Cardioprotection of the aged rat heart by GSK-3β inhibitor is attenuated: age-related changes in mitochondrial permeability transition pore modulation

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Zhu J, Rebecchi MJ, Glass PS, Brink PR, Liu L. Cardioprotection of the aged rat heart by GSK-3β inhibitor is attenuated: age-related changes in mitochondrial permeability transition pore modulation. Am J Physiol Heart Circ Physiol 300: H922–H930, 2011. First published January 7, 2011; doi:10.1152/ajpheart.00860.2010.—It is well established that inhibition of glycogen synthase kinase (GSK)-3β in the young adult myocardium protects against ischemia-reperfusion (I/R) injury through inhibition of mitochondrial permeability transition pore (mPTP) opening. Here, we investigated age-associated differences in the ability of GSK-3β inhibitor [SB-216763 (SB)] to protect the heart and to modulate mPTP opening during I/R injury. Fischer 344 male rats were assigned from their respective young or old age groups. Animals were subjected to 30 min ischemia following 120 min reperfusion to determine myocardial infarction (MI) size in vivo. Ischemic tissues were collected 10 min after reperfusion for nicotinamide adenine dinucleotide (NAD+) measurements and immunoblotting. In parallel experiments, ventricular myocytes isolated from young or old rats were exposed to oxidative stress through generation of reactive oxygen species (ROS), and mPTP opening times were measured by using confocal microscopy. Our results showed that SB decreased MI in young SB-treated rats compared with young untreated I/R animals, whereas SB failed to significantly affect MI in the old animals. SB also significantly increased GSK-3β phosphorylation in young rats, but phosphorylation levels were already highly elevated in old control groups. There were no significant differences observed between SB-treated and untreated old animals. NAD+ levels were better maintained in young SB-treated animals compared with the young untreated group during I/R, but this relative improvement was not observed in old animals. SB also significantly prolonged the time to mPTP opening induced by ROS in young cardiomyocytes, but not in aged cardiomyocytes. These results demonstrate that this GSK-3β inhibitor fails to protect the aged myocardium in response to I/R injury or prevent mPTP opening following a rise in ROS and suggest that healthy aging alters mPTP regulation by GSK-3β.

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THE FIRST MINUTES OF REPERFUSION are critical for salvaging ischemic myocardium, but reperfusion also paradoxically worsens ischemic damage (37, 42). Ischemia-reperfusion (I/R) injury causes a wide array of functional and structural alterations in the affected cardiomyocytes and their mitochondria. In recent years, great interest has been devoted to the mitochondrial permeability transition pore (mPTP). Crompton and Costi (7) first postulated the possible involvement of the mPTP located in the cardiomitocondria during reoxygenation-injury. Griffiths and Halestrap (17) discovered that the mPTP remained closed during myocardial ischemia and only opened in the first few minutes of myocardial reperfusion (17). It is now recognized that formation and opening of mPTP and resulting mitochondrial dysfunction are the major determinants of cardiomyocyte death following an episode of I/R injury (8, 9, 20, 22, 23, 43).

Glycogen synthase kinase-3β (GSK-3β) is a constitutively active Ser/Thr protein kinase, the function of which is regulated by phosphorylation, intracellular translocation, and complex formation with other proteins (6, 24). GSK-3β has recently received attention as a possible regulator of mPTP opening, since this kinase is a common target of multiple signal pathways that lead to myocardial protection from infarction. It has been demonstrated that GSK-3β inhibition provides a strategy for limiting myocardial infarction size at the time of myocardial reperfusion in pharmacological preconditioning, as well as ischemic preconditioning, and all of these interventions induce Ser19 phosphorylation of GSK-3β (19, 33, 40). Evidence for a regulatory role of GSK-3β in mPTP opening was first reported by Juhaszova et al. (25). They determined the threshold for opening of the mPTP by monitoring mitochondrial membrane potential (ΔΨm) in isolated cardiomyocytes and used ROS generated by laser irradiation of tetramethylrhodamine ethylester (TMRE) as a trigger to induce mPTP opening, which was shown to be regulated by GSK-3β activity. Other studies have demonstrated that pretreatment with GSK-3β inhibitor, SB-216763 (SB) or lithium, protected against infarction during early reperfusion via an mPTP-dependent mechanism (35, 40).

