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SHORT COMMUNICATION

PIOGLITAZONE MAY RECONSTITUTE THE HMGB1 PROTEIN EXPRESSION LOWERED BY HIGH GLUCOSE CONDITION IN INSULINOMA CELLS.

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Abstract: Pioglitazone is a Peroxisome proliferator-activated receptor-gamma (PPARγ) agonist that has been widely used as an insulin-sensitizing drug to reduce insulin resistance in type II diabetic patients. Previous studies demonstrated that the high mobility group box-1 (HMGB1) protein level of insulinoma cell line (INS-1) cells was reduced by high glucose treatment. The study, therefore, determined that pioglitazone is able to reconstitute the expression of HMGB1 protein in a high glucose condition. For this purpose, INS-1 cells were incubated in medium without serum, supplemented with either 10 mM or 30 mM glucose, with or without an addition of 40 μM of pioglitazone. The HMGB1 protein expression from each treated cells was then examined by Western blot. The results demonstrated that pioglitazone may be potentially useful to reconstitute the HMGB1 protein expression in high glucose condition, which may provide important information on the mechanism of action of pioglitazone in diabetes treatment.

Keywords: TZDs, Type II Diabetic, HMGB1

INTRODUCTION

Thiazolidinediones (TZDs) are insulin-sensitizing agents that improve glycemic control primarily by reducing fasting plasma glucose levels in type II diabetic patients (Lebovitz *et al*. 2001; Philips *et al*. 2001; Fonseca *et al*. 2003). Pioglitazone as well as troglitazone, rosiglitazone and ciglitazone are members of the TZD family (Willson *et al*. 1996; Zingarelli & Cook 2005). Like other members of the TZD, pioglitazone is a specific PPARγ agonist (Fig. 1) (Lehmann *et al*. 1997). PPARγ represents a member of a large family of ligand-activated transcription factors that regulate gene expression in target tissues (Kliewer *et al*. 1992; Palmer *et al*. 1995; Zingarelli & Cook 2005). Activation of PPARγ with pioglitazone has been demonstrated to reduce the insulin-resistance in type II diabetic patients (Mathews *et al*. 1999) and has been approved for the treatment of type II diabetes (Zander *et al*. 2004).

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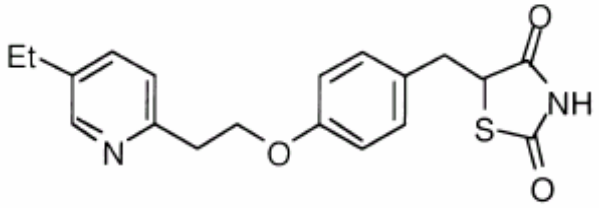

Figure 1: Molecular structure of pioglitazone, a PPARγ ligand.

In this study, the effect of pioglitazone was determined on whether it was able to reconstitute the expression of high mobility group box-1 (HMGB1) protein in a high glucose condition. HMGB1 was originally identified as a nuclear DNA-binding protein that functions as a structural cofactor critical for proper transcriptional regulation and gene expression (Mantell *et al*. 2006). It is released by activated macrophages and monocytes whereby inducing the release of proinflammatory cytokines from various immune cells (Mantell *et al*. 2006). Previous studies have demonstrated that the HMGB1 protein expression of INS-1 cells was reduced in a high glucose condition. The INS-1 cells (isolated from rat pancreatic-cell) are the common model cells for diabetes study. HMGB1 may play an important role in diabetes patients where a decrease of the gene expression is likely to affect the INS-1 cell growth indirectly. Consistently, studies in autopsies found decreased pancreatic β-cell mass in type II diabetic patients because high glucose inhibits fat oxidation and consequently lipid detoxification (Federici *et al*. 2001; El-Assaad *et al*. 2006). Although the usefulness of pioglitazone in diabetes has been widely demonstrated, its role in gene regulation of insulin sensitivity has not been studied extensively. The study may provide important information on the mechanism of pioglitazone action which may be useful in diabetes treatment.

MATERIALS AND METHODS

Maintenance of Cell Culture

The INS-1 was cultured in Roselle's Park Memorial Institute Medium (Gibco BRL) medium supplemented with serum (10% FBS, Gibco BRL) and maintained at 37^{o}C in a humidified atmosphere of 5% CO_2. The culture medium was changed every 2–3 days.

