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RESEARCH ARTICLE

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Morphine induces physiological, structural, and molecular benefits in the diabetic myocardium

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Abstract

The obesity epidemic has increased type II diabetes mellitus (T2DM) across developed countries. Cardiac T2DM risks include ischemic heart disease, heart failure with preserved ejection fraction, intolerance to ischemia-reperfusion (I-R) injury, and refractoriness to cardioprotection. While opioids are cardioprotective, T2DM causes opioid receptor signaling dysfunction. We tested the hypothesis that sustained opioid receptor stimulus may overcome diabetes mellitus-induced cardiac dysfunction via membrane/mitochondrial-dependent protection. In a murine T2DM model, we investigated effects of morphine on cardiac function, I-R tolerance, ultrastructure, subcellular cholesterol expression, mitochondrial protein abundance, and mitochondrial function. T2DM induced 25% weight gain, hyperglycemia, glucose intolerance, cardiac hypertrophy, moderate cardiac depression, exaggerated postischemic myocardial dysfunction, abnormalities in mitochondrial respiration, ultrastructure and Ca^{2+}-induced swelling, and cell death were all evident. Morphine administration for 5 days: (1) improved glucose homeostasis; (2) reversed cardiac depression; (3) enhanced I-R tolerance; (4) restored mitochondrial ultrastructure; (5) improved

Abbreviations: COX IV, cytochrome C oxidase IV; DRP1, dynamin-related protein 1; GPCR, G protein-coupled receptor; I-R, ischemia-reperfusion; IPC, ischemic preconditioning; K_{ATP}, ATP-sensitive K^+ channel; LDH, lactate dehydrogenase; MFF, mitochondrial fission factor; MPTP, mitochondrial permeability transition pore; OPA1, mitochondrial dynamin-related protein 120 kDa; PI3K, phosphatidylinositol-3-kinase; Stat3, signal transducer and activator of transcription 3; TEM, transmission electron microscopy; Tom20, mitochondrial import receptor subunit TOM20 homolog; T2DM, type 2 diabetes mellitus; VDAC1, voltage-dependent anion-selective channel 1.

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mitochondrial function; (6) upregulated Stat3 protein; and (7) preserved membrane cholesterol homeostasis. These data show that morphine treatment restores contractile function, ischemic tolerance, mitochondrial structure and function, and membrane dynamics in type II diabetic hearts. These findings suggest potential translational value for short-term, but high-dose morphine administration in diabetic patients undergoing or recovering from acute ischemic cardiovascular events.

KEYWORDS

cardioprotection, diabetic cardiomyopathy, ischemia-reperfusion injury, mitochondria, opioid receptors

1 | INTRODUCTION

Three epidemics–obesity, chronic pain, and opioid abuse–challenge health-care systems and impair the lives of patients affected. Obesity poses an enormous health-care burden and correlates with the high incidence of T2DM thereby leading to increased comorbidities and impaired quality of life.[1,2] In the United States, the prevalence of T2DM has increased over the last two decades, with 12-14% of adults having a diagnosis of T2DM, and 38% diagnosed as prediabetic.[3] Since 1980, the T2DM prevalence in adults almost quadrupled worldwide, with a faster increase in low- and middle-income countries than in high-income countries.[4]

Chronic T2DM may lead to vascular dysfunction, nephropathy, retinopathy as well as diabetic cardiomyopathy. Diabetic cardiomyopathy can arise independently of coronary artery disease or hypertension, and clinically presents as cardiac hypertrophy, fibrosis, and dysfunction.[5-7] A critical barrier to studying diabetic complications is the plethora of cellular mechanisms involved, including inflammation, lipotoxicity, posttranslational modifications ranging from advanced glycation end-product generation and protein glycosylation, to ATP-sensitive K^+ channel (K_{ATP}) dysfunction, autophagy dysregulation, altered Ca^{2+}-signaling, and hyperglycemia-dependent oxidative stress, with key involvement of mitochondrial dysfunction.[8] Due to this multifactorial nature of diabetic cardiomyopathy, no specific treatments have yet been identified.

The risk of ischemic heart disease, myocardial outcomes post ischemia, and responses to "cardioprotective" stimuli are all worsened in diabetic patients.[9-12] Experimental T2DM models suggest the diabetic heart to be broadly refractory to "cardioprotective" interventions, including ischemic pre- or post-conditioning and responses to protective G protein-coupled receptor (GPCRs).[7,12] Few studies directly address the conundrum of ischemic intolerance and cardioprotective refractoriness in diabetic hearts. The question of how reversible the myocardial abnormalities of T2DM are, and how to best limit cardiac I-R injury remain unresolved. However, changes in opioid receptor signaling may both participate in the cardiac abnormalities of T2DM, and offer a potential protective target.

Opioid receptors belong to the GPCR family, are expressed in the heart, and facilitate cardiac stress tolerance.[7,13] Indeed, opioid agonists induce cardioprotection, while antagonists attenuate ischemic preconditioning and increase the development of sublethal arrhythmias.[13,14] Diabetes mellitus suppresses cardiac[15-17] and extra-cardiac[18,19] opioid receptor responses, and many implicated mechanisms of diabetic cardiomyopathy appear to be sensitive to intrinsic opioid receptor activity.[6,7] Opioids can induce cardiac protection in humans.[20-22] Impairment of this signaling could thus contribute to the cardiac abnormalities in T2DM, while presenting a novel target for therapy. Although acute opioid receptor responses appear impaired in diabetes mellitus,[15,16] a mechanistically distinct protective effect of sustained opioid receptor agonism has been identified[17,23] and appears conserved in aged hearts[24] that, as in diabetes mellitus, are resistant to conventional opioid receptor and ischemic conditioning stimuli.[7,12] While primarily a clinically used analgesic, the non-selective opioid receptor agonist morphine has been recently reported to alleviate dyspnea in patients with chronic heart failure,[25] chronic obstructive pulmonary disease (COPD),[26] and cancer.[27] Such data reveal a unique ability of opioid receptor agonism to have utility beyond pain relief. The impact of sustained opioid receptor activation during T2DM is currently unknown.

Since T2DM suppresses acute opioid receptor responses[15,16] that normally protect the heart,[28-31] we theorized that a novel sustained opioid receptor stimulus via morphine administration might benefit the diabetic myocardium. We examined the impact of 5-day opioid receptor agonism with morphine, testing for the first time the impact of sustained opioid receptor activation on in vivo myocardial function (echocardiography), ex vivo I-R intolerance (Langendorff analysis), cardiac ultrastructure, and mitochondrial function. These preclinical studies harbor novel implications on how to limit I-R injury in the diabetic heart.

2 | MATERIAL AND METHODS

2.1 | Pharmacological agents

Chemicals were purchased from Sigma Chemical Co (St Louis, MO) unless otherwise indicated. Morphine (75 mg morphine) or placebo tablets were acquired from Murthy Pharmaceuticals Inc. (Lexington, KY).

2.2 | High-fat diet-induced T2DM model

Experimental procedures followed the Guide for the Care and Use of Laboratory Animals (National Research Council, Washington, DC), with protocols approved by the Veteran Affairs San Diego Healthcare System Institutional Animal Care and Use Committee. A murine T2DM model was studied, involving high-fat feeding (60% fat of total caloric intake; TD-06414 adjusted calorie diet, Harlan Laboratories, Madison, WI, USA) combined with low-dose streptozotocin (STZ).[32,33] Specifically, 3-month-old C57Bl/6J male mice received a single STZ injection (75 mg/kg in 0.1 M citrate buffer, pH = 4.5, i.p, during non-fasting conditions; S0130, Sigma, St. Louis, MO) and were switched to high-fat chow for 12-14 weeks to induce obesity, hyperglycemia, insulin resistance, and hyperinsulinemia (Figure 1). Control mice received injections of 0.1 M citrate buffer and were maintained on standard chow (4% of calories from fat, LFD, Teklad 7001). Four individual cohorts of mice (n = 60 total, entailing n = 30 control and n = 30 T2DM, with n = 15 treated with either morphine or placebo) were investigated to ensure reproducibility of phenotype and experimental data. For serum analysis in non-fasted mice, blood was collected via the retro-orbital route for glucose and insulin measurements. Glucose tolerance tests (GTT) were performed in a subset of fasted mice from each cohort 12-14 weeks after high-fat/normal chow feeding. For GTT, mice were fasted for 11-12 hours, fasting glucose levels were assessed via tail snip, followed by i.p injection of 1 g/kg glucose in 0.9% NaCl and monitoring of blood glucose at 30 minutes intervals for up to 3 hours (ONETOUCH Ultra 2 glucometer, LifeScan Inc., Milpitas, CA, USA). To perform insulin tolerance testing (ITT) mice were fasted as above, fasting glucose levels assessed, followed by i.p injection of 0.4 mU/g insulin in 0.9% NaCl. Blood glucose levels were monitored as noted above. To measure serum insulin levels an ultrasensitive mouse insulin ELISA kit was used (Cat #80-INSHU-E01.1, ALPCO Diagnostics, Salem, NH).

2.3 | Morphine treatment

Prior to implantation, both placebo and morphine pellets had a nitrocellulose-based coating applied to one side (~50%) and were allowed to cure, aiming to slow drug release through limitation of contact in the subcutaneous space. Mice were anesthetized with isoflurane and placebo or morphine pellets were inserted into the dorsal subcutaneous space via a small incision, subsequently closed with 9 mm wound clips. Pellets remained in place for 5 days, when mice were assessed via echocardiography and cardiac tissue analyses/perfusion.[17,23] Based on dried pellet mass after 5 days, mice receive 60-100 mg/kg of morphine/day.

2.4 | Echocardiography

After GTT, animals recovered for 1 week before "pre-treatment" echocardiography (M-mode, two-dimensional, and Pulse Wave Doppler). This was performed under isoflurane anesthesia in animals from each experimental group using a small-animal, high-resolution Vevo 2100 imaging unit with a MS400 18-38 MHz transducer (VisualSonics Inc., Toronto, Canada, Sonosite, Fuji). Five days after morphine administration the echocardiography was repeated. Specifically, mice were anesthetized with isoflurane via nose cone (1% isoflurane, at 1 L/min O_2 flow), ECG traced heart rates maintained between 500 and 600 bpm, and cardiac function assessed before (pre) and 5 days after (post) drug treatment. Left-ventricular ejection fraction (%EF), fractional shortening (%FS), mean circumferential fiber shortening rate (V_{CF}),[34] and wall thicknesses were assessed as previously described.[35]

2.5 | Picrosirius red staining

To assess fibrosis, picrosirius red staining was performed by the UCSD Comparative Phenotyping Core using standard techniques. Stained sections were imaged using a Keyence Microscope BZ-X710 (Keyence, Laguna Hills, CA, USA).

