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The GSK-3 Inhibitor BIO Promotes Proliferation in Mammalian Cardiomyocytes

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Summary

The maintenance of self-renewal in stem cells appears to be distinct from the induction of proliferation of the terminally differentiated mammalian cardiomyocytes because it is believed that the latter are unable to divide. However, proliferation is a necessary step in both processes. Interestingly, the small molecule 6-bromoindirubin-3′-oxime (BIO) is the first pharmacological agent shown to maintain self-renewal in human and mouse embryonic stem cells. To determine whether a molecule that can maintain stem cell properties can also participate in controlling the proliferative capability of the highly differentiated cardiomyocytes, we examine the effect of BIO in postmitotic cardiac cells. Here, we show that BIO promotes proliferation in mammalian cardiomyocytes. Our demonstration of a second role for BIO suggests that the maintenance of stem cell self-renewal and the induction of proliferation in differentiated cardiomyocytes may share common molecular pathways.

Introduction

Heart disease results in the loss of cardiomyocytes and is a leading cause of death [1]. It has been a significant challenge to develop effective treatments for cardiac repair because adult mammalian cardiomyocytes are highly differentiated cells and have been believed to be unable to proliferate. Mammalian cardiomyocytes withdraw from the cell cycle soon after birth and have lowered levels of Cyclin A [2]. The fact that primary cardiac tumors rarely occur supports the notion that adult cardiomyocytes are highly restricted in their ability to divide. Because of its lack of proliferative potential, the primary response of the mammalian heart to injury is scar formation, which prevents cardiac repair. Thus, the loss of cardiomyocytes after damage caused by events such as myocardial infarction generally results in a compensatory response that is inadequate to restore function. Current therapies are also limited in their effectiveness. In order to sufficiently repair cardiac injury, it is necessary to provide a source of new cardiomyocytes.

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A major focus of potential therapeutic designs has been to identify and expand cardiac stem and/or progenitor cells, or to utilize known stem cell populations to generate functional cardiomyocytes [3, 4]. However, the difficulty in identifying cardiomyocytes derived from these transplanted sources in animal models raises the question of whether this approach will result in effective therapy. Alternatively, we have sought to identify pathways that could induce proliferation of cardiomyocytes as a potential route to restore cardiac function. Recently, our laboratory has shown that proliferation in differentiated rat cardiomyocytes can be achieved [5].

Interestingly, the maintenance of self-renewal in stem cells has generally been thought of as a process that is distinct from that of the induction of proliferation in postmitotic mammalian cardiomyocytes even though the requirement for proliferation is a necessary step in both events. Whether these two processes share common molecular pathways is an important question that has not been addressed by existing studies and has strong implications for future therapeutic approaches. The recently identified small molecule 6-bromoindirubin-3′-oxime (BIO) [6], a specific inhibitor of glycogen synthase kinase-3 (GSK-3), has been shown to maintain self-renewal and pluripotency in human and mouse embryonic stem cells (ESCs) [7]. Treatment of ESCs with BIO results in increased β-catenin activity, indicating that activation of canonical Wnt signaling promotes maintenance of stem cell properties [7]. The characterization of the function of BIO in ESCs has therefore allowed us to examine the question of whether a molecule that can maintain stem cell properties can also participate in controlling the proliferative capability of the highly differentiated cardiomyocyte. Here, we show that BIO promotes proliferation in mammalian cardiomyocytes. Stimulation of neonatal rat cardiomyocytes with BIO induced S phase entry and the upregulation of positive cell cycle regulators, while the level of the CDK inhibitor p27 was decreased. BIO treatment elevated β-catenin activity in cardiomyocytes, suggesting that the increase in proliferative ability may be due, in part, to the activation of the canonical Wnt pathway. In both neonatal and adult rat cardiomyocytes, BIO stimulation promoted cell cycle progression and increased the number of mitoses. Our demonstration of a second role for BIO suggests that the maintenance of stem cell self-renewal and the induction of proliferation in differentiated cardiomyocytes may share common molecular pathways.