Preparation of Drug and Treatment

Pioglitazone (Sigma-Aldrich) was prepared as stock solutions of 40 mM, in dimethyl sulfoxide (DMSO) (Sigma-Aldrich). The solutions were frozen at –20^{o}C until use. Before treatment, cells (1×10^5 cells/ml) were seeded into a 15 mm culture dish and allowed to grow in the 10 ml RPMI (Gibco BRL) medium without serum, but supplemented with either 10 mM or 30 mM of glucose (Table 1) until they were approximately 70% confluent. Subsequently, 10 μl of stock

pioglitazone was added to the cells and the culture flasks were incubated for 24, 48 and 72 hours. Control samples contained medium and DMSO only. The final concentration of DMSO in all experimental samples, including controls, did not exceed 0.1% (v/v). Following incubation, the cells were subjected to protein extraction as described below.

Table 1: The INS-1 cell culture set up.

During cells treatment, the cells were cultured in 10 ml RPMI medium without serum supplemented with:

30 mM glucose (30G)		10 mM glucose (10G)	
Pioglitazone (P)	Control (D)	Pioglitazone (P)	Control (D)

G: glucose; P: pioglitazone; D: DMSO.

Western Blot

The treated and control cells were scraped off the dish with a plastic cell scraper. Subsequently, the scraped cells were transferred into a centrifuge tube. The cells were then washed twice with ice-cold PBS and lysed with sonication. The protein concentration was determined using a Protein Assay Kit (Bio-Rad) before samples were subjected to a Western blot. Protein samples of 1 μg/μl were separated on 10% SDS-polyacrylamide gels. Proteins were then electrophoretically transferred onto nitrocellulose membranes and incubated in 5% dry milk powder blocking buffer for 60 minutes at room temperature with constant shaking. The membrane was then washed and reacted with 1:500 rabbit polyclonal antibody for HMGB1 (Santa Cruz) at 4°C overnight. After three washes with washing buffer (10 minutes each), the membrane was incubated at room temperature for 1 hour with 1:5000 horseradish peroxidase (HRP)-conjugated mouse anti-rabbit antibody. The membrane was then washed and the peroxidase signals were detected using an ECL™ Western Blotting Analysis System (Amersham) according to the manufacturer's instructions. The blot was then exposed to X-ray film. The human β-actin was used as a control to verify equal samples loading.

RESULTS AND DISCUSSIONS

The result demonstrated that pioglitazone (40 μM) was likely to reconstitute the HMGB1 protein expression in high glucose treatment after 48 hours incubation onward. The concentration of 40 μM pioglitazone was used because it is the concentration of pioglitazone optimized in the group for diabetes study. To get this insight, the INS-1 cells were grown in medium without serum, but supplemented with either 10 mM or 30 mM of glucose. The medium with no serum was used in order to exclude the side effects by several growth factors existing in the serum. The previous MTT assays showed reduced cell numbers in medium with 30 mM glucose (high glucose) in comparison to 10 mM glucose (low glucose) (unpublished results), while the HMGB1 identified by 2D-gel

electrophoresis was found to be reduced upon high glucose condition (un-published results).

The current study demonstrated that there was no apparent difference in the HMGB1 protein levels could be seen after 24 hours treatment of the cells with 10 mM or 30 mM glucose, with or without pioglitazone [Fig. 2(a)]. Indeed, no apparent difference in the HMGB1 protein levels were observed after 48 hours and 72 hours treatment of the cells in 10 mM glucose medium, with or without pioglitazone [Fig. 2(b) and 2(c)]. In contrast, a clear decrease of HMGB1 protein levels was observed after 48 hours and 72 hours of culture in 30 mM glucose medium without pioglitazone [Fig. 2(b) and 2(c)]. However, the reduction of HMGB1 protein levels was likely to be reconstituted following incubation of the cells in 30 mM glucose and in the presence of pioglitazone [Fig. 2(b) and 2(c)].

