Absence of pyruvate anti-oxidant effect on granulocytes stimulated toll-like receptors

Jamille Silveira Fernandes Chamon, Fernanda Sarmento Fagundes-Netto, Raquel Miranda Gonzaga, Pollyanna Stephanie Gomes, Caroline Maria de Oliveira Volpe, José Augusto Nogueira-Machado

Núcleo de Pesquisa e Pós-Graduação, Hospital Santa Casa de Belo Horizonte, 30150-240 Belo Horizonte, MG, Brazil

Abstract

Aim & background: Pyruvate is considered as an anti-inflammatory and anti-oxidant produced from glucose metabolism. We examined the effects of sodium pyruvate on reactive oxygen species (ROS) production induced by opsonized particles in phagocytosing or toll-like receptor 4 (TLR4)-stimulated granulocytes obtained from type 2 diabetic (T2DM) patients compared with those from healthy individuals.

Methods: Luminol-dependent chemiluminescence was used to quantify the ROS generated. The phagocytosis by granulocytes obtained from T2DM patients or healthy individuals was evaluated in the absence of stimulation and in the presence of opsonized particles (zymosan complex recovered using the complement fragment C3b, ZC3b) or LPS as a TLR4 activator. Pyruvate (10^{-4} M) markedly inhibited ROS generation in unstimulated and ZC3b-stimulated granulocytes from T2DM patients and healthy individuals.

Results: Our results showed 370.0% and 199.0% activation of ROS generation during phagocytosis in healthy subjects and T2DM patients, respectively. In the presence of pyruvate, these percentages were reduced to 81.0% and 80.0%, respectively. Thus, pyruvate exhibited similar suppressive activity during granulocyte phagocytosis in healthy individuals and T2DM patients (p > 0.05). In contrast, pyruvate did not inhibit or down-regulate ROS generation in granulocytes stimulated with LPS. LPS-induced ROS production in granulocytes from healthy subjects (309%) and T2DM patients (62.5%). In the presence of pyruvate, the ROS generation in LPS-stimulated granulocytes was greater (64.0%) in the cells obtained from T2DM patients compared with cells obtained from healthy individuals (38.0%) (p < 0.05; chi-square test).

Conclusion: The dual effect of pyruvate might be associated with a metabolic signaling pathway that depends on the oxidizing profile of the target cell. However, the effects of pyruvate must be further studied before using this compound as an anti-inflammatory or anti-oxidant therapeutic resource.

1. Introduction

Pyruvate acts as an endogenous anti-oxidant and anti-inflammatory substance under various pathological conditions, such as cerebral ischemia and sepsis, in animal models. Ju et al demonstrated that pyruvate reduces albuminuria and attenuates NADPH-oxidase-dependent ROS (reactive oxygen species) production in diabetic rats, suggesting that this enzymatic complex is a possible target for pyruvate action. Ethyl pyruvate reduced adhesion of neutrophils to activated human umbilical vein endothelial cells (HUVEC), generation of IL-8 or G-CSF, surface expression of the adhesion molecules, prevent the release of pro-inflammatory cytokines, attenuate LPS-induced HMGB-1 release and inhibits p38 MAPK and NF-kappaB activation. The anti-inflammatory effect of ethyl pyruvate was inhibited through treatment with glutathione ethyl ester (GSH-Et) or SB203580 (p38 MAPK inhibitor).

However, it has also been demonstrated that hyperglycemic diabetes, as a consequence of direct osmolarity and/or alterations in...
signaling pathways, induces several modifications in cellular reactivity, tissue damage, inflammation, pyruvate dehydrogenase inhibition, increased ROS generation and the formation of advanced glycation end products (AGEs). AGEs interact with their respective receptors (RAGEs) to induce a cascade of metabolic responses leading to the production and secretion of pro-inflammatory cytokines. Similarly, toll-like receptors (TLR) are associated with innate immunity and inflammatory cytokines. It has been suggested that both RAGEs and TLRs use similar metabolic signaling routes to generate inflammatory cytokines. Several studies have provided evidence to support the hypothesis that the activation of the innate immune system is associated with type 2 diabetes complications. Specifically, TLR4 has been considered as an important factor in the pathogenesis of diabetes through ROS and inflammatory cytokines production. Lipopolysaccharides (LPS) are the primary activators of TLR4. Dasu et al. demonstrated that insulin exerts an anti-inflammatory effect through the suppression of TLR expression. Granulocytes are effectors of innate immunity and express the majority of TLR family members, except the intracellular receptors TLR3 and TLR7. It is well known that phagocytosis by granulocytes is a major cellular arm of the innate immune system.

During phagocytosis, a burst in the oxidative response is induced, which contributes to host defense. But also results in collateral damage and inflammation of the host tissue from the infiltration of granulocytes and peripheral blood mononuclear cells (PBMCs) into the reaction site. However, it has been suggested that hyperglycemia reduces phagocytosis and consequently downregulates innate immunity and increases susceptibility to infection. Both phagocytosis and the activation of toll-like receptors play a dual role in the protection and inflammation associated with innate immunity.

Whether pyruvate is an endogenous anti-oxidant and anti-inflammatory substance to modulate the oxidizing metabolic response of the immunological parameters associated with innate immunity remains unknown. The aim of this study was to evaluate the modulation of ROS production in phagocytosing and TLR4-stimulated granulocytes from type 2 diabetes patients.

2. Material and methods

2.1. Diabetic patients and healthy volunteers

The ethics committee of the Santa Casa Hospital of Belo Horizonte, Brazil, approved this study, and informed consent was obtained from all participants. Patients with T2DM (diagnosed according to the criteria of the American Diabetes Association) and healthy volunteers, all within the age range of 30–60 years, were recruited from the endocrinology department of the Santa Casa Hospital. The diabetic patients were currently being treated with statins, beta-blockers and hypoglycemic drugs. All volunteers were subjected to a detailed physical examination, and the medical history and laboratory data for each participant were evaluated before entering the study. The subjects were excluded if they were presented with one or more of the following conditions or pathologies: smoking, pregnancy, alcoholism, dementia, inflammation, malignant disease, and infection.

2.2. Reagents

Sodium pyruvate 99% PA (Pyruvate 10^{-4} M; Sigma Co - St. Louis, MO, USA).

2.3. Separation of granulocytes

The granulocytes were purified from 10.0 mL of heparinized venous blood, using a Ficoll-Hypaque gradient according to the methods of Bicalho et al., with slight modifications. The cell viability in each sample was greater than 95%, as determined using the trypan blue exclusion test.

2.4. Determination of ROS production during phagocytosis and under TLR4 stimulation with LPS

2.4.1. Effect of pyruvate on ROS generation in unstimulated granulocytes from type 2 diabetes patients or healthy individuals

The ROS generation was measured quantitatively using chemiluminescence. An aliquot (100.0 μl) of Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum (FBS), containing granulocytes (1 × 10^{5}/mL cells) previously washed in PBS, was transferred to an unsealed luminescence tube together with 200.0 μl of luminol (dissolved in 0.4 M dimethyl sulfoxide). The final volume was adjusted to 700.0 μl with PBS (pH 7.3). The chemiluminescence [expressed in Relative Light Units (RLU)/min] was recorded every minute for 20 min (basal reading). Subsequently, 100.0 μl of sodium pyruvate (10^{-4} M) was added in the same luminometer tube, and a new reading was performed for an additional 40 min.

2.4.2. Effect of pyruvate during phagocytosis of opsonized zymosan-C3b particles or under TLR4 stimulation of granulocytes in patients and healthy individuals

To evaluate phagocytosis and TLR4 stimulation after ROS generation (2.4.1), either opsonized particles (100.0 μl of a 3.6 mg/ml zymosan-C3b suspension) or LPS (50.0 μg/50.0 μl) was added to the tube and an additional reading was performed for 20 min. A 100.0 μl aliquot of 10^{-4} M sodium pyruvate was added, and the reaction was performed for 60 min. The total chemiluminescence was recorded after the 60 min reaction was completed.

2.5. Statistical analysis

The statistical analysis was performed using the Mann–Whitney test, and a value of p < 0.05 was considered significant. In same experiment, we also used the chi-square test.

3. Results

3.1. Suppressive effect of pyruvate on ROS production

The results on ROS production are shown in Table 1. The granulocytes obtained from T2DM patients produced higher levels of ROS compared to healthy subjects. The ROS production in healthy subjects was significantly lower than in diabetic patients. The pyruvate significantly reduced the ROS production in both healthy and diabetic groups.

<table>
<thead>
<tr>
<th>Experiments</th>
<th>ROS production (RLU/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy subjects</td>
<td>Type 2 diabetes patients</td>
</tr>
<tr>
<td>1-G + PBS</td>
<td>2174^a</td>
</tr>
<tr>
<td>2-G + pyruvate (10^{-4} M)</td>
<td>1050^a</td>
</tr>
<tr>
<td>3-G + ZC3b</td>
<td>10,199^a</td>
</tr>
<tr>
<td>4-G + ZC3b + pyruvate</td>
<td>1989^a</td>
</tr>
<tr>
<td>5-G + LPS</td>
<td>8900^b</td>
</tr>
<tr>
<td>6-G + LPS + pyruvate</td>
<td>14,690^b</td>
</tr>
</tbody>
</table>

Different letters denote significance at p < 0.05 using the Mann–Whitney non-parametric test. The values represent the medians; n = 15 experiments; G = Granulocytes; ZC3b = Zymosan recovered using C3b fragments (opsonized particles); RLU = Relative Light Units; ROS = Reactive Oxygen Species.
ROS than those from healthy individuals. The values differed significantly at $p < 0.05$ (non-parametric Mann–Whitney test). In the presence of $10^{-4}$ M pyruvate, the reduction in ROS production was significantly greater in cells from healthy subjects ($p < 0.05$) than that observed in T2DM patients. The results, expressed as a percentage of ROS inhibition, were 52.0% and 36.0% for healthy control and T2DM patients, respectively ($p < 0.05$) (chi-square test).

However, pyruvate-induced down-regulation of phagocytosis. The phagocytosis (ROS production in the presence of opsonized particles) of granulocytes was 10,199 and 11,178 RLU/min, and in the presence of pyruvate was 1998 and 2322 RLU/min, for healthy individuals and T2DM patients, respectively. We also observed 370.0% and 199.0% activation of ROS generation during phagocytosis in healthy subjects and T2DM patients, respectively. In the presence of pyruvate, these percentages were reduced to 81.0% and 80.0%, respectively. Thus, pyruvate exhibited similar suppressive activity during granulocyte phagocytosis in healthy individuals and type 2 diabetic patients ($p > 0.05$). Surprisingly, pyruvate does not inhibit ROS production in TLR4-stimulated granulocytes. The following results, expressed as the respective medians (RLU/min) for healthy subjects and T2DM patients, were evaluated using the Mann–Whitney test: granulocytes + PBS = 1960 and 2700 ($p < 0.05$); granulocytes + LPS = 8931 and 9160 ($p > 0.05$); and granulocytes + LPS + pyruvate = 12,773 and 14,690 ($p < 0.05$). Thus, pyruvate did not suppress ROS production in TLR4-stimulated granulocytes in healthy individuals or type 2 diabetes patients. TLR4 activation through LPS showed similar results for granulocytes from both healthy subjects and diabetes patients ($p > 0.05$); however, in the presence of pyruvate, the ROS generation was greater (64.0%) in the cells obtained from type 2 diabetic patients compared with that in the granulocytes obtained from healthy individuals (38.0%) ($p < 0.05$; chi-square test).

