Adrenergic Control of Cardiac Fatty Acid Oxidation in Diabetes

Vijay Sharma and John H. McNeill

Abstract Diabetes produces a direct and continuous myocardial insult even in the absence of ischemic, hypertensive or valvular disease. β-blocking agents have been shown in large-scale randomized controlled trials to reduce heart failure mortality. In this chapter, we summarise the results of our studies investigating how β-adrenergic signalling controls cardiac metabolism, and the significance of these mechanisms in diabetes. Metoprolol inhibits fatty acid oxidation but does not prevent lipotoxicity; its beneficial effects are more likely to be due to anti-apoptotic effects of chronic treatment. The range of effects produced by β-adrenergic blockade are broad and illustrate how interconnected the signalling pathways of function and metabolism are in the heart. Our initial hypothesis that inhibition of fatty acid oxidation would be a key mechanism of action was disproved. However, unexpected results have led us to some new and hitherto unexpected regulatory mechanisms of cardiac metabolism. The first is USF-2-mediated repression of PGC-1α, most likely occurring as a consequence of improved function. The second is the identification of covalent modifications which directly regulate carnitine palmitoyltransferase-1 (CPT-1) at the level of the mitochondria. We also found that β-adrenergic signalling interacts with caveolins, which could be a key mechanism of action of β-adrenergic blockade. Our experience of studying this labyrinthine signalling web illustrates that it is not necessary for initial hypotheses to be correct, and all ends foreseen, in order for valid lines of inquiry to be opened and new information revealed.

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1 Introduction

Diabetes produces a direct and continuous myocardial insult even in the absence of ischemic, hypertensive or valvular disease. The resulting pathology is diabetic cardiomyopathy, which can act synergistically with hypertension or ischemia to damage heart muscle, but can also cause heart failure independently. The disease process decreases both the compliance of the heart wall (due to increased cross-linking of collagen, cardiac hypertrophy and fibrosis [1, 2]) and contractility. Diabetic cardiomyopathy has a long clinical course which evolves in three stages [3]:

1. In the initial phase, the combination of hyperglycemia and a shift in cardiac metabolism to almost 100 % fatty acid oxidation produces oxidative stress and cellular damage due to the cytoplasmic accumulation of toxic intermediates of glucose and fatty acid metabolism (glucotoxicity and lipotoxicity) [2]. Calcium handling is impaired and the fetal gene program is activated. Ultrastructural changes in tissue architecture occur in parallel, and the extent to which these are causes or consequences of metabolic and contractile remodelling is still not entirely clear. These changes are sufficient to induce a mild diastolic dysfunction, detectable only with the use of Doppler echocardiography.
2. Left ventricular hypertrophy develops, and apoptosis and necrosis of cardiomyocytes, with ensuing myocardial fibrosis, also occurs. This results in more severe diastolic dysfunction and mild systolic dysfunction, sufficient to activate the renin-angiotensin system. The situation is further exacerbated by the development of a mild autonomic neuropathy.
3. In the final stage, microvascular disease appears, and the autonomic neuropathy becomes severe. Hypertension and ischemic heart disease frequently occur as well [3]. The result is a combined systolic and diastolic dysfunction, severe enough to cause systemic sympathetic nervous system activation.

This pathogenesis appears to be driven by the combination of hyerglycemia and shifts in substrate utilisation by the heart. It is an example of maladaptive metabolic plasticity begetting maladaptive metabolic and contractile remodelling. The main pathways involved in cardiac metabolism are summarised in Fig. 1.

All forms of heart failure are associated with activation of the sympathetic nervous system. A failing resting heart is subjected to a sympathetic drive equivalent to the maximum drive a normal heart is subjected to during severe exercise; there is increased spillover of catecholamines (as much as 50-fold), leading to an extremely large increase in cardiac and systemic catecholamine levels [4–7]. The aim of this response is to maintain systemic perfusion, but it is maladaptive and correlates inversely with survival [8]. It is for this reason that the β-blocking agents bisoprolol,