2.6 | Langendorff heart model

Mice were anesthetized with sodium pentobarbital (60 mg/kg i.p.) and blood sampled for non-fasting [glucose] and [insulin] before heart excision. Aortic cannulation and Langendorff perfusion of the coronary circulation were performed as previously described.[17,23,36] Perfusion pressure was fixed at 80 mm Hg, and a modified Krebs-Henseleit buffer was employed, bubbled with 95% O_2/5% CO_2 at 37°C (giving a pH of 7.4), and containing (in mM): NaCl, 120; $NaHCO_3$, 25; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.2; KH_2PO_4, 1.2; D-glucose, 15; and EDTA, 0.5. Following 20 minutes stabilization at intrinsic heart rates, ventricular pacing at 7 Hz was initiated, and after 10 minutes baseline

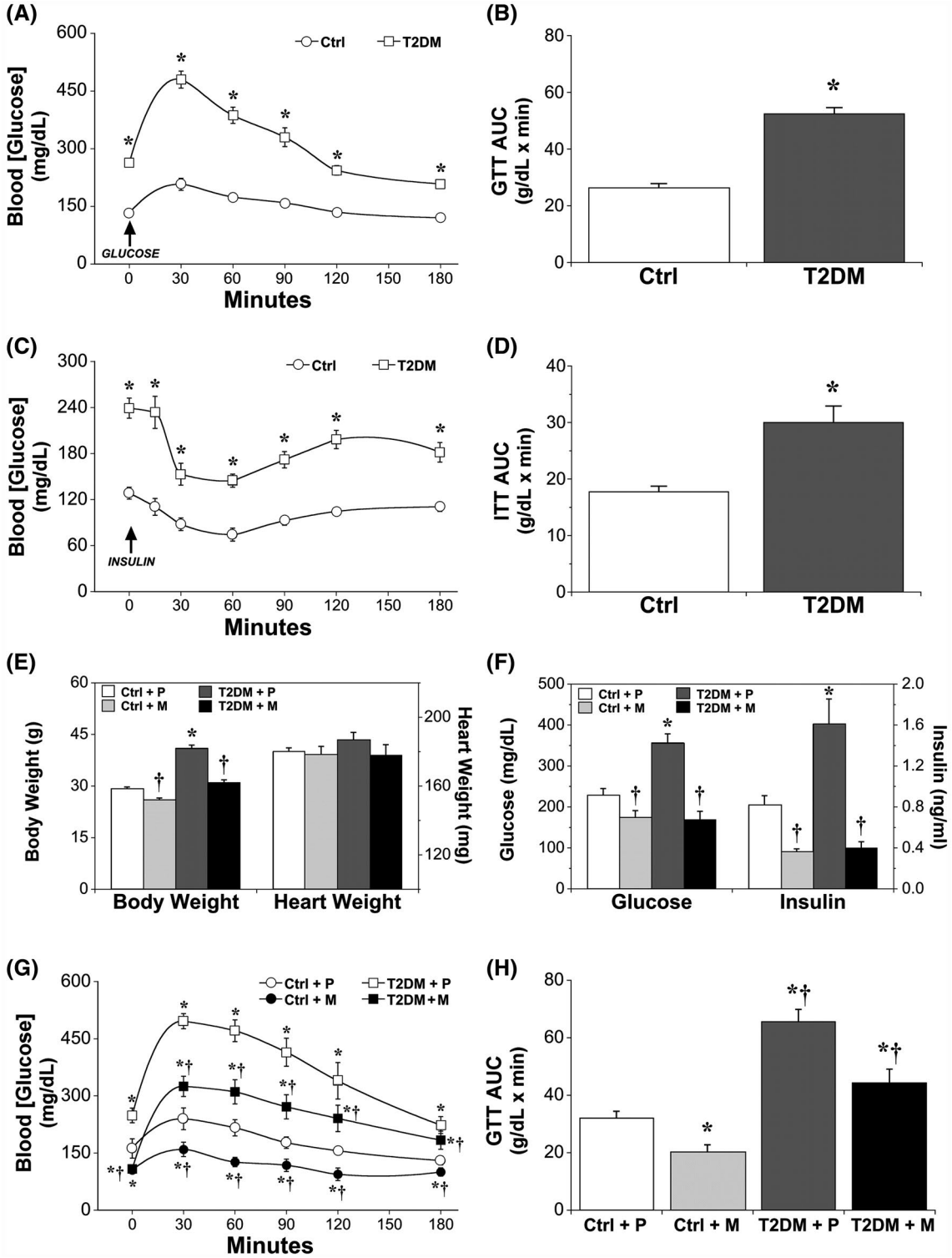

FIGURE 1 Characterization of a T2DM mouse model, and effects of morphine in T2DM. Evolution of a T2DM phenotype is evidenced by changes in: A, Glucose tolerance testing (GTT) in control (Ctrl, 4% kcal fat chow) and T2DM (60% kcal fat chow) mice 12 weeks after high-fat feeding; B, area under the curve (AUC) of GTT; C, Insulin tolerance testing (ITT); and D, AUC of ITT. Morphine (M) or placebo (P) effects in Ctrl (Ctrl +P and Ctrl +M) and T2DM (T2DM + P and T2DM + M) mice reveal changes in: E, Body weight (BW) and heart weight (HW); F, Serum glucose as well as insulin levels, G, GTT; and H, AUC of GTT. Data are presented as means ± SEM and were analyzed via two-way ANOVA and Tukey's multiple comparison test

measures were made before subjecting hearts to 25 minutes of global normothermic ischemia and 45 minutes aerobic reperfusion. Coronary effluent was collected on ice throughout reperfusion for determination of total postischemic LDH efflux (IU/mL × mL/g total postischemic volume). IPC was assessed in subsets of control and T2DM

hearts (placebo and morphine groups), subjected to 2 ×5 minutes ischemic episodes interposed by 5 minutes reperfusion prior to the index 25 minutes ischemia.

2.7 | Cardiac tissue fractionation and cholesterol assay

Frozen hearts (n = 6 per group) were pulverized on dry ice and homogenized on ice in detergent-free lysis buffer (150 mM Na_2CO_3, 1 mM EDTA, 1× protease and phosphatase inhibitor, pH 11) utilizing a Qiagen Beat TissueLyser (LT beat mill, 50 oscillations/min for 5 minutes). Lysates were sonicated on ice (40% intensity) three times for 20 seconds each, and incubated on ice for 30 minutes between sonication steps. Protein concentration was determined in order to normalize to 500 μg of protein loaded in each sucrose gradient column. Sucrose gradients (2.2 mL) were prepared as follows: 183 μL of normalized lysate (500 μg protein) were mixed with equal volume of 90% sucrose inside centrifugation tube, mixed with vortex until homogenous and overlaid with 1.1 mL of 35% sucrose. The sucrose gradient was overlaid with 734 μL of sucrose 5%, with a final volume of 2.2 mL. All sucrose fractions were prepared in MBS buffer (25 mM MES, 150 mM NaCl, 5 mM EDTA, pH 6.5). Sucrose gradients were centrifugated in a Beckman Coulter Optima Max-TL bench top ultracentrifuge (TLS-55 rotor) at 175,000 g for 18 hours. Twelve fractions of 183 μL were collected from top (fraction 1) to bottom (fraction 12). In addition, cholesterol concentration of fractions was determined using the Amplex Red Cholesterol Assay (Thermo Fisher, Waltham, MA).

2.8 | Immunoblotting

Protein concentrations were normalized between samples for whole cell lysates or loaded per volume for fractions (15 μL per fraction), and then, SDS-PAGE was performed using NuPAGE gels in MES running buffer (55 mM MES, 10 mM Tris-base, 1 mM EDTA, 0.1% SDS). Gels were resolved at 150 V for 70 minutes, and then, subsequently transferred to 0.45 μM PVDF membranes (200 mA/blot for 60 minutes). Membranes were blocked using 5% BSA in 1× PBS–0.05% Tween (PBST) for 60 min, then, primary antibodies were incubated overnight at 4°C in PBST or 1× TBS–0.1% Tween20 (TBST) at the concentrations specified (Cav-1 (1:1000), Cav-3 (1:1000), Vinculin (1:1000), and GAPDH (1:1000)). Antibodies utilized for mitochondrial protein detection included anti-OPA1 (Cell Signaling Technology), anti-VDAC1 (Santa Cruz), anti-MFF (Cell Signaling Technology), anti-Mitofusin-1 (Abcam), anti-Tom20 (Cell Signaling Technology), anti-DRP1 (Cell Signaling Technology), anti-Mitofusin-2 (Cell Signaling Technology), and anti-Stat3 (Cell Signaling Technology) all diluted to 1:1000. Anti-COX IV and anti-GAPDH (Cell signaling, 1:1000) were used as a protein loading controls. Immunoblot images were acquired and analyzed using the UVP imager system.

2.9 | Mitochondrial isolation and analysis

Mitochondria were isolated from hearts at 10 minutes of reperfusion for analysis of postischemic respiratory function and Ca^{2+}-induced swelling, an indicator of mitochondrial permeability transition pore (MPTP) opening.[37] Left ventricular tissue was placed in ice-cold mitochondrial isolation medium (MIM: 0.3 M sucrose, 10 mM HEPES, 250 μM EDTA) and homogenized with a Tissuemiser (Fisher Scientific, Waltham, MA, USA). Homogenates were rinsed in MIM and samples centrifuged at 600 ×*g* to clear nuclear/membrane debris. Supernatants were spun at 8000 ×*g* for 15 minutes, and pellets suspended in MIM with 1 mM BSA before recentrifuging15 minutes at 8000 ×*g*. Centrifugation was repeated and metabolically active mitochondria resuspended in 150 μL MIM.

Mitochondrial analysis was performed as previously described [37]. For Ca^{2+}-induced swelling, mitochondria solutions (0.5 μg/μL) lacking Ca^{2+} were loaded into clear flat-bottomed 96-well plates and challenged with 250 μM $CaCl_2$. Mitochondrial O_2 consumption was measured using an oxygraph with a Clark-type O_2 electrode (Hansatech, Norfolk, UK) during sequential addition of substrates and inhibitors [10]. Briefly, mitochondria (100-200 μg protein) were added to an oximeter chamber in a solution containing 100 mM KCl, 75 mM mannitol, 25 mM sucrose, 5 mM H_3PO_4, 0.05 mM EDTA, and 10 mM Tris-HCl, pH 7.2 at 37°C. After a 2 minutes equilibration, 5 mM pyruvate and 5 mM malate were added and O_2 consumption followed for 1-2 minutes (state 4). ADP (250 μM) was added for measurement of state 3 (phosphorylating) respiration. To switch from NAD^+- to FAD^+-linked respiration, complex I was eliminated by inhibition of back electron transfer with 0.5 mM rotenone, and complex II activity triggered by addition of 10 mM succinate. Complex III was then inhibited by addition of 5 mM antimycin A. Complex IV activity was measured in the presence of 0.5 mM 2,2,4-trimethyl-1,3-pentanediol and 2 mM ascorbate. O_2 utilization was normalized to protein content. The respiratory control ratio (RCR), indicative of oxidative phosphorylation uncoupling, was calculated from the state 3:state 4 complex I activity ratio.