3.2. Suppression of phagocytosis by pyruvate

Typical curves are shown in Fig. 1, panels A–D. Panels A and B show the results from the evaluation of the kinetics studies on ROS production during the phagocytosis of opsonized particles in the presence of pyruvate. Panels C and D show the results from similar kinetics studies for LPS-stimulated granulocytes in the presence of pyruvate. Taken together, these results showed that pyruvate suppresses ROS generation in phagocytosing granulocytes but not in LPS-stimulated cells.

4. Discussion

Pyruvate is considered as an anti-inflammatory and antioxidant produced from glucose metabolism. In the present study, we demonstrated that pyruvate suppresses ROS generation in phagocytosing, but not in TLR4-stimulated, granulocytes obtained from type 2 diabetic patients and healthy individuals.

The anti-inflammatory effects of pyruvate have been implicated in several pathologies, such as severe acute pancreatitis, liver injury in diabetic rats, cataracts down-regulation of oxidative stress, albuminuria and glomerular injury in animal models of nephropathy. The anti-inflammatory activities of pyruvate have

![Fig. 1. Kinetics studies on the effect of pyruvate on ROS production in phagocytosing and TLR4-stimulated granulocytes obtained from Type 2 diabetes patients. Each point represents the average of 15 experiments ± standard deviation; RLU = Relative Light Units; panels A and B show the results obtained from experiments with opsonized particles, and panels C and D show the results obtained from experiments with LPS (TLR4)-stimulated granulocytes.](image-url)
been associated with i) the inhibition of cellular adhesion to the endothelium, ii) the inhibition of NADPH-oxidase in kidney lesion, iii) the association between the up-regulation of HO-1 and the inhibition of nitric oxide synthase expression associated with high-mobility group box 1 (HMGB-1) release in RAW 264.7 cells, and iv) the down-regulation of oxidative stress and HMGB-1 expression during lung injury in C57BL/mice.  

The results obtained in the present study demonstrate the effects of pyruvate on ROS generation during phagocytosis or toll-like receptor activation. Pyruvate suppressed the phagocytosis of opsonized particle by granulocytes obtained from type 2 diabetic patients and healthy individuals in a similar manner (p > 0.05; Table 1). Phagocytosis depends on the diacylglycerol (DAG)-PKC-NADPH-oxidase metabolic pathway. The pyruvate-mediated inhibition of ROS production in granulocytes might reflect the action of pyruvate on NADPH-oxidase, as previously suggested or the direct effect of pyruvate on extracellular ROS during phagocytosis. We propose that the inhibition of NADPH-oxidase is a more coherent explanation. TLR4 has an innate intrinsic metabolic signaling. Different ligands induce multiple MyD88-dependent pathways for the activation of several kinase families and adapters, resulting in the nuclear translocation of one or more transcription factor (NF-kappaB) and the expression of effector protein (cytokines and chemokines). It is difficult to infer the mechanism underlying the absence of the pyruvate-mediated suppression on ROS production in TLR4-stimulated granulocytes, as a similar response was observed for cells from either type 2 diabetic patients or healthy individuals.

Initially, signaling was considered as a linear cascade of sequential reactions that culminate in the up or down-regulation of proteins through the activities of kinases and phosphatases. Recently, it has been shown that the metabolic response to stimuli involves an integrated network of signaling pathways, and the balance between phosphorylation and dephosphorylation reactions defines the exact response and types of effectors involved. ROS inactivate protein tyrosine phosphatases (PTP), which constitute a specific family of proteins that dephosphorylate Ser/Tyr residues and non-protein substrates, such as inositol phospholipids in response to stimuli. Thus, after stimulation, the balance between kinases and phosphatases might become altered. The difference between ROS response in granulocytes after either stimulation with ZC3b or LPS (TLR4) might suggest a difference in the signaling pathways or ROS modulation during TLR4 activation, which might interfere with action of pyruvate. The intracellular increase in anti-oxidant effector molecules, such as glutathione (GSH), or the imbalance between kinases and phosphatases might further explain the results obtained in this study.

