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Differential Regulation of Bcl-x_L Gene Expression by Corticosterone, Progesterone and Retinoic Acid

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Abstract

Corticosterone (CT), progesterone (PG), and retinoic acid (RA) are capable of inhibiting Doxorubicin (Dox) from inducing apoptosis in rat cardiomyocytes. Mechanistically, CT, PG and RA induce increases of Bcl-x_L protein and mRNA, and activate a 3.2 kb bcl-x gene promoter. CT and RA, but not PG induced the activity of a 0.9 kb bcl-x promoter, containing sequences for AP-1 and NF-κB binding. RA, but not CT or PG, induced NF-κB activation. CT, but not PG or RA induced AP-1 activation, and induction of the 0.9 kb bcl-x reporter by CT was inhibited by dominant negative c-Jun TAM-67 or removal of AP-1 binding site. Therefore, although CT, PG and RA all induce Bcl-x_L mRNA and protein, three independent mechanisms are in operation: while CT induces Bcl-x_L via AP-1 transcription factor, and RA induces NF-κB activation and bcl-x promoter activity, PG induces Bcl-x_L via a mechanism independent of NF-κB or AP-1.

Keywords

Apoptosis; cell survival; nuclear receptor; steroids; retinoids

Introduction

The majority of the myocytes in the myocardium cease cell replication within a few days after the birth of a mammal. In adult myocardium, loss of cardiomyocytes occurs as a result of chemotherapy, cardiac disease and aging, contributing to decline in cardiac contractile function and eventually heart failure [1–9]. Apoptotic-like cell death plays a critical role in cardiomyopathy induced by the antineoplastic drug Doxorubicin (Dox), an anthracycline quinone used for treatment of a variety of leukemia, lymphomas and solid tumors [5, 10, 11]. Although the protocol for administering Dox has been improved to minimize acute cardiac toxicity, a dilated cardiomyopathy may develop two to ten years after. During pathological analysis of the failing hearts, apoptosis has been observed in a significant proportion of cardiomyocytes [5, 10, 11]. Previous works from our laboratory have found that corticosterone (CT) and progesterone (PG) were able to protect cardiomyocytes from

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Dox induced apoptosis [12, 13]. Among the multiple signaling pathways and genes altered by these steroids, induction of Bcl-x_L gene appears to mediate the anti-apoptotic effect [12–20].

Bcl-2 family members are known as a major player in apoptosis. Although members of bcl-2 family share consensus sequence and structural domains, some act to promote apoptosis (pro-apoptotic) whereas others inhibit apoptosis (anti-apoptotic). The relative levels of pro versus anti-apoptotic bcl-2 family members are believed to be a key determinant for cell survival [21, 22]. Bcl-x_L, an anti-apoptotic member, is encoded by bcl-x gene, which produces several isoforms of proteins due to alternative mRNA splicing with Bcl-x_L transcript as the major product [23–25]. Bcl-x gene promoter contains nuclear receptor binding site upstream of –3 kb region, and consensus elements for binding of Sp1, AP-1, Oct-1, Ets, Rel/NF-κB, STATs and GATA-1 transcription factors [23, 24, 26]. These elements suggest multiple mechanisms of transcriptional regulation of bcl-x gene.

The nuclear receptors accept incoming signals from agonists such as steroids or retinoids to alter gene expression or affect cellular metabolism [27, 28]. The Glucocorticoid Receptor (GR) and Progesterone Receptor (PR) each is encoded by a single gene that produces two isoforms from alternative promoters [29, 30]. These receptors undergo dimerization, can become phosphorylated, and bind to the Hormone Response Element (HRE) in the promoter of downstream target genes. There are two classes of retinoid receptors, retinoic acid receptors (RAR) and retinoid X receptors (RXR), which can form homodimers or heterodimers for binding to the Retinoid Response Elements in the promoter of target genes [31, 32]. We have found that glucocorticoids and progesterone are capable of inhibiting Dox from inducing apoptosis and inducing Bcl-x_L expression in a receptor dependent manner [12, 13, 20]. Whether retinoic acid can induce Bcl-x_L in cardiomyocytes is not known. We compare the induction of Bcl-x_L to determine if these nuclear receptor ligands induce Bcl-x_L through distinct mechanisms.

