Little is known regarding the role of hyperglycaemia on histone H3 modifications and, in turn, altering the expression of genes during the development of diabetes-associated complications. In the present study, we have investigated the hyperinsulinaemia/hyperglycaemia-induced epigenetic changes and alteration of Fbn1 (fibrillin 1) and Col3A1 (collagen type III α1) gene expression. Insulin resistance and Type 2 diabetes in male Sprague–Dawley rats was developed by feeding rats an HFD (high-fat diet) and administering a low dose of STZ (streptozotocin). Hyperglycaemia induced deacetylation and dephosphorylation of histone H3 in the heart and kidneys of diabetic rats. Furthermore, mRNA expression of Fbn1 and Col3A1 increased in the kidneys and decreased in the heart under hyperglycaemic/hyperinsulinaemic conditions. Similar to mRNA expression, chromatin immunoprecipitation also showed an increase in the level of histone H3 acetylation of the Fbn1 gene, but not of the Col3A1 gene. Our present findings suggest that the change in expression of the Fbn1 gene is epigenetically regulated, but the expression of the Col3A1 gene may either be independent of epigenetic regulation or may involve other histone modifications. We provide the first evidence regarding the role of hyperglycaemia/hyperinsulinaemia in altering histone H3 modifications, which may result in the alteration of extracellular matrix gene expression.

Key words: diabetic nephropathy, fibrosis, hyperglycaemia, histone H3 modification, insulin resistance.

INTRODUCTION

DN (diabetic nephropathy) affects 30% of diabetic patients and has become the leading cause of end-stage kidney disease worldwide [1,2]. Patients with different stages of DN frequently present with cardiac disease, for example myocardial ischaemia and/or diabetic cardiomyopathy [3]. Both kidney disease and diabetes are major and independent risk factors for the development of cardiac changes, such that individuals with DN are at high risk. Such patients are not only likely to have coronary artery disease and hypertension, but may suffer from diabetic cardiomyopathy [4]. Diabetic cardiomyopathy is characterized by cardiomyocyte hypertrophy, perivascular or interstitial fibrosis, and the interstitial accumulation of glycoprotein [5].

The molecular mechanisms that link renal failure to the progression of cardiac disease remain unclear. Hyperglycaemia alone has been reported to be involved in the development of cardiomyopathy and activation of a sequence of maladaptive stimuli that results in myocardial fibrosis and collagen deposition [6]. Furthermore, increased TAGs [triacylglycerols (triglycerides)], NEFAs (non-esterified ‘free’ fatty acids) and hyperinsulinaemia eventually lead to hyperglycaemia, which is responsible for affecting cardiac structure and function [5]. In addition, several studies have proposed a role for the renin–angiotensin system, hypertension, dyslipidaemia, endothelial dysfunction, oxidative stress and inflammation [7]. Moreover, involvement of different cellular mechanisms, such as MAPK (mitogen-activated protein kinase) activation [8], Akt activation [9] and a reduction in PPAR-β/δ (peroxisome-proliferator-activated receptor-β/δ) expression during the development of diabetic cardiomyopathy [10] have been reported. All of these alter the function and, eventually, the structure of the cardiac vasculature and cardiomyocytes by activating different signalling pathways that specifically drive transcription of downstream pathogenic factors and genes.

Covalent modifications of histones are involved in the regulation of chromatin dynamics, which in turn regulates a variety of biological processes, such as replication, repair, transcription and genome stability [11,12]. For example, the transcriptional activity of CAMTAs (calmodulin-binding transcription activators) (which promote cardiomyocyte hypertrophy) is governed by their association with class II histone deacetylases, which negatively regulate cardiac growth [13]. Furthermore, trimethylation of histone H3 on Lys4 (K4) or Lys9 (K9) is affected in cardiomyocytes during the development of heart failure [14]. A recent study has also reported the role of elevated acetylated histone levels in vascular injury and remodelling in Type 1 diabetic patients [15]. In addition, we have recently reported that insulin under hyperglycaemic conditions alters multiple histone modifications and alters gene expression in variety of biological processes in L6 skeletal muscle myoblasts [16]. These studies imply the involvement of global epigenetic changes in the pathogenesis of diabetic complications.