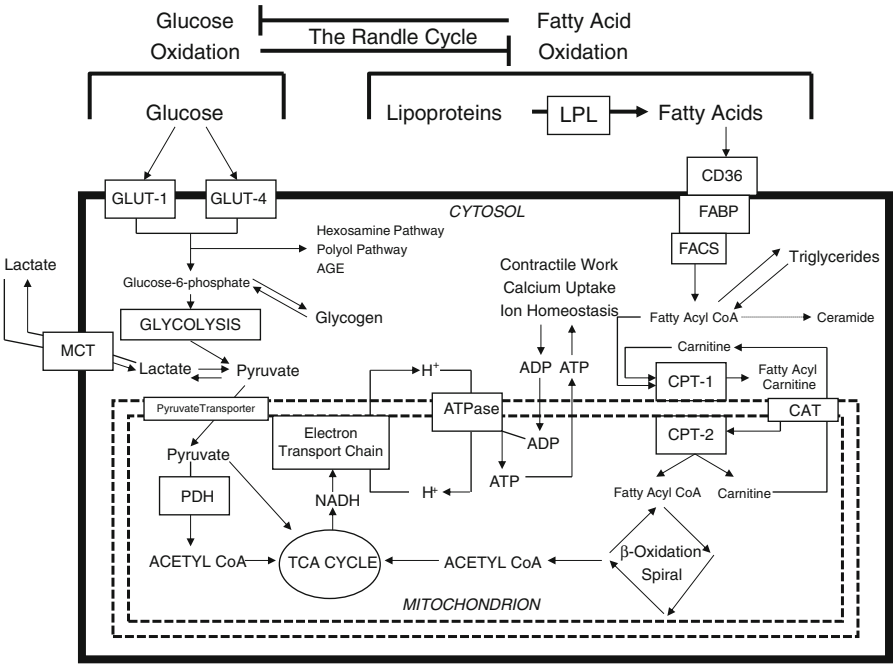

Fig. 1 Summary of fatty acid and glucose metabolism. Glucose is taken up by Glut-1 and Glut-4 transporters and is converted by glycolysis to pyruvate which enters the mitochondria to be oxidized, producing acetyl CoA. Fatty acids are liberated from lipoproteins by LPL and taken up by CD36 and FABP. LCAS converts the fatty acid to a CoA ester which is then taken up by the carnitine shuttle system to the mitochondria. The fatty acyl CoA undergoes β-oxidation, removing two carbons per turn of the cycle and generating acetyl CoA. Acetyl CoA, generated by either pathway, enters the TCA cycle to generate reducing equivalents (NADH). These pass electrons to the electron transport chain which creates an electrochemical proton gradient to drive ATP synthesis. ATP synthesis is coupled to the systems which create the ATP demand (*LPL* lipoprotein lipase, *CD36* fatty acid translocase, *FABP* fatty acid binding protein, *FACS* fatty acyl CoA synthase, *CPT* carnitine palmitoyltransferase, *CAT* carnitine acyl transferase, *CoA* coenyme A, *TCA* cycle tricarboxylic acid cycle, *AGE* advanced glycosylation endproduct, *PDH* pyruvate dehydrogenase, *MCT* monocarboxylate transporter, *PDH* pyruvate dehydrogenase, *NADH* reduced nicotinamide adenide dinucleotide, *ATP* adenosine triphosphate, *ADP* adenosine monophosphate. (Modified from Figure 1 of [117])

carvedilol and metoprolol reduce mortality, an effect which has been consistently seen in large-scale randomized controlled trials [9]. Acute administration of β-blocking drugs produces negative chronotropic and inotropic responses, and they were contraindicated in heart failure for many years for this reason. However, β-blockers were pioneered as heart failure treatments in the 1970s [10] and they are now among the agents of choice for the treatment of heart failure [9].

There are several putative mechanisms for the chronic effect of β-blockers, which are inter-related by a complex signalling web. These include antiarrhythmic effects, amelioration of cardiomyocyte hypertrophy, necrosis and apoptosis, reversal of the

fetal gene program (thereby improving calcium handling and force of contraction), increases in cardiac receptor density, anti-inflammatory effects and partial restoration of cardiac glucose oxidation [11, 12]. Metoprolol [13, 14], carvedilol [15] and bucindolol [16] induce a switch from fatty acid to glucose oxidation in non-diabetic patients with heart failure. A study in dogs with microembolism-induced heart failure revealed a potential mechanism for this effect: CPT-1 was inhibited by chronic treatment with metoprolol [17].

2 β-Adrenoceptor Signalling

The existence of two broad subtypes of adrenoceptors, α-adrenoceptors and β-adrenoceptors, was first demonstrated by Ahlquist in 1948 [18]. In the 1960s, two subtypes of β-adrenoceptors, β1 and β2, were characterized [19], and the third, β3, was characterized in 1989 [20]. In the heart, all three subtypes are expressed in the heart, but the major subtypes are β1 and β2, with the ratio of β1: β2 being approximately 60–70 %: 40–30 %, and very low β3 expression [21]. The absolute expression levels are in the femtomolar range (50–70 fmol/mg protein for the β1 adrenoceptor) so the receptor reserve is low [11]. The receptors show varying affinities for their ligands [22]:

1. β1 (adrenaline = 4 μM, noradrenaline = 4 μM, isoproterenol = 0.2 μM)
2. β2 (adrenaline = 0.7 μM, noradrenaline = 26 μM, isoproterenol = 0.5 μM)
3. β3 (adrenaline = 130 μM, noradrenaline = 4 μM, isoproterenol = 2 μM).

β-adrenoceptors are G-protein coupled receptors which, in the classical β-adrenoceptor pathway, act via Gs to produce an acute positive inotopic response mediated by increased cAMP levels and stimulation of protein kinase A (PKA). PKA subsequently phosphorylates and activates L-type calcium channels and ryanodine receptors, increasing calcium uptake and release, and phospholamban, relieving inhibition of SERCA and increasing sarcoplasmic reticulum calcium uptake [23–25]. In parallel, phosphorylation of troponin I and myosin binding protein B by PKA increases the calcium sensitivity of myofilaments [26, 27]. Phosphorylation of protein phosphatase inhibitor-1 by PKA prevents dephosphorylation of PKA target proteins and sustains its effects [28].

β-adrenoceptor signaling is temporally and spatially organized. β-adrenoceptors, and most particularly the β2-adrenoceptor, desensitize by uncoupling from their G-proteins. This dissociation is initiated by receptor phosphorylation and is mediated by β-arrestins acting together with G protein-coupled receptor kinases or PKA itself [29–31]. β-adrenoceptors can also change their coupling to downstream signaling pathways, usually in response to prolonged activation. At the β1 adrenoceptor, this causes a switch from PKA to calcium/calmodulin dependent protein kinase-II (CAMK II)—dependent signaling which is maladaptive, leading to CAMK-II mediated apoptosis and pathological hypertrophy [32]. In contrast, at the β2-adrenoceptor, it causes a switch in G-protein coupling from Gs to Gi, which is cardioprotective

due to activation both of phosphodiesterase-4, which ameliorates cAMP/PKA signaling, and of the pro-survival PI3 kinase/Akt pathway [33, 34]. A role for the extracellular-signal-regulated kinase (ERK) 1/2 in mediating β2-adrenoceptor-Gi cardioprotection has also been proposed [35]. Gi-mediated signaling therefore produces functional antagonism of Gs-mediated signaling. β1 adrenoceptor signaling is widely disseminated throughout the cell, whereas β2 adrenoceptor signaling is compartmentalized, partly due to the enrichment of β2 adrenoceptors in caveolae [36, 37]. The positive inotopic effect elicited by β2/Gs signaling is therefore comparatively small [34, 38]. It is possible that translocation of β2 adrenoceptors out of caveolae causes the switch from Gs to Gi association during prolonged activation [39]. Overall, it is clear that the coupling of β-adrenoceptors to their downstream signaling pathways is compartmentalized and time-dependent. Sustained activation of β1 adrenoceptors is harmful, whereas sustained activation of β2 adrenoceptors is cardioprotective, but the latter effect is constrained by the limited compartmentalization of the pathways.

