Polychlorinated biphenyls (pcb)-induced changes in the expression of insulin downstream signalling molecules in cardiac tissue of adult male rats.

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Abstract

Persistence and accumulation of numerous toxic substances found in the environment solely resulting from human activity causes abnormality in normal growth and developmental functions of endocrine systems in mammals. Among these xenobiotic endocrine disruptors, PCBs' (Polychlorinated Biphenyls) effect on living organisms is of great interest. Its immense durable properties are the cause of extensive usage in various industries, which resulted in their wide distribution throughout the ecosystem. Our previous studies have shown that PCB exposure alters glucose homeostasis in skeletal muscle.

Keywords: PCB, diabetes, Vitamin C and E, Adult male Wistar albino rats, Innovative technology; Novel method.

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Introduction

Polychlorinated Biphenyls (PCB) are a type of organochlorines which are stable and are resistant to temperature and pressure extremes. There has been an association of diabetes with exposure to a wide variety of organochlorines like dioxins, dioxin-like compounds, PCBs and also persistent pesticides.

PCBs act on a number of endogenous hormones, which in turn, shows its relationship with diabetes [1-5]. A previous research done on this topic by Persky et al., studied the relationship of PCB exposure at a capacitor manufacturing plant with diabetes and endogenous hormones in post-menopausal women [6].

Insulin and its downstream signalling molecules are present in the brain tissues especially in the regions of cortex and hippocampus [6-10]. IRS, PI3K, Akt and GSK-3β are the major molecules involved in insulin signalling. These major molecules are activated or deactivated through increased or decreased phosphorylation which plays a role in insulin signalling abnormalities or insulin resistance.

This research is needed to find a safe drug for diabetes so that it can be used by human beings. This research aims to fill the lacunae in literature on the expression of insulin downstream signalling molecules in cardiac tissue of adult male rats.

Our team has extensive knowledge and research experience that has translate into high quality publications. The aim of this study is to identify the Vitamin C and E as a therapeutic drug against PCB- induced diabetic rats [11-15].

Materials and Methods

Chemicals

All chemicals and reagents used in this study were purchased from Sigma Chemical Company St. Louis, MO, USA; Invitrogen, USA; Eurofins Genomics India Pvt Ltd, Bangalore, India; New England Biolabs (NEB), USA; Promega, USA.

PCB was procured from Sigma Chemical Company St. Louis, MO, USA; Total RNA Isolation Reagent (TRIR) was purchased from Invitrogen, USA. The reverse-transcriptase enzyme (MMuLv) was purchased from Genet Bio, South Korea purchased from Promega, USA.

Animals

The present experimental study was approved by the institutional animal ethics committee (IAEC no.: BRULAC/SDCH/SIMATS/IAEC/12.2019/048). Adult male Wistar albino rats, weighing 180–200 g, were obtained and maintained in clean propylene cages at the Biomedical Research Unit and Laboratory Animal Centre (BRULAC), Saveetha Dental College and Hospitals, Saveetha University, India) in an air-conditioned animal house, fed with standard rat pelleted diet (Lipton India Ltd., Mumbai, India), and clean drinking water was made available ad libitum. Rats were divided into 3 groups, each consisting of 6 animals.

Experimental Design

Group 1: Control (Vehicle control, rats were intraperitoneally (ip) administered with the vehicle (corn oil) for 30 days.

Group 2: Rats received PCB (PCB was dissolved in corn oil at a dose of 2 mg/kg body weight (b.wt) intraperitoneally daily at 10:00 am for 30 days.

Group 3: PCB and vitamin E (dissolved in olive oil at a dose of 50 mg/kg body weight), and vitamin C treated (100 mg/kg body weight dissolved in distilled water daily at 10 AM through gastric intubation for 30 days).

At the end of treatment, animals were anesthetized with sodium thiopental (5 mg/kg, ip), and 20 ml of normal saline was perfused through the left ventricle, to clear blood from the liver, and other organs. Cardiac tissue was dissected out and used for the assay of various parameters.

Gene Expression Analysis

Isolation of total RNA

Total RNA was isolated from control and experimental samples using TRIR (total RNA isolation reagent) kit. Briefly, 100 mg fresh tissue was homogenized with 1 ml TRIR and the homogenate was transferred immediately to a microfuge tube and kept at -80°C for 60 min to permit the complete dissociation of nucleoprotein complexes.

Then, 0.2 ml of chloroform was added, vortexed for 1 min and placed on ice at 4°C for 5 min. The homogenates were centrifuged at 12,000 xg for 15 min at 4°C. The aqueous phase was carefully transferred to a fresh microfuge tube and an equal volume of isopropanol was added, vortexed for 15 sec and placed on ice at 4°C for 10 min.

The samples were centrifuged at 12,000 xg for 10 min at 4°C. The supernatant was discarded and RNA pellet was washed with 1 ml of 75% ethanol by vortexing and subsequent centrifugation for 5 min at 7,500 xg (4°C). The supernatant was removed and RNA pellets were mixed with 50 µl of autoclaved Milli-Q water and dissolved by heating in a water bath for 10 min at 60°C.