Results

BIO Stimulates Neonatal Cardiomyocytes to Enter S Phase

To assess the ability of BIO to induce cell cycle entry in neonatal cardiomyocytes, cells were treated once with increasing concentrations of the small molecule for 48 hr and were pulse labeled with 5-bromo 2′-deoxy-uridine (BrdU) for the last 24 hr. BrdU can be incorporated during DNA synthesis in lieu of thymidine and thus has been used as a marker for S phase entry. To distinguish

cardiomyocytes from non-myocytes, cells were stained with an antibody to sarcomeric tropomyosin. Neonatal cardiomyocytes stimulated with the control compound dimethyl sulfoxide (DMSO) incorporated BrdU in 4.9% ± 1.9% (mean ± SD) of cells assayed, as detected by immunofluorescence staining. In the presence of 5 μM BIO, 51.7% ± 3.6% of cardiomyocytes assayed showed BrdU incorporation, representing an approximate 10-fold increase when compared to the control ($p = 0.0004$) (Figure 1A and Table S1; see the Supplemental Data available with this article online). The ability of BIO to stimulate BrdU incorporation was dosage dependent, and a maximum effect was seen at 5 μM, demonstrating that this effect is specific to the compound. At concentrations of 10 μM or greater, cell toxicity is observed, resulting in decreased BrdU incorporation. In addition, treatment of cells with 1-methyl-BIO (MeBIO), a kinase-inactive analog of BIO that does not inhibit GSK-3 activity [6], failed to induce BrdU incorporation in cells (Figure 1A). To further verify the specificity of BIO action, we also tested the ability of a cell-permeable and substrate-competitive peptide inhibitor of GSK-3 [8] to induce S phase entry. Cells treated with the GSK-3 peptide inhibitor and labeled with BrdU for 72 hr showed a 2-fold increase in BrdU incorporation (26.8% ± 6.7%) when compared to control (14.6% ± 2.2%, $p = 0.037$) (Figure 1C). Thus, the ability of BIO to promote S phase entry is dependent upon its inhibition of the kinase activity of GSK-3.

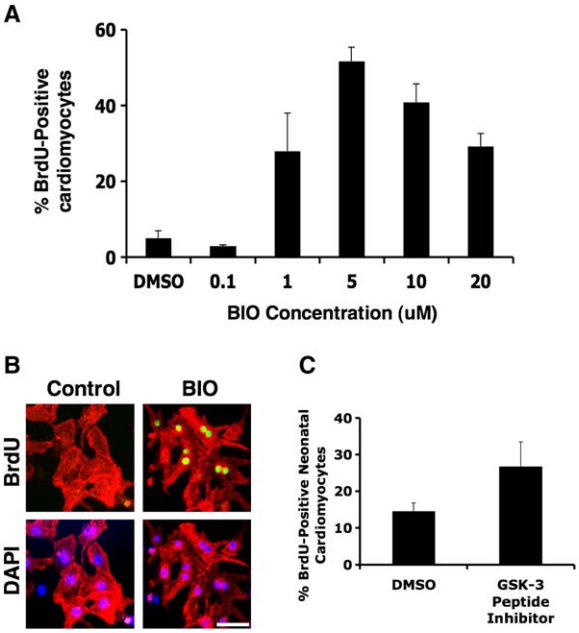

Figure 1. BIO Induces S Phase Entry in Neonatal Cardiomyocytes

(A) Neonatal cardiomyocytes were stimulated with either DMSO (control), MeBIO (kinase-inactive small molecule), or BIO for 48 hr, pulsed with BrdU from 24–48 hr, fixed, and stained to detect DNA synthesis. BIO stimulates BrdU incorporation in a dosage-dependent manner (mean ± SD). The optimal effect was achieved at 5 μM BIO and represents a 10-fold increase over the control.

(B) Stainings of neonatal cardiomyocytes treated with either DMSO (control) or 5 μM BIO in the same conditions as those described in (A) to detect DNA synthesis (anti-BrdU, green) and cardiomyocytes (anti-tropomyosin, red). DAPI (blue) stains nuclei. The scale bar is 50 μm.

(C) Neonatal cardiomyocytes were stimulated with either DMSO (control) or 20 μM GSK-3 peptide inhibitor and labeled with BrdU for 72 hr. Cells treated with the GSK-3 peptide inhibitor showed a 2-fold increase in BrdU incorporation when compared to control.

Stimulation with BIO Increases the Levels of Positive Cell Cycle Regulators in Neonatal Cardiomyocytes

To better understand the effect of BIO on neonatal cardiomyocyte proliferation in vitro, we assayed changes in protein levels of genes associated with cell proliferation by using immunofluoresence staining. As expected, stimulation of cells with MeBIO showed effects similar to control (Figure 2A). In contrast, the percentage of cells expressing high levels of Ki67, a proliferation marker [9], increased by 20-fold compared to control (1.7% ± 1.1%, $p = 0.01$) when stimulated with BIO (34.7% ± 13.8%) (Figure 2A).

