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Research Article

Regulation of mitochondrial aconitase by phosphorylation in diabetic rat heart

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Abstract. Mitochondrial dysfunction and protein kinase C (PKC) activation are consistently found in diabetic cardiomyopathy but their relationship remains unclear. This study identified mitochondrial aconitase as a downstream target of PKC activation using immunoblotting and mass spectrometry, and then characterized phosphorylation-induced changes in its activity in hearts from type 1 diabetic rats. PKCβ_2 co-immunoprecipitated with phosphorylated aconitase from mitochondria isolated from diabetic hearts. Augmented phosphorylation of mitochondrial aconitase in diabetic hearts was found to be associated with an increase in its reverse activity (isocitrate to aconitate), while the rate of the forward activity was unchanged. Similar results were obtained on phosphorylation of mitochondrial aconitase by PKCβ_2 *in vitro*. These results demonstrate the regulation of mitochondrial aconitase activity by PKC-dependent phosphorylation. This may influence the activity of the tricarboxylic acid cycle, and contribute to impaired mitochondrial function and energy metabolism in diabetic hearts.

Keywords. Aconitase 2, TCA cycle, PKC, phosphorylation, diabetes

Introduction

The development of a specific form of cardiomyopathy, unrelated to coronary artery disease or hypertension, is one of the most common complications of diabetes in both human patients and in experimental animal models of chronic type 1 and type 2 diabetes. This is manifested by decreased contractility and impaired relaxation of the heart, which contribute to the eventual development of heart failure [1, 2]. Although the mechanisms underlying the condition remain unclear, a series of complex changes have been identified. These include abnormalities in cellular morphology, calcium homeostasis and contractile protein composition, as well as mitochondrial dysfunction. The latter is associated with increased production of reactive oxygen species (ROS), altered substrate metabolism and reduced efficiency of respiration and energy metabolism in the diabetic heart [3 – 5].

Alterations in signal transduction resulting from hyperglycemia have been considered an important factor in the pathogenesis of diabetic cardiomyopathy. One of the most well-characterized changes in signaling in the cardiovascular system in diabetes is activation of specific isoforms of the serine/threonine kinase, protein kinase C (PKC). There are at least 12

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isoforms of PKC that exert their effects in a tissue- and isoform-dependent manner [6]. Although hyperglycemia has been shown to increase the activation of PKCβ, PKCδ and PKCε [7–10], the PKCβ_2 isoform appears to be preferentially activated by high glucose levels and has been shown to be closely associated with diabetic complications [11, 12]. The selective PKCβ inhibitor, ruboxistaurin, has been found to be effective in reducing diabetic complications, including diabetic retinopathy, nephropathy, and neuropathy [7, 13–15], and was recently reported to improve cardiac function in diabetic rats [16]. In addition to diabetic cardiomyopathy, PKCβ_2 has been also implicated in ischemia-reperfusion injury [17]. Consistent with the above observations, transgenic overexpression of PKCβ_2 causes left ventricular hypertrophy, cardiomyocyte necrosis and decreased left ventricular performance, which were improved by treatment with ruboxistaurin [18]. However, the downstream targets phosphorylated by activated PKC and how they consequently contribute to diabetic cardiomyopathy remain unclear. Using an antibody that selectively recognizes a phosphoserine PKC substrate motif, we recently found reciprocal changes in PKC-mediated phosphorylation of two proteins in diabetic hearts. The protein with decreased phosphorylation was identified, by mass spectrometry (MS), as muscle creatine kinase (M-CK) [19]. The reduced PKC-mediated phosphorylation of Ser128 in M-CK causes a reduction in the forward reaction that transfers high-energy phosphate from ATP to generate phosphocreatine, and an increase in the reverse reaction that shuttles high-energy phosphate from phosphocreatine to ATP. In the present study, we characterized the target with increased phosphorylation as mitochondrial aconitase. We found that phosphorylation of mitochondrial aconitase alters its activity such that it may limit the tricarboxylic acid (TCA) in mitochondria and impact energy metabolism in diabetic hearts.

Material and methods

Type 1 diabetic rats. Wistar rats (200–250 g) were lightly anesthetized with isoflurane and given a single tail vein injection of 60 mg/kg streptozotocin (STZ) in 0.1 M citrate buffer (pH 4.5) or citrate buffer alone as control. STZ-treated rats with blood glucose levels over 13 mmol/L 1 week after injection were considered diabetic. All animals were housed under identical conditions and were given free access to food and water for 12–14 weeks. To obtain tissues for experiments, rats were deeply anesthetized with sodium pentobarbital, and hearts and other tissues were rapidly removed. Hearts from 15 control and diabetic rats were used immediately for isolation of mitochondria as described below. In addition, hearts and livers from another 15 control and 15 diabetic rats were rapidly frozen in liquid nitrogen and stored at –70°C before being processed for preparation of tissue homogenate (see below). The use of animals in this investigation conformed to the Canadian Council on Animal Care Guidelines on the Care and Use of Experimental Animals and was approved by the University of British Columbia Animal Care Committee.

Tissue homogenate. Frozen tissues were powdered and homogenized in buffer containing 20 mM Tris-HCl (pH 7.5), 50 mM β-mercaptoethanol, 5 mM EGTA, 2 mM EDTA, 10 mM NaF, 1 mM PMSF, 25 μg/ml leupeptin, 2 μg/ml aprotinin, 0.1 % NP40, 0.1 % SDS, 0.1 % deoxycholic acid and 1 % phosphatase inhibitor cocktail (Sigma). The tissue homogenates were spun at 700 *g* for 5 min to remove debris and unbroken cells, and the protein content of the supernatants was determined by the Bradford protein assay. The samples were frozen in small aliquots at –70°C for further experiments.

Mitochondria isolation. Rat heart mitochondria were prepared from freshly excised hearts by differential centrifugation according to the procedure described in [20]. Briefly, rats were deeply anesthetized with sodium pentobarbital and hearts were rapidly removed and cleaned in ice-cold PBS. The left ventricle was cut into small pieces and mixed with 1.5 volumes of mitochondrial buffer containing 10 mM Tris (pH 7.8), 0.2 mM EDTA, 0.25 M sucrose, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 0.3 mM PMSF and 1 % phosphatase inhibitor cocktail (Sigma). The tissue was ruptured by a polytron for 10 s and the resulting homogenate was spun at 1000 *g* for 10 min at 4°C. The supernatant was then centrifuged at 12 000 *g* for 15 min. The resulting pellet was washed twice by resuspension in 1 ml mitochondrial buffer and centrifuged again at 12 000 *g* for 15 min, followed by further purification by discontinuous gradient centrifugation using 30 % Percoll [21, 22]. The mitochondria were collected and washed with mitochondrial buffer. The final pellet was collected by centrifugation at 12 000 *g* for 15 min and re-suspended in 0.20 ml mitochondrial buffer. Protein concentration was determined by the Bradford protein assay and mitochondrial purity was assessed by Western blotting with the mitochondrial marker prohibitin, the plasma membrane marker pan cadherin, the 58 K Golgi marker and the cytosolic marker GAPDH. The mitochondrial samples were frozen at –70°C until use.

