Inhibition of Mas G-protein signaling improves coronary blood flow, reduces myocardial infarct size, and provides long-term cardioprotection

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Zhang T, Li Z, Dang H, Chen R, Liaw C, Tran TA, Boatman PD, Connolly DT, Adams JW. Inhibition of Mas G-protein signaling improves coronary blood flow, reduces myocardial infarct size, and provides long-term cardioprotection. Am J Physiol Heart Circ Physiol 302: H299–H311, 2012. First published October 14, 2011; doi:10.1152/ajpheart.00723.2011.—The Mas receptor is a class I G-protein-coupled receptor that is expressed in brain, testis, heart, and kidney. The intracellular signaling pathways activated downstream of Mas are still largely unknown. In the present study, we examined the expression pattern and signaling of Mas in the heart and assessed the participation of Mas in cardiac ischemia-reperfusion injury. Mas mRNA and protein were present in all chambers of human hearts, with cardiomyocytes and coronary arteries being sites of enriched expression. Expression of Mas in either HEK293 cells or cardiac myocytes resulted in constitutive coupling to the Gq protein, which in turn activated phospholipase C and caused inositol phosphate accumulation. To generate chemical tools for use in probing the function of Mas, we performed a library screen and chemistry optimization program to identify potent and selective nonpeptide agonists and inverse agonists. Mas agonists activated Gq signaling in a dose-dependent manner and reduced coronary blood flow in isolated mouse and rat hearts. Conversely, treatment of isolated rat hearts with Mas inverse agonists improved coronary flow, reduced arrhythmias, and provided cardioprotection from ischemia-reperfusion injury, an effect that was due, at least in part, to decreased cardiomyocyte apoptosis. Participation of Mas in ischemia-reperfusion injury was confirmed in Mas knockout mice, which had reduced infarct size relative to mice with normal Mas expression. These results suggest that activation of Mas during myocardial infarction contributes to ischemia-reperfusion injury and further suggest that inhibition of Mas-Gq signaling may provide a new therapeutic strategy directed at cardioprotection.

G-protein-coupled receptor; Gq protein; ischemia and reperfusion

THE MAS RECEPTOR (Mas or alternatively Mas1) is a class I rhodopsin-like G-protein-coupled receptor (GPCR). In mammals, Mas is expressed predominantly in brain and testis with moderate levels of expression in heart and kidney and lower expression in several other tissues (2, 24, 36, 41).

Although it was suggested in early studies that Mas is an angiotensin II (ANG II) receptor (19), later studies (3) demonstrated that ANG II-mediated intracellular signaling in Mas-transfected cells was only observed in cells endogenously expressing the ANG II type 1 (AT1) receptor. Currently, there is still uncertainty about the endogenous ligand for the Mas receptor, although the peptide angiotensin-1–7 [ANG-(1–7)], which is derived from ANG II, has recently been described as an endogenous agonist of Mas (28). However, the intracellular signaling pathways activated by Mas are still only partially characterized. It has been reported that Mas expression in NIH 3T3 cells can activate G11 and Gq pathways (32), resulting in activation of Rac-dependent signaling pathways and cell transformation (42). More recently, others (5) have demonstrated that expression of Mas in HEK293 cells could upregulate AT1 receptor levels and that this was due to the constitutive capacity of Mas to activate the Gq/G11-PKC pathway. In addition, a synthetic peptide ligand MBP7 has been reported to induce Mas internalization and stimulate both phospholipase C (PLC) activity and intracellular Ca2+ mobilization in Chinese hamster ovary cells expressing Mas (4), indicating that Mas may signal through the Gq-PLC-Ca2+ pathway. Interestingly, while ANG-(1–7) has been shown to increase arachidonic acid levels (28), it does not appear to modulate Gq-PLC signaling in Mas-expressing cells (4, 31).

For the most part, the biological functions of Mas have been interrogated using the putative ligand ANG-(1–7). However, since ANG-(1–7) is known to also regulate cardiovascular function independently of Mas via the ANG II receptors AT1 and AT2 (7, 15, 37), the role of Mas in these processes remains somewhat unclear. In addition, the complex nature of possible Mas/AT1 heterodimerization (20, 27) makes interpretation of in vivo studies using ANG-(1–7) challenging. Nonetheless, the Mas receptor itself has been observed to play a role in regulating cardiovascular functions. For example, when hearts isolated from Mas knockout mice were compared with wild-type controls, they exhibited alterations in systolic and diastolic tension during global ischemia and reperfusion (6). However, the in vivo function of the Mas receptor during ischemia-reperfusion injury has not previously been determined.

The aim of this study was to examine the proximal signaling pathways activated by Mas and to assess the role of the Mas receptor in cardiac ischemia-reperfusion injury in vivo. In addition, we describe, for the first time, small molecule, nonpeptide modulators of the Mas-Gq-PLC signaling pathway. Our results demonstrate that Mas is a Gq-coupled receptor. Reduction of Mas signaling activity, either by genetic alteration or with the pharmacological use of Mas inverse agonists, was found to be cardioprotective during ischemia-reperfusion injury. Our results indicate that therapies aimed at reducing Mas receptor Gq-PLC signaling may represent a promising new strategy for treatment of cardiac ischemia-reperfusion injury.

MATERIALS AND METHODS

Cloning of human and rat mas genes. The cDNA for human and rat mas genes were obtained by PCR using genomic DNA as templates. The following were used as primer sets: 5′-TGGATGGGT-CAACGTTGACATCATT-3′ (human mas sense primer); 5′-CGCG-
GATCCTGACGAGCATCTCATGATGACC-3' (human mas antisense primer); 5' -ACCAAGCTTGACAAATGTAATGATCATCTGG-3' (rat mas sense primer); and 5'-CAAGATTCCAGACACGTCTCTAATGATGACC-3'. PCR was performed using pfu polymerase (Stratagene, San Diego, CA) with the buffer system provided by the manufacturer plus 10% DMSO, 2.5 mM of each primer, and 300 µM each of the four nucleotides. After an initial denaturation at 95°C for 4 min, 30 cycles of 95°C for 40 s, 60°C for 50 s, and 72°C for 1 min 40 s were performed, which was followed by a final extension at 72°C for 7 min. The 986 bp human mas PCR fragment was digested with BamHI and cloned into blunted HindIII(5')-BamHI(3') sites of expression vector pHM6 (Invitrogen, Carlsbad, CA), while the 988 bp rat mas PCR fragment was cloned into HindIII(5')-EcoRI(3') sites of pHM6 after digestion with HindIII and EcoRI.

Chemicals. Mas agonist (AR234960) and inverse agonists (AR244555 and AR305532) were synthesized at Arena Pharmaceuticals (San Diego, CA). They were dissolved in DMSO for in vitro and ex vivo assays and in 20% hydroxypropyl β-cyclodextrin for in vivo experiments. ANG-(1–7) was purchased from Tocris Bioscience (Ellisville, MO) and dissolved in water. MBP7 and P61 were synthesized at Peptides International (Louisville, KY) and dissolved in water. AVE0991 was synthesized at Arena Pharmaceuticals and dissolved in DMSO. The PLC inhibitor U-73122 was purchased from Sigma (St. Louis, MO) and dissolved in DMSO.

Animals. Animal studies were performed according to the Guide for the Care and Use of Laboratory Animals published by the National Academy of Sciences (1996). All study protocols were reviewed and approved by the Arena Pharmaceuticals Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (270–330 g) were purchased from Harlan (Placentia, CA). The Mas knockout mouse line was purchased from Deltagen (San Mateo, CA), and confirmation of Mas mRNA deletion was performed by RT-PCR using gen-specif primers (sense: ATCTTTGAAAGCCCTGGTCA; and antisense: ATCTTTGAAAGCCCTGGTCA). All animals were housed in standard cages and were maintained at 25°C under 12-h light-dark cycles. The animals were fed standard diet and water ad libitum.

Homogeneous time-resolved fluorescence inositol-1-phosphate assay. Human and rat Mas receptors were either transiently or stably expressed in HEK293 cells (ATCC, Manassas, VA). For transient transfections, human or rat Mas genes and the neor gene were transfected into HEK293 cells using Lipofectamine (Invitrogen). HEK293 cells transfected with empty pHM6 vector were used as a control. For generation of stable cell lines, cDNA expression plasmids encoding human or rat Mas genes and the neo' gene were transfected into HEK293 cells using Lipofectamine (Invitrogen) according to the manufacturer’s instructions. Stable receptor-expressing pools were then generated over 3 wk by standard techniques in the presence of 500 µg/ml genetin (Invitrogen). Individual stable receptor pools were dilution cloned using standard techniques, and clones were comparatively evaluated in inositol phosphate accumulation assays. Preferred clones were banked and cultured as needed. HEK293 cells were used as controls. The 384-well inositol-1-phosphate (IP1) homogeneous time-resolved fluorescence (HTRF) assay (Cisbio, Bedford, MA) was performed as described by the manufacturer’s protocol. Cells were plated at 100,000 cells per well in 15 µl DMEM (Invitrogen) and incubated in the CO2 incubator at 37°C for 2 h. Five microliters of compounds diluted in 2× stimulation buffer plus 0.4% BSA (Sigma) were added to each well, and serial diluted IP standards (Cisbio) were also added to corresponding wells at this step. The cells were incubated for 4 h in the CO2 incubator at 37°C, d2-labeled IP1 and cryptate-labeled anti-IP1 monoclonal antibody diluted in lysis buffer were added sequentially in 10 µl per well. The assay plates were kept in dark at room temperature overnight. Ratiometric measurements of fluorescence emission at 665 and 620 nm were obtained using a Perstarr fluorometer (BMG Labtech, Ortenberg, Germany). IP1 levels in each well were calculated according to the standard curves on each plate. IC50 values were obtained by fitting data to a nonlinear curve-fitting program (GraphPad Software, La Jolla, CA).

cAMP assay. CAMP accumulation in HEK293 cells stably expressing human or rat Mas receptors was determined by the 384-well cAMP Dynamic2 HTRF assay (CisBio) following the manufacturer’s protocol. Briefly, cells were plated at 30,000 (G, activity) or 1,000 (G, activity) cells per well in 5 µl stimulation buffer (PBS containing 500 µM IBMX and 0.1% BSA), 5 µl of Mas compounds diluted in PBS were added to each well, and serial-diluted cAMP standards were also added to corresponding wells at this step. For detecting G-coupled activities, 10 µM forskolin were included with the compounds for a final concentration of 5 µM at the stimulation step. Following a 1-h stimulation at room temperature, d2-labeled cAMP and anti-cAMP cryptate conjugate diluted in detection buffer (included in the kit) were added to cells sequentially at 5 µl per well. The plates were incubated further for 1 h at room temperature. Ratiometric measurements of fluorescence emission at 665 and 620 nm were obtained by Pherasear fluorometer (BMG Labtech), and cAMP levels in each well were calculated according to the standard curves on each plate. IC50 values were obtained by fitting data to a nonlinear curve-fitting program (GraphPad Software).

Ca2+ measurements by fluorometric imaging plate reader assay. HEK293 cells stably expressing human Mas receptors were monitored for changes in intracellular Ca2+ using a fluorometric imaging plate reader (FLIPR-384; Molecular Devices, Sunnyvale, CA). Cells were seeded into black-walled clear-base 384-well plates at a density of 2 × 104 cells per well and incubated with HBSS containing 20 mM HEPES pH 7.4, 2 mM calcium 3 dye (Molecular Devices, Sunnyvale, CA), and 2.5 mM probenecid at 37°C for 60 min. Cells were washed with HBSS containing 20 mM HEPES pH 7.4 and 2.5 mM probenecid, and the plates were then placed into the FLIPR instrument to monitor cell fluorescence before and after the addition of the agonists at different concentrations.

Preparation of adenoviral constructs and adenoviral infection of cultured cardiomyocytes. Adenoviral constructs were prepared from expression plasmids encoding β-galactosidase (AdLaCZ, as a control) or wild-type human Mas receptor (AdMas). Homologously recombinant adenoviruses were generated by Qbiogene (Carlsbad, CA). Neonatal rat ventricular myocytes (NRVMs) isolated from 1- to 2-day-old rats were cultured from Cell Applications, (San Diego, CA) and plated overnight in serum-containing media at a density of 0.3 × 106 cells per well in 24-well plates for inositol phosphate assays or at a density of 0.25 × 106 cells per well in 2-well chamber slides for immunocytochemistry. After overnight culture, the cells were washed and the medium was replaced with serum-free medium supplemented with insulin/transferrin/selenium (Sigma). Cells were infected for 6 h with AdLaCZ or AdMas adenoviruses (1,000 viral particles/cell). Using the control adenovirus encoding “LacZ” (AdLaCZ) and β-galactosidase staining of AdLaCZ-infected myocytes, we determined that a viral titer of 1,000 viral particles per cell resulted in nearly 100% infection efficiency without cytotoxicity. Cells were subsequently washed and maintained in serum-free medium with supplements for inositol phosphate assay or immunocytochemical staining.

Inositol phosphate assay in NRVMs. NRVMs were plated on 24-well plates and infected as described above. 3H-labeled myoinositol was added, and the cells were incubated overnight. After 24 h, inositol-free media containing 10 mmol/l LiCl were added and cells were stimulated with vehicle (DMSO) or Mas compounds for 3 h. Cells were then lysed in ice-cold 0.1 mol/l formic acid and left at 80°C for 30 min to freeze and 37°C for thaw. Cell lysates were transferred to MultiScreen plates (Millipore, Billerica, MA) containing formate-form resin beads. The beads were washed with distilled H2O. Total inositol phosphates were eluted with 1 mol/l ammonium formate and 0.1 mol/l formic acid. Total 3H-labeled inositol phosphate was quantified by liquid scintillation counting.

Immunocytochemistry in NRVMs. NRVMs were plated on 2-well chamber slides and infected with adenoviruses as described above. Six
hours after adenovirus infection, cells were washed and then incubated with either vehicle or the Mas inverse agonist AR24-555 at 10 μM for another 42 h. Cells were then fixed with 3.7% formaldehyde, washed with PBS, permeabilized with 0.3% Triton X-100 in PBS, and blocked with 10% normal goat serum in PBS. Myocyte sarcomeres (F-actin) were stained with rhodamin-phalloidin (Invitrogen) and visualized on a Zeiss fluorescence microscope. Cell size was quantitated by digital planimetry using Adobe Photoshop.

**Semiquantitative RT-PCR.** Semiquantitative RT-PCR for mRNA expression of human Mas receptor was performed in a human cardiovascular cDNA panel (AMS Biotechnology, Abingdon, UK) using actin as a control. Human Mas primer sequences were as follows: sense: ACGGGGCTCTATCTGCTGACG; and anti-sense: AAGGGTTGGCGGCTACTGTGATT.

**Immunohistochemistry.** Snap-frozen heart tissues from male Sprague-Dawley rats were cryosectioned at a thickness of 8 μm and stored at −80°C. Sections were removed from the freezer and allowed to come to room temperature. Sections were fixed with cold acetone, washed with PBS, and blocked with 10% normal goat serum in PBS containing 0.2% Tween (PBST). Sections were incubated with the primary rabbit Mas antibody (Novus Biologicals, Littleton, CO) diluted 1:100 in PBST containing 1% BSA for overnight at 4°C. Half of the primary antibody solution was preabsorbed for 30 min at room temperature. Sections were fixed with cold acetone, washed with PBS, and blocked with 10% normal goat serum in PBS. Myocyte sarcomeres (F-actin) were stained with rhodamin-phalloidin (Invitrogen) and visualized on a Zeiss fluorescence microscope. Cell size was quantitated by digital planimetry using Adobe Photoshop.

**Coronary artery ligation model.** Occlusion and reperfusion of the coronary artery was performed in male Mas knockout (Mas−/−) mice and wild-type (Mas+/+) controls or in male Sprague-Dawley rats as previously reported (23). Briefly, mice or rats were anesthetized with an intraperitoneal injection of pentobarbital (70 mg/kg) and placed in a supine position under body-temperature control. Each animal was endotracheally intubated and ventilated with a tidal volume of 0.8 ml at a rate of 120 strokes/min (mice) or 2.5 ml at a rate of 70 strokes/min (rats) by using a rodent respirator (Harvard Apparatus, Holliston, MA). After left thoracotomy, a 8–0 (mice) or 7–0 (rats) surgical suture was placed underneath the left anterior descending coronary artery (LAD) at a position 2 mm from the tip of the left auricle using the aid of a Nikon stereoscope. PE-10 tubing (1–2 mm in length) was placed along the vessel as a cushion and was secured around the tubing to occlude the LAD. For the sham-operated control animals, the procedure was performed as above except that the suture was not secured around the LAD to occlude the vessel. Myocardial ischemia was verified by blanching of the LV and by change in electrocardiogram. To induce ischemia-reperfusion injury and determine infarction size, the LAD was occluded for 30 min and then the heart was reperfused for 2 h. In experiments using rats with Mas inverse agonist, vehicle (20% hydroxypropyl-β-cyclodextrin) or Mas compound (10 mg/kg) was injected as a bolus through jugular vein either 10 min before ischemia or 3 min before reperfusion. For long-term functional studies, vehicle or Mas inverse agonist was given 3 min before reperfusion. Following reperfusion, the incision was closed and benzathine penicillin administered intramuscularly as a prophylaxis against infection. The rats were extubated and allowed to recover in individual cages. After 3 mo, cardiac function was measured and infarct size was determined.

**Assessment of LV area at risk and infarct size.** For acute studies, following 2 h of reperfusion, the LAD was reoccluded and 5% Evans blue dye was injected into the LV cavity with a 27-gauge needle to define the nonischemic zone (blue area). The heart was excised immediately and rinsed in saline to remove excess dye, and the LV was cut transversely into five slices of equal thickness. These samples were incubated in 1% 2,3,5-triphenyltetrazolium chloride-containing Tris-HCl buffer (pH 7.8) at 37°C for 2 × 10 min to stain the viable myocardium (red area). The unstained (white) area inside the red area defined the infarcted area. The area at risk (i.e., the ischemic area) was defined as the white infarcted necrotic tissue plus the red viable salvaged tissue. Each slice was photographed from both sides using a microscope equipped with a high-resolution digital camera. The area at risk, infarcted area, and the total LV area were measured by digital planimetry using Adobe Photoshop. Infarct size was expressed as percentage of area at risk. For long-term studies, after measurements of cardiovascular hemodynamics (see below), the LV was cut transversely into five slices of equal thickness and the sections were stained with the 2,3,5-triphenyltetrazolium chloridesolution. Infarct size was expressed as a ratio of the infarcted area over the total LV area.

**In vivo cardiac functional analysis at 3 mo following ischemia-reperfusion.** Rats were anesthetized with pentobarbital, intubated, and ventilated, and the right carotid artery was cannulated with a Millar catheter (model SPR-320; Millar Instruments, TX). The tip of the catheter was advanced into the cavity of the LV to assess LV pressure. LV end-systolic and end-diastolic pressures were recorded with the sampling frequency of 1 kHz on an eight-channel, multipurpose data-acquisition system (Power Lab; AD Instruments, Sydney, AU) connected to a computer. LV developed pressure (LVPD; calculated as LV end-systolic pressure – LV end-diastolic pressure) was used as an index of cardiac function.

**Ex vivo coronary flow measurements.** Coronary flow was measured in male Mas−/− and Mas+/+ mice or in male Sprague-Dawley rats using Langendorff-perfused isolated hearts. Freshly isolated hearts were placed on a Langendorff apparatus (Harvard Apparatus) and perfused at a constant pressure of 80 mmHg with a modified Krebs-Henseleit buffer solution (Sigma) and aerated with 95% oxygen-5%
carbon dioxide pH 7.35–7.4. The temperature was maintained at 37°C by surrounding the heart with a water-heated glassware chamber. Coronary flow was measured using a flow measurement system (Harvard Apparatus), which included the transit time flow meter and a flow probe built into an adaptor block located at the perfusate inflow port. Data were recorded continuously using an ISOHEART data acquisition system (Harvard Apparatus). After a 20-min equilibration period, Mas compounds were added to the perfusion buffer reservoir at the desired concentration (agonist AR234960 at 1 μM or inverse agonist AR244555 at 5 μM) and coronary flow was recorded for 10 min.

To determine the role of the Mas-Gq-PLC pathway in regulating coronary flow, the Mas inverse agonist AR244555 (5 μM) or the PLC inhibitor U-73122 (0.5 μM) was added to the perfusion buffer for 10 min and then the agonist AR234960 (1 μM) was added to the perfusion buffer. Coronary flow was recorded for another 10 min. Changes in coronary flow induced by AR234960 were calculated as percentage of the coronary flow at 10 min following AR234960 treatment, relative to the coronary flow measured immediately before addition of AR234960. This protocol allowed for measurement of agonist-mediated vasoconstrictor activity, and accounted for changes in baseline coronary flow due to inverse agonist treatment alone.

To determine whether Mas agonist-induced changes in coronary flow were endothelium dependent, we measured responses in Langendorff-perfused hearts after chemical removal of endothelium with sodium deoxycholate. After a 20-min equilibration period, sodium deoxycholate was added to the perfusion buffer at 0.2 mg/ml for 3 min and then washed out for 10 min. Mas compounds were added and coronary flow was recorded for 10 min. Adenosine (1 μM), a coronary vasodilator that targets the endothelium, was used as a control to verify effective removal of endothelium.

For ischemia-reperfusion experiments, hearts were equilibrated for 20 min and then baseline coronary flow was recorded for 10 min. Thereafter, all hearts were subjected to 30 min of global ischemia by stopping perfusate flow, followed by 30 min of reperfusion with either vehicle (0.01% DMSO), Mas agonist AR234960 (1 μM), or Mas inverse agonist AR244555 (5 μM) added to the perfusion buffer. Electrocardiography was also continuously recorded during the observation period through electrodes attached directly to the surface of the ventricles to detect cardiac arrhythmias during reperfusion.

To determine whether the Mas receptor has constitutive G-protein coupling activity, we expressed human or rat Mas in HEK293 cells by transient transfection. An antibody to the hemagglutinin epitope tag on the Mas expression constructs was used to confirm expression by flow cytometry 48 h posttransfection (data not shown). Gq coupling in these cells was measured by HTRF IP1 assays. Expression of either human Mas or rat Mas receptor in HEK293 cells resulted in a significant increase in IP1 accumulation compared with cells transfected with empty vector (Fig. 3A), indicating constitutive Gq coupling of the receptor. Similar results were seen when dog and pig Mas orthologs were transfected into HEK293 cells (data not shown).

The constitutive Gq coupling of the Mas receptor provided a suitable assay signal with which to screen small molecule libraries for Mas receptor modulators. Using this assay, we were able to identify and optimize both agonists and inverse agonists to the Mas receptor. Functional Gq agonism and inverse agonism for representative compounds (agonist AR234960, and inverse agonists AR244555 and AR305352) were demonstrated in HEK cells stably expressing either human or rat Mas receptor (Fig. 3, B and C, and Table 1). We were unable to detect any effect of these compounds in control HEK293 cells (data not shown).

Ang-(1–7) did not stimulate IP1 accumulation in these Mas-expressing cells (Fig. 3, B and C), indicating that Ang-(1–7) does not modulate Mas signaling through Gq. We also evaluated other putative peptide or nonpeptide ligands for Mas in the HTRF IP1 assays, including MBP7 (4), P61 (31), and AVE 0991 (26). None of these ligands stimulated significant IP1 accumulation in the Mas-expressing cells (Table 1).

Since GPCR activation of the Gq-PLC pathway typically results in increased intracellular calcium, we measured Ca2+ in HEK293 cells stably expressing the human Mas receptor.
Consistent with its effect on inositol phosphate accumulation, AR234960 elicited a significant increase in intracellular Ca\(^{2+}\) in a dose-dependent manner (Fig. 3D), further verifying that Mas activates the downstream Gq-PLC-Ca\(^{2+}\) pathway.

In contrast, there was no detectable constitutive coupling to Gs or Gi in cells stably expressing human or rat Mas receptor (data not shown). Furthermore, we were unable to detect activation of the Gs/cAMP pathway by AR234960, AR305352, AR244555, or any of the other purported Mas receptor agonists (Fig. 3E and Table 2). However, although no constitutive Mas-Gi signaling was evident in our cAMP assays, the Mas agonist AR234960 was able to stimulate Mas-Gi activity resulting in a dose dependent reduction in forskolin stimulated cAMP levels (Fig. 3F and Table 2).

**Mas signaling in neonatal rat cardiomyocytes.** To study Mas signaling in biologically relevant cells, we examined Mas signaling and function in NRVMs. Since isolated adult rat cardiomyocytes are difficult to maintain in culture, NRVMs are the most suitable cellular model to investigate receptor pharmacology in cardiac cells; however, the expression of Mas at this stage of development is very low and does not support signaling studies via the endogenous receptor. Thus we used adenoviral constructs designed to boost expression of Mas in NRVMs. Low-level Mas receptor mRNA expression was detected in sham-infected NRVMs, but robust expression was
detected in myocytes infected with adenovirus encoding the human Mas receptor (AdMas; data not shown). Expression of Mas in NRVMs resulted in a significant increase in $[^{3}H]$inositol phosphate accumulation (Fig. 4A) compared with AdLacZ infected cells, demonstrating functional activation of Gq-coupled pathways. Furthermore, the Mas inverse agonist AR244555 caused a dose-dependent inhibition of inositol 1,4,5-trisphosphate accumulation in AdMas-infected cells (Fig. 4A).

It has been previously demonstrated (1) that enhanced Gq signaling in cardiac myocytes results in hypertrophic growth. To test whether signaling through the Gq-coupled Mas receptor elicited hypertrophy in cardiomyocytes, NRVMs were infected
Table 1. Summary of IP assay IC$_{50}$ data

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Human IC$_{50}$ μM</th>
<th>Rat IC$_{50}$ μM</th>
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<tbody>
<tr>
<td>AR234960</td>
<td>0.351 ± 0.055 (agonist)</td>
<td>0.172 ± 0.009 (agonist)</td>
</tr>
<tr>
<td>AR244555</td>
<td>0.186 ± 0.011 (inverse agonist)</td>
<td>0.348 ± 0.067 (inverse agonist)</td>
</tr>
<tr>
<td>AR305352</td>
<td>0.166 ± 0.006 (inverse agonist)</td>
<td>0.320 ± 0.053 (inverse agonist)</td>
</tr>
<tr>
<td>ANG-(1–7)</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>MBP7</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>P6i</td>
<td>NR</td>
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<tr>
<td>AVE0991</td>
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Values are means ± SE. IP, inositol phosphate; NR, no response.

with AdMas, and then typical hypertrophic responses including increased cell size and myofilament organization were measured. Increased myofilament organization into sarcomeres and a significant 2.2-fold increase in myocyte size were observed in Mas overexpressing cells compared with AdLacZ expressing cells (Fig. 4B). Moreover, treatment of myocytes with the Mas inverse agonist AR244555 attenuated the sarcomeric organization and cell enlargement observed in Mas overexpressing myocytes (Fig. 4B).

Regulation of coronary flow by Mas. Since Mas expression is enriched in coronary arteries, we wanted to determine whether the Mas receptor plays a role in the regulation of coronary flow. In isolated perfused hearts from genetically altered Mas knockout (Mas$^{-/-}$) and wild-type (Mas$^{+/+}$) mice, there was no detectable difference in coronary flow at baseline (Fig. 5A) or after vasoconstriction with ANG II or endothelin-1 (data not shown). However, treatment of Mas$^{+/+}$ mice with the Mas agonist AR234960 resulted in a significant reduction (64% of baseline) in coronary flow (Fig. 5A). This response was absent in Mas$^{-/-}$ hearts, indicating that the AR234960-mediated decrease in coronary flow is Mas-receptor dependent. A decrease in coronary flow was also observed in isolated perfused rat hearts upon treatment with the agonist AR234960 (Fig. 5B). Furthermore, the Mas receptor inverse agonist AR244555 caused a modest but significant increase in coronary flow in rat hearts. Pretreatment with the inverse agonist AR244555 prevented the decrease in coronary flow caused by the agonist AR234960 (Fig. 5B). These data demonstrate that agonist stimulation of the Mas receptor causes vasoconstriction, whereas inverse agonist treatment reverses the vasoconstriction and promotes dilatation of the coronary arteries.

To determine if the Mas agonist-induced decrease in coronary flow was endothelium mediated or smooth muscle mediated, we measured the change in coronary flow following treatment with sodium deoxycholate, a chemical that removes the endothelial layer but leaves the smooth muscle intact. To validate this procedure, we used adenosine as an experimental control, since it is known to cause vasodilation via activation of adenosine A$_2$ receptors on endothelial cells (10). The adenosine-mediated increase in coronary flow was abolished after sodium deoxycholate treatment (data not shown), verifying effective removal of endothelium. In contrast, the AR234960-mediated decrease in coronary flow was preserved in hearts denuded of endothelium (Fig. 5B), indicating that vasoconstriction is mediated through Mas receptors on smooth muscle cells. To confirm the role for G$_q$-PLC signaling in the vasoconstriction response, isolated rat hearts were treated with a PLC inhibitor (U-73122) before Mas agonist AR234960 treatment. PLC inhibition blocked the decrease in coronary flow caused by AR234960 (Fig. 5B).

To examine whether Mas activation might promote reperfusion injury following ischemia, we subjected isolated perfused rat hearts to 30 min of global ischemia followed by 30 min of reperfusion with perfusate containing vehicle or drug. During reperfusion, coronary flow in vehicle-treated rats initially returned to preischemia levels but then progressively decreased thereafter (Fig. 5C). Treatment with the Mas agonist AR234960 during reperfusion resulted in a trend toward decreased coronary flow during reperfusion. In contrast, treatment of hearts with the Mas inverse agonist AR244555 during reperfusion resulted in significantly elevated coronary flow at all time points during reperfusion compared with vehicle-treated hearts. These results suggest that in isolated perfused rat hearts Mas receptor activity causes decreased coronary flow during reperfusion following ischemia and that inhibition of Mas during reperfusion can significantly increase coronary flow under these conditions.

Electrocardiography was also continuously recorded during the observation period to detect cardiac arrhythmias during reperfusion. Two out of six hearts (33.3%) in the vehicle group exhibited prolonged (>10 min) ventricular arrhythmias, mainly ventricular fibrillation, during reperfusion. The frequency of arrhythmias was increased to three out of seven hearts (42.9%) with treatment with the Mas agonist AR234960. In contrast, no arrhythmias were observed in the six hearts treated with the Mas inverse agonist AR244555 during reperfusion.

Decreased infarct size in mas$^{-/-}$ mice after ischemia-reperfusion injury. To determine whether the Mas receptor activation might contribute to ischemia-reperfusion injury in vivo, we compared Mas$^{+/+}$ with Mas$^{-/-}$ mice using a well-established model of regional myocardial ischemia-reperfusion injury. Myocardial infarction size was measured in hearts exposed to 30 min of left anterior descending coronary occlusion followed by 2 h of reperfusion. The infarct size, expressed as a percentage of the area at risk, was significantly decreased in Mas$^{-/-}$ mice (34%) compared with Mas$^{+/+}$ mice (47%; Fig. 6A). Thus genetic ablation of the Mas receptor provides protection against in vivo myocardial ischemia-reperfusion injury in the mouse.

Reduction of myocardial ischemia-reperfusion injury by pharmacological inhibition of mas. To verify the role of the Mas-G$_q$ signaling pathway in ischemia-reperfusion injury, we also used a pharmacological approach. Since reduced Mas activity in the Mas-G$_q$ signaling pathway in ischemia-reperfusion injury, we subjected isolated perfused hearts from genetically altered Mas knockout (Mas$^{-/-}$) and wild-type (Mas$^{+/+}$) mice to 30 min of global ischemia followed by 30 min of reperfusion with perfusate containing vehicle or drug. During reperfusion, coronary flow in vehicle-treated rats initially returned to preischemia levels but then progressively decreased thereafter (Fig. 5C). Treatment with the Mas agonist AR234960 during reperfusion resulted in a trend toward decreased coronary flow during reperfusion. In contrast, treatment of hearts with the Mas inverse agonist AR244555 during reperfusion resulted in significantly elevated coronary flow at all time points during reperfusion compared with vehicle-treated hearts. These results suggest that in isolated perfused rat hearts Mas receptor activity causes decreased coronary flow during reperfusion following ischemia and that inhibition of Mas during reperfusion can significantly increase coronary flow under these conditions.

Table 2. Summary of cAMP assay IC$_{50}$ data

<table>
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<th>Rat IC$_{50}$ μM</th>
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</thead>
<tbody>
<tr>
<td>AR234960</td>
<td>0.719 ± 0.012 (agonist)</td>
<td>1.710 ± 0.011 (agonist)</td>
</tr>
<tr>
<td>AR244555</td>
<td>NR</td>
<td>NR</td>
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Values are means ± SE.
Mas inverse agonist decreases apoptotic cell death during myocardial ischemia-reperfusion injury. Apoptotic cell death is thought to be a major contributor to myocardial ischemia-reperfusion injury. To determine if Mas inverse agonist treatment can reduce apoptosis, rats were subjected to regional myocardial ischemia for 30 min, treated with either vehicle or Mas inverse agonist AR305352 (10 mg/kg) for 3 min, and then reperfused for 2 h. TUNEL staining was performed on heart sections to assess apoptosis in the LV. As shown in Fig. 7A, the number of TUNEL-positive cardiomyocytes was significantly increased in vehicle-treated rat hearts after ischemia-reperfusion compared with sham rats. Mas receptor inverse agonist treatment before reperfusion significantly reduced apoptotic myocyte death, suggesting that inhibition of Mas-Gq signaling in cardiomyocytes during ischemia-reperfusion provides cardioprotection via reduction in cardiomyocyte apoptosis.

**Improvement of long-term cardiac function by acute treatment with Mas inverse agonists during ischemia-reperfusion.** To determine whether the acute cardioprotection observed in Mas inverse agonist-treated animals after ischemia-reperfusion injury had positive long-term effects on cardiac function, we treated rats with vehicle or a single dose of the Mas inverse agonist AR305352 administered immediately before reperfusion. The animals were then allowed to recover for a period of 3 mo. Cardiac function and infarct size were measured at that time. The LVDP, an index of cardiac function, was 130 ± 5 mmHg in sham-operated rats but was decreased to 110 ± 4 mmHg in vehicle-treated rats, indicating that ischemia-reperfusion resulted in impaired cardiac function 3 mo later (Fig. 7B). In the group of animals given a single bolus administration of AR305352 just before reperfusion, the LVDP was improved relative to the vehicle group, and was similar to the sham-treated group (129 ± 5 mmHg; Fig. 7B).

Infarct sizes were also measured 3 mo following ischemia-reperfusion. The results were similar to the acute assessment, with infarct sizes reduced significantly in the AR305352 treatment group compared with the vehicle group (16% of total LV vs. 27% in vehicle group) (Fig. 7B). These results demonstrate that Mas inverse agonist treatment during ischemia-reperfusion provides long-term reduction in infarct size and results in long-term improvement in cardiac function.

**DISCUSSION**

Cardiovascular tissue expression of Mas. In the mouse heart, low levels of Mas mRNA transcripts have been detected in cardiomyocytes and higher concentrations in the endothelium of coronary arteries (2). Our data confirm Mas protein expression in rat hearts, with cardiomyocytes and coronary arteries as sites of enriched expression. Additionally, by colocalization studies, we determined that both smooth muscle cells and endothelial cells in coronary arteries express Mas. Importantly, we have also demonstrated, for the first time, that Mas is expressed in human heart. Consistent with its expression in rodents, Mas is expressed in all human cardiac chambers and in both human cardiomyocytes and human coronary arteries, suggesting that Mas plays a role in human heart function.

Mas is a Gq-coupled receptor. The Mas receptor was discovered more than two decades ago. Based on its sequence, Mas was predicted to be a GPCR; however, an understanding
of its intracellular signaling pathways has been slow to develop. Some studies have suggested that Mas may couple to Gq or Gi (4, 5, 32), but direct evidence of the intracellular signaling pathways activated by Mas is still limited. In the present study, we examined the G-protein coupling of Mas in both HEK293 cells and the more biologically relevant cardiac myocytes. Our data demonstrate that in both cell types Mas constitutively couples to the Gq protein, which in turn activates PLC and causes inositol phosphate accumulation. We confirmed Mas-Gq coupling using novel Mas agonists and inverse agonists discovered in our laboratory. These ligands modulated Mas-dependent IP accumulation and calcium mobilization in a dose-dependent manner. The absence of constitutive adenylyl cyclase activity in the same cellular context suggests that Mas couples preferentially to Gq, although Gi coupling was activated by high concentrations of the Mas agonist. Our results demonstrate higher sensitivity for Gq coupling (IC50 = 0.351 ± 0.055 μM) with agonist AR234960 compared with Gi coupling (IC50 = 0.719 ± 0.012 μM). In addition, the preferred Gq coupling observed with Mas agonists was also observed in cells expressing rat Mas (Tables 1 and 2) and was confirmed with additional Mas-Gq agonists discovered in our laboratories but not reported in these studies.

Although there is currently no direct evidence that Mas signals via activation of G12/G13, it has been reported that Mas transformation in NIH 3T3 cells is mediated through Rac1, a member of the Rho family proteins (42). Our data do not rule out the possibility that Mas also couples to G12/G13 in the heart.

While the ANG II peptide fragment ANG-(1–7), and more recently CGEN-856S/P61, have been reported to provide cardioprotection in rodents via the Mas receptor (29, 31, 38), their ability to activate proximal signaling downstream of the receptor is poorly defined. A clear understanding of the relationship between ANG-(1–7) and Mas is complicated by its binding to and signaling through the angiotensin AT1 and AT2 receptors (7, 11, 15, 17, 37) and the possibility that Mas and AT1 may heterodimerize and alter the receptor pharmacology (20, 27). Previous studies (12, 14, 16, 28) have shown that ANG-(1–7) causes internalization of the Mas receptor and induces the release of arachidonic acid, bradykinin, and prostaglandins and increases the activation of endothelial nitric oxide synthetase and Akt. However, ANG-(1–7) has not been shown to elicit Ca2+ changes or stimulate inositol phosphate accumulation in cardiomyocytes or Mas-overexpressing cells (4, 12, 31). Our results are consistent with these studies and demonstrate conclusively that ANG-(1–7) does not modulate Mas signaling through Gq, Gs, or Gi. Thus the sequence of events following ANG-(1–7) binding to Mas in heart cells remains unclear, and
its protective effects appear to occur via a non-G-protein mechanism.

By utilizing adenoviral infection to boost Mas expression levels in cultured NRVMs, we demonstrated that Mas expression, and its constitutive Gq signaling activity, significantly increases IP accumulation and lead to increased cell size and myofilament organization, which are hypertrophic responses typically observed upon activation of Gq-coupled receptors in this cell type (1). Treatment of Mas-expressing NRVMs with a Mas inverse agonist decreased IP accumulation and blocked the increase in cell size and myofilament organization into sarcomeres. These findings indicate that Mas can constitutively couple to Gq in cardiomyocytes and that Mas inverse agonists can modulate Gq signaling in these cells and inhibit the development of the hypertrophic phenotype. However, since the virally enhanced level of Mas expression in these experiments is likely to be supraphysiological, a direct role for the endogenous Mas receptor in regulating cardiac hypertrophy in vivo is still to be determined.

**Role of Mas signaling in the heart.** While we have observed Gq coupling of the Mas receptor in Mas-expressing cells in vitro, it was also important to confirm the signaling pathway of the endogenous Mas receptor in the heart. To address this, and the relationship between Mas signaling and biological function in the heart, we utilized the ex vivo coronary flow functional assay and the in vivo coronary artery ligation model.

Agonist stimulation of arterial smooth muscle Gq-coupled receptors (e.g., endothelin ETa), results in increased cytosolic Ca2+, vasoconstriction, and decreased arterial blood flow (30, 40). Conversely, pharmacological blockade of the ETa receptor results in vasodilation and increased coronary flow (18, 21). Therefore, we hypothesized that activation of the Gq-coupled Mas receptor in coronary arteries would lead to vasoconstriction and decreased coronary flow. In the present study, we have demonstrated that Mas-Gq agonist treatment causes vasoconstriction resulting in decreased coronary flow and conversely, Mas inverse agonist treatment results in vasodilation and increased coronary flow. Inhibition of PLC attenuated the effect of the Mas agonist, confirming that vasoconstriction via the endogenous Mas receptor is mediated through the Gq-PLC pathway. The observation that Mas agonist-induced vasoconstriction is preserved in endothelium-denuded hearts suggests that this response is mediated by Mas receptors on smooth muscle cells in coronary arteries.

The Mas receptor has been implicated in the regulation of cardiac function during ischemia-reperfusion in isolated hearts (6). To determine whether the Mas receptor also plays a role in regional ischemia-reperfusion injury in vivo, we performed coronary artery ligation studies in Mas+/+ and Mas−/− mice. Our data demonstrate that infarct size is significantly reduced in Mas−/− mice after ischemia-reperfusion. Interestingly, ablation of Mas expression in mice also renders the kidneys resistant to ischemia-reperfusion injury (13). Finally, we confirmed a role for Mas in myocardial ischemia-reperfusion injury by treating rats with Mas inverse agonists either before ischemia or immediately before reperfusion. Both treatment

**Fig. 6. Reduced activity of Mas receptor is cardioprotective during ischemia-reperfusion injury.** A: ablation of Mas receptor in mice is cardioprotective during I/R injury. Regional ischemia-reperfusion injury was produced in mice by ligation of the left anterior descending coronary artery for 30 min followed by release of the ligation (reperfusion). After 2 h of reperfusion, hearts were removed and infarct size was measured as a percentage of the area at risk (AAR). **P < 0.01 vs. WT; n = 7–9 mice per group. B: inverse agonist of the Mas receptor AR244555 was cardioprotective in rats when administered 10 min before ligation (Preischemia) or 3 min before reperfusion (Prereperfusion). Infarct size was measured as a percentage of the area AAR. LV, left, ventricle. ***P < 0.001 vs. vehicle; n = 8 rats per group.
protocols resulted in reduced infarct size, suggesting that excessive Mas-Gq signaling occurs both during ischemia and during reperfusion. These data are in agreement with the cardioprotection observed with other inhibitors of other myocardial Gq-coupled receptors in the setting of reperfusion injury (9, 39). Importantly, the decreased infarct size observed with Mas-Gq inverse agonist treatment in vivo resulted in improved long-term cardiac function.

Mechanisms of Mas cardioprotection. One mechanism whereby Mas inverse agonists provide cardioprotection is by improving coronary blood flow. It has been suggested that reduced coronary flow is an important factor that contributes to ischemia-reperfusion injury (8). The Mas receptor present on arterial smooth muscle cells promotes vasoconstriction, whereas inhibition of Mas signaling by Mas inverse agonists promotes vasodilation, resulting in improved blood flow. Thus it is likely that the improved coronary flow resulting from Mas inverse agonist treatment accounts, in part, for the cardioprotective properties of these compounds.

A second mechanism whereby Mas inverse agonists can provide cardioprotection is by reducing apoptosis. Mas is expressed in cardiomyocytes where ischemia-reperfusion is known to cause a marked increase in intracellular Ca2+ content (25, 35). Cytosolic and subsequent mitochondrial Ca2+ overload results in cell death during myocardial ischemia-reperfusion injury (34). We propose that the activation of Mas in cardiomyocytes during ischemia-reperfusion leads to activation of Gq-PLC-inositol 1,4,5-trisphosphate-Ca2+ signaling, which contributes to elevated cytosolic and mitochondrial Ca2+ loading and thus contributes to cell death by either apoptosis or necrosis. Cardiomyocyte loss by apoptosis has been recognized as a major factor contributing to ischemia-reperfusion injury (22). Indeed, we see marked increases in cardiomyocyte apoptosis in rat hearts after ischemia-reperfusion injury. Our observation that administration of Mas receptor inverse agonists before reperfusion reduces cardiomyocyte apoptosis supports this mechanistic hypothesis.

Fig. 7. Pharmacological inhibition of Mas receptor inhibits cardiomyocyte apoptosis and provides long-term cardioprotection following ischemia-reperfusion injury. A: decreased apoptotic cell death after myocardial ischemia-reperfusion injury by administration of Mas inverse agonist AR305352 immediately before reperfusion. Deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) staining was performed to assess apoptosis in frozen sections prepared from sham-operated rat hearts or rat hearts subjected to 30-min ischemia and 2-h reperfusion. Either vehicle (20% HPBCD) or Mas inverse agonist (AR305352, 10 mg/kg iv) was administered 3 min before reperfusion. TUNEL-labeled cardiomyocyte nuclei were counted to determine the percentage of apoptotic cells. **P < 0.01 vs. sham; *P < 0.05 vs. MI/vehicle; n = 4 rats per group. B: reduced infarct size and improved long-term cardiac function in rats after myocardial ischemia-reperfusion injury by acute administration of Mas inverse agonist AR305352. Regional ischemia-reperfusion injury was produced in rats by ligation of the left anterior descending coronary artery for 30 min followed by reperfusion. Vehicle (20% HPBCD iv) or the Mas inverse agonist (AR305352, 10 mg/kg iv) was administered 3 min before reperfusion. Following reperfusion the rats were allowed to recover for 3 mo, then cardiac function (left ventricular developed pressure, LVDP) and infarct size were analyzed. Infarct size was measured as a percentage of the total left ventricular area (%ventricle). **P < 0.01 vs. vehicle; *P < 0.05 vs. sham; n = 9–12 rats per group.
In addition to reducing infarct size, we hypothesized that the improved coronary flow and Ca^2+ handling during reperfusion would result in fewer ventricular arrhythmias. This is consistent with our observation that the Mas inverse agonist decreased the incidence of ventricular arrhythmias during reperfusion. This may be a third mechanism whereby Mas inverse agonists provide cardioprotection.

In conclusion, our data demonstrate that the Mas receptor is expressed in cardiomyocytes and coronary arteries across multiple species including humans. However, our studies have focused on a previously underappreciated aspect of Mas receptor pharmacology; G-protein signaling. We find that the Mas receptor preferentially couples to G_q resulting in PLC activation, and increased intracellular calcium and that G_q coupling can also occur when the receptor is exposed to high concentrations of agonist. Ex vivo coronary flow studies in isolated hearts support this signaling mechanism. Our discovery of small molecule modulators of Mas G-protein signaling provides an important tool for future studies aimed at understanding the biology of this receptor and incorporates the known benefits of small molecule therapies over ANG-(1–7) peptides, including oral bioavailability, improved pharmacokinetics, selectivity, and ease of synthesis and manufacturing. In addition, we demonstrate that inhibition of Mas signaling provides cardioprotection through mechanisms involving improved coronary flow, reduced apoptosis, and reduced incidence of arrhythmias. Furthermore, we show that inhibition of Mas receptor G_q signaling in the heart protects against ischemia-reperfusion injury in vivo, as demonstrated by reduced infarct size and improved long-term myocardial function. Together, these results reveal a previously unrecognized pathological role for excessive Mas-G_q signaling in the setting of myocardial ischemia-reperfusion injury and suggest that inhibition of Mas-G_q signaling may be therapeutically beneficial.

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DISCLOSURES

All contributing authors are employed by Arena Pharmaceuticals, who sponsored these studies.

AUTHOR CONTRIBUTIONS


REFERENCES
