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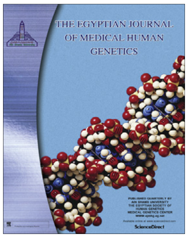

ORIGINAL ARTICLE

Cyclooxygenase 1 (COX1) expression in Type 2 diabetes mellitus: A preliminary study from north India

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KEYWORDS

Type 2 diabetes mellitus;
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Reactive oxygen species

Abstract *Background and purpose:* The 6th edition of International Diabetes Federation, 2014 shows an estimate of 387 million people with Type 2 diabetes mellitus (T2DM) worldwide, expected to rise to 592 million by 2035. T2DM is a metabolic disorder, one of the reasons being oxidative stress due to impairment in antioxidant enzymes. It leads to several complications such as micro and macrovascular diseases. Cyclooxygenase1 (COX1) enzyme is the rate limiting factor for the arachidonic pathway leading to vascular wall contraction with angiotensin II occurring in heart diseases resulting from T2DM. COX1 determines 6-Keto Prostaglandin $F_{1\alpha}$ (6-k-PGF1α) level, plays a major role in vasodilation and restricts macrophage platelet aggregation. The aim of the present study was to compare the COX1 expression and level of reactive oxygen species (ROS) in T2DM patients and controls at different time periods in human macrophages in order to find a biomarker or drug target.

Subjects and methods: The study subjects consisted of 100 individuals, 50 each from T2DM patients and healthy sex/age matched controls. Cell proliferation by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and ROS measurement by 2′,7′-dichlorofluorescein diacetate (DCFDA) staining were performed at different time periods (24, 48, 72 h). COX1 mRNA expression was checked by relative quantification method after real-time polymerase chain reaction (RT-PCR).

Results: The MTT assay showed that cell viability was significantly higher at 48 h ($P < 0.05$). ROS production was found to be lowest at 24 h by DCFDA staining. ROS levels were raised in T2DM patients as compared to controls. The quantitative RT-PCR analysis showed that the COX1 expression was higher in T2DM patients as compared to healthy controls although not significant ($P > 0.05$).

Abbreviations: COX1, cyclooxygenase 1; DCFDA, 2′,7′-dichlorofluorescein diacetate; h, hours; ITS-A, insulin transferin selenium A; MCSF, macrophage colony stimulating factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ROS, reactive oxygen species; RT-PCR, real time polymerase chain reaction

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Conclusion: Although COX1 is known to be a “housekeeping” gene, our study showed that its expression can be correlated with the disease condition and be used as a marker. However, further studies are required in more number of samples from other ethnic populations to confirm the findings.

1. Introduction

Diabetes is a metabolic disorder characterized by chronic hyperglycemia, leading to many complications *viz*. atherosclerosis, coronary heart disease, micro and macrovascular diseases. The 6th edition of International Diabetes Federation showed that prevalence of diabetes was 8.3% in 2013 and is expected to rise to 10.1% in 2035. The estimates in India showed 65.1 million diabetics in 2013 which has been predicted to reach 109 million till 2035 [1]. Susceptibility to diabetes depends on age, environmental factors and genetic makeup. Cyclooxygenase (COX) has several isoforms, COX1 and COX2 being the two major ones. They show 60% homology but have different localizations [2–3]. COX1 is found in the endoplasmic reticulum whereas COX2 is found in the nuclear envelope [4].

COX1 is expressed constitutively in bronchial epithelial, smooth muscles, gastric mucosa and monocyte and any change in its level affects various organs *viz*. the kidney, stomach and central nervous system [2,4–6]. It is a rate limiting enzyme in the arachidonic to prostanoid pathway [5,7,8]. COX1 is expressed in inflammatory cells and restricts the macrophage platelet aggregation [9]. It also leads to vasoconstriction and vasodilation [10] with the help of angiotensin II (angII) derived reactive oxygen species (ROS) [11]. Sometimes in the case of high ROS production, it exerts vascular damage by initiating the redox signaling pathway [12]. COX1 gene is 22 kb in size with 11 exons located on chromosome 9 [5]. The expression of *COX1* gene is induced by proinflammatory stimuli *viz* growth factors, cytokines and mitogens in various cells [13]. However, some workers suggested that it is unaffected by cytokines and inflammatory molecules [9]. The COX1 expression level increases at the time of onset of diabetes [7] and is associated with excess cardiovascular morbidity. There has been a constant search for biomarkers and drug targets for T2DM and its complications. The aim of the present study was to establish a system for gene expression studies and search for a T2DM marker in north Indian population. Therefore, an attempt was made to determine the COX1 expression and levels of reactive oxygen species (ROS) in T2DM patients and controls at different time periods in human macrophages.

