ORIGINAL ARTICLE

DPP-4 Inhibitors Repress NLRP3 Inflammasome and Interleukin-1beta via GLP-1 Receptor in Macrophages Through Protein Kinase C Pathway

Yao Dai · Dongsheng Dai · Xianwei Wang · Zufeng Ding · Jawahar L. Mehta

Abstract

Background Anti-atherosclerotic effects of dipeptidyl peptidase-4 (DPP-4) inhibitors have been shown in many studies. Since inflammation and immune response play a key role in atherogenesis, we examined the effect of DPP-4 inhibitors on the expression of nod-like receptor family, pyrin domain containing 3 (NLRP3) Inflammasome and Interleukin-1beta (IL-1β) in human macrophages.

Methods and Results THP-1 macrophages were incubated with oxidized low density lipoprotein (ox-LDL) with or without DPP-4 inhibitors (sitagliptin and NVPDPP728). The effects of DPP-4 inhibitors on the expression of NLRP3, toll-like receptor 4 (TLR4) and pro-inflammatory cytokine IL-1β were studied. Both DPP-4 inhibitors induced a significant reduction in NLRP3, TLR4 and IL-1β expression; concurrently, there was an increase in glucagon like peptide 1 receptor (GLP-1R) expression. Simultaneously, DPP-4 inhibitors reduced phosphorylated-PKC, but not PKA, levels. To determine the role of PKC activation in the effects of DPP-4 inhibitors, cells were treated with PMA- which blocked the effect of DPP-4 inhibitors on NLRP3 and IL-1β as well as TLR4 and GLP-1R. Over-expression of GLP-1R in macrophages with its agonist liraglutide also blocked the effects of PMA.

Conclusion DPP-4 inhibitors suppress NLRP3, TLR4 and IL-1β in human macrophages through inhibition of PKC activity. This study provides novel insights into the mechanism of inhibition of inflammatory state and immune response in atherosclerosis by DPP-4 inhibitors.

Keywords DPP-4 · Inflammation · GLP-1 · PKC

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Introduction

Atherogenesis is a complex process including interaction of vascular cells and lipids and inflammatory mediators [1]. Oxidized low density lipoprotein (ox-LDL) play a critical role in atherogenesis [2]. Ox-LDL binds to the scavenger receptors on membrane of macrophages, and stimulates a number of pro-inflammatory cytokines such as IL-1β that play a role in the progression of atherosclerosis [3,4]. NLRP3 inflammasomes are involved in inflammation when the immune system sends signaling molecules and inflammatory cells to the site of injury to facilitate tissue repair. There is evidence for the activation of NLRP3 inflammasome by islet amyloid polypeptide as a mechanism for enhanced IL-1β generation in type 2 diabetes [3]. IL-1β and NLRP3 inflammasome activation are involved in apoptosis and foam cell formation, which are hallmarks of atherosclerosis [5,6]. Recently, Liu et al. [7] showed that ox-LDL induces IL-1β secretion in human macrophages through activating NLRP3 expression.

Protein kinases (PK) are a cluster of enzymes that modulate other proteins by phosphorylating and

activating them [8]. A wide range of steps in atherogenesis are regulated by PKC and PKA [9]. PKC and PKA also play an important role in vascular inflammation [10,11].

Dipeptidyl peptidase-4 (DPP-4) is an enzyme that degrades native glucagon-like peptide 1 (GLP-1), an incretin that postpones gastric emptying, inhibits food intake, and stimulates insulin release [12]. GLP-1 agonists and DPP-4 inhibitors are widely used in the treatment of patients with type-2 diabetes. Due to their potential to prevent cardiovascular diseases, DPP-4 inhibitors are regarded as a major milestone in the treatment of patients with type-2 diabetes [13]. A number of studies have shown that these agents can modify many of the steps leading to atherosclerosis, a major complication of diabetes [14,15].

Studies show that DPP-4 inhibitors repress proliferation of vascular smooth muscle cells and inflammatory reactions [14], improve endothelial function [15] and reduce thrombogenesis [16]. We designed this study to examine if DPP-4 inhibitors would inhibit ox-LDL-induced inflammation and immune activation with particular reference to the generation of NLRP3 inflammasomes and IL-1β.