Western blotting analysis and immunoprecipitation. Equal amounts of total protein (60 μg) or mitochondrial protein (10 μg) from control and diabetic hearts, or phosphatase PP2A-treated and untreated protein (30 μg) were separated by SDS-PAGE on a 12% gel and transferred to PVDF membrane. The membranes were blocked for 1 h in a solution of 5% skim milk powder and then incubated overnight at 4°C with 1:1000 dilution (0.2 μg/ml) of anti-PKCβ_2 phospho T641 (Abcam, Cambridge, MA), anti-phospho-(Ser) PKC substrate (Cell Signaling Technology, MA), or anti-PKCα, β_2, δ or ε antibodies (Santa Cruz, Santa Cruz, CA). Membranes were washed three times and then incubated with 1:10 000 dilution (0.04 μg/ml) of secondary antibody conjugated to horseradish peroxidase for 1 h. Protein bands were identified by a standard ECL method (Amersham Inc, Québec, Canada). Anti-aconitase 2 mouse monoclonal antibody (Abnova) was incubated with 500 μg of mitochondria isolated from control or diabetic hearts, and then was immunoprecipitated with the Catch and Release® kit 2 (Upstate) according to the manufacturer's instructions. The resulting samples were separated by SDS-PAGE and subsequently detected by Western blotting with corresponding antibodies.

Mass spectrometry. The experiments were carried out as described in our recent study [19]. The candidate proteins were identified by Western blotting with anti-phospho-(Ser) PKC substrate antibody. The bands were excised from the gel and were subjected to in-gel digestion with the trypsin profile IGD kit (Sigma) according to the manufacturer's protocol. The resulting peptides were analyzed at the Proteomics Core Facility of the University of British Columbia with a AB MDS-SCIEX API QSTAR Pulsar i mass spectrometer (Sciex, Thornhill, ON, Canada). Briefly, peptides were separated by reverse-phase C18 column on an Ultimate HPLC system (LC Packings, Amsterdam, The Netherlands) interfaced with the mass spectrometer. For LC/MS experiments, proteolytic digests of protein were loaded onto a C18 column (LC Packings, 75 μm i.d.×150 mm PepMap) and eluted with a gradient of 5–40% generated from solvent A and B over the course of 90 min at a flow rate of 0.2 μl/min (solvent A: 0.1% formic acid and 2% acetonitrile in water; solvent B: 0.1% formic acid and 85% acetonitrile in water). The TOF mass analyzer was scanned over a mass-to-charge ratio (*m/z*) range of 360–2000 amu, with a step size of 0.1 amu and a scan time of 1 s. The ion source potential was set at 2.2 kV; the orifice energy was 50 V.

To determine the amino acid sequence, the mass spectrometer operated in an IDA (Information Determined Acquisition) tandem mass mode. A 1-s survey scan was first done from 360 to 1500 *m/z*. The three most intense doubly and triply charged ions were selected to undergo tandem mass fragmentation in 3-s scans from 70 to 2000 *m/z*. The collision energies were determined automatically by the instrument based on the *m/z* values and charged states of the selected peptides. The sequences obtained were subsequently searched against the Swiss-Prot database with Mascot software.

Enzymatic activity assays and metabolite determinations. The enzymatic activities of aconitase, isocitrate dehydrogenase and α-ketoglutarate dehydrogenase and levels of citrate, isocitrate and α-ketoglutarate were determined as described in [23]. Briefly, a 10-μl sample of protein extract from control and diabetic hearts was used for each enzyme assay. The activity of aconitase was determined by measuring aconitate absorbance at a wavelength of 240 nm in buffer containing 90 mM Tris (pH 7.4) and 20 mM citrate (forward reaction) or 20 mM DL-isocitrate (reverse reaction). Isocitrate dehydrogenase activity was determined by measuring NADH absorbance at a wavelength of 340 nm in buffer containing 25 mM Tris (pH 7.4), 18 mM $MgCl_2$, 20 mM citrate, 5 mM isocitrate, 5 mM ADP and 2 mM NAD. α-ketoglutarate dehydrogenase activity was determined by measuring NADH absorbance at a wavelength of 340 nm in buffer containing 25 mM Tris (pH 7.4), 5 mM $MgCl_2$, 40 μM rotenone, 2.5 mM α-ketoglutarate, 0.1 mM CoA, 0.2 mM thiamine pyrophosphate and 1 mM NAD. To determine levels of metabolites in the TCA cycle, frozen heart powder (100 mg) was extracted with 60% acetonitrile in cold water (vol/vol) and then centrifuged at 13 000 *g* for 20 min to collect the supernatant [24]. Acetonitrile was removed by N_2 gas and the sample was dried by lyophilization. The tissue extract obtained was resuspended in 100 μl 50 mM Tris (pH 7.4) and a 5-μl sample was then used for metabolite assay. Citrate levels were determined with citrate lyase and malate dehydrogenase by measuring NADH oxidation at 340 nm in 50 mM Tris (pH 7.4), 0.2 mM NADH and 40 μM $ZnCl_2$. The isocitrate concentration was determined with NADP-dependent isocitrate dehydrogenases by measuring NADPH formation at 340 nm in 50 mM Tris (pH 7.4), 0.2 mM $MnCl_2$ and 0.6 mM NADP. α-Ketoglutarate levels were determined with glutamate dehydrogenase by measuring NADH oxidation at 340 nm in 40 mM imidazole acetate, 25 mM ammonium acetate (pH 7.0), 0.1 mM ADP and 0.1 mM NADH.

PP2A dephosphorylation. Protein extract (100 μg) from diabetic hearts was incubated without and with 0.5 U PP2A (Upstate) in buffer containing 20 mM

MOPS, pH 7.4, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA and 1 mM DTT at room temperature for 60 min. The resulting samples were then used for aconitase activity assay and Western blotting with the anti-phospho-(Ser) PKC substrate antibody.

Phosphorylation of mitochondrial aconitase by PKCβ_2 *in vitro*. Partially purified mitochondrial aconitase (Sigma) was incubated at a molar ratio of 60:1 with active PKCβ_2 (Upstate) in a total volume of 25 µl reaction mixture containing 0.4 mM ATP at 37°C for 30 min. The reaction mixture contained 20 mM Tris pH 7.4, 0.03% Triton X-100, 0.5 mM $CaCl_2$, 2.5 mM glycerol 2-phosphate, 1 mM DTT and 2 mM $MgCl_2$. At the end of the incubation, SDS gel loading buffer (5 µl) was added and the samples were boiled for 5 min. Proteins were separated by SDS-PAGE and transferred to PVDF membrane. Phosphorylated aconitase was detected by Western blot with anti-phospho-(Ser) PKC substrate antibody (Cell Signaling).

Immunogold labeling and electron microscopy. A standard protocol for transmission electron microscopy was used as described in [25]. Briefly, a 2.5×2.5 mm^2 piece of left ventricular tissue was removed from hearts and was fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 4 h. The fixed heart tissues were dehydrated with graded ethanol, infiltrated with L-R white, and dried in a vacuum. Semi-vertical thin sections of embedded cells were cut on an Ultrome III. The thin sections were placed in the middle of a 200-mesh copper grid (Pelco International, Clovis, CA). The primary antibody, either rabbit anti-PKCβ_2 phospho T641 antibody (Abcam) or anti-phospho-(Ser) PKC substrate antibody (Cell Signaling), diluted 1:1000 (final concentration 0.2 µg/ml) with phosphate-buffered saline containing 5% bovine serum albumin (PBS/BSA), was incubated with the grid overnight at 4°C. Following the reaction, the grids were washed four times with PBS/BSA at room temperature and incubated with secondary antibody goat anti-rabbit IgG conjugated with 10-nm colloidal gold at 1:50 dilution (final concentration at 4×10^{11} gold particles/ml) with PBS/BSA for 1 h at room temperature. The grids were washed four times with PBS, then washed once with distilled water. The specimens were double stained with uranyl acetate and lead citrate, dried in air, and examined with a transmission electron microscope (model 100, Philips Electron Optics USA) at an accelerating voltage of 60 kV.