2.10 | Pair feeding studies

Since 5-day morphine therapy reduced body weight in association with improved cardiac function in T2DM mice,

paired-feeding in non-morphine-treated mice was assessed. Briefly, to investigate the "true morphine effect" versus morphine-induced weight loss effect on cardiac recovery, we restricted food intake of placebo-treated mice to the food intake in morphine-treated animals. To reduce stress from single housing, animals were group-housed from 7 am to 7 pm (inactive phase), and then, single housed from 7 pm to 7 am (active phase) to monitor/control food intake. Water was provided ad libitum throughout the study, and placebo-treated animal received daily food quantities matching those consumed by morphine-treated mice

2.11 | Transmission electron microscopy (TEM)

Cardiac tissue was assessed via TEM in three hearts of each group ($n = 12$ total), with specific emphasis on mitochondrial morphology. Transcardial perfusions (5 mL/min) were performed as previously described.[38] Morphometric analysis was performed with free hand tracing of mitochondria/cristae to estimate number and size per visual field using ImageJ as previously described[38,39]:

$$\%Mitochondrialvolumedensity = \left[Sumofmitochondrialarea\left(\mu m^2\right)\right] / totalfield\left(\mu m^2\right) \times 100$$

$$\%Cristeavolumedensity = \left[Sumofcristeaarea\left(\mu m^2\right)\right] / mitochondrialarea\left(\mu m^2\right) \times 100$$

2.12 | Statistical analyses

Experimental replicates are listed for each individual experiment. Presented data was analyzed with GraphPad Prism software (San Diego, CA, USA). Differences between groups were analyzed with one-, two-, or three-way ANOVA where appropriate as described in the figure legends. This was followed by post hoc Tukey's multiple comparison tests to evaluate differences between specific groups. Data are presented as means ± SEM. Significance was accepted for $P < .05$.

3 | RESULTS

3.1 | Effects of opioid receptor activation on the systemic T2DM phenotype

Diet-induced obesity models are commonly employed to investigate molecular mechanisms underlying T2DM risks. Combined high-fat feeding with single low-dose STZ treatment is widely employed to induce T2DM in a nongenetic manner in rats and mice.[32,33] After a single STZ injection in non-fasted animals followed by 12-14 weeks of a 60% kcal HFD, we detected impaired glucose-tolerance in fasted mice (Figure 1A,B) when compared to mice fed 4% kcal fat control chow (low fat). Hyperglycemia was evident in fasted HFD mice when compared to controls, and i.p. insulin administration reduced serum glucose in control and STZ/HFD animals (Figure 1C,D). Based on documented hyperglycemia, and responsiveness to insulin, the HFD fed mice were considered type 2 diabetic (T2DM). Prior to drug treatment, T2DM mice exhibited 25% weight gain, while morphine administration for 5 days induced significant body weight loss of 2-3 g (or ~10%) in healthy and 7-9 g (~20%) in T2DM animals when compared to placebo-treated groups, without changing heart weight (Figure 1E). Morphine-exposed T2DM mice had significant reductions in non-fasting hyperglycemia and hyperinsulinemia (Figure 1F), compared to placebo-treated control groups. Morphine caused a dramatic fall in T2DM fasting glucose levels (~55% reduction) when compared to control mice (~35% fall), with decreased serum insulin levels (Figure 1F). Glucose tolerance testing after morphine administration showed reduced glucose elevation and faster return to baseline (ie, lower area under the curve) in morphine-treated T2DM and control mice when compared to placebo-treated groups (Figure 1G,H).

3.2 | Effects of opioid receptor activation via morphine on in vivo cardiac function

Opioids have been shown to produce cardiac protection during I-R in humans,[20,21] yet effects of sustained opioid receptor activation on the diabetic myocardium are currently unclear. Echocardiography was performed prior to (Pre) and 5 days after (Post) morphine or placebo administration. M-mode, B-mode, and Pulse Wave Doppler analyses identified mild contractile dysfunction in T2DM mice, with 15-25% reductions in ejection fraction (%EF) and mean velocity of circumferential fiber shortening (Vcf), with morphine administration for 5 days restoring cardiac function (Figure 2A,B). We documented unchanged left ventricular inner dimensions during diastole (LVIDd), with widened left ventricular inner dimensions during systole (LVIDs) in placebo-treated T2DM mice (Figure 2C,D), indicating compromised systolic contractile function. Morphine reversed this latter change in LVIDs (Figure 2D). T2DM hearts showed increased intraventricular septal wall thicknesses in diastole (IVSd) (Figure 2E), and a decreased relative wall thickness (LV radius/wall thickness ratio, R/Th) when compared to controls (Figure 2F), demonstrating cardiac hypertrophy in T2DM mice. Taken together, 5 day opioid receptor activation by morphine selectively reversed the T2DM-dependant contractile dysfunction without influencing cardiac hypertrophy and wall thickness. T2DM resulted in increased picrosirius red staining relative to controls; however, morphine treatment had no effect and T2DM hearts with placebo treatment showed similar levels

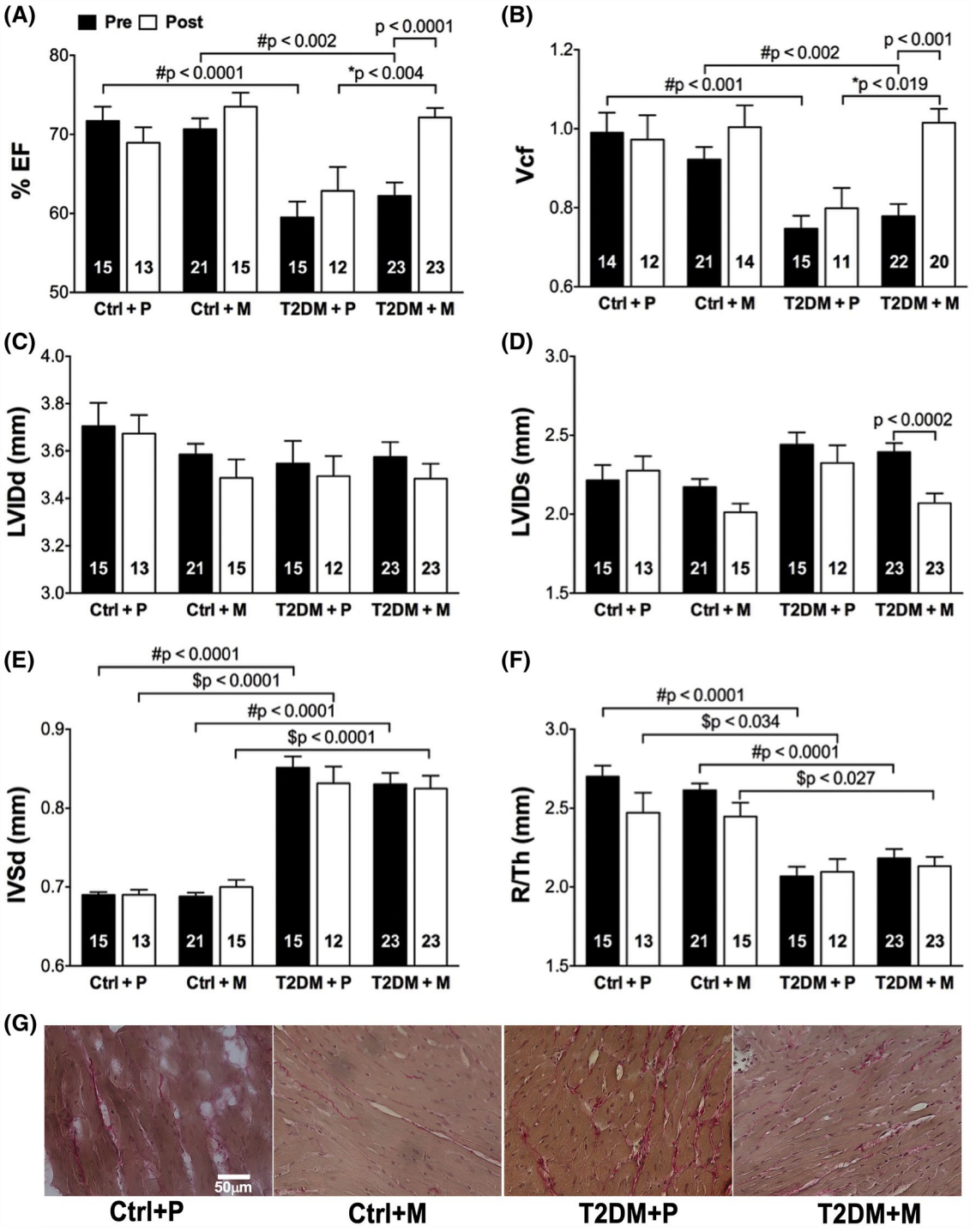

FIGURE 2 Morphine treatment improves cardiac function in T2DM mice, but does not affect cardiac hypertrophy. Echocardiographic analysis of Ctrl and T2DM mice was performed before (Pre) and 5 days after morphine (M) or placebo (P) administration (Post): A, % ejection fraction (%EF); B, velocity of circumferential fiber shortening (Vcf); C, left ventricular inner dimension in diastole (LVIDd); D, left ventricular inner dimension in systole (LVIDs); E, interventricular septum dimensions in diastole (IVSd); and F, calculated relative wall thickness (LV radius/wall thickness ratio). Data are presented as means ± SEM and were analyzed via two-way ANOVA and Tukey's multiple comparison tests. Pre-Tx vs Post-Tx per group ($P < .05$), Pre-Tx Ctrl vs Pre-Tx T2DM (#$P < .05$), Post-Tx Ctrl vs Post -Tx T2DM ($$P < .05$), and Placebo vs M. G, Picrosirius red stain to determine cardiac fibrosis at 5 days post P or M treatment

of fibrosis when compared to morphine-treated T2DM hearts (Figure 2G).

3.3 | Morphine effects on ex vivo myocardial function and I-R tolerance

Failure of preconditioning in diabetic human myocardium was first described by Ghosh et al.[9] Clinical evidence reveals dysfunctional protection in diabetic patients and myocardial sensitization to I-R injury.[10-12] Multiple studies report that diabetic hearts are broadly refractory to cardioprotective interventions, including ischemic pre- or post-conditioning.[40] We investigated effects of sustained opioid receptor activation with morphine in T2DM hearts using an ex vivo Langendorff approach to focus specifically on intrinsic myocardial changes.

