Modern Research in Inflammation, 2025, 14(3), 89-103
https://www.scirp.org/journal/mri
ISSN Online: 2169-9690
ISSN Print: 2169-9682

Effect of Inflammatory Environment on NADPH Oxidase 2 Expression in Pancreatic Islets of Diabetic Rats

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How to cite this paper: Mayassi, F.K.H., Elenga-Bongo, C.L., Loubano-Voumbi, G., Gondo, J.B.K., Kimpolo, C.N., Bouenizabila, E., Makele, J.J.M., Got, F.E.B., Missamou, G.L.E., Bactchy, V.A.J.A. and Moukassa, D. (2025) Effect of Inflammatory Environment on NADPH Oxidase 2 Expression in Pancreatic Islets of Diabetic Rats. *Modern Research in Inflammation*, **14**, 89-103.
https://doi.org/10.4236/mri.2025.143007

Received: July 5, 2025
Accepted: August 15, 2025
Published: August 18, 2025

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Abstract

Background: Persistent low-grade inflammation is a hallmark of type 2 diabetes (T2D), a chronic disease that poses a serious threat to health and leads to the gradual degeneration of islets pancreatic β-cells. The purpose of the current study was to assess how the inflammatory environment affected the expression of NADPH oxidase 2 (NOX2) in the pancreatic islets of diabetic rats and to investigate the connections between NOX2 activity and systemic inflammatory markers. **Methods:** Male Wistar rats weighing 250 - 300 g and aged 8 - 10 weeks were given an intraperitoneal injection of streptozotocin (50 mg/kg) to induce diabetes. After being separated by enzymatic digestion using collagenase P and purified using a Ficoll gradient, pancreatic islets were cultivated and incubated for 24 hours at different glucose concentrations (5.5 mM, 15 mM, and 25 mM). Apocynin and diphenyleneiodonium (5, 15, 25 µM) were used to pharmacologically inhibit NOX2. Following that, ELISA and immunoturbidimetry methods were used to measure the levels of NOX2, interleukin-6, and CRP. **Results:** The study showed that NOX2 activity increased in response to rising glucose concentrations in a dose-dependent manner. At 25 mM, a notable peak was seen ($p < 0.01$ vs. 5.5 mM), suggesting

a marked rise in NOX2 activity at this concentration. This activation was accompanied by a proportional elevation of inflammatory markers in which IL-6 demonstrated an approximately eight-fold increase from baseline levels, while CRP exhibited a comparable progression. Pharmacological inhibition of NOX2 by both DPI and apocynin reduced significantly its enzymatic activity, IL-6 and CRP levels. Both NOX2 and CRP ($r = 0.41$, $p = 0.1$) and NOX2 and IL-6 ($r = 0.41$, $p = 0.2$) showed moderate but non-significant correlations. **Conclusion**: This study shows that in the pancreatic islets of diabetic rats, hyperglycemia directly causes NOX2 activation and ensuing inflammation. This discovery shows NOX2 as an intriguing therapeutic target worth pursuing and offers important new insights into the mechanisms underlying the progression of diabetes.

Keywords

NADPH Oxidase 2, Diabetes, Inflammation, Islets β-Cell

1. Introduction

Globally, type 2 diabetes (T2D) affects over 537 million adults and is one of the most important health issues of our time [1]. Two main dysfunctions "insulin resistance and the progressive degeneration of the pancreatic β cells that secrete insulin" are the cause of this complex metabolic pathology [2].

According to recent studies, chronic low-grade inflammation plays a key role in the pathophysiology of type 2 diabetes (T2D), establishing a pathological link between insulin resistance, obesity, and the degeneration of the pancreatic islets [3]. This inflammation is characterized at the pancreatic level by a phenomenon called "insulitis", which is characterized by immune cell infiltration into the islets and the release of pro-inflammatory cytokines like C-reactive protein (CRP) and interleukin-6 (IL-6), creating a toxic environment for β cells [4].

This inflammation is closely linked to oxidative stress, which is a major mechanism that destroys β cells [5]. The reactive oxygen species (ROS) have been shown to cause direct cellular damage while also triggering signaling pathways that intensify inflammation, resulting in a harmful cycle [6]. As the main source of ROS in many tissues, especially the pancreas, NADPH oxidase (NOX) enzymes are essential to this process [7].