Statistics. All values are expressed as means ± SEM. Statistical analysis was performed using unpaired student's *t*-test for comparison of two groups, one-way ANOVA followed by the Newman-Keuls test when more than two groups were compared, or two-way ANOVA followed by the Bonferroni test when more than two factors were compared, using GraphPad Prism (GraphPad Software). For all results the level of significance was set at $p<0.05$.

Results

PKC phosphorylation targets in diabetic hearts. By 12–14 weeks after STZ injection, diabetic rats had blood glucose levels of 23.4 ± 0.4 mM (mean ± SEM, n=30) *versus* 5.9 ± 0.05 mM (n=30) in controls. Diabetic rats also showed other symptoms of diabetes, including reduced weight gain, polydipsia, polyuria and increased food intake. The typical characteristics of diabetic cardiomyopathy in these animals include reduced left ventricular developed pressure and rate of pressure development and decline (+dp/dt and -dp/dt), demonstrated in isolated working hearts, and significantly reduced percent fractional shortening, an index of systolic function, observed by echocardiography in our recent study [26].

Upon activation, PKC phosphorylates its protein targets containing a sequence motif of serine or threonine, with arginine or lysine at the –3, –2 and +2 positions and a hydrophobic amino acid at the +1 position [27, 28]. Using Western blotting with an antibody selectively recognizing a phospho-serine PKC substrate motif, we identified two bands with major changes in phosphorylation (Fig. 1A), an 80-kDa protein with increased phosphorylation and a 40-kDa protein with reduced phosphorylation, in hearts from diabetic rats. The 40-kDa protein was identified as M-CK, and further characterization was recently reported [19]. To identify the 80-kDa protein, the corresponding band was cut from the gel, subjected to in-gel digestion and the resulting peptides were analyzed by MS. The data generated was used to search the Swiss-Prot database with Mascot, and the 80-kDa protein was identified as mitochondrial aconitase (aconitase 2, Table 1), which consists of a [4Fe–4S] cluster and a single peptide with apparent molecular mass of 84 kDa [29]. Further analysis indicated that Ser471 (NTIVTS$_{471}$YNR) and Ser690 (AIITKS$_{690}$FAR) residues were phosphorylated, based on detection of an 80-Da shift of the molecular mass of the corresponding unphosphorylated peptides in the mass spectrum.

To investigate whether the increase in phosphorylated aconitase 2 in diabetic hearts was associated with increased total expression of the protein, Western blotting with anti-aconitase 2 antibody was used to

Table 1. Peptide Data Obtained by Mass Spectrometry

Peptide sequences
R.YDLLEK.N
R.IHETNLK.K
K.LTIQGLK.D
R.AIITKSFAR.I
K.QALAHGLK.C
K.VAGILTVK.G
R.DGYAQILR.D
K.EGWPLDIR.V
R.EHAALEPR.H
R.IHETNLKK.Q
R.EHAALEPR.H
K.NTIVTSYNR.N
R.LQLLEPFDK.W
K.DLEDLQILIK.V
R.VDVSPTSQR.L
R.LNRPLTLSEK.I
K.FNPETDFLTGK.D
K.RLNRPLTLSEK.I
R.ADIANLAEEFK.D
K.LEAPDADELPR.S
K.LTGTLSGWTSPK.D
R.ATIERDGYAQILR.D
K.DFAPGKPLNCIIK.H
K.SQFTITPGSEQIR.A
K.VAMSHFEPSEYIR.Y
R.LQLLEPFDKWDGK.D
K.CTTDHISAAGPWLK.F
K.NTIVTSYNRNFTGR.N
K.FKLEAPDADELPR.S
K.WDGKDLEDLQILIK.V
K.FNPETDFLTGKDGK.K
R.SDFDPGQDTYQHPPK.D
R.WVVIGDENYGEGSSR.E
K.DINQEVYNFLATAGAK.Y
K.IVYGHLDDPANQEIER.G
R.NAVTQEFGPVPDTAR.Y
K.QGLLPLTFADPSDYNK.I
K.LTGTLSGWTSPKDVILK.V
K.KQGLLPLTFADPSDYNK.I
R.VGLIGSCTNSSYEDMGR.S
R.AKDINQEVYNFLATAGAK.Y
R.GHLDNISNNLLIGAINIENGK.A
R.DVGGIVLANACGPCIGQWDR.K
K.VAVPSTIHCDHLIEAQLGGEK.D
R.SDFDPGQDTYQHPPKDSSGQR.V
K.HPNGTQETILLNHTFNETQIEWFR.A
R.NDANPETHAFVTSPEIVTALAIAGTLK.F

Access No. Q9ER34, aconitase 2, coverage 79.30 %

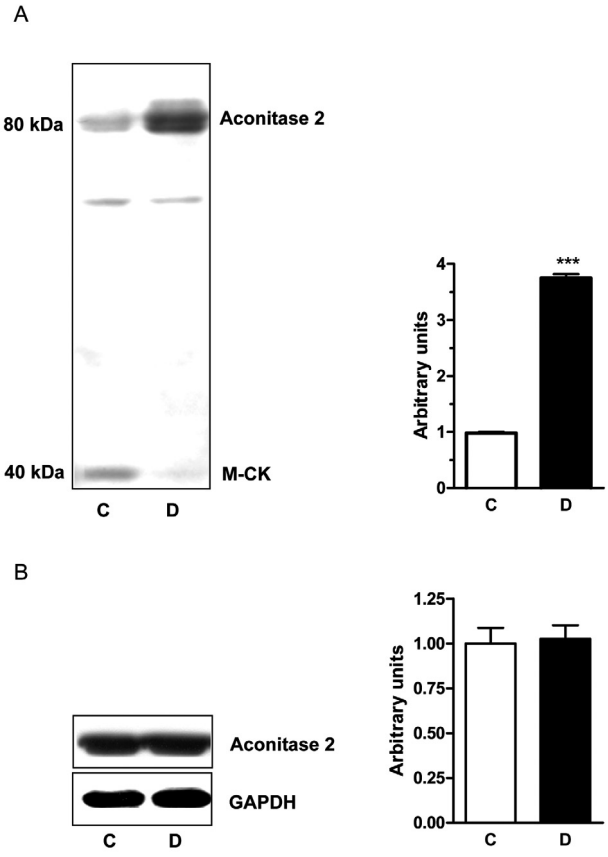

Figure 1. Protein kinase C (PKC) substrate phosphorylation in diabetic hearts. (*A*, left) Representative Western blot showing increased phosphorylation of aconitase and reduced phosphorylation of muscle creatine kinase (M-CK) protein in total cellular protein from a diabetic (*D*) heart compared to a control (*C*) heart. (*A*, right) Quantification of increased phosphorylation of the 80-kDa protein in diabetic hearts relative to control. ***$p<0.001$ compared to control by Student's unpaired t-test, $n=5$ in each group. (*B*, left) Representative Western blot showing similar levels of total aconitase in control and diabetic hearts. (*B*, right) Quantification of total aconitase in diabetic hearts, normalized to GAPDH and expressed relative to levels in control hearts, $n=5$ in each group.

determine the total enzyme levels. The results showed that levels of total aconitase 2 were similar in control and diabetic hearts (Fig. 1B).