On the basis of this, we hypothesized that hyperinsulinaemia (pre-diabetic) and hyperglycaemia (Type 2 diabetes) may change histone H3 modifications, which in turn alter the expression of the Fbn1 (fibrillin 1) [17] and Col3A1 (collagen type III α1) [18] genes that initiate fibrosis during the development of cardiac–renal

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Abbreviations used: BUN, blood urea nitrogen; ChIP, chromatin immunoprecipitation; Col3A1, collagen type III α1; DN, diabetic nephropathy; ECL, enhanced chemiluminescence; ECM, extracellular matrix; Fbn1, fibrillin 1; HFD, high-fat diet; HRP, horseradish peroxidase; NPD, normal pellet diet; PCR, plasma creatinine; PGL, plasma glucose; PI, plasma insulin; PTC, plasma total cholesterol; qPCR, quantitative PCR; RT–PCR, reverse transcription–PCR; STZ, streptozotocin; TAG, triacylglycerol; PTAG, plasma TAG.

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syndrome. Most of the genetic defects in genetic models of Type 2 diabetes have not been well-characterized [19], and the epidemic of Type 2 diabetes calls for an animal model that more closely mimics human pathophysiology. Hence, in the present study, we have used a non-genetic model of Type 2 DN [HFD (high-fat diet) feeding plus administering a low dose of STZ (streptozotocin)], which mimics the metabolic abnormalities very similar to those seen in human Type 2 diabetes [20].

MATERIALS AND METHODS

Animal studies

The male adult Sprague–Dawley rats (160–180g) were procured from the central animal facility of the National Institute of Pharmaceutical Education and Research (NIPER). Animals were maintained under standard environmental conditions and were provided with feed and water ad libitum. All the animals were fed on a NPD (normal pellet diet) 1 week prior to experimentation. Our protocol is in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Environment, Government of India. Rules of CPCSEA are laid down as per ILAR (Institute of Laboratory Animal Resources, U.S.A.) guidelines and prior permission was sought from the institutional animal ethics committee (IAEC #08/06) for conducting the study. All experimental procedures had been approved by the local government authorities.

Insulin resistance and Type 2 diabetes in male Sprague–Dawley rats was developed by HFD feeding and administering a low dose of STZ respectively, as described previously [21]. Briefly, the rats were allocated to two dietary regimens either an NPD or HFD (58% fat, 25% protein and 17% carbohydrate, as a percentage of total kcal) ad libitum respectively for an initial period of 2 weeks. The composition and preparation of the HFD used was as described by Srinivasan et al. [22]. Each rat in the NPD and HFD groups consumed approx. 64 kcal/day and 105 kcal/day respectively for an initial period of 2 weeks of dietary manipulation. The rats from the HFD-fed group were injected with a low dose of STZ (35 mg/kg of body weight, intraperitoneally), whereas the respective control rats were given vehicle citrate buffer (pH 4.4). The rats were allowed to continue to feed on their respective diets until the end of the study (24 weeks). Body weight was measured and biochemical estimations were carried out after 24 weeks of dietary manipulation. Food intake, urine output and water intake were also measured after 24 weeks. For these measurements, rats were placed in metabolic cages as described previously [23]. Heart and kidney weights were taken at the end of the study.

Assessment of insulin resistance, diabetes and renal function

The blood samples were collected, plasma was separated and analysed for PGL (plasma glucose), PTAGs (plasma TAGs), PTC (plasma total cholesterol), PI (plasma insulin), BUN (blood urea nitrogen) and PCR (plasma creatinine), as described previously [21,24]. Insulin was measured using an ELISA kit with rat insulin as the standard (Linco Research).

Measurement of blood pressure

Blood pressure (systolic, mean and diastolic) was recorded after 24 weeks of feeding in all of the groups using a tail cuff blood pressure recorder (Model No. 29,229; Life Science Instruments), as described previously [21,23].

Measurement of blood pressure

Blood pressure (systolic, mean and diastolic) was recorded after 24 weeks of feeding in all of the groups using a tail cuff blood pressure recorder (Model No. 29,229; Life Science Instruments), as described previously [21,23].