Neither T2DM nor morphine significantly altered baseline function in control or T2DM hearts (Table 1). A 25 min ischemic insult resulted in profoundly depressed mechanical function throughout reperfusion (Figure 3A,B), with diastolic pressure elevated to ~40 mm Hg and only 40-50% recoveries of ventricular pressure development and ±dP/dt in placebo-treated healthy hearts. Coronary flow returned to ~75% of baseline, with impaired mechanical recovery in T2DM hearts not apparently stemming from reduced reflow (Figure 3C,D); the modest differences in postischemic flow correlating with (likely reflecting) effects of T2DM and morphine on diastolic dysfunction (vascular compression) and contractile work. Interestingly, T2DM did not influence diastolic dysfunction (Figure 3A), peak contracture or contracture time (Figure 3E,F) during I-R. While morphine administration reduced the rate and extent of contracture in controls (Ctrl + M), T2DM selectively negated this effect on ex vivo contracture (Figure 3E,F). The reduced recovery of left ventricular developed pressure (LVDP) in T2DM + P compared to Ctrl + P hearts (Figure 3B) thus reflects selective impairment of systolic function (Figure 3G). Morphine administration improved LVDP recoveries in both groups (Figure 3G). Recoveries for +dP/dt and −dP/dt were not significantly altered by T2DM and were improved by morphine in both groups, whereas recovery of coronary flow was unaltered by either T2DM or morphine (Figure 3G). To assess postischemic cellular injury (necrosis) LDH washout throughout reperfusion was measured. Placebo-treated T2DM hearts exhibited substantially exaggerated LDH release (by ~60%) when compared to Ctrl + P hearts (Figure 3H). Morphine treatment significantly reduced necrotic cell death in control (Ctrl + M) as well as in T2DM (T2DM + M) hearts. These data collectively suggest a primary impact of T2DM on postischemic cell death. T2DM increased necrotic cell death (LDH efflux) by >60% without altering diastolic dysfunction, and only moderately depressing postischemic pressure development. Although no changes in contractile function were detected in isolated T2DM hearts ex vivo, morphine treatment significantly reduced postischemic LDH release.

3.4 | Impact of morphine on myocardial stunning and ischemic preconditioning

While T2DM and morphine significantly modify cell death and contractile outcomes from prolonged ischemia, it is less clear whether the process of myocardial stunning–contractile dysfunction with transient sublethal ischemia–is modified. Functional recoveries from brief 5 min ischemic episodes in hearts from control and T2DM mice (Figure 4A,B) show that stunning is not modified in T2DM hearts, and that a significant anti-stunning effect of morphine apparent in healthy hearts is negated in T2DM hearts. The efficacy of ischemic preconditioning (IPC) was assessed in placebo- and morphine-treated Langendorff perfused hearts from Ctrl and

TABLE 1 Neither T2DM nor morphine altered baseline cardiac function in isolated hearts.

	Heart weight (mg)	Heart/Body Wt ratio	Intrinsic HR (bpm)	EDP (mm Hg	LVDP (mm Hg)	+dP/dt (mm Hg/s)	−dP/dt (mm Hg/s)	Coronary flow (mL/min)
Ctrl + P ($n = 12$)	180 ± 5	5.89 ± 0.17	341 ± 28	5 ± 1	126 ± 6	5196 ± 294	−2967 ± 145	2.7 ± 0.3
Ctrl + M ($n = 9$)	178 ± 5	6.41 ± 0.19†	363 ± 17	6 ± 1	128 ± 7	5168 ± 433	−3109 ± 108	3.1 ± 0.3
T2DM + P ($n = 9$)	187 ± 9	4.85 ± 0.16*	327 ± 6	5 ± 1	119 ± 5	5123 ± 277	−3120 ± 185	3.4 ± 0.3
T2DM + M ($n = 9$)	178 ± 6	5.66 ± 0.15†	303 ± 16	6 ± 1	119 ± 6	4960 ± 422	−2913 ± 183	4.3 ± 1.0

Note: Following T2DM and/or morphine exposure in mice, hearts were isolated and Langendorff-perfused. Heart weights and baseline functional parameters for ex vivo perfused hearts are represented in Table 1. Data are means ± SEM. *$P < .05$ T2DM vs respective Ctrl (nondiabetic); †$P < .05$ Morphine vs respective Placebo. EDP, end-diastolic pressure; HR, heart rate; LVDP, left ventricular developed pressure; +dP/dt, differential of ventricular pressure development relative to time; M, morphine; P, placebo.

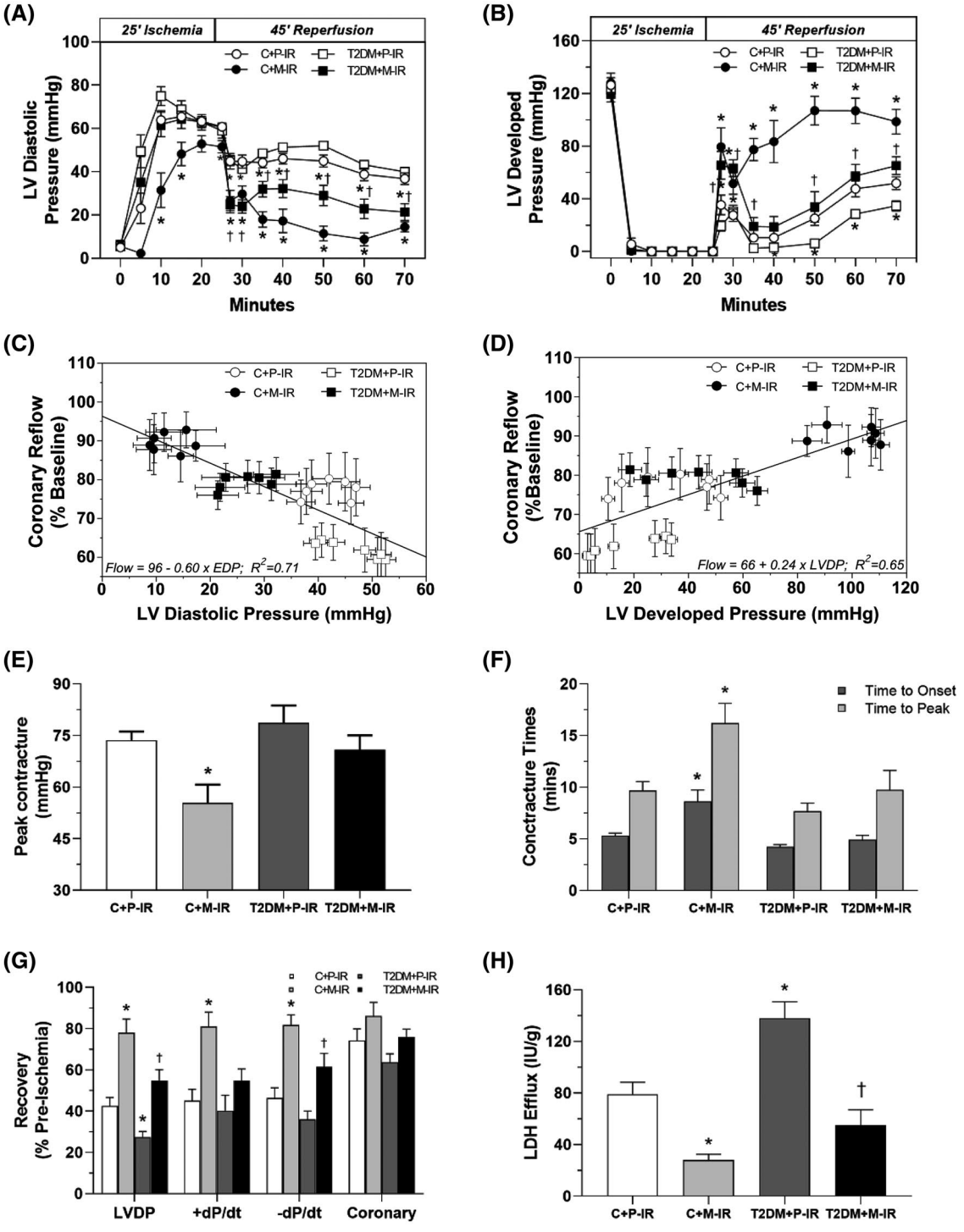

FIGURE 3 Effects of T2DM and morphine on myocardial functional, myocardial contracture, recovery, and cell death responses to I-R. Langendorff analysis detected changes in: A, Left ventricular (LV) end diastolic pressure; B, LV developed pressure throughout I-R; relationships between coronary reflow and postischemic recoveries of C, Left ventricular (LV) end diastolic pressure; D, LV developed pressure. Changes in ischemic contracture include: E, peak contracture development during ischemia; F, times to onset of (rise to 20 mm Hg) and peak contracture development. Also shown are: G, relative postischemic recoveries of contractile parameters and coronary flow; and H, total postischemic efflux of LDH. Data are means ± SEM, and analyzed with two-way ANOVA, followed by post hoc Tukey's multiple comparison tests. Data presented in Figure 3A,B were first analyzed using a three-way ANOVA, followed by two-way ANOVA, and post hoc Tukey's multiple comparisons test. $^{*}P < .05$ versus Control; $^{\dagger}P < .05$ vs respective untreated group. Group sizes are as follows: C+P-IR, $n = 17$ for all parameters except LDH where $n = 15$; C+M-IR, $n = 9$; T2DM+P-IR, $n = 18$ for all parameters except LDH where $n = 17$; T2DM+M-IR, $n = 8$

