PKC-mediated toxicity of elevated glucose concentration on cardiomyocyte function

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Sims MW, Winter J, Brennan S, Norman RI, Ng GA, Squire IB, Rainbow RD. PKC-mediated toxicity of elevated glucose concentration on cardiomyocyte function. Am J Physiol Heart Circ Physiol 307: H587–H597, 2014. First published June 20, 2014; doi:10.1152/ajpheart.00894.2013.—While it is well established that mortality risk after myocardial infarction (MI) increases in proportion to blood glucose concentration at the time of admission, it is unclear whether there is a direct, causal relationship. We investigated potential mechanisms by which increased blood glucose may exert cardiotoxicity. Using a Wistar rat or guinea-pig isolated cardiomyocyte model, we investigated the effects on cardiomyocyte function and electrical stability of alterations in extracellular glucose concentration. Contractile function studies using electric field stimulation (EFS), patch-clamp recording, and Ca2+ imaging were used to determine the effects of increased extracellular glucose concentration on cardiomyocyte function. Increasing glucose from 5 to 20 mM caused prolongation of the action potential and increased both basal Ca2+ and variability of the Ca2+ transient amplitude. Elevated extracellular glucose concentration also attenuated the protection afforded by ischemic preconditioning (IPc), as assessed using a simulated ischemia and reperfusion model. Inhibition of PKCα and β, using Gö6976 or specific inhibitor peptides, attenuated the detrimental effects of glucose and restored the cardioprotected phenotype to IPC cells. Increased glucose concentration did not attenuate the cardioprotective role of PKC, but rather activation of PKCα and β masked its beneficial effect. Elevated extracellular glucose concentration exerts acute cardiotoxicity mediated via PKCα and β. Inhibition of these PKC isoenzymes abolishes the cardiotoxic effects and restores IPC-mediated cardioprotection. These data support a direct link between hyperglycemia and adverse outcome after MI. Cardiac-specific PKCα and β inhibition may be of clinical benefit in this setting.

protein kinase C; hyperglycemia; glucose; arrhythmia; ischemic preconditioning

IN PATIENTS HOSPITALIZED WITH acute myocardial infarction (AMI), acute hyperglycemia is common and is associated with adverse outcome irrespective of a prior diagnosis of diabetes mellitus (DM) (19, 34, 39). In this setting, mortality risk increases in proportion to blood glucose concentration at admission (2, 19, 34, 39). Indeed, in many reports, for a given concentration of admission glucose, mortality risk is increased to a greater extent in patients without, compared with those with, an established diagnosis of DM (2, 4, 6, 12, 19, 34, 40). Furthermore, we have reported, in patients with ST-elevation MI, that admission blood glucose holds stronger association with risk of mortality than does the diagnosis of DM (10, 34).

It has been suggested that hyperglycemia after MI is simply a manifestation of the stress response to the event or a marker of the extent of myocardial damage. However, there are several mechanisms through which acute hyperglycemia may exert direct, potentially harmful, effects on myocardial function (3, 4) and, via protein kinase C (PKC)-mediated vasoconstriction (27), on vascular tone. In cardiac myocytes, elevated extracellular glucose concentration activates PKC (21, 33), including isoforms known to modulate cardiac calcium and potassium currents (8). Furthermore, we (27) and others (36) have demonstrated that PKC activation inhibits potassium currents in vascular smooth muscle, suggesting that high glucose may inhibit cardiac potassium currents via the same mechanism. These findings translate to human physiology where an increase in blood glucose from 5 to 15 mM causes QT interval prolongation (11). Such QT disturbances, which can occur in both hypo- and hyperglycemia, are proarrhythmic and have been associated with sudden cardiac death in patients with poorly controlled blood glucose (14, 23, 24).

Observations from clinical studies also support a possible causal link between blood glucose concentration at the time of MI and subsequent outcome. In particular, such observations indicate a link between persisting elevation of blood glucose after admission and adverse prognosis (4, 6, 12, 19) and, conversely, between effective glucose lowering and improved outcome (40). Most significantly, in randomized controlled trials of active management of elevated blood glucose after MI, prognosis was improved only when the intervention lowered blood glucose effectively (22).

