Sex-dependent dysregulation of human neutrophils responses by bisphenol A

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Abstract

Background: The study objective was to assess the impact of a xenoestrogen—bisphenol A (BPA) (at the environmental concentration and 100-fold increased dose)—on selected functions of neutrophils, as well as to compare the effect of this compound and 17-β-estradiol (E2) (at the physiological concentration) on the studied functions of these cells in women and men.

Methods and Results: Analysis of the results obtained for both sexes demonstrated a reduction in the chemotactic potential of neutrophils after exposure to BPA. In the presence of BPA, the phagocytic activity, as measured via the SCORE index, was found to be elevated in the neutrophils from women and reduced in those isolated for men. Exposure to BPA resulted in an increased percentage of neutrophils expressing CD14 and CD284 (TLR4), as well as an increased percentage of NET-forming cells, in the case of both sexes. In addition, a reduced capacity to release NETs was observed in cells that were preincubated with xenoestrogen and then stimulated with LPS. The stimulating role of BPA and 17-β-estradiol in the activation of NADPH oxidase, evaluated using NBT stimulation test, was evident only in the neutrophils from women. No influence of E2 could be observed on the expression of CD14 and CD284 by neutrophils, their chemotactic potential and phagocytic activity, and the amount of NETs for both sexes. The study further showed that BPA intensified the NO production and iNOS expression in the neutrophils from both sexes. In addition, an increase in expression was found for all the tested proteins of the PI3K/AKT pathway for men.

Conclusions: The conducted study demonstrated that BPA influenced the functions of neutrophils associated with locomotion and pathogen elimination, which may disturb the response of these cells in both women and men. Furthermore, neutrophils isolated from women were found to be more susceptible to the effect of BPA in terms of oxygen-dependent killing, compared to those obtained from men. The variable results of the tests conducted in the presence of BPA indicated the stronger effect of xenoestrogen on human neutrophils compared to that of 17-β-estradiol.

Introduction

The primary cell components of the innate immune system are phagocytes, including neutrophils (polymorphonuclear leukocytes—PMNs). Owing to a wide spectrum of mechanisms, these cells constitute the first line of defense against pathogenic microorganisms and neoplastic cells. PMNs also serve as regulatory and effector cells in the mechanisms of the acquired immune response. These cells that are present in the bloodstream are metabolically inactive and their cytotoxic properties are inhibited [1–6]. On receiving the chemical signal, neutrophils are stimulated, which enables their movement (chemotaxis) to the site of infection/inflammation. Due to the presence of receptors recognizing molecular patterns of pathogens (PRMs) on the surface of neutrophils, these cells can recognize foreign particles and differentiate them from their own. The PRMs include, *inter alia*, Toll-like receptors (e.g. TLR4), the ligand for which is lipopolysaccharide—LPS [1, 5, 7, 8]. One of the strategies used by neutrophils to kill pathogens is phagocytosis, in which we can distinguish the stages of “recognition” and “absorption” of the pathogen and formation of a phagolysosome. Inside the phagolysosome, the absorbed pathogen is killed by two mechanisms: aerobic (associated with the production of reactive oxygen (ROS) and nitrogen species) and anaerobic (associated with the presence of proteins contained in the granules) [7, 9–13]. Another strategy that neutrophils use as the first line of defense in the body against pathogenic microorganisms is the creation of neutrophil extracellular traps—NETs. This phenomenon can be understood as the release of the content of cellular nucleus (decondensed chromatin and histone proteins) together with components of granularity (including MPO) and cell cytoplasm into extracellular space. In addition to neutralizing and destroying pathogens, NETs create a physical barrier against their proliferation [10, 14–20]. It is suggested that neutrophils prefer a fast pathway of phagocytosis when they come in contact with small microorganisms, whereas

when recognizing larger pathogens or aggregates of microorganisms, neutrophils activate mechanisms resulting in the formation of NETs [21].

One of the reactive nitrogen radicals produced by activated neutrophils is nitrogen oxide (NO). It is produced with the participation of nitric oxide synthase enzymes including, among others, inducible nitric oxide synthase (iNOS) by oxidation of L-arginine. The expression and activity of iNOS can be controlled by both endogenous and exogenous factors [22, 23]. Previous studies demonstrated that Bisphenol A (BPA) affects the production of NO with iNOS participation by human PMNs. Moreover, this process is associated with a different activity of the NF-κB transcription factor in neutrophils depending on sex [24].

Currently, people are well versed with BPA because of its widespread use in the manufacturing of everyday products. BPA can be transferred from packaging to foodstuffs [25, 26]. Its presence has been demonstrated in many tissues and fluids including whole blood, umbilical cord blood, and urine [26–28]. The analysis conducted by the *National Health and Nutrition Examination Survey* showed BPA concentrations in the range of 1.8–660 nM in urine samples of the majority of US residents [29, 30].

BPA is a xenoestrogen—a chemical compound that resembles steroid hormones in its structure. It is classified as an endocrine active compound (EDC), which can interfere with the functioning of the endocrine system by acting through various mechanisms such as imitating natural hormones, inhibiting their action, and/or changing the proper functioning of the endocrine system [31, 32]. In a study, it was demonstrated that BPA can affect signaling pathways related to the nucleus (ERα and ERβ) as well as membrane receptors (GPR30) of estrogens [33]. Research studies indicate the presence of these nuclear receptors in both female and male neutrophils [34].

In the light of current knowledge, it is assumed that human neutrophils are also the target cells for BPA. However, the molecular mechanisms of this xenobiotic action on neutrophils are not fully characterized. Previous studies demonstrated the effect of BPA on neutrophil immunophenotypes as well as gender-dependent effects of BPA on the mechanism of intracellular killing of PMNs associated with the release of serine proteases [35, 36]. Therefore, we conducted further studies in women and men to answer the question: Can BPA affect the basic neutrophil functions? If so, do they depend on gender? To gain a better understanding of the basis of the molecular mechanism of this xenobiotic action on the functions of the studied leukocyte population related to NO synthesis and release, we have assessed the role of another intracellular signaling pathway—PI3K-Akt, which is crucial for the activation of the signal pathway of the NF-κB transcription factor. As the PI3K-Akt pathway activation is expressed by the presence of phospho-PI3K kinase, phospho-Akt (T308), and phospho-Akt (S473) proteins, the evaluation of their expression in the cytoplasmic fraction of neutrophils exposed to BPA can confirm or deny the participation of this signaling pathway in iNOS activation.

Methodology

Reagents

Bisphenol A (BPA), Superoxide Dismutase from bovine erythrocytes, Cytochrome C from equine heart, Griess reagent, nitroblue tetrazolium (NBT), N-Formyl-Met-Leu-Phe (fMLP) and Latex were from Merck Millipore, Burlington, USA. May-Grunwald and Giemsa were from Aqua-med; Polimorphoprep™ (Axis Shield), PBS with or without ions of $CaCl_2$ and $MgCl_2$ (Thermo Fisher Scientific, Waltham, USA). Wortmannin was from Calbiochem (San Diego, CA); ICI 182.780 (ICI/Fulvestrant, 98% pure) and 17-β-estradiol (E2) were from SigmaAldrich.

Material

The study involved a group of 15 healthy voluntary blood donors [5 women (in the follicular phase of the menstrual cycle) and 10 men] aged 22–30 years, from Regional Centre for Transfusion Medicine, Bialystok, Poland. Blood donors were healthy people, who did not smoke and did not drink alcohol for 48 hours before blood collection. All donors gave written informed consent before to blood donations. Material to study was whole blood taken from a vein in the arm into the test-tube with anticoagulant - Heparin (10 U/ml).

All of the experiments were performed in accordance with good laboratory practice.