Of all the NOX isoforms, NOX2 (also known as $gp91^{phox}$) has garnered the most attention from academics. The enzyme under investigation has been shown to be expressed in pancreatic β cells and immune cells, indicating a direct role in metabolic and inflammatory processes [8]. Several experimental models of diabetes have shown elevated NOX2 expression and activity, as shown in earlier research [9]. It is still unclear exactly how the inflammatory milieu controls NOX2 expression in the pancreatic islets.

As described in Brownlee's unifying theory of diabetic complications, chronic

hyperglycemia, a defining feature of diabetes, has been proposed as a key catalyst for NOX2 activation via protein kinase C (PKC) stimulation [6]. This activation could create a direct mechanistic connection between pancreatic inflammation, ROS generation, and hyperglycemia.

Animal models, especially diabetic rodents, are useful research tools for clarifying these intricate mechanisms because they enable in-depth examination of the molecular and cellular alterations taking place in the pancreatic islets [10]. The streptozotocin-induced diabetic model is still widely used to study oxidative stress and pancreatic inflammation mechanisms, despite its tendency to cause rapid β cell destruction [11].

The current study intends to investigate the connections between systemic inflammatory markers (CRP and IL-6) and NOX2 activity, as well as the effects of the inflammatory environment on NOX2 expression in the pancreatic islets of diabetic rats. The current study postulates that, in pancreatic islets, NOX2 overexpression coincides with an increase in inflammatory markers, resulting in a particularly harmful pro-oxidant environment for β cell survival and function.

2. Materials and Methods

2.1. Experimental Model and Housing Conditions

2.1.1. Animal Characteristics

Male Wistar rats, aged 8 - 10 weeks and weighing 250 - 300 g at the time of experimentation, were selected from the breeding facility of the Faculty of Science and Technology (Marien Ngouabi University, Congo).

2.1.2. Standardized Housing Conditions

In accordance with international standards, the animal facility was kept at a consistent temperature of 22˚C ± 2˚C, with a relative humidity of 55% ± 10% and an artificial circadian cycle of 12 hours of light and 12 hours of darkness. The animals were given free access to drinking water and a typical rodent pellet diet.

2.1.3. Diabetic Induction Procedure

The administration of streptozotocin (50 mg/kg body weight) intraperitoneally resulted in diabetic hyperglycemia. The specific and irreversible destruction of pancreatic β-cells has been shown to be induced by this alkylating compound [11]. The diagnosis of diabetes was made using multiple glycemic measurements, and the diagnostic criterion was determined to be the persistence of fasting hyperglycemia >250 mg/dL.

2.2. Pancreatic Islet Isolation Protocol

2.2.1. Tissue Collection

Before being put to death by beheading, the animals were anesthetized by inhaling isoflurane at a concentration of 5% for induction and 2% for maintenance. To maintain cell viability, pancreas were removed and placed in ice-cold HBSS (Gibco, Thermo Fisher Scientific) devoid of calcium and magnesium.

2.2.2. Enzymatic Digestion

Pancreatic tissue was then digested with collagenase P (Roche Diagnostics, 0.5 mg/ml) for 15 to 20 minutes at 37˚C while being gently stirred. To maximize islet release without sacrificing their integrity, this crucial step necessitates rigorous temporal control.

2.2.3. Density Gradient Purification

The cell suspension was purified by differential centrifugation on a Ficoll gradient (Sigma-Aldrich) at 400 g for 20 minutes after being filtered through sterile gauze. Before being resuspended, islets that were recovered from the intermediate fraction underwent two HBSS washes.

2.3. Culture and Experimental Treatments

A total of 24 male Wistar rats were used in this study. For each condition (5.5 mM, 15 mM, 25 mM glucose), 8 rats were included. Approximately 10 pancreatic islets were isolated per rat, representing 80 islets analysed per condition.

2.3.1. Culture Configuration

In 24-well Corning plates, isolated islets (10 - 20 per well) were cultivated in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin, with the pH adjusted to 7.4. The "incubation" phase, which involves laying eggs, was carried out at 37˚C in a controlled environment with saturated humidity and 5% carbon dioxide.

2.3.2. Glycemic Stimulation

After being acclimated for 24 hours, islets were exposed to progressively higher glucose concentrations. The concentrations shown are as follows: moderate hyperglycemia (15 mM), severe hyperglycemia (25 mM), and normoglycemia (5.5 mM). These levels are suggestive of both healthy and unhealthy states.

2.3.3. Pharmacological Treatments

Apocynin or DPI (Sigma-Aldrich) at concentrations of 5, 15, and 25 μM were pre-incubated for 30 minutes in order to inhibit NOX2. To avoid the solvent's possible cytotoxic effects, stock solutions were made in DMSO with a final concentration of ≤0.1% v/v.