Materials and Methods

Materials

THP-1 cells, 1640 medium and FBS for cell culture were purchased from ATCC (Manassas, VA), and phorbol 12-myristate 13-acetate (PMA) from Sigma Aldrich (St. Louis, MO). Liraglutide was bought from ThermoFisher Scientific (Pittsburgh, PA). Ox-LDL (TBARS 50.00 ± 0.54 nmol/mg) was obtained from Biomedical Technologies Inc (Stoughton, MA). Sitagliptin and NVPDPP728 were purchased from Santa Cruz Biotecholgy (Santa Cruz, CA). Sources of primary antibodies for Western blotting were as follows: anti-rabbit GLP-1R and anti-mouse β-actin (Abcam, Cambridge, MA); anti-rabbit NLRP3, anti-rabbit IL-1β and anti-mouse TLR4 (Santa Cruz Biotechnology, Santa Cruz, CA); anti-rabbit phosphorylated-PKC (Ser) and PKA (Ser/Thr) substrates (Cell Signaling Technology, Danvers, MA).

Cell Cultures

RPMI-1640 medium containing 10 % FBS (Sigma Aldrich) was used to culture THP-1 cells in 75 mm^2 flask. After seeding onto multiwell plates, THP-1 cells were incubated
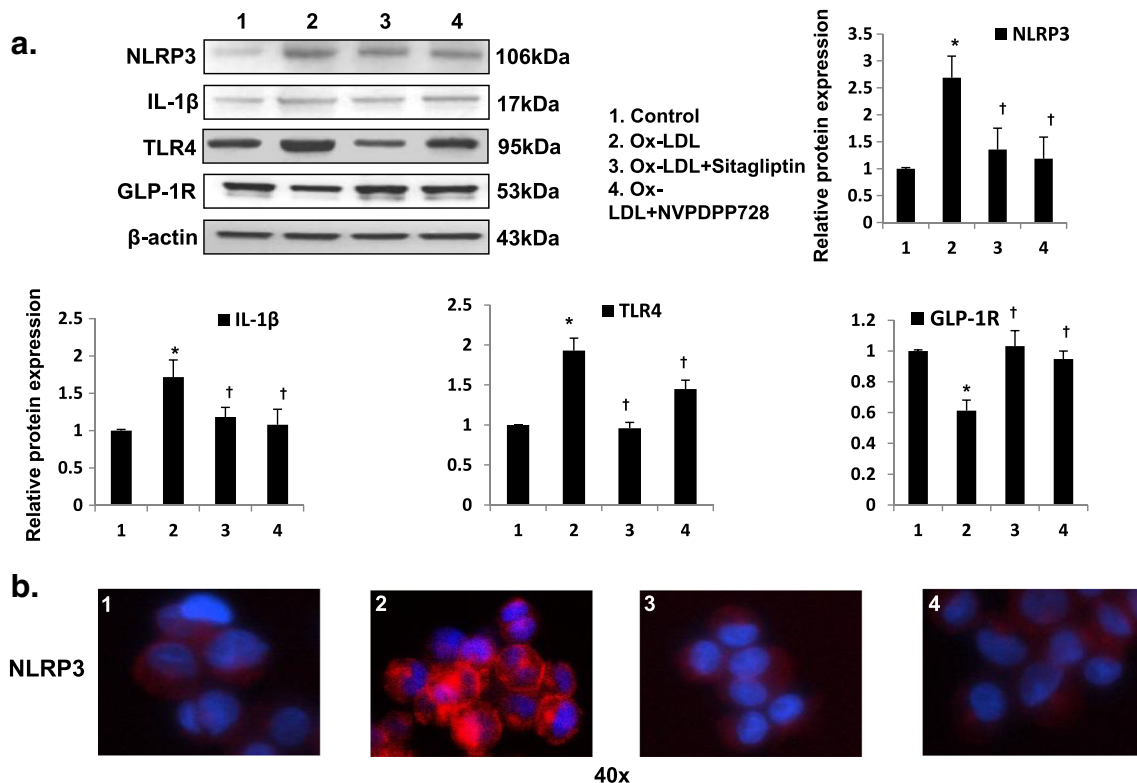
Fig. 1 Effects of DPP-4 inhibitors on the expression of NLRP3, TLR4, IL-1β and GLP-1R. **a**. expression of NLRP3, TLR4 and IL-1β was repressed, and that of GLP-1R was enhanced after treatment with DPP-4 inhibitors (Western blotting). **b**. Immunofluorescence