Neutrophils Isolation

Polymorphonuclear leukocytes (PMNs) were isolated by centrifugation in density gradient Polymorphoprep™. This method enables to obtain 91% pure fraction of PMNs [36].

To purify neutrophils (up to 99,9%), cells positive selection using Midi-MACS magnetic separation system (Miltenyi Biotec, Bergisch Gladbach, Germany) that employed MicroBeads conjugated to monoclonal anti-human CD16 antibodies were performed [36].

The neutrophils survival was evaluated with use of trypan blue and light microscope and was equal 97%.

Assessment Of Cell Capacity To Chemotaxis By Boyden Chamber

Previously isolated neutrophils were suspended in PBS with $CaCl_2$ and $MgCl_2$ ions and incubated for 30 minutes in an incubator with 5% CO_2 flow and 37 °C without stimulants, or in the presence of BPA (16 nM or 1.6 μM) or E2 (100 pM). Using the Boyden chamber (Neuro Probe, Gaithersburg, USA) enables the quantitative assessment of chemotaxis-capable cells using cell movement towards a chemotactic agent. Boyden chamber consists of two compartments separated by a filter with a pore diameter of 5 μm. The lower part of the chamber was filled with a chemoattractant solution – N-Formyl-Met-Leu-Phe (fMLP). Upper part of the chamber was filled out with suspension of 50,000 cells incubated with or without tested compounds. After an hour incubation in an incubator with a 5% CO_2 flow and a temperature of 37 °C, the filter was removed by placing it on a slide. The preparation was stained using the May-Grunwald-Giemza method and evaluated under a light microscope using immersion oil by counting all the cells in the filter. Result were presents as percentage of cells which presents ability to chemotaxis.

Assessment of cell capacity to phagocytosis by Park's method with latex

Blood collected on heparin was centrifuged at 2000 rpm/5 minutes. Then, neutrophils collected from the "leukocyte coat" fraction were incubated (5% CO_2, 37 °C) for 30 min in the presence of BPA (16 nM or 1.6 μM) or E2 (100 pM). Latex (an exogenous beads) was added to the cells and absorbed into the cytoplasm via cells capable of phagocytosis. After incubation (55 minutes; (5% CO_2, 37 °C)), smears were made, which were fixed and stained using the May-Grunwald-Giemza method. The preparations were evaluated under immersion in a light microscope by differentiating neutrophils in terms of the amount of absorbed latex beads into 4 classes: 0 class - neutrophils without absorbed latex deads, I class - neutrophils containing 1–10 latex beads, II class - neutrophils containing 11–30 latex beads, and III class - neutrophils containing more than 30 latex beads in cytoplasm. The application of this method

allows to quantitative (percentage of cells capable of phagocytosis) and qualitative (social cohesion and reconciliation (SCORE) index)) assessment of phagocytosis. SCORE index is a result of multiplying the group's name and number of neutrophils in that group.

Assessment of nicotinamide adenine dinucleotide phosphate hydrogen oxidase (NADPH) activity by Park's test with nitroblue tetrazolium (NBT)

The use of the by Park's test with nitroblue tetrazolium (NBT) allows to assess the effect of BPA and E2 on NADPH oxidase activity in human neutrophils. The method is based on the phenomenon of reduction of the absorbed into cells cytoplasm NBT into insoluble blue formazan crystals.

Whole blood samples were incubated for 30 minutes with BPA (16 nM or 1.6 μM) or E2 (100 pM). Then, NBT was added; moreover, latex beads which stimulate cells to absorption were added into NBT stimulated samples. The samples were then incubated for 15 minutes at 37 °C with a constant flow of 5% CO_2 and 15 minutes at room temperature. Then, smears were made on slides, which were fixed and stained using the May-Grunwald-Giemza method. The preparations were evaluated using immersion oil using a light microscope. The results were expressed as percent of cells with formazan in spontaneous test or as percent of cells with formazan and latex in stimulated test.

Neutrophil Extracellular Traps

Isolated neutrophils were suspended in culture medium containing Rosewell Park Memorial Institute (RPMI) 1640, antibiotic, and serum (4%). Neutrophils were stimulated to form NETs on 96-well culture plates. Cells were introduced in the amount of 5×10^4 per well. Neutrophils were preincubated for 30 min at 37°C, 5% CO_2 in the absence or presence of BPA (16 nM or 1.6 μM) or E2 (100 pM). Then, LPS was added to part of the samples to initiate NET formation by neutrophils. Neutrophil stimulation was performed for 1 h at 37°C, 5% CO_2. Hoechst 3342 dye (Invitrogen) (at a concentration of 1 μg/ml prepared in phosphate-buffered saline (PBS), providing DNA detection) and antihuman myeloperoxidase (MPO) antibody (Life Technologies), which allowed detection of the main NET component, were added to the samples. The analysis of NET formation in wells was carried out under the confocal microscope–IN Cell Analyzer 2200 (GE Healthcare Life Sciences) using the IN Cell Analyzer Workstation program.

Cluster of Differentiation

The expression of cluster of differentiation (CD) antigens on cells suspended in PBS was assessed by the direct fluorescence method using Canto II flow cytometer (Becton Dickinson) and monoclonal antibodies (Becton Dickinson). 20 μl/5 μl of monoclonal antiCD14/antiCD284 antibodies, respectively, were added to 50 μl of the mixture. After 30 min of incubation in the dark, the samples were suspended in PBS and analyzed for 30 min using the FASCSDiva software.

Some of the cells were previously preincubated for 60 min with a wortmannin inhibitor (0.1 μM), Calbiochem (San Diego, CA), or an estrogen-receptor antagonist—ICI 182.780 (1 μM, ICI/Fulvestrant, Sigma Aldrich, 98% pure).

Assessment of total NO concentration in PMN supernatants by Griess reaction

Isolated neutrophils were suspended in Hank's Salt Solution 1X (HBSS) culture medium (5×10^5 cells/ml) in the presence of donor serum, antibiotics: 100 U penicillin/ml and 50 ng streptomycin/ml. The microplates (Microtest III-Falcon) was incubated for 2 hours (temperature – 37 °C, with a constant flow of 5% CO_2 (Nuarie™ US Autoflow, Plymouth, MN) in presence of BPA (16 nM and 1.6 μM) and E2 (100 pM).Some cells were previously preincubated 60 min with a wortmannin inhibitor (0.1 μM) Calbiochem (San Diego, CA) or ICI 182.780 (1 μM, ICI/Fulvestrant, SigmaAldrich, 98% pure). Then, the plate was centrifuged and the culture supernatants obtained were used to assess total nitric oxide (NO) concentration. Cells precipitate was collected and used to assess protein expression by Western Blot.

The assessment of NO production by neutrophils was performed using an indirect method based on measuring the concentration of NO_2^- ions in culture supernatants according to the Griess reaction. The total NO concentration is equal to the sum of the nitrate (III) and nitrate (V) concentrations. The Griess reaction detects only nitrates (III) and consists of a two-stage reaction. At first stage, neutrophils supernatants were incubated for 30 minutes with cadmium to reduce nitrates (V) to nitrates (III). Then, Griess reagent was added, and absorbance was read at 540 nm in spectrophotometer. Nitric oxide products were expressed as mM (5×10^5 cells in 270 ml supernatant).