According to the previous study, moderate amounts of HMGB1 induce a beneficial immune response to confine infection or tissue damage and promote wound healing and tissue regeneration (van der Poll & Lowry 1995; Ulloa *et al*. 1999; Ulloa *et al*. 2002; Yang *et al*. 2004; Wang *et al*. 2004; Mantell *et al*. 2006). Excessive levels of HMGB1 result in an un-controlled inflammatory response that can produce tissue injury and organ failure (Lotze & Tracey 2005; Scaffidi *et al*. 2002; Ulloa *et al*. 2003; Ulloa & Tracey 2005). Indeed, extra cellular HMGB1 can cause multiple organ failure and contribute to the pathogenesis of diverse disorders including diabetes and cancer (Mantell *et al*. 2006). However, in the previous study, the HMGB1 protein expression was found decreased in INS-1 cells cultured in high glucose condition indicated that HMGB1 expression was likely to play role in normal biological function. Consistently, the study of Calogero *et al*. (1999) demonstrated that HMGB1 knockout mice died within 24 hours after birth.

This may be associated with pyruvic acid, an intermediate metabolite of glucose, also an effective scavenger of reactive oxygen species (ROS) which had been proven to reduce significantly circulating levels of HMGB1 (Das 2006). As the study had proven the important effect of pioglitazone on HMGB1 gene regulation, pioglitazone is not only useful to reduce insulin-resistance and prevent the development of atherosclerosis but it can probably be used to reconstitute the HMGB1 protein expression to the moderate level in type II diabetic patients. Therefore, this study may provide useful information on the action of pioglitazone and emphasises the impact of this drug for therapeutic approaches in the treatment of type II diabetes.

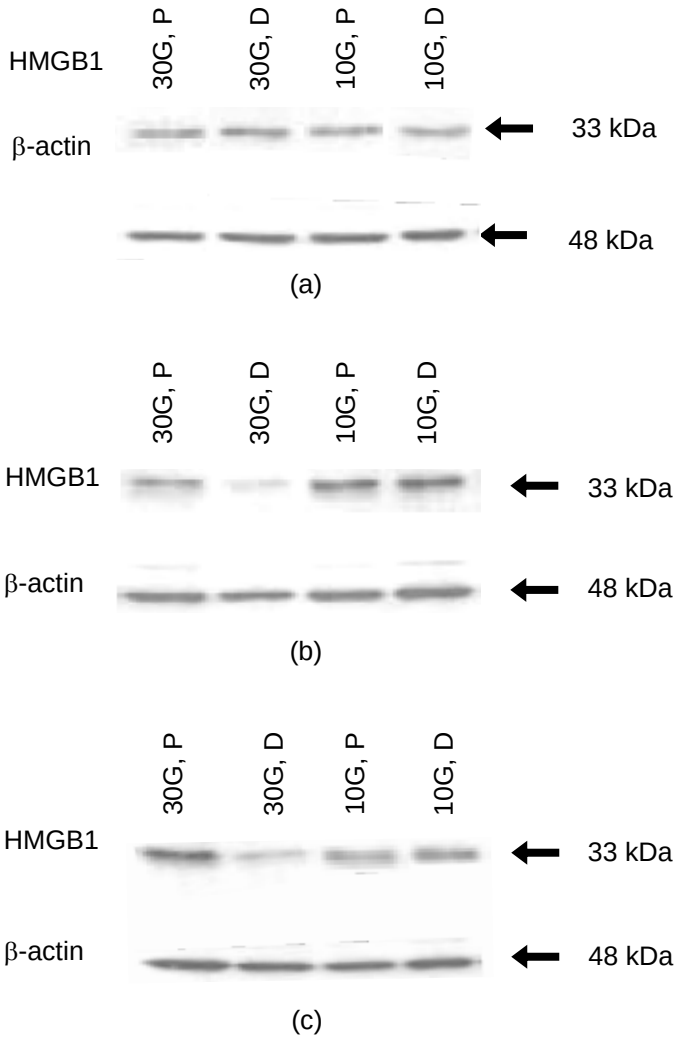

Figure 2: The effect of glucose and pioglitazone on HMGB1 protein expression in INS-1 cells. The expression was analysed using Western blot. Human β-actin was used as an internal control to verify equal protein loading. (a) 24 hours (b) 48 hours (c) 72 hours.
G: glucose; P: pioglitazone; D: DMSO.

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