for NLRP3 (magnification 40×) confirms Western blotting data. *P <0.05 vs. control, †P <0.05 vs. Ox-LDL alone treatment group. *Abbrev*: Ox-LDL, oxidized low density lipoprotein; NLRP3, Nod-like receptor family, pyrin domain containing 3; IL-1β, Interleukin-1β; GLP-1R, glucagon like peptide 1 receptor
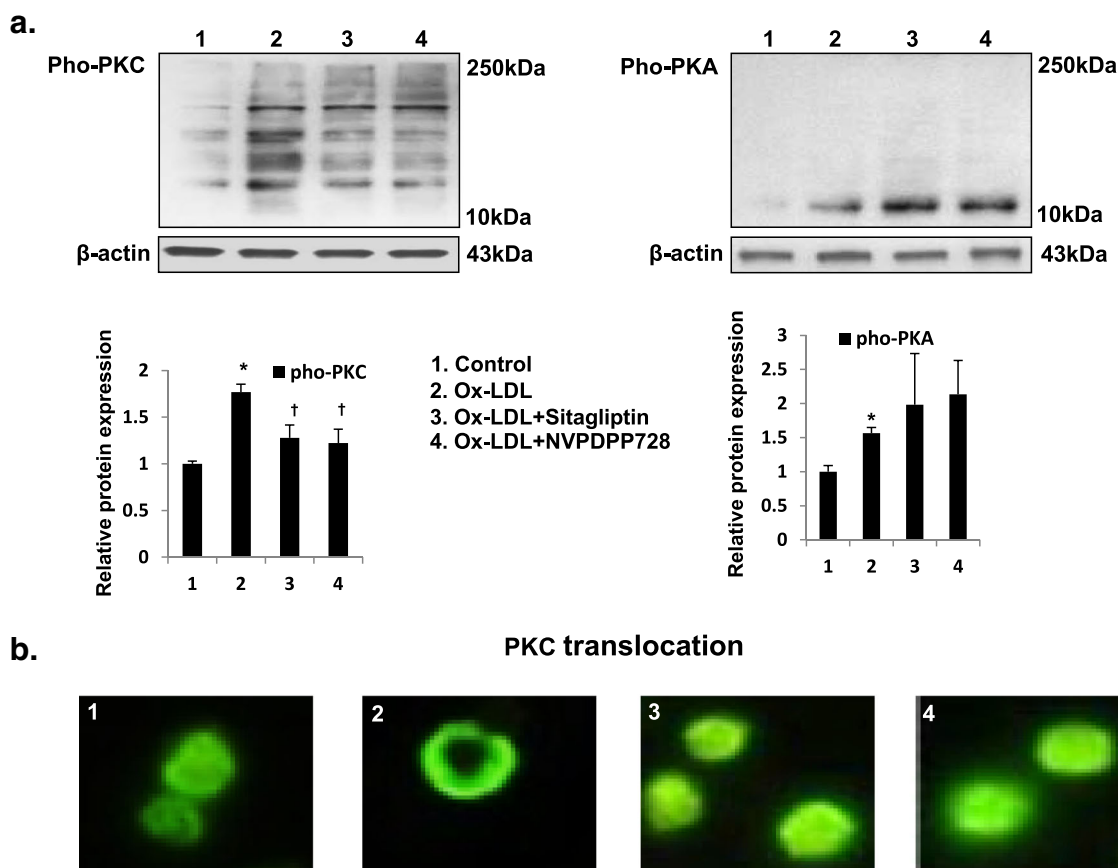
Fig. 2 Effects of DPP-4 inhibitors on the expression of PKC and PKA. **a**. Phosphorylated-PKC, but not PKA, was repressed by DPP-4 inhibitors (Western blotting). **b**. Shift in the location of PKC in macrophages from cytoplasm to the membrane treated with DPP-4 inhibitors. (magnification 40×) *$P < 0.05$ vs. control, †$P < 0.05$ vs. Ox-LDL alone treatment group. *Abbrev*: Ox-LDL, oxidized low density lipoprotein; pho-PKC, Phosphorylated Protein Kinase C; pho-PKA, Phosphorylated Protein Kinase A

with low concentration of PMA (5 ng/mL) for 24 h to induce macrophage formation [17]. Cells (≈1.5×10^6/mL per plate) were then washed with PBS to remove unattached cells, and were re-incubated with fresh medium and treated with ox-LDL (50 μg/mL) for another 24 h at 37 °C to obtain foam cells and stimulate expression of inflammatory signals. This concentration of ox-LDL was selected based on our previous studies [18]