Hyperglycemia may adversely influence cardioprotective stimuli, such as ischemic preconditioning (IPC), the process by which short nonlethal periods of myocardial ischemia and reperfusion result in reduced infarct size following a later, prolonged ischemic insult (26). While attenuation of IPC by hyperglycemia has been demonstrated in vivo (18), the mechanisms remain unclear. Cardioprotection can also be impaired by a number of pharmacological agents and volatile anesthetics, the latter being attenuated by hyperglycemia (16). Should IPC and other cardioprotective stimuli act through common pathways attenuated by hyperglycemia, this would provide a direct link between hyperglycemia at the point of MI and adverse prognosis.

The aim of the current study was to investigate the effects of alterations in extracellular glucose on contractile and electrical function of isolated cardiac ventricular myocytes. We assessed the effect on cardiomyocyte action potential and intracellular calcium concentration ([Ca2+]i) of changing extracellular glucose and the role of PKC in this context. Furthermore, we investigated the effects of extracellular glucose on the cardio-

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Our observations demonstrate clear, toxic effects of acute elevations of extracellular glucose concentration on cardiomyocyte contractile function, including perturbation of action potential duration (APD), Ca\(^{2+}\) homeostasis, and cardioprotection, mediated via specific PKC isoforms, PKC\(\alpha\) and \(\beta\). These data have potential clinical implications for the management of hyperglycemia in the context of AMI.

**METHODS**

**Solutions.** All extracellular solutions were based on a modified Tyrode's solution (29). The basic solution contained the following (in mM): 135 NaCl, 5 KCl, 0.33 NaH\(_2\)PO\(_4\), 5 Na-pyruvate, 10 HEPES, 1 MgCl\(_2\), and 2 CaCl\(_2\) (pH 7.4). Glucose was added to the solution as indicated in the text and is referred to as normal Tyrode's (NT) solution. Nominally Ca\(^{2+}\)-free Tyrode's solution used during cardiomyocyte isolation was as outlined above with no added Ca\(^{2+}\). Substrate-free 2 mM Ca\(^{2+}\) Tyrode's (SFT) contained the following (in mM): 140 NaCl, 5 KCl, 0.33 NaH\(_2\)PO\(_4\), 10 HEPES, 1 MgCl\(_2\), and 2 mM CaCl\(_2\) (pH 7.4). Sucrose was also added to the SFT solution to match the concentration of glucose in the NT solution (e.g., 10 mM glucose in NT = 10 mM sucrose in SFT).

The pipette solution for whole cell recording contained the following (in mM): 30 KOH, 5 EGTA, 110 KCl, 10 HEPES, 1 MgCl\(_2\), 1 ATP, 0.1 ADP, and 0.1 GTP (pH 7.2).

For all experiments, NT with 5 mM glucose was considered as the control glucose concentration as it is within the physiological fasting range.

**Isolation of ventricular myocytes.** Adult male Wistar rats (300–400 g), or Dunkin-Hartley guinea pigs (up to 500 g), were killed by stunning and cervical dislocation. The care and death of the animals conformed to the requirements of the UK Animals (Scientific Procedures) Act 1986. All procedures were approved by the University ethical review board. The protocol for isolation of cardiomyocytes was as described previously (15, 29). Briefly, the heart was rapidly excised and placed into cold, nominally Ca\(^{2+}\)-free NT solution. The heart was then cannulated via the aorta on a Langendorff type apparatus, and warmed Ca\(^{2+}\)-free NT (37°C) was perfused through the heart in a retrograde fashion for 6 min to clear residual blood. The solution was then exchanged for a Ca\(^{2+}\)-free NT with enzyme mix (containing 15 mg collagenase type II; Worthington), with 50 mg BSA prepared from factor V albumin and 18 mg protease (type XIV 15% Ca\(^{2+}\) in 30 ml; Sigma-Aldrich) for 8–15 min. Identification of rod shaped cardiomyocytes in the perfusate was used as an indication of digestion being complete. The solution was then exchanged for a 2 mM Ca\(^{2+}\) NT solution, the heart was cut down, and the cardiomyo-
cytes were mechanically dispersed from the tissue in a shaking water bath. Typically this method yielded 70–90% rod-shaped cardiomyocytes, which were stored in NT solution at room temperature and used within 18 h of isolating. IPC was imparted to cardiomyocytes using a protocol involving three cycles of halted perfusion followed by reperfusion, adapted from Rodrigo and Samani (31). IPC cardiomyocytes were used up to 6 h after isolation.