Western Blot

The cytoplasmic fraction of neutrophils was isolated by the NucBuster™ Protein Extraction Kit (Merck). The isolated cytoplasmic protein was suspended in 2x Laemmli Sample Buffer (Bio-Rad) with βME (Bio-Rad). Protein in the amount of 10 μg/well was applied to polyacrylamide gel and electrophoresis under denaturing conditions (SDS-PAGE) was performed in Mini-PROTEAN® Tetra Cell (Bio-Rad). The Transfer Buffer solution was used to transfer the electrophoretically separated proteins from gel to nitrocellulose membranes on a 0.45 m roll (Bio-Rad) of the Mini-PROTEAN ® Tetra Cell apparatus. The next stages of the procedure were carried out in SNAP i.d.® 2.0 (Millipore) apparatus. The membrane was blocked in 1 × TBS 1% Casein Blocker (Bio-Rad). Nitrocellulose was incubated for 10 min at room temperature with primary antibodies: anti-p-PI3K goat polyclonal, anti-p-Akt1/2/3 (B-5) mouse monoclonal, anti-p-Akt1/2/3 (Ser473) rabbit polyclonal, anti-iNOS rabbit polyclonal (1:100, Santa Cruz Biotechnology). The membrane was rinsed three times in Tris Buffered Saline (Bio-Rad) with Tween® 20 (Sigma). Nitrocellulose was incubated for 10 min at room temperature with appropriate secondary antibodies labeled with alkaline phosphatase (1:5000, Santa Cruz Biotechnology). Again, the membrane was rinsed three times. Immunoreactive strips were obtained by adding BCIP®/NBT Liquid Substrate System (Sigma). The intensity of the stained strips was evaluated using ImageJ software (NIMH, Bethesda, MD). The obtained results are presented in arbitrary units.

Statistical analysis

All data are reported as the mean ± standard deviation (SD). Untransformed variables were statistically analysed if two criteria were met: data were normally distributed (assumption: SD was less than one-half of the mean of non-negative variables) and the Shapiro-Wilk W-test was insignificant. For the comparison between groups, the t-Student test was used. A significance level of $p < 0.05$ was considered statistically significant. STATISTICA version 13.3 program (StatSoft, Inc., Tulsa, OK) was used for all analyses.

Results

Evaluation Of The Ability Of Cells To Chemotaxis

A reduction in the percentage of neutrophils capable of chemotaxis after the exposure to BPA (16 nM and 1.6 μM) was observed in both women and men (Fig. 2). There were no differences in the percentage of neutrophils capable of chemotaxis in both groups in the presence of E2 (100 pM) as compared to the cells incubated without the compound. There were no statistically significant differences between female and male cells with respect to one of the main functions of neutrophils—chemotaxis (Table 1).

Table 1
Chemotaxis of neutrophils of women and men. Value significantly different between: * – cells without and with BPA ($p < 0.05$); d – women and men ($p < 0.05$).

			PMN	PMN + BPA (16 nM)	PMN + BPA (1.6 μM)	PMN + E2 (100 pM)
Women	**percentage of cells capable to chemotaxis (%)**	Mean	8.19	4.14*	4.34*	7.11
		± SD	± 1.25	± 1.33	± 1.4	± 1.01
Men		Mean	7.59	2.14*[d]	2.15*	4,06
		± SD	± 0.61	± 0.71	± 0.61	± 2.11

Examination Of The Phagocytic Ability Of Neutrophils

Exposure of female and male neutrophils to BPA at all tested concentrations and E2 (100 pM) did not lead to changes in the percentage of phagocytosis-capable cells (Table 2) .

Table 2
Phagocytosis—the percentage of phagocytic cells in women and men.

		PMN	PMN + BPA (16 nM)	PMN + BPA (1.6 μM)	PMN + E2 (100 pM)
Women	Mean	86	87	92	89
	± SD	± 13.89	± 18.35	± 26.17	± 22.81
Men	Mean	86	83	69	82
	± SD	± 19.79	± 25.85	± 27.23	± 27.49

In women, it was observed that in the presence of BPA (1.6 μM) there was increased phagocytic activity, measured by the SCORE index, in comparison with the unexposed cells (Fig. 2). In contrast to the results obtained in female neutrophils, incubation of male neutrophils with BPA (1.6 μM) resulted in a decrease in phagocytic activity as measured with SCORE (Table 3).

Table 3
Phagocytosis—SCORE index in women and men Value significantly different between: * – cells without and with BPA ($p < 0.05$); d – women and men ($p < 0.05$).

		PMN	PMN + BPA (16 nM)	PMN + BPA (1.6 µM)	PMN + E2 (100 pM)
Women	Mean	215	222	274*	220
	± SD	± 26.8	± 37.9	± 34.12	± 39.67
Men	Mean	219	211	152*[d]	220
	± SD	± 35.23	± 38.25	± 32.89	± 43.36

There was no effect of BPA (16 nM) and E2 (100 pM) on phagocytosis of the examined cells, in both women and men (Table 3).

Analysis of the results of both sexes showed reduced phagocytic activity of male cells incubated in the presence of BPA (1.6 µM) measured by the SCORE index as compared to female neutrophils (Table 3).

Evaluation Of Nadph Oxidase Activity In Neutrophils

After incubation with BPA (16 nM and 1.6 µM) and E2 (100 pM), the results did not show an increased proportion of nitroblue tetrazolium (NBT) positive cells in the spontaneous NBT test in women as compared to the cells incubated without this compound (Fig. 2). On the other hand, in men, the percentage of NBT positive cells in the presence of BPA at 1.6 µM was significantly higher than those of both female neutrophils and cells incubated without the compound (Table 4).

Table 4
Spontaneous NBT test in women and men. Value significantly different between: * – cells without and with BPA ($p < 0.05$); d – women and men ($p < 0.05$).

		PMN	PMN + BPA (16 nM)	PMN + BPA (1.6 µM)	PMN + E2 (100 pM)
Women	Mean	11	15	12	16
	± SD	± 3.27	± 4.61	± 3.71	± 4.64
Men	Mean	9	10	35*[d]	11
	± SD	± 3.24	± 3.79	± 10.88	± 4.25

The stimulated test in women showed an increased proportion of NBT positive cells in the presence of all applied BPA as well as E2 concentrations compared to the cells incubated without the compound. Contrary to the results obtained in female neutrophils, no changes in the percentage of NBT positive cells in the presence of BPA (16 nM and 1.6 µM) and E2 (100 pM) were observed in men (Table 5).

Table 5
Stimulated NBT test in women and men. Value significantly different between: * – cells without and with BPA ($p < 0.05$); a – cells without and with E2 ($p < 0.05$).

		PMN	PMN + BPA (16 nM)	PMN + BPA (1.6 µM)	PMN + E2 (100 pM)
Women	Mean	32	68*	62*	59[a]
	± SD	± 9.21	± 23.35	± 24.4	± 21.02
Men	Mean	48	45	67	67
	± SD	± 15.92	± 17.03	± 25.2	± 23.42

No statistically significant differences in the percentage of NBT positive cells between female and male neutrophils were found (Table 5).

Evaluation Of Neutrophil Extracellular Traps Formation

Stimulation of female and male neutrophils with LPS caused an increase in the number of NETs compared to networks generated by nonstimulated cells. In contrast to E2 (100 pM), in the presence of BPA (16 nM and 1.6 µM), an increased percentage of NET-forming cells as compared to untreated cells was found in both women and men. Moreover, it was observed that the neutrophils of both sexes preincubated with BPA (16 nM and 1.6 µM) and then stimulated with LPS produced smaller amounts of NETs in comparison to cells stimulated with LPS only (Fig. 3, Table 6).

Table 6
Summary of average values of NET-forming neutrophils. Value significantly different between * – cells without and with BPA ($p < 0.05$); b – cells incubated only with LPS and cells incubated without BPA and LPS ($p < 0.05$); e – cells incubated only with LPS and cells preincubated with BPA and LPS ($p < 0.05$).