To study the regulation of inflammatory markers by DPP-4 inhibitors, THP-1 cells were treated with two different agents: sitagliptin (25 μM) or NVPDPP728 (270 μM) for 24 h before incubation with ox-LDL for additional 24 h. The concentrations of DPP-4 inhibitors were selected based on the results of previous studies [19,20].

To study the role of PKC in the effect of DPP-4 inhibitors, THP-1-induced macrophages were co-incubated with PMA (100 ng/mL, PKC activator) and sitagliptin or NVPDPP728 for 1 h before incubation with ox-LDL for additional 24 h [21].

To study the role of GLP-1R in DPP-4/PKC/inflammation pathway, cells were seeded onto 6-well plates. When

confluent (≈70 %), the above process was repeated and cells were treated with liraglutide (1,000 ng/mL) [17] for 1 h followed by another 24 h of ox-LDL treatment.

Western Blotting

NLRP3, IL-1β, TLR4, GLP-1R, phosphorylated-PKC and PKA were measured by Western blotting. Concentrations of primary antibodies were as follows: NLRP3 (1:1000), IL-1β (1:1500), TLR4 (1:1500), GLP-1R (1:1000), phospho-PKC (1:1000), and phospho-PKA (1:1200). The protocol described in a previous study was followed [22]. β-actin was used for normalizing expression of proteins The expression of phospho-PKC and phospho-PKA substrates was quantified in relation to β-actin.

Immunofluorescence for NLRP3

Immunofluorescence was used to detect NLRP3 and PKC (FITC) expression in macrophages. The protocol described previously was followed [23].
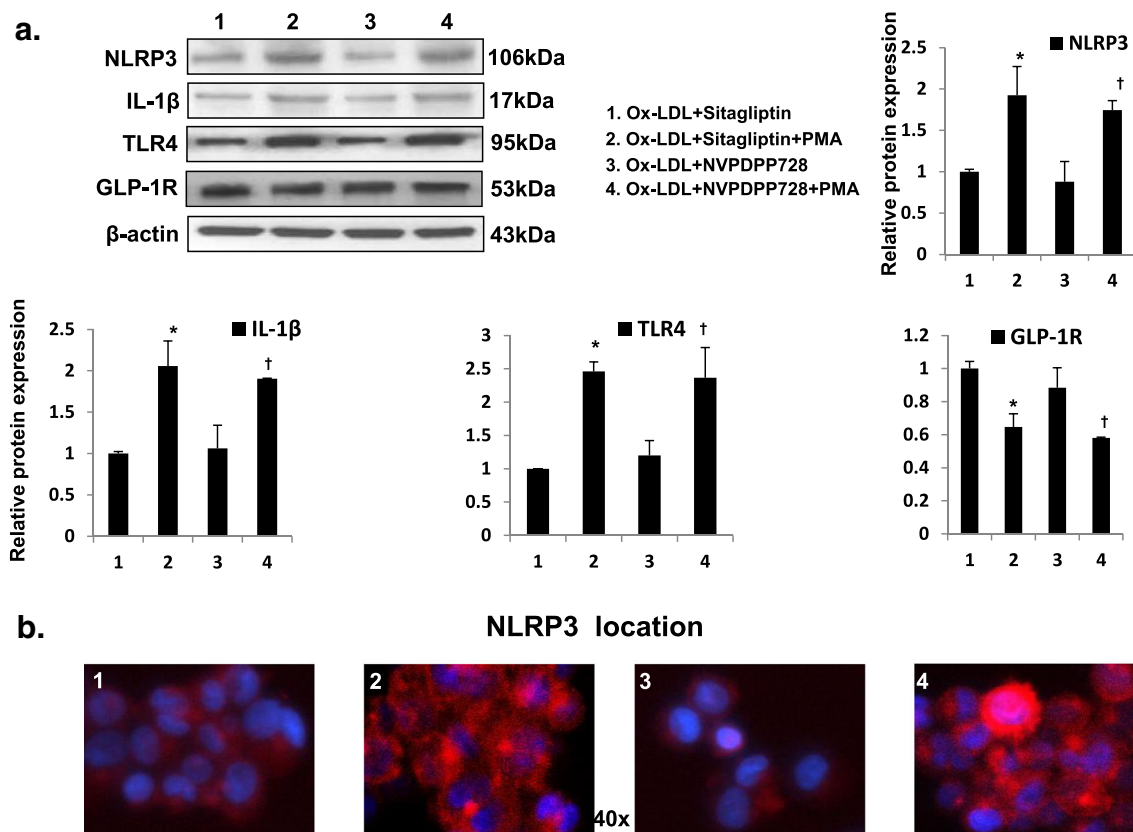
Fig. 3 Modulation of PMA the effects of DPP-4 inhibitors on the expression of NLRP3, TLR4, IL-1β and GLP-1R. **a**. PMA reversed the inhibitory effects of DPP-4 inhibitors on the expression of NLRP3, TLR4 and IL-1β. **b**. Immunofluorescence for NLRP3 (magnification 40×) confirms Western blotting data. *P <0.05 vs. Ox-LDL plus sitagliptin treatment, [†]P <0.05 vs. Ox-LDL plus NVPDPP728 treatment. Abbreviations same as in previous figures

