Effect of vitamin D₃ in reducing metabolic and oxidative stress in the liver of streptozotocin-induced diabetic rats

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Abstract

Diabetes mellitus is a growing health problem worldwide and is associated with severe liver complications. The aim of the present study is to analyse the status of metabolic and free-radical-scavenging enzymes and second messengers in the liver of streptozotocin (STZ)-induced diabetic rats, and to determine the hepatoprotective role of vitamin D₃. All studies were performed using the liver of adult male Wistar rats. Gene expression studies were carried out using real-time PCR with specific probes. Second messenger levels were determined using ³H-labelled Biotrak assay kits, and glucose uptake assay with D-[¹⁴C]glucose. The present results show that there was a decrease in hepatic glucose uptake, malate dehydrogenase activity, glycogen content, inositol triphosphate (IP₃) and cyclic GMP levels, and superoxide dismutase, glutathione peroxidase, phospholipase C, cyclic AMP-responsive element-binding protein, vitamin D receptor (VDR) and insulin receptor (INSR) gene expression in the diabetic rats when compared with the controls (all $P<0.05$), while cyclic AMP levels and GLUT2 expression were increased ($P<0.05$). Treatment of the diabetic rats with vitamin D₃ and insulin reversed the altered parameters to near control values. In conclusion, the data suggest a novel role of vitamin D₃ in restoring impaired liver metabolism in STZ-induced diabetic rats by regulating glucose uptake, storage and metabolism. We demonstrated that the restoring effect of vitamin D₃ is mediated through VDR modulation, thereby improving signal transduction and controlling free radicals in the liver of diabetic rats. These data suggest a potential role for vitamin D₃ in the treatment of diabetes-associated hepatic complications.

Key words: Vitamin D: Diabetes: Liver: Oxidative stress: Metabolism: Insulin

Diabetes-associated complications are major causes of morbidity and mortality. The liver plays a unique role in glucose homeostasis. Because of its anatomical location, it is ideally suited to control the systemic supply of absorbed nutrients, and it is one of only two organs that both consume and produce substantial amounts of glucose. The central role of the liver in the maintenance of blood glucose levels is assured by complex regulation by metabolic substrates, insulin and other hormones[1]. Diabetes-associated hyperglycaemia and hypoinsulinaemia lead to the impairment of hepatic glucose and lipid metabolism. Virtually, the entire spectrum of liver diseases is seen in patients with diabetes, including liver cirrhosis, a significant cause of death in diabetic patients, even more relevant than CVD[2]. Interest in the development of novel antidiabetic agents has been fuelled by the intense complications due to therapeutic treatment of diabetes and associated liver failure[2,3]. An increased prevalence of diabetes has been observed in vitamin D-deficient individuals[4], and our previous study has shown that vitamin D₃ treatment restores blood glucose homeostasis in streptozotocin (STZ)-induced diabetic rats[5]. Binding of vitamin D₃ promotes the vitamin D receptor (VDR) to form a heterodimer with the retinoid X receptor and transactivates vitamin D-responsive elements present in target genes[6]. VDR mRNA and protein have previously been detected and studied in several rat tissues including liver[7,8].

Diabetes-associated liver complications are widely studied using STZ-induced diabetic rats[9]. Net glucose uptake and its metabolism in hepatocytes depend on intracellular metabolic status, which is determined by metabolic enzymes, second messengers and transcription factors. Evidence for the role of free radicals and oxidative stress in diabetes-associated complications has been established. Implication of oxidative stress in the pathogenesis of diabetes mellitus is

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suggested not only by oxygen free radical generation but also due to non-enzymatic protein glycosylation, auto-oxidation of glucose, impairment of antioxidant enzymes and the formation of peroxides[10]. Free radical-mediated lipid peroxidation (LPO) is a key factor in the development of diabetic liver complications[11].

Although vitamin D₃ is considered as a potential antidiabetic agent, no studies have reported the effect of vitamin D₃ on hepatic LPO, free-radical-scavenging systems, insulin sensitivity, glucose uptake and metabolism at the molecular level. Therefore, the present study was undertaken to investigate the effect of vitamin D₃ in the liver of STZ-induced diabetic rats. The therapeutic possibilities of vitamin D₃ for diabetes-associated physiological, biochemical and pathological alterations in the liver were assessed in the present study.