Previous studies have shown that levels of cyclins are decreased in neonatal cardiomyocytes [5, 10]. In mouse fibroblasts, GSK-3β can directly phosphorylate Cyclin D1, leading to its degradation [11]. In addition, targeting of Cyclin D1 and CDK4 to the nucleus was sufficient to induce cell cycle entry in neonatal rat cardiomyocytes, and the expression of a dominant-negative form of GSK-3β in cardiomyocytes resulted in an increase in nuclear accumulation of Cyclin D1 [12]. Treatment with BIO increased the percentage of cardiomyocytes expressing Cyclin D1 by 13-fold (16.4% ± 1.8%) compared to control (1.3% ± 0.2%, $p = 0.0001$) (Figure 2A). Moreover, the increase in Cyclin D1 level was detected in the nucleus (Figure 2B). Similarly, Cyclin A mRNA and protein levels have been shown to rapidly decrease in rat hearts postbirth such that it is undetectable in adults [2, 13]. Treatment with BIO also increased the percentage of cells that show strong Cyclin A expression in cardiomyocytes by 13-fold (13.9% ± 1.6%) compared to control (1.1% ± 0.7%, $p = 0.0002$). The CDK inhibitor p27 is a negative regulator of cell cycle progression that is highly expressed in rat hearts postbirth and has been hypothesized to act as a block in cardiomyocyte cell cycle re-entry [14–16]. When treated with BIO, the number of cells that stain positively for p27 diminished by 22-fold (2.1% ± 1.7%) compared to control (47.2% ± 11.1%, $p = 0.002$) (Figure 2A). Together, our data suggest that BIO has the ability to promote cell cycle progression by increasing the levels of positive cell cycle regulators and decreasing the level of a negative effector.

Stimulation of Neonatal Cardiomyocytes with BIO Induces Cell Division

Our previous work has demonstrated that stimulation of neonatal cardiomyocytes with FGF1 is sufficient to induce S phase entry, but passage through mitosis and cytokinesis requires the concomitant inhibition of p38 kinase activity [5]. This result suggests that perhaps modulation of multiple pathways is needed to achieve proliferation. To determine whether BIO is sufficient to promote cell division, we assayed for G2/M progression by staining for phospho-Histone H3 (H3P), a marker of mitosis. BIO-stimulated cells (2.25% ± 0.74%) showed a 5-fold increase in the percentage of H3P-positive cells over control (0.42 ± 0.09; $p = 0.013$) (Figure 2C). We

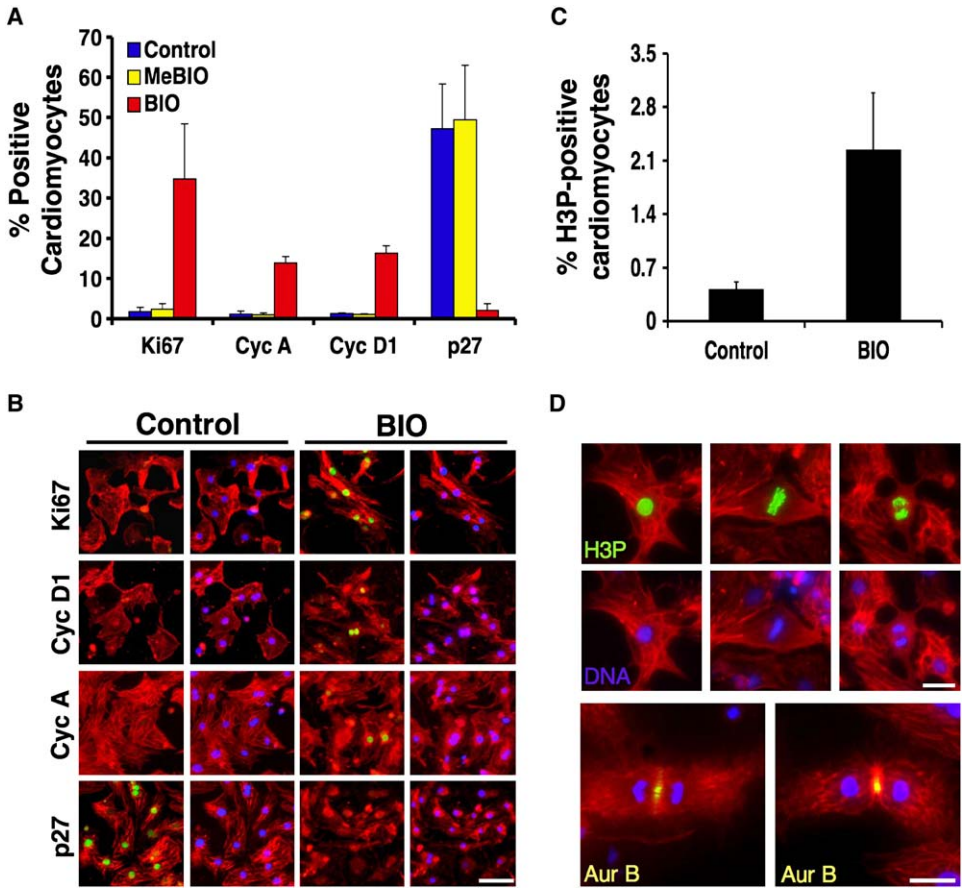

Figure 2. Stimulation of Cardiomyocytes by BIO Increased the Proliferative Potential of Cardiomyocytes

