ABSTRACT

Type 2 diabetes (T2D) entitled itself as a global pandemic with the manifold increase in the affected population reaching to approximately 400 million in year 2014 as confirmed by the International Diabetes Federation (IDF). Sedentary life attributes add on to individual's body mass index (>30 kg/m^2) which is marked as 'obese' state contributing to the high plasma levels of saturated fatty acids (SFA) or free fatty acid (FFA) that poorly catabolised to glucose in the insulin sensitive organs like liver, adipose tissues and skeletal muscle. Adipose tissue (AT) is the main player to maintain lipid homeostasis by sequestrating lipids inside adipocytes and balanced adipokine secretion. Chronic low-grade adipose tissue inflammation of the adipose tissue results in the adipocyte dysfunction characterized by the presence of inflamed hypertrophic adipocytes and increased accumulation of macrophages, which leads to the development of insulin resistance. Thus, obesity and insulin resistance are the prime factors resulting in T2D progression with time and age. Toll-Like Receptor 4 (TLR4) signalling pathway and Adenosine Receptor (AR) signalling pathway play pivotal role in regulating adipose tissue inflammation.

Traditional medicine acquired from natural sources particularly different plants species has been explored since old times due to their easy availability and potential to mitigate different diseases. Natural products or small molecules such as phytochemicals have been traditionally utilized for the management of various human diseases and to develop various derivatives with reduced toxic side effects, improved pharmacokinetics and enhanced efficacy. Though, small molecules are capable of treating various health disorders but gap exists in unravelling the specific molecular targets and their mechanism of action which could be helpful in treating different metabolic disorders. Thus, the aim of the present study was to investigate the efficacy of small molecules in the prevention of lipid-induced adipocyte inflammation and lipopolysaccharide-induced macrophage inflammation and the improvement of insulin sensitivity and also to explore their potential molecular targets in combating insulin resistance and inflammation.

Accumulating evidence support the role of adenosine receptor activation in improving insulin sensitivity regulated particularly via $A_{2A}AR$ signalling pathway by adenosine

agonist 5'-N-ethylcarboxamidoadenosine (NECA) in zebra fish model thus restoring compromised diabetic state. Thus, agonistic activation of AR signalling pathway has a potential ground in improving insulin sensitivity. Using a combination of different in silico approaches, screening potential of anti-diabetic molecules were investigated for their efficacy in binding with the A_{2A} adenosine receptor (A_{2A}AR). Ligplot analysis of the molecules docked with the receptor suggested three small molecules namely indirubin-3`-monoxime (I3M), vanillin (VNL) and cytisine (CYT) having optimal binding energy, hydrogen bonding and non-bonded interaction with the active site residues, have a potential binding affinity towards the A_{2A}AR. Although all these three molecules displayed efficacy in preventing lipid-induced insulin resistance, however, considering the cytotoxic nature of CYT, further study was carried out with VNL and I3M. VNL and I3M incubation considerably prevented lipid-induced impairment of glucose uptake, activation of insulin signalling pathway molecules and GLUT-4 migration from cytosol to cell membrane in adipocytes. Also, these molecules mediated significant stimulation of the phosphorylation status of A_{2A}AR signalling pathway markers namely ERK1/2 and CREB. The above observations were compromised in cells when treated with A_{2A}AR antagonist SCH-58261 suggesting the insulin sensitive effect of VNL and I3M requires A_{2A}AR activation. Palmitate-induced adipocyte inflammation was successfully resolved by VNL incubation as evident from the RT-PCR gene expression analysis of different inflammatory cytokines (MCP-1, IL-6, IL-1β and TNF-α). VNL treatment significantly promoted CREB activation and also resulted in the induction of IL-10 anti-inflammatory cytokine gene expression which was confirmed by the ChIP assay and RT-qPCR analysis. To explore the direct involvement of A_{2A}AR signalling activation in I3M and VNL mediated effect on insulin sensitivity and anti-inflammatory properties, A_{2A}AR silenced cells were treated without or with VNL and I3M. Intriguingly, VNL incubation maintained A_{2A}AR activation with a static level of phosphorylated CREB while I3M couldn't mediate CREB phosphorylation in the A_{2A}AR silenced cells. Moreover, VNL didn't exhibit direct binding with A_{2A}AR as supported by radio-ligand binding experiment, whereas I3M substantiate as a potential A_{2A}AR agonist as I3M display a strong binding affinity with A_{2A}AR.

