

Metoprolol exerts a non-class effect against ischaemia–reperfusion injury by abrogating exacerbated inflammation

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Aims

Clinical guidelines recommend early intravenous β -blockers during ongoing myocardial infarction; however, it is unknown whether all β -blockers exert a similar cardioprotective effect. We experimentally compared three clinically approved intravenous β -blockers.

Methods and results

Mice undergoing 45 min/24 h ischaemia–reperfusion (I/R) received vehicle, metoprolol, atenolol, or propranolol at min 35. The effect on neutrophil infiltration was tested in three models of exacerbated inflammation. Neutrophil migration was evaluated *in vitro* and *in vivo* by intravital microscopy. The effect of β -blockers on the conformation of the β_1 adrenergic receptor was studied *in silico*. Of the tested β -blockers, only metoprolol ameliorated I/R injury [infarct size (IS) = 18.0% \pm 0.03% for metoprolol vs. 35.9% \pm 0.03% for vehicle; $P < 0.01$]. Atenolol and propranolol had no effect on IS. In the three exacerbated inflammation models, neutrophil infiltration was significantly attenuated only in the presence of metoprolol (60%, 50%, and 70% reductions vs. vehicle in myocardial I/R injury, thioglycolate-induced peritonitis, and lipopolysaccharide-induced acute lung injury, respectively). Migration studies confirmed the particular ability of metoprolol to disrupt neutrophil dynamics. *In silico* analysis indicated different intracellular β_1 adrenergic receptor conformational changes when bound to metoprolol than to the other two β -blockers.

Conclusions

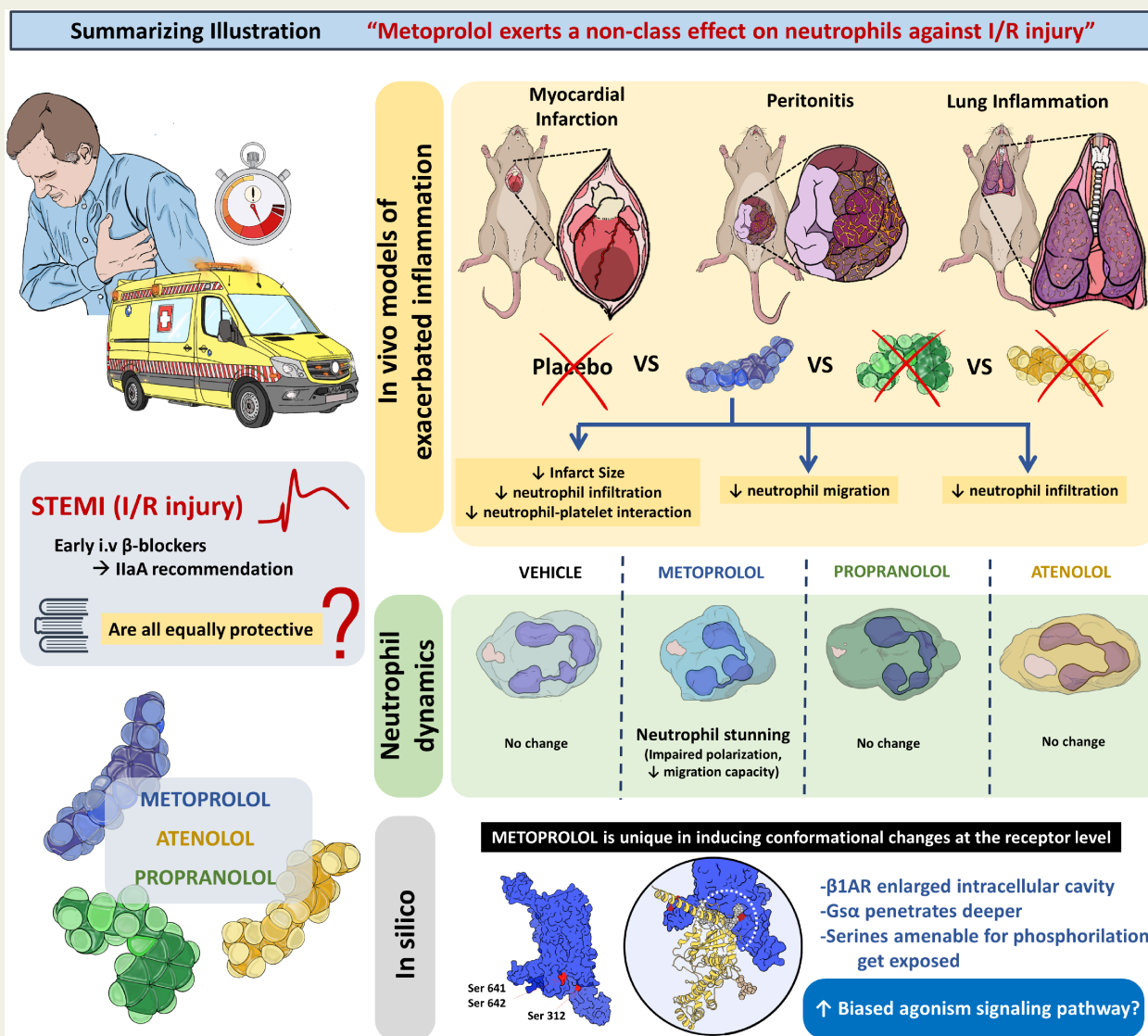
Metoprolol exerts a disruptive action on neutrophil dynamics during exacerbated inflammation, resulting in an infarct-limiting effect not observed with atenolol or propranolol. The differential effect of β -blockers may be related to distinct conformational changes in the β_1 adrenergic receptor upon metoprolol binding. If these data are confirmed in a clinical trial, metoprolol should become the intravenous β -blocker of choice for patients with ongoing infarction.

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Graphical Abstract



Keywords

β-blockers • β1AR • I/R injury • Neutrophils • Inflammation

Translational perspective

Early administration of intravenous (i.v.) β-blockers is recommended for patients with an ongoing myocardial infarction; however, it is unknown whether all approved drugs exert the same cardioprotective effect. Here, we show that metoprolol, but not atenolol or propranolol, has an ameliorative effect on neutrophil-induced tissue damage during exacerbated inflammation, including myocardial ischaemia–reperfusion injury. Metoprolol disrupts deleterious neutrophil dynamics during reperfusion, and this translates into a significant infarct-limiting effect not shared by the other tested β-blockers. Modelling shows that metoprolol binding triggers a unique conformational change in the β1 adrenergic receptor intracellular domain. If confirmed at the clinical level, early intravenous metoprolol, but not other β-blockers, should be used to treat patients with ongoing infarction before reperfusion.

Introduction

Acute myocardial infarction (AMI) is a leading cause of morbidity and mortality worldwide. The advent of reperfusion technologies has dramatically reduced acute mortality associated with AMI. However, the size of the infarct often leaves survivors with severe heart damage, and these patients are at high risk of future heart failure and readmission.^{1,2} Reperfusion, despite being essential for myocardial salvage, triggers an exacerbated sterile inflammatory process that contributes to final infarct size (IS). This inflammation is driven by neutrophils, which infiltrate the damaged myocardium through interactions with platelets contribute to ischaemia–reperfusion (I/R) injury (IRI).^{3–5} Paradoxically, blood flow restoration in the large epicardial coronary artery many times is not accompanied by efficient tissue perfusion due to the obstruction of the microvasculature. Endothelial swelling, external compression of small vessels secondary to oedema formation, and cellular aggregates (neutrophils, platelets, and erythrocytes) generating plugs that restrict tissue perfusion at the capillary level contribute to the phenomenon known as microvascular obstruction (MVO).^{6–8} The latter is a main contributor to IRI and final IS.^{1,2} The β 1-selective blocker metoprolol has been demonstrated to reduce myocardial IS in several species, including humans.^{9–11} Metoprolol appears to limit IS largely through its inhibitory effect on neutrophils.⁹ Based partly on the cardioprotective effect of metoprolol injection,^{10,12} current clinical practice guidelines recommend early intravenous administration of β -blockers (as a drug class) to patients with an ongoing AMI.¹³ However, it is unknown whether different β -blockers exert the same cardioprotective effect, and a trial in patients with ongoing AMI undergoing reperfusion showed no infarct-limiting effect of the β 1-selective blocker atenolol.¹⁴ In this study, we explored the cardioprotective effect of three β -blockers approved for clinical i.v. administration (metoprolol, atenolol, and propranolol) in a mouse model of IRI. We further explored the effect of these β -blockers on neutrophil migration and infiltration in three models of exacerbated inflammation: myocardial I/R, thioglycolate-induced peritonitis, and lipopolysaccharide (LPS)-induced acute lung injury (ALI). *In silico* studies were conducted to evaluate conformational changes in the β 1 adrenergic receptor upon binding the different β -blockers. Our results show that metoprolol has a particular action on neutrophils during exacerbated inflammation that affords a cardioprotection not provided by other β -blockers.

Methods

Full section of material and methods can be found in the [Supplementary material online](#).