Table 1 Primer sequences used for mRNA expression and ChIP assay

<table>
<thead>
<tr>
<th>For RT–PCR (mRNA expression)</th>
<th>Right (5′→3′)</th>
<th>Left (5′→3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fbn1</td>
<td>GCCAGCTCGCCAGTGACAGGG</td>
<td>CGTGGCGACATCGACGTT</td>
</tr>
<tr>
<td>Col1A1</td>
<td>AACGGAGCTCGCCAGTGACAGGG</td>
<td>ATTGGCCTCGACGACCACC</td>
</tr>
<tr>
<td>tS</td>
<td>AGGGCCTCTAACCAAACTCC</td>
<td>GCAATTATGCCCAGTAGACG</td>
</tr>
<tr>
<td>For ChIP assay</td>
<td>GCCAGCTCGCCAGTGACAGGG</td>
<td>CGTGGCGACATCGACGTT</td>
</tr>
<tr>
<td>Fbn1</td>
<td>AACGGAGCTCGCCAGTGACAGGG</td>
<td>ATTGGCCTCGACGACCACC</td>
</tr>
<tr>
<td>Col1A1</td>
<td>AACGGAGCTCGCCAGTGACAGGG</td>
<td>ATTGGCCTCGACGACCACC</td>
</tr>
</tbody>
</table>

Histopathological evaluation and immunostaining

From each rat, portions of heart and kidney tissue were fixed in 10% (v/v) formalin in PBS and embedded in paraffin. Sections (2-μm thick) were stained with haematoxylin/eosin. Glomerular damage was assessed using a semi-quantitative score by an observer blinded to the study groups as follows: 0 = no lesion, 1 = < 25% damage, 2 = 25–49% damage, 3 = 50–74% damage, and 4 = 75–100% damage, as described previously [25]. A total of 15 glomeruli were analysed per section. All immunohistochemical studies were performed on paraffin-embedded sections as described previously [25]. A rabbit anti-fibronectin antibody (1:50 dilution; Santa Cruz Biotechnology) was used as the primary antibody, and an HRP (horseradish peroxidase)-conjugated anti-rabbit antibody (1:1000 dilution; Santa Cruz Biotechnology) was used as the secondary antibody. The intensity of the spots was graded from 1–4 (1, slight or no colour; 2, very low colour; 3, moderate brown colour; and 4, very intense brown colour). The immunohistochemistry score is expressed as means ± S.E.M. for each experimental group. The number of cardiomyocyte nuclei was counted in each high-power field.

Histone extraction and immunoblotting

Heart and kidneys were dissected manually, and histone isolation and Western blotting were performed as described previously [24]. Immunoblot analysis was performed using a rabbit anti-[acylated histone H3 (Lys8/14)] antibody (1:5000 dilution; Upstate Biotechnology), a rabbit anti-[phospho-histone H3 (Ser10)] antibody (rabbit 1:2500, Santa Cruz Biotechnology), a rabbit anti-histone H3 antibody (1:5000 dilution; Upstate Biotechnology) and HRP-conjugated anti-rabbit secondary antibodies (diluted 1:20000; Santa Cruz Biotechnology). Proteins were detected with the ECL (enhanced chemiluminescence) system and ECL Hyperfilm (Amersham Biosciences). Immunoblots were quantified by densitometric analysis and the exposures were in linear dynamic range. The densitometric analysis was performed by Image J software.

PCR

RNA was isolated from the heart and kidneys using RNA extraction kit (Qiagen). After reverse transcription with Superscript II (Invitrogen) real-time RT–PCR (reverse transcription–PCR) was performed on a Light Cycler 480 using a Light Cycler Fast Start DNA master plus kit (Roche Diagnostics) and the specific forward and reverse primers (Midland Certified Reagent). The sequences of the primers used are listed in Table 1. After amplification, a melting curve analysis was performed to verify the specificity of the reaction. The 18S gene was used as an internal control, and the results are expressed as the fold changes over those in NPD-fed rats.
**CHI** (chromatin immunoprecipitation) assay

