RESEARCH PAPER

In-Vitro Evaluation of the Anti-Inflammatory Potential of Empagliflozin Via Nf-Kb Pathway Inhibition

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Received: 17th Mar, 2026 | Revised: 29th Mar, 2026 | Accepted: 19th Apr, 2026 | Available Online: 5th May, 2026

ABSTRACT

Background: Empagliflozin, a sodium-glucose cotransporter-2 (SGLT2) inhibitor, exhibits profound cardioprotective and renoprotective benefits that extend beyond its primary role in glycemic control. Emerging evidence suggests these pleiotropic effects are driven by intrinsic immune-modulating properties. This study aimed to systematically evaluate the in vitro anti-inflammatory efficacy and the underlying molecular mechanisms of empagliflozin in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages.

Methods: Macrophages were pre-treated with safe, non-cytotoxic concentrations of empagliflozin (10, 25, and 50 μM), determined via CCK-8 assay, prior to stimulation with LPS (1 μg/mL). Extracellular nitric oxide (NO) production was quantified using the Griess reagent, and the secretion of primary pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β) was measured via highly sensitive enzyme-linked immunosorbent assays (ELISA). Compartmentalized protein extraction and Western blotting were executed to evaluate the modulation of the nuclear factor-kappa B (NF-κB) signaling axis.

Results: Empagliflozin maintained macrophage viability (>96%) at all tested doses. Upon LPS stimulation, empagliflozin dose-dependently and significantly ($p < 0.001$) attenuated the hyper-secretion of NO, TNF-α, IL-6, and IL-1β. Mechanistic evaluations revealed that empagliflozin robustly rescued cytosolic IκBα from degradation and simultaneously suppressed the phosphorylation of the p65 subunit (p-p65). Consequently, the subsequent nuclear translocation of p65 was dramatically abrogated.

Conclusion: Empagliflozin exerts potent, direct anti-inflammatory effects by systematically stabilizing IκBα and paralyzing the NF-κB signaling cascade. These findings provide critical mechanistic insights into its pleiotropic properties, further substantiating its therapeutic utility in mitigating chronic inflammation associated with severe cardio-metabolic disorders.

Keywords: Empagliflozin; Sglt2 Inhibitor; Inflammation; Nf-Kb Pathway; Macrophages; Cytokines.

How to cite this article: Raj P, Karandikar P, Kadam N N, Jha A K, Lone A A, Supriya, Padhi D K, Joel Mart E., In-Vitro Evaluation of the Anti-Inflammatory Potential of Empagliflozin Via Nf-Kb Pathway Inhibition. Int J Drug Deliv Technol. 2026;16(43s): 780-788; Doi: 10.25258/Ijddt.16.43s.79