2.4. Analytical Techniques

2.4.1. Sample Preparation

The analyses were conducted on 24 biological samples from pools of 3 rats per experimental condition, with technical duplicates for each assay. The islets (n = 24) were enriched with protease inhibitors after being treated with a detergent buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% SDS, and 0.5% deoxycholate). Prior to clarifying centrifugation (12,000 g, 10 min, 4˚C), the samples were sonicated lysed (3 × 10 s, 20% amplitude, on ice).

2.4.2. NOX2 Quantification by ELISA Technique

The ELISA kit used quantifies the abundance of the NOX2 protein, not its enzymatic activity. It is a sandwich test based on immunological detection by specific antibodies, without direct measurement of catalytic activity.

The quantification of NOX2 concentrations in islet lysates was performed by specific ELISA kit (MyBiosource, San Diego, CA, USA), following the instructions provided by the manufacturer. ELISA was performed by placing samples and standards in a 96-well plate pre-coated with anti-NOX2 antibodies. After the incubation and washing steps, a horseradish peroxidase (HRP)-conjugated secondary antibody was added. The reaction was developed with TMB substrate, and the absorption was measured at 450 nm using a microplate reader (Sinothinker, Guangdong). The concentrations of NOX2 were calculated by reference to a standard curve and expressed as picograms per milligram of total protein.

2.4.3. IL-6 Quantification

The interleukin-6 (IL-6) was measured by ELISA technique (MyBiosource, San Diego, CA, USA), the manufacturer's guidelines. Subsequently, the samples were subjected to incubation with primary and secondary antibodies, by the standard protocol. Subsequent to this, the addition of chromogenic substrate was employed to reveal the samples. Spectrophotometric readings (450 nm) were performed on an automated reader (Sinothinker, Guangdong), with quantification by interpolation on standard curves and normalization by total proteins. Assays were performed in duplicate for each sample.

2.4.4. CRP Quantification

Using a Cobas c111 analyzer (Roche Diagnostics), C-reactive protein (CRP) was measured by immunoturbidimetry in compliance with the manufacturer's instructions. In milligrams per liter, the results were reported. Each assay was run twice for every sample.

2.5. Ethical Considerations

All experimental procedures involving animals were carried out in accordance with the Animal Research: Reporting of *in vivo* Experiments guidelines and institutional regulations on animal care and use, approved by the Health Ethics Committee under number 053-25/MESRSIT/DGRST/CERSSA/-25.

2.6. Statistical Analysis

The data were analysed using GraphPad Prism software (version 5.0). Normally distributed quantitative variables are expressed as mean ± standard deviation and compared using Student's t-test for pairwise comparisons, or one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons. Non-normally distributed data are presented as median and analysed using the Mann-Whitney or Kruskal-Wallis test, as appropriate. Correlations were assessed using Spearman's coefficient. Sample sizes were determined based on preliminary studies and power

analyses, with a fixed α risk of 5%. All measurements were performed in duplicate, and outliers identified by Grubbs' test were excluded from the analysis. Differences were considered statistically significant when $p < 0.05$. The power analysis was performed with a significance level of $\alpha = 0.05$ and a statistical power $(1 - \beta)$ of 0.80. The estimated effect size was moderate (Cohen's $d \approx 0.6$), which justified the inclusion of 8 animals per group.

3. Results

3.1. Effect of Glucose on CRP Expression in Pancreatic Islets

Exposure of pancreatic islets to increasing concentrations of glucose (5.5 mM, 15 mM, 25 mM) induced a gradual and significant increase in CRP concentration ($p = 0.032$, ANOVA). The maximum value was observed at 25 mM, reflecting activation of the inflammatory response in a hyperglycaemic context, as shown in Figure 1.

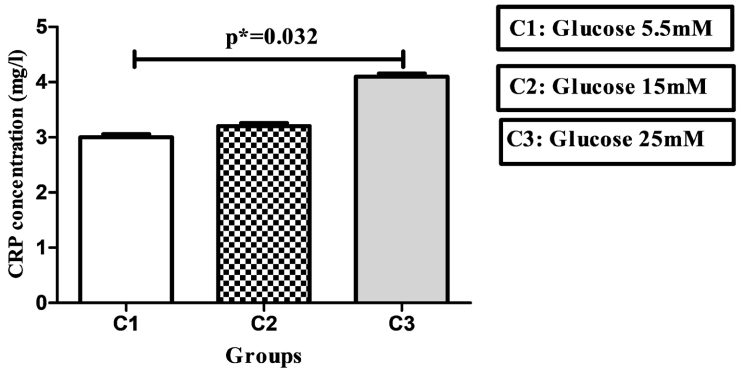


Figure 1. CRP concentration (mg/L) as a function of glucose (n = 8 rats, 100 islets per condition). Results are expressed as mean ± standard deviation.

