Calcitonin gene-related peptide protects the myocardium from ischemia induced by endothelin-1: Intravital microscopic observation and $^{31}$P-MR spectroscopic studies☆

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A B S T R A C T
Aims: Calcitonin gene-related peptide (CGRP) is a potent vasodilator neuropeptide. We investigated the ameliorating effect of CGRP in myocardial ischemia induced by endothelin-1 (ET-1), with special emphasis on myocardial microvascular hemodynamics and levels of energy-related metabolites.

Main methods: The Langendorff preparations of rat isolated heart were perfused at a constant flow rate. Microvascular blood flow was also visualized in the anterior epicardium of the left ventricle by means of an intravital fluorescence microscope system. Energy-related metabolite contents in the myocardium were measured by means of $^{31}$P-magnetic resonance spectroscopy ($^{31}$P-MRS).

Key findings: Intracoronary bolus injections of CGRP caused dose-dependent decreases in coronary perfusion pressure (CPP) in the hearts exposed to ET-1 (30 pmol). The vasodilator potency of CGRP was about 10,000-fold greater than that of nitroglycerin and 1,000-fold greater than that of isobutylmethylxanthine. Vasodilation of the small-sized arterioles (10–40 μm in diameter) in response to CGRP (100 pmol) was confirmed by direct microscopic observation. After ET-1 (30 pmol) plus vehicle administration, high energy phosphates (phosphocreatine (PCr), ATP) were markedly reduced (p < 0.05). CGRP administration significantly (p < 0.05) attenuated the anaerobic changes in the myocardium (decrease in PCr) and macrohemodynamic alterations (increase in CPP, decrease in dP/dt etc.) induced by ET-1.

Significance: We conclude that CGRP effectively confers hemodynamic and metabolic protections to isolated beating hearts against ET-1-induced myocardial ischemia.

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Introduction
Cumulative intracoronary application of the potent vasoconstrictor peptide endothelin-1 (ET-1), discovered by Yanagisawa et al. (1988), has been reported to reduce coronary blood flow even in the absence of detectable vasospasm of large epicardial arteries (Kurihara et al., 1989; Larkin et al., 1989; Kolettis et al., 2013). This observation indicates that small resistance vessels are more sensitive to ET-1 than large conduit arteries in the heart (Miyachi and Goto, 2013). Using intravital microscopy, we previously found that intracoronary administration of ET-1 in the Langendorff perfusion preparation elicited more prominent vasoconstriction of the small-sized arterioles than the large ones (Homma et al., 1992). In view of these findings, among previously reported vasoactive agents, ET-1 is believed to be the most potent microvascular constrictor of the small-sized microvessels (Tang and Vanhoutte, 2010; Kolettis et al., 2013; Miyachi and Goto, 2013). The endothelin (ET) system consists of two G protein coupled-receptors, namely ET A and ET B receptors, and three endogenous ligands, ET-1, ET-2, and ET-3 (Horinouchi et al., 2013).

Although the vasoactive properties of ET-1 are widely recognized, minimal attention has been given to potential intrinsic physiological antagonistic mechanisms which may limit the vasoconstrictor’s effects. Accumulation of recent knowledge in vascular pharmacology has revealed that several endogenously formed vasoactive agents, such as prostacyclin, vasoactive intestinal peptide (VIP), atrial natriuretic polypeptide (ANP) and the endothelium-dependent relaxing factor (EDRF) nitric oxide (NO), might control vascular smooth muscle tonus and act as vasodilators (Brayet and Bevan, 1985; Furchgott and Vanhoutte, 1989). However, to date there have been

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very few demonstrations of the actions of these agents as physiological antagonists of ET-1. Compared with these vasodilator agents, calcitonin gene-related peptide (CGRP) is of particular interest as a possible physiological antagonist of ET-1 (Homma et al., 1991a) because of the following facts: 1) it is an extremely potent vasodilator of isolated coronary arteries (McEwan et al., 1986; Greenberg et al., 1987; Shoji et al., 1987); 2) perivascular plexuses of CGRP-like immunoreactive fibers are widely distributed in the cardiovascular system (Mulderry et al., 1985; Wharton et al., 1986); 3) like ET-1, CGRP exerts slow stable effects on the heart (Goto et al., 1987); and 4) myocardial ischemia enhances local intravascular CGRP release from peri-coronary arterial nerves (Franco-Cereceda et al., 1989; Mair et al., 1990). Although it was reported that CGRP acts as a physiological antagonist of the vascular effects of ET-1 in the systemic circulation in the rat (Meens et al., 2009, 2010, 2011, 2012; De Mey et al., 2013), the antagonistic action of CGRP to the vasoconstrictive effect of ET-1 at the microcirculation level in the heart has never been studied.