2. Subjects and methods

2.1. Participants

Blood samples were collected from T2DM patients ($n = 50$) and control subjects ($n = 50$) at Balarampur Hospital, Lucknow, India under the supervision of expert clinicians. The study was approved by the Institutional Ethics Committee and written informed consent was taken from all subjects. The work has been carried out in accordance with the Code of Ethics of the world Medical association (Declaration of Helsinki) for experiments in humans. Controls showed a normal oral glucose tolerance test and no family history of coronary artery disease or other metabolic disorders, while diabetic cases showed fasting glucose concentration of $\geqslant 126$ mg/dl or 2-h glucose concentration of $\geqslant 200$ mg/dl after a 75-g oral glucose tolerance test. Clinical details of patients and controls were recorded and diabetes-associated complications were reviewed [14].

2.2. Culture of macrophages

Mononuclear cells were isolated by centrifugation of EDTA-blood at 1200 rpm by Histopaque density gradient separation using HiSep 1077 (HiMedia) at room temperature. The mononuclear cell suspension (0.5 ml) was transferred to 6 well culture plates containing macrophage serum free (MSF) media (Thermo Fisher, USA), colony stimulating factor (MCSF, 100 ng/ml), 1.0% media supplement, insulin transferin selenium A (ITS-A) and antibiotics. The plates were incubated in a CO$_2$ incubator (37 °C; 5.0% CO$_2$) for cell proliferation [15].

2.3. MTT assay

Cell viability was performed using tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (MTT) assay at 24, 48, and 72 h and optical density was measured at 490 nm using Enzyme Linked Immunosorbent Assay (ELISA) microplate reader (Biorad, USA).

2.4. ROS measurement

Reactive oxygen species (ROS) generation in monocyte cell derived macrophages were measured by fluorescent 2′,7′-dichlorofluorescein diacetate (DCFDA) staining with some modifications at 24, 48, and 72 h [16]. Images were taken in an inverted fluorescent microscope (Nikon ECLIPSE Ti-S, Japan).

2.5. Gene expression

RNA was isolated from monocyte-derived macrophages with the help of RNeasy kit (Qiagen, The Netherlands). Complimentary DNA (cDNA) was synthesized by commercially available SuperScript$^{\circledR}$ VILO™ cDNA synthesis kit (Biosciences, USA) as per manufacturers protocol. Quantitative real-time PCR (RT-PCR) with SYBR green I master mix was performed in a Light Cycler 480 real-time PCR machine (Roche, Germany) using specific primers for *COX1* and the housekeeping gene, *GAPDH* as internal control. Primers were designed using Primer3 software and further checked by OligoAnalyser (ver. online) (Table 1). Relative quantification methodology was used to study the expression levels [15].

2.6. Statistical analysis

Students' *t*-test (two-tailed) was used for cell viability analysis and evaluation of variation in expression levels between controls and T2DM cases using Prism software (V 5.0). Difference between variables was considered significant at $P < 0.05$.

3. Results

Cell viability analysis by MTT assay clearly indicated that cells at 48 h (h) were more viable than those at 24 and 72 h. Student paired *t*-test showed that cell viability decreased significantly at 48 and 72 h ($P < 0.0034$; 0.0085 respectively) (Fig. 1).

DCFDA staining at different time periods showed that ROS production was lower at 24 h in comparison to 48 and 72 h in both normal and T2DM subjects. ROS production increased with time and when the levels were compared between normal and T2DM subjects, the difference was clearly visible as shown in Fig. 2. The cultured macrophages from T2DM individuals appeared brightly stained showing significant ROS production.

The COX1 mRNA expression was higher in cells grown for 48 h in comparison to other time periods. The COX1 expression was higher in diabetic samples as observed in 24 and 48 h (Fig. 3). However, the difference in mRNA expression levels in the normal and T2DM subjects was not significant.

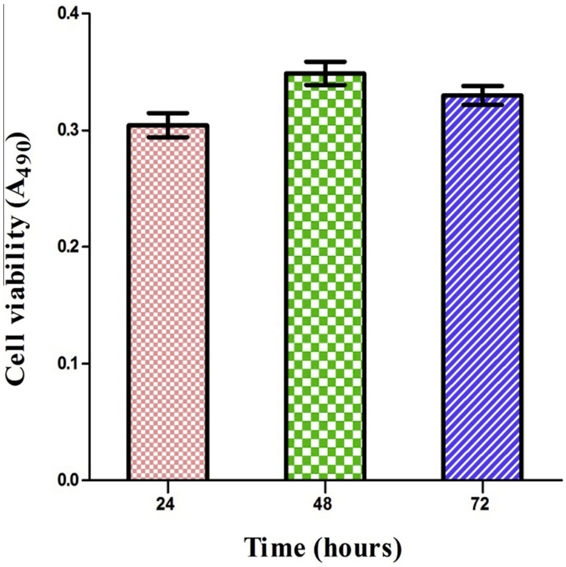

Figure 1 Bar diagram showing cell viability (A_{490}) of human macrophages at 24, 48, and 72 h.