(A and B) BIO stimulation promotes cell cycle progression in neonatal cardiomyocytes. Neonatal cardiomyocytes were stimulated with DMSO (control), 5 μM MeBIO, or 5 μM BIO for 48 hr. (A) A greater percentage of cells that were stimulated with BIO showed highly elevated levels of Ki67, Cyclin A, and Cyclin D1 and decreased levels of the CDK inhibitor p27 when compared to control (mean ± SD). (B) Examples of cardiomyocytes stained with tropomyosin (red) or Troponin I (red) that show increased expression of Ki67, Cyclin D1, and Cyclin A (green) as compared to control. BIO-stimulated cells showed significantly lowered levels of p27 (green) expression. DAPI staining is shown in blue. The scale bar is 50 μm.

(C and D) Neonatal cardiomyocytes were stimulated with DMSO (control) or 5 μM BIO for 3 days and were assayed for karyokinesis. The scale bar is 20 μm. (C) Treatment with BIO showed a 5-fold increase in the number of mitoses when compared to control (mean ± SD). (D) Visualization of BIO-treated cardiomyocytes identified by tropomyosin staining (red) undergoing stages of mitosis marked by phospho-Histone H3 antibody (green), including prophase, metaphase, and anaphase. Cardiomyocytes undergoing cytokinesis after BIO treatment (tropomyosin, red; DAPI, blue). The cleavage furrow is marked by Aurora B staining (yellow).

observed BIO-treated cells undergoing all phases of mitosis, including prophase, metaphase, and anaphase (Figure 2D). To further confirm that BIO-treated cells are capable of cell division, we assayed for cytokinesis by performing immunostaining with anti-Aurora B (AurB) antibody [5]. AurB is a mitotic protein kinase that is involved in chromosome segregation and is essential for cytokinesis, during which it is located at the midbody [17]. AurB staining showed that BIO-stimulated cells formed contractile rings and can undergo proper cytokinesis (Figure 2D).

BIO Increases β-Catenin Activity in Cardiomyocytes

BIO has been shown to reduce GSK-3β kinase activity and thus signal through the canonical Wnt pathway by causing an increase in β-catenin levels [6]. Stimulation of the Wnt pathway induces cytoplasmic β-catenin to translocate to the nucleus, where it acts with T cell factor (Tcf) to regulate transcription of Wnt target genes [18]. In other systems, an increase in β-catenin levels, whether through mutational activation or overexpression, has been associated with elevated rates of proliferation [19]. To determine whether the same mechanism exists in neonatal cardiomyocytes, we stimulated cells with BIO and examined β-catenin levels. In control cells, β-catenin staining is weak and appears to concentrate at cell borders (Figure 3A). In contrast, BIO-stimulated cells show strong nuclear staining, suggesting that there is an increased level of β-catenin (Figure 3A). To determine whether the increase in nuclear accumulation of β-catenin led to an increase in functional activity, we performed luciferase assays by using a Tcf reporter as an indicator of β-catenin transcriptional activity. Neonatal cardiomyocytes were transfected either with a wild-type Tcf reporter carrying multimeric Tcf sites (TOPflash) or a mutant reporter carrying nonfunctional Tcf sites (FOPflash) [20]. Cells stimulated with BIO showed significantly higher reporter activity compared