	NETs	
	women Mean ± SD	men Mean ± SD
PMNs	7.76 ± 1.83	7.6 ± 1.45
PMNs + LPS	14.73[b] ± 4.21	15.38[b] ± 3.85
PMNs + BPA (16 nM)	13.13* ± 3.55	14.13* ± 2.97
PMNs + BPA (1.6 µM)	15.56* ± 3.86	16.1* ± 4.19
PMNs + E2 (100 pM)	9.98 ± 2.59	9.83 ± 2.56
PMNs + 30' BPA (16 nM) + LPS	8.27[e] ± 2.17	9.11[e] ± 2.16
PMNs + 30' BPA (1.6 µM) + LPS	8.14[e] ± 2.12	8.5[e] ± 2.23

Evaluation Of Neutrophil Surface Markers

The neutrophil cell membrane of men and women displayed an expression of TLR4, CD284, and CD14 (Fig. 4).

A higher percentage of neutrophils with TLR4 was shown in neutrophils of both women and men after BPA (16 nM and 1.6 µM) application in comparison to cells incubated without xenoestrogen. There were no changes in the percentage of neutrophils with this molecule expression in the presence of E2 (100 pM).

Exposure of female and male neutrophils to BPA (16 nM and 1.6 µM) led to an increase in the percentage of neutrophils with CD14 expression as compared to untreated cells. In the presence of E2 (100 pM), there were no changes in the percentage of neutrophils with CD14 expression.

LPS stimulation of neutrophils in women and men resulted in an increase in the percentage of neutrophils with an expression of TLR4 and CD14 compared to cells not stimulated by LPS (Table 7).

Table 7
Alterations in CD284 (TLR4) and CD14 in human PMN. PMN were treated for 2 hours with BPA (16 nM or 1.6 µM) or LPS (10 µg/ml). Value significantly different between * – cells without and with BPA ($p < 0.05$); b – cells incubated only with LPS and cells incubated without BPA and LPS ($p < 0.05$).

	CD284 (TLR4)		**CD14**	
	women	**men**	**women**	**men**
	Mean ± SD	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**
PMN	6.27% ± 2.03	6.14 ± 1.99	52.15% ± 13.2	54.6 ± 14.58
PMN + LPS	32.8^{b} ± 8.41	28.3^{b} ± 8.09	84.7^{b} ± 17.12	92.8^{b} ± 18.07
PMN + BPA (16 nM)	16.3* ± 4.29	15.33* ± 4.99	78.1* ± 16.03	79.2* ± 16.65
PMN + BPA (1.6 µM)	24.75* ± 7.99	22.7* ± 6.13	59.6 ± 18.01	55.1 ± 15.64
PMN + E2	7.95 ± 1.97	6.2 ± 1.78	58.12 ± 19.22	60.6 ± 21.3

Evaluation Of Nitric Oxide Concentration In Neutrophil Supernatants

Exposure of female and male neutrophils showed an increased release of NO in the presence of BPA (16 nM and 1.6 µM) and E2 (100 pM) compared to neutrophils incubated without the compound. There were no statistically significant differences in total NO concentrations between male and female neutrophil supernatants (Table 8).

Table 8
Concentrations of total NO in PMNs supernatants. PMNs were treated with BPA (16 nM or 1.6 μM) or with E2 (100 pM) for 2 hours, and then the supernatants were subjected to nitrite assay. Value significantly different between: * – cells without and with BPA ($p < 0.05$) or a – cells without and with E2 ($p < 0.05$).

		PMN	PMN + BPA (16 nM)	PMN + BPA (1.6 μM)	PMN + E2 (100 pM)
Women	Mean	17.4	30.12*	36.84*	34.65[a]
	± SD	± 4.73	± 7.74	± 8.94	± 7.27
Men	Mean	18.18	34.66*	34.74*	38.91[a]
	± SD	± 5.22	± 8.18	± 6.97	± 7.49

However, in the presence of BPA (16 nM and 1.6 μM) and E2 (100 pM), higher concentrations of total NO were observed in male neutrophil supernatants compared to supernatants incubated without the compound (Table 8).

In order to determine the participation of PI3K kinase in iNOS production by neutrophils exposed to BPA, its concentration in the presence of a selective inhibitor was evaluated. Moreover, experiments with the estrogen receptor ER inhibitor were performed (ICI) to learn the role of estrogen receptors in this process.

In the presence of wortmannin inhibitor or ICI inhibitor, no changes in total NO concentration was demonstrated in female supernatants compared to supernatants of the cells without inhibitor. In turn, lower concentrations of this radical were found in male neutrophil supernatants compared to the cells without an inhibitor (Table 9).

Table 9
Concentrations of total NO in PMNs supernatants. PMNs were treated with BPA (16 nM) or coincubated with wortmanin (0.1 μM) or ICI 180.720 (1 μM) for 2 hours, and then the supernatants were subjected to nitrite assay. Value significantly different between: * – cells without and with BPA ($p < 0.05$) or c – BPA -treated pre-incubated with or without inhibitor ($p < 0.05$).

		PMN	PMN + BPA (16 nM)	PMN + Wortmanin + BPA (16 nM)	PMN + ICI + BPA (16 nM)
Women	Mean	17.4	30.12*	25.93	26.4
	± SD	± 4.73	± 7.74	± 6.43	± 7.62
Men	Mean	18.18	34.66*	19.89[c]	19.04[c]
	± SD	± 5.22	± 8.18	± 5.51	± 5.09

Evaluation of Protein Expression

The incubation of women and men neutrophils with 16 nM BPA showed a higher expression of iNOS and phospho-Akt (T308) in the cytoplasmic fraction as compared to cells incubated without xenoestrogen. In contrast to the results obtained in female neutrophils, the exposure of male neutrophils to BPA also led to an increased expression of phospho-PI3K and phospho-Akt (S473) (Fig. 5, Table 10).

Table 10

Band intensity was quantified using ImageJ software and expressed in arbitrary units (A.U). Data shown are mean and standard deviation (SD) of five independent experiments. Value significantly different between: * – cells without and with BPA ($p < 0.05$) or c – BPA -treated pre-incubated with or without inhibitor ($p < 0.05$).

arbitrary units (A.U).								
	Women				**Men**			
	PMN	**PMN + BPA (16 nM)**	**PMN + wormanin + BPA (16 nM)**	**PMN + ICI + BPA (16 nM)**	**PMN**	**PMN + BPA (16 nM)**	**PMN + wormanin + BPA (16 nM)**	**PMN + ICI + BPA (16 nM)**
	mean ± SD	**mean ± SD**	**mean ± SD**	**mean ± SD**	**mean ± SD**	**mean ± SD**	**mean ± SD**	**mean ± SD**
iNOS	73602 ± 16664.48	121391* ± 19436.22	173411^{c} ± 17211.74	195392^{c} ± 18359.31	79979 ± 15221.77	138417* ± 18654.85	99982^{cd} ± 18963.14	101273^{cd} ± 15469.23
phospho-PI3K	74673 ± 18580.1	83177 ± 17469.22	82881 ± 165487.53	71545 ± 17365.74	91245 ± 18362.84	143493* ± 22145.66	99005^{c} ± 178965.21	91727^{c} ± 21518.1
phospho-Akt (T308)	165281 ± 36346.2	215469* ± 48176.63	365813^{c} ± 67795.12	273775^{c} ± 58443.75	350054^{d} ± 63511.34	437066*^{d} ± 8615.52	370979^{c} ± 56128.76	361734^{c} ± 69581.48
phospho-Akt (S473)	227547 ± 50060.34	237535 ± 39882.35	300272^{c} ± 41046.24	299886^{c} ± 52976.06	369437^{d} ± 70581.77	418813*^{d} ± 8786.34	534478^{cd} ± 72441.78	496401^{cd} ± 89208.22

To confirm the participation of PI3K kinase in the induction of iNOS expression in PMNs exposed to BPA, experiments with a selective inhibitor of this kinase (wortmannin) were carried out.