CHI assays were performed using an EZ-ChIP™ kit (Upstate Biotechnology), and CHI-enriched DNA samples were analysed by real-time qPCR (quantitative PCR) as described previously [16]. Briefly, the heart and kidneys were cut into small pieces, resuspended in serum-free medium (Dulbecco’s modified Eagle’s medium) and cross-linked with 1% (v/v) formaldehyde for 10 min. The cross-linking reaction was stopped with 0.125 M glycine, washed twice with PBS containing protease inhibitors and lysed in SDS lysis buffer (from the kit). Chromatin was sonicated to a size of 0.5–2 kb and was incubated overnight with 10 μg of the respective antibody. DNA–protein complexes were collected on Protein A–agarose beads. Protein A–agarose pellets were washed and DNA–protein complexes were eluted, treated with RNaseA (Amersham Bioscience) and cross-links were reversed. Proteins were degraded by proteinase K (Amersham Bioscience), and cross-links were reversed. Proteins were degraded by proteinase K (Amersham Bioscience), and DNA was extracted, purified and resuspended in water. CHI (chromatin immunoprecipitation) assay

Chromatin immunoprecipitation (ChIP) with SYBR reagent in a Light cycler 2 real-time PCR enriched DNA samples and input DNA samples were analysed by qPCR. DNA was extracted, purified and resuspended in water. ChIP-enriched DNA samples and input DNA samples were analysed by qPCR with SYBR reagent in a Light cycler 2 real-time PCR machine (Roche Diagnostics) using primers specific for the Fbn1 and Col3A1 genes (the sequences are listed in Table 1), qPCR data were analysed using the 2–ΔΔct method and were normalized to the input samples. Results are expressed as the fold change over those in NPD-fed rats.

**Statistical analysis**

Experimental values are expressed as means ± S.E.M. Comparison of the mean values between various groups was performed using one-way ANOVA, followed by a Dunnett test. Histopathological scores were analysed using Kruskal–Wallis ANOVA on ranks, followed by the Tukey test. A P value <0.05 was considered to be significant.

**RESULTS**

**Development of renal dysfunction under hyperinsulinaemic and hyperglycaemic conditions**

Injection of STZ after 2 weeks of dietary manipulation to HFD-fed rats significantly increased PGL, thus producing frank hyperglycaemia. However, only a small, but statistically significant, increase in PGL was observed in HFD-alone-fed rats compared with NPD-fed rats (Table 2). In addition, a significant increase was observed in basal PTAG and PTC levels along with a significant increase in PI levels in HFD-fed rats compared with NPD-fed rats, whereas PI levels were decreased along with a significant increase in basal PTAG and PTC levels in HFD+STZ-treated rats compared with HFD-fed rats (Table 2). These results demonstrate the development of insulin resistance and Type 2 diabetes in the HFD-fed and HFD+STZ-treated animals respectively. Moreover, HFD+STZ-treated rats also had significant increases in BUN and PCR levels compared with HFD- and NPD-fed rats (Table 2). Similarly, a significant increase in BUN was also observed in HFD-fed rats compared with NPD-fed rats. However, there was no significant increase in PCR levels in the HFD-fed group. In addition, HFD+STZ-treated rats developed symptoms including polydipsia and polyuria compared with NPD-fed control rats (Table 2). There was a significant increase in kidney weight in HFD+STZ-treated animals compared with HFD-fed and control NFD-fed rats (Table 3). At a tissue level, the extent of glomerular damage was more in the HFD+STZ group (Type 2 diabetic) compared with the NPD (control) and HFD (pre-diabetic) groups (Figures 1A and 1B). In addition to glomerular damage, nephropathy was confirmed further by the expression of fibronectin in the kidneys of these animals. We found an increase in fibronectin expression in HFD-fed and HFD+STZ-treated rats, suggesting the development of renal fibrosis in these animals (Figure 1C). These observations clearly support the development of renal dysfunction under insulin-resistant and Type 2 diabetic conditions.

**Development of cardiomyopathy, and increases in systolic and diastolic blood pressure under hyperinsulinaemic and hyperglycaemic conditions**

