Pharmacologic modulation of intracellular Na⁺ concentration with ranolazine impacts inflammatory response in humans and mice

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Changes in Ca²⁺ influx during proinflammatory stimulation modulates cellular responses, including the subsequent activation of inflammation. Whereas the involvement of Ca²⁺ has been widely acknowledged, little is known about the role of Na⁺. Ranolazine, a piperazine derivative and established antianginal drug, is known to reduce intracellular Na⁺ as well as Ca²⁺ levels. In stable coronary artery disease patients (n = 51) we observed reduced levels of high-sensitive C-reactive protein (CRP) 3 mo after the start of ranolazine treatment (n = 25) as compared to the control group. Furthermore, we found that in 3,808 acute coronary syndrome patients of the MERLIN-TIMI 36 trial, individuals treated with ranolazine (1,934 patients) showed reduced CRP values compared to placebo-treated patients. The antiinflammatory effects of sodium modulation were further confirmed in an atherosclerotic mouse model. LDL⁻/⁻ mice on a high-fat diet were treated with ranolazine, resulting in a reduced atherosclerotic plaque burden, increased plaque stability, and reduced activation of the immune system. Pharmacological Na⁺ inhibition by ranolazine led to reduced expression of adhesion molecules and proinflammatory cytokines and reduced adhesion of leukocytes to activated endothelium both in vitro and in vivo. We demonstrate that functional Na⁺ shuttling is required for a full cellular response to inflammation and that inhibition of Na⁺ influx results in an attenuated inflammatory reaction. In conclusion, we demonstrate that inhibition of Na⁺⁻Ca²⁺ exchange during inflammation reduces the inflammatory response in human endothelial cells in vitro, in a mouse atherosclerotic disease model, and in human patients.

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Significance

Inflammation is a key process accompanying cardiovascular disease. Reducing inflammation is therefore an important therapeutic option. We provide evidence, that Na⁺ and Ca²⁺ modulation regulate the inflammatory response. Reducing intracellular Na⁺ pharmacologically using the drug ranolazine reduced the influx of Ca²⁺ during inflammation and thereby reduced the cellular production of inflammatory mediators. Similarly, reduction of extracellular Na⁺ and knockdown of a Na⁺⁻Ca²⁺ exchanger led to reduced inflammation. Our in vitro finding translated to in vivo experiments as ranolazine treatment led to reduced atherosclerotic plaque growth, increased plaque stability, and diminished inflammation in a mouse model. Finally, we were able to observe the antiinflammatory effect of Na⁺ modulation in human patients, demonstrating that inflammation was reduced after treatment with ranolazine.

Cardiovascular diseases (CVD) remain the number one cause of mortality in the Western world. According to the European Society of Cardiology, ischemic heart disease caused 45% of all deaths in females and 39% in males during the year 2019 (1). Similarly, the leading cause of death within the United States was heart disease (2). Patients with an underlying atherosclerotic process represent a substantial number among those individuals. The chance of recurrent vascular events was found to be of moderate to high risk of 59% in a large-scale model analysis (3, 4). The fact that atherosclerosis is not just a mere storage disease but rather a chronic inflammatory process has been known since the end of the last century (5), and numerous studies have shown that inflammatory markers like highsensitive C-reactive protein (hsCRP) predict cardiovascular events (6). The CANTOS trial was able to show that antiinflammatory therapy targeting the interleukin-1β (IL-1β) innate immunity pathway can lower the rate of recurrent cardiovascular events as compared to treatment with placebo in patients with a history of myocardial infarction and hsCRP ≥ 2mg/L. Importantly, these findings were independent of lipid levels and substantiate the importance of inflammation in the atherosclerotic process (7). However, the CANTOS trial also demonstrated the usability of hsCRP as an indirect marker as the significant reduction of vascular event rates was in proportion to the magnitude of hsCRP reduction achieved (8). The mode of action of canakinumab, the monoclonal antibody used in the CANTOS trial, is to prevent the downstream activation of IL-1β targets and the nuclear factor “kappa-light-chain-enhancer” of activated B cells (nuclear factor κB [NFκB]) pathway. A similar antiinflammatory strategy in CVD was presented in the Colchicine Cardiovascular Outcomes Trial (COLCOT) in patients within 30 d after myocardial infarction and in the Low-Dose Colchicine 2 (LoDoCo2) Trial involving patients with chronic coronary disease which indicated a positive effect of the antiinflammatory drug colchicine on the development of ischemic cardiovascular events (9). In a following substudy hsCRP levels were found lower in patients treated with colchicine compared to placebo (10).
The NFκB pathway plays a major role in the cellular inflammatory response and can be activated by numerous triggers, including IL-1β (11). Intracellular electrolyte shifts, especially Ca\(^{2+}\) influx, and the Ca\(^{2+}\) concentration in itself are thought to be among those factors propagating the inflammatory signal. The association of intracellular Ca\(^{2+}\) levels with the activation of the NFκB pathway was shown in various cell lines (12–14). Intracellular calcium signaling might require several proteins, including stromal interaction molecule (STIM) and Orai (15). Interestingly, Orai signaling is also associated with Na\(^{+}\) influx (16). To our knowledge, there are no clear data on the influence of intracellular Na\(^{+}\) concentration on NFκB activation in vascular cells or on the effects of modulation of Na\(^{+}\) to control inflammation in vivo or in vitro.

