# Bisphenol A impacts cardiomyocyte differentiation in vitro by modulating cardiac protein expression

Vimarsha Swami*[1,2], Lorraine E. Chalifour[1,2]

[1]Lady Davis Institute for Medical Research, Sir Mortimer B. Davis - Jewish General Hospital, 3755 chemin Côte Ste Catherine, Montréal, Québec, Canada, H3T 1E2.
[2]Division of Experimental Medicine, McGill University, Lady Meredith House, 1110 Pine Avenue West, Montreal, Quebec, Canada, H3A 1A3

## ABSTRACT

**Introduction**: Bisphenol A (BPA) is an environmental toxin commonly found in plastics and is able to mimic the actions of endogenous steroid hormones. BPA binds and activates intracellular estrogen receptors (ERα and ERβ) and estrogen related receptor γ (ERRγ), all of which are present in cardiomyocytes. However, it is unclear how BPA impacts the heart. We hypothesized that BPA modulates the expression of proteins regulating cardiac structure, energy and calcium homeostasis during cardiomyocyte differentiation in vitro. **Methods**: We differentiated H9C2 cells into cardiomyocytes in hormone-replete (RM) or hormone-depleted (HD) media. We co-treated the cells with graded amounts of BPA and pure anti-estrogen ICI 182,780, which blocks ERα and ERβ activity. Immunoblotting measured the expression of the structural protein β-myosin heavy chain (βMHC), calcium homeostasis protein sarcoendoplasmic reticulum calcium ATPase (SERCA2a), and the cardiac energy-producing protein creatine kinase (CK). **Results**: Expression of these proteins was hormone-dependent during cardiomyocyte differentiation, with expression highest in RM media after 72 or 96 hours of differentiation. Adding $10^{-8}$ M BPA to HD media increased cardiac structural (βMHC), energy (CK), and calcium homeostasis (SERCA2a) protein expression. Conversely, $10^{-7}$ M BPA added to RM media decreased protein expression. Co-treatment with ICI 182,780 reduced BPA-mediated induction of SERCA2a and CK expression in HD media. **Discussion**: BPA modulates cardiac structure, calcium and energy homeostasis protein expression during cardiomyocyte differention in vitro. Moreover, the data suggest that BPA mediates these changes in protein expression through activation of cardiomyocyte ERα, ERβ, or ERRγ.

**KEYWORDS**

*Endocrine disruptor, Estrogen receptors, Cardiomyocytes, Anti-estrogen*

*Corresponding author:
vimarsha.swami@mail.mcgill.ca

Received: 4 January 2010
Revised: 5 March 2010

## INTRODUCTION

Bisphenol A (BPA) is a known endocrine disruptor prevalent in the environment. Endocrine disruptors are environmental chemicals that can mimic the action of endogenous steroid hormones with some shown to alter reproductive tissue development (*1*). BPA is a component of polycarbonates and other plastics, and can leach into stored foods and drinks. BPA is one of the most common chemicals used in industry today and has the potential to become a major environmental toxin (*2*). Recent measurements indicate that greater than 92% of people have detectable levels of BPA in urine, indicating continued and chronic exposure (*3*). In 2008, BPA was judged by Health Canada to be a substance

that may be entering the environment in quantities sufficient to constitute a danger to Canadians' health (*4*). The daily exposure in Canada, mostly through diet, is estimated to be 0.67 μg/kgBW/day for adults, between 0.8 and 2.27 μg/kgBW/day for children and 0.92 to 4.3 μg/kgBW/day for infants up to 18 months old (*4*).

Studies have shown that when pregnant rodents were treated with low dosages of BPA, amounts commonly found in human blood and tissue, BPA entered the rodent placenta, accumulated in the fetus, and altered reproductive and non-reproductive tissues (*1*). BPA has been shown to impact the development of both the male and female reproductive tracts in developing fetuses (*1*). Chronic exposure to BPA from conception is therefore a widely-prevalent phenomenon. Researchers have shown that BPA acts in a manner similar to sex hormones, and modulates protein expression by binding estrogen receptor α (ERα) and estrogen receptor β (ERβ) (*5*). The activated ERs then activate ER-dependent target transcription (*6*). Alternatively, studies have also shown that BPA behaves as an endocrine disruptor by binding estrogen related receptor γ (ERRγ) (*7*). Finally, BPA also inhibits the activity of the thyroid hormone triiodothyronine (T3) (*8*).