Treatment with STZ produced a significant reduction in the body weights of the HFD-fed rats. A significant increase was observed in the body weight of HFD-fed rats compared with control NPD-fed rats. Body weights remained largely unaltered between control STZ-treated and control NPD-fed rats (Table 3). There was a significant increase in heart weight of the HFD-fed rats compared with the NPD-fed rats, whereas there was a decrease in heart weight in the HFD+STZ-treated rats compared with HFD-fed rats. However, there was an increase in the heart weight/body weight ratio in HFD+STZ (hyperglycaemia) animals (Table 3). Moreover, the heart weight/body weight ratio in HFD-fed rats remained unchanged (Table 3). Histomorphometrical analysis of cross-sections from the heart of insulin-resistant and Type 2 diabetic animals revealed that the number of cardiomyocyte nuclei in a given high-power field was significantly reduced compared with control NPD-fed rats (Figures 2A and 2B). These findings support the presence of cardiomyocyte hypertrophy. To confirm the development of cardiac fibrosis associated with cardiomyopathy, we assessed the expression of fibronectin. We found an increase in fibronectin expression in the myocardium in HFD-fed and HFD+STZ-treated rats (Figure 2C), which confirmed further the development of cardiac fibrosis in these animals. To correlate the structural changes with a cardiac functional parameter, we measured the blood pressure in these animals. Systolic, mean and diastolic blood pressure were significantly elevated in both pre-diabetic (HFD-fed rats) and diabetic (HFD+STZ-treated rats) condition compared with control NPD-fed rats (Table 3). The increase in blood pressure was greater in HFD+STZ-treated rats (hyperglycaemia) compared with HFD-alone-fed rats (hyperinsulinaemia). These results indicate the development of cardiac fibrosis associated with cardiomyopathy and altered cardiovascular functions in pre-diabetic and diabetic states.
Differential pattern of histone H3 modifications in the heart and kidneys under hyperinsulinaemic and hyperglycaemic conditions

To understand the effects of hyperinsulinaemia and hyperglycaemia on chromatin remodelling (nuclear events) in the heart and kidneys, we determined the levels of acetylation (on Lys\(^9/14\)) and phosphorylation (on Ser\(^10\)) of histone H3 in these animals. An increase in acetylation (on Lys\(^9/14\)) and phosphorylation (on Ser\(^10\)) of histone H3 was observed in the hearts of HFD-fed and HFD+STZ-treated rats (Figure 3, lanes c and d) in comparison with NPD-fed rats (Figure 3, lane a). However, a decrease in acetylation and phosphorylation was observed in the kidneys of HFD-fed and HFD+STZ-treated rats compared with NPD-fed rats (Figure 3). Similar results were also found when the levels of dimethylation (on Lys\(^4\)) were determined (see Supplementary Figure S1 at http://www.biochemj.org/bj/432/bj4320333add.htm). Interestingly, when comparing hyperinsulinaemic (insulin-resistant) and hyperglycaemic (Type 2 diabetic) conditions, there was a decrease in the acetylation and phosphorylation in both the heart and kidneys in the Type 2 diabetic condition (Figure 3, lanes c and d). These results suggest a differential pattern of histone H3 modification in the heart and kidneys under both conditions and that hyperglycaemia/hyperinsulinaemia has a definite role in altering the histone H3 modifications.

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Table 3  Development of cardiomyopathy and altered cardiac function in insulin-resistant and Type 2 diabetic rats.

Body weight, heart weight, kidney weight and blood pressure were taken after 24 weeks of HFD feeding. All the values are means ± S.E.M. (n = 10). *P < 0.05 compared with NPD; and †P < 0.05 compared with HFD.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NPD</th>
<th>NPD+STZ</th>
<th>HFD</th>
<th>HFD+STZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>508 ± 8</td>
<td>497 ± 6</td>
<td>554 ± 11*</td>
<td>366 ± 11†</td>
</tr>
<tr>
<td>Kidney weight (g)</td>
<td>2.96 ± 0.02</td>
<td>2.85 ± 0.08</td>
<td>2.45 ± 0.11</td>
<td>3.65 ± 0.23†</td>
</tr>
<tr>
<td>Kidney weight/body weight (g)</td>
<td>(5.82 ± 0.08) x 10⁻³</td>
<td>(5.74 ± 0.19) x 10⁻³</td>
<td>(4.41 ± 0.13) x 10⁻³</td>
<td>(9.98 ± 0.53) x 10⁻³†</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>1.17 ± 0.05</td>
<td>1.04 ± 0.22</td>
<td>1.36 ± 0.08*</td>
<td>1.09 ± 0.12†</td>
</tr>
<tr>
<td>Heart weight/body weight (g)</td>
<td>(2.31 ± 0.10) x 10⁻³</td>
<td>(2.09 ± 0.43) x 10⁻³</td>
<td>(2.44 ± 0.11) x 10⁻³</td>
<td>(2.98 ± 0.30) x 10⁻³†</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>116 ± 3.81</td>
<td>118 ± 2.69</td>
<td>148 ± 0.87*</td>
<td>154 ± 1.59†</td>
</tr>
<tr>
<td>Mean arterial pressure (mmHg)</td>
<td>91 ± 1.63</td>
<td>91 ± 2.18</td>
<td>115 ± 1.66*</td>
<td>124 ± 1.89†</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>79 ± 0.68</td>
<td>80 ± 1.25</td>
<td>101 ± 1.82*</td>
<td>110 ± 1.9†</td>
</tr>
</tbody>
</table>