Methods

Cell Culture and Drug Treatment

Cardiomyocytes were prepared from 1 to 2 days old neonatal Sprague-Dawley rats (Harland, Indianapolis, IN) as described [33]. The myocytes were seeded at a density of 0.3×10^6 cells per well in 6-well plates or 7.5×10^4 cells per well in 24-well plates. Cells were cultured in low glucose DMEM with 1 mM pyruvate, 10% fetal bovine serum, 100 units/ml penicillin and 100 units/ml streptomycin for 3–4 days before being placed in fresh DMEM containing 0.5% FBS and CT, PG or RA. After another 24 hrs, cells were harvested for Western blot analyses or the media were changed to fresh DMEM containing 0.5% FBS and 1 μM Dox. Cells were harvested 16 hours later for caspase activity assays.

Caspase Activity Assay

Detached cells were collected by centrifugation and were combined with attached cells from the same well in 6-well plates. The cells were dissolved in 250 μl of lysis buffer (0.5% Nonidet P-40, 0.5 mM EDTA, 150 mM NaCl, and 50 mM Tris pH 7.5). Cell lysates (50 μl)

were incubated for 1 hour at 37°C with 40 µM of N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC, Alexis Biochemicals, San Diego, CA) in 100 µl reaction buffer (10 mM HEPES, pH 7.5, 0.05 M NaCl and 2.5 mM DTT). The released AMC was measured using a 96-well fluorescence plate reader (Cambridge Bioresearch Model 7620) with an excitation wavelength of 365 nm and an emission wavelength of 450 nm.

Western Blot

Cells in 100 mm dishes were lysed by scraping in Laemini buffer [0.5 M Tris, pH 6.8, 2.4% (w/v) SDS, 50% (v/v) glycerol] with freshly added protease inhibitors: 10 µg/ml aprotinin and 1 mM phenylmethanesulfonyl fluoride. Protein concentration was determined using the bicinchoninic acid (BCA) method according to the manufacturer's instruction (Pierce, Rockford, IL). Proteins were separated by SDS polyacrylamide gel electrophoresis using a mini-Protean II electrophoresis apparatus (Bio-Rad, Richmond, CA) run at 60 volts. The separated proteins were transferred to immobilon-P membranes (Millipore, Bedford, MA) by electrophoresis. The membrane was incubated with primary antibodies, anti-Bcl-x_L (Santa Cruz Biotechnology, sc#8392) and then secondary antibodies conjugated with horseradish peroxidase. The bound antibodies were detected using an enhanced chemiluminescent reaction.

RT-PCR

Total RNA extracted using Trizol was used as a template for RT-PCR. Superscript II was used for reverse transcription at 37–50°C for 1 hr. For PCR, the denaturation and primer extension were carried out at 94°C and 72°C. The annealing temperature was calculated based on GC content of the primers, 5'-CCCCAGAACTGAACCA-3' (forward) and 5'-AGTTTACCCCATCCCGAAAG-3' (reverse) as suggested by the oligonucleotide synthesis company (Biosynthesis, Lewisville, TX). The products were detected by real-time PCR (BioRad CFX96) using SYBR Green dye.

Bcl-x_L Promoter Luciferase Constructs, Transient Transfection and Luciferase

Cardiomyocytes were seeded at 0.5×10^6 per well in 6-well plates (~960 mm² culture area per well). At 24 h after plating, cells were incubated 5 h with 0.8 µg DNA of luciferase reporter construct per well using 3 µL of Fugene-6 liposomes (Roche, Mannheim, Germany). The transfection efficiency is generally low (3–10%) in cardiomyocytes and can vary due to the nature of primary culture. To control for transfection efficiency, we cotransfected cardiomyocytes with the pRL-TK plasmid (0.04µg/well), which encodes a Renilla luciferase gene under the control of a Thymidine Kinase promoter. After 5 h incubation under tissue culture condition, cells were replenished with fresh DMEM containing 10% FBS for overnight recovery. After 24 h serum starvation followed by experimental treatments, cells were rinsed with cold PBS and lysed in a passive lysis buffer provided in a luciferase assay kit for measurements of luciferase activity according to the manufacturer's protocol (Promega, Madison, WI). The luciferase activity was normalized to the Renilla activity and expressed as fold induction over control unless indicated otherwise. All treatments were performed in triplicate.

