Deletion of the mouse \(\alpha\)-calcitonin gene-related peptide gene increases the vulnerability of the heart to ischemia-reperfusion injury

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Huang R, Karve A, Shah I, Bowers MC, DiPette DJ, Supowit SC, Abela GS. Deletion of the mouse \(\alpha\)-calcitonin gene-related peptide gene increases the vulnerability of the heart to ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol 294: H1291–H1297, 2008. First published January 11, 2008; doi:10.1152/ajpheart.00749.2007.—Calcitonin gene-related peptide (CGRP), a potent vasodilator released from capsaicin-sensitive C-fiber and A\(\delta\)-fiber sensory nerves, has been suggested to play a beneficial role in myocardial ischemia-reperfusion (I/R) injury. Because most previous studies showing a cardioprotective role of CGRP employed pharmacological experiments, the purpose of this study was to utilize a genetic approach by using mice with a targeted deletion of the \(\alpha\)-CGRP gene to determine whether this neuropeptide had a modulatory function on the severity of I/R injury. To accomplish this goal, isolated, perfused hearts from \(\alpha\)-CGRP knockout (KO) and wild-type (WT) mice were subjected to 30 min of ischemia followed by 5, 15, and 30 min of reperfusion. Cardiac functional parameters, including coronary flow rates, left ventricular developed pressure, maximum rates of pressure development, and left ventricular end-diastolic pressure, were measured before and after I/R injury, as were levels of creatine kinase, to assess myocardial damage, and malonaldehyde, to assess oxidative stress. Following I/R injury, cardiac performance was significantly reduced in the hearts from the \(\alpha\)-CGRP KO mice compared with their WT counterparts. The decrease in myocardial function in the \(\alpha\)-CGRP KO hearts compared with WT hearts after I/R injury was associated with a significant elevation in creatine kinase release into the perfusates and malonaldehyde production in the cardiac tissue. Therefore, these data indicate that, in this in vitro setting, deletion of \(\alpha\)-CGRP makes the heart more vulnerable to I/R injury, possibly due, at least in part, to increased oxidative stress.

\(\alpha\)-CGRP is by far the predominant product in dorsal root ganglia sensory neurons and appears to be the CGRP gene product that has markedly greater activity in the regulation of cardiovascular function (5, 11, 31).

Immunoreactive CGRP and its receptor are widely distributed in the nervous and cardiovascular systems (8, 13, 26, 38). In the peripheral nervous system, a prominent site of CGRP (and substance P) synthesis, are the dorsal root ganglia. These structures contain the cell bodies of sensory nerves that terminate peripherally on blood vessels and centrally in laminae I/II of the dorsal horn of the spinal cord. Indeed, both CGRP and substance P are often colocalized in the same nerve terminals. A dense perivascular CGRP-containing neural network is seen around the blood vessels in all vascular beds. Receptors for CGRP have been identified in the media, intima, and endothelium of resistance vessels, veins, and multiple tissues and organs (26, 33, 38).

CGRP is the most potent vasodilator discovered to date, ~100 to 1,000 times more potent than other vasodilators, such as adenosine, acetylcholine, and substance P (1, 6, 7, 12). CGRP has been shown to selectively dilate multiple vascular beds, with the coronary vasculature being a particularly sensitive target (1, 6, 12). In multiple mammalian species, immunoreactive CGRP has been localized in all regions of the heart, particularly in the coronary arteries and around the sinoatral and atrioventricular nodes (6, 13, 38). A more recent developmental study in the mouse indicates that CGRP-containing sensory nerves are also abundant in the ventricular myocardium, especially at epicardial sites (17). Under conditions of myocardial ischemia-reperfusion (I/R), the unmyelinated C-fiber and thinly myelinated A\(\delta\)-fiber nerves play an afferent role, resulting in pain perception and a possible efferent cardioprotective role that is mediated through the release of CGRP and/or substance P (25, 37). This release of these sensory neuropeptides appears to be caused by activation of the vanilloid receptor 1, which is also known as transient receptor potential vanilloid type 1 (TRPV1), by the I/R-evoked production of protons, bradykinin, and other stimuli (4, 29).