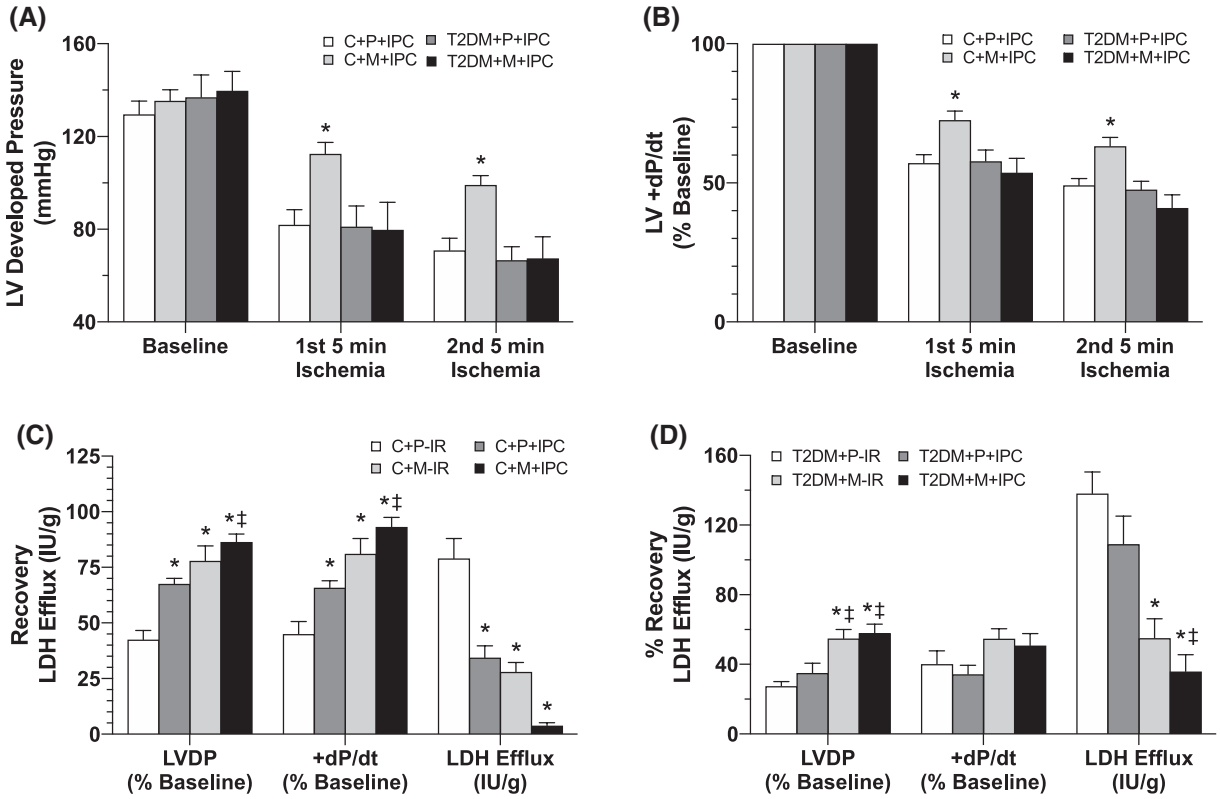

FIGURE 4 Effects of T2DM and morphine on myocardial stunning responses, and cardioprotection via ischemic preconditioning (IPC). Data are shown for recoveries following repeated transient (5 min) ischemic periods for: A, LVDP (mm Hg) and B, +dP/dt (% of baseline). Effects of IPC on relative recoveries of LVDP and +dP/dt, and postischemic efflux of LDH following 25 minutes ischemia are shown for: C, control hearts; and D, T2DM hearts (note differing scales for panels C and D). Data are presented as means ± SEM and analyzed using two-way ANOVA, with post hoc Tukey's multiple comparison tests. $^{*}P < .05$ vs Ctrl + P; $^{‡}P < .05$ versus IPC alone. Group sizes are as follows: C+P+IR, $n = 17$ for all parameters except LDH where $n = 15$; C+M-IR, $n = 9$; C+P+IPC, $n = 10$ for all parameters except LDH where $n = 9$; C+M+IPC, $n = 10$ for all parameters except LDH where $n = 8$; T2DM+P-IR, $n = 18$ for all parameters except LDH where $n = 17$; T2DM+M-IR, $n = 8$; T2DM+P+IPC, $n = 9$; T2DM+M+IPC, $n = 8$

T2DM mice. Induction of IPC significantly enhanced I-R tolerance in hearts from healthy mice, improving both mechanical recovery and LDH efflux (Figure 4C). There was also evidence of additive effects or synergy of IPC- and morphine-dependent protection in healthy hearts, with a greater decline in LDH efflux with morphine+IPC than either intervention alone (Figure 4C). In contrast, T2DM negated protection via IPC, which failed to influence function and LDH efflux in these diabetic hearts (Figure 4D). Once again, morphine significantly reduced LDH release in T2DM hearts; however, an additive effect of IPC and morphine was not detected. IPC failed to further modify I-R outcomes in hearts from T2DM mice treated with morphine.

3.5 | Effects of morphine treatment on T2DM cardiac mitochondrial ultrastructure, function, and biochemistry

Maintenance of physiological mitochondrial function and structure is critical for postischemic recovery of cardiac muscle. In T2DM, blunted mitochondrial plasticity and reduced mitochondrial function may accelerate organ dysfunction via increased production of reactive oxygen species.[41] We therefore assessed effects of T2DM on myocardial mitochondrial ultrastructure by TEM, and investigated cardiac myocyte morphology, mitochondrial localization/organization and cristae structure ($n = 3$ hearts per group), mitochondrial function (ie, oxygen consumption and Ca^{2+} tolerance), and biochemical changes.

At a magnification of 2,500×, cardiac mitochondria in T2DM+P hearts exhibited a "disorganized" and clustered appearance (Figure 5A, T2DM + P, arrows) when compared to mitochondria localized in lines along longitudinally cut sarcomeres ("pearls on a string appearance") in healthy hearts (Figure 5A, Ctrl + P, and Ctrl + M). Morphine treatment reversed the clustered mitochondrial appearance in T2DM hearts (Figure 5A, T2DM + M, arrows). Higher magnification (25,000×) revealed that cristae structure in T2DM + P lacked physical cohesion with the inner mitochondrial membrane, with indistinct membrane structure when compared to Ctrl + P (Figure 5B, T2DM + P, arrowheads, and asterisk).

Morphine treatment increased distinct cristae membrane formation in T2DM hearts and pronounced inner and outer mitochondrial membrane distinction (Figure 5B, T2DM + M, arrowheads, and asterisk). In general, T2DM + M cardiac mitochondria displayed "crisp" (distinct) cristae structure when compared to an indistinct ("fuzzy") cristae structure of placebo-treated T2DM hearts (Figure 5B, T2DM + P vs T2DM + M, asterisk). Mitochondrial as well as cristae number and area were analyzed in 2,500× and 25,000× scans as described in the Methods. We found that T2DM did not alter cardiac mitochondrial number but increased mitochondrial size (area) when compared to control hearts (Figure 5C +

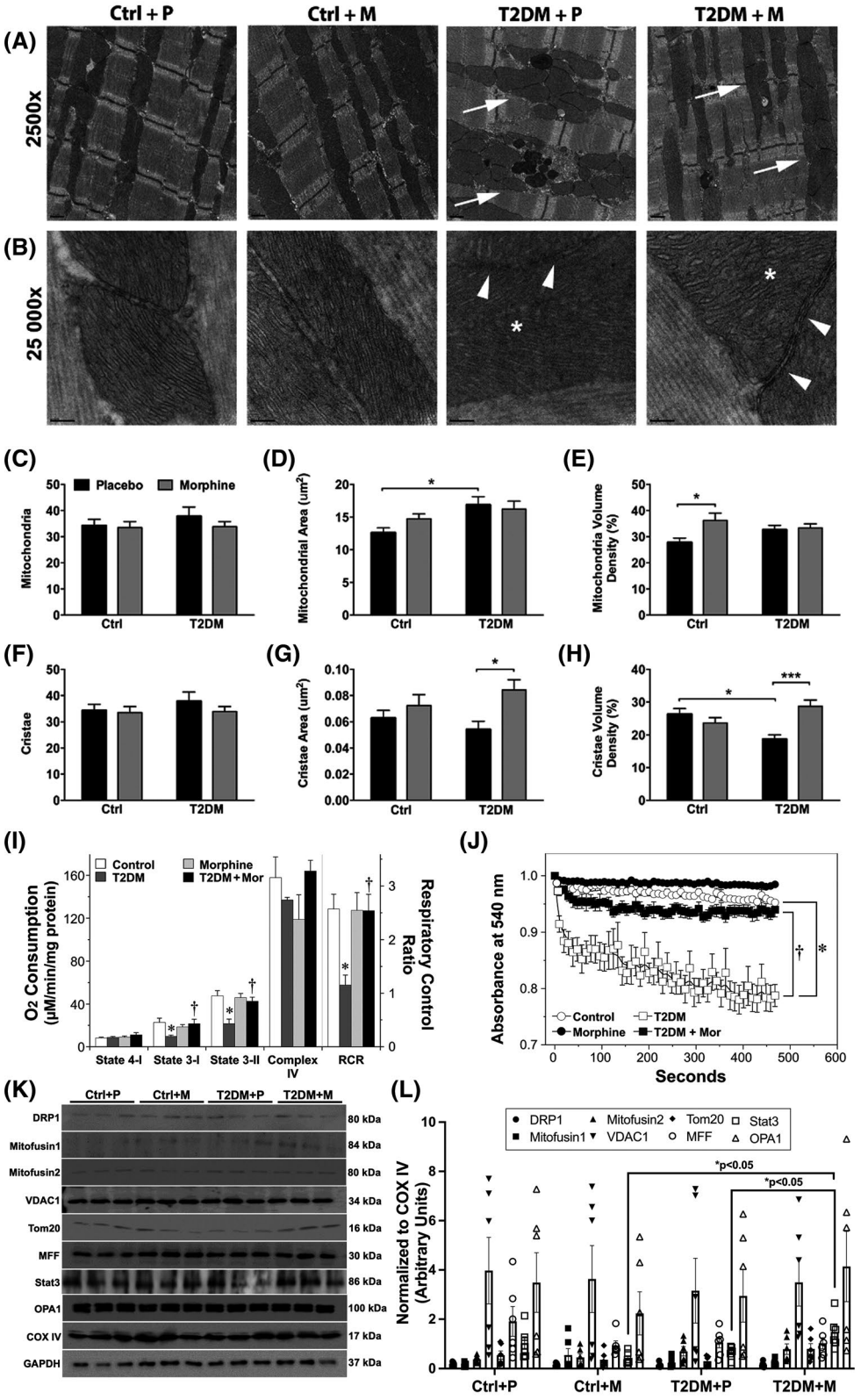

FIGURE 5 Morphine treatment improves cardiac mitochondrial ultrastructure, function, and biochemistry in T2DM hearts. Cardiac tissue from Ctrl and T2DM after placebo or morphine administration were assessed. A, Representative TEM images per group displaying mitochondrial morphology at 2,500×; and B, cristae morphology at 25,000× (n = 3 hearts per group, n = 15 images per animal and magnification). Arrows indicate clustered mitochondria and T2DM + P versus physiologically organized mitochondria in T2DM + M. Arrowheads point at mitochondrial membrane structure, and asterisk indicate cristae formation. Panels C-H show morphometric TEM analysis: C, Mitochondrial number, D, Mitochondrial area (size, μm^2), E, Mitochondrial volume density, F, Cristae number, G, Cristae area (size, μm^2), and H, Cristae volume density analysis. Data are presented as mean ± SEM; $*P < .05$; $**P < .01$; $***P < .001$, and analyzed with two-way ANOVA and Tukey's multiple comparison tests. Assessment of mitochondrial function was performed in purified cardiac mitochondria: I, Respiratory function; and J, Ca^{2+}-induced swelling. Data are presented as mean ± SEM; $*P < .05$; $**P < .01$; $***P < .001$, and analyzed with two-way ANOVA and Tukey's multiple comparison tests. Assessment of mitochondrial structural and signaling proteins was performed on whole heart lysates: K, Representative images of total heart lysates that underwent immunoblot analysis. L, Densitometry analysis showed that morphine administration did not alter the protein abundance of DRP1, mitofusin-1, mitofusin-2, VSDAC1, TOM20, MFF, OPA1, and GAPDH, when normalized to COX IV, but increased Stat3. Data are presented as mean ± SEM; $*P < .05$ with one-way ANOVA and Tukey's multiple comparison tests

D). Interestingly, total mitochondrial volume density was not altered in morphine-treated compared to placebo-treated T2DM hearts, while morphine increased mitochondrial volume density in healthy hearts (Ctrl + M) (Figure 5E). Five-day morphine treatment increased cristae area and cristae volume density in T2DM hearts, without altering cristae number (Figure 5F-H). The 20% fall in cristae volume density during T2DM was reversed by morphine treatment, while morphine did not modify cristae volume density in control hearts (Figure 5H). The observation that morphine itself augmented mitochondrial volume density only in healthy and not T2DM hearts suggests T2DM negates this beneficial opioid receptor effect on mitochondrial plasticity, whereas T2DM-dependent cristae disruption was specifically reversed by morphine treatment. These data reveal a profound remodeling of mitochondrial ultrastructure after morphine treatment.