Based on our findings that ET-1 induces pronounced changes in the luminal diameter of heart microvasculature (Homma et al., 1991b, 1992), we suggested at the time that most likely metabolic alterations were important determinants of the hemodynamic microcirculation responses to ET-1 administration. Thus, in the current study we investigate the pharmacological interactions between CGRP and ET-1, with respect to their influences on myocardial hemodynamic and metabolic functions. The results presented herein show, for the first time, that CGRP potently alleviates the vasoconstrictor and metabolic effects of ET-1 in the heart.

Materials and methods
Animal preparation to investigate the effect of CGRP and ET-1

Male Wistar rats (n = 17) weighing 250–350 g were anesthetized with an i.p. injection of pentobarbital sodium (50 mg/kg), injected with sodium heparin (200 units/kg) into the jugular vein, for the purpose of anticoagulation, and the heart was removed and immediately immersed in ice-cold Krebs-Ringer solution. The Langendorff perfusion preparation was made by inserting a polyethylene catheter into the aorta, in order to retrogradely perfuse the heart with Krebs-Ringer solution aerated with 95% and 5% CO₂ mixture at 37 °C by means of a roller pump at a constant flow rate of 3.0 ml/min, as described previously (Homma et al., 1991b, 1992). Heart arrest was promptly reversed once perfusion with warm oxygenated perfusate was initiated. Composition of the Krebs-Ringer solution was (in mM): NaCl 113, KCl 4.8, CaCl₂ 2.2, MgSO₄ 1.2, NaHCO₃ 25, KH₂PO₄ 5.5 and Glucose 5.5. After an equilibration period of 15 min, a bolus injection of ET-1 (30 pmol; Peptide Institute, Osaka, Japan) was given to increase perfusion pressure. Once the response to ET-1 plateaued (usually in about 7 min after injection), boluses of other vasoactive agents were injected cumulatively into the perfusate, in increasing doses, through a catheter placed in the perfusion circuit. These vasoactive agents and respective doses used were: CGRP (human CGRP, 1–1000 pmol; Peptide Institute, Osaka, Japan), followed either by nitroglycerine (NTG; 10–3000 nmol; Nippon Kayaku Co., Tokyo, Japan) or isobutylmethylxanthine (IBMX; 10 pmol–3000 nmol; Wako Pure Chemicals Industries Ltd., Osaka, Japan). The changes in perfusion pressure accompanying administration of CGRP, NTG or IBMX were measured as previously described (Homma et al., 1992). In other experiments, after examining the effects of CGRP (100 pmol) on the ET-1-enhanced coronary perfusion pressure in the Langendorff circuit, its accompanying effects on microcirculatory blood flow in the myocardium were visualized under an intravital microscope system, to enable measurement of changes in microvessel diameter. The intravital television microscope system used in such experiments was previously described in detail elsewhere (Ohshima and Sato, 1987) (Sato and Ohshima, 1990). In short, the heart was placed on a specially designed holder to prevent lateral sliding motions. To attain clear visualization of the microvessels, FITC-labeled dextran was dissolved in the perfusate, and its fluorescence was visualized through a CCD video camera under an epi-illuminated fluorescence microscope system. A final magnification of 500–2000 was attained on a monitor TV screen by use of a plain or water-immersion objective lens, which was focused on the microvessels at the late diastolic phase of the cardiac cycles. Blood flow observation was made in the anterior epicardium of the left ventricle, in particular, at the site of the bifurcation of arterioles (10–40 μm in diameter). The dose of ET-1 chosen (30 pmol) as use in this study is maximally effective in promoting vasoconstriction of arterioles in the rat myocardium (Homma et al., 1991a), whereas the dose of CGRP (100 pmol) was selected because it corresponds roughly to the ED₅₀ value for decreasing perfusion pressure in the preparation.