I3M exhibited a stable binding with A_{2A}AR, thus further investigation was designed to confirm the downstream effect of I3M upon A_{2A}AR binding. cAMP assay supported I3M efficiently bind A_{2A}AR and induce A_{2A}AR dependent signalling (EC_{50} = 0.12µM).

I3M binding stimulated the activation of $A_{2A}AR$ as well as insulin signalling marked by the upregulated protein levels of phosphorylated CREB and Akt (T308) in a dose dependent manner. RT-qPCR analysis suggested that while I3M restrains lipid-induced adipocyte inflammation by inhibiting NF-κB dependent pro-inflammatory cytokines expression (MCP-1, TNF-α, IL-6 and IL-1β), it also augments cAMP-mediated CREB activation. I3M incubation facilitated an anti-inflammatory state in FFA-induced adipocytes which was proven by the RT-qPCR analysis of anti-inflammatory cytokines (IL-10, IL-13, IL-4 and TGF-β). The preventive effect of I3M on addressing FFA-induced adipocyte inflammation and insulin resistance was significantly compromised in $A_{2A}AR$ silenced cells as well as when cells were pre-treated with the $A_{2A}AR$ antagonist, SCH 58261 which supports the direct binding of I3M to $A_{2A}AR$. Thus, the overall observations gathered from this study suggests the significance of I3M as a valuable option to intervene adipocyte inflammation and thus showing promise for the management of insulin resistance and type 2 diabetes.

Several experimental studies proved that FFA promotes inflammation by activating TLR4 signalling pathway in L6 myotubes and in mice models of obesity. This leads to the transcriptional activation of several pro-inflammatory genes including TNF-α, IL-1β, IL-6 and MCP-1. These results suggest the blockage of TLR4 signalling pathway could protect the chronic low-grade inflammation in the adipocytes and macrophages of obesity induced insulin resistant conditions. VNL treatment markedly prevent TLR1/2, TLR2 and TLR4 agonists-induced NF-kB activation in THP1 macrophages. VNL significantly inhibited LPS-induced pro-inflammatory cytokines expression without any noticeable change in anti-inflammatory cytokines expression. However, expression of type 1 interferons was not affected by VNL. Moreover, VNL markedly inhibits NF-kB and MAPK signaling molecules activation without any significant alteration of LPS-induced IRF3 activation. These results indicate that VNL effect could possibly target the MyD88-dependent pathway. In this report, we provide evidence that vanillin inhibits agonists-induced TLR4 activation in macrophages by targeting the MyD88-dependent pathway through direct interaction and suppression of interleukin-1 receptor-associated kinase 4 (IRAK4) activity. VNL notably inhibits LPS-induced NF-kB activation and its nuclear translocation as indicated by immunofluorescence study. ChIP assay showed that LPS-induced increased binding of pNF-kB to IL6 promoter was strikingly inhibited by VNL. Also, LPS-induced macrophage M1 polarization was prevented by VNL with

significant up-regulation of M2 state as indicated by flow cytometric analysis of CD80 (M1 marker) and CD206 (M2 marker). Moreover, incubation of vanillin in cells expressing constitutively active forms of different TLR4 signalling molecules revealed that vanillin could only able to block the ligand-independent constitutively activated IRAK4/1 or its upstream associated NF-κB activation and its transactivation potential along with the impairment of various proinflammatory cytokines expression. We also noticed a significant blockage of LPS-induced IRAK4/MyD88, IRAK4/IRAK1, and IRAK1/TRAF6 association in response to vanillin treatment. Furthermore, the in-silico study signifies the Tyr262, Val263, and Asp329 residues in IRAK4 are mainly responsible for their interaction with the 3-OCH3 and 4-OH side-groups of vanillin. Mutations of these residues in IRAK4 or altering the side-groups in vanillin structure significantly abolished IRAK4 kinase activity. Mice pre-treated with vanillin followed by LPS challenge markedly impaired LPS-induced TLR4 activation and inflammation in peritoneal macrophages. Thus, the present study posits vanillin as a novel and potent IRAK4 inhibitor and its therapeutic application in the management of various inflammatory diseases.

In conclusion, this study reports I3M and VNL notably promotes A_{2A}AR signalling and attenuates lipid-induced adipocyte inflammation. Although radioligand binding assay supports the efficient binding of I3M with A_{2A}AR, however, VNL did not directly engaged with A_{2A}AR. I3M thus alleviates lipid-induced adipocyte insulin resistance through interaction-dependent stimulation of A_{2A}AR signalling. Vanillin attenuates LPS-induced TLR4 signalling and inflammation in macrophage by inhibiting the myddosome assembly of IRAK4 and its kinase activity.