Simulated ischemia and reperfusion model. Cardiomyocytes were perfused at a rate of 5 ml/min at 32°C and stimulated to contract via 1-Hz electric field stimulation (EFS), and contractile function was observed via a JVC CCTV camera and recorded to DVD. For simulated ischemia and reperfusion (I/R) experiments, cardiomyocytes were perfused with NT solution containing glucose concentrations as indicated in the text for 2 min and substrate-free metabolic inhibition Tyrode’s solution (SFT-MI; containing 2 mM cyanide and 1 mM iodoacetic acid; Refs. 15, 30) for 7 min, followed by 10 min of washout (reperfusion) with NT. Analysis was performed on the video files recording contractile and morphological changes of cardiomyocytes. For experiments not using I/R, the number of contractile cardiomyocytes was counted every 30 s of the recording. The number of asynchronous contractions (contractions additional to the 1-Hz EFS) was also counted.

Whole heart Langendorff. Hearts were isolated from adult male guinea pigs (n = 9) and perfused in constant flow (15 ml/min) Langendorff mode with oxygenated Tyrode’s solution (37°C, pH 7.4) containing the following (in mM): 138 Na+, 4 K+, 124 Cl−, 24 HCO3−, 1.0 Mg2+, 1.6 Ca2+, and 0.4 H2PO4− with 5 or 20 mM glucose. Low glucose (5 mM) solutions were osmotically balanced to 20 mM by addition of mannitol (i.e., 5 mM glucose + 15 mM mannitol). Sodium octanoate (2.4 mM), a medium-chain fatty acid, was added to the perfusate to provide metabolic support (20). Left ventricular (LV) functional parameters and monophasic action potentials (MAPs) were recorded and analyzed as described previously (41). LV function and MAP duration (MAPD) was assessed during constant right intraventricular pacing at a cycle length of 250 ms. Analyzed data represent a mean average of 100 beats.

Patch-clamp electrophysiology. Action potential recordings were made from isolated cardiomyocytes using an Axopatch 200B amplifier, digitized using a Digidata 1440, and recorded and analyzed using pCLAMP 10.3 software (Axon Instruments, Scientifica, Uckfield UK) (29). Cardiomyocytes were perfused with NT solution containing glucose concentrations as indicated in the RESULTS. In current-clamp recording, action potentials were stimulated via the patch pipette with a 5-ms depolarizing trigger set to 130% of that required to stimulate an action potential (29). Action potential duration to 90% repolarized (APD90) and membrane potential were calculated within pCLAMP software.

Fura-2 measurement of [Ca2+]i. Cardiomyocytes were loaded with 5 mM fura-2 AM for 20 min at room temperature and allowed to settle for 5 min in a heated perfusion chamber before being washed with NT solution before the start of the experiment. Cardiomyocytes were stimulated to contract by 1 Hz EFS and illuminated with 340- and 380-nm wavelengths. Fluorescence emissions were captured above

![Fig. 2. Glucose-induced perturbation in electrical signaling in cardiomyocytes is attenuated by PKCα and β inhibition. A: example MAP traces in 5 and 20 mM glucose from guinea pig whole heart Langendorff recording perfused with 500 nM Gö6976. Aii: MAPD prolongation seen in Fig. 1A was attenuated by Gö6976 perfusion (n = 6; P < 0.05, ANOVA). B: example action potentials recorded from guinea pig isolated cardiomyocytes in 5 (black) and 20 mM glucose (gray) after pretreatment with 300 nM Gö6976. Histogram showing the mean APD90 prolongation (Bii) and depolarization (Biii) of membrane potential with increasing extracellular glucose was attenuated by Gö6976. C: as B in rat isolated cardiomyocytes preincubated with Gö6976 or with 100 nM cell permeant Tat-PK20-28 nonselective PKC inhibitor peptide (P < 0.01, ***P < 0.001, ANOVA with Bonferroni’s post hoc test; n = 8 cardiomyocytes from 3 animals for each species).](http://ajpheart.physiology.org/)