Statistical Analysis

All experiments were performed at least in triplicate. Values were analyzed by using 2-tailed Newman-Keuls-Student t-test. Data are presented as means±SD. A P value<0.05 was considered significant.

Results

DPP-4 Inhibitors and Inflammation

As shown in Fig. 1a, the expression of inflammasome NLRP3 and IL-1β increased following treatment with ox-LDL (P <0.05). Simultaneously, there was an increase in the expression of TLR4, a well-known mediator in inflammation and innate immune system [24]. Not unexpectedly, there was a reduction in GLP-1R expression (P <0.05). Importantly, these effects of ox-LDL were blocked by both DPP-4 inhibitors, sitagliptin and NVPDPP728 (P <0.05 vs. Ox-LDL alone treatment group). The effect of DPP-4 inhibitors on NLRP3 expression determined by Western blotting was confirmed by immunofluorescence (Fig. 1b).

DPP-4 Inhibitors and Protein Kinases

PKs' activity plays a role in inflammation [10,11]. Accordingly, we measured phospho-PKC and -PKA protein levels and their regulation by DPP-4 inhibitors. As shown in Fig. 2a, ox-LDL stimulated the expression of phospho-PKC, and this effect was blocked by both DPP-4 inhibitors (P <0.05 vs. Ox-LDL alone treatment group). PKC was noted to move from cytoplasm to the membrane (on fluorescence imaging), when macrophages were incubated with ox-LDL. This effect was blocked by DPP-4 inhibitors (Fig. 2b). On the other hand, while the expression of phospho-PKA increased after ox-LDL treatment; this effect was not affected by either DPP-4 inhibitor.

PKC Stimulation and DPP-4 Inhibitors

To confirm if PKC regulates the inhibitory effect of DPP-4 inhibitors, we treated DPP-4 inhibitor-treated cells with PMA, a PKC activator [9]. As shown in Fig. 3a, the inhibitory effect of DPP-4 inhibitors on NLRP3 inflammasome, IL-1β and TLR4 all were reversed by PKC stimulation with PMA. (P <0.05 vs. non-PMA treatment). Immunofluorescence for NLRP3 confirmed the Western blot results (Fig. 3b).