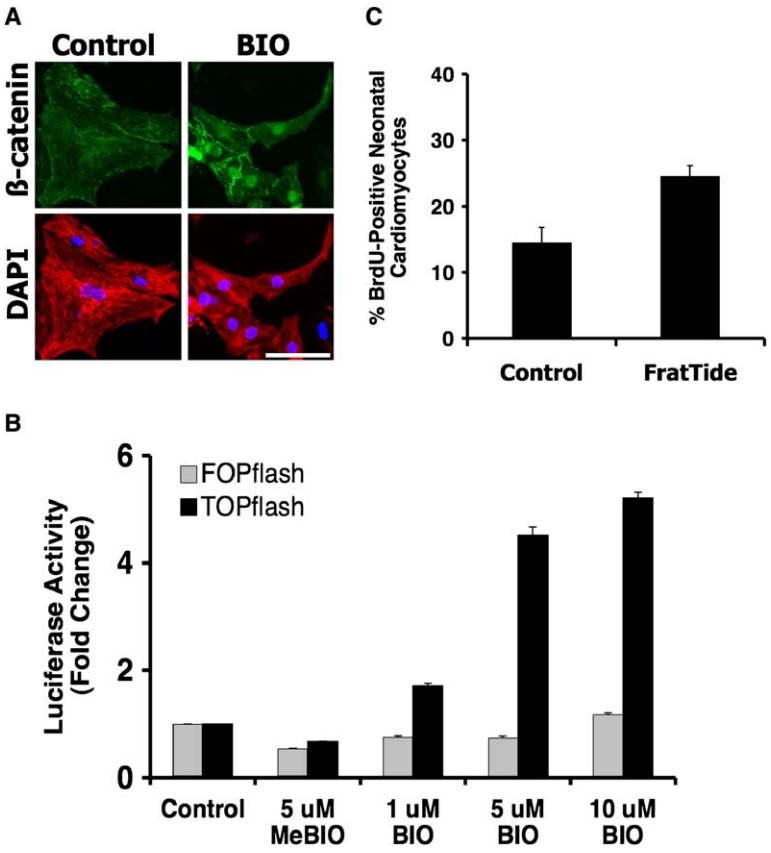

Figure 3. BIO Increased β-Catenin Activity in Neonatal Cardiomyocytes

(A–C) Neonatal cardiomyocytes were treated with either DMSO (control) or the indicated compound for 24 hr. (A) BIO treatment causes nuclear accumulation of β-catenin (green), which is not observed in control cells (tropomyosin, red; DAPI, blue). The scale bar is 50 μm. (B) Cells were assayed for β-catenin-mediated transcriptional activation by using a reporter containing either functional (TOPflash) or mutated (FOPflash) Tcf binding sites. Cells stimulated with increasing dosages of BIO showed a corresponding rise in the levels of β-catenin activity, as measured by luciferase activity (mean ± SD). Treatment with 5 μM BIO resulted in the highest fold change (almost 6-fold) when TOPflash activity was normalized against FOPflash activity. There was no significant difference in activities in the presence of the mutated FOPflash reporter. Cells treated with DMSO (control) or MeBIO did not have a significant effect in modulating β-catenin activity. (C) Cardiomyocytes were treated with either DMSO (control) or 20 μM FRATtide and were labeled with BrdU for 72 hr (mean ± SD).

to control ($p < 0.05$), indicative of increased β-catenin function (Figure 3B). Importantly, the increased activity was dosage dependent and was not observed in cells transfected with the mutant reporter, FOPflash.

Because elevated β-catenin activity can positively influence proliferation [19, 21], we examined whether increasing the β-catenin level would be sufficient to drive neonatal cardiomyocytes into cell cycle entry. FRATtide [22] is a peptide fragment corresponding to residues 188–226 of the gene named *frequently rearranged in advanced T cell lymphomas 1* (*FRAT1*). FRATtide binds to the carboxy terminus of GSK-3 and inhibits the ability of GSK-3 to phosphorylate and thus inactivate β-catenin [22, 23]. Interestingly, FRATtide does not appear to affect other phosphorylation substrates of GSK-3, such as glycogen synthase [22]. Cardiomyocytes treated with FRATtide and labeled with BrdU for 72 hr exhibited a BrdU incorporation rate of 24.6% ± 1.5%, representing an almost 2-fold increase over the control (14.6% ± 2.2%, $p = 0.0045$) (Figure 3C). Our results suggest that BIO stimulation elevates β-catenin activity in neonatal cardiomyocytes, and that this may account, in part, for the increased proliferative ability of the cells.

BIO Induces Adult Mammalian Cardiomyocytes to Dedifferentiate and Undergo Mitosis

In neonatal cardiomyocytes, the presence of BIO induces the cells to undergo cell cycle progression. Thus, we examined whether BIO has a similar effect in adult rat cardiomyocytes. To determine if BIO can induce S phase entry, adult cells were labeled with BrdU for 6 days. Cardiomyocytes were identified by their specific stainings for the cardiac contractile apparatus proteins tropomyosin or Troponin T. Control cells had a low rate of BrdU incorporation (1.2% ± 0.2%) after 6 days of BrdU labeling (Figure 4A). Treatment with MeBIO shows a comparably low rate. In contrast, stimulation of cardiomyocytes with 5 μM BIO increased the BrdU incorporation rate 10-fold to 13.1% ± 1.7% compared to the control ($p = 0.0003$). Similarly, results were observed after 9 days of labeling (data not shown). Thus, the stimulation of adult cardiomyocytes with BIO can induce S phase entry.