Materials and methods

Biochemicals used in the present study were purchased from Sigma Chemical Company. All other reagents of analytical grade were purchased locally. Real-time PCR Taqman probe assays were from Applied Biosystems. D-[^{14}C]Glucose and RIA kits for insulin were purchased from the Bhabha Atomic Research Centre. [^{3}H] inositol triphosphate (IP₃), [^{3}H] cyclic GMP (cGMP) and [^{3}H] cyclic AMP (cAMP) Biotrak Assay Systems were purchased from GE Healthcare UK Limited.

Adult male Wistar rats of 180–240 g body weight were used for all experiments. They were housed in separate cages under 12 h light–12 h dark periods. Rats had free access to standard food and water *ad libitum*. All animal care and procedures were carried out in accordance with the Institutional, National Institute of Health and CPCSEA guidelines. All efforts were made to minimise the number of animals used and their suffering. Diabetes was induced in rats by a single intrafemoral injection of STZ, which was freshly dissolved in 0·1 M-citrate buffer (pH 4·5) under anaesthesia[12]. STZ was given at a dose of 55 mg/kg body weight[13]. Control rats were injected with citrate buffer alone. Diabetes in rats was confirmed on the third day by checking the fasting blood glucose concentration. The animals were divided into the following four groups: (1) control, (2) diabetic, (3) insulin-treated diabetic, (4) vitamin D₃-treated diabetic. Each group consisted of six to eight animals. The insulin-treated diabetic group received subcutaneous injections (1 U (45·5 μg)/kg body weight) of Lente and Plain insulin daily during the entire period of the experiment. The last injection was given 24 h before killing the rats. The vitamin D₃-treated group received 12 μg cholecalciferol/kg dissolved in 0·3 ml of coconut oil by oral administration. Maximum care was given in order to avoid stress during the oral supplementation of vitamin D₃. Rats underwent 14 d of treatment, so that they were habituated to vitamin D₃ supplementation[14,15]. The last dose of vitamin D₃ and insulin was given 24 h before killing to minimise stress-induced endocrine changes due to oral administration. Rats were killed on day 15 by decapitation. The liver was quickly dissected out and the tissues collected were stored at −80°C until the performance of the assay.

Estimation of blood glucose concentration

Blood samples were collected from the tail vein before STZ injection on days 1 and 15 of the treatment. Fasting blood glucose was estimated by a spectrophotometric method using glucose oxidase–peroxidase reactions. The results were expressed in terms of mg/l blood.

Estimation of blood insulin concentration

Blood samples were collected on day 15 of the treatment. The assay was performed according to the procedure of the Bhabha Atomic Research Centre RIA kit. The RIA method was based on the competition of unlabelled insulin in the standard or samples and [^{125}I]insulin for limited binding sites on a specific antibody. At the end of incubation, antibody-bound and free insulin were separated by a second antibody–polyethylene glycol-aided separation method. Measurement of radioactivity associated with the bound fraction of samples and standards allowed the quantification of the insulin concentration of samples.

Determination of hepatic glycogen content and lipid peroxidation

Liver glycogen content was determined according to the method of Hargreaves *et al.*[16]. This method is based on acid digestion of the liver sample, followed by neutralisation of the solution and subsequent glucose determination. LPO products were measured as thiobarbituric acid-reactive substances and were used to determine the index of LPO[17,18].

Measurement of glucose uptake

Glucose uptake activity was measured using D-[^{14}C]glucose by the modified procedure of Crane & Mandelstam[19]. Liver cells were isolated by perfusion with collagenase according to the modified procedure of Seglen[20]. The isolated cells were resuspended in William's media E and cell viability was determined. For the *in vitro* study, the liver cells were isolated from normal rats and 10^{6} cells were added to four different culture plates. Together with William's media E, each group of plates contained (1) 4 mM-glucose (control group), (2) 20 mM-glucose (diabetic group), (3) 20 mM-glucose with insulin (insulin-treated diabetic group) and (4) 20 mM-glucose with vitamin D₃ (vitamin D₃-treated diabetic group). For the *in vivo* experiments, the liver cells were isolated from the control, diabetic, insulin-treated diabetic and vitamin D₃-treated diabetic rats on day 15 of the treatment. The liver cells of the experimental rats were transferred to 3 ml of standard William's media E alone. The isolated liver cells for both *in vitro* and *in vivo* experiments were then incubated for 1 h at 37°C with the addition of 20 000 dpm D-[^{14}C]glucose/tube. The gas phase in the flask during the incubation period contained 5 % CO_2. After incubation, cells were removed from the culture plates and washed in the incubation medium to remove the unbound D-[^{14}C]glucose. The cells were hydrolysed in 1 M-NaOH (85°C, 1 h) and ^{14}C radioactivity was counted with cocktail

in a Wallac 1409 liquid scintillation counter. D-[^{14}C]glucose uptake was expressed as nmol/mg protein.

Second messenger assay

The livers of the experimental rats were homogenised in a polytron homogeniser with twenty volumes of cold 50 mM-Tris–HCl buffer (pH 7·4) containing 1 mM-EDTA. The homogenate was then centrifuged at 30 000 g for 30 min and the supernatant was transferred to fresh tubes for IP$_3$, cAMP and cGMP assays using [^{3}H]IP$_3$, [^{3}H]cAMP and [^{3}H]cGMP Biotrak assay system kits.

Malate dehydrogenase assay

Liver mitochondria were isolated by the modified method of Jonshon & Lardy[21]. Subcellular fractions of the liver were separated by differential centrifugation[22]. Mitochondrial and cytosolic fractions were separated and purified in several stages. Total protein concentration was estimated by the method of Lowry *et al.*[23] using bovine serum albumin as the standard. Malate dehydrogenase (MDH) was assayed according to the method of Mehler *et al.*[24] in the cytoplasmic and mitochondrial fractions, and kinetic parameters were measured. The reaction mixture contained phosphate buffer (pH 7·4), NADH, oxaloacetate and tissue extract. The reaction mixture of 1 ml was assayed at 340 nm in a spectrophotometer by measuring the decrease in optical density due to the oxidation of NADH measured at 15 s intervals for 1 min at room temperature. A unit of enzyme activity is equal to the change in the optical density of 0·1 for 100 s at 334 nm. Kinetic parameters such as V_{max} and K_m were calculated from the data of MDH activity measured at substrate concentrations of 0·0125–0·2 mM.

Analysis of gene expression by real-time PCR

RNA was isolated from the liver of the experimental rats using the Tri-reagent (Molecular Research Centre) according to the procedure of Chomczynski & Sacchi[25]. Total complementary DNA synthesis was performed using the ABI PRISM complementary DNA archive kit in 0·2 ml microfuge tubes. The reaction mixture of 20 µl contained 0·2 µg total RNA, 10 × RT buffer, 25 × dNTP mixture, 10 × random primers, MultiScribe RT (50 U/µl) and RNase-free water. The complementary DNA synthesis reactions were carried out at 25°C for 10 min and 37°C for 2 h using an Eppendorf Personal Cycler. Real-time PCR assays were performed in ninety-six-well plates in an ABI 7300 Real-time-PCR instrument (Applied Biosystems). The specific primers of GPx (Rn00577994_g1), SOD (Rn01477289_m1), insulin receptor (INSR) (Rn00567070_m1), GLUT2 (Rn00563565_m1), PLC (Rn01647142_m1), CREB (Rn00578826_m1) and VDR (Rn00566976_m1) were purchased from Applied Biosystems. The TaqMan reaction mixture of 20 µl contained 25 ng of total RNA-derived complementary DNA, 200 nm each of the forward primer, reverse primer and TaqMan probe for assay on demand and endogenous control β-actin and 12·5 µl of Taqman 2X Universal PCR Master Mix (Applied Biosystems),

and the volume was made up with RNase-free water. The following thermal cycling profile was used (forty cycles): 50°C for 2 min, 95°C for 10 min, 95°C for 15 s and 60°C for 1 min. Fluorescence signals measured during amplification were considered positive if fluorescence intensity was 20-fold greater than the standard deviation of baseline fluorescence. The $^{\Delta\Delta}$CT method of relative quantification was used to determine the fold change in expression. This was done by normalising the resulting threshold cycle (CT) values of the target mRNA to the CT values of the internal control β-actin in the same samples ($^\Delta$CT = CT target − CT β-actin). It was further normalised with the control ($^{\Delta\Delta}$CT = $^\Delta$CT − CT control). The fold change in expression was then obtained as ($2^{-\Delta\Delta}$CT) and the graph was plotted using $\log 2^{-\Delta\Delta}$CT.

Statistics

Data are presented as means with their standard errors. Statistical evaluations were done by ANOVA using In Stat (version 2.04a; GraphPad Software Inc.). The Student–Newman–Keuls *post hoc* test was used to compare data among the groups. Relative Quantification Software (Relative Quantification Software) was used for analysing real-time PCR results. In all cases, $P < 0.05$ was taken to indicate statistical significance.

Results

Fasting glucose and insulin concentrations

Fasting blood glucose concentrations of all rats before STZ administration were within the normal range. STZ administration led to a significant increase in blood glucose and a significant decrease in blood insulin level compared with the non-diabetic controls. In the vitamin D$_3$- and insulin-treated rats, blood glucose and insulin levels were significantly reversed towards control when compared with the diabetic group (Table 1). The body weight of the diabetic group showed a significant decrease and the percentage of liver weight showed a significant increase when compared with the controls. This was significantly reversed in the vitamin D$_3$- and insulin-treated rats (Table 1).

Malate dehydrogenase activity

Mitochondrial and cytoplasmic MDH activities showed a significant decrease in the liver of diabetic rats. During diabetes, the V_{max} values of MDH showed a significant decrease compared with the control in the mitochondria and cytoplasm. Compared with the diabetic group, the V_{max} of the cytoplasmic fraction showed a significant decrease in the insulin-treated group, but no significant decrease was observed in the vitamin D$_3$-treated group (Table 2). Both vitamin D$_3$ and insulin significantly reversed the V_{max} of the mitochondrial MDH fraction to near control values compared with the diabetic group (Table 2). K_m showed no significant change in the mitochondrial fraction but in the cytoplasmic fraction, it showed a significant increase in the diabetic group. The vitamin D$_3$ and insulin treatments significantly reversed K_m to near control values (Table 2).

Table 1. Blood glucose, blood insulin, body weight and percentage of liver weight in the experimental rats

(Mean values with their standard errors of six to eight separate experiments (n 8–10 animals per group))

Animal status	Blood glucose (mg/l), day 1 (initial)		Blood glucose (mg/l), day 15 (final)		Blood insulin (mg/l), day 15 (final)		Body weight (day 15) (g)		Percentage of liver weight	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Control	862	14	891	18	625·1	33·1	234·1	8·5	4·54	0·23
Diabetic	2629*	15	3149*	11	213·2*	20·3	159·6*	6·1	5·18*	0·14
Diabetic + insulin	2596*	12	1418*†	17	581·7†	21·2	194·8*†	3·4	4·47†	0·14
Diabetic + vitamin D₃	2564*	19	1917*†	20	557·1†	33·2	199·4*†	7·3	4·62†	0·21

* Mean values were significantly different when compared with the control group ($P < 0.05$).
† Mean values were significantly different when compared with the diabetic group ($P < 0.05$).

Real-time PCR analysis of superoxide dismutase, glutathione peroxidase, phospholipase C, cyclic AMP-responsive element-binding protein, vitamin D receptor, insulin receptor and GLUT2

Real-time PCR analysis showed a significant decrease in SOD, GPx, PLC, CREB, VDR and INSR gene expression in the liver of diabetic animals, which was reversed to near control values with the treatment of vitamin D₃ or insulin (Figs. 1 and 2). There was a significant increase in GLUT2 gene expression in the liver of the diabetic group; however, it was significantly reversed to near control values in the vitamin D₃- and insulin-treated diabetic rats (Fig. 2).

Second messenger content in the liver of the experimental rats

IP₃ and cGMP contents were significantly decreased in the liver of the diabetic rats, whereas the cAMP level was significantly increased when compared with the control. The vitamin D₃ and insulin treatments significantly reversed the IP₃, cGMP and cAMP contents near to control values (Table 3).

Lipid peroxidation in the liver of the experimental rats

There was a significant elevation in thiobarbituric acid-reactive substances in the liver of the diabetic rats when compared with the control. The treatment of vitamin D₃ and insulin significantly reversed thiobarbituric acid-reactive substances in the liver of the diabetic rats to near control values (Table 3).

Glycogen content in the liver of the experimental rats

There was a significant decrease in glycogen in the liver of the diabetic rats when compared with the control. The vitamin D₃ and insulin treatments significantly reversed the glycogen content in the liver of the diabetic rats to near control values (Table 3).

Glucose uptake activity in the liver of the experimental rats

In the in vivo and in vitro studies, diabetes led to a significant decrease in glucose uptake by hepatocytes when compared with the control. The vitamin D₃ and insulin treatments significantly increased glucose uptake to near control values (Table 3).

Discussion

The present study investigated the effects of vitamin D₃ treatment on diabetes-associated liver metabolic complications and the possible mechanisms involved. STZ-induced diabetic rats have proved to be useful in trying to determine the underlying cause of liver complications during diabetes[9]. Similar to previous reports, in the present study, STZ-induced diabetes was characterised by hypoinsulinaemia, hyperglycaemia and decreased body weight[5,12]. Treatment with vitamin D₃ significantly improved hypoinsulinaemia and hyperglycaemia and prevented body-weight loss, which indicated amelioration in diabetic complications. Moreover, there were neither

Table 2. Malate dehydrogenase (MDH) activity in the liver of the experimental rats

(Mean values with their standard errors of six to eight separate experiments (n 8–10 animals per group))

Animal status	Cytoplasmic MDH				Mitochondrial MDH			
	V_{max}		K_m		V_{max}		K_m	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Control	6·75	0·91	0·0189	0·001	14·71	1·56	0·0298	0·002
Diabetic	4·29*	0·72	0·0252*	0·002	11·17*	0·86	0·0278	0·003
Diabetic + insulin	2·67*†	0·34	0·0171†	0·001	14·69†	1·19	0·0302	0·002
Diabetic + vitamin D₃	4·15*	0·45	0·0214†	0·003	16·04†	1·25	0·0302	0·005

V_{max}, K_m, kinetic parameters.
* Mean values were significantly different when compared with the control group ($P < 0.05$).
† Mean values were significantly different when compared with the diabetic group ($P < 0.05$).
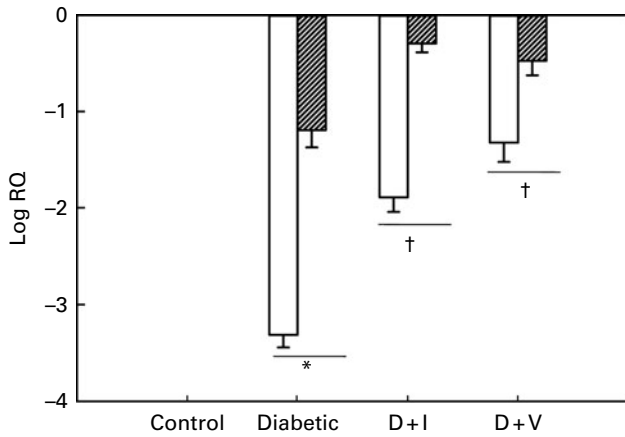
Fig. 1. Real-time amplification of glutathione peroxidase (GPx, □) and super-oxide dismutase (SOD, ▨) mRNA from the liver of the experimental rats. Values are means of six to eight separate experiments (n 8–10 animals per group), with standard errors of the mean represented by vertical bars. * Mean values were significantly different when compared with the control group ($P<0.05$). † Mean values were significantly different when compared with the diabetic group ($P<0.05$). RQ, relative quantification; D + I, insulin-treated diabetic rats; D + V, vitamin D$_3$-treated diabetic rats.

significant changes in food intake nor any sign of toxicity. Since STZ is known to destroy pancreatic β-cells, the possible mechanism of action of vitamin D$_3$ is mediated through influencing glucose uptake and utilisation, especially in the liver.

During diabetes, total body weight was significantly reduced, but the percentage of liver weight was significantly increased. This was most probably due to fat accumulation in the liver as a result of the increased release of NEFA from adipose tissue[26]. Increased accumulation of fat in the form of TAG and reduced protein and stored glycogen contribute to the development of fatty liver disease. Non-alcoholic fatty
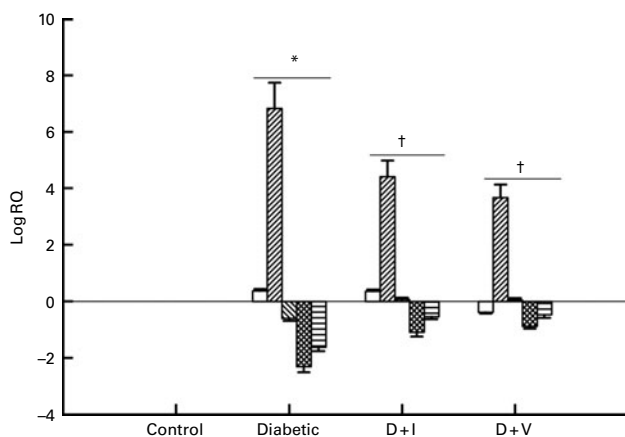

Fig. 2. Real-time amplification of insulin receptor (INSR, □), GLUT2 (▨), phospholipase C (PLC, ◣), cyclic AMP-responsive element-binding protein (CREB, ▩) and vitamin D receptor (VDR, ▤) mRNA from the liver of the experimental rats. Values are means of six to eight separate experiments (n 8–10 animals per group), with standard errors of the mean represented by vertical bars. * Mean values were significantly different when compared with the control group ($P<0.05$). † Mean values were significantly different when compared with the diabetic group. RQ, relative quantification; D + I, insulin-treated diabetic rats; D + V, vitamin D$_3$-treated diabetic rats.

liver disease is considered as the hepatic manifestation of the metabolic syndrome, and is present in approximately 80 % of type 2 diabetic patients[27]. The *in vitro* and *in vivo* results showed that there was a significant reduction of net glucose uptake into liver cells in diabetic rats. The significant increase in the uptake of glucose *in vitro* in the presence of vitamin D$_3$ indicates its direct action on hepatocytes. Hepatic glucose uptake was regulated by the glucose load to the liver, arterial–portal glucose gradient and insulin level[28]. The present study showed decreased INSR gene expression in the liver of STZ-induced diabetic rats, which subsequently would lead to the deterioration of INSR-mediated cell signalling[29]. This finding is not in accordance with previous results showing increased hepatic INSR levels in a similar diabetic rat model[30]. However, a reduced expression of INSR, a decreased intracellular pool of receptors and diminished insulin-stimulated protein kinase activity have been observed in the liver of diabetic human subjects[31–33]. The present findings agree with previous reports that treatment with vitamin D$_3$ causes transcriptional activation of the INSR gene[34]. Maestro *et al.*[34] observed that vitamin D$_3$ induced a 1·8-fold increase in both INSR gene expression and insulin receptor number, and enhanced cellular responsiveness to insulin in terms of glucose transport (1·3-fold increase) and glucose oxidation (1·6-fold increase) in human cells. Kong *et al.*[35] and Zhang *et al.*[36] suggested that INSR up-regulators have clinical benefit in improving insulin sensitivity and lowering blood glucose. The vitamin D-responsive element is present in the INSR gene promoter and accounts for the transcriptional induction of the INSR gene by vitamin D$_3$. This vitamin D-responsive element is overlapped by a downstream activator protein 2 (AP-2) sequence. Together, these *cis*-elements form a locus that can respond to vitamin D$_3$ via the activation of the VDR. This locus could mediate crosstalk between vitamin D and insulin signalling pathways[34]. Impaired insulin action in the liver resulted in decreased glucose uptake, decreased oxidative and non-oxidative glucose metabolism[37]. These defects of hepatic glucose uptake and metabolism contribute to the development of diseases such as liver cirrhosis[38].

GLUT2 ensures large bidirectional fluxes of glucose in and out of the liver cell due to its low affinity and high capacity[39]. GLUT2 gene expression is regulated at the transcriptional level by its promoter, which is capable of a rapid, dose-dependent glucose response[40]. Although GLUT2 mRNA expression was significantly up-regulated in the liver of diabetic rats, it cannot facilitate a net glucose entry into hepatocytes because of diabetes-induced metabolic alterations. The reduced glucose uptake has profound effects on metabolic flux, adenine nucleotide potential ((ATP)/(ADP), (NAD(P)H/(NAD(P) +)), cellular electrical activity and cytosolic (Ca^{2+}), and cells even commit to programmed cell death[41].

MDH enzyme activity is a useful parameter to evaluate the metabolic status of both the mitochondria and the cytoplasm. Mitochondrial MDH is a tricarboxylic acid cycle marker enzyme. Cytoplasmic MDH plays a crucial role in the malate-aspartate shuttle, lipogenesis and gluconeogenesis. The reduced activity of MDH during diabetes results from

Table 3. Concentrations of malondialdehyde (MDA), glycogen, second messengers and D-[^{14}C]glucose uptake in the liver of the experimental rats

(Mean values with their standard errors of six to eight separate experiments (n 8–10 animals per group))

| Animal status | Concentration of MDA (nmol/mg) | | Liver glycogen (mg/g) | | D-[^{14}C]Glucose uptake (nmol/mg protein) | | | | Second messenger (pmol/mg protein) | | | | | |
| | | | | | In vitro | | In vivo | | IP$_3$ | | cAMP | | cGMP | |
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Control	47·56	4·53	36·27	3·51	15·08	1·91	47·62	2·91	52·95	3·56	8·41	0·69	7·62	0·41
Diabetic	67·81*	6·16	22·54*	1·41	8·09*	1·84	28·13*	3·84	24·17*	4·13	15·06*	0·93	4·31*	0·22
Diabetic + insulin	54·30†	4·61	40·85†	3·20	12·60†	1·30	40·98†	3·30	63·69†	2·45	10·85†	0·53	6·54†	0·35
Diabetic + vitamin D$_3$	51·77†	3·77	34·98†	2·38	14·69†	1·37	44·41†	4·37	52·01†	3·87	8·97†	0·62	7·51†	0·58

* Mean values were significantly different when compared with the control group ($P < 0.05$).
† Mean values were significantly different when compared with the diabetic group ($P < 0.05$).

reduced glucose uptake, decreased INSR-mediated cell signalling, free radical-induced inactivation and glycation of the enzyme[17]. Decreased MDH enzyme activity reflects depression of energy metabolism in the cytoplasm and mitochondria. The reversal of the decreased mitochondrial MDH activity to near control during the vitamin D$_3$ treatment is attributed to the reversal of altered glucose metabolism, increased hepatic antioxidant enzymes and decreased ROS levels. Due to the central role of the liver in glucose homeostasis, impairment in liver glucose transport and metabolism lead to the impairment of blood glucose and fat level. Treatment with vitamin D$_3$ significantly reversed the INRS and GLUT2 mRNA expression levels and glucose uptake to near control to conserve hepatocyte metabolism which would help to maintain blood glucose homeostasis.

Hepatic glycogen storage is promoted by hyperglycaemia and insulin[42]. In the present study, decreased hepatic glycogen levels during diabetes indicate a decreased ability of the liver to convert glucose to glycogen. Inability to synthesise hepatic glycogen and reduced glucose uptake cause postprandial hyperglycaemia[43] and post-absorptive or fasting hyperketonaemia and hypoglycaemia[44]. Acute therapy of diabetes with insulin causes excessive glycogen storage in the liver and hence can result in hepatomegaly[45]. The present results show that treatment with vitamin D$_3$ significantly increased stored glycogen level in the liver of rats. Hence, it facilitated the maintenance of normal blood glucose level in absorptive, post-absorptive and fasting periods. Diabetes-associated reduction in hepatic glucose uptake, metabolism and storage in spite of elevated blood glucose and GLUT expression were reversed by vitamin D$_3$ administration. The potential of vitamin D$_3$ in reversing metabolic alterations during diabetes to near control contributed to its hepatoprotective and anti-diabetic properties.

Liver VDR upon activation induces changes in gene expression via classical transactivation by nuclear receptors and intracellular signalling pathways[46]. The vitamin D hormone system is involved in the regulation of more than 800 genes[47]. Studies have shown that in the liver, vitamin D$_3$ confers benefits on cholesterol metabolism, bile acid metabolism and Ca^{2+} homeostasis[46]. The present study showed that VDR gene expression was modulated by diabetes, confirming a role in the development of diabetes-associated complications.

Therefore, the present results provide the first demonstration of an in vivo tissue-specific regulation of VDR gene expression levels by its ligand, vitamin D$_3$, in STZ-induced diabetic rats. Vitamin D$_3$-induced changes in gene expression through the VDR seemed to have a role in reversing impaired hepatic metabolism during diabetes to near control. These vitamin D$_3$-induced changes in VDR expression are suggested to contribute to its hepatoprotective and antidiabetic effects.

Several pathways are thought to be impaired in the pathogenesis of hyperglycaemia-induced liver complications. Our studies showed that the gene expression of CREB and cAMP levels was significantly increased in the liver of diabetic rats, which indicates the stimulated cAMP transduction pathway due to hyperglucagonaemia, a common hormonal perturbation during diabetes[48]. Increased cAMP-mediated cell signalling resulted in cholangiocyte proliferation and fluid secretion, which led to the development of polycystic liver diseases[49]. Insulin and vitamin D$_3$ treatments significantly reversed the changes in cAMP transduction pathway, which in turn contributed to the normalisation of liver metabolic alterations. Impaired Ca^{2+} signalling plays an important role in the long-term complications observed in the diabetic liver[50]. Decreased IP$_3$ and PLC levels in diabetic rats resulted in poor cell signalling due to the impaired Ca^{2+} release from intracellular stores. STZ-induced diabetic rats were associated with a decreased Ca-dependent regulator activity which played a major role in the distortion of cAMP metabolism[51]. Del Pino-Montes et al.[52] showed that vitamin D$_3$ treatment did not change serum Ca levels in STZ-induced diabetic rats. During diabetes, increased reactive oxygen species levels resulted in the down-regulation of guanylate cyclase expression and activity by tyrosine phosphorylation, leading to a reduction in apoptotic inhibition mediated by cGMP[53]. Treatment with vitamin D$_3$ reverses the cGMP content to near control and inhibited NADPH oxidase activity, mitochondrial permeability transition and cytochrome c release through the activation of the cGMP-dependent protein kinase pathway, and thus reduced TNF-α-induced apoptosis in hepatocytes[54]. The differential regulation of cAMP and cGMP observed during diabetes is through the action of phosphodiesterases. The phosphodiesterases allow cross-talk between the cGMP and cAMP signalling pathways because they cause the concentration of one cyclic nucleotide to influence the

degradation of the other[54]. Treatment with vitamin D_3 and insulin significantly reversed IP_3 and cGMP contents in the liver of diabetic rats to near control.

The present study showed that peroxidation of lipids was significantly increased in the liver of diabetic rats, which in turn resulted in elevated production of other free radicals that are harmful to the cell[55]. Hypoinsulinaemia during diabetes increased the oxidation of fatty acids and resulted in decreased membrane fluidity, changed the activity of membrane-bound enzymes and receptors, impaired Ca metabolism[56], elevated the level of TAG and LDL-cholesterol and reduced the level of HDL-cholesterol, which led to liver dysfunction[57]. Antioxidant enzymes, SOD and GPx, have an important role in maintaining physiological levels of free radicals by hastening the dismutation of free radicals and eliminating organic peroxides and H_2O_2[10]. The observed decrease in SOD activity in the liver of diabetic rats is suggested to result from the inactivation by free radicals or by glycosylation of the enzyme, which have been reported to occur during diabetes[58]. Reduced gene expression of GPx and SOD during diabetes suggests impairment of hepatic cell signalling, and is responsible for the inadequacy of antioxidant defence in combating free radical-mediated damage[10]. GPx has been regarded as a major determinant of hepatic antioxidant status, and it protects the tissue from highly reactive hydroxyl radicals by catalysing the reduction of H_2O_2. In the present study, lower levels of hepatic antioxidant enzymes and increased LPO in the liver of diabetic rats were observed when compared with the control. Vitamin D_3 and insulin treatments reduced hepatic oxidative stress by minimising the imbalance between the rate of reactive oxygen species generation and activity of antioxidant enzymes. An increase in GPx and SOD expression protects cellular proteins against inactivation by free radicals[59]. Therefore, treatment with vitamin D_3 could reduce free radical generation and increase the activity of antioxidant enzymes, and hence help to control free radical-mediated hepatic complications.

The major strengths of the present study include its prospective design and the range of parameters that have been evaluated regarding cellular metabolism, cell signalling and oxidative stress, thus promoting a better overview of the effects of diabetes, insulin and vitamin D_3. However, one limitation of the study is that the dosage of vitamin D_3 used is difficult to extrapolate to the human setting because humans have multiple sources of vitamin D.

In conclusion, the present study suggests that vitamin D_3 treatment ameliorates impaired liver metabolism during diabetes by regulating glucose uptake, storage and metabolism. Vitamin D_3 acts via VDR in the liver of diabetic rats, which in turn normalises signal transduction by improving the expression of IP_3, cAMP, cGMP, PLC and CREB. Vitamin D_3 treatment reduced diabetes-associated hepatic LPO and oxidative stress. Thus, the results provide evidence for antidiabetic and hepatoprotective properties of vitamin D_3. Considering that the treatment of diabetes is hampered by the well-known ability of insulin to induce profound and life-threatening hypoglycaemia, vitamin D_3 represents a therapeutic possibility for the better management of diabetes-mediated hepatic complications and other liver diseases. Our investigations add to the understanding of the mechanisms of the antidiabetic effects of vitamin D_3. The effect of vitamin D_3 pretreatment should be evaluated and further studies should focus on signal transduction to elucidate the detailed mechanisms of the antidiabetic effect of vitamin D_3.

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