Another consequence of PI3K/Akt activation is stimulation of nitric oxide (NO) production by nitric oxide synthase (NOS), which catalyses the generation of NO is from the terminal guanidine nitrogen atom of the amino acid L-arginine and molecular oxygen. Tetrahydrobiopterin (BH_4) is required as a cofactor; without BH_4, eNOS becomes 'uncoupled' and produces reactive oxygen species such as peroxynitrite. In adult cardiomyocytes, endothelial nitric oxide synthase (eNOS) is constitutively expressed and produces physiological NO signaling in the nanomolar range. Inducible nitric oxide synthase (iNOS) is expressed in response to inflammatory stimuli [40, 41] and produces higher levels of NO that mediate pathophysiological effects [42, 43]. In a well-characterised signaling pathway, soluble guanylyl cyclase is activated following nitrosylation, stimulating the production of cyclic 3′, 5′guanosine monophosphate (cGMP) from guanosine triphosphate [44]. Just as cAMP activates PKA, cGMP activates protein kinase G (PKG) isoforms. In the heart, the NO/cGMP pathway has a negative inotropic effect [40]. β2 adrenoceptor-Gi signaling and β3 adrenoceptor-Gi signaling both stimulate NO production [45, 46].

The effects of diabetes on cardiac β-adrenergic responsiveness have been studied for many years, but the picture remains confusing. Studies have variously a decrease in the cardiac relaxant effects without an effect on heart rate or contractility in rat hearts [47], a decrease in the chronotropic response in rabbit heart *in vivo* [48], an increased chronotropic response and a decreased inotropic response in atria [49] and decreased sensitivity to β-adrenergic stimulation in cardiac tissues [50, 51]. Similar controversy surrounds the effects of diabetes on β-receptor expression and downstream signalling, but an overall picture of a shift away from β1-adrenoceptor signalling seems to be emerging [51–55]. The chronotropic response to noradrenaline was blunted by 14 weeks but not 8 weeks of diabetes, with preservation of the response to fenoterol, a selective β2 agonist, suggesting that β1-mediated responses are selectively blunted [56]. Consistent with this observation, the expression of β1 adrenoceptors is markedly decreased and that of β2 adrenoceptors modestly decreased in the diabetic heart, whereas the expression of β3 adrenoceptors is increased twofold [51]. A similar increase in β3 adrenoceptor expression has also

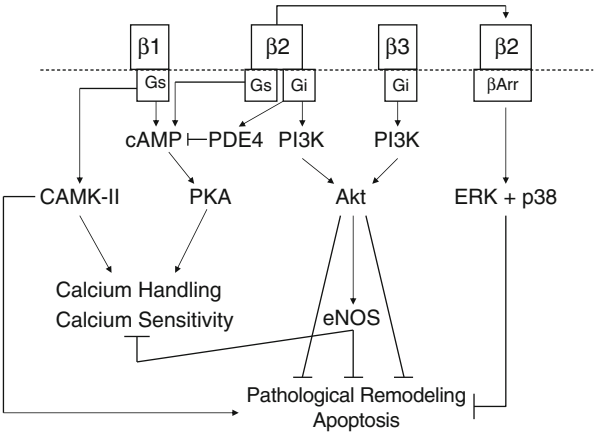

Fig. 2 β-adrenergic signaling pathways. β1-adrenergic receptors activate PKA, which regulates calcium sensitivity and calcium handling. Prolonged activation of this receptor activates a harmful CAMK-II pathway which is pro-apoptotic and induces pathological remodeling. β2-adrenergic receptors also activate PKA, but prolonged activation causes a switch to Gi signaling which activates PDE4, inhibiting cAMP formation, and activates the cardioprotective PI3K/Akt pathway. Desensitization of β2-adrenergic receptors by β-arrestin can recruit p38 and ERK, which protect the cell from apoptosis. β3-adrenergic receptors produce a negative inotopic effect which is mediated by NO produced via the PI3K/Akt pathway (Modified from Figure 2 of [117])

been found in failing human hearts [57]. The significance of this shift in receptor subtypes is unclear; an increase in NO-mediated signalling could produce a harmful negative inotropic effect, but, if β3 adrenoceptor-mediated activation of the PI3K/Akt pathway also prevents apoptosis, the shift could be cardioprotective (Fig. 2).

3 Effects of Metoprolol on Cardiac Function and Metabolism

We have shown that the β1-adrenoceptor-selective β-blocker metoprolol ameliorates the cardiac dysfunction produced by diabetic cardiomyopathy [58]. This improvement is evident both from Starling curves generated by direct left ventricular pressure measurements and from measurements of cardiac output and hydraulic power at constant preload and afterload. However, a robust improvement in function was not seen when we repeated the measurements *in vivo* using echocardiography; although metoprolol improved stroke volume and cardiac output, it also increased end-diastolic volume, so it was unclear whether the underlying cardiac dysfunction was being attenuated or worsened [59].

The effects of β1-adrenoceptor-blockade on *ex vivo* fatty acid and glucose metabolism in the heart are complex and depend on the disease state and the duration of the blockade [58]. Surprisingly, chronic metoprolol treatment increases palmitate

oxidation and decreases glucose oxidation in control hearts [58]. However, in diabetic hearts, chronic metoprolol treatment lowers fatty acid oxidation and increasing glucose oxidation. Short term perfusion with metoprolol inhibits fatty acid oxidation and produces marked stimulation of glucose oxidation in both control and diabetic hearts [58]. Before attempting to resolve this apparent paradox, it is important to note that the main target of metoprolol is in the fatty acid oxidation pathway, because it is preserved in the absence of insulin, whereas the effect on glucose oxidation is not [58]. Furthermore, metoprolol inhibits the activity of pyruvate dehydrogenase, so any stimulatory effect on glucose oxidation would have to be mediated by the Randle cycle. The β-blocker propanolol has been reported to induce an increase in CPT-1 activity in normal Sprague–Dawley rats [60], whereas metoprolol decreases CPT-1 activity in conscious dogs with micro-embolism-induced heart failure [17]. In dogs with pacing-induced heart failure, glucose uptake is improved by carvedilol but not metoprolol [61]. However, in clinical studies, metoprolol, carvedilol and bucindolol have all been shown to inhibit fatty acid oxidation [13, 15, 16].