It is well known that men and women develop heart disease differently. Sex hormone activation is thought to play an important role in determining cardiac structure and function, and could account for differences between men and women in developing heart disease, though this is largely unresolved (*9*). Cardiomyocytes express androgen receptors (ARs), ERα, ERβ, and ERRγ (*10*). Consequently, cardiomyocytes can respond to steroid hormones, and thus can also respond to environmental endocrine disruptors such as BPA. Although the impact of BPA on various mammalian tissues is well documented, little is known about the effects of this endocrine disruptor on the heart structure and function during development. In particular, BPA's possible effects on cardiomyocyte development are largely unexplored and unresolved. Cardiac differentiation requires expression of structural proteins involved in contraction, such as β-myosin heavy chain (βMHC); calcium homeostasis proteins that are also involved in the control of contraction, such as sarcoendoplasmic reticulum calcium ATPase (SERCA2a); and proteins involved in cardiac-specific energy production, such as creatine kinase (CK).

We hypothesize that BPA modulates the expression of cardiac structural, energy and calcium homeostasis proteins during cardiomyocyte differentiation *in vitro*.

### MODEL AND EXPERIMENTAL PLAN

We used H9C2 cells in our experiments. This permanent cell line is derived from rat cardiac ventricular tissue (*11*), and can be differentiated into a cardiomyocyte lineage in vitro. We measured the expression of cardiomyocyte structural (βMHC), energy (CK) and calcium homeostasis (SERCA2a) proteins.

To test our hypothesis, we cultured H9C2 cells in hormone-replete (RM) and hormone-depleted (HD) media, and determined the conditions for optimal differentiation. To test the influence of BPA on differentiation and protein expression, we added graded amounts of BPA to RM and HD media. To test the mechanism of BPA action, we treated cells with both BPA and pure anti-estrogen ICI 182,780, which binds both ERα and ERβ with high affinity and blocks putative ER activity.

We expect expression of these important cardiac proteins to rise as differentiation progresses in cells treated with hormone-replete media. We also expect lower protein expression in cells treated with hormone-deficient media, compared with cells treated in hormone-replete media, since we hypothesize that sex hormones modulate gene expression during cardiomyocyte differentiation. We also anticipate increased expression of cardiac-specific proteins in cells treated with both BPA and hormone-deficient media, compared with hormone-deficient controls lacking BPA, as we expect BPA to bind and activate ERs or ERRγ. If BPA does indeed bind the ERs, we anticipate that the effects of BPA on protein expression will be reversed back to control levels when ICI 182,780 is added to cells in culture.

## METHODS

#### CELL CULTURE

H9C2 cells were plated in 60 mm cell culture dishes and cultured in Dulbecco's Modified Eagle Medium (DMEM). Penicillin, streptomycin, and 10% Fetal Calf Serum (FCS) were added (all from Invitrogen). At 90-95% confluency, the media was changed to differentiation media. Hormone-replete differentiation media (RM) contained DMEM, 1% FCS and antibiotics. Hormone-deficient differentiation media (HD) contained phenol-free DMEM, 1% charcoal stripped-FCS and antibiotics. $10^{-8}$ M Retinoic Acid (RA) (Sigma-Aldrich) was added daily to induce cardiac differentiation. Media were changed every 48 hours for all experiments.

#### PROTEIN ISOLATION AND IMMUNOBLOT ANALYSIS

Cells were homogenized in SDS lysis buffer containing 62.5mM Tris pH 6.8, 2% weight/volume SDS, 10% glycerol, 50 mM dithiothreitol and 0.01% w/v bromophenol blue. Proteins were separated using 8% SDS-polyacrylamide gel at 80 mV, and then transferred to a Immobilon P blotting membrane (Millipore) at 90 mV for 2 hours at 4 °C using a wet transfer apparatus.

The membranes were stained with Ponceau S, and then blocked with 10% TBS-T (βMHC, CK) or 10% PBS-T (SERCA2a) in

milk for 1 hour. Membranes were then incubated overnight at 4 ºC in 5% TBS-T or PBS-T milk containing primary antibody. βMHC primary antibody (MF-20 culture supernatant) was diluted 1:10, SERCA2a antibody (Santa Cruz) 1:100 and CK antibody (Sigma-Aldrich) was diluted 1:1000.