1. Introduction

Inflammation is a fundamental biological defense mechanism deployed by the innate immune system to eradicate pathogens and repair tissue injury; however, when this acute response fails to resolve, it culminates in chronic, low-grade inflammation, serving as a primary pathogenic driver for severe metabolic and cardiovascular diseases, including heart failure and type 2 diabetes (Manabe, 2011). Central to the propagation and maintenance of this chronic inflammatory state is the nuclear factor-kappa B (NF-κB) signaling pathway (Tak & Firestein, 2001). Under physiological resting conditions, NF-κB dimers, predominantly the p50 and p65 (RelA) subunits, are sequestered in the cytoplasm by inhibitory IκB proteins (Tak & Firestein, 2001). Upon encountering cellular stressors or pathogen-associated molecular patterns, such as the binding of lipopolysaccharide (LPS) to Toll-like receptors (TLRs), an intracellular signaling cascade is initiated that rigorously activates the IκB kinase (IKK) complex (Chen et al., 2017). The activated IKK complex then phosphorylates IκB, targeting it for rapid proteasomal degradation and thereby liberating the NF-κB complex to undergo nuclear translocation (Chen et al., 2017). Once within the nucleus, NF-κB binds to specific DNA elements to actively trigger the robust transcription of a broad array of pro-inflammatory cytokines, including interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α), alongside critical downstream inflammatory mediators such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) (Tak & Firestein, 2001). Due to its profound role in tissue-damaging immune responses, modulating the NF-κB pathway is considered a vital therapeutic strategy. In recent years, empagliflozin, a highly selective sodium-glucose cotransporter-2 (SGLT2) inhibitor initially developed for glycemic control, has emerged as a groundbreaking pharmacological agent with properties expanding well beyond its primary indication (Vallon & Verma, 2021). By selectively inhibiting renal glucose reabsorption in the proximal convoluted tubule, empagliflozin promotes glycosuria; however, extensive clinical outcome trials have unequivocally demonstrated that its therapeutic advantages extend significantly beyond glucose lowering (Mashayekhi et al., 2024; Vallon & Verma, 2021). Empagliflozin exerts profound pleiotropic effects, conferring robust cardio-renal protection characterized by marked reductions in cardiovascular mortality, heart failure exacerbations, and the progression of chronic kidney disease (Vallon & Verma, 2021). Emerging mechanistic evidence strongly suggests that these cardioprotective and renoprotective benefits are largely independent of HbA1c reduction and are instead heavily reliant on the drug's intrinsic anti-inflammatory and anti-fibrotic properties (Mashayekhi et al., 2024; Rolski & Mączewski, 2025). Despite a wealth of systemic clinical data underscoring these protective benefits, the precise intracellular molecular mechanisms governing empagliflozin's immune-modulating effects particularly its direct influence on macrophage behavior and the NF-κB pathway remain an area requiring detailed investigation. Macrophages act as the principal orchestrators of metabolic inflammation, and recent studies have established that SGLT2 inhibitors can directly suppress the inflammatory activation of tissue macrophages, actively preventing their polarization into the classically activated, pro-inflammatory M1 phenotype (Rykova et al., 2025). Specifically, empagliflozin has been shown to mitigate severe inflammatory immune responses during pathogenic infections by decreasing the intracellular expression of IL-1β, IL-6, and iNOS in macrophage models (Constantinesco et al., 2023). Furthermore, while the NLRP3 inflammasome acts in concert with NF-κB to process and secrete mature IL-1β, empagliflozin exhibits a remarkable capacity to inhibit these intersecting inflammatory networks, thus curtailing atherosclerotic and cardiovascular pathogenesis (Yang et al., 2022). Previous investigations have also confirmed that empagliflozin effectively attenuates the production of prostaglandin E2 (PGE2) and suppresses the mRNA expression of COX-2 in LPS-stimulated RAW 264.7 macrophages by directly downregulating the IKK/NF-κB signaling axis (Lee et al., 2021). Additional in vivo and in vitro evidence supports this mechanism, demonstrating that empagliflozin reduces nuclear translocation of NF-κB and inhibits downstream neuro-inflammation and cardiomyocyte pyroptosis (Kim et al., 2024; Lv et al., 2024). Consequently, recognizing the critical research gap between empagliflozin's recognized systemic clinical benefits and its exact cellular anti-inflammatory dynamics, there is a compelling

scientific rationale to systematically isolate its effects at the molecular level. Therefore, a comprehensive evaluation of the *in vitro* anti-inflammatory activity of empagliflozin utilizing an LPS-induced macrophage model is highly warranted to definitively map its inhibitory impact on the NF-κB signaling cascade, thereby solidifying its status as a multifaceted cardio-metabolic therapeutic.

2. Materials and Methods

2.1 Chemicals and Reagents:

High-purity Empagliflozin (>98%) was procured from Century Pharmaceuticals Ltd. (Vadodara, Gujarat, India) for all *in vitro* cellular assays. Lipopolysaccharide (LPS) utilized for macrophage stimulation was formally obtained from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). Validated quantitative enzyme-linked immunosorbent assay (ELISA) kits required for cytokine detection were supplied by Krishgen Biosystems (Mumbai, India). Additionally, all analytical-grade primary and secondary antibodies necessary for Western blotting were sourced through DSS Takara Bio India Pvt. Ltd. (New Delhi, India).