3.2. Influence of Glucose on IL-6 Expression

A marked increase in IL-6 levels was detected in response to elevated glucose, suggesting activation of the local inflammatory network. The increase was dose-dependent, reaching a peak at 25 mM, as presented in Figure 2.

3.3. NOX2 Activity in Response to Glucose

Quantification by ELISA revealed significant activation of NOX2 proportional to glucose concentration. Maximum activity was observed at 25 mM, consistent with increased oxidative stress in islets exposed to hyperglycemia, as shown in Figure 3.

3.4. Effect of Pharmacological Inhibitors (DPI and Apocynin) on Inflammatory Markers

Pretreatment of islets with DPI or apocynin significantly reduced IL-6 and CRP concentrations, as well as NOX2 enzyme activity. Inhibition was more pronounced with DPI. These results are presented in Figures 4(a)-(f).

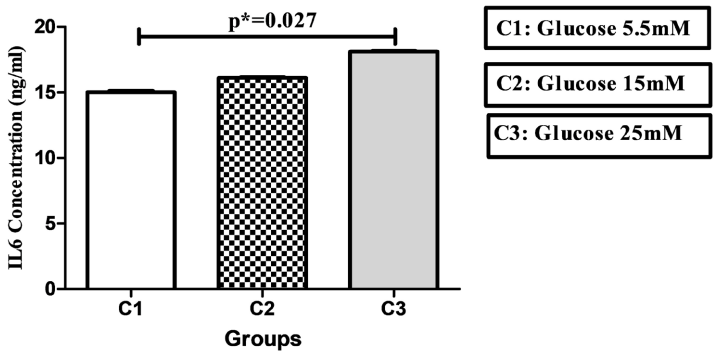


Figure 2. IL-6 expression according to glucose concentration. Means ± SD, n = 8 rats/condition.

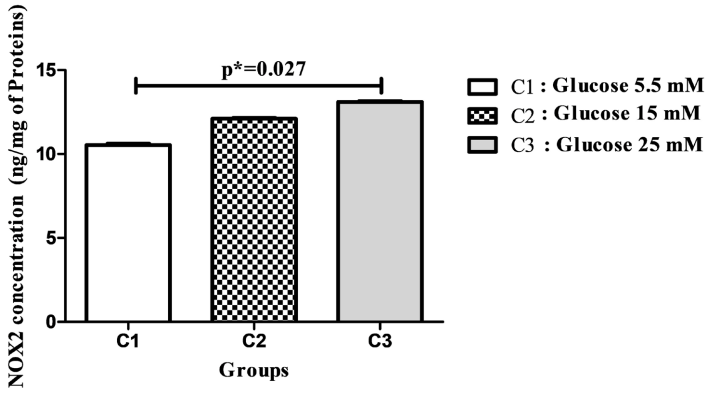
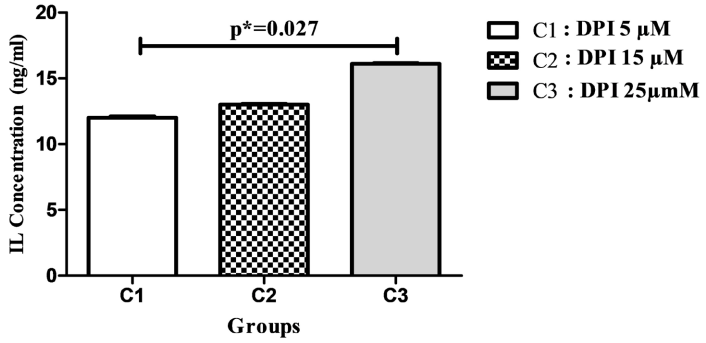
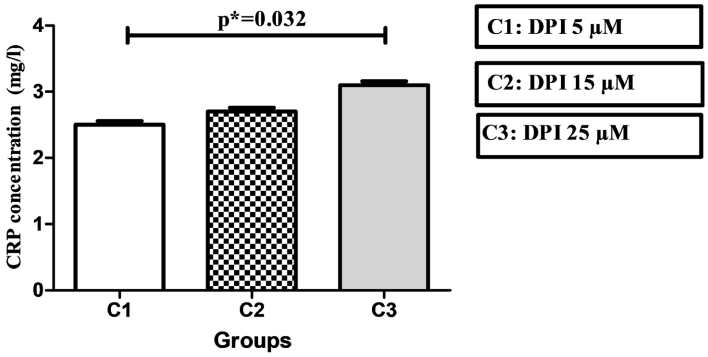


Figure 3. NOX2 activity (pg/mg total protein) measured by ELISA. Means ± SD, n = 8 rats.