Ranolazine, a piperazine derivative and inhibitor of Na\(^{+}\) channel currents, is known to reduce intracellular Na\(^{+}\) and Ca\(^{2+}\) levels in cardiomyocytes (17). Even though ranolazine is indicated in patients with stable angina [class IIa recommendation for symptom relief in the American College of Cardiology/American Heart Association guidelines (18)], no information of ranolazine treatment regarding inflammation or atherosclerotic disease progression is available and a potential finding of inflammation-modulating effects might be highly relevant in such patients. The purpose of this study was to investigate possible pleiotropic effects of an established antianginal drug in vivo and to evaluate a potential Na\(^{+}\)- and Ca\(^{2+}\)-dependent inflammatory response via the NFκB pathway in endothelial cells in vitro.

Results

Ranolazine Reduces CRP in Patients with Stable Coronary Artery Disease and in Patients after Non-ST-Elevation Acute Coronary Syndromes. Ranolazine is a well-described antianginal drug, its main function being the reduction of intracellular Na\(^{+}\) content by inhibition of late Na\(^{+}\) channel currents. To evaluate possible antiinflammatory effects of the Na\(^{+}\)-modulating drug ranolazine in humans, we included 51 patients with chronic coronary artery disease (CAD) that were at least 12 mo after a myocardial infarction and analyzed serum levels of hscRP at baseline and after 12 wk of treatment. Patients were randomized to ranolazine (375 mg twice daily for 4 wk with 500 mg twice daily for the remainder of the study, \(n = 25\)) or to control treatment (\(n = 26\)). Baseline characteristics of the patients are presented in SI Appendix, Table 1. We observed significantly reduced circulating levels of hsCRP in patients 12 wk after receiving ranolazine (\(P = 0.01\) baseline vs. 12 wk; Fig. 1A) whereas the control group did not show changes in hsCRP serum levels (\(P = 0.90\) baseline vs. 12 wk), resulting in a significant higher decrease in the ranolazine group by −37.1%, interquartile range (IQR) −77.5% to +23.6% as compared to the control group (+ 0.2%, IQR −39.1 to +107.6%; \(P = 0.044\)).

To further support this antiinflammatory finding, we analyzed data from the MERLIN-TIMI 36 trial that determined the effect of ranolazine in patients with non-ST-elevation acute coronary syndrome (ACS) (19). CRP was determined in a total of 3,808 patients at baseline and after 2 wk of treatment with ranolazine or placebo. Patients were included within 48 h after the ACS, a condition that is well known to activate the acute-phase reaction. As expected, a decrease of CRP was observed in both groups 2 wk after the ACS. However, this decrease was more pronounced in patients treated with ranolazine (−18.7%, IQR −63.5% to +95.7%) compared to placebo (−7.2%, IQR −57.5% to +98.1%; \(P = 0.013\)), resulting in significantly lower CRP levels in patients treated with ranolazine as compared to controls (\(P = 0.029\); Fig. 1B).

![Fig. 1. Data from CVD patients treated with ranolazine.](https://doi.org/10.1073/pnas.2207020119)

| CRP at baseline | Ranolazine (\(n = 25\)) | CRP after treatment | P-value |
|-----------------|------------------------|---------------------|---------|
| hsCRP serum levels | 5.35 (2.38 - 12.56) mg/l | 5.35 (2.38 - 11.87) mg/l | \(P = 0.009\) |
| % change | 4.95 (3.11 - 10.83) mg/l | 4.53 (1.96 - 10.40) mg/l | \(P = 0.029\) |

![Fig. S1A](https://doi.org/10.1073/pnas.2207020119)

| Control (\(n = 25\)) | Ranolazine (\(n = 25\)) | \(P\)-value |
|-----------------------|------------------------|----------|
| CRP at baseline | 5.35 (2.38 - 12.56) mg/l | 5.35 (2.38 - 11.87) mg/l | - |
| CRP after treatment | 4.95 (3.11 - 10.83) mg/l | 4.53 (1.96 - 10.40) mg/l | \(P = 0.029\) |
| % change | 7.22% (57.5% - 98.0%) | 18.74% (63.49% - 95.68%) | \(P = 0.013\) |

Ranolazine Reduces Plaque Size and Increases Plaque Stability in Low-Density-Lipoprotein Receptor Knockout Mice. To understand the effect of ranolazine treatment on inflammatory disease progression, we determined the effect of ranolazine in an atherosclerosis model employing low-density-lipoprotein receptor (LDL-R) knockout mice. We started a high-fat diet 6 wk before ranolazine treatment in order to use an animal model with an already established atherosclerotic plaque. We did not observe differences in weight, LDL, or triglyceride levels comparing control and ranolazine-treated animals at the end of the study (SI Appendix, Fig. S1A). We further found no difference in liver histology samples including apoptosis of liver cells or changes in fibrosis in animals treated with ranolazine compared to control animals (SI Appendix, Fig. S1B–D). Similarly, oxidized LDL was not altered in the liver (SI Appendix, Fig. S1E).