In the presence of the PI3K kinase inhibitor, a higher expression of iNOS and phospho-Akt308 in the cytoplasmic fraction of PMNs was found in women while a lower expression of the same was found in men compared to cells without an inhibitor. In contrast to the results obtained in female neutrophils, the exposure of male neutrophils to BPA led to a decrease in phospho-PI3K expression compared to cells without the inhibitor. Both female and male neutrophils showed increased phospho-Akt473 expression (Fig. 5, Table 10). Experiments with the estrogen receptor inhibitor (ICI) were carried out to examine the role of estrogen receptors in the induction of the tested proteins.

In the presence of the ICI inhibitor, a higher expression of iNOS in the cytoplasmic fraction of PMNs exposed to BPA was demonstrated in women, while a lower expression of iNOS was displayed in men. In the case of phospho-PI3K, there were no changes in the expression of this protein in the cytoplasmic fraction in women. In contrast to the results obtained in female neutrophils, after ICI inhibitor application, men showed a lower expression of phosphor-PI3K in the

cytoplasmic fraction of PMNs exposed to BPA compared to cells incubated without an inhibitor. No changes in phospho-Akt (T308) expression were shown in both female and male neutrophils. However, a higher expression of phospho-Akt (S473) was observed in the cells of both sexes compared to cells incubated without the inhibitor (Fig. 5, Table 10).

Discussion

Due to numerous controversies concerning the toxicity and safety of BPA, the so-called low dose hypothesis was put forward. It says that EDCs may have an undesirable effect on animals in doses considered safe for humans till date. It is suggested that humans may also experience undesirable effects at theoretically low doses [37, 38]. To verify this hypothesis, we cross-checked several research studies that state that, during the exposure of neutrophils to different doses of BPA, a different xenoestrogen effect on these cells is observed. In this study, we extended these observations and demonstrated that BPA also modulates neutrophil functions at the lowest environmental concentration (16 nM).

The study carried out in our laboratory demonstrated, both in women and men, the opposite effect of BPA on neutrophil chemotaxis as compared to estradiol. The inhibitory effect of BPA on the chemotaxis of these cells observed by us was also presented by the Balistrieri team and in the studies carried out on PMNs isolated from people exposed to chronic dichlorodiphenyltrichloroethane—an insecticide included in EDCs. It was demonstrated that EDC compounds, which also include BPA, lead to a reduced ability of these cells to adhere to chemotaxis, phagocytosis, and aerobic killing. These researchers also observed that the impairment of PMN functions in these individuals was associated with an increased incidence of diseases such as upper respiratory tract infections [39, 40].

The results of our study on the influence of estradiol (in the physiological concentration applied by us) on the phagocytic activity of neutrophils are also consistent with the results obtained by other scientists. The Shibuya team did not observe any effect of estradiol on the zymosan-induced phagocytic activity of neutrophils. The researchers also showed that high estradiol concentrations inhibited MPO degranulation from cytoplasmic neutrophil granules, while physiological estrogen concentrations reinforced this process [41].

It is suggested that the stimulating or inhibitory effect of BPA may depend not only on its dose and the mechanism in which it acts on the cells but also on the estrogen concentration in the human body [25]. Depending on the sex, this concentration varies, and, in women, it changes during each phase of the menstrual cycle. Therefore, in the case of cell exposure to BPA, we observed a different direction of BPA action to estradiol related to the phagocytic activity of neutrophils in women and men. The inhibitory effect of BPA (16 μM) on neutrophil phagocytic activity measured by SCORE is consistent with the results presented by Balistrieri et al. [39]. These researchers demonstrated the inhibitory effect of BPA on the neutrophils' ability to phagocytosis and killing ability of *Staphylococcus aureus*. However, a different trend was found in women.

Liao et al. [42] suggest that the mechanism of BPA action on neutrophil activity may result from the direct influence of this xenoestrogen on TLR receptors, including TRL4, or its indirect influence leading to a change in cell signaling. Our study demonstrated that BPA leads to the activation of TLR4 receptors in neutrophils. Therefore, it can be assumed that one of the mechanisms of BPA action on these cells is its direct action, which can lead to an inappropriate immune response associated with neutrophils in case of defense against pathogens. It is important to note that, in this study, we demonstrated in women the regulatory stimulating role of BPA (at doses of 16 nM, 1.6 μM) and estradiol in NADPH oxidase activation measured by the NBT stimulated test. On the other hand, while evaluating the NADPH oxidase activity in the NBT spontaneous test, we observed the stimulating effect of BPA (at 1.6 μM) only in men. The abovementioned results indicating the stimulating effect of BPA on the generation of superoxide anion radical can be explained by the possibility of BPA influence on messenger RNA (mRNA) expression of NADPH oxidase subunits:

p47phox and p67phox. Watanabe et al. [38] demonstrated the effect of BPA (at concentrations of 2–12 μM) on mRNA expression of these subunits.

Therefore, the obtained results indicate that different phagocytic activity and NADPH oxidase activation by female and male neutrophils under the influence of BPA may result from a stronger affinity of this compound to ERβ receptor than ERα. Molero et al. [34] showed that estradiol upregulated both ERα and ERβ receptors in female neutrophils but only ERα in male cells. BPA is considered to be a relatively weak xenoestrogen as compared to estradiol 17β and its affinity to ERα and ERβ is 1000–10000 times smaller. However, it has ten times higher affinity to the ERβ receptor than to the ERα one [43].

Efficient production of superoxide anion radical is crucial for the oxygen killing mechanism involving the production of ROS, which has a direct cytotoxic effect on pathogens. Moreover, NADPH oxidase activation is an important element of the NETosis process regulation—generation of NETs that neutralize pathogens immobilized in them [44, 45]. In this study, in contrast to estradiol, we observed the influence of BPA on the process of NET formation by neutrophils of both women and men. Considering the disadvantages or ill effects of traps, excessive network generation in people exposed to this xenoestrogen may lead to the development of inflammatory diseases, including autoimmune disorders [46, 47]. Reduction of neutrophil traps, observed in our study, after preincubation with xenoestrogen in response to LPS, may be important in the infectious environment. Additionally, previous studies indicate that BPA modulates epigenetic DNA modification by influencing DNA methylation and posttranslational modification of histones. It is suggested that EDCs may cause intergenerational hereditary susceptibility to DNA-related disorders. It is suggested that maternal exposure to EDCs may contribute to changes in the fetus that will have genetic consequences in future generations [48].

Experiments with BPA also showed the same stimulating effect of BPA as estradiol on the generation of nitric oxide by neutrophils of both sexes. Therefore, we were looking for an answer to the question: Does the generation of NO with iNOS participation result from the activity of the PI3K/Akt pathway in these cells? Interestingly, in the case of BPA at the lowest environmental concentration (16 nM), we observed the involvement of the PI3K/AKT pathway in this process only in male cells. In the case of female neutrophils, we found BPA involvement in the phosphorylation of threonine 308 of Akt kinase. Based on available data, it should be noted that the phosphorylation of the residues of threonine and the serine of the Akt kinase may not only occur independently with the participation of kinases but also autocatalytically [49]. Moreover, based on the changes in NO production and iNOS expression in the presence of wortmannin inhibitor (PI3K pathway inhibitor) in neutrophils of women and men in the presence of BPA, it can be assumed that NO synthesis with iNOS involvement in these cells is associated with PI3K kinase. However, depending on the sex, it plays a different role in this process. More importantly, the results obtained in the presence of ICI suggest that BPA affects NO regulation in female and male cells by acting on estrogen receptors. However, the observed different effects of BPA on iNOS regulation based on sex, which is largely related to ERα and ERβ, seem to result from the activation of separate control pathways by BPA for both receptors.