Membranes were then washed 3 x 15 minutes in TBS-T or PBS-T, and incubated for 2 hours at room temperature in 5% TBS-T or PBS-T milk containing horseradish-peroxidase coupled secondary antibody. Anti-mouse secondary antibody (Pierce) was used for βMHC (1:10000 dilution) and Creatine Kinase (1:8000 dilution). Anti-goat secondary antibody (Pierce) was used for SERCA2a (1:5000). After incubation, membranes were washed 3 x 15 minutes in TBS-T or PBS-T. We then performed chemiluminescent detection of specific binding, following the manufacturer's instructions (Pierce, Rockford IL). We then normalized protein levels using the bands obtained from the loading control gels (discussed below).

### LOADING CONTROL FOR IMMUNOBLOT ANALYSIS

For immunoblot analysis, 25 µL of homogenate was loaded in each well. For a loading control, protein samples separated on 8% SDS-Polyacrylamide gels were stained with Coomassie Brilliant Blue 250 to visualize bands. We quantified bands to compare the relative amounts of protein loaded in each well, and all experimental results were normalized to these values.

### STATISTICAL ANALYSIS

Normalized and vehicle-corrected values for relative protein expression are shown in Figures 1 to 3. Paired t test was used to compare relative protein expressions when needed. Values of $P<0.05$ were considered statistically significant.

## RESULTS

### EXPERIMENT 1: DIFFERENTIATION TIME COURSE FOR H9C2 CELLS IN HORMONE-REPLETE AND HORMONE-DEPLETED MEDIA

To test the impact of sex hormones on the expression of cardiac structural (βMHC), energy (CK) and calcium homeostasis (SERCA2a) proteins during the time course of cardiomyocyte differentiation, a first experiment (Experiment 1) was performed in which protein expression was measured using immunoblotting at 24, 48, 72, and 96 hour intervals after the change to differentiation media (Fig. 1) in HD and RM.

βMHC expression increased with time in RM as the H9C2 cells differentiated into cardiomyocytes (RM 72: $P<0.002$, RM 96: $P<0.04$ compared with RM 24). Also, the RM 72 and RM 96 treated cells showed higher βMHC expression than HD treated cells ($P < 0.002$ at 72 hours, $P < 0.035$ at 96 hours). We observed a similar pattern when the expression of SERCA2a and CK was measured. We observed higher SERCA2a and CK expression in RM treated samples than in HD treated samples, though this difference in expression was less pronounced than what was observed with βMHC. During the differentiation time course for RM treated cells, SERCA2a expression appeared to be highest after 96 hours, and CK expression appeared to be stronger after both 72 and 96 hours.

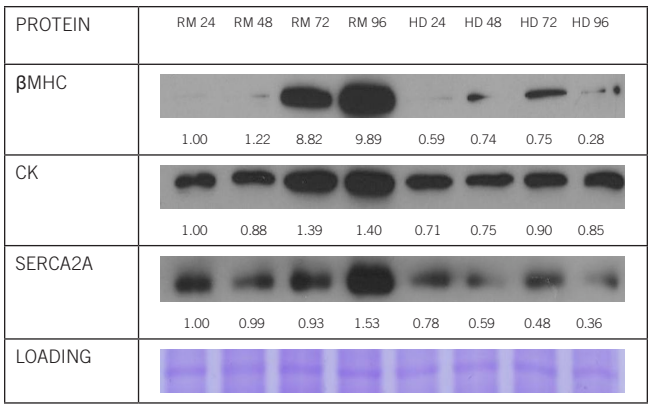


**Fig. 1**. Differentiation Time Course for H9C2 cells cultured in hormone-replete (RM) media and hormone-depleted (HD) media. Daily additions of retinoic acid induced cardiomyocyte differentiation. Cells were harvested after 24, 48, 72, or 96 hours. Relative protein expression (βMHC, CK, SERCA2a) was measured using immunoblotting. Relative protein expression, quantified using ImageJ software and normalized to Coomassie-stained gel used as loading control, is shown below each immunoblot (values in arbitrary units). These values are averages taken from all repeats conducted. The loading control is shown below the data for the proteins.

### EXPERIMENT 2: EFFECTS OF BPA ON CARDIOMYOCYTE PROTEIN EXPRESSION IN H9C2 CELLS CULTURED IN RM AND HD MEDIA

To test the hypothesis that BPA modulates the expression of cardiomyocyte structural, energy and calcium homeostasis proteins, we performed a second experiment to compare protein expression in H9C2 cells treated with BPA with control groups not treated with the endocrine disruptor. Cells in the control group were differentiated in RM or HD media alone, while cells in the experimental groups had $10^{-7}$ M or $10^{-8}$ M BPA in methanol (MeOH) added to the culture at the time of differentiation induction. Controls for both types of media were treated with an equal volume of MeOH only. Cells were harvested after 72 or 96 hours. Results are shown in Fig. 2.