It is well known that, during the aging process, cardiomyocytes undergo complex changes that finally result in loss of contractile function and loss of endogenous protection against irreversible injury (3). In the aged myocardium, reductions in cellular cardioprotective reserves contribute to the diminished ability of the aged heart to respond and adapt to mechanical and oxidative stresses, rendering the aged heart more vulnerable to ischemic insult. Our previous studies and those of others have shown that the benefits of anesthetic preconditioning (APC) are significantly reduced by age in the rat I/R heart model (31, 39), as well as in human atrial myocytes (29). Our studies also have demonstrated that failure of cardioprotection by APC in the aged myocardium may be linked to apparently higher, constitutive levels of ROS in vivo. To our knowledge, no studies have reported on measures of GSK-3β effectives, mPTP opening, and their relationship to aging in vivo or in vitro. In this study, we used a specific inhibitor of GSK-3β, SB, to investigate 1) whether cardioprotection can be induced by inhibiting GSK-3β in the aging rat heart and 2) whether there are age-associated differences in modulation by GSK-3β of...
mPTP opening during I/R injury in vivo and mPTP opening induced by ROS in vitro.

MATERIALS AND METHODS

All experimental procedures and protocols used in this investigation were reviewed and approved by the Animal Care and Use Committee of the State University of New York Stony Brook. Furthermore, all procedures conformed to the *Guiding Principles in the Care and Use of Animals* of the American Physiological Society and were in accordance with the *Guide for the Care and Use of Laboratory Animals*.

**General preparation and surgery protocol.** Male Fischer 344 rats of the following age groups were obtained: 3–5 mo (240–300 g) and 20–24 mo (420–490 g) (Harlan Laboratories). Each group has seven rats. Animals were housed in the Division of Laboratory Animal Resources until the day of study. Anesthesia was induced with an intraperitoneal injection of sodium thiobutabarbital (120–135 mg/kg) (Sigma-Aldrich), with additional maintenance doses given as needed. Rats were tested for the absence of pedal reflexes throughout the experimental protocol to ensure adequate anesthesia. Heparin-filled (10 U/ml) (APP Pharmaceuticals) catheters were inserted in the right jugular vein for fluid and drug administration. The right carotid artery was cannulated to measure arterial blood pressure. A tracheotomy was performed, and the animal’s lungs were ventilated using a Harvard Apparatus model 638 rat ventilator with an air-and-oxygen (1:1) mixture and 5 cmH$_2$O of positive end-expiratory pressure. Inspired oxygen concentrations were maintained at 50%, and end-tidal carbon dioxide concentration was maintained at 35–40 mmHg by adjusting the respiratory rate or tidal volume throughout the experiment. Arterial blood gas tension and acid-base status were monitored at regular intervals and maintained within a normal range (pH, 7.35–7.45; PaCO$_2$, 30–40 mmHg; and PaO$_2$, 90–150 mmHg). End-tidal concentrations of carbon dioxide and inspired oxygen concentrations were measured using a Poet IQ2 infrared gas analyzer (Criticare Systems). Body temperature was maintained at 37.0 ± 0.2°C using a heating pad and radiant warmer. The surgery protocol followed our previous work (31). In brief, a 6–0 Prolene suture (Ethicon) was placed around the proximal left anterior descending (LAD) coronary artery. Coronary artery occlusion was produced by clamping the snare on the epicardial surface of the heart with a hemostat and was confirmed by the appearance of epicardial cyanosis. Reperfusion was achieved by loosening the snare and was verified by observing an epicardial hyperemic response. At the end of the experiment, the animal was euthanized with an overdose of sodium thiobutabarbital.