Myocardial metabolism

To evaluate metabolic state of the myocardium accompanying ET-1 administration, intramuscular pH was first measured by directly inserting a micro-pH sensor (model PH-2135, Kuraray Co., Ltd., Okayama, Japan) into the myocardium of the anterior wall of the left ventricle. The Langendorff preparations of rat heart (n = 5) were set up and given ET-1 (30 pmol) as described before, and the changes in intramuscular pH were monitored for 60 min. In a separate series of experiments (n = 20), both myocardial contractility and metabolic state of the myocardium were examined simultaneously. To measure the left ventricular pressure (LVP) in such preparations, a latex balloon was introduced into the left ventricle (LV) through the left atrium and this parameter were monitored throughout the experiment alongside its first derivative (dP/dt), coronary perfusion pressure (CPP) and heart rate (HR). Each preparation was mounted inside the magnetic coil of a 31P-MRS apparatus (GX-400, JEOL, Tokyo, Japan) and carefully maintained at 37 °C. Preliminary experiments confirmed that phosphocreatine (PCr) content in the myocardium attained a stable value 15 min after initiating perfusion. After the 15 min-stabilization period, baseline measurements of high energy-phosphates content (PCr and ATP) and pH in the myocardium were carried out for 12 min. After the baseline measurements, the ET-1 (30 pmol) with vehicle (0.02% albumin solution in PBS) or the ET-1 with CGRP (100 pmol) were injected into the perfusate, and the high-energy phosphate contents and pH were continuously monitored for 30 min. An MRS was averaged from data of 45 spectra acquired under conditions of a pulse width of 85 μs and a recycle time of 4 s.

Statistical analysis

In all experiments statistical differences of % change from the control state between two groups were analyzed using analysis of variance (ANOVA) followed by an unpaired t-test (Wallenstein et al., 1980). Differences with values of p less than 0.05 were considered statistically significant in all tests. All experimental data are presented as mean ± SEM.

Results

Dose–response curves to ET-1, CGRP, NTG and IBMX

Curves for the depressor effects of CGRP (n = 7), NTG (n = 5) and IBMX (n = 5) following perfusion with 30 pmol ET-1 were obtained from a total of 17 Langendorff heart preparations. Fig. 1 illustrates typical time course tracings of the CPP responses to ET-1 alone and upon injections of CGRP or NTG to preparations pre-exposed to ET-1 (30 pmol). It should be noted that ET-1 caused an elevation in CPP which was stable for more than 30 min after injection (Fig. 1a), and that after precontraction with ET-1 cumulative application of increasing bolus doses of CGRP (3–1000 pmol; Fig. 1b) or NTG (10–1000 nmol; Fig. 1c) each induced...
dose-dependent reductions in CPP. Moreover, although CGRP was effective at far lower doses than NTG, addition of the highest dose of NTG (1000 nmol) after completing the CGRP curve caused an additional reduction in CPP to a level similar to that recorded prior to ET-1 injection. The mean dose–response curves for the depressor effects of CGRP, NTG and the phosphodiesterase inhibitor IBMX on CPP are depicted in Fig. 2. Although the ED$_{50}$ value of CGRP ($2.82 \times 10^{-11}$ mol) was about four orders of magnitude smaller than that of NTG ($2.19 \times 10^{-7}$ mol) and three orders of magnitude smaller than that of IBMX ($1.74 \times 10^{-8}$ mol), the maximal decrease in CPP induced by CGRP was smaller than that produced by NTG or IBMX.

Microvascular responses

Intravital microscopic observation of myocardial microcirculation also revealed prominent effects of ET and CGRP. Fig. 3 presents a series of photomicrographs showing typical microscopic images of an arteriole under control conditions (Fig. 3A) and after consecutive injections of ET-1 (Fig. 3B) and CGRP (Fig. 3C). The actual CPP values recorded at the specific time points when each image was obtained by an arrow head are indicated in the time course tracing of the CPP response to CGRP. It is evident that ET-1 caused a marked and diffuse vasoconstriction of the arteriole, allied to a localized vasospasm (near the bifurcation indicated by an arrow in Fig. 3B). Subsequent injection of CGRP promoted vasodilation over the entire length of the arteriole, except at the point of vasospasm. Similar findings were observed in cardiac arterioles preconstricted with ET-1 of another 6 preparations, yielding a mean relaxant effect of CGRP of $47.1 \pm 5.4\%$.

Changes in heart rate and myocardial contractility

The tracings depicted in Fig. 4 illustrate the typical changes in hemodynamic parameters recorded in the Langendorff preparations following injection of ET-1 alone (Fig. 4a) or together with CGRP (Fig. 4b). It can be seen that ET-1 alone promoted a sustained increase in CPP and transient increases in LVP and dP/dT followed by a more prolonged decrease in both parameters, accompanied by an early small and short-lived decrease in HR. In comparison, when ET-1 was injected concomitantly with CGRP, the resulting increase in CPP was much smaller, but the increases in both LVD and dP/dT were clearly sustained with no evident signs of depression at later stages, and HR was modestly and persistently increased.

The mean results of some of these experiments (CPP, HR and dP/dT), recorded over the first 30 min after the injections, are shown in Fig. 5. It should be noted that the magnitude of CPP changes induced by each of the injections was significantly different over the full length of the observation period, whereas the differences in HR and dP/dT only achieved statistical significance at few time points. On the other hand, the CGRP injection together with ET-1 clearly induced the increase of the products of dP/dt and HR (i.e. cardiac work index) compared to only the ET-1 application (Fig. 5d).

Metabolic changes induced in the myocardium

Fig. 6 shows a typical tracing of the decrease in myocardium pH, as recorded by use of a micro pH sensor, following the injection of ET-1 (30 pmol). As it was possible that this finding could reflect a state of severe metabolic acidosis, due to the vasoconstriction produced by ET-1 and consequent poor myocardial perfusion, we used 31P-magnetic resonance spectrum (SIP-MRS) analysis to examine in more detail the metabolic changes of isolated and perfused hearts induced by injection of ET-1 alone or plus CGRP. Typical tracings of the NMR spectra obtained at various time points prior to and following each of the two types of injection are illustrated in Fig. 7, whereas the mean results obtained by integrating the peaks of the 31P-MRS spectra of seven hearts per
Fig. 4. Typical examples of macrohemodynamic parameters. CPP, LVP, dP/dt and HR for the runs administered ET-1 with vehicle (a), and a concurrent injection of ET-1 with CGRP (b). CPP, coronary perfusion pressure; LVP, left ventricle pressure; dP/dt, first derivative of left ventricle pressure; HR, heart rate.

Fig. 5. Time course changes in perfusion pressure (a), heart rate (b), dP/dt (c), and work index (d) before and after the injection of ET-1 with vehicle (●) or the concurrent injection of ET-1 with CGRP (○). ET-1, 30 pmol; CGRP, 100 pmol; vehicle, 100 µl of 0.02% albumin in Krebs-Ringer solution. Difference between the ET-1 with vehicle and the concurrent injection of ET-1 with CGRP.
Discussion

The present investigation revealed that CGRP exerts potent cardiac microvascular vasodilator effects and can act as an intrinsic physiological antagonist of ET-1 in the rat isolated perfused heart. Moreover, it has characterized several features of myocardial ischemia induced by ET-1, including alterations in cardiac metabolic state. Finally, it also demonstrated that exogenous CGRP protects the heart from the microcirculatory and myocardial metabolic disturbances promoted by ET-1.

CGRP has been shown to display potent vasodilator actions in large isolated blood vessels, such as rat aorta, porcine coronary arteries, and human skeletal muscle arteries (Brain et al., 1985; Shoji et al., 1987; Pernow, 1989), as well as small arteries, resistance vessels and microvessels in various perfused organs and tissues including human skin and rabbit skeletal muscle microcirculation and mesenteric microvessels (Brain et al., 1985; Ohlén et al., 1987; Kawasaki et al., 1988). It was reported that CGRP acts as a physiological antagonist of the vascular effects of ET-1 in the systemic circulation in the rat (Meens et al., 2009, 2010, 2011, 2012; De Mey et al., 2011). However, there are very few reports on the actions of CGRP in the coronary circulation (Holman et al., 1986; McEwan et al., 1986; Ludman et al., 1991), and virtually no direct demonstrations by intravital microscopy of the effect of CGRP on coronary microcirculation. In this regard, the current study reveals, for the first time, that CGRP is a remarkably potent microvascular vasodilator in the myocardium, capable of dose-dependently reversing the elevation of CPP and arteriolar constrictions induced by ET-1 in isolated heart Langendorff preparations. It is also interesting to note that the ED50 values for the CPP enhancing effect of ET-1 (Homma et al., 1992) and the CPP depressor effect of CGRP (present study) in the rat isolated and perfused heart are quite similar, indicating that its potency in promoting vasodilation of the coronary vasculature is comparable to that of ET-1 inducing vasoconstriction.

Several studies have shown that ET-1 exerts positive inotropic actions in freshly isolated and cultured myocardial cells or in isolated cardiac atrial or ventricular preparations (Moravec et al., 1989; Shah et al., 1989; Kelly et al., 1990; Kaoukis et al., 2013; Miyauchi and Goto, 2013). Interestingly and in marked contrast, we observed herein that ET-1 application to rat isolated perfused spontaneously beating hearts promoted a transient increase in LVP which was rapidly replaced by a long-lasting negative inotropic effect. One of the potential reasons which might account for the distinct inotropic effects of ET-1 is that it may promptly and severely exhaust the myocardial energy stores utilized to sustain contractile function in the Langendorff preparation. In an earlier study in the same preparation (Homma et al., 1992), we showed that ET-1 potently constricted small sized arterioles and at higher doses completely blocked capillary blood flow in the myocardium. Mechanistically, a plausible hypothesis is that the reduced coronary perfusion on exposure to ET-1 could block the aerobic metabolic pathway in the local myocardium, thus accelerating anaerobic metabolism and decreasing myocardial contractility.

Indeed, this view is substantiated by the results we obtained in the myocardial metabolism analysis experiments using 31P-MRS. This method enables real-time in situ detection of the free high energy phosphate contents in the isolated beating heart (Argov et al., 1987; Aussedat et al., 1991). Moreover, PCR, ATP and Pi contents detected by this method quantitatively reflect the aerobic conditions of the specimen (Aussedat et al., 1991), and correlate well with the values obtained using classical biochemical methods of analysis (Humphrey and Garlick, 1991). When compared to the effects promoted by ET-1 alone, the concomitant administration of CGRP (together with ET-1) clearly counteracted the long-lasting negative inotropic effect, increased cardiac work index and lessened the decrease of high energy phosphate content of the whole heart. These actions of CGRP might be partly due to its enhancing effects on capillary blood flow, and partly because the peptide exerts a direct positive inotropic action of its own in isolated myocardial muscle (Ishikawa et al., 1988a, 1988b). Thus, our results suggest that: 1) impairment of cardiac contractility by ET-1 is due to
a reduction in levels of high energy metabolites, as a consequence of insufficient local myocardial perfusion; and 2), in part by its relaxant effect on the coronary microvasculature, CGRP can counteract these actions of ET-1 to dramatically improve myocardial metabolic conditions and performance.

Coronary blood flow is believed to be finely tuned, by a precise control mechanism, to meet the metabolic demand of the myocardium, thus affording strong protection against ischemic insults (Berne, 1980). Since, in the present study, the preparations were perfused at a fixed flow rate, it is likely that ET-1 might redistribute perfusate flow in the coronary microcirculation to affect cardiac metabolism or contractile function heterogeneously, and this action is modulated by CGRP. In other words, significant metabolic and functional alterations may occur well in restricted loci of the myocardium even in conditions when the volumetric flow rate supplied to the large coronary arteries is unchanged. Few studies have described the effects of other vasoactive agents on metabolic conditions of the heart, all of them conducted in dogs. In this regard, net myocardial lactate production was unchanged by adrenergic vasoconstriction even when coronary artery stenosis was present (Bufﬁngton and Feigl, 1981). Likewise, no change in arteriovenous lactate extraction was detected following intracardiac vasopressin administration (Corliss et al., 1968; Wilson et al., 1980). Moreover, doses of vasopressin sufﬁcient to decrease coronary blood flow and myocardial contractile force caused no essential change in the myocardial contractile force when the coronary artery stenosis was present (Buffington and Feigl, 1981). Likewise, no change in arteriovenous lactate extraction was detected following intracardiac vasopressin administration (Corliss et al., 1968; Wilson et al., 1980).

In conclusion, the present study has shown that, in the perfused rat heart, ET-1 enhances anaerobic metabolism in the myocardium, most likely via a marked reduction in coronary perfusion, and that these effects are potently alleviated by CGRP. These results strongly suggest that CGRP may act as an intrinsic physiological antagonist of ET-1 to control not only vasomotor tone in the coronary microcirculation, but also the metabolic condition of the myocardium. CGRP has been reported to exert its actions by binding to speciﬁc CGRP receptors coupled to cAMP (Shoji et al., 1987). However, several lines of evidence point to a closer relationship between CGRP and ET-1, whereby CGRP could counteract the effects of ET-1 by acting as an allosteric antagonist of endothelin ETA receptors (De Mey et al., 2011). Further investigations are thus needed to elucidate in greater detail the mechanisms underlying the interactions between ET-1 and CGRP seen in the rat isolated perfused heart.

Conflict of interest

There are no conﬂicts of interests.

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