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520 nm using a Roper Cascade CCD camera. At the end of 3 min of the 5 mM glucose NT perfusion and 10 min of the 20 mM glucose NT perfusion, images were acquired for 30 s at a rate of 16 ratio images/s to measure single Ca^{2+} transients. Data are expressed as the ratio of the 340:380 nm signals.

**PKC inhibition.** Tat-linked PKC inhibitor peptides, soluble in NT solution, were used at a concentration of 100 nM as described previously (28). Isolated cardiomyocytes were preincubated for 15 min with Tat-linked peptides, or the Tat-peptide with no linked peptides as a control, before experimentation. PKCα and β were also pharmacologically inhibited in isolated cardiomyocytes and whole hearts using 300–500 nM Gö6976 made from a 1 mM stock in DMSO (Tocris, Bristol, UK). Preliminary investigations in whole heart recordings showed that an elevated concentration of the PKC inhibitor (500 nM Gö6976) was required to attenuate the prolongation in MAPD90. This may represent a difficulty in the compound gaining access to the cardiomyocytes in the intact preparation. Equivalent concentrations of DMSO had no effect on cardiomyocyte function.

**Confocal imaging of PKC localization in cardiomyocytes.** Freshly isolated cardiomyocytes were settled on coverslips in 24-well plates in either 5 or 20 mM glucose NT for 30 min. Cardiomyocytes were fixed using methanol at −20°C for 15 min and washed three times in PBS before incubating with PBST (PBS + 0.1% Triton-X 100 + 1% BSA) for 1 h at room temperature. Cells were then incubated in PBST overnight at 4°C with one of the anti-PKCα, PKCB, PKCα, or PKCε primary antibodies (BD Transduction Laboratories). Cells were then incubated for 1 h at room temperature with Alexa Fluor 488 goat anti-mouse Ig (Invitrogen). Mounted coverslips were then visualized using an Olympus FV500 laser scanning confocal inverted microscope at an excitation wavelength of 488 nm.

**Statistical analyses.** All patch-clamp data were analyzed using pCLAMP10 and Microsoft Excel 2010 software. Whole heart recordings were analyzed using the bundled Chart software (v8; ADInstruments, Oxford, UK), with the exception of the MAPD, which was analyzed using custom written software (NewMap; Dr. Francis Burton, Glasgow, UK). Calcium imaging data were analyzed using WinFluo3.4.9. (University of Strathclyde). Contractile function data were analyzed in Microsoft Excel. All figures were made in Graphpad Prism 6, and statistical analysis was performed in Prism 6 as indicated in the text.

**RESULTS**

Elevated extracellular glucose prolongs cardiac APD and is reversed by PKC inhibition. The effects of high glucose (20 mM) on LV function and electrophysiology were assessed in the isolated guinea pig heart. Perfusion in high glucose caused reversible prolongation of LV MAPD90, with a similar trend for MAPD50 (Fig. 1A; n = 6). Additional experiments (n = 3), completed without the inclusion of octanoate in the perfusate, demonstrated a similar increase in MAPD90 (96 ± 2 vs. 103 ± 3 ms; P < 0.05) and MAPD50 (113 ± 3 vs. 118 ± 3 ms; P < 0.01). Switching from low to high glucose had no effect on LV pressure (71 ± 4 vs. 71 ± 4 mmHg; NS), perfusion pressure (58 ± 4 vs. 59 ± 4 mmHg; NS), and either maximum (dP/dt max; 1,315 ± 84 vs. 1,316 ± 76 mmHg/s; NS) or minimum (dP/dt min; −840 ± 59 vs. −838 ± 54 mmHg/s; NS; n = 6) rates of change of LV pressure.