To sum up, the results of our study, both in women and men, indicate a significant influence of BPA (even at the lowest environmental concentration) on the basic functions of neutrophils, which may lead to disorders of the first phase of the immune response associated with defensive reactions against pathogens and cancer cells. Furthermore, neutrophils isolated from women were found to be more susceptible to the effect of BPA in terms of oxygen-dependent killing, compared to those obtained from men. The variable results of the tests conducted in the presence of BPA indicated the stronger effect of xenoestrogen on human neutrophils compared to that of 17-β-estradiol. Moreover, different results of tests on the presence of BPA in women and men indicate sex-dependent, different mechanisms of the influence of xenoestrogens on neutrophils.

Considering the results presented above, one of the characteristic features of EDCs is the nonmonotonic dose-effect relationship, which makes it difficult to determine the specific direction of the effect of BPA on neutrophils, and in particular, the mechanism that could probably lead to the effects obtained. The possibility of simultaneous interaction of multiple xenoestrogen compounds should also be taken into account, which may result in overlapping of effects, their intensification, or mutual inhibition. Further research is, therefore, necessary to clarify the changes that may occur under the influence of BPA and its potential effects on human health. Therefore, it appears that to reduce exposure to BPA, we should handle plastic packaging properly, including the avoidance of heavy-duty cleaners as well as rough scrubbers damaging the surface of the packaging.

Declarations

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in paper.

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Authors contribution

statement

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Ethical Approval and Consent to participate

The Ethics Committee of the Medical University of Bialystok (R-I-002/396/2019; R-I-002/333/2017) approved this study.

Consent to participate

Informed consent was obtained from all participants prior to blood donations. All of the experiments were performed in accordance with good laboratory practice.

Consent for publication

Not applicable.

Availability of supporting data

The datasets used and/or analyzed during the current study are availablefrom the corresponding author on reasonable request.

Authors contribution statement

All authors have contributed substantially to this work. WRW wrote the manuscript with critical comments and input from EJ, MG, MD, SW and PR. WRW, KN, KW and MG designed the experimental protocol. WRW, MG, MR, KN, JC and KW performed experiments and collected date. WRW performe the statistical analysis. WRW, MG, EJ and KN drafted the manuscript. All authors read and approved the manuscript.