Our experimental design is illustrated in Fig. 1. Rats of similar age were randomly assigned to one of the groups. Protocol A was designed for infarction size measurements. In this protocol, all animals underwent 30 min of LAD coronary artery occlusion followed by 120 min of reperfusion. Protocol B was designed for nicotinamide adenine dinucleotide (NAD$^+$) measurements and GSK-3β Western immunoblotting analysis, since mPTP opening is observed during the early reperfusion. The ischemic area tissues were collected after 10 min of reperfusion. These left ventricular (LV) samples were immediately frozen in liquid nitrogen and stored in a freezer at −80°C for subsequent analysis. In the SB groups, SB (0.6 mg/kg) (Sigma-Aldrich) is administrated by intravenous injection 5 min before reperfusion. The dose of SB was chosen based on experimental data of Pagel et al. (35). Protocol C was designed for oxidative stress studies in isolated cardiomyocytes (25, 38).

**Myocardial infarction size experiment.** Myocardial infarction size was measured as previously described (31). Briefly, at the end of each experiment, the LAD coronary artery was reocluded, and patent blue dye was injected intravenously to stain the normal region of the left ventricle. The heart was rapidly excised, and the left ventricle was isolated. The LV area at risk (AAR) was separated from surrounding blue-stained normal areas, and the two regions were incubated at 37°C.
for 15 min in 1% 2,3,5-triphenyltetrazolium chloride in 0.1 M phospho-
buffer adjusted to a pH of 7.4. After overnight fix in 10% formalin (Poly Scientific), infiltrated (unstained) and noninfarcted (stained red) myocardium samples within the AAR were carefully
dissected, separated, and weighed. Infarct size was expressed as a
percentage of the LV AAR.

Western immunoblotting. In protocol B (Fig. 1), the rat heart tissues
were collected from rats 10 min after reperfusion; the LV samples
were homogenized using a Polytron homogenizer (Kinematica) in
ice-cold lysis buffer containing (in mM): 20 Tris-HCl (pH 7.4), 150
NaCl, 1 EDTA, 1 NaVO<sub>3</sub>, 1 NaF, 2.5 Na<sub>2</sub>PO<sub>4</sub>, 1% Nonidet P-40,
0.1% SDS, 1 mM diethiothreitol, 1 mM phenylmethylsulfonyl fluoride,
and a complete protease inhibitor cocktail (1 tablet/10 ml; Sigma-
Aldrich). The samples were then centrifuged at 13,000 g for 30 min at
4°C to isolate total soluble protein. The clarified supernatant was used to
quantify protein expression. Protein concentrations were determined
using the BCA Protein Assay Kit (Bio-Rad). Equivalent amounts (30 μg) of protein were mixed with 2X Laemmler buffer (Bio-Rad) and heated at 95°C for 5 min before electrophoretic
separation as described below. All samples were separated on a 10%
polyacrylamide gel and transferred (Trans-Blot SD Semi-Dry Trans-
fer Cell; Bio-Rad) to a polyvinylidene difluoride (PVDF) membrane.
After blocking with 5% nonfat dry milk in TBS containing 0.1%
Tween 20, PVDF membranes were incubated with the rabbit poly-
clonal anti-phospho-GSK-3β (Ser9) (dilution 1:1,000; Cell Signaling
Technology) at 4°C overnight. The primary antibody binding was
detected with a secondary anti-rabbit antibody (1:2,000; Bio-Rad) and
visualized with ECL (Amersham ECL Plus Western Blotting Detec-
tion Reagents; GE Healthcare, Buckinghamshire, UK). To determine
total GSK-3β, the membrane was stripped with restore stripping
buffer (100 mM β-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl,
PH 6.8) and reprobed with GSK-3β antibody (Cell Signaling
Technology). Quantitative analysis of the band densities from X-ray film
was performed using NIH ImageJ 1.43 (National Institutes of Health).
Band densities obtained from phosphorylated proteins were normal-
ized against the concentrations of total GSK-3β in the same samples.

Determination of NAD<sup>+</sup>, a marker of mPTP opening. In protocol B
(Fig. 1), the rats' heart tissues were collected after 10 min reperfusion.
NAD<sup>+</sup> was extracted from LV tissue using perchloric acid as previ-
ously described (10, 12). Briefly, NAD<sup>+</sup> was released from inactive and
dysfunctional mitochondria upon opening of the mPTP pore and was
washed out during reperfusion. Therefore, low concentrations of
NAD<sup>+</sup> in postischemic cardiac tissue indicate mPTP opening. For these
determinations, 30 μl of each frozen tissue sample were
powdered in liquid N<sub>2</sub> using a mortar and pestle and then thoroughly
mixed with 150 μl perchloric acid, 0.6 M. The mixture was then
homogenized and centrifuged at 13,000 g for 5 min. After being neutralized with 3 M potassium hydroxide, NAD<sup>+</sup> concentrations were determined fluorometrically using alcohol dehydrogenase activ-
ity (Sigma-Aldrich). Excitation was at 339 nm and emission wave-
length at 460 nm monitored in a Multi-frequency Phase Spectroflu-
orometer (ISS K2; ILC Technology).