To further investigate the glucose-dependent perturbation of electrical signaling in single cells, APD90 was measured in cardiomyocytes isolated from guinea pig and rats perfused with increasing glucose concentrations. Compared with 5 mM glucose, APD90 was significantly prolonged in 15 and 20 mM glucose in both guinea pig (Fig. 1B) and rat (Fig. 1C) cardiomyocytes where there was also a glucose concentration-dependent depolarization of membrane potential in both species. These experiments were carried out in solutions balanced osmotically to 20 mM with mannitol. Removing mannitol had no influence on the deleterious effects of glucose, indicating an osmolarity-independent effect (data not shown).

The prolongation in MAPD or APD in 20 mM glucose was fully reversed by treatment of whole guinea pig hearts, or isolated cardiomyocytes, with the conventional PKC isoenzyme inhibitor Gö6976 (Fig. 2, A and B). Similar results were seen in rat cardiomyocytes using cell permeant Tat peptide-linked rat PKC isoenzyme–specific peptide inhibitors against PKCα and β or nonselective PKC20–28 peptide inhibitor (Fig. 2C). The membrane potential depolarization seen in both species with elevated glucose was also reversed by nonselective PKC inhibition in rat cardiomyocytes or by PKCα and β inhibition in both species.

To determine whether elevated glucose was able to cause PKCα and β to translocate (a surrogate measure of activation), the localization of PKC isoforms was investigated using confocal microscopy (Fig. 3). In 5 mM glucose NT, all PKC

![Fig. 3. PKCα and β translocate from the cytosol after incubation in 20 mM glucose. Example confocal images of isolated cardiomyocytes labeled with anti-PKCα (A), PKCβ (B), PKCδ (C), or PKCe (D) antibodies after 30 min of incubation in either 5 (i) or 20 (ii) mM glucose normal Tyrode’s (NT) solution. All isoforms appeared diffuse in the cytoplasm of the cell in 5 mM NT (Ai–Di). After a 30-min incubation with 20 mM glucose NT, PKCα was predominantly located at the nucleus (Aii), while PKCβ was more diffuse with some longitudinal alignment along the length of the cell (Bii). PKCδ remained diffuse in the cytoplasm in 20 mM glucose (Cii), whereas PKCe (Dii), although predominantly cytoplasmic, showed some transverse alignment suggestive of association with the T-tubule network. Dotted white line on each image represents where the pixel profile was measured. Scale bar on each image = 10 μm.](http://ajpheart.physiology.org/)
isoforms investigated (PKCα, β, δ, and ε) were diffuse in the cytoplasm and not associated with any membranes or intracellular structures (Fig. 3, A–Di). After a 30-min incubation in 20 mM glucose NT, PKCα was located predominantly at the nuclei (Fig. 3Aii), whereas PKCβ appeared in a longitudinal striated pattern, suggestive of contractile filaments (Fig. 3Bii). PKCδ (Fig. 3Cii) remained diffuse while PKCε (Fig. 3Dii), although largely cytoplasmic, also showed some evidence of localizing with the T-tubule network after incubation in 20 mM glucose consistent with previous findings from our group on activation of PKCε (15).

To further investigate the PKC isoenzyme specificity of the glucose-induced perturbation in electrical activity, specific Tat-PKC inhibitor peptides were used in rat isolated cardiomyocytes. The APD90 response to a 1-Hz stimulation via the patch pipette was recorded in response to changing extracellular glucose concentration from 5 to 20 mM. Prolongation in 20 mM glucose was unaffected by pretreatment with 100 nM Tat-peptide alone. Again, pretreatment with Tat-PKCα and β inhibitor (Fig. 4Ai) but not Tat-PKCβII, γ, or ε attenuated APD90 prolongation in 20 mM glucose (Fig. 4, Bi and Bii). Inhibition of PKCε had severe effects on APD90 where electrical activity became very unstable in 20 mM glucose (Fig. 4, Aii and B; APD not measurable). Figure 4Bi shows the mean change in APD90 and Fig. 3Bii the change in membrane potential between 5 and 20 mM glucose. These data further confirm that PKCα and βI, but not PKCβII, γ, or ε, or unlinked Tat-peptide control, have a role in glucose-induced, PKC-mediated, membrane depolarization.