Results

Metoprolol, but not atenolol or propranolol, limits myocardial infarct size

The cardioprotective activity of three clinically approved intravenous β -blocker agents was assessed in an established *in vivo* model of IRI

(*Figure 1A*).⁹ In brief, mice were anaesthetized by i.p. administration of ketamine, xylazine, and atropine, and were placed on mechanical ventilation. The left anterior descending coronary artery was accessed by a small thoracotomy and then fully occluded by tying a silk knot around the proximal segment of the artery. After 45 min of coronary artery occlusion, the knot was released to allow reperfusion. Before reperfusion, mice were randomly allocated to i.v. metoprolol, atenolol, propranolol (all 12.5 mg/kg) or vehicle (0.9% NaCl). Operators were blinded to treatment allocation. Drug or vehicle was injected as a single bolus through the retro-orbital sinus 10 min before reperfusion (35 min after ischaemia onset). The metoprolol, atenolol, and propranolol dose was based on a dose–response study, in which 12.5 mg/kg was identified as the highest dose with moderate haemodynamic effect (<20%) for the three β -blockers ([Supplementary material online, Figure S1](#)). At 24 h post-reperfusion, mice were euthanized, and area at risk (AAR)-normalized IS was calculated.⁹

Confirming previous studies, i.v. metoprolol resulted in smaller IS (% AAR) than in vehicle-treated mice (metoprolol, 18.0% \pm 8.11%; vehicle, 35.9% \pm 10.7%; $P=0.0142$). In contrast, atenolol and propranolol had no effect on IS (atenolol, 38.0% \pm 20.9%; propranolol, 36.0% \pm 10.3%; vehicle, 35.9% \pm 8.11%) (*Figure 1B–D*).

Metoprolol is the only tested β -blocker that attenuates post-acute myocardial infarction neutrophil infiltration

In previous studies in pigs and mice, we showed that pre-reperfusion metoprolol injection results in reduced myocardial neutrophil infiltration,^{9,15} accounting for its cardioprotective effect. Here, assessment of myocardial Ly6G protein levels at 24 h post-reperfusion revealed significantly lower neutrophil density in metoprolol-injected mice than in vehicle-treated mice, whereas atenolol and propranolol had no effect (*Figure 1A, E, and F*). Ly6G protein levels in metoprolol-treated mice were almost 60% lower in left ventricles of metoprolol-treated mice than those of controls.

Metoprolol, but not atenolol or propranolol, inhibits neutrophil–platelet interactions during myocardial ischaemia–reperfusion

Neutrophil–platelet interactions are crucial for neutrophil tissue infiltration during sterile inflammation.^{3–5} Because the cardioprotective effect not shared by the other β -blockers was expected to be driven by altered neutrophil dynamics, we next explored neutrophil interactions with platelets in peripheral blood 24 h after reperfusion. The percentage of circulating neutrophils interacting with platelets was significantly reduced only in the case of metoprolol (metoprolol, 37.5% \pm 20.9%; vehicle, 80.2% \pm 10.1%; $P=0.0166$), whereas atenolol (67.6% \pm 17.5%) and propranolol (67.3% \pm 28.1%) had no statistically significant effect (*Figure 1G and H*). The percentage and number of circulating neutrophil population were not affected by any of the treatment conditions ([Supplementary material online, Figure S2](#)).

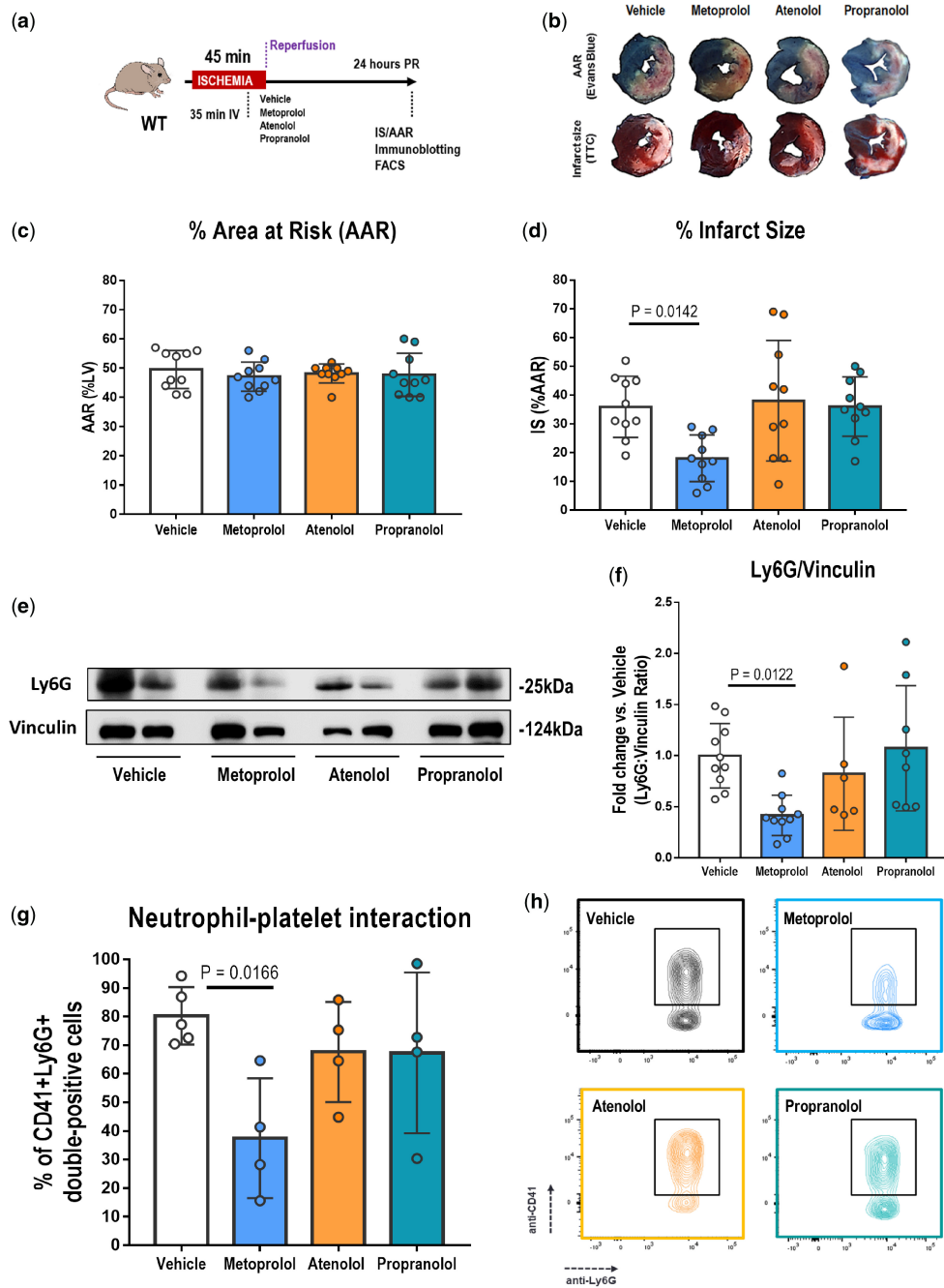


Figure 1 The infarct-limiting effect of metoprolol is not shared by atenolol or propranolol. (A) Mouse model of myocardial ischaemia–reperfusion for the estimation of area at risk (AAR) and infarct size (IS) by Evans Blue and triphenyl tetrazolium chloride (TTC) staining and the collection of left ventricle tissue and blood for immunoblotting and flow cytometry analysis. Mice were randomized to receive the indicated i.v. treatments 10 min before reperfusion. (B) Representative images of 1-mm-thick transverse left ventricle slices showing area at risk (negative for Evans Blue, white) and the extent of necrosis (triphenyl tetrazolium chloride-negative area). (C, D) Histological analysis of AAR (% left ventricle) and IS (% area at risk) in mice subjected to ischaemia–reperfusion and randomized to receive vehicle (white), metoprolol (blue), atenolol (orange), or propranolol (green). $n = 10$ for each condition. (E, F) Immunoblot analysis of Ly6G (25 kDa) and vinculin (124 kDa) protein expression at 24 h post-reperfusion in myocardium of mice subjected to ischaemia–reperfusion and randomized to pre-reperfusion treatments as above: vehicle, $n = 10$; metoprolol, $n = 10$; atenolol, $n = 6$; propranolol, $n = 8$. Quantified Ly6G levels in (F) are normalized to vinculin and expressed as the fold change relative to vehicle-treated mice. (G, H) Flow cytometry analysis of neutrophil–platelet interaction in peripheral citrated blood of mice subjected to ischaemia–reperfusion and randomized to receive one of the four indicated treatments. Data are presented as the percentage of neutrophils (Ly6G⁺) staining doubly positive for Ly6G and the platelet marker CD41. The representative flow cytometry plots in (H) illustrate the reduction in neutrophil–platelet interactions (boxed areas) in metoprolol-treated mice: $n = 4$ for all treatments except vehicle, $n = 5$. Data are presented as mean \pm SD.

Metoprolol has a particular inhibitory effect on neutrophil migration *in vitro* and *in vivo*

We previously showed that metoprolol exerts its cardioprotective effect during I/R by targeting neutrophils.⁹ Here, we wanted to explore whether the action on neutrophils was a drug class effect, and thus shared by other β -blockers, or was particular to metoprolol. The effect of the tested β -blockers on neutrophil migration was assessed in a chemokine-induced transwell migration assay (Figure 2A). Mouse neutrophils were exposed across the transwell filter to the chemoattractant CXCL1 in the presence or absence of metoprolol, atenolol, or propranolol (10 μ M for every condition), and the number of cells migrating across the transwell membrane was quantified by flow cytometry after 90 min. Metoprolol inhibited baseline neutrophil migration along the CXCL1 gradient (0.73 ± 0.31 vs. vehicle; $P = 0.0095$), whereas no effect on chemokine-induced migration was seen with either atenolol (1.04 ± 0.27) or propranolol (1.01 ± 0.19) (Figure 2B and C).

To confirm these results, we investigated whether atenolol or propranolol could mimic the ability of metoprolol to inhibit neutrophil tissue infiltration *in vivo* in a validated mouse model of thioglycolate-induced peritonitis⁹ (Figure 2D). Thioglycolate induces massive leucocyte migration into the peritoneal cavity within the first 6 h, with most infiltrating cells being neutrophils. The i.v. metoprolol bolus (12.5 mg/kg) steeply inhibited thioglycolate-induced neutrophil infiltration into the mouse peritoneal cavity ($4.03 \pm 4.70 \times 10^5$ vs. $7.84 \pm 5.01 \times 10^5$ neutrophils/mL for metoprolol and vehicle, respectively; $P = 0.0336$) and reduced neutrophils as a percentage of viable cells ($55.2\% \pm 23.3\%$ vs. $78.5\% \pm 17.1\%$ for metoprolol and vehicle, respectively; $P = 0.0053$). In contrast, atenolol and propranolol (12.5 mg/kg each) had no anti-migratory effect on neutrophil infiltration ($10.8 \pm 5.06 \times 10^5$, $7.26 \pm 4.14 \times 10^5$, and $7.84 \pm 5.01 \times 10^5$ neutrophils/mL for atenolol, propranolol, and vehicle, respectively) or neutrophils as a percentage of viable cells ($82.9\% \pm 5.76\%$, $77.9\% \pm 11.23\%$, and $78.5\% \pm 17.1\%$ for atenolol, propranolol, and vehicle, respectively) (Figure 2E–G).

To exclude potential dose-dependent effects and differential potency of the three tested β -blockers, we halved and doubled the β -blocker dose in the thioglycolate-induced peritonitis model to a single 6.25 or 25 mg/kg i.v. bolus, respectively. At these β -blocker doses, the same pattern was maintained, with neutrophil migration inhibited only by metoprolol, and atenolol and propranolol having no effect (Supplementary material online, Figure S3).

Metoprolol attenuates neutrophil infiltration during lipopolysaccharide-induced acute lung injury

We next tested the differential effects of i.v. β -blockers on neutrophil migration and infiltration in a mouse model of infection-induced inflammation: LPS-induced ALI (Figure 3A). At 24 h after LPS instillation, Broncho-alveolar lavage fluid (BALF) from metoprolol-treated mice contained significantly fewer neutrophils than BALF from vehicle-treated mice ($1.03 \pm 0.81 \times 10^5$ vs. $3.44 \pm 2.71 \times 10^5$ neutrophils/mL for metoprolol and vehicle, respectively; $P = 0.0060$). Neither atenolol nor propranolol had any effect on the BALF neutrophil count ($3.78 \pm 1.36 \times 10^5$, $4.06 \pm 1.05 \times 10^5$, and $3.44 \pm 2.71 \times 10^5$

neutrophils/mL for atenolol, propranolol, and vehicle, respectively) (Figure 3B and C). Tissue damage in response to an acute inflammatory response is known to involve neutrophil release of nuclear chromatin, known as neutrophil extracellular traps (NETs).¹⁶ Given that the LPS challenge increases citH3,¹⁶ which is strongly implicated in NET formation,¹⁷ we assessed whether β -blocker treatment affects this process. Immunoblot analysis showed that mice receiving i.v. metoprolol exhibited a 65% attenuation of H3 citrullination (on R2 + R8 + R17) compared with those receiving vehicle, whereas atenolol and propranolol had no effect (Figure 3D and E). Confocal microscopy analysis of lung tissue revealed that metoprolol significantly reduced the area of lung tissue covered by citH3 and the area of co-localization between citH3 and neutrophils (Ly6G+ cells) (Figure 3F and G). Moreover, reduced H3 citrullination in the lungs of metoprolol-treated mice was accompanied by reductions in neutrophil-elastase and myeloperoxidase (Supplementary material online, Figure S4A and B), neutrophil granule proteins involved in NET generation.¹⁷ These changes were accompanied by a protection against lung tissue damage in metoprolol-treated mice (Supplementary material online, Figure S4C and D). These results confirm attenuation of NET production and the amelioration of ALI in mice receiving metoprolol.

Metoprolol has a disruptive effect on neutrophil dynamics *in vivo* not shared by the other β -blockers tested

Myocardial I/R is a paradigm of acute sterile inflammation, in which chemotactic recruitment of inflammatory cells is predominantly mediated by neutrophils. To initiate an acute inflammatory response, neutrophils adhering to the activated endothelium undergo morphological rearrangements that allow them to interact with and recruit other cell types to infiltrate the tissue.⁴ Having observed that, unlike metoprolol, atenolol and propranolol showed no effect on neutrophil recruitment, we next explored the effect of these drugs on neutrophil dynamics. For this, we used 2D intravital microscopy (IVM) to image migration in the cremaster muscle vessels of mice injected with tumour necrosis factor α (TNF α), which triggers massive neutrophil recruitment⁴ (Figure 4A). Of the tested β -blockers, only metoprolol reduced neutrophil migratory velocity ($0.16 \pm 0.07 \mu\text{m/s}$ vs. $0.29 \pm 0.15 \mu\text{m/s}$ for metoprolol and vehicle, respectively; $P < 0.0001$), accumulated distance ($9.75 \pm 4.33 \mu\text{m}$ vs. $17.7 \pm 8.82 \mu\text{m}$ for metoprolol and vehicle, respectively; $P < 0.0001$), and euclidean crawling distance ($5.90 \pm 3.66 \mu\text{m}$ vs. $10.2 \pm 8.18 \mu\text{m}$ for metoprolol and vehicle, respectively; $P < 0.0010$). Moreover, metoprolol reduced the percentage of neutrophils interacting with platelets through the uropod ($42.5\% \pm 17.6\%$ vs. $59.4\% \pm 12.3\%$ for metoprolol and vehicle, respectively; $P < 0.0014$). Neither atenolol nor propranolol had any effect on any of the *in vivo* neutrophil dynamics parameters evaluated (Figure 4B–F and Supplementary material online, Videos S1A–D).

3D IVM studies were performed (Figure 5A) to test whether metoprolol specifically altered neutrophil shape or polarization during the acute inflammatory response. Consistent with the disrupted crawling dynamics observed in the 2D analysis, 3D reconstructions revealed that metoprolol impaired neutrophil polarization in TNF α -inflamed cremaster vessels, reducing neutrophil length and preventing the adoption of the typical cigar-like prolate spheroid cell shape