Isolation of cardiomyocytes. Ventricular myocytes were obtained by
enzymatic dissociation as previously described (34). Briefly, rats were injected with heparin (3,000 U/kg ip; APP Pharmaceuticals) to
inhibit blood coagulation. Thirty minutes later, rats were killed by
overdose of sodium thiobutabarbital (250 mg ip; Sigma-Aldrich), and
the hearts, with major blood vessels attached, were removed. The
aorta of each animal was cannulated, and the heart was perfused ex
vivo for 15–20 min with a perfusion buffer containing (in mM): 140
NaCl, 5.4 KCl, 5 HEPES, 1.0 MgCl<sub>2</sub>, and 10 glucose with 50 μM
CaCl<sub>2</sub> (pH 7.4) gassed with 95% O<sub>2</sub>-5% CO<sub>2</sub>, at 37°C. The protocel
disperse II (0.1 mg/ml; Roche) and collagenase type 2 [331 U/mg
(Worthington) and 0.4 and 0.5 mg/ml for young and old rat hearts]
were added. After perfusion with the enzyme solution, the ventricles
were separated from the atria and minced in high-potassium substrate-
enriched buffer containing (in mM): 83 KCl, 30 K<sub>2</sub>HPO<sub>4</sub>, 5 MgSO<sub>4</sub>,
5 sodium pyruvic acid, 5 β-hydroxybutyric acid, 5 creatine, 20
taurine, 0.5 EGTA, 5 HEPES, and 10 glucose (pH 7.2, adjusted with
KOH). The supernatant was then decanted and filtered through 225
μm polyethylene mesh to remove large particles. The isolated cardi-
omyocytes were suspended in Tyrode buffer containing (in mM): 140
NaCl, 5.4 KCl, 5 HEPES, 1.0 MgCl<sub>2</sub>, and 10 glucose with 1.2 CaCl<sub>2</sub>
(pH 7.4). Only quiescent, rod-shaped myocytes with visible striations
and no visible membrane damage were used experimentally. As in
previous work, a decrease in myocyte yield with age was observed (13, 28). Following isolation, ventricular myocytes were then plated in
Tyrode buffer on laminin precoated glass-bottom dishes (In Vitro
Scientific). Myocytes were allowed to recover for 1 h and were used for
experiments within 5 h.

Opening of mPTP in cardiomyocytes. freshly isolated cardiomyo-
cytes were loaded with the fluorescent probe TMRE (100 nM) (Invitrogen) for 25 min at room temperature and then incubated with
SB (3 μM) for 15 min. On laser-illumination, TMRE generates ROS
within mitochondria, which leads to opening of mPTP (21). In some
experiments, after incubation with TMRE, adult rat myocytes were
loaded with calcein AM (1.0 μM) (Invitrogen) and cobalt chloride
(2.0 mM) (Sigma-Aldrich) for 15 min at room temperature. Calcein
AM is deesterified and distributed in mitochondria and cytosol, where
cytosolic calcine fluorescence is quenched by cobalt chloride so that
only the mitochondrial dye is visible. ROS scavenger Trolox (2 mM)
(Calbiochem) and mPTP inhibitor cyclosporin A (CSA, 0.5 μM
(Sigma-Aldrich) were used to determine the changes in TMRE and
calcine fluorescence that were due to ROS generation and mPTP
opening, respectively.