2.2 Cell Culture and Maintenance

The accurate evaluation of anti-inflammatory pharmacological agents necessitates a highly stable, reproducible, and robust *in vitro* cellular model. In this experimental framework, the RAW 264.7 murine macrophage cell line is utilized due to its well-established pathophysiological relevance and extensive validation in both immunological and metabolic research. Derived from an Abelson murine leukemia virus-induced tumor, RAW 264.7 cells exhibit profound phenotypic and functional similarities to primary tissue macrophages, particularly in their capacity to robustly activate the nuclear factor-kappa B (NF-κB) signaling pathway and secrete copious amounts of pro-inflammatory cytokines upon stimulation with pathogenic agonists such as lipopolysaccharide (LPS). The cells are strictly maintained in high-glucose Dulbecco's Modified Eagle Medium (DMEM), which provides a biochemically optimized microenvironment rich in essential amino acids, vitamins, and inorganic salts vital for rigorous cellular metabolism and proliferation. To support optimal logarithmic growth and cellular adherence, the DMEM is uniformly supplemented with 10% (v/v) heat-inactivated Fetal Bovine Serum (FBS), supplying indispensable exogenous growth factors, carrier proteins, and vital trace elements. Furthermore, to rigorously preclude opportunistic microbial contamination without interfering with baseline macrophage functionality, the culture medium is fortified with a standardized dual-antibiotic admixture comprising 100 U/mL penicillin and 100 µg/mL streptomycin. The RAW 264.7 cells are cultivated in sterile, tissue-culture treated polystyrene flasks and maintained in a specialized humidified incubator at an exact temperature of 37°C under an atmospheric condition of 5% CO2 and 95% air, ensuring that the physiological bicarbonate buffering system of the DMEM sustains the ambient pH at precisely 7.2 to 7.4. The cell cultures are meticulously monitored utilizing inverted phase-contrast microscopy to evaluate cellular morphology, adherence characteristics, and overall confluency. Upon reaching approximately 70% to 80% confluency, the macrophages are gently harvested utilizing a sterile cell scraper deliberately avoiding the use of enzymatic dissociation agents such as trypsin, which can profoundly alter macrophage surface receptor integrity and subsequent mechanotransduction cascades and subcultured at defined split ratios to guarantee uninterrupted exponential growth prior to pharmacological intervention (Constantinesco et al., 2023; Lee et al., 2021).

2.3 Cell Viability Assay (CCK-8)

Prior to assessing the anti-inflammatory efficacy of empagliflozin, it is a fundamental methodological requisite to definitively distinguish true pharmacological target-pathway inhibition from non-specific cytotoxicity. Consequently, the quantitative evaluation of empagliflozin's impact on RAW 264.7 macrophage viability is conducted utilizing the highly sensitive Cell Counting Kit-8 (CCK-8) assay, a sophisticated and superior methodological evolution of the classic MTT assay. The CCK-8 assay is predicated on the biochemical reduction of a highly water-soluble tetrazolium salt, WST-8, by endogenous cellular mitochondrial dehydrogenases. RAW 264.7 cells are carefully seeded into 96-well microtiter plates at a precisely calibrated density and incubated overnight to allow for complete cellular attachment and metabolic equilibration. Subsequently, the macrophages are exposed to a carefully titrated gradient of empagliflozin concentrations alongside an appropriate vehicle control for a predetermined