(a)



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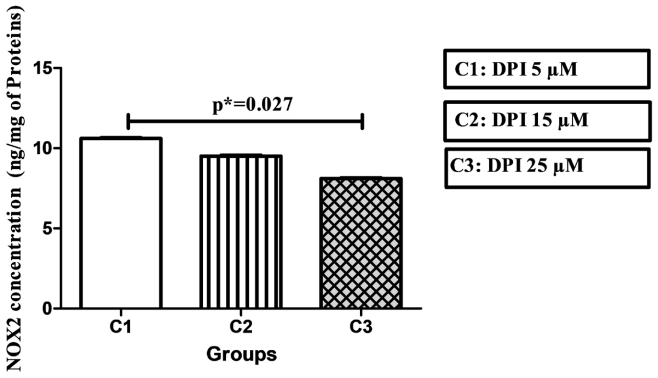

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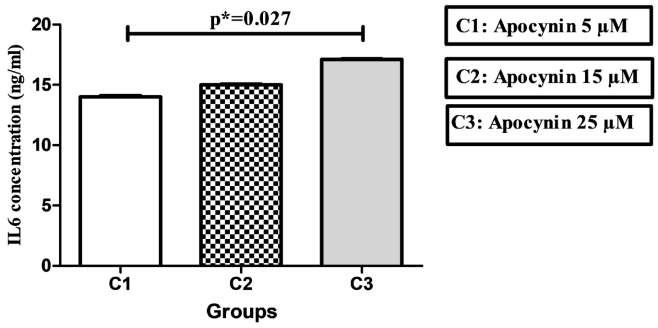

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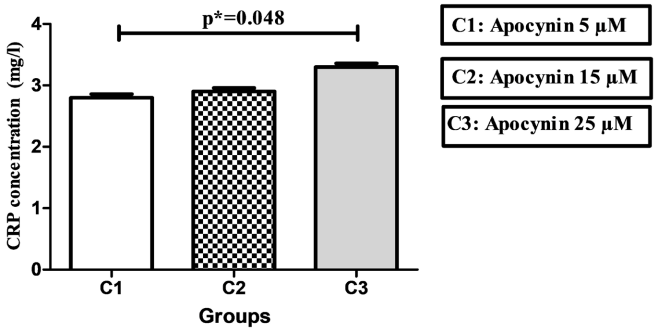

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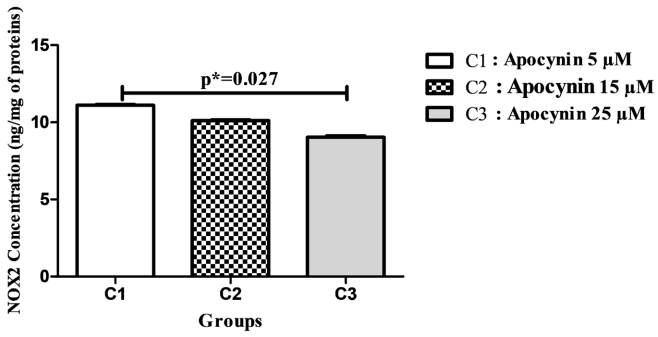


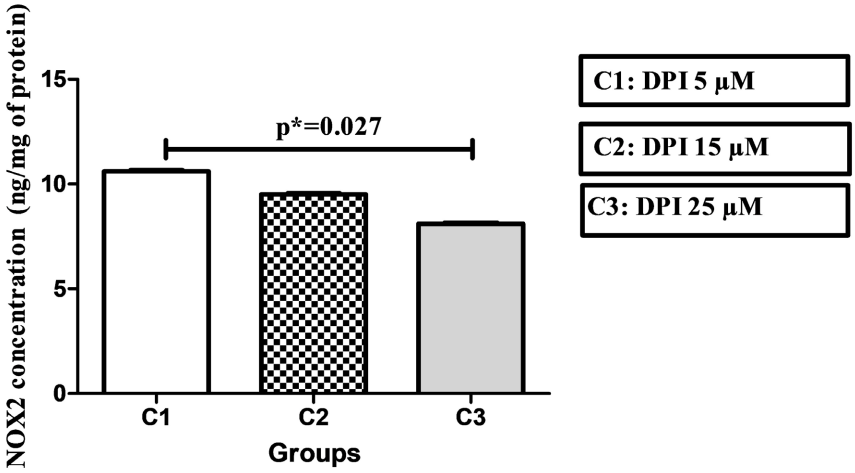
(f)