Confocal microscopy and image processing. Cardiomyocytes were
selected according to the criteria that they be rod shaped and free of
membrane blebs, which are associated with cell stress and imminent
cell death. Experiments were performed using a laser-scanning con-
focal microscope (Fluoview, FV1000; Olympus, Tokyo, Japan) and
×60 oil-immersion objective lens. Isolated cardiomyocytes were placed in a recording chamber on the stage of the confocal micro-
scope, and cells were allowed to settle for 10 min. GSK-3β inhibitor
SB (final concentration 3 μM) (14) was added 15 min before imaging.
All experiments were conducted at room temperature. The experimen-
tal protocol is shown in Fig. 1. For TMRE fluorescence, cells were
scanned with the 543-nm emission line of a HeNe laser. The emitted
fluorescence signal was collected at 590 nm. To stimulate the localized
production of ROS, selected regions of the myocyte (30 × 30 μm<sup>2</sup>)
were subjected to laser-induced oxidative stress that induced mPTP
opening during which the collapse of ΔΨ<sub>mt</sub> could be visualized (4), as
well as release of the fluorescent dye calcine (620 Da) from mito-
chondria (36). Calcine release was used to verify the opening of mPTP
independently from changes of ΔΨ<sub>mt</sub>. The mean calcine signal
decreased with time of illumination concomitant with the loss of
TMRE signal, indicating the opening of mPTP. Each region of
interest was scanned at 3-s intervals, and the pixel dwell time was 2 μs.
The image sequences (each 512 × 512 pixels) were used to
record changes in signal throughout. For all photosensitization
experiments, all settings of the confocal microscope (laser power,
confocal pinhole, and pixel dwell time) were identical to ensure
comparability between experiments. Images were analyzed using NIH
Image J 1.43. Intensity of a cell-free area was subtracted as back-
ground. After background subtraction, image series were corrected for
photobleaching by normalization to a predetermined monoeponential
decay that was calculated from changes in the average intensities for
the whole recording in the absence of mPTP opening. The peak
corrected signal recorded over the region (30 × 30 μm<sup>2</sup>) was
normalized to 100% and the lowest value to 0%. After normalization,
the time required for a 50% decrease in TMRE fluorescence emission
signal was calculated and denoted as mPTP opening (t<sub>50,ptp</sub>) (38).
Cardiomyocytes were isolated from at least five individual animals
from each age group, and the results of at least five cells from each
animal in each treatment group were analyzed.
Table 1. Hemodynamics

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Baseline</th>
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<th>10</th>
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<tr>
<td>YI/R</td>
<td>7</td>
<td>376 ± 27</td>
<td>372 ± 21</td>
<td>346 ± 27</td>
<td>322 ± 22</td>
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</tr>
<tr>
<td>YI/R + SB</td>
<td>7</td>
<td>372 ± 22</td>
<td>366 ± 21</td>
<td>342 ± 15</td>
<td>332 ± 15</td>
<td></td>
</tr>
<tr>
<td>OI/R</td>
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<td>349 ± 26</td>
<td>358 ± 23</td>
<td>341 ± 16</td>
<td>323 ± 35</td>
<td></td>
</tr>
<tr>
<td>OI/R + SB</td>
<td>7</td>
<td>357 ± 12</td>
<td>363 ± 27</td>
<td>350 ± 20</td>
<td>347 ± 31</td>
<td></td>
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<tr>
<td>YI/R</td>
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<td>125 ± 14</td>
<td>123 ± 12</td>
<td>83 ± 13</td>
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<tr>
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<td>99 ± 16</td>
<td>90 ± 13</td>
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<td>344 ± 18</td>
<td>328 ± 25</td>
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<tr>
<td>YI/R + SB</td>
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<td>331 ± 21</td>
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<td>OI/R</td>
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<tr>
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<td>142 ± 32</td>
<td>116 ± 27</td>
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</tr>
<tr>
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<td>7</td>
<td>135 ± 15</td>
<td>125 ± 26</td>
<td>117 ± 19</td>
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Data were means ± SD; n, no. of experiments. SB, SB-216763 (glycogen synthase kinase-3β inhibitor); HR, heart rate; I/R, ischemia-reperfusion; MAP, mean arterial blood pressure; OCC, occlusion coronary artery; O, old; SC, sham control; Y, young. *P < 0.05, significantly different from baseline within the same group.