When $10^{-7}$ M BPA was added to RM media for 72 hours, expression of the structural protein βMHC was decreased ($P<0.03$) compared with the RM 72 MeOH control. However, when $10^{-8}$

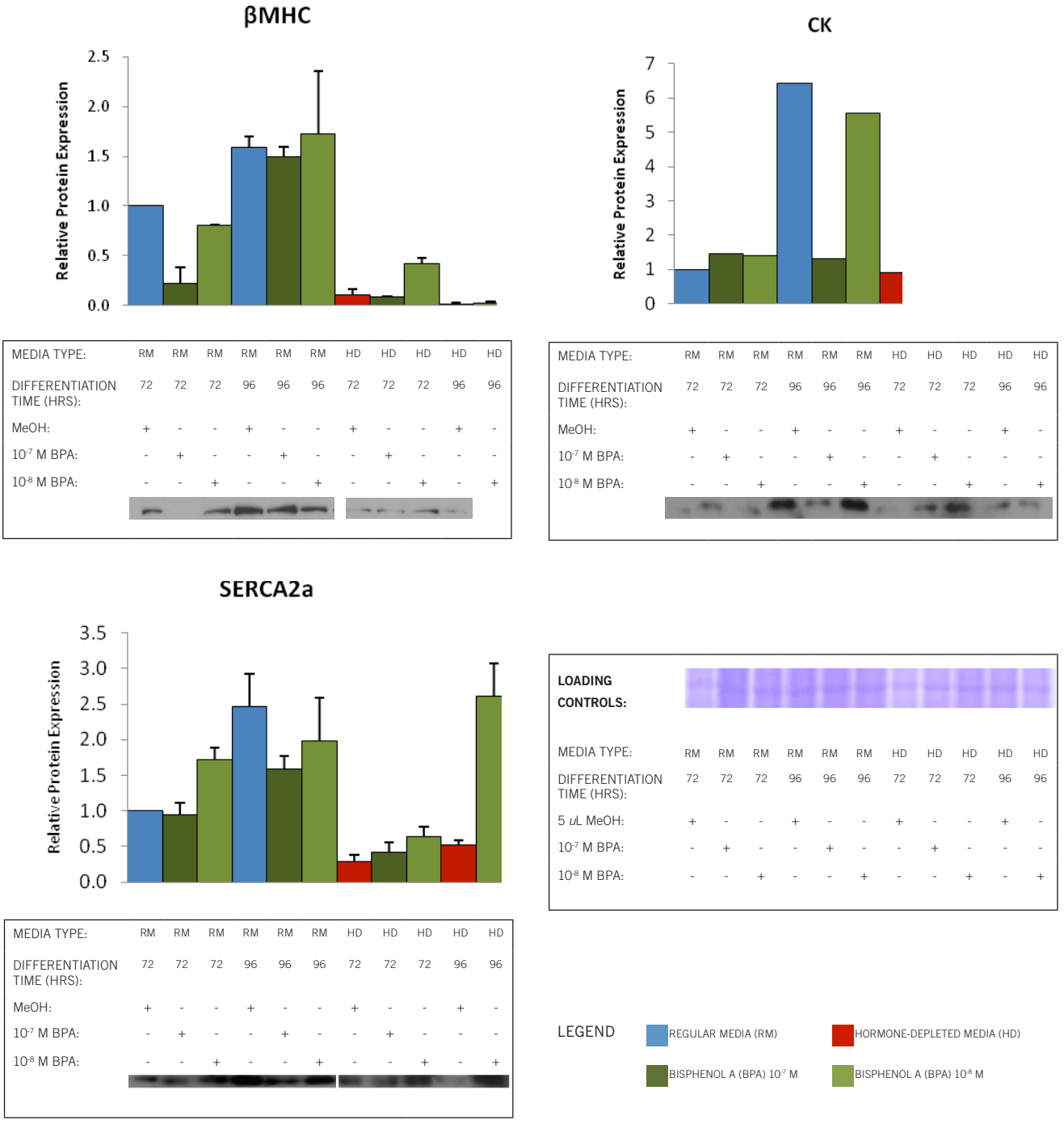


**Fig. 2**. Impact of Bisphenol A on Cardiomyocyte Differentation. H9C2 cells cultured in hormone-replete (RM) media and hormone-depleted (HD) media; cells were treated with $10^{-7}$ M BPA, $10^{-8}$ M BPA, or MeOH (controls). Daily additions of retinoic acid induced cardiomyocyte differentiation. Cells were harvested after 72 or 96 hours. Relative protein expression (βMHC, CK, SERCA2a) was measured using immunoblotting. For each protein, immunoblots are shown along with a graphical representation of relative protein expression, quantified using ImageJ software and normalized to Coomassie-stained gel used as loading control. The gel used as a loading control for all 4 proteins is shown below the data for the proteins. The immunoblot images for βMHC and SERCA2a show a 1 minute film exposure for RM samples, and a 5 minute film exposure of the same gel for HD samples. Error bars are shown for βMHC and SERCA2a, for which replicates were performed.

M BPA was added to HD media for 72 hours, βMHC expression was increased (P<0.05) when compared with the HD 72 MeOH control.

CK is a protein involved in energy production. When the expression of CK was measured, expression increased when cells were treated with $10^{-8}$ M BPA in HD media for 72 hours, compared with the HD 72 MeOH control. For cells cultured in RM media for 96 hours, $10^{-7}$ M BPA treatment decreased CK expression compared to the RM 96 MeOH control.