Data presented so far indicate that 20 mM extracellular glucose leads to electrical instability in isolated cardiomyocytes. Spontaneous contractions may occur due to early or delayed afterdepolarization or may be due to spontaneous Ca2+ release triggered by increased [Ca2+]i. To investigate the...
relationship between Ca\(^{2+}\) homeostasis and extracellular glucose concentration, cardiomyocytes were loaded with the ratiometric Ca\(^{2+}\) indicator fura-2 AM and perfused with 5 mM, followed by 20 mM, glucose. In 20 mM glucose without any PKC inhibitor, cardiomyocytes displayed disordered Ca\(^{2+}\) transients, showing asynchronous peaks in fluorescence, increase in basal [Ca\(^{2+}\)]\(_i\) (Fig. 5A), and marked variability in the amplitude of Ca\(^{2+}\) transients (Fig. 5B). Pretreatment of cardiomyocytes with Gö6976, or with Tat-PKC\(\alpha\) and \(\beta\) together, attenuated the increase in basal [Ca\(^{2+}\)]\(_i\), and the perturbations in Ca\(^{2+}\) transients.

**Elevated extracellular glucose promotes asynchronous contraction in isolated cardiomyocytes.** Whether elevated extracellular glucose was able to elicit asynchronous contractile activity was assessed. Cardiomyocytes were perfused at 32°C with NT containing 5 mM glucose for 5 min and 20 mM glucose for 10 min, returning to 5 mM NT for 10 min. Figure 6A shows an example recording of contractions using video edge detection from a single cardiomyocyte at the time points indicated in Fig. 6Ai. These example recordings show asynchronous contractions, determined as contractions additional to those elicited by 1-Hz EFS, and are recorded as an asynchronous event if the duration was longer than 2 s. The mean data for a number of cardiomyocytes are shown in Fig. 6Ai. During perfusion with 20 mM glucose, asynchronous contractions increased, concurrent with reduction in the number of cardiomyocytes in rhythmic contraction with EFS. Both phenomena were markedly attenuated by pretreatment with Tat-PKC\(\alpha\) and \(\beta\) inhibitor peptides (Fig. 6B).

Specific Tat-PKC inhibitor peptides were used to confirm the isoenzyme specificity of the glucose-induced changes in contractile behavior. Tat-PKC20-28, \(\alpha\), \(\beta\)I, and Gö6976, but not Tat-PKC\(\beta\)II or unlinked Tat-peptide control, markedly attenuated the contractile failure and asynchronous contractions induced by 20 mM glucose (Fig. 6C). Pretreatment with Tat-PKCe inhibitor was devastating to cardiomyocytes in 20 mM glucose, where all entered hypercontracture (Fig. 6Bii and C). This effect was glucose concentration-dependent and was not evident after PKCe-inhibitor pretreatment in 5 mM glucose alone (Fig. 6Bii).

**Cardioprotection by IPC is restored in elevated extracellular glucose by inhibition of PKCa and \(\beta\).** Previous data from us (15), and others (1, 5, 7), suggest PKC activation to be important in the process of IPC. To determine the involvement of PKC in cardioprotection in our system, cardiomyocytes were treated with 1 \(\mu\)M phorbol 12-myristate 13-acetate (PMA) to directly activate PKC. With the use of a model of I/R on a whole heart Langendorff preparation, cardioprotection was imparted to cardiomyocytes and was maintained in the isolated cells for up to 6 h after enzymatic isolation (31). Cardioprotection was quantified using an I/R protocol, and PMA-treated cells exhibited a cardioprotected phenotype equivalent to IPC cardiomyocytes (Fig. 7B). Using Tat-PKC inhibitors, we confirmed that Tat-PKCe reduced the cardioprotection imparted by PMA stimulation or IPC (Fig. 7, A and B).