Fig. 4 Enhancement of GLP-1R and PKC activation. **a.** confirmation of PKC activation by PMA. *$P <0.05$ vs. Ox-LDL plus sitagliptin treatment, †$P <0.05$ vs. Ox-LDL plus NVPDPP728 treatment. **b.** Liraglutide reversed the modulation by PMA of the inhibitory effect of DPP-4 inhibitors on PKC activation (Western blotting). *$P < .05$ vs. Ox-LDL plus sitagliptin plus PMA treatment, †$P <0.05$ vs. Ox-LDL plus NVPDPP728 plus PMA treatment. Abbreviations same as in previous figures
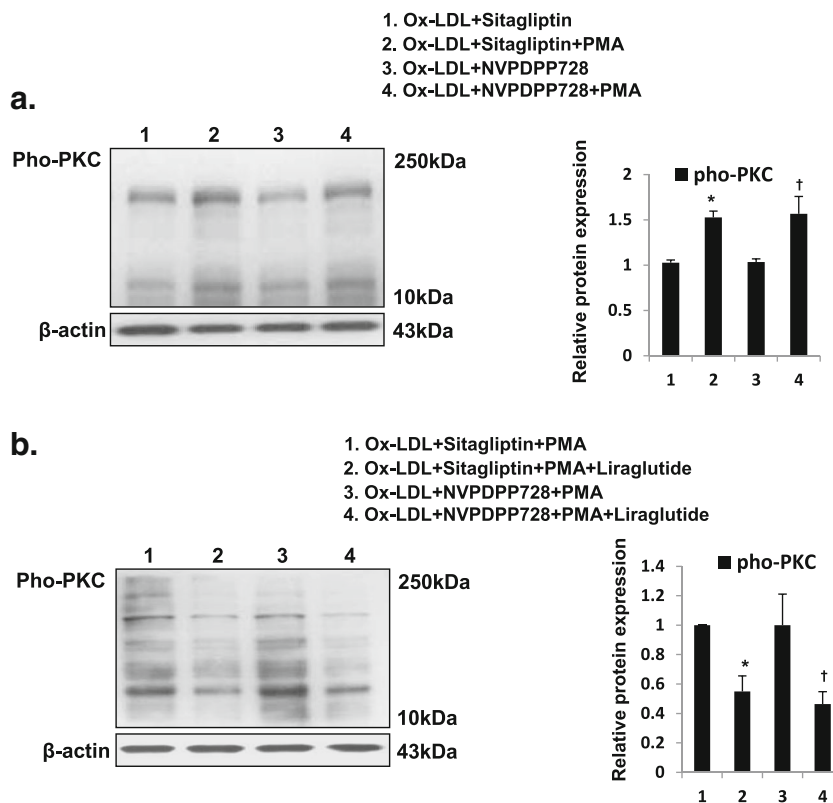
Interestingly, the expression of GLP-1R protein did not change with PKC upregulation (Fig. 3a). PMA treatment also reversed the effect of DPP4 inhibitors on phosphor-PKC (Fig. 4a).

DPP-4 Inhibitors Repress PKC Activity Through GLP-1R Activation

DPP-4 inhibitors exhibit their effect via activation of GLP-1 receptor, and at times independent of GLP-1R activation [25,26]. To examine regulation of the effect of DPP-4 inhibitors by GLP-1R activation, we incubated macrophages with GLP-1R agonist, liraglutide, after the cells had been treated with DPP-4 inhibitors. As shown in Fig. 4b, PKC activity was blocked when macrophages were treated with liraglutide ($P <0.05$ vs. ox-LDL+PMA+DPP-4 inhibitors). Further, the protein expression of NLRP3, IL-1β and TLR4 was inhibited by liraglutide treatment ($P <0.05$ vs. ox-LDL+PMA+DPP-4 inhibitors) (Fig. 5a). Immunofluorescence for NLRP3 inflammasome supported the results of Western blotting (Fig. 5b).

Discussion

Diabetes is one of the major risk factors for atherogenesis. This heightened risk probably originates from high circulating levels of ox-LDL and AGEs, both of which induce endothelial dysfunction, create a pro-inflammatory milieu and induce inflammation [27]. Because much of the focus in diabetes care is on preventing and treating atherosclerosis-related diseases, any therapy that modulates atherogenesis would be important. DPP-4 inhibitors carry much promise in this regard.

Inflammation is a major contributor to atherogenesis from a number of perspectives as discussed recently [4]. Inflammatory cytokines induce a number of alterations in key steps leading to vascular injury, such as endothelial dysfunction, thrombosis and apoptosis. Much of the inflammatory cascade is related to the activation of the immune system, both innate and acquired [28]. Inflammasome is a complex which belongs to the innate immune system that plays a key role in atherogenesis [29]. NLRP3, the most intensively studied NLRs family member, is a key component of inflammasome complex. Part of the NLRP3 inflammasome complex is involved in the activation of caspase-1, leading to the formation of IL-1β. IL-1β has been shown to exert important pro-inflammatory and pro-apoptotic effects [6]. Liu et al. [7] recently showed that ox-LDL per se can activate NLRP3 inflammasome in THP1 macrophages. It is of note that in addition to generation of NLRP3 inflammasome, macrophages treated with ox-LDL generate a number of cytokines, including IL-1β [30].