Quantification of RNA

Diluted RNA samples were quantified spectrophotometrically by measuring the absorbance (A) at 260/280 nm. 40 µg of RNA in 1 ml gives one absorbance at 260 nm. Therefore, the concentration of RNA in the given sample can be determined by multiplying its A260 by 40 and dilution factor.

The purity of RNA preparation can be calculated using the ratio between its absorbance at 260 and 280 nm. A ratio of absorbance at 260/280 nm>1.8 is generally considered as good quality RNA. The purity of RNA obtained was 1.8.

Reverse Transcriptase – Polymerase Chain Reaction (RT – PCR)

RT-PCR is an approach for converting and amplifying a single stranded RNA template to yield abundant double stranded DNA products. 1. First strand reaction: Complementary DNA (cDNA) is made from the mRNA template using Oligo dT, dNTPs and reverse transcriptase. 2. Second strand reaction: After the reverse transcriptase reaction is complete, standard PCR (called the "second strand reaction") is initiated.

Principle RT-PCR is a method used to amplify cDNA copies of RNA. It is the enzymatic conversion of mRNA into a single cDNA template. A specific oligodeoxynucleotide primer hybridizes to the mRNA and is then extended by an RNA dependent DNA polymerase to create a cDNA copy.

First strand DNA synthesis The RT kit was purchased from Eurogentec (Seraing, Belgium). Reagents 1. 10X RT buffer: One vial containing 1.4 ml of 10X RT buffer. 2. Euroscript reverse transcriptase: One tube containing 75 µl of Moloney Murine leukemia virus reverse transcriptase (3750 U at 50 U/µl).

Quantitative real time PCR principle

The purpose of a PCR (Polymerase Chain Reaction) is to make a huge number of copies of a gene. There are three major steps in a PCR, which are as follows: Denaturation at 94°C for 3 min: During the denaturation at 94°C for 2-5 min, the double strand melts open to single stranded DNA, all enzymatic reactions stop.

Annealing at 54°C- 65°C for 30 sec: Ionic bonds are constantly formed and broken between primer and the single stranded template to ensure the extension process. Extension at 72°C for 30 sec: Primers that are in positions with no exact match get loose again (because of the higher temperature) and don't give an extension of the fragment.

The bases (complementary to the template) are coupled to the primer on the 3' side (the polymerase adds dNTP from 5' to 3', reading the template from 3' to 5' side; bases are added complementary to the template). Because both strands are copied during PCR, there is an exponential increase of the number of copies of the gene.

Reagents

1. 2X Reaction buffer: The PCR master mix kit was purchased from Takara

Bio Inc., Japan. Contains TaKaRa Ex Taq HS (a hot start PCR enzyme) dNTP

Mixture, Mg2+, Tli RNase H (a heat-resistant RNase H that minimizes PCR

inhibition by residual mRNA), and SYBR Green I.

2. Forward primer (10 µM).

3. Reverse primer (10 µM).

4. cDNA- Template.

5. Autoclaved milli Q water.

6. Primers: The following gene specific oligonucleotide primers were used.

Details of Primers Used in the Present Study

Rat IRS-1

FW: 5′-GCC AAT CTT CAT CCA GTT GCT-3′

RW: 5′-CAT CGT GAA GAA GGC ATA GGG-3′

Rat GLUT4

FW: 5′- GGG CTG TGA GTG AGT GCT TTC - 3′

RW: 5′- CAG CGA GGC AAG GCT AGA – 3

Procedure

Procedure Real Time PCR was carried out on the CFX 96 Real Time system (Bio-Rad). The reaction mix (10 µl) was prepared by adding 5 µl of 2X reaction buffer, 0.1 µl of sense and

antisense primer, 1 µl of cDNA and 3.8 µl of sterile water. The thermal cycler protocol was as follows: Initial denaturation at 95°C for 3 min, followed by 40 cycles of PCR, denaturation at 95°C for 10 sec, annealing at 60°C for 20 sec and extension at 72°C for 20 sec.

All reactions were performed in triplicate along with No Template Control (NTC). Melt curve analysis was performed using the thermal cycling programmed at 50°C-95°C for each sample to determine the presence of multiple amplicons, non-specific products and contaminants.

The results were analysed using CFX 96 Real Time system software (Bio-Rad). As an invariant control, the present study used rat β-actin.

The triplicate analysis results of the experiments performed on control and treated rat were expressed as mean standard deviation.

Statistical Analysis

Results were analyzed statistically by a one-way analysis of variance (ANOVA) and significant differences between the mean values were measured using Duncan's multiple range test using Graph Pad Prism version 5.

The results with the p<0.05 level were considered to be statistically significant.

Results

Effect of vitamin C and E on IRS-1

The first group is the control group. When PCB was induced, it down regulated the levels of IRS-1, whereas, when Vitamin C and E were induced, it upregulated the levels of IRS-1 and brought it back to the controlled level which shows that Vitamin C and E can increase the levels of insulin downstream signalling molecules (IRS-1), bringing back the insulin level to normal which results in a decrease in diabetes.