Statistical analysis. Results are expressed as means ± SD. Two-way ANOVA with Student-Newman-Keuls posttest was performed on baseline hemodynamics, myocardial infarction size, Western blotting, and NAD⁺ and cardiomyocyte oxidative stress data. ANOVA for repeated measures was used to test for time-related differences in hemodynamics within groups. Differences of P < 0.05 were considered significant. Analyses were performed on Sigmastat (Version 3.5; Systat Software).

RESULTS

Ninety-three animals were instrumented to obtain 90 successful experiments, one old rat was excluded because of intractable ventricular arrhythmias, and two young rats were excluded because of severe bleeding. The ages, body weights, and AAR were similar within old and young groups.

Systemic hemodynamics. No differences in the baseline hemodynamics were observed among experimental groups (Table 1), and no differences were observed in mean arterial blood pressures (MBP) between the young SB and old SB groups. There were no differences in MBP and heart rate during coronary artery occlusion compared with their respective baseline period. In protocol A, all MBPs significantly (P < 0.05) decreased during reperfusion compared with the baseline. There was no significant difference in MBP or heart rate between the groups at the beginning or throughout the experiment in protocol B.

Myocardial infarct size. SB significantly reduced (P < 0.001) the infarct-to-AAR ratios in the young animals (YI/R + SB = 34.6 ± 5.5%) compared with the YI/R group (53.7 ± 7.3%). By contrast, there was no significant reduction in infarct/AAR in older animals (OI/R + SB = 44.8 ± 8.0%) vs. OI/R (51.3 ± 10.7%) (Fig. 2). AAR values were similar among all groups as reflected in the AAR/LV values among groups.

Phosphorylated Ser⁹-GSK-3β levels. The expression of GSK-3β and their phosphorylated forms pSer⁹-GSK-3β during myocardial ischemia and reperfusion with or without prior SB administration are shown in Fig. 3. All data are expressed as the ratios of phospho-GSK-3β/GSK-3β, and these ratios were normalized to young sham control (YSC). YI/R + SB increased this ratio (2.01 ± 0.22-fold) when compared with either the YSC groups (P < 0.001) or YI/R (1.14 ± 0.14-fold, P < 0.001). There was no difference in phospho-GSK-3β-to-GSK-3β ratios between YI/R and YSC groups. In old sham control rats, an increased level of phospho-GSK-3β/GSK-3β (2.13 ± 0.28-fold) relative to YSC (P < 0.001) was observed. SB, however, did not increase phospho-GSK-3β/GSK-3β levels in OI/R + SB groups (1.95 ± 0.19-fold) compared with the
old sham control (2.13 ± 0.28-fold) and OI/R (2.23 ± 0.31-fold) groups. Total GSK-3β levels were similar between young and old groups.

**NAD⁺ loss from the myocardium.** To determine mPTP opening, we measured AAR myocardial NAD⁺, which is released from damaged mitochondria upon opening of mPTP and subsequently washed out from cardiac tissue (12). In the young groups, SB clearly reduced the release of NAD⁺ from myocardial tissue, indicating inhibition of the mPTP (YI/R = 49.0 ± 7.9, YI/R + SB = 110.1 ± 17.5, *P < 0.001, unit = nmol/g tissue; Fig. 4). In contrast, in the old groups, NAD⁺ release and washout were not inhibited by SB (OI/R = 91.7 ± 13.2, OI/R + SB = 88.3 ± 3.0, *P > 0.05, unit = nmol/g tissue).

**Measurements of mPTP opening in vitro.** Opening of mPTP can be detected in intact cardiomyocytes by measuring permeability of the inner mitochondrial membrane to the fluorescent dye calcein (36). Figure 5A shows a typical recording of photoexcitation-induced dissipation of ΔΨₘ coincides with calcein leakage from the mitochondria. Fluorescence at 590 nm (TMRE) and between 515 and 525 nm (calcein) was recorded simultaneously from the same region. In the cytosol, calcein fluorescence was quenched by cobalt chloride. To investigate whether ROS formation was involved in the loss of ΔΨₘ and the role of mPTP, we examined the effect of the ROS scavenger Trolox and the mPTP inhibitor CsA. As shown in Fig. 5B, dissipation of ΔΨₘ was significantly delayed in the presence of 0.5 μM CsA or 2 mM Trolox.