Figure 2  Development of cardiomyopathy and associated fibrosis under hyperinsulinaemic and hyperglycaemic conditions

(A) Heart sections of rats from the different groups were stained with haematoxylin/eosin, as described in the Materials and methods section (original magnification, ×100). (B) The mean ± S.E.M. percentage of nuclei under the high-power field is shown. *P < 0.05 compared with NPD-fed rats; and †P < 0.05 compared with HFD-fed rats. (C) Images show representative fibronectin immunostaining in heart sections (original magnification, ×100). A decrease in the number of nuclei and an increase in fibronectin expression suggests the development of cardiac hypertrophy in these rats. A total of six animals were analysed from each group.
Change in expression of *Fbn1* and *Col3A1*, and the level of histone H3 acetylation in these genes in the heart and kidneys under hyperinsulinaemic and hyperglycaemic conditions

Several reports have suggested that global changes in acetylation and phosphorylation of histone H3 alter chromatin structure and, in turn, gene expression [11,12]. Therefore the expression of the *Fbn1* [17] and *Col3A1* [18] genes and the level of histone H3 acetylation on these ECM (extracellular matrix)-formation and fibrosis-related genes was determined by real-time qPCR and ChIP assays in the pre-diabetic and diabetic heart and kidneys. Cardiac gene expression of *Fbn1* and *Col3A1* was decreased in HFD+STZ-treated rats in comparison with control NPD-fed rats (Figure 4 and 5). However, the expression of *Fbn1* was increased and *Col3A1* expression was decreased in hearts of HFD-fed rats (Figures 4 and 5). ChIP assays also revealed an increase in the levels of histone H3 acetylation of the *Fbn1* gene in hearts of HFD-fed and HFD+STZ-treated rats (Figure 4). In contrast, there was no change in levels of histone H3 acetylation in the *Col3A1* gene in hearts of HFD-fed and HFD+STZ-treated rats (Figure 5). These results indicate that *Fbn1* plays a critical role in the insulin-resistance condition and may be involved in the transformation of adaptive to maladaptive hypertrophy in Type 2 diabetic conditions. Renal expression of the *Fbn1* and *Col3A1* genes was increased in HFD-fed and HFD+STZ-treated rats (Figures 4 and 5). Levels of histone H3 acetylation were increased in the *Fbn1* gene under hyperglycaemic and hyperinsulinaemic conditions in kidneys compared with control NPD-fed rats (Figure 4). However, we did not find any change in levels of histone H3 acetylation in the *Col3A1* gene in kidneys under hyperglycaemic and hyperinsulinaemic conditions compared with control NPD-fed rats (Figure 5). These results show the differential expression of *Fbn1* and *Col3A1* and levels of histone H3 in these genes in the heart and kidneys under hyperinsulinaemic and hyperglycaemic conditions.

**DISCUSSION**

Our present findings clearly demonstrate that insulin resistance and Type 2 diabetes alter post-translational modifications of histone H3 in the heart and kidney which, in turn, are involved in modulating the expression of genes related to cardiac and renal fibrosis in these animals. Our present study demonstrates that rats fed on an HFD for 24 weeks exhibited all of the hallmark features of insulin resistance, as characterized by hyperinsulinaemia, mild hyperglycaemia, hypertriglyceridaemia, hypercholesterolaemia and hypertension. However, HFD-fed rats upon injection with a low dose of STZ produced frank hyperglycaemia in the presence of a circulating insulin concentration almost comparable with the metabolic characteristics of human Type 2 diabetes, along
with an increase in BUN and PCR. Renal dysfunction was more pronounced in HFD+STZ-treated rats compared with HFD-alone-fed rats.