The calcium homeostasis protein SERCA2a showed decreased expression in RM media at 96 hours when treated with $10^{-7}$ M BPA, though the difference was not statistically significant. Cells treated in RM media for 72 hours showed increased expression when treated with $10^{-8}$ M BPA (P<0.053). Additionally, treating cells in HD media for 96 hours with $10^{-8}$ M BPA caused a significant increase (P<0.05) in SERCA2a expression compared to HD 96 MeOH controls.

### EXPERIMENT 3: TREATMENT WITH BPA AND ANTI-ESTROGEN ICI 182,780

Finally, to investigate whether BPA modulates cardiomyocyte protein expression by binding to ERs, we performed a third experiment. H9C2 cells differentiated in RM or HD media for 72 hours, with both $10^{-8}$ M BPA and pure anti-estrogen ICI 182,780 ($10^{-6}$ M or $10^{-8}$ M) in DMSO, were compared with two sets of controls: cells treated with BPA alone, and cells treated with media lacking both BPA and ICI 182,780. Controls were treated with MeOH, DMSO, or both to account for any possible effects the solvents may have on the cells. Immunoblot results are shown in Fig. 3. We concentrated on SERCA2a and CK expression as these were the most strongly influenced by BPA induction in Experiment 2. Statistical analysis was not performed.

Treatment with BPA and ICI 182,780 ($10^{-6}$ M and $10^{-8}$ M) reduced SERCA2a expression in HD treated cells when compared with both HD 72 BPA $10^{-8}$ M and HD 72 controls, most particularly when $10^{-8}$ M ICI 182,780 was added. However, the inclusion of ICI 182,780 treatment increased SERCA2a expression in RM treated cells when compared with both RM 72 BPA $10^{-8}$ M and RM 72 controls. A similar trend was observed with CK in RM treated cells, with highest expression in cells treated with ICI 182,780 $10^{-6}$ M. Treatment with BPA and ICI 182,780 also appeared to reduce CK expression in HD treated cells when compared with both HD 72 BPA $10^{-8}$ M and HD 72 controls.