Figure 4C shows a typical recording of TMRE fluorescence obtained from a 30 × 30-μm² region, in isolated cardiomyocytes, as assessed by confocal microscopy. ROS were rapidly produced from laser excitation of TMRE, and TMRE fluorescence at 590 nm was recorded as described in MATERIALS AND METHODS. SB prolonged the tₘPTP in the young rats in the setting of oxidative stress, which suggests an increase in the...
ROS threshold required to induce mPTP opening (young TMRE $279.6 \pm 50.2$, young TMRE + SB $438.0 \pm 70.0$, $P < 0.05$, unit = s). In contrast, SB lost the ability to inhibit mPTP opening in myocardiocytes isolated from old heart ventricles (old TMRE $207.6 \pm 71.5$ vs. old TMRE + SB $164.5 \pm 53.1$, $P > 0.05$, unit = s). The data are summarized in Fig. 5D.

**DISCUSSION**

As it is currently understood, myocardial aging is associated with reduced functional reserve and altered responsiveness of the heart to I/R injury, but the molecular basis for this deficiency has not been elucidated. The study presented here is the first, to our knowledge, to examine age-associated response differences in cardioprotection and mPTP modulation by an inhibitor of GSK-3β.

Experimental evidence suggests that both pharmacological and genetic treatments designed to prevent mPTP opening at the onset of myocardial reperfusion are capable of reducing myocardial infarct size by 30–50% (1, 2, 16, 30). GSK-3β is also critically involved in the fate of cells subjected to extracellular stress, including I/R (15, 19, 25, 32, 33, 41). This suggests that GSK-3β may play a central role in a final pathway of cardioprotection. Furthermore, previous work has suggested that effects of GSK-3β inhibition are primarily focused on the mitochondria and limited opening of mPTP (25), a putative end-effector that may be responsible for protection against ischemia reperfusion injury.

GSK-3β inhibitor SB is a potent, cell-permeable competitive inhibitor of the ATP-binding site of GSK-3β that, in turn, inhibits GSK-3β activity (5). Our study compared the in vivo effects of SB in young and old rat hearts. We found ~40%...
reduction in myocardial infarction size in young animals receiving SB compared with the young control group. In contrast, there was no reduction in myocardial infarction size in the old animals exposed to the same dose of SB and compared with their respective control group.

In the second part of our study, hearts were collected after 10 min reperfusion. We found that ratios of p-GSK-3β-to-GSK-3β in young animal hearts after SB treatment were increased ~50% compared with young control animals, whereas p-GSK-3β-to-GSK-3β ratios were not significantly elevated in aged rats after SB treatment, although higher p-GSK-3β-to-GSK-3β ratios were found in old control animals compared with the young sham controls. We suggest that the GSK-3β pathway is constitutively upmodulated in the old myocardium in vivo so that the GSK-3β inhibitor SB has no effect and also that mPTP regulation by SB is dysfunctional in the old rat heart. Indeed, our previous work showing constitutive upmodulation of the protein kinase B/GSK-3β pathway in aged myocardium supports this view (44). Because phospho-GSK-3β appears to be required for cardioprotection in the young animals (15, 18, 35, 40), our new results imply that a response pathway distal to GSK-3β is somehow desensitized in the older animals.

In our study, NAD+ was measured from whole tissue extracts in the different treatment protocols to assess indirectly mPTP opening in vivo. Di Lisa et al. (10) devised a method to determine the loss of mitochondrial NAD+ that accompanies reperfusion as a surrogate indicator of pore opening in vivo. Mitochondria represent the major stores of NAD+, possessing >90% of the total cellular content, and both mitochondrial and cytosolic NAD+ are lost during reperfusion. Therefore, NAD+ tissue content can be used as a surrogate indicator of mPTP pore opening (11). On the other hand, mitochondrial release of NAD+ per se may aggravate reperfusion damage because NAD+ becomes a substrate of the cytosolic glycohydrolase forming cyclic adenosine diphosphate-ribose and nicotinic acid adenine dinucleotide phosphate, which in turn promote Ca2+ release from the sarcoplasmic reticulum (26). Rat hearts collected in protocol B were obtained after 10 min reperfusion. SB administrated before reperfusion injury greatly reduced the loss of NAD+ in young rats compared with the young I/R group. However, NAD+ levels were not significantly changed in old rats after SB treatment compared with old untreated animals. These results suggest that SB reduces mPTP opening in young animals but not in old animals. Whether this reduced sensitivity of mPTP to modulation by GSK-3β inhibitor is the result of age-related changes in the mPTP itself or to other changes in mitochondrial function remains to be determined. Although the NAD+ levels in young and old hearts were the same, it is interesting to note that the amounts of NAD+ retained following reperfusion were somewhat greater in the old untreated hearts during I/R injury compared with the young untreated I/R group. The reason for this concentration difference is unclear.