Several reports have indicated the involvement of covalent modifications of histones in a variety of biological processes [11,12]. These histone modifications lead to the recruitment of protein complexes/transcription factors that regulate the transcription of a variety of genes [26]. Little is known about the histone H3 modifications in the heart under insulin-resistant and Type 2 diabetic conditions, but a recent study has suggested that high glucose levels activate the histone acetylases CBP (cAMP-response-element-binding protein-binding protein) and p/CAF (p300/cAMP-response-element-binding protein-binding protein) and increment. Inactivation of the transcription of the genes responsible for the pathophysiology of ECM and calcium ion homeostasis [31]. Our present results also support these findings in that, under hyperglycaemia and insulin-resistant conditions, there must be a change in transcription of genes related to cardiac and renal fibrosis in the heart and kidneys. Alteration of histone H3 modifications under Type 2 diabetic conditions in the heart and kidneys may be responsible for altering the expression of the genes responsible for the pathophysiology of the disease. Hence we studied profibrotic ECM gene expression in the heart and kidneys. A number of studies have reported
the involvement of Fbn1 [17] and Col3A1 [18] genes in ECM formation and development of tissue fibrosis, but, to date, no reports are available regarding its epigenetic regulation under insulin-resistant and Type 2 diabetic conditions in the heart and kidneys, which results in the development of cardio-renal syndrome. Our present result indicates that hyperglycaemia differentially regulates the gene expression of Fbn1 and Col3A1 during the development of cardio-renal syndrome in the heart and kidneys.

To understand further the epigenetic regulation of these genes, we performed real-time qPCR and ChIP assays with an antibody against acetylated histone H3 and studied the level of histone H3 acetylation of these genes. Our results show that the Fbn1 gene in the heart was acetylated more under insulin-resistant conditions and the level of acetylation was decreased under hyperglycaemic conditions compared with the controls. Increased histone H3 Lys9/14 acetylation of the Fbn1 gene in the heart may be responsible, at least in part, for the persistent expression of these genes by rendering chromatin near these genes more accessible to transcription factors. However, there was no change in the acetylation level of histone H3 in the Col3A1 gene in the heart or kidneys under both of the conditions. These results suggest that either the change in expression of the Col3A1 gene is not epigenetically regulated or may be regulated by other histone modifications. High insulin levels cause the development of adaptive hypertrophy which, in turn, leads to the development of maladaptive hypertrophy by the presence of stress signal, i.e. hyperglycaemia in Type 2 conditions (Figure 6). Our present results suggest that the expression of Fbn1 was increased under high insulin conditions and decreased under high glucose conditions. Furthermore, expression of Fbn1 is epigenetically regulated under these conditions. This transformation involves epigenetic changes which, in turn, alter the expression of genes such as Fbn1 that control hypertrophy. The switching of Fbn1 gene expression under hyperinsulinaemic/hyperglycaemic conditions can be used as a predictive marker, which acts as a switch between adaptive and maladaptive hypertrophy during diseases development.

The pathogenesis of Type 2 diabetes is multifaceted, involving the progressive development of insulin resistance in the liver and peripheral tissues accompanied by defective insulin secretion from pancreatic β-cells, which leads to overt hyperglycaemia [32]. Suitable experimental models are vital tools for understanding the pathogenesis and complications of Type 2 diabetes. Attempts have been made to develop models of Type 2 diabetes (non-insulin-dependent diabetes mellitus) that closely simulate the metabolic abnormalities of the human disease and are also cost-effective compared with the genetic models currently available. In 1998, Lou et al. [33] reported that a combination of dietary-induced insulin resistance and a relatively low dose of STZ resulted in mouse models that could be of use in studying the pathophysiology of Type 2 diabetes. In 2000, Reed et al. [34] demonstrated an animal model of Type 2 diabetes (a combination of HFD-fed and low-dose STZ-treated rats) in a non-obese out-bred rat strain that replicates the natural history and metabolic characteristics of the human syndrome and is suitable for pharmaceutical research [34]. Srinivasan et al. [35] in 2005 described a rat model which is unique in so far as the approach adopted towards the development of the model as well as its suitability for pharmacological screening is concerned [35]. To this, on the basis of the methods established previously, Zhang et al. [36], in 2008, demonstrated that an HFD combined with multiple low doses of STZ (30 mg/kg of body weight at weekly intervals for 2 weeks) proved to be a better way to develop a stable animal model of Type 2 diabetes.