Plasmid Constructs

The -3.2kb murine bcl-x gene promoter-reporter construct was generously provided by the laboratory of Dr. Gabriel Nunez (University of Michigan, Ann Arbor, MI). The construct encodes a 3.2kb fragment of the Bcl-x_L promoter fused to the promoterless pGL2 firefly luciferase promoter [23]. Within 1 kb upstream of transcription start side, the sequence homology between mouse bcl-x and human bcl-x gene is 71%. The cis-elements in this region of promoter is well characterized for human bcl-x gene [24]. The 0.9 kb human bcl-x gene promoter-reporter pGL3 construct was generously provided by the laboratory of Dr. G.T. Bowden (University of Arizona, Tucson, AZ). This construct contains a 905 bp region of the Bcl-x_L promoter (gene bank number D30746) fused to the promoterless pGL3 firefly luciferase promoter and was first cloned by Dr. Michael A. Bachelor from genomic DNA of Hela cells when studying the cause of bcl-xL protein increase due to UV-A exposure in keratinocytes [34]. The luciferase reporter construct for NF-κB transcription factor was a generous gift of Dr. Zigang Dong (University of Minnesota, The Hormel Institute, Austin, MN)[35]. The NF-κB reporter construct encodes a 196 bp fragment of the HIV promoter containing 2x NF-κB binding elements driving a luciferase reporter gene. The AP-1 luciferase reporter construct has four consensus AP-1 binding sites from collagenase gene was obtained from Stratagene [36]. The dominant negative c-Jun mutant TAM67 is composed of amino acids 123–331 of c-Jun cloned into the expression vector pcDNA3 [37]. Mutant 0.9 kb bcl-x promoter with eliminated NF-κB or AP-1 binding site was obtained using QuickChange II XL site-directed mutagenesis kit (Stratagene) using the primer of 5' GGA GTG GGG GCG GGG AGG GAG TGA CTT TCC for NF-κB mutation and 5' CAC TGG TGC TTT CGA TGA AGT ATC TTG GAA for AP-1 mutation. The vectors containing mutated sequence were verifying by DNA sequencing.

Statistics

Stata Statistics/Data Analysis software was used for one-way analysis of variance (ANOVA) with a Bonferroni correction to compare multiple means. A p-value of <0.05 was used as the cutoff for statistical significance. When a mean is statistically different from another, the two means were labeled with two different letters, e.g. *a* and *b*. A mean labeled with two letters, e.g. *ab*, indicates no significant difference between the mean labeled with *a* or *b*, although the mean labeled *a* is significantly different from that labeled with *b*.

Results

CT, PG and RA Inhibit Dox Induced Caspase-3 Activity in Rat Cardiomyocytes

We have previously shown that CT and PG can decrease Dox induced apoptosis in cardiomyocytes as measured by several markers of apoptosis including caspase-3 activation [12, 13]. Caspase-3 activity serves well as a quantitative measurement for apoptosis [12, 13]. When screening a number of nuclear receptor agonists for cytoprotective effect, we found that all-trans retinoic acid (RA) also inhibited Dox from inducing apoptosis based on morphology examination and caspase assays. Figure 1A shows that CT, PG, and RA at 1 μM concentration all decreased activation of caspase-3 by Dox. The amount of protection was 46 ± 2.0 % for 1 μM CT, 30 ± 1.0 % for 1 μM PG, and 52 ± 0.03 % for 1 μM RA. CT and PG are known to bind to GR and PR, respectively. Mifepristone (MF) is an antagonist of GR

and PR, and was able to cancel the cytoprotective effect of CT and PR [12, 13]. To document that the protective effect of RA is not through MF sensitive receptors, we tested the effect of MF on RA induced cytoprotection. As expected, MF was not able to cancel the protective effect of RA against Dox induced caspase activation (Fig 1B), suggesting a GR or PR independent mechanism of cytoprotection.