Significance at P<0.05, a-significantly different from the control group ab-significantly different from PCB-induced (Figure 1).

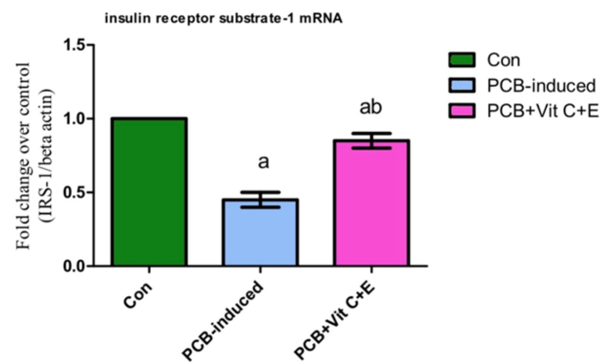

Figure 1. *The bar graph depicts the mRNA levels of insulin receptor substrate-1. The X-axis represents the control group and experimental group and the Y-axis represents the amount of insulin receptor substrate-1. Green denotes the control group, Blue denotes that PCB is induced and Purple denotes that along with induced PCB, Vitamin C and E are also supplemented. It is observed that when PCB induction takes place, the mRNA level significantly decreases, but when Vitamin C and E are supplemented the level is up regulated.*

Effect of vitamin C and E on GLUT4

The first group is the control group. When PCB was induced, it down regulated the levels of GLUT4, whereas, when Vitamin C and E were induced, it upregulated the levels of GLUT4 and brought it back to the controlled level which shows that Vitamin C and E can increase the levels of insulin downstream signalling molecules (GLUT4), bringing back the insulin level to normal which results in a decrease in diabetes.

Significance at P<0.05, a-significantly different from control group. b-significantly different from PCB-induced (Figure 2).

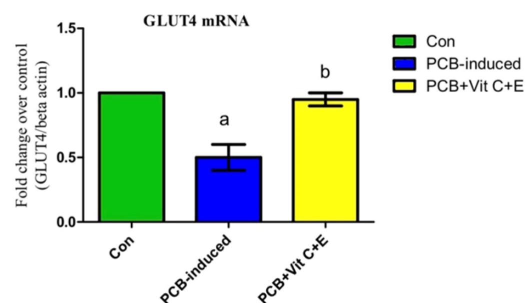

Figure 2. *The bar graph depicts the mRNA levels of GLUT4. The X-axis represents the control group and experimental group and the Y-axis represents the amount of GLUT4. Green denotes the control group, Blue denotes that PCB is induced and Yellow denotes that along with induced PCB, Vitamin C and E are also supplemented. It is observed that when PCB induction takes place, the mRNA level significantly decreases, but when Vitamin C and E are supplemented the level is up regulated.*

Discussion

The present research showed that PCB exposure downregulates insulin downstream signalling molecules in cardiac tissue such as Insulin Receptor Substrate-1 (IRS-1) and Glucose Transporter-4 (GLUT4) mRNA expression suggesting that PCB negatively influences the insulin signalling in cardiac tissue that leads to development of diabetes. However, Vitamin C and E supplementation to PCB-induced rats could effectively restore the IRS-1 and GLUT4 expression to that of control levels. This study clearly indicates that antioxidant vitamins play a therapeutic role in diabetes due to its potential antioxidant capacity [16-19]. In accordance with the present finding, study from our laboratory has shown that Vitamin C

and E treatment given to PCB exposed rats could effectively normalise the hyperglycemia.

In this study, it is reconfirmed that Vitamin C is an antioxidant. In a previous study done by Linster it was seen that Vitamin C is a reducing agent and has antioxidant property. In a previous study done by Brigelius-Flohé et al. it was seen that Vitamin E has antioxidant property, it has persuaded many researchers to study its ability to prevent chronic diseases especially the ones which are said to have an oxidative stress component like cardiovascular diseases, atherosclerosis and cancer. The Cambridge Heart Antioxidant Study (CHAOS) conducted in 1996, reported in over 2000 patients who had angiographically proven atherosclerosis that Vitamin E supplementation reduced the incidence of cardiovascular death and nonfatal myocardial infarction by 77%. In a previous study, PCB with supplementation of Vitamin C and E maintained normal serum LH levels [20-24].

The limitations of this study were that more parameters having protein expression of insulin downstream molecules need to be studied. Human cell-line modem has to be checked with Vitamin C and E. The future scope involved with this study is that Vitamin C and E can be a therapeutic drug for management of diabetes [25-29].

Conclusion

It is concluded that PCB induces diabetes and supplementation of antioxidant Vitamin C and E can bring down the level of diabetes by bringing back the levels of IRS-1 and GLUT4 to the control level thus, suggesting that these vitamins play a protective role by inhibiting diabetes.

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Statement of Conflict of Interest

The author declares that there is no conflict of interest in the present study.

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