Figure 4. (a) Effect of DPI inhibitor on IL-6 concentration. (b) Effect of DPI inhibitor on CRP concentration. (c) Effect of DPI inhibitor on NOX2 activity, CRP, and IL-6. (d) Effect of apocynin inhibitor on interleukin-6 concentration. (e) Effect of apocynin inhibitor on CRP concentration. (f) Effect of apocynin inhibitor on NOX2 expression.

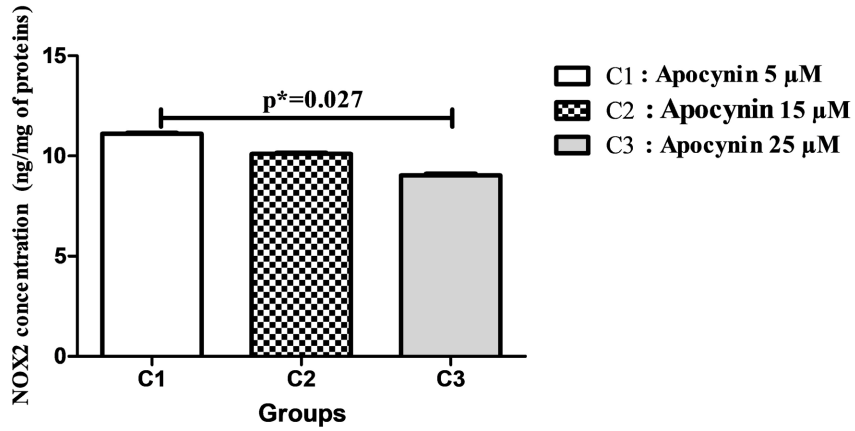
Figures 4(a)-(f): Effects of DPI and apocynin (5 - 25 μM) on IL-6, CRP, and NOX2. Means ± SD, n = 3 biological isolates/condition.

3.5. Specific Inhibition of NOX2 Activity

The isolated assessment of NOX2 enzyme activity confirmed a significant reduction after treatment with both inhibitors, with DPI showing more consistent and pronounced inhibition, as illustrated in Figure 5(a) and Figure 5(b).



(a)



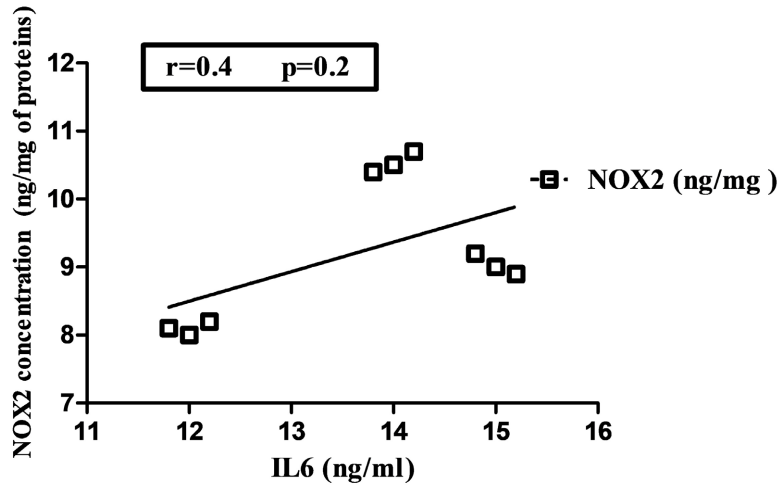
(b)

Figure 5. (a) NOX2 activity after treatment with DPI. (b) NOX2 activity after treatment with apocynin.

