EXPERIMENTAL STUDIES OF BISPHENOL A IN CARDIOVASCULAR CELLS AND TISSUES – EFFECTS ON GENES THAT REGULATE ANGIOGENESIS AND VASCULAR TONE

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Introduction

A growing scientific literature has raised concerns about the health effects of human exposure to the suspected endocrine disruptor bisphenol A (BPA). Humans are continuously exposed to BPA, mainly through consumption of plastic packaged and canned foods and beverages but other possible exposure sources, such as dermal exposure from thermal papers and oral exposure from children's books have also been recognized [1, 2]. Some large epidemiological studies suggest that high levels of exposure to BPA are associated with increased risk of cardiovascular disease. In a study of subjects in the Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS) cohort we report of a positive correlation between serum BPA and atherosclerotic changes in the vascular wall [3]. However, another study of the PIVUS cohort did not show an association between serum BPA and cardiovascular risk according to the Framingham Risk Score [4, 5]. Furthermore, two cross-sectional studies of the National Health and Nutrition Surveys (NHANES) 2003/06 cohort and one prospective study of the European Prospective Investigation of Cancer-Norfolk UK cohort report associations between elevated levels of urinary BPA and cardiovascular disease [6-8].

To elucidate possible mechanisms by which environmental exposure to BPA may increase the risk for cardiovascular disease, more experimental studies are needed addressing the effects of BPA in cardiovascular cells and tissues. The aim of these studies were to investigate the effects of BPA in human primary cells of cardiovascular origin *in vitro* and also to study the effects of long-term exposure to BPA on cardiovascular markers *in vivo* in rat cardiovascular tissues.

Materials and methods

For the *in vitro* studies, human umbilical vein endothelial cells (HUVEC) were treated with 0.1-1000 nM BPA and human cardiomyocytes (HCM) were treated with 0.01-10 μM BPA for 6 hours. The mRNA expression of genetic markers for cardiovascular function was then examined using qRT-PCR in HUVEC and HCM. In addition the effects of BPA on the nitric oxide system and also on *in vitro* angiogenesis were examined in HUVEC using western blot, the DAF-FM nitric oxide assay and the tube formation assay.

For the *in vivo* studies female Fisher rats were exposed to 0.025, 0.25 or 2.5 μg/ml BPA in the drinking water from five to fifteen weeks of age. To mimic BPA exposure in a human westernized diet, a modest dose of fructose (5%) was added to the drinking water. After the exposure period, the rats were killed and the heart was dissected. Then the mRNA expression of genetic markers for cardiovascular function was investigated in cardiac tissue using qRT-PCR.

The concentrations of BPA for the *in vitro* studies were selected based on the concentrations found in human blood samples [3, 9]. For the *in vivo* study the concentrations of BPA were selected based on human exposure levels and the current oral reference dose. Exposure of rats to BPA at 0.025, 0.25 or 2.5 μg/ml in the drinking water resulted in an average intake of 5.5, 58 and 521 μg/kg/body weight per day, respectively. The lowest dose is comparable to the estimated human intake and the middle dose is similar to RfD [10, 11]. Since the mechanisms and effects of BPA are reported to be different at low concentrations compared to high

concentrations, i.e. a non-monotonic dose response, it is critical to use environmentally relevant concentrations and a large dose interval in experimental settings to be able to pick up relevant effects and mechanisms.

Results and discussion

The results from the *in vitro* studies revealed that 1 nM-1000 nM BPA increased the mRNA expression of endothelial nitric oxide synthase (eNOS), vascular endothelial growth factor (VEGF), VEGF receptor 2 (VEGFR2), connexin 43 and angiotensin converting enzyme 1 (ACE1) in HUVEC [12] and that 10 μM BPA increased the mRNA expression of eNOS, ACE1, RELA (NFκβ) and interleukin 8 (IL-8) in HCM. See table 1 for the full list of genetic markers. These findings show that BPA partially affects the same genes in HUVEC as in HCM and further suggests that HUVEC are more susceptible to BPA compared to HCM.