The heart consumes more than five times its mass in ATP per day,[42] and ATP-generating mitochondria comprise nearly 30% of myocyte volume.[43] Cardiac mitochondria not only generate energy, but serve as major checkpoints for cell death and survival. Several studies provide evidence that mitochondrial dysfunction underlies diabetic complications.[8] We therefore assessed effects of sustained opioid receptor activation during T2DM on cardiac mitochondrial function. Oxygen consumption was measured in isolated mitochondria during sequential addition of substrates and inhibitors. Mitochondria from T2DM hearts exhibited reduced state 3 activities at complexes I and II, and evidence of uncoupling based on a reduced respiratory control ratio (RCR) and O_2 consumption (Figure 5I). Morphine selectively improved postischemic respiratory function in T2DM mitochondria, without any effect on healthy cardiac mitochondria (Figure 5I). Mitochondria also serve as high capacity sinks for Ca^{2+}, thus, buffering cytoplasmic Ca^{2+} and functioning as a sensor of cellular pathology.[44] Ischemic intolerance in T2DM myocardium was associated with exaggerated Ca^{2+}-induced mitochondrial swelling, an indicator of MPTP opening (Figure 5J). Morphine inhibited Ca^{2+}-induced swelling in both healthy and T2DM mitochondria (Figure 5J). Mitochondrial functional data (showing morphine counters T2DM effects on respiration and Ca^{2+}-induced swelling, without influencing these in otherwise healthy hearts) suggest differing mechanisms of benefit via morphine in T2DM vs healthy hearts.

Finally, we assessed changes in proteins related to mitochondrial structure, dynamics (ie, fusion and fission), and signaling in whole heart lysates. Between the four groups, we did not observe specific changes in mitochondrial fusion proteins (mitofusin-1, mitofusin-2, and OPA1), proteins involved in mitochondrial fission (DRP-1, and MFF), and mitochondrial-specific signaling proteins (VDAC1 and TOM20) (Figure 5K,L). However, we observed a significant increase in the protein abundance of signal transducer and activator of transcription 3 (Stat3) in the setting of T2DM with morphine treatment (Figure 5K,L). Taken together our data suggest that morphine in the setting of T2DM preserves mitochondrial ultrastructure, resulting in preserved cristae to likely improve mitochondrial O_2 consumption and Ca^{2+} tolerance. Though these effects do not appear to result from changes in mitochondrial structural and dynamic proteins, the improvement could be explained by upregulation of Stat3, specifically in the setting of diabetic stress and morphine treatment to impact mitochondrial signaling and function.

3.6 | Effects of morphine treatment on T2DM cardiac membrane dynamics

Recent data suggest the involvement of cholesterol rich microdomains and caveolins in modulation of cardioprotection in the setting of I-R injury.[45] We sought to determine the impact of morphine on sarcolemmal membrane biochemical changes in the setting of T2DM. Total heart lysates showed no change in total cholesterol (Figure 6A); however, when heart lysates were subjected to sucrose density fractionation to separate buoyant (fractions 4-6) from heavy, non-buoyant membranes (fractions 10-12), we observed that T2DM altered

membrane cholesterol distribution, and this was attenuated by morphine treatment (Figure 6B). We next determined if there were changes in caveolin protein expression relative to a stable membrane-associated protein, vinculin. We observed no change in the total expression (Figure 6C,D) or fractionated distribution (Figure 6E-H) of caveolin proteins (Cav1 and 3) critical in the formation of caveolae.

3.7 | Systemic and cardiac impact of morphine vs matched caloric restriction

Myocardial I-R tolerance correlated with body weight, blood [glucose], and glucose tolerance in healthy and T2DM mice. To further explore metabolic effects of morphine-associated reductions in caloric intake and their relevance to myocardial

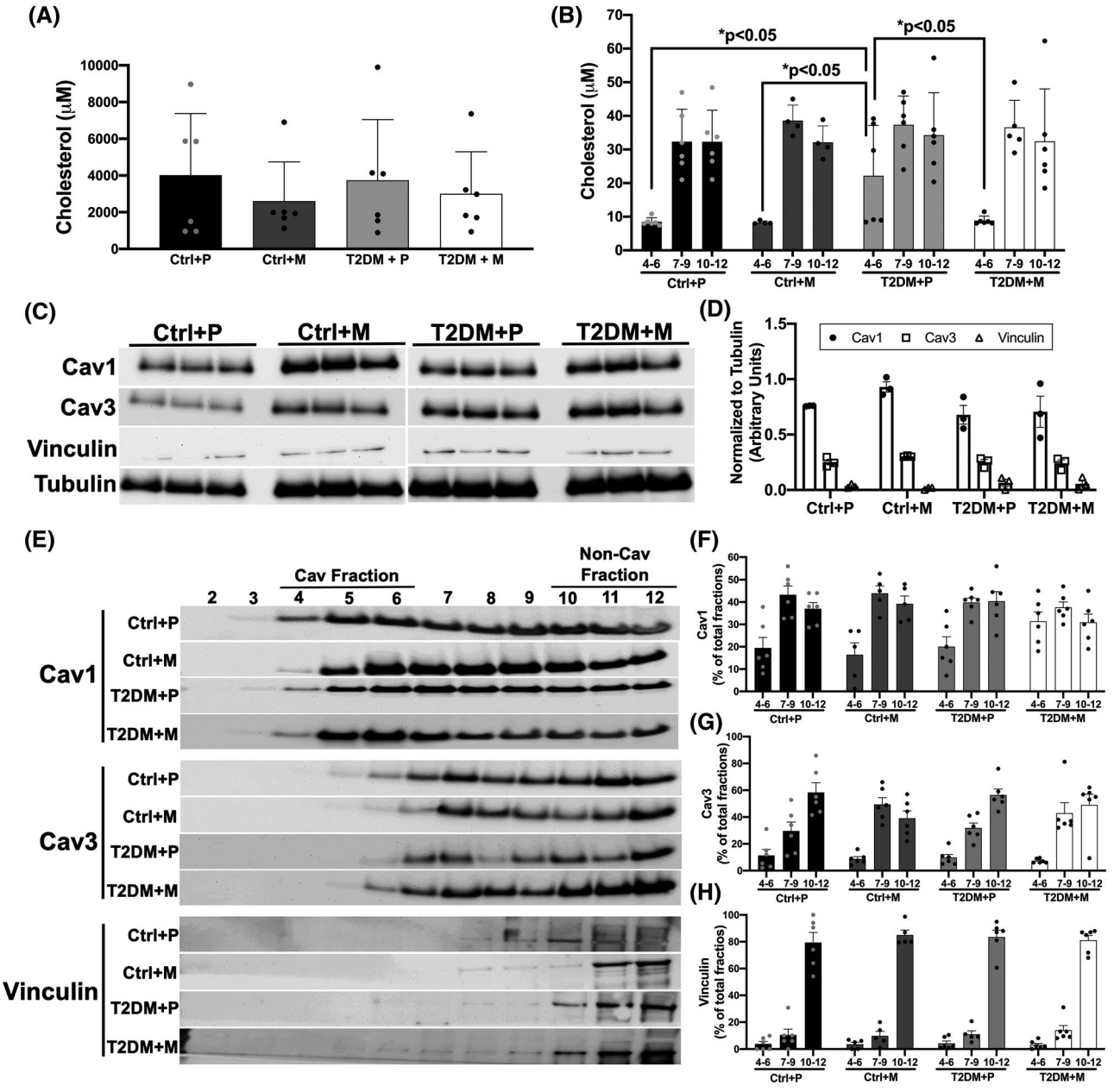

FIGURE 6 Morphine treatment preserves cardiac membrane dynamics in T2DM hearts. Cardiac tissue from Ctrl and T2DM after placebo or morphine administration were assessed. Total heart lysates or sucrose density fractions to separate buoyant, caveolar membranes from non-buoyant membranes were prepared and assessed for cholesterol and caveolin proteins. A, Total cholesterol in heart lysates. B,Cholesterol distribution in density fractions. C, Representative immunoblots of whole heart lysates for expression of caveolin (Cav1 and Cav3) and vinculin with densitometry analysis (D). E, Representative immunoblots of density fractions to determine distribution of Cav1, Cav3, and Vinculin. F-G, Densitometry analysis of distribution of Cav1, Cav3, and Vinculin, respectively. Data are presented as mean ± SEM; $^*P < .05$ with one-way ANOVA and Tukey's multiple comparison tests

changes, we monitored food intake and body weight in morphine-treated mice and pair-fed placebo-treated animals from the same group. Morphine was strongly anorexigenic in the initial 48 hrs, and reduced 5-day caloric intake by ~35% in healthy and ~80% in T2DM mice (Figure 7A,B). This was associated with 10% (~2 g) vs 20% (7-9 g) reductions in weight in healthy vs T2DM mice, respectively. Paired-feeding replicated morphine-induced weight loss in healthy and T2DM mice (Figure 7B), whereas reductions in blood glucose with morphine in healthy (30%) and T2DM mice (50-55%) were only partially replicated by paired feeding (15% and ~35%, respectively). We further assessed the effects of morphine-induced fasting, monitoring caloric efficiency, and glucose levels (Figure 7B) as well as cardiac outcomes (Figure 7C) with morphine, and compared outcomes with those arising from a paired-feeding regime in placebo-treated mice (matching daily caloric intake to the morphine-treated animals). We observed that pair feeding did not improve cardiac function in control nor T2DM mice (Figure 7C). Morphine-dependent improvements in glucose may thus involve both direct opioid receptor effects and benefit via caloric limitation. Thus, caloric limitation due to anorexigenic effects of morphine may contribute in part to cardioprotection in T2DM, but not improved ventricular ejection in vivo.