When diastolic filling increases, cardiac work and oxygen consumption also increase in direct proportion via the Frank-Starling mechanism. However, in the normal heart, ATP supply is maintained regardless of cardiac work or oxygen consumption, indicating that cardiac metabolism is driven by cardiac function [62]. The mechanisms which underlie this exquisite coupling are manifold. However, how cardiac function influences cardiac energy substrate *selection* is less clear. Some of the effects of metoprolol on cardiac metabolism could conceivably be *attributable* to, rather than responsible for, its effects on cardiac function. Normalisation of palmitate and glucose oxidation rates to cardiac function, the does not eliminate the pattern of changes observed, suggesting that the shifts cannot be solely explained on the basis of cardiac function. However, to be definitive, it is important to establish whether the metabolic effects are preserved in isolated cardiomyocytes, in which the effects of cardiac function and the Frank-Starling mechanism do not apply.

4 CPT-1 Activity and Regulation by Malonyl CoA

β1-adrenoceptor-blockade has no effect on the activities of acyl-CoA dehydrogenase or citrate synthase, and does not decrease CD36 translocation [59, 63]. The major target of β1-adrenoceptor-signaling is CPT-1. In the heart, the major mechanism by which CPT-1 is regulated is through modulation of malonyl CoA levels. Isoproterenol has previously been shown to lower malonyl CoA levels by increasing PKA-mediated phosphorylation of acetyl CoA carboxylase (ACC) [64]. Furthermore, a study in isolated cardiomyocytes using activators and inhibitors of cAMP showed that stimulation of fatty acid oxidation by contraction was PKA-dependent [65]. We therefore expected that β-adrenergic blockade could have the opposite effect, preventing ACC phosphorylation and increasing malonyl CoA levels, but this turned out not to be the case. Chronic β1-adrenoceptor-blockade decreases malonyl CoA

levels in control hearts and has no effect in diabetic hearts. The mechanism of this effect is unclear, because there is no effect on ACC and malonyl CoA decarboxylase (MCD) expression, AMP-activated protein kinase (AMPK) or PKA-mediated phosphorylation of ACC. Dobutamine, a non-selective β-agonist, was previously found to decrease malonyl CoA levels without an effect on AMPK, ACC or MCD, so there must be other mechanisms by which malonyl CoA levels are regulated [66, 67]. Malonyl CoA levels are known to be dependent on the cytosolic supply of acetyl CoA, which is derived from peroxisomal β-oxidation, citrate and acetylcarnitine [67–69]. It is interesting to note that acute inhibition of CPT-1 has been shown to produce a fall in malonyl CoA levels independent of ACC and MCD [69], raising the possibility that the fall in malonyl CoA levels observed in control hearts could, therefore, have been *secondary* to the inhibition of CPT-1. Why such a mechanism would only lower malonyl CoA levels in control hearts is unclear. It is possible that fatty acid oxidation rates, and therefore the acetyl CoA/CoA ratio, are higher in the diabetic heart, and the fall in cytosolic acetyl CoA levels produced by CPT-1 inhibition in this context may not be sufficient to decrease malonyl CoA levels. Metoprolol tends to decrease tissue acetyl CoA, but measurements of the cytosolic and mitochondrial acetyl CoA pools are required to confirm these speculations. Overall, however, malonyl CoA levels do not correspond with the observed changes in fatty acid oxidation and cannot, therefore, be used to explain the effects of β1-adrenoceptor-blockade [58]. β1-adrenoceptor-blockade decreases the maximum capacity of CPT-1 activity as measured *in vitro* following both short-term and chronic exposure in both control and diabetic hearts. Surprisingly, metoprolol also decreases the sensitivity of CPT-1 to malonyl CoA. Incubation of metoprolol with CPT-1 *in vitro* has no effect on the maximum activity or malonyl CoA sensitivity of the enzyme.

Overall, the time and disease-dependent changes in fatty acid oxidation can be summarised as follows. In control hearts, acute metoprolol perfusion lowers malonyl CoA levels. The sensitivity of CPT-1 to malonyl CoA falls, and the activity of CPT-1 is markedly reduced. With chronic treatment, malonyl CoA levels remain low but the sensitivity of CPT-1 to malonyl CoA is restored and the inhibition of CPT-1 activity is less marked. Fatty acid oxidation is therefore inhibited following short-term β1-adrenoceptor-blockade, but this effect is lost with time. In diabetic hearts, short-term β1-adrenoceptor-blockade markedly lowers CPT-1 activity. This reduction is sustained with chronic blockade and produces inhibition of fatty acid oxidation despite a concomitant decrease in malonyl CoA sensitivity. The major determinants of the fatty acid oxidation rate are CPT-1 activity and malonyl CoA levels. In our studies, fatty acid oxidation was always inhibited if CPT-1 activity was inhibited by approximately 50 %, which is consistent with previous observations that CPT-1 only becomes rate-limiting when its activity is inhibited by approximately 50 % [70]. The decrease in malonyl CoA sensitivity would be expected to increase flux through CPT-1; however, this may represent a fine tuning mechanism since at no point do changes in malonyl CoA sensitivity hold sway over the overall fatty acid oxidation rate.

Both CPT-1A (the liver isoform) and CPT-1B (the muscle isoform) are present in the heart [71, 72]. We found that the IC_{50} of control hearts was approximately 30 μM

malonyl CoA, which is intermediate between the high sensitivity of CPT-1B and the low sensitivity of CPT-1A [58, 73]. The N- and C- termini of CPT-1 both face the cytosol and are separated by a loop region containing two membrane spanning domains inserted into the outer mitochondrial membrane. The catalytic region is within the C-terminus and residues which regulate malonyl CoA sensitivity have been found within the C-terminus, the N-terminus and the loop region [74–77]. In the liver, regulation of CPT-1A sensitivity is more important than regulation of malonyl CoA levels, and has been attributed to regulation by cytoskeletal elements [78], changes in the membrane environment [79] and direct phosphorylation of CPT-1 [80]. Peroxynitrite-mediated nitration of CPT-1B has been shown to decrease CPT-1B catalytic activity following endotoxemia in the heart [81]. Our own studies have revealed that both changes in expression and covalent modifications of CPT-1 occur within the heart, and provide an explanation for the short and long-term effects on CPT-1 activity and malonyl CoA sensitivity we observed. However, the relationship of these effects to β1-adrenergic signalling remains incompletely characterised.