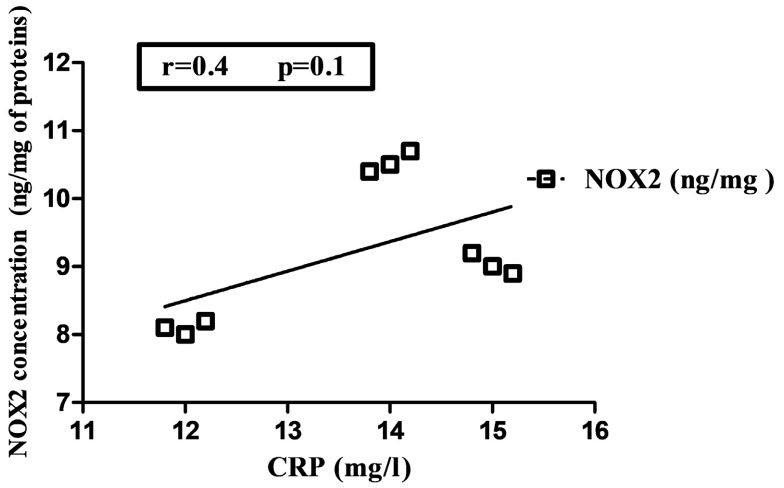
3.6. Correlation between NOX2 and Inflammatory Markers

Correlation analyses between NOX2 activity and IL-6 levels (r = 0.41; p = 0.20) or CRP levels (r = 0.41; p = 0.10) did not reveal any statistically significant relationship, as shown in Figure 6(a) and Figure 6(b).

Figure 6(a) and Figure 6(b): Correlation between NOX2 and IL-6 or CRP. No significant association observed (r = 0.41 and p = 0.20 for Figure 6(a) and r = 0.4 and p = 0.1 for Figure 6(b)).



(a)



(b)

Figure 6. (a) Correlation between IL-6 and NOX2. (b) Correlation between CRP and NOX2.

4. Discussion

Our research has revealed an intriguing facet of pancreatic biology: we have demonstrated that glucose levels and NOX2 enzyme activation in the pancreatic islets of diabetic rats are directly and proportionately correlated. This discovery is especially noteworthy because it connects inflammation and hyperglycemia, two processes that are already known to play a significant role in diabetes. But up until now, the mechanism underlying these two phenomena has been unclear.

Although the streptozotocin model more closely replicates type 1 diabetes, it was used here for its ability to rapidly induce oxidative stress and a marked inflammatory response, thereby allowing the effect of NOX2 to be assessed in an acute hyperglycemic context [9].

When the pancreatic islets were exposed to increasing glucose levels (5.5 - 25 mM), a significant and proportionate increase in NOX2 activity was observed,

along with increased inflammatory indicators, such as CRP and IL-6. This finding supports the hypothesis more credence to our original theory that an integrated "hyperglycemia-NOX2-inflammation" pathway may be responsible for the progressive degradation of pancreatic β-cells in diabetics.

This result aligns with the groundbreaking study by Syed *et al.* [12] and Elumalai *et al.* [13], who had previously shown NOX2 expression in a variety of experimental diabetes models. However, proving that this activation is dose-dependent is a new contribution. In conclusion, it has been demonstrated that higher glucose levels cause NOX2 activation to rise, which starts a harmful cycle of negative effects.

Furthermore, as Brownlee [6] expertly outlined in his unifying theory of diabetic complications, the underlying mechanism may involve the activation of protein kinase C (PKC). According to this model, PKC is activated by diacylglycerol accumulation brought on by hyperglycemia. The production of reactive oxygen species is then set off by PKC's phosphorylation and activation of NOX2 subunits [14]. Elumalai *et al.* [13] further elaborated on this mechanism, explaining that in type 2 diabetes, metabolic stress negatively impacts pancreatic β-cell function and survival, with an imbalance in redox homeostasis causing abnormal tissue damage and β-cell dysfunction, emphasizing that NADPH oxidase targeting represents a promising therapeutic approach for preserving β-cell function.

At the same time, the significant increase in IL-6 and CRP in our model shows that a complex inflammatory response has begun in the pancreatic islets. The ambivalent role that IL-6 plays in this process is especially noteworthy; Kristiansen and Mandrup-Poulsen [15] thoroughly examined this aspect. It has been shown that IL-6 activates suppressor proteins like SOCS3, which makes the environment unfavorable to insulin action and contributes to insulin resistance. Choi *et al.* [16] have shown that, ironically, this same cytokine has short-term protective effects on β-cells. The idea that biological reactions are rarely clear-cut is further supported by this duality, which reflects the complexity of the biological processes being studied. The vulnerability of pancreatic β-cells to oxidative stress is particularly well-documented, as Gurgul-Convey and Lenzen [9] noted that β-cells are characterized by relatively low antioxidant capacity, making them extremely sensitive to oxidative stress and particularly susceptible to NOX-mediated oxidative damage.

Additionally, we found local production of CRP in our islet cultures, which is surprising given that the liver is the primary site of this protein's synthesis. This finding points to an extra-hepatic synthesis capacity in vascular and adipose tissues that has been documented by other research teams before [17]-[19]. The inflammatory response within the pancreatic microenvironment may therefore be amplified by this local production, resulting in a localized "inflammatory storm".

However, one particularly positive finding of our research concerns the exceptional effectiveness of NOX2 inhibitors. In fact, enzymatic activity and inflammatory markers were both significantly and simultaneously reduced when DPI and

apocynin were administered to the islets. The fact that NOX2 is a key participant in this pathological cascade rather than just a passive observer of inflammation makes this observation extremely important. This therapeutic potential is strongly supported by the work of Elumalai *et al.* [13], who provided comprehensive evidence that NOX targeting in diabetes, with special emphasis on pancreatic β-cell dysfunction, represents a viable therapeutic strategy, highlighting that selective NOX inhibition could preserve β-cell function and mass.

Furthermore, DPI (diphenyleneiodonium) outperformed apocynin in the model, exhibiting unique efficacy. This difference in effectiveness sheds light on the mechanisms of action: apocynin takes a more subtle approach by preventing the formation of the active NOX2 complex, whereas DPI acts as a true "switch" by directly preventing electron transfer at the flavin-FAD complex level [20]. This finding implies that direct inhibition of enzymatic activity may be more effective in therapeutics than blocking complex assembly. The therapeutic implications of our findings are further reinforced by the work of Song *et al.* [21], who demonstrated that modulation of NOX2 activity can influence insulin secretion, showing that interventions targeting the BMAL1-NOX2 pathway could potentially restore normal insulin secretion patterns.

Similarly, Jha *et al.* [22] showed that NOX2 inhibition in a mouse model prevented the development of diabetic nephropathy, and these results are in line with their findings. Similarly, Gao's group [23] showed that genetic NOX2 suppression protected mice from β-cell dysfunction brought on by a high-fat diet. Our faith in this approach's therapeutic potential is strengthened by these convergences.

Although there is no statistically significant association between NOX2 and inflammatory markers (CRP: $r = 0.41$, $p = 0.1$; IL-6: $r = 0.41$, $p = 0.2$), our results indicate a moderate trend towards a positive correlation between NOX2 and inflammation. These findings suggest that NOX2 may be important in controlling the inflammatory response. Our findings support those of Lambeth and Neish [24], who claimed that NOX2 is a major modulator of cellular redox signaling and has the ability to directly affect the expression of genes that promote inflammation.

On the other hand, the finding that hyperglycemia activates NOX2 points to the involvement of a complex web of signaling pathways that converge on this important enzyme. It has been shown that the polyol pathway weakens the cell's built-in antioxidant defenses by causing sorbitol accumulation and NADPH depletion [25]. At the same time, it has been shown that the production of advanced glycation end products (AGEs) activates the RAGE receptor, which starts the NF-κB transcription factor pathway and causes the expression of pro-inflammatory cytokines [26].

The harmful nature of the system, however, stems from the direct activation of NF-κB by NOX2-mediated oxidation of its inhibitor IκB, creating a loop of inflammatory amplification that perpetuates itself [27]. The current study clarifies the fundamental processes through which the simultaneous decrease of ROS gen-

eration and cytokine expression is made possible by NOX2 inhibition. The comprehensive evidence from our study, combined with the insights from Song *et al.* [21], Gurgul-Convey and Lenzen [9], and Elumalai *et al.* [13], strongly supports the concept that NOX2 represents a critical therapeutic target in diabetes, with the convergence of evidence suggesting that targeting NOX2 activity could address multiple pathological mechanisms simultaneously: oxidative stress, inflammation, circadian dysfunction, and β-cell death.

5. Conclusion

In the pancreatic islets of diabetic rats, the current study shows that hyperglycemia directly causes NOX2 activation and ensuing inflammation. NOX2 is an intriguing therapeutic target that merits further investigation, and this discovery offers important new insights into the mechanisms underlying the progression of diabetes. It is understood, nevertheless, that it will take a great deal of time and work to convert these findings into patient treatments. It is suggested that in order to build on the results of this study, future research should focus on developing diabetes models that more closely mimic human disease, developing more selective NOX2 inhibitors, and incorporating larger patient populations.

Acknowledgements

We would like to thank the doctoral programm at the Faculty of Health Sciences for giving us the opportinity to write these scientific articles. Our thanks also go to the research laboratory of the Faculty of Health Sciences.

Conflicts of Interest

The authors do not declare any conflict of interest.

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