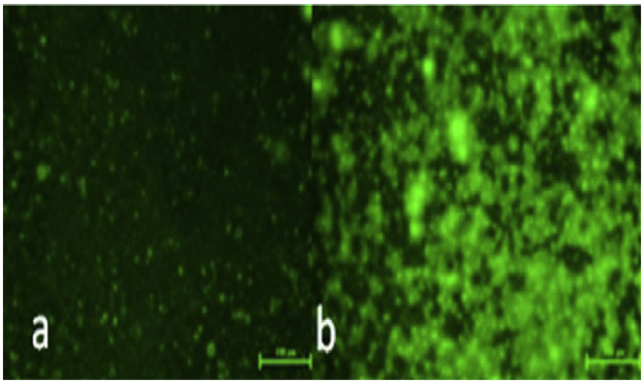

Figure 2 Human macrophages stained with DCFDA showing levels of ROS (a) normal and (b) T2DM cases.

4. Discussion

T2DM is a metabolic syndrome with increased glucose levels, abdominal obesity, high cholesterol and blood pressure. These conditions create stress in the biological system due to inflammation and ROS generation. It was suggested that poor glycemic control in subjects increases ROS production which leads to stimulation of the redox pathway [7,17]. ROS is managed by enzymatic and non-enzymatic mechanisms. Different antioxidant enzymes *viz*. glutathione S-transferase (GST), superoxide dismutase (SOD), catalase (CAT) and COX maintain homeostasis in the cell [18]. As mentioned earlier, COX are key regulators in the conversion of arachidonic acid into prostanoids which mediate inflammation, immunomodulation, apoptosis and blood flow [19].

As reported earlier, our study also showed the expression of COX1 in monocytes isolated from peripheral cells [4]. In this study, we found that ROS generation increased with time and was higher in T2DM subjects as compared to controls. We found highest ROS generation at 72 h and lowest at 24 h. Our results also indicated that COX1 expression was upregulated in T2DM cases as compared to controls but the change was not significant. The respective ROS and COX1 levels at different time periods showed that ROS production is directly proportional to COX1 levels (Fig. 3).

There are controversial views regarding COX1 expression such as no expression was observed in rat alveolar macrophages while a marked over expression was reported in mice and human macrophages [20–23]. However, there are several studies which suggest that COX1 expression can be modulated differentially by different cells under various conditions

Table 1 Specific primers, PCR conditions and amplicon sizes of *COX1* and *GAPDH* genes.

Gene	Primers (5′–3′)	Annealing temp (°C)	Cycles	Amplicon size (bp)
COX1 F′	AAGGAGATGGCAGCAGAGTT	57	55	236
COX1 R′	GTGGCCGTCTTGACAATGTT			
GAPDH F′	TCATCCCTGAGCTGAACGG	61	55	230
GAPDH R′	GTCAAAGGTGGAGGAGTGGG			

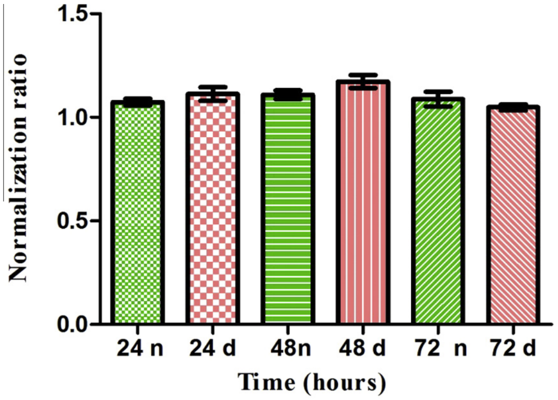

Figure 3 Bar diagram showing *COX1*-mRNA expression levels in (n) normal and (d) T2DM cases at different time periods.

[24–26]. Some workers have found that the *COX1* gene expression goes down with glucose concentration under conditions of increased ROS, whereas, others suggested that ROS upregulates the COX1 expression [7,19].

Unlike the study suggesting COX1 down regulation in the presence of ROS [7], an increase in COX1 expression was observed in the north Indian diabetic patients. However, the increase was not significant when compared to controls. Our study also supported the report of Kottireddy et al. [27]. These findings suggested that there are several factors and different genes in various pathways which may be influencing the expression of COX1. However, further studies are required in more number of samples in order to establish a correlation between COX1 expression and T2DM.

Disclosure statement

The authors declare no conflict of interest.

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