IPC-induced cardioprotection was attenuated by glucose in a concentration-dependent manner and abolished in 20 mM glucose (Fig. 8A). Pretreatment of IPC cardiomyocytes with PKCa and \(\beta\), or Gö6976, restored the proportion of cardiomyocytes regaining contractile function after the I/R protocol to control levels (Fig. 8B). These data show that cardioprotection

![Fig. 5. Inhibition of PKCa and \(\beta\) attenuate 20 mM glucose-induced disruption of intracellular Ca\(^{2+}\) homeostasis. Cardiomyocytes were stimulated to contract at 1 Hz and perfused continuously at 32°C with 5 mM glucose for 5 min and 20 mM glucose for 10 min. Recordings were made at the end of a 5-min perfusion with 5 mM glucose and the end of perfusion with 20 mM glucose where the acquisition rate was 32 frames/s (16 ratio images) for 30 s to record Ca\(^{2+}\) transients. A: example traces of Ca\(^{2+}\) transients recorded from 3 cardiomyocytes in control, 50 mM of both Tat-PKCa and \(\beta\) inhibitor peptides, or 300 nM Gö6976-treated cardiomyocytes. B: scatter-plot showing the variability in the peak of the Ca\(^{2+}\) transients as measured from the cardiomyocytes used in example traces (A). Transient amplitude was measured as the diastolic fura-2 ratio subtracted from the peak of fluorescence (F/F\(0\)). These data show that the amplitude of Ca\(^{2+}\)-transients were less defined in 20 mM glucose, manifesting as a reduced peak amplitude \([n = 6 (40 for each experiment)]; **P < 0.01, ANOVA with Bonferroni’s post hoc test]. Tat-PKCa and \(\beta\), or Gö6976, pretreatment attenuated this variability.

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imparted by IPC, lost in 20 mM glucose, was restored by inhibition of PKCα and β.

DISCUSSION

The current study demonstrates a number of deleterious effects of acute elevation of extracellular glucose concentration on cardiomyocyte function and electrical stability. First, and importantly, hyperglycemia prolonged cellular APD, increased [Ca\textsuperscript{2+}]\textsubscript{i}, and elicited spontaneous contraction of cardiomyocytes. Secondly, the time course of decay in numbers of contractile cardiomyocytes mirrored the increase in spontaneous contractions in high extracellular glucose. Thirdly, IPC-imparted cardioprotection was attenuated by increases in extracellular glucose concentrations. Finally, the deleterious effects of hyperglycemia were attenuated by inhibition of PKCα and β.

Cardiotoxicity of elevated glucose—clinical implications. While there is unequivocal evidence of an association between hyperglycemia at the time of AMI and subsequent poor prognosis (12, 34, 37, 39), a causal relationship has not been established. Active management of hyperglycemia is not routine in the setting of AMI; indeed in both the UK (40) and North America (19) only a minority of patients with hyperglycemia at admission with AMI receive such treatment. While likely to be multifactorial, this phenomenon reflects doubts regarding the clinical benefit of active management of hyper-
Glycemia, arising from apparently conflicting results from randomized control trials (22). However, current clinical guidelines from the National Institute for Health and Care Excellence in the UK (26a), the European Society of Cardiology (35), and North America (9) all recommend consideration of active management of elevated glucose after AMI, in some circumstances, including significant hyperglycemia.

In this study, we provide evidence of a specific pathophysiological mechanism by which hyperglycemia adversely influences cardiomyocyte function. High extracellular glucose concentrations, in the range seen frequently in the context of AMI in humans (34), were directly toxic to, and promoted electrical instability in, cardiomyocytes in vitro. Such phenomena have been demonstrated in in vivo in humans in response to hyperglycemia (11) and may contribute to the early mortality associated with admission hyperglycemia.

Hyperglycemia at admission with AMI may be part of the stress response to the event or may reflect previously unidentified, chronic dysglycemia. While both points have merit, we suggest that published evidence indicates the relevance of acute hyperglycemia to outcomes after AMI. Active management of hyperglycemia is associated with improved outcome after AMI (32, 40), and prognosis is poor for patients in whom hyperglycemia persists over the 24 h after admission (12, 19). Importantly, in controlled trials of active management of hyperglycemia after AMI, the clinical benefit is apparent only when the intervention results in lowering of elevated blood glucose concentration (22).