incubation period of 24 to 48 hours. Following the pharmacological exposure, the CCK-8 reagent is directly introduced into the culture medium. In viable, metabolically active cells, mitochondrial dehydrogenases cleave the tetrazolium ring of WST-8, generating a highly water-soluble orange formazan dye that is uniformly secreted into the extracellular medium. This critical biochemical reaction is directly proportional to the aggregate number of living cells, offering a significant advantage over traditional MTT assays by eliminating the necessity for cytotoxic organic solubilization solvents like dimethyl sulfoxide (DMSO). The optical density (OD) of the resulting formazan solution is subsequently quantified using a high-throughput microplate spectrophotometer at an absorbance wavelength of 450 nm. By systematically calculating the percentage of viable macrophages in the empagliflozin-treated groups relative to the untreated control group, a precise cellular cytotoxicity profile is constructed. This rigorous viability screening is an absolute prerequisite for high-impact research, as it definitively establishes the maximum non-toxic concentration of empagliflozin, ensuring that subsequent observations of suppressed NF-κB nuclear translocation and diminished cytokine secretion are strictly attributed to specific anti-inflammatory pathway inhibition rather than a generalized suppression of translational machinery due to compromised cell survival (Constantinesco et al., 2023; Lee et al., 2021).

2.4 Evaluation of Pro-Inflammatory Mediators

2.4.1 Nitric Oxide (NO) Production via Griess Assay

To quantitatively assess the extracellular release of nitric oxide (NO) a critical physiological hallmark of pro-inflammatory macrophage activation the colorimetric Griess reagent assay is systematically employed. RAW 264.7 macrophages are pre-treated with established, non-cytotoxic concentrations of empagliflozin for 1 hour, followed by stimulation with lipopolysaccharide (LPS, 1 μg/mL) for an additional 24 hours. Following the incubation period, 100 μL of cell-free culture supernatant is carefully aspirated from each well, transferred to a pristine 96-well microplate, and combined with an equal volume (100 μL) of Griess reagent, which comprises 1% sulfanilamide in 5% phosphoric acid and 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride in distilled water. The microplate is then incubated in the dark at room temperature for precisely 10 to 15 minutes to facilitate the diazotization reaction, resulting in the formation of a stable, magenta-colored azo dye. The optical density is immediately measured at a wavelength of 540 nm using a high-throughput microplate spectrophotometer. The absolute concentration of nitrite (a stable aqueous decomposition product of NO) in the experimental samples is precisely calculated by interpolating the recorded absorbance values against a standard calibration curve generated from serial dilutions of a reference sodium nitrite ($NaNO_2$) solution (Lee et al., 2021).

2.4.2 Cytokine Quantification via ELISA

The secretory levels of primary pro-inflammatory cytokines, specifically tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and interleukin-1 beta (IL-1β), are quantitatively evaluated using highly sensitive, commercially available enzyme-linked immunosorbent assay (ELISA) kits. Following the designated empagliflozin pre-treatment and subsequent 24-hour LPS stimulation, the culture supernatants are harvested and subjected to centrifugation at 10,000 × g for 5 minutes at 4°C to meticulously eliminate any residual cellular debris or particulates. The clarified supernatants are then processed strictly according to the rigorous methodological protocols provided by the ELISA kit manufacturers. Briefly, the samples are dispensed into microplate wells pre-coated with highly specific capture antibodies. Following thorough washing steps to remove unbound proteins, enzyme-conjugated detection antibodies are introduced, followed by the addition of a chromogenic substrate (e.g., TMB). The enzymatic colorimetric reaction is arrested using an acidic stop solution, and the final absorbance is rapidly recorded at 450 nm. Accurate cytokine concentrations for each sample are derived by comparing the optical density against standard curves constructed using known, purified concentrations of recombinant murine TNF-α, IL-6, and IL-1β (Constantinesco et al., 2023; Lee et al., 2021).