The attenuation of pharmacological preconditioning in the aged myocardium may be attributed to multiple factors. Inhibition of the mPTP is a general mechanism of cardiomyocyte protection against I/R (20). It is possible that SB-induced phosphorylation of GSK-3β or inhibition of mPTP in the aged myocardium is not sufficient to trigger cardioprotection. In the former case, stress/survival pathways may already be maximally activated; therefore, protection against further damage may not be possible through this mechanism. Alternatively, GSK-3β kinase activity may be maximally inhibited in the untreated aged myocardium by the oxidative stress during the life time, thereby rendering GSK-3β incapable of further modulating the mPTP opening. Finally, the mPTP may be altered by aging so that GSK-3β is unable to downmodulate its opening, thereby rendering the old heart insensitive to SB.

The results of this study demonstrate that there is no significant protection by SB against myocardial I/R-induced changes in infarction size and inhibition of mPTP pore opening in the aged heart. These results may explain past difficulties in translating promising animal studies of cardioprotective efficacy into clinically applicable treatment strategies. It is well known that the aging myocardium is subjected to enhanced oxidative stress, which damages mitochondria (3). Indeed, we have previously reported high levels of ROS in the myocardium from aged rats (31). Oxidative damage to mitochondria in concert with mitochondrial calcium overload favors the onset of mPTP opening and subsequent release of cytochrome c (27). Hence, it is plausible that multiple defects in the mitochondria themselves accumulate during aging of the myocardium and may account for the lack of SB-induced myocardial protection. In our in vitro study, we used a cellular model of oxidative stress to study the mechanism of SB-induced delay of mPTP opening (25, 38, 45). SB inhibited mPTP opening in the setting of oxidative stress, represented by an increase in the ROS threshold required to induce mPTP opening. In contrast, SB lost its ability to inhibit mPTP opening in myocytes isolated from the aged ventricles. Our results suggested that mPTP pore opening in the young and aged cardiomyocytes responded differently to laser-induced ROS production. Whether this also involves changes in antioxidant reserves, including enzymes that clear these toxic metabolites, is not known. It should be pointed out that this in vitro model only simulates ROS production during the reperfusion of ischemic myocardium and may not include other contributors to mPTP opening in cardiomyocytes during reperfusion, particularly the increased influx of Ca2+.

It is important to note that we have not directly addressed causality in the relationship of cardioprotective mechanisms, aging, and mPTP and that, in the aged myocardium, this causality remains inferential. This study is also limited in that only one dose of SB was examined, which was selected based on an acute cardioprotective dose from a previous study (35); nonetheless, this dose was well within the effective ranges used previously to inhibit GSK-3β. Moreover, the possibility that this drug may have inhibited other protein kinases involved in myocardial protection cannot be completely excluded, although SB has previously been reported to selectively inhibit GSK-3β in vitro with little effect on activities of phosphatidylinositol 3-kinase and p70-S6 kinase, or multiple other protein kinases (5).

In conclusion, our findings demonstrate an aging-related loss of cardioprotection by SB in the rat myocardium. These in vivo results are consistent with a failure to reduce mPTP opening in cardiomyocytes isolated from old but not young hearts. These results suggest that mPTP regulation is dysfunctional in the aged myocardium and could account for loss of cardioprotec-
mitigation with aging. Dysfunctional regulation of mPTP appears to be the key to understanding how to protect the aged myocardium. Hopefully, future studies of mPTP and aging will lead to the development of improved protective therapeutic interventions that preserve I/R tolerance in the elderly.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES