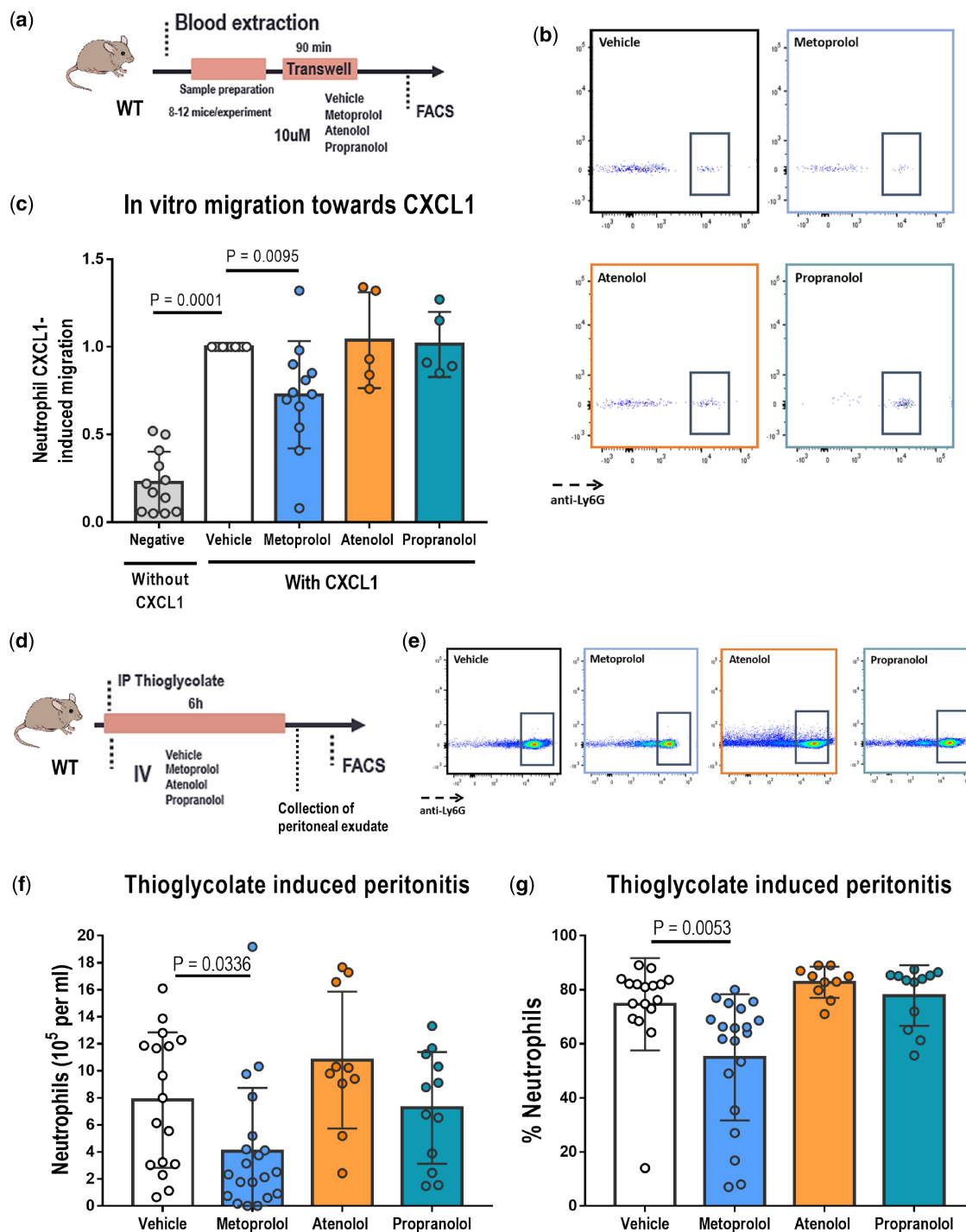


Figure 2 Metoprolol has a particular ability to inhibit neutrophil migration. (A) Experimental scheme for CXCL1-induced transwell migration analysis. (B) Flow cytometry plots illustrating reduced migration of neutrophils (Ly6G⁺ cells) upon treatment with metoprolol. To allow comparison between experiments, neutrophil migration for all treatments was normalized to the mean positive control (vehicle) value in each independent experiment. (C) Particular limiting effect of metoprolol on chemokine-induced neutrophil migration. Each independent experiment was conducted with leucocytes pooled from 8 to 12 animals, and each condition was run with three to four technical replicates: $n = 12$ for all conditions except for atenolol and propranolol, $n = 5$ each. (D) Experimental scheme for thioglycolate-induced peritonitis. Mice received a 12.5 mg/kg i.v. β -blocker dose immediately after i.p. thioglycolate administration. (E) Flow cytometry plots illustrating reduced peritoneal infiltration of neutrophils (Ly6G⁺ cells) in metoprolol-treated mice. (F, G) Specific limiting effect of metoprolol on thioglycolate-induced peritoneal infiltration in wild-type mice. (F) Absolute number of neutrophils/mL of infiltrate 6 h after thioglycolate injection in wild-type mice. (G) Neutrophils in intraperitoneal exudate calculated as a percentage of total viable cells. Vehicle, $n = 16$; metoprolol, $n = 18$; atenolol, $n = 10$; propranolol, $n = 12$. Data are presented as mean \pm SD.

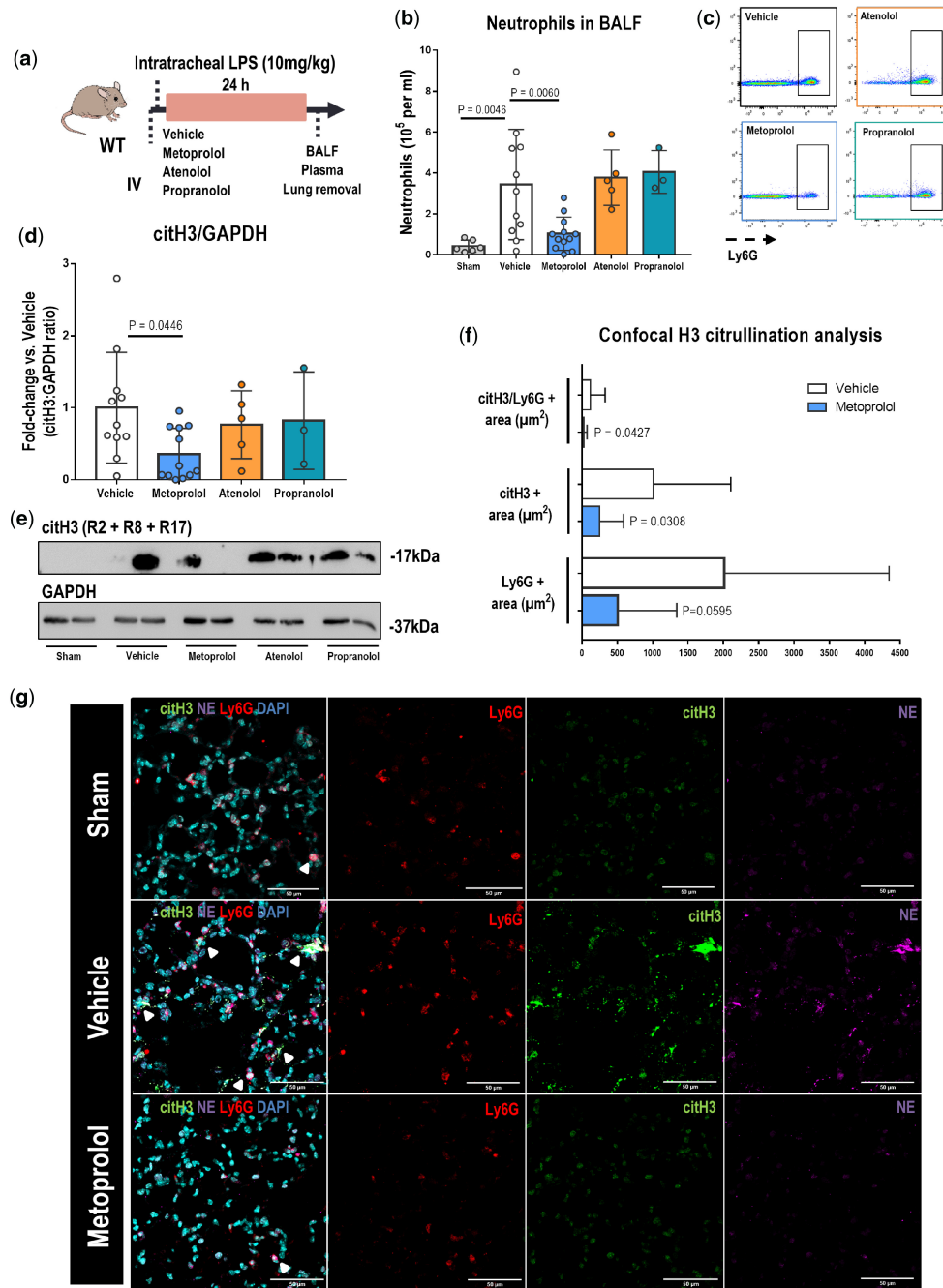


Figure 3 Metoprolol attenuates broncho-alveolar lavage fluid (BALF) neutrophil counts and H3 citrullination in lipopolysaccharide (LPS)-treated lungs. (A) Model of LPS-induced acute lung injury (ALI). Mice received an intratracheal instillation of LPS immediately after i.v. injection with the indicated treatments. (B, C) Flow cytometry analysis of neutrophil counts in BALF at 24 h after LPS instillation. The flow cytometry plots illustrate the reduced presence of neutrophils (Ly6G⁺ cells) in BALF of metoprolol-treated mice. (D, E) Immunoblot analysis of Histone 3 hypercitrullination (citH3) (17 kDa) and GAPDH (37 kDa) protein expression in the lungs of mice with acute lung injury and receiving the indicated treatments. Quantified citH3 levels in (D) are normalized to GAPDH and expressed as the fold change relative to vehicle-treated mice. Data in (B) and (D) are means \pm SD. Sham (no LPS), $n = 6$; vehicle, $n = 11$; metoprolol, $n = 12$; atenolol, $n = 5$; propranolol, $n = 3$. (F, G) Confocal microscopy analysis of histone 3 citrullination in acute lung injury. (F) Total area of lung tissue covered by citH3 and neutrophils (Ly6G⁺ cells) and the area of neutrophil–citH3 co-localization 24 h after lipopolysaccharide instillation. (G) Representative confocal images of lung sections from sham-treated mice (Control, no lipopolysaccharide), and lipopolysaccharide-instilled mice receiving i.v. vehicle (saline) or metoprolol. Areas of co-localization (arrowheads) between histone 3 citrullination (citH3, green) and neutrophils (Ly6G⁺ cells, red) indicate generation of neutrophil extracellular traps (NETs). The neutrophil granule protein neutrophil–elastase (NE, purple) is a marker of neutrophil activation. $n = 10$ –12 mice per condition. Data are presented as mean \pm SD.