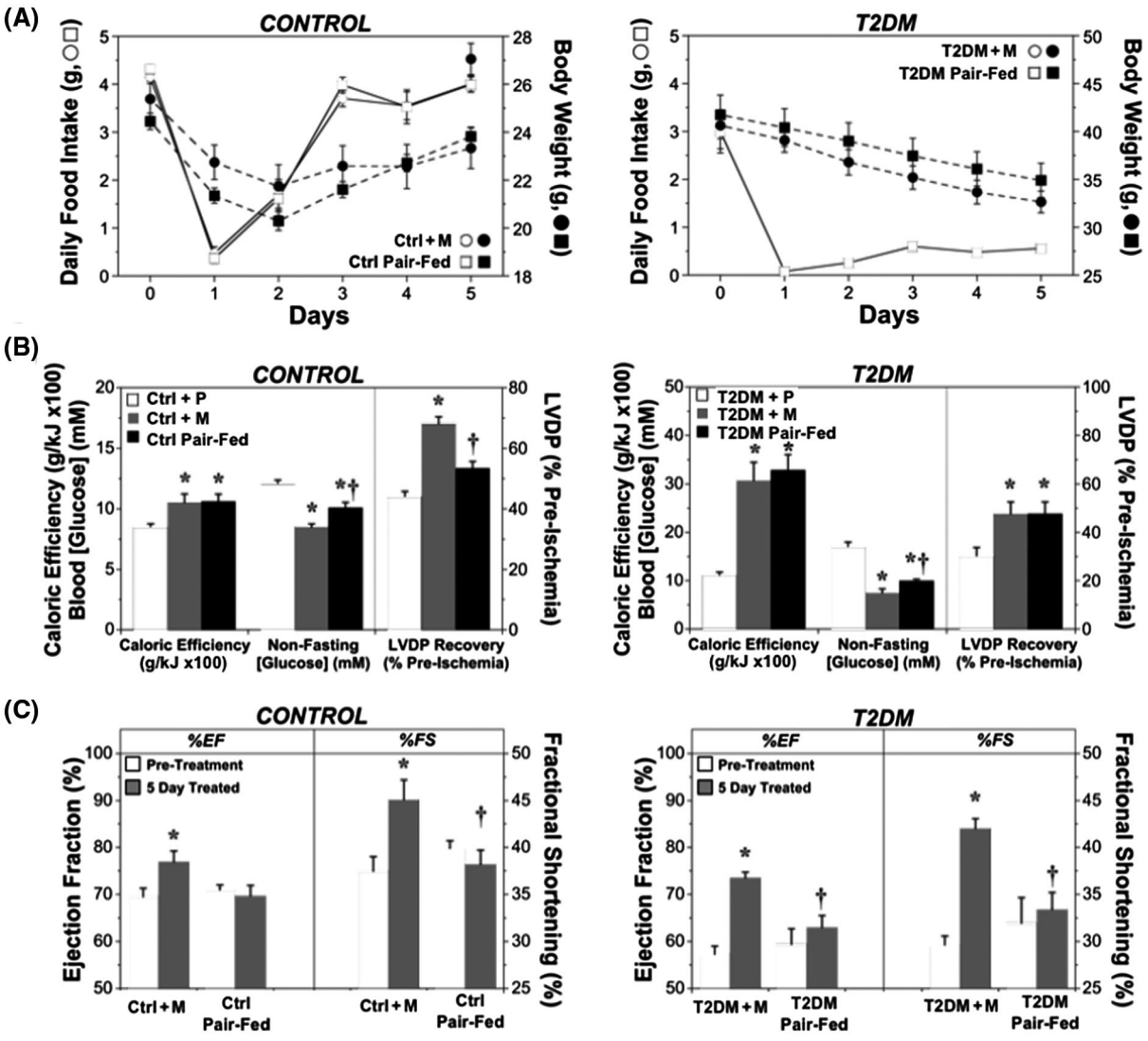

FIGURE 7 Morphine effects on food intake, body weight, and cardiac phenotype in healthy and T2DM mice compared to caloric restriction by pair feeding. Data are shown for morphine-treated and pair-fed Ctrl (left panels) and T2DM (right panels): A, Daily food intake and body weight; B, caloric efficiency, glucose levels, and cardiac I-R tolerance; and C, cardiac ejection parameters in vivo in controls and T2DM; for morphine-treated and pair-fed mice. Data are means ± SEM (n = 6-9) and analyzed with two-way ANOVA and Tukey's multiple comparison tests. *$P < .05$ versus Untreated; †$P < .05$ Pair-Fed versus Morphine

3.8 | Matched caloric intake by pair feeding did not alter mitochondrial ultrastructure in T2DM hearts

Since morphine therapy reduced body weight in association with improved cardiac function in T2DM mice, paired-feeding in non-morphine (placebo)-treated mice was assessed (Figure 7) and the effect of caloric restriction on myocardial ultrastructure analyzed by TEM (Figure 8). Pair-fed T2DM (T2DM + PF) hearts presented a mitochondrial disarray with mitochondria and cristae of variable size (Figure 8A,B). However, morphine improved mitochondrial arrangement (Figure 8A), inner and outer mitochondrial membrane separation, and cristae organization (Figure 8B) in T2DM mice. Thus, the impact on morphine-induced improved mitochondrial ultrastructure is independent of calorie restricted weight loss.

4 | DISCUSSION

We identify profound myocardial protection with morphine therapy in a murine T2DM model that exhibits cardiac depression, I-R intolerance, refractoriness to IPC, and abnormalities in mitochondrial ultrastructure and function. A 5-day period of opioid receptor agonism with morphine largely reversed this abnormal cardiac phenotype, countering cardiac depression, enhancing I-R tolerance, and improving mitochondrial ultrastructure and function, in association with improved systemic glucose homeostasis and body weight. Thus, our findings suggest morphine-specific effects on T2DM myocardial tissue independent of caloric restriction. Unraveling the basis of these "protective" morphine effects may reveal novel approaches to countering myocardial and systemic pathologies in T2DM.

We show that combining high-fat feeding with partial islet cell damage via STZ[32,33] induces features relevant to human disease, including impaired glucose tolerance, hyperglycemia, and hyperinsulinemia coupled with responsiveness to insulin administration–akin to serological findings in patients with T2DM. Elevated insulin levels in T2DM mice confirms that pancreatic β-cells, initially challenged by single-dose STZ, then, substantially increase their insulin production, a hallmark of T2DM compared to type I diabetes mellitus. Obese wild-type mice do not develop arteriosclerosis. Since diabetic cardiomyopathy is a heart muscle disorder, independent of coronary artery disease or hypertension, this mouse model is well suited to studying direct myocardial effects of obesity, hyperglycemia, and hyperinsulinemia.

While there is evidence that neither high-fat feeding nor hyperglycemia independently depress in vivo myocardial function,[46] the combination of these two factors (with obesity) in T2DM mice is clearly sufficient to depress cardiac systolic function. Despite clinical evidence of diabetic inhibition of cardiac stress-resistance,[11,12] findings in animal models (and translation to clinical benefit) are mixed.[12] Our data reveal T2DM not only suppresses function in hypertrophied T2DM heart, but also substantially reduces myocardial I-R tolerance, exaggerating cell death, and contractile and mitochondrial dysfunction, consistent with worsened I-R outcomes in diabetic patients.[11,12] Since contracture is unaltered, T2DM does not appear to influence the factors governing this

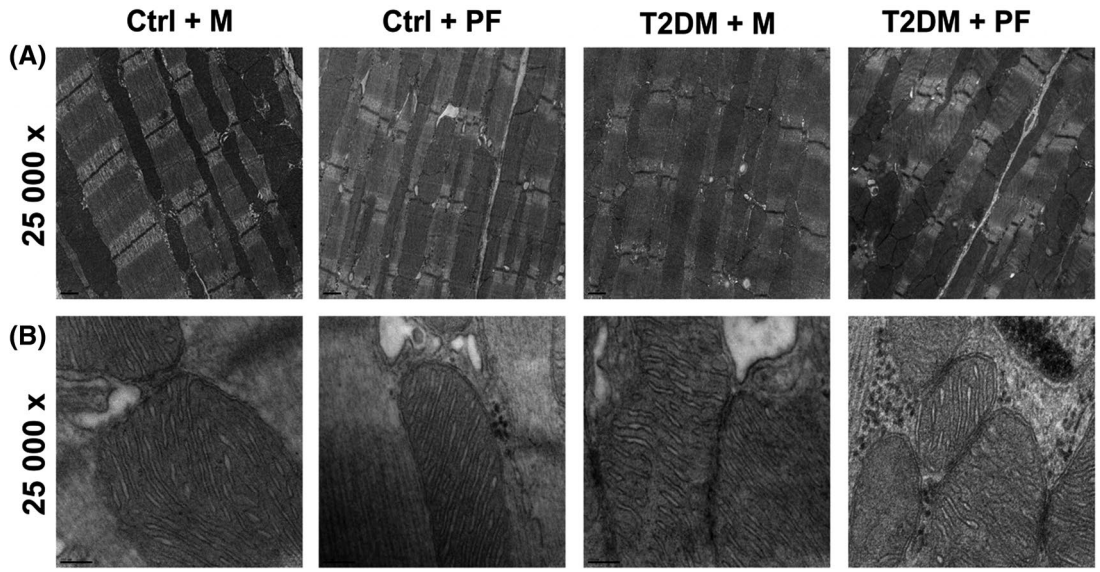

FIGURE 8 Pair feeding did not alter mitochondrial ultrastructure in T2DM hearts. We performed TEM analysis on cardiac tissue from Ctrl and T2DM after morphine administration or pair feeding (caloric restriction) during placebo administration. A, Representative TEM images per group displaying mitochondrial morphology at 2,500×; and B, mitochondrial structure at 25,000× (n = 5 hearts per group, n = 15 images per animal and magnification)

injury process during ischemia itself (ischemic Ca^{2+} overload and bioenergetic depression). However, exaggerated postischemic damage shows T2DM strongly influences determinants of reperfusion injury. This profound reduction in I-R tolerance was largely reversed by 5-day morphine treatment, implicating a morphine produced "preconditioning like effect," distinguishing this novel intervention from conventional opioidergic and other protective interventions that are impaired/negated in diabetes mellitus.[7]

Opioid receptor agonism has been shown to: (1) protect cardiac mitochondria,[14,47] (2) mediate cardiac protection via ischemic conditioning[28,30] and exercise,[29] (3) promote an anti-inflammatory cardiac phenotype,[31] and (4) improve systemic glucose homeostasis and cellular (albeit extracardiac) glucose uptake.[48-51] Improved glucose homeostasis with morphine is consistent with opioidergic enhancement of glucose handling in healthy[52] and obese/prediabetic rats,[18,50] together with systemic glucose dysregulation in opioid receptor knockout mice.[50] However, blood glucose is reportedly morphine-insensitive in healthy rats,[53] suggesting species-dependence of this action. Since morphine reduced circulating levels of both glucose and insulin, improved glucose homeostasis could involve augmented cellular uptake[48,51] and/or sensitization to lowered insulin. Indeed, opioids have been shown to promote glucose uptake in noncardiac cells via δ- and μ-opioid receptor sensitive Glut1/AMPK[51] and Glut4/PI3K[48] mechanisms, and can promote tissue insulin binding.[52]

Improvements in glucose homeostasis induced by morphine may also involve reduced caloric intake, since paired feeding partially replicates hypoglycemic effects of morphine. Chronic morphine induces anorexigenic and weight loss effects in healthy rodents,[18,53] though prominence of this effect with significantly reduced food intake was observed in obese T2DM mice. A preserved (or exaggerated) response in T2DM supports the postulated role for opioid receptors in inhibiting insulin-dependent growth,[50] with morphine significantly countering hyperinsulinemia. Why the anorexigenic response itself is more pronounced in T2DM animals (75-80% reduction in caloric intake) vs healthy animals (35% reduction) is unclear, though increased body fat in T2DM mice may present an effective energy store, facilitating feeding suppression. In any case, this pronounced effect suggests potential to selectively manipulate whole body metabolism in diabetes mellitus via opioid receptor-targeted interventions.

