Inflammasome activation by NLRP1 and NLRC4 in patients with coronary stenosis

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ABSTRACT

Objective and design: We performed an experimental, analytical and prospective study to evaluate the systemic activation of inflammasome in atherosclerosis' patients, in order to shed light into responsible mechanisms for plaque formation.

Subjects: We included sixty individuals distributed into 3 groups: 2 groups based on the report from the angiography (severe lesions - SL and primary lesions - PL) and 1 group enclosing healthy individuals (HC).

Methods: The expression assays of inflammasome genes NLRP1, NLRC4, CASP-1 and IL-1β were performed using Real Time qPCR, with specific Taqman Assays. IL-1β serum levels were analysed by commercial kit. Were applied the Shapiro-Wilk and Student's T-test as statistical tests. Statistical significance was set to p ≤ 0.05.

Results: Upregulation of NLRP1 (+3.47 FC, p = 0.0001), NLRC4 (+7.06 FC, p = 6.792 × 10^{-09}) and IL-1β (+2.43 FC, p = 0.005) was observed in all atherosclerosis patients when compared to HC. According to stenosis severity, patients with primary lesions showed upregulation of inflammasome genes NLRP1 (+2.87 FC, p = 0.0008), NLRC4 (+6.34 FC, p = 4.134 × 10^{-07}) and IL-1β (+3.39 FC, p = 0.0012) with respect to the HC group. No statistical difference was found in IL-1β serum levels according the assessed groups.

Conclusions: Inflammasome activation in atherosclerosis’ patients can be systemic altered and may be triggered by NLRP1 and NLRC4 receptors. IL-1β gene expression was identified in our study as an important systemic detectable marker of plaque severity.

1. Introduction

Cardiovascular diseases (CVDs) are the world’s most frequent cause of death (Gisterå and Hansson, 2017). The most common CVDs are caused by atherosclerosis, a multifactorial, chronic and inflammatory process featured by plaque formations within arterial vessel’s wall (Alie et al., 2014). Several risk factors contribute to atherosclerosis development, among them, the systemic arterial hypertension, diabetes mellitus and dyslipidemia are the most representatives (Bleda et al., 2016; Soares et al., 2017).

Initial lesions in vessels’ endothelial cells, derived from shear stress and presence of reactive oxygen species (ROS) may act as Damage Associated Molecular Patterns (DAMPs). These lesions trigger an inflammatory response which initially has a protective effect, however the maintenance of this environment leads to a chronic and progressive process of endothelial damage (Gisterå and Hansson, 2017). DAMPs and Pathogen Associated Molecular Patterns (PAMPs) activate inflammatory response through pathogen recognition receptors (PRRs), the NOD-like receptors (NLRs), activators of the inflammasome complex (Zheng et al., 2011).

The inflammatory complex comprises accessory and self-oligomerizing scaffold proteins belonging to nucleotide-binding oligomerization domain receptors (NLR Family) (Zheng et al., 2011). Five of these receptors have been confirmed to assemble inflammasome complex: the leucine-rich repeat (LRR)-containing protein family members NLR Family Pyrin domain-containing protein 1 (NLRP1), NLR Family Pyrin domain-containing protein 3 (NLRP3) and NLR Family CARD domain-containing protein 4 (NLRC4), the proteins absent in melanoma 2 (AIM2) and pyrin, as well the nucleotide-binding oligomerization domain (NOD) (Broz and Dixit, 2016). By means of homotypic interactions, the NLRs bind to the signaling domains pyrin (PYD) or CARD, which in turn, interact with the caspase recruitment protein (ASC), that
recruits pro-caspase-1, leading to its self-proteolytic dimerization and processing. This mechanism generates caspase-1 (CASP-1), a protease that triggers pyroptosis, a regulated cell death characterized by cell lysis and release of the mature proinflammatory cytokine Interleukin-1β (IL-1β) (Shen et al., 2010). Recent evidence reveals that other less well characterized inflammasome pathways as NLRP2, NLRP6, NLRP7 and NLRP12 may promote caspase-1 activation, but additional studies are needed to understand their role in inflammasome activation (Broz and Dixit, 2016).

During atherosclerosis, macrophage death is strongly associated with inflammation and when these cells are not effectively eliminated they accumulate resulting in plaque progression. Macrophage death at the beginning of the atherosclerotic lesion may decrease the inflammatory response; however, the same type of death of these cells in lesions’ later stages may promote the formation of a necrotic nucleus that may contribute to plaque instability (Moore and Tabas, 2011). The orchestration of how cell death pathways are activated and the exact moment each pathway is triggered is crucial for the establishment, progression, and stability of atherosclerotic lesion.

Therefore, our study aimed at evaluating the systemic activation of inflammasome complex in atherosclerosis by NLRP1 and NLRC4, analysing NLRP1, NLRC4, CASP-1 and IL-1β gene expression. We also assessed IL-1β cytokine serum levels in patients with atherosclerosis with different levels of severity and in healthy individuals.

2. Methods

2.1. Study population and design

We performed an experimental, analytical and prospective study and included a total of sixty individuals. The individuals enrolled were distributed into three groups: patients were stratified into two groups based on the report from the angiography performed and one group enclosing healthy individuals. The inclusion and exclusion criteria are reported in Table 1. The patient’s group with atherosclerosis (AP) accounted forty individuals who had atherosclerotic lesions confirmed by the image exam and then subdivided into two groups according to disease’s severity. The group of patients with severe lesions (SL, n = 20) was characterized by the presence of three or more stenosis that affected at least seventy percent of the vessel, whereas for the group of patients with primary lesions (PL, n = 20) a maximum of three stenosis involving up to 30% of the artery was included, as shown in Table 1. The control group consisted of twenty healthy subjects (HC) who did not present any of the exclusion criteria for atherosclerosis namely obesity, diabetes mellitus, systemic arterial hypertension and dyslipidemia or any chronic inflammatory and infectious as well as autoimmune conditions. Both groups were recruited from hemodynamic laboratory from a local hospital from Recife, a Brazilian Northeast city, from September to October 2017. We did not find any statistical association between individuals’ gender and the disease’s presence (SL vs. HC, p = 0.416; PL vs. HC, p = 0.092). When comparing individuals’ age we found a statistical difference between the analysed groups. This difference may be due to the fact that the control group is composed by overall younger individuals with no disease/ condition history (as seen in Table 1) (SL vs. HC: p = 1.63 × 10⁻⁹; PL vs. HC: p = 1.6 × 10⁻¹⁰). The demographic and clinical characteristics from study participants are shown in Table 2.

2.2. Ethical statements

All procedures performed herein were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards (National Ethical committee for research using human biological samples – PLATAFORMA BRASIL, under reference number: 16441813.1.0000.5208). Informed consent was obtained from all individual participants included in the study. Participation in the study was voluntary and had no impact on medical treatment.

2.3. Relative gene expression assay

2.3.1. RNA extraction and cDNA synthesis

The peripheral blood samples were collected in EDTA tubes and RNA was isolated using TRizol® by Invitrogen (Thermo Fisher Scientific, USA), following the manufacturer’s protocols. The concentration and the integrity analyses were evaluated using Nanodrop 2000 (Thermo Fisher Scientific, USA) and gel electrophoresis, respectively. The samples were stored as −80 °C. Further, for cDNA synthesis we used the GoScript™ Reverse Transcription Mix, Oligo(dT) kit (Promega, USA). For each sample a standardized amount of 500 ng total RNA was used. Samples were stored at −20 °C until further use, following the standard protocol.

2.3.2. Gene expression assay

Inflammasome gene expression of NLRP1, NLRC4, CASP-1 and IL-1β were performed using specific TaqMan Gene Expression assays (Hs00248187_m1, Hs00892666_m1, Hs00354836_m1 and Hs01555410_m1, respectively). As endogenous reference and gene for normalization β-glucoronidase (GUSB) was selected using the following primers with the SyBR Green methodology: forward: CACTGTGGCTG − β−80 °C. Further, for cDNA synthesis we used the GoScript™ Reverse Transcription Mix, Oligo(dT) kit (Promega, USA). For each sample a standardized amount of 500 ng total RNA was used. Samples were stored at −20 °C until further use, following the standard protocol.

2.4. IL-1β measurement

The serum levels of IL-1β were measured by the Human IL-1β

| Table 1 |
| Inclusion and exclusion criteria of study subjects. |
| Patients with atherosclerosis (PA) | Control group (HC) |
| Inclusion criteria | Exclusion criteria | Inclusion criteria | Exclusion criteria |
| Patients who had atherosclerotic lesions confirmed by angiography exam. | Patients with another inflammatory or infectious diseases and/or autoimmune disorders | Healthy individuals | Individuals with obesity, systemic arterial hypertension, dyslipidemia, diabetes mellitus, chronic inflammation, infectious diseases or autoimmune conditions |
| **Subgroups:** | | | |
| Patients with severe lesions (SL) | Patients with ≥3 stenosis affecting ≥70% of the vessel | Patients with < 3 stenosis affecting ≥70% of the vessel |
| Patients with primary lesions (PL) | Patients with ≤3 stenosis affecting ≤30% of the vessel | Patients with > 3 stenosis affecting ≤30% of the vessel |
Table 2
Demographic and clinical manifestations of the patients and controls. *SD: Standard devi.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Patients with severe lesions (SL) n (%)</th>
<th>Patients with primary lesions (PL) n (%)</th>
<th>Control group (HC) n (%)</th>
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<tr>
<td>Sex</td>
<td>Male (13 (65 %))</td>
<td>8 (40 %)</td>
<td>11 (55 %)</td>
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<tr>
<td></td>
<td>Female (7 (35 %))</td>
<td>12 (60 %)</td>
<td>9 (45 %)</td>
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<td>Ethnicity</td>
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<td></td>
<td>Brown (8 (40 %))</td>
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<td>10 (50 %)</td>
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<td></td>
<td>Caucasian (8 (40 %))</td>
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</tr>
<tr>
<td>Age (years)</td>
<td>Mean (SD*) 604 (± 1075)</td>
<td>583 (± 913)</td>
<td>3615 (± 638)</td>
</tr>
<tr>
<td>Body Mass Index (kg/m²)</td>
<td>Mean (SD*) 2809 (± 376)</td>
<td>2878 (± 509)</td>
<td>262 (± 291)</td>
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<tr>
<td>Abdominal circumference (cm)</td>
<td>Man (9946 (± 1037))</td>
<td>10,207 (± 1076)</td>
<td>9018 (± 995)</td>
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<td></td>
<td>Woman (10,323 (± 793))</td>
<td>9973 (± 1008)</td>
<td>8968 (± 918)</td>
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<td></td>
<td>No (15 (75 %))</td>
<td>19 (95 %)</td>
<td>19 (95 %)</td>
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<td>Ex-smoker</td>
<td>Yes (3 (15 %))</td>
<td>1 (5%)</td>
<td>0 (0%)</td>
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<td>5 (25 %)</td>
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<td></td>
<td>No (18 (90 %))</td>
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<td>9 (45 %)</td>
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<tr>
<td>Systemic Arterial Hypertension</td>
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<td>13 (65 %)</td>
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<td></td>
<td>No (1 (5 %))</td>
<td>7 (35 %)</td>
<td>20 (100 %)</td>
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<tr>
<td>Type 2 Diabetes Mellitus</td>
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<td></td>
<td>No (12 (60 %))</td>
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<td>Dyslipidemia</td>
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<td>20 (100 %)</td>
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<tr>
<td></td>
<td>No (12 (60 %))</td>
<td>20 (100 %)</td>
<td>20 (100 %)</td>
</tr>
</tbody>
</table>

Screening Set (Thermo Scientific, USA) commercial kit, following the manufacturer’s instructions. The results were expressed in picograms per millilitre (pg/mL).

2.5. Statistical analysis

The statistical analyses were performed using R software, version 3.6.1. To verify the type of sample’s distribution we performed the Shapiro-Wilk test, applying One-Way ANOVA test for analysis of variance. The statistical tests applied were performed considering as statistically significant p < 0.05 in a 95 % confidence interval (95 % CI).

3. Results

3.1. Inflammasome gene expression profile in atherosclerosis

We evaluated the expression of NLRC4 and CASP-1 genes, both inflammasome receptors and the activation products of this complex, CASP-1 and IL-1β. When mRNA levels were evaluated we found an up-regulation of NLRC4 (+3.47 FC, p = 0.0001), NLRC4 (+7.06 FC, p = 6.792 × 10⁻⁰⁸) and IL-1β (+2.43 FC, p = 0.0066) expression levels in atherosclerosis patients compared to HC. When we evaluated CASP-1 expression, no statistically significant results were observed between AP and HC groups (Fig. 1).

3.2. Inflammasome gene expression and atherosclerosis severity

We also evaluated the expression of inflammasome genes according to atherosclerosis progression, comparing severe lesion group (SL) (n = 20) and primary lesion group (PL) (n = 20), with HC group (n = 20). When we compared NLRC1 gene mRNA expression we found an increased expression in SL patients compared to HC group (+2.92 FC, p = 0.0006). The same expression profile was found when the assessing the PL versus HC groups (+2.81 FC, p = 0.0008). When mRNA levels were compared between SL and PL groups, we found no statistical differences (Fig. 2a).

When the expression of the NLRC4 gene was evaluated comparing SL to HC group an up-regulation was observed (+5.45 FC, p = 4.158 × 10⁻⁰⁸). The same profile was found when comparing the PL and HC groups (+6.34 FC, p = 4.134 × 10⁻⁰⁸). We did not find statistical differences between SL and PL groups (Fig. 2b).

When evaluating the expression of the CASP-1 considering the degree of atherosclerosis’ severity we found no statistically significant differences amongst the assessed groups (Fig. 2c).

For the IL-1β gene, an increase in its expression was observed when comparing PL and HC (+3.39 FC, p = 0.0012). We found no expression differences in patients from SL versus HC groups, however when mRNA levels were compared between the different degrees of injury (severe lesions vs. primary lesions) we observed that patients belonging to SL group presented IL-1β down regulation when compared to the PL group (-2.81 FC, p = 0.009) (Fig. 2d).

3.3. Serum levels of IL-1β

IL-1β cytokine serum levels in patients with atherosclerosis (82.02 pg/mL) and control subjects (86.36 pg/mL) were measured but no statistically significant differences were detected between the assessed groups (p = 0.0538) (Fig. 3a).

No statistically significant differences were observed between the groups of patients with severe (84.79 pg/mL, p = 0.052) and primary (79.25 pg/mL, p = 0.195) lesions compared to HC group. When compared according to the severity of the atherosclerotic lesion (SL vs. PL) no statistically differences were found (p = 0.473) (Fig. 3b).
4. Discussion

The expression profile analysis of inflammasome genes allowed the detection of complex activation involved in the atherosclerotic plaque formation not only limited to the NLRP3 receptor. Herein we showed that the inflammasome complex is activated by the “NOD-like” receptors NLRP1 and NLRC4 in atherosclerosis patients. When comparing the expression of NLRP1 and NLRC4 in patients with atherosclerosis and healthy controls, we report for the first time, a systemic upregulation from those genes.

It is known that during the atherosclerotic process NLRP3 is an important receptor in the activation of the inflammatory response. Studies demonstrate that this receptor can be activated by DAMPs present in the plaque formation like cholesterol crystals generated from oxidized low-density lipoprotein (oxLDL) (Karasawa and Takahashi, 2017). Animal studies shows that the lack of IL-1β can decrease atherosclerosis severity in ApoE -/- mice (Kirii et al., 2003). On the other hand, in a study conducted in a same animal model reported that neither NLRP3 nor ASC deficiency could inhibit macrophage infiltration and consequently atherosclerosis process (Menu et al., 2011), demonstrating that others inflammasome receptors may be activating IL-1β release.

Each receptor of the inflammasome complex is able to recognize different molecular patterns thus triggering an inflammatory response (Broz and Dixit, 2016). High concentrations of glucose and/or glycated albumin contributes increasing NLRP1 expression through transcription ATF-4 factor, already mediating inflammation (Soares et al., 2017). Since about 30 % of our studied patients with atherosclerosis had type 2 diabetes mellitus, we suggest that this condition may contribute to the increase in this gene expression. Moreover, there was already been identified by Bleda et al. study that there is a positive relation between NLRP1 expression levels and a dyslipidemic profile (Bleda et al., 2016).

Based on our finding and the ones described by Bleda et al. we also suggest that the increased NLRP1 expression may be due to the fact that about 50 % of our patients present dyslipidemia. Bleda et al. also demonstrated that NLPR1 activation is necessary for triggering a proinflammatory state in endothelial cells, reflecting the hypothesis that this NLR may contribute to the triggering of the inflammatory response and its maintenance (Bleda et al., 2014). These data corroborate our findings, since expression of NLRP1 remains increased in the initial and more severe phases of the atherosclerotic lesion when compared to the control group. Noteworthy, patients with severe lesions in our study presented stable atherosclerosis plaques which justify the absence of different expression levels according to the disease severity.

Pyroptosis is a fundamental cell death process involved in plaque formation, since as a first defense mechanism, the body attempts to decrease the amount of inflammatory cells at the lesion site, leading to pyroptosis (Xu et al., 2018). Unlike other NLRs, NLRC4 does not need to interact with an adapter protein as ASC to activate caspase-1, it only needs to interact with an apoptosis inhibitor protein (NAIP), thus being a scaffolding structure. This possibility of direct activation favors towards pyroptosis process (Xu et al., 2018). This sensitive activation of NLRC4 would explain the increased gene expression detected in our study.

Atherosclerosis plaque formation has been associated with infectious agents, that may contribute to chronic inflammatory process (Campbell and Rosenfeld, 2015). Inflammasome activation by NLRP1 and NLRC4 is hypothesized to be induced by the interaction with infectious agents such as *Toxoplasma gondii* and gram-negative bacteria, however this hypothesis can not be confirmed by our study since the assessed individuals have not informed any infectious diseases (Campbell and Rosenfeld, 2015; Chavarría-Smith and Vance, 2015; Zhao et al., 2011).

Activation of the inflammatory complex results in the release of active caspase-1 (Broz and Dixit, 2016). Recent studies have shown that

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**Fig. 2.** Inflammasome gene expression stratified according to atherosclerosis severity. The results were normalized using GUSB as endogenous reference. Target gene expression in control group were set at 1. HC: Healthy controls; SL: Severe lesions; PL: Primary lesions. *p-value < 0.05. (a) NLRP1; (b) NLRC4; (c) CASP-1; (d) IL-1β.
the components of this complex are more expressed in patients with atherosclerosis (Xu et al., 2018). In a study performed by Lee et al., as well in our findings, no statistically significant difference was found in the expression of CASP-1 in atherosclerosis’s patients (Lee et al., 2017). Lee et al., hypothesizes that this result is possibly due to a negative uptake of NLRPs, such as the use of statins by about 50% of the patients with atherosclerosis involved in our study, thus corroborating these findings (Lee et al., 2017; Yu et al., 2017).

The final product of pyroptosis activation pathway by the inflammasome is the IL-1β. Its action undergoes a cleavage process by caspase-1. Pyroptosis is a fundamental cell death process at the beginning of plaque formation, since as a first defence mechanism, the body attempts to decrease the amount of inflammatory cells at the site of the lesion (Xu et al., 2018). Similarly to our findings, Lee et al. identified that patients with initial atherosclerotic lesions present increased IL-1β gene expression, while those with severe lesions were not significantly different from controls (Lee et al., 2017).

IL-1β is a proinflammatory cytokine that to play its role requires pre-activation by caspase-1. Pyroptosis is a fundamental cell death process at the beginning of plaque formation, since as a first defence mechanism, the body attempts to decrease the amount of inflammatory cells at the site of the lesion (Xu et al., 2018). Similarly to our findings, Lee et al. identified that patients with initial atherosclerotic lesions present increased IL-1β gene expression, while those with severe lesions were not significantly different from controls (Lee et al., 2017). The numerous factors influencing the serum levels of this particular cytokine difficult to interpret the results, thus further studies are needed to investigate its activation profile in atherosclerosis and lesion severity (Lee et al., 2017).

In conclusion, we report for the first time the increased expression of NLRP1 and NLRC4 genes in patients with atherosclerosis. We also emphasize, confirming previous findings, IL-1β as an important marker against the onset of atherosclerotic plaque formation that is systemic detectable. However, we understand the limitation of this work because since we assessed at gene levels only. We believe further analysis including all the proteins involved in the study is needed. As well, to better understand the role of NLRP1 and NLRC4 in coronary stenosis development, animal studies would be clarifying.

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which has been configured to accept email from j.azvedo@gmail.com.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imbio.2020.151940.

References