In cells treated with growth factors, transient increases in ROS concentrations have been observed during enhanced cell proliferation through the inhibition of phosphotyrosine phosphatases, thereby facilitating the amplification of tyrosine kinase and phosphatidylinositol-3 kinase (PI-3K) signaling pathways. The detoxification of ROS and the repair of oxidatively damaged proteins primarily depend on the availability of reduced glutathione. Glucose metabolism through the pentose phosphate pathway provides NADPH to maintain glutathione in the reduced state. Jang et al demonstrated that glutathione ethyl ester (GSH-Et), SB203580 (p38 MAPK inhibitor), siHO-1, or p38-siRNA transfection abolished the anti-inflammatory effect of ethyl pyruvate. Kamata et al showed that the treatment of mice with concanavalin A (Con A) induces liver lesions due to the prolonged activation of JNK (c-Jun N-terminal Kinase). However, when the mice were fed the anti-oxidant butylated hydroxyanisole (BHA), the JNK activation was reduced, and the Con A-mediated induction of liver lesion was suppressed. These results suggest that cellular anti-oxidant status plays an important role in the definition of metabolic signaling, and the intracellular redox balance plays a pivotal role in pyruvate activity. Thus, we suggest that pyruvate could act as anti-oxidant and/or anti-inflammatory agent in the dependence of activation of specific signaling pathway in conjunction with the intracellular redox profile.

5. Conclusion

Based on the results obtained in the present study, we propose that the suppression of ROS production through pyruvate depends on the metabolic oxidation profile, as observed in the oxidizing burst during phagocytosis, and potentially involves the NADPH-oxidase system. In contrast, the ROS produced in TLR4-stimulated granulocytes might involve a different signaling pathway, with increased mitochondrial ROS generation and a high reductive/anti-oxidant profile, which suppresses the pyruvate-mediated inhibition of ROS generation. Thus, pyruvate could preferentially act as an anti-inflammatory and anti-oxidant in cells with an oxidizing profile. Further research is needed to clarify the precise mechanism of pyruvate before using this agent as a therapeutic resource.

Conflicts of interest

All authors have none to declare.

Acknowledgments

This investigation was supported with grants from FAPEMIG, CAPES, CNPq, and Rede Fapemig 262012 Toxinas Peptídicas com ação Terapêutica. Brasil.

Abbreviations

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGEs</td>
<td>advanced glycation end products</td>
<td></td>
</tr>
<tr>
<td>Con A</td>
<td>concanavalin A</td>
<td></td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
<td></td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
<td></td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione</td>
<td></td>
</tr>
<tr>
<td>GSH-Et</td>
<td>glutathione ethyl ester</td>
<td></td>
</tr>
<tr>
<td>HMGB-1</td>
<td>high-mobility group box 1</td>
<td></td>
</tr>
<tr>
<td>HO-1</td>
<td>heme oxygenase-1</td>
<td></td>
</tr>
<tr>
<td>HUVEC</td>
<td>human umbilical vein endothelial cells</td>
<td></td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
<td></td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal Kinase</td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharides</td>
<td></td>
</tr>
<tr>
<td>PBMCs</td>
<td>peripheral blood mononuclear cells</td>
<td></td>
</tr>
<tr>
<td>PI-3K</td>
<td>phosphatidylinositol-3 kinase</td>
<td></td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
<td></td>
</tr>
<tr>
<td>PTP</td>
<td>protein tyrosine phosphatases</td>
<td></td>
</tr>
<tr>
<td>RAGEs</td>
<td>receptor for advanced glycation end products</td>
<td></td>
</tr>
<tr>
<td>RLU</td>
<td>Relative Light Units</td>
<td></td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
<td></td>
</tr>
<tr>
<td>T2DM</td>
<td>type 2 diabetes</td>
<td></td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptors</td>
<td></td>
</tr>
<tr>
<td>ZC3b</td>
<td>Zymosan recovered using C3b fragments</td>
<td></td>
</tr>
</tbody>
</table>

References