Our experimental model demonstrates the clear deleterious effects on cardiomyocyte function of an acute increase in extracellular glucose. Moreover, these deleterious effects are maintained for the duration of exposure to “hyperglycemia.” If the elevated glucose (20 mM) is not washed off at the end of the 25-min experiment, then the proportion of cells able to contract to EFS remains constant at 47% compared with 81% in 5 mM glucose. Furthermore, if the extracellular glucose remains elevated for a prolonged period of time (6 h), then the deleterious effect also remains and can be attenuated with concurrent incubation with Gö6976 or Tat-PKCε (data not shown).

Our data are in keeping with the observation that after AMI in humans hyperglycemia is associated with adverse outcome irrespective of diabetes status. In our nondiabetic animal models, changes in extracellular glucose caused pronounced acute changes in cardiomyocyte function. Such observations may have implications for the development of pharmacological agents relevant to acute coronary syndromes, particularly in the context of elevated glucose.

Our data in the whole heart Langendorff recording demonstrated attenuation of a 20 mM glucose-induced MAPD pro-
longation. In these recordings, however, our preliminary investigations showed that PKCα and β inhibition with 300 nM Gö6976 was not sufficient to attenuate the effects of glucose but was achieved with 500 nM Gö6976. We cannot fully account for this disparity, but it may relate to the requirement for the compound to cross the glycocalyx and vascular endothelium. The concentration “seen” by the myocyte is not known.

These observations cast light on one mechanism by which acute hyperglycemia is likely to impact adversely on survival after AMI, exerting a deleterious effect on myocyte contractile function, at least in part through PKC activation. Previous reports demonstrated involvement of activation of specific PKC isoforms (PKCε) in cardioprotection afforded by ischemia or pharmacological agents (1, 7, 15). However, other studies have implicated hyperglycemia in activation of PKC with resultant detrimental effects on cardiac tissue (21, 33). Our data show a translocation of PKCα and β on incubation with 20 mM glucose, suggesting an activation of these isoforms with this concentration. There was no clear translocation of PKCβ or ε after treatment with 20 mM glucose. Thus it would appear that specific and distinct PKC isoforms are involved in both IPC and in the cardiotoxicity associated with hyperglycemia.

The mechanism behind such glucose-induced activation is unclear; however, it may be due to a shift in diacylglycerol (DAG) metabolism (25). A change in DAG, or DAG kinase, regulation may promote conventional PKC isoform activation in hyperglycemia providing a mechanism by which PKCα and β were activated in this study. While previous studies have hypothesized a role for PKCβ inhibition in improving revascularization after reperfusion in an animal model of diabetes (17), our data suggest PKCβ activation by hyperglycemia has a deleterious effect during the ischemic period.

Previous studies have hypothesized a role for elevated glucose in inhibition, or downregulation, of sarco(endo)plasmic reticulum Ca2+-ATPase (38). While we have not investigated this in our system, our data support these findings where an increased Ca2+ influx leads to an increased [Ca2+]; the removal of which is perhaps slowed by an increased PKCα and/or β activity. Such observations may have implications for the development of pharmacological agents relevant to acute coronary syndromes, particularly in the context of elevated glucose.

Cardioprotection by IPC is attenuated by elevated glucose and restored by inhibition of PKCα and β. All of the deleterious effects reported in this study were attenuated by specific inhibition of PKCα and β isoforms, which also restored IPC in 20 mM extracellular glucose. This suggests that glucose-induced modulation of signaling involves PKC isoforms other than those demonstrated to be cardioprotective. Furthermore, since inhibition of PKCα and β in 20 mM glucose restored cardioprotection, this suggests that the cardioprotection imparted by PKCε activation during IPC was largely unaffected by increased extracellular glucose but overridden by the activation of PKCα and β.
In summary, our findings provide a potential mechanistic link between hyperglycemia at the time of AMI and subsequent adverse prognosis. Elevated glucose leads to cardiomyocyte death and electrical instability before, and during, simulated ischemia in vitro, effects that are mediated via PKCα- and β-dependent mechanisms. Our data suggest specific PKCs to be potential therapeutic targets in the management of AMI and strengthen the case for the active management of elevated glucose in MI in human. Further experimental and clinical studies are merited of the cardiotoxic effects of hyperglycemia in AMI.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS


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