5 Regulation of CPT-1 Expression

Chronic metoprolol treatment reduces total CPT-1 expression in the diabetic heart only, and this decrease is entirely attributable to a fall in CPT-1B expression. Intringuingly, there is a modest increase in CPT-1A expression which provides a partial explanation for the decrease in CPT-1 malonyl CoA sensitivity. CPT-1 expression is known to be controlled by PPAR-α, but the PPAR-α/RXR complex is only a modest inducer of CPT-1 when acting alone [82–84]. The induction of CPT-1 by PPAR-α is greatly enhanced by PGC1α, but PGC1α can also induce CPT-1 independently by binding to MEF-2A [85]. PGC1α-mediated expression of CPT-1 is repressed in isolated cardiomyocytes by upstream stimulatory factor (USF)-2. Upstream stimulatory factors are transcription factors of the basic helix-loop-helix leucine zipper family which bind to the E-box consensus sequence CANNTG and which, in the heart, respond to sustained increases in electrical stimulation by increasing the expression of sarcomeric genes such as sarcomeric mitochondrial creatine kinase and MHC [86–88]. PGC1α occupancy of the CPT-1 promoter is increased in diabetes. Chronic β1-adrenoceptor-blockade increases the binding of USF-2 to PGC1α, and this is associated with a decrease in the occupancy of the CPT-1 promoter by the PGC1α complex [63]. No such effect is observed in control hearts. We propose that USF-2 maintains a constant level of tonic repression of CPT-1 expression in the normal heart. In the diabetic heart, this tonic repression is clearly lost, because USF expression, and USF activity as indicated by MHC expression, are both decreased. Restoration of USF-2 repression in the diabetic heart produces marked changes in CPT-1 expression (Fig. 3), and we believe that this effect is most likely due to an increase in electrical stimulation produced by the improvement in function; in other words, it is a *consequence* of improved function.

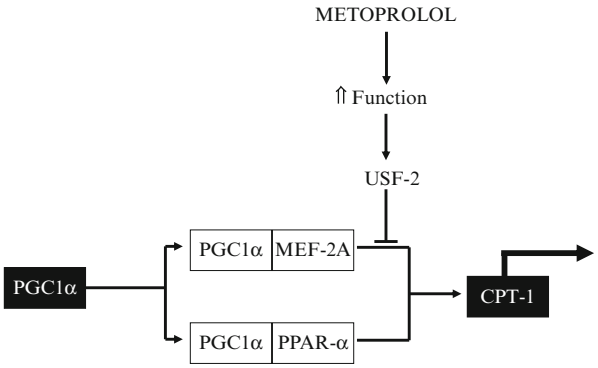

Fig. 3 Repression of CPT-1 by metoprolol. Improved contractile function leads to stimulation of USF-2, which represses PGC1-α transcriptional complex and reduces the expression of CPT-1 (Modified from Figure 3 of [117])

However, β1-adrenoceptor-blockade in the diabetic heart is associated with a more global regulation of the PGC1α transcriptional complex which is not explicable solely on the basis of USF binding. β1-adrenoceptor-blockade produces a decrease in the association of PGC1α with PPAR-α and MEF2A. Although this could be an indirect effect of the acute changes in fatty acid metabolism, it is more likely that active regulation of the complex is occurring. β1-adrenoceptor-blockade, by promoting glucose oxidation, reduces pyruvate levels, leading to a decrease in the binding of the pyruvate-activated deacetylase SIRT-1 to PGC1α. Furthermore, the binding of the acetylase p300 was increased by metoprolol. One would expect both of these changes to increase acetylation state of PGC1α, but we observed the opposite effect; β1-adrenoceptor-blockade decreased PGC1α acetylation! The acetylation site is very close to the USF-2 binding region, so it is possible that binding of USF-2 to PGC1α interferes with the acetylation reaction. This may be another mechanism of the repressive effect of USF-2, and one which could produce a more global repression of PGC1α target genes. Surprisingly, the phosphorylation of PGC1α by PKA, was increased by β1-adrenoceptor-blockade in the diabetic heart. We have no explanation for this. It is conceivable that PGC1α is a low priority target of PKA, and that binding of PGC1α to PKA only occurs when PKA is not interacting with its primary targets. Phosphorylation of p38 mitogen-activated protein kinase (MAPK) increases both PGC1α/PPAR-α coactivation and downstream signaling to PGC1α and PPAR-α targets [89–92]. It has been suggested that phosphorylation by p38 MAPK may serve to integrate and coordinate contractile and metabolic gene expression [85]. Activation of β2-adrenoceptors in the heart has been shown to increase signaling through the p38 MAPK pathway [93]. It is therefore possible that metoprolol decreases p38 phosphorylation by blocking β2-adrenoceptors, leading to a decrease in the association of PGC1α with its coactivators. Whether p38 MAPK-mediated phosphorylation of PGC1α is affected in this way in the diabetic heart remains to be determined.

The association of MEF-2A with the CPT-1 promoter is obliterated by both β1-adrenoceptor-blockade and diabetes. In diabetes, this may reflect generalised repression of MEF-2A targets, which is known to occur. With β1-adrenoceptor-blockade, it may represent sequestration of MEF-2A to higher priority gene targets, covalent modifications of MEF-2A or PGC1α, perhaps mediated by p38, or displacement of MEF-2A from its consensus site by PPAR-α, whose own consensus site overlaps with that of MEF-2A.

α-MHC and SERCA expression are decreased in the diabetic heart as part of the fetal gene program; both effects are reversed by β1-adrenoceptor-blockade [58]. α-MHC is regulated by USF's, while SERCA is regulated by MEF-2A [94] and possibly by PPAR-α [95]. It is therefore conceivable that the PGC1α/PPARα/MEF2A/USF complex can regulate and reverse induction of the fetal gene program. In other words, contractile and metabolic remodelling could be regulated in parallel by the same transcriptional complex.