The observed metabolic effects of morphine raise a key question: to what extent does morphine-dependent anorexigenesis improve myocardial phenotype in T2DM? Defects in protein folding were shown to be at the core of physiological adaptations during T2DM,[54] and dietary restriction suppresses proteotoxicity in *C. elegans*.[55,56] However, we are unaware of studies of brief caloric restriction demonstrating acutely enhanced I-R tolerance and mitochondrial viability, and none have assessed cardiac effects of brief restriction in diabetes mellitus. Severe caloric restriction for 11 days generates unique I-R tolerance in nondiabetic rats,[57] while outcomes in the limited studies of calorie restriction in T2DM are mixed: ≥2 mo of 30% caloric restriction improves glucose homeostasis and markers of systemic or cardiac oxidative stress in rodent models of T2DM[58,59]; in contrast 30% caloric restriction for ~3 mo fails to influence I-R tolerance in models of T2DM and metabolic syndrome, though benefit via IPC was restored.[60] Opioid receptor effects on mitochondria together with "voluntary" dietary restriction may influence proteostasis (as presented by TEM), which is known to be compromised in the setting of T2DM. Although prolonged caloric limitation is a well-established protective stimulus, effects of brief or moderate fasting await detailed study in healthy and diabetic animals. Given this paucity of data, we studied paired-feeding, which did not influence in vivo or ex vivo contractile function yet improved I-R tolerance in T2DM (not healthy) hearts. Anorexigenic effects of opioid therapy together with sustained opioid receptor activation may thus contribute in part to cardioprotection in T2DM mice.

Mitochondrial abnormalities are considered centrally important to the cardiac dysfunction of T2DM.[6,8] The current data reinforce this notion, demonstrating significant disruption of mitochondrial ultrastructure and respiratory function in T2DM which likely contributes to a pro-death phenotype, postischemic Ca^{2+}-dependent mitochondrial swelling, and a propensity for mitochondrial permeability transition pore (MPTP) activation in T2DM. Conventional opioid responses confer cardiac protection via mitochondrial modulation in nondiabetic tissue,[14,47] with this receptor signaling reportedly impaired in diabetes mellitus.[15,16] Nonetheless, our data reveal a 5-day opioid stimulus, applied well after establishment of T2DM abnormalities, effectively reverses mitochondrial ultrastructural abnormalities, selectively improves respiratory function, and counters the increased propensity for mitochondrial swelling/MPTP activity in T2DM hearts. A select effect of morphine on mitochondrial volume density in Ctrl but not in T2DM hearts suggests reduced mitochondrial plasticity in the setting of T2DM. Conversely, cristae volume density was only significantly altered with morphine in T2DM mice, increasing cristae surface area and potentially respiratory capacity. The remarkable mitochondrial protection documented likely underpins beneficial cardiac effects of morphine in T2DM, though precisely how opioid therapy induces mitochondrial benefit remains to be established. Previous reports document that morphine protects the heart against I-R through inhibition of glycogen synthase kinase 3-beta (GSK3β), thus, improving MPTP opening.[61,62] Since HFD itself does not appear to modify respiratory function, and may in fact desensitize mitochondria to Ca^{2+}-dependent swelling[46] these mitochondrial impacts of T2DM may largely reflect hyperglycemic dysfunction. Further, unraveling the

mechanisms of mitochondrial protection and defining opioid subtype involvement may reveal novel, more targeted approaches to ameliorating myocardial and systemic sequelae of diabetes mellitus and protecting "at-risk" diabetic hearts.

When assessing proteins involved in mitochondrial structure, dynamics, and signaling, we do not observe changes in fusion/fission or structural proteins, but do observe potential involvement of Stat3 in regulating the morphine response to T2DM. A recent study showed that the protective effects of remifentanil, a short-acting μ-opioid receptor agonist used clinically, were dependent on caveolin-3 (Cav3) and Jak2/Stat3; however, protection was abrogated by hyperglycemia in an oxidant-dependent mechanism.[63] We show significant improvement of mitochondrial function as well as post-morphine treatment-induced Stat3 expression, suggesting possible coupling between these two observations in the molecular mechanism of morphine-induced protection in our T2DM model. Stat3 regulates a number of cellular responses, including intracellular communication, signal transduction, and gene transcription to impact mitochondrial function, as well as to regulate autophagy, apoptosis, and oxidative stress.[64] Involvement of Stat3 in downstream responses in diabetes, particularly with opioid treatment, will be important for future therapeutic considerations and exploration of novel areas of signaling.

Overall, we observe multiple beneficial outcomes encompassing improved lipid membrane structure/integrity at the sarcolemma and mitochondria. The sarcolemmal membrane may also be a target for physiological and pathophysiological regulation. Our group has advanced the hypothesis that membrane microdomains–caveolae–enriched in cholesterol, glycosphingolipids, and caveolin structural proteins, are critical to cardioprotection.[45] We do not see a direct effect of morphine in the setting of T2DM on caveolin protein expression and localization in buoyant fractions, suggesting the regulation of membrane microdomains may be more complex in disease settings and dependent on basal versus overexpression of caveolin proteins on subsequent stress response. Opioidergic protection was shown to involve translocation of caveolins to mitochondria,[65] as we have suggested for cardiac ischemic preconditioning.[37] Since we investigated whole heart lysates, we did not specifically explore distribution of caveolin to mitochondria, but will consider experiments on isolated cardiac mitochondria in future studies. However, as we have suggested previously,[66] the effect of long-term morphine treatment may involve non-caveolar mechanisms. Interestingly, we do see effects of morphine on membrane cholesterol stabilization in T2DM. Data are limited in the literature on impact of T2DM on cardiac membrane cholesterol; however, published results suggests that patients with T2DM have elevated levels of red blood cell membrane cholesterol that may predispose such individuals to cardiovascular risk.[67] We detect no change in total cholesterol, but see specific elevations in buoyant fractions in placebo-treated T2DM hearts that are attenuated by morphine treatment. This elevated cholesterol may impact specific signaling pathways which are protected by morphine, an area for future investigation.

Beneficial effects of morphine treatment have been shown in patients with heart failure.[25] It is also reported that breathing difficulties in patients with COPD are alleviated with morphine therapy.[26] These clinically valuable studies describe symptomatic relief with opioids in patients suffering different diseases, though underlying mechanisms remain unclear. Morphine side effects include (among others) central nervous system and respiratory depression, and gastrointestinal effects. The clinical safety and tolerability profile of morphine needs to be carefully evaluated and monitored by slow dose titration and sustained release formulations. Whether T2DM patients with chronic morphine treatment experience improvement in mitochondrial function and glycemic control is currently unknown. Our study suggests that beneficial effects of morphine on mitochondrial function and structure may confer benefit in other organ systems, though this promising theory requires further clinical and mechanistic exploration.

4.1 | Study limitations

A broad limitation of our study is that a single definitive mechanism has not been identified for this profoundly protective intervention (which may stem from both direct cardiac and indirect systemic/metabolic impacts of morphine). Nonetheless, data collectively reveal involvement of select mitochondrial protection, including preservation of structure, function, and signaling, and a suppression of MPTP activity. Moreover, since morphine was employed in this initial study, the identity of the receptor subtype(s) involved is not revealed (though the drug has much higher affinity for μ- versus δ- or κ-opioid receptors). Our prior work supports δ-opioid receptor involvement in chronic morphine-dependent cardioprotection in healthy mice.[17] In this regard, there are also potential differences in rodent versus human opioid receptor biology that may influence the translatability of the current findings. Finally, although we assessed feeding and body weight responses here, animal behavior and activity were not specifically measured. Morphine can also induce excitability in rodents,[68] and we observed increased activity in the initial 1-2 days of treatment. However, the relevance of such transient behavioral changes is unclear. Beneficial effects of long-term physical activity have been reported in mice with diet-induced obesity,[69] and 7 days of voluntary running can protect hearts of healthy mice,[70] while others show single forced exercise bouts improve cardiac NOS

signaling[71] and protein O-GlcNAcylation.[72] Whether 1-2 days of increased "voluntary" activity might mediate cardiac benefit in T2DM remains to be tested.

5 | CONCLUSIONS

Our study reveals powerful cardioprotection via sustained morphine therapy in a T2DM model exhibiting cardiac depression, I-R intolerance and mitochondrial dysfunction/disruption. Morphine therapy decreases voluntary food intake and body weight, improves glucose homeostasis, and effectively reversed T2DM-dependent cardiac abnormalities in association with improved mitochondrial function, structure, and signaling as well as impact on membrane lipids. Further work is warranted in delineating mechanisms underlying these myocardial and antidiabetic outcomes, which may involve both direct cardiac (mitochondrial modulation) and systemic changes (improved glucose homeostasis, reduced calorie intake). Our study shows that a 5-day-long opioid agonism potently improved cardiac function, mitochondrial abnormalities, and systemic glucose homeostasis in diabetic animals.

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CONFLICT OF INTEREST

The authors have no financial conflict of interest.

AUTHOR CONTRIBUTIONS

A.E. Zemljic-Harpf, L.E. See Hoe, J.N. Peart, H.H. Patel, and J.P. Headrick codesigned the experiments. A.E. Zemljic-Harpf performed in vivo physiological assessments including diabetes mellitus induction, GTT, ITT, serum analysis, and echocardiography, as well as ultrastructural image analysis. L.E. See Hoe, J.M. Schilling, and J.P. Headrick conducted ex vivo Langendorff experiments and data analysis. A. Nguyen and Y.J. Vaishnav performed statistical analysis of echocardiography, GTT, and ITT data. S.K. Mahata performed in vivo physiological assessments (GTT, ITT, and serum analysis), TEM perfusions and photography, and morphometric measurements on TEM scans. A.E. Zemljic-Harpf, H.H. Patel, and J.P. Headrick cowrote the manuscript. A.E. Zemljic-Harpf, J.P. Zuniga-Hertz, and G.J. Belza were involved in biochemical assays to fractionate the hearts and run immunoblots. W.H. Dillmann was involved in the design of the diabetic model. P.M. Patel, B.P. Head, W.H. Dillmann, J.N. Peart, and D.M. Roth contributed to scientific discussions and reviewed/edited the manuscript. H.H. Patel and J.P. Headrick are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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