CT, PG and RA Induce Transcriptional Activation of Bcl-x_L

We have previously shown that CT and PG can induce the anti-apoptotic gene Bcl-x_L [12, 13]. To determine whether RA induces Bcl-x_L, we incubated cardiomyocytes with varying doses of RA for 24 hours and then harvested the cells for Western blot analysis. PG and CT were included for comparisons. The level of Bcl-x_L protein was increased by all three steroids in a dose dependent manner (Fig 2A). With 1 μ M of CT, PG or RA, the induction was 2.14, 2.23, and 4.24 fold respectively. An increase of Bcl-x_L mRNA was observed with 1 μ M of CT, PG or RA (Fig 2B).

To determine whether increase of Bcl-x_L mRNA is due to transcriptional activation of Bcl-x gene, we tested the effect of CT, PG and RA on a -3.2 kb Bcl-x_L promoter using a luciferase reporter construct [23]. MF was included to block Bcl-x_L promoter activation by CT or PG. Cardiomyocytes transfected with the reporter plasmid were treated with 1 μ M CT, 10 μ M PG or 1 μ M RA for 24 hours. RA is most effective in activating Bcl-x_L promoter-reporter (Fig 3A). The activity of the 3.2 kb bcl-x reporter plasmid was increased by RA (1.63 ± 0.18 fold induction $p < 0.05$) and RA+MF (1.84 ± 0.11 fold induction $p < 0.05$). As expected, MF did not block RA induced bcl-x promoter activation. Although CT and PG induced the activity of 3.2 kb bcl-x promoter and as expected MF blocked bcl-x promoter activation by CT or PG, induction of bcl-x promoter activity is not statistically significant as compared to control without any drug treatment (Fig 3A).

Role of NF- κ B and AP-1 in Bcl-x Promoter Activation

Sequence alignment of 1 kb human bcl-x gene promoter with mouse bcl-x gene promoter indicate 71% sequence homology. Several cis-elements have been characterized within this region in human bcl-x gene [24]. There is an NF- κ B binding site, GGGGGGACTGCCCC, between 522 and 536 bp, and an AP-1 binding site, TGACTTAA, between 508 and 528 bp. To determine whether 0.9 kb promoter of bcl-x is sufficient for activation by CT, PG or RA, primary cardiomyocytes were transfected with 0.9 kb promoter-luciferase reporter construct. The activity of the reporter was increased by CT (1.39 ± 0.05 fold induction $p < 0.05$), RA (1.52 ± 0.10 fold induction $p < 0.05$) and RA+MF (1.48 ± 0.08 fold induction $p < 0.05$), but not PG. MF inhibited the induction of CT, but not RA (Fig 3). Therefore, CT and RA but not PG was able to activate the 0.9 kb bcl-x promoter.

To examine the involvement of NF- κ B or AP-1 in Bcl-x_L induction, we tested NF- κ B activation using NF- κ B reporter construct. When NF- κ B was transfected into cardiomyocytes, RA was able to activate NF- κ B (1.96 ± 0.17 $p < 0.05$), which was not inhibited by MF (Fig 4A). Neither CT nor PG was able to activate NF- κ B (Fig 4A). However, when NF- κ B binding site was eliminated in the 905 bp bcl-x promoter, RA

remained capable of activating the bcl-x promoter (Fig 4B), pointing to additional mechanisms beyond NF- κ B in bcl-x promoter activation by RA.