Overall plaque burden was evaluated using en face staining of the total aorta. Ranolazine treatment led to a significant reduction of overall plaque burden (Fig. 2A). When analyzing the composition of the atherosclerotic plaque we found a reduction in cholesterol clefts in ranolazine-treated animals (Fig. 2B). This reduction was accompanied by an increased thickness of the fibrous cap area (Fig. 2C) and a reduced content of the atherosclerotic plaque. Consequently, also the total collagen content was increased in ranolazine-treated mice (SI Appendix, Fig. S1E). Structural integrity was increased in ranolazine-treated animals as indicated by a reduction of total collagen–positive cells within the atherosclerotic region (Fig. 2D). However, increased stability was not caused by an enhancement of smooth muscle content within the plaque region, as ranolazine-treated animals showed a reduced number of α smooth muscle actin–positive cells within the atherosclerotic region (Fig. 2E). Supporting a more stable plaque phenotype are data on cell apoptosis, as atherosclerotic lesions from ranolazine-treated animals showed a strong reduction in apoptotic cells as indicated by cleaved caspase 3 staining (Fig. 2F).
For a closer analysis on changes of the immune cell compartment within the atherosclerotic lesion, we stained the plaque for CD45 to determine the global infiltration of immune cells into the lesion. We observed a reduction in CD45+ cells within the plaque in ranolazine-treated animals (Fig. 3A). This reduction was also mimicked when analyzing atherosclerotic lesions for CD68+ cells (Fig. 3B). Similarly, foam cells indicated by CD146 staining were reduced in ranolazine-treated animals (Fig. 3C). Reduction of foam cells was paralleled by a reduction in oxidized LDL (oxLDL) within the lesion (SI Appendix, Fig. S1F). Neutrophils characterized by myeloperoxidase staining also showed a significant reduction in ranolazine-treated animals (Fig. 3D). In contrast, we did not observe a difference for CD4+ immune cells within the atherosclerotic region but for CD8+ cells (Fig. 3E and F). The interaction and migration of immune cells into the atherosclerotic lesion is mediated by interacting with endothelial cell expressed adhesion molecules, including intercellular adhesion molecule 1 (ICAM-1) and endothelial selectin (E-selectin). We found that plaques from mice treated with ranolazine displayed reduced content of both adhesion molecules (Fig. 3G and H).

**Ranolazine Treatment Reduces Endothelial Expression of Adhesion Molecules and Inflammatory Cytokines In Vitro.**

The data so far indicated reduced content of the atherosclerotic lesion with immune cells and reduced expression of adhesion molecules. To test if the observed effect is directly related to ranolazine treatment, we analyzed the effects of ranolazine...
on inflammation-induced adhesion molecule expression in endothelial cells in vitro. Ranolazine treatment of human umbilical vein endothelial cells (HUVEC) led to a reduction of E-selectin (P = 0.026), vascular adhesion molecule (VCAM-1) (P < 0.01), and ICAM-1 (P < 0.01) messenger RNA (mRNA) 2 h after IL-1β stimulation (Fig. 4A). We were able to confirm mRNA data on protein level using flow cytometry (Fig. 4B). The observed reduction of adhesion molecules in HUVEC was dose-dependent, with increasing concentrations of ranolazine showing a dose-dependent reduction of the respective adhesion molecule’s expression (Fig. 4C). In addition, this reduction was not only true for HUVEC but also for human coronary endothelial cells (hCAECs; Fig. 4E). A robust reduction of adhesion molecules could also be observed using immunohistochemistry (Fig. 4D).

To determine if the reduced expression of adhesion proteins would lead to a reduction of endothelial cell interaction with polymorphonuclear leukocytes (PMN), we performed a static adhesion assay in vitro. Indeed, ranolazine treatment led to a strong reduction of PMN adhering to the endothelial cell layer after IL-1β challenge of HUVEC (Fig. 4E). To confirm that this in vitro phenotype can also be observed in vivo, we used intravital microscopy on mesenteric arterioles. The arterioles were exteriorized and IL-1β was applied topically in animals with or without previous ranolazine treatment (treatment period 7 d). The number of rolling and/or adhesive leukocytes was reduced in arterioles over a 30-min observation time, resulting in an overall reduction of average adhesion of leukocytes in vivo (Fig. 4G and Movie S1).

The main induction pathway of endothelial adhesion molecules by IL-1β is via the activation of NFκB. To understand further possible modulations within this pathway we analyzed the expression of proinflammatory cytokines IL-6, IL-8, and monocyte chemoattractant protein-1 (MCP-1) as well as the procoagulatory protein tissue factor (TF). All analyzed cytokines were robustly induced by IL-1β and ranolazine reduced their expression (Fig. 5A and B and SI Appendix, Fig. S2A and B). RNA data were further confirmed on protein level for IL-6, IL-8, and TF (Fig. 5C and D and SI Appendix, Fig. S2C). Given this close interaction with NFκB targets we analyzed the phosphorylation of IκB-α, which is required for activation of the NFκB downstream signaling pathway. Indeed, phosphorylation was reduced in endothelial cells treated with ranolazine (Fig. 5E). In addition, translocation of the p65 subunit of NFκB into the nucleus was reduced after treatment with ranolazine (Fig. 5F). To determine if these changes in proinflammatory cytokines would also be reflected in vivo we analyzed the plasma levels of MCP-1, tumor necrosis factor α (TNF-α), IL1-β, IL-18, and IL-6. Overall, we observed a trend for lower levels of inflammatory cytokines in ranolazine-treated mice reaching statistical significance for IL-18 and IL-6 (Fig. 5G). Furthermore, ranolazine did not exhibit toxic properties in the performed lactate dehydrogenase (LDH) cytotoxicity assay (SI Appendix, Fig. S2D).