We performed H3P analyses to determine whether BIO-stimulated adult rat cardiomyocytes are able to undergo mitosis. After 6 days of culture, only 0.1% ± 0.1% of control cells stained positive for H3P. When stimulated with BIO, the percentage of H3P-positive cells increased to 2.41% ± 0.8% ($p = 0.007$) (Figure 4B). Furthermore, we observed adult cardiomyocytes at different stages of mitosis (Figure 4C). Importantly, the adult cardiomyocytes that are induced to undergo mitosis have similar morphology to the nondividing cardiomyocytes and thus do not appear to represent a specific subpopulation of cells. Together, our data indicate that adult cardiomyocytes stimulated with BIO can undergo cell cycle progression.

Previously, we showed that FGF1-mediated proliferation of adult cardiomyocytes enabled by p38 MAPK inhibition results in a transient dedifferentiation of myofibrillar structure during cell division [5]. In culture, there is a gradual spreading of the adult rat cardiomyocytes,

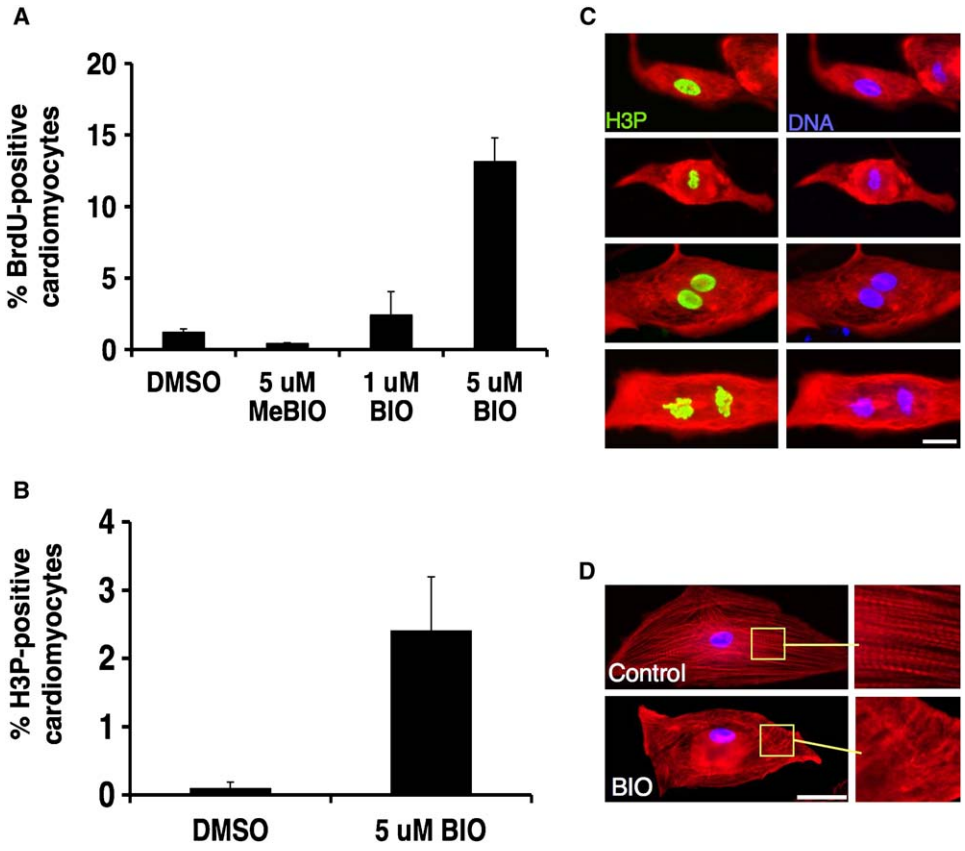

Figure 4. BIO Increases the Proliferative Potential of Adult Rat Mammalian Cardiomyocytes