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Figures

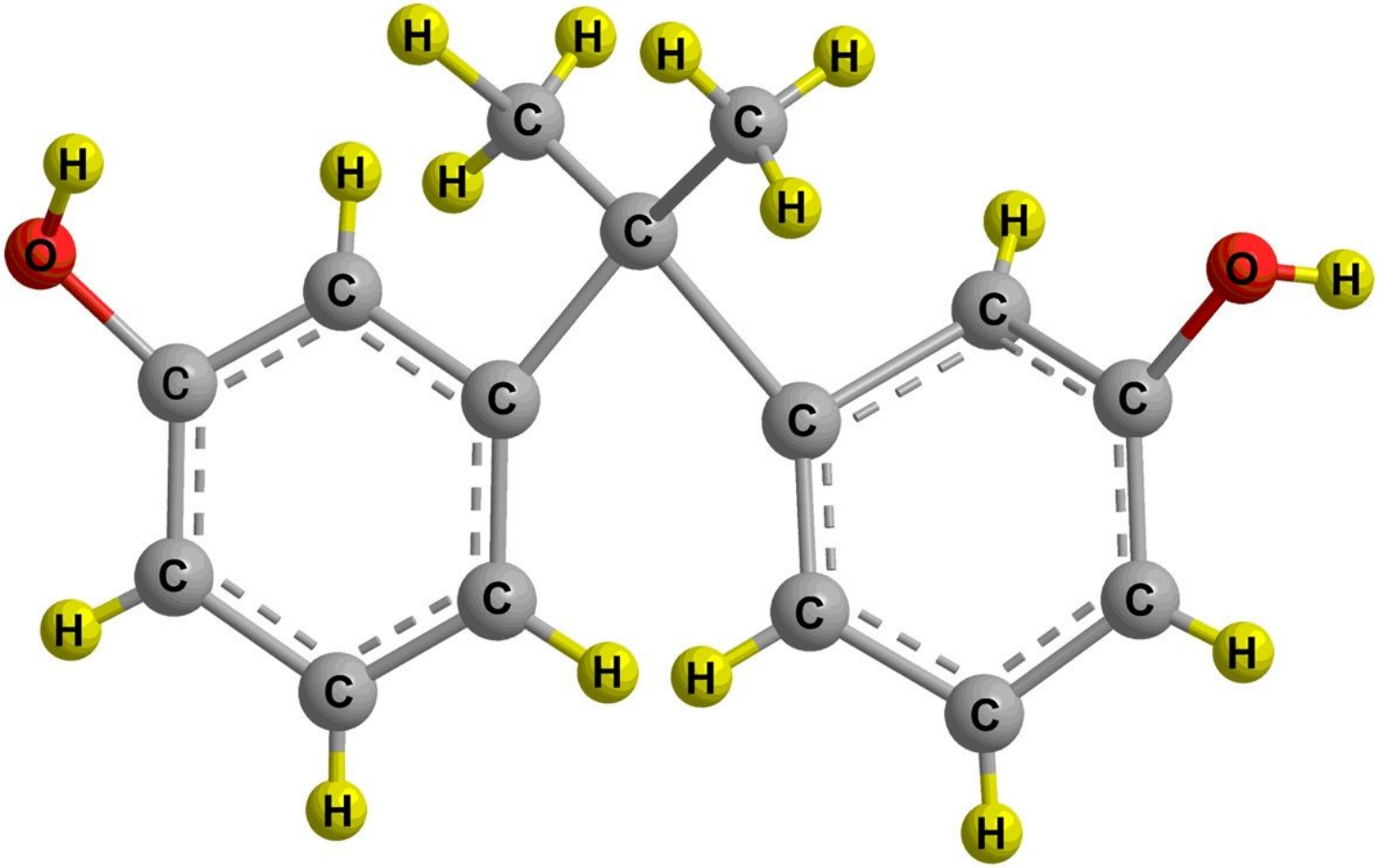

Figure 1

Structural Formula of Bisphenol A

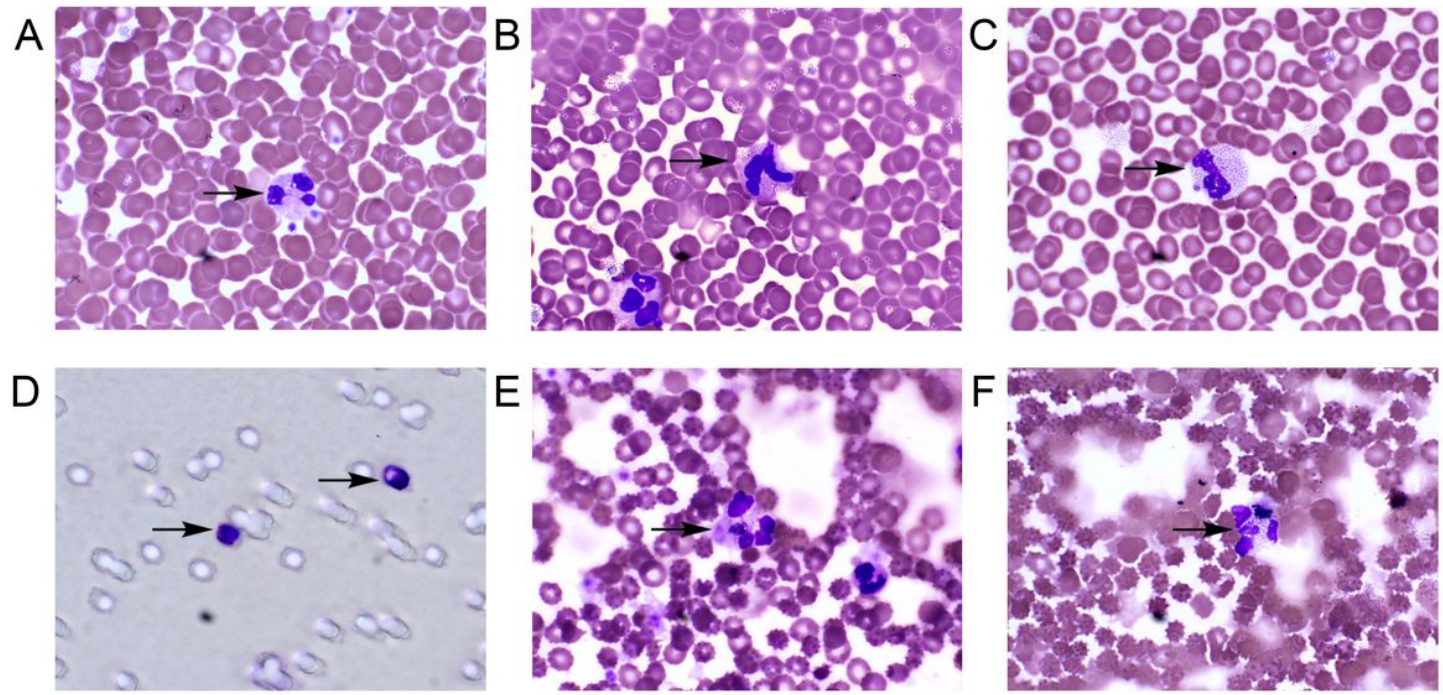

Figure 2

Representatives images of neutrophils after 60 min incubation with 16 μM BPA and assessed neuthophils' functions: phagocytosis, chemotaxis and NADPH oxidase activity. Cells were stained by May-Grünwald-Giemsa and evaluated in light microscope. Malignation × 100. A-C Neutrophils ability to phagocytosis was assessed in Park's test with latex beads. A. Neutrophil in the 1st group with up to 10 latex beads in cytoplasm. B. Neutrophil in the 2nd group with 11-30 latex beads in cytoplasm. C. Neutrophil in the 3rd group with more than 30 latex beads in cytoplasm. D. Neutrophils ability to chemotaxis after BPA treatment was evaluated in the Boyden chamber. The arrow marks cell which passed through pore in membrane. The arrow with star labels cell which stays in membrane pore. E-F. NADPH oxidase activity in neutrophils after BPA treatment was evaluated in NBT test. E. Neutrophil with formazan cristal in spontaneous test. F. Neutrophil with latex beads and formazan cristal in cytoplasm in stimulated test.

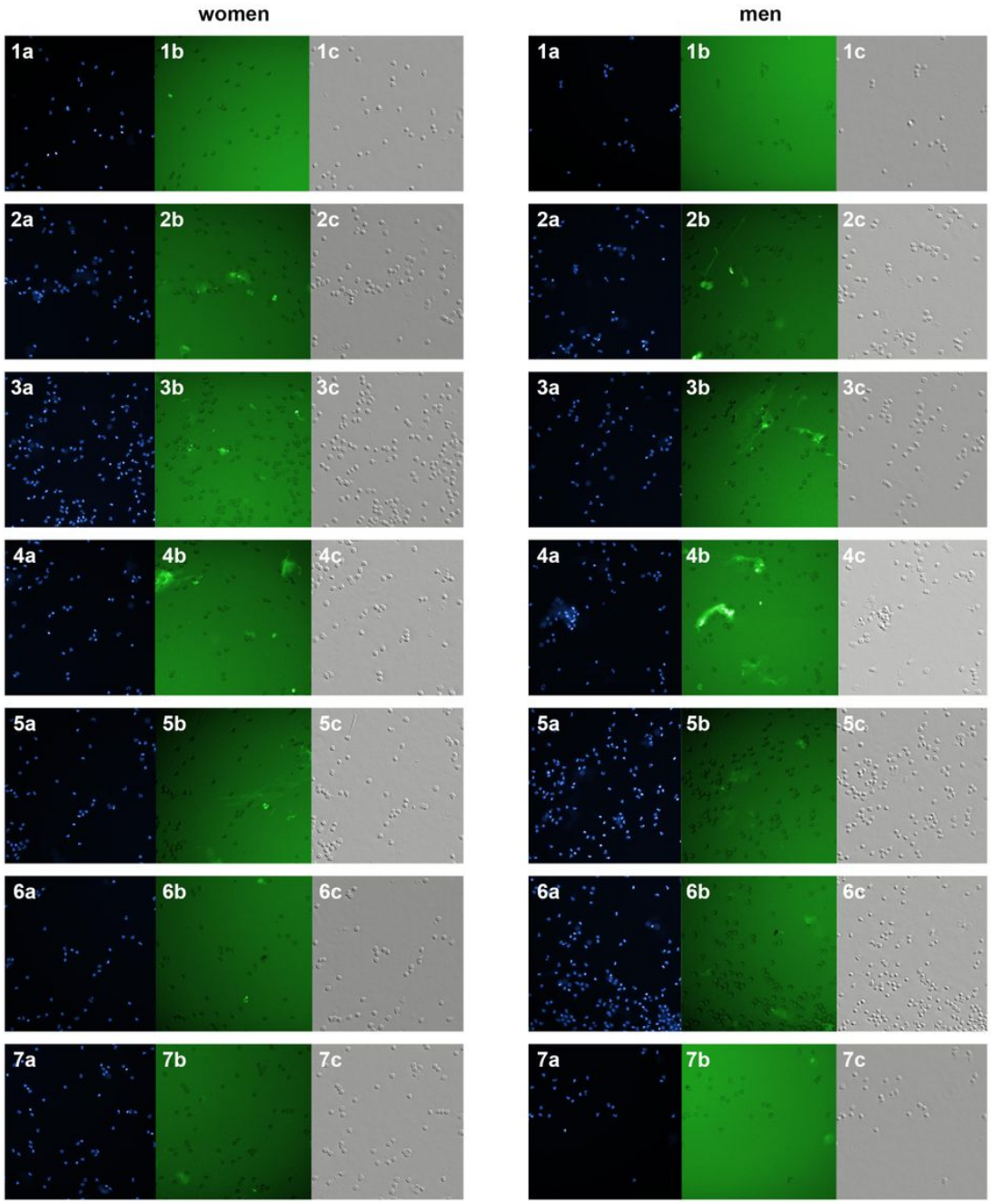

Figure 3

Induction of NET formation by BPA or LPS. Neutrophils from healthy donors (women and men) were stimulated with the indicated reagents and analyzed by confocal microscopy and In Cell Analyzer Workstation (c - differential interference contrast (DIC) setting). Cells were stained with Hoechst 3342 against chromatin (a - blue) and anti-MPO antibody (b - green) to verify NET production. Original magnification ×20. Representative images of neutrophils are shown. 1 - unstimulated PMNs; 2 - LPS (10 μg/ml) stimulation; 3 - BPA (16 nM) stimulation; 4 - BPA (1.6 μM) stimulation; 5 – E2 (100 pM) stimulation; 6 - 30-minutes BPA (16 nM) preincubation and LPS (10 μg/ml) stimulation; 7 - 30-minutes BPA (1.6 μM) preincubation and LPS (10 μg/ml) stimulation.