Toll-like receptors (TLRs) have evolved to recognize conserved products unique to microbial metabolism in mammals. This character of TLR protein allows them to induce innate immune responses and inflammation [24]. Increasing
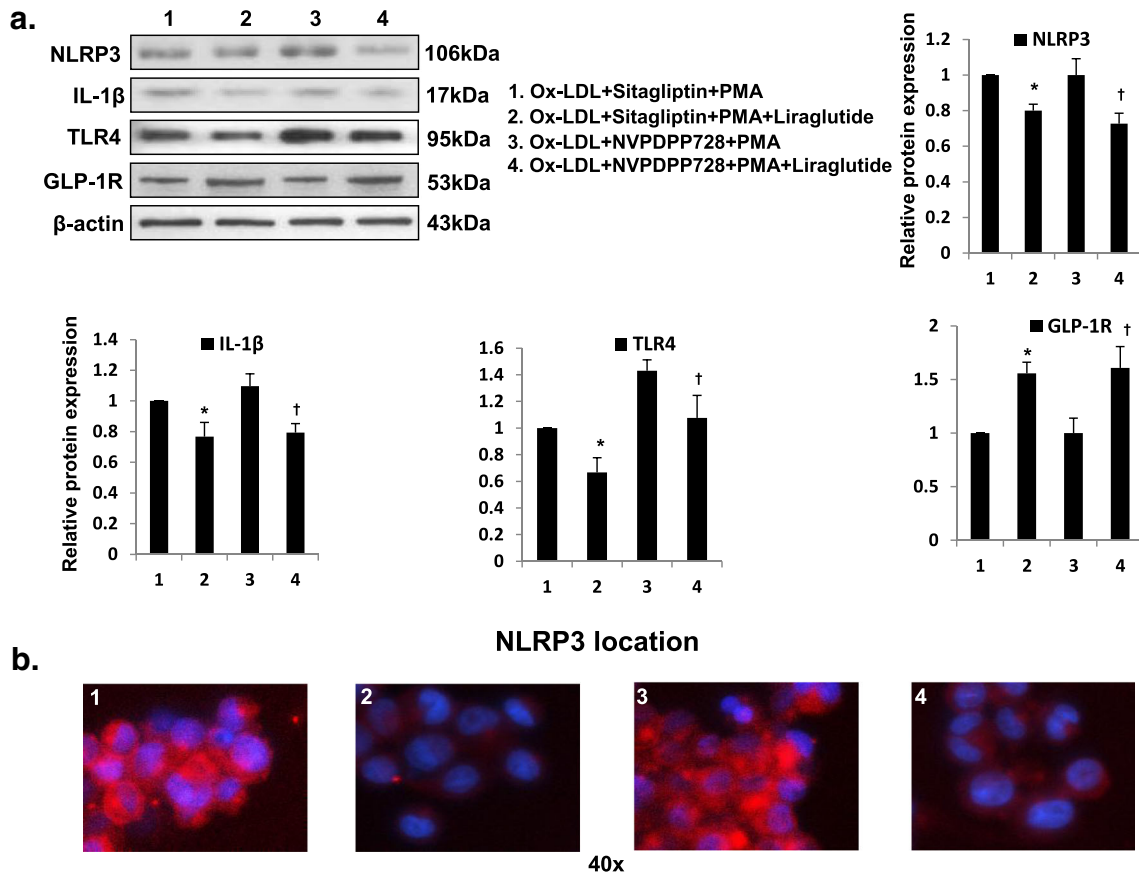
Fig. 5 Enhancement of GLP-1R and inflammation and immune mediators. **a**. Liraglutide reversed the modulatory effect of PMA repression of inflammatory mediators (NLRP3, IL-1β and TLR4) and GLP-1R (Western blotting). **b**. Immunofluorescence for NLRP3 (magnification 40×) confirms Western blotting dat. *P <0.05 vs. Ox-LDL plus sitagliptin plus PMA treatment, †P <0.05 vs. Ox-LDL plus NVPDPP728 plus PMA treatment. Abbreviations same as in previous figures

evidence has shown that TLRs are key regulator in atherogenesis [31,32]. Liu et al. [33] showed that TLR4 is mainly expressed by macrophages and can be induced by ox-LDL. Recent studies link TLR4 expression to atherogenesis [34]. Kaur et al. [35] have recently identified enhanced expression and activity of TLR4 in hyperglycemia. In their studies, enhanced TLR4 expression was associated with downstream signaling cascade such as MyD88, IRF3, and TRIF-related adaptor molecule (TRAM) as well as in NF-κB activation. Thus it appears that TLR4 is an important component of the immune response in vascular disease.