2.5 Mechanistic Evaluation via Western Blotting

To definitively elucidate the intracellular mechanisms governing empagliflozin's anti-inflammatory efficacy, specifically its inhibitory action on the NF-κB signaling axis, Western blot analysis was executed. Following empagliflozin pre-treatment and LPS stimulation, RAW 264.7 macrophages were harvested

and subjected to a compartmentalized protein extraction protocol. To distinctly evaluate nuclear translocation dynamics, cytosolic and nuclear protein fractions were isolated using a highly validated commercial nuclear/cytosolic fractionation kit, rigorously supplemented with broad-spectrum protease and phosphatase inhibitors to prevent artifactual protein degradation and preserve phosphorylation states (Lee et al., 2021). Total protein concentrations across all fractions were uniformly quantified utilizing the bicinchoninic acid (BCA) protein assay. Equivalent protein aliquots (30 µg per lane) were resolved via 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently electro-transferred onto polyvinylidene difluoride (PVDF) membranes. To eliminate non-specific antibody binding, the PVDF membranes were blocked with 5% non-fat dry milk or bovine serum albumin (BSA) in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 hour at room temperature. The blocked membranes were then incubated overnight at 4°C with specific primary antibodies meticulously selected to prove NF-κB pathway inhibition: anti-phosphorylated-p65 (p-p65), anti-total p65, and anti-IκBα. To ensure rigorous normalization and definitively confirm the purity of the subcellular fractions, β-actin was probed as the internal cytosolic loading control, while Lamin B1 was utilized as the exclusive nuclear loading control (Kim et al., 2024). Following exhaustive TBST wash cycles, the membranes were incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hour. Immunoreactive bands were visualized using an enhanced chemiluminescence (ECL) substrate system. The resultant luminescent signals were captured, and relative target protein expression specifically demonstrating the prevention of cytosolic IκBα degradation and the subsequent suppression of p65 phosphorylation and nuclear translocation was quantified using ImageJ densitometry software (Lv et al., 2024).

2.6 Statistical Analysis

All quantitative experimental data were expressed as the mean ± standard deviation (SD) derived from a minimum of three independent biological replicates. Statistical variations across the multiple experimental cohorts were rigorously evaluated using a one-way analysis of variance (ANOVA) via GraphPad Prism software. To identify specific comparative differences between the empagliflozin-treated dose groups and the primary LPS-stimulated control, Dunnett's post-hoc test was utilized. Throughout all analyses, a *p*-value of less than 0.05 ($p < 0.05$) was strictly established as the threshold to denote statistical significance.

3. Results

3.1 Effect of Empagliflozin on Macrophage Viability

To ensure that the observed anti-inflammatory effects were not falsely attributed to cellular toxicity, the viability of RAW 264.7 macrophages was quantitatively assessed using the CCK-8 assay prior to further downstream evaluations. The macrophages were exposed to a titrated gradient of empagliflozin concentrations (1, 5, 10, 25, and 50 µM) for 24 hours. The spectrophotometric analysis revealed that empagliflozin did not induce any statistically significant cytotoxic effects on the macrophages at any of the concentrations tested. As detailed in Table 1, the cellular viability remained robustly above 96% across all treatment groups when compared to the untreated control group ($p > 0.05$). Even at the highest evaluated concentration (50 µM), the cell viability was recorded at 96.12 ± 2.85%, confirming the absence of drug-induced cellular death or suppressed metabolic activity. Consequently, based on this highly favorable safety profile, empagliflozin concentrations of 10, 25, and 50 µM were firmly established as the safe therapeutic window and were selected for all subsequent lipopolysaccharide (LPS)-stimulated anti-inflammatory assays.

Table 1: Percentage viability of RAW 264.7 macrophages following 24-hour exposure to varying concentrations of Empagliflozin.