In 2005, Danda et al. [20] reported that rats with non-insulinopenic (Type 2) diabetes developed lesions of DN significantly more prominently than those seen in classic insulinopenic (Type 1) diabetic rats. Kidney lesions in this Type 2 model appear to be more pronounced than in Type 1 diabetic rats, despite lower blood glucose levels and proteinuria. The authors presented this as a non-genetic rat model of Type 2 diabetes and nephropathy. Their study showed that the body weight of the animals fed on the HFD and treated with a low dose of STZ decreased compared with control rats at 14 weeks. In our present study, we maintained the animals until 24 weeks, and we also observed a decrease in body weight in these animals compared with control animals. Hence our results are in accordance with the published model. Recently, we have also performed hepatic gene expression profiling of these Type 2 diabetic rats and have shown the involvement of PKCε (protein kinase Cε), DGKη (diacylglycerol kinase η), Tnfα (tumour necrosis factor-α-induced protein) and Rho-kinase with DN [37].

In the present study, we used a diet and chemically induced Type 2 diabetic model. These models of diabetes are common in elucidating the possible role of environmental factors involved in the endocrine pancreatic destructive processes and subsequent development of diabetes. However, these models have certain limitations such as mostly requiring long periods of dietary treatment, as no frank hyperglycaemia develops upon simple dietary treatment in genetically normal animals [38]. Hence there is a need to conduct similar studies in genetic models of Type 2 DN such as in db/db animals. Recently, we have reported that progressive glomerulosclerosis in Type 2 diabetes (genetic model, db/db animals) is associated with renal histone H3 Lys9 and Lys23 acetylation, histone H3 Lys4 dimethylation and phosphorylation at Ser10, and treatment with an Mcp-1/Ccl2 (monocyte chemoattractant protein-1/CC chemokine ligand 2) antagonist prevented these histone H3 modification abnormalities associated with DN [39]. Furthermore, we also reported that renal failure in these animals increased cardiac histone H3 acetylation, dimethylation and phosphorylation and the induction of cardiomyopathy-related genes in the heart [40].

In summary, the present study is the first report to demonstrate the involvement of covalent modifications of histone H3 under insulin-resistant and Type 2 diabetic conditions in the heart and kidneys. In addition, hyperglycaemia regulates profibrotic gene expression by changing histone H3 acetylation in cardiac

![Figure 6 Proposed signalling events that contribute to a switch between adaptive and maladaptive hypertrophy](image-url)
and renal fibrosis. Results from this non-genetic model will have profound implications because it replicates the human metabolic syndrome and can be utilized for testing anti-diabetic agents in Type 2 diabetes. Further studies need to be carried out with phospho-acetylation of histone H3 and other histone modifications to better understand the alteration of gene expression in the pathogenesis of cardio–renal syndrome.

AUTHOR CONTRIBUTION
Anil Gaikwad designed and performed the experiments, analysed the data and wrote the manuscript. Jeena Gupta designed and performed the experiments. Kubhushan Tikoo designed the study, analysed the data and wrote the manuscript.

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SUPPLEMENTARY ONLINE DATA

Epigenetic changes and alteration of Fbn1 and Col3A1 gene expression under hyperglycaemic and hyperinsulinaemic conditions

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Figure S1  Dimethylation (on Lys4) of histone H3 in the heart and kidneys under hyperinsulinaemic and hyperglycaemic conditions

Histone H3 dimethylation (on Lys4) was determined by Western blot analysis in total heart and kidneys from the NPD, NPD + STZ, HFD and HFD + STZ groups. The blots shown are representative of three different blots from histone isolates from the different groups. Lane a, NPD; lane b, NPD + STZ; lane c, HFD; and lane d, HFD + STZ. For quantification, each band of each isolate was normalized to the respective total histone H3 content (lower blot). The values are means ± S.E.M. from three separate blots. *P <0.05 compared with NPD-fed rats. Similar results were obtained in three independent sets of experiments.

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