In contrast, when AP-1 involvement was determined by transfection of AP-1 reporter construct, CT but not PG or RA was able to activate AP-1 reporter (Fig 5A). Such activation was sensitive to MF (Fig 5A), supporting a role of GR in AP-1 mediated transcriptional event. TAM67 is a dominant negative mutant of c-Jun, encoding 121–331 amino acids of c-Jun protein. Cotransfection of 0.9 kb bcl-x promoter with TAM67 was able to block CT induced bcl-x promoter activation (Fig 5B). With 0.9 kb bcl-x promoter containing mutations eliminating AP-1 binding, CT was not effective in activating the promoter (Fig 5C). In contrast, RA, which was not able to activate AP-1 reporter, remained capable of activating AP-1 null bcl-x promoter (Fig 5C).

Discussion

We found that 3.2 kb or 0.9 kb promoter of bcl-x gene was induced by CT and RA, whereas PG is able to activate 3.2 kb but not 0.9 kb promoter of bcl-x gene. The induction of 0.9 kb bcl-x promoter by CT was blocked by a dominant-negative c-Jun, TAM-67. CT was able to activate AP-1 promoter-reporter and such activation could be inhibited with MF. RA induced Bcl-x_L, possibly via NF- κ B in conjunction with additional mechanisms. Activation of NF- κ B cis-element was detected with RA, but not CT or PG.

In cardiomyocytes, we have previously shown that CT or PG can inhibit apoptosis in a Bcl-x_L dependent mechanism [12, 13, 20]. The existence of HREs upstream of 3 kb bcl-x promoter region has been previously described [38]. PG and CT were reported to activate bcl-x promoter through two HREs located in –3040 and –3001 region [26]. The fact that 3.2 kb bcl-x promoter is activated by PG in our study suggests the possibility of HRE involvement.

The discovery of CT inducing bcl-x gene promoter through AP-1 response element is novel. Bcl-x gene promoter contains AP-1 responsive elements [39, 40], and it has been shown that induction of AP-1 can stimulate Bcl-x_L transcription [41]. The role of AP-1 in CT induced Bcl-x_L has been demonstrated by: 1) CT is capable of activating AP-1 reporter, and 2) expression of TAM67, the dominant negative of c-Jun, was capable of preventing 0.9 kb bcl-x promoter activation by CT. Contradictory to our finding, Jacobs-Helber et. al. [40] discovered that erythroid cells undergoing apoptosis had increased AP-1 activity and decreased Bcl-x_L expression. TAM67 expression resulted in an increased level of Bcl-x_L [40]. This may reflect differential regulation of Bcl-x_L by steroids among different tissues or cell types. In most hematopoietic cells, CT induces apoptosis, while in cardiomyocytes CT inhibits apoptosis [42]. Nevertheless, it appears that whether CT induces or inhibits apoptosis correlates with its ability to inhibit or induce Bcl-x_L respectively.

Like CT, whether RA induces or inhibits apoptosis is cell type dependent [43]. In a leukemia T-cell line, retinoic acids induce apoptosis through down regulation of Bcl-x_L [44]. In contrast, in naïve T-thymocytes, RA has been shown to increase bcl2a1 expression and decrease apoptosis without affecting the expression of Bcl-x_L [45]. Retinoic acids have been

shown to mediate cell survival response in neuroblastoma cells, neural crest cells, myeloid leukemia blasts, lung cancer cells, islet-epithelial cells, and olfactory sensory neurons [46–51]. In cardiomyocytes, high glucose induced apoptosis can be inhibited by RA in NF- κ B dependent manner [52].

The ability of RA to activate NF- κ B suggests the possibility of additional mechanism of cell survival besides Bcl-x_L. The transcription factor NF- κ B has previously been shown to exhibit antiapoptotic effects through induction of anti-apoptotic genes, including cIAP1, cIAP2 [53–55], xIAP [56], and Bcl-2 homolog Bfl-1/A1 [57, 58]. Nevertheless, NF- κ B dependent Bcl-x_L expression has been reported in hippocampal neurons or in polymorphonuclear leukocytes [59, 60]. Glasgow, et al., identified NF- κ B binding sites in the Bcl-x_L promoter that are responsive to p65 containing NF- κ B transcription factor [61]. The fact that RA activates NF- κ B reporter construct but remains capable of activating bcl-x promoter when NF- κ B is mutated suggests multiple mechanisms of transcriptional regulation of bcl-x gene by RA.