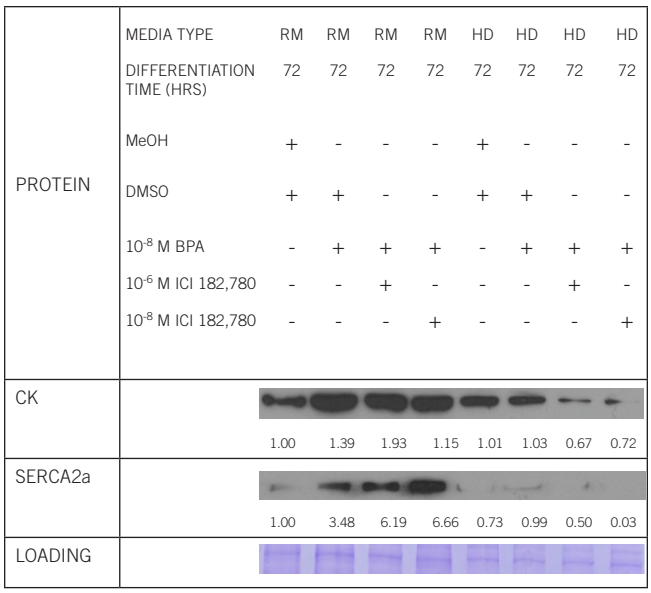


**Fig. 3**. H9C2 cells cultured in hormone-replete (RM) media and hormone-depleted (HD) media; cells were treated with $10^{-8}$ M BPA + $10^{-6}$ M ICI, $10^{-8}$ M BPA + $10^{-8}$ M ICI, $10^{-8}$ M BPA + DMSO, or DMSO + MeOH (controls). Daily additions of retinoic acid induced cardiomyocyte differentiation. Cells were harvested after 72 hours. Relative protein expression (CK, SERCA2a) was measured using immunoblotting. Relative protein expression, quantified using ImageJ software and normalized to Coomassie-stained gel used as loading control, is shown below each immunoblot (values in arbitrary units). Values shown were obtained from one experimental trial. The loading control is shown below the data for the proteins.

## DISCUSSION

Our H9C2 cell culture system is an effective model to measure changes in cardiac structural, energy and calcium homeostasis protein expression during cardiomyocyte differentiation, and offers an appropriate basis for our experiments. In particular, since treatment with BPA produced noticeable and quantifiable effects on protein expression patterns detected through immunoblotting, our cell culture model could be useful for future research exploring the effects of endocrine disruptors on cardiomyocyte protein expression.

Our results from Experiment 1 show that cells differentiated in the presence of hormones in RM have increased expression of βMHC, SERCA2a and CK during the later stages of the time course experiment, suggesting that H9C2 cells have differentiated into cardiomyocytes by this point. As we expected, HD treated cells show lower cardiac protein expression, likely due to incomplete differentiation. This result confirms that the expression of important cardiac proteins is hormone-dependent

during cardiomyocyte differentiation, and is consistent with our hypothesis that sex hormones play a role in modulating cardiac gene expression. However, HD media contains reduced estrogen, progesterone, androgen, glucorticoid, thyroid hormone and cortisol. It is unclear if all or only one of these factors contributes to cardiac differentiation. Further experimentation must be conducted to confirm our belief that changes in sex hormone levels specifically contribute to the observed differences in protein expression between RM and HD treated cells.

Our results in Experiment 2 strongly support our hypothesis that BPA modulates cardiac protein expression in cardiomyocytes *in vitro*. Differentiation in HD media with $10^{-8}$ M BPA, a concentration detectable in human urine (*12*), for 72 hours caused considerable increases in the expression of the structural protein βMHC and the cardiac energy-producing protein CK, compared to HD controls. We observed the same trend with the calcium homeostasis protein SERCA2a, except the increased expression in HD media due to BPA treatment was most pronounced after 96 hours of differentiation. These results show that BPA modulates the expression of cardiac structural, energy and calcium homeostasis proteins in differentiating cardiomyocytes. Furthermore, these results are consistent with our hypothesis that BPA influences cardiac protein expression by binding and activating ERs or ERRγ. BPA could be binding cardiomyocyte ERRγ and activating ERRγ-driven transcription, as demonstrated in previous studies (*13*). ERRγ is thought to exclusively drive the transcription of proteins involved in metabolism and energy production (*14*); however, our results show that BPA modulates the expression of cardiac structural and calcium homeostasis proteins as well. This suggests that BPA may also be activating cardiomyocyte ERs, which would then bind genomic DNA to regulate transcription.

Additionally, treating cells with $10^{-7}$ M BPA in RM media for 72 or 96 hours noticeably decreased βMHC, SERCA2a and CK expression compared to RM controls in Experiment 2. It is possible that the higher concentration of BPA was toxic, causing decreased ER- and/or ERRγ-driven transcription of cardiac proteins. This is consistent with the notion that BPA is an estrogen-like compound, as it is well known that steroid hormones function optimally in a non-monotonic fashion (*15*). This means that a low concentration can appear to be more toxic than a higher concentration and vice versa. BPA was effective and modulated cardiac protein expression even in hormone-replete media with all other factors for differentiation present. This suggests that ingested BPA might affect cardiac differentiation *in vivo*.