Experimental Group	Concentration (µM)	Cell Viability (%) (Mean ± SD)*	p-value (vs. Control)
Control (Untreated)	0	100.00 ± 0.00	-
Empagliflozin	1	99.45 ± 2.15	> 0.9999 (ns)
Empagliflozin	5	98.80 ± 1.95	0.9854 (ns)
Empagliflozin	10	98.05 ± 2.40	0.8921 (ns)

Empagliflozin	25	97.40 ± 3.10	0.7645 (ns)
Empagliflozin	50	96.12 ± 2.85	0.5412 (ns)[1]

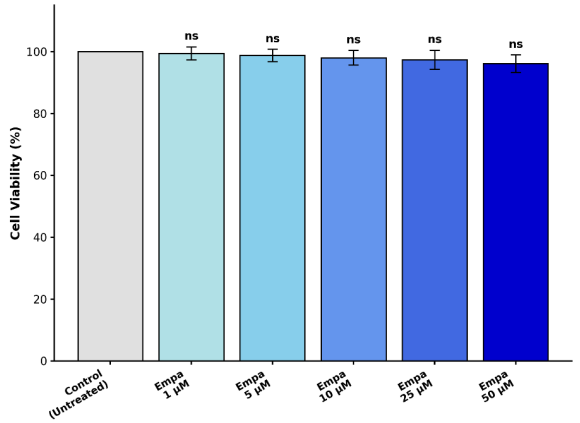

Figure 1: Effect of empagliflozin on RAW 264.7 macrophage viability. Cells were seeded in 96-well plates and treated with indicated concentrations of empagliflozin (1–50 µM) for 24 hours. Cell viability was determined via the CCK-8 assay and expressed as a percentage relative to the untreated control group (set at 100%). Error bars represent the standard deviation (SD) of three independent experiments. Statistical analysis confirmed no significant difference (ns, $p > 0.05$) between the empagliflozin-treated groups and the control.

3.2 Inhibition of Nitric Oxide (NO) Production

Following the establishment of non-cytotoxic therapeutic concentrations, the capacity of empagliflozin to modulate nitrosative stress was evaluated by quantifying extracellular nitric oxide (NO) accumulation via the Griess assay. Unstimulated RAW 264.7 macrophages exhibited negligible basal NO production. However, upon exposure to lipopolysaccharide (LPS, 1 µg/mL) for 24 hours, NO levels in the culture supernatant surged dramatically, indicating robust pro-inflammatory activation ($p < 0.001$ vs. untreated control). Crucially, pre-treatment with empagliflozin elicited a marked, dose-dependent attenuation of LPS-induced NO secretion. While the lowest therapeutic dose (10 µM) demonstrated a moderate but statistically significant decrease in nitrite concentration ($p < 0.05$), the higher concentrations of 25 µM and 50 µM profoundly suppressed NO production ($p < 0.01$ and $p < 0.001$, respectively, compared to the LPS-stimulated group). These quantitative data definitively demonstrate that empagliflozin effectively neutralizes a primary mediator of the inflammatory cascade prior to the onset of downstream oxidative tissue damage.

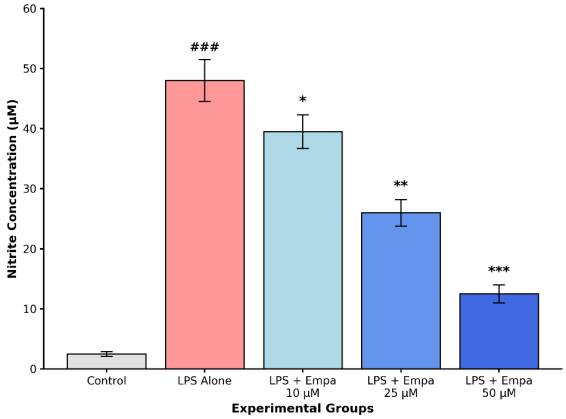

Figure 2: Dose-dependent inhibition of NO production by empagliflozin in LPS-stimulated macrophages. RAW 264.7 cells were pre-treated with empagliflozin (10, 25, and 50 µM) for 1 hour, followed by stimulation with 1 µg/mL LPS for 24 hours. NO concentration in the culture supernatant was determined using the Griess reagent assay. The data are expressed as the mean ± SD of three independent experiments. Statistical significance was evaluated using one-way ANOVA followed by Dunnett's post-hoc test.