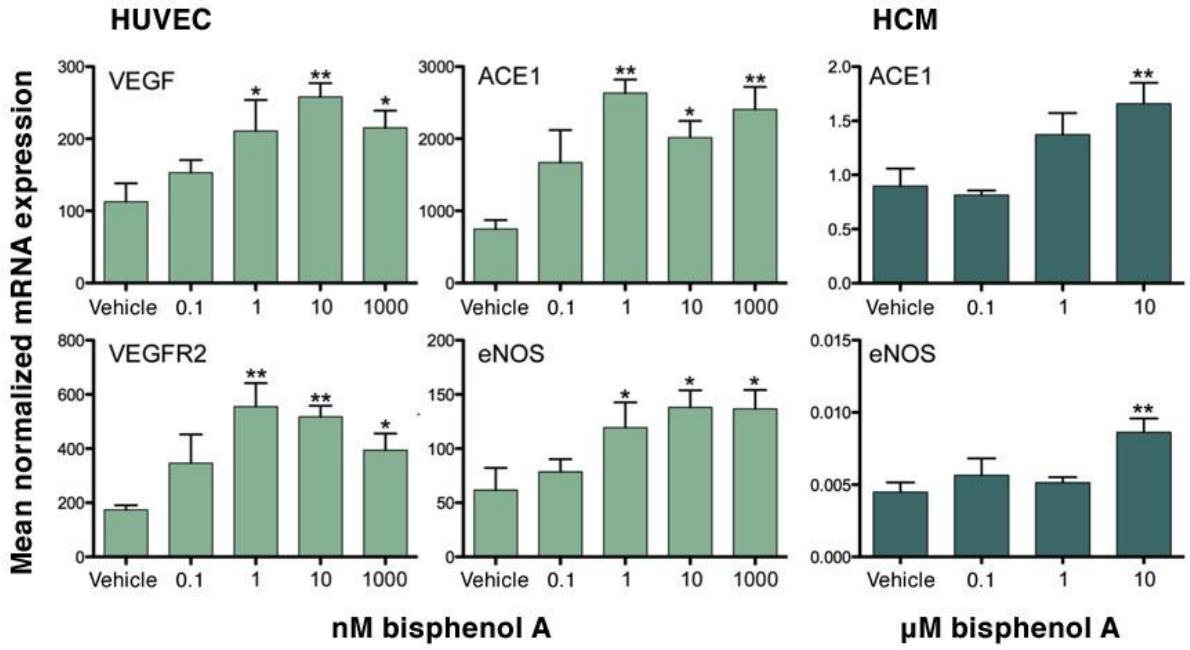

Figure 1. In vitro effects of BPA on genetic markers of cardiovascular function in HUVEC and HCM as demonstrated by qRT-PCR. Bars represents mean normalized mRNA expression ± SEM of three cell cultures. *p<0.05, **p<0.01 compared to vehicle.

Further studies of BPA's effects on the nitric oxide system in HUVEC demonstrated that exposure of HUVEC to 10 nM and 1000 nM BPA increased the expression of activated eNOS ((P-eNOS(ser1177)) and the production of nitric oxide. The expression of activated eNOS in BPA-treated HUVEC was increased in nuclei whereas the expression pattern remained unchanged. P-eNOS(ser1177) was mainly expressed in the nucleus with very weak expression in the plasma, only occasional cells showed expression in the plasma membrane.

Based on the findings that BPA increased the expression of genes encoding VEGF and VEGFR2 and also enhanced the production of nitric oxide, which is known to stimulate angiogenesis down stream of VEGF, we investigated whether BPA could stimulate *in vitro* angiogenesis in HUVEC. The results demonstrated that 1 nM and 10 nM BPA increased HUVEC tube formation suggesting that BPA can act directly on the endothelial cells and stimulate angiogenesis.

Table 1. *Genetic markers of cardiovascular function included in the in vitro and in vivo studies of BPA*

Genetic biomarker	Linked to
VEGF	Angiogenesis
VEGFR2	Angiogenesis
eNOS	Angiogenesis/Endothelial dysfunction
ET-1	Endothelial dysfunction
RELA (NFκβ)	Inflammation
IL-6	Inflammation
IL-8/CXCL1/CXCL2[a]	Inflammation
COX-2	Inflammation, vasoconstriction
HO-1	Oxidative stress
ACE1	Vasoconstriction
Connexin 43	Vasoconstriction/angiogenesis
VCAM1	Endothelial activation
E-selectin	Endothelial activation

a, CXCL1 and CXCL2 are murine IL-8 homologs

The BPA *in vivo* studies revealed that exposure of rats to environmentally relevant levels of BPA, from preadolescence to adulthood, increased the cardiac mRNA expression of genes previously associated with coronary artery disease. Ten weeks exposure of rats to BPA (0.025-2.5 μg/ml) in combination with 5% fructose in the drinking water increased the expression of genes that regulate vasoconstriction and angiogenesis eNOS, VEGF, VEGFR2 and ACE1, compared to rats exposed to vehicle only and cehicle in combination with 5% fructose. The mRNA expression of VEGF, VEGFR2, ACE and eNOS was similar in fructose controls and water controls. Exposure to BPA did not change the cardiac mRNA expression of the other genetic markers included in the study, listed in Table 1, compared to fructose controls and water controls.

Over all, these studies show that the genes that were up-regulated in rat cardiac tissues *in vivo* (eNOS, VEGF, VEGFR2 and ACE1) were also up-regulated in human endothelial cells and cardiomyocytes *in vitro*, following exposure to BPA. According to the *in vitro* studies, the effects of BPA on cardiomyocytes is restricted to eNOS and ACE1 and endothelial cells appear to be more susceptible to BPA compared to cardiomyocytes. The heart is a heavily vascularized tissue that consists mainly of cardiac endothelial cells and cardiomyocytes and although cardiomyocytes dominate the volume of the myocardium the number of endothelial cells exceeds the number of cardiomyocytes by approximately three to one [13]. Thus, the effects of BPA on eNOS VEGF, VEGFR2 and ACE1 mRNA expression in rat cardiac tissues are most likely to be related to an effect of BPA on cardiac endothelial cells but may also involve cardiomyocytes.

VEGF and ACE1 are known as oestrogen responsive genes in cardiovascular cells and tissues and BPAs oestrogen mimicking mode of action may mediate the effects of BPA on ACE1 and VEGF [14, 15]. In addition, VEGF up-regulates ACE1 and eNOS mRNA expressions in human endothelial cells and angiotensin II up-regulates VEGF mRNA expression in rat cardiac endothelial cells, thus, the BPA-induced mRNA expression of eNOS, VEGF and ACE1 in cardiovascular cells and tissues may be associated events [16-18]. ACE1 is responsible for the formation of angiotensin II that exerts vasoconstricting and prothrombotic effects in the vasculature and is strongly implicated in the development of human coronary artery disease [19]. Furthermore, endothelial VEGF signalling and angiogenesis is increasingly being recognized to promote the early stages of atherosclerotic plaque progression and rupture of vulnerable plaques the onset and progression of human coronary artery disease have been associated with angiogenesis in coronary arteries [20].

In conclusion, these findings suggest that the association between elevated environmental exposure to BPA and cardiovascular disorders may be related to the stimulating effects of BPA on pro-angiogenic and vasoconstriction factors in cardiac tissues.

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