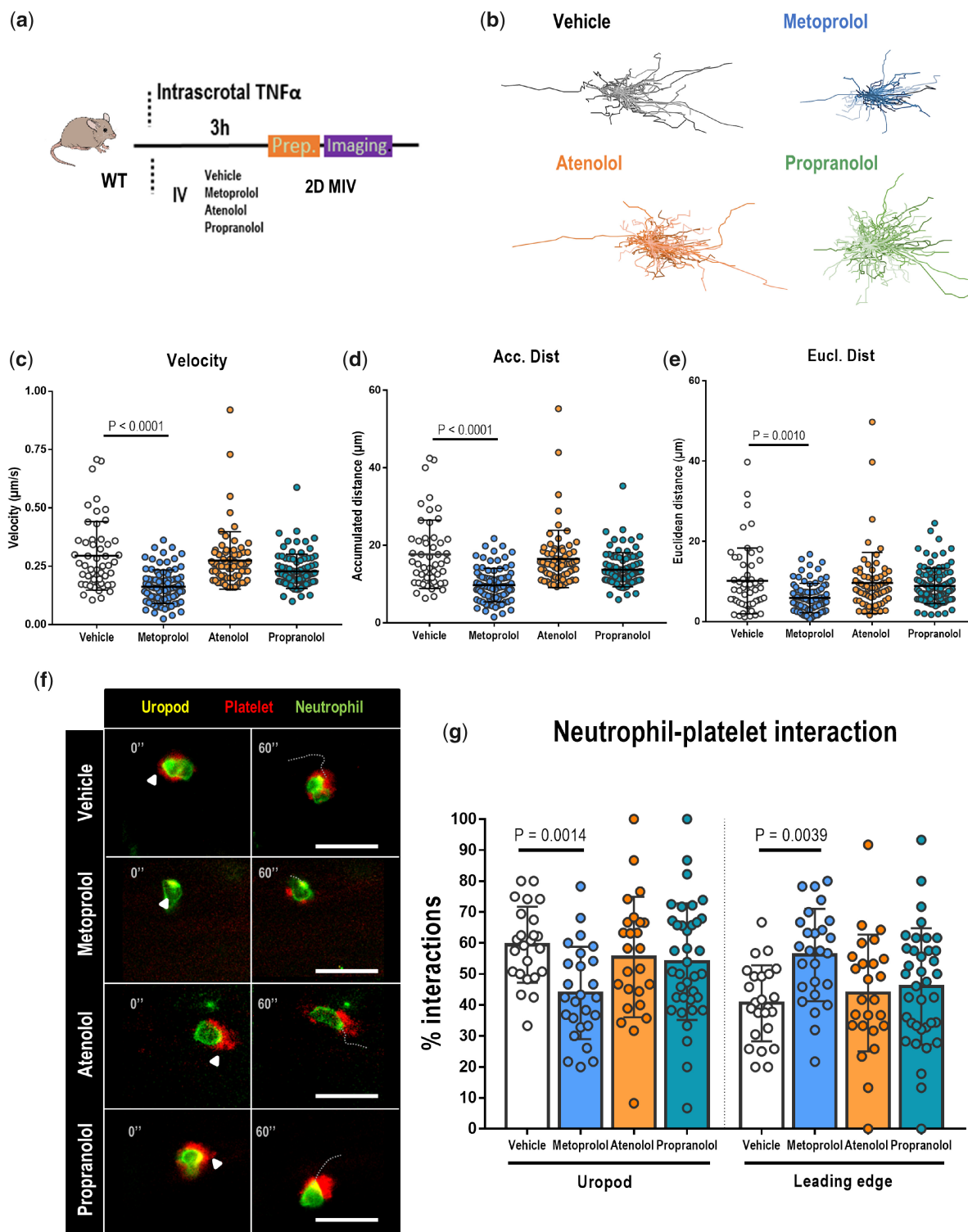


Figure 4 Metoprolol has a particular disruptive effect on neutrophil dynamics *in vivo*. (A) Experimental scheme for 2D intravital microscopy (IVM) of neutrophil motility in inflamed cremaster muscle. (B) Representative tracks of crawling neutrophils within inflamed vessels of mice treated with vehicle, metoprolol, atenolol, or propranolol. (C–E) Two-dimensional intravascular motility parameters: velocity ($\mu\text{m/s}$), accumulated distance (μm), and euclidean distance (μm); $n = 52\text{--}89$ cells from 5 to 6 mice per condition. (F) Representative time-lapse images of platelets (CD41^+ cells, red) with the polarized neutrophil uropod (CD62L^+ domain, yellow) or leading edge (Ly6G^+ domain, green) in the different conditions. Arrowheads indicate interactions with the uropod domain, and dotted lines indicate displacement of the neutrophil over 60s. (G) Percentage of platelet interactions with the neutrophil uropod or leading edge; $n = 24\text{--}37$ cells from three mice per condition. Data are presented as means \pm SD.

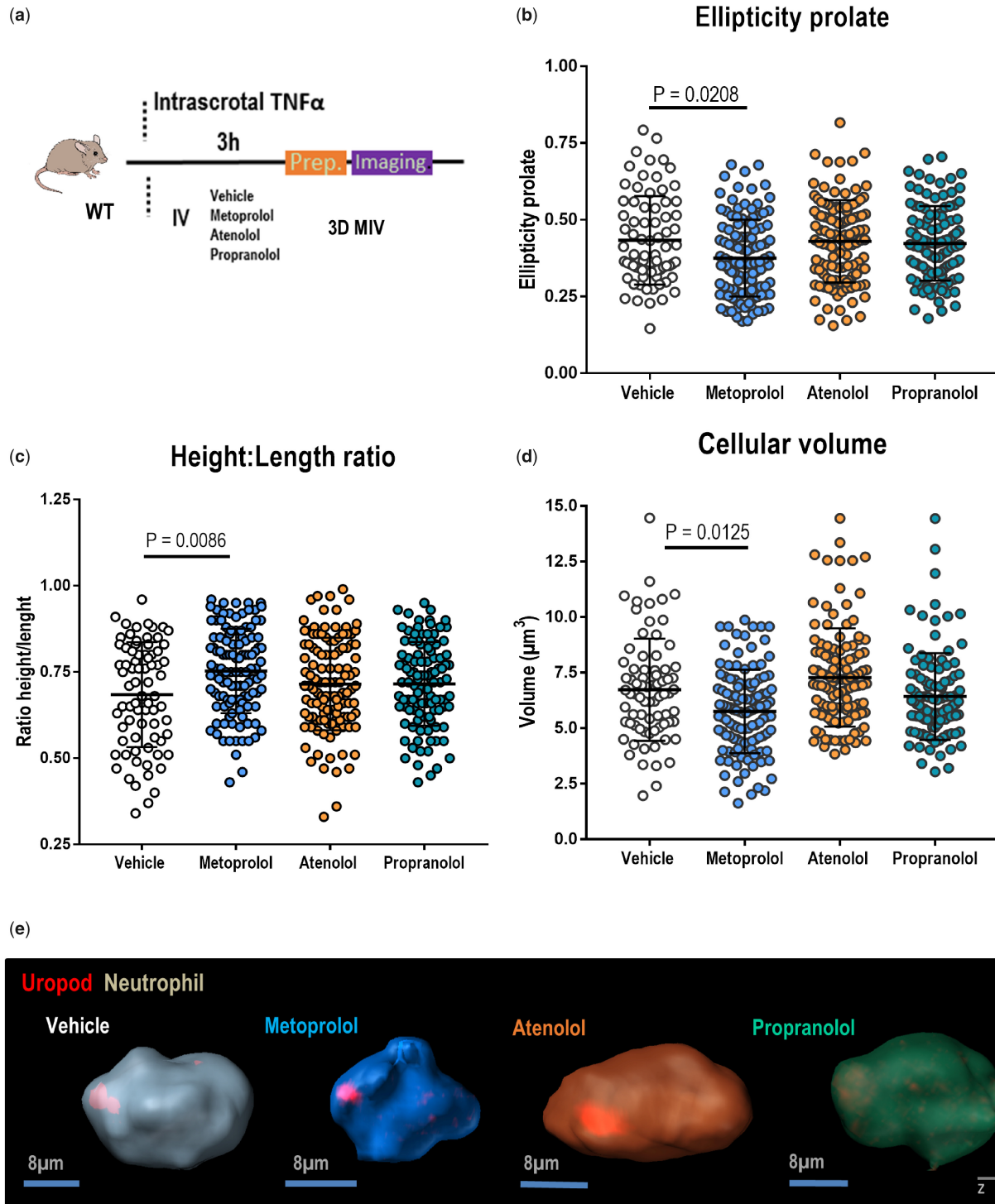


Figure 5 Metoprolol alters neutrophil polarized morphology. (A) Experimental scheme for 3D intravital microscopy (IVM) of neutrophil morphology in inflamed cremaster muscle. (B–D) Three-dimensional intravascular cell morphology parameters: ellipticity prolate, height: length ratio, and volume. $n = 75$ –118 cells from 3 to 4 mice per condition. Data are presented as mean \pm SD. (E) Representative 3D reconstructions of polarized neutrophils (uropod, red) within live cremaster vessels of mice treated with vehicle (grey), metoprolol (blue), atenolol (orange), or propranolol (green).