**The Antiinflammatory Effects of Ranolazine Are Mediated by Modulation of Intracellular Sodium and Calcium Levels.** To determine if the observed antiinflammatory effects of ranolazine are due to modulation of Ca²⁺ and Na⁺ levels, we analyzed the respective ions’ concentrations in endothelial cells. Treatment of endothelial cells with ranolazine for 10 min led to a significant reduction of intracellular Na⁺ (SI Appendix, Fig. S3A and B). When endothelial cells were stimulated with IL-1β we observed a gradual decrease of Na⁺ signaling over time. This effect was augmented by ranolazine treatment and the reduction of Na⁺ was significantly stronger (Fig. 6A and B). This resulted in significant lower Na⁺ concentrations in endothelial cells treated with ranolazine after 10 min of observation (Fig. 6C). When analyzing Ca²⁺ levels in endothelial cells stimulated with IL-1β with and without ranolazine treatment, we found lower Ca²⁺ levels in ranolazine-treated cells already at baseline (Fig. 6D). Furthermore, in contrast to untreated cells, we did not observe an increase of intracellular Ca²⁺ upon stimulation with IL-1β in ranolazine-treated cells, which is reflected by a significantly smaller area under the curve of Ca²⁺ concentrations in ranolazine-treated cells during the IL-1β stimulation period compared to IL-1β effects alone (Fig. 6E). When analyzing the relative change of Ca²⁺ over time normalized to its respective starting value, a clear inhibition of Ca²⁺ cellular entry due to ranolazine treatment was observed (Fig. 6F). Given our data on sodium and calcium entry and exit during inflammatory stimulation, we hypothesized that sodium is required for calcium entry into the cells as reducing levels of sodium by ranolazine affects calcium influx during IL-1β stimulation.

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**Fig. 4.** In vitro effects of ranolazine treatment on inflammation in endothelial cells. Ranolazine treatment of HUVEC reduces E-selectin, VCAM-1, and ICAM-1 expression after IL-1β stimulation on mRNA (A) as well as protein level as detected by flow cytometry (B and C) or immunohistochemistry (D). Flow cytometry was confirmed in hCAECs (E). Ranolazine treatment reduced granulocyte adhesion to HUVEC in vitro (F). Intravital microscopy on mesenteric arterioles of C57BL6 mice showed a reduction of rolling and/or adhesive leukocytes in vivo upon topical application of IL-1β after treatment with ranolazine as compared to placebo for 7 d (G and Movie S1). Data are presented as mean ± SD and P values <0.05 are considered statistically significant. *P < 0.05; **P < 0.01.
stimulation. To further analyze the direct effect of sodium on propagation of inflammation we used an extracellular solution (ES) with a defined Na⁺ content with or without a 70% reduction of sodium by replacement with N-methyl-D-glucamine (NMDG). When stimulating endothelial cells with IL-1β in ES we observed a significant reduction of induction of E-selectin and VCAM-1 in Na⁺-reduced conditions (black bars) compared to physiological sodium concentrations (white bars, Fig. 6 G and H). Treatment of cells with ranolazine in ES before stimulating with IL-1β in Na⁺-reduced medium did not further increase the reduction of E-selectin and VCAM-1 observed in ES medium alone (Fig. 6 G and H). However, under ES conditions we did not observe an alteration of ICAM-1 induction (Fig. 6 I). To support our data indicating a requirement of Na⁺ shunting during inflammatory stimulation, we performed small interfering RNA (siRNA)-mediated knockdown of the Na⁺-Ca²⁺ exchanger SLC8A1. To test for a possible inflammation modulating capacity of SLC8A1 knockdown, we analyzed endothelial cells 2 h after stimulation with IL-1β for E-selectin, VCAM-1, and ICAM-1. A significant drop in the respective mRNA levels after SLC8A1 knockdown was observed when compared to the control siRNA group (E-selectin, reduction by 30.0 ± 17.85%, P < 0.01; VCAM-1, reduction by 36.19 ± 25.54%, P < 0.01; ICAM-1, reduction by 19.20 ± 15.28%, P < 0.01).

Discussion
Ranolazine showed antiinflammatory properties in patient cohorts with chronic CAD and after non-ST-elevation myocardial infarction, in in vivo mouse models, and in endothelial cells in vitro. We suggest that ranolazine reduces inflammation via depletion of Na⁺ in cells and hence a decreased capacity of Ca²⁺ entry triggered by inflammatory cell stimulation.