A number of in vivo and in vitro studies indicate that sensory nerves, through CGRP, can significantly attenuate cardiac I/R injury and also play a role in cardiac preconditioning (both early and late) and remote preconditioning (2, 4, 9, 14, 18–22, 24, 25, 28, 29, 34, 36, 39, 40). However, not all of the reports are in agreement, and the mechanisms are still unclear. In

CALCITONIN GENE-RELATED PEPTIDE (CGRP), a 37-amino acid neuropeptide, is derived from the tissue-specific splicing of the primary transcript of the calcitonin/\(\alpha\)-CGRP gene (6, 8, 13, 38). Whereas calcitonin is produced mainly in the C cells of the thyroid, CGRP synthesis is limited almost exclusively to specific regions of the central and peripheral nervous systems. There is a second CGRP gene (\(\beta\)-CGRP) that does not produce calcitonin, which is also synthesized primarily in neuronal tissues. The two CGRP genes, \(\alpha\) and \(\beta\) in the rat and I and II in humans, differ in their protein sequences by one and three amino acids, respectively, and the biological activities of the two peptides are quite similar in most vascular beds. However,
addition, other investigators have reported that substance P release from cardiac sensory nerves can act in a paracrine manner to reduce I/R injury (4, 10, 35, 37). In light of the aforementioned studies, and given our extensive experience with the α-CGRP knockout (KO) mouse, notably studies demonstrating that α-CGRP possesses significant protective activity against hypertension-induced heart and kidney damage, with inhibition of oxidative stress being one of the underlying mechanisms (5, 31), it was logical to use this genetic model to assess the role of α-CGRP in cardiac I/R injury. Thus isolated perfused heart preparations from α-CGRP KO and wild-type (WT) mice were subjected to an I/R protocol, in conjunction with quantification of cardiac performance, myocardial cellular damage, creatine kinase (CK) release, and tissue generation of malonaldehyde (MDA), a presumptive marker of oxidative stress.

**MATERIALS AND METHODS**

**Preparation of isolated mouse hearts and measurement of flow rates and contractile function.** The animal protocols used for this study were approved by the University Animal Care and Use Committee of Michigan State University and were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The mice lacking the α-CGRP/calcitonin gene were generated as described elsewhere (42) and were generously provided by Dr. Robert F. Gagel (University of Texas M. D. Anderson Cancer Center, Houston, TX). The α-CGRP KO mice were subsequently back-crossed into C57/BL6 mice. This strain of mouse was used for the WT controls.

The α-CGRP KO mouse are fertile, grow normally, and have a normal life span. They exhibit a normal phenotype, with the exception of an elevated basal blood pressure (16, 31, 42). As described previously (16, 31), a comprehensive pathological evaluation was performed to determine whether there were any significant developmental or pathological changes in the absence of treatment. No significant gross postmortem or histopathological alterations were detected in the body cavities, or integumentary, alimentary, respiratory, circulatory, urogenital, endocrine, hematopoetic, musculoskeletal, or nervous systems of the α-CGRP KO mice compared with their WT counterparts. In addition, there was no microscopic evidence of vascular alterations or vascular variations among the mice examined. The one exception to the results described above was that the heart-to-body weight ratio was increased ~10% in the α-CGRP KO mice compared with the WT mice. The characterization of the α-CGRP mice with regard to α-CGRP, β-CGRP, substance P expression, and blood pressure phenotype has also been characterized (16, 31). It should be noted that, to delete the α-CGRP gene, it was also necessary to inactivate the calcitonin gene as well as kastacalcin, which is derived from the processing of the calcitonin peptide precursor (38). It has been clearly demonstrated that endogenous calcitonin or kastacalcin do not play a role in cardiovascular regulation (13, 38).

The mice were anesthetized with pentobarbital (100 mg/kg), and the hearts were removed after thoracotomy and placed in ice cold buffer. The aortae were cannulated, and the hearts perfused at 37°C with oxygenated (95% O2−5% CO2) modified Krebs-Henseleit buffer (118 mM NaCl, 4.7 mM KCl, 25 mM NaHCO3, 2 mM CaCl2, 1.2 mM MgCl2, 1.2 mM Na2HPO4, 0.5 mM Na2-EDTA, 11 mM glucose) using the Langendorff technique (32, 37). Perfusion was performed from the aorta to the coronary arteries, then through the left atrium. Once the isolated hearts were in the Langendorff apparatus, they were perfused for 60 min before any experimental manipulation. Myocardial ischemia was induced by stopping flow for 30 min followed by up to 30 min of reperfusion. For the functional studies, the isolated hearts were perfused at 55 mmHg of pressure and paced using a MP001 electrophysiological catheter (NuMed, Hopkinton, NY) at a rate of 450 beats/min. Pacing was stopped during the ischemic period. To measure contractility, a fluid-filled balloon was inserted into the left ventricle via the left atria. The balloon was connected to a pressure sensor and an HPA 410 Heart Performance Analyzer (Micro-Med, Louisville, KY). The data obtained by this method included heart rate, maximum rates of pressure development (dP/dT, and −dP/dT), left ventricular developed pressure (LVDP), and left ventricular end-diastolic pressure (LVEDP). For LVENDP determination, the ventilricular pressure was adjusted to 5−10 mmHg for the 60-min stabilization period. For the experiments employing the CGRP receptor antagonist, CGRP4-37 (Sigma-Aldrich, St. Louis, MO), the peptide was added to the perfusate at a final concentration of 10−6 M 5 min before induction of ischemia. The antagonist was kept in the perfusate for the duration of the ischemia and reperfusion periods. Coronary flow rates were derived by correlating pressure differences on both sides of a glass capillary tube in the perfusion line with flow. It has been established that the flow rate is proportional to the pressure difference on both sides of the capillary tube (32). The correlation between flow rate and pressure difference was calibrated before experiments.

**Measurement of CK and MDA.** CK release from the heart was quantified in the perfusate to assess myocardial injury. At the indicated time points, the perfusion buffer was collected, and CK activity was determined using a kit (Synchron CX System) from Beckman Coulter (Fullerton, CA) under conditions recommended by the supplier. To determine the development of oxidative stress following I/R injury in the isolated heart preparations, MDA was quantified in the heart tissues using a colorimetric assay for lipid peroxidation (Oxford Biomedical Research, Oxford, MI). These studies were performed in separate groups of α-CGRP KO and WT mouse heart preparations that were subjected to the I/R protocol in the absence of the functional studies, except for coronary flow measurements.

**Statistical analysis.** The differences among groups were determined by the unpaired Student’s t-test. When necessary, the results were analyzed by two-way ANOVA followed by the Bonferroni test. The results were considered to be statistically significant at P < 0.05.

**RESULTS**

**Measurement of myocardial performance in α-CGRP KO and WT mouse hearts, with and without I/R injury.** There were no significant differences in basal coronary flow rates, LVDP, LVEDP, and dP/dT between WT and α-CGRP KO hearts in control nonischemic groups. Basal values are shown at the 60-min stabilization period, just before the initiation of 30 min of no-flow ischemia. During the 30 min of global ischemia, the heartbeat would slow and then stop completely. Although after reperfusion was initiated the hearts would begin to beat spontaneously, electrical pacing of the heart was resumed at this time. As shown in Fig. 1, subsequent to ischemic injury, the flow rates in the WT hearts were significantly reduced ~13, 18, and 21% at the 5-, 15-, and 30-min time points, respectively, while, over the same time course of reperfusion, the hearts from the α-CGRP KO displayed a significantly larger decrease (~30%) in flow rates compared with their WT counterparts.

As shown in Fig. 2A, at the 5-min reperfusion time point, there was a significant reduction in LVDP in the α-CGRP KO hearts (21 ± 2 mmHg) compared with those from the WT (31 ± 2 mmHg) mice. At the later two time points, some recovery of function was observed in isolated hearts from both groups of animals; however, the degree of recovery in LVDP was significantly less in the α-CGRP KO compared with WT hearts.

As described previously, the LVEDP was adjusted to between 5 and 10 mmHg during the 60-min stabilization period.
Thus basal LVEDP levels were not different in the hearts from the two strains of mice (Fig. 2B). After the ischemic insult, 5 min of reperfusion produced a significant elevation of LVEDP in the WT hearts (48 ± 4 mmHg). There was, however, an improvement in diastolic pressure, as evidenced by a reduction in LVEDP, at the 15-min (36 ± 6 mmHg) and 30-min (31 ± 5 mmHg) time points. A similar pattern in LVEDP was seen in the hearts from the α-CGRP KO mice (56 ± 6, 48 ± 5, and 43 ± 4 mmHg at 5, 15, and 30 min, respectively); however, the LVEDP in the α-CGRP KO hearts was higher than that in the WT hearts, achieving statistical significance following 15 and 30 min of reperfusion.

The basal dP/dT values were not significantly different between the hearts from the WT and α-CGRP KO hearts after the 60-min stabilization period (Fig. 3). At the 5-min time point following ischemia, the dP/dT values were reduced to 44% of basal levels in the WT hearts. As seen previously, the hearts displayed improved systolic function at the 15- (56%) and 30-min (64%) time points. Although the hearts from the α-CGRP KO mice showed the same pattern as the WT hearts, they were significantly more vulnerable to I/R injury at all three reperfusion time points.

Figure 4 shows the results of the assessment of the coronary flow rates, LVDP, and dP/dT (Figs. 1, 2A, and 3) between the Langendorff preparations from the α-CGRP KO and WT mice when expressed as percent recovery (following normalization to basal levels) from cardiac ischemia at the end of the reperfusion period. As expected, these data demonstrate that the α-CGRP KO hearts exhibit a significant attenuation of recovery from I/R injury compared with the WT hearts.

Cardiac function studies were also performed in the presence or absence of the CGRP receptor antagonist CGRP8–37. It has been previously reported that this antagonist, at a concentration of 10–6 M, can block the cardioprotective effect of exogenous CGRP in mouse heart Langendorff preparations (37). Postischemic recovery of flow rates, LVDP, and dP/dT was impaired in the WT hearts when the CGRP receptor was blocked by the antagonist (Table 1). CGRP8–37 had no effect on cardiac function in WT hearts without ischemia (data not shown).

Determination of CK release from the heart. Total CK activity in the perfusate was used to assess cellular injury in the isolated heart preparations. As shown in Fig. 5, after the ischemic insult, CK release was increased in the WT hearts after 5 min (30 ± 2 IU/ml), 15 min (42 ± 4 IU/ml), and 30 min (47 ± 3 IU/ml) of reperfusion. However, CK levels in the perfusates from the α-CGRP KO hearts were markedly higher at all three time points (141 ± 8, 130 ± 6, and 118 ± 5 IU/ml).
These results are supported by the finding that CK release after 30 min of reperfusion is increased ~1.8-fold in WT hearts in the presence of the CGRP receptor antagonist compared with the absence of CGRP8-37.

Production of MDA in the heart. The generation of MDA is widely accepted as a presumptive marker of oxidative stress (40). At baseline, before ischemia, the amount of MDA was similar in hearts from both the α-CGRP KO and WT mice (Fig. 6). Following the 30-min ischemia period, there was a significant fourfold increase in MDA content in the hearts from both strains of mice after 5 min of reperfusion. This increase in MDA formation continued to increase after 15 min (α-CGRP KO, 12-fold vs. WT, 9-fold) and 30 min (α-CGRP KO, 19-fold vs. WT, 9-fold), with the levels of MDA being significantly higher in the α-CGRP KO hearts following 15 and 30 min of reperfusion.

DISCUSSION

The objective of this study was to employ, for the first time, isolated perfused heart preparations from mice that have a permanent deletion of α-CGRP to determine whether this sensory neuropeptide plays a cardioprotective role in I/R injury. The major findings to come from these in vitro experiments are as follows. 1) Hearts from α-CGRP KO mice display a significant reduction in myocardial performance after 30 min of ischemia followed by up to 30 min of reperfusion compared with their WT counterparts. Similar results were observed in WT hearts treated with the CGRP receptor antagonist CGRP8-37. 2) This decrease in cardiac function is associated with a marked increase in myocardial cell damage, as evidenced by an enhanced release of CK and higher levels of MDA generation, which is indicative of an elevated rate of reactive oxygen species (ROS) production. If the deleterious effects of the absence of α-CGRP on coronary flows and cardiac function are taken without any assessment of myocardial damage, then one cannot distinguish whether α-CGRP is protective against myocardial stunning, a reversible injury, and/or myocardial infarction (4). However, the marked increase in CK release in the α-CGRP KO hearts compared with WT hearts provides strong evidence that this neuropeptide attenuates cell death after I/R injury, thus providing a more fundamental and ongoing protective activity. In addition, it is well established that reperfusion of the ischemic myocardium generates ROS that exert harmful effects on heart function and structure (3). Therefore, the increased generation of MDA in the α-CGRP KO hearts suggests that the α-CGRP-mediated inhibition of oxidative stress is responsible, at least in part, for its cardioprotective effects.
The results presented herein are consistent with several in vivo and in vitro studies in rats (9, 10, 14, 22, 35, 39, 40), mice (28, 29, 37), guinea pigs (15), dogs (2), pigs (18–20), and humans (21, 24, 34, 36), demonstrating that cardiac sensory nerves possess protective activities in I/R injury and that these effects are mediated by CGRP and/or substance P. Major endpoints assessed in these studies include determination of cardiac hemodynamics and function, infarct size, CK release, and levels of CRP and/or substance P in the systemic and coronary circulations. Although most of these reports suggest that CGRP is the primary candidate, it must be noted that many of these studies make extensive use of capsaicin (the main pungent ingredient in chili peppers) treatment. The major difficulty associated with this drug is that sensory nerve degeneration produced by capsaicin is very nonspecific and results in the loss of a variety of receptors, ion channels, and neuropeptides expressed in this class of nerves (25, 37). Therefore, there is some inconsistency in this area regarding the role(s) of endogenous CGRP and substance P in cardioprotection, as well as which of these sensory peptides is most effective in mitigating I/R injury. These discrepancies have come mainly from studies involving the use of pigs (18–20) and humans (21, 24, 34, 36).

For example, in the human studies, patients who underwent coronary bypass surgery who experienced 10–20 min of local ischemia (as evidenced by lactate production) had significantly increased levels of CGRP in coronary sinus blood (21). In patients with acute myocardial infarction, an almost twofold increase in circulating CGRP was detected within 24 h, suggesting that CGRP is released in response to the reduction in myocardial perfusion (24). Similarly, in patients with stable angina pectoris, intracoronary infusion of CGRP delayed the onset of ischemia-evoked pain and increased the work tolerance during treadmill exercise (34). In contrast, another study showed that acute ischemic chest pain is not associated with increased CGRP in the systemic or coronary circulations (36).

In addition, the results from the present study are not in complete agreement with a report that employed mice that had a targeted deletion of the TRPV1 (vanilloid receptor 1) gene (4, 37). In this study, isolated perfused heart preparations from TRPV1 KO and WT mice were subjected to 40 min of ischemia followed by 30 min of reperfusion. Cardiac functional parameters (coronary flows, LVDP, and LVEDP) were then quantified in the presence or absence of agonists and antagonists of TRPV1, the CGRP receptor, and substance P receptor. No markers of myocardial damage, such as CK release or measurement of infarct size, were assessed. As expected, postischemic recovery of cardiac performance was significantly reduced in the hearts from the TRPV1 KO mice compared with WT controls. However, in this study, the cardioprotective mechanisms initiated by activation of TRPV1 were thought to be mediated primarily by substance P rather than CGRP. Due to the absence of any markers for myocardial damage, these investigators were unable to determine if the beneficial effects of substance P on cardiac function were more transient in nature (attenuation of myocardial stunning) or were more permanent effects, such as inhibition of cell death. Although we do not have sufficient data to explain the discrepancy between the two studies, one cannot rule out the possibility inherent in any experiments involving conventional KO mouse strains that the α-CGRP KO and/or the TRPV1 KO animals have an unknown developmental alteration(s) that could confound the experimental findings.

Although isolated perfused hearts are a widely used methodology to study cardiac function, because it has the advantages of performing experiments free of the influences of hemodynamic factors and blood constituents, there are two major caveats that must be considered when interpreting these data. First, Langendorff preparations are for acute experiments that provide reliable data for only a few hours (32). Therefore, it is not possible to assess long-term changes in oxidative stress, inflammatory responses, and cardiac remodeling evoked by I/R injury. Second, one can never be certain that the results obtained in vitro can be extrapolated to the in vivo situation. For example, there is accumulating evidence that the sensory neuropeptides CGRP and substance P contribute to immune responses (4, 6, 37). Briefly, because CGRP is such a potent vasodilator, the increased blood flow in the affected region also increases the number of circulating cells and other chemotactic factors that are present in the area. However, this mechanism can have either pro- or anti-inflammatory actions, depending on the local physiological status. In addition, a range of in vivo and in vivo studies that are sometimes contradictory suggest that, while the majority of the effects of CGRP on neutrophils and lymphocytes are inhibitory, its actions on monocytes and macrophages are a mixture of both stimulatory and inhibitory activities (6, 37). In regards to substance P, it has been shown to activate macrophages and neutrophils, thereby releasing inflammatory mediators and generating free radicals (4). Thus the potential cardioprotective effects exhibited by α-CGRP and (substance P) in vitro may be negated by potential in vivo sensory neuropeptide-mediated proinflammatory actions.

There are several lines of indirect evidence that point to an in vivo protective role for endogenous α-CGRP in the pathophysiology of cardiovascular disease and also add support to the idea that one of the mechanisms that contributes to the cardioprotective effects of this peptide in I/R injury is through the inhibition of oxidative stress. As described previously, our laboratory has demonstrated that deletion of the α-CGRP gene (using the same strain of α-CGRP mice employed in the present study) enhances deoxycorticosterone-salt hypertension-induced end organ damage and dysfunction in the heart and kidney (5, 31). Moreover, these adverse effects are mediated, in part, by an increase in the local tissue production of ROS and inflammatory mediators. Although we cannot directly compare the longer term in vivo hypertension-induced end-organ damage results to the present in vitro acute I/R injury studies, the marked increase in oxidative stress is a critical pathological mechanism that is common to both cardiovascular disease states. Through the use of primary cultures of rat aortic endothelial and vascular smooth muscle cells, we have also shown that CGRP is a potent inhibitor of ROS generation evoked by angiotensin II or high glucose treatment and that the primary mechanism underlying this effect is inhibition of NADPH oxidase activity (23). Similarly, it has been demonstrated that CGRP can inhibit oxidative stress-induced apoptosis in cultured rat vascular smooth muscle cells (27) and rat cardiomyocytes (30).

Given the exquisite sensitivity of the coronary vasculature to the dilator effects of CGRP (1, 6, 12, 38), it is logical to speculate that the protective actions of this peptide are also likely to involve the enhancement of coronary perfusion and/or...
myocardial metabolism and reduced oxygen consumption (1, 6, 7, 12, 13, 38). Coronary vasodilation induced by CGRP is mediated by endothelium-independent mechanisms, resulting in increased levels of cAMP (6). A third potential mechanism that could be operative under in vivo conditions is a CGRP-mediated inhibition of platelet aggregation and neutrophil adhesion to endothelial cell infiltration of the myocardium, thereby ameliorating endothelial dysfunction and myocardial injury (6).

In summary, these data demonstrate that, in isolated perfused heart preparations subjected to I/R injury, cardiac performance is significantly reduced in the hearts from the α-CGRP KO mice compared with their WT counterparts. This enhanced reduction in myocardial function was associated with a significant increase in cell death and oxidative stress, as evidenced by a marked elevation of CK release and MDA production. These results suggest that, through the effenter function of cardiac sensory nerves, α-CGRP plays a fundamental and ongoing cardioprotective role against I/R injury.

GRANTS
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REFERENCES