In Experiment 3, cells were treated with the anti-estrogen ICI 182,780 and BPA. The results we obtained provide further insight on the possible mechanisms of BPA action. If BPA functions by activating ERs, we expect ICI 182,780 to block the ERs and reverse the changes in protein expression caused by adding $10^{-8}$ M BPA. In HD media, ICI 182,780 appeared to drive SERCA2a and CK expression to even lower levels than those of the controls, or when BPA alone was added to the HD media. This trend suggests that BPA could be modulating SERCA2a and CK transcription by activating ERs. Further experiments should treat cells with a wider range of ICI 182,780 concentrations. The same experiment should also be repeated using other anti-estrogens that block putative ER activity, to test whether they impact results in a similar manner as ICI 182,780.

Our research shows that cardiac protein expression during differentiation is steroid hormone-dependent, and that BPA modulates cardiac protein expression in cardiomyocytes *in vitro*. Future research must be conducted to further understand the mechanisms by which sex hormones and BPA modulate cardiac gene expression. First, we plan to treat cells with higher or lower doses of BPA to identify concentrations at which the estrogenizing effects of BPA are most strongly seen. To identify the main receptor through which BPA functions, the genes encoding ERRγ, ERα and ERβ receptors in H9C2 cells can be over-expressed and under-expressed using our cell culture model. An siRNA approach can be employed to downregulate the expression of these same proteins. We can then measure changes in protein expression in BPA-treated cells as receptor expression is varied. We also plan to test whether performing mutagenesis on the consensus binding elements for ERα, ERβ and ERRγ in the promoters of cardiac target genes, such as SERCA2a, significantly impacts BPA function in H9C2 cells. Additionally, chromatin immunoprecipitation (ChIP) can be used to assay for ER receptor-DNA binding interactions at SERCA2a or other gene promoters when cells are treated with BPA. Finally, an outstanding question is whether BPA regulates transcription epigenetically in cardiomyocytes, in a manner similar to sex hormones (*16*). Sodium bisulfite analysis can be used to sequence possible DNA methylations in CpG islands upstream of genes encoding cardiac proteins after BPA treatment.

The results of our study offer valuable insight into the impacts of sex hormones and endocrine disruptors on cardiac tissue. Given the exposure of pregnant women, children and adults to BPA on a daily basis, further research on the impact of BPA on the cardiovascular system is of major importance to public health. The ability of the environmental toxin BPA to impact the heart by functioning as an estrogenizing compound makes it an important target for future research, which could lead to a greater understanding of how sex hormones and environmental endocrine disruptors influence the development of heart disease.

## ACKNOWLEDGEMENTS

We thank Amanda Kasneci, Rami Haddad and William Noiles, and the rest of the Chalifour laboratory at the Lady Davis Institute for their guidance, support, and encouragement. Funding support is from the Canadian Institutes of Health Research (CIHR).

## REFERENCES

1. C.A. Richter, *Reprod. Toxicol.* **24**, 199-204 (2007).
2. E. Burridge, *Eur. Chem. News*. **17**, 14-20 (2003).
3. A.M. Calafat, X. Ye, L.Y. Wong, J.A. Reidy, L.L. Needham, *Environ. Health Perspect*. **116**, 39-44 (2008).
4. Canada Gazette. Vol 142, 17. April 19, 2008.
5. A. Vivacqua *et al., Endocrine*. **22**, 275-84 (2003).
6. D.C. Dolinoy, D. Huang, R.L. Jirtle, *PNAS*. **104**, 13056-61 (2007).
7. J. Barrett, *Environ. Health Perspect.* **116**, A36 (2008).
8. M. Kruger *et al., Circ. Res*. **102(4),** 439-47 (2008).
9. J.E. Rossouw, *Cardiovasc. Res*. **53**, 550-57 (2002).
10. C. Grohé *et al., FEBS LETTS*. **416**, 107-112 (1997).
11. J. Hescheler *et al., Circulation Research*. **69**, 1476-86 (1991).
12. K. Becker *et al., Int J Hyg Environ Health*. **212(6)**, 685-92 (2009).
13. Liu X *et al., FEBS J*. **274(24)**, 6340-51 (2007).
14. Giguère V, *Endocr. Rev.* **29(6)**, 677-96 (2008).
15. R. Conolly, W. Lutz, *Toxicological Sciences*. **77**, 151-57 (2004).
16. J.A. Moolman, *Cardiovasc. Res*. **69**, 777-780 (2006).