6 NO/RNS-Induced Covalent Modifications of CPT-1

There is growing interest in the ability of RNS to directly regulate protein function [96–98]. Physiological levels of NO and RNS typically produce the following reversible modifications to thiol groups within critical cysteine residues: S-nitrosylation (addition of NO), glutathiolation (formation of mixed disulphides between the thiol group and glutathione) or oxidation from thiol to sulfenate. Glutathiolation and S-nitrosylation have been most frequently implicated in the regulation of enzyme activity, and the effects can be inhibitory or stimulatory [96]. The unique redox chemistry of protein thiol groups confers specificity and reversibility to these covalent modifications. The specificity is mediated by a consensus sequence, analogous to kinase consensus sequences [99]. However, this consensus sequence is not "recognised" as such; instead, it creates the correct reaction conditions for the covalent modification to occur. Reversibility is conferred by a number of enzymatic and non-enzymatic reactions [100–102]. The list of proteins proposed to be regulated by these modifications is growing, and, in the heart, includes GAPDH and SERCA [103]. Higher pathological levels of RNS induce further oxidation of the sulfenate (one oxygen) to sulfinate (two oxygens) and sulfonate (three oxygens). This causes irreversible loss of function and is therefore toxic. Glutathiolation, by committing the thiol to an alternate reaction pathway, protects critical thiol residues against irreversible oxidation [96] (Fig. 4).

Another covalent modification produced by RNS is tyrosine nitration. Peroxynitrite is the best characterised inducer of tyrosine nitration; indeed, tyrosine nitration is frequently used as a biomarker of peroxynitrite [104, 105]. Although classically described as an inhibitory modification, some proteins are activated by tyrosine nitration including cytochrome C, fibrinogen and PKC [105–108]. Tyrosine nitration, like the thiol modifications, also exhibits site-specificity via a similar mechanism.

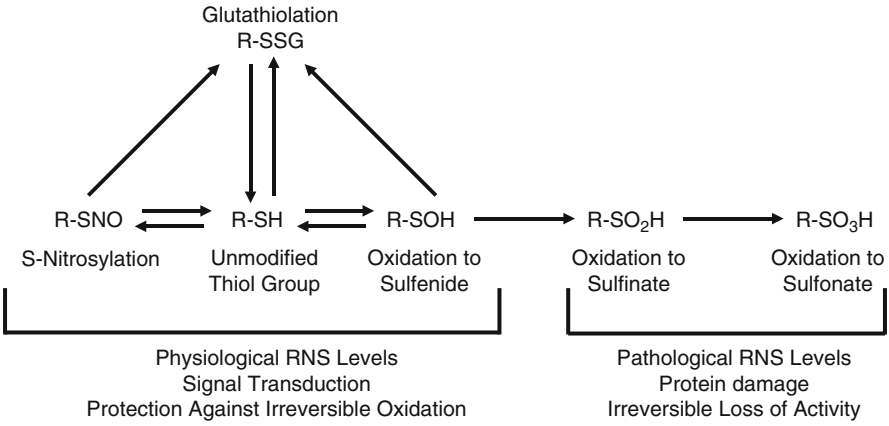

Fig. 4 NO and RNS-mediated modifications of thiol residues. Thiol (SH) residues undergo a series of reversible modifications in response to changes in the redox potential or exposure to physiological levels of reactive nitrogen species or nitric oxide. Oxidation of the thiol to the corresponding sulfenide or the formation of a disulphide bond between the thiol and glutathione (glutathiolation) are reversible either by changes in the equilibrium, or enzymatic restoration of the thiol group by thiol transferases. Further oxidation of a glutathiolated residue is not possible, so glutathiolation confers protection against oxidative damage for as long as it persists. However, exposure of the thiol group or the sulfenide to pathological levels of reactive nitrogen or oxygen species results in the formation of sulfinate and then sulfonate; these are irreversible modifications which result in protein damage and loss of activity (Modified from Figure 4 of [117])

Incubation of CPT-1 with continuous peroxynitrite, NO or hydrogen peroxide producing systems *in vitro* produces a decrease in CPT-1 activity which is associated with tyrosine nitration [109]. Furthermore, endotoxemia produces inhibition and nitration of CPT-1 in suckling rats [81]. Cysteine-scanning mutagenesis of CPT-1 revealed that cysteine 305 is critical for catalytic activity of the enzyme [110]. In our own studies, we incubated isolated mitochondria both with increasing concentrations of peroxynitrite ranging from 100 nM to 1 mM, and with a peroxynitrite-generating system, and dithiothreitol to remove the resulting covalent modifications. We found that CPT-1 was stimulated by peroxynitrite at low physiological levels but inhibited at high levels, and that peroxynitrite could induce tyrosine nitration, cysteine nitrosylation and cysteine glutathiolation. Activation of CPT-1 was most consistently associated with glutathiolation of CPT-1B. We hypothesised that the key residue involved was cysteine 305, but our efforts to confirm this by Mass Spectroscopy were unsuccessful [73].

We also successfully detected cysteine-nitrosylation, glutathiolation and nitration of CPT-1 in whole heart homogenates. Short-term β1-adrenoceptor-blockade increased nitrosylation and glutathiolation, but decreased tyrosine nitration, in diabetic hearts. In control hearts, nitrosylation was low and glutathiolation increased only following chronic treatment. This increase in CPT-1 glutathiolation would be expected to increase CPT-1 activity based on our *in vitro* studies, so CPT-1 glutathiolation does not explain the changes in CPT-1 activity seen following short-term

β1-adrenoceptor-blockade. Furthermore, it is not clear how the changes in CPT-1 nitrosylation, glutathiolation and nitration are linked to β1-adrenoceptor signalling; the patterns in systemic NO/RNS and CPT-1 covalent modifications which we observed do not match. The mitochondrial isoform of NOS (mtNOS) affects targets within the mitochondrial matrix and the inner mitochondrial membrane [111]. CPT-1 predominantly faces the cytosol, so it is likely that regulation of CPT-1 by NO/RNS is mediated by eNOS and possibly iNOS. eNOS has been proposed to translocate to the mitochondria [112, 113], so it is conceivable that mitochondrial eNOS translocation could determine NO/RNS mediated effects on CPT-1.