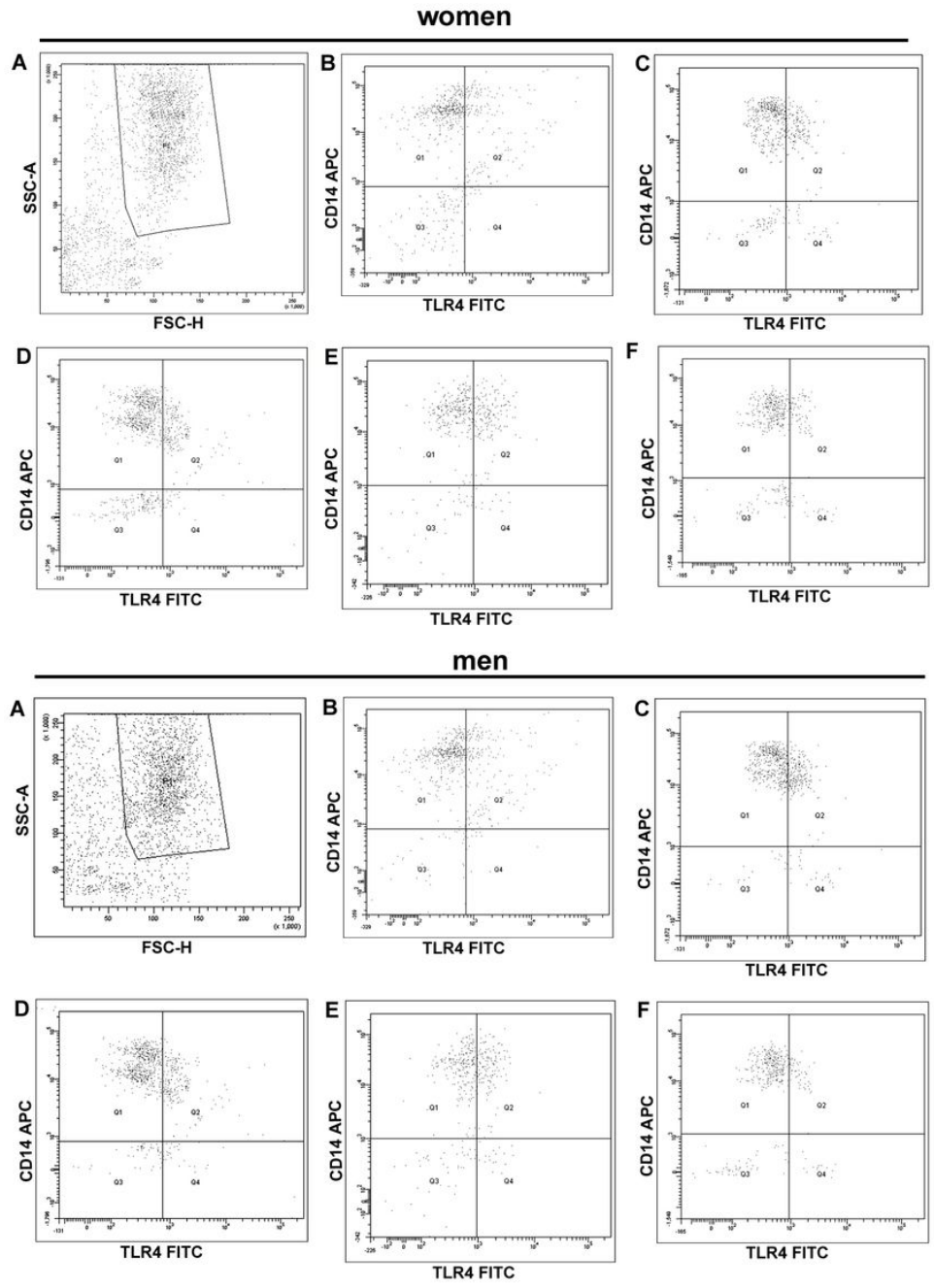

Figure 4

Representative FCAS plots demonstrating of CD antigens (CD14 and CD284 (TLR4)) expression on the PMNs: A – PMNs; B – PMNs without BPA and LPS; C – cells incubated only with LPS; D – cells incubated only with BPA (16 nM); E – cells incubated only with BPA (1.6 μM); F – cells incubated only with E2 (100 pM).

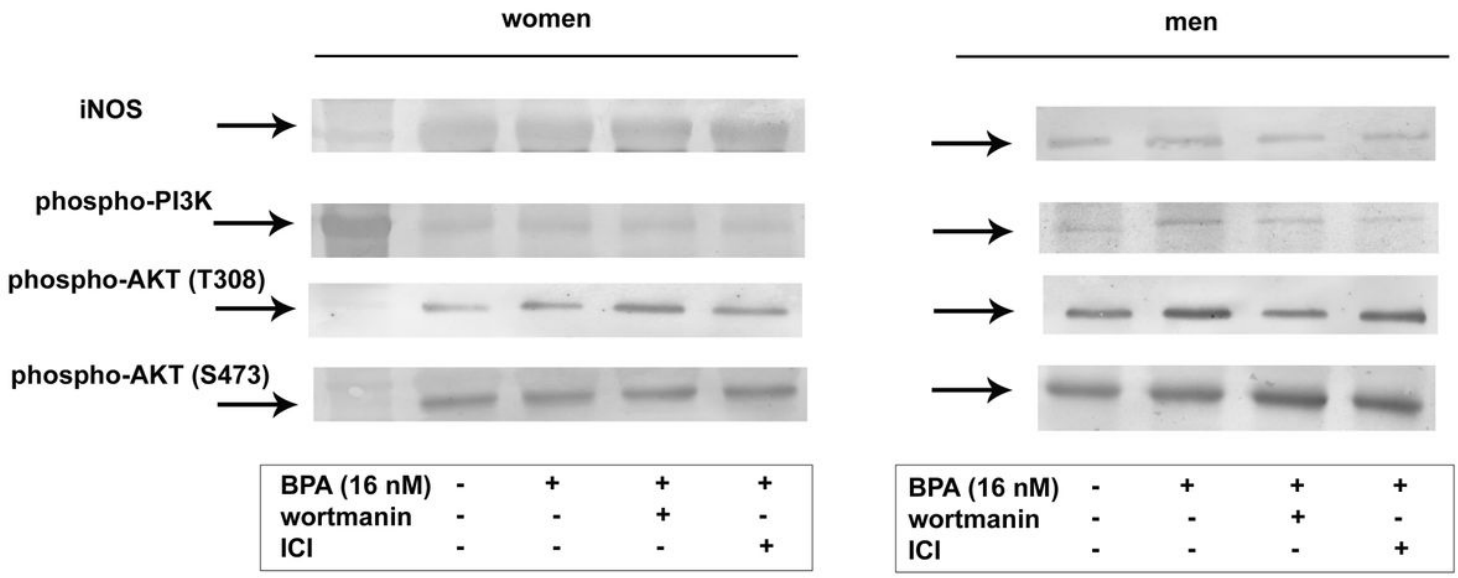

Figure 5

Expression of iNOS, phospho-PI3K, phospho-Akt (T308) and phospho-Akt (S473) in human PMNs. PMNs were treated with or without wortmannin (0.1 mM) or ICI 180.720 (1μM) for 1 h before addition of BPA (16 nM). The cytoplasmic fractions obtained from the cells were used to detect for iNOS, phospho-PI3K, phospho-Akt (T308) and phospho-Akt(S473) protein levels by western blot. These results are representative of five independent experiments.