The CANTOS, COLOCOT, and LoDoCo2 trials demonstrated that antiinflammatory therapy is successful in patients with CAD (7, 9, 20). Mechanistically, due to the use of a specific antibody versus IL-1β, the CANTOS trial further demonstrated a causal link between inflammation, in particular IL-1β, and the development of ischemic cardiovascular events (7). Given this interest in antiinflammatory therapy, we therefore tested the effect of ranolazine treatment on patients with stable CAD prior to and 12 wk after ranolazine treatment on circulating levels of hsCRP. After 12 wk of treatment hsCRP was significantly reduced. In 3,808 ACS patients of the MERLIN-TIMI 36 trial hsCRP serum levels at baseline and after 2 wk of treatment were available. As expected, hsCRP levels decreased in both groups 2 wk after the ACS. However, the reduction of hsCRP was much more pronounced in patients with ranolazine treatment. The MERLIN-TIMI 36 trial did not show a significant effect of ranolazine treatment on major acute cardiovascular events. However, the survival curve of ranolazine showed a trend for an overall reduction of cardiovascular events after one and a half years, similar to the survival curves in the CANTOS trial at this time. The CANTOS trial was evaluated after a median follow-up of 44.4 mo, whereas the median follow-up in the MERLIN-TIMI 36 trial was only 11.5 mo. Similarly, the LoDoCo2 trial was conducted over a 60-mo time span. We speculate that antiinflammatory treatment needs to be observed for a rather long period in order to show effects on cardiovascular events and that possible antiinflammatory effects on cardiovascular events in the MERLIN-TIMI 36 would have become evident only after several years of treatment and follow-up. However, in the MERLIN-TIMI 36 trial ranolazine was associated with a significant reduction of recurrent ischemia that could possibly reflect positive effects of ranolazine on plaque remodeling (19).

In addition, we tested in an animal model if the observed reduction of inflammation in human patients might be reflected in reduced atherosclerotic plaque progression and inflammation in a mouse model using a dose comparable to 1,500 mg/d in human patients whereby the drug dose was calculated according to Reagan-Shaw et al. from the human daily dose using weight and body surface factors for human-to-mouse conversion rather than relying on a simple extrapolated human-equivalent dose (21). Our results demonstrated reduced atherosclerotic plaque burden and increased plaque stability in animals receiving ranolazine compared to the control group. Specifically, we found that the reduced lesion size was paralleled with an increase in fibrous cap thickness. This, however, was not paralleled with an influx of smooth muscle cells, as we found reduced numbers of α smooth muscle actin–positive cells within the lesion. This reduction might further benefit the stability of the atherosclerotic plaque as smooth muscle cells were reported to be plastic within the lesion and transdifferentiate into proinflammatory, macrophage-like cells (22). A...
less-inflammatory phenotype of smooth muscle cells is also indicated by a reduction in elastin fiber fragmentation in our ranolazine-treated mice, which is related to inflammation and might reflect a more stable smooth muscle cell phenotype (23, 24). Furthermore, an overall less-active atherosclerotic lesion in these mice is also suggested by the reduced numbers of apoptotic cells within the atherosclerotic lesion, which is linked to an increased atherosclerotic plaque stability (25). In addition, lesions of animals treated with ranolazine showed reduced infiltration with innate and adaptive immune cells. In parallel to the observed reduction in cholesterol clefts we also found a reduction in plaque-resident foam cells. Especially, innate immune cells including monocytes and macrophages are associated with amplifying inflammation in atherosclerosis in part by IL-1β production (24, 26). A key event in atherosclerotic plaque progression is the migration of immune cells into the lesion. This entry is dependent on vascular adhesion molecules including ICAM, VCAM, and E-selectin (27). When analyzing ICAM and E-selectin protein in atherosclerotic lesions we observed a significant reduction in both proteins in the

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**Fig. 6.** Role of sodium and calcium during inflammation in endothelial cells in vitro. IL-1β stimulation of HUVEC leads to a decrease of Na⁺ signaling over time which was more pronounced in ranolazine-treated cells (A and B), resulting in lower Na⁺ concentrations in ranolazine-treated cells after 10 min (C). Cytoplasmic Ca²⁺ levels over time in endothelial cells under ranolazine treatment and IL-1β stimulation are depicted in D. Ranolazine treatment leads to a significant reduction of Ca²⁺ at baseline and following IL-1β stimulation (D–F). Following IL-1β stimulation, adhesion molecule expression in HUVEC is found decreased in Na⁺ reduced medium (black bars) as compared to medium containing physiological concentrations of Na⁺ (white bars, G–I). Additional ranolazine treatment did not further decrease adhesion molecule expression (black bars, G–I). The dotted lines represent adhesion molecule expression in unstimulated HUVEC under physiological concentrations of Na⁺. Data are presented as mean ± SD (G–I) or SEM (A–F) and P values <0.05 are considered statistically significant. Sodium data were generated from 986 control cells and 844 cells treated with ranolazine, calcium staining was evaluated in 1,562 control cells and 1,187 ranolazine-treated cells.
ranolazine-treated animals, suggesting a possible mode of action of ranolazine in modulating the atherosclerotic lesion.

