-induced cardiac hypertrophy. Activation of Ucp3 has been considered a mechanism of protection against ROS damage. In fact, Ucp3-null mice develop exaggerated apoptotic cell death and enhanced signs of cardiac damage. Thus, the data presented here are consistent with an important role of Ucp3 induction in the protective action of Fgf21 against cardiac oxidative stress under conditions of cardiac hypertrophy.

In the heart, LPS also induces oxidative stress by stimulating the production of ROS. In the current study, we demonstrated that LPS induces Fgf21 expression and secretion by cardiac cells; secreted Fgf21, in turn, acts in an autocrine manner to induce the expression of antioxidant genes encoding uncoupling proteins and Sod2. The fact that Fgf21 protein levels in plasma from LPS-treated mice were unchanged further suggests an autocrine role for Fgf21 in the heart.

Collectively, these results indicate that the secretion of Fgf21 in the context of cardiac damage may serve as an endogenous, auto-regulatory, cardioprotective signalling pathway against pro-oxidative damage. Our observation that Fgf21 is induced in parallel with genes encoding antioxidant proteins in humans is consistent with this scenario. Moreover, a recent study in mice has shown protective effects of Fgf21 on myocardium after ischaemia/reperfusion injury, a known oxidative stress condition.

We further showed that the antioxidant properties previously described for the protein Sirt1 are partially mediated by Fgf21. Previous studies have demonstrated the involvement of Sirt1 in controlling antioxidant gene expression in cardiac tissue, and the Sirt1/FoxO3a pathway has been recently reported to control the transcription of Sod2. In the current study, we showed that Fgf21 is involved in antioxidant-dependent changes in Sirt1 in the heart. The extracellular signal-regulated kinase (ERK) pathway is involved in the transcriptional control of Sod2, and Fgf21 activates the ERK pathway in the heart. Thus, the secreted Fgf21 may act through the ERK pathway to activate Sod2 and prevent ROS formation in the context of Sirt1 overexpression. Sirt1 activity can be modulated by mechanisms other than changes in its expression level (e.g., by the action of specific metabolic activators). Although Sirt1 mRNA levels were not found to be induced in failing human hearts, it remains possible that the up-regulation of Fgf21 in failing hearts may still be mediated by post-transcriptional changes in Sirt1.

Improving the prognosis of patients with heart failure will require therapeutic strategies based on novel insights into the pathophysiology of myocardial remodelling and failure. Approaches based on controlling mitochondrial oxidative stress may contribute to the development of effective treatment strategies for patients with heart failure. In this context, our demonstration that Fgf21 acts in an autocrine manner to regulate genes involved in antioxidant pathways and prevents ROS production in cardiac cells point to Fgf21 as a potential tool in the development of strategies for preventing and/or treating cardiac oxidative stress associated with several cardiac diseases.

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**References**


**Supplementary material**

Supplementary material is available at *Cardiovascular Research* online.