(Figure 5B–D). These effects were not observed with atenolol and propranolol, indicating that the neutrophil morphological changes needed to initiate intercellular interactions and subsequent tissue infiltration remain intact in mice treated with these drugs. This result might explain the lack of a cardioprotective effect with these drugs during I/R. Conversely, metoprolol blocks neutrophil infiltration and migration through an effect on neutrophil dynamics, and this neutrophil-stunning effect confers a cardioprotective effect during myocardial I/R.

Metoprolol-binding triggers β 1 adrenergic receptor intracellular conformational change exposing phosphorylation targets involved in β -arrestin signalling cascade

The interaction of the three β -blockers with the β 1 adrenergic receptor (β 1AR) was investigated by *in silico* approaches. All three selected β -blockers belong to the same pharmaceutical class, signal through G-protein coupled receptors (GPCRs), have high affinity for the β 1AR, and are currently authorized for intravenous administration to patients. Simulated ligand binding did not substantially alter the overall topology of the human β 1AR, which showed only minor differences upon binding the different β -blockers. As expected for drugs belonging to the same class, the extracellular drug-binding pocket has a small solvent accessible surface, and this pocket was moved slightly and to a similar extent with respect to the unbound protein upon binding of all tested ligands.

The model was refined by submitting it to the positioning of proteins in membranes (PPM) server, which positioned the receptor–drug complex more precisely in the membrane. The energy and stability of the ligand– β 1AR complex was similar for all drugs; however, metoprolol binding induced an affinity-independent increase in the size of the internal cavity significantly greater than seen with the other tested drugs (63 759.34 Å² for metoprolol, 37 571.32 Å² for epinephrine, 50069.59 Å² for atenolol, and 44 371.77 Å² for propranolol) (Figure 6A and B and Supplementary material online, Tables S1 and S2). Modelling of the mouse β 1AR yielded proportionally similar differences in internal cavity size (Supplementary material online, Figure S6, Tables S3 and S4). These results strongly suggest that metoprolol binding induces a bigger conformational change in the receptor that opens the intracellular cavity, likely modifying its interactions with intracellular effectors.

To elucidate whether the opening of the intracellular cavity of the receptor when bound to metoprolol translates into differences in G_s protein signalling, we modelled the binding of the G_s α subunit to the complexes established upon docking of the different ligands to the human β 1AR. Although large differences in the energy of interface were not documented, the G_s α subunit penetrates more in the cavity of metoprolol– β 1AR than in the rest of the ligand– β 1AR complexes (Figure 7), possibly making it more difficult for the G_s α subunit to interact with other effectors to perpetuate the classical adenylate cyclase–AMPc signalling cascade.

We further explored the impact of the β 1AR–G_s α interaction (upon binding to different β -blockers) on biased agonism signalling pathways. We focused our computational analyses on the study of two conserved sites (regions) of the intracellular region of the β 1AR

experimentally described as containing putative phosphoryl-Ser that initiate the receptor signalling and deactivation cascade [Ser461 and Ser462, which are susceptible to being phosphorylated by G-protein-coupled receptor kinases (GRKs); and Ser312 that is susceptible to being phosphorylated by protein kinase A].

Qualitatively, it is noticeable that the Ser 461–462 positions are more exposed when the G_s α subunit binds the metoprolol– β 1AR complex (Figure 7), potentially being more prone to be phosphorylated by GRKs, triggering a β -arrestin-mediated signalling cascade.

Discussion

In this study, we have evaluated the cardioprotective effect of different clinically approved i.v. β -blockers to reduce IS in a mouse model of myocardial IRI. We have explored the effect of these drugs on the hyperactive immune response during exacerbated inflammation in models of acute injury in the heart, peritoneum and lung, and neutrophil migration *in vitro*. Finally, we have studied *in silico* structural changes occurring in the β 1AR when bound to the different β -blockers.

Our results show that while metoprolol significantly ameliorates myocardial IRI, atenolol and propranolol have no cardioprotective effect. Our *in vitro* and *in vivo* studies show that metoprolol is the only studied β -blocker that impairs neutrophil migration and infiltration during exacerbated inflammation, and 2D and 3D IVM studies show that metoprolol exerts a particular disruptive effect on neutrophil dynamics. The *in silico* analysis reveals that, upon binding to the β 1AR, metoprolol provokes a significant conformational change in the intracellular domain that is not observed with atenolol or propranolol. Taken together, these results show that metoprolol has a unique ability among the β -blockers tested to target neutrophils and stun the neutrophil immune response during exacerbated inflammation (Take home figure). These findings have important clinical implications, given that since clinical practice guidelines on the use of β -blockers during AMI assume that the cardioprotective effect of metoprolol is shared by other drugs of this class.¹³

The METOCARD-CNIC clinical trial demonstrated that reperfusion injection of metoprolol in AMI patients significantly reduces IS and the incidence of long-term heart failure.^{10,12} In another trial in AMI patients undergoing reperfusion, atenolol administration showed no association with reduced IS.¹⁴ While these starkly different outcomes could reflect differences in trial design, they also point to possible differences in the ability of these β -blocker agents to counter injurious mechanisms. The leading mechanism of tissue injury during sterile inflammation is exaggerated neutrophil activation and tissue infiltration,^{18–20} and myocardial I/R serves as a paradigm of this process. A recent study showed that metoprolol ameliorates myocardial IRI through a direct action on neutrophils that prevents intercellular interactions and the cell morphological changes needed to initiate tissue infiltration.⁹ This prompted us to explore the potential cardioprotective effect of three clinically approved β -blockers, as well as their effect on neutrophil biology during exacerbated inflammation.

We previously showed that metoprolol-induced cardioprotection involves a ‘stunning’ effect on neutrophils. This effect is β 1AR-mediated, since metoprolol did not reduce migration in neutrophils from

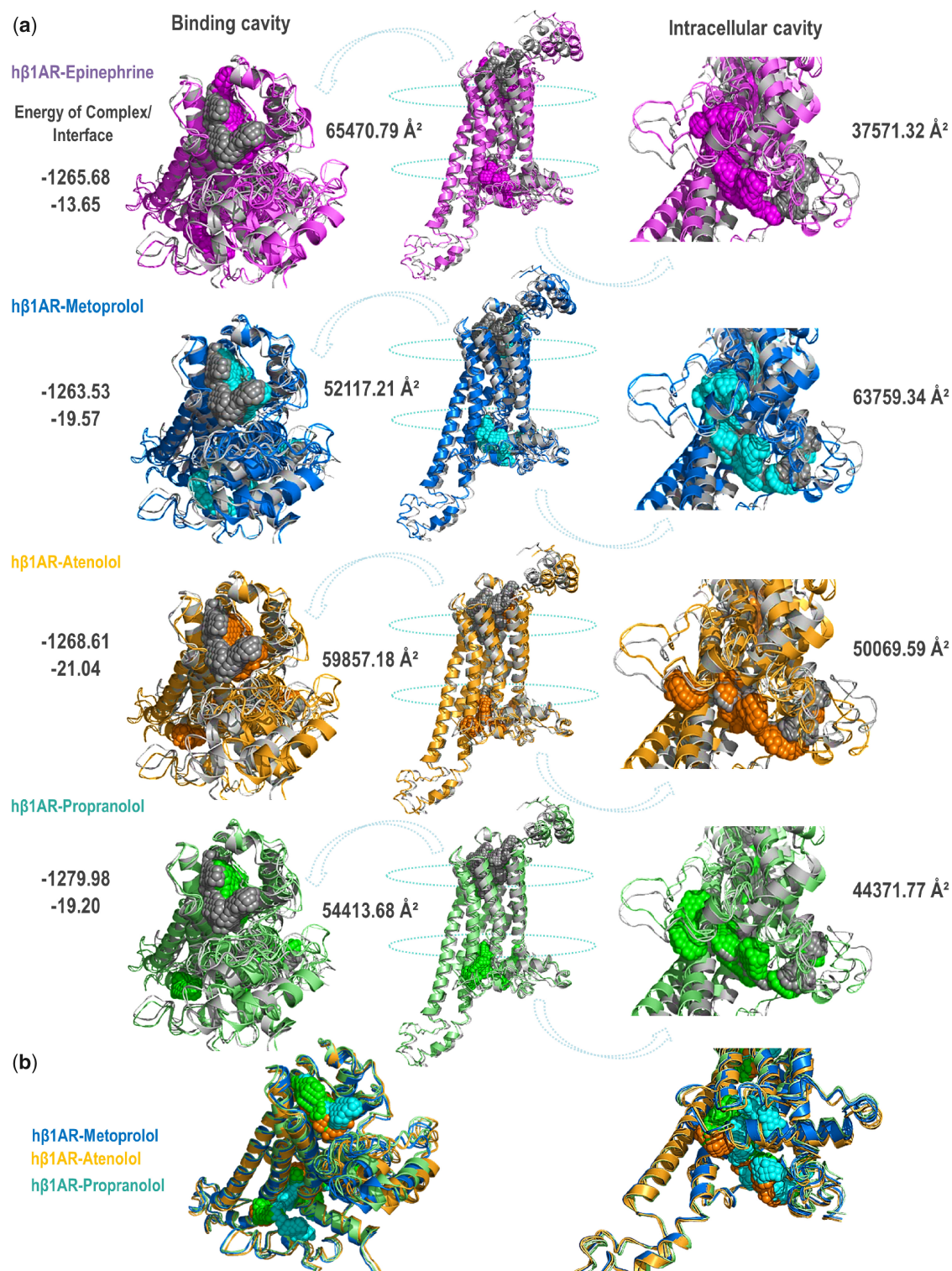


Figure 6 Metoprolol induces a conformational change in the human β 1AR that increases the size of the intracellular cavity. (A) Modelling of the human β 1AR modelling alone (grey) and bound to epinephrine (purple), metoprolol (blue), atenolol (orange), or propranolol (green). Each ligand-bound β 1AR conformation was compared to the unbound β 1AR conformation. Images were obtained with the PyMOL molecular visualization system. *In silico* analysis indicates that β 1AR conformational changes induced by metoprolol binding differ from those induced by the other ligands, producing an enlarged intracellular receptor cavity that is more open than that of the epinephrine-, atenolol-, or propranolol-bound receptor. (B) Superposition of all β -blocker-induced β 1AR conformations. The energies of the complex and interface are shown in Rosetta Energy Internal Units, whereas cavity sizes are shown in square Ångström Units (\AA^2).