Ranolazine is a well-documented antianginal drug, its main function being the reduction of intracellular Na+ content by inhibition of late Na+ channels in cardiac myocytes (28). The major mechanism of ranolazine is to prevent the Na+ overload of the cell inhibiting late INa and as a consequence preventing reverse-mode Na+-Ca2+ exchange (28, 29). To confirm that ranolazine has a direct effect on modulating inflammation, we used an in vitro cell culture approach to determine the effect of cotreatment of endothelial cells with ranolazine and IL-1β. We observed a reduction of adhesion molecules and proinflammatory cytokines when cells were treated with ranolazine concomitantly with the inflammatory agent. The increased adhesion of cells to endothelial cells both in vitro and in vivo was reduced after treatment with ranolazine, suggesting that the reduced occurrence of immune cells within the atherosclerotic lesion is in part mediated by the reduction of adhesion molecules in inflammatory activated endothelial cells. Through the reduced recruitment of these cells, subsequently the lesion has reduced inflammatory potential and therefore shows a more stable phenotype.

To confirm that the antiinflammatory effect of ranolazine is a direct consequence of a modulation of Ca2+ and Na+ levels, we analyzed respective ion concentrations in endothelial cells. Indeed, we were able to observe a reduction of baseline Na+ levels in ranolazine-treated endothelial cells. Besides this basal reduction we also observed a significantly increased Na+ loss from endothelial cells when treated concomitantly with IL-1β and ranolazine compared to cells treated with IL-1β alone. In parallel, Ca2+ levels did not increase after inflammatory stimulation but remained at baseline levels in ranolazine-treated cells. Previously, reports already demonstrated that Ca2+ shuttling is closely intertwined with NfKB activation (30). Specifically, the oscillation of the Ca2+ signaling reduces the effective Ca2+ threshold for activating transcription factors, whereby NfKB is already activated by infrequent oscillation (31). Na+ was already proposed to promote the movement of Ca2+ into the cell via a Na+-Ca2+ exchange during cell activation (32). Indeed, reducing Na+ content within a cell leads to Ca2+ influx (33). To further test the importance of active Na+ shuttling during inflammatory stimulation we used a defined buffer environment to reduce extracellular sodium and hence inhibit the pool of available Na+, mimicking the pharmacologic effect of ranolazine. These experiments confirmed that Na+ shuttling is crucial for a full inflammatory response. This cross-talk requirement of Ca2+ and Na+ was also observed when we analyzed cells with a knockdown of the Na+-Ca2+ exchanger SLC8A1 and could show an induction of adhesion molecules after IL-1β stimulation. The exchangers operate in a bidirectional mode of the cell inhibiting late INa and as a consequence preventing reverse-mode Na+-Ca2+ exchange (28, 29). To confirm that ranolazine has a direct effect on modulating inflammation, we used an in vitro cell culture approach to determine the effect of cotreatment of endothelial cells with ranolazine and IL-1β. We observed a reduction of adhesion molecules and proinflammatory cytokines when cells were treated with ranolazine concomitantly with the inflammatory agent. The increased adhesion of cells to endothelial cells both in vitro and in vivo was reduced after treatment with ranolazine, suggesting that the reduced occurrence of immune cells within the atherosclerotic lesion is in part mediated by the reduction of adhesion molecules in inflammatory activated endothelial cells. Through the reduced recruitment of these cells, subsequently the lesion has reduced inflammatory potential and therefore shows a more stable phenotype.

Materials and Methods

Mouse Treatment. Twenty-five male LDL-R knockout mice [LDL-R (−/−) mice] on a C57/Bl6 background were used. At the age of 12 wk, mice were put on a high-fat diet (Sniff). At the age of 18 wk, they were randomly allocated to a treatment/control group consisting of 12 and 13 animals, respectively. The treatment group received ranolazine 300 mg kg−1·d−1 via tap water. The drug dose was calculated according to Reagan-Shaw et al. from the maximum human daily dose using weight and body surface rather than relying on a simple extrapolated human equivalent dose (ranolazine 1,500 mg/d) (21). Mice in the control group received tap water only. At the age of 26 wk, mice were killed and the extent of the aortic plaque was analyzed. Additionally, serum was stored frozen and livers were harvested for paraffin embedding and later analysis. Furthermore, 11 wild-type mice on a C57/Bl6 background (5 female, 6 male at the age of 5 wk) were randomly allocated to receive the same dose of ranolazine (n = 5) or tap water (n = 6) for 1 wk. They were subsequently used to determine leukocyte adhesion in vivo. Experiments were carried out in accordance with the ethics committee (GZ.66.009/0172-WF/V/3b/2016 and GZ2020-0.547.806). The three R’s of ethics in animal testing were followed and taken into account while planning all experiments.

Atherosclerotic Plaque Staining: Size and Composition. Harvesting of the whole aorta was done according to a prior established protocol (36). After the process of cleaning and pinning the aorta to a paraffin plate, Sudan IV (Merck) staining was used to visualize the atherosclerotic plaque. Evaluation of the en face stainings was carried out by a blinded member of the study team using ImageJ software (NIH). Preparation of the aortic root was done according to a prior established protocol (37). Collagen content, necrotic core size, and fibrous cap thickness were determined by Masson’s trichrome staining (Merck). Elastin fragmentation was visualized using a respective staining kit (Merck). Moreover, anti-α smooth muscle actin antibody (anti-α-Sma; 1; Abcam) and cleaved caspase-3 antibody (Thermo Fisher Scientific) were utilized according to the manufacturer’s instructions.

