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Translocator Protein Modulation by PK11195 and NO Synthase Inhibition Affect Cardiac Oxidative Stress and Cardiometabolic and Inflammatory Markers in Isoprenaline-Induced Rat Myocardial Injury

Ana Ilic [1,†], Nina Radisavljevic [2,†], Slavica Mutavdzin Krneta [2], Dusan Todorovic [2], Novica Boricic [3], Sanja Stankovic [4,5], Biljana Bozic Nedeljkovic [6], Marija Matić [7], Marija Stojanovic [2], Ranko Skrbic [8,9,10] and Dragan Djuric [2,*]

1 Department of Cardiology, University Clinical Hospital Center "Dr. Dragisa Misovic—Dedinje", 11000 Belgrade, Serbia; ana.stevanovic15@yahoo.com
2 Institute of Medical Physiology "Richard Burian", Faculty of Medicine, University of Belgrade, 11000 Belgrade, Serbia; nina_radisavljevic@outlook.com (N.R.); slavica.mutavdzin@gmail.com (S.M.K.); t.dusan@hotmail.com (D.T.); marija.stojanovic@med.bg.ac.rs (M.S.)
3 Institute of Pathology, Faculty of Medicine, University of Belgrade, 11000 Belgrade, Serbia; boricic.novica@gmail.com
4 Centre of Medical Biochemistry, University Clinical Centre of Serbia, 11000 Belgrade, Serbia
5 Faculty of Medical Sciences, University of Kragujevac, 34000 Kragujevac, Serbia
6 Institute for Physiology and Biochemistry "Ivan Djaja", Faculty of Biology, University of Belgrade, 11000 Belgrade, Serbia; najbiljana@yahoo.com
7 Institute of Medical and Clinical Biochemistry, Faculty of Medicine, University of Belgrade, 11000 Belgrade, Serbia; marija.matic@med.bg.ac.rs
8 Centre for Biomedical Research, Faculty of Medicine, University of Banja Luka, 78 000 Banja Luka, Bosnia and Herzegovina; ranko.skrbic@med.unibl.org
9 Department of Pharmacology, Toxicology and Clinical Pharmacology, Faculty of Medicine, University of Banja Luka, 78 000 Banja Luka, Bosnia and Herzegovina
10 Department of Pathologic Physiology, I.M. Sechenov First Moscow State Medical University, 119435 Moscow, Russia
* Correspondence: dragan.djuric@med.bg.ac.rs
† These authors contributed equally to this work.

Abstract

Translocator protein (TSPO) regulates mitochondrial function, inflammation, and oxidative stress; however, its role in acute myocardial injury (MI) remains incompletely understood. While previous studies have examined TSPO ligands in cardiac injury, the interplay between TSPO modulation and nitric oxide (NO) signaling in AMI has not been systematically investigated. The aim of this study was to investigate the effects of TSPO modulation by PK11195, alone or in combination with nitric oxide synthase (NOS) inhibition by Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME), on cardiometabolic, inflammatory, oxidative stress, and histopathological parameters in an experimental model of isoprenaline-induced MI in rats. Male Wistar albino rats were divided into four groups: control (C); isoprenaline + saline-treated (ISO); isoprenaline + PK11195-treated (IP); and isoprenaline + PK11195 + L-NAME-treated (IPLN) groups. Isoprenaline administration induced MI, evidenced by elevated cardiac biomarkers, electrocardiographic (ECG) alterations, and histopathological damage. PK11195 treatment significantly attenuated MI and reduced pro-inflammatory cytokine levels while increasing anti-inflammatory cytokine levels, indicating protective effects. Nevertheless, TSPO modulation was associated with adverse metabolic effects, notably elevated fibrinogen and plasma homocysteine levels. Co-administration of L-NAME mechanistically demonstrated that NO availability is essential for PK11195 cardioprotective effects, as NOS inhibition partially abolished cardioprotection and modified

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oxidative stress parameters. Overall, TSPO modulation exerts complex actions in acute MI through regulating mitochondrial function, inflammatory signaling, and NO pathways, suggesting that TSPO is a potential, multifaceted therapeutic target.

Keywords: cardioprotection; isoprenaline; myocardial injury; nitric oxide (NO); oxidative stress; PK11195; translocator protein (TSPO)

1. Introduction

Acute myocardial infarction (AMI) is associated with high morbidity and mortality. Although major advances in prevention and treatment have been achieved, the disease remains a significant clinical and public health problem worldwide. The prevalence of AMI ranges from 3.8% in populations under the age of 60 to nearly 10% in elderly individuals, underscoring the persistent public-health burden of ischemic heart disease (IHD) [1].

The development and progression of AMI is strongly driven by oxidative stress and inflammation. Acute interruption of coronary blood flow leads to mitochondrial dysfunction and excessive formation of reactive oxygen species (ROS) and nitrogen species (RNS). These reactive species damage lipids, proteins, and nucleic acids, thereby amplifying cardiomyocyte injury. The inflammatory response further contributes to additional myocardial damage and impairs cardiac function. Importantly, oxidative stress and inflammation form a *circulus vitiosus* that accelerates post-infarction pathological remodeling and leads to poorer clinical outcomes [2,3].

Translational cardiovascular research depends on experimental models that accurately reproduce the pathophysiology of myocardial injury (MI). Among these, the isoprenaline-induced MI model in rats is the most widely used experimental model for investigating AMI [4]. Isoprenaline is a synthetic catecholamine and β-adrenergic agonist that triggers oxidative damage, leading to cardiomyocyte necrosis and lesions resembling those observed in clinical infarction [5].

Considering the critical involvement of oxidative injury, inflammatory cascades, and mitochondrial dysfunction in the development of AMI, research efforts have progressively shifted towards molecular targets that modulate these processes. Among these, 18-kDa translocator protein (TSPO) has emerged as a promising molecular candidate [6]. TSPO, originally identified as a peripheral benzodiazepine receptor, is a ubiquitously expressed five-transmembrane protein, primarily localized in the outer mitochondrial membrane. In eukaryotic cells, TSPO resides in close physical association with key mitochondrial channels, including the voltage-dependent anion channel (VDAC) as a part of the mitochondrial permeability transition pore (mPTP) complex and the inner membrane ion channel (IMAC), positioning it as an important regulator of mitochondrial communication and stress signaling [7]. TSPO is highly expressed in steroid-synthesizing tissues, including the adrenal glands, gonads, brain, and heart. Beyond its classical role in steroidogenesis, accumulating evidence suggests that TSPO participates in the regulation of oxidative stress and in key aspects of mitochondrial physiology and metabolism [8].

Under conditions of oxidative and metabolic stress, TSPO interacts with mPTP components to maintain mitochondrial stability. However, elevated ROS production and Ca^{2+} overload trigger prolonged mPTP opening, leading to cardiomyocyte death, arrhythmogenesis, and impaired post-ischemic recovery. Experimental evidence suggests that pharmacological modulation of TSPO can moderate these harmful processes: TSPO ligands reduce mitochondrial ROS generation, limit mPTP opening, and ultimately protect against reperfusion-induced arrhythmias and contractile dysfunction. These findings highlight

TSPO as a therapeutically relevant target in cardiac injury and underscore the potential utility of its ligands in mitigating oxidative, inflammatory, and metabolic disturbances characteristic of AMI [6,9,10].

PK11195 is a first-generation TSPO ligand widely used to study TSPO function in vitro and in vivo, and its radiolabeled form, [^{11}C]-PK11195, has been employed to visualize inflammatory processes such as atherosclerosis [6]. Experimental evidence indicates that PK11195 modulates mitochondrial function during ischemia–reperfusion injury, exerting protective effects specifically when administered at reperfusion by reducing mitochondrial ROS production, preserving membrane potential, and limiting cell death [11]. In addition, PK11195 may modulate vascular tone, possibly via inhibition of voltage-operated Ca^{2+} channels [12].

Oxidative stress in MI is tightly linked to disturbances in gasotransmitter signaling. Nitric oxide (NO) emerges as a key regulator of vascular function, mitochondrial homeostasis, and redox balance. In addition to NO, other gasotransmitters such as hydrogen sulfide (H_2S) and carbon monoxide (CO) also contribute to cardiovascular redox signaling and may interact with NO-dependent protective pathways [13]. However, the present study focuses specifically on NO signaling as it can be directly and pharmacologically modulated by NOS inhibition and represents a well-established regulator of mitochondrial and vascular function in AMI.

Given the important roles of NO in regulating vascular tone, mitochondrial homeostasis, and redox signaling during myocardial injury, alterations in NO bioavailability may represent an important link between oxidative stress and TSPO-dependent mitochondrial responses. However, the involvement of NO-related mechanisms in the cardioprotective effects of TSPO modulation by PK11195 in isoprenaline-induced MI remains insufficiently explored. In particular, it remains unclear whether NO bioavailability is required for TSPO-mediated cardioprotection or whether TSPO effects occur independently of NO signaling.

We hypothesized that PK11195-mediated TSPO modulation exerts cardioprotective effects in isoprenaline-induced MI and that these effects are, at least partially, influenced by NO-dependent mechanisms.

This study aimed to investigate the potential cardioprotective effects of TSPO modulation by its ligand, PK11195, in an isoprenaline-induced rat model of MI, either alone or in the presence of nitric oxide synthase (NOS) inhibition by Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME).

2. Results

2.1. Biomarkers of Myocardial Injury and Other Biochemical Parameters Determination

Serum markers of myocardial injury demonstrated a significant increase in high-sensitivity troponin T (hsTnT) in the ISO group compared to the control group (ISO = 173.4 (70.4–358.5) ng/L vs. C = 12.0 (8.0–13.0) ng/L, $p < 0.05$) (Figure 1). Administration of PK11195 in the IP group markedly reduced hsTnT levels compared to the ISO group (IP = 61.5 (25.0–95.0) ng/L vs. 173.4 (70.4–358.5) ng/L, $p < 0.01$). Notably, co-administration of PK11195 and L-NAME in the IPLN group caused a significant increase in hsTnT relative to both the IP group (IPLN = 942.0 (228.0–2850.0) ng/L vs. IP = 61.5 (25.0–95.0) ng/L, $p < 0.01$) as well as the ISO group (IPLN = 942.0 (228.0–2850.0) ng/L vs. ISO = 173.4 (70.4–358.5) ng/L, $p < 0.05$). Regarding aspartate aminotransferase (AST), a non-specific cardiac marker, a significant increase was observed in the IPLN group compared to the IP group (IPLN =165.0 (132.0–259.0) U/L) vs. (IP = 113.5 (101.0–143.0) U/L, $p < 0.05$). In contrast, serum concentrations of AST were significantly lower in the ISO group in comparison to the control group (ISO = 112.6 (82.8–223.8) U/L) vs. (C = 170.0 (115.00–265.0) U/L, $p < 0.05$). On the other hand, there were no statistically significant differences in levels of

creatine kinase (CK) between the ISO group (ISO = 1608.0 (801.0–3111.0) U/L), IP group (2100.5 (1151.0–2695.0) U/L), and IPLN group (IPLN = 1638.5 (1004.0–2078.0) U/L, $p > 0.05$). Similarly, no significant differences were observed in levels of lactate dehydrogenase (LDH) (ISO = 2855.0 (1399.0–5675.0) U/L vs. C = 4820.5 (4500.0–4978.0) U/L, $p > 0.05$; IP = 4556.5 (2327.0–5879.0) U/L) vs. IPLN = 3453.0 (2585.0–4390.0) U/L, $p > 0.05$).

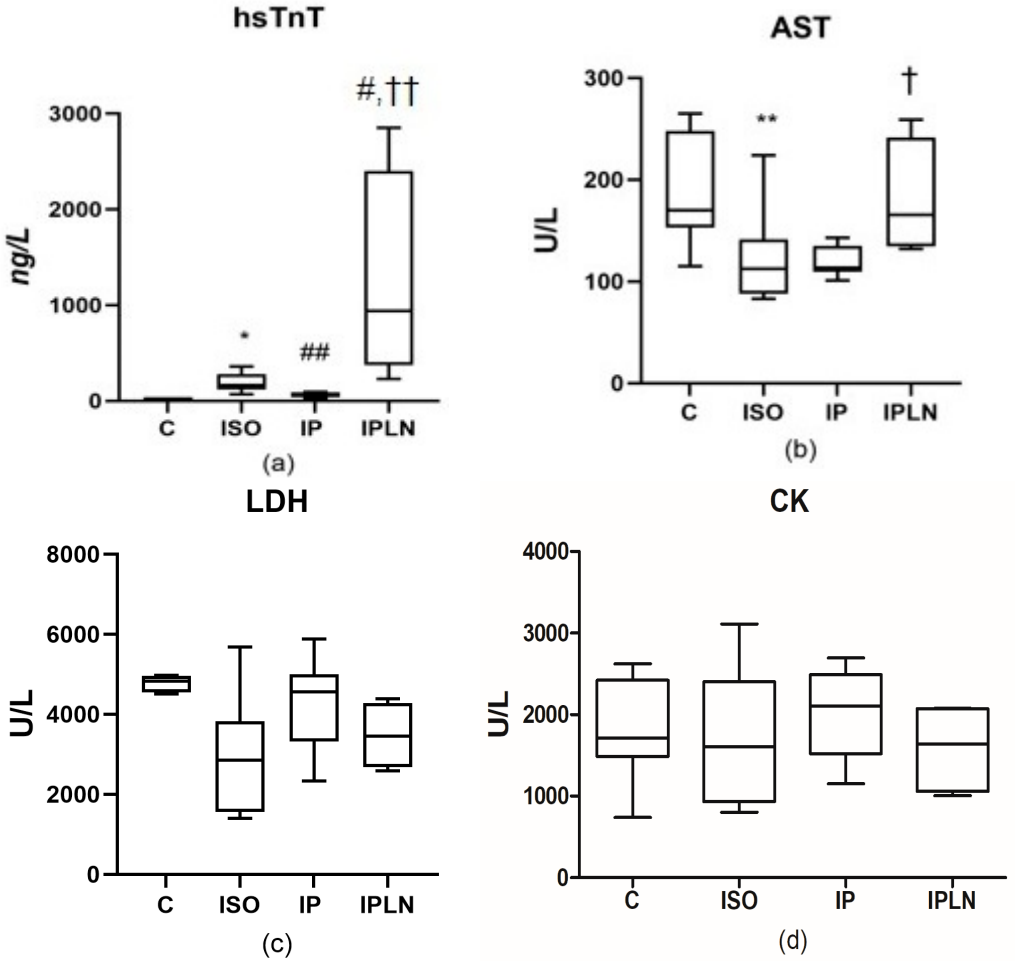


Figure 1. Biomarkers of myocardial injury. Serum levels of high-sensitivity troponin T (hsTnT ng/L) (**a**) serum levels of aspartate aminotransferase (AST U/L) (**b**) serum levels of lactate dehydrogenase (LDH U/L) (**c**) serum levels of creatine kinase (CK U/L) (**d**). C—control group; ISO—isoprenaline + saline-treated group; IP—isoprenaline + PK11195-treated group; IPLN—isoprenaline + PK11195 + L-NAME-treated group. Data are presented as box plot showing the median (horizontal line within the box), interquartile range (box boundaries), and minimum to maximum values (whiskers); * $p < 0.05$ vs. C group; ** $p < 0.01$ vs. C group; # $p < 0.05$ vs. ISO group; ## $p < 0.01$ vs. ISO group; † $p < 0.05$ vs. IP group; †† $p < 0.01$ vs. IP group. Statistical analysis was performed using the Kruskal–Wallis test followed by the Mann–Whitney U test.

The biochemical parameters of serum and plasma are presented in Table 1. The concentration of total cholesterol (TC) was significantly lower in the ISO group compared to the control (ISO = 1.69 (1.4–2.1) mmol/L vs. C = 2.32 (2.0–2.7) mmol/L, $p < 0.01$). In contrast, TC levels in both IP and IPLN groups were significantly higher compared to the ISO group (IP = 2.11 (1.9–2.6) mmol/L, $p < 0.01$; IPLN = 2.29 (1.9–2.5) mmol/L, $p < 0.05$). high density lipoprotein cholesterol (HDL) levels were significantly higher in the control group compared to the ISO group (C = 1.6 (1.3–1.9) mmol/L vs. ISO = 1.0 (0.8–1.4) mmol/L, $p < 0.01$). Both the IP and IPLN groups exhibited significantly lower HDL concentrations compared to the ISO group (IP = 0.7 (0.6–0.7) mmol/L; IPLN = 0.6 (0.6–0.6)

mmol/L $p < 0.01$). Furthermore, HDL was significantly lower in the IPLN group compared to the IP group, ($p < 0.05$). In terms of other lipid profile parameters, serum triglyceride levels were significantly higher in the control group compared to the ISO group (C = 0.87 (0.5–1.3) mmol/L vs. ISO = 0.41 (0.3–0.6) mmol/L, $p < 0.01$). No significant differences in triglyceride concentrations were observed between the ISO, IP, and IPLN groups.

Table 1. Serum and plasma biochemical parameters in control and experimental groups.

	Groups			
Parameter	**C**	**ISO**	**IP**	**IPLN**
TC (mmol/L) [s]	2.32 (2.0–2.7)	1.69 (1.4–2.1) **	2.11 (1.9–2.6) ##	2.29 (1.9–2.5) #
HDL (mmol/L) [s]	1.6 (1.3–1.9)	1.0 (0.8–1.4) **	0.7 (0.6–0.7) ##	0.6 (0.6–0.6) ##,†
TG (mmol/L) [s]	0.87 (0.5–1.3)	0.41 (0.3–0.6) **	0.44 (0.43–0.6)	0.46 (0.3–0.9)
Hcy (µmol/mL) [s]	11.8 (8.5–20.1)	13.30 (12.70–14.85)	26.99 (22.30–42.30) ##	33.39 (17.80–41.60) ##
Urea (mmol/L) [s]	10.2 (8.8–13.3)	10.4 (9.1–11.9)	6.9 (5.5–9.9) ##	8.6 (7.9–11.6) †
Creatinine [s] (µmol/L)	28.5 (25.0–33.0)	33.0 (26.0–43.0) *	34.0 (30.0–38.0)	44.5 (37.0–48.0) #,†
Uric acid (µmol/L) [s]	69.0 (57.0–86.0)	72.5 (50.0–93.0)	70.0 (60.0–80.0)	80.0 (80.0–100.0) #,†
ALT (U/L) [s]	77.5 (62.0–104.0)	49.3 (30.2–66.8) **	57.0 (48.0–69.0)	65.5 (53.0–149.0)
ALP (U/L) [s]	299.0 (248.0–424.0)	166.0 (139.0–192.0) **	282.0 (169.0–418.0) ##	225.0 (213.0–276.0) ##
α-Amylase (U/L) [s]	2418.0 (2013.0–3603.0)	2396.5 (1576.0–2997.0)	1321.5 (742.0–2632.0) #	1048.5 (816.0–1317.0) ##
TP (g/L) [s]	59.0 (57.0–64.0)	48.8 (45.4–57.5) **	47.0 (42.0–48.0) #	49.5 (48.0–51.0) †
Albumin (g/L) [s]	25.0 (25.0–30.0)	31.4 (30.2–37.5) **	21.5 (20.0–24.0) ##	22.5 (20.0–24.0) ##
FIB (g/L) [p]	2.07 (1.6–2.4)	1.95 (1.80−2.5)	2.10 (1.8–3.1) #	3.05 (2.4–3.7) #
vWF (%) [p]	215.0 (50.0–225.0)	214.1 (136.1–259.6)	211.8 (59.7–268.1)	242.4 (232.2–270.3)

Data are expressed as median and interquartile range (IQR). C—control group; ISO—isoprenaline + saline-treated group; IP—isoprenaline + PK11195-treated group; IPLN—isoprenaline + PK11195 + L-NAME-treated group; TC—total cholesterol; HDL—high-density lipoprotein cholesterol; TG—triglycerides; Hcy—homocysteine; TP—total protein; FIB—fibrinogen; vWF—von Willebrand factor; ALT—alanine aminotransferase; ALP—alkaline phosphatase. Statistical significance: * $p < 0.05$ vs. C group; ** $p < 0.01$ vs. C group; # $p < 0.05$ vs. ISO group; ## $p < 0.01$ vs. ISO group; † $p < 0.05$ vs. IP group. s—serum, p—plasma. Statistical analysis was performed using the Kruskal–Wallis test followed by the Mann–Whitney U test.

Homocysteine levels were significantly elevated in both the IP and IPLN groups compared to the ISO group (IP = 26.99 (22.30–42.30) µmol/mL; IPLN = 33.39 (17.80–41.60) µmol/mL vs. ISO = 13.30 (12.70–14.85) µmol/mL $p < 0.01$), while no statistical difference was registered between the ISO and control groups.

There were statistically significant differences in serum urea and creatinine levels among the groups. Urea concentrations were significantly lower in the IP group compared to the ISO group (IP = 6.9 (5.5–9.9) mmol/L vs. ISO = 10.4 (9.1–11.9) mmol/L, $p < 0.01$). On the other hand, the IPLN group showed significantly higher levels of urea compared to the IP group (IPLN = 8.6 (7.9–11.6) mmol/L, $p < 0.05$). Serum creatinine levels were significantly increased in the ISO group (ISO = 33.0 (26.0–43.0) µmol/L) compared to the control group (C = 28.5 (25.0–33.0) µmol/L, $p < 0.05$). Moreover, creatinine concentrations were significantly higher in the IPLN group than in both the IP and ISO groups ($p < 0.05$). Co-administration of PK11195 and L-NAME significantly increased uric acid levels in the IPLN group compared to both the IP and ISO groups ($p < 0.05$).

Alanine aminotransferase (ALT) and alkaline phosphatase (ALP) activities were significantly reduced in the ISO group compared with the control group ($p < 0.01$). In contrast, ALP activity was significantly higher in both the IP and IPLN groups compared to the ISO group ($p < 0.01$). Serum α-amylase levels were significantly reduced in the IP group ($p < 0.05$) and further decreased in the IPLN group ($p < 0.01$) compared to the ISO group.

Total protein (TP) levels also showed significant variations among the groups. TP levels were significantly lower in the ISO group than in the control group (ISO = 48.5 (45.4–57.5) g/L vs. C = 59.0 (57.0–64.0) g/L, $p < 0.01$). In addition, TP levels were significantly reduced in the IP group compared to the ISO group ($p < 0.05$). However, the IPLN group showed substantially higher levels of TP compared to the IP group ($p < 0.05$). The serum albumin concentration was significantly lower in the control group compared to the ISO group

($p < 0.01$), while both IP and IPLN groups exhibited significantly lower albumin levels compared to the ISO group ($p < 0.01$).

Regarding hemostatic parameters, a statistically significant elevation in fibrinogen levels was observed in both the IP and IPLN groups compared to the ISO group ($p < 0.05$). At the same time, there were no significant differences in von Willebrand factor (vWF) levels between the groups.

Overall, the biochemical profile revealed significant alterations in lipid, metabolic, and hemostatic parameters across experimental groups, indicating differential responses to TSPO modulation and NOS inhibition.

2.2. Inflammatory Parameters Determination

A notable difference in pro-inflammatory cytokine levels was observed among the investigated groups (Figure 2). Serum concentrations of interleukin-1β (IL-1β) were significantly increased in the ISO group compared to the control group (ISO = 37.5 (29.2–42.5) pg/mL vs. C = 28.3 (23.3–31.7) pg/mL, $p < 0.05$). In contrast, treatment with PK11195 alone (IP group) resulted in significantly lower levels of IL-1β compared to the ISO group (IP = 20.0 (16.7–23.3) pg/mL, $p < 0.01$). The tumor necrosis factor-α (TNF-α) concentration was significantly elevated in the ISO group relative to the control group (ISO = 40.6 (33.1–49.3) pg/mL vs. C = 15.0 (9.4–19.4) pg/mL, $p < 0.05$). Administration of PK11195, either alone (IP group) or in combination with L-NAME (IPLN group) resulted in a significant reduction in TNF-α levels compared to the ISO group (IP = 10.5 (1.6–17.2) pg/mL, $p < 0.01$; IPLN = 8.3 (7.2–11.7) pg/mL, $p < 0.05$). Serum levels of interleukin-6 (IL-6) did not differ significantly between the ISO and the control group (C = 33.8 (13.9–81.6) pg/mL vs. ISO = 36.0 (21.7–52.9) pg/mL), $p > 0.05$). However, IL-6 concentrations were significantly reduced in both the IP and IPLN groups compared to the ISO group (IP = 20.8 (10.4–33.0) pg/mL, $p < 0.01$; IPLN = 13.9 (10.4–33.0) pg/mL, $p < 0.05$). Regarding anti-inflammatory cytokine levels, interleukin-10 (IL-10) levels were significantly reduced in the ISO group compared to the control group (ISO = 111.2 (70.0–155.0) pg/mL vs. C = 6327.0 (6317.0–6857.0) pg/mL, $p < 0.05$). In contrast, IL-10 levels were significantly increased in both the IP and IPLN groups relative to the ISO group (IP = 6252.0 (5957.0–6892.0) pg/mL, $p < 0.01$ vs. IPLN = 5972.0 (5422.0–6442.0) pg/mL, $p < 0.05$).

Collectively, the data reveal persistent reductions in TNF-α in the IPLN group despite NOS inhibition, as well as large quantitative differences in IL-10 across groups, which are notable and within the expected assay range.

2.3. Oxidative Stress Parameters Determination

Activities of antioxidant enzymes, including superoxide dismutase (SOD), glutathione peroxidase (GPx), total glutathione concentrations, as well as total protein S-glutathionylation in cardiac tissue homogenates from the groups were measured (Figure 3). SOD activity was significantly reduced in the ISO group relative to the control (ISO = 205.06 (174.20–497.20) U/ML vs. C = 601.12 (519.70–640.40) U/mL), $p < 0.05$). Notably, administration of PK11195 further decreased SOD activity in the IP group compared to the ISO group (IP = 70.22 (61.80–78.70) U/mL, $p < 0.05$). In marked contrast, co-administration of PK11195 and L-NAME resulted in a substantial increase in SOD activity in the IPLN group compared to both the ISO and IP groups (IPLN = 515.45 (497.20–533.70) U/mL), $p < 0.05$), restoring SOD activity to near-control levels. GPx activity was significantly reduced in both the IP and IPLN groups compared to the ISO group (IP = 273.35 ± 9.67 U/mL; IPLN = 361.37 ± 58.19 U/mL vs. ISO = 507.07 ± 22.90 U/ML, $p < 0.01$), while no significant difference was observed between the ISO group and the control ($p > 0.05$). No significant differences in total glutathione levels were observed among the investigated groups despite

observed alterations in antioxidant enzyme activities. Densitometric analysis revealed significantly elevated total protein S-glutathionylation in the ISO group compared to the control group (ISO = 4,413,910.8 ± 316,042 vs. C = 3,178,634.4 ± 250,697, $p < 0.05$). It should be noted that the relatively small sample size in the IPLN group may limit the statistical power to detect differences.

2.4. Histopathological Findings

There were statistically significant differences in the frequency of histopathological findings among the investigated groups (Table 2, Figure 4). The histopathological grades were described as follows: (a) Grade 0—no changes; (b) Grade 1—mild–focal myocyte damage or small multifocal degeneration with a slight degree of inflammatory process; (c) Grade 2—moderate–extensive myofibrillar degeneration and/or diffuse inflammatory process; and (d) Grade 3—severe–necrosis with a diffuse inflammatory process. All cardiac tissue samples from the control group exhibited Grade 0 changes, whereas the majority of samples from the ISO group (80%) showed Grade 3 lesions. No statistically significant differences in histopathological grades were observed among the remaining groups. However, the limited sample size in the IPLN group may have reduced the statistical power to detect differences.

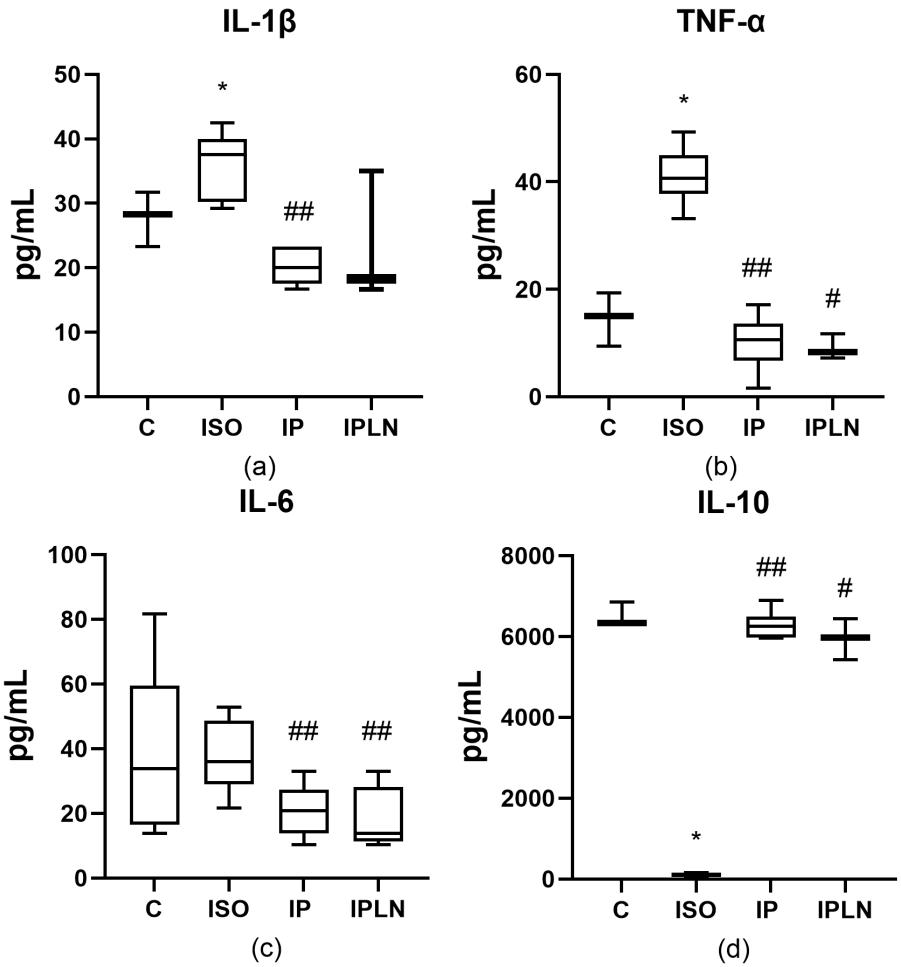


Figure 2. Inflammatory parameters. Serum levels of interleukin 1β (IL-1β pg/mL) (**a**) serum levels of tumor necrosis factor-α (TNF- α pg/mL) (**b**) serum levels of interleukin-6 (IL-6 pg/mL) (**c**) serum levels of interleukin-10 (IL-10 pg/mL) (**d**). C—control group; ISO—isoprenaline + saline-treated group; IP—isoprenaline + PK11195-treated group; IPLN—isoprenaline + PK11195 + L-NAME-treated group. * $p < 0.05$ vs. C group; # $p < 0.05$ vs. ISO group; ## $p < 0.01$ vs. ISO group. Statistical analysis was performed using the Kruskal–Wallis test followed by the Mann–Whitney U test.

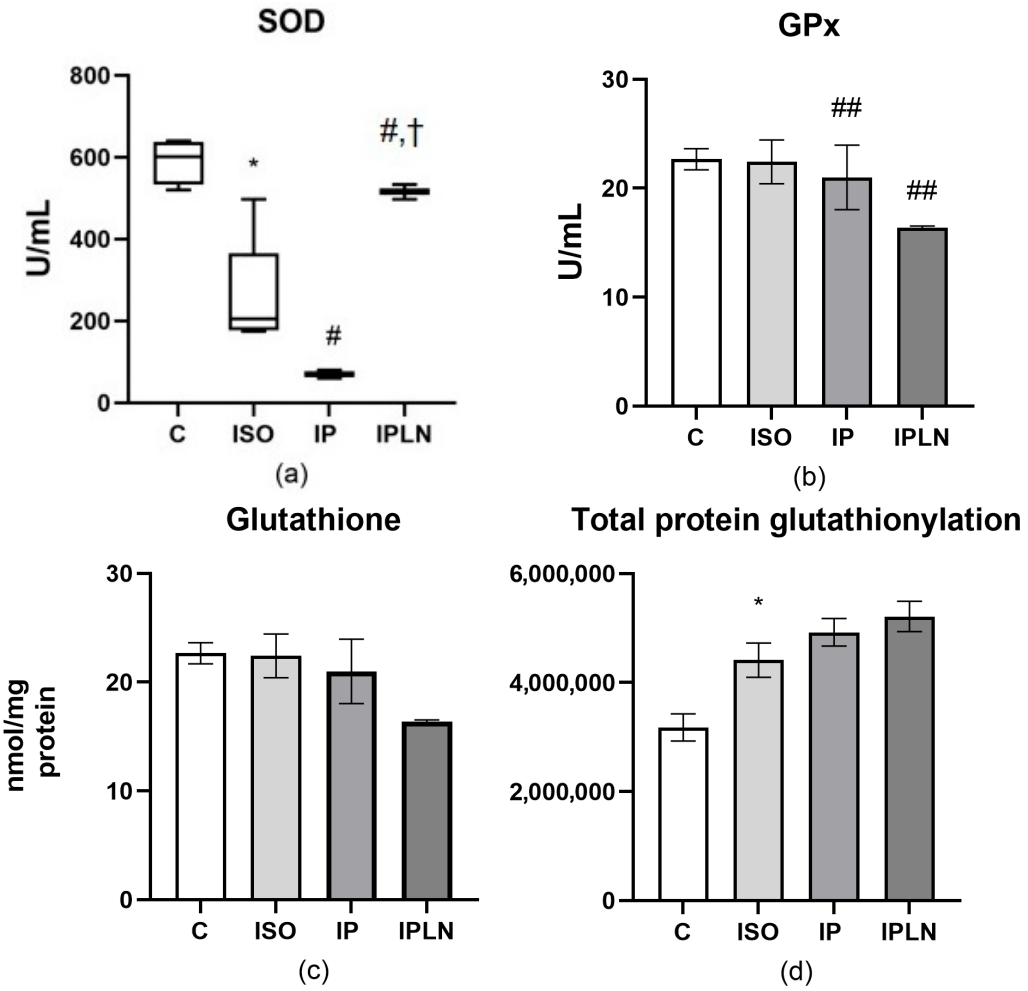


Figure 3. Oxidative stress parameters. Levels of superoxide dismutase (SOD, U/mL) (**a**) glutathione peroxidase (GPx, U/mL) (**b**) total glutathione (nmol/mg protein) (**c**) total protein glutathionylation (**d**). C—control group; ISO—isoprenaline + saline-treated group; IP—isoprenaline + PK11195-treated group; IPLN—isoprenaline + PK11195 + L-NAME-treated group. (**a**) Data are presented as box plot showing the median (horizontal line within the box), interquartile range (box boundaries), and minimum to maximum values (whiskers). (**b**–**d**) Data are expressed as mean ± standard error of the mean (SEM). * $p < 0.05$ vs. C group; # $p < 0.05$ vs. ISO group; ## $p < 0.01$ vs. ISO group; † $p < 0.05$ vs. IP group. Statistical analysis: (**a**) Kruskal–Wallis test followed by the Mann–Whitney U test; (**b**–**d**) one way ANOVA followed by Tukey's post hoc test.

Table 2. Distribution of histopathological injury grades among experimental groups.

Group	Grade 0	Grade 1	Grade 2	Grade 3	Σ
C	4 (100%)	0 (0%)	0 (0%)	0 (0%)	4 (100%)
ISO *	0 (0%)	0 (0%)	1 (20%)	4 (80%)	5 (100%)
IP	0 (0%)	2 (50%)	1 (25%)	1 (25%)	4 (100%)
IPLN	0 (0%)	0 (0%)	2 (100%)	0 (0%)	2 (100%)
Σ	4 (26.7%)	2 (13.3%)	4 (26.7%)	5 (33.3%)	15 (100%)

Data are presented as a number of animals (%). C—control group; ISO—isoprenaline + saline-treated group; IP—isoprenaline + PK11195-treated group; IPLN—isoprenaline + PK11195 + L-NAME-treated group. Histopathological grading was performed on a semiquantitative scale: Grade 0—no changes; Grade 1—mild–focal myocyte damage or small multifocal degeneration with a slight degree of inflammatory process; Grade 2—moderate–extensive myofibrillar degeneration and/or diffuse inflammatory process; Grade 3—severe–necrosis with a diffuse inflammatory process. Fisher's exact test: * $p < 0.05$ vs. C group.

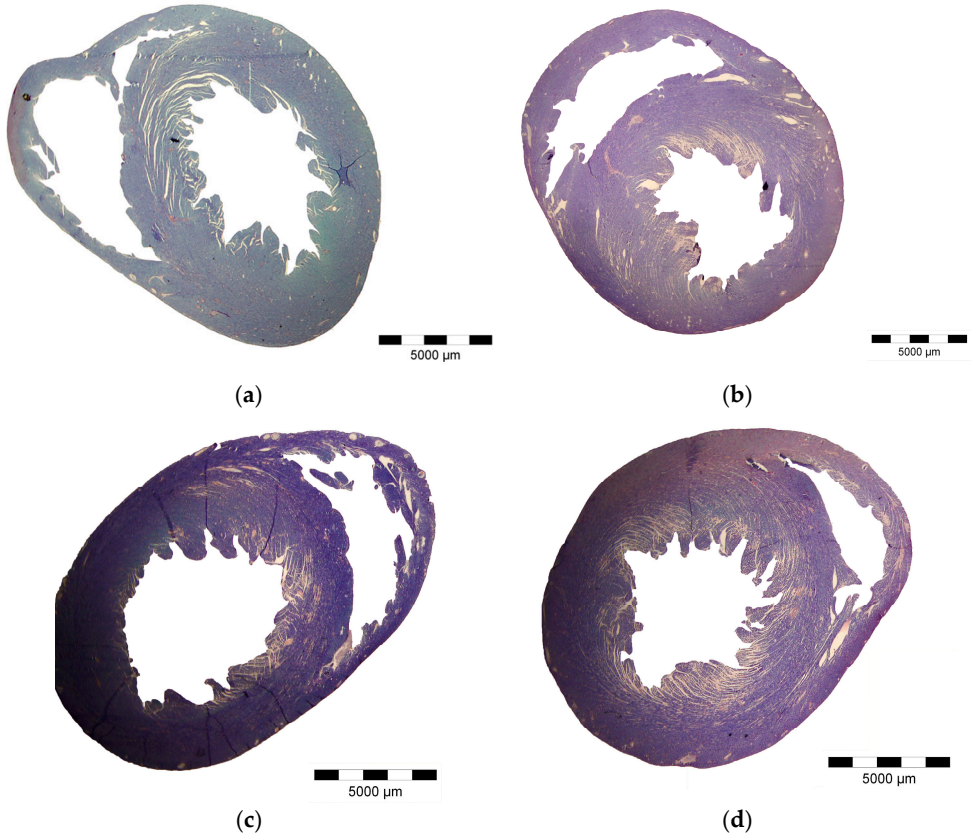


Figure 4. Representative transverse sections of rat heart tissue stained with phosphotungstic acid hematoxylin (PTAH) showing left and right ventricles across groups. (**a**), Control group—normal myocardial architecture with no pathological changes (Grade 0); (**b**) ISO group—multiple foci of MI with severe necrosis and diffuse inflammatory infiltration (Grade 3); (**c**) IP group—rare foci of myocardial infarction with multifocal myocyte degeneration with mild inflammatory process; (**d**) IPLN group—several foci of myocardial infarction are visible in both the left and right ventricles, with moderately extensive myofibrillar degeneration. ISO—isoprenaline + saline-treated group; IP—isoprenaline + PK11195-treated group; IPLN—isoprenaline + PK11195 + L-NAME-treated group.

Microscopic examination revealed no myocardial damage in the control group. The ISO group demonstrated multiple foci of MI with severe necrosis and diffuse inflammatory infiltration. In the IP group, rare foci of MI with multifocal myocyte degeneration and mild inflammatory process were observed. The IPLN group showed several foci of MI in both ventricles with moderately extensive myofibrillar degeneration.

3. Discussion

The present study investigated the effects of pharmacological modulation of the TSPO on myocardial injury induced by isoprenaline, with a particular focus on biochemical alterations, inflammatory responses, oxidative stress, and histopathological changes in cardiac tissue. To address this aim, a well-established experimental model of AMI induced by a high dose of isoprenaline administration was employed, allowing for controlled evaluation of myocardial damage and potential cardioprotective mechanisms. The isoprenaline-induced MI model has been extensively validated and is widely accepted as a reliable experimental approach for mimicking key pathophysiological features of acute myocardial injury, including catecholamine overstimulation, relative myocardial ischemia, oxidative stress, inflammation, and cardiomyocyte necrosis [14,15]. Excessive β-adrenergic stimulation caused by isoprenaline leads to increased myocardial oxygen demand, Ca^{2+} overload, mitochondrial dysfunction, and enhanced generation of ROS, thereby creating a complex

pathophysiological milieu characteristic of acute ischemic myocardial injury [16,17]. Within this framework, modulation of mitochondrial function through TSPO ligands, such as PK11195, alone or in combination with NOS inhibition, represents a relevant strategy for exploring novel mechanisms involved in myocardial injury and cardioprotection.

To further validate the applied experimental model, biochemical markers of myocardial injury (hsTnT, AST, LDH, and CK), histopathological alterations in cardiac tissue, and cardiac functional parameters assessed by ECG were analyzed. Changes in these parameters in the ISO-treated group confirmed the presence of pronounced myocardial injury, which is consistent with previous reports describing isoprenaline administration as a reliable and reproducible model of AMI in rats [15]. Collectively, these findings support the validity of the isoprenaline model of MI. This provides an appropriate experimental platform for evaluating the cardioprotective potential of TSPO modulation.

NO is synthesized from L-arginine through the enzymatic action of three NOS isoforms: neuronal (nNOS), inducible (iNOS), and endothelial (eNOS). These enzymes catalyze the oxidation of L-arginine to release NO and l-citrulline and requires NADPH and oxygen as cofactors. The availability of intracellular L-arginine is the rate-limiting step in these processes [18]. NO participates as a mediator in several physiological processes, including vasodilation, inhibition of platelet aggregation, and regulation of apoptosis, inflammation, and angiogenesis [19]. However, because NO readily reacts with superoxide, one of its multiple effects is the nitrosylation of protein thiol groups and the formation of RNS, such as peroxynitrite. Consequently, the balance between NO and superoxide determines whether NO exerts protective or detrimental effects in the cardiovascular system [20]. Pharmacological inhibition of NOS using L-NAME has been employed to elucidate the contribution of NO signaling to TSPO-mediated cardioprotection and to assess potential interactions between mitochondrial function and NO bioavailability in the setting of acute MI.

Growing evidence suggests the potential cardioprotective role for TSPO and its ligands, including a marked reduction in infarct size in ischemia/reperfusion injury, prevention of reperfusion-associated arrhythmias, and inhibition of post-infarction cardiac hypertrophy [21]. Accordingly, cardiac injury biomarkers were evaluated to determine the effects of PK11195 alone or in combination with L-NAME on the severity of isoprenaline-induced MI. The significant reduction in hsTnT levels following administration of PK11195 alone suggests a protective effect on cardiomyocyte integrity. Although PK11195 has been described as a TSPO ligand capable of promoting heart mitochondrial permeability transition in a dose-dependent manner [22], its cardioprotective action observed in the present in vivo model may involve mechanisms beyond direct induction of mPTP opening. The mechanisms may include modulation of intracellular Ca^{2+} homeostasis and inhibition of L-type Ca^{2+} channels, thereby attenuating Ca^{2+} overload-induced cardiomyocyte necrosis [23]. On the other hand, co-administration of both PK11195 and L-NAME abolished these protective effects, resulting in a marked increase in hsTnT levels, suggesting that NO availability is involved in the cardioprotective effects of PK11195. Direct interactions between PK11195 and NO signaling in the myocardium have not been thoroughly characterized. However, experimental studies on non-cardiac models show functional cross-talk between these pathways. In glioblastoma cells, PK11195 and TSPO knockdown attenuated NO donor-induced cell death by stabilizing mitochondrial membrane potential and reducing apoptosis, suggesting that TSPO participates in NO-related mechanisms of mitochondrial dysfunction [24]. As for the other cardiac injury biomarkers, AST activity was increased in the IPLN group compared to the IP group; however, these changes should be interpreted with caution due to the limited cardiac specificity of AST [25].

In the present study, isoprenaline-induced MI was associated with marked alterations in serum lipid profile, reflected by reduced TC and HDL cholesterol levels, as well as

decreased triglyceride levels. Although some studies have reported increased cholesterol and triglyceride levels following isoprenaline administration [26,27], the lipid reduction observed in this study may reflect the increased metabolic demands and altered lipid utilization in ischemic cardiomyocytes. Administration of PK11195, either alone or in combination with L-NAME, resulted in partial restoration of TC levels compared to the ISO group. However, this treatment was paradoxically associated with a further reduction in HDL cholesterol levels, with both the IP and IPLN groups exhibiting significantly lower HDL cholesterol concentrations than the ISO group. The divergent effects on TC and HDL may reflect TSPO-mediated alterations in mitochondrial cholesterol utilization or redistribution rather than enhanced lipid synthesis [28], potentially indicating a metabolically unfavorable shift that could limit the overall cardioprotective efficacy of TSPO modulation. However, the precise mechanisms underlying these changes remain to be elucidated and warrant further investigation.

Homocysteine has been widely recognized as an independent risk factor and/or biomarker for cardiovascular disease and has been associated with MI, atherosclerosis, and endothelial dysfunction. Elevated homocysteine levels promote oxidative stress through enhanced generation of ROS and impair NO bioavailability by inducing NOS uncoupling, thereby contributing to endothelial injury and vascular dysfunction [29,30]. In the present study, isoprenaline-induced MI was not associated with a statistically significant increase in plasma homocysteine levels compared to the control group, suggesting that acute isoprenaline plus saline administration did not markedly affect systemic homocysteine levels. However, pharmacological modulation with PK11195, both alone and in combination with L-NAME, resulted in a significant elevation of homocysteine concentrations compared to the ISO group.

The interplay between homocysteine and NO signaling is complex and bidirectional: NO directly inhibits methionine synthase (the enzyme responsible for homocysteine remethylation) [31,32], while hyperhomocysteinemia promotes the accumulation of asymmetric dimethylarginine (ADMA), an endogenous NOS inhibitor, thereby creating a feedback loop where elevated homocysteine further reduces NO bioavailability [33]. Although the direct interactions between TSPO modulation and homocysteine metabolism have not been previously characterized, this finding may reflect secondary effects related to altered mitochondrial function, increased oxidative stress, and disturbed NO signaling.

Urea and creatinine are routinely used biomarkers for assessing renal function and are closely linked to cardiovascular outcomes. This is particularly evident in the setting of AMI, where impaired cardiac output and hemodynamic instability may secondarily affect renal perfusion. Elevated urea and creatinine levels have been associated with worse prognosis and increased mortality following MI, reflecting the complex interplay between cardiac injury and renal dysfunction [34,35]. Isoprenaline-induced MI resulted in increased serum creatinine levels, suggesting possible transient renal function impairment secondary to acute hemodynamic alterations. PK11195 administration alone was associated with lower urea concentrations compared to the ISO group, whereas combined PK11195 and L-NAME administration led to increased urea and creatinine levels. This effect may be partially explained by NOS inhibition-related alterations in renal hemodynamics since NO plays an important role in maintaining renal blood flow and oxygenation, particularly in the medulla. Inhibition of NO synthesis has been shown to reduce renal perfusion and increase renal oxygen consumption, thereby predisposing to renal hypoxia [36]. Interestingly, in the present study, a significantly increased level of uric acid was observed in the IPLN group compared to the IP and ISO groups. In this setting, elevated uric acid in the IPLN group is more likely a consequence of impaired NO signaling and oxidative stress, which may in turn further exacerbate NO deficiency and redox imbalance [37].

Although ALT and ALP are not cardiac-specific biomarkers, alterations in their serum activities have been reported in experimental models of isoprenaline-induced myocardial injury. Experimental studies have shown that isoprenaline administration can increase ALT and ALP levels [38,39], which is interpreted as a reflection of altered cellular membrane integrity and permeability under conditions of acute cardiac damage, as well as secondary systemic effects involving other organs such as the liver and kidneys [39]. However, in our study, ALT and ALP levels were decreased in the ISO group compared to the control, while ALP was higher in the IP and IPLN groups relative to the ISO group. These divergent findings may reflect differences in the systemic stress responses and the modulatory effects of TSPO modulation and NOS inhibition on enzyme activity, rather than myocardial injury per se, and warrant further investigation.

Upon assessment of serum biochemical parameters, the ISO group exhibited a significant elevation in albumin levels compared to the control group, accompanied by a marked reduction in TP concentrations. Albumin is a well-recognized negative acute phase reactant, and reduced serum albumin levels have been consistently associated with an increased cardiovascular risk and adverse outcomes following AMI [40]. However, the relatively higher albumin levels observed in the ISO group may reflect the acute phase of isoprenaline-induced injury, before the development of a sustained inflammatory response and redistribution of plasma proteins. In contrast, the reduction in TP levels likely reflects enhanced protein catabolism and oxidative modification of plasma proteins under conditions of acute systemic stress, as previously reported in experimental studies [41]. Both the IP and IPLN groups exhibited significantly lower albumin concentrations compared to the ISO group, accompanied by a reduction in TP levels in the IP group and a partial restoration of TP levels in the IPLN group. Pharmacological modulation with PK11195, particularly in combination with NOS inhibition, appears to alter protein metabolism and plasma protein balance in a manner distinct from isoprenaline-induced injury alone.

In both the IP and IPLN groups, fibrinogen levels were significantly increased relative to the ISO group, indicating enhanced pro-inflammatory and pro-thrombotic activity. As a positive acute phase reactant, fibrinogen plays an important role in acute coronary events by promoting platelet aggregation, cross-linking, and clot formation, and elevated fibrinogen levels have been consistently associated with an increased risk of AMI [42]. On the other hand, another hemostatic parameter, vWF, did not significantly differ between the investigated groups.

Systemic and local inflammation play a central role in the initiation and progression of cardiovascular diseases, contributing to endothelial dysfunction, plaque instability, and myocardial injury. Inflammatory processes are not only involved in the development of atherosclerosis and AMI but also significantly influence the development of heart failure, recurrent pericarditis, and other cardiovascular diseases. Numerous inflammatory biomarkers have been shown to predict cardiovascular risk and adverse outcomes independently of traditional risk factors [43,44]. In this context, assessment of circulatory inflammatory mediators provides valuable insight into the systemic inflammatory response associated with myocardial injury and its potential pharmacological modulation. Comparing the ISO and the control group, we observed a significant elevation in pro-inflammatory cytokines, including IL-1β and TNF-α, accompanied by a reduction in the anti-inflammatory cytokine IL-10. This cytokine profile reflects a pronounced systemic inflammatory response induced by isoprenaline-mediated myocardial injury, consistent with previous reports describing the activation of innate immune pathways following acute myocardial damage [45,46]. Pharmacological modulation with TSPO ligand PK11195, administered either alone or in combination with L-NAME, was associated with a significant attenuation of this inflammatory response. Both treatment protocols were associated with a significant reduction

in pro-inflammatory cytokines (IL-1β, TNF-α, and IL-6), along with a marked increase in IL-10 concentrations relative to the ISO group. Given the established role of excessive cytokine signaling in myocardial remodeling, cardiomyocyte apoptosis, and progression toward heart failure [47], the observed shift toward an anti-inflammatory cytokine profile may represent a relevant cardioprotective mechanism of TSPO modulation. Notably, this favorable cytokine response occurred despite persistent elevation in fibrinogen levels (as discussed above), suggesting that different components of the acute phase response may be regulated through distinct, partially independent pathways. Studies indicate that TSPO is directly involved in the regulation of inflammatory responses, particularly under a pro-inflammatory phenotype. Increased TSPO expression has been observed in immune cells exposed to pro-inflammatory cytokines such as IL-1β and IFN-γ. Moreover, TSPO ligands have been shown to modulate cytokine secretion profiles through mechanisms involving mitochondrial ROS production and NF-κB signaling pathways [24]. In line with these findings, experimental evidence suggests that PK11195 attenuates LPS-induced inflammatory activation by reducing the production of TNF-α, as well as Ca^{2+} influx [48]. Although these observations originate predominantly from neuroinflammatory and immune cell models, they support the concept that TSPO modulation exerts broad immunoregulatory effects including conditions of myocardial injury. In conclusion, our findings suggest that TSPO modulation, either alone or in combination with NOS inhibition, effectively attenuates the systemic inflammatory response associated with isoprenaline-induced MI.

Oxidative stress, defined as an imbalance in the production of free radicals and antioxidants that favors the production of free radicals, plays a significant role in the development of cardiovascular diseases [49,50]. Acute myocardial ischemia leads to oxygen deprivation of cardiac tissue, resulting in mitochondrial dysfunction and excessive generation of ROS, predominantly at complexes I and III of the mitochondrial electron transport chain [51]. These ROS induce oxidative damage to lipids, proteins, and nucleic acids, thereby contributing to cardiomyocyte injury and cell death while activating inflammatory and apoptotic signaling pathways [3]. Under physiological conditions, mitochondrial ROS are tightly controlled by antioxidant defense systems. Superoxide anion, generated during oxidative phosphorylation, is rapidly converted to hydrogen peroxide (H_2O_2) by SOD, highlighting the critical role of this enzyme in mitochondrial redox homeostasis. H_2O_2 is subsequently neutralized by GPx, catalase, and other antioxidant enzymes. However, when the antioxidant capacity is overwhelmed, H_2O_2 can give rise to highly reactive species such as hydroxyl radicals, amplifying oxidative injury [51,52]. While disruption of antioxidant defenses is a recognized feature of isoprenaline-induced myocardial injury [53,54], in the present study, this was reflected by reduced SOD activity, whereas GPx activity remained unchanged in the ISO group compared to the control. On the other hand, administration of PK11195 resulted in a significant reduction in GPx activity in the IP group while SOD activity was further decreased compared to the ISO group. Interestingly, previous studies have shown that PK11195 can exert cardioprotective effects under certain specific conditions. Exposure of isolated cardiomyocytes to PK11195 only during the reperfusion was reported to reduce ROS production, prevent ROS-induced ROS release (RIRR), and preserve mitochondrial Ca^{2+} handling, limiting cell death. These effects involve modulation of the mPTP and possibly inhibition of the c subunit of the ATP synthase complex [11]. Thus, the cardioprotective potential of PK11195 may depend on the timing of administration and the specific cellular context, particularly under conditions of severe mitochondrial dysfunction and oxidative stress. Additionally, densitometric analysis revealed a significantly elevated total protein S-glutathionylation in the ISO group compared to the control. S-glutathionylation is a reversible post-translational modification in which a disulfide bond is formed between protein cysteine residues and glutathione. This modification serves a dual role: it protects

functionally important protein thiols from irreversible oxidation under oxidative stress conditions while also regulating key physiological processes in the myocardium, including myocyte contraction, oxidative phosphorylation, and Ca handling [55,56]. The observed elevation in protein S-glutathionylation in the ISO group indicates enhanced oxidative protein modification that may contribute to isoprenaline-induced cardiac dysfunction.

Several other TSPO ligands have been reported to limit myocardial oxidative stress in cellular and animal models [21]. Similarly, our previous study demonstrated a related cardioprotective effect of 4′-Chlorodiazepam (4′-ClDzp), suggesting that TSPO modulation can reduce oxidative damage of the myocardium [57]. Critically, co-administration of PK11195 and L-NAME resulted in a marked increase in the SOD activity in the IPLN group, demonstrating NO-dependent regulation of this key antioxidant enzyme. Under oxidative stress conditions, NO rapidly reacts with superoxide anion, leading to the formation of peroxynitrite, a redox mediator implicated in oxidative tissue damage. Importantly, the magnitude of peroxynitrite formation is dependent on the relative levels of NO and superoxide anion [58]. Inhibition of NO synthesis by L-NAME may therefore limit peroxynitrite generation, shifting the redox balance toward enhanced superoxide anion detoxification by SOD. In contrast, the observed reduction in GPx activity in the IPLN group may reflect altered downstream redox signaling under conditions of modified NO-superoxide anion interplay. Importantly, despite SOD restoration, the IPLN group exhibited marker elevation of troponin levels and loss of cardioprotection, underscoring that NO availability is essential for PK11195-mediated cardioprotective effects through mechanisms extending beyond antioxidant enzyme modulation alone. However, the exact mechanisms underlying these observations remain to be clarified.

These findings reveal a complex interplay between TSPO modulation and NO signaling. First, PK11195 reduces antioxidant enzyme activities yet provides cardioprotection through mitochondrial mechanisms. Second, L-NAME co-administration restores SOD activity by limiting peroxynitrite formation but abolishes cardioprotection by reducing NO bioavailability. Third, the cardioprotective efficacy of TSPO modulation is critically dependent on intact NO signaling, independent of antioxidant enzyme status.

Histopathological evaluation of myocardial tissue was performed to assess the presence and severity of myocardial injury using a semi-quantitative grading system, in accordance with previously published criteria [59]. In the ISO group, severe myocardial damage was predominantly observed, with the majority of samples exhibiting Grade 3 lesions characterized by severe necrosis and diffuse inflammatory infiltration, confirming the successful induction of myocardial injury when compared with the control group. These findings are in line with previous reports describing extensive necrosis and inflammatory infiltration in experimental models of isoprenaline-induced MI [60,61].

In the groups receiving PK11195 alone or in combination with L-NAME, myocardial tissue exhibited variable degrees of structural damage; however, no statistically significant differences in histopathological grades were observed compared to the ISO group. This suggests that, under the present experimental conditions, PK11195 administration, either alone or combined with L-NAME, was insufficient to induce robust structural myocardial protection detectable by conventional histopathological scoring despite the observed biochemical and inflammatory modulation.

Several limitations of the present study should be acknowledged. First, our investigation employed a single dose of PK11195, and dose–response relationships were not explored. It remains uncertain whether higher or lower doses might yield more favorable cardioprotective outcomes or mitigate some of the observed adverse metabolic effects, such as elevated homocysteine and reduced HDL cholesterol levels. Second, our study assessed myocardial injury at a single time point following isoprenaline administration. Although

this approach allowed for the characterization of acute biochemical and inflammatory responses, it does not capture the temporal dynamics of myocardial remodeling, functional recovery, or long-term outcomes. Third, histopathological assessment was based on a semi-quantitative grading system, which may lack sensitivity in detecting subtle structural changes or variations in myocardial damage. Moreover, the timing of tissue sampling may precede the development of detectable structural remodeling. Alternatively, PK-11195-mediated cardioprotection may primarily operate through functional preservation rather than preventing structural damage under present experimental conditions. Finally, while this study provides evidence for both cardioprotective and potentially detrimental effects of TSPO modulation, further research is needed to fully understand the underlying mechanisms and identify strategies to optimize therapeutic outcomes in IHD.

To the best of our knowledge, this is the first study to investigate the effects of TSPO modulation by PK11195 in combination with NOS inhibition in an isoprenaline-induced MI model. Our findings provide novel insights into the interplay between mitochondrial function, inflammatory signaling, and NO-dependent pathways. Importantly, TSPO may represent a multifaceted therapeutic target in acute MI, which could inform future preclinical and clinical studies.

4. Materials and Methods

4.1. Animal Ethics Statement

The study was approved by the Ethical Council for the Welfare of Experimental Animals, Ministry of Agriculture, Forestry and Water Management, Veterinary Directorate, Republic of Serbia (number: 323-07-00412/2020-05; date: 22 January 2020). All experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals, European Directive for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (86/609/EEC) and ethics principles [62].

4.2. Experimental Animals

This study was conducted using adult male Wistar albino rats (200–250 g body weight, 6–8 weeks of age, total number: 32) obtained from accredited vivarium of Military Medical Academy (Belgrade, Serbia). Only male rats were used to minimize biological variability related to hormonal fluctuations during the estrous cycle. The animals were housed paired in transparent Plexiglas cages with wood-chip bedding, with free access to standard laboratory chow and water ad libitum. Ambient conditions were maintained at a temperature of 21 $\pm$ 2 °C, a relative humidity of 55 $\pm$ 5%, and a 12/12 h light–dark cycle with the light period beginning at 07:30 a.m.

4.3. Experimental Design

Male Wistar albino rats were randomly divided into four groups (eight animals in each group): 1. control group C (0.2 mL of 0.9% NaCl (saline) solution subcutaneously (sc.) twice with an interval of 24 h), 2. experimental group ISO (85 mg/kg b.w. ISO in 1 mL saline solution sc. twice with an interval of 24 h plus 0.5 mL of saline intraperitoneally (ip.)), 3. experimental group IP (85 mg/kg b.w. ISO in 1 mL of saline solution sc. twice with an interval of 24 h plus 5 mg/kg b.w. PK-11195 in saline ip.), 4. experimental group IPLN (85 mg/kg b.w. ISO in 1 mL of saline solution sc. twice with an interval of 24 h plus 5 mg/kg b.w. PK-11195 in saline ip. plus 50 mg/ kg b.w. L-NAME in saline ip.). The dose of PK11195 was selected based on previous in vivo studies demonstrating cardioprotective and antiarrhythmic effects of TSPO ligands in experimental models of cardiac ischemia–reperfusion injury, with reported effective doses ranging from 5 to 25 mg/kg [11,63].

4.4. Induction of Myocardial Injury in Rats

Myocardial injury or infarction (MI) in experimental animals was induced by subcutaneous administration of isoprenaline in the dorsal region at a dose of 85 mg/kg, administered twice with a 24 h interval over two consecutive days. The experimental model of MI was assessed based on the temporal dynamics of serum myocardial ischemia biomarkers, the presence of electrocardiographic (ECG) signs of MI, and histopathological examination of cardiac tissue following animal sacrifice. Serum levels of ischemic markers (hsTnT, AST, LDH, and CK) were determined from blood samples collected from the rat tail vein on the 0th and 2nd day of the experiment. ECG recordings were obtained using standard ECG leads (i.e., ST segment elevation (>1 mm) or T wave inversion) on the 0th and 2nd days, and cardiac histopathology analysis was performed.

Sedation for ECG recording and blood sampling was achieved with 2.5 mg/kg b.w. acepromazine + 0.01 mL of 10% ketamine hydrochloride (100 mg/mL) at the beginning of the experiment and on day 2, while terminal anesthesia was performed with ketamine hydrochloride (50 mg/kg b.w.), followed by guillotine sacrifice and collection of blood and cardiac tissue for further analysis.

4.5. Biomarkers of Myocardial Injury and Other Biochemical Parameters Determination

Serum concentrations of high-sensitivity cardiac troponin T (hs-cTnT) were determined using a highly sensitive Roche Cobas e601 automated analyzer (Roche Diagnostics, Mannheim, Germany). Additional cardiac biomarkers, including aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and creatine kinase (CK), along with serum lipid profile parameters (total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and triglycerides (TG)), renal function markers (urea, creatinine), hepatic enzymes (alanine aminotransferase (ALT), alkaline phosphatase (ALP)), pancreatic markers (α-amylase (α-AMY)), as well as total protein and albumin levels, were measured using spectrophotometry commercial kits (Siemens Healthcare Diagnostics Inc., Newark, NJ, USA) and an automatic analyzer (Dimension Xpand, Siemens, Erlangen, Germany). Plasma fibrinogen concentration was measured using the modified Clauss assay (Siemens Healthineers Headquarters, Erlangen, Germany), while von Willebrand factor (vWF) activity was determined by the INNOVANCE® VWF Ac particle-enhanced, using a BCS XP analyzer (Siemens Healthineers, Erlangen, Germany).

4.6. Inflammatory Parameters Determination

Serum levels of inflammatory cytokines, including tumor necrosis factor α (TNF-α), interleukin (IL-1β), interleukin-6 (IL-6), and interleukin-10 (IL-10), were measured using commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Blood samples were collected from the rat tail vein immediately prior to euthanasia, and samples were stored at −70 °C until analysis. Cytokine concentrations were determined based on standard curves prepared from known concentrations of recombinant cytokines provided with the kits.

4.7. Oxidative Stress Parameter Determination and Cardiac Tissue Preparation

Serum homocysteine was measured by competitive immunoassays using direct, chemiluminescent technology and an ADVIA Centaur XP system (Siemens Healthcare Diagnostics, Tarrytown, New York, NY, USA). Serum levels of uric acid (UA) were determined spectrophotometrically using commercial kits (Siemens Healthcare Diagnostics Ltd., Frimley, Camberley, UK) and an automatic biochemical analyzer (Dimension Xpand, Siemens, Washington, DC, USA, USA).

After isolation, hearts were rinsed in 0.9% NaCl and gently dried on filter paper. Cardiac tissue was homogenized in 50 mmol/L RIPA (Radio-Immunoprecipitation Assay) buffer (pH 7.4) containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged at 14,000 rpm for 30 min at 4 C. The resulting supernatant was collected and stored at −80 °C until analysis. Cytosolic superoxide dismutase (Cu, Zn SOD) activity was determined spectrophotometrically [64,65] based on the ability of SOD to inhibit autooxidation of epinephrine at alkaline pH. One unit of SOD activity was defined as the amount of enzyme that inhibits the oxidation of epinephrine by 50%. Glutathione peroxidase (GPx) activity was measured using the coupled assay procedure [66], with one unit of enzyme activity expressed as nmol NADPH oxidized per minute, assuming a molar absorbance of NADPH at 340 nm of 6.22×10^3/L/mol/cm. Total glutathione (GSH) was determined spectrophotometrically and expressed as nanomoles per milligram of protein [67]. Protein concentration was determined using a bicinchoninic acid protein assay kit (BCA-1) (Sigma-Aldrich).

Total protein S-glutathionylation was assessed by Western blot analysis. Cardiac tissue homogenates were prepared in RIPA(buffer (50 mM TRIS-HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 50 mM NaF, 150 mM NaCl, 1 mM PMSF, 0.2 mM sodium orthovanadate) supplemented with a protease inhibitor cocktail and N-ethylmaleimide (NEM), followed by centrifugation at 10,000× *g* at 4 °C. Protein samples (30 µg) were denatured in 2× Laemmli buffer at 95 °C for 5 min and separated on 4–15% Criterion™ TGX precast gels (Bio-Rad, Hercules, CA, USA) at 150 V (4 °C). Proteins were transferred onto nitrocellulose membranes using a Bio-Rad Criterion™ transfer system (100 V, 4 °C). Membranes were incubated with a mouse monoclonal anti-glutathione antibody (1:500; Sigma-Aldrich, USA), followed by goat anti-mouse secondary antibody (1:8000; Abcam, Cambridge, UK). Protein bands were visualized using Clarity™ Western ECL substrate (Bio-Rad, USA) and a ChemiDoc™ MP Imaging System (Bio-Rad, USA), and densitometric analysis was performed using ImageLab 5.1 software (Bio-Rad, USA).

4.8. Histopathological Analysis

Cardiac tissue was used for histopathological analysis. After proper orientation, the hearts were transversely sectioned into 3 mm-thick slices and fixed by immersion in 4% neutral buffered formaldehyde for 24 h. The samples were subsequently dehydrated through increasing concentrations of alcohol, cleared in xylene, and embedded in paraplast using a tissue embedding system (Tissue Tech II Tissue Embedding Center). Paraffin blocks were carefully trimmed, and serial sections with a thickness of 5 µm were obtained using a microtome (Leica Reinhart Austria and Leica SM 2000 R, Heidelberg, Germany). Sectioning was continued until the entire myocardial wall thickness was visualized. The sections were stained with hematoxylin–eosin (H&E) and phosphotungstic acid hematoxylin (PTAH). All histological slides were examined under a light microscope (Olympus BX41, Tokyo, Japan) equipped with an Olympus C5060-ADU "wide zoom" digital camera (Olympus C-5060-ADU, Tokyo, Japan). Histopathological evaluation was performed by a pathologist blinded to the experimental groups. Histological findings were graded to establish a myocardial injury scoring system as follows: (0) no histological changes; (1) mild focal myocyte damage or small multifocal degeneration with minimal inflammatory infiltration; (2) moderate to extensive myofibrillar degeneration and/or diffuse inflammatory process; (3) severe myocardial necrosis accompanied by a diffuse inflammatory response [68].

4.9. Drugs

During the experimental phase of the study, the following drugs were used: isoproterenol hydrochloride, PK-11195, Nω-Nitro-L-arginine methyl ester hydrochloride

(L-NAME), all obtained from Sigma-Aldrich Chemie GmbH, Germany. Acepromazine (Neurotranq^R was obtained from Alfasan International B.V., JA (Woerden, The Netherlands), and ketamine hydrochloride (Ketamidor^R) was obtained from Richter Pharma AG, Wels, Austria.

4.10. Statistical Analysis

Statistical analyses were performed using the SPSS 19.0 software package for Windows. A p-value < 0.05 was considered statistically significant. Data are presented as mean ± standard deviation (SD) or as median with interquartile range (IQR), as appropriate. Normality of data distribution was assessed using the Shapiro–Wilk test. For normally distributed data, one-way analysis of variance (ANOVA), followed by the Tukey post hoc test was applied. For non-normally distributed data, the Kruskal–Wallis test followed by Mann–Whitney U test was used. Fisher's exact test was applied for categorical data. Based on the study design, post hoc comparisons were selectively conducted between the ISO and C groups, IP and ISO groups, IPLN and ISO groups, and between the IPLN and IP groups.

5. Conclusions

The present study demonstrated that pharmacological modulation of the TSPO significantly influences myocardial injury-associated biochemical, inflammatory, and oxidative stress responses in an experimental model of isoprenaline-induced MI. Administration of the TSPO ligand PK11195 attenuated myocardial damage, evidenced by reduced serum hsTnT levels, decreased pro-inflammatory cytokine production, and modulation of oxidative stress parameters. These findings indicate a potential cardioprotective role of TSPO targeting under conditions of acute catecholamine-induced cardiac injury. However, TSPO modulation was also accompanied by heterogeneous effects on cardiometabolic and hemostatic markers, including increased fibrinogen and homocysteine levels. This suggests that TSPO modulation differentially affects inflammatory, redox, and metabolic pathways. The concomitant inhibition of NOS partially abolished these beneficial effects, highlighting the importance of NO bioavailability in TSPO-mediated cardioprotection. In conclusion, our findings suggest that TSPO modulation exerts integrated mitochondrial, redox, and immunomodulatory effects in the injured myocardium, while also revealing complex interactions between TSPO signaling and NO-dependent pathways. Further studies are warranted to elucidate the precise molecular mechanisms underlying TSPO-NO crosstalk and to explore the therapeutic potential of TSPO modulation in myocardial ischemic injury.

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