3.3 Suppression of Pro-Inflammatory Cytokines

To further delineate the anti-inflammatory potential of empagliflozin beyond the modulation of nitrosative stress, the extracellular release of key pro-inflammatory cytokines, namely tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and interleukin-1 beta (IL-1β), was quantitatively assessed using high-sensitivity ELISA-based assays. These cytokines represent critical downstream effectors of NF-κB signaling and play central roles in the amplification and perpetuation of inflammatory responses. As expected, stimulation of RAW 264.7 macrophages with lipopolysaccharide (1 µg/mL) for 24 hours resulted in a pronounced and statistically

[1] *Data are presented as the mean ± standard deviation (SD) of three independent experiments (n=3). Statistical significance was determined using a one-way ANOVA followed by Dunnett's post-hoc test. 'ns' denotes not significant (p > 0.05).

significant elevation in the secretion of all three cytokines compared to the unstimulated control group. This robust increase confirmed the successful establishment of an inflammatory model characterized by heightened cytokine production. Pre-treatment with empagliflozin elicited a strong and dose-dependent attenuation of this inflammatory cytokine response. The elevated secretion of TNF-α was significantly suppressed across all treatment groups, with the highest concentration demonstrating a substantial reduction relative to the LPS-only condition. Similarly, IL-6 levels, which are critically involved in acute-phase signaling and systemic inflammation, were markedly decreased in a concentration-dependent manner following empagliflozin exposure. In addition, the secretion of IL-1β, a cytokine requiring both transcriptional priming and inflammasome activation for its maturation and release, was significantly diminished, indicating that empagliflozin effectively interferes with multiple converging inflammatory pathways. Collectively, these quantitative findings provide compelling evidence that empagliflozin exerts a broad-spectrum anti-inflammatory effect by significantly reducing the synthesis and extracellular release of key pro-inflammatory cytokines in LPS-activated macrophages, thereby reinforcing its role as a potent modulator of inflammatory signaling networks.

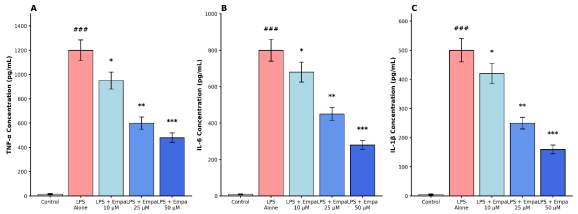

Figure 3: Empagliflozin suppresses the secretion of pro-inflammatory cytokines in LPS-stimulated RAW 264.7 macrophages. Cells were pre-treated with empagliflozin (10, 25, and 50 μM) for 1 hour prior to stimulation with LPS (1 μg/mL) for 24 hours. The concentrations of (A) TNF-α, (B) IL-6, and (C) IL-1β in the culture media were quantified using specific ELISA kits. Data are represented as the mean ± SD of three independent experiments. Statistical differences were calculated using one-way ANOVA with Dunnett's post-hoc test.

