BAY 58-2667, a nitric oxide-independent guanylyl cyclase activator, pharmacologically post-conditions rabbit and rat hearts

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Aims

BAY 58-2667 (BAY-58) directly activates soluble guanylyl cyclase without tolerance in a nitric oxide (NO)-independent manner, and its haemodynamic effect is similar to that of nitroglycerin. We tested whether BAY-58 could make both rabbit and rat hearts resistant to infarction when given at the end of an ischaemic insult.

Methods and results

All hearts were exposed to 30 min regional ischaemia followed by 120- (isolated hearts) or 180- (in situ hearts) min reperfusion. BAY-58 (1–50 nM) infused for 60 min starting 5 min before reperfusion significantly reduced infarction from 33.0 ± 3.2% in control isolated rabbit hearts to 9.5–12.7% (P, 0.05). In a more clinically relevant in situ rabbit model, infarct size was similarly reduced with a loading dose of 53.6 μg/kg followed by a 60 min infusion of 1.25 μg/kg/min (41.1 ± 3.1% infarction in control hearts to 16.0 ± 4.4% in treated hearts, P, 0.05). BAY-58 similarly decreased infarction in the isolated rat heart, and protection was abolished by co-treatment with a protein kinase G (PKG) antagonist, or a mitochondrial KATP channel antagonist. Conversely, Nω-nitro-L-arginine-methyl-ester-hydrochloride, a NO-synthase inhibitor, failed to block BAY-58’s ability to decrease infarction, consistent with the latter’s putative NO-independent activation of PKG. Finally, BAY-58 increased myocardial cGMP content in reperfused hearts while cAMP was unchanged.

Conclusion

When applied at reperfusion, BAY-58 is an effective cardioprotective agent with a mechanism similar to that of ischaemic pre-conditioning and, hence, should be a candidate for treatment of acute myocardial infarction in man.

Keywords

BAY 58-2667 • Cardioprotection • Guanylyl cyclase • Protein kinase G • Reperfusion

Introduction

The intracellular second messenger cyclic guanosine 3’-5’-monophosphate (cGMP) plays a role in various physiological processes. cGMP is generated by two distinct enzymes: the cytoplasmatic soluble guanylyl cyclase (sGC), a heterodimer consisting of α- and β-subunits, and the membrane-bound particulate guanylyl cyclase. While sGC acts as a receptor for the biological messenger nitric oxide (NO), particulate guanylyl cyclase is the receptor for extracellular natriuretic peptides. cGMP effectors include the cGMP-dependent protein kinase (protein kinase G (PKG)), cGMP-modulated cation channels, and cGMP-regulated phosphodiesterases (PDEs).

In recent years, there has been accumulating evidence that cGMP and its downstream target PKG play crucial roles in the survival signalling of pre- and post-conditioning.1 While pre-conditioning leads to infarct size reduction following several short ischaemic cycles preceding a lethal ischaemic insult,2 post-conditioning represents a comparable phenomenon in which brief cycles of coronary flow interruption are applied after the prolonged ischaemic period.3 Pharmacological post-conditioning can also be accomplished by giving an agent that activates ischaemic...
post-conditioning’s pro-survival pathways at the time of reperfusion. Because of their obvious clinical potential in the treatment of acute myocardial infarction in man, both ischaemic and pharmacological post-conditioning have received much attention in recent years. While clinical trials with ischaemic post-conditioning are quite encouraging, the ideal pharmacological post-conditioning agent has yet to be identified. The signalling pathway of ischaemic pre-conditioning is complex and can be divided into two phases: a pre-ischaemic trigger phase and a post-ischaemic mediator phase. During the trigger phase, ischaemic myocardium releases agonists to G-protein-coupled receptors, including adenosine, bradykinin, and opioids. Although there are some differences between signalling triggered by these three agonists, the target is activation of protein kinase C (PKC). For bradykinin and opioids, the signalling involves activation of nitric oxide synthase (NOS) with subsequent activation of PKG. Protein kinase G opens particulate guanylyl cyclase by atrial natriuretic peptide or B-type natriuretic peptide (BNP) at reperfusion all at reperfusion limit infarction. That may be because haemodynamics of the heart, and these same components are needed in ischaemic preconditioning. Activating these elements at reperfusion proved to be equally protective. For example, it could be shown that activation of particulate guanylyl cyclase by atrial natriuretic peptide or B-type natriuretic peptide or direct activation of PKG at reperfusion all result in profound protection comparable to that seen in preconditioning. Furthermore, activation of PKG by increasing cGMP levels as a result of PDE Type 5 inhibition at reperfusion also result in profound protection comparable to that seen in pre-conditioning. Ischaemic post-conditioning's pro-survival pathways at the time of reperfusion during an acute myocardial infarction in man, both ischaemic and pharmacological post-conditioning have received much attention in recent years.

**Methods**

Infarct size studies were conducted in Mobile, Alabama and Greifswald, Germany. Rabbits were used in Mobile, whereas rats were studied in Greifswald. These studies were performed in accordance with The Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, DC, 1996). The experimental protocols used in the rat experiments were approved by the local authorities of the state of Mecklenburg-Vorpommern (LALLF M-V/TSĐ/7221.3-2.3-014/08), and protocols in rabbits by the IACUC committee at the University of South Alabama.

**Isolated rabbit heart**

Briefly, New Zealand white rabbits were anesthetized with pentobarbital sodium (30 mg/kg i.v.) and ventilated with 100% oxygen. After a left thoracotomy in the fourth interspace, a suture was passed around a major anterior epicardial coronary arterial branch coursing towards the apex. The heart was excised and perfused on a Langendorff apparatus with Krebs—Henseleit bicarbonate buffer containing (mM) 118.5 NaCl, 24.8 NaHCO₃, 4.7 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 2.5 CaCl₂, and 10 glucose, and bubbled with 95% O₂/5% CO₂ to a pH of 7.35—7.45 at 38°C. A fluid-filled latex balloon measured pressure in the left ventricle as the heart beat spontaneously and isometrically.

**Experimental protocol**

Hearts of four experimental groups were studied. The snared coronary branch was occluded for 30-min and reperfused for 2 h in all groups. In the control group, no other treatment was given. In the drug-treated groups, BAY-58 was added to the perfusate for 1 h starting 5 min prior to reperfusion to yield concentrations of either 50, 10, or 1 nM in the buffer perfusate.

**Measurement of risk zone and infarct size**

At the end of the experiments, the coronary artery was re-occluded, and fluorescent microscopes were infused to demarcate the ischaemic zone. Hearts were weighed, frozen, and then cut into 2 mm thick transverse slices. The slices were incubated in 1% triphenyltetrazolium chloride (TTC) in sodium phosphate buffer. Triphenyltetrazolium chloride stains the non-infarcted myocardium brick-red indicating the presence of dehydrogenase enzymes. The slices were then immersed in 10% formalin to enhance the contrast between stained (viable) and unstained (necrotic) tissue. After illumination with UV light, the risk zone was identified as the non-fluorescent region. The areas of infarct and risk zone were determined by planimetry of each slice and volumes were calculated by multiplying each area by the slice thickness and summing them for each heart. Infarct size was expressed as a percentage of the risk zone.

**In situ rabbit heart**

New Zealand white rabbits were anesthetized as above and through-out the experiment, additional anaesthesia was administered as needed (~4 mg/kg pentobarbital every 15 min). A heating pad maintained rectal temperature between 38.5 and 39.5°C. Animals were intubated through a tracheotomy and ventilated with 100% O₂. Arterial pH, pO₂, and pCO₂ were measured with a blood gas analyzer. Respiratory rate was adjusted to keep pH between 7.35 and 7.45. In this preparation, arterial pO₂ averages 180–200 mmHg and pCO₂ 25–30 mmHg. A PE 50 catheter filled with heparinized saline (10 U/mL) was placed in the right carotid artery to monitor arterial blood pressure and to measure arterial pH and gases. To administer drugs, a butterfly needle was placed in an ear vein.

After a left thoracotomy in the fourth interspace, a prominent branch of the left coronary artery was surrounded with a suture (2-0 silk) to form a snare. The rabbits were allowed to stabilize for 15 min after surgery before the protocols were begun. In all cases, the coronary branch was occluded for 30 min and reperfused for 3 h.
BAY-58 was started 5 min before the onset of reperfusion. BAY-58 was given as a bolus of 53.6 μg/kg followed by a 60 min infusion of either 5.36 or 1.25 μg/kg/min. The doses and schedules were based on pharmacokinetic data from laboratory animals provided by the manufacturer (volume of distribution approximates whole body water and τ1/2 is 30 min). Finally, we tested the vehicle which was a mixture of dimethyl sulfoxide (DMSO), polyethylene glycol, and water.

Measurement of risk zone and infarct size
After completion of the experiment, all hearts were excised and the aortic root was perfused with 0.9% saline. The coronary artery was re-occluded, and infarct size was determined as outlined above.

Isolated rat heart
We wanted to extend our study to a second species so we chose the rat. Hearts from male Wistar rats were quickly excised and mounted on a Langendorff apparatus, as described for the isolated rabbit hearts above. We decided to use the isolated rat hearts for the mechanistic studies for two reasons. First, we wanted a crystalloid-perfused preparation so we could control the dose of the inhibitors as tissue concentration of drugs in situ preparations are unknown due to uncertainties in their volume of distribution, half life, and protein binding. Secondly, the smaller coronary flow rate reduces the cost of inhibitor drugs such as KT5823 which are quite expensive.

Experimental protocol
Five groups of hearts were subjected to regional ischaemia for 30 min followed by 2 h of reperfusion. Control hearts were subjected to ischaemia and reperfusion with no treatment. One group of hearts was treated with 50 nM BAY-58 starting 5 min prior to reperfusion and continuing for the entire reperfusion period. In the remaining three groups, one of three inhibitors was co-infused with BAY-58. Either the NOS inhibitor N\textsuperscript{-}nitro-L-arginine methyl ester hydrochloride (L-NAME, 200 μM), the mK\textsubscript{ATP} channel blocker 5-hydroxydecanoate (5-HD, 100 μM), or KT5823 (1 μM), a PKG inhibitor, was added to the perfusate. At the end of reperfusion, infarct size was determined as described above for isolated rabbit hearts.

Radioimmunoassay for cAMP and cGMP
Isolated rat hearts were perfused on a Langendorff apparatus as described above. A transmural biopsy of the left ventricle was obtained after 30 min of global ischaemia followed by 10 min of reperfusion and immediately frozen in liquid nitrogen. Two groups were studied: control hearts received no treatment while the second group was treated with 50 nM BAY-58 during reperfusion. The frozen tissue lines were evaluated with ANCOVA. A value of P < 0.05 was considered significant.

Results
Isolated rabbit hearts
There were no significant differences in heart rate or developed pressure at baseline among the experimental groups (data not shown). As expected, developed pressure was reduced during coronary occlusion, and only partially recovered after reperfusion. Hearts treated with BAY-58 showed a significantly higher coronary flow at 30 min of reperfusion. As shown in Figure 1, BAY-58 reduced infarct size from 33.0 ± 2.8% for 50 nM, 11.2 ± 1.5% for 10 nM, and 12.7 ± 2.8% for 1 nM. Since all doses tested showed significant protection (P < 0.05 vs. control), we could not determine an EC50 which must be in the picomolar range.

In situ rabbit hearts
No group differences in systolic and diastolic pressure, mean arterial pressure, or heart rate were observed at baseline. At the end of reperfusion, no difference was seen between the groups. Figure 2 shows mean blood pressure data. Blood pressure declined mildly and comparably during coronary occlusion in all groups. In rabbits treated with the low-dose infusion of BAY-58, there was a trend toward lower arterial blood pressure during drug infusion, but it

Materials
BAY 58-2667 was kindly provided by Bayer HealthCare GmbH, Wuppertal, Germany. KT5823 was obtained from Alexis Pharma, Lörrach, Germany. All other chemicals were from Sigma-Aldrich Chemical Co. For rabbit studies, BAY-58 was dissolved in the company vehicle of DMSO, polyethylene glycol, and water. For rat studies, BAY-58 and KT5823 were dissolved in DMSO before being diluted in buffer resulting in a DMSO concentration of less than 0.01%. L-NAME and 5-HD were diluted directly in Krebs–Henseleit buffer.
is not significantly different than that seen in vehicle-treated animals. The decline in rabbits treated with the higher dose of BAY-58 was more noticeable and became significant following discontinuation of the infusion. Although hypotension persisted to the end of the experiment in these animals, the difference in mean blood pressure during drug infusion compared with vehicle-treated group, although changes were not significant. Mild hypotension persisted in animals treated with high infusion of BAY-58. \( P < 0.05 \) with Bonferroni correction vs. vehicle-treated rabbits.

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**Isolated rat hearts**

We chose to do the mechanistic studies of BAY-58’s protective effect in isolated rat hearts because its lower coronary flow rate would greatly reduce the cost of materials. The results are summarized in Figure 5. As expected, BAY-58 at a concentration of 50 nM given at reperfusion reduced infarct size from 34.6 ± 3.4% of the risk zone in the control group to 11.1 ± 3.5% (\( P < 0.001 \) vs. control). Co-administration of the NOS inhibitor L-NAME failed to block BAY-58’s protection (15.4 ± 3.0% infarction, \( P < 0.001 \) vs. control and \( P = \text{n.s.} \) vs. BAY-58), indicating an NO-independent mechanism. On the other hand, KT5823 as well as 5-HD abolished protection (29.8 ± 1.8 and 29.4 ± 2.7% infarction, resp., \( P = \text{n.s.} \) vs. control), indicating that the effect of BAY-58 occurred upstream of both PKG and the opening of mKATP. Neither L-NAME, KT5823 nor 5-HD alone have any effect on infarct size in untreated isolated rat hearts when given at reperfusion.

**Measurement of cyclic nucleotides**

The cyclic nucleotides cAMP and cGMP were measured in biopsies of isolated rat hearts undergoing 30 min of global ischaemia and 10 min of reperfusion. There was no difference between the cAMP levels in hearts treated with BAY-58 at reperfusion and the control hearts (0.11 ± 0.018 vs. 0.11 ± 0.014 pmol/mg wet weight), whereas BAY-58 at reperfusion caused a significant increase in cGMP from 0.14 ± 0.058 fmol/mg wet weight in the untreated controls to 4.12 ± 0.81 fmol/mg (\( P = 0.0014 \)) (Figure 6).

**Discussion**

An intervention that limits myocardial infarction when administered just before reperfusion has great clinical appeal. This investigation examined the efficacy of the novel NO-independent sGC activator BAY-58 which is currently in early clinical testing for the long-term treatment of heart failure. BAY-58 dramatically decreased infarction in both isolated and in situ rabbit hearts as well as isolated rat hearts when administered just prior to
reperfusion. Although direct sGC activators including BAY-58 have been reported to reduce cardiac preload and afterload in an animal model of congestive heart failure, reverse pulmonary hypertension, and attenuate vascular atherosclerosis and restenosis, our study is, to our knowledge, the first to suggest that they could also be clinically relevant cardioprotectants.

Guanylyl cyclase generates cGMP which in turn activates PKG and indeed BAY-58 raised the cGMP content in ventricular tissue. BAY-58’s protection was abolished by the PKG inhibitor KT5823 indicating that PKG was indeed responsible for the protection. Previous studies have shown that activation of PKG opens mKATP, and this mechanism was confirmed when 5-HD, a putatively selective mKATP antagonist, blocked BAY-58’s protection. Thus, BAY-58 likely protects by activating PKG and opening mKATP as seen in ischaemic pre-conditioning. Recent studies reveal that a direct PKG activator given at reperfusion also protects against infarction. The sequence of signalling was found to be opening of mKATP, generation of ROS, and activation of PKC. Re-introduction of oxygen during reperfusion would provide fuel for the generation of the reactive oxygen species and the subsequent activation of PKC at reperfusion would lead to activation of the survival kinases.

BAY-58 was recently discovered to activate a NO- and haem-independent regulatory site on sGC in the region of the amino acids 371 (α-subunit) and 231–310 (β-subunit). Concentrations of BAY-58 as low as 1 nM activate sGC sufficiently to yield a biologically important increase in cGMP. In agreement, we observed an infarct reducing effect of BAY-58 at a concentration of as little as 1 nM in the isolated rabbit heart. We did not test concentrations below 1 nM as our in vivo studies revealed that a plasma concentration of 65 nM was already low enough to avoid haemodynamic effects. The NOS inhibitor L-NAME was not able to block BAY-58’s protection, consistent with the latter’s NO-independent activation of PKG. Activated sGC is known to generate cGMP which in turn has various physiological effects through its downstream effector, PKG. In the present study, BAY-58 increased cGMP in reperfused cardiac tissue, but had no effect on cAMP. Because there is extensive cross-talk between cGMP and cAMP at the level of the PDEs, we cannot exclude some role for cAMP, but increased cGMP seems to be the most likely factor causing BAY-58’s anti-infarct effect. This was supported by our finding that the PKG
inhibitor KT5823 abolished BAY-58’s protection in isolated rat hearts. Although there are some concerns about the selectivity of KT5823, we are not aware of a more suitable PKG inhibitor that we could have used. Unfortunately, there is no current way to directly assay for PKG activity in a tissue sample as activity depends on the amount of cGMP added to the assay mixture which would be unrelated to that in the tissue. Phosphorylation of the protein VASP has been used as a reporter for PKG activity, but unfortunately cardiomyocytes do not express VASP. Thus, cGMP concentration is currently the best indicator of PKG activation. Although we considered blocking the cAMP-dependent kinase, PKA, with the widely used inhibitor H89, this strategy is not suitable for the present model since H89 has cardioprotective properties which appear to be unrelated to PKA inhibition.20

We have reported that activation of PKG leads to opening of mKATP, and this event is thought to be an important step in preconditioning’s trigger pathway.7 When the cell-permeant cGMP analog 8-(4-chlorophenylthio)-guanosine 3’5’-cyclic monophosphate was administered to rabbit hearts just before reperfusion, it protected against infarction and that protection could be prevented by two blockers of mKATP 5-HD, and glibenclamide,18 suggesting that the opening of mKATP by PKG can protect at reperfusion as well. We could also block the protective effects of BAY-58 with 5-HD, suggesting again that PKG and mKATP are in series in the present protective pathway. Clearly, further investigations are necessary to clarify the exact role played by the channel. Finally, it must be remembered that there has been some criticism about the specificity of 5-HD as an antagonist of mKATP.21

PKG dilates vascular smooth muscle. We, therefore, were concerned that it might not be possible to protect the heart without an unacceptable level of peripheral dilatation. The haemodynamic effect of the BAY infusion shown in Figure 2 turned out to be only transient during drug infusion and also very mild in the group receiving the lower infusion rate of 1.25 μg/kg/min. BAY-58 proved to be protective in an in situ rabbit heart model with only modest hypotension, and the decline in blood pressure in animals treated with the low dose infusion was not significant. Since our low infusion rate protocol still produced plasma levels of BAY-58 well above the very protective dose of 1 nM used in isolated hearts, it should be possible to preserve cardioprotection while substantially decreasing the loading bolus and maintenance infusion doses thus eliminating any tendency to produce hypotension.

In past studies, protection afforded at reperfusion is critically dependent on the timing of the intervention and delaying the onset of a post-conditioning stimulus, either pharmacological or ischaemic,23 sometimes for as little as 1 min, leads to a loss of protection. We gave a bolus of BAY-58 5 min before reperfusion to increase blood levels quickly so that blood entering the ischaemic zone with the onset of reperfusion would have a high concentration of drug. Based on the pathways involved, BAY-58 most likely activates the pre-conditioning mechanism which protects by preventing destructive mitochondrial permeability transition pores from forming in the first minutes of reperfusion.24 Early treatment would be required to prevent pore formation. We did not test whether delaying the loading dose until a minute or two after reperfusion would cause a loss of protection, but in our pilot studies we did find that infusions without the loading dose were not protective (data not shown). That failure probably was because plasma levels were not in a protective range during the first critical moments of reperfusion. It is not known whether the infusion had to be maintained for a full hour, but our pilot data demonstrated that the loading dose alone was not protective either (data not shown). AMP 579, a drug that also protects at reperfusion by activating the pre-conditioning pathways did require a full hour of infusion to protect. Stopping the AMP579 infusion after only 30 min led to the loss of protection.22

The present data indicate that BAY-58 is highly protective to the ischaemic heart in the clinically relevant scenario in which its administration begins just prior to reperfusion. Infusion of BAY-58 leads to increased levels of cGMP in cardiac tissue, and the protection is independent of NO, but is dependent on both PKG and mKATP. In a rabbit in situ model, low dose intravenous BAY-58 elicits an infarct-reducing effect with an acceptable haemodynamic profile. BAY-58 may, therefore, be a promising candidate for the limitation of infarct size at reperfusion in man.

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**Conflict of interest:** J.-P.S. is currently a full-time employee of Bayer HealthCare.

**References**

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An asymptomatic 65-year-old woman was transferred to our hospital with a hook-wire breast marker migrated into the chest. PA Chest X-Ray film (Panel A) shows a small linear structure projecting on the right ventricle (curved arrow); on the lateral film (Panel B) a linear opacity is appreciable projecting on the sternum (black arrow).

Electrocardiographically gated computed tomography angiography (CTA) was performed to localize the foreign body. Axial CTA (Panel C) and 3D volume rendered (VR) images (Panel D) demonstrate the presence of two foreign bodies located in the subcutaneous pre-cardiac region (small arrow) and in the right ventricular (RV) wall protruding into the RV cavity (curved arrow). Three-dimensional VR image (Panel E) demonstrates normal right and left anterior descending coronary arteries (black arrows). There is a tiny tip protruding into the RV wall (white arrow). ‘Transparent blood’ 3D VR image (Panel F) defines the localization of the deeper foreign body and its extension into the RV wall (curved arrow) (AR, aortic root; LV, left ventricle).

An endovascular attempt to remove the fragment embedded in the RV wall was tried, but the fragment fractured so only a small part could be removed.

The patient was clinically and haemodynamically stable and refused any further intervention.

The penetration of foreign bodies and their retention in the heart can be due to chest trauma or to secondary venous embolization from peripheral injuries.

The rarity of such events precludes standardized diagnostic and therapeutic protocols; approaches must be tailored in accordance with clinical conditions and surgical risks.