(A–D) Adult rat cardiomyocytes were stimulated with DMSO (control) or 5 μM BIO every 3 days and were assayed for cell cycle progression after 6 days. (A) Cells were labeled with BrdU for 6 days. Treatment of cardiomyocytes with BIO significantly increased the percentage of cells that labeled positively with BrdU as compared to control cells (mean ± SD). (B) H3P analyses were performed to determine whether BIO-stimulated cells are able to undergo mitosis. After 6 days, 0.1% ± 0.09% of the control cells stained positive for H3P. When treated with BIO, the percentage of H3P-positive cells increased 20-fold (mean ± SD). (C) Adult cardiomyocytes at different stages of mitosis detected with H3P (green), tropomyosin (red), and DNA (blue). (D) Cells treated with DMSO (top panels) contain well-organized myofibrils and show striations formed by Z-discs when stained with the sarcomeric marker Troponin T (red). Cells treated with BIO have disorganized myofibrils oriented randomly with no apparent striations (lower panels). DNA is shown in blue. The scale bars are 20 μm in (C) and 40 μm in (D).

leading to a loss of the rod-shaped morphology. Importantly, control adult cardiomyocytes maintain a well-organized contractile apparatus (myofibrils) with striations, as visualized with the sarcomeric marker Troponin T (Figure 4D, top panel). In contrast, BIO-treated cells have a disorganized myofibrillar architecture with few striations, independent of cell cycle state (Figure 4D, bottom panel). It has been postulated that the well-organized contractile architecture of adult cardiomyocytes may physically impede the kinetics of cell division [24]. For example, newt cardiomyocytes completely lose striation during division [25]. Moreover, the loss of the hallmark myofibrillar structure has been described as "dedifferentiation" [26–28]. Thus, BIO-induced dedifferentiation may potentiate proliferation of adult cardiomyocytes by facilitating cell division.

Discussion

Our results demonstrate that the small molecule BIO can increase the proliferative ability of mammalian cardiomyocytes. Furthermore, the results reported in this paper, together with the work of Sato and colleagues, provide proof of principle that a pharmacological inhibitor, BIO, can maintain self-renewal and pluripotency in ESCs and promote dedifferentiation and proliferation in terminally differentiated cardiomyocytes. The effect of BIO can be attributed to the inhibition of GSK-3 function in cardiomyocytes; thus, we have identified a previously uncharacterized function for GSK-3 in regulating mammalian cardiomyocyte proliferation.

Several lines of evidence have indicated that GSK-3β can act as a negative regulator of hypertrophy in mammalian hearts both during development and in response to several forms of pathological stress (for a review, see [29]). However, these studies all utilize overexpression technology. To date, the native role of GSK-3 in the heart has not been examined. If GSK-3 acts endogenously as a negative growth regulator, then it can be predicted that loss of GSK-3 activity in cardiomyocytes would cause an increase in cell size. Surprisingly, cardiomyocytes that are treated with BIO are not larger than control cells in vitro. Rather, they appear to be slightly smaller in size (data not shown). This observation suggests that endogenous GSK-3 may not normally function to inhibit growth, at least in our culture system. Our data also

suggest that the inhibition or downregulation of canonical Wnt signaling may play a role in preventing cardiomyocytes from undergoing cell division postbirth.

One potential pathway with which BIO may interact to regulate cardiomyocyte proliferation is the p38 MAPK pathway. Inhibition of p38 MAPK activity has been shown to promote mammalian cardiomyocyte proliferation [5]. To test this possibility, we assayed for changes in p38 MAPK in response to BIO treatment in vitro. There were no detectable differences in the phosphorylation state of p38 between BIO, MeBIO, or control-treated cardiomyocytes (data not shown). Similar results were obtained when we assayed for p38 MAPK activity. While we have not observed any detectable changes in p38 MAPK under the conditions tested, it is possible that BIO may interact with the p38 MAPK pathway downstream of the kinase itself. However, our results suggest that BIO does not regulate p38 MAPK activity in promoting proliferation in mammalian cardiomyocytes.

Our findings that BIO increases the proliferative capacity of cardiomyocytes in vitro validates the use of chemical genetics as an excellent and rapid approach by which to identify and modulate pathways involved in cardiac regeneration. The use of pharmacological tools bypasses the requirement for generating genetic and molecular reagents and will expand our ability to characterize the biology of mammalian cardiomyocytes.

Finally, our identification of a mechanism that is able to increase cardiac proliferative potential in vitro is an important step toward the understanding of signaling pathways and their interactions that are required for heart regeneration. Furthermore, the results of this study, together with the work of Sato and coworkers [7], demonstrate that a pharmacological inhibitor, BIO, can maintain pluripotent self-renewal in ESCs and promote dedifferentiation and proliferation in terminally differentiated cardiomyocytes. Additional studies are needed to determine if BIO's functions are recapitulated both in stem cells and cardiomyocytes in animals. Nevertheless, the identification of a dual role for BIO suggests that the maintenance of stem cell properties and the induction of proliferation in differentiated cardiomyocytes may share common molecular pathways. The demonstration of this concept might alter our approach to identify therapeutic compounds that can restore function in the diseased heart.