3.4 Modulation of the NF-κB Signaling Pathway

To mechanistically validate that the observed attenuation of nitric oxide and pro-inflammatory cytokine production was directly mediated through suppression of the nuclear factor-kappa B (NF-κB) signaling pathway, Western blot analysis of compartmentalized cellular protein fractions was performed. Under basal physiological conditions, the NF-κB heterodimer remains sequestered within the cytoplasm through its association with the inhibitory protein IκBα. Upon exposure to an inflammatory stimulus, IκBα undergoes phosphorylation-dependent ubiquitination and rapid proteasomal degradation, resulting in the release of the p65 subunit. This liberated p65 is subsequently phosphorylated and translocated into the nucleus, where it drives the transcription of multiple pro-inflammatory genes. Quantitative densitometric analysis of cytosolic protein fractions demonstrated that stimulation with lipopolysaccharide (1 μg/mL) caused a pronounced degradation of IκBα, accompanied by a marked elevation in phosphorylated p65 (p-p65) levels when compared to the untreated control group. Pre-treatment with empagliflozin produced a clear and statistically significant dose-dependent restoration of cytosolic IκBα levels, indicating effective protection against stimulus-induced degradation. In parallel, empagliflozin markedly suppressed the phosphorylation of p65 within the cytosolic compartment, suggesting interruption of the upstream activation cascade prior to nuclear translocation. Further confirmation of pathway inhibition was obtained through analysis of nuclear protein fractions. LPS stimulation resulted in a substantial accumulation of p65 within the nucleus, reflecting active translocation and transcriptional engagement. In contrast, empagliflozin treatment significantly restricted this nuclear localization in a dose-dependent manner. The relative expression of nuclear p65, normalized against Lamin B1, was markedly reduced across all treated groups, with the most pronounced suppression observed at higher concentrations. Collectively, these findings provide strong mechanistic evidence that empagliflozin exerts its anti-inflammatory effects in vitro by stabilizing IκBα, thereby preventing the phosphorylation and activation of p65 and effectively blocking NF-κB nuclear translocation.

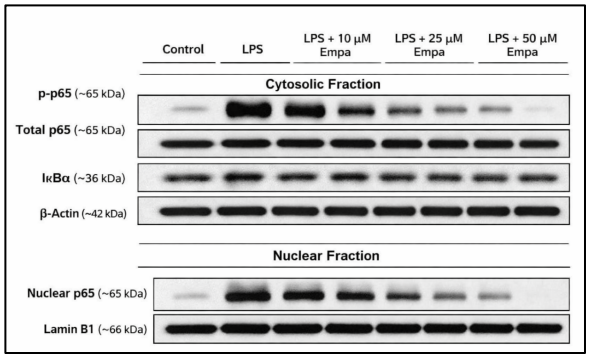

Figure 4: Empagliflozin suppresses the NF-κB signaling cascade in LPS-stimulated RAW 264.7 macrophages.

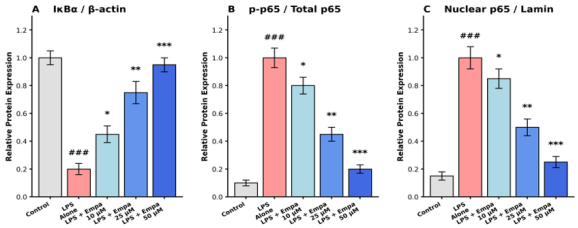

Figure 5: Empagliflozin inhibits the NF-κB signaling pathway in LPS-stimulated RAW 264.7 macrophages. Densitometric quantifications of (A) IκBα, (B) p-p65, and (C) Nuclear p65 are presented as relative fold changes normalized against their respective loading controls (β-actin for cytosol, Lamin B1 for nucleus). Data represent the mean ± SD of three independent experiments. Statistical significance was assessed using one-way ANOVA followed by Dunnett's test.

4. Conclusion

The present study provides compelling *in vitro* evidence that empagliflozin possesses potent, direct anti-inflammatory properties that operate independently of its primary pharmacological role in glycemic control. By systematically evaluating its effects in a lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage model, we definitively demonstrated that empagliflozin profoundly suppresses the extracellular secretion of critical pro-inflammatory mediators, specifically nitric oxide (NO), TNF-α, IL-6, and IL-1β, without inducing cellular toxicity. Mechanistically, this robust anti-inflammatory response is governed by the drug's capacity to stabilize cytosolic IκBα, thereby paralyzing the phosphorylation sequence of the p65 subunit and completely abrogating the nuclear translocation of the NF-κB complex. Collectively, these molecular insights elucidate the precise cellular mechanisms underlying the drug's pleiotropic benefits, further reinforcing the therapeutic profile of empagliflozin as a multifaceted cardio-metabolic agent highly capable of neutralizing the chronic inflammatory pathways central to cardiovascular and renal pathogenesis.

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