Atherosclerotic Plaque Staining: Immune Cell Composition. CD45-positive cells were stained using a suitable antibody (Biolegend). Macrophage content of the atherosclerotic lesion was determined using an anti-CD68 antibody (Abcam). Additionally anti-CD146, anti-CD4 (both Abcam), anti-CD8 (Bioss Antibodies), and MPO (R&D Systems) antibodies were used for the differentiation of cell populations within the plaque. ICAM-1 as well as E-selectin (both Biolegend) staining was performed to visualize adhesion molecules on the plaque surface/area. A Zeiss Axio observer microscope equipped with a Hamamatsu Orca Flash (Hamamatsu) was used for fluorescence microscopy, whereas a Zeiss LSM 700 (Carl Zeiss AG) was utilized for broad-field imaging. Tissue sections were automatically analyzed using TissueFAXS (TissueGnostics) and ImageJ (NIH). All analyses were carried out by a blinded member of the study team.

Cell Culture: HUVEC and Coronary Endothelial Cells. HUVEC were isolated from fresh umbilical cords and cultured thereafter (38). hCAECs (Lonza Bioscience) were cultivated similarly, in accordance with a preestablished protocol (39). For all experiments, three different pools, consisting of cells from at least five independent donors, were used. All human material was obtained and processed according to the recommendations of the hospital’s ethics committee and security board. In particular, HUVEC were obtained and processed in accordance with the requirements of the local ethics committee including informed consent.
Flow Cytometry: HUVEC and hCAECs. Following 30 min of preincubation with rano-
lazine at indicated concentrations, cells were stimulated with IL-1β (200 U/mL) for a duration of 2 h. mRNA was isolated using High Pure RNA Isolation Kit (Roche). For obtaining complementary DNA (cDNA), reverse transcription was performed using Transcripter First Strand cDNA Synthesis Kit (Roche). qPCR was performed using a LightCyclerTaqMan Master (Roche) system according to the manufacturers’ instructions. Primer sequences are given in SI Appendix, Table 3.

Adhesion Assay In Vitro. PMN adhesion to HUVEC under static conditions was determined as described previously (40). For isolation, a human whole-

mRNA Purification and qPCR. Following 30 min of preincubation with rano-
lazine at indicated concentrations, cells were stimulated with IL-1β (200 U/mL) for a duration of 2 h. mRNA was isolated using High Pure RNA Isolation Kit (Roche). For obtaining complementary DNA (cDNA), reverse transcription was performed using Transcripter First Strand cDNA Synthesis Kit (Roche). qPCR was performed using a LightCyclerTaqMan Master (Roche) system according to the manufacturers’ instructions. Primer sequences are given in SI Appendix, Table 3.

Adhesion Assay In Vivo. Intravital microscopy of IL-1β-induced leukocyte adhesion was performed according to an adapted protocol (41). Eleven 5-wk-old C57/BL6 mice were anesthetized; leukocytes were labeled in vivo with Alexa Fluor 488 anti-mouse CD45 antibody (0.2 μg/g; Biolegend) and mesenteric ves-
s was exteriorized through abdominal incision under total anesthesia. Inflamm-
atory activation of mesenteric arterioles was induced by topical application of one drop of IL-1β (200 U/mL; Merck) with a Whatman filter paper. Adhesive and/or rolling leukocytes were defined as labeled cells being traceable within the field of observation for a duration of 10 or more seconds. All arterioles (35 to 60 μm) were monitored with an Olympus IX71 microscope (Visirot Systems) using a Cach N 10x/0.25 PhN22 UIS-2 objective (Olympus) and iXON Life (Andor) for 40 min. Two mesenteric arterioles were analyzed per animal.

Immunofluorescence Microscopy. Staining of adhesion molecules on endo-

thelial cells was performed as published previously (40). Cells were incubated with primary antibodies for E-selectin (R&D Systems), ICAM-1 (R&D Systems), and VCAM-1 (BD Pharmingen) and Alexa Fluor 488-labeled goat anti-mouse IgG (Thermo Fisher Scientific) as a secondary antibody.

Protein Determination. Enzyme-linked immunosorbent assays (ELISAs) for IL-6 (R&D Systems), IL-8, and hωx (both Thermo Fisher Scientific) were used to measure the respective protein in HUVEC supernatants (4 h), whereas intranuclear p65 (Active Motif) was determined by ELISA from nuclear extract according to the manufacturer’s instructions. ELISAs for MCP-1, TNF-α, IL-1β (all LEGEND-
plex; Biolegend), IL-18, and IL-6 (both R&D Systems) were used to measure the respective protein in mouse serum. Furthermore, triglycerides (Abcam), LDL lev-
els (MyBioSource), and LDH (CytoTox 96; Promega) were determined by colori-
metric assays.

Measurement of Intracellular Sodium and Calcium. The ES used for cellular experiments (contained in millimolar) 145 NaCl, 5 KCl, 10 glucose, 10 Hepes, 1.25 CaCl2, and 1 MgCl2 (Merck), buffered to pH 7.4 with NaOH and had an osmolarity of 300 mosm. The 30% sodium-containing buffer was obtained by correspondingly replacing NaCl with NMDG and contained (in milli-
molar) 43.5 NaCl, 101.5 NMDG, 5 KCl, 10 glucose, 10 Hepes, 1.25 CaCl2, and 1 MgCl2 (Merck).

The coverslips were incubated with Fura-2 AM ester (Biotium) for 30 min at 37 °C and 5% CO2, before placement in glass-bottom 35-mm dishes in ES and a recovery period of 10 min. Then, they were mounted onto an Olympus IX73 inverted microscope and imaged using a 10x objective. Cells were permanently superfused with ES using a software-controlled, eight-channel, gravity-driven, common-outlet system (ALA Scientific Instruments Inc.). This superfusion was switched to different solutions after adequate baseline recording. A positive con-
trol detecting viable cells was added at the end of each recording, using ionomy-
cin 3 μM. Fura-2 was alternatingly excited for 30 ms by a 340-nm light-emitting diode (LED) (50 mW, used at 100%) and by a 385-nm LED (1,435 mW, used at 5%) using an Omicron LEDHub (Laserage-Laserprodukte GmbH). Fluorescence emission was long-pass filtered at 495 nm, and pairs of images were acquired at a rate of 1 Hz with a 4.2-megapixel 16-bit charge-coupled device camera (6.5 μm pixel edge length, 18.8 mm sensor diameter, Prime BSI; Teledyne Pho-
tometers). The hardware was controlled by the μManager 1.4 plugin in ImageJ (NIH). The background intensity was subtracted before calculating the ratio between the fluorescence emitted when the dye was excited at 340 nm and at 385 nm (F340/F385 nm). The time course of this ratio was analyzed for regions of interest adapted to individual cells. Sodium green was excited for 30 ms by a 455-nm LED (2,875 mW, used at 25%). The background intensity was subtracted before calculating the fluorescence time course adapted for regions of interest.

Liver Staining and oxLDL. Following sacrifice the of the LDL-receptor knockout mice, livers were harvested and paraffin-embedded. After subsequent sectioning (5 μm thickness), hematoxylin/eosin staining, Masson’s trichrome staining (both Merck), and tunnel staining (DeadEnd; Promega) were used according to the manufacturer’s instructions and using protocols as published recently (42). Futhermore, oxidized LDL-antibody (Immundiagnostik) was utilized in liver and aor-
tic root sections to visualize oxLDL content.

SLC8A1 Knockdown. siRNA-mediated knockdown of SLC8A1 was achieved by elec-
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troporation of an siRNA pool (Dharmacon) into HUVEC as published previ-
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SI Appendix Table 3.

MERLIN-TIMI 36 Cohort. The MERLIN-TIMI 36 study was a randomized,
double-blind, placebo-controlled, multinational clinical trial of 6,560 patients within 48 h of ischemic symptoms who were treated with rano-
lazine (initiated intravenously and followed by oral rano-
lazine extended-release 1,000 mg twice daily) or placebo (19). CRP measurement at baseline and after 2 wk of treatment was available in 3,808 patients (1,934 in the placebo group and 1,874 in the rano-
lazine treatment group). Baseline characteristics of these patients are avail-
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SI Appendix Table 2.

Exploratory Human Cohort. Human samples were analyzed for CRP in patients undergoing rano-
lazine treatment within a previous study (44). Briefly, 51 patients aged between 32 and 65 y were included in a chronic phase of CAD at least 12 mo after acute myocardial infarction. All patients were treated with β-blockers, platelet therapy, angiotensin-converting inhibitors or sartans, and statins. The therapy was not changed at least 6 wk prior to inclusion and during the study. In this double-blind study, patients were randomized to rano-
lazine 375 mg twice daily for the first 4 wk and 500 mg twice daily for 8 wk or to con-
trol treatment. Baseline patient characteristics including age, body mass index, cholesterol, high-density lipoprotein, LDL, and triglyceride levels can be found in SI Appendix Table 1. Blood was drawn before and after 12 wk of treatment. High-sensitivity CRP was measured with a fully automated, latex-enhanced nephel-
ometric immunoassay (N High Sensitivity CRP; Dade Behring).

Statistical Analysis. Mouse data and in vitro culture data are presented as mean ± SD or mean ± SEM as indicated in the figure legends. When analyzing two conditions Student’s t test was used, and multiple comparison was achieved by ANOVA followed by Bonferroni correction. In human samples nonparametric data (determined by Kolmogorov-Smirnov test) are presented as median and IQR. The Wilcoxon test was used for paired samples and the Mann-Whitney U test was used for independent samples. Values of P < 0.05 (two-tailed) were considered as statistically significant.

Data Availability. All study data are included in the article and/or supporting information.

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TIMI 36 cohort including CRP values and patient characteristics. This work was

(approval number 1616/2013). Furthermore, all samples were processed anony-
mously so that no identification of individual patients was possible for the involved scientists. For cell culture setups with reduced sodium concentrations ES contained (in millimolar) 145 NaCl, 5 KCl, 10 glucose, 10 4-(2-hydroxyethyl)piperazine-1-
ethanesulfonic acid (Hepes), 1.25 CaCl2, and 1 MgCl2 (Merck), buffered to pH 7.4 with NaOH and had an osmolarity of 300 mosm.