Significance

Heart disease results in the loss of cardiomyocytes and is a leading cause of death. It is generally believed that the adult mammalian cardiomyocytes are unable to divide; thus, it is a significant challenge to develop effective therapies. A major approach is to generate new cardiomyocytes from stem cells. Alternatively, we have demonstrated that it is possible to induce proliferation in existing mammalian cardiomyocytes, and we sought to identify mechanisms involved in this process. Interestingly, the maintenance of self-renewal in stem cells has generally been thought of as a process that is distinct from that of the induction of proliferation in postmitotic mammalian cells, even though the requirement for proliferation is a necessary step in both events. Whether these two processes share common molecular pathways is an important question that has not been addressed by existing studies, and it has strong implications for future therapeutic approaches. To our knowledge, the small molecule GSK-3 inhibitor, BIO, is the first pharmacological agent shown to maintain self-renewal in human and mouse ESCs. We show that BIO promotes proliferation in mammalian cardiomyocytes. Stimulation of neonatal rat cardiomyocytes with BIO induced cell cycle entry and the upregulation of positive cell cycle regulators, while the level of a CDK inhibitor p27 was decreased. BIO treatment elevated β-catenin activity in cardiomyocytes, suggesting that the increase in proliferative ability may be partly due to the activation of the canonical Wnt pathway. In both neonatal and adult rat cardiomyocytes, BIO stimulation promoted cell cycle progression and increased the number of mitoses. The ability of BIO to promote proliferation of cardiomyocytes defines a new signaling pathway, GSK-3, in regeneration. Furthermore, our demonstration of a second role for BIO suggests that the maintenance of stem cell self-renewal and the induction of proliferation in differentiated cardiomyocytes may share common molecular pathways.

Experimental Procedures

Culture of Mammalian Cardiomyocytes

Animal experiments were performed in accordance with guidelines of Children's Hospital, Boston. Rat ventricular cardiomyocytes from 2- to 3-day-old Wistar rats were isolated and cultured as described [5]. Adult cardiomyocytes from 3-month-old male rats were cultured for 1 day after isolation and were treated every 3 days with the appropriate compounds in the same medium supplemented with 2 mM glutamine as described [5]. BIO was purchased from EMD Biosciences and was dissolved in DMSO. In our primary cultures, more than 90% of the cells are myocytes, as evaluated by indirect immunofluorescence staining with an antibody to sarcomeric tropomyosin [5].

Immunofluorescence Stainings

Primary antibodies used include: rat anti-BrdU (Abcam), rabbit anti-Cyclin A (Cell Signaling), mouse anti-Cyclin D1 (Cell Signaling), mouse anti-p27 (BD Transduction Laboratories), rabbit anti-Ki67 (Abcam), rabbit anti-phospho-Histone H3 (Upstate Signaling), rabbit anti-β-catenin (BD Transduction Laboratories), and mouse anti-tropomyosin (Developmental Hybridoma Studies Bank). Secondary antibodies conjugated to Alexa fluorophores were used for signal detection (Invitrogen). DNA was visualized with DAPI (4′,6′-diamidino-2-phenylindole, 0.5 μg/ml; Sigma). Stainings were performed as described [5].

Luciferase Assays

A total of 40 μg TOPflash or FOPflash reporter plasmids (Upstate Signaling) were cotransfected with 4 μg pGL4.75 [*hRluc*/CMV] vector (Promega) into 2×10^6 neonatal cardiomyocytes using the Nucleofector Kit (Amaxa) and were plated in 24-well plates. Cells were cultured for 3 days and were subsequently stimulated with selected compounds for 24 hr. Reporter activity was measured by using the Dual Luciferase Assay System (Promega). TOPflash or FOPflash activity was normalized to the measured *Renilla* luciferase activity of pGL4.75, an internal standard for transfection efficiency.

Statistical Analysis

Data were analyzed with a two-tailed Student's t test. Each experiment was performed at least three times ($n \geq 3$), and the results reported are statistically significant ($p \leq 0.05$). Standard deviations (±SD) are given for each mean value. For immunofluorescence analyses, 500 or more cells were analyzed per experiment for neonatal

cardiomyocytes, and at least 500 (BrdU) or 1000 cells (H3P) per experiment were analyzed for adult cardiomyocytes.

Supplemental Data

Supplemental Data include a table showing the percentage of BrdU incorporation of cardiomyocytes under conditions tested and are available at http://www.chembiol.com/cgi/content/full/13/9/957/DC1/.

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