Aconitase activities and effect of dephosphorylation. Aconitase reversibly catalyzes the stereo-specific isomerization of citrate to isocitrate *via cis*-aconitate in the TCA or Krebs cycle in mitochondria. To determine whether phosphorylation of mitochondrial aconitase alters its enzymatic activity, the forward activity (citrate → *cis*-aconitate) and the reverse activity (isocitrate → *cis*-aconitate) were determined in total cellular protein extracts from control and diabetic hearts since almost all aconitase activity in the heart (98 %) is due to the mitochondrial form of the enzyme [30]. The results showed that the reverse activity (isocitrate → aconitate) in diabetic heart was double that in the control, while the forward activity (citrate → aconitate) was similar in control and diabetic hearts (Fig. 2A). The reverse aconitase activity was also measured in livers from diabetic rats to determine whether similar changes in activity were seen in other organs. However, aconitase activity in the reverse direction was similar in livers from control (0.08±0.02 μmol/mg protein/min) and diabetic (0.08±0.03 μmol/mg protein/min) rats.

If altered phosphorylation of mitochondrial aconitase accounts for its changed activity in diabetic hearts, we expected that dephosphorylation of the enzyme would normalize the higher reverse activity. To test this

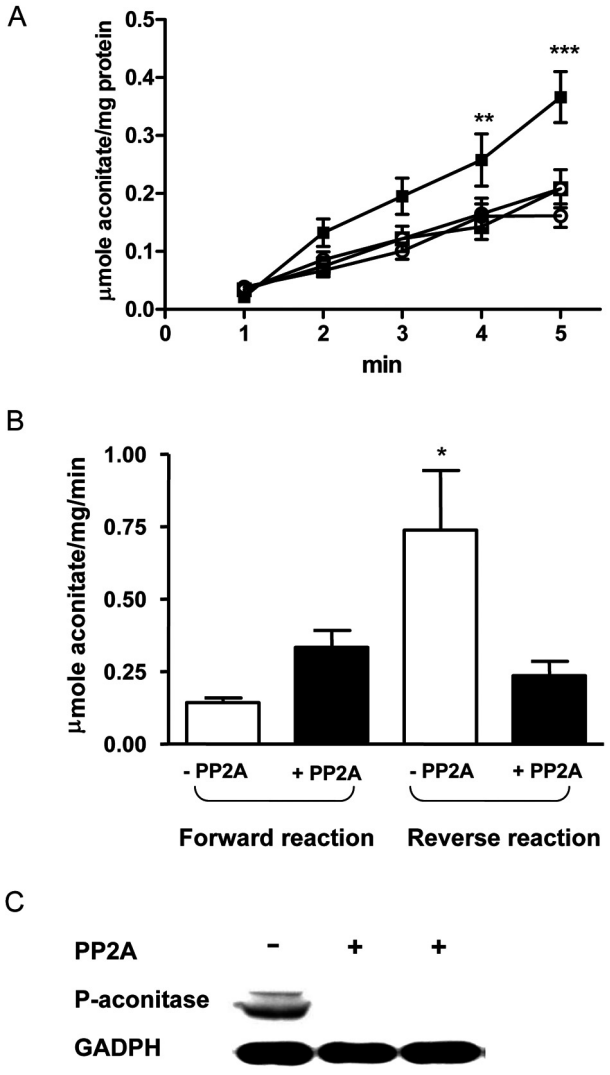

Figure 2. Alteration of aconitase activity in hearts from diabetic rats. (*A*) Aconitase activity was measured in the forward (circles) and reverse (squares) directions in control (open symbols) and diabetic (closed symbols) hearts. $^{**}p<0.01$, $^{***}p<0.001$ compared to the control, two-way ANOVA, followed by the Bonferroni test. $n=5$ in each group. (*B*) Effect of dephosphorylation *in vitro* on aconitase activity in diabetic hearts. The forward and reverse aconitate activities were measured after incubation of total protein from diabetic hearts with or without phosphatase PP2A for 60 min, $^{*}p<0.05$ compared to group treated with PP2A, one-way ANOVA, followed by the Newman-Keuls test. $n=5$ in each group. (*C*) Representative Western blot with the phospho-PKC substrate antibody confirming dephosphorylation of aconitase after treatment with the phosphatases and GAPDH showing equal loading in the Western blot.

hypothesis, we determined aconitase activity in both directions after incubation of the protein extract from diabetic hearts with phosphatase PP2A. The results showed that the reverse activity was significantly reduced, while the rate of the forward reaction was not significantly altered (Fig. 2B). Dephosphorylation of aconitase was confirmed by Western blotting with the phospho-PKC substrate antibody in the same samples treated with PP2A (Fig. 2B).

PKC levels in mitochondria. The major PKC isoforms expressed in normal rat heart are PKCα, PKCδ and PKCε; $PKC\beta_2$ has also been detected. All major PKC isoforms are also detected in mitochondria isolated by Percoll purification from normal hearts [31]. We and others have reported that levels of $PKC\beta_2$ and PKCε are increased in total cellular protein from diabetic hearts [11, 12, 19]. However, whether this is associated with changes in the levels of these isoforms in mitochondria from diabetic hearts is not known. We therefore determined the levels of each isoform in mitochondria isolated by Percoll gradient from control and diabetic hearts. We were able to detect all four isoforms in mitochondria from both control and diabetic hearts. Levels of $PKC\beta_2$ were increased, PKCε levels were reduced and levels of PKCα and PKCδ were unchanged in mitochondria from diabetic hearts compared to control (Fig. 3A). Western blotting of mitochondrial preparations from control and diabetic with different cellular markers revealed a high degree of mitochondrial enrichment as indicated by the mitochondrial marker prohibitin, and the absence of contamination with sarcolemma, Golgi apparatus or cytosol (Fig. 3B).

The phosphorylation of Thr641 in $PKC\beta_2$ is required for its correct subcellular localization and catalytic activity, and is often used as indication of $PKC\beta_2$ activation. We next used immunogold electron microscopy to detect Thr641-phosphorylated $PKC\beta_2$ as well as PKC-phosphorylated proteins in mitochondria from control and diabetic hearts. In hearts from control rats, mitochondria had intact ultrastructure and few gold particles were detected in cytosol or mitochondria (Fig. 4A, B). On the other hand, diabetic hearts showed deterioration of the myocardial mitochondrial integrity, and both Thr641-phosphorylated $PKC\beta_2$ and PKC-phosphorylated proteins were detected by gold labeling mainly in mitochondria (Fig. 4C, D).

Lastly, we found that both phosphorylated aconitase and Thr641-phosphorylated $PKC\beta_2$ could be co-immunoprecipitated with anti-aconitase antibody from mitochondria isolated from diabetic but not from control hearts (Fig. 5A). This suggests that aconitase 2 and $PKC\beta_2$ are physically associated in the mitochondria from diabetic hearts.

Phosphorylation of aconitase 2 *in vitro* by $PKC\beta_2$. We next investigated whether $PKC\beta_2$ could directly phosphorylate aconitase 2 *in vitro*, and whether this was associated with changes in the activity of the enzyme. Partially purified aconitase 2 was used as a

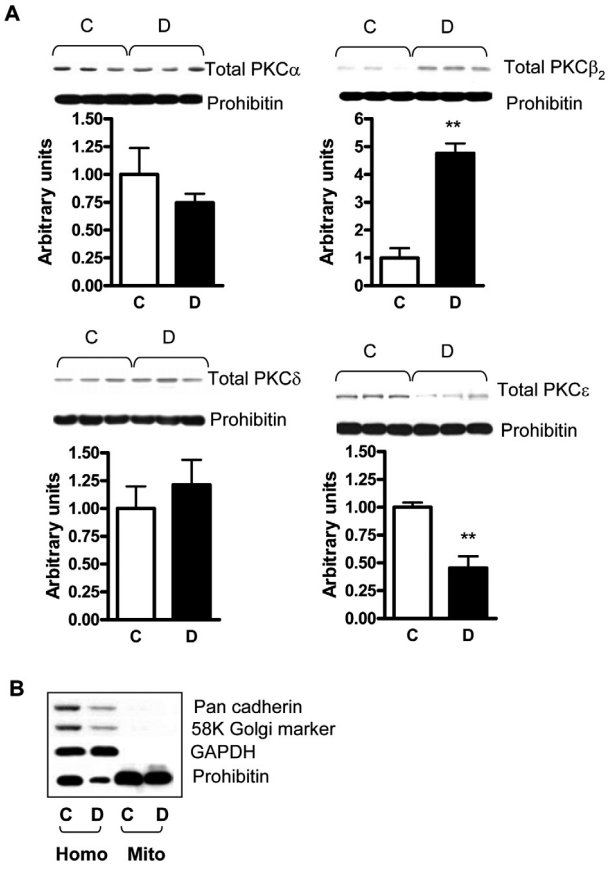

Figure 3. Levels of PKC isoforms in mitochondria. (*A*) Representative Western blots showing each PKC isoform, with the mitochondrial marker prohibitin as a loading control, in mitochondria from control (*C*) and diabetic (*D*) hearts. The quantification of each PKC isoform normalized to prohibitin and expressed relative to levels in mitochondria from control hearts is shown immediately below the Western blot. $^{**}p<0.01$, Student's unpaired *t*-test. (*B*) Representative Western blot showing various cellular markers in total homogenate (Homo) and isolated mitochondria (Mito) prepared from control (*C*) and diabetic (*D*) hearts.

substrate for the *in vitro* phosphorylation assay at a molar ratio of kinase to substrate of 1:60. $PKC\beta_2$ was very robust in phosphorylating aconitase 2 (Fig. 5B). This had no effect on the forward aconitase activity, but produced a marked and significant increase in the activity in the reverse direction (Fig. 5C). These results were very similar to the changes in aconitase activity found in diabetic hearts (Fig. 2A).

Activities of the rate-limiting enzymes and levels of metabolites in the TCA cycle. Since aconitase catalyzes the first step in the TCA cycle, changes in its activity due to increased phosphorylation may affect metabolism of the pathway. We measured the activities of isocitrate dehydrogenase and α-ketoglutarate dehydrogenase, the classic rate-limiting enzymes of the TCA cycle in both control and diabetic hearts. No significant change in the activity of either enzyme was detected in diabetic hearts compared to control (Fig. 6). We then determined the concentrations of the metabolites citrate, isocitrate and α-ketoglutarate in both control and diabetic hearts. The results showed that the concentration of citrate was similar in control and diabetic hearts (Fig. 7, top). However, the concentration of isocitrate, the product of the aconitase forward reaction, was significantly lower in diabetic than in control hearts (Fig. 7, middle), such that the ratio of citrate to isocitrate increased from 5 in control to 29 in diabetic hearts. The concentration of α-ketoglutarate, the product of isocitrate dehydrogenase, was also significantly lower in diabetic than in control hearts (Fig. 7, bottom). Taken together, these results suggest that the increased reverse activity of aconitase 2 reduced the concentration of downstream metabolites in the TCA cycle.

Phosphorylation of ATP citrate lyase. Our next objective was to investigate why the citrate concentration did not increase in diabetic hearts, as would be expected from the increased reverse activity of aconitase. One possibility is that there is also increased activity of ATP citrate lyase in diabetic hearts. This enzyme catalyzes the cleavage of citrate to produce acetyl-CoA for fatty acid synthesis in the presence of ATP and Coenzyme A. Western blotting was used to determine levels of phosphorylated ATP citrate lyase, since the activity of the enzyme is increased by phosphorylation. The phosphorylated form of the enzyme was significantly greater in diabetic than in control hearts (Fig. 8), suggesting that its increased activity may explain why levels of citrate in diabetic hearts did not increase despite the increased reverse activity of aconitase.

Discussion

The current study demonstrates that the activity of mitochondrial aconitase is subject to regulation by PKC-dependent phosphorylation in diabetic hearts. Furthermore, the results suggest that the phosphorylation of aconitase may impair the function of the TCA cycle. This could contribute to mitochondrial dysfunction and disturbances in energy metabolism in the diabetic heart.

The TCA cycle is a vital metabolic pathway in the late stage of converting carbohydrates, fats and proteins into CO_2 and H_2O to generate ATP in all aerobic organisms. Citrate synthase, isocitrate dehydrogenase and α-ketoglutarate dehydrogenase are classically considered as the rate-limiting steps in the TCA cycle, under negative feedback regulation mainly by their products, abundant ATP and a high ratio of

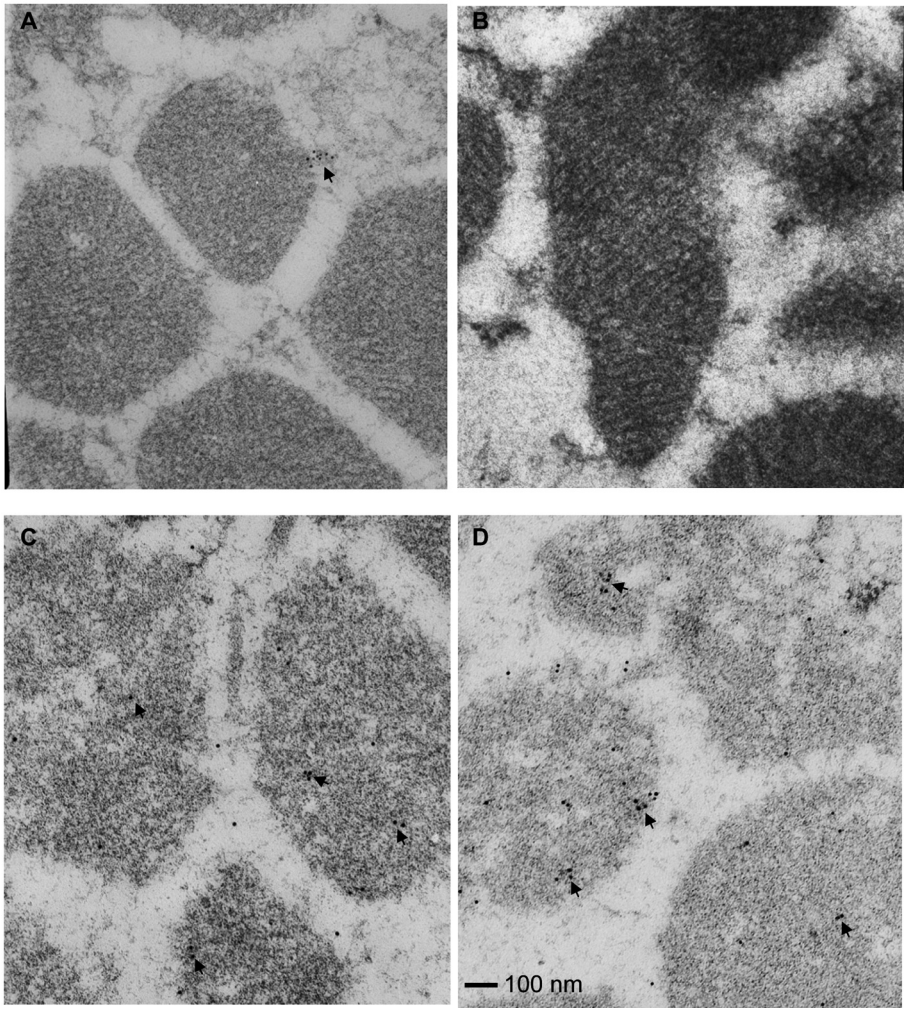

Figure 4. Detection of phosphorylated PKCβ_2 and PKC substrate in mitochondria by immunogold electron microscopy. Representative images of labeling of hearts from control and diabetic rats with anti-phosphorylated Thr641 PKCβ_2 antibody (*A*, control; *C*, diabetic) and anti-phospho-Ser PKC substrate antibody (*B*, control; *D*, diabetic). Original magnification ×60 000.