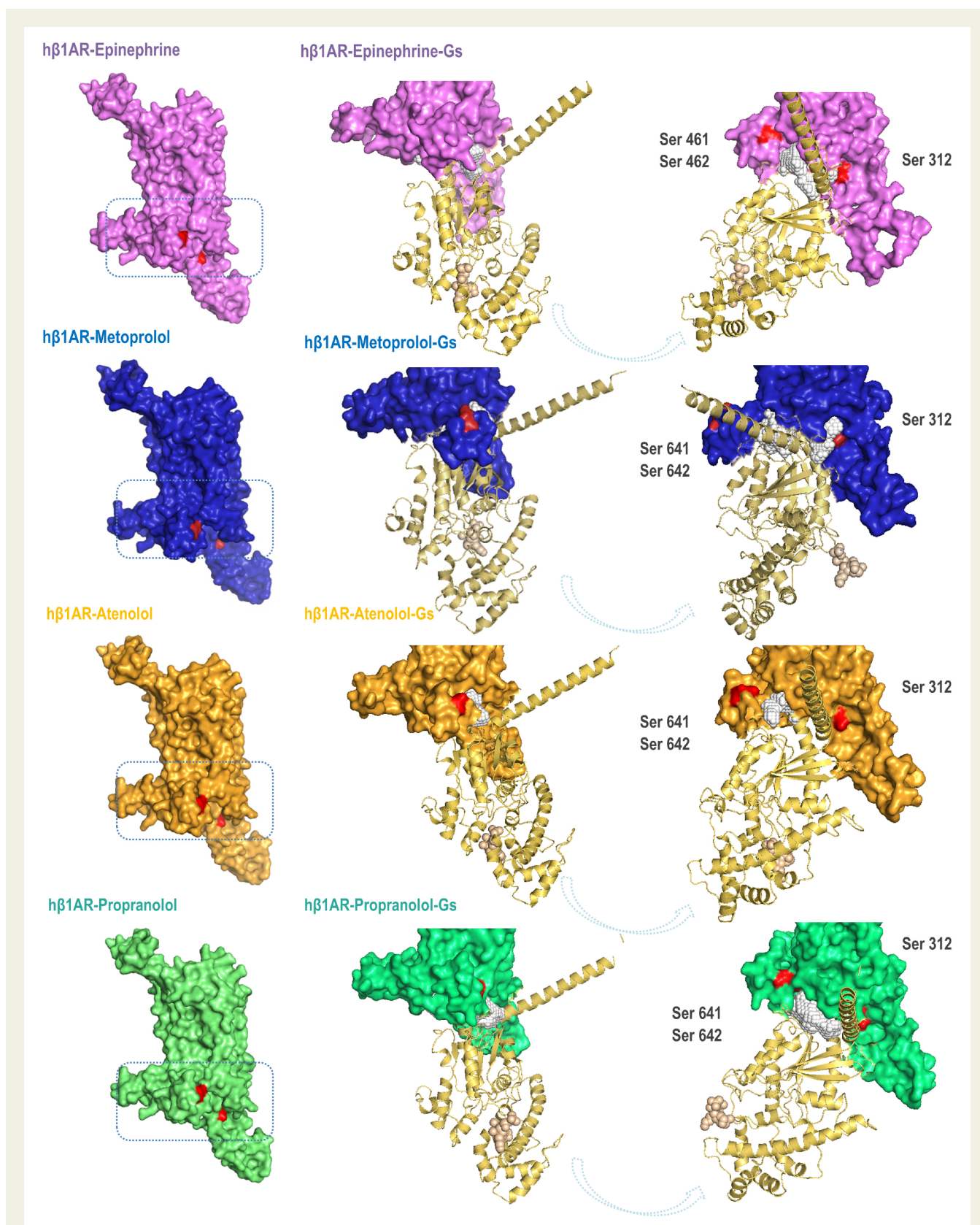
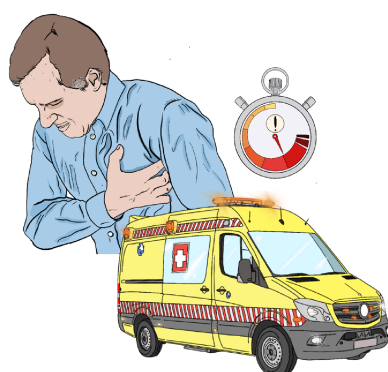


Figure 7 Ligand-human $\beta 1\text{AR-G}_s$ protein α subunit conformational changes. Modelling of the binding of the $\text{Gs}\alpha$ subunit to the complexes established upon docking of the different ligands (epinephrine, purple; metoprolol, blue; atenolol, orange; and propranolol, green) to the human $\beta 1\text{AR}$. The binding of the $\text{Gs}\alpha$ subunit to the metoprolol- $\beta 1\text{AR}$ complex exposes phosphorylation sites in the receptor potentially triggering GRK/ β -arrestin signalling cascade (see text). Red areas indicate Ser phosphorylation sites.

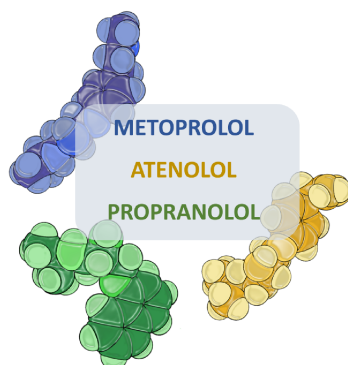
Summarizing Illustration "Metoprolol exerts a non-class effect on neutrophils against I/R injury"



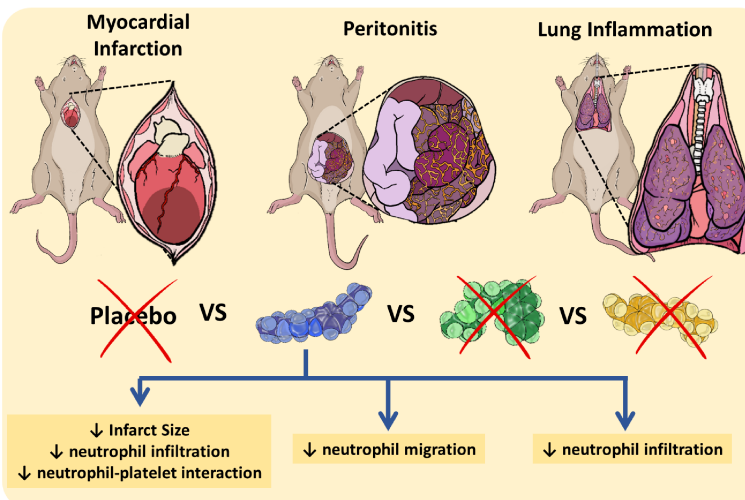
STEMI (I/R injury)

Early i.v β -blockers
→ IlaA recommendation

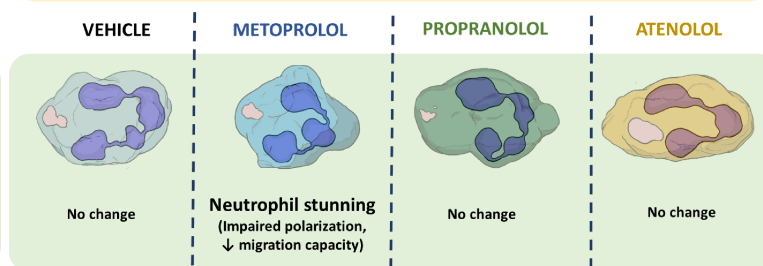
Are all equally protective?



In vivo models of exacerbated inflammation

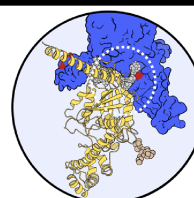
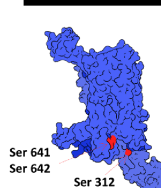


Neutrophil dynamics



In silico

METOPROLOL is unique in inducing conformational changes at the receptor level



- β 1AR enlarged intracellular cavity
-G α penetrates deeper
-Serines amenable for phosphorylation get exposed

↑ Biased agonism signaling pathway?