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Fig. 1. CT, PG and RA decrease Dox induced caspase-3 activity

Primary cultured rat neonatal cardiomyocytes were pretreated 24 hours with 1 μ M PG, CT or RA prior to 0.8 μ M Dox treatment for 24 hrs (A). MF (1 μ M) was added to cells 10 mins prior to addition of 1 μ M RA. At 24 hours after incubation with RA, cells were treated with 0.8 μ M Dox for 24 hours (B). At the end of 24 hours of Dox treatment, cells were harvested for measurements of caspase activity using DEVD-AMC as a substrate. The data represent means $\pm$ standard deviations from triplicates of one representative experiment and were analyzed by ANOVA. A letter indicates significant difference from the means labeled with different letters. The mean labeled “ac” is not significant different from that labeled with “a” or “c”, although the mean labeled with “a” is significant different from that labeled with “c”.

Fig. 2. CT, PG and RA induce the expression of Bcl-x_L protein and mRNA

Primary cultured rat neonatal cardiomyocytes were treated with CT, PG or RA at indicated doses and were harvested at 24 hours after for measurements of Bcl-x_L protein using Western blot (30 μ g protein/lane, A). Vinculin from RA treated cells was used as a loading control (A). Cells were treated with 1 μ M CT, PG or RA for 24 hr before harvesting for measurement of Bcl-x_L mRNA using real time RT-PCR with triplicate samples (B). A letter indicates significant difference from the means labeled with different letters.

Fig. 3. Activation of Bcl-x gene promoter by CT, PG or RA

Primary cultured cardiomyocytes were transfected with 3.2kb (A) or 0.9kb (B) bcl-x promoter-luciferase reporter construct. At 24 hrs after transfection, cells were treated with 1 μ M CT, 10 μ M PG or 1 μ M RA in the absence or presence of 1 μ M MF, which was added to cells 10 mins prior to CT, PG or RA. Cells were harvested 24 hours after for measurements of luciferase activities. The data represent means $\pm$ standard deviations from triplicates as fold increases when the luciferase activity for control untreated cells was set at 1. A letter indicates significant difference from the means labeled with different letters. The mean labeled "ab" is not significant different from that labeled with "a" or "b", although the mean labeled with "a" is significant different from that labeled with "b".

Fig. 4. RA but not CT or PG activates NF-κB cis-elements

Primary cultured cardiomyocytes were transfected with 2x NF-κB luciferase reporter construct (A) or -0.9 kb bcl-x promoter construct with NF-κB binding site being mutated (B). Cells were treated with 1 μM CT, 10 μM PG, or 1 μM RA in the absence or presence of 1 μM MF for 24 hours before harvesting for measurements of luciferases. Data are expressed as means ± standard deviations of triplicate samples with fold induction when the activity of luciferase for control was set at 1. A letter indicates significant difference from the means labeled with a different letter.

Fig. 5. CT but not PG or RA activates AP-1 cis-elements

Primary cultured cardiomyocytes were transfected with 4xAP-1 reporter construct (A), wild type 0.9 kb bcl-x promoter-reporter construct (B), or 0.9 kb bcl-x promoter-reporter construct with AP-1 binding site being mutated (C). TAM67 (0.08 μ g) was cotransfected with the bcl-x promoter-reporter construct (B). At 24 hours after treatment with 1 μ M CT, 10 μ M PG, or 1 μ M RA in the absence or presence of 1 μ M MF, cells were harvested for measurements of luciferases. Data are expressed as means $\pm$ standard deviations of triplicates with fold induction when the activity of luciferase for control was set at 1. A letter indicates significant difference from the means labeled with different letters. The mean labeled “ac” is not significant different from that labeled with “a” or “c”, although the mean labeled with “a” is significant different from that labeled with “c”.