NADH/NAD through an allosteric mechanism [32, 33]. In *E. coli*, the activity of the rate-limiting enzyme isocitrate dehydrogenase is reversibly controlled by the phosphorylation and dephosphorylation of Ser113 through isocitrate dehydrogenase kinase and phosphatase, respectively [34, 35]. Phosphorylation of isocitrate dehydrogenase prevents isocitrate from binding to its active sites, resulting in inhibition of the activity of the enzyme. However, to the best of our knowledge protein phosphorylation has not previously been shown to regulate the activities of the major enzymes in the TCA cycle in mammalian cells.

Previous studies have shown that aconitase 2 is phosphorylated, although neither the phosphorylation site or sites, nor the effects of phosphorylation on aconitase activity were investigated [26, 36, 37]. The results of the MS analysis in the present study indicating that Ser471 and Ser690 are phosphorylated in aconitase 2 are consistent with the predicted PKC phosphorylation sites based on the algorithm developed by Blom et al. [29]. Using the 3-D model for aconitase 2 (Swiss-Model Repository, Q9ER34 and the Swiss-PdbViewer), we found that Ser690 is located adjacent to the active center of the enzyme and is also exposed to the surface, which makes it accessible to PKCβ_2 upon its activation (Fig. 9). Once phosphorylated, the negative charge on the serine side chain may affect binding and/or orientation within the active center, leading to the altered catalytic activity of the enzyme.

Activation of PKCβ_2 has been shown in isolated vascular cells cultured in high glucose, and in cardiovascular tissues from diabetic animals [11]. This is believed to result, at least partly, from increased *de novo* production of diacylglycerol as a result of hyperglycemia [11, 12]. PKCβ_2 has been shown to undergo translocation to the cell membrane as the final step of activation [38–40], but to our knowledge has not previously been demonstrated to be present in mitochondria. In the present study, we have used three different approaches to demonstrate that levels of PKCβ_2 are increased in mitochondria from diabetic

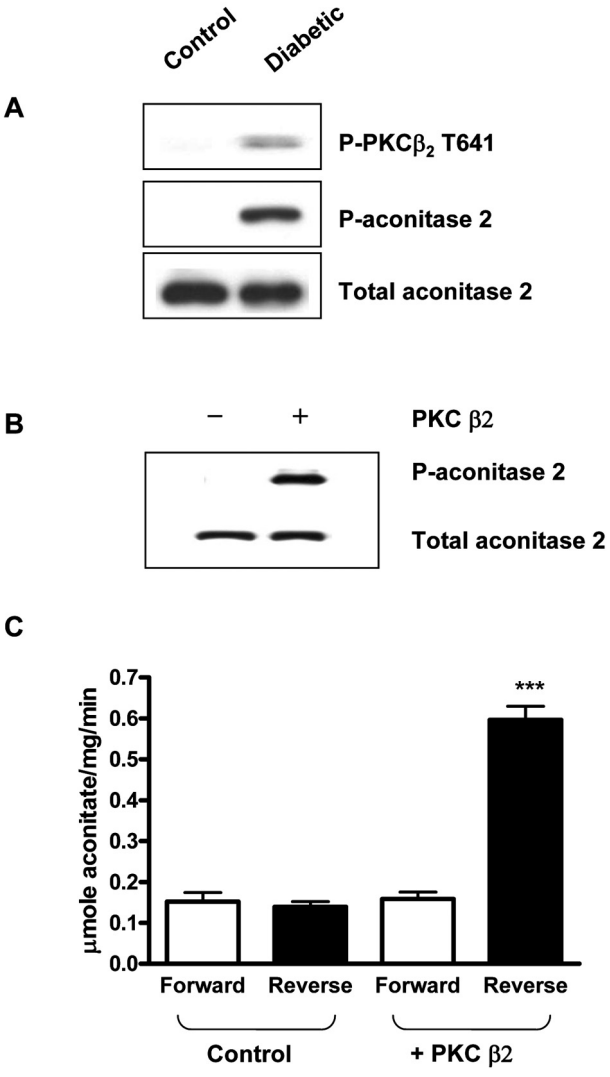

Figure 5. (*A*) Co-immunoprecipitation of phosphorylated aconitase and Thr641-phosphorylated PKCβ$_2$ from mitochondria isolated from diabetic but not control hearts. Re-probe of the same membrane with rabbit anti-aconitase 2 antibody showed precipitation of equal amounts of total aconitase 2 from control and diabetic mitochondria. (*B*) *In vitro* phosphorylation of partially purified aconitase 2 by PKCβ$_2$. The molar ratio of PKCβ$_2$ to aconitase was 1:60 in the assay. Phosphorylated aconitase 2 was detected by Western blotting with PKC substrate antibody. The re-probe of the same membrane with rabbit anti-aconitase antibody showed equal amount of total aconitase 2 in each condition. (*C*) Parallel determination of aconitase activity following in vitro phosphorylation. ***p=0.001 by Student's unpaired t-test, n=4.

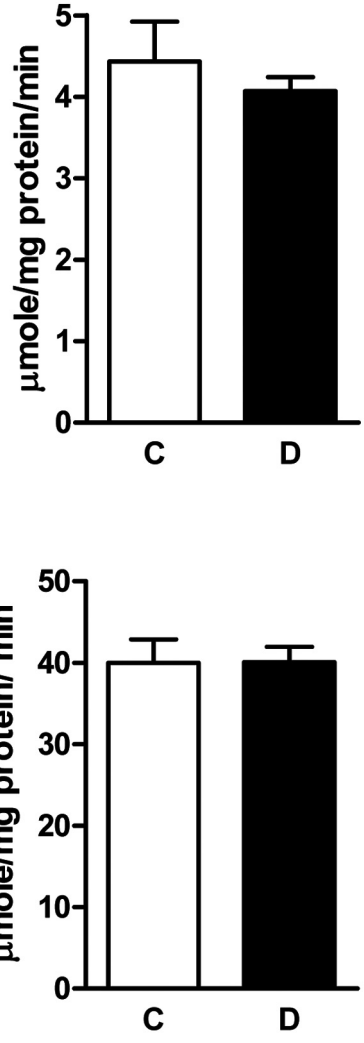

Figure 6. Comparison of isocitrate dehydrogenase (top) and α-ketoglutarate dehydrogenase activities (below) in control (*C*) and diabetic (*D*) hearts; n=5 in each group.

hearts: Western blotting with anti-PKCβ$_2$ antibody, immunogold electron microscopy with anti-T641 phosphorylated PKCβ$_2$ antibody and co-immunoprecipitation with anti-aconitase 2 antibody. The detection of phosphorylated PKCβ$_2$ in association with phosphorylated aconitase in mitochondria from diabetic but not control hearts suggests that the increased activation of this PKC isoform in diabetic hearts is associated with its translocation into mitochondria, where it phosphorylates the enzyme and alters its activity. That PKCβ$_2$ is the kinase responsible for the phosphorylation of aconitase 2 in diabetic hearts is also supported by the observation that PKCβ$_2$ phosphorylated aconitase 2 *in vitro* at a very low concentration (the molar ratio of the kinase to the target being 1:60).

Interestingly, despite our previous observation of elevated levels of PKCε in total protein from diabetic hearts [19], levels of PKCε in mitochondria were significantly lower than in the control. This is consistent with a previous study in diabetic mouse hearts, in which mitochondrial levels of PKCε were also significantly reduced [41]. In the latter study, overexpression of a PKCε translocation activator restored mitochondrial PKCε levels to control, suggesting that in diabetic hearts, the translocation of PKCε to mitochondria may be impaired. Translocation of

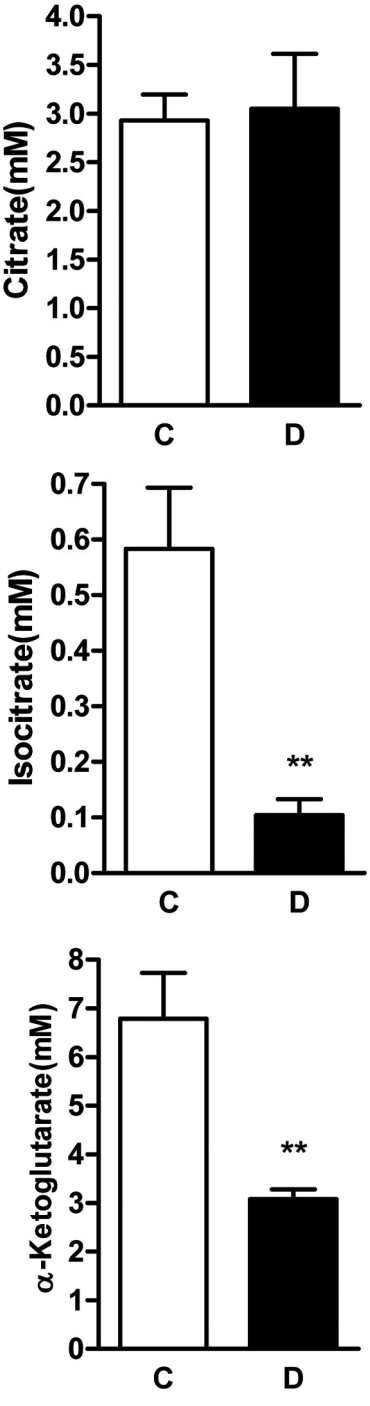

Figure 7. Concentrations of the metabolites citrate (top), isocitrate (middle) and α-ketoglutarate (bottom) in control (*C*) and diabetic (*D*) hearts. $^{**}p<0.01$ *versus* control, Student's unpaired *t*-test, $n=5$ in each group.

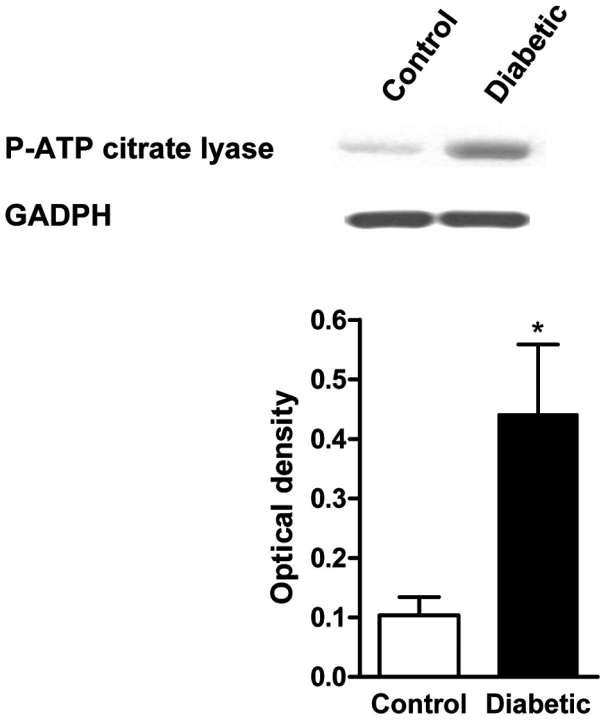

Figure 8. Levels of phosphorylated ATP citrate lyase in control and diabetic hearts. Representative Western blot showing levels of phosphorylated ATP citrate lyase in total cellular protein with GAPDH as a loading control (top). Quantification of phosphorylated ATP citrate lyase in diabetic hearts, normalized to GAPDH and expressed relative to levels in control hearts (below). $^{*}p<0.05$, Student's unpaired *t*-test, $n=5$.

both PKCδ and PKCε into cardiac mitochondria following ischemia-reperfusion and ischemic preconditioning, respectively, has also been reported [42–44]. In all circumstances, the mechanisms by which PKC isoforms translocate into mitochondria are still unknown and remain to be investigated.

Aconitase is a redox-sensitive enzyme that has been shown to be inactivated *in vitro* by ROS due to oxidation of the [4Fe–4S] center and release of the labile Fe [45, 46]. Indeed, loss of aconitase activity (measured in the forward direction, from citrate to aconitate) has been used as an indicator of oxidative stress in the heart and other tissues [47]. Increased oxidative stress due to overproduction of superoxide, H_2O_2 and/or peroxynitrite has been demonstrated in diabetic hearts [48], suggesting the possibility that the integrity of mitochondrial aconitase might be compromised in these hearts. However, our data demonstrating that the activity of aconitase in the forward direction was unchanged in diabetic compared to control hearts does not support this suggestion. Recently it was found that despite marked elevations in oxidative stress, mitochondrial aconitase activity was actually unchanged or increased rather than decreased in skeletal muscle subjected to exercise [49]. Although the mechanism underlying these results was not determined, it was suggested that either levels of reactive oxygen species were not high enough to inactivate aconitase, or that its modulation by phosphorylation acted to maintain its activation during periods of oxidative stress [49]. Studies in

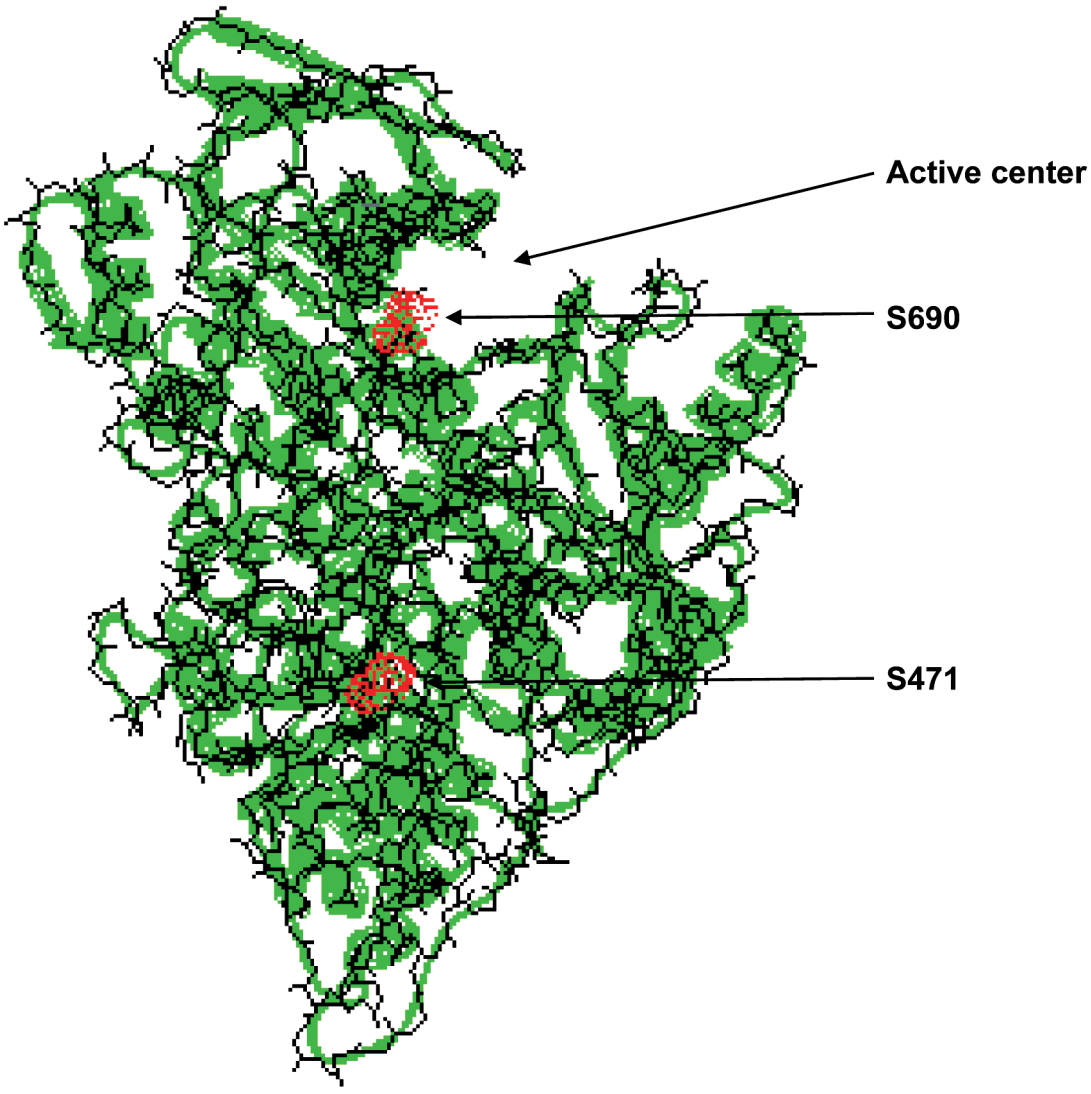

Figure 9. Model showing location of phosphorylated Ser690 adjacent to the active center of aconitase 2. Coordinates used were obtained from Swiss-model Repository, Q9ER34.

hearts subjected to ischemia-reperfusion *in vivo* suggest that the association of aconitase with the iron-binding protein frataxin is an additional mechanism for its protection from inactivation by oxidative stress [50]. Whether this is also the case in hearts from diabetic rats remains to be investigated.

Metabolism of carbohydrates, fats and proteins converges at the TCA cycle, which then directly generates ATP and provides NADH as well as $FADH_2$ as substrates for ATP production through the electron transport chain. A major mechanism for the regulation of cellular energy metabolism is achieved through the TCA cycle enzymes citrate synthase, isocitrate dehydrogenase and α-ketoglutarate dehydrogenase, which are classically considered as under negative allosteric inhibition by their products, abundant ATP and a high ratio of NADH/NAD in mammalian cells. Previous studies did not find differences in the activity of these rate-limiting enzymes in the TCA cycle in diabetic hearts [51, 52]. Our results are consistent with these earlier studies since the activities of isocitrate dehydrogenase and α-ketoglutarate dehydrogenase were similar in both control and diabetic hearts. However, we also found that the reverse activity of aconitase was increased in hearts from diabetic rats due to its increased phosphorylation. This was associated with significantly lower concentrations of isocitrate and α-ketoglutarate, while the ratio of citrate to isocitrate increased from 5 in control to 29 in diabetic heart. These data suggest that on its phosphorylation, aconitase 2 may reduce the overall activity of the TCA cycle in diabetic heart.

Previous studies have reported that the citrate concentration is increased in the heart in both acute and

chronic diabetes [51]. This is consistent with existence of inhibition of the TCA cycle in diabetic hearts, leading to the accumulation of upstream metabolites. Acute treatment with fluoroacetate (FA), a metabolically activated aconitase inhibitor, was also found to cause a 15-fold increase in heart citrate concentrations and a 46% depletion in heart ATP concentrations [53]. However, in the current study, we did not find an increased concentration of citrate in diabetic hearts. The explanation for this may be a phosphorylation-induced increase in ATP citrate lyase activity in cytosol [54, 55], resulting in increased conversion of citrate into oxaloacetate and acetyl-CoA for fatty acid synthesis when citrate is shuttled into cytosol from mitochondria, since our results demonstrate that levels of phosphorylated ATP citrate lyase activity are increased in diabetic hearts.

The heart is the greatest oxygen-consuming organ in the body, and up to 90% of oxygen available to the heart is used for oxidative phosphorylation to generate energy in mitochondria [56]. Oxidation of fatty acids contributes to 60–70% of ATP production in normal hearts [57–60], with the remainder coming from the oxidation of pyruvate from glycolysis and lactate. One of the characteristic metabolic alterations in diabetic hearts is that fatty acids become nearly the sole substrate for energy production [61], while the rate of glycolysis is significantly reduced both *in vitro* and *in vivo*. One reason for this is low expression or activity of glucose transporters due to lack of, or resistance to, insulin, leading to a reduction in glucose uptake and intracellular glucose levels [62, 63]. Another important mechanism is the inhibition of pyruvate dehydrogenase that catalyzes the decarboxylation of pyruvate to acetyl-CoA, the substrate entering the TCA cycle. Pyruvate dehydrogenase is phosphorylated into an inactive form by pyruvate dehydrogenase kinase that is activated by increased ratios of acetyl-CoA/CoA from fatty acid oxidation [64, 65]. Administration of the pyruvate dehydrogenase kinase inhibitor dichloroacetate (DCA) to diabetic hearts results in an increase in myocardial glucose oxidation and improved contractile function, suggesting that altered energy metabolism may contribute to impaired contractility in diabetic cardiomyopathy [66]. However, DCA was found to produce only a twofold stimulation of glucose oxidation in diabetic hearts compared to a fivefold increase in control hearts [67], suggesting that inhibition of downstream metabolic pathways, in particular the TCA cycle, may ultimately control the rate of glucose oxidation in diabetic hearts. Our finding that phosphorylation of aconitase limits the production of downstream metabolites in the TCA cycle in hearts from diabetic rats suggests a possible explanation for this observation.

In summary, the current study provides evidence for molecular mechanisms that may interconnect mitochondrial dysfunction and PKC activation in diabetic cardiomyopathy. The phosphorylation of aconitase, which increases its reverse activity, may reduce the overall activity of the TCA cycle, contributing to disturbances in energy metabolism in the diabetic hearts. Although these studies were conducted in hearts from type 1 STZ-diabetic rats, we have evidence that similar changes in phosphorylation of $PKC\beta_2$ and mitochondrial aconitase also occur in hearts from a type 2 diabetic rat model, the Goto-Kakizaki rat (data not shown). This suggests that phosphorylation of aconitase may be a common mechanism of regulation of aconitase activity in the heart in both type 1 and type 2 diabetes.

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