Take home figure Metoprolol exerts a particular protective effect against neutrophil-mediated ischaemia–reperfusion injury. The cardioprotective properties of metoprolol derive from its particular ability to target neutrophils and reduce ischaemia–reperfusion injury, whereas atenolol and propranolol have no effect on this cell population or on IS. Conformational changes induced in the β 1AR upon binding to metoprolol differ significantly from those induced by atenolol and propranolol, and this difference may underlie the neutrophil-stunning action of metoprolol. These data have important implications because clinical practice guidelines currently recommend the use of β -blockers during acute myocardial infarction as a drug class, making no distinction among them.

β 1KO mice.⁹ In the present study, we show that other β 1AR-selective β -blockers do not inhibit neutrophil migration *in vitro* or *in vivo*. This result is in line with a previous *in vitro* study, in which metoprolol but not atenolol reduced neutrophil migration.²¹ The lack of an inhibitory effect with another β 1AR-selective blocker prompted the authors to conclude that the metoprolol effect was independent of the β 1AR. However, the lack of an anti-migratory effect of metoprolol in β 1KO neutrophils suggests that the discrepancy between the effects of metoprolol and atenolol might be due to differences in the outcome of β -blocker– β 1AR interaction. Our *in silico* studies confirm

that the β 1AR undergoes different conformational changes upon binding to these different β -blockers.

The lack of an IS-reducing effect with propranolol appears to contradict a classical analysis showing smaller IS upon propranolol injection in a dog model of chronic coronary occlusion.^{22,23} However, there are important differences between that study and ours, the most important being that the canine model did not include reperfusion and thus did not examine IRI.^{22,23} Our work shows that metoprolol achieves its protective effect by targeting neutrophils, which are prominent mediators of reperfusion injury. In the absence of

reperfusion, the leading mechanism of death is ischaemic damage, in which neutrophils do not play such significant role.

The drugs used in this study were selected on the basis of their availability in i.v. formulations and their shared affinity for the β 1AR, with no other direct vasodilatory effect and with metoprolol and atenolol being more selective than propranolol.²⁴ This selectivity was particularly important to avoid interference from non-specific effects. Our results with the mouse IRI model unexpectedly establish that cardioprotection is not a β -blocker class effect and that metoprolol has a differential ability to limit IS by reducing neutrophil migration to cardiac tissue and impeding neutrophil–platelet interactions (Figure 1). The inhibitory effect of metoprolol on neutrophil–platelet interactions has been previously shown to be associated with less MVO⁹ (a major contributor to IS). The fact that atenolol and propranolol did not show any effect on these cell-to-cell interactions probably resulted in no effect on MVO. Unfortunately, in the present study we have not performed thioflavin-based MVO measurements to definitely demonstrate that only metoprolol breaks the axis neutrophil–platelet interactions-MVO-IS. This non-class effect was confirmed in the other *in vitro* and *in vivo* models of exacerbated inflammation examined. The transwell and acute peritonitis results show a characteristically strong blocking effect of metoprolol on neutrophil migration and infiltration that was not observed with atenolol or propranolol even at double the i.v. dose (Figure 2 and Supplementary material online, Figure S2). The ability of metoprolol to reduce neutrophil counts in BALF from mice with LPS-induced ALI (Figure 3) confirms that the protective effect is exportable to any inflammation setting. It is also significant that metoprolol attenuated histone three hypercitrullination in the ALI model (Figure 3 and Supplementary material online, Figure S4). Histone 3 hypercitrullination is a hallmark of the generation of NETs, extracellular fibrillary networks primarily composed of neutrophil chromatin. Neutrophil extracellular trap generation is a key feature of the acute inflammatory response in a variety of settings, such as atherothrombosis.²⁵ The ability to form NETs has recently been implicated in the organ damage and mortality associated with COVID-19.²⁶ Impaired NET formation in the ALI model appears to be due to the scarcity of neutrophils in the inflamed lung resulting from the disruptive effect of metoprolol on neutrophil recruitment.

The single-cell *in vivo* 2D and 3D IVM analyses confirm that metoprolol directly targets neutrophils. Metoprolol specifically induced erratic behaviour in neutrophils and altered morphological features required for tissue infiltration. The lack of any effect on these properties in the presence of atenolol or propranolol excludes any effect of atenolol and propranolol on this immune cell type (Figures 4 and 5).

Our previous results showed that the cardioprotective effect of metoprolol is mediated by the β 1AR, with no involvement of the β 2AR.⁹ We therefore focused the *in silico* analysis exclusively on the β 1AR. β -blockers are believed to act by occupying the β AR extracellular domain, thereby blocking ligand-dependent downstream cascade activation. Nevertheless, β -blockers with similar receptor affinities have been suggested to trigger different downstream effects. Our finding that neutrophil migration and infiltration are inhibited only with metoprolol suggests that its protective effect might involve more than simply blocking catecholamine interaction with the receptor. Indeed, the ability of metoprolol to inhibit neutrophil migration

in the *in vitro* transwell assays shows that this metoprolol action is not dependent on the presence of catecholamines.

Our *in silico* analysis clearly shows that metoprolol is the tested β -blocker able to induce a more significant change in β 1AR conformation, increasing the size of the intracellular cavity (Figure 6 and Supplementary material online, Tables S1 and S2). Large-scale rearrangement of GPCR residue side-chains can produce different receptor conformations that influence G-protein selectivity and generate differential effects on downstream signalling proteins.^{27,28} Our *in silico* analysis suggests that when metoprolol- β 1AR complex binds to G_s α protein induces a specific conformational change in β 1AR that affects its intracellular coupling interface, exposing Ser 461 and 462 phosphorylation sites and potentially modifying its interaction with diverse intracellular-binding partners, such as GRKs. A greater exposition of this site could boost the phosphorylation of these Serines by GRK2 (complex GRK2-G $\beta\gamma$) and mediate the recruitment of β -arrestins to the receptor, which uncouples the receptor from its G protein and initiates receptor internalization and desensitization. We speculate that activated β -arrestin through these conformational changes at the receptor level might initiate a biased agonism signalling pathway.

This conformational change may deactivate constitutive β 1AR function²⁹ or activate a specific signalling profile that eventually produces cardioprotection by neutrophil stunning. These *in silico* outcomes suggest recent pharmacological concepts, such as inverse or biased agonism^{30–32} as possible mechanisms underlying metoprolol-induced neutrophil stunning through β 1AR.

To date, no intervention aimed at reducing IS has demonstrated a solid clinical benefit in terms of hard endpoints reduction.³³ For the case of i.v. β -blockers in the acute phase of STEMI, the acute benefits in terms of cardioprotection and primary ventricular fibrillation reduction¹⁰ have not been translated into long-term clinical benefits, as shown in a recent meta-analysis including 1150 patients.³⁴ Several reasons might explain the lack of translation of cardioprotection into improved clinical benefits.^{35,36} The most obvious reason is the small sample size of all trials on the topic performed in the primary angioplasty era.³⁴ In addition, key aspects, such as type, dose, and timing of β -blocker administration varied significantly between trials included in the meta-analysis.³⁴ According to experimental data,¹¹ the trial using the ideal dose and timing of i.v. metoprolol administration was the METOCARD-CNIC study.¹⁰ While in this trial, acute infarct-limiting effect was associated with a reduction in long-term heart failure, the small sample size ($N = 270$) precludes a definite conclusion. Based on these clinical data, and supported by the results provided in the present study, a definite trial with adequate dose and timing of i.v. metoprolol administration (not other β -blocker) powered to detect clinical benefits is needed to determine the clinical benefits (hard endpoints) of this strategy in haemodynamically stable STEMI patients.

In summary, the present study indicates that β -blockers should not be considered a single drug class in the treatment of myocardial IRI. The cardioprotective effect of metoprolol is mediated by a targeting of neutrophils that is not shared by other β -blockers. These findings refine cardiovascular pharmacotherapy and have major implications for clinical cardiology.

Limitations

Extrapolation of our data to the clinical scenario is limited by the fact that we have used mouse models only. Validation of these data in a more translational animal model such as the pig would have been desirable, but beyond the scope of the present mechanistic study. The *in silico* studies performed have intrinsic limitations, such as the lack of modelling of all molecular dynamics occurring in the *in vivo* setting or the lack of consideration for dose–response effects. In addition, *in silico* findings were not biochemically validated. Future biological studies (e.g. study of GRK2-mediated Ser 461/462 phosphorylation) should confirm the proposed mechanism responsible for the differential effect of β -blockers on IS and other exacerbated inflammation outcomes observed here. In our study, we focused on the effect of β -blockers on neutrophils, but other cell types such as macrophages play a role in final IS. Dynamics of different macrophage subtypes, and the crosstalk between these and neutrophils,³⁷ impact post-MI healing, and it is plausible that metoprolol can affect these as well.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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Data availability

The individual data will be shared on reasonable request to the corresponding authors.

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Corrigendum

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