Our study lends support to the observations of Liu et al. [7] who showed induction of NLRP3 inflammasome and IL-1β in ox-LDL treated human macrophages. The novelty of the

Fig. 6 Mechanism of DPP-4 inhibitor controlling inflammation in macrophages. DPP-4 inhibitors repress GLP-1 degradation via upregulating GLP-1R in macrophages, which blocks ox-LDL induced PKC activation and subsequent TLR4, NLRP3 and IL-1β expression. These effects may contribute to the prevention of atherogenesis. Abbreviations same as in previous figures
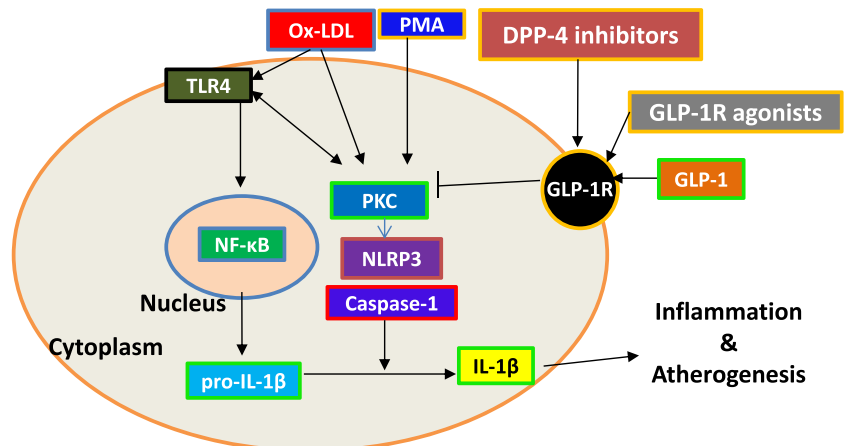
present studies lies in the fact that two different DPP-4 inhibitors were shown to reduce the expression of NLRP3 inflammasome, TLR4 and IL-1β in ox-LDL treated human macrophages. This effect appears to due to a suppressive effect on PKC activation. A previous study showed that PKC activation is related to TLR4 expression [36]. However, the underlying mechanism of repression of TLR4 by DPP-4 inhibitor still remains unclear, and needs to be examined. In a previous study [20], we showed that internalization of ox-LDL was blocked by DPP-4 inhibitors. Of note, PKC activation seems to play a critical role in ox-LDL internalization via scavenger receptors. These observations collectively may have significant implications in the anti-atherosclerotic effects of DPP-4 inhibitors.

In order to confirm the role of PKC in the modulation of DPP-4 inhibitors inflammatory process, macrophages were incubated with PMA, a specific activator of PKC. As shown in Fig. 3, PKC activation reversed the inhibitory effect of DPP-4 inhibitors on the expression of NLRP3 inflammasome, TLR4 and IL-1β. PKC was recently found to promote vascular inflammation and to accelerate atherogenesis [10]. This study showed NLRC4, another inflammasome family member, was activated via PKC pathway [37].

Most DPP-4 inhibitors exert their effect by blocking the catabolism of GLP-1R agonists, although some of the effects of DPP-4 inhibitors are independent of GLP-1R [25,26]. To ascertain if the effects of DPP-4 inhibitors on macrophage function relate to GLP1 activity, we incubated cells with a GLP-1R agonist, liraglutide, and found that this GLP-1R agonist overcame the effect of DPP-4 inhibitors (Fig. 5). Interestingly, the expression of GLP-1R also decreased with PKC activation, implying a link between PKC activity and TLR4 activation (Fig. 3).

Collectively, these observations suggest that DPP-4 inhibitors modulate many of the pro-inflammatory effects mediated via macrophages. The mechanism of DPP-4 inhibitor regulating inflammation is summarized in Fig. 6.

Statement of conflicts of interest None.

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