7 Phosphorylation of CPT-1

Phosphorylation of CPT-1A is known to occur *in vitro* [80] and is prevented by a specific inhibitor of CAMK II [114]. Activation of the sympathetic nervous system centrally by cerulinin stimulates CPT-1B activity in soleus muscle within 3 h [115]. This effect must have been mediated by an as-yet unidentified covalent modification of CPT-1B which could conceivably be phosphorylation. We speculated that the reason phosphorylation of CPT-1B had never been reported is because the kinases involved require other mediators to be present in order to bind their targets. We examined the effects of the main downstream kinases of β1-adrenoceptor signaling on CPT-1 activity, malonyl CoA sensitivity, and on the association of CPT-1 with known scaffolding proteins of these kinases. For example, A-kinase anchoring proteins (AKAPs) are a group of proteins which bind to PKA targets in order to regulate PKA-dependent phosphorylation of those targets [116]. We found that, when PKA was incubated with isolated mitochondria, it bound and phosphorylated CPT-1A, and increased the binding of AKAP-149 to CPT-1A; the functional effect was a decrease in CPT-1 sensitivity without any effect on catalytic activity.

When CAMK-II was incubated with isolated mitochondria, it bound and phosphorylated CPT-1B; however, the functional effect in this case was an increase in CPT-1 sensitivity without any effect on catalytic activity [73]. Serendipitously, our mitochondrial isolation produced a variation in the initial sensitivity of CPT-1, most likely as a result of membrane effects. Thanks to this unintended effect, we found that CAMK-II tended to restore CPT-1 sensitivity to a "set-point" represented approximately by an IC_{25} of 20 μM, an IC_{50} of 100 μM and an IC_{75} of 150 μM malonyl CoA. In this case, the scaffolding protein turned out to be α-actinin, whose binding was decreased by CAMK-II phosphorylation of CPT-1. We believe it unlikely that α-actinin, which is a cytoskeleton protein, is anchored in the mitochondrial membrane; CPT-1B probably forms a point of attachment of the cytoskeleton to the mitochondria. We speculate that AKAP-149 and α-actinin limit access of malonyl CoA to its binding site, and produce changes in malonyl CoA sensitivity by varying the strength of their association with their respective isoforms.

The MAPK p38 bound and phosphorylated CPT-1B via the scaffolding protein JIP-2, stimulating CPT-1 catalytic activity without affecting malonyl CoA

sensitivity [73]. By contrast, Akt did not bind or phosphorylate CPT-1, and had no effect on the activity or sensitivity of the enzyme.

Short and long-term β1-adrenoceptor-blockade increase the total phosphorylation state of CPT-1. β1-adrenoceptor-blockade increases PKA-mediated desensitization of CPT-1 to malonyl CoA in control hearts, and decreases CAMK-II-mediated sensitization in diabetic hearts [117]. Both effects decrease malonyl CoA sensitivity. Short, but not long-term, β1-adrenoceptor-blockade abolishes the association of p38 with CPT-1, providing a partial explanation for the inhibition of CPT-1 activity following short term β1-adrenoceptor-blockade in control hearts. A similar effect on p38 is not seen in diabetic hearts, because diabetes itself abolishes the association of p38 with CPT-1 [117]. The binding of PKA, CAMK-II and p38 to CPT-1 bear no relation to the overall activities of these kinases in the whole heart. It appears that the translocation of these kinases to CPT-1 on the mitochondrial surface is the important mechanism. Precisely how β-adrenoceptors might regulate such a translocation process is not known. Clearly the compartmentalisation and regulation of the signalling pathway are altered in the setting of diabetes.

Overall, we propose that changes in CPT-1 sensitivity produced by short-term β1-adrenoceptor-blockade are due to decreased CAMK-II phosphorylation of CPT-1 (in diabetic hearts) and/or increased phosphorylation of CPT-1 by PKA (in control hearts), mediated by changes in the association of CPT-1 with scaffolding proteins (Fig. 5). Obliteration of the CPT-1/p38 interaction could explain the decrease in CPT-1 activity in control hearts, but not in diabetic hearts, which show an increase in CPT-1 activity despite loss of this interaction. There is no explanation for the acute decrease in CPT-1 catalytic activity seen in the diabetic heart. The association with p38 MAPK is obliterated in the diabetic heart to begin with, and glutathiolation of CPT-1 is increased by short term β1-adrenoceptor blockade. The *in vitro* effects of the covalent modifications we identified may not reflect their effects *in vivo*. Also, it is possible that these covalent modifications produce more complex and varied effects when acting together as opposed to in isolation. There may also be other mechanisms for regulating CPT-1 catalytic activity which have not yet been identified.

8 Pro-Survival Signalling

Diabetes produces a decrease in β1-adrenoceptor expression and a marked increase in β3-adrenoceptor-expression. Long-term β1-adrenoceptor blockade increases the expression of all three adrenoceptor subtypes. In the whole heart, the major effect of short-term β1-adrenoceptor blockade is, unsurprisingly, to decrease classical cAMP/PKA signaling. Long-term blockade, in addition, increases PI3K/Akt signaling, probably due to the marked increase in β3-adrenoceptor-expression. This is associated with pro-survival effects. The pro-apoptotic factors FOXO-3 and Bad are inhibited, and the anti-apoptotic factor BCl-2 is stimulated [59, 118]. Another intriguing pro-survival effect is that long-term β1-adrenoceptor blockade increases

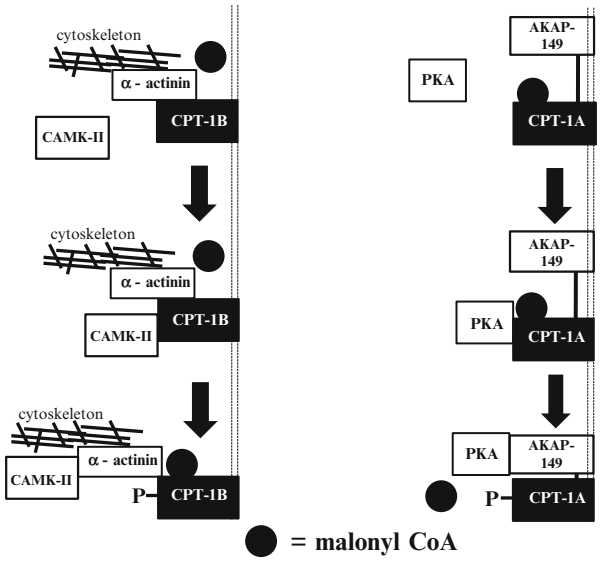

Fig. 5 Proposed model of the actions of PKA and CAMK-II. Left panel: Exogenously applied PKA phosphorylates CPT-1A and is then captured by its scaffolding protein. Phosphorylation produces a conformational change tightening the interaction between AKAP-149 and CPT-1A. As a result, malonyl CoA is denied access to its binding site, and the sensitivity of CPT-1 to malonyl CoA is reduced. Note that CPT-1 and AKAP-149 are both anchored in the mitochondrial membrane. Right panel: Exogenously applied CAMK-II phosphorylates CPT-1B and is then captured by its scaffolding protein α-actinin. Phosphorylation produces a conformational change, loosening the interaction between α-actinin and CPT-1B. As a result, malonyl CoA has improved access to its binding site and the sensitivity of CPT-1 to malonyl CoA is increased. Note that CPT-1B is anchored to the mitochondrial membrane whereas α-actinin is anchored to the cytoskeleton (Modified from Figure 10 of [117])

the sequestration of activated caspase-3 by caveolins [59]. These pro-survival effects provide the key to the beneficial effects of β1-adrenoceptor blockade on cardiac function. Although β1-adrenoceptor blockade inhibits CPT-1, it has no effect on CD-36 translocation and it therefore increases the cytoplasmic accumulation of long-chain acyl CoAs. It also does not prevent oxidative stress or the resulting DNA damage. The stimulus for cell damage therefore remains unaltered. β1-adrenoceptor blockade improves function by preventing the sequelae of this stimulus [59] (Fig. 6).

9 Clinical Significance

The fact that β1-adrenoceptor blockade improves cardiac function in diabetic hearts raises the question as to whether the drug should be used earlier in diabetic patients. However, its *in vivo* effects in this setting are equivocal. There are also clinical

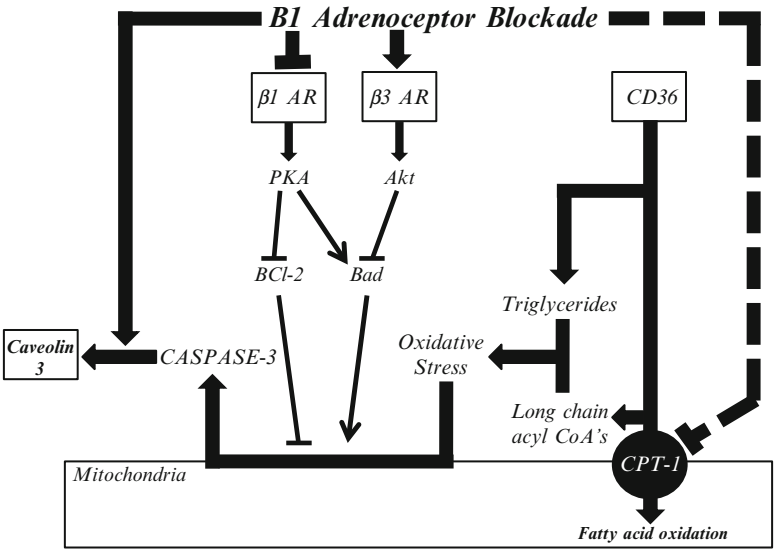

Fig. 6 Mechanisms of action of metoprolol. Metoprolol inhibits fatty acid oxidation by inhibiting carnitine palmitoyltransferase-1 (CPT-1), but has no effect on CD36. Triglyceride and long chain acyl CoA accumulation, and stimulation of oxidative stress, are therefore unaltered. Metoprolol also promotes β3 adrenoceptor signaling, leading to inhibition of Bad and stimulation of BCl-2, and inhibition of caspase-3 activation. Finally, metoprolol stimulates sequestration of caspase-3 by caveolins. The net effect is a prevention of caspase-3 activation (Modified from Figure 7 of [73])

concerns with the use of β-blockers in diabetic patients which need to be carefully weighed against the benefits of introducing the drug so early. First and foremost are concerns about the effects of β-blockers on glycemic control. The use of β-blockers as antihypertensive agents has been associated with an increased risk of new-onset diabetes [119]. Hepatic glucose output is controlled by the β2 adrenoceptor, and blockade of this receptor, which does occur with the β1-selective agents, lowers hepatic glucose output, delaying recovery from hypoglycemia [120, 121]. Attenuation of hypoglycaemic symptoms by β-blockade is no longer considered to be a problem, because sweating and paresthesias are preserved, and these are signs which patients can be taught to recognize [121, 122]. Another concern with chronic β-blockade is the presence of sustained unopposed α1-adrenoceptor stimulation, because activation of the sympathetic nervous system by hypoglycemia can increase unopposed α1-adrenoceptor stimulation to the point where a hypertensive crisis occurs [121]. Also, unopposed α1-adrenoceptor stimulation produces peripheral vasoconstriction which could worsen peripheral vascular disease and, by decreasing muscle flow, also worsen insulin resistance [12, 123]. These concerns are not sufficient to deny β-blockers to patients with systolic heart failure because these drugs are lifesaving in this setting. However, the risks and benefits of earlier β-blocker use must be weighed carefully. There is currently no evidence on which to base these considerations.

10 Conclusion

The range of effects produced by β-adrenergic blockade are broad and illustrate how interconnected the signalling pathways of function and metabolism are in the heart. Our initial hypothesis that inhibition of fatty acid oxidation would be a key mechanism of action was disproved. However, unexpected results have led us to some new and hitherto unexpected regulatory mechanisms of cardiac metabolism. The first is USF-2-mediated repression of PGC-1α, most likely occurring as a consequence of improved function. The second is the identification of covalent modifications which directly regulate CPT-1 at the level of the mitochondria. We also found that β-adrenergic signalling interacts with caveolins, which could be a key mechanism of action of β-adrenergic blockade. Our experience of studying this labyrinthine signalling web illustrates that it is not necessary for initial hypotheses to be correct, and all ends foreseen, in order